

Livia Leoni
Giordano Rampioni
Editors

Quorum Sensing

Methods and Protocols

METHODS IN MOLECULAR BIOLOGY

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Edited by

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Foreword

Since the term “Quorum Sensing” was coined in 1994, the number of papers on the topic has steadily risen. This continual interest over the last quarter century is a testament to the fascination of bacterial communication and the promising scientific advances it represents. For many researchers, understanding bacterial communication represents the promise of new treatment modalities for infections. For others, untangling complex networks of quorum sensing regulatory systems is a tantalizing puzzle to solve. And still other groups of researchers hope to apply their knowledge of bacterial communication to address important environmental issues such as bioremediation and sustainability.

Quorum sensing researchers are a broad and multidisciplinary group of scientists. Thus the field itself has traditionally intersected and combined microbiology, engineering, math, medicine, and molecular biology. As we approach the 25th year of formal Quorum Sensing research, disciplines such as evolution and ecology are also emerging as areas ripe for advancing our understanding. Many researchers are now focusing on the complexities of bacterial communication in the context of heterogeneous populations and spatially structured communities. The methods needed to accomplish these studies are of course also consistently changing and evolving; thus practical guidelines for experimental methods are needed.

Seven years ago I had the privilege of compiling the expertise of some of the foremost Quorum Sensing researchers in the world into a volume of experimental methods. In this new version of *Quorum Sensing: Methods and Protocols*, editors Livia Leoni and Giordano Rampioni have done an excellent job of incorporating a broad range of topics that include traditional techniques and new, cutting-edge approaches. Many of the essential techniques, such as autoinducer detection by mass spectrometry and biosensors, are revisited and updated and new approaches, such as rapid electrochemical detection, are presented for the first time.

I am also delighted that this edition includes many of the pioneering researchers who contributed to the first volume and continue to make substantial contributions to the field, as well as new investigators who are beginning to make their mark and transform the field of Quorum Sensing. Undoubtedly, the information compiled here will continue to provide excellent instruction to an ever expanding and diverse group of scientists who are all joined in their curiosity and passion to better understand this ancient mode of cell-to-cell signaling used by our microbial relatives.

Lubbock, TX, USA

Kendra P. Rumbaugh

Preface

Since van Leeuwenhoek's descriptions of the microbial world up to few decades ago, bacteria were considered as deaf-mute individual cells designed to proliferate but unable to communicate and interact with each other. The first evidence of bacterial social behaviors can be traced back to 1965, when Alexander Tomasz reported that the ability of a *Streptococcus pneumoniae* population to enter the competence state is governed by a self-produced extracellular factor. With a remarkable and inspired intuition, Tomasz stated: "Since the activator—a cell-produced chemical—seems to impose a high degree of physiological homogeneity in a pneumococcal population with respect to competence, one is forced to conclude that in this case a bacterial population can behave as a biological unit with considerable coordination among its members. One wonders whether this kind of control may not be operative in some other microbial phenomena also" [1].

Few years later, Kenneth H. Nealson, Terry Platt, and J. Woodland Hastings unraveled the population density dependency of light emission in the bioluminescent marine bacterium *Vibrio fischeri* [2], thus paving the way for the identification of the first autoinducer used by bacteria to coordinate gene expression at the population level [3].

These pioneer works suggested that certain bacteria coordinate behaviors at the population level in response to variations in cell density and that this phenomenon is mediated by self-produced chemical signals. It is now acknowledged that this communication system, which has been named quorum sensing by Clay Fuqua, Stephen C. Winans, and E. Peter Greenberg in 1994 [4], is widespread in eubacteria, archaea and fungi rather than a curiosity restricted to few bacterial species. In the last decades, a rich vocabulary of quorum sensing signals belonging to distinct chemical classes has been compiled, by which microorganisms exploit complex beneficial or competitive interactions at the intraspecies, interspecies, and interkingdom levels. In this context, it is not surprising that sociomicrobiology has recently emerged as a fascinating research topic involving a growing community of scientists worldwide.

The aim of this book is to provide scientists interested in quorum sensing with a broad spectrum of methods and protocols useful for studying bacterial communication processes at the chemico-physical, molecular, and physiological level. In addition to wet-lab approaches, the book also contains bioinformatic methods and a discursive description of mathematical models (Chapter 20) to investigate quorum sensing.

The book is structured in three main parts: (1) Detection and quantification of quorum sensing signal molecules; (2) Methods for the studying of quorum sensing at the molecular, physiological, and population level; (3) Identification and characterization of anti-quorum sensing agents.

Overall, we think that this book could be helpful for a broad community of scientists interested in quorum sensing, ranging from biochemists to microbial ecologists, including molecular microbiologists, biotechnologists, bioinformaticians, and synthetic biologists.

Our first thanks go to Kendra Rumbaugh, the editor of the previous book "*Quorum Sensing: Methods and Protocols*" (Springer, 2010) [5]. We acknowledge her outstanding work by maintaining the original organization in three main parts, since we could not find a better rationale to follow, and by including some chapters present in the previous edition. Indeed, some of the methods and protocols reported in the book edited by Kendra can be

still considered as references in quorum sensing studies. As the quorum sensing research proceeds swiftly, a number of new approaches for quorum sensing investigations have been published in the last years; hence new methods and protocols have been included in this collection in the attempt to provide a comprehensive coverage of the methodology available in the field.

Special thanks also go to our dear friends and brilliant scientists Vittorio Venturi, for introducing us into the quorum sensing world, and Roman Popat, for providing the initial inspiration in editing this book.

Equally, we thank the Series Editor John M. Walker for the encouraging support along this editorial adventure.

Last but not least, thanks to all the experts who decided to share the precious protocols developed in their laboratories with the quorum sensing scientific community, and who kindly contributed their time and expertise for realizing this book.

Special thoughts go to our dear colleague Burkhard Hense, who passed away while this book was in preparation. This book is dedicated to him.

Rome, Italy

*Livia Leoni, Ph.D.
Giordano Rampioni, Ph.D.*

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Part I

Detection and Quantification of Quorum Sensing Signal Molecules

Chapter 1

Use of Whole-Cell Bioassays for Screening Quorum Signaling, Quorum Interference, and Biofilm Dispersion

Starla G. Thornhill and Robert J.C. McLean

Abstract

In most bacteria, a global level of regulation, termed quorum sensing (QS), exists involving intercellular communication via the production and response to cell density-dependent signal molecules. QS has been associated with a number of important features in bacteria including virulence regulation and biofilm formation. Consequently, there is considerable interest in understanding, detecting, and inhibiting QS. *N*-acylated homoserine lactones (AHLs) are used as extracellular QS signals by a variety of Gram-negative bacteria. *Chromobacterium violaceum*, commonly found in soil and water, produces the characteristic purple pigment violacein, regulated by AHL-mediated QS. Based on this readily observed pigmentation phenotype, *C. violaceum* strains can be used to detect various aspects of AHL-mediated QS activity. In another commonly used bioassay organism, *Agrobacterium tumefaciens*, QS can be detected by the use of a reporter gene such as *lacZ*. Here, we describe several commonly used approaches incorporating *C. violaceum* and *A. tumefaciens* that can be used to detect AHL and QS inhibitors. Due to the inherent low susceptibility of biofilm bacteria to antimicrobial agents, biofilm dispersion, whereby bacteria reenter the planktonic community, is another increasingly important area of research. At least one signal, distinct from traditional QS, has been identified and there are a variety of other environmental factors that also trigger dispersion. We describe a microtiter-based experimental strategy whereby potential biofilm dispersion compounds can be screened.

Key words Quorum sensing, *N*-acyl homoserine lactones, Violacein, *Chromobacterium violaceum*, *Agrobacterium tumefaciens*, Biofilm dispersion

1 Introduction

This chapter has been revised and updated from an earlier version [1].

1.1 Quorum Sensing Detection in Gram-Negative Bacteria

Population-dependent gene expression, commonly referred to as quorum sensing (QS), was first associated with light production in *Vibrio fischeri* [2]. QS is now recognized as a major component of gene regulation and intercellular communication in bacteria [3, 4] and is considered to be involved in other aspects of microorganisms including competition and biofilm structure [5]. QS is an environmental sensing system that allows bacteria to monitor population

density and to connect cell population density with gene expression. As part of their normal metabolic activities, bacteria produce several small diffusible signaling molecules. These signal molecules often positively regulate their own synthesis and were originally described as autoinducers (AI). The best known QS systems are based on the signal molecules *N*-acyl homoserine lactones (AHLs), of which *N*-3-oxo-hexanoyl homoserine lactone (3-o-C₆ HSL) represents the signal associated with *V. fischeri* [6]. AHLs represent the first class of AI to be described [7]. The genes associated with the production of these signals include an AHL synthase and an AHL-binding transcriptional regulator. In *V. fischeri* these genes are *luxI* and *luxR*, respectively [8]. Similar genes in other Gram-negative organisms having AHL-regulated QS are *luxI* and *luxR* homologues even though the QS-mediated gene functions in these organisms do not involve bacterial luminescence [7]. Table 1 shows a list of representative AHLs.

Other representative AHLs include the *N*-butyryl HSL (C₄-HSL) and *N*-3-oxo-dodecanoyl HSL (3-o-C₁₂ HSL), both

Table 1
Commonly used bioassay strains

Strain	Use	Reference
<i>Agrobacterium tumefaciens</i>		
A136 (pCF218)(pCF372)	Detection of broad range of AHLs	[29, 35]
(also referred to as WCF47(pCF218) (3-o-C ₄ to 3-o-C ₁₂ HSLs, C ₅ to C ₁₀ HSLs) ^{a, b} (pCF372))		
NTL4 (pCF218)(pCF372)	Detection of broad range of AHLs (3-o-C ₄ to 3-o-C ₁₂ HSLs, C ₅ to C ₁₀ HSLs) ^{a, b}	[36, 37]
KYC55 (pJZ372)(pJZ384) (pJZ410)	Detection of broad range of AHLs (3-o-C ₄ to 3-o-C ₁₈ HSLs, C ₄ -C ₁₈ HSLs) ^{a, c}	[30]
KYC6 (pCF218)(traM-tn5-gusA, Km ^R)	3-o-C ₈ HSL overproducer, positive control for bioassay	[51]
<i>Chromobacterium violaceum</i>		
ATCC 12472	wt, used in quorum signal inhibition screens, indirect AHL detection	[11, 33]
ATCC 31532	C ₆ HSL overproducer, used as positive control for CVO26 bioassay	[11]
CVO26	Detection of C ₄ - and C ₆ -HSLs	[11]

^aFor details, see [30, 35]. All strains grow at 30 °C

^bFor plasmid maintenance, grow on LB + spectinomycin (50 µg/ml) and tetracycline (4.5 µg/ml)

^cFor plasmid maintenance, grow on LB + spectinomycin (50 µg/ml), tetracycline (4.5 µg/ml), and gentamycin (15 µg/ml)

produced by the opportunistic pathogen *Pseudomonas aeruginosa* [9], N-3-oxo-octanoyl HSL (3-o-C8 HSL) produced by *Agrobacterium tumefaciens* [10], and N-hexanoyl HSL (C6-HSL) produced by *Chromobacterium violaceum* [11]. AHL-based QS is reviewed in [7, 12].

AHLs are associated with a number of important microbial activities including virulence gene regulation [13, 14], antibiotic resistance [15, 16], and aspects of biofilm formation in *P. aeruginosa* [17, 18]. Quorum interference by brominated furanones and other compounds [19–21] as well as AHL-degrading enzymes has shown promise as a novel antibacterial treatment strategy due to its effectiveness against highly resistant biofilm populations [22, 23]. As a result, there is considerable interest in the identification of QS systems and QS inhibitors (QSIs). There are a number of chemical approaches including chromatography and mass spectrometry that can be used to characterize AHLs (e.g., [24–27]); however, the equipment and expertise needed for these approaches can be quite significant. In contrast, QS bioassays (reviewed in [28]) are relatively inexpensive and thus allow screening for QSIs and AHLs in regions of the world including places with high biodiversity, such as the tropics, and limited financial resources.

Bioassay organisms for QS have a transcriptional response regulator (*luxR* homologue in the case of AHLs) coupled to a reporter gene allowing a readily observable phenotype. In the case of the widely used *A. tumefaciens* AHL bioassays [29, 30], the *traR* gene (*luxR* homologue) is coupled to *lacZ*. In this fashion, AHLs can be detected on the basis of β-galactosidase activity often using 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). The normal phenotype associated with QS in *A. tumefaciens* is gene conjugation [29], which is readily visualized. In contrast to *A. tumefaciens*, several organisms, including *Serratia marscescens* [31], *Pseudomonas aureofaciens* [32], and *C. violaceum* [11, 33] naturally produce pigmented compounds in response to QS. We shall focus on the use of *A. tumefaciens* and *C. violaceum* in this chapter.

1.2 *Agrobacterium tumefaciens* as a Biosensor

A. tumefaciens is a Gram-negative opportunistic plant pathogen that causes crown gall formation on plants through the transmission of DNA fragments into the nuclei of infected plants [29]. Conjugation-based gene transfer is under the regulation of 3-o-C8 HSL-dependent QS. The genes responsible for synthesis and response regulation of this signal molecule are *traI* and *traR*, respectively, which are homologues of *luxI* and *luxR* [34]. During their investigations of QS-related functions of *A. tumefaciens*, S.C. Winans, C. Fuqua, J. Zhu, and colleagues developed several reporter strains, notably A136 (pCF218)(pCF372) (also referred in literature as WCF47(pCF218)(pCF372)) [29, 35], NTL4 (pCF218)(pCF372) [36, 37], and KYC55 (pJZ372)(pJZ384)

(pJZ410) [30] (listed in Table 1) that have become widely used in the detection of AHLs. These strains lack the *traI* gene (AHL synthesis), overexpress the *traR* gene, and employ *lacZ* as a reporter gene. They require supplementation with a β-galactosidase substrate such as X-gal in order to visualize AHL recognition. These strains recognize a wide range of AHLs [30, 35] with the KYC55 (PJZ372)(PJZ384)(PJZ410) strain being able to detect a wider range of AHLs with increased sensitivity [30]. It has been our experience that all strains will detect their cognate AHL (3-o-C8 HSL) at subpicomole concentrations, with other AHLs being detected in the micromole to nanomole range. *A. tumefaciens* bioassays have been incorporated into AHL detection by reverse-phase thin-layer chromatography [38] and high-performance liquid chromatography [24]. Alternatively, assays for β-galactosidase (LacZ gene product) can be employed to gain quantitative data [30].

1.3 Chromobacterium violaceum as a Biosensor

C. violaceum is a Gram-negative bacterium that produces a purple pigment, violacein, under regulation of C6-HSL-dependent QS. N-decanoyl homoserine lactone (C10-HSL) has also been identified as a signal in *C. violaceum* [39]. The C6-HSL synthesis and response regulator genes are *cviI* and *cviR*, respectively, and are homologues of *luxI* and *luxR* [40]. Several strains are commonly used for bioassays (Table 1). These strains allow the direct detection of short-chain AHLs through the induction of pigmentation in strain CVO26 [11], which is unable to produce AHLs but is fully capable of producing violacein in response to its cognate signal molecule (C6-HSL) or the short-chain C4-HSL. As a positive control for the bioassay, a C6-HSL-producing, nonpigmented strain (ATCC 31532), is used in association with the CVO26 bioassay strain [33]. Using a plate streaking protocol (Subheading 3.2.1), the pigmentation is readily visible after overnight culture (Fig. 1a) and is absent if C6 HSL is not present (Fig. 1b). We have observed that some pigmentation will occur in older (>48 h) CVO26 cultures. AHLs can be extracted from cultures (process described below), synthesized in the lab [26] or alternatively purchased from commercial sources. Several investigators have modified the *C. violaceum* assay by extracting the violacein with a solvent (typically acetone, ethanol, or butanol) and then measuring absorption using a spectrophotometer [40]. This approach enables to gain quantitative data from the bioassay.

AHL-based QS is very specific in that the response regulator (*cviR* in the case of *C. violaceum*) will only respond to the cognate AHL (C6-HSL) or a closely related AHL such as *N*-butyryl HSL (C4-HSL). Other AHLs will bind ineffectively to the CviR receptor and competitively interfere with its ability to activate genes associated with violacein production, which is seen as a loss in pigmentation. A strategy for investigating whether quorum inhibition is

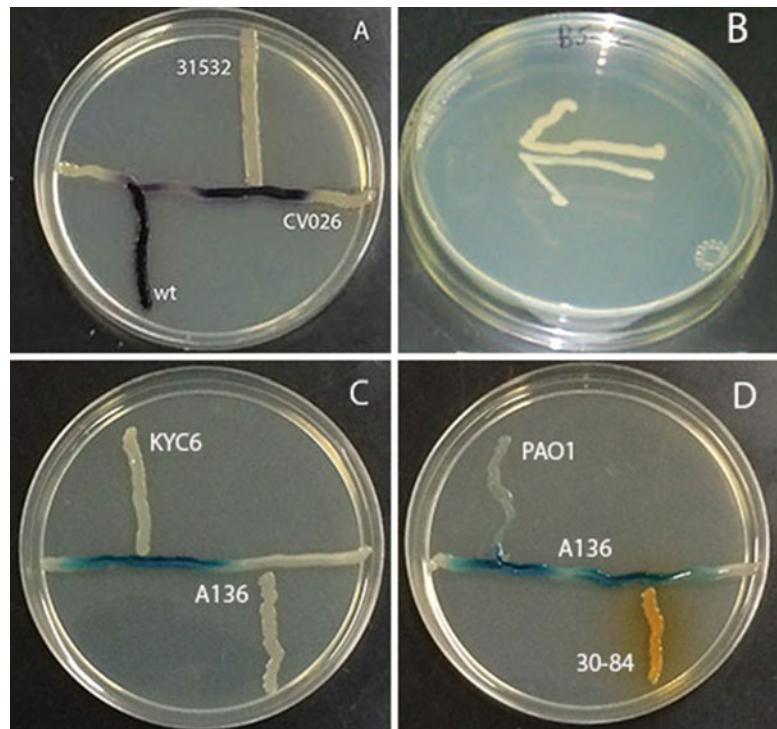


Fig. 1 Cross-feeding plate assays for AHL detection. **(a)** shows indicator strain *C. violaceum* CV026 producing violacein in response to *C. violaceum* 31532 (C6-HSL overproducer, positive control) and *C. violaceum* ATCC12472 (wt) (**a**) [11, 33]. No violacein is produced when strain CV026 (*cvi* mutant) is streaked against itself (**b**). The *A. tumefaciens* plate assay is shown in **(c)** and **(d)**. **(c)** *A. tumefaciens* A136 (PCF218)(PCF372) (bioassay strain) expressing *lacZ* in response to *A. tumefaciens* KYC6 (3-o-C8 HSL overproducer), but not when streaked against itself; **(d)** strain A136 (PCF218)(PCF372) expressing *lacZ* in response to *P. aeruginosa* PA01 and *Pseudomonas chlororaphis* (formerly *P. aureofaciens*) 30-84 [33]

based on AHL synthesis (i.e., *luxI*-directed inhibition) or AHL regulation (i.e., *luxR*-directed inhibition) is shown in Fig. 2 and examples of quorum inhibition using *C. violaceum* are shown in Fig. 3. *A. tumefaciens* and *C. violaceum* biosensors are compared in Figs. 4 and 5.

Here we describe several approaches in which *A. tumefaciens* and *C. violaceum* can be used for QS detection. The first section describes extraction and concentration of AHLs using an ethyl acetate protocol [35, 38]. Chemical extraction is not required but can increase the sensitivity of bioassays and also provides material for downstream applications, including the detection of individual AHLs from environmental samples. The second section describes the *A. tumefaciens* bioassays, which are generally used for detecting C6-C12 HSLs, although C14-C18 HSLs have been detected at

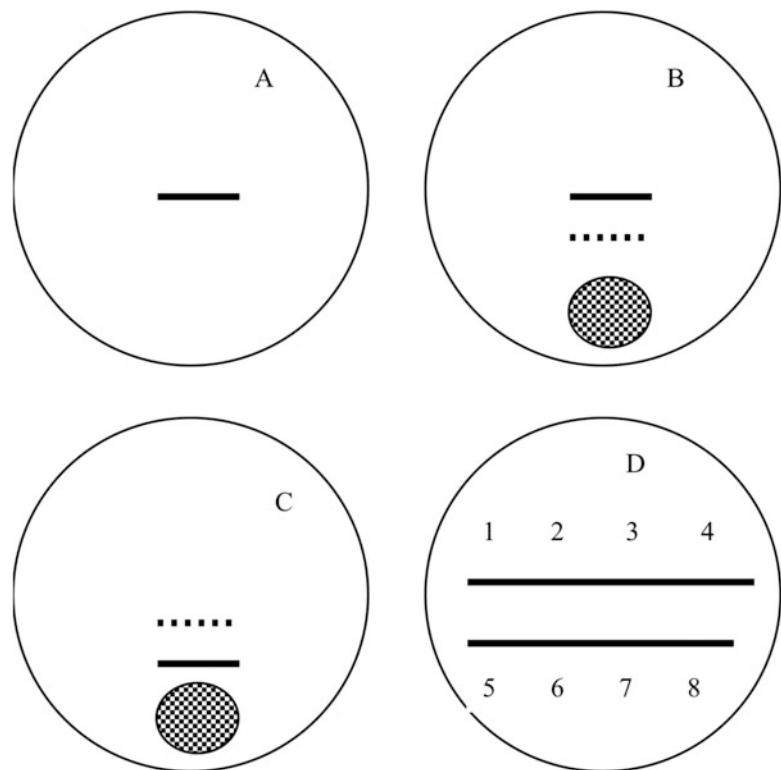


Fig. 2 Inoculation strategies for bioassays on Petri plates. (a) shows inoculation of a test organism prior to an overlay assay (Subheading 3.2.2). (b) and (c) show the inoculation pattern of a test organism or extract (*patterned small circle*) and varied location of AHL-producing (*dashed line*) and AHL-detecting (*solid line*) strains to differentiate interference with AHL production or AHL response (details in Subheading 3.2.3). (d) shows an inoculation strategy for high-throughput screens of test organisms (indicated by *numbers*) near a bioassay organism (*solid line*)

micromolar concentrations. The third section describes the *C. violaceum* AHL bioassay for detecting C4- and C6-HSLs on the basis of pigment induction in the reporter strain, CVO26. The fourth section describes a pigmentation inhibition assay, using the type strain 12472, which can be used as an indirect detection approach for other AHLs (other than C4- or C6-HSLs). Alternatively this fourth approach can be used as an initial screen for QS-inhibiting compounds or AHL-degrading (quorum-quenching) enzymes [33]. The fifth section describes a thin-layer chromatography (TLC) protocol that can be used to detect AHLs [38]. Here, *A. tumefaciens* A136 (pCF218)(pCF372) is typically used as a biosensor (Fig. 5).

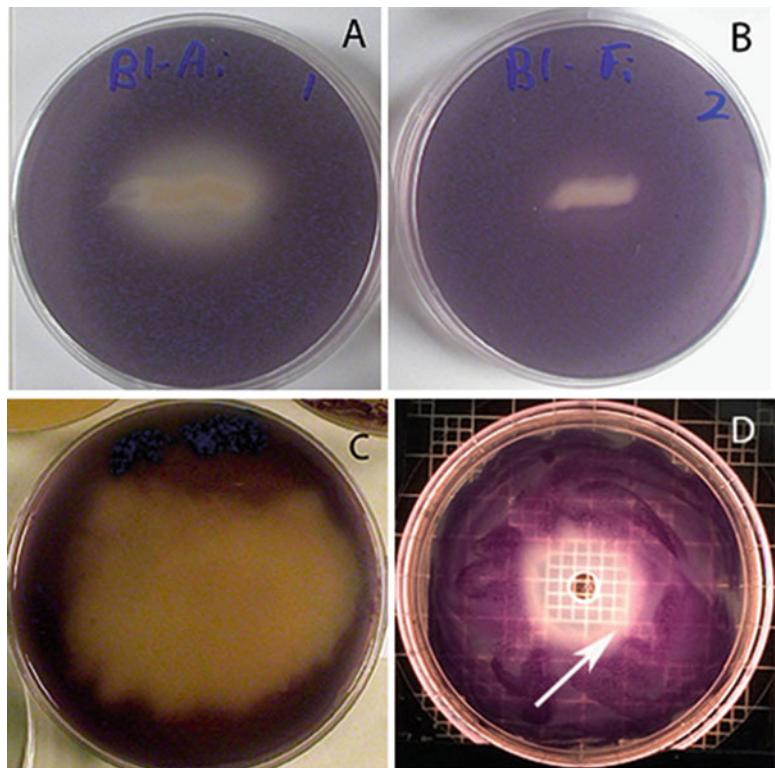


Fig. 3 *C. violaceum* pigmentation inhibition assay is used to detect QS disruption in *C. violaceum* 12472 ((1)) as modified [33]. Here, AHLs or enzymes that disrupt the binding of the *C. violaceum* cognate AHL (C6 HSL) cause a loss of pigmentation in the vicinity of the test organism in the center of the plate. In (a) (positive control), *P. aeruginosa* PA01 AHLs (C4-HSL, and 3-o-C12 HSL) interfere with the *C. violaceum* CviR receptor. Pigmentation inhibition is absent (negative control, (b)) in the vicinity of C6-HSL-producing *C. violaceum* strain 31532, although the 31532 strain itself is nonpigmented. (c) shows the pigmentation inhibition, due to an uncharacterized quorum-disrupting compound produced by an environmental isolate [58] (RJC McLean and C Fuqua, unpublished). (d) shows a plate in which a potential quorum-inhibiting compound has been placed into a well in the center of the plate and *C. violaceum* 12474 spread across the plate. Growth inhibition is seen close to the quorum inhibitor, and quorum inhibition (arrow) is indicated by the nonpigmented zone. More details are given in the text

1.4 Biofilms and Biofilm Dispersion

Adhesion to and growth on surfaces as biofilms is a common feature of bacteria and other microorganisms in their natural environments [41]. Growth as surface-adherent biofilm communities confers significant antimicrobial protection to microorganisms, which in some cases can approach values of 1000-fold reduced susceptibility in comparison to planktonic values [42]. Unlike traditional resistance mechanisms which tend to persist regardless of growth mode, the reduced susceptibility of biofilm bacteria to antibiotics is classified as

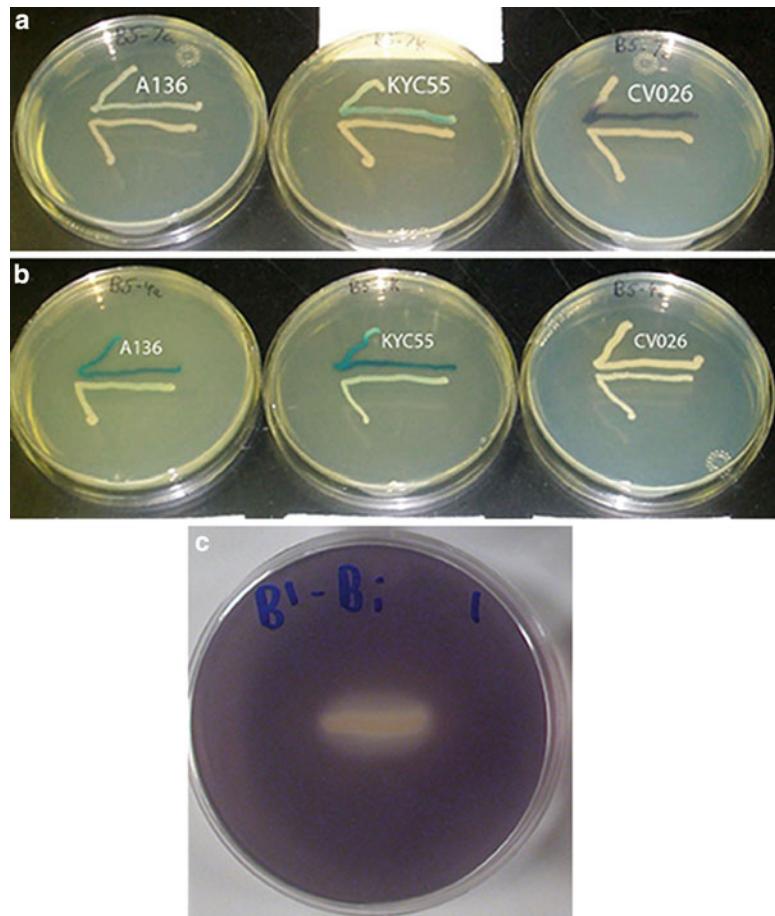


Fig. 4 Comparison of *C. violaceum* with *A. tumefaciens* biosensor activity. (a) shows reaction of *A. tumefaciens* A136 (pCF 218)(pCF 372) [29], *A. tumefaciens* KYC55 (pJZ372)(pJZ384)(pJZ410) [30], and *C. violaceum* CV026 [11] biosensors (top streak) to C6-HSL production by *C. violaceum* 31532 (bottom streak) in a plate bioassay (Subheading 3.2.1). (b) shows response of same three biosensor strains to 3-o-C8-HSL production by *A. tumefaciens* strain KYC6 [51]. However, the 3-o-C8-HSL, produced by *A. tumefaciens*, can be detected indirectly by *C. violaceum* 12472 pigmentation inhibition (c, Subheading 3.2.2), bioassay

tolerance, since microorganisms regain planktonic levels of susceptibility once they leave the biofilm community [43]. Just as QS has been associated with biofilm formation and some biofilm phenotypes [44], there is now increasing evidence of different signals and chemical stimuli being associated with biofilm dispersion [45]. One example of a dispersant signal is *cis*-decanoic acid [46]. Other dispersion triggers include nitric oxide, altered nutrition, starvation, and anthranilate (reviewed in [43, 45]). A number of mechanisms have been proposed including a reduction of the second messenger, 3',5'-cyclic di-guanylate (c-di-GMP) [47] via phosphodiesterases.

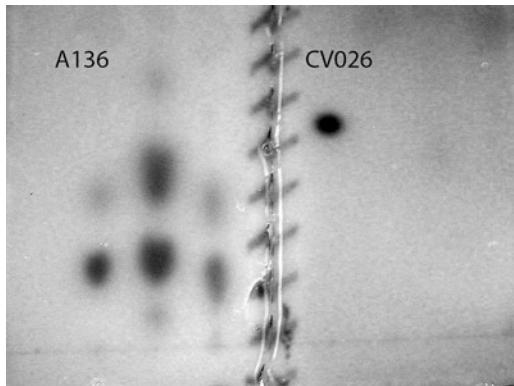


Fig. 5 TLC analysis of AHL extracts from several strains of *P. aeruginosa*. Of note, *A. tumefaciens* A136 (PCF218)(PCF372) (*left*) detected many more AHLs than did the *C. violaceum* CV026 (*right*)

While intracellular c-di-GMP concentrations become elevated in biofilms via diguanylate cyclases, the levels decrease notably in planktonic populations. Phosphodiesterases are coupled directly or indirectly to environmental sensors, which in turn are activated by a variety of cues. Reduced c-di-GMP levels trigger gene expression related to the return to planktonic growth. Other mechanisms besides alterations of c-di-GMP levels have been described including activation of phage and other genes that can act as mechanisms of apoptosis [48–50] of a portion of the organisms within a biofilm. While there is considerable work related to the identification of quorum inhibition in prevention of biofilms, there is a need for work in identifying compounds that may trigger biofilm dispersion. We address a microtiter plate strategy for screening and identifying potential biofilm dispersion molecules. This concept is adapted from the flow-cell protocol by Karin Sauer and colleagues [48], and representative data is shown in Fig. 6.

2 Materials

2.1 AHL Extraction [35, 38]

1. Centrifuge capable of $4000 \times g$, and solvent-resistant tubes.
2. Ethyl acetate (containing 0.1% (v/v) acetic acid) (store in the dark in volatile chemical storage).
3. Overnight broth culture (stationary phase) of organism to be investigated.
4. Alternatively, if naturally occurring biofilms are to be investigated, then prepare small glass containers with water—fill half full with water, cover, and autoclave. Container size is chosen based on environmental sample size.

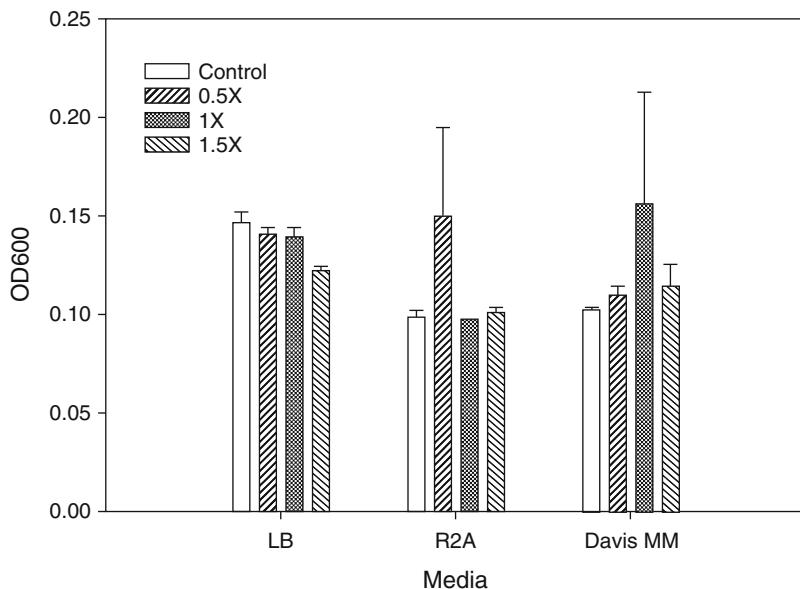


Fig. 6 Representative dispersion screen showing the ability of a potential novel dispersion agent at various concentrations [59] to detach preformed *P. aeruginosa* biofilms that were grown in Luria-Bertani (LB) broth, R2A broth, or Davis minimal media (Davis MM). Detachment is indicated by increased turbidity

5. Bath sonicator (for environmental biofilm samples): We have found it important to use glass vials rather than plastic as the latter substance (plastic) dampens the shear forces generated by the sonicator and reduces biofilm disruption.
6. Small glass vials for storing extracted AHLs (scintillation vials with autoclavable polycarbonate lids are excellent general-purpose containers in this regard).
7. Water aspirator or vacuum source (use water trap to avoid contamination of vacuum line).
8. Rotary evaporator.
9. Freezer (preferably -80°C).

2.2 *A. tumefaciens* Assay for AHLs

1. *A. tumefaciens* A136 (pCF218)(pCF372) (biosensor strain): Maintain on LB supplemented with spectinomycin (50 $\mu\text{g}/\text{ml}$) and tetracycline (4.5 $\mu\text{g}/\text{ml}$) [29, 35].
2. *A. tumefaciens* KYC55 (pJZ372)(pJZ384)(pJZ410) (alternate, highly sensitive biosensor strain): Maintain on LB supplemented with spectinomycin (50 $\mu\text{g}/\text{ml}$), tetracycline (4.5 $\mu\text{g}/\text{ml}$), and gentamycin (15 $\mu\text{g}/\text{ml}$) [30].
3. *A. tumefaciens* NTL4 (pCF218)(pCF372) (biosensor strain that does not spontaneously mutate for tetracycline resistance): Maintain on LB supplemented with spectinomycin (50 $\mu\text{g}/\text{ml}$) and tetracycline (4.5 $\mu\text{g}/\text{ml}$) [36, 37].

4. *A. tumefaciens* KYC6 (pCF218)(*traM*-tn5-*gusA*, Km^R) (3-o-C8-HSL overproducing strain used as positive control for *A. tumefaciens* AHL bioassay) [51].
5. Luria-Bertani broth (LB): 10 g/l NaCl, 10 g/l tryptone, 5 g/l yeast extract.
6. LB agar: LB plus 1.5% (w/v) agar.
7. LB soft agar: LB plus 0.3% (w/v) agar.
8. ATGN medium (gives increased sensitivity for *A. tumefaciens* bioassay) [52] (composition described in Subheading 2.2.1).
9. Glass or metal spreading rod and 250–500 ml beaker filled to a depth of 2–3 cm with 100% ethanol: Alcohol sterilize spreading rod before use by dipping in 100% ethanol and flame (caution: flammable—if ethanol in beaker catches fire, cover the top of beaker with a suitable object, such as a book, in order to quickly smother the flame).
10. 20 mg/ml X-gal in dimethylformide: Add 50 µl and spread over surface of agar plate prior to inoculating bacteria for bioassay.
11. Other agar as appropriate for organism to be investigated (as a cautionary note, ensure that this medium does not inhibit *A. tumefaciens*).
12. 50 °C Water bath (needed for overlay assay).
13. Test tubes with caps and rack for preparing soft agar (5 ml aliquots).
14. Vortex mixer.
15. Pipette and sterile tips (adjustable 10–200 µl).

2.2.1 ATGN Medium for Enhanced *A. tumefaciens* Bioassay [52]

1. Prepare and autoclave the following stock solutions:
 - (a) 20× AT buffer: Mix 214 g of KH₂PO₄ in 800 ml H₂O (adjust pH to 7.0 with approximately 35 g solid NaOH), and then bring the volume to 1000 ml with H₂O. **As a safety precaution, please use suitable eye protection when handling solid NaOH.**
 - (b) 20× AT salts: 40 g/l (NH₄)₂SO₄, 3.2 g/l MgSO₄ · 7H₂O, 0.2 g/l CaCl₂ · 2H₂O, 24 mg/l iMnSO₄ · H₂O.
 - (c) 100× Iron stock (2.2 mM): Dissolve 0.611 g FeSO₄ · 7H₂O in 1 l of 10 mM HCl to prevent precipitation.
 - (d) 50% Glucose: Dissolve 50 g of glucose in 100 ml H₂O.
2. 1× ATGN medium (per liter): Mix 50 ml 20× AT buffer, 50 ml 20× AT salts, 10 ml 50% glucose, and 10 ml 100× iron stock. If needed, add 15 g agar. Bring total volume to 1000 ml with distilled water and autoclave.

**2.3 Chromo-
bacterium violaceum
AHL Detection
(See Note 1)**

1. *C. violaceum* CVO26 (biosensor strain) [11].
2. *C. violaceum* ATCC 31532 (C6 HSL overproducer, used as positive control) [11].
3. Luria-Bertani broth (LB): 10 g/l NaCl, 10 g/l tryptone, 5 g/l yeast extract.
4. LB agar: LB plus 1.5% (w/v) agar.
5. LB soft agar: LB plus 0.3% (w/v) agar.
6. Other agar as appropriate for organism to be investigated (as a cautionary note, ensure that this medium does not inhibit *C. violaceum*).
7. Other materials as shown in Subheading 2.2 (above).

**2.4 Indirect *C. violaceum* Assay
for AHLs and QS
Inhibition**

1. *C. violaceum* ATCC 12472 type strain used for pigmentation inhibition [33].
2. *P. aeruginosa* PAO1 (used as positive control) [33].
3. Other materials as listed in Subheading 2.2 (above).

**2.5 Thin-Layer
Chromatography (TLC)
Detection of AHLs**

1. Ethyl acetate (containing 0.1% (v/v) acetic acid) (store in the dark in volatile chemical storage).
2. C18 TLC plate (see Note 2).
3. C6-HSL and C8-HSL standards (see Note 3), dissolve 5 µM in ethyl acetate.
4. TLC glass tank (see Note 4).
5. Paper towels: We use these to line tank during run, and the resulting elevated humidity within the tank will prevent an uneven migration of solvent front (“smile” or “frown” pattern).
6. Methanol/water mixture (60:40)—need at least 200 ml.
7. Laboratory-adjustable temperature hot plate.
8. Labeling tape (approximately 2 cm wide).
9. Plastic tub and cover (big enough to incubate TLC plate with indicator bacteria).
10. Incubator (30 °C).
11. *A. tumefaciens* A136 (pCF218)(pCF372) bioassay strain.
12. X-gal solution (20 mg/ml in dimethylformamide).

**2.6 Biofilm
Dispersion Screen**

1. Sterile microtiter plate with lid.
2. Appropriate growth media for organism being tested.
3. Test organism(s).
4. Candidate-dispersing agents.
5. Incubator with orbital shaker.

6. Microtiter plate reader (preferably with temperature control and ability to agitate samples).
7. Multichannel pipets are very useful (200 μl volume for 96-well plates).

3 Methods

3.1 Ethyl Acetate Extraction [35, 38]

1. Grow 20 ml broth culture to stationary phase. If biofilm from pebbles or other surfaces is desired, sonicate pebbles in sterile H₂O for 15 min, and remove 20 ml of the sonicated liquid to a centrifuge tube.
2. Centrifuge to remove bacterial cells, at $3000 \times g$ for 10 min at 4 °C.
3. Transfer supernatant to clean bottle.
4. Extract the cell-free supernatant three times with three volumes of ethyl acetate.
5. Pool the ethyl acetate fractions (top layers) and evaporate to dryness using a rotary evaporator (with water bath set at 40 °C).
6. Suspend residue in 1 ml ethyl acetate and transfer to a small glass vial. Again evaporate the ethyl acetate, this time using a Pasteur pipet attached to a vacuum source (aspirator, linked to a water tap works fine for this) that is placed above the liquid (careful: it's easy to suck up liquid here).
7. Resuspend the residue in 100 μl ethyl acetate and store at -80 °C until needed.

3.2 AHL Reporter Agar-Based Plate Bioassays [53, 54]

3.2.1 Plate-Based Assay

(Works well if both bioassay *C. violaceum* or *A. tumefaciens* and test organism grow on same medium)

1. If using *A. tumefaciens*-based bioassay, pipet 50 μl X-gal solution onto agar and spread across surface with alcohol-sterilized spreading rod, prior to inoculating with bacteria.
2. Streak test organism and bioassay organism beside each other on LB agar plate using a "T" shape. The cross of the "T" would represent strain CVO26 and the vertical line would represent the test organism (Fig. 1). Following 16–48-h incubation at 30 °C, you should see a positive (blue coloration (due to X-gal hydrolysis) in the case of *A. tumefaciens* A136 (pCF218) (pCF372) or KYC55 (pJZ372)(pJZ384)(pJZ410) bioassay, and purple violacein in the case of *C. violaceum* bioassay) production in the bioassay in the region closest to the intersection of the two strains and a lack of pigmentation away from this intersection. An alternative streaking design is shown in Fig. 1, which is intended to give a concentration gradient of

exposure to the bioassay organism. Although antibiotics are required to maintain the plasmids in strains A136 (pCF218) (pCF372) or KYC55 (pJZ372)(pJZ384)(pJZ410), these strains are sufficiently stable to be grown without antibiotics for the duration of the bioassay.

3. As a positive control in the *C. violaceum* bioassay, streak CVO26 (bioassay strain) and *C. violaceum* 31532 (C6-HSL overproducer) in this same manner. For *A. tumefaciens* bioassay, first spread 50 µl X-gal solution on the surface of the LB agar plate, and then streak the bioassay strain (A136 (pCF218) (pCF372) or KYC55 (pJZ372)(pJZ384)(pJZ410)) and KYC6 (3-o-C8 HSL overproducer) in this same manner. For a negative control, we simply streak the reporter strains (CVO26, A136 (pCF218)(pCF372) or KYC55 (PJZ372)(PJZ384) (PJZ410)) against itself (both as reporter and test) as these strains do not produce AHLs.

3.2.2 Soft Agar Overlay Assay [33]

This approach gives more sensitivity than the plate-based assay (Subheading 3.2.1), and also enables the observation of potential competition between the test organism and the bioassay organism (*C. violaceum*), which may indicate production of an antibacterial compound. This test typically takes 3 days:

1. *Day One:* Streak the test organism (see Notes 5 and 6) onto suitable agar using the patterns shown (Fig. 2a), and then grow in incubator overnight. (For freshwater aquatic isolates, we typically use R2A agar [55]).
2. As a positive control, streak *P. aeruginosa* onto LB agar in the same pattern and grow overnight at 37 °C. If desired, a negative control would consist either of strain 12472 on the plate, or alternatively strain 31532 (C6-HSL overproducer).
3. Grow an overnight LB broth culture of *C. violaceum* (30 °C with shaking).
4. If some other substance (example would be a plant leaf or other tissue) is to be examined, then place the object onto a plate of LB agar and immediately proceed to steps 5 and 6 (agar overlay).
5. *Day Two:* Prepare and autoclave test tubes containing 5 ml LB soft agar. Store tubes in 50 °C water bath once they come out of the autoclave.
6. For each test plate (from step 1 or 2), mix 5 µl overnight *C. violaceum* 12472 culture with 5 ml soft agar, vortex for 5 s, and then pour the mixture over plate. Incubate at 30 °C overnight. (It is important that step 6 be done as quickly as possible as *C. violaceum* is susceptible to heat.)

7. *Day Three:* Examine and photograph plates. A positive result is indicated by a loss of pigmentation in the vicinity (*see* **Note 6**). An example is shown in Fig. 3c.

3.2.3 Alternative Plate-Based Assays

The aforementioned assays in Subheadings 3.2.1 and 3.2.2 are useful at screening various microorganisms and other tissues for AHL- and QS-inhibiting materials. QS bioassays also lend themselves to the examination of other substances including plant and food extracts [56]. Here, one can examine pigment induction in an AHL-responsive strain (CVO26), or pigmentation inhibition (strain 12472). Here the test material can be placed onto a small sterile disk of filter paper (we typically use a three-hole office punch to generate these, and then autoclave before use):

1. Prepare overnight broth cultures of *Chromobacterium* strains as described in **step 3**, Subheading 3.2.2 (use CVO26 for pigment induction, and 12472 for pigment inhibition).
2. Take 100 µl overnight culture and spread over the surface of LB agar (forming a bacterial lawn).
3. Using alcohol-sterilized forceps, place filter paper disk (containing test substance) onto plate. The substance can be added to the filter paper disk with a pipet after the disk has been placed onto the agar.
4. Incubate overnight at 30 °C and examine for pigmentation. Again the effect should be most noticeable in the vicinity of the test material.
5. As an alternative, one can use two reporter strains simultaneously [57] and investigate whether QS inhibition might be due to interference with AHL production (*luxI*-effect) or a transcription response (*luxR*-effect). In the case of the *C. violaceum* bioassay, two reporters are used: 31532 (C6-HSL overproducer) and CVO26 (AHL biosensor). Overnight cultures of the reporters are prepared as described above and the samples inoculated as shown in Fig. 2b, c.
6. If larger amounts of the test substance need to be delivered, 100 µl of an aqueous solution of the compound can be added to the agar plate rather than using a paper disk. Use the wide end of a 200 µl pipette tip to create a 6 mm punch in the agar plate after plating the lawn. Add 100 µl of the aqueous solution to the punch, and incubate for 24 h at the appropriate temperature (Fig. 2d). Ensure that replicate plates are of uniform thickness by adding the same amount of agar media to each poured plate.

The test material is shown as a darkened circle within the Petri plate, the bioassay strain CVO26 is shown as a solid line, and the C6-HSL-producing strain 31532 is shown as a dashed line.

Normally these two strains will cross-feed each other (positive control for AHL detection) such that strain CVO26 will produce violacein in the presence of 31532. Using the first pattern (Fig. 2b), the test material is closest to strain CVO26 and so pigmentation inhibition results could be interpreted as transcription inhibition (*luxR*-effect). In the case of the second pattern (Fig. 2c), the acyl-HSL-producing strain, 31532, is closest to the test material and so a pigmentation inhibition effect could be interpreted as interference with C6-HSL production (*luxI*-effect) [57].

A schematic of a plate-based protocol for a high-throughput bioassay is shown in Fig. 2d. Here, the bioassay strain is shown as a solid line, and the test organisms (indicated by numbers) can be spotted near the bioassay strain. Due to possible interactions between test organisms during the high-throughput assays, we recommend that positive results be confirmed using the plate-based assay (Subheading 3.2.1) or overlay assay (Subheading 3.2.2).

3.2.4 Thin-Layer Chromatography (TLC) Detection of AHLs

1. The resuspended sample and standards are added to a C18 TLC plate (see Note 2). Apply 1 or 2 μ l dot-wise, approx. 2 cm apart, along a line which is 2 cm from the bottom of the plate. (If necessary, use a pencil for labeling the TLC plate as ink will dissolve in the organic phase).
2. For standards, use C6 and 3-o-C8 standards (cognate AHLs of *C. violaceum* and *A. tumefaciens*, respectively), 5 μ M.
3. Line chromatography chamber with white paper towel or filter paper. Add methanol/water (60:40, vol/vol), 200 ml. Let towels saturate with the solvent. This prevents uneven solvent front migration.
4. Place TLC plate into the chamber and cover with glass lid. Develop chromatogram until the solvent front is approximately 15 cm from the starting line.
5. Remove TLC plate and let solvent evaporate in the fume hood. Then place labeling tape (upright orientation) around the edge of the plate to form a wall and prevent spills from the indicator bacteria-agar mixture.
6. Overlay dried plate with indicator bacterium, as follows:
 - (a) From a fresh 5 ml overnight culture, inoculate 50 ml media and grow to late exponential phase. It is important that steps 6b and c be done quickly as *A. tumefaciens* is sensitive to elevated temperatures.
 - (b) Add this 50 ml culture to 100 ml media plus 1.12 g melted agar plus 10 mg X-gal (500 μ l of 20 mg/ml stock in dimethylformamide), tempered at 45 °C.
 - (c) Prewarm TLC plate by putting on heater (low setting) for approximately 5 min (allows bacteria-agar mixture to

spread evenly over TLC plate). Immediately pour mixture over pre-warmed plate and spread evenly to cover. Layer should be approx. 3 mm thick.

- (d) Allow the agar to solidify, and then place in a plastic tub (avoids dehydration during incubation). Cover and place in a 30 °C incubator for 12–18 h. Check for blue spots. The most hydrophobic (largest) AHLs will be near the origin (bottom of the TLC plate) with the smaller (less hydrophobic) AHLs migrating a longer distance. Sample results are shown in Fig. 5.
- (e) Plastic tub can be disinfected after use with bleach or 70% (v/v) ethanol.

3.3 Biofilm Dispersion Screen

The method listed below is based on the use of a 96-well microtiter polystyrene plate and was adapted from a flow cell assay. Other microtiter plates can be used and colonization substrata can be added (to test for adhesion and release from surfaces in addition to polystyrene). Specific culturing and dispersion conditions will need to be determined empirically.

1. Prior to the screen, plan the arrangement of the cultures and tests on the microtiter plate. (We often use an Excel spreadsheet to plan the distribution of the various tests and controls). Given the 96-well format, one can perform replicates, using different organisms, culture conditions, and candidate-dispersing agents, as well as including controls.
2. Prepare culture inoculum (typically we grow fast-growing organisms such as *P. aeruginosa*, *Staphylococcus aureus*, *Escherichia coli* for 18–24 h in broth culture and use this culture as an inoculum).
3. Partially fill wells on microtiter plate with 200 µl media (assuming that well capacity is 300 µl).
4. Inoculate some (but not all the wells) with culture inoculum (it is very important to have uninoculated controls). We typically use a 1% (v/v) inoculum.
5. Cover the microtiter plate and seal the edge with parafilm.
6. Incubate on an orbital shaker. The swirling will cause biofilms to form along the edges of the wells and is useful for organisms such as *P. aeruginosa* that typically form pellicles at air–liquid interfaces. Incubation time can be determined empirically (typically older biofilms >48-h incubation [48] will detach more readily than younger biofilms).
7. Using a multichannel pipettor, remove the culture (and place into a container where it can be autoclaved for safe disposal).

8. Add 200 μ l of candidate dispersant solution (a buffer such as PBS can be used as a negative control).
9. Place in plate reader (if the plate reader has an agitation capability), then allow that to happen for a short period (1 min or so), and then measure OD₆₀₀. If the plate reader does not have agitation capability, then expose the plate to some type of gentle vibration for a few minutes before measuring OD₆₀₀.
10. Release of biofilm bacteria into the planktonic population will be indicated by an increased OD reading in the respective wells. This increase should be evident within 1–5-min exposure. Increased OD₆₀₀ readings at longer times may reflect the growth of planktonic cultures in the liquid.

4 Notes

1. For long-term storage of *C. violaceum* strains, grow an overnight culture in broth with shaking at 30 °C, then mix 1.2 ml of the overnight strain with 0.4 ml sterile 50% (v/v) glycerol, place into a 2-ml sterile cryogenic storage tube, and store at –80 °C. To revive the frozen culture, simply scrape some cells from the top of the frozen culture onto LB and incubate overnight at 30 °C. For short-term (2–3 days) storage, leave at room temperature (20–25 °C), since *Chromobacterium* strains do not survive very well at 4 °C.
2. We routinely use Si-C18F TLC Plate Reversed Phase Octadecyl, JT Baker (Cat no. 7013-04).
3. Some AHLs can be purchased commercially through companies such as Sigma-Aldrich (St. Louis, MO).
4. We routinely use a TLC glass tank Z204161 from Sigma-Aldrich (17.5 cm × 16.0 cm × 6.2 cm). We have found the *C. violaceum* CVO26 bioassay to respond to low micromole to high nanomole concentrations to its cognate AHL (C6-HSL). This strain is best suited to detecting short-chain AHLs (C4- and C6-HSLs). In contrast, another AHL bioassay strain, *A. tumefaciens* A136 (pCF218)(pCF372) developed by Fuqua and Winans [29], is much more sensitive than CVO26, in that it can detect its cognate AHL (3-o-C8 HSL) in the low-picomole range. *A. tumefaciens* NTL4 (pCF218)(pCF372) has a similar sensitivity as strain A136 (pCF218)(pCF372) [36, 37]. These *A. tumefaciens* strains will detect a wider variety of AHLs with acyl groups ranging in size from 6 carbons to 12 carbons [35]. Zhu and Winans developed an even more sensitive *A. tumefaciens* strain, designated KYC55 (PJZ372) (PJZ384)(PJZ410) [30] capable of detecting long-chain AHLs (C6- to C14-HSLs). Both *A. tumefaciens* biosensors

employ *lacZ* as a reporter gene and require supplementation of X-gal to the medium. A comparison of the sensitivity of the *C. violaceum* CVO26 assay to the aforementioned *A. tumefaciens* bioassays is shown in Fig. 4. Here, the CVO26 assay readily shows the presence of C6-HSL, whereas this AHL is weakly detected by strain KYC55 (PJZ372)(PJZ384)(PJZ410) and not detected by strain A136 (PCF218)(PCF372) (Fig. 3a). In contrast, the two *A. tumefaciens* biosensors readily detect their cognate AHL (3-o-C8-HSL), whereas CVO26 does not.

5. Pigmentation inhibition (Fig. 3d) can give a useful indication of potential QS inhibition. However, the CviR transcriptional activator (LuxR homologue) in *C. violaceum* can be blocked by other AHLs (Fig. 4c) [33] and so identification of inhibitory substances should not be made on the basis of a *C. violaceum* bioassay alone. We recommend that potential QS inhibitors be screened for AHLs with a broad-range bioassay system such as the *A. tumefaciens* reporter strains mentioned in Subheading 2.2 (above).
6. If using a solvent to extract AHLs (typically acidified ethyl acetate) or potential QS-inhibiting compound (usually an organic solvent such as acetone), test the solvent on the bioassay strain to ensure that any pigmentation or viability alteration due to the solvent is measured. As a safety matter, wear gloves (we recommend nitrile gloves) and perform any solvent-based studies in a fume hood.

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Chapter 2

Detection of 2-Alkyl-4-Quinolones Using Biosensors

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Abstract

2-Alkyl-4-quinolones (AQs) such as 2-heptyl-3-hydroxy-4-quinolone (PQS) and 2-heptyl-4-hydroxyquinoline (HHQ) are quorum-sensing signal molecules. Here we describe two methods for AQ detection and quantification that employ thin-layer chromatography (TLC) and microtiter plate assays in combination with a *lux*-based *Pseudomonas aeruginosa* AQ biosensor strain. For TLC detection, organic solvent extracts of bacterial cells or spent culture supernatants are chromatographed on TLC plates, which are then dried and overlaid with the AQ biosensor. After detection by the bioreporter, AQs appear as both luminescent and green (from pyocyanin) spots. For the microtiter assay, either spent bacterial culture supernatants or extracts are added to a growth medium containing the AQ biosensor. Light output by the bioreporter correlates with the AQ content of the sample. The assays described are simple to perform, do not require sophisticated instrumentation, and are highly amenable to screening large numbers of bacterial samples.

Key words *Pseudomonas aeruginosa*, Biosensor, 2-Alkyl-4-quinolones, *Pseudomonas* quinolone signal (PQS), 2-Heptyl-4-hydroxyquinoline (HHQ), *pqsA*

1 Introduction

In *Pseudomonas aeruginosa*, cell-cell communication (quorum sensing, QS) controls the production of multiple virulence factors and secondary metabolites and promotes biofilm maturation. The QS system consists of two *N*-acylhomoserine lactone (AHL) regulatory circuits (*las* and *rhl*), linked to a 2-alkyl-4-quinolone (AQ) system [1]. In the *las* system, the *lasI* gene product directs the synthesis of *N*-(3-oxo-dodecanoyl)-L-homoserine lactone (3-oxo-C₁₂-HSL), which interacts with the transcriptional regulator LasR and activates target promoters. In the *rhl* system, RhlII directs the synthesis of *N*-(butanoyl)-L-homoserine lactone (C₄-HSL), which interacts with the cognate regulator RhlR and activates target gene promoters. The *las* and *rhl* systems are hierarchically connected and regulate the timing and production of multiple virulence factors including elastase, alkaline protease, exotoxin A,

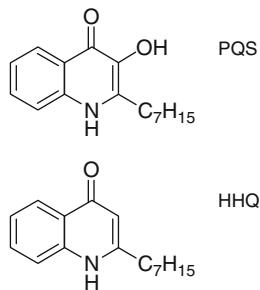


Fig. 1 Structures of the AQ signal molecules; PQS and HHQ

rhamnolipids, pyocyanin, lectins, superoxide dismutases, and biofilm development [1].

Pesci et al. [2] demonstrated that addition of a spent culture medium extract from a *P. aeruginosa* wild type (PAO1) caused induction of *lasB* (elastase) expression in a PAO1 AHL-deficient *lasR* mutant [2]. This data suggested that a non-AHL signal produced by the bacterium was capable of activating *lasB* expression which required LasR and 3-oxo-C₁₂-HSL for its biosynthesis. It was also shown that the novel signal required a functional *rhl* system for its bioactivity since *lasB* could not be activated in a *rhlII/rhlR* double mutant by PAO1 spent culture extracts. The molecule responsible for the non-AHL-mediated QS signaling pathway was purified and chemically identified as 2-heptyl-3-hydroxy-4-quinolone and termed the *Pseudomonas* quinolone signal (PQS) (Fig. 1) [2]. PQS belongs to the AQ family of compounds, which were first chemically identified in the 1940s and studied for their antibacterial properties. In addition to PQS, other AQ molecules produced by *P. aeruginosa* include 2-heptyl-4-hydroxyquinoline (HHQ) (Fig. 1), 2-nonyl-4-hydroxyquinoline (NHQ), and 2-heptyl-4-quinolone N-oxide (HQNO) [3]. PQS and HHQ enhance their own biosynthesis by binding to the cognate transcriptional regulator protein, PqsR, to activate the expression of the AQ biosynthetic operon, *pqsACBDE* [4, 5], that is responsible for producing over 50 AQ congeners in conjunction with the *pqsH* and *pqsL* gene products. PqsH is a mono-oxygenase that converts 2-alkyl-4-quinolones such as HHQ into 2-alkyl-3-hydroxy-4-quinolones such as PQS while PqsL is required for the biosynthesis of N-oxides such as HQNO [3].

In *P. aeruginosa*, the AQ signaling pathway plays a role in the regulation of production of several virulence factors including the blue-green pigment pyocyanin, rhamnolipids, and lectin A and is involved in extracellular DNA release during biofilm development [6, 7]. AQs are also produced by other bacterial genera including *Burkholderia pseudomallei*, the causative organism of melioidosis [8], suggesting that AQ signaling may be more widespread than previously thought.

For the detection and unequivocal chemical identification of AQs, both NMR (Nuclear Magnetic Resonance) spectroscopy and LC-MS (Liquid Chromatography-Mass Spectrometry) have been used [9–11]. These methods are extremely sensitive but rely on access to expensive instrumentation and require considerable expertise. A simple alternative is to use thin-layer chromatography (TLC) to detect AQs and previously TLC assays have been used to detect PQS under UV light [6, 12, 13]. However, this method is not sufficiently discriminatory in complex bacterial supernatants that contain multiple fluorescent compounds and is not particularly suitable for 2-alkyl-4-quinolones such as HHQ, the fluorescence of which is much weaker than PQS under UV light. A third option is to use a specific AQ biosensor [14–16] as described in this chapter.

2 Materials

2.1 Bacterial Bioreporters, Growth Media, and AQ Compounds

1. The bioreporter used in this assay is PAO1 $\Delta pqsA$ CTX-*lux*::*pqsA* [16]. This strain is *P. aeruginosa* PAO1 with a chromosomal deletion in the *pqsA* gene. It is AQ negative but responds via light and pyocyanin production to the presence of AQs such as PQS and HHQ. The reporter gene construct in this strain is the *pqsA* promoter (which is sensitive to both PQS and HHQ) fused to the CTX-*luxCDABE* cassette [17]. This construct was conjugated into PAO1 $\Delta pqsA$, resulting in a stable, chromosomal single-copy reporter. The strain can be maintained in tetracycline 125 µg/ml and stored at –80 °C in 25% (vol/vol) glycerol.
2. PQS standard, 10 mM in methanol (PQS MW 259).
3. HHQ standard, 10 mM in methanol (HHQ MW 243).
4. Lysogeny broth (LB): 1% (wt/vol) bacto-tryptone, 0.5% (wt/vol) yeast extract, 1% (wt/vol) sodium chloride in distilled water.
5. LB agar: Lysogeny broth with the addition of 2% (wt/vol) agar technical No. 3.
6. Tetracycline, 50 mg/ml in methanol.

2.2 Extraction of AQs

1. Potassium phosphate monobasic (KH_2PO_4).
2. Methanol—HPLC gradient grade.
3. Ethyl acetate—HPLC gradient grade.
4. Acetone—HPLC gradient grade.
5. Dichloromethane—HPLC gradient grade.
6. Glacial acetic acid—analytical grade.
7. Rotary evaporator or centrifugal evaporator.

2.3 Detection of AQs Using TLC

1. Normal-phase 20 × 20 cm silica 60_{F254} TLC plates.
2. TLC developing tank.
3. Soft top agar: 0.65% (wt/vol) agar technical No. 3, 1% (wt/vol) tryptone, 0.5% (wt/vol) sodium chloride.
4. UV transilluminator (312 nm).
5. Luminograph photon video camera.
6. X-ray film.

2.4 Detection of AQs Using Microtiter Plates

1. 96-Well plate spectrophotometer/luminometer.
2. 96-Well, white or black, clear bottom microtiter plates.

3 Methods

3.1 Preparing Bacterial Cultures for AQ Extraction

1. Under sterile conditions, streak out a 10 µl loop of the test bacterium, *P. aeruginosa* PAO1 (AQ positive control), the AQ-negative mutant PAO1 *pqsA* (AQ-negative control) and the PQS-negative (but HHQ positive) PAO1 *pqsH* (PQS-negative control) onto fresh LB agar plates. Grow overnight at 37 °C with any appropriate antibiotics.
2. On the following day, inoculate 5 ml of LB medium containing appropriate antibiotics with single colonies of the relevant strains. Grow the cultures overnight at 37 °C with shaking at 200 rpm. It is not essential that LB medium is used for this stage; any appropriate growth medium can be used, although *P. aeruginosa* strains produce high concentrations of AQs in LB.
3. The following day, the optical densities (OD) of the cultures should be measured at 600 nm (OD₆₀₀). Using these readings, standardize the cultures to OD 1.0 by diluting with the growth medium used in step 2.
4. Transfer 0.25 ml of standardized culture into 250 ml Erlenmeyer flasks containing 25 ml LB broth (or alternative growth medium). A small volume of culture in a large flask allows good aeration of the medium. Incubate at 37 °C, with shaking at 200 rpm for 8 h (see Note 1).

3.2 AQ Extraction of Bacterial Cultures

1. Transfer a defined volume of each culture (in this example 10 ml) to a 50 ml centrifuge tube and centrifuge at 10,000 × *g* for 10 min. Extract the cells (see step 2) or the supernatant (see step 3). It is possible to carry out both procedures together.
2. For extraction of AQs from cells, centrifuge the culture at 10,000 × *g* for 10 min, remove the supernatant (save if required for supernatant extraction), and resuspend the cells

in 10 ml of fresh LB or relevant medium. Centrifuge again at $10,000 \times g$ for 10 min and discard the supernatant. Repeat the media wash steps twice to remove all traces of the supernatant AQs from the cells. Add 10 ml of methanol to the cell pellet and vortex until fully resuspended. Allow to stand for 10 min to allow the cells to lyse before centrifuging again at $10,000 \times g$ for 10 min. Filter the extract through sterile 0.2 μm filters into clean centrifuge tubes to remove all cell debris from the extraction mixtures. At this stage it is possible to store the cell extractions in the freezer at -20°C for several days if required.

3. For supernatant extraction, centrifuge the culture at $10,000 \times g$ for 10 min and filter the supernatants through sterile 0.2 μm filters into clean centrifuge tubes to remove any cells. Add 10 ml of acidified ethyl acetate (glacial acetic acid 0.01% (vol/vol) in ethyl acetate) to the supernatant and vortex for 30 s so the two phases are well mixed. Transfer the extraction mixtures into a separating funnel that has been previously washed with acetone and allow the extraction mixtures to settle and the two phases to separate. Transfer the top organic layer into a fresh centrifuge tube. Repeat the extraction procedure on the bottom layer twice before discarding. Pool the collected organic layers. If time is limiting, supernatant extraction mixtures can be stored in the freezer at -20°C for several days.
4. For the cell and supernatant procedures, evaporate the mixtures to dryness, e.g., using a centrifugal evaporator or a rotary evaporator (transfer the extraction mixtures to 50 ml round-bottom flasks that have been previously washed with acetone).
5. Add 0.5 ml of methanol to the round-bottom flasks and agitate for 30 s before transferring the liquid to 2 ml glass sample vials. Repeat this step with two further additions of 0.5 ml methanol and pool each sample in each vial. If time is limiting, both cell and supernatant extraction mixtures can be stored in the freezer at -20°C for several days.
6. Dry down the extraction mixtures in the sample vials using, e.g., a centrifugal evaporator or under a stream of nitrogen gas. Dry cell and supernatant extraction residues can be stored in the freezer at -20°C for several months.

3.3 Preparation of TLC Plates and Running of Samples

1. Prepare normal-phase silica $20 \times 20 \text{ cm } 60_{\text{F}254}$ TLC plates (*see Note 2*) by soaking in a 5% (wt/vol) solution of KH_2PO_4 for 30 min before activating at approximately 100°C for 1 h, e.g. in a hybridization oven.
2. Draw a faint line in pencil approximately 2.5 cm from the bottom of the silica TLC plate to use as a guide for spotting sample extracts.

3. Reconstitute sample extracts in 100 μ l of methanol and spot 5 μ l of each onto the TLC plate. The amount spotted onto the TLC depends on the concentration of AQs in the sample. *P. aeruginosa* produces high amounts of AQs and in this case 5 μ l is a good starting quantity. As positive controls, 2 μ l of 10 mM stock solutions of PQS and HHQ (or other AQs) in methanol can be spotted onto the TLC plate. Space each spot at 2 cm intervals along the line. A hairdryer can be used to dry samples during spotting to give a tighter spot.
4. When dry, place the TLC plate into a developing tank and run the TLC using a mixture of dichloromethane:methanol (95:5) as the mobile phase until the solvent front is 1–2 cm from the top of the plate. The TLC plate can be visualized using a UV transilluminator at 312 nm and photographed at this point (Fig. 3a).
5. Allow the TLC plate to dry and apply autoclave tape to both the underside of the TLC plate and around the edges so that the tape creates a well at least 0.5 cm deep around the TLC plate into which the nutrient agar containing the biosensor will be poured. Make sure that the autoclave tape is firmly pressed down and forms a tight seal (Fig. 2).

3.4 Overlay of TLC Plates and Detection of AQs Using a Bioreporter

1. Streak out a 10 μ l loop of the AQ biosensor (PAO1 $\Delta pqsA$ CTX-lux::*pqsA*) [16] onto fresh LB agar plates containing tetracycline 125 μ g/ml and grow overnight at 37 °C.
2. The following day, inoculate a single colony into 5 ml LB medium containing tetracycline 125 μ g/ml and grow overnight at 37 °C with shaking at 200 rpm.
3. The following day, gently melt 100 ml of soft top agar in a microwave and allow to cool to approximately 50 °C. Add 1 ml of the overnight culture to the soft top agar and mix gently (see Note 3).
4. Pour the agar mixture slowly into the well made around the TLC plate, being careful to minimize bubble formation in the agar and on the TLC plate (see Note 4).
5. Allow the agar to solidify around a Bunsen flame (to help keep sterile) and then incubate the plate at 37 °C for 6–8 h to view light production, or overnight to view pyocyanin production. Visualize the plates for light production using a luminograph photon video camera (or similar) (Fig. 3b) or develop using X-ray film. Alternatively simply view production of the blue/green phenazine pigment pyocyanin by eye (Fig. 3c) (see Note 5).

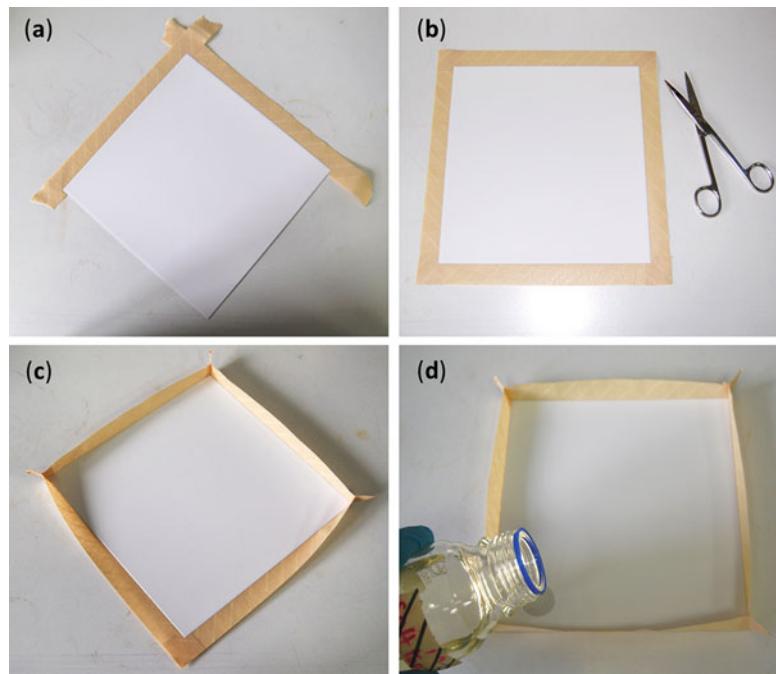


Fig. 2 Preparation of the biosensor TLC plate agar overlay. **(a)** Attach a strip of autoclave tape to the aluminum backing along each edge of the TLC plate, pressing down firmly to ensure a good seal. **(b)** Neatly trim the excess autoclave tape using scissors. **(c)** Pinch the autoclave tape at each corner of the TLC plate, creating a barrier of autoclave tape along each side of the TLC plate to create a well. **(d)** Place the TLC plate into an appropriate dish and pour into the well the molten soft top agar containing the biosensor bacteria

3.5 Testing for AQ Production Using a Microtiter Plate Assay

1. Prepare crude bacterial culture supernatants by growing the test bacterium as described in Subheading 3.1.
2. Remove 5 ml of culture and spin at $10,000 \times g$ for 5 min before collecting the supernatant and passing it through a sterile 0.2 μm filter into clean tubes (*see Note 6*). If time is limiting, this supernatant extract can be frozen at -20°C for a few days.
3. Grow the AQ biosensor overnight and dilute with LB medium to OD_{600} 1.0. Further dilute this standardized biosensor culture with LB medium to give (a) 1 in 50 and (b) 1 in 100 dilutions.
4. Take a sterile 96-well plate (*see Note 7*) and for each well mix 100 μl of the sterile test bacterial supernatant with 100 μl of the 1 in 50 dilution of the biosensor to give a final culture dilution of 1 in 100. For each negative control well, add 200 μl of the 1 in 100 dilution of the AQ biosensor. A positive control well containing a PQS or a HHQ standard at a concentration of 10 μM can be added or alternatively a *P. aeruginosa* wild-type culture supernatant (100 μl) can also be included in a well during the assay.

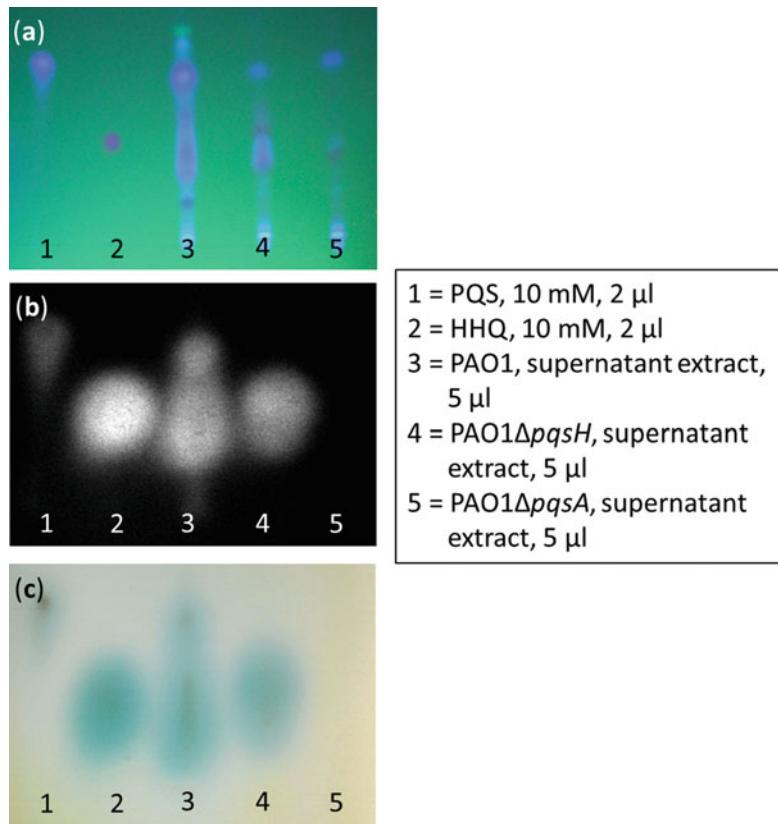


Fig. 3 TLC assay for AQS. **(a)** TLC plate run with standards of PQS and HHQ and supernatant extracts of PAO1, PAO1 $\Delta pqsH$, and PAO1 $\Delta pqsA$ visualized under UV at 312 nm. **(b)** Overlay of TLC plate with soft top agar containing biosensor bacteria showing the production of light in response to AQS visualized using a luminograph photon video camera. **(c)** Overlay of TLC plate with soft top agar containing the biosensor bacteria showing production of the blue/green pigment pyocyanin, in response to AQS. TLC lanes: (1) PQS 10 mM, 2 μ l; (2) HHQ 10 mM, 2 μ l; (3) PAO1 supernatant extract, 5 μ l; (4) PAO1 $\Delta pqsH$ supernatant extract, 5 μ l; (5) PAO1 $\Delta pqsA$ supernatant extract, 5 μ l. The AQ biosensor emits light over spots identified as PQS and HHQ in PAO1 and HHQ only in PAO1 $\Delta pqsH$. Light spots are absent for the AQ-negative $pqsA$ mutant

5. Monitor bioluminescence and OD at 37 °C using a combined spectrophotometer/luminometer. This measures OD and bioluminescence from all wells every 30 min for 24 h. Luminescence is recorded as relative light units (RLU) per unit of OD. If an automated combined spectrophotometer/luminometer is unavailable, readings can be taken manually at defined time points by growing the bacterial cultures under specific conditions and measuring the OD and bioluminescence of culture samples using a spectrophotometer and tube luminometer, respectively.

4 Notes

1. Growth of *P. aeruginosa* cultures for 8 h in LB medium results in the bacteria reaching mid-stationary phase, which is sufficient for high quantities of AQs to be produced. If using an alternative growth medium, the time of incubation may have to be altered to account for growth rate differences. We recommend that stationary-phase cells be used when attempting to detect AQs produced by bacterial species.
2. We routinely use normal-phase 20 × 20 cm silica 60_{F254} TLC plates from Merck.
3. Make sure that the agar has cooled sufficiently before adding the bacterial reporter strain. Addition at too high a temperature will attenuate bacterial growth.
4. Pour the agar promptly or it will begin to solidify. Bubbles can be removed by gently passing a Bunsen burner flame over the surface of the agar.
5. Both PQS and HHQ will activate light production in the reporter, as both control the expression of the *pqsA* gene. In addition, both PQS and HHQ activate the production of pyocyanin.
6. In addition to cell-free culture supernatants, the solvent-extracted culture extracts described in Subheading 3.2 may also be analyzed via this method. Simply dilute 5 µl of the solvent extract in 100 µl of LB and add to 100 µl of 1 in 50 dilution of the AQ biosensor per well.
7. Specialized 96-well white or black plates need to be used when monitoring bioluminescence. The plates should not be clear-sided to reduce light scatter between wells but should have clear plastic bottoms so that an automated spectrophotometer/luminometer can detect and measure both light output and absorbance accurately.

Acknowledgements

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Chapter 3

“Hot Stuff”: The Many Uses of a Radiolabel Assay in Detecting Acyl-Homoserine Lactone Quorum-Sensing Signals

Amy L. Schaefer, Caroline S. Harwood, and E. Peter Greenberg

Abstract

Many *Proteobacteria* synthesize acyl-homoserine lactone (AHL) molecules for use as signals in cell density-dependent gene regulation known as quorum sensing (QS) and response. AHL detection protocols are essential to QS researchers and several techniques are available, including a ^{14}C -AHL radiolabel assay. This assay is based on the uptake of radiolabeled methionine by living cells and conversion of the radiolabel into *S*-adenosylmethionine (SAM). The radiolabeled SAM is then incorporated into AHL signal by an AHL synthase enzyme. Here we describe a methodology to perform the AHL radiolabel assay, which is unbiased, relatively fast, and very sensitive compared to other AHL detection protocols.

Key words ^{14}C -carboxy-methionine, Quorum sensing, QS, LuxI, Acyl-homoserine lactone, AHL, Acyl-HSL

1 Introduction

1.1 Quorum Sensing Overview

It is now appreciated that bacteria are able to sense one another and coordinate their group activities. Many bacteria monitor their population densities, and change their gene expression patterns as appropriate, using a process known as quorum sensing and response [1, 2]. Over 100 species of *Proteobacteria* synthesize small diffusible chemicals, originally named “autoinducers” but now called “quorum-sensing (QS) signals,” to mediate cell density-dependent gene regulation. The QS signal accumulates in the surrounding medium during growth. Because QS signals are free to diffuse into and out of the cytoplasm into the environment [3, 4], environmental and cellular concentrations are equal. At high bacterial populations, a QS signal can accumulate to high levels and once a threshold concentration is reached, the QS signal interacts with a cognate receptor protein. Once the receptor protein is

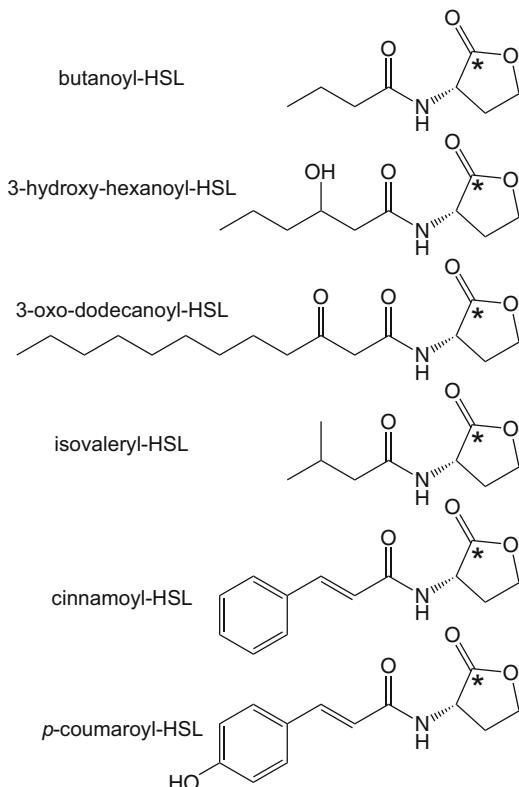


Fig. 1 Examples of AHL QS signal structures. The *top three* compounds are representative of the “typical” AHL molecule, which has a side chain derived from fatty acid biosynthesis (acyl-acyl carrier protein substrates). The *bottom three* compounds are the more recently discovered AHL signals derived from branched chain amino acid biosynthesis (isovaleryl-HSL) and aromatic acid degradation (cinnamoyl-HSL, *p*-coumaroyl-HSL), which utilize acyl-coenzyme A (CoA)-linked substrates [6, 9]. The asterisk indicates the location of the ^{14}C -label incorporated in the ^{14}C -AHL during the radiolabel assay protocol described in this chapter

bound by QS signal, it controls gene expression as activator or repressor, depending upon the particular system [1, 2].

Two genes are required for such QS systems in *Proteobacteria*: *luxR*- and *luxI*-type genes. The *luxR*-encoded proteins (R proteins) are the cognate receptors for the QS signals and function as transcriptional regulators. The *luxI* homologs encode AHL QS signal synthases (I proteins), which use the common metabolites S-adenosylmethionine (SAM) and organic acids activated via acyl carrier protein (ACP)- or coenzyme A (CoA)-linkage [5–10] as AHL substrates (Figs. 1 and 2). Most of the AHLs described have acyl side chains comprised of fatty acyl groups of varying carbon lengths [4–18] and substitutions (ACP derived), but more recently AHL signals comprised of aromatic acid [9, 11] and branched amino acid [6] side chains (CoA derived) have been discovered (Fig. 1).

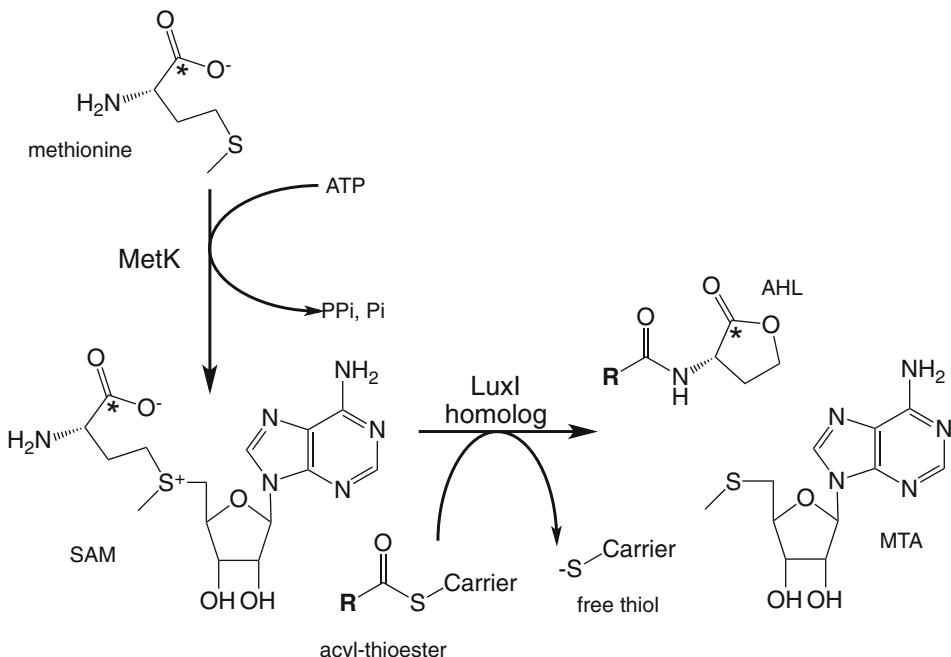


Fig. 2 Pathway illustrating the flow of radiolabel into AHL from methionine, via S-adenosylmethionine (SAM). The radiolabeled carbon is indicated by asterisk. Bacteria convert some labeled methionine to SAM via the enzymatic action of MetK, a SAM synthetase. A portion of the ^{14}C -SAM is used by a LuxI-type AHL synthase as substrate for AHL synthesis. The acyl-thioester carrier is either acyl carrier protein or coenzyme A, depending upon the LuxI enzyme. MTA is the coproduct 5'-methylthioadenosine

1.2 Techniques to Detect and Identify AHL Compounds

A variety of protocols have been developed to detect and quantify AHL signals: bioassays [12], enzyme-linked immunosorbent assay (ELISA) [13], mass spectrometry [14], and radiolabel assay [15, 16] described here. Each assay has strengths and weaknesses, and in practice all of these techniques are used in some combination by the field. Detailed descriptions of AHL detection by bioassay (Chapter 1), mass spectrometry (Chapter 4), and ELISA (Chapter 5) are provided elsewhere in this volume, but we briefly discuss these techniques here in order to better understand the relative benefits (and limitations) of the radiolabel assay.

Most often, AHLs are detected using a series of bioassay reporter strains. These strains contain a LuxR-type regulator and a gene promoter fusion that requires R protein bound to an AHL signal for expression (for examples see Chapter 1 and refs. 6, 9, 12, 17). Bioassays can quantitate the total AHL levels present in a sample if a standard curve is generated using known amounts of purified AHL compound. The simple-to-perform and inexpensive bioassay reporters are important tools for the QS field, but they do have limitations. Each reporter responds only to a subset of known AHLs; therefore multiple reporters must be employed to screen for an undefined AHL signal. Also when the nonfatty acyl-linked AHLs

were first identified, the *p*-coumaroyl- and isovaleryl-linked AHLs (Fig. 1), they were not detected by the existing repertoire of bioassay reporters [6, 9]. There are also examples of non-HSL compounds activating reporter strains [18, 19], which could confound results. Additionally, bioassays are sensitive to inhibition by other compounds sometimes present in sample extracts (e.g., we had difficulties detecting AHLs in cystic fibrosis patient sputum extracts using bioassays, but not using the radiolabel assay described here [15]).

Mass spectrometry (MS) analyses are essential to QS research (see Chapter 4), especially with regard to structural elucidation of undefined AHL compounds, and can be quantitative when an internal deuterated AHL standard is included [14]. However, not all laboratories have ready access to a mass spectrometer and many of the MS techniques rely on the presence of a fragment ion at ($m/z + H$) 102 (corresponding to aminobutyrolactone), which is observed in most, but not all [9], AHLs. Recently ELISA techniques (see Chapter 5) have been used to detect acyl-HSLs, but require custom monoclonal antibodies and cannot differentiate between closed HSL ring compounds (acyl-HSL, active QS signal) and lactonized ones (acyl-homoserine, inactive QS signal) [13].

The ^{14}C -radiolabel assay detailed here is similar to that first described by Eberhard et al. [20]. AHL-synthesizing bacteria are fed radiolabeled L-[1- ^{14}C]-methionine (^{14}C -met), which is transported into the cell, and a portion is converted into SAM via enzymes like the *Escherichia coli* MetK SAM synthetase. The ^{14}C -SAM is used by LuxI-type synthases as the substrate for the conserved HSL ring in the AHL QS signal (diagrammed in Fig. 2). ^{14}C -AHLs are solvent extracted from cell-free supernatants, separated by HPLC, and radioactivity in the HPLC fractions is measured by liquid scintillation counting. Because all LuxI-synthesized QS signals utilize SAM as the HSL ring substrate, all HSL-type signals (including novel AHL structures) are detected by the radiolabel technique so long as the bacterium tested is capable of assimilating exogenous ^{14}C -met. The presence of a single radiolabeled carbon in the HSL ring, regardless of side-chain moiety, allows for easy comparison of relative HSL compounds in a single sample (Fig. 3 illustrates the detection of both major and minor AHL compounds present in *Pseudomonas* cultures). It is possible that some bacteria could incorporate ^{14}C -met into other non-AHL, extracellular, solvent-extractable compounds; however, we have not found this to be the case for the dozens of *Proteobacteria* species we have tested using the radiolabel assay.

Although some labs prefer to avoid using radioactivity due to licensing and disposal cost issues, the radiolabel protocol for monitoring AHL production is a powerful technique that shows no bias with regard to AHL side chain and is faster and more sensitive than many traditional bioassays. Variations of this protocol have been

applied in many ways: to detect novel AHL signals [6, 11]; to detect AHL production *in situ* for complex environments such as cystic fibrosis patient sputum and biofilm reactors [15, 16]; to generate significant amounts of radiolabeled AHL compound [21]; to test for positive autoregulation of AHL synthase genes [20, 22]; to study AHL synthesis patterns during bacterial growth [23]; to identify preferred side-chain substrates [11]; and to assess inhibitors of AHL synthesis in whole bacterial cells [24]. Here we provide detailed protocols for AHL detection during bacterial growth and identification of preferred side-chain substrates using the radiolabel assay.

2 Materials

2.1 ^{14}C -Labeling of AHLs During Bacterial Growth

1. L-[1- ^{14}C]-methionine in sterile water (50–60 mCi/mmol, *see Notes 1 and 2*).
2. Bacterium of interest (*see Note 3*).
3. Methionine-free medium appropriate for growing your bacterium of interest (*see Notes 4 and 5*).
4. (Optional, for aromatic substrate specificity assay) Mixture of aromatic compounds at a stock concentration of 0.1 M (*see Note 6*) or other naturally occurring mixtures (*see Note 7*).

2.2 Extraction and Separation of ^{14}C -AHLs

1. Acidified ethyl acetate (0.1 ml glacial acetic acid per 1 l ethyl acetate).
2. HPLC-grade methanol and water solvents.
3. 1.5-ml Plastic snapcap tubes.
4. 15-ml Plastic centrifuge tubes (ethyl acetate resistant).
5. 3-ml Polyethylene transfer pipettes (ethyl acetate resistant).
6. N-evap nitrogen evaporator; alternatively incubate your extract-containing tubes in a warm water bath without cap in a fume hood until solvent has evaporated.
7. High-pressure liquid chromatography (HPLC) system (200 μl loading loop) using a C₁₈-reverse-phase HPLC column.
8. Fraction collector outfitted with solvent-resistant, 7-ml polyethylene scintillation vials.
9. AHL standards or defined AHL-positive bacteria (*see Note 8*).

2.3 Detection of ^{14}C -AHLs

1. Scintillation counting cocktail (*see Note 9*).
2. Liquid scintillation counter.
3. (Alternative) Some laboratories have an in-line scintillation detector, which can be used in lieu of fraction collection and liquid scintillation counting.

3 Methods

3.1 Protocol for AHL Detection During Bacterial Growth

This is a general protocol for ^{14}C -radiolabeling of AHL signals during bacteria growth, but it should be modified according to the needs of the particular strain as noted below. To illustrate the range of labeling times and ^{14}C -HSL production among strains, we performed the radiolabel assay using three different bacteria (Fig. 3, see Note 4): *Pseudomonas aeruginosa* PAO1 [25], *Pseudomonas chlororaphis* GM17 [26], and *Bradyrhizobium japonicum* USDA110 [27].

1. Grow your bacterium of interest in a small volume (usually 5 ml) of methionine-free medium with the appropriate growth conditions (e.g., temperature, aerobic with shaking, anaerobic, photosynthetic) until the culture reaches desired density, usually late logarithmic phase or early stationary phase (see Note 10).

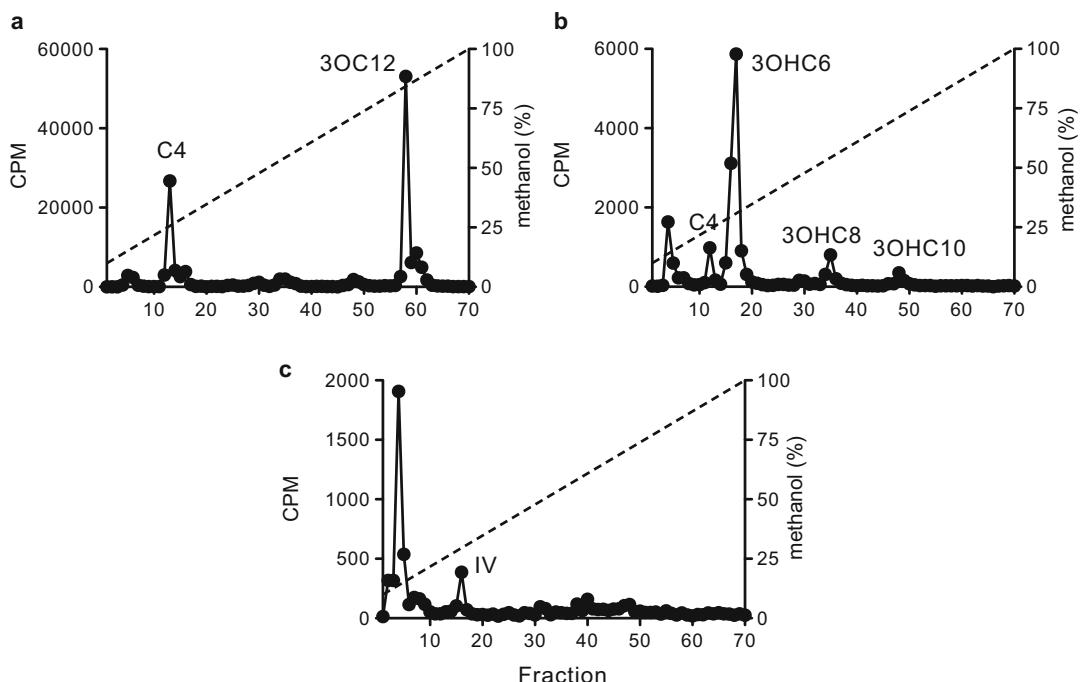


Fig. 3 HPLC profiles of ^{14}C -AHLs synthesized by (a) *Pseudomonas aeruginosa* [25] (labeled for 30 min), (b) *Pseudomonas chlororaphis* GM17 [26] (labeled for 2 h), and (c) *Bradyrhizobium japonicum* USDA110 [27] (labeled for 1 day). In all graphs the x-axis indicates the fraction numbers that were collected over a 10–100% methanol-in-water gradient. The left y-axis denotes the counts per minute (CPM) of radiolabel in each fraction (black circles) and the right y-axis indicates the methanol concentration of the HPLC run (dashed line). The synthetic AHL compound that co-elutes with the observed radiolabel peak is abbreviated as follows: butanoyl-HSL (C4), 3-oxo-dodecanoyl-HSL (3OC12), 3-hydroxy-hexanoyl-HSL (3OHC6), 3-hydroxy-octanoyl-HSL (3OHC8), 3-hydroxy-decanoyl-HSL (3OHC10), and isovaleryl-HSL (IV). Radioactivity that eluted in the column void volume (fraction 4) is presumed to be unincorporated methionine

2. Radiolabel cells by adding 5 μ Ci (~90 nmol, *see Note 1*) of [$1\text{-}^{14}\text{C}$]-methionine (^{14}C -met) to the culture for an appropriate labeling time. The radiolabeling duration can vary greatly, from minutes to days, depending on the organism and growth conditions. For the experiments in Fig. 3 shorter labeling times were used for the fast-growing bacteria *P. aeruginosa* (30 min) and *P. chlororaphis* (2 h), while a longer labeling time was required for the slow-growing *B. japonicum* (1 day).

You can test whether the labeling incubation time is sufficient by monitoring the amount of ^{14}C -met incorporated into the cell pellet: (1) sample a small amount (50 μ l) of culture and aliquot to a 1.5-ml snapcap tube; (2) determine the amount of radioactivity in culture by pipetting a 5 μ l sample into a scintillation vial containing 4 ml of scintillation cocktail fluid and then count ^{14}C -radioactivity using a liquid scintillation detector; (3) take the remaining 45 μ l of culture and pellet the cells by centrifugation, aliquot 5 μ l of the cell-free supernatant to a scintillation vial containing 4 ml of scintillation cocktail fluid, and count ^{14}C -radioactivity using a liquid scintillation detector; and (4) compare ^{14}C -counts from the cell-free supernatant vs. the total culture to estimate the amount of radioactivity incorporated into cell material. If ~10% (or more) of the ^{14}C -counts are associated with the cell pellet, this is usually sufficient for a successful radiolabel assay. However, longer incubation times typically yield increased ^{14}C -AHL product. This step also confirms that your organism is capable of ^{14}C -methionine transport (*see Note 11*).

3. After labeling for a sufficient time (determined as described in the previous step), you are ready to extract the ^{14}C -AHLs from the bacterial culture. Pellet cells by centrifugation and transfer the cell-free supernatant to a solvent-resistant tube using a 3-ml polypropylene transfer pipette. Extract the supernatant twice with equal volumes of acidified ethyl acetate (EtAc), and collect and combine both organic phases.
4. EtAc solvent is evaporated and AHLs dried under a gentle stream of N_2 gas with warming (using N-evap nitrogen evaporator or similar). Resuspend the dried sample in 100 μ l of 50% methanol-in-water.
5. Separate the 100- μ l sample by HPLC on a C₁₈ reverse-phase column by using a 10–100% (vol/vol) methanol in water gradient (1 ml/min flow, 70-min profile). Collect 70 1-ml (1-min) fractions in scintillation vials using an automated fraction collector.
6. Add 4 ml of scintillation cocktail to each fraction and count with a scintillation detector. AHL assignment is determined by co-elution with known AHL standards. As illustrated

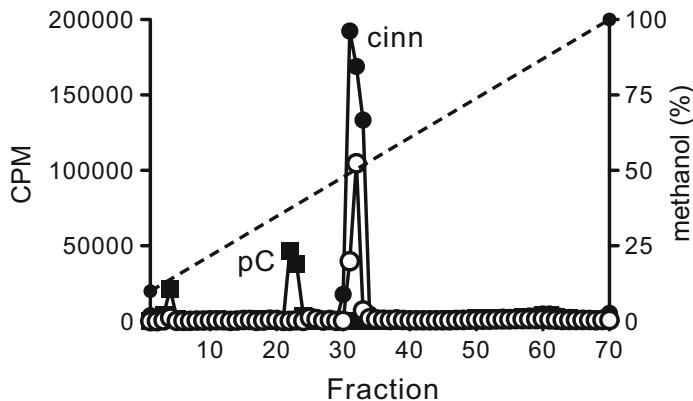


Fig. 4 HPLC profiles of ^{14}C -AHLs synthesized by AHL synthases when grown in the presence of potential substrates added exogenously. Cells expressing the LuxI-type AHL synthases from either *Rhodopseudomonas palustris* CGA009 [9] (Rpal, squares) or *Bradyrhizobium* ORS278 [11] (Bral, circles) were grown in the presence of a mixture of 13 aromatic acids (black-filled shapes) or cinnamic acid only (white-filled shapes) (see Note 13). The x-axis indicates the fraction numbers that were collected over a 10–100% methanol-in-water gradient. The left y-axis denotes the counts per minute (CPM) of radiolabel in each fraction (circles or squares) and the right y-axis indicates the methanol concentration of the HPLC run (dashed line). The synthetic AHL compound that co-elutes with the observed radiolabeled peak is abbreviated as follows: *p*-coumaroyl-HSL (pC) and cinnamoyl-HSL (cinn). Radioactivity that eluted in the column void volume (fraction 4) is presumed to be unincorporated methionine

in Figs. 3 and 4, the amount of ^{14}C -AHL produced can vary by ~10,000-fold depending upon the bacterium, labeling time, and experimental setup (see Note 12).

3.2 Protocol for Aromatic Substrate Specificity Assay

Some bacteria, such as *Rhodopseudomonas* [9] and *Bradyrhizobium* [11] species, utilize exogenous aromatic substrates as the AHL side chain (Fig. 1). To screen for the preferred aromatic substrate of a particular LuxI homolog, the radiolabel assay can be performed using cells grown in the presence of aromatic acid mixtures. This allows the AHL synthase enzyme to select its preferred side-chain substrate [11]. To illustrate this technique here (Fig. 4), we used a heterologous host (see Note 13) to express the AHL synthase from either *R. palustris* CGA009 (RpaI) or *Bradyrhizobium* sp. ORS278 (BraI).

1. Grow the bacterium in a small volume (usually 5 ml) of methionine-free, minimal medium with the appropriate growth conditions (e.g., temperature, aerobic with shaking, anaerobic, photosynthetic) until the appropriate culture density is reached (mid-logarithmic phase, A_{660} of 0.5 for the example shown in Fig. 4).

2. Dilute the culture (~1:5) in fresh medium. For each experimental comparison condition (e.g., no addition, aromatic acid mixture addition) use 5 ml of freshly diluted culture in a 15-ml plastic tube and then add the potential substrates to be tested to the culture (*see Note 7*). For the experiments represented in Fig. 4, we added either a mixture of 13 aromatic acids (*see Note 6*) or cinnamate alone (0.1 mM final concentrations).
3. Radiolabel cells by adding 5 µCi (~90 nmol, *see Note 1*) of [$1\text{-}^{14}\text{C}$]-methionine (^{14}C -met) to the culture for an appropriate labeling time. As described above, the labeling duration can vary greatly depending on the bacterium and growth conditions. For the experiments in Fig. 4 we radiolabeled cells for 20 h.

You can test whether the labeling incubation time is sufficient by monitoring the amount of ^{14}C -met incorporated into the cell pellet: (1) sample a small amount (50 µl) of culture and aliquot to a 1.5-ml snapcap tube; (2) determine the amount of radioactivity in culture by pipetting a 5 µl sample into a scintillation vial containing 4 ml of scintillation cocktail fluid and then count ^{14}C -radioactivity using a liquid scintillation detector; (3) take the remaining 45 µl of culture and pellet the cells by centrifugation, aliquot 5 µl of the cell-free supernatant to a scintillation vial containing 4 ml of scintillation cocktail fluid, and count ^{14}C -radioactivity using a liquid scintillation detector; and (4) compare ^{14}C -counts from the cell-free supernatant vs. the total culture to estimate the amount of radioactivity incorporated into cell material. If ~10% (or more) of the ^{14}C -counts are associated with the cell pellet, this is usually sufficient for a successful radiolabel assay. However, longer incubation times typically yield increased ^{14}C -AHL product. This step also confirms that your organism is capable of ^{14}C -methionine transport (*see Note 11*).

4. After labeling for a sufficient time (determined as described in the previous step), you are ready to extract the ^{14}C -AHLs from the bacterial culture. Pellet cells by centrifugation and transfer the cell-free supernatant to a solvent-resistant tube using a 3-ml polypropylene transfer pipette. Extract the supernatant twice with equal volumes of acidified ethyl acetate (EtAc), and collect and combine both organic phases.
5. EtAc solvent is evaporated and AHLs dried under a gentle stream of N_2 gas with warming (using N-evap nitrogen evaporator or similar). Resuspend the dried sample in 100 µl of 50% methanol-in-water.
6. Separate the 100-µl sample by HPLC on a C₁₈ reverse-phase column by using a 10–100% (vol/vol) methanol in water

gradient (1 ml/min flow, 70-min profile). Collect 70 1-ml (1-min) fractions in scintillation vials using an automated fraction collector.

7. Add 4 ml of scintillation cocktail to each fraction and count with a scintillation detector. Examples of ^{14}C -radiolabel profiles of cells grown in the presence of exogenous aromatic substrates are shown in Fig. 4. AHL assignment is determined by co-elution with known AHL standards.

4 Notes

1. Previous experiments using similar protocols have used [$2-^{14}\text{C}$]-methionine [20] or uniformly labeled ^3H -methionine [22], but these compounds are no longer commercially available. To our knowledge, the only current supplier of L-[$1-^{14}\text{C}$]-methionine is American Radiolabel Chemicals, St. Louis, MO (ARC-0271A, 50–60 mCi/mmol, 0.1 mCi/ml).
2. Confirm the purity of the ^{14}C -methionine when you first receive a new lot by separating a small amount (1 μl) over the HPLC column; all radioactive counts should pass through the column in the void volume.
3. LuxI-type AHL synthases have only been identified in members of the *Proteobacteria*. As the number of bacterial genomes sequenced increases, so does the number of potentially interesting AHL systems. We often identify potentially interesting strains for AHL screening by searching their sequenced genomes for the AHL synthase motif pfam00765 [28].
4. The growth medium is specific to the bacterium you are testing and, if possible, should be methionine free. For the experiments used in this chapter (Figs. 3 and 4) we used the following media: Jensen's medium with 0.3% glycerol [29] (*Pseudomonas aeruginosa* PAO1), M9 minimal medium [30] with 10 mM succinate (*Pseudomonas chlororaphis* GM17), AG medium [31] (*Bradyrhizobium japonicum* USDA110), and PM medium with 10 mM succinate [32] (*Rhodopseudomonas palustris* CGA009).
5. In some cases, we have also grown bacteria in rich (methionine-replete) medium, pelleted cells by centrifugation, and resuspended the cell pellet in phosphate-buffered saline with a carbon and energy source (e.g., 10 mM glucose) for the ^{14}C -labeling reaction [21].
6. We made filter-sterilized stock solutions of 13 individual aromatic salts, at a concentration of 0.1 M and pH 7, including benzoate, *p*-coumarate, *m*-coumarate, *o*-coumarate, cinnamate, caffeoate, ferulate, methoxycinnamate, sinapate, *p*-hydroxybenzoate, vanillate, phenylalanine, and tryptophan. Compounds were diluted 1000-fold in culture medium to a

final concentration of 0.1 mM for substrate labeling experiments.

7. Although here we use defined aromatic acid mixtures, one could imagine using naturally occurring mixtures of potential substrates such as extracts of plant tissue or sediments.
8. Many synthetic AHL standards can be purchased from a variety of vendors including Sigma-Aldrich (St. Louis, MO), Cayman Chemical Co. (Ann Arbor, MI), or the University of Nottingham (Nottingham, UK, <https://www.nottingham.ac.uk/quorum/compounds.htm>). Alternatively, one could extract AHLs from bacteria with well-defined AHL QS systems, such as *P. aeruginosa* PAO1 (Fig. 3a).
9. Over the years we have tested a variety of scintillation counting cocktails, including those marketed as nonhazardous or biodegradable fluids, but we obtained the best results using a xylene-based scintillation fluid.
10. Keep in mind that the AHL synthase must be expressed under the laboratory growth conditions employed for the ¹⁴C-radiolabel (and other AHL detection methods) to work. Some AHL-type systems have additional regulatory controls, such as the presence of plant metabolites [9, 33] or host redox conditions [34], that must be satisfied before AHL signals will accumulate. If the genome sequence is available for the bacterium of interest, cloning the LuxI-homolog gene into a heterologous host under a constitutive or an inducible promoter can circumvent some of these issues.
11. There are some bacteria that cannot transport exogenous methionine, for example the chemolithoautotroph *Acidithiobacillus ferrooxidans*. Although this organism synthesizes an AHL molecule, we were unable to utilize the radiolabel protocol to detect AHL in this organism because the ¹⁴C-met is not assimilated into the AHL signal.
12. The amount of ¹⁴C-AHL produced is influenced by several things including rates of bacterial growth and methionine incorporation, presence of AHL-degrading enzymes [35], and AHL synthesis and accumulation rates. For example, *P. aeruginosa* PAO1 typically produces low micromolar levels of its AHL signals [36], while *B. japonicum* USDA110 maximally produces isovaleryl-HSL (IV-HSL) in the low nM range [6].
13. For the experiments represented in Fig. 4, we used an *rpaI* mutant of *R. palustris* [9] as the heterologous host because this bacterium can metabolize a wide variety of aromatic compounds, no longer produces any endogenous AHL signal, and can constitutively express LuxI homologs using the vector pBBR1MCS-5 [37]. Cells were grown photoheterotrophically in PM succinate plus 100 µg/ml of gentamycin [9].

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Chapter 4

Liquid Chromatography/Mass Spectrometry (LC/MS) for the Detection and Quantification of *N*-Acyl-L-Homoserine Lactones (AHLs) and 4-Hydroxy-2-Alkylquinolines (HAQs)

François Lépine, Sylvain Milot, Marie-Christine Groleau, and Eric Déziel

Abstract

High-performance liquid chromatography (HPLC) coupled in-line with mass spectrometry (MS) permits rapid and specific identification and quantification of *N*-acyl-L-homoserine lactones (AHLs) and 4-hydroxy-2-alkylquinolines (HAQs). We are presenting here methods for the analysis of these molecules directly from biological samples using LC/MS.

Key words Bacteria, Quorum sensing, Acyl-homoserine lactone, 4-Hydroxy-2-alkylquinoline, Analysis, Quantification, Mass spectrometry, Liquid chromatography

1 Introduction

Most Gram-negative bacteria produce *N*-acyl-L-homoserine lactones (AHLs), such as *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C₁₂-HSL) and *N*-butanoyl-L-homoserine lactone (C₄-HSL) for *Pseudomonas aeruginosa* [1, 2]. A few species, such as *P. aeruginosa*, *Burkholderia pseudomallei*, and *Burkholderia thailandensis* also release a number of 4-hydroxy-2-alkylquinolines (HAQs), also known as 2-alkyl-4-quinolones [3–6] and derivatives, such as 3-methyl analogs (HMAQs).

Although bioassays are essential for screening and for rapid qualitative determination of quorum-sensing signaling molecules, more precise and sensitive methods are required to provide definitive confirmation of their presence and allow formal structural confirmation and accurate quantification. Because of the very large number of structurally related signaling molecules, a preliminary separation is required using high-performance liquid chromatography (HPLC). Identification and quantification are then performed by mass spectrometry coupled in-line with the HPLC.

2 Materials

2.1 Bacterial Cell Cultures

1. Bacterial strain: The methods presented here use cultures of the Gram-negative bacterium *P. aeruginosa*, strain PA14 [7].
2. Tryptic soy broth (TSB) medium.
3. 18 × 150 mm borosilicate glass tubes.
4. Roller drum for culture tubes.

2.2 LC/MS Analysis

1. Triple-quadrupole mass spectrometer (MS) equipped with a Z-spray interface.
2. Nitrogen is used for drying and argon is used as collision gas in multiple reaction monitoring (MRM) mode.
3. HPLC equipped with a 4.6 × 150 or 3.0 × 100 mm C8 or C18 column: the MS is connected to the HPLC through a T splitter. The third output of the splitter is fitted with a tube of internal diameter and length such as only 10% of the initial flow goes to the electrospray probe.
4. Solvent A: Distilled water containing 1% ACS-grade acetic acid.
5. Solvent B: HPLC-grade acetonitrile (or 2-propanol), containing 1% ACS-grade acetic acid.

2.3 Internal Standards

1. 5,6,7,8-Tetradeutero-4-hydroxy-2-heptylquinoline (HHQ-d₄) and 5,6,7,8-tetradeutero-3,4-dihydroxy-2-heptylquinoline (PQS-d₄) are synthesized as described in [8].
2. Standard stock solution: 10 mg/l HHQ-d₄ and 20 mg/l PQS-d₄ are prepared in HPLC-grade methanol and kept at -20 °C.

3 Methods

Mass spectrometric analysis of the samples is performed under positive electrospray ionization conditions, a process that adds a proton to the analytes, thus producing pseudomolecular ions which correspond to the mass of the neutral molecule with the addition of one proton.

The methods presented here mostly deal with the quantification of 4-hydroxy-2-heptylquinoline (HHQ), 3,4-dihydroxy-2-heptylquinoline (*Pseudomonas* quinolone signal; PQS), and 4-hydroxy-2-alkylquinoline N-oxide (HQNO), each the best studied HAQ congener of its family [3], and of C₄-HSL and 3-oxo-C₁₂-HSL as the most representative AHLs of *P. aeruginosa* (Fig. 1). Within each family, the various congeners only differ by the length of their alkyl side chain. These molecules can thus be analyzed by the same methods by taking into account the mass difference due to the successive addition (or subtraction) of one methylene

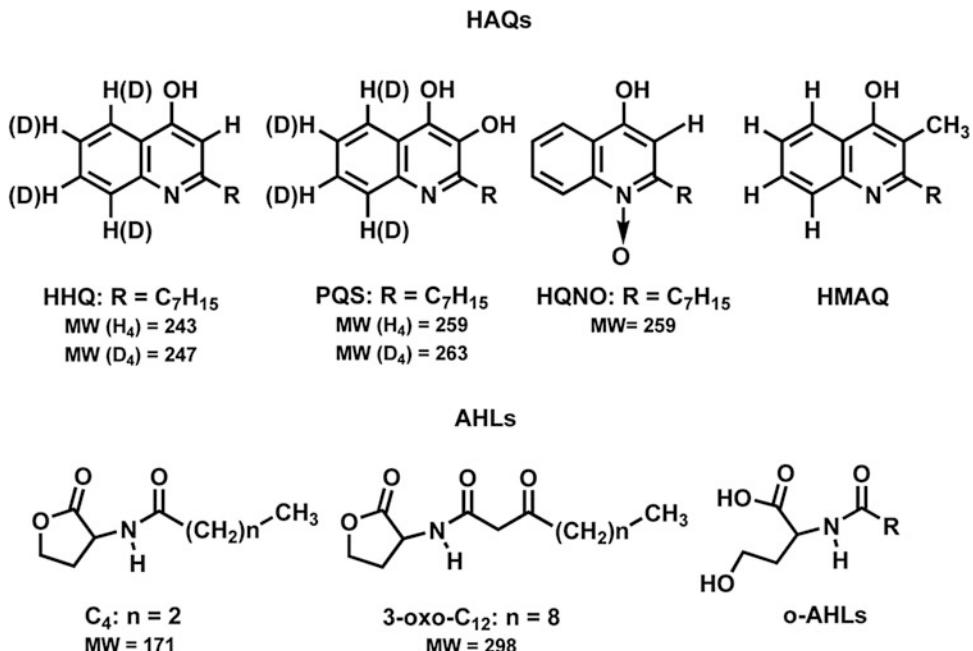


Fig. 1 Chemical structure of HAQs and AHLs

unit (14 Da) going from one congener to the next. The most abundant HAQs, contrary to AHLs, contain an odd number of carbons on their side chain. Going from the most abundant congener to the next most abundant ones generally entails successive addition (or subtraction) of 28 Da. These methods can also be used to quantify the open form of the AHLs (o-AHLs) (Fig. 1), in which the lactone ring is hydrolyzed, which corresponds to the addition of one molecule of water (18 Da) to the unmodified AHLs. Depending on the growth conditions and age of the cultures, these o-AHLs can be found in abundance. The methods presented can be adapted to perform the desired analysis. For instance, some *Burkholderia* species produce modified HAQs carrying an additional methyl substitution [6]; detection and quantification of these 4-hydroxy-3-methyl-2-alkylquinolines (HMAQs) are possible by taking into account the additional mass of 14 Da resulting from the replacement of a proton by a methyl group on the quinoline ring (Fig. 1).

Various scanning modes can be used with a triple quadrupole to acquire quantitative data, each mode having its advantages and limitations. Hence, in full-scan mode, a wide range of masses can be acquired, allowing for subsequent analysis of other compounds of interest aside from the intended HAQs and AHLs. In this scanning mode, the sensitivity is not maximal if only quantification of a few molecules is needed. On the other hand, it is very useful for detecting other members of the various families of HAQs or AHLs,

or other metabolites directly in culture broth. In multiple reaction monitoring (MRM) mode, one takes advantages of the ability of the instrument to select and fragment specific pseudomolecular ions, and monitor the intensity of one specific resulting fragment ion. This scanning mode is limited to a preset series of fragmentation reactions (called transitions), but due to the specificity of the fragmentation, it provides a much better signal-to-noise ratio than the full-scan mode, thus increasing the sensitivity of the analysis. The MRM mode is especially interesting for the analysis of AHLs, or for low concentrations of HAQs produced in complex matrices, such as from infected animal tissue samples.

3.1 Direct Quantification of HAQs from Culture Broth in Full-Scan Mode

1. *P. aeruginosa* PA14 cultivated in TSB medium at 37 °C and 200 rpm overnight (*see Note 1*) is used to inoculate 3 ml of fresh TSB medium at a starting OD₆₀₀ of 0.05 (*see Note 2*). The cultures are then incubated under the same conditions, typically until they reach an OD₆₀₀ of 3.0 (*see Note 3*). A 300 µl culture sample is then transferred to a microcentrifuge tube and 300 µl methanol containing the internal standards is added (*see Note 4*). Vortex briefly.
2. The tube is centrifuged at 13,000 × *g* for 15 min to pellet the cells and debris, and then 500 µl of the supernatant is pipetted in a borosilicate HPLC vial, from which 20 µl is injected in the HPLC.
3. The solvent gradient for the chromatographic run is as follows: from 0 to 1 min 70% solvent A; from 1 to 13 min 100% solvent B; from 13 to 23 min 100% solvent B; from 23 to 25 min 70% solvent A; and from 25 to 28 min 70% solvent A (*see Note 5*). Flow rate is set at 400 µl/min split to 40 µl/min by the T splitter.
4. The MS parameters are positive mode; needle voltage 3.0 kV; cone 30 V; block temperature 120 °C and drying gas 150 °C; nebulizing gas 20 l/min; and drying gas 200 l/min.
5. In full-scan mode, the scanning range is set to *m/z* 100–400. The chromatogram of all the ions monitored (total ion chromatogram or TIC) is presented in Fig. 2.
6. Figure 3 shows the chromatogram of the pseudomolecular ions ($M + H^+$) of HHQ, PQS, and HQNO at 244, 260, and 260, respectively, and those of the internal standards HHQ-d₄ and PQS-d₄ at 248 and 264, respectively. With this column the retention time of PQS is 21.8 min while the one of the isobaric (an ion with the same nominal *m/z* value) HQNO is 19.9 min. All members of the HQNO family have a retention time shorter than those of the corresponding alkyl chain length in the PQS family.

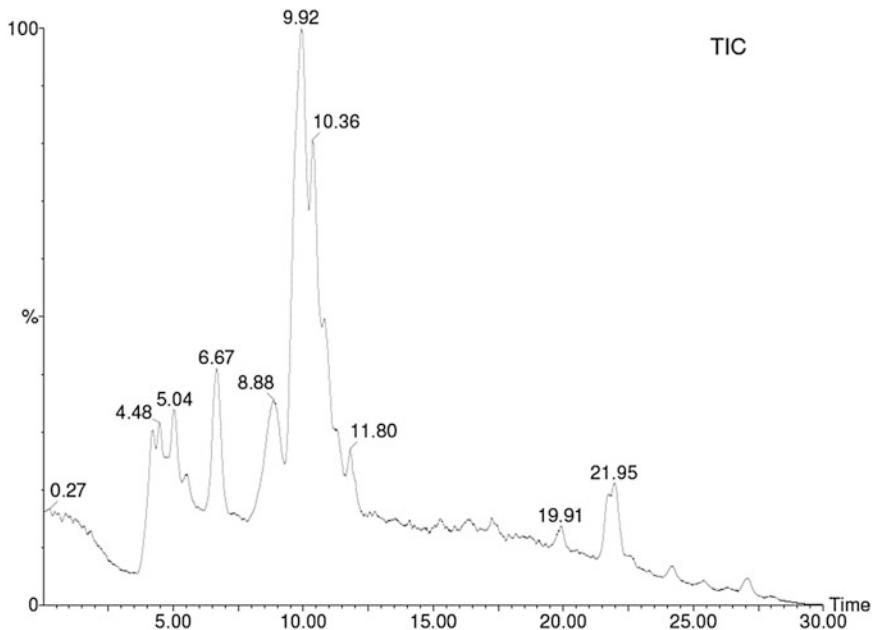


Fig. 2 Total ion chromatogram of the supernatant of a *P. aeruginosa* strain PA14 culture grown to an OD₆₀₀ of 3.0

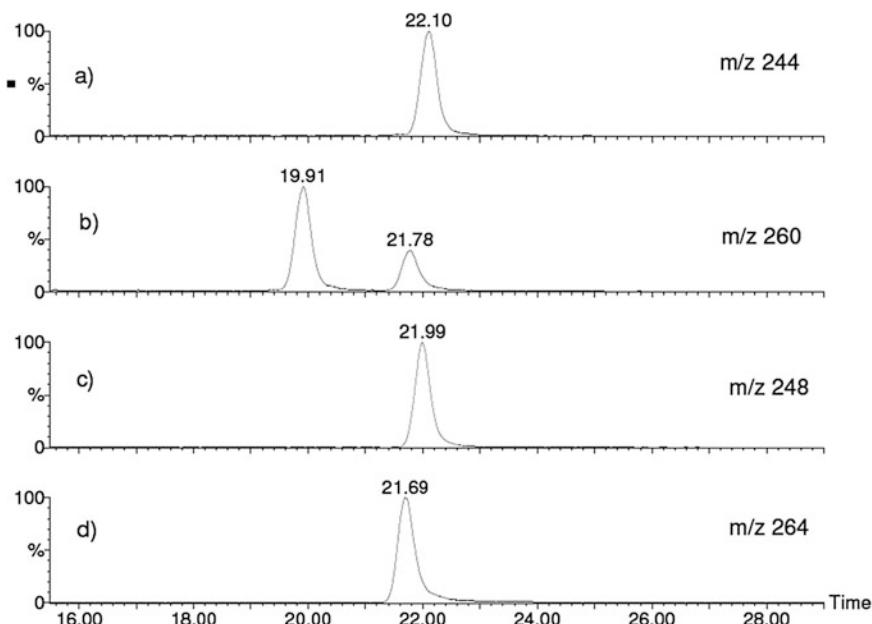


Fig. 3 Ion chromatograms of the supernatant of a *P. aeruginosa* strain PA14 culture grown to an OD₆₀₀ of 3.0. (a) HHQ; (b) HQNO and PQS; (c) HHQ-d₄; (d) PQS-d₄

7. The area under each of these chromatographic peaks is integrated. The concentration of the analyte A in the culture medium is given by the equation:

$$C \times A \times 2/H$$

where C = concentration of internal standard; A = area of the HAQ peak; and H = area of the internal standard peak. The factor of 2 is to keep into account the dilution factor due to the addition of the methanol containing the internal standard. For HHQ and HQNO and the other members of these families with different chain lengths, the internal standard used is HHQ-d₄, while for the congeners of the PQS family the internal standard used is PQS-d₄ (*see Note 6*).

3.2 Quantification of HAQs from a Complex Matrix in MRM Mode

1. HAQs can be analyzed from a sample of muscle tissue from a mouse infected with *P. aeruginosa* [9]. One hundred milligram of muscle tissues are put in a 2 ml microcentrifuge tube to which is added 500 µl of methanol containing 0.2 mg/l of HHQ-d₄ and PQS-d₄. The tissues are homogenized with a Polytron and then centrifuged at 13,000 × g for 15 min. The supernatant is then collected and 80 µl injected in the HPLC under the same conditions as in Subheading 3.1.
2. The source operating parameters are the same as in full-scan mode. In MRM mode the following transitions are monitored: for HHQ 244 → 159; HHQ-d₄ 248 → 163; HQNO 260 → 159; PQS 260 → 175; and PQS-d₄ 264 → 179. The pressure of the collision gas (argon) is set at 2×10^{-3} mTorr and the collision energy at 30 V for all transitions.
3. The area of each chromatographic peak is integrated and the concentration of each compound is calculated as above (*see Note 7*).

3.3 Direct Quantification of Targeted AHLs from Culture Broth in MRM Mode

1. The bacteria are cultivated as in Subheading 3.1 (*see Note 8*). To 480 µl of culture is added 120 µl of anhydrous acetonitrile containing 15 mg/l HHQ-d₄ and the mixture is vortexed and then centrifuged at 13,000 × g for 10 min. A 500 µl aliquot is collected in a HPLC vial and 15 µl is injected (*see Note 9*).
2. The solvent gradient is as follows: from 0 to 1 min 100% solvent A; from 1 to 5 min 50% solvent B; from 5 to 13 min 100% solvent B; from 13 to 23 min 100% solvent B; from 23 to 25 min 100% solvent A; and from 25 to 28 min 100% solvent A. Flow rate is set at 400 µl/min split to 40 µl/min by the splitter. Under these conditions the open form of each AHL always has a shorter retention time than the corresponding closed form.

3. The following transitions are monitored: C₄-HSL 172 → 102; o-C₄-HSL 190 → 120; HHQ-d₄ 248 → 163; 3-oxo-C₁₂-HSL 298 → 102; and o-3-oxo-C₁₂-HSL 316 → 120. The pressure of the collision gas is set at 2×10^{-3} mTorr and the collision energy is 15 V for the AHLs and 33 V for HHQ-d₄.
4. A calibration curve is performed to obtain the response factor of C₄-HSL and 3-oxo-C₁₂-HSL relative to HHQ-d₄. To do so, a series of solutions with increasing concentration of the AHL from 0 to 15 mg/l are made with a constant concentration of 10 mg/l of HHQ-d₄. The same response factor is used for the open and closed forms of each AHL.
5. The concentration of the AHLs in the solution is calculated as follows:

$$R \times C \times A \times 2/H$$

where R = response factor, C = concentration of internal standard (HHQ-d₄), A = area of the AHL peak, and H = area of the internal standard (HHQ-d₄) peak.

3.4 Detection of Non-targeted AHLs from Culture Broth in Parent Mode

Knowing that the majority of AHLs generate a 102 Da fragment (e.g., *see* Subheading 3.3, step 4), it is possible to detect and quantify non-targeted AHLs by looking for the parent ion of daughter ion 102 using the Parent mode.

1. The samples are prepared as in Subheading 3.3.
2. The source operating parameters are the same as in full-scan mode (*see* Subheading 3.1). In Parent mode, the internal standard is Parent of 163 → 248 (HHQ-d₄). The pressure of the collision gas (argon) is set at 3×10^{-3} mBar (collision gas flow of 0.35 (ml/min) and a collision energy of 15 V are used).
3. Identify the AHLs corresponding to the parent ions of 102 in the list of Table 1.

3.5 Quantification of Low Concentrations of HAQs or AHLs by Solvent Extraction

To obtain a better sensitivity or to quantify molecules present in minute concentrations, it is optimal to extract the AHLs from the cultures and concentrate.

1. Internal standard (HHQ-d₄) is added to a 10 ml culture sample to get a final concentration of 0.2–0.5 mg/l. The sample is then transferred to a 15 ml glass tube closed with a PTFE-lined twist cap.
2. Two milliliter ethyl acetate (or other appropriate solvent) is added and the mixture is strongly vortexed for 30 s. If the emulsion is stable (the phases do not separate), the tubes can be centrifuged to separate the phases.

Table 1
Mass of various AHLs [11]

AHLs	Mass [M + H] ⁺
C ₄ -HSL	172
C ₆ -HSL	200
3-oxo-C ₆ -HSL	214
C ₈ -HSL	228
3-oxo-C ₈ -HSL	242
C ₁₀ -HSL	256
3-OH-C ₁₀ -HSL	272
3-oxo-C ₁₀ -HSL	270
C ₁₂ -HSL	284
3-oxo-C ₁₂ -HSL	298

3. Using a Pasteur pipette, the organic phase (top when ethyl acetate is used) is transferred to a HPLC vial, being very careful not to carry over any emulsion from the interface.
4. Especially if one wants to precisely quantify the concentration of AHL present, **steps 2–4** should be repeated twice. The organic phases are pooled together.
5. The solvent is then evaporated under a gentle stream of nitrogen gas, and finally the dry residue is kept in a tightly closed vial at –20 °C.
6. At the time of injection, the extract is solubilized in 500 µl anhydrous acetonitrile (*see Note 10*). Vortex for 30 s to fully dissolve the residue (*see Note 11*).
7. Injection and quantification are performed as described above.

4 Notes

1. HAQs have been detected in most *P. aeruginosa* wild-type strains we have tested, except PAK and PA7.
2. The cultivation can also be performed in larger volumes, such as 50 ml of TSB in 250 ml flasks. However, we found that upon scaling up, the concentration of AHLs and HAQs tends to decrease significantly with larger culture volumes for the same final cell density.
3. HAQs such as PQS or HQNO accumulate up to concentrations in excess of 15 mg/l in the culture medium under the described culture conditions and are thus easily detected in

normal scanning mode. However, HHQ and the other members of the same family of compounds are the precursors of PQS and its congeners and thus show a decrease in their concentration following an initial increase. It is thus important to select the proper cultivation time if HHQ and its congeners are to be measured.

4. HAQs such as PQS and HHQ have a limited solubility in water. For example the solubility of PQS is <5 mg/l in TSB. However, concentrations in excess of 15 mg/l have often been measured in whole cultures. This is an indication that these compounds are either pseudosolubilized by bacterial exoproducts or adsorbed on the cell surface. As bacteria must be removed from the medium prior to analysis, in order to avoid plugging the HPLC column, this can have an important effect on the concentration of HAQs detected in the samples. To dissociate HAQs from the bacteria cell surface, methanol is added to the culture at a 50% (v/v) concentration prior to removing the cells by centrifugation [8]. In fact, we measure typically 50% less HAQs if the cells are centrifuged prior to adding methanol. Thus addition of methanol (which contains the internal standards) to the culture samples serves two purposes: to release bound HAQs and to act as carrier for the internal standards for quantification purposes.
5. Because of occasional uncertainties in the supply of acetonitrile, an alternative gradient method can be used with 2-propanol as solvent according to the following steps: at the moment of injection 90% solvent A; from 0 to 3 min 40% solvent B (2-propanol containing 1% acetic acid); from 3 to 10 min 65% solvent B; from 10 to 21 min 70% solvent B; from 21 to 23 min 100% solvent B; from 23 to 25 min 100% solvent B; from 25 to 26 min 90% solvent A; and from 26 to 30 min 90% solvent A.
6. One problem with PQS quantification is that when the HPLC column ages, the shape of the PQS peaks tends to present tailing and the area of the corresponding peak often varies considerably from one injection to another, which makes the quantification more difficult without using an appropriate deuterated internal standard. The PQS-d₄ internal standard fluctuates in the same manner, thus correcting for these variations.
7. The most abundant members of each families of compound can be quantified using their respective internal standard (PQS-d₄ for the PQS family and HHQ-d₄ for all the other HAQs) by adding (or subtracting) 28 Da to the weight of the corresponding pseudomolecular ion of the internal standard while monitoring the same fragment ion. Under these conditions, fragmentation of all HAQs produces a fragment ion common to all the congeners of a given family [10].

8. Because the Las system, which mediates 3-oxo-C₁₂-HSL, is expressed before the Rhl system which produces the C₄-HSL, the maximal concentration of 3-oxo-C₁₂-HSL in cultures is achieved prior to the one of C₄-HSL. Thus timing of sampling will critically affect the concentrations of AHLs obtained.
9. Because the lactone ring of AHLs can spontaneously open at high pH or the open form can close at low pH, care should be taken to avoid these conditions if the samples are stored for a certain period of time prior to analysis.
10. Keep in mind the concentration factor. At the end, the final concentration of the internal standard in the injection vial should be between 1 to 20 mg/l; if it is too high, the detector signal will be saturated, and a too low concentration will result in large variations. For instance, in the present example, the 0.2 to 0.5 mg/l HHQ-d4 added to the 10 ml culture sample before the extraction will end up at a final concentration of 4 to 10 mg/l in the injection vial if the residue is dissolved in 500 µl acetonitrile.
11. If a sample is turbid, transfer to a microcentrifuge tube and centrifuge at 12,000 × g for 5–10 min. Transfer the cleared supernatant to a new vial.

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Chapter 5

Detection of the Bacterial Quorum-Sensing Signaling Molecules *N*-Acyl-Homoserine Lactones (HSL) and *N*-Acyl-Homoserine (HS) with an Enzyme-Linked Immunosorbent Assay (ELISA) and via Ultrahigh-Performance Liquid Chromatography Coupled to Mass Spectrometry (UHPLC-MS)

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Abstract

Quick and reliable quantitative methods requiring low amounts of sample volume are needed for the detection of *N*-acyl-homoserine lactones (HSL) and their degradation products *N*-acyl-homoserines (HS) in order to elucidate the occurrence and dynamics of these prevalent quorum-sensing molecules of Gram-negative bacteria in natural samples and laboratory model experiments. A combination of ELISA and UHPLC-MS is presented here which has proven to meet these requirements. Both methods can not only precisely detect and quantify HSLs but also their degradation products HS and thereby enable studying signaling dynamics in quorum sensing, which have been identified to play an essential role in bacterial communication.

Key words *N*-acyl-homoserine lactone, Quorum sensing, Bacterial signaling, ELISA, UHPLC-MS

1 Introduction

For the analysis of quorum-sensing (QS) signaling in Gram-negative bacteria it is mandatory to measure exactly what kind of signaling substances are present in a certain habitat and at what concentration. For the best known QS-signaling substance, *N*-acyl-homoserine lactones (HSLs), several detection methods have been developed, e.g., biosensor assays, thin-layer chromatography, and gas and liquid chromatography without or coupled with mass spectrometry [1–7]. All these methods have certain advantages and drawbacks, including difficulties in quantification of autoinducers produced at low level, although low concentrations of signal

molecules have been shown to be relevant in natural systems. Furthermore, it is often not possible to detect the degradation products of HSLs, the *N*-acyl-homoserines (HSs), which are formed due to the activity of lactonases cleaving the lactone ring or due to abiotic degradation when the pH rises above 7. This often leads to an underestimation of produced HSLs and prevents to monitor the fate of HSLs over a longer time period. It has become more apparent in recent years that HSL degradation is an integral part of the QS-signaling process and facilitates a reset of the auto-inducer system in order to respond quickly to environmental changes [7–9].

As one method to quantitatively detect HSLs and their degradation product HSs, a competitive ELISA in the coating antigen format was established in our labs [1]. The applied monoclonal antibody (mAb) had the advantage to bind to both HSL and HS with different affinities (quantified by the cross-reactivity value, CR). By measuring each sample before and after a hydrolysis step, the signal intensity of the untreated sample consisting of an unknown ratio of HSLs and HSs can be compared to the signal intensity of the hydrolyzed sample consisting of only HSs. In this way, the individual amounts of HSL and HS in each sample can be calculated. For the ELISA, a microtiter plate is coated with a BSA-conjugated antigen (HSL-BSA). Sample and standard HSL or HS are incubated with a defined amount of monoclonal antibody (mAb) specific for the HSL/HS present in the sample. This mixture is then transferred to the antigen-coated plates, where only mAbs not saturated with the acyl-HSL/HS molecules from the sample/standard can bind to the antigens on the plates. These mAbs are then detected by a peroxidase-coupled secondary antibody, which facilitates a colorimetric quantification. Thus, the stronger the color intensity, the less analyte was present in the sample/standard solution. The described method provides a fast, sensitive, and cost-efficient way to detect acyl-HSLs/HSs in biological samples, omitting complex sample preparation. Another advantage is the low amount of sample required (<1 ml). One critical point is that the method is prone to attenuating matrix effects.

To become independent from the sample matrix and to precisely quantify even low concentrations of acyl-HSL/HS molecules within small amounts of cell cultures, a UHPLC-MS method was established [7]. The sample preparation is adapted for culture supernatants and intact cellular compartments separately to reach the most efficient extraction effect for HSLs/HSs. A relatively long (10 min) reversed-phase separation is used to clearly isolate different side-chain lengths of HSLs/HSs, which is confirmed by the MS detection in positive ionization mode.

2 Materials

All chemicals should be at least of analytical grade and LC-MS-grade solvents needed for chromatographic separations must be filtered through 0.2 µm membrane filters. Aquatic solutions are prepared using ultrapure water with a conductivity of 18 MΩ·cm and a total organic carbon lower than 5 ppb at 25 °C. Read the material safety data sheets of the used chemicals and diligently follow all regulations regarding personal protection, exposure controls, and disposal considerations.

2.1 ELISA

Unless otherwise indicated, store all reagents at 4 °C.

1. 40 mM PBS: 5 mM Na₂HPO₄, 35 mM Na₂HPO₄, 100 mM NaCl in H₂O_{ultrapure}, pH 7.2.
2. 40 mM PBST: PBS plus 0.05% (v/v) Tween-20, pH 7.6.
3. Carbonate buffer: 15 mM Na₂CO₃, 35 mM NaHCO₃ in H₂O_{ultrapure}, pH 9.6.
4. Primary antibody and coating antigen: For the preparation of suitable AHL-specific antibodies and BSA-conjugated coating antigens please refer to [1, 10]. The primary antibody has to be selected according to the size of the *N*-acyl side chain (long, with more than 6 C atoms, or short, with 6 C atoms or less) and the substitution at the C3 atom.
5. Secondary antibody: 5 µg/ml of goat-anti-rat-POD (0.4 mg/ml stock concentration) in 40 mM PBST.
6. Coating antigen solution: 0.2 µg/ml HSL-BSA antigen in 50 mM carbonate buffer.
7. Wash solution 4 mM PBST: 4 mM PBS (1:10 dilution of 40 mM PBS stock), 0.05% (v/v) Tween-20 in H₂O_{ultrapure}, pH 7.2.
8. Blocking solution: 1 g Casein in 100 ml 40 mM PBS, pH 7.6. Prepare fresh every time and stir until needed.
9. HSL standard stock solution: 1 mg/ml solution in acetonitrile of the HSL in question.
10. HS standard stock solution: Hydrolyze HSL solution with 1 M NaOH as described in the method section for the samples to obtain the 1 mg/ml HS standard stock solution.
11. Standard dilution series: This depends largely on the sensitivity of the antibody in use and has to be optimized for each application. The values given here are just an example. The antibodies characterized by [1] were more sensitive to HS; therefore the concentration of the HS standard could be five times lower. HSL and HS stock solutions are diluted preferably in the growth medium used for the bacterial culture (or in an appropriate buffer) according to Tables 1 and 2.

Table 1
HSL standards

µl Buffer	950	960	500	960	500	960	500	800	900	900
µl Pipetted from previous dilution	50	240	500	240	500	240	500	200	100	
Conc. [ng/ml]	50,000	10,000	5000	1000	500	100	50	10	1	0

Table 2
HS standards

µl Buffer	990	1080	500	960	500	960	900	900	900	900
µl Pipetted from previous dilution	10	120	500	240	500	240	100	100	100	
Conc. [ng/ml]	10,000	1000	500	100	50	10	1	0.1	0.01	0

12. Hydrolysis: 1 M NaOH, 1 M HCl.
13. Substrate: 0.4 mM 1-Step Ultra Tetramethylbenzidine (TMB)-ELISA, 1.3 mM H₂O₂ in 100 mM sodium acetate buffer, pH 5.5.
14. Stop solution: 2 M H₂SO₄.
15. Plates: MaxiSorp plate, U-bottom microplate low binding.

2.2 Hybrid Magnetic Microparticles-Solid-Phase Extraction (HMP-SPE) of Culture Supernatants

1. Hybrid magnetic microparticles (HMP): Weigh 0.27 g iron (III)-chloride in a glass vial and add 0.70 g sodium acetate. Dissolve the mixture in 8 ml ethylene glycol (*see Note 1*). Add 100 mg of the copolymer Oasis HLB (*see Note 2*) to the solution and homogenize it by stirring. To form HMP, place the glass vial in a polytetrafluoroethylene (PTFE)-lined stainless steel digestion vessel and keep it in an oven for 12 h at 200 °C (*see Note 3*). Separate HMP and reaction solvent by using an external magnet and wash them at least five times with 5 ml purified water. Repeat washing steps with methanol. Finally, keep HMP at 60 °C until dryness [[7](#)].
2. Methanol, LC-MS grade.
3. Purified deionized water.
4. Elution solvent: Fill 7.5 ml of 3-propanol in a glass vial and add 2.5 ml hexane (*see Note 4*).
5. H₂O/ACN mixture: Fill 9.0 ml purified deionized water in a glass vial and add 1.0 ml acetonitrile (*see Note 4*).
6. HSL standard solutions: Prepare standard solutions in a concentration range of 0.1–10.0 µM using cell culture medium or acetonitrile for dilution (*see Note 5*).

2.3 HSL/HS**Extraction of Cell Pellets**

1. Extraction solvent: Prepare 100 ml of anhydrous ethyl acetate and acidify the solution with 10 µl glacial acetic acid.
2. EtOAc/ACN mixture: Prepare a 100 ml mixture of 50% (v/v) anhydrous ethyl acetate and 50% (v/v) acetonitrile (*see Note 4*).

2.4 UHPLC-ESI-QToF-MS Analysis

1. UHPLC Column: Use a Waters Acquity Ethylene Bridged Hybrid (BEH) C₁₈ column with the dimensions 1.0 × 150 mm and a particle size of 1.7 µm or a column with similar separation properties.
2. Eluant A: Prepare a solution of purified deionized water with 10% (v/v) acetonitrile and 0.1% (v/v) formic acid.
3. Eluant B: 100% Acetonitrile.

3 Methods**3.1 Sample Preparation**

In principle, liquid samples from any given habitat can be analyzed. However, the matrix can lead to a considerable inhibition of the assay and needs to be thoroughly tested. Especially rich bacterial culture media, like nutrient broth (NB), are likely to cause a significant matrix effect and should be avoided.

1. Separation of cell fraction and culture supernatant should be achieved by centrifugation or ultracentrifugation at 4 °C (*see Note 6*).
2. Subsequently, the supernatant should be filtered through a 0.22 µm nitrocellulose membrane and frozen at -80 °C for further extraction and analysis (*see Note 7*).

3.2 ELISA

The given amounts are calculated for three plates. Measure all standards and samples at least in triplicates and take mean. Mix all components in glass beakers and then pour in specifically labeled plastic reservoirs for multi-pipettes. If used for one component only, these plastic reservoirs can be reused after rinsing with tap water. Plates should be covered with PCR foil (reusable if clean) during every incubation step. It is advisable to process not more than three plates in parallel and always finish one complete step with one plate before repeating the step with the next one.

1. Coating: Prepare coating antigen solution by diluting 1 µl stock solution of HSL-BSA in 34.5 ml of 50 mM carbonate buffer. Add 100 µl of antigen solution per well into a Maxi Sorp microtiter plate and incubate overnight at 4 °C without shaking.
2. Preparation: On the next day put all buffers out of the refrigerator to equilibrate to room temperature. If you are using an automated washer (*see next step*) attach vacuum and buffer bottles, and perform a washing cycle with an empty 96-well plate.

3. General washing procedure: Wash the coated plate three times with 200 µl of 4 mM PBST buffer per well. This can be done manually with a multichannel pipette or with an automated washer. After washing empty the plate completely by tapping it down with open wells on a paper towel. Do not let the plate dry out after the washing process, but immediately proceed to next step.
4. Blocking step: Prepare 1% blocking solution by adding 1 g of casein to 100 ml of 40 mM PBS, pH 7.6, and stir until needed. Add 300 µl blocking solution per well. Place the plate on a plate incubator at room temperature with shaking at 500 rpm until needed (not longer than 3–4 h).
5. Standard preparation: Prepare HSL and HS standards in bacterial culture medium (or appropriate buffer) according to the list under point 8 in the Materials section. Hydrolyze part of samples by mixing 480 µl of sample with 60 µl 1 M NaOH and incubate at room temperature with shaking at 500 rpm for 15 min. Neutralize with 60 µl 1 M HCl.
6. Preincubation with mAb: Transfer 75 µl of each sample and standard dilution onto a fresh U-bottom microplate low binding. Prepare mAb depending on its specificity and concentration. Add 75 µl of mAb per well. Place on plate incubator at room temperature with shaking at 500 rpm for 1 h.
7. Transfer of analyte and mAB: Wash the blocked MaxiSorp microtiter plate as in **step 3**. Transfer 100 µl mixture of preincubated analyte plus mAB per well from U-bottom microplate to blocked MaxiSorp plate. Place on plate incubator at room temperature with shaking at 500 rpm for 1 h.
8. Goat-anti-rat-POD: Add 5 µl of GAR-POD to 40 ml of 40 mM PBST. Wash plate as in **step 3** and add 100 µl GAR-POD per well. Place on plate incubator at room temperature with shaking at 500 rpm for 1 h.
9. Substrate: Prepare substrate by adding 600 µl TMB and 150 µl H₂O₂ to 37.5 ml substrate (acetate) buffer. Wash plate as in **step 3** and add 100 µl substrate per well for the HRP reaction. Incubate in the dark (e.g., in a drawer) for 5–25 min (*see Note 8*).
10. Stop reaction: Add 50 µl of 2 M H₂SO₄ per well (*see Note 9*).
11. Absorbance measurement: Measure the optical density of the plate wells at 450 nm wavelength (reference 650 nm), and auto mix before measurement with microplate reader.
12. Turn off washer: If you are using a washer, put washing buffer and all other buffers back in the refrigerator. Attach a bottle with ultrapure water to the washer tubing and wash until no foam is visible in the exhaustion tube. Empty the vacuum bottle and turn off the machine.

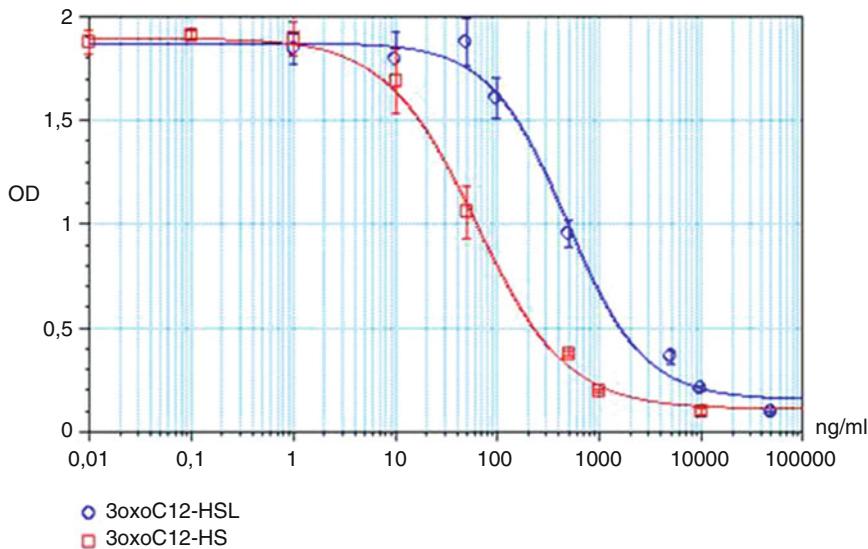


Fig. 1 Standard curves for the competitive ELISA detecting 3oxoC12-HSL and 3oxoC12-HS

13. Evaluation: Curve fitting of the standard curve can be performed using the four-parameter fit according to the equation [10]

$$y = \frac{A - D}{1 + \left(\frac{x}{C}\right)^B} + D$$

where x is the sample concentration and y the measured optical density. A is the y value corresponding to the asymptote at low values of the x -axis and D is the y value corresponding to the asymptote at high values of the x -axis. The coefficient C is the x value corresponding to the midpoint between A and D (IC_{50}). The coefficient B describes how rapidly the curve makes its transition from the asymptotes in the center of the curve (Fig. 1).

After A , B , C , and D have been determined experimentally, the equation can be used to calculate the sample concentration x from the measured optical densities:

$$x = \left(\frac{A - D}{y - D} - 1 \right)^{\frac{1}{B}} \times C$$

From these sum parameters the individual concentrations of HSL and HS can be calculated like this (for more details see [7]):

$$c(HSL) = \frac{S(HSL)_{\text{hydrolyzed}} - S(HSL)_{\text{non-hydrolyzed}}}{CR_{HS} - 1}$$

$$c(HS) = S(HS)_{\text{non-hydrolyzed}} - c(HSL) \times CR_{HSL}$$

$c(\text{HSL})$ and $c(\text{HS})$ are the individual concentrations of HSL and HS in the samples, while $S(\text{HSL})$ hydrolyzed and non-hydrolyzed and $S(\text{HS})$ non-hydrolyzed are the actually measured sample concentrations as sum parameters for the hydrolyzed and non-hydrolyzed samples. CR_{HSL} and CR_{HS} are the cross-reactivity values calculated for each measured plate by dividing the IC_{50} values (equal to C in the equation above) of the standard curve for HS by the IC_{50} of the standard curve for HSL for CR_{HSL} or vice versa for CR_{HS} (see Note 10).

3.3 Acyl-HSL/HS Extraction from Cell Pellets [11]

1. Add 3 ml of acidified ethyl acetate to a cell pellet and stir for 10 min at room temperature.
2. Evaporate the solvent under a continuous nitrogen stream (see Note 11) at room temperature.
3. Reconstitute extract in 500 μl EtOAc/ACN mixture and keep it at 4 °C for further analysis.

3.4 Solid-Phase Extraction (SPE) of Supernatants [7]

1. Weigh 25 mg of HMP in a glass vial for extraction of 2.5 ml cell culture supernatant or HSL/HS standards, respectively.
2. Capture HMP at the edge of the vial using an external magnet to discard wash solutions or culture medium after each step.
3. Condition HMP with 1 ml methanol and 1 ml purified water for 2 min, respectively.
4. Add 2.5 ml of cell culture supernatant or HSL/HS standard and incubate the mixture for 20 min at room temperature, stirring occasionally.
5. Wash HMP with 2 ml of purified water.
6. Elute HSL with a 2 ml solvent mixture of 3-propanol and hexane (75:25% v/v) and transfer the eluant into a 2 ml tube.
7. Dry the eluants using a centrifugal vacuum concentrator or evaporate solvent mixture under a low stream of nitrogen.
8. Redissolve extract in 250 μl H₂O/ACN mixture (90:10% v/v) and keep it cooled for further analysis.

3.5 UHPLC-ESI-QToF-MS Analysis

We routinely use a Waters Acquity UPLC, consisting of binary solvent manager, tempered sample manager, tempered column manager, and photo diode array detector, coupled to an electrospray ionization quadrupole time-of-flight mass spectrometer (ESI-QToF-MS, Bruker Daltonik).

1. Install reversed-phase C₁₈ column in the column manager and set the temperature to 40 °C.
2. Install eluants A and B on the binary solvent manager and purge all lines for 4 min with a flow rate of 8 ml/min. Equilibrate the C₁₈ column starting with 100% eluant B and a flow

rate of 0.1 ml/min. Change solvent composition after 20 min first to 50% eluant B and second to 0.5% eluant B.

3. Set sample temperature to 4 °C to keep samples stable for the analysis.
4. Use the following settings for each chromatographic run: apply a linear gradient from 0 to 90% eluant B within 5 min and with a flow rate of 0.1 ml/min. Keep the solvent composition stable for 2.5 min before equilibrating the column with 0.5% eluant B. Inject 5.0 µl of sample. Perform UV detection at 195 nm with a scan rate of 10 Hz.
5. Acquire mass spectra in positive ionization mode within a mass range of 50–1000 m/z (see Note 12). Measure HSL/HS standards from lower to higher concentrations first and then analyze samples in randomized order.
6. In order to ensure mass accuracy calibrate acquired spectra according to reference mass signals, e.g., from solvent impurities.
7. Create extracted ion chromatograms for protonated masses of HSL/HS molecules and integrate peaks to calculate peak areas (Fig. 2).
8. Plot peak areas of HSL/HS standards and make a linear equation. The coefficient of determination should be bigger than 0.95.
9. Calculate quantities of samples according to linear equation of HSL/HS standards.

4 Notes

1. Put the solution for 5 min into an ultrasonic bath to ensure complete dissolution.
2. Oasis® HLB is a hydrophilic-lipophilic balanced reversed-phase sorbent found and supplied by Waters GmbH, Milford, USA. It was tested to be the best sorbent for extracting acyl-HSL/HS from cell culture supernatants.
3. The reaction at 200 °C is forming high pressure according to boiling retardation of the solution. Therefore, usage of a tightly closed stainless steel digestion vessel is indispensable. Keep the vessel closed until it is cooled down to room temperature before continuing.
4. Use all solvents always at the same temperature (best room temperature) to prevent volumetric differences. To ensure correct volumetric percentages of organic solvents in mixtures, measure volumes by use of graduate flasks. Shake or stir organic solvent mixtures carefully to ensure complete mixing.

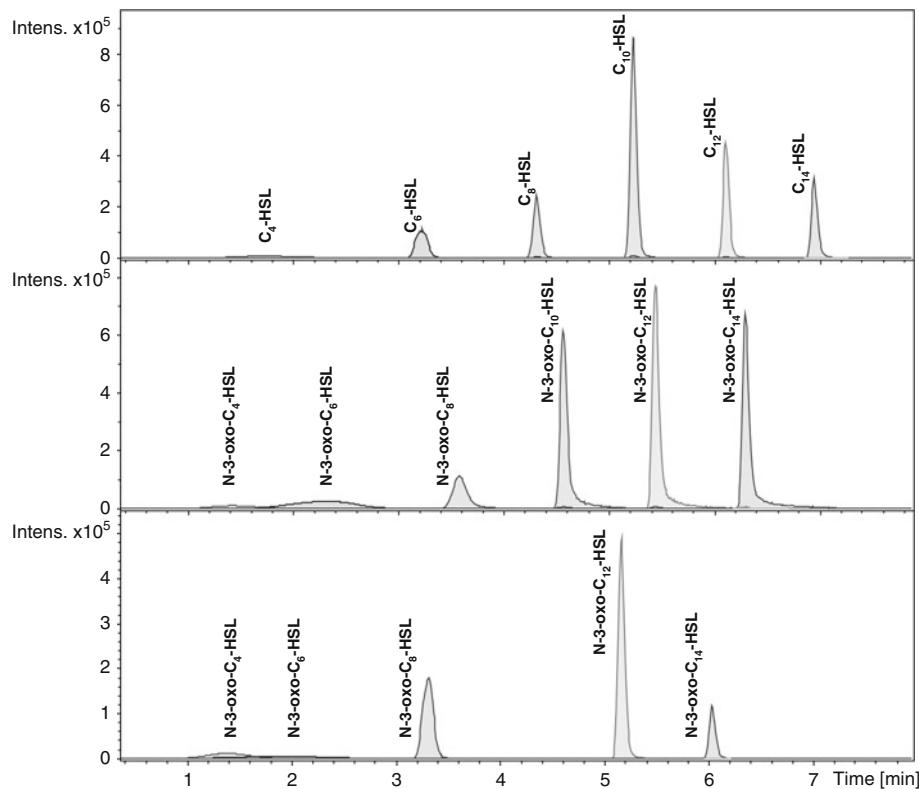


Fig. 2 Extracted ion chromatograms of *N*-butyryl-L-homoserine lactone, *N*-hexanoyl-L-homoserine lactone, *N*-octanoyl-L-homoserine lactone, *N*-decanoyl-L-homoserine lactone, *N*-dodecanoyl-L-homoserine lactone, *N*-tetradecanoyl-L-homoserine lactone (C₄-C₁₄; *top*), *N*-(3-oxobutyryl)-L-homoserine lactone, *N*-(3-oxohexanoyl)-L-homoserine lactone, *N*-(3-oxooctanoyl)-L-homoserine lactone, *N*-(3-oxododecanoyl)-L-homoserine lactone, *N*-(3-oxotetradecanoyl)-L-homoserine lactone (C₄-C₁₄; *middle*) and *N*-(3-hydroxybutyryl)-L-homoserine lactone, *N*-(3-hydroxyhexanoyl)-L-homoserine lactone, *N*-(3-hydroxyoctanoyl)-L-homoserine lactone, *N*-(3-hydroxydodecanoyl)-L-homoserine lactone, *N*-(3-hydroxytetradecanoyl)-L-homoserine lactone (C₄-C₈, C₁₂-C₁₄; *down*)

5. The concentration range depends on the limit of detection (LOD). It might be necessary to adjust it to the analytical instrument used for quantification. For correct quantification of HSL/HS from supernatant, standard solutions should be prepared following the same protocol for HMP-SPE. Standards for the quantification of HSL/HS from cell pellets can be prepared by dilution of a stock solution with acetonitrile. It is best to prepare fresh standard solutions each time. Mixing different HSL or HS standards in one solution is possible. HSL standards are commercially available as *N*-“R”-L-homoserine lactones, *N*-3-oxo-“R”-L-homoserine lactones, and *N*-(3-hydroxy-“R”)-L-homoserine lactone with “R” as the aliphatic side-chain length differing from C₄ to C₁₆.

6. Ultracentrifugation is only needed if HSLs/HSSs should be quantified in intact cells. A detailed description is given by [12]. For the quantification of excreted molecules in culture supernatants, a centrifugation at $15,000 \times g$ for 30 min is sufficient.
7. Samples should be stored no longer than a month depending on sample type and acyl-HSL concentration. For details see [10].
8. The incubation time varies depending on the assay. The darkest wells should be about the color of a transparent blue 1 ml pipette tip (equals approximately to an OD₄₅₀ of 1.0). It is critical not to incubate for too long, as then the color signal will be in saturation and the standard curve will be flawed.
9. No washing step should be carried out before adding the stop solution. If you are handling more than one plate, make sure that the incubation time—from adding the substrate until stopping the reaction—is the same for every individual plate. Usually it is sufficient to always keep the same order in handling the plates.
10. Make sure not to forget any dilution of the samples you might have applied. For example, the hydrolysis of the samples in Subheading 3.2, step 5, results in a dilution factor of 1.25. In highly concentrated samples, or samples with problematic matrix effect, dilution of the samples with PBS to up to 10% of the original concentration might overcome these difficulties.
11. You may also use a centrifugal vacuum concentrator at 30–35 °C.
12. Settings for MS detection: dry gas flow 10 l/min, dry gas temperature 200 °C, nebulizer gas flow 2.0 bar, capillary voltage 4500 V, end plate offset –500 V, ion energy 3.0 eV, and collision energy 8.0 eV. The MS was first calibrated on a reference standard including five masses within a mass range of 100–1600 m/z.

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Chapter 6

Biosensors for the Detection and Quantification of AI-2 Class Quorum-Sensing Compounds

Sathish Rajamani and Richard Sayre

Abstract

Intercellular small-molecular-weight signaling molecules modulate a variety of biological functions in bacteria. One of the more complex behaviors mediated by intercellular signaling molecules is the suite of activities regulated by quorum-sensing molecules. These molecules mediate a variety of population-dependent responses including the expression of genes that regulate bioluminescence, type III secretion, siderophore production, colony morphology, biofilm formation, and metalloprotease production. Given their central role in regulating these responses, the detection and quantification of QS molecules have important practical implications. Until recently, the detection of QS molecules from Gram-negative bacteria has relied primarily on bacterial reporter systems. These bioassays though immensely useful are subject to interference by compounds that affect bacterial growth and metabolism. In addition, the reporter response is highly dependent on culture age and cell population density. To overcome such limitations, we developed an *in vitro* protein-based assay system for the rapid detection and quantification of the furansyl borate diester (BAI-2) subclass of autoinducer-2 (AI-2) QS molecules. The biosensor is based on the interaction of BAI-2 with the *Vibrio harveyi* QS receptor LuxP. Conformation changes associated with BAI-2 binding to the LuxP receptor change the orientation of cyan and yellow variants of GFP (CFP and YFP) fused to the *N*- and *C*-termini, respectively, of the LuxP receptor. LuxP-BAI2 binding induces changes in fluorescence resonance energy transfer (FRET) between CFP and YFP, whose magnitude of change is ligand concentration dependent. Ligand-insensitive LuxP mutant FRET protein sensors were also developed for use as control biosensors. The FRET-based BAI-2 biosensor responds selectively to both synthetic and biologically derived BAI-2 compounds. This report describes the use of the LuxP-FRET biosensor for the detection and quantification of BAI-2.

Keywords Autoinducer, Quorum sensing, LuxP, Ligand, AI-2, BAI-2, DPD, FRET, Biosensor, GFP, CFP, YFP, Dissociation constant, Quantification, Fluorescence

1 Introduction

V. harveyi bioassays for the autoinducer 2 (AI-2) class of QS compounds have been used for over a decade to monitor QS signals in biological samples. *V. harveyi* uses a two-component sensor kinase system to detect autoinducer 1 (AI-1, *N*-(3-hydroxybutanoyl)-*L*-homoserine lactone) and the boron derivative of autoinducer

2 (BAI-2, (2*S,4S*)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuranborate). These QS compounds regulate the expression of genes involved in bioluminescence, type III secretion, siderophore production, colony morphology, and metalloprotease production [1–4]. Until recently, the detection of the BAI-2 class of QS compounds was based on BAI-2-induced bioluminescence using *V. harveyi* bioassays. The BAI-2 bioassay system, however, was notoriously difficult to standardize, takes several hours to complete, and is subject to substantial environmental and biological perturbations [5, 6]. Fluctuations in culture pH, metabolites, and growth inhibitor concentrations can all affect BAI-2 bioassays. These shortcomings necessitated the need for the development of a more rapid, ligand-specific assay for the detection and quantification of BAI-2. With this knowledge we developed an *in vitro* LuxP FRET-based biosensor (CLPY) consisting of a cyan fluorescent protein (CFP) and a yellow fluorescent protein (YFP) fused to the surface-exposed *N*- and *C*-termini of the BAI-2 receptor protein LuxP, devoid of its *N*-terminal periplasmic targeting peptide (23 amino acids).

LuxP belongs to a large family of bacterial periplasmic-binding proteins (bPBP) [7, 8]. The LuxP class of bPBPs is highly conserved among many *Vibrio* species including several potential human pathogens [9]. The bPBPs are ideally suited for the development of FRET-based biosensors with their *N*- to *C*-termini distances between 10 and 100 Å [10, 11]. They typically have two globular protein domains tethered by a flexible hinge region that encompasses the ligand-binding site [8]. Structure-function analyses of bPBPs have demonstrated that the binding of the ligand induces substantial conformational changes in the receptor protein [12–14] including changes in the protein radius of gyration, and the distance between the *N*- and *C*-termini of the protein [7, 8, 12–14].

The CLPY biosensor (MW: 98 kD, Fig. 1) is conveniently expressed in *E. coli* using an inducible T5-promoter/*lac* operator vector construct and purified using an *N*-terminal 6xHistidine tag. Herein, we describe a rapid, highly sensitive, BAI-2 biosensor, CLPY, and accompanying control biosensors (M2CLPY and M3CLPY) for the FRET-based detection and quantification of BAI-2 from biological samples.

2 Materials

2.1 Bacterial Strains and Plasmids

1. *Escherichia coli* BL21 (*luxS*) and *Vibrio harveyi* strains BB120 (wild type), MM30 (*luxS*), and MM32 (*luxS*, *luxN*) (generously provided by Dr. Bonnie L. Bassler—Princeton University).

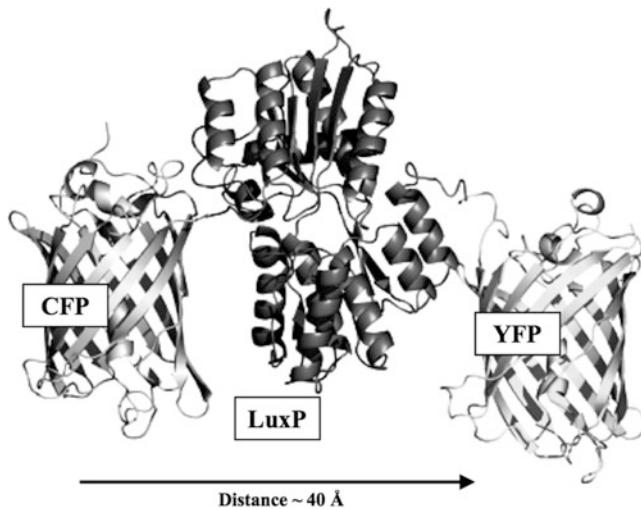


Fig. 1 LuxP protein biosensor (CLPY). Schematic representation of LuxP protein biosensor with CFP and YFP proteins attached to the *N*- and *C*-termini of LuxP. The calculated distance between the fluorophores of ~40 Å is optimum measuring conformational changes within LuxP. The ensemble measure of LuxP-BAI-2 binding induced distance and conformational changes in LuxP is observed by proportional decrease in the CLPY FRET ratio (YFP/CFP fluorescence ratio) changes

2. Strain BL21 (*luxS*⁻) carrying plasmid constructs pQE30-CLPY (wild-type LuxP biosensor), LuxP mutant biosensors: pQE30-M2CLPY (Q77A and S79A) and pQE30-M3CLPY (Q77A, S79A, and W82F) [9].

2.2 Bacterial Culture Media

1. Unless otherwise stated, media, stocks, and solutions are made with deionized distilled water.
2. Luria-Bertani (LB) medium: 5.0 g/l Yeast extract, 10.0 g/l bacto tryptone, and 10.0 g/l sodium chloride are dissolved in water and autoclaved.
3. Luria-Marine (LM) medium [15]: 5.0 g/l Yeast extract, 10.0 g/l bactotryptone, and 20.0 g/l sodium chloride are dissolved in water and autoclaved.
4. Autoinducer bioassay (AB) medium [2]: Prepare solutions A, B, C, and D first as detailed below.

Solution A: 0.3 M NaCl (17.53 g/l), 0.05 M MgSO₄·7H₂O (12.32 g/l), 0.2% casamino acids (2.0 g/l) in 960 ml water. Adjust pH to 7.5 with 10.0 M KOH, autoclave, and cool to room temperature.

Solution B: 1.0 M NaH₂PO₄-Na₂HPO₄ buffer (pH 7.0) is prepared by mixing autoclaved stocks of 1.0 M NaH₂PO₄ (39 ml) and Na₂HPO₄ (61 ml) prepared in water.

Solution C: 50% Glycerol in water, autoclave.

Solution D: 0.1 M Arginine dissolved in water, filter sterilize.

To 960 ml of Solution A, add 10 ml of Solution B, 20 ml of Solution C, and 10 ml of Solution D to make 1.0 l of AB medium.

5. Antibiotics: Stocks of ampicillin (100 mg/ml) and kanamycin (100 mg/ml) prepared in water are used with growth media to the desired final concentrations by diluting the stocks to 1000× fold in growth media (e.g., add 1 ml of antibiotic stock to 1.0 l of growth medium). Before use, stocks are filtered sterilized with 0.22 µm PVDF membrane syringe filter disc. Aliquots of 1.0–5.0 ml are stored at –20 °C.
6. Solid agar media plates: Prepare using 15 g/l of select agar in desired bacterial growth media and autoclave for 20–30 min.
7. Incubator shaker fitted with desired adaptors for bacterial culture growth.

2.3 Protein Overexpression and Purification

1. 1.0 M Isopropyl thiogalactoside (IPTG) is dissolved in distilled water and filter sterilized with 0.22 µm PVDF membrane syringe filter disc.
2. Lysozyme stock of 50 mg/ml in distilled water, stored as 25 µl aliquots at –20 °C.
3. 0.1 M Phenylmethyl sulfonyl fluoride (PMSF) in isopropanol, stored at –20 °C (*see Note 1*).
4. Following stocks in water are prepared and autoclaved: 1.0 M NaH₂PO₄, 1.0 M Na₂HPO₄, and 3.0 M NaCl.
5. A buffer stock of 1.0 M NaH₂PO₄-Na₂HPO₄ (pH 8.0) is prepared by mixing appropriate volumes of 1.0 M NaH₂PO₄ and 1.0 M Na₂HPO₄. For 100 ml of stock add 94.7 ml of Na₂HPO₄ and 5.3 ml of NaH₂PO₄ and check the final pH using pH electrode.
6. 2.5 M Imidazole in water, adjust pH to 7.5 with HCl and filter sterilize. Store at 4 °C.
7. Buffer A (column equilibration/wash buffer): 25 mM NaH₂PO₄-Na₂HPO₄ (pH 8.0), 35 mM NaCl, and 10 mM imidazole.
8. Buffer B (lysis buffer): To buffer A solution add 15 mM 2-mercaptoethanol (2-ME), 1 mM PMSF, and 0.2 mg/ml lysozyme (*see Note 1*).
9. Buffer C (elution buffer): 25 mM NaH₂PO₄-Na₂HPO₄ (pH 8.0), 35 mM NaCl, and 50 mM imidazole.
10. Ni-NTA affinity gel (*see Note 2*) and empty 2.5 × 30 cm (or similar) chromatography column for affinity gel packing.

11. Spectrofluorometer with cuvette holder and integrated with analysis software.
12. Ultrasonic cell homogenizor/sonicator fitted with a microtip.
13. Refrigerated floor centrifuge with rotors, centrifuge tubes, bottle, and adaptors.

2.4 Protein Estimation, Denaturing Gel Electrophoresis, and Fluorescence Detection

1. Bradford protein assay kit: Store at 4 °C.
2. Other components needed for protein assay: Clean 13 × 100 mm test tubes, test tube racks, vortex mixer, filter paper, 1 cm path length cuvettes.
3. Bovine serum albumin (BSA) standard stock solution at 2 mg/ml in water: Store at 4 °C for up to a month or at –20 °C for long-time storage.
4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) procedure is described assuming the use of protein mini gel apparatus setup.
5. Precast 10% SDS-PAGE (*see Note 3*), electrode, protein molecular weight standard, pipettes, and 1–200 µl gel loading tips.
6. SDS-PAGE Tris-glycine electrophoresis buffer (1×): 25 mM Tris, 250 mM glycine, 0.1% (w/v) SDS (pH 8.3) in water. A 5× stock of the above buffer can be prepared by dissolving 15.1 g/l of Tris base and 94.0 g/l of glycine in 950 ml water and 50 ml of 10% (w/v) of SDS. This can be stored at 4 °C until use.
7. In water, 1.0 M Tris-HCl (pH 6.8) and 1.0 M dithiothreitol are prepared for making 2× protein gel loading dye: 100 mM Tris-HCl (pH 6.8), 200 mM dithiothreitol, 4% (w/v) SDS, 0.2% (w/v) bromophenol blue, 20% (v/v) glycerol. 100 µl aliquots can be made and stored at –20 °C.
8. For preparing protein samples for gel electrophoresis, boiling water bath or heat block capable of reaching temperatures of 100 °C.
9. For SDS-PAGE Coomassie Brilliant Blue staining (detection range 100–1000 ng):
 - (a) Coomassie Brilliant Blue Stain solution: 40% (v/v) methanol, 50% (v/v) water, 10% (v/v) acetic acid, 0.025% (w/v) Coomassie Brilliant Blue R-250. This solution can be stored at room temperature.
 - (b) Destain solution: 40% (v/v) methanol, 50% (v/v) water, 10% (v/v) acetic acid. Store at room temperature.
 - (c) Other requirements include a rocking shaker and a gel imager.
10. Spectrofluorometer with integrated analysis software, four-side clear quartz cuvette, or disposable plastic cuvettes for fluorescence measurements.

**2.5 Boron-Depleted
Media and Conditioned
Media Preparation**

1. Amberlite® IRA743 borate-specific chelating resin.
2. Chromatography column 5 cm × 10 cm (or equivalent).
3. Following stocks in water: 3.0 M Ammonium hydroxide, 1.0 M hydrochloric acid, 0.16 M nitric acid, and 10.0 M potassium hydroxide for pH adjustment.
4. 3.0 kDa Membrane cutoff filter with omega membrane: 3 K Microsep™ centrifugal device and 0.2 µm HT Tuffryn® membrane syringe filter.

3 Methods

For best results, the use of borosilicate glassware should be avoided for the entire procedure. BAI-2 is formed from the cyclization of DPD in the presence of borate. It has been previously determined that a DPD:borate ratio of 1:4 leads to a yield of 10% BAI-2 and potentially other borate derivatives of AI-2 (not LuxP ligand) [16]. All the buffer and media preparations should be stored in clean polystyrene/polypropylene containers. Bacterial culturing is done using sterile polystyrene 14 ml culture tubes. To avoid changes in the DPD:borate ratio that potentially alter the detected BAI-2 concentrations the media should be cleaned of any contaminating borate prior to use using the procedure described in Subheading 3.4.

**3.1 Biosensor
Overexpression
and Purification**

1. An overnight (O/N) starter culture of BL21 (*luxS*) cells transformed with pQE30-CLPY or LuxP mutant constructs is started as single-colony inoculum in 14 ml sterile tubes containing 3.0 ml of LB broth supplemented with 100 µg/ml ampicillin (see Note 4). The cultures are grown in a roller drum or shaker O/N at 37 °C (see Note 5).
2. A 500 ml conical flask with 75.0 ml LB broth supplemented with 100 µg/ml ampicillin is inoculated with 1% (v/v) of the above O/N culture (750 µl) to begin second O/N shake cultures (250 rpm) at 28 °C for 16 h.
3. The O/N culture is then transferred to 1.5 l of LB in 4.0 l volume Erlenmeyer flask (5% (v/v) inoculum) supplemented with 100 µg/ml ampicillin.
4. The culture is grown at 28 °C, shaking at 200 rpm. At about 3–4 h the optical density at 600 nm wavelength (OD₆₀₀) is measured using a spectrophotometer. A small volume of culture is then aliquoted into 1 cm path length cuvette and OD₆₀₀ is measured with fresh LB medium as blank control. When the OD₆₀₀ is 0.6, 1 ml of culture is removed and cell pellet collected by centrifugation (benchtop centrifuge—15,500 × g/3 min) and stored at –20 °C for subsequent SDS-PAGE

analysis. The remaining culture is induced for CLPY expression by adding 0.3 mM IPTG and grown for additional 6 h.

5. After 6.0-h growth, 300 μ l of culture is removed to collect the cell pellet by centrifugation for use with SDS-PAGE analysis. The rest of the bacterial cells are then harvested by centrifugation using a floor-top centrifuge at $8000 \times g$ for 10 min and protein purifications carried out at 4 °C (*see Notes 6 and 7*).
6. Cell pellet is resuspended in 35 ml of 25 mM NaH₂PO₄-Na₂HPO₄ (pH 8.0), 35 mM NaCl, 10 mM imidazole, 15 mM 2-mercaptoethanol, and 1.0 mM phenylmethyl sulfonyl fluoride (Buffer B), placed in an ice bath and lysed by sonication as follows. A sonicator fitted with microtip is used for six rounds of sonication with power set at 40 and pulsed ten times with a 1-min pause between each round to allow for cooling.
7. Cell lysate supernatant is separated from cell debris and unlysed cells by centrifugation at $12,000 \times g$ for 20 min. About 200 μ l of clarified lysate is removed and stored at -20 °C for SDS-PAGE analysis (Subheading 3.2). The rest of the clarified cell lysate is then loaded onto a 2.5 cm × 30 cm column containing 7.5 ml bed volume of Ni-NTA affinity gel equilibrated with five column volumes of buffer A.
8. The protein-bound resin is washed with five column volumes of buffer A and eluted by adding three column volumes of 25 mM NaH₂PO₄-Na₂HPO₄ (pH 8.0), 35 mM NaCl, and 50 mM imidazole (Buffer B). In clean tubes, eluate containing biosensor protein (characteristics pale to brighter yellow color) is collected as 3.0 ml fractions.
9. The same column is now ready to be equilibrated and reused or can be stored in 70:30 ethanol:water (v/v) for later use (*see Note 2*).

3.2 Biosensor Quantification and SDS-PAGE Analysis

1. The purified CLPY biosensor fractions are quantified using BioRad protein assay method using BSA as standard. The assay is carried out as detailed in the user's manual with some modifications.
2. The dye reagent is prepared by diluting 1 part dye reagent concentrate with 4 parts distilled water. The solution is filtered through filter paper to remove particulates. This diluted dye reagent can be used for approximately 2 weeks when stored at room temperature.
3. Prepare six dilutions of a protein BSA standard (0.2, 0.4, 0.5, 0.6, 0.8, and 0.9 mg/ml): store at 4 °C or -20 °C for long-term storage.

4. Add 1.0 ml of diluted dye reagent to clean-dry test tubes; add 20 μ l of each standard and CLPY fraction solution and vortex for 5 s.
5. Incubate at room temperature for 5 min and measure absorbance at 595 nm using a spectrophotometer (*see Note 8*).
6. The protein concentration is determined using the standard curve. Typically the protein yields in visibly colored fractions range from 0.1 to 0.3 mg/ml.
7. For SDS-PAGE analysis, a precast 10% (w/v) acrylamide gel is assembled in the minigel apparatus as per the user's manual. Immediately add 1 \times Tris-glycine electrophoresis buffer to fill the gel reservoir to limit idling and drying of the gel. Using both hands carefully remove the comb in a single vertical motion (*see Note 9*).
8. The samples for SDS-PAGE are prepared as follows. Bacterial cell pellet, cell lysate, and purified protein (Subheading 3.1) are left on ice to provide sufficient time for frozen samples to thaw entirely.
9. Resuspend the cell pellets in 50 μ l of water. 50 μ l of clarified cell lysate and purified protein (say concentration ~50 ng/ μ l) are aliquoted in 1.5 ml centrifuge tubes. To all the samples, add 50 μ l of 2 \times SDS-PAGE loading dye and gently mix with the pipette tip. Place the tubes in boiling water bath for 10 min. Remove the tubes and leave at RT to cool. In a benchtop centrifuge spin down (15,500 \times *g*/1 min) the sample tubes that contain bacterial cells. Carefully remove 30 μ l of sample using the gel-loading tip and load onto designated wells. Add 10–15 μ l of protein standard for use as molecular weight marker.
10. Once the sample is loaded, connect the apparatus to the power supply and initially run at 20 mA (about 2 h to allow sample through stacking gel) and then increase to 40 mA for protein separation in running gel. Run the gel until the bromophenol blue loading dye starts running out of the gel. At this point remove the assembly and carefully remove the gel for staining.
11. Place the gel in Coomassie staining solution so it immerses the gel fully. The gel is left on a rocking shaker for 2 h to O/N in the staining solution.
12. The staining solution is transferred to a new container and can be reused later. Rinse the gel with water, destain by adding sufficient volume of destain solution, and let incubate for additional 2–3 h. Following destaining remove two-thirds of the destain solution and replace the volume with water, which allows the shrunk gel (during staining and destaining) to swell back to its original size.
13. The gel image can be captured using a gel imager or document scanner layered with thin transparent plastic foil (Fig. 2a).

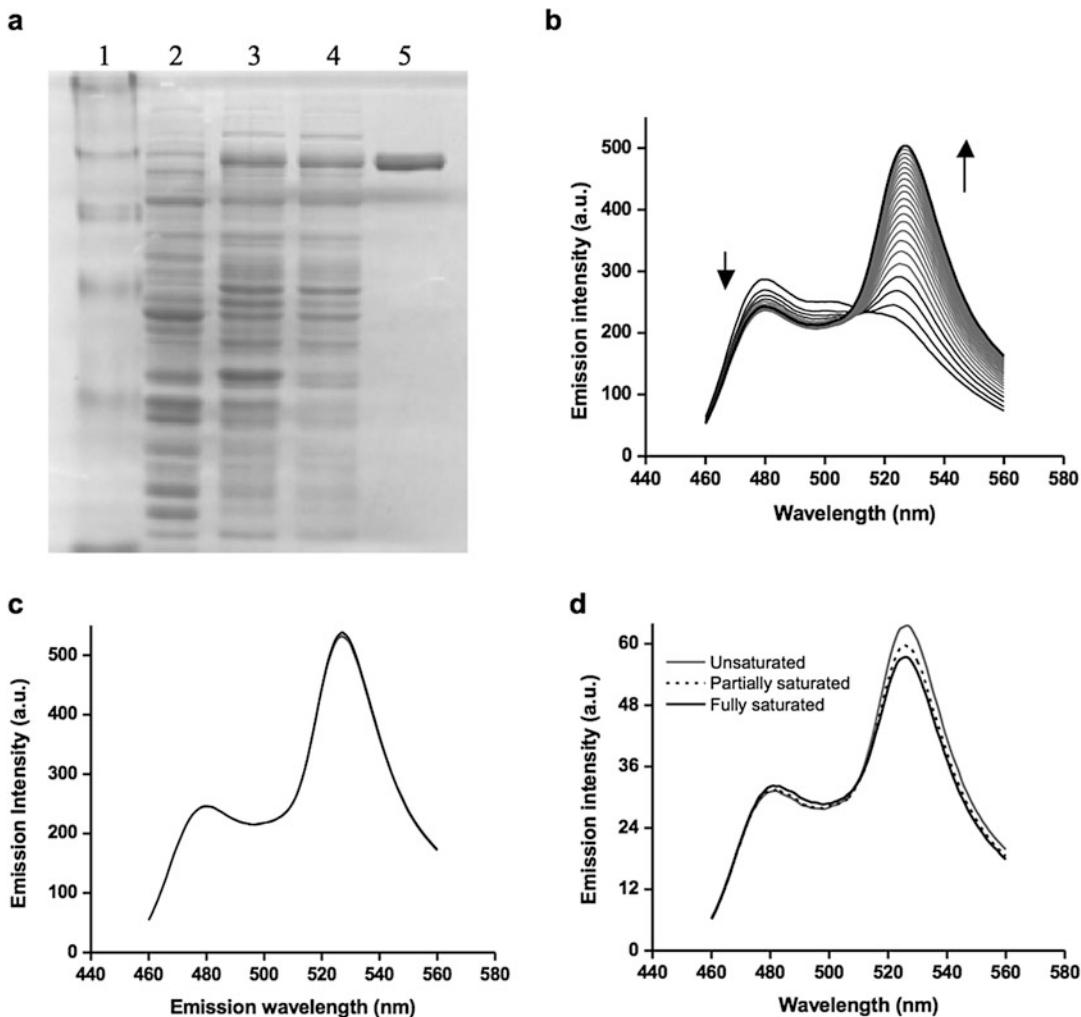


Fig. 2 Characterization of biosensor. (a) SDS-PAGE analysis of CLPY purification. Lanes: 1—see Blue protein standard; 2—uninduced BL21(*luxS*)-pQE30-CLPY; 3–6-h induced BL21(*luxS*)-pQE30-CLPY; 4—cell lysate supernatant; 5—purified CLPY (~98 kD). (b) Time-dependent change in FRET associated with YFP maturation. CLPY protein immediately after purification when monitored at room temperature [λ_{ex} 440 nm (slit 5 nm) and λ_{em} 460–560 nm (slit 5 nm)] shows time-dependent FRET increase. (c) Mature FRET sensor. The purified CLPY stored at room temperature for 5 h or at 4 °C for 48 h showed no time-dependent FRET changes. Shown here is an overlay of several emission spectrum scans recorded for mature CLPY protein over 30 min. (d) CLPY responses to AI-2 ligand. Concentrations: unsaturating (grey line), partially saturating (dotted black line), and fully saturating (black line) concentrations of AI-2 ligand

3.3 Biosensor Characterization and Fluorophore Maturation

Fluorescence measurements are carried out at room temperature using spectrofluorometer set in a scanning mode. CLPY and LuxP mutants are monitored by CFP excitation (λ_{ex} 440 nm/slit 5 nm) and the emission spectrum (λ_{em}) is measured from 460 to 560 nm using a 5 nm slit width. Alternatively, the FRET ratio (527 nm/485 nm) is recorded using an excitation wavelength of 440 nm.

1. A ligand-binding independent increase in FRET is observed with recently purified biosensor (Fig. 2b). This is due to the delayed maturation of YFP over CFP, which is characterized by faster fluorophore maturation.
2. To eliminate this problem, immediately after purification, the biosensor should be left for maturation at RT (5 h) or 4 °C (2 days) before beginning ligand binding studies.
3. Following this step, the FRET change does not happen in the absence of the ligand (Fig. 2c). At this time the biosensor is ready for use for BAI-2 ligand binding studies.
4. The biosensor response to the detection of BAI-2 is characterized by a decrease in the FRET ratio (YFP/CFP ratio). This decrease is proportional to the amount of BAI-2 ligand concentration present in a given test sample. As reported elsewhere [9], Fig. 2d shows a representative spectrum of biosensor responses to unsaturating, partially saturating, and fully saturating BAI-2 concentrations in test sample.

3.4 Boron-Depleted Media Preparation

1. The protocol is adapted from Bennett et al. [17], with some modifications.
2. 30 ml of Amberlite® IRA743 resin is used per liter of media. Here we use AB media as an example for describing the procedure.
3. The column is treated with 150 ml 3.0 M ammonium hydroxide, 600 ml distilled water, 180 ml of 1.0 M hydrochloric acid, 150 ml distilled water, 180 ml of 0.16 M nitric acid, and 300 ml distilled water.
4. Following these treatments, a liter of AB medium is passed through the column and the pH adjusted to 6.75 using 10 M potassium hydroxide.

3.5 BAI-2 Ligand Detection and Quantification from *V. harveyi* Cultures

1. As an example, we show here the determination of *V. harveyi* BAI-2 ligand concentrations as a function of culture age. This procedure can be suitably followed for the determination of BAI-2 from other *Vibrio* strains and other bacteria that synthesize and use BAI-2 as a signal molecule.
2. *V. harveyi* BB120 (wild-type) grown overnight at 28 °C for 16 h is used to make 2% (v/v) inoculum in fresh AB medium (2.0 ml, 0.5 mM borate) in round-bottom polystyrene tubes. Set up 36 tubes of 2.0 ml cultures for using three tubes at any given time point.
3. For this experiment, every 2.5 h (including the 0-h time point) the cell density is determined by plating serial dilutions of *V. harveyi* cultures on LM agar plates. Before removing cultures for plating, the culture tubes are vortexed for 10–15 s to

eliminate any visible cell clumps. The dilutions are made in fresh LM medium and different volumes are plated out on LM agar (*see Note 10*). The agar plates are left at 30 °C for 24 h before counting the colonies.

4. At the same time the BB120 cell-free supernatant is collected using a refrigerated benchtop centrifuge ($15,500 \times g/5$ min).
5. The supernatant is collected, and to remove proteins (proteases) and cell debris, it is passed through 3 K Microsep™ centrifugal device, pretreated with 2.0 ml water by centrifugation at $5000 \times g$ for 20 min (*see Notes 11 and 12*).
6. The flow through of the culture supernatant containing the BAI-2 signal is collected after centrifugation at $5000 \times g$ for 30 min.
7. Working stock of 0.015 mg/ml of CLPY is made in buffer C. For triplicate measurements three different batches of CLPY preparations are used.
8. In a 1.5 ml centrifuge tube aliquot 1.0 ml of 0.015 mg/ml of CLPY and leave at room temperature for 15 min to equilibrate.
9. The YFP/CFP fluorescence or FRET ratio (527 nm/485 nm) response of CLPY for the given sample is measured at room temperature after 5-min incubation of the biosensor with the sample. The sample is mixed with 1.0 ml of CLPY by gently inverting the tube 4–5 times and incubating before transferring the contents to a cuvette for FRET measurements (*see Notes 13 and 14*).
10. For instance 150 µl of *V. harveyi* BB120 culture filtrate is mixed with 1.0 ml of CLPY and used for generating a graph representative of FRET response versus time and cell number. This assay provides an indication of the BAI-2 concentration in the medium as a function of culture age (Fig. 3a).

3.6 BAI-2 Quantification from *V. harveyi* Cultures

1. For a given culture age fixed volume of diluted culture filtrate is added to the CLPY biosensor (0.015 mg/ml).
2. For instance to determine the concentration of BAI-2 from 7.5-h-old 2% (v/v) *V. harveyi* in AB medium (0.5 mM borate), 200 µl of various dilutions of culture supernatant (prepared as detailed in the previous section) is made in borate-free AB medium (Subheading 3.3) and added to CLPY (1.0 ml, 0.015 mg/ml) to generate a biosensor response saturation curve. The following volumes, 5, 10, 20, 40, 60, 80, 100, 120, 150, and 200 µl, are made to 200 µl with borate-free AB medium and used for generating the CLPY biosensor saturation curve.
3. The biosensor response curve is plotted as a function of dilution of cell supernatant versus the FRET ratio response

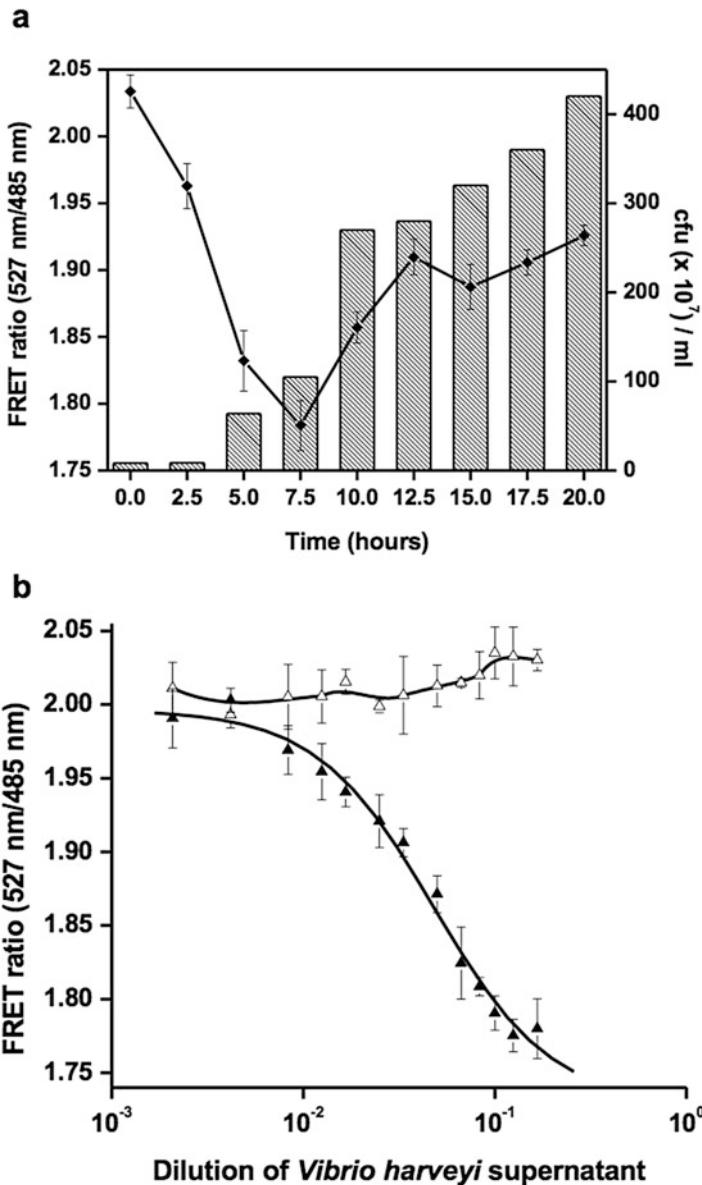


Fig. 3 Quantification of BAI-2 from *Vibrio harveyi* cultures using the CLPY biosensor. (a) Wild-type *V. harveyi* BAI-2 levels monitored as a function of time and culture density. Cell-free culture filtrates were prepared by passing the bacterial cell culture media through a 3 kDa MW cutoff filter. The filtrate (150 μ l) was added to CLPY (0.015 mg/ml). The YFP/CFP FRET ratio (closed diamonds) is plotted as a function of cell density ($\text{CFU} \times 10^7/\text{ml}$; bars). The error bars represent the standard deviations from three independent experiments. (b) CLPY fluorescence response to culture filtrate from wild-type (BB120) *V. harveyi* (closed triangles) and *luxS* mutant (MM30, open triangles) culture filtrate. The error bars represent the standard deviations from three independent experiments (In this Figure a and b, Adapted with permission from [9]; Copyright[©] 2007 American Chemical Society)

(Fig. 3b, *x*-axis shown in \log_{10} scale). The dilution volume for each data point is determined by dividing the volume of conditioned medium used (do not include the volume of fresh borate-free AB medium used for diluting) by the total volume of the FRET assay mix (this volume will remain the same when calculating all the dilutions).

4. Dilutions in borate-free AB medium help maintain the ligand DPD and boron ratios the same in the bacterial growth medium. This is important since changes in DPD and boron ratio might affect the equilibrium of BAI-2 ligand.
5. Cell-free supernatant from 7.5-h-old *V. harveyi* MM32 (a *luxS* mutant devoid of BAI-2 synthesis) culture is used as a control (Fig. 3b) (see Note 15).
6. Using a software program (such as “OriginLab”) capable of fitting a nonlinear regression relationship shown in Eq. 1, the CLPY response to various dilutions of *V. harveyi* supernatant “v” can be used for determining half saturation volume “h,” where “R” is the observed CLPY FRET ratio response for different dilutions of *V. harveyi* supernatant “v,” with “R_{max}” and “R_{min}” representing the ligand-free and ligand-saturated FRET ratios, respectively:

$$R = R_{\max}(((R_{\max} - R_{\min}) \times n \times v) / (h + v)) \quad (1)$$

7. By fitting the above equation, the “h” value is determined and used for calculating the unknown BAI-2 concentration “M” using the following relationship given in Eq. 2. As reported previously, the BAI-2 and LuxP binding affinity “K_d”—270 nM [9] is used for determining “M” in a given culture supernatant:

$$M = K_d/h \quad (2)$$

8. Other controls for these experiments include using M2CLPY and M3CLPY biosensors to confirm if the CLPY FRET response is specifically due to LuxP-BAI-2 binding (see Note 16).

4 Notes

1. PMSF has a short half-life in aqueous solutions (~30 min at pH 8.0). PMSF should be added to the lysis buffer immediately before initiation of cell lysis to avoid loss of PMSF protease inhibitory activity.
2. Ni-NTA affinity resin can be reused 3–4 times depending on the column condition. Once the column looks faded (initially

bright nickel blue), the resin can be cleaned and recharged with nickel for reuse. Following is a modified procedure from Qiagen Inc. that is used regularly for obtaining good protein purification (refer to their procedure for more stringent treatment). Our modified procedure includes the following steps: wash resin with one column volume of water, followed by five column volumes of 100 mM Na-EDTA (pH 8.0) to remove nickel and bound impurities and three column volumes of water and recharge the resin with two column volumes of 100 mM NiSO₄; wash with two column volumes of water; and equilibrate with three column volumes of Buffer A before use. Always store the column in small volumes of buffer for short-time storage or with 70% ethanol for long-time storage.

3. Precast gels are recommended to be used within a certain period of time as designated by the manufacturer. Also, in case if there is a need, SDS-PAGE gels can be conveniently prepared in the lab following published procedures. We recommend you refer standard laboratory manuals, e.g., Molecular Cloning: A laboratory manual by E.F. Fritsch, J. Sambrook, and T. Maniatis (1989) for detailed procedure.
4. In case the *E. coli* strain BL21(*luxS*) is not available DH5 α can be used. However, appropriate protease inhibitors should be added, as DH5 α is not a protease-deficient strain unlike BL21 derivatives.
5. A single-colony culture can be used to make 15% glycerol stock and stored at -80 °C. For starting the cultures, scrape out small amount of frozen stock into fresh media. Care must be taken not to freeze-thaw stock vials that may result in loss of cell viability.
6. At this point the culture flasks can be moved to 4 °C and left standing O/N before harvesting. Next day, the cells can be noticed to have settled to the bottom. The culture flask can be gently removed and ~500 ml of the culture can be decanted. The rest of the culture can be resuspended in the remaining media and the cells harvested using a refrigerated centrifuge set at 4 °C.
7. The cell pellet can be stored at -80 °C for prolonged periods of time without thawing. To thaw the cells, remove the cell pellet from -80 °C and leave it O/N at -20 °C. Transfer to ice next day to enable slow thawing of cells.
8. It is important to make the protein assay reads within minutes of each other. Over time the response intensifies, so care must be taken not to incubate for a prolonged time. It is also recommended to go through user's manual for troubleshooting.

9. To have good sample runs clean the wells carefully. Using a pipette set at 100 μl and fitted with 1–200 μl gel-loading tip, pipette in electrophoresis buffer into wells (3–5 times) to remove any residual polymerized acrylamide on the loading well walls that might stick to sample and create a drag during sample run.
10. Under the given growth conditions for *V. harveyi* BB120, dilutions ranged from 10^6 -fold at start (0 h) to 5×10^7 -fold at 10 h and later. It is recommended that a pilot experiment be conducted to determine the dilution series required for the strain of bacteria you are working with before you take up the biosensor quantification.
11. Filtering and centrifugal devices fitted with OmegaTM and HT Tuffryn membranes (Pall Life Sciences) were found to have less interference with BAI-2 ligand sticking to membrane. So care must be taken with the choice of filtration/centrifugation device to identify the best membrane for this application.
12. If later time point cultures have cell debris that clogs the 3 kD membrane, try including an additional step of passing the cell supernatant through 0.2 μm HT Tuffryn[®] membrane syringe filter before 3 kD membrane filtration.
13. Care must be taken not to vortex the contents; this may result in CLPY protein denaturation and loss of biosensor functionality.
14. The assay has also been successfully adapted for use in a 96-well plate format [18]. Such adaptation could increase the number of assays that can be run from a single CLPY biosensor preparation and has potential for use in high-throughput assays.
15. In case the bacterial strain you are working with has no *luxS* deletion derivative, try using borate-free AB medium-grown culture filtrate as control.
16. Chloride ions (Cl^-) can affect YFP fluorescence [19]. You will note that the FRET ratios are lower in the presence of chloride due to YFP fluorescence (CFP remains unaffected). So care must be taken to minimize changes in buffer and media concentrations between samples. FRET ratio of mature CLPY with the choice of bacterial media can be initially determined as your reference FRET ratio for the experiments involving culture supernatants. For spent media experiments, to achieve same chloride ion concentrations in all dilutions of test samples, use desired volumes of fresh media with spent media and monitoring the biosensor response.

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Chapter 7

Detection of *Agr*-Type Autoinducing Peptides Produced by *Staphylococcus aureus*

Ewan J. Murray and Paul Williams

Abstract

Strains of the Gram-positive pathogen *Staphylococcus aureus* can be divided into four quorum sensing (QS) groups. Membership of each group is defined by the amino acid sequence of the autoinducing peptide (AIP) QS signal molecule that is encoded within the *agrBDCA* genetic locus and specifically within *agrD*. This chapter describes the use of simple, in-cell, *lux*-based, bio-reporters that can be used to identify/confirm the specific *agr* group to which a particular *S. aureus* isolate belongs, as well as to assess the timing and quantity of AIP produced.

Key words *Staphylococcus aureus*, *Agr*, Bio-reporter, Autoinducing peptide, Quorum sensing

1 Introduction

S. aureus is a human pathogen of major clinical concern [1]. The emergence of multi-antibiotic-resistant *S. aureus* and other clinically relevant bacteria has led to calls for the development of new antibacterial strategies that target nonessential cellular processes that would reduce the selective pressures that drive the emergence of antibiotic-resistant isolates [2]. Bacterial cell-to-cell communication or quorum sensing (QS) is one such nonessential process that has been targeted in an attempt to generate novel antibacterial agents. This is because QS regulates virulence factor production in many different pathogens. Ideally, any new therapy that targets QS should silence bacterial communication, thus blocking the production of virulence factors and reducing disease, and so offer the host immune system extra time to clear an infection [3].

S. aureus and related Gram-positive bacteria including the coagulase-negative staphylococci, enterococci, clostridia, and listeria all use an autoinducing peptide (AIP)-based QS system to “sense” their local population density [4–6]. In *S. aureus*, AIP-dependent QS incorporates a typical auto-activation circuit that is encoded by the *agrBDCA* genetic locus. The timing of activation of

agr varies between different *S. aureus* isolates but tends to initialize in mid-exponential phase after the external concentration of the AIP reaches a threshold concentration. The four-gene *agrBDCA* operon encodes a classical two-component signaling system; AgrC, a sensor kinase, auto-phosphorylates upon binding of its cognate AIP. The phosphoryl group is transferred to AgrA, a cytosolic transcriptional response regulator, resulting in a conformational change that enhances the binding of AgrA to specific DNA promoter elements. For example, phosphorylated AgrA binds to and enhances expression from the divergent P2 and P3 promoters. P2 regulates expression from the *agr* operon while P3 regulates expression of RNAIII, a regulatory RNA known as the “effector” of virulence factor production in *S. aureus*. The final two genes of the *agr* operon encode the pro-peptide AgrD and the integral membrane endopeptidase AgrB. AgrB cleaves the carboxy tail of AgrD and also catalyzes the cyclization of AgrD via the formation of a thiolactone bond. The mature cyclic AIP is exported from the cell by an as-yet unknown secretion mechanism [5, 7, 8].

S. aureus can be separated into four *agr* groups (I–IV) where *agr* classification is dependent on the primary amino acid sequence of the cognate AIP [9]. AIPs from *agr* groups I and IV, i.e., AIP1 and AIP4, differ by just one amino acid yet maintain a high degree of selectivity for their respective AgrC receptor. However, AIP1 can weakly activate AgrC4 and vice versa [10]. The amino acid sequence and therefore the structures of AIP2 and AIP3 have diverged so that they only activate their cognate AgrC receptor (Fig. 1). Moreover, non-cognate AIPs display competitive, cross-group antagonism, a phenomenon that has been shown *in vivo* to prevent disease progression [9, 11]. Subsequently, the cross-group inhibition phenomenon has been successfully exploited and a number of AIP mimetics that display potent cross-group antagonism have been synthesized [10, 12, 13]. Although AIP mimetics show

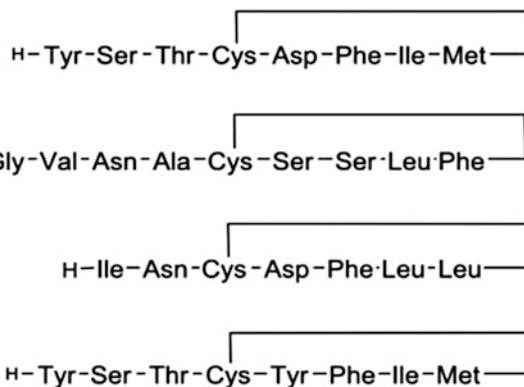


Fig. 1 Chemical structure of AIPs 1–4

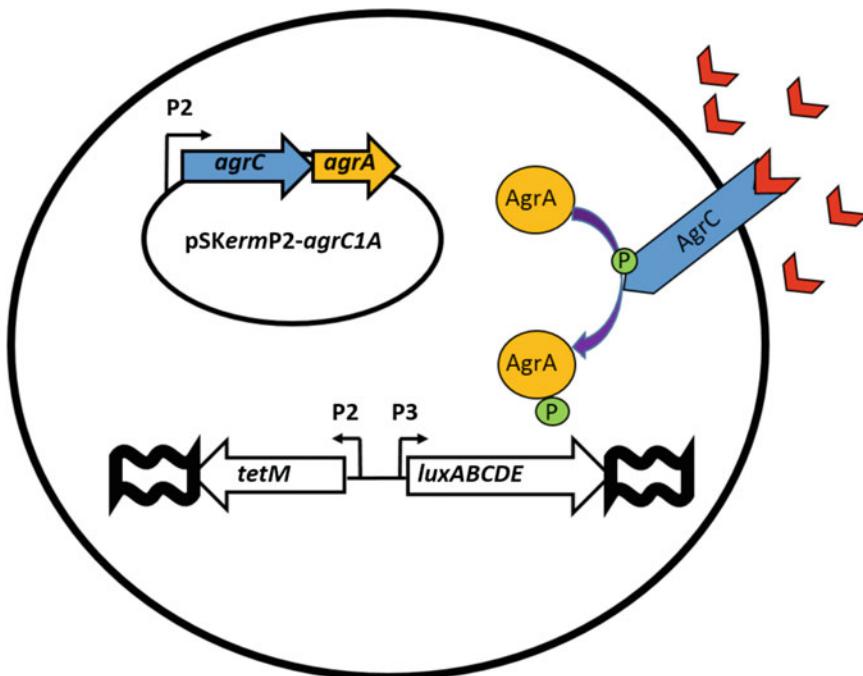


Fig. 2 Schematic representation of *S. aureus* reporter strain ROJ48 harboring the expression construct pSKermP2-*agrC1A*. Incubation with exogenously supplied AIP-1 (red chevron) from either filtered culture supernatant or a synthetic supply drives AgrA phosphorylation. Phosphorylated AgrA binds to and further increases expression from both the P2 and P3 promoters resulting in the production of bioluminescence

great antivirulence potential in vitro, their efficacy in vivo has yet to be determined.

Since QS in *S. aureus* is an excellent model system for AIP-based signaling and as inhibition of *S. aureus* QS has therapeutic potential, methods to assess AIP production and inhibition are essential. Although AIPs can be detected directly in culture supernatants by LC-MS, the cost of equipment and the required technical expertise can be prohibitive [14]. Here, the use of simple and relatively inexpensive *lux*-based bio-reporters, genetically engineered so that they emit light in a concentration-dependent manner only when provided with an exogenous AIP (Fig. 2) [15], is described. These assays can be used to identify the *agr* group to which a given strain belongs, as well as to assess the kinetics of AIP production and the concentration of AIP produced by a given *S. aureus* strain.

2 Materials

2.1 Culture Medium

1. Brain heart infusion broth (BHI).
2. Brain heart infusion agar: BHI broth plus 1.5% (wt/vol) agar.
3. Antibiotics: Chloramphenicol 10 mg/ml in ethanol (1000× stock concentration).

2.2 Reporter Strain and Plasmids (See Note 1)

- Strain ROJ48: RN4220 $\Delta agr::ErmB$ p $\Delta agr-lux$ (integrated as a single crossover); tetracycline (tet^R) and erythromycin (erm^R) resistant [15].
- Plasmids required to generate bio-reporters for *agr* groups 1, 2, 3, and 4 are available upon request from this laboratory: pAgrC1*agrA*, pAgrC2*agrA*, pAgrC3*agrA*, and pAgrC4*agrA*, all chloramphenicol (cml^R) resistant [15].

2.3 AIP Sample Preparation

- 1 ml of culture supernatant to be tested.
- 0.22 μ m Sterile filter with syringe attachment.
- 1 ml Sterile syringes.
- Sterilized 1.5 ml microfuge tubes.

2.4 Reporter Assay for Detection and Quantification of AIPs 1–4 from Cell Free Culture Supernatants

- The appropriate *agr* group reporter.
- Microtiter plate: Sterile, 96 well, black, 200 μ l well volume, transparent flat bottom.
- AIP1–4: To be used as a standard (synthesized to >95% purity) dissolved 1 mM in dimethyl sulfoxide (DMSO) (see Note 2). Filtered culture supernatant of a strain with a known *agr* group can also be used as a positive control if synthetic AIP is unavailable (see Note 1).
- Plate reader: Multi-mode plate reader capable of optical density (OD_{600}) and luminescence [relative light unit (RLU) detection].
- Sample: Filtered supernatants to be tested.

3 Methods

3.1 Collection of *S. aureus* Cell-Free Culture Supernatants for Assays of AIP Production and Kinetics

- Streak the *S. aureus* strain(s) to be tested for AIP production, and include strains of known *agr* groups as their supernatants will act as positive and negative controls if synthetic AIP(s) are unavailable (see Note 1 for *agr* groups of common lab strains), onto a BHI agar plate using standard microbiological sterile technique. Incubate agar plates at 37 °C overnight.
- Inoculate a single colony of the strain to be tested, as well as appropriate *agr* group control strains if synthetic AIP is unavailable, into 5 ml BHI broth. Incubate at 37 °C overnight with shaking, 200 rpm.
- Remove a 1 ml sample from each overnight culture, pellet by centrifugation (14,000 $\times g$ for 1 min), and discard the supernatant. Wash the cell pellets three times by successive rounds of centrifugation and suspension in fresh sterile medium (1 ml) (see Note 3).

4. Inoculate the washed cells (25 µl) into 10 ml BHI in a sterile 50 ml Falcon tube.
5. Grow cells at 37 °C, 200 rpm, and measure optical densities (OD₆₀₀) at regular intervals to monitor growth. At selected time points, remove 1 ml samples and immediately filter through a 0.22 µm filter.
6. Strains used as *agr* group controls are grown until stationary phase (≥ 6 h in the growth conditions described). Supernatants should be collected and filtered as described above.
7. Use supernatants directly (Subheading 3.2) or store at -20 °C until required.

3.2 Semiquantitative Reporter Assay for AIPs in Culture Supernatants

1. Streak the specific *agr* reporter strain required onto a BHI agar plate supplemented with 10 µg/ml chloramphenicol, and incubate the plate overnight at 37 °C. If the *agr* group of the strain is unknown all four *agr* reporters may be used in parallel to identify the correct *agr* group (see Note 3).
2. Inoculate a single colony of the reporter strain in 5 ml BHI supplemented with 10 µg/ml chloramphenicol and incubate overnight at 37 °C with shaking, 200 rpm.
3. Dilute the overnight culture 200-fold in 10 ml BHI supplemented with 10 µg/ml chloramphenicol and incubate at 37 °C with shaking for 1 h.
4. Thaw frozen test supernatants.
5. Prepare a series of AIP-I stock solutions over a range of concentrations in BHI. Add 10 µl of each concentration in triplicate to a sterile 96-well microtiter plate. Include a negative control, e.g., 10 µl of BHI (see Notes 4–6). If the appropriate synthetic AIP is unavailable, supernatant from a strain with a known *agr* group can be used as a positive control.
6. Add 10 µl of each supernatant collected from Subheading 3.1 in triplicate, into the empty wells of a sterile, black, 96-well microtiter plate.
7. After 1 h of growth, dilute the reporter strain tenfold in fresh, pre-warmed BHI to a final volume of at least 25 ml (see Note 7).
8. Use a multichannel pipette to add 190 µl of diluted reporter strain culture to all 96 wells of the microtiter plate (see Note 6).
9. Use a suitable multi-mode plate reader to measure the OD₆₀₀ and RLU every 15 min for a period of up to 10 h at 37 °C (see Note 8).
10. Use a suitable statistical software package (see Note 9) to plot RLU/OD₆₀₀ against time so that peak values can be obtained, Fig. 3a.

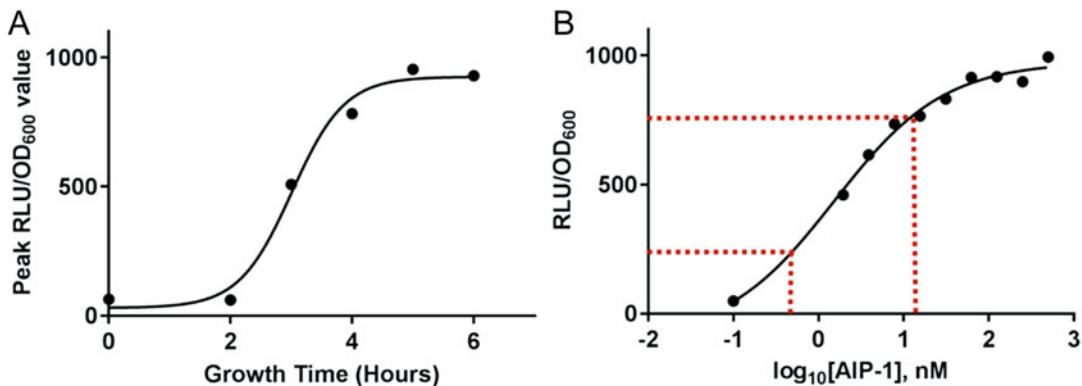


Fig. 3 (a) P3 lux expression as an indicator of AIP production by *S. aureus* over time, peak values can be used in combination with the calibration curve shown in b to extrapolate AIP concentration in the tested sample. (b) AIP calibration curve. Dashed red lines indicate linear section that can be used to estimate AIP concentration

11. Use a suitable statistical software package (see Note 9) to draw a standard curve of AIP concentration relative to maximum RLU/OD₆₀₀, Fig. 3b.
12. Extrapolate the AIP concentration for each sample using the linear section of the AIP standard curve, Fig. 3a, b. Multiply the concentration 20-fold to account for the dilution of supernatant (5%) in the reporter assay. This should give an estimate of the AIP concentration at a given time point during growth, allowing comparisons to be made between different strains and mutants and growth conditions.

4 Notes

1. The reporter strain ROJ48 described in this protocol is suitable for detecting AIPs from all *agr* groups when combined with the correct AgrC(X)A plasmid. Reporter strain and plasmids for all *agr* groups are available from our laboratory on request. Examples of strains which belong to each *agr* group can therefore be used as positive or negative controls in reporter assays:
 - Group 1: 8325, USA300, SH1000.
 - Group 2: Mu50, Mu3.
 - Group 3: MN8, MW2.
 - Group 4: RN4850, H560.
2. We routinely purchase synthetic AIPs from Cambridge Research Biochemicals.
3. To determine whether a particular *S. aureus* strain can produce AIP or to identify the *agr* group to which a given strain belongs to, supernatant prepared from an overnight culture is adequate

as an activator of the reporter assay. DNA sequencing of the *agrD* gene can be utilized to identify the specific *agr* group a strain belongs to; however, this will not confirm that the strain is an AIP producer.

4. A sufficient number of AIP concentrations should be used to generate a smooth EC₅₀ curve. A minimum of eight different concentrations (0.1, 0.5, 1, 10, 50, 250, 500, and 1000 nM) is recommended. N.B. the AIP added to the plate must be 20 times more concentrated. For example, to generate a final concentration of 50 nM in the plate, 10 µl of a 1 µM AIP stock solution must be used.
5. High concentrations of solvents such as DMSO can affect the *lux* reaction. For that reason, the DMSO concentration is maintained constant in all samples ($\leq 0.5\%$) in the microtiter plate assay.
6. The 60 internal wells of a 96-well plate are used for samples. This limits the loss of sample volume by dehydration that occurs in the outside wells of the 96-well plate. Outside wells should be filled with sterile media. If you are attempting to quantify AIP, a standard curve must be generated using synthetic AIP for each individual plate/experiment.
7. Chloramphenicol is not required to maintain the pAgrC(X)A plasmid from this point onwards in the assay.
8. Plate readers from different manufacturers have different sensitivities; therefore detection parameters should be optimized to suit the specific equipment available.
9. We routinely use the software package Graphpad Prism for data analysis.

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Chapter 8

Ultra-Performance Liquid Chromatography/Mass Spectrometry for the Detection and Quantification of Diffusible Signal Factor (DSF) Family Quorum-Sensing Signals

Lian Zhou, Shuang Sun, Wei Zhang, and Ya-Wen He

Abstract

Molecules of the diffusible signal factor (DSF) family represent a class of widely conserved quorum-sensing signals used by many Gram-negative bacterial pathogens. The measurement of DSF family signals is essential for understanding their biological roles, signaling pathway, and regulatory network. We are presenting here methods for extraction and purification of DSF family signals from culture supernatants, and further quantification of members of DSF family signals using an ultra-performance liquid chromatographic system (UPLC) coupled with an accurate time-of-flight mass spectrometry (TOF-MS) analysis.

Key words Quorum sensing, Diffusible signal factor, *Xanthomonas*, Quantification, Ultra-performance liquid chromatographic system, Mass spectrometry

1 Introduction

The diffusible signaling factor (DSF)-based quorum-sensing (QS) system, which was initially identified in the plant bacterial pathogen *Xanthomonas campestris* pv. *campestris* (*Xcc*), has emerged as a widely conserved cell-cell communication mechanism in diverse Gram-negative bacteria, such as *Xylella fastidiosa*, *Lysobacter enzymogenes*, *Stenotrophomonas maltophilia*, *Burkholderia cepacia*, and *Pseudomonas aeruginosa* [1, 2]. Typically, the DSF family signals are *cis*-2-unsaturated fatty acids (Fig. 1), which regulate diverse biological functions such as bacterial virulence, biofilm formation, antibiotic resistance, adaptation, and fitness. DSF activity was first detected based on its ability to restore endoglucanase production in a *Xcc rpfF* mutant [3]. The detection system was further improved by fusing the promoter of an endoglucanase gene to the GUS (β -glucuronidase) reporter gene [4, 5]. By using this improved

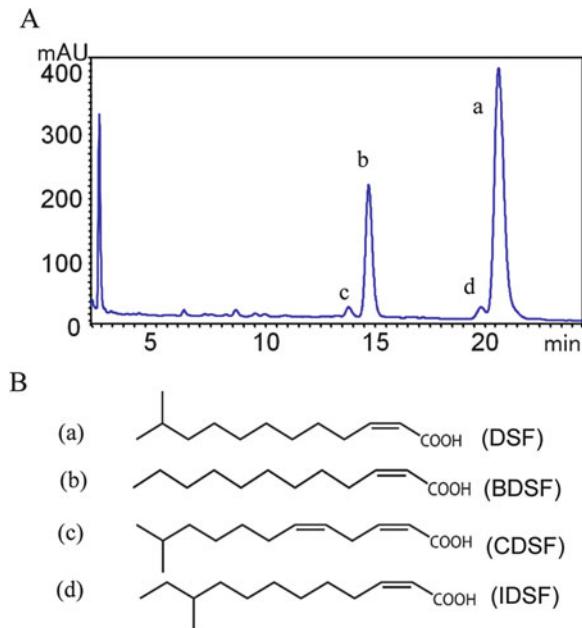


Fig. 1 HPLC chromatogram of ethyl acetate extract of the culture supernatant of the DSF hyperproduction mutant *ΔrpfC* of *Xcc* strain XC1. (a) Four members of DSF family QS signals are detected in the supernatant of *ΔrpfC*, among which DSF and BDSF are the major signal molecules. (b) The chemical structures of DSF, BDSF, CDSF, and IDSF

DSF biosensor and the *Xcc* DSF overproduction mutant *ΔrpfC*, methods for DSF extraction and purification were established [5, 6].

Biosensor-independent methods for DSF extraction and purification were also established [2, 7]. In order to improve detection sensitivity, a quantitative detection of DSF family signals via LC-MS was developed [8]. The condensed crude extracts from *Xcc* supernatants were separated by an ultra-performance liquid chromatographic (UPLC) system on a C₁₈ reverse-phase column, followed by negative electrospray ionization (ESI) and detection by a time-of-flight mass spectrometry (TOF-MS) analysis. As a result, the minimal detectable level of DSF or BDSF was reduced to as low as 1 μM. This allowed fast and convenient determination of DSF and BDSF levels in bacterial cultures and reaction mixtures [8, 9].

2 Materials

Prepare all solutions using ultrapure water (prepared by purifying deionized water, to attain a sensitivity of 18 MΩ·cm at 25 °C) and analytical grade reagents. Prepare and store all reagents at room temperature (unless indicated otherwise). Diligently follow all waste disposal regulations when disposing waste materials.

2.1 Bacterial Cell Cultures

1. Bacterial strain: The methods presented here use the *rpfC* mutant ($\Delta rpfC$) of the Gram-negative bacterium *X. campestris* pv. *campestris* (*Xcc*) strain XC1 [10]. The mutant strain $\Delta rpfC$ overproduces DSF family signals (see Notes 1 and 2).
2. NA broth: 5 g/l Peptone, 3 g/l beef extract, 10 g/l sucrose, 1 g/l yeast extract. Fill up to 1 l with bidistilled H₂O and adjust the pH to 6.8 with NaOH. Sterilize the medium by autoclaving for 20 min at 115 °C.
3. NA agar: NA broth plus 15 g/l agar. Also in this case fill up to 1 l with bi-distilled H₂O and adjust the pH to 6.8 with NaOH. Sterilize the medium by autoclaving for 20 min at 115 °C.
4. 50 ml and 250 ml glass Erlenmeyer flasks.
5. Heated/refrigerated shaker.
6. 1.5 ml Cuvette.
7. UV-visible spectrophotometer.
8. Rifampicin stock: 50 mg/ml in ethanol.

2.2 Extraction of DSF Family Signals

1. pH test strips (pH 0.0–6.0).
2. 1.5, 5, and 50 ml centrifuge tubes.
3. Refrigerated centrifuge capable of 13,800 × *g* and solvent-resistant tubes.
4. Ethyl acetate (ACS reagent grade, purity ≥99.5%) containing 0.1% (v/v) acetic acid (store in the dark in volatile chemical storage).
5. Rotary evaporator or centrifugal evaporator.
6. Circulation cooling-water system.
7. Vortex mixer.
8. 0.2 µm Syringe filters.
9. Acetic acid (ACS reagent grade, purity ≥99.7%).

2.3 DSF and BDSF

DSF (*cis*-11-methyl-dodecenoic acid) (HPLC grade, purity ≥90.0%); BDSF (*cis*-2-dodecenoic acid) (HPLC grade, purity ≥90.0%) (see Note 3). The stock solutions (5 mM) are prepared using methanol as solvent. The stock solution is further diluted using methanol to get a range of diluted DSF or BDSF solutions (1 µM, 5 µM, 10 µM, and 50 µM, respectively) for UPLC analysis.

2.4 UPLC/MS Analysis

1. 1.5 ml Tubes.
2. HPLC screw-cap vials.
3. Polypropylene flat-bottom inserts for HPLC screw-cap vials.
4. Pipette tips (volume ranges 50–1000 µl).

5. Zorbax Eclipse XDB-C18 reverse phase column (Analytical, 4.6 × 150 mm, 5-Micron).
6. -20 °C Refrigerator.
7. UPLC/MS system: UPLC system coupled with an accurate mass TOF-MS equipped with a Jet Stream (JS) electrospray ionization (ESI) source.
8. Diode array detector (model: G4212A).
9. Software: MassHunter Workstation Data Acquisition Software (revision B.04).
10. Methanol (HPLC grade).
11. Mobile phase or elution buffer for UPLC analysis: 80:20 methanol:water (v/v).

3 Methods

3.1 Preparation of *Xcc* Cultures

1. Streak the *Xcc* mutant strain $\Delta rpfC$ on NA agar plate supplemented with 50 µg/ml rifampicin. Incubate the plate at 28 °C for 3 days (*see Note 4*).
2. Pick a single colony and inoculate in 10 ml of NA broth supplemented with 50 µg/ml rifampicin in a 50 ml Erlenmeyer flask. Incubate the culture at 28 °C with shaking at 200 rpm for 36 h.
3. Measure the optical density at 600 nm wavelength (OD₆₀₀) of the $\Delta rpfC$ pre-culture with a spectrophotometer. Adjust the OD₆₀₀ of the pre-culture to approximately 1.0 with NA broth.
4. Add 1 ml of the adjusted pre-culture to 50 ml of NA broth in a 250 ml Erlenmeyer flask with vent cap. Incubate the culture at 28 °C with shaking at 200 rpm for approximately 36 h, until it reaches an OD₆₀₀ of 2.8. This culture will be used for DSF extraction.

3.2 DSF Signal Extraction and Sample Preparation for UPLC/MS Analysis

1. Centrifuge at 8000 × g for 15 min at room temperature 1.0 ml of the $\Delta rpfC$ culture from Subheading 3.1, point 4, into a 1.5 ml tube.
2. Transfer the supernatant into a 5 ml tube and adjust the supernatant's pH to 4.0 by adding 10 µl of 6 M hydrochloric acid. Check the pH with pH test strips (*see Notes 5 and 6*).
3. Add 1 ml of ethyl acetate to the adjusted supernatant and vortex the tube vigorously for 5 min to extract DSF family signals. Centrifuge at 8000 × g for 10 min to separate the ethyl acetate fraction from the aqueous fraction.
4. Transfer the ethyl acetate fractions (upper layer, about 0.9 ml) into a clean 1.5 ml tube.

5. Evaporate ethyl acetate of the extracts with a rotary evaporator or centrifugal evaporator (maximum temperature 40 °C) (*see Note 7*).
6. Add 120 µl of methanol (HPLC grade) to dissolve the dried extract and mix by vortexing for 10 s.
7. Centrifuge the crude extract at 13,800 × *g* for 15 min at 4 °C to remove particles.
8. Transfer the supernatant (approximately 100 µl) into a chromatography vial fitted with a flat-bottom insert and cap the vial.

3.3 UPLC/MS Analysis

1. Maintain the column temperature at 30 °C and keep the flow rate at 0.4 ml/min.
2. Balance the column for 15 min or longer until a stable baseline.
3. Inject 5 µl of DSF or BDSF standard solutions, or 5 µl of the extract from Subheading 3.2, step 8, to a 1290 Infinity UPLC equipped with a Zorbax Eclipse XDB-C18 column.
4. Detect DSF family signals in a diode array detector under the detection wavelength of 220 nm with a bandwidth of ±4 nm.
5. The UPLC elutes are further introduced into an accurate mass TOF-MS equipped with a Jet Stream (JS) electrospray ionization (ESI) source in the negative ionization mode.
6. MS source operating parameters are as follows: (1) gas temperature: 325 °C; (2) drying gas: 8 l/min; (3) nebulizer: 35 psig; (4) sheath gas temperature: 350 °C; (5) sheath gas flow: 11 l/min; (6) capillary voltage (Vcap): 3500 V; (7) nozzle voltage: 200 V; and (8) mass range: m/z 100–1700.

3.4 Quantification of DSF Family Signals

1. Use the MassHunter Workstation Data Acquisition Software (revision B.04) to acquire data in the centroid mode. Typical mass spectra of DSF, BDSF, CDSF, and IDSF are shown in Figs. 2–4.
 - (a) To detect [BDSF-H]⁻, set the monoisotopic exact value (m/z) as 197.1547 (Fig. 2).
 - (b) To detect [CDSF-H]⁻, set the monoisotopic exact value (m/z) as 209.1547 (Fig. 3).
 - (c) To detect [DSF-H]⁻ and [IDSF-H]⁻, set the monoisotopic exact value (m/z) as 211.1704 (Fig. 4).
2. Integrate and quantify the area under each of the chromatographic peaks.
3. Create a standard curve for DSF or BDSF by plotting the peak area vs. the known concentrations. Example standard curves are shown in Fig. 5, in which the DSF or BDSF standards at the concentrations of 1 µM, 5 µM, 10 µM, and 50 µM were used (*see Note 8*).

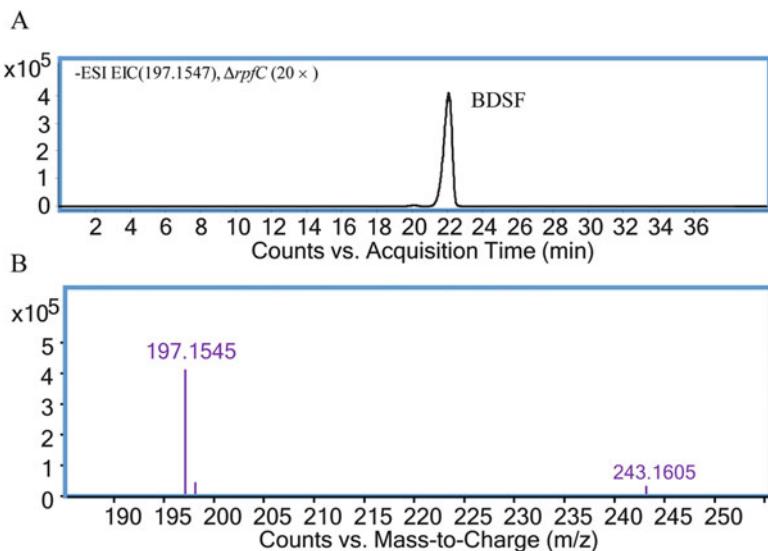


Fig. 2 Typical MS spectrum of BDSF. (a) Ion chromatograms of BDSF in the culture supernatant of $\Delta rpfC$ strain (20 times concentrated). (b) MS analysis showing an exact molecular weight of 198.1543 Dalton for the detected BDSF signal

4. The concentration of DSF and BDSF in the extract is calculated based on the peak area and the standard curve as shown in Fig. 5.

4 Notes

1. In this protocol, the DSF-overproducing strain $\Delta rpfC$ was used as an example. This protocol is also applicable for extraction of DSF family signals from the cell cultures of other bacterial species. The QS signals are usually biosynthesized in a growth phase-dependent manner. As a result, to detect DSF family signals in cell cultures, it is essential to collect cell cultures in the right growth stage (usually early stationary phase). Therefore, it is advised to do pilot experiments to determine the optimal growth stage for the extraction of DSF family signals.
2. As mass spectrometry is a very sensitive technique, a DSF-deficient strain is recommended to be included as a negative control for DSF extraction and quantification in each individual experiment. For example, the DSF-deficient mutant $\Delta rpfF$ of *Xcc* [10] could be used as a control.
3. DSF and BDSF signal molecules can be purchased from Sigma-Aldrich.

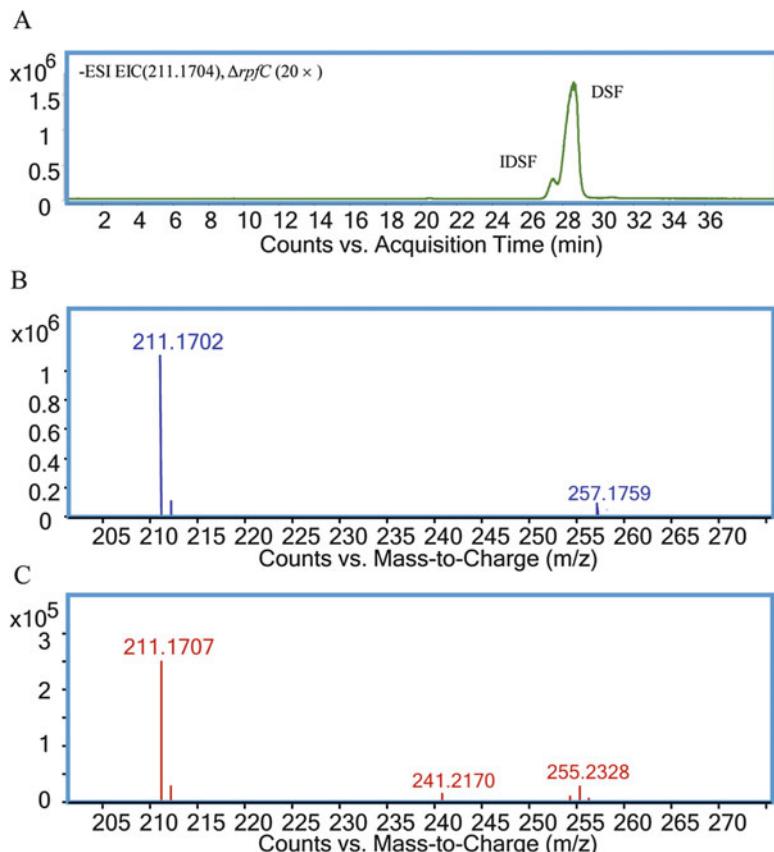


Fig. 3 Typical mass spectrum of CDSF. **(a)** Ion chromatograms of CDSF in the culture supernatant of $\Delta rpfC$ strain (20 times concentrated). **(b)** MS analysis showing an exact molecular weight of 210.1547 Dalton for the detected CDSF

4. This protocol is optimized for measuring the levels of DSF family signals in the cultures of *Xcc* or *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). It is also applicable for other bacterial species. Please use the appropriate growth medium, antibiotics, and growth conditions.
5. Acidification prior to the extraction is a critical step for DSF/BDSF extraction in this protocol. Make sure that the culture supernatant is acidified (pH lower than 4.0).
6. The methods described here are also applicable for detecting DSF family signals in the reaction mixtures. The reaction mixture should also be adjusted to a pH < 4.0 by adding adequate volume of 6 M hydrochloric acid. DSF family signals in the reaction mixtures are then extracted and analyzed following the protocol described in Subheadings 3.3 and 3.4.
7. At this point, the samples can be frozen at -20°C or immediately analyzed, as described in the following steps.

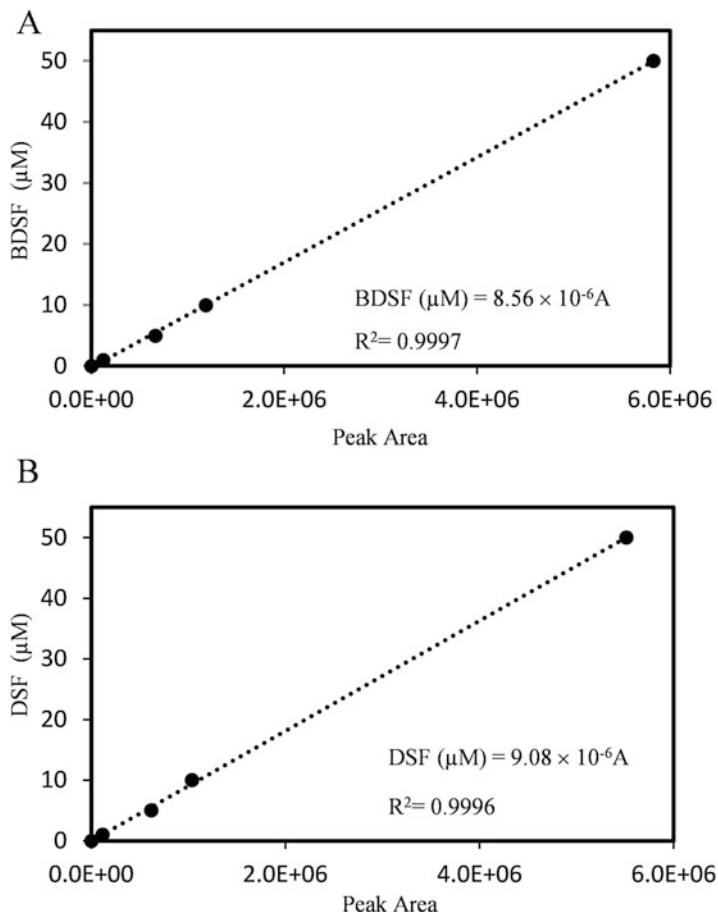


Fig. 4 Typical mass spectrum of DSF and IDSF. **(a)** Ion chromatograms of DSF and IDSF in the culture supernatant of $\Delta rpfc$ strain (20 times concentrated). **(b)** MS analysis showing an exact molecular weight of 212.1700 Dalton for the detected DSF. **(c)** MS analysis of IDSF showing an exact molecular weight of 212.1704 Dalton for the detected IDSF

8. The signal intensity or peak area of each of the DSF family signals may vary in different LC-MS systems. We recommend to generate system-specific standard curves using the commercially available DSF and BDSF. Since IDSF and CDSF are not commercially available, the standard curves for these two signal molecules were not generated in the previous studies. Their relative concentrations can be roughly calculated using the standard curves for DSF or BDSF.

Acknowledgments

This protocol is adapted from [8, 9]. This work was supported by the research grants from the National Natural Science Foundation of China (No. 31471743 to HYW, No. 31301634 to ZL).

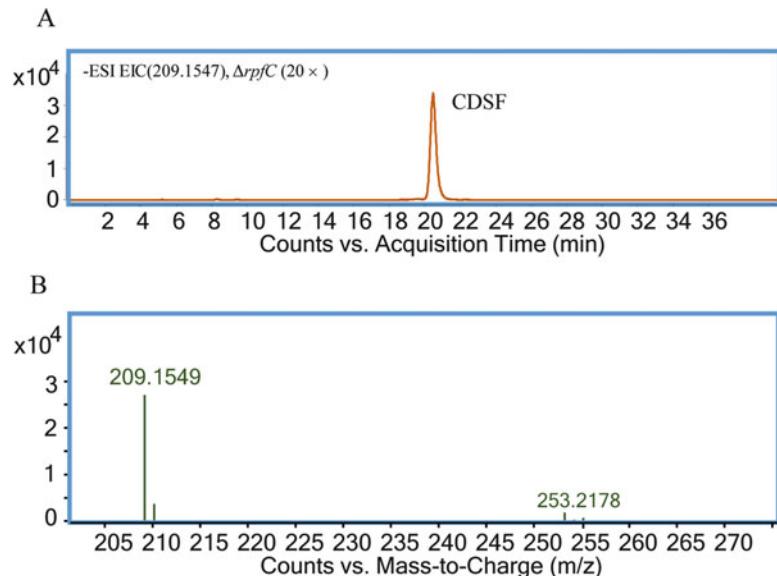


Fig. 5 Standard curves generated by measuring the peak area of BDSF (a) or DSF (b) at different concentrations. These standard curves were then applied to analytical data

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Chapter 9

Rapid Electrochemical Detection of *Pseudomonas aeruginosa* Signaling Molecules by Boron-Doped Diamond Electrode

Alyah Buzid, John H.T. Luong, F. Jerry Reen, Fergal O'Gara, Jeremy D. Glennon, and Gerard P. McGlacken

Abstract

As the leading cause of morbidity and mortality of cystic fibrosis (CF) patients, early detection of *Pseudomonas aeruginosa* (PA) is critical in the clinical management of this pathogen. Herein, we describe rapid and sensitive electroanalytical methods using differential pulse voltammetry (DPV) at a boron-doped diamond (BDD) electrode for the detection of PA signaling biomolecules. Monitoring the production of key signaling molecules in bacterial cultures of *P. aeruginosa* PA14 over 8 h is described, involving sample pretreatment by liquid-liquid and solid-phase extraction. In addition, direct electrochemical detection approach of PA signaling molecules is also reported in conjunction with hexadecyltrimethylammonium bromide (CTAB) to disrupt the bacterial membrane.

Key words *Pseudomonas aeruginosa*, Signaling molecules, Cystic fibrosis, Electrochemical detection, Extraction, Cationic surfactants, Boron-doped diamond electrode

1 Introduction

As an intrinsically antibiotic-resistant human pathogen, the Gram-negative bacterium *Pseudomonas aeruginosa* (PA) is associated with hospital-acquired infections and causes acute and chronic lung infections in cystic fibrosis (CF) patients [1, 2]. The ability of PA to form biofilms, with their associated resistance to antibiotic penetration in nosocomial infections, underpins its classification as a life-threatening infection [3, 4]. PA also causes bovine mastitis in cows which in turn poses a serious health risk for the consumption of infected dairy products [5–7].

Quorum sensing (QS) allows bacteria to coordinate gene expression at a multicellular level in response to changes in population density and/or the environment. Therefore, bacteria can behave as a community and express phenotypes that are beneficial

for the entire population [8]. PA also utilizes QS for cell-to-cell communication to enable biofilm formation, and regulate the production of virulence factors which challenge the host defense systems and provoke acute and eventually chronic infections [4, 9, 10]. Among >50 different quinolones produced by PA, the most predominant are 2-heptyl-3-hydroxy-4(*IH*)-quinolone, classified as *Pseudomonas* quinolone signal (PQS), and its biosynthetic precursor 2-heptyl-4-hydroxyquinoline (HHQ). PQS and HHQ function as signaling molecules and boost pathogenicity, for instance, in the production of pyocyanin (PYO) and biofilm formation [11]. Other potential biomarkers such as a thiazolyl-indole alkaloid have also been isolated from PA, but have been far less studied [12].

Typically, PA infections are diagnosed in the clinic using plate culturing and polymerase chain reaction (PCR) which are not quantitative and require time-consuming sample preparation, respectively. Despite sensitive, PCR is open to significant interference by contamination, while mispriming and false negatives can present a challenge to the clinical implementation of this technology [13]. Rapid, sensitive, and selective electrochemical strategies would offer advantages over these and other techniques.

The protocols described here involve the detection of PYO, HHQ, and PQS by simple electrochemical methods using a boron-doped diamond (BDD) electrode [14, 15]. We have also reported on the detection of barakacin, derived from a ruminal PA strain [16]. However, this compound is less documented and therefore is not discussed further here.

This chapter describes the detection of PA signaling molecules PYO, HHQ, and PQS as standards (Fig. 1) using the BDD electrode. Detection of PYO, HHQ, and PQS in bacterial cultures of PA14 is then described (Fig. 2) using liquid-liquid extraction (LLE) followed by solid-phase extraction (SPE) [14] as a preconcentration step for the improvement of detection sensitivity. Finally, an improved procedure, involving rapid and direct detection of PYO, HHQ, and PQS, is described using cationic surfactant hexadecyltrimethylammonium bromide (CTAB) to disrupt the bacterial membrane, aiding the release of biomarkers (Fig. 3) [15].

2 Materials

Prepare all buffer solutions in deionized water (DW) using an appropriate water purification system and analytical grade reagents. Store all buffer solutions at room temperature (unless otherwise stated). Prepare 2 mM of the stock solutions of HHQ, PQS, or PYO in acetonitrile (ACN) (*see Note 1*) and store them in a refrigerator at 4 °C. Follow all regulations when disposing of waste materials.

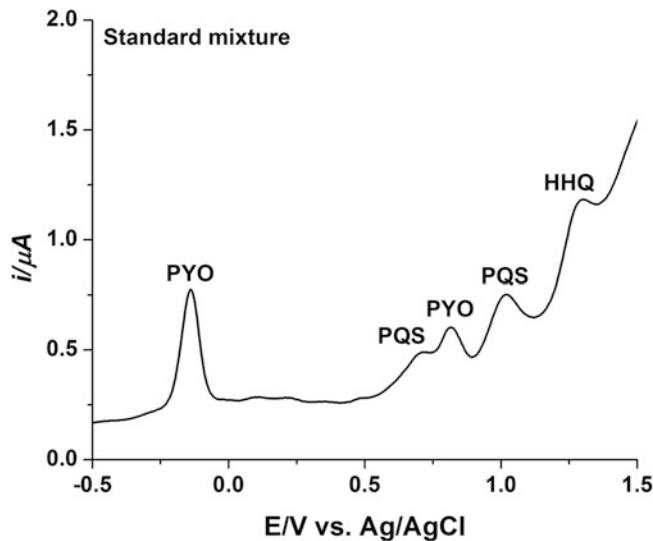


Fig. 1 DPV of 5 μM PYO, 20 μM HHQ, and 20 μM PQS using 50 mM acetate buffer (pH 5.0) consisting of 20 % ACN as the electrolyte. Detection was performed on the BDD electrode vs. Ag/AgCl [14]

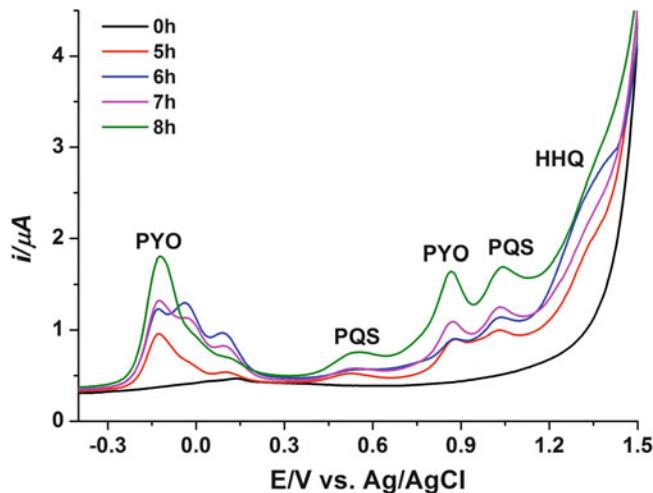


Fig. 2 Monitoring the production of HHQ, PQS, and PYO in the bacterial strain *P. aeruginosa* PA14 in LB media was carried out for 8 h. The bacterial culture was extracted twice with acidified ethyl acetate and then with MCX SPE. Electrolyte: 50 mM acetate buffer at pH 5.0 containing 20 % ACN. Detection was performed on the BDD electrode vs. Ag/AgCl [14]

2.1 Electrochemical Workstation

1. The electrochemical cell consists of the BDD electrode (a diameter of 3 mm) as the working electrode, a Pt wire as a counter electrode, and an Ag/AgCl as a reference electrode (see Note 2).

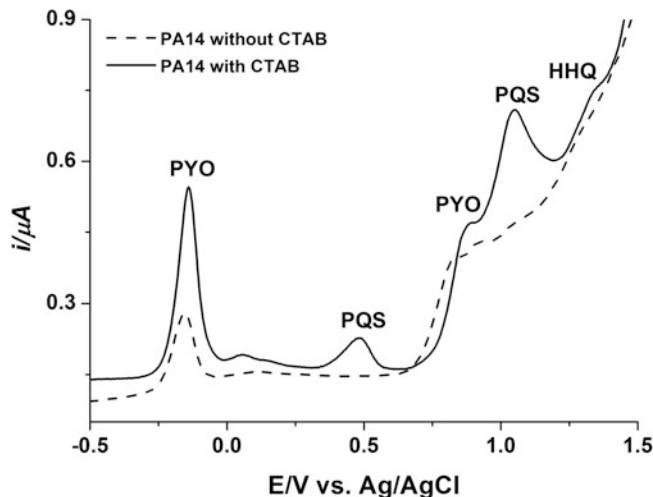


Fig. 3 DPV response towards the PA14 strains without (dashed line) and with CTAB (solid line) which was grown for 7 h. 50 mM acetate buffer pH 5.0 consisting of 20 % ACN was used as an electrolyte for detection on the BDD electrode vs. Ag/AgCl [15]

2. Electroanalysis is performed by differential pulse voltammetry (DPV) (*see Note 3*).
3. Glass beakers (10 or 5 ml).
4. Cleaning the BDD electrode with cyclic voltammetry (CV) (*see Note 4*) is performed using a 50 mM acetate buffer at pH 5.0 (*see Note 5*). A fresh buffer is prepared daily (*see Note 6*).
5. The acetate buffer used as the electrolyte solution for the detection of PYO, HHQ, and PQS: 50 mM acetate buffer (pH 5.0) consisting of 20 % ACN (*see Note 5*).
6. Control run: Use DPV in the presence of a buffer only (**step 5**) without added analytes to ensure that the BDD electrode is optimized for further measurements (*see Note 7*).
7. Standard run of 5 μM PYO, 20 μM HHQ, and 20 μM PQS: Use DPV (*see Note 3*) in the presence of the analytes in 50 mM acetate buffer (pH 5.0) consisting of 20 % ACN (*see Note 8*) (Fig. 1).
8. Rinse all three electrodes with DW between runs.

2.2 Sample Preparation from Bacterial Cultures

2.2.1 Liquid-Liquid Extraction (LLE) of Bacterial Cultures

1. *P. aeruginosa* PA14 bacterial cultures: PA14 cultures are grown overnight in LB broth [1 % (wt/vol) bacto-tryptone, 0.5 % (wt/vol) yeast extract, and 1 % (wt/vol) sodium chloride in distilled water] at 37 °C with shaking at 200 rpm. Overnight cultures are diluted into fresh Luria-Bertani (LB) broth to an absorbance at 600 nm wavelength (A_{600}) of 0.05, and then incubated at 37 °C (total volume 20 ml) [17]. Sample aliquots (1 ml) are taken at different time intervals (e.g., 0, 5, 6, 7, and 8 h).

2. The whole-cell cultures are used for the monitoring study.
3. Round-bottom flasks (25 ml).
4. Acidified ethyl acetate: [0.01 % (v/v) acetic acid in ethyl acetate] is utilized for the liquid-liquid extraction of PYO, HHQ, and PQS from bacterial cultures. Add 10 µl of acetic acid (99.7 %) to 100 ml of ethyl acetate (*see Note 9*).

2.2.2 Solid-Phase Extraction (SPE)

1. Solid-phase extraction (SPE): Using a mixed-mode strong cation-exchange (MCX) SPE cartridge (30 mg) (*see Note 10*). All SPE procedures are performed manually using a 5 ml plastic syringe (*see Note 11*) in a fume hood.
2. The formate buffer: 0.5 M formate buffer (HCOOH) at pH 2.0 is used for equilibration and washing buffer for MCX SPE. Prepare a stock solution of 1 M formate buffer. Add about 25 ml DW to a 100 ml volumetric flask, then slowly add 3.850 ml of concentrated formic acid (98 %) (*see Note 12*), and adjust the final volume to 100 ml with DW. Then, dilute 50 ml of 1 M formate buffer to 100 ml with DW to obtain a 0.5 M HCOOH buffer, pH 2.0 (*see Note 6*).
3. Ammonium formate: 5 % of 4.5 M ammonium formate (NH_4HCO_2) in methanol (95 % CH_3OH : 5 % DW, v/v) is used as an elution solvent for MCX SPE. Prepare a stock solution of 10 M ammonium formate in a 10 ml volumetric flask. Weigh 6.306 g of ammonium formate, then add 6 ml of DW, sonicate until the ammonium formate is dissolved, and adjust the final volume to 10 ml with DW. Subsequently, 4.5 M ammonium formate is obtained by the dilution of 0.45 ml of 10 M ammonium formate to 1 ml with DW. Prepare 95 % CH_3OH : 5 % DW, v/v. Add 4.75 ml of 100 % CH_3OH to 0.25 ml DW. Lastly, add 0.25 ml of 4.5 M ammonium formate to 4.75 ml of 95 % CH_3OH : 5 % DW to obtain 5 % of 4.5 M ammonium formate in methanol (95 % CH_3OH : 5 % DW, v/v).

2.3 Direct Detection of PYO, HHQ, and PQS in Bacterial Cultures Based on In Situ Cationic Surfactant-Assisted Membrane Disruption

1. Phosphate buffer: 50 mM phosphate buffer, pH 7.0 is used to dilute the CTAB stock solution (**step 2**). Prepare a stock solution of 0.1 M of sodium phosphate dibasic (Na_2HPO_4). Weigh 1.419 g sodium phosphate dibasic and transfer to a 100 ml volumetric flask. Add DW to a volume of 90 ml, sonicate until sodium phosphate dibasic is dissolved, and then adjust the final volume to 100 ml with DW. Also, prepare a stock solution of 0.1 M of sodium phosphate monobasic (NaH_2PO_4). Weigh 1.560 g sodium phosphate monobasic and transfer to a 100 ml volumetric flask. Add DW to a volume of 90 ml, sonicate until sodium phosphate monobasic is dissolved, and then adjust the final volume to 100 ml with DW. 50 mM phosphate buffer,

pH 7.0 can be obtained by diluting 0.1 M sodium phosphate dibasic and 0.1 M sodium phosphate monobasic. Add 4 ml of 0.1 M sodium phosphate dibasic to 5 ml of 0.1 M sodium phosphate monobasic and adjust the final volume to 50 ml with DW.

2. CTAB solution: 1 mM CTAB is used for the bacterial membrane disruption. Prepare a stock solution of 25 mM CTAB. Weight 0.911 g CTAB and transfer to a 200 ml glass bottle. Add DW to around 80 ml and sonicate for 15–25 min until CTAB is dissolved. Then, adjust the final volume to 100 ml with DW (*see Note 13*). 1 mM CTAB is obtained by diluting 25 mM CTAB in 50 mM phosphate buffer, pH 7.0. Dilute 40 µl of 25 mM CTAB to 1 ml with 50 mM phosphate buffer at pH 7.0.
3. *P. aeruginosa* PA14 bacterial cultures: PA14 cultures are grown overnight in LB and then diluted into 20 ml of fresh LB as described in Subheading 2.2.1, step 1. Diluted PA14 cultures are grown at 37 °C for 7 h with shaking at 200 rpm (total volume 20 ml) [17].

3 Methods

3.1 Monitoring the Production of PYO, HHQ, and PQS in PA14 Cultures

1. 1 ml sample aliquot of the bacterial culture before incubating at 37 °C is extracted with 1 ml of acidified ethyl acetate (1:1, whole-cell culture:acidified ethyl acetate, v/v) (*see Note 14*). Then, separate and collect the top layer of the ethyl acetate into a 25 ml round-bottom flask.
2. Add another 1 ml of the acidified ethyl acetate to the 1 ml of the bacterial culture and follow the same procedure as mentioned in step 1.
3. Evaporate the ethyl acetate using a rotary evaporator, reconstitute the residue by dissolution in 1 ml of 0.5 M formate buffer, pH 2.0, and sonicate for 5 min.
4. Wash the SPE cartridge with 1 ml of 100 % methanol followed by conditioning with 1 ml of 0.5 M formate buffer, pH 2.0.
5. Load 1 ml of the reconstituted sample (**step 3**) to the preconditioned SPE cartridge, applying gentle pressure (*see Note 11*) to ensure the maximum retention of the analytes on the stationary phase.
6. Wash the cartridge with 0.2 ml of a 0.5 M formate buffer, pH 2.0, to assist the retention of the analytes and then wash with 0.2 ml of 100 % methanol to remove any neutral contaminants.

7. Elute PYO, HHQ, and PQS from the SPE cartridge using 0.5 ml of 5 % of 4.5 M ammonium formate in methanol (95 % CH₃OH:5 % DW, v/v).
8. Add 0.3 ml of the eluent (**step 7**) to the 0.3 ml of electrolyte, 50 mM acetate buffer at pH 5.0 consisting of 20 % ACN, and perform the DPV detection on the BDD electrode vs. Ag/AgCl.
9. Repeat the same procedure (**step 1–8**) at each different time interval (5, 6, 7, and 8 h) (Fig. 2).

3.2 Direct Detection of PYO, HHQ, and PQS in PA14 Bacterial Cultures Based on In Situ Cationic Surfactant-Assisted Membrane Disruption

1. Add a 0.25 ml sample of the bacterial culture to 0.75 ml of 1 mM CTAB prepared in 50 mM phosphate buffer, pH 7.0 (1:4, culture:CTAB, v/v).
2. Equilibrate this bacterial culture, CTAB, and buffer mixture for 5 min at room temperature.
3. Add 0.2 ml of the bacterial culture, CTAB, and buffer mixture to 0.8 ml of electrolyte, 50 mM acetate buffer (pH 5.0) consisting of 20 % ACN, and perform the DPV detection at the BDD electrode vs. Ag/AgCl (Fig. 3).

4 Notes

1. Stock solutions of PYO, HHQ, and PQS: 2 mM each in ACN. Weight 2.1 mg of PYO (C₁₃H₁₀N₂O) and then add 5 ml of ACN. Weight 2.4 mg of HHQ (C₁₆H₂₁NO) and then add 5 ml of ACN. Weight 2.6 mg of PQS (C₁₆H₂₁NO₂) and then add 5 ml of ACN.
2. The BDD electrode was polished with wet polishing (nylon and MasterTex) pads. Always add some DW to polishing pads before the addition of alumina. First, use wet nylon paper with 0.3 µm alumina slurries and make figure-8 motions on the polishing pad to clean the surface. Then, use wet MasterTex paper with 0.05 µM alumina and make figure-8 motions on the polishing pad to further polish the rough surface of the BDD electrode. After rinsing the electrode with DW, ethanol or propanol can be used to sonicate the electrode for 5 min to remove any residual organic species. Subsequently, sonicate the electrode in DW for 10 min to remove any residual alumina particles.
3. The DPV potential is swept between –0.6 V and +2.0 V for the electrochemical detection of PYO, HHQ, and PQS.
4. Insert the BDD electrode into the electrochemical cell, connect the Pt and Ag/AgCl electrodes. Then, use CV between –1.0 and +2.0 V vs. Ag/AgCl (3 M KCl) at a scan rate of 100 mV s^{–1} in a 50 mM acetate buffer (pH 5.0) (*see Note 5*) until a steady CV profile is obtained.

5. Electrolyte solution for PYO, HHQ, and PQS detection: 50 mM acetate buffer (pH 5.0) with 20 % ACN. Prepare a stock solution of 0.2 M sodium acetate (CH_3COONa). Weigh 1.640 g of sodium acetate and transfer to a 100 ml volumetric flask. Add DW up to 90 ml. Sonicate until the sodium acetate is dissolved and then adjust the final volume to 100 ml with DW. Also, prepare a stock solution of 0.2 M acetic acid (CH_3COOH). Add about 25 ml of DW to a 100 ml volumetric flask. Add 1.144 ml of acetic acid (99.7 %) to the volumetric flask (*see Note 12*). Then, make up to 100 ml with DW. 50 mM acetate buffer at pH 5.0 can be obtained by diluting 0.2 M sodium acetate and 0.2 M acetic acid. Add about 40 ml of DW to a 250 ml graduated cylinder, then add 17.85 ml of 0.2 M acetic acid, 32.15 ml of 0.2 M sodium acetate, and make up to 200 ml with DW. Similarly, 50 mM acetate buffer (pH 5.0) with 20 % ACN can be obtained by diluting 0.2 M sodium acetate and 0.2 M acetic acid. Add about 20 ml of DW to a 100 ml graduated cylinder; then add 8.925 ml of 0.2 M acetic acid, 16.07 ml of 0.2 M sodium acetate, and 20 ml of 100 % ACN; and make up to 100 ml with DW.
6. Prepare daily electrolyte of acetate buffer and formate buffer as will evaporate and affect the desired concentration.
7. Run the DPV control using the only electrolyte 50 mM acetate buffer (pH 5.0) consisting of 20 % ACN prior to the electrochemical detection of PYO, HHQ, and PQS. This step aims to ensure that the BDD electrode is clean, uncontaminated, and ready for analysis. In a blank electrolyte, DPV should give a clean background. If the DPV shows any oxidation/reduction peaks in the blank buffer, polish and clean the BDD electrode with CV (*see Notes 2 and 4*).
8. Run the DPV for the standard mixture of 5 μM PYO, 20 μM HHQ, and 20 μM PQS. 5 μM PYO, 20 μM HHQ, and 20 μM PQS can be obtained by diluting 2 mM stock solutions of PYO, HHQ, and PQS (*see Note 1*). 12.5 μl of the 2 mM PYO stock solution, 50 μl of the 2 mM HHQ stock solution, 50 μl of the 2 mM PQS stock solution, and makeup with 5 ml of 50 mM acetate buffer (pH 5.0) consisting of 20 % ACN (*see Note 5*).
9. The acetic acid is added to ethyl acetate to release any complex metal ion from PQS and facilitate the partitioning of PQS into the ethyl acetate layer [17] (PQS is a ferric iron chelator [18]).
10. The MCX sorbents have a polymeric structure (hydrophilic N-vinylpyrrolidone and lipophilic divinylbenzene (PVP-DVB)) that is modified with sulfonic groups. The MCX SPE was the best cartridge to extract PYO, HHQ, and PQS based on the electrostatic interaction between the positively charged analytes at low pHs and the negatively charged stationary phase.

11. An SPE tube adapter is used to connect the syringe plunger to the SPE sorbent for applying air pressure.
12. Always prepare the acids under a fume hood, and use protective gloves, safety glasses, and a mask.
13. Since CTAB forms a foam during sonication, using a glass bottle with a volume significantly higher than the liquid volume is recommended. As an example, if the desired final volume is 100 ml, a 200 ml glass bottle should be used. Also, CTAB can be crystallized during storage of the stock solution, and just sonicate the solution again for ~15 min until a clear solution is obtained.
14. Always wear safety glasses and protective gloves especially when handling organic solvents and bacterial cultures, and carry out the extraction in the fume hood.

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Chapter 10

Detection and Quantification of Butyrolactones from *Streptomyces*

Marc Biarnes-Carrera, Rainer Breitling, and Eriko Takano

Abstract

In *Streptomyces*, the onset of antibiotic production and sporulation is coordinated through small diffusible molecules known as γ -butyrolactones (GBLs). These are active in very low amounts, and their extraction and characterization are challenging. Here we describe a rapid, small-scale method for the extraction of GBL from *Streptomyces coelicolor*, from both solid and liquid cultures, which provides sufficient material for subsequent bioassays and partial characterization. We also present two different bioassay techniques for the detection and quantification of the GBL content in the extracts: the antibiotic bioassay and the kanamycin bioassay.

Key words Gamma-butyrolactones, Antibiotic bioassay, Kanamycin bioassay, Small-scale gamma-butyrolactone extraction, Antibiotic production regulation

1 Introduction

The *Streptomyces* species undergo a complex life cycle and are prolific producers of a battery of clinically relevant compounds [1]. The onset of secondary metabolite production and/or morphological differentiation occurs at the transition from vegetative growth to stationary phase and is coordinated by bacterial hormones known as γ -butyrolactones (GBLs) [2]. Biosynthesis of GBLs occurs at the nanomolar scale and involves a first condensation of dihydroxyacetone phosphate with a beta-ketoacid [3], which can be followed by further modifications by tailoring enzymes [4]. The final GBLs are known to interact with DNA-binding repressors, known as ScbR in *S. coelicolor*, that act as master regulators of the primary cascade leading to secondary metabolite production.

Currently, at least 15 different GBLs have been isolated and characterized from different *Streptomyces* species [2], as well as

from various non-Streptomyces [5]. However, even more GBL synthases and repressors have been identified in genome sequence data [2, 6], suggesting the presence of more GBLs yet to be discovered. Moreover, recent publications [7, 8] have shown that GBLs have cross-species activities and signals from one *Streptomyces* species can induce antibiotic production in another one; consequently, their potential role in synthetic biology has been proposed [2].

Butyrolactones are very potent signaling molecules, and *Streptomyces* species produce them in low amounts. Therefore, complete purification and structural determination (e.g., by NMR) are not the first choice when attempting to isolate GBLs for bioactivity assays, as this may require culture volumes of up to 300 l [9]. Instead, we here describe a rapid method for GBL extraction from solid medium and a small-scale GBL extraction from liquid medium, which provide sufficient amounts of GBLs for sensitive bioassays and allow partial structural characterization and quantification using relatively small quantities of crude extract using current chromatography and mass spectrometry techniques [10, 11].

We then describe two bioassays that are used for GBL detection: the traditional antibiotic bioassay [12] and the kanamycin bioassay [13]. The antibiotic bioassay is based on the observation of precocious antibiotic production as a response to the exogenous addition of GBLs. In *S. coelicolor* M145, the antibiotic bioassay measures the enhanced production of pigmented antibiotics prodigiosins (RED: red) and actinorhodin (Act: blue) [9, 12]. Other antibiotic bioassays have been described for other strains, such as *Streptomyces griseus* [14], which involve extraction of antibiotic in a disk and evaluation of antibiotic activity measuring growth inhibition of a *Bacillus subtilis* indicator strain.

An alternative to the antibiotic bioassay is the kanamycin bioassay, developed by Hsiao et al. [13]. This method is based on the repression of *cpkO* promoter activity by the GBL receptor ScbR [15]. In the presence of GBLs, this repression is released, allowing transcription of the *cpkO* gene, a master regulator of the *cpk* cluster [15, 16].

Based on this concept, plasmid pTE1062 (Fig. 1), an analogue of plasmid pTE134 [13], was built and integrated into strain LW16 (M145 *scbR/A::aac3(IV)*), which does not produce butyrolactones, to generate the reporter strain LW18. The pTE1062 plasmid contains the *scbR* gene, regulated by its own promoter region, and the *neo* gene, regulated by the *cpkO* promoter. Thus, the reporter strain is sensitive to kanamycin unless GBLs are present in the medium. Interestingly, this system allows for detection of GBLs other than the ones from *S. coelicolor* [13], even though a higher concentration of these GBLs is required.

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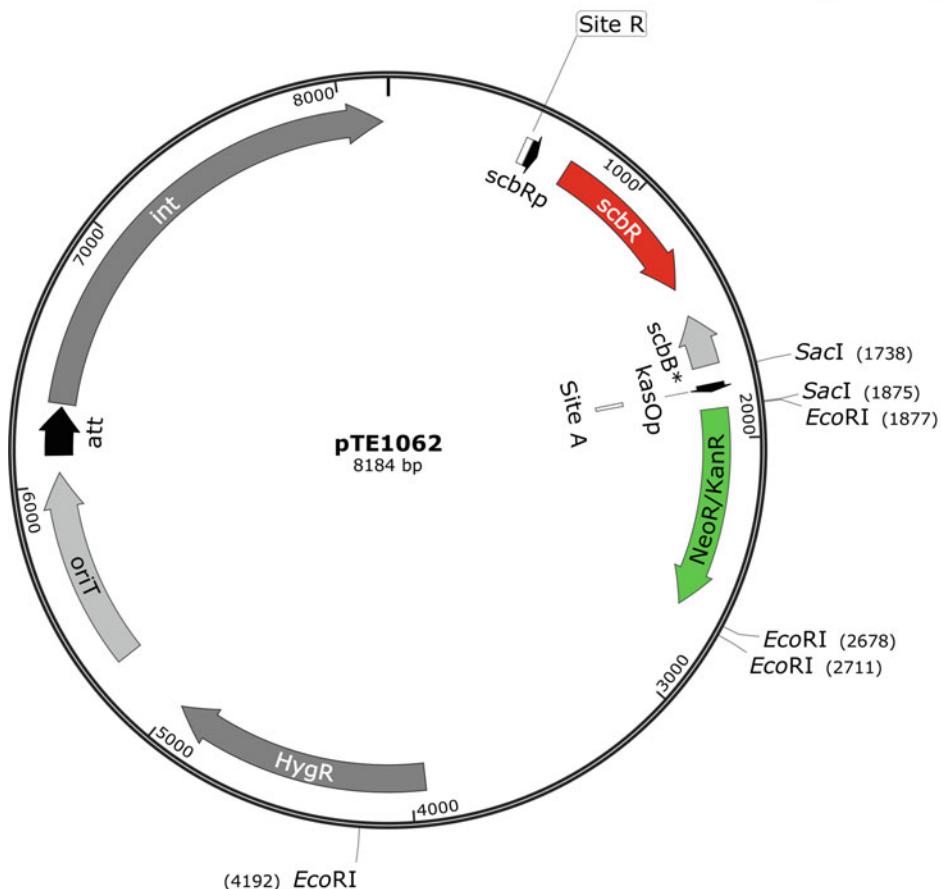


Fig. 1 Schematic representation of a kanamycin bioassay reporter plasmid (pTE1062). The gene product of *scbR* (red arrow) binds to Site R and Site A (open boxes). In the presence of GBLs, ScbR is released from Site A and allows transcription of aminoglycoside phosphotransferase from Tn5 (green arrow) downstream of the *kasO* promoter (*kasOp*)

2 Materials

2.1 Extraction of Butyrolactones from Solid Culture

1. Trace element solution [17]: 0.1 g/l ZnSO₄·7H₂O, 0.1 g/l FeSO₄·7H₂O, 0.1 g/l MnCl₂·4H₂O, 0.1 g/l CaCl₂, 0.1 g/l NaCl. Sterilize by filtration. Store at 4 °C and prepare fresh every 6 months.
2. Supplemented minimal medium solid (SMMS) [17]: Prepare 1000 ml of a 0.2% (w/v) casamino acids (Difco) and 25 mM TES buffer at pH 7.2 and transfer 200 ml into five different 250 ml conical flasks containing 3 g of agar (Difco) each. Autoclave all five flasks. Right before usage, melt the medium and add 0.5 mM NaH₂PO₄, 0.5 mM K₂HPO₄, 5 mM MgSO₄, 50 mM glucose, and 0.2 ml of trace element solution (this section, item 1).

3. 10^7 Spores of *S. coelicolor* M1152 [18] or *S. coelicolor* M145 [12].
4. Sterile water.
5. Razor blades.
6. Ethyl acetate, HPLC grade (*see Note 1*).
7. Methanol, HPLC grade.
8. 30 °C Incubator.
9. Round-bottom flask.
10. Rotary evaporator.
11. 250-ml conical flasks.
12. 500-ml conical flask.
13. 2-ml plastic tubes.
14. Speed vacuum concentrator.
15. Vortex.

2.2 Extraction of Butyrolactones from Liquid Culture

1. Supplemented minimal medium [17]: 5% (w/v) PEG 6000, 0.5 mM MgSO₄·7H₂O, 25 mM TES buffer at pH 7.2, 0.5 mM NaH₂PO₄, 0.5 mM K₂HPO₄, 50 mM glucose, 0.01% (w/v) antifoam 289, 0.1% (v/v) trace element solution (Subheading 2.1, item 1), 0.2% (w/v) casamino acids (Difco). Autoclave all components individually before mixing, except the glucose and the trace elements, which are filter-sterilized before mixing.
2. 10^{10} Spores of *S. coelicolor* M1152 [18] or *S. coelicolor* M145 [12].
3. 250-ml conical flask with stainless steel spring.
4. 500-ml conical flask.
5. Ethyl acetate, HPLC grade (*see Note 1*).
6. Methanol, HPLC grade.
7. 30 °C shaking incubator.
8. Round-bottom flask.
9. Rotary evaporator.
10. Separation funnel.
11. Spectrophotometer.
12. Disposable 1-ml cuvettes.
13. 50-ml plastic tubes.
14. Centrifuge.
15. 2-ml plastic tubes.
16. Vortex.
17. MgSO₄ (anhydrous).
18. Filter paper.

2.3 Detection of Butyrolactones by Means of Antibiotic Bioassay

1. SFM medium [17]: 20 g/l Agar (Difco), 20 g/l mannitol, 20 g/l soya flour. Autoclave the medium.
2. Spore stock of *S. coelicolor* M145 strain. Evenly spread one single colony of *S. coelicolor* M145 on an SFM plate (25 ml of SFM/100 mm diameter plate) using a sterile cotton bud, followed by incubation at 30 °C for 4–5 days (see Note 2). Add 1 ml of 20% (v/v) glycerol to the plates containing spores and gently rub the spores off the surface of the plate with a sterile cotton pad (see Note 3). Carefully, collect the spores with a 5-ml syringe and transfer to a 2-ml sterile screw-cap tube. Store at –20 °C (see Notes 4 and 5).
3. SMMS (see Subheading 2.1, item 2).
4. Methanol extract containing GBLs.
5. Methanol, HPLC grade.
6. Previously validated GBL extract or 0.25 µg/µl of *S. coelicolor* GBL SCB1 [13] (see Note 6).
7. Laminar flow hood.
8. 30 °C incubator.
9. Petri dishes.

2.4 Detection of Butyrolactones by Means of Kanamycin Bioassay

1. Spore stocks of LW18 or LW94, prepared as previously described for M145 spore stock (Subheading 2.3, item 2).
2. DNAgar [17]: Add 9.2 g of Difco Nutrient Agar into 400-ml of deionized H₂O in a 500-ml bottle and autoclave.
3. Kanamycin.
4. Methanol extract containing GBLs.
5. Methanol, HPLC grade.
6. Previously validated GBL extract or 0.25 µg/µl of *S. coelicolor* GBL SCB1 [13] (see Note 6).
7. Laminar flow hood.
8. 30 °C Incubator.
9. Petri dishes.

3 Methods

3.1 Extraction of Butyrolactones from Solid Medium

GBLs are best extracted in batches of 10–20 plates, as more plates would be complicated to handle and fewer plates might not yield sufficient GBLs for later bioassay and characterization steps.

1. Prepare SMMS plates (25 ml/100 mm diameter plate). Rich media can also be used, but GBLs are less stable in these, potentially reducing the extraction yields.

2. Add 10^7 *Streptomyces* spores/plate, diluted with sterile water in 100 μl total volume, and spread evenly across the entire plate using a sterile cotton bud to generate confluent lawns. Grow for about 20 h (when using SMMS medium) or until the onset of Red production (when using other types of medium).
3. Cut the agar into pieces of about 25–50 mm in diameter, using a clean razor blade, and transfer into a 500-ml conical flask. Cutting the agar into too small pieces might cause difficulties in **step 5**.
4. Add enough ethyl acetate into the flask containing the pieces of agar to just cover the entire amount of agar, and gently swirl and allow resting for 5 min at room temperature.
5. Carefully transfer the ethyl acetate into a round-bottom flask (using pipettes if required), avoiding the transfer of any piece of agar, and remove solvent by rotary evaporation at room temperature.
6. Resuspend the remaining brown oil in 5 ml of 100% methanol and transfer to three 2-ml plastic tubes. Bring the solution to dryness using a speed vacuum concentrator and resuspend in a total volume of 50–150 μl of 100% methanol per each tube, making sure that the entire remaining brown oil dissolves, by gentle vortexing for 5–10 s. Pool all the samples in one tube.

3.2 Extraction of Butyrolactones from Liquid Medium

The following protocol is intended for 50 ml of *S. coelicolor* M145 culture. Where higher yields of GBLs are required, add more cultures or scale-up accordingly.

1. Add 10^{10} spores (*see Note 5*) to 50 ml of SMM medium in a 250-ml sterile conical flask containing a stainless steel spring to avoid cells to clump and provide good aeration [17].
2. Incubate at 30 °C until early stationary phase. Monitor growth every 2 h after the initial 12 h of inoculation by measuring the optical density at 450 nm using a spectrophotometer.
3. Transfer culture to a 50 ml plastic tube, centrifuge the cells at 10,000 $\times g$, and transfer the supernatant to a 500-ml conical flask. Add 50 ml of ethyl acetate to the supernatant and mix vigorously. Transfer mixture to a separation funnel and allow for both phases to separate (the lower one will be the aqueous phase and the upper one the ethyl acetate).
4. Transfer the aqueous phase to a beaker and the upper organic phase into a clean round-bottom flask. Transfer the aqueous phase to the separation funnel again, add 50 ml of ethyl acetate, and shake vigorously. Discard the aqueous phase and pool the organic phases.
5. Dry the organic phase by adding two spatulas of MgSO₄ (anhydrous)/50 ml of organic layer and vortex vigorously.

Allow the MgSO₄ to sediment at the bottom of the beaker and then filter.

6. Remove the solvent by rotary evaporation at room temperature.
 7. Resuspend the remaining brown oil in 5 ml of 100% methanol and transfer to three 2-ml plastic tubes. Dry the solution using a speedy vacuum concentrator and resuspend in a total volume of 50–150 µl of 100% methanol per each tube. Make sure that the entire remaining brown oil dissolves, by gentle vortexing for 5–10 s. Pool all the samples.
- 3.3 Detection of Butyrolactones: Antibiotic Bioassay**
1. Prepare plates of SMMS (25 ml/100 mm diameter plate).
 2. From the M145 spore stock (*see* Subheading 2.3, item 2), add 10⁸ spores of M145/plate, diluted in H₂O to 100 µl final volume, and spread evenly over the plate using sterile cotton buds (*see* Note 7).
 3. Under a sterile laminar flow hood, allow plates to dry for 3 min at room temperature, keeping the lid open.
 4. Add 2–3 µl of methanol extract to the dried plates by carefully pipetting a drop on the center of the plate and let dry for 1 min. If more sample needs to be added, because of the low expected concentration of GBL, repeat the operation. Do not add more than 3 µl of sample at once (*see* Notes 8 and 9). When necessary (e.g., when comparing the relative bioactivity of different GBLs or serial dilutions of methanol extract), multiple samples can be tested on the same plate, ensuring that enough space is left between them (around 5 cm) so that there is no interaction between different diffusing GBL samples.
 5. Incubate at 30 °C for 16–18 h. Evaluate precocious antibiotic production. This can be seen as a red halo surrounding the area where the sample was applied (Fig. 2), due to the induced production of prodigiosins. Record results using a conventional flatbed scanner or camera.
 6. If no antibiotic production was detected after 18 h, check plates every 2–3 h to monitor precocious antibiotic production, until the onset of prodigiosin production, which would result in all the plate acquiring a red color. Use a conventional scanner to record results on final plates as soon as precocious antibiotic production is evident (Fig. 2).
 7. The concentration of GBLs can be approximately determined by interpolating the diameter of the red antibiotic halo of the sample to a calibration curve obtained by performing the bioassay using standards of known concentration (e.g., purified SCB1).

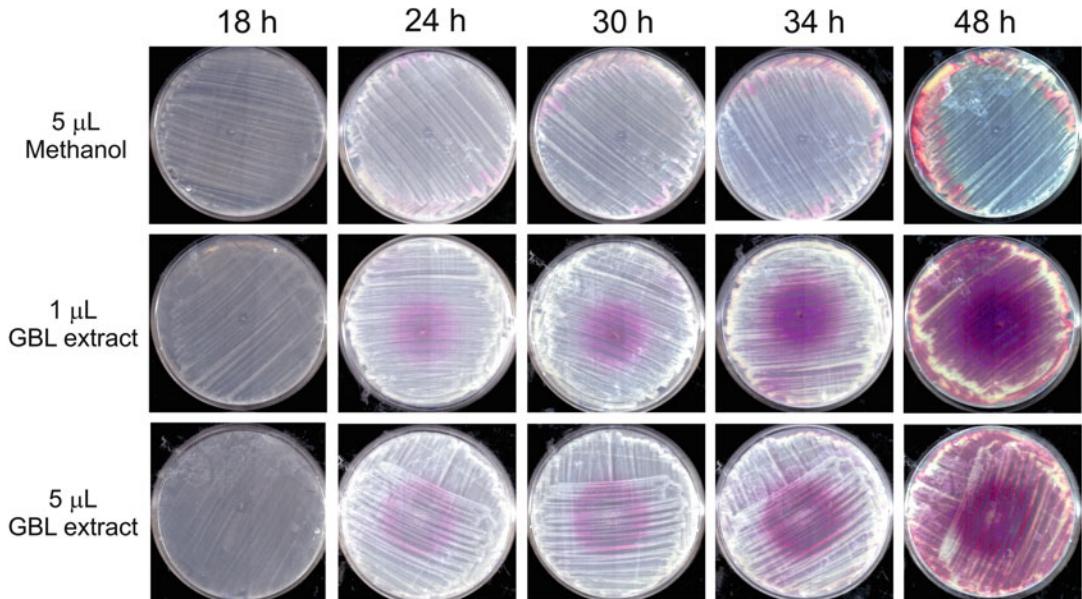


Fig. 2 Antibiotic bioassay results in SMMS medium after incubation at 30 °C for up to 48 h, using two different volumes of methanol extract containing GBL from *S. coelicolor* M145 compared to a methanol control

3.4 Detection of Butyrolactones: Kanamycin Bioassay

The kanamycin bioassay is based on the use of *S. coelicolor* LW18 or LW94 strains [13]. Strain LW18 contains a replacement of the GBL system in *S. coelicolor* by the apramycin resistance cassette and a genomic insertion of plasmid pTE1062 (unpublished). Therefore, this strain does not produce GBLs and will develop kanamycin resistance in the presence of exogenous GBLs. LW94 is a derivative of LW18, with the apramycin resistance cassette removed (unpublished).

1. Prepare one DNA agar plate with 5 µg/ml kanamycin for each sample, one for the negative control (with methanol only), one for the positive control (e.g., 0.25 µg of SCB1; *see Note 6*), and one plate of DNA agar without antibiotic, which will be used as growth control to check the indicator strain growth.
2. Prepare a LW18 or LW94 spore solution with 2.6×10^6 spores/100 µl sterile water (*see Subheading 2.4, item 1*). Spread 100 µl of the spore solution evenly over each plate using sterile cotton buds previously wetted with sterile deionized H₂O to avoid the spores to stick to the dry cotton bud. Allow plates to dry for 3 min under a sterile laminar flow hood, keeping the lid open.
3. Add 2–3 µl of methanol extract and let dry for 1 min (*see Notes 8 and 9*). In the negative control, add 2–3 µl of methanol and let dry for 1 min, and in the positive control add 1 µl of a

0.25 µg/µl solution of SCB1 or 2–3 µl of a previously validated methanol extract and let dry for 1 min.

4. Incubate at 30 °C incubator for 24 h. Check plates every 12 h for 3 days monitoring growth using a conventional scanner (Fig. 3) (see Note 10). A confluent lawn should be visible in the growth control after incubation at 30 °C for 24 h.
5. To estimate the concentration of GBLs in the methanol extract prepare serial dilutions of the same and perform the bioassay by adding 1 µl of each prepared sample. Estimate the concentration of GBLs by multiplying the dilution factor of the minimum concentration that show growth of the reporter strain, in the presence of 5 µg/ml kanamycin after 3 days of incubation at 30 °C, by 0.025 µg/µl (see Note 11). This quantification method has also been used in previous studies [19].

4 Notes

1. The ethyl acetate used for extraction should be previously tested in the same bioassay that the extracted sample will be subjected to before using it for extracting the butyrolactones, as it might have background bioactivity.
2. Depending on the soya flour used sporulation may take longer. When *S. coelicolor* sporulates, the culture will acquire a grey color. Attempting to harvest them before the shift to grey will result in no spores being collected or a very low spore concentration.
3. To prevent contamination of the spore stock, use previously sterilized tweezers to pick up the cotton pad.
4. Keep spore stocks frozen (−20 °C), and when thawing keep at low temperature (4 °C) and on ice.
5. The number of viable spores in the spore stock can be determined using a serial dilution (up to 10^{−10}) on Lysogeny Broth (LB) agar plates and incubation at 37 °C for 20–24 h.
6. The sensitivity of both bioassays towards SCB1 has been reported to be the same, being the optimal amount for the positive control of 1 µl of a 0.25 µg/µl solution of SCB1 [12, 13]. Note that SCB1 is not commercially available, although its chemical synthesis has been described in [12].
7. In the antibiotic bioassay, add the methanol extract within 8 h of plating. Otherwise, no induction of Red or Act will be seen [12].
8. For the bioassays, the added volume of methanol extract onto bioassay plates should never exceed 3 µl. If larger volumes are necessary, because of the very low expected concentration, add

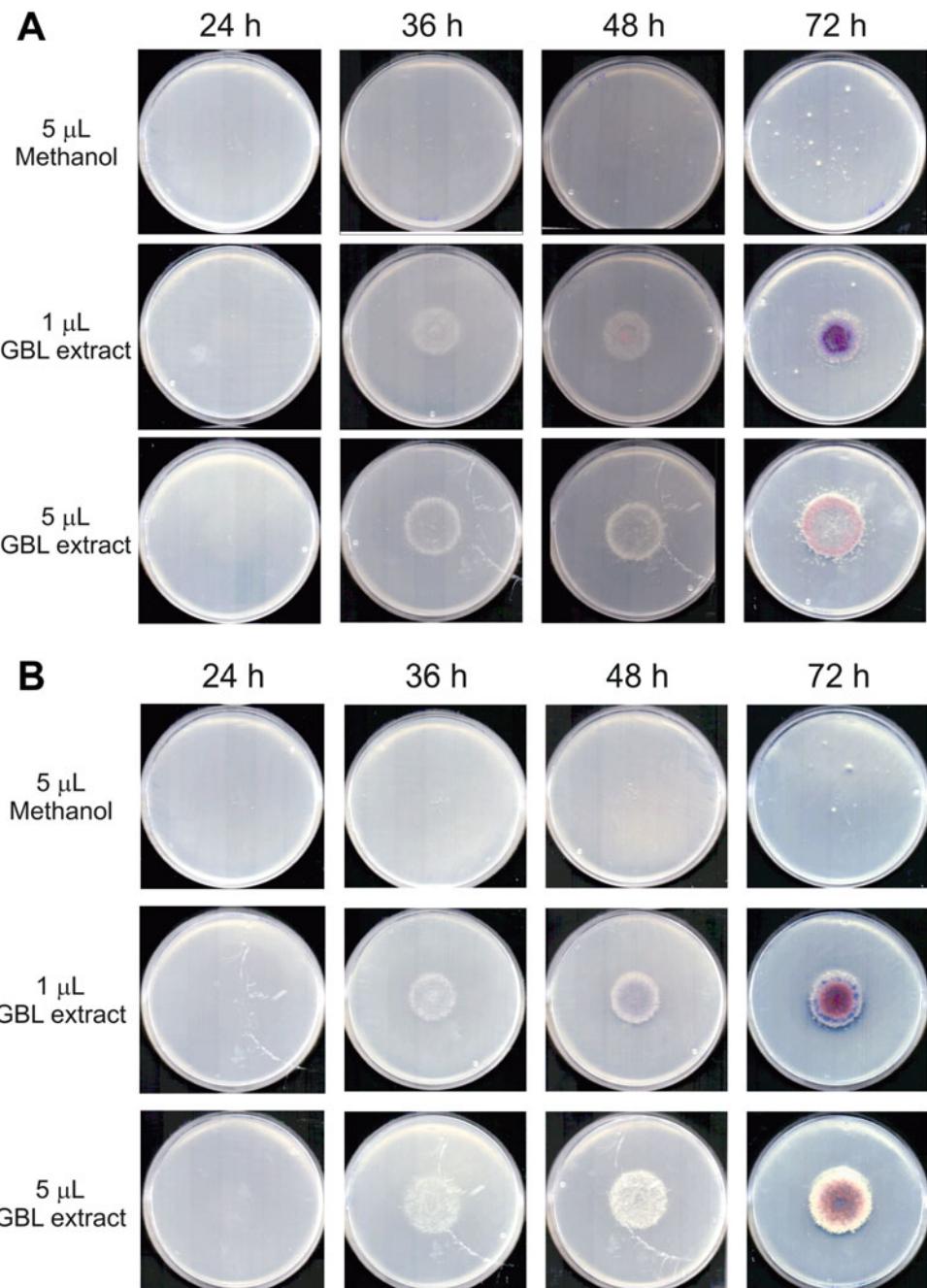


Fig. 3 Kanamycin bioassay results in DNA agar containing 5 µg/ml of kanamycin after incubation at 30 °C for up to 3 days with two different volumes of methanol extract containing GBL from *S. coelicolor* M145 compared to a methanol control. (a) Bioassay performed using the LW18 indicator strain; (b) bioassay performed using the LW94 indicator strain. The extracts used in the bioassay are the same as those used in Fig. 2

aliquots of at most 3 µl sequentially and let the plates dry in between additions, to avoid excessive spreading of the extract on the plate.

9. If far more than 3 µl of extract has to be used and the sample cannot be reconcentrated (e.g., when using directly culture supernatant instead of ethyl acetate extract), a hole can be made on the agar plate using the top of a glass Pasteur pipette (diameter approximately 7 mm) and then resealing the bottom with 0.5 ml of melted DNAagar. The extract can then be placed in the resulting well, avoiding spilling. In this case, the butyrolactone extract in methanol must be diluted adding TE buffer (10 mM Tris, 1 mM EDTA) pH 7.0 with at least threefold volume of the extract used.
10. For the kanamycin bioassay, the intensity of the halo can be increased by using a higher concentration of inoculum of LW18 or LW94 strain.
11. Hsiao et al. [13] determined the minimum concentration of several different GBLs required to induce the kanamycin resistance phenotype. For SCB1, the minimum concentration was of 0.025 µg/µl. Using the halo diameter for quantification is discouraged, as this shows a sigmoidal response to the amount of GBLs [13].

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Part II

Methods for the Studying of Quorum Sensing at the Molecular, Physiological, and Population Level



Chapter 11

Fluorescence Quenching Studies of γ -Butyrolactone-Binding Protein (CprB) from *Streptomyces coelicolor A3(2)*

Jessy Mariam and Ruchi Anand

Abstract

Fluorescence spectroscopy is an important analytical tool which is widely employed to study biological systems. This technique can be applied to qualitatively and quantitatively probe protein-ligand interactions primarily because of its sensitivity, selectivity, nondestructive and rapid form of analysis. In this chapter we describe the utility of this technique to establish a label-free, universal screening protocol for putative γ -butyrolactone (GBL) receptors by exploiting the intrinsic fluorescence of a highly conserved tryptophan residue that constitutes the hydrophobic pocket for GBL binding, a unique feature possessed by this family of receptors. Here we demonstrate this technique using a combination of steady-state fluorescence quenching methods and fluorescence lifetime decay kinetics using CprB protein from *Streptomyces coelicolor A3(2)* as a model system. Interaction data between CprB and two chemically synthesized GBLs involved in quorum sensing, Cp1 and Cp2, have been used as example.

Key words Quorum sensing, γ -Butyrolactones, CprB, Fluorescence quenching, Potassium iodide quenching, Time-resolved fluorescence lifetime

1 Introduction

Quorum sensing is a networking mechanism in bacteria that enables synergistic regulation of gene expression [1, 2]. This community behavior involves cell-to-cell communication via production of small signaling molecules called autoinducers, followed by their release in the surrounding environment. When the concentration of the signaling molecule exceeds the threshold level (as a result of high cell density) a downstream response is elicited, activating pathways attuned to survival conditions [3]. Bacteria use this mechanism to control processes such as antibiotic and virulence factor production, sporulation, biofilm formation, and competence. *N*-acylhomoserine lactones (AHLs) [4], 4-hydroxy-2-alkyl

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quinolines (HAQs) [5], γ -butyrolactones (GBLs) [6], and cyclic and linear oligopeptides [7] are various chemical classes of signaling molecules. Since these signaling molecules trigger response at very low concentrations (pM– μ M ranges) techniques that can accurately capture their binding profile in a similar range would be greatly beneficial. Moreover, the receptors that these molecules bind to are also difficult to produce and purify at high concentration. Hence, sensitive methods that can detect and probe into the mechanistic details of these interactions at the nano-micromolar scale are needed. Fluorescence spectroscopy, a robust complementary biophysical technique to investigate structure-function relationships, serves as an ideal platform to study these systems. Other techniques like isothermal titration calorimetry and crystallization are limited by the amount of reagents required, sensitivity, and scope of multiplexing.

Fluorescence spectroscopy is a trending technique in biological sciences that has been employed to unravel a plethora of cellular mechanisms and interactions [8, 9]. The timescale of bimolecular dynamics (nanoseconds) and heterogeneity observed in biological systems makes fluorescence spectroscopy an apt technique that can reveal a huge wealth of information. The utility of this analytical tool is augmented by the spectral selectivity that allows one to derive specific information in the biological structure. For instance, selective excitation at 295 nm in proteins reveals the conformational and microenvironment changes of the tryptophan residues [10, 11] and masks the signal arising from the fluorescent tyrosine residues. Another advantage of this technique is that even small spectral changes (fluorescence intensity or peak shifts) can be quantitated by employing sophisticated instrumentation like time-correlated single-photon counting (TCSPC), which can probe variations in spectral properties of excited states of both the ligand and protein of interest. Furthermore, the field of fluorescence spectroscopy can be expanded to encompass fluorescently silent molecules such as proteins and nucleic acids, which can be customized by site-specific labeling using various synthetic fluorophores [12, 13]. For example, cysteine and lysine reactive dyes [14] are commonly coupled to proteins to make them fluorescent. Similarly, fluorophores appended at the 5' end of oligonucleotides like fluorescein and carboxytetramethylrhodamine (TAMRA) dyes as well as nucleic acid base-specific analogs like 2-aminopurine [15] and 3-methylisoxanthopterin [16] have been widely used to study binding properties and dynamics in these systems [16]. However, a drawback of introduction of foreign chemical groups is the potential alteration of structure. Therefore, wherever possible using natural tryptophan residues that serve as label-free probes is an excellent alternative.

In this chapter, we describe a simple, rapid, label-free approach using fluorescence spectroscopy to screen ligands of the γ -

butyrolactone (GBL) receptor proteins such as CprB. GBLs are small diffusible quorum-sensing molecules used by *Streptomyces* and its related genera for triggering secondary metabolism. The mode of binding of GBLs to their cognate receptor family of proteins remains elusive as they are difficult to purify and there is a general paucity of X-ray structural information available in this regard. However, the structure of the apo [17] and DNA-bound forms of CprB [18] from *S. coelicolor* A3(2) provided information about the GBL pocket and aided in design of the screening assay presented here. Similar to all tetracycline receptors, CprB is a Ω -shaped molecule possessing two domains: an N-terminal DNA-binding domain, and a divergent C-terminal ligand-binding domain (the regulatory domain) that is proposed to bind the cognate quorum-sensing molecule. The protein exists in a dimeric state with the two units being related by a pseudo-2-fold axis. The regulatory domain is composed of an antiparallel bundle of five helices ($\alpha 5-\alpha 10$) with helix $\alpha 6$ forming the base of the cavity. The large cavity has a depth of approximately 20 Å and a diameter of 5 Å that is lined by hydrophobic residues. This cavity contains a tryptophan residue (W127) (Fig. 1a) that is known to be conserved among the members (Fig. 2). Docking studies of CprB with γ -butyrolactones have shown that the indole ring of tryptophan interacts with the γ -butyrolactone ring via hydrogen bonding and hydrophobic base stacking interactions [19] (Fig. 1b). Therefore, here the intrinsic fluorescence of this conserved tryptophan was exploited to screen GBLs. The beauty of this method lies in exploring the structure in its innate form, without having to attach

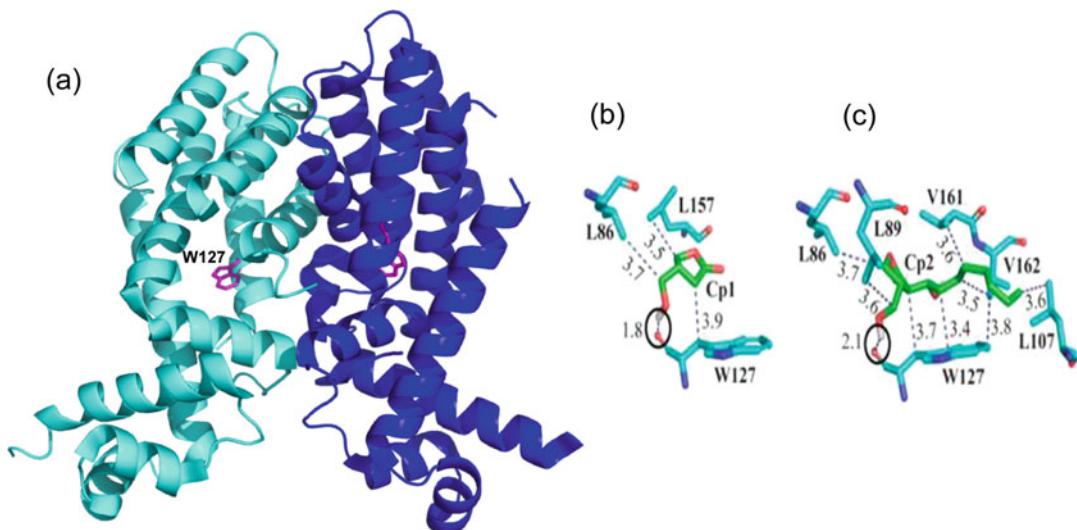


Fig. 1 (a) Structure of the dimeric protein CprB (PDB ID: 1UI5) depicting the hydrophobic pocket containing the conserved tryptophan, W127, (b) and (c) docking of Cp1 and Cp2 with CprB, respectively, revealing the interaction of tryptophan with the butyrolactone ring. Adapted from [19]

CprB	GVPVRPPLPHPFTEWREIATSRLDAVRQSDVHQDIDVDSVAHTLVCSVVGTRVGGTLE
ArpA	G--VFLGGPHPWGDWIDATARMLELGQERGEVFPQIDPMVSAKIIIVASFTGIQLVSEADS
BarA	QGAVIDFSDANPFGEWGDICAQLLAEAQERGEVLPHVNPKKTGDFIVGCFTGLQAVSRVTS
ScbR	QQAHGLDRRGPFRRWHETLLKLLNQAKENGELLPHVVTTDSADLYVGTFAQIQVVSQTVS
FarA	VNAGGLDRSAPFRNWVDKFTDLLEKAQAQGELLPHVVPAETADVITGAYGGVQSMSQALT

Fig. 2 Sequence alignment of GBL family of receptor proteins. The conserved tryptophan is shown in red

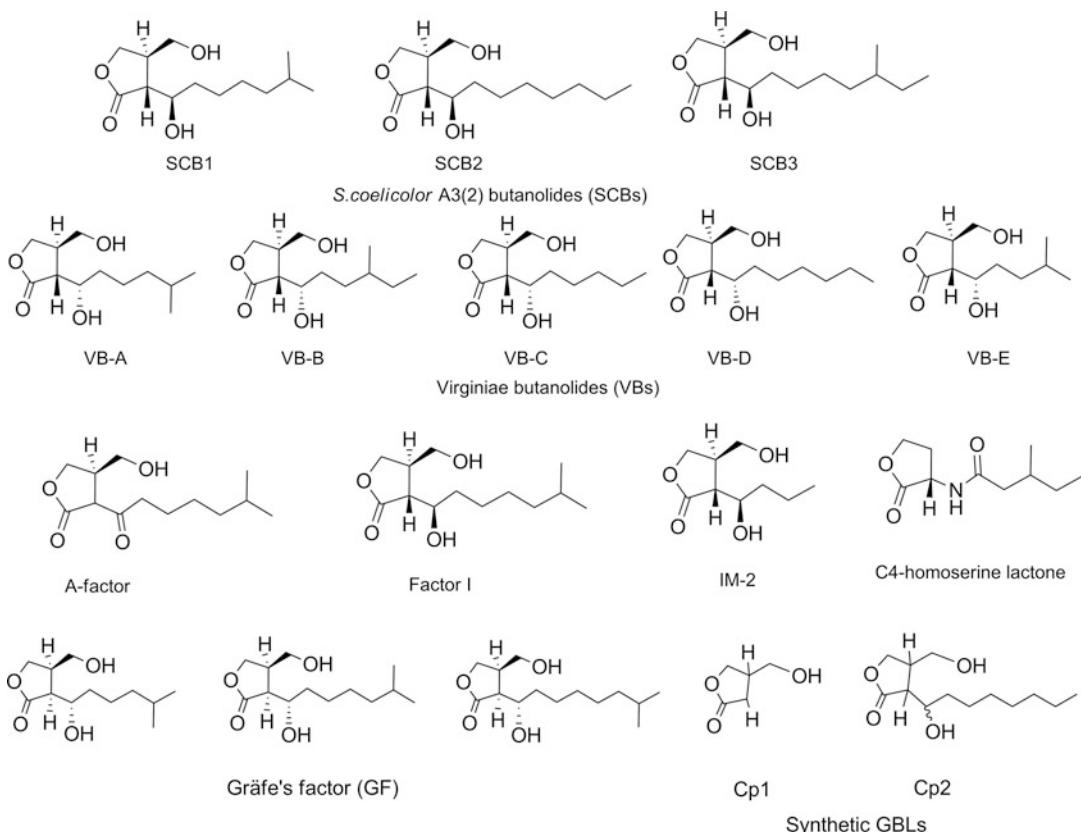


Fig. 3 γ -Butyrolactones and their variants

extrinsic fluorophores that could potentially lead to conformational changes in the protein.

The ligand binding studies in this protocol are performed with two chemically synthesized GBLs, Cp1 and Cp2 (Fig. 3). Cp1 (3-hydroxymethylbutanolide) is a basic butyrolactone moiety whereas Cp2 (2-(1'-hydroxyoctyl)-3-hydroxymethylbutanolide) has an additional aliphatic chain with eight carbon atoms. If a library of γ -butyrolactones and their variants is available, then an array of these ligands (Fig. 3) and various γ -butyrolactone receptors can be screened rapidly in a fluorescence microplate reader setup.

Thus large-scale screening with small quantities of ligands can be undertaken efficiently with microplate reader systems.

In order to ascertain the entry of the ligand in the pocket of the regulatory domain and the ligand binding capacity of CprB, we have adopted fluorescence quenching and lifetime decay kinetics for ligand screening that would be universally applicable to the family of GBL receptors. Fluorescence quenching can be described as a process that decreases the fluorescence intensity of a sample. Quenching can be either dynamic or static or a combination of both. Dynamic, i.e., collisional quenching, occurs when excited fluorophore is deactivated upon contact with the quencher molecule in solution. In this case, the fluorophore returns to the ground state during a diffusive encounter with the quencher. Static quenching on the other hand occurs due to the formation of a nonfluorescent ground-state complex between the fluorophore and the quencher. Dynamic and static quenching can be distinguished by their differing dependence on temperature. Higher temperature results in faster diffusion and larger amounts of collisional quenching. The quenching constant increases with increasing temperature for dynamic quenching; however, it decreases with increasing temperature for static quenching [20]. Steady-state intrinsic tryptophan fluorescence is an excellent indicator of conformational changes in proteins and interaction with ligands that can act as quenchers. Time-resolved fluorescence lifetime studies provide complementary information in addition to the excited-state lifetimes which is determined from the slope of the decay curve. Dynamic or static mechanism of quenching can be distinguished by estimating τ_o/τ , where τ_o and τ are the average fluorescence lifetimes in the absence and presence of the quencher, respectively. For static quenching $\tau_o/\tau = 1$. In contrast, for dynamic quenching there is a decrease in lifetime as depopulation of the excited state occurs. Apart from dynamic and static mechanism, quenching could be a result of other processes such as energy transfer and molecular rearrangements.

In the protocol described here changes in the fluorescence emission characteristics of the conserved tryptophan (W127) are employed to assess the ligand binding in both the native and W185L mutant (single-tryptophan system) forms of the protein. Furthermore, to confirm whether quenching is static or dynamic in nature and to gauge the surface accessibility of the tryptophan residues, fluorescence lifetime and potassium iodide (KI) quenching studies are performed, respectively. KI is a bulky molecule that is used as a marker to determine accessibility of the intrinsic tryptophan. This protocol can be extended for the development and study of quorum-sensing molecule inhibitors against pathogenic virulent strains, which can have a profound pharmacological relevance and impact.

2 Materials (See Notes 1 and 2)

Milli-Q water is used throughout. Buffers are stored at 4 °C and protein stocks at –80 °C.

1. Luria-Bertani (LB) broth: 1% (wt/vol) casein enzymic hydrolysate, 0.5% (wt/vol) yeast extract, 1% (wt/vol) sodium chloride in distilled water. Sterilize by autoclaving.
2. Antibiotic stock solutions: 100 mg/ml Chloramphenicol and 100 mg/ml kanamycin dissolved in ethanol or water, respectively. Filter sterilize and store at –20 °C.
3. Isopropyl β-D-1-thiogalactopyranoside (IPTG) stock solution: 1 M IPTG in sterile Milli-Q water. Store at –20 °C.
4. Binding buffer: 50 mM Sodium phosphate, pH 7.
5. Elution buffer 1: 50 mM Sodium phosphate, 0.1 M NaCl, pH 7.
6. Elution buffer 2: 50 mM Sodium phosphate, 0.2 M NaCl, pH 7.
7. Elution buffer 3: 50 mM Sodium phosphate, 0.3 M NaCl, pH 7.
8. Elution buffer 4: 50 mM Sodium phosphate, 0.4 M NaCl, pH 7.
9. Desalting buffer: 50 mM Sodium phosphate, 100 mM NaCl, pH 7.
10. Ligands Cp1 and Cp2 [19].
11. Potassium iodide (KI) solution: Dissolve KI in desalting buffer to 1 M concentration (*see Note 3*).
12. SP-Sepharose beads.
13. Desalting columns.
14. Fluorescence cuvettes (*see Note 4*).
15. Cary Eclipse spectrofluorimeter.
16. Time-correlated single-photon counting system, IBH Horiba-JY fluorocube.
17. Spectrophotometer.
18. Two liter conical flasks.
19. Shaking incubator.

3 Methods

3.1 Protein Expression and Purification

1. The clone of native CprB in the pET26b(+) expression vector was a gift to us by Ryo Natsume (JBIC, Japan) [19]. These plasmids are transformed in *Escherichia coli* BL21(DE3) pLysS cells, after which the cultures are grown at 37 °C with 250 rpm shaking in 1 l of LB broth supplemented with the 30 µg/ml chloramphenicol and 35 µg/ml kanamycin, in 2 l conical flasks.
2. Measure the optical density of the cultures by spectrophotometry at 600 nm wavelength (OD_{600}). When the OD_{600} of the cultures reaches ~0.6, the expression of the proteins is induced by adding 1 mM IPTG.
3. The induced cells are cultured for 3 h at 37 °C, and then for additional 3 h at 25 °C. Harvest the cells by centrifugation.
4. The harvested cells are resuspended in 10 ml of binding buffer and lysed by sonication.
5. Cell debris is removed by centrifugation at 17,500 × g at 4 °C, and the supernatant is added to 500 µl of SP-Sepharose slurry previously equilibrated with 15 ml of binding buffer.
6. The bead suspensions are gently stirred for 1.5 h. The beads are subsequently separated by centrifugation at 200 × g and mounted on columns followed by a slow wash for 6–8 h with 200 ml of binding buffer.
7. Proteins are gradient eluted with 1 ml each of elution buffer 1, 2, and 3 and final elution is performed with elution buffer 4 till all the protein is eluted out from the beads.
8. The eluted proteins are buffer exchanged in columns with 5 ml of desalting buffer, and used for fluorescence studies.
9. The purity of the protein is verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis with 15% polyacrylamide gel followed by Coomassie blue staining. Protein concentrations are quantified in a spectrophotometer by measuring the absorbance at 280 nm.

3.2 Steady-State Fluorescence Measurements

Fluorescence quenching is an efficient method to probe interactions between biomolecules. The most common method to quantitate the information obtained from quenching data is to plot changes in fluorescence intensity versus quencher concentration in the form of a Stern-Volmer plot that allows one to determine the relative accessibility of the fluorophore (K_{SV}) which in turn can be used to derive binding constants. In the system described here, the conserved active-site tryptophan residue of CprB is the fluorophore and the γ -butyrolactone ligands Cp1 and Cp2 are quenchers. Comparison of quenching constants K_{SV} is used as a measure of quencher strength where high K_{SV} signifies stronger quenching (plausibly enhanced binding) and similar K_{SV} values are reflective of comparable binding affinities of the screened ligands.

3.2.1 Instrument Setup

1. Turn on the spectrofluorimeter. Depending on the instrument, several minutes could be required in order to stabilize the light source before using it.
2. To probe the intrinsic fluorescence of tryptophan in the protein, set the excitation wavelength to 295 nm. Set the emission scan range from 305 to 450 nm.
3. Set the excitation and emission slit widths to 10 nm (*see Notes 5 and 6*).
4. The voltage is set to medium, i.e., 600 V.

3.2.2 Recording the Spectrum

1. Record the baseline fluorescence by placing the desalting buffer in a 1 cm pathlength cuvette. This spectrum is later used to subtract from the sample emission spectrum.
2. All measurements are carried out at 24 °C (*see Note 7*).
3. Record the emission spectrum of 1 ml of CprB solution which is at a final concentration of 3.75 μM (*see Notes 8 and 9*).
4. Dissolve the ligands Cp1 and Cp2 to a concentration of 200 μM in desalting buffer supplemented with 2% (vol/vol) dimethyl sulfoxide (DMSO).
5. Cp1 titration is performed by successive additions of 5 μl of the Cp1 solution prepared in **step 4** into the cuvette already containing the protein. Incubate for 5 min. After each titration, thoroughly mix the solution and record the fluorescence spectrum (Fig. 4).
6. Repeat the above titration **step 5** till the fluorescence intensity saturates.
7. Note down the intensity values at the peak emission wavelength. The values are corrected for dilution (*see Note 10*).
8. Plot a graph of F_0/F versus quencher concentration, [Q] where F_0/F are the fluorescence intensities in the absence and presence of the quencher (Fig. 4).
9. In accordance to the Stern-Volmer equation (Eq. 1), the slope of this graph represents K_{SV} , the Stern-Volmer quenching constant (*see Note 11*):

$$\frac{F_0}{F} = 1 + K_{SV}[Q] \quad (1)$$

10. The K_{SV} value is an average of at least three independent experiments.
11. The same procedure is followed with ligand Cp2 and the K_{SV} values are estimated.
12. Similarly the entire protocol is repeated with the mutant version of CprB, W185L. This tryptophan residue is located away

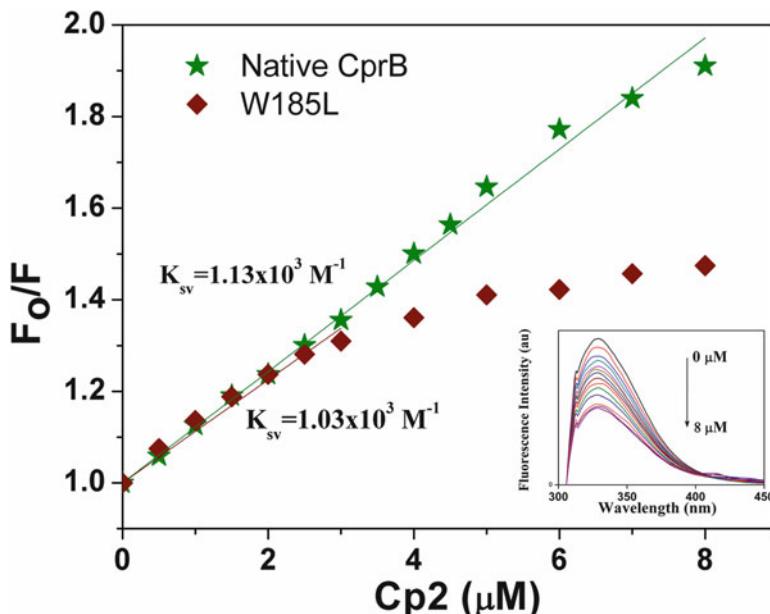


Fig. 4 Stern-Volmer plot for native CprB protein and mutant W185L in the presence of quencher Cp2 (inset: Steady-state emission spectra of CprB (3.75 μ M) in the presence of increasing concentrations (0–8 μ M) of Cp2)

from the binding pocket and will enable to delineate the contribution of fluorescence quenching by W185 from that of the tryptophan residue of interest W127.

3.3 KI Quenching Studies

Fluorescence quenching experiment using KI is an excellent indicator of solvent accessibility of tryptophans. Iodide ions being bulky are unable to penetrate into the protein core and hence K_{SV} values obtained using iodide as a quencher are indicative of the location of tryptophan residues, i.e., surface or buried. Here, very low K_{SV} values obtained via KI quenching indicated that the intrinsic tryptophan residues are deeply buried in the core of the protein, which the smaller and specific γ -butyrolactones could selectively access.

1. Record the emission spectrum of 1.5 μ M CprB as described in Subheading 3.2.
2. Perform a series of titration of KI solution into the CprB solution, such that the final concentration of KI varies from 0 to 50 μ M.
3. Record the fluorescence spectrum after each titration.
4. Determine the K_{SV} values as described in Subheading 3.2.

3.4 Time-Resolved Fluorescence Lifetime Measurements

Time-resolved fluorescence lifetime measurements are performed in the same timescale as the lifetime of the fluorophore. Hence conformational changes of the fluorophore and its plausible interactions with the neighboring environment can be additionally

deduced. In static quenching, formation of a nonfluorescent ground-state complex occurs between the fluorophore and the quencher. Thus the lifetime of the free (τ_0) or complexed fluorophore (τ) remains approximately the same. Contrastingly, in dynamic quenching, collisions between the fluorophore and the quencher occur during the lifetime of the excited state and a decrease in lifetime will be observed upon increasing quencher concentrations.

3.4.1 Instrument Setup

1. Time-resolved fluorescence experiments are performed on an IBH Horiba-JY fluorocube.
2. Attach the NanoLED 295 nm and switch on the instrument. Keep it on for 30 min to stabilize the source before using.
3. To avoid polarization artifacts decays are recorded under magic angle conditions. Emission polarizer is kept at 54.7° and excitation polarizer is kept in vertical position, i.e., 0° .
4. Emission slit width is set to 12 nm.
5. Time per channel is set to 7 ps.
6. The instrument response function (IRF) viz. prompt is done at the excitation wavelength 295 nm using a glass light scatterer. The FWHM is found to be 700 ps.

3.4.2 Recording the Lifetime Decay

1. $5 \mu\text{M}$ CprB is placed in a 1 cm pathlength quartz cuvette and excited at 295 nm and the emission is collected at 328 nm.
2. Decay is recorded till the number of counts in the peak channel is at least 3000.
3. Lifetime decay kinetics is then collected for native CprB in the presence of varying concentrations of Cp2, ranging from 1 to $10 \mu\text{M}$ (Fig. 5).

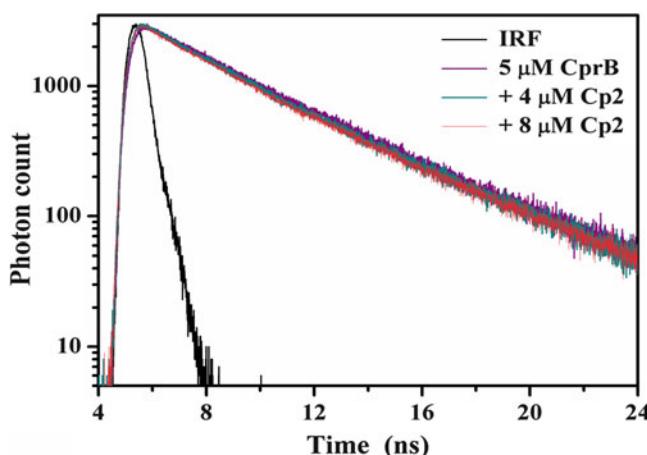


Fig. 5 Time-resolved fluorescence decay curve of CprB ($5 \mu\text{M}$) and CprB complexed with Cp2. Adapted from [19]

3.4.3 Data Analysis

1. The decay obtained is fitted by the global deconvolution fitting technique using the software FluoFit (Global Fluorescence Decay Data Analysis Software, Pico Quant).
2. The analysis is a nonlinear least-squares iterative deconvolution based on the Levenberg-Marquardt algorithm and expressed as a sum of exponentials with Eq. 2:

$$I(t) = \sum \alpha_i \exp(-t/\tau_i) \quad (2)$$

where α_i represents the amplitude of the i th component associated with fluorescence lifetime τ_i such that $\sum \alpha_i = 1$. $\sum \alpha_i \tau_i$ gives the mean lifetime τ_m of the system.

3. The goodness of fits is determined from the reduced χ^2 values (~ 1) as well as from the randomness of the residuals.

4 Notes

1. Solvents and chemicals must be devoid of fluorescent contaminants that can act as a quencher or alter the background signal.
2. Buffers and solutions must be free of particles/tissue fibers or aggregates that can scatter light and produce artifacts in the readings. In such situations it is ideal to filter all the solutions.
3. KI solutions should be freshly prepared as they are sensitive to light degradation.
4. All the faces of the fluorescence cuvette should be completely clean. Avoid cleaning of cuvettes with detergent solutions as they could potentially contain fluorescent compounds. Clean the cuvettes thoroughly with chromic acid on a regular basis. Before placing the cuvette in the cell holder, wipe the surface of the cuvette with a fiber-free tissue and be careful to not touch the sides of the cuvette. Position the cuvette reproducibly in the cell holder each time. During the titration process avoid spillage of sample on the sides of the cuvette.
5. Initial optimization of the instrument parameter settings such as slit widths for your sample will have to be done in order to obtain a good signal-to-noise ratio. Slit widths are chosen based on fluorescence yield of the sample. Ideally larger slit widths are not selected due to interference of noise which may result in loss of data resolution. In such cases it is preferable to increase the concentration of the fluorophore and maintain narrow slit widths.
6. Once the optimal instrument parameter settings are known all the steady-state fluorescence measurements are performed at the same settings for all the samples. Do not compare results that have different parameter settings. This can lead to a wrong interpretation of the results.

7. As fluorescence measurements are sensitive to temperature it is ideal to use thermostatted cell holders.
8. Ideally measure absorbance of each sample before taking fluorescence measurements to reduce inner filter effects. It is also crucial to measure the absorbance of the quencher sample alone. If the quencher has an absorption peak at the excitation wavelength, the decrease in fluorescence intensity could be wrongly interpreted as quenching. Under such circumstances appropriate correction factors need to be applied and then interpret the data carefully.
9. Monitor if the protein chosen for the study is stable under the given experimental conditions and the time course of the experiment. It should not form aggregates or be sensitive to photodegradation.
10. Make correction for the concentrations of the fluorophore at each point of titration. At this point interpret the data carefully. The changes in fluorescence intensity should not be a result of dilution. If small amounts of titrant are added the changes in concentration of fluorophore can be considered to be negligible.
11. K_{SV} values signify the extent of accessibility of the quencher to tryptophan. While determining the slope from the Stern-Volmer plot fix the intercept at 1. If the plot deviates from linearity then calculate K_{SV} only from the linear part of the plot. Deviation from linearity could represent various conditions such as fractional accessibility to quencher and conformational changes that lead to exposure of previously shielded tryptophan residues. Hence deviations in Stern-Volmer plot must be carefully interpreted.

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Chapter 12

Methods to Study Solo/Orphan Quorum-Sensing Receptors

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Abstract

LuxR solos/orphans are very widespread among Proteobacteria; however they are surprisingly under-studied given that they are likely to play a major role in cell-cell communication in bacteria. Here we describe three simple methodologies/approaches that can be used in order to begin to study this subgroup of quorum sensing-related LuxR receptors.

Key words *E. coli*, *Salmonella*, LuxR solos, LuxR orphans, SdiA, LuxI, Quorum sensing, Regulon, Transposon, Transcriptional fusion

1 Introduction

Quorum sensing (QS) is a signaling mechanism that mediates regulation of bacterial behavior in response to environmental changes in a population density-dependent manner. In Gram-negative bacteria the most common and best studied signals thus far are the *N*-acyl-homoserine lactones (AHLs); these vary in structure having acyl chains of different lengths (4–20 carbons) and different oxidation states at position C3. An archetypical AHL QS system consists of a LuxI family synthase, which synthesizes the AHL signal, and a LuxR family regulator, which directly binds the cognate AHL and consequently affects gene expression [1]. Typically, LuxI/R AHL QS systems are usually genetically linked either on the chromosome or the plasmids. AHL QS systems have been investigated in many Gram-negative proteobacteria and have been linked with phenotypes that benefit the bacterial AHL-producing community including production of virulence factors, motility, nodulation, plasmid transfer, antibiotic production, conjugation, competence, bioluminescence and biofilm formation [2, 3]. The increasing availability of new bacterial genomes and their analyses has revealed several LuxR proteins that lack a cognate LuxI synthase; these have been named LuxR solos or orphans [4–8].

The purpose of this chapter is to describe simple ways to identify these LuxR solos, detect their ligands, and study their regulon.

The LuxR regulatory family is large and consists of several subfamilies, one being the AHL-binding QS-LuxRs. Proteins of the QS-LuxR subfamily are about 250 amino acids long and have two domains bridged by a short linker region. One is an autoinducer (AHL)-binding domain located at the *N*-terminus [9, 10] and the other is a DNA-binding helix-turn-helix (HTH) domain positioned at the *C*-terminus [11–13]. The two domains can function independently of each other. These proteins bind to an inverted DNA repeat recognition sequence that consists of a conserved 20 bp palindrome site called *lux* box [14–17]. QS LuxR-type proteins can act as either a transcriptional activator or a repressor [1]. In the case of activation, binding to the AHL brings about a conformational change that allows the HTH domain to bind DNA at *lux* boxes of its target genes, which then allows the LuxR/AHL complex to recruit RNA polymerase [18] and activate transcription [19]. When the protein is a repressor, it is bound to target promoters in the absence of the cognate AHL, thereby blocking access to RNA polymerase. When AHL concentrations reach quorum threshold concentrations and bind to the LuxR-type protein, conformational changes cause it to release the promoter and in turn relieve repression [20]. Structural analysis of TraR of *Agrobacterium tumefaciens* [19, 21] and SdiA of *Escherichia coli* [22] revealed that the AHL-binding cavity is composed of five-stranded antiparallel β -sheets with three α -helices on each side. AHL binding to some QS-LuxRs is crucial for their folding and stability and the absence of AHLs leads to fast degradation by proteases [23].

Surprisingly, QS-LuxRs display rather low homology in their primary structure; however almost all share nine highly conserved amino acid residues [3, 19]. Six of these residues are hydrophobic or aromatic and form the cavity of the AHL-binding domain and three are in the HTH domain. Evolutionarily, two distinct groups of QS-LuxRs are emerging; one group is found in proteobacteria from different divisions (α , β , and γ) and the second is found only in γ -proteobacteria. Interestingly, proteins from the first family behave as activators whereas members of the second group are mainly repressors [16]. The latter could have evolved relatively recently probably at the divergence of the *Xanthomonads* and *Xylellas*, which lack QS LuxI/R homologs, but before the divergence of the pseudomonads which harbors them [24].

Many sequenced proteobacterial genomes have evidence of the widespread presence of QS-related LuxR AHL sensors/regulators which lack a cognate LuxI AHL synthase [4, 6, 7]. These unpaired QS LuxR family proteins have been called orphans and more recently solos [5, 8] and possess the typical modular structure having an acyl-homoserine lactone-binding domain at their *N*-

terminus and a helix-turn-helix DNA-binding domain at their C-terminus. LuxR solos have so far been implicated in intraspecies, interspecies, and interkingdom communication and are likely to play a major role in cell-cell communication in bacteria. Some of them have been shown to bind AHLs and expand the regulatory repertoire of a classical quorum-sensing system by responding to endogenous AHLs [8]. Alternatively they can also regulate genes by “eavesdropping” on exogenous AHLs produced by neighboring bacteria. QscR of *Pseudomonas aeruginosa* is a very-well-studied example of a LuxR solo responding to endogenously produced AHLs [25] and SdiA of *E. coli* and *Salmonella enterica* is a well-studied LuxR solo which binds exogenous AHLs produced by neighbors [26]. Recently a group of non-AHL-binding LuxR solos has been identified that bind yet-unknown plant-derived molecules, and these have been studied in several plant-associated bacteria [27]. Finally, some LuxR solos have been reported to regulate target genes in a ligand-independent manner [8, 28].

This chapter intends to highlight three inexpensive and simple ways to begin to investigate this family of proteins that are currently under-studied and are likely to play important roles in different types of cell-cell bacterial communication. These approaches can then lead to other methodologies allowing further characterization of LuxR solos (Fig. 1). We first describe criteria that can be used for *in silico* discrimination of LuxR solos from other QS LuxRs and to classify them into different LuxR solo types based on certain sequence features that reflect ligand specificities. In the next section

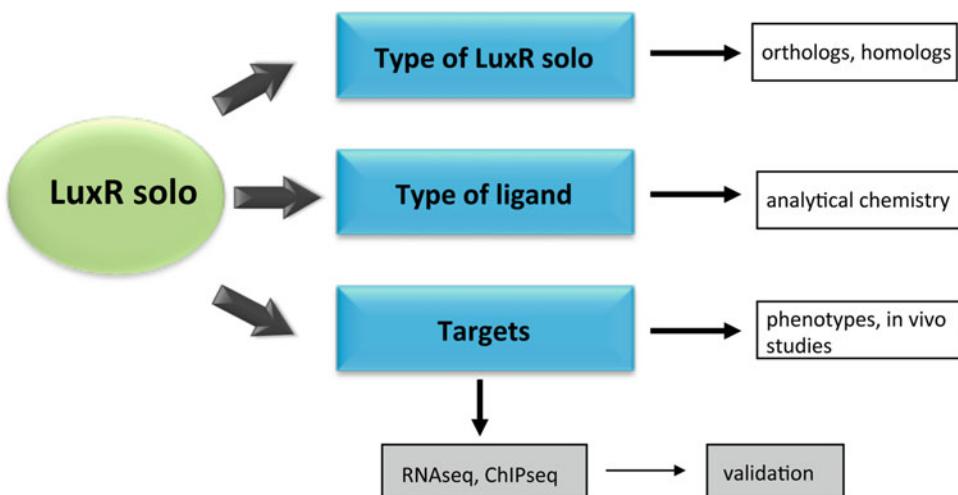


Fig. 1 The three simple methodologies allow a classification of the LuxR solo, information on the ligand, and identification of potential targets. This could then lead to further studies involving analytical chemistry in order to precisely determine the structure of the ligand, to study phenotypes and role *in vivo*. More modern approaches like RNAseq and ChIPseq which are expensive and which require extensive validation studies can also be used for determination of LuxR solo targets

we describe a simple method to experimentally confirm ligand binding specificities of the LuxR solo protein. Finally, a method that can be used to identify possible gene targets of LuxR solos is described.

2 Materials

2.1 Hardware and Software for In Silico Identification of LuxR Solos/Orphans

1. A personal computer connected to the Internet.
2. Complete genome sequences available at public databases like NCBI, GeneDB (<http://www.genedb.org/>), and TIGR (<http://www.tigr.org/>) or genome sequences of strains of interest like *Pseudomonas* Genome Database (<http://www.pseudomonas.com/>), EcoCyc (<http://ecocyc.org/>), or sequences obtained from samples of interest.
3. The InterPro database freely available at <http://www.ebi.ac.uk/interpro>.
4. SynTax: <http://archaea.u-psud.fr/SyntTax>.
5. FGENESB: <http://www.softberry.com/berry.phtml?topic=fgenesb&group=programs&subgroup=gfindb>.
6. Clustal W: <http://embnet.vital-it.ch/software/ClustalW.html>.
7. Clustal Omega: <https://www.ebi.ac.uk/Tools/msa/clustalo/>.
8. BPROM: <http://www.softberry.com/berry.phtml?topic=beprom&group=programs&subgroup=gfindb>.
9. MEME Suite: <http://meme-suite.org/>.

2.2 Studies on the Ligand of the LuxR Solo/Orphan

1. Plasmids pQE30, pQE31 and pQE32.
2. Structurally different AHLs.
3. *E. coli* M15 (pREP-4).
4. Antibiotic stock solutions: 100 mg/ml ampicillin (Ap), 25 mg/ml kanamycin (Km), and 25 mg/ml nalidixic acid (Nal) dissolved in distilled water and filter sterilized. A few drops of 10 M NaOH are required to dissolve Nal in distilled water before sterilization by filtration.
5. Lysogeny broth (LB): 1% (wt/vol) bacto-tryptone, 0.5% (wt/vol) yeast extract, 1% (wt/vol) sodium chloride in distilled water. Sterilize by autoclaving.
6. LB agar: LB plus 1.5% (wt/vol) agar. Sterilize by autoclaving.
7. 1 M Isopropyl-β-D-thiogalactoside (IPTG) dissolved in distilled water.
8. Binding/lysis buffer: 50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole; adjusted to pH 8.0 using NaOH.

9. SDS-PAGE sample buffer: 0.09 M Tris-HCl pH 6.8, 20% (vol/vol) glycerol, 2% (wt/vol) sodium dodecyl sulfate (SDS), 0.02% (wt/vol) bromophenol blue, 0.1 M dithiothreitol (DTT).
10. Donor strain: This is the strain that contains the transposon (*see Note 1* and *2*) that has to be mobilized into your recipient strain of interest (*see Note 3*). We will use the *E. coli* strain BW20767 carrying the suicide vector, pUT mTn5 *lux kan2*, as an example.
11. Recipient strain: This is the strain that you wish to mutagenize. We will use the *Enterobacter cloacae* strain JLD401 as an example (*see Note 4*).
12. 100 and 250 ml Erlenmeyer flasks.
13. Shaking incubator.
14. Refrigerated centrifuge capable of 10,000 $\times g$.
15. Sonicator.
16. 1.5 and 15 ml plastic tubes.
17. 96-Well microtiter plates.
18. Sterile toothpicks or pipet tips.
19. Spectrophotometer/luminometer.
20. Sequencing and PCR primers.

3 Methods

3.1 In Silico Analyses for Identification of a LuxR Solo/Orphan

3.1.1 Identifying a LuxR Solo

Identification of a LuxR solo (*see Note 5*) involves first determining if the protein has sequence features typical of a QS LuxR protein and then ascertaining the characteristics of the genome hosting this protein to find out whether it is part of a LuxI/LuxR QS system or occurs alone (*see Note 6*). In case of metagenome sequences these analyses might be limited if a complete genome sequence is not available. In that case, a particular contig covering several 100 Kbs might be used as the template for analyses of the context of the LuxR-coding region. The details for use of each bioinformatic tool mentioned here are available in the respective web sites and they should be referred to for further information.

1. Identification of QS LuxR proteins: Go to the InterProScan web page. Enter the sequence/s of interest (either protein or DNA) in the search box or upload from a file. Choose the sequence type as either protein or DNA and if latter choose the translation table. Enter an e-mail address and submit job to begin the search for protein signatures of the sequence. In the results page, identify protein sequences showing IPR005143 with the signature “transcriptional factors LuxR-like,

autoinducer-binding domain” at the *N*-terminal end. Examine protein sequences positive for IPR005143 for the presence of any of the three signatures namely IPR000792 (transcription regulator LuxR, C-terminal), IPR016032 (signal transduction response regulator, C-terminal effector), or IPR011991 (winged HTH DNA-binding domain). A typical QS LuxR protein can be identified by the presence of one of the three combinations of different domain architectures in the InterPro collection: IPR005143 (ABD)-IPR000792, IPR005143-IPR016032, and IPR005143-IPR011991.

2. Determining QS-related characteristics of the QS LuxR-coding region/genome: If the protein sequence of interest has been identified as a QS LuxR protein by in silico analyses, the next step is to analyze the remainder of the genome for the presence of genes that could code for other LuxI or LuxR homologs. Carry out a BLASTP or a PFAM search using the predicted protein sequences of the genome of interest to identify any proteins that might code for LuxI homolog. Take the putative LuxI homolog protein and carry out InterProScan of the identified sequence to verify the presence of IPR001690 (autoinducer synthesis protein) and IPR018311 (autoinducer synthesis, conserved site) domain, the protein signatures of the LuxI homolog. To analyze the neighboring regions for a LuxR homolog, retrieve flanking sequences and analyze using SynTax [29] or annotate using tools available at FGENESB [30].
3. Interpretation of results of the InterProScan search and the context search in the genomes/sequences of interest: If no gene coding for a LuxI homolog was found in the genome or in the genomic locus near the gene coding for QS domain LuxR protein and if no unpaired or extra genes coding for LuxI homologs were present in the remainder of the genome, the QS LuxR protein of interest may be classified as a LuxR solo or orphan.

3.1.2 Classification of LuxR Solo Types

AHL-binding QS LuxR proteins are characterized by the presence of all nine key residues which have been reported to be invariant previously, six of which are in the AHL-binding domain and three in the DNA-binding region (W57, Y61, D70, P71, W85, G113, E178, L182, G188) with respect to TraR (*see Note 7*) of *A. tumefaciens* [3, 19]. In contrast, non-AHL-binding LuxR solos are characterized by variation in at least one of the six key residues in the autoinducer-binding domain. Although it is not clear if these differences alone are sufficient to determine altered ligand specificity, the changes in the key amino acid residues could be a way to distinguish types of LuxR solos. Therefore, by analyzing the protein sequence of a newly identified LuxR solo, it is possible to classify it putatively into AHL-binding or non-AHL-binding

LuxR solo types. Here we describe steps to distinguish and methods to identify AHL-binding LuxR solos, plant-associated bacterial (PAB) LuxR solos, and non-AHL-binding LuxR solos by *in silico* analyses.

1. Go to Clustal W [31–33] and align the protein sequence of the LuxR solo of interest against the TraR amino acid sequence.
2. Inspect the alignment and evaluate the presence or absence of invariant amino acids at the designated positions of the LuxR solo protein sequence. No change indicates that the LuxR solo likely binds AHLs.
3. Further inspect the alignment at positions W57 and Y61 (with respect to TraR). LuxR solos of PAB are known to carry substitutions at these positions (*see Note 8*).
4. Next, note the conservation of amino acids at Y61 and D70 (with respect to TraR). These are the only two amino acids at the AHL-binding domain that remain invariant in the non-AHL-binding LuxR solos.
5. Having determined tentatively the LuxR solo type, analyze the genomic locus adjacent to the gene encoding the LuxR solo. LuxR solos and non-AHL LuxR proteins are known to regulate adjacent target genes, which encode for proteins that synthesize secondary metabolites or enzymes. Identify any genes/operons using FGENESB and analyze promoter regions using prokaryotic promoter search software such as BPROM [30]. Retrieve the promoter region of the adjacent locus and ascertain if it has the 20-bp palindromic *lux* box using tools available at MEME [34]. Examine the annotated genes to determine if they encode protein(s) responsible for the synthesis of any secondary metabolite. The promoter analysis information and the identity of the adjacent gene may be used for wet-lab validation of *in silico* predictions.

3.2 Studies on the Ligand of the LuxR Solo/Orphan

QS-LuxRs are highly insoluble when overexpressed; however, in the presence of their cognate AHL molecule, which they usually bind with very high affinity, they often become soluble (*see Note 9*). Cognate AHL is required for the proper folding of the nascent protein, for formation of homomultimers, and for protection from proteases [23, 35–38]. AHL-free QS-LuxRs instead misfold, and are highly unstable and prone to proteolysis. Similarly, it has been observed, for the subfamily of LuxR solos that bind a plant-derived molecule, that plant extracts can solubilize the protein [27, 39], the reason being that instead of binding AHLs they bind a plant low-molecular-weight compound. This clear biochemical feature can therefore be exploited to indirectly demonstrate that a LuxR solo binds AHLs or a plant-derived molecule. This methodology consists of a simple and fast protocol for the expression and purification

of the His-tagged LuxR solo in *E. coli* in the presence and absence of AHLs or plant extracts and for testing their solubility and stability under native non-denaturing conditions.

1. Clone the gene coding for a LuxR solo in any one of the pQE vectors for expression as an *N*-terminal His-tagged protein. The gene has to be cloned in frame with the His-encoding tag; plasmid vectors pQE-30, pQE-31, and pQE-32 provide the multiple cloning site (MCS) in all three reading frames; use one of these depending on the desired reading frame for generating the His-LuxR fusion protein. For more detailed information see the QIAexpressionist Handbook (Qiagen, Hilden, Germany).
2. Transform the pQE-based His-tagged-*luxR* solo (pQELUXRSOLO) plasmid expression construct in heterologous *E. coli* M15(pREP4) and select on LB agar plates supplemented with 100 µg/ml ampicillin and 50 µg/ml kanamycin. The presence of pREP4 allows control via the LacI repressor of the promoter controlling the expression of the *luxR* solo and allowing induction of expression upon the addition of inducer (*see step 4* below).
3. Use *E. coli* M15 (pREP-4) (pQELUXRSOLO) to inoculate 10 ml of LB broth supplemented with 100 µg/ml ampicillin and 50 µg/ml kanamycin in a 100 ml Erlenmeyer flask and grow overnight at 37 °C with shaking.
4. For determination of whether it binds an AHL, add 1 ml of the overnight culture to two independent 250 ml Erlenmeyer flasks containing 50 ml of prewarmed LB broth; of these, one is supplemented with 20 µM of the AHL(s) of interest (*see Note 10*). Induce expression of the LuxR solo protein by adding 1 mM IPTG at an OD₆₀₀ of 0.6 and allow growth to continue for 3 h at 37 °C. The culture then needs to be rapidly chilled on ice and the cells are harvested by centrifugation (8000 × *g* for 10 min at 4 °C) and frozen at -80 °C.
5. For determination whether it binds a plant-derived molecule, instead of adding AHLs to the media, plant macerated material is provided to LB as follows: 10 g of plant material (leaves, stems, or roots depending on the location of the plant-associated bacteria which is being studied, *see Note 11*) is frozen with liquid nitrogen and macerated. The resultant plant powder is added to 100 ml LB broth and autoclaved (alternatively it can be mixed/vortexed with LB at room temperature for 30 min and then filter sterilized). This media containing plant extract can then be used for LuxR solo protein solubilization studies as described in **step 4**.
6. Resuspend the cell pellet in 5 ml of binding/lysis buffer and lyse the cells by sonication using 5 × 10 s pulses (200–300 W) pausing for 20 s in between each pulse. During sonication the sample should be kept on ice at all times.

7. Centrifuge the lysate at $10,000 \times g$ at 4 °C for 30 min. Take and save the cleared supernatant: it represents the soluble fraction. Resuspend the pellet in 5 ml of binding/lysis buffer: this, instead, represents the insoluble fraction.
8. Analyze the samples representing soluble and insoluble proteins in SDS-PAGE as follows: add 5 µl of SDS-PAGE sample buffer to 5 µl of the two protein extracts. Microcentrifuge at maximum speed for 2 min and load each sample on a 12% SDS-PAGE gel. Run the gel at 200 V for ~45 min and determine whether the overexpressed QS-LuxR solo is located in the soluble or insoluble fraction by visual inspection of the gel after Coomassie staining (more details on SDS-PAGE analysis can be found in Chapter 6 of this book).

3.3 Identifying LuxR Solo Regulon Members Using Random Transcriptional Fusions

3.3.1 Generation of Fusions

Once a LuxR solo has been identified, the regulon can be identified by either screening random transcriptional fusions or using RNA-seq and/or ChIPseq. RNAseq has the advantage of more complete coverage of the genome, but the genes that appear to be regulated by the LuxR solo must be confirmed at a later time, either with qRT-PCR or with transcriptional fusions. The advantage to screening random transcriptional fusions is that the resulting fusions can be used for confirmation and further studies immediately. In this chapter we describe the screening of random transcriptional fusions, while RNAseq and ChIPseq are the topics of other reviews; the experiment described will highlight genes that respond to AHL(s) which have to be confirmed as belonging to the LuxRsolo regulon by further analysis as checking their response to AHLs in the LuxRsolo mutant.

1. Grow 5 ml overnight cultures of the donor strain (*E. coli* BW20767 carrying pUT mTn5 *lux kan2*) and your recipient strain of choice (for example, *E. cloacae* JLD401) at 37 °C with shaking. In this case, both cultures can utilize LB broth as growth medium. The donor culture should include 100 µg/ml Km and the recipient culture should include 25 µg/ml Nal to insure that those markers are maintained.
2. The following day, remove the antibiotics from the cultures. To do this, centrifuge the 5 ml of both cultures for 10 min at $10,000 \times g$ to achieve pelleting of the bacteria. Remove the supernatant from each pellet and resuspend the pellet in 5 ml fresh LB.
3. To begin the mating of the strains, mix 100 µl of the donor and the recipient together in a microcentrifuge tube (final volume 200 µl); set up between 10 and 50 matings. Spot the entire volume of each tube, 200 µl, on an LB agar plate and incubate overnight at 37 °C. Having each plate represent a separate mating insures that mutants obtained from separate plates are

independent transposition events and not siblings. As a control, and to check the quality of the plates that will be used the following day, streak both your donor and recipient cultures on LB plates supplemented with 100 µg/ml Km or 25 µg/ml Nal. The donor should not grow on LB Nal and the recipient should not grow on LB Km.

4. The following day, pipet 5 ml of LB onto each of the mating plates. Scrape the bacteria from the surface and remove 1 ml of the LB and bacteria mixture into a 1.5 ml microcentrifuge tube. You should now have between 10 and 50 microcentrifuge tubes of mated bacteria. From each tube, plate 100 µl onto each of the ten selective plates (*see Note 12*). In our case, the selective plates are LB agar containing 100 µg/ml Km and 25 µg/ml Nal. Incubate overnight at 37 °C.
5. The following day, each selective plate should have hundreds of colonies, each ideally representing a recipient strain containing a single-transposon insertion (*see Note 13* for troubleshooting).
6. Repeat this procedure until the required number of mutants is achieved (*see Note 14*).

3.3.2 Screening and Characterization of Fusions

1. To screen large numbers of fusions for AHL responsiveness, we typically use 96-well plates. The wells are pre-filled with a growth medium that is permissive for the expression and activity of the LuxR homolog of interest (*see Note 15*). Alternating rows contain one or more AHLs or solvent control.
2. Using toothpicks or pipet tips, stab each isolated colony and stab it into the 96-well microtiter plate, first into the solvent control well and then the AHL-containing well (to prevent carryover of AHLs). The toothpick will easily carry enough bacteria to inoculate both wells and does not need to be re-stabbed into the original colony between wells.
3. After the bacteria have achieved stationary phase, or at multiple time points during growth, depending on your reporter, compare the fusion activity of the adjacent wells to identify those that are AHL responsive. In our case, we used a spectrophotometer/luminometer to measure luciferase activity after 9 h of growth.
4. Fusions that appear to be AHL responsive in the well of the 96-well plate need to be streaked to isolate single colonies and retested.
5. The location of the transposon insertion point in the genome must be determined. There are a variety of methods to do this, including direct sequencing of genomic DNA, inverse PCR, and cloning of the transposon.

4 Notes

1. Fusions can be generated in two main ways. One method is to clone fragments of DNA into a plasmid vector that contains a promoterless reporter gene(s) downstream of the multiple cloning site. This library of fusions is then transformed or electroporated into your organism of choice [40]. Plasmid-based fusions can behave oddly based on their high copy number but they are easy to recover and characterize. If the vector has conditional replication it can be used to generate fusions in the chromosome by Campbell recombination (single crossover) [41]. In this report, we utilize another method of making chromosomal fusions: random transposon integration.
2. There are numerous choices of transposon [42]. The transposon should have random integration characteristics and a selectable marker that functions in your organism of choice. It should also create transcriptional fusions to a suitable reporter gene(s). In this report we are using an mTn5 encoding kanamycin resistance that creates fusions to the *luxCDABE* operon of *Photobacterium luminescens* [43].
3. Transposons can be delivered as a purified protein complex by electroporation (a transposome) [44], or delivered as a genetic construct on a plasmid with conditional replication (a suicide vector) either by electroporation, transduction, or mobilization. In this report, we are using mobilization in which the donor strain encodes the conjugation machinery. The suicide vector has an RP4 origin of transfer (*oriT*) allowing passage through the RP4-derived conjugative apparatus. The vector has an R6K origin of replication lacking the *pir* gene that is required for replication [45]. Thus, it can only replicate in host strains that provide the *pir* gene [46]. Upon arrival in the recipient strain the plasmid will fail to replicate.
4. Your recipient strain needs to have a characteristic that is not present in the donor strain, i.e., a metabolic capability or antibiotic resistance. For this reason, most donor strains have auxotrophies so that a recipient strain can be distinguished by simply being prototrophic. Alternatively, your recipient strain can encode an antibiotic resistance that is not present in the donor strain. In our example, we selected for a spontaneous resistance to nalidixic acid in our recipient strain by plating on agar containing nalidixic acid.
5. In this chapter LuxR solos or orphans are identified in the context of AHL signals. Therefore genomes lacking a cognate LuxI for a LuxR would be classified as carrying a LuxR solo. However, these LuxR solos might bind to novel non-AHL signals, and once these new signals are identified and verified

to be encoded by the same genome, it is not appropriate to refer to these LuxR proteins as LuxR solos. Instead, they are non-AHL-binding LuxR proteins that sense endogenous ligands and together constitute the non-AHL QS systems. However, they might differ from typical AHL QS system in lacking the feedback effect of LuxR homolog on the signal synthase, for example the PluR/PpyS system [47].

6. LuxR solos identified in this manner might be occurring alone or together with one or more complete QS system(s).
7. Here TraR is used as an example of a canonical AHL-binding QS LuxR protein. Other well-studied QS LuxR proteins that may be used for the purpose of identification of invariant amino acid residues in the LuxR solo include well-characterized LuxR homologs such as LasR of *P. aeruginosa* and LuxR of *Vibrio fischeri*.
8. The two amino acid substitutions for PAB LuxR solos are methionine for tryptophan at position 57 and tryptophan for tyrosine at position 61 in case of OryR [39]. Depending on the type of LuxR solo, different well-studied representatives of each type may be used for alignment. For example, for PAB LuxRs, XccR of *Xanthomonas campestris*, OryR of *Xanthomonas oryzae*, PsOR of *Pseudomonas protegens*, or NesR of *Sinorhizobium meliloti* may be aligned with the protein of interest. For non-AHL LuxR solo, PluR of *P. luminiscens* and PauR of *P. asymbiotica* may be used for protein sequence alignment.
9. It cannot be excluded that some LuxR solo proteins when purified as unbound to AHLs are soluble and stable [48].
10. Since there are many structurally different AHLs available, a cocktail can be used always ensuring that each AHL is used at a concentration of 20 µM. Subsequently they can be independently used in order to establish which one(s) are causing solubilization of the protein.
11. It is possible that some plant extracts could be toxic for bacterial growth at these concentrations: in this case reduce the amount of macerated material accordingly.
12. The selective conditions must prevent growth of the donor and recipient strains, but allow growth of transconjugants in which the transposon has transposed from the suicide vector into the chromosome of the recipient. Most commonly, this is done with either nutrient source limitations or antibiotics. In the case illustrated here, the donor is killed by nalidixic acid while the recipient is killed by kanamycin. Only transposition events in the recipient can lead to resistance to both antibiotics.
13. If no colonies are obtained there may be a problem with the suicide vector. Plasmids encoding transposons are not stable

and rearrange quite frequently. Find a new source of the vector. Another issue is that the RP4 conjugative apparatus must be able to initiate conjugation with your recipient strain. The RP4 apparatus does exhibit phase variable host specificity, which can be problematic. In this case, try other donor strains such as SM10 λ *pir* or S17 λ *pir* [49]. If too many colonies are obtained, repeat the mating and plate dilutions of the mating mix onto the selective plates until isolated colonies are obtained.

14. Assuming 4400 genes in the typical bacterial genome, and given the fact that transposons can insert into reading frames in two orientations, 8800 fusions are required to provide roughly 1 \times coverage of the genome. However, this does not account for the Poisson distribution of insertions. When accounting for the Poisson distribution, screening 8800 fusions will give 63% coverage (a 63% chance that any given gene is screened at least once), screening 17,000 fusions will give 86% coverage, and screening 27,000 fusions will give 95% coverage.
15. It is helpful to know which growth conditions are permissive for the LuxR solo in question. If the fusions are screened for AHL responsiveness during growth in conditions in which the LuxR of interest is not expressed, or not stable, etc., the AHL-responsive fusions will test as nonresponsive. This is somewhat of a chicken-and-egg problem as permissive conditions are not usually known until a fusion can be obtained and tested. In this case, best guesses based on the environment of the bacterium are required. If no AHL-responsive fusions are obtained, one can adopt an alternative screening strategy in which the LuxR solo is overexpressed, in the hope that this provides activity under nonideal conditions. For instance, we identified our first *sdiA*-dependent fusions in *Salmonella* using overexpression because we did not know the proper AHLs to use or the best growth conditions [50]. Optimizing conditions with these fusions led to later screens being performed without overexpression and using what was an optimal condition for SdiA of *Salmonella* growth in motility agar [51, 52]. However, this optimal condition did not turn out to be optimal for *E. coli* [53].

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Chapter 13

Enzymatic Assays to Investigate Acyl-Homoserine Lactone Autoinducer Synthases

Daniel Shin and Rajesh Nagarajan

Abstract

Bacteria use chemical molecules called autoinducers as votes to poll their numerical strength in a colony. This polling mechanism, commonly referred to as quorum sensing, enables bacteria to build a social network and provide a collective response for fighting off common threats. In Gram-negative bacteria, AHL synthases synthesize acyl-homoserine lactone (AHL) autoinducers to turn on the expression of several virulent genes including biofilm formation, protease secretion, and toxin production. Therefore, inhibiting AHL signal synthase would limit quorum sensing and virulence. In this chapter, we describe four enzymatic methods that could be adopted to investigate a broad array of AHL synthases. The enzymatic assays described here should accelerate our mechanistic understanding of quorum-sensing signal synthesis that could pave the way for discovery of potent antivirulence compounds.

Key words Quorum sensing, *N*-acyl-homoserine lactone, AHL synthase, *S*-adenosyl-L-methionine, 5'-Deoxy-5'-(methylthio)adenosine, Acyl carrier protein, Coenzyme A, Methylthioadenosine nucleosidase, Xanthine oxidase, HPLC assay, Colorimetric assay, Spectrophotometric assay

1 Introduction

Autoinducers are signal molecules that enable individual bacterial cells to sense if a “quorum” has been reached before promoting behaviors that are reminiscent of multicellular species [1]. In most Gram-negative bacteria, the *N*-acyl-L-homoserine lactone autoinducers, synthesized by AHL synthases or I-proteins, diffuse out to bind to transcriptional regulator R-proteins to initiate a quorum-sensing cascade that simultaneously activates both signal autoinduction loop and virulent gene expression [2–4]. Due to the importance of quorum-sensing signals in virulence, any approach that limits signal synthesis should provide us a handle to manipulate bacterial pathogenicity. Therefore, AHL synthase enzymes are attractive targets for controlling virulence in several pathogenic bacteria [5]. Unfortunately, most AHL synthases still remain uncharacterized, perhaps due to challenges associated with studying

this class of enzymes such as (1) the substrates for AHL synthase have low turnover (k_{cat}) and high nonenzymatic background rates, which affect the accuracy in the determination of true initial rates; (2) neither the substrates nor the products absorb in a unique region in the electromagnetic spectrum necessitating the need for a reporter system to measure enzymatic rates; and (3) signal synthases are slow enzymes that do not appear to be selected for catalytic efficiency [6]. As a result, determination of enzymatic rates for mutant enzymes/substrate analogs becomes nontrivial. Assays developed for signal synthases must therefore be robust and versatile for characterizing a wide array of AHL synthases. In this chapter, we describe methods that specifically address these deficiencies.

AHL synthases are bi-ter enzymes that react with an acyl-substrate such as acyl-acyl carrier protein (acyl-ACP) or acyl-CoenzymeA (acyl-CoA) and S-adenosyl-L-methionine (SAM), to produce CoA/holo-ACP, 5'-deoxy-5'-(methylthio)adenosine (MTA), and the AHL signal (Fig. 1) [7–10]. AHL synthases can be further classified into several categories based on their acyl-substrate preference such as acyl-CoA (RpaI:Coumaryl-CoA, BjaI: Isovaleryl-CoA), acyl-ACP (BmaII:C8-ACP), short-chain (RhlI: C4-ACP), medium-chain (TofI:C8-ACP), long-chain (LasI:3-oxo-C12-ACP), linear-chain (BpsI:C8-ACP), branched-chain (BjaI:Isovaleryl-CoA), aromatic acyl-chain (RpaI:Coumaryl-CoA),

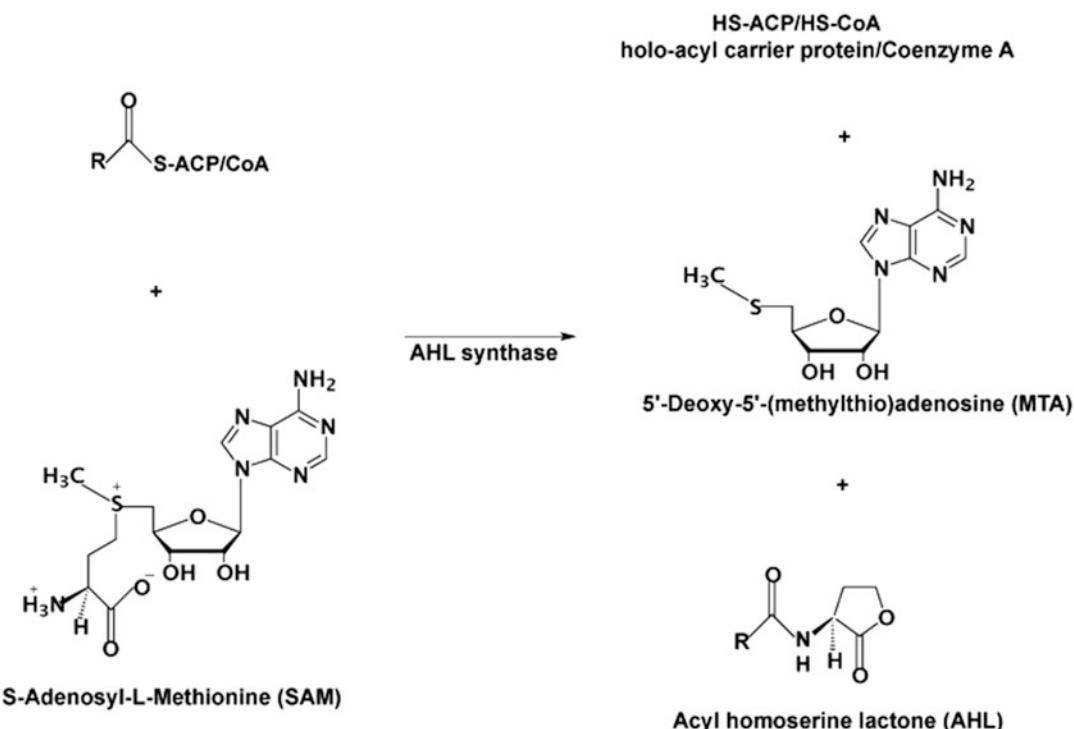


Fig. 1 AHL synthase reaction. Substrates and products of AHL synthase are shown in this figure

and substituted acyl-chain (EsaI:3-oxo-C6-ACP, BmaI3:3-hydroxy-C8-ACP) [8–16]. Despite the diversity in the type of AHL synthases identified so far, each enzyme recognizes a specific acyl-substrate to synthesize a specific quorum-sensing signal for the bacterium [10, 11]. After the initial substrate binding to the enzyme, the following two distinct chemical transformation steps occur to complete AHL synthesis: (1) nucleophilic attack of SAM α -amino group to the C-1 carbonyl carbon of the acyl-substrate (acylation), and (2) nucleophilic attack of α -carboxylate oxygen to the γ -carbon in SAM (lactonization). The acylation step cleaves the thioester bond to release CoA or holo-ACP and the lactonization step relieves the positive charge on the sulfonium ion in SAM to form MTA. A reliable enzymatic assay involves accurate quantification of either products accumulated or substrate depleted over time. In this chapter, we describe four methods for investigating AHL synthases: (1) a colorimetric assay that quantifies the rate of CoA/ holo-ACP thiol released upon acylation of SAM-alpha-amine (Fig. 2); (2) a spectrophotometric assay to measure the amount of intact vs. cleaved C-S thioester linkage in acyl-substrate over time

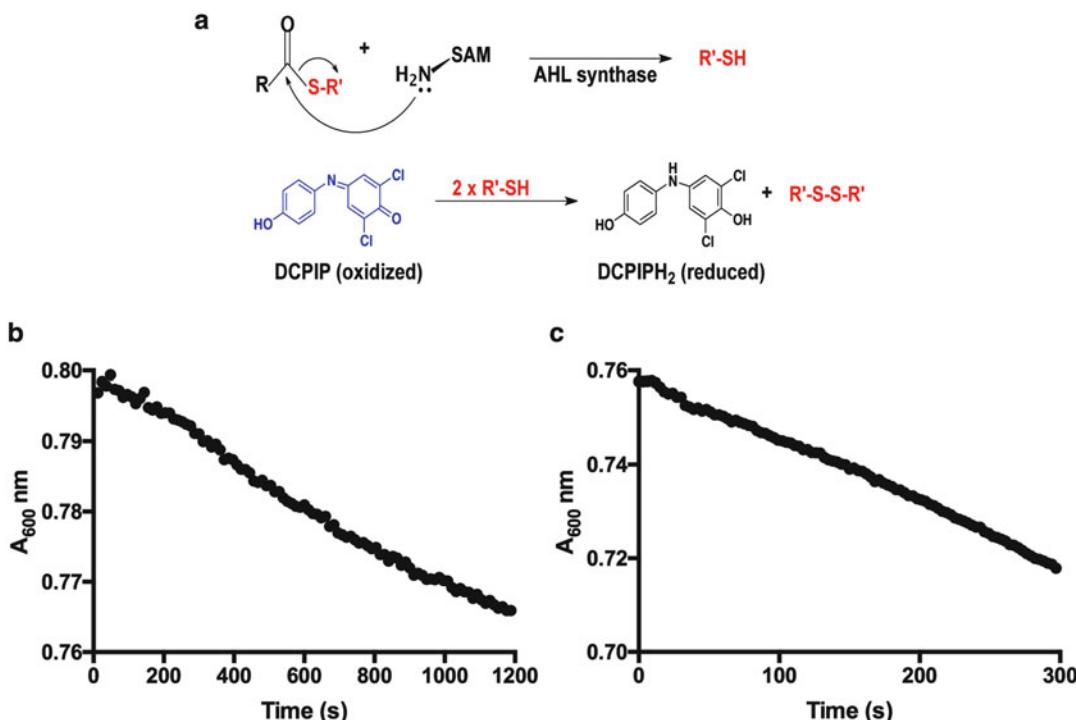


Fig. 2 DCPIP assay. **(a)** Assay principle. Holo-ACP or coenzyme A released upon acylation reduces DCPIP dye, which is monitored as reduction in absorbance at 600 nm. **(b)** Background reaction progress curve. Enzyme was excluded in this run. The concentrations of substrates were 14 μ M (butyryl-ACP) and 300 μ M (SAM). **(c)** Enzyme reaction progress curve. The concentrations of RhII, SAM, and C4-ACP are, respectively, 0.3 μ M, 300 μ M, and 14 μ M

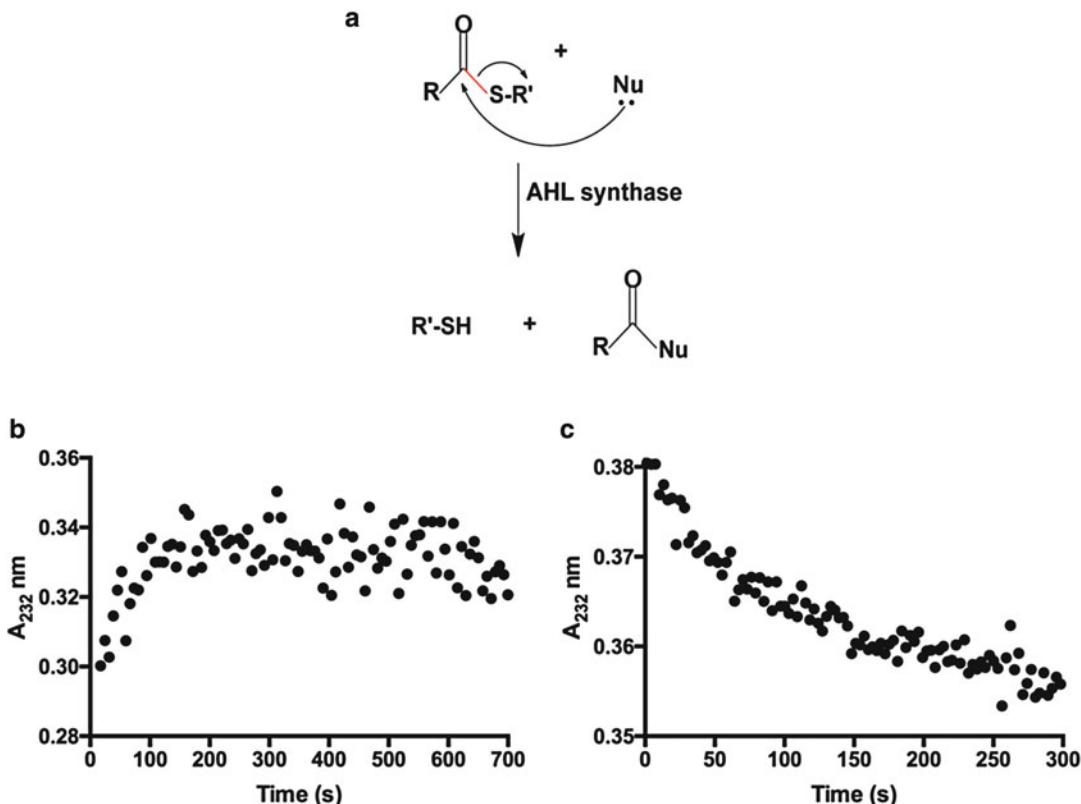


Fig. 3 C-S bond cleavage assay. (a) Assay principle. The C-S thioester bond breaking at the acyl-transfer step was monitored as change in absorbance at 232 nm. (b) Background reaction progress curve for 104 μ M isovaleryl-CoA and 394 μ M SAM. (c) Enzyme reaction progress curve. The concentrations of isovaleryl-CoA, SAM, and Bjal are, respectively, 104 μ M, 394 μ M, and 0.48 μ M

during acylation (Fig. 3); (3) a nucleosidase-xanthine oxidase-coupled assay to determine the amount of MTA released upon lactonization (Fig. 4); and (4) HPLC-based assay to independently monitor acylation and lactonization half-reactions (Fig. 5) [6, 11, 16–18]. The benefits and limitations of each assay have been discussed elsewhere [6]. The methods presented below are amenable to conduct enzymological investigations on any AHL synthase, which should expedite the development of quorum-sensing signal synthesis inhibitors in Gram-negative bacteria.

2 Materials

2.1 Enzymes and Substrates

1. S-adenosyl-L-methionine chloride dihydrochloride (*see Note 1*).
2. Acyl-CoA or Acyl-ACP (*see Note 2*).
3. AHL synthase (*see Note 2*).

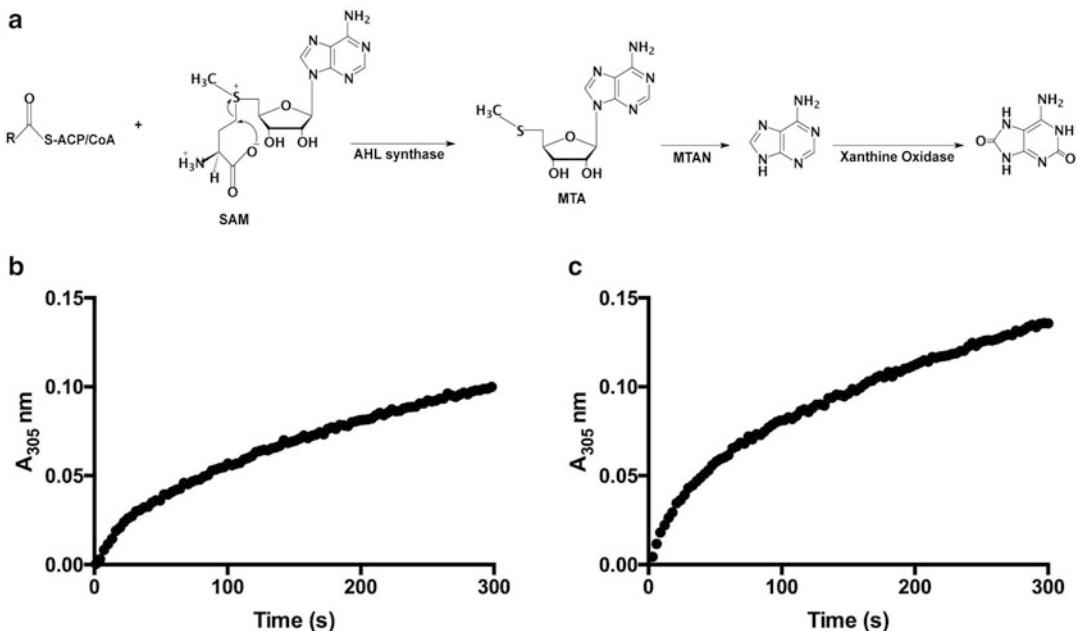


Fig. 4 Coupled assay. (a) Assay principle (b) Background reaction progress curve for 300 μM SAM, 50 μM C4-ACP, 0.5 μM MTAN, and 2 units/ml xanthine oxidase. (c) Enzyme reaction progress curve. The assay mixture includes all components for background curve described in (b) and 0.3 μM RhII

2.2 Reagents and Equipment

1. 300–500 μM Dichlorophenolindophenol (DCPIP) stock solution (10 \times) in nanopure water.
2. 3–10 mM SAM stock solution (10 \times) in nanopure water.
3. 100 μl Cuvettes or 96/384-well plates (*see Note 3*).
4. Acyl-CoA stocks (5–20 \times) in nanopure water to cover a range of 1–200 μM final acyl-CoA concentration in the assay (*see Note 4*).
5. 1 M HEPES buffer pH 7.3 (10 \times).
6. 1–10 μM Stock *Bacillus subtilis* Sfp phosphopantetheinyl transferase.
7. 5 μM Stock *Escherichia coli* methylthioadenosine nucleosidase (MTAN).
8. 20 units/ml xanthine oxidase.
9. Other reagents: Glycerol, 10 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), 0.5 M potassium chloride, acetic acid, HPLC-grade acetonitrile, HPLC-grade methanol, 2 M sodium acetate pH 4, trifluoroacetic acid (TFA).
10. UV-visible spectrophotometer.
11. HPLC instrument with autosampler and PDA detector.

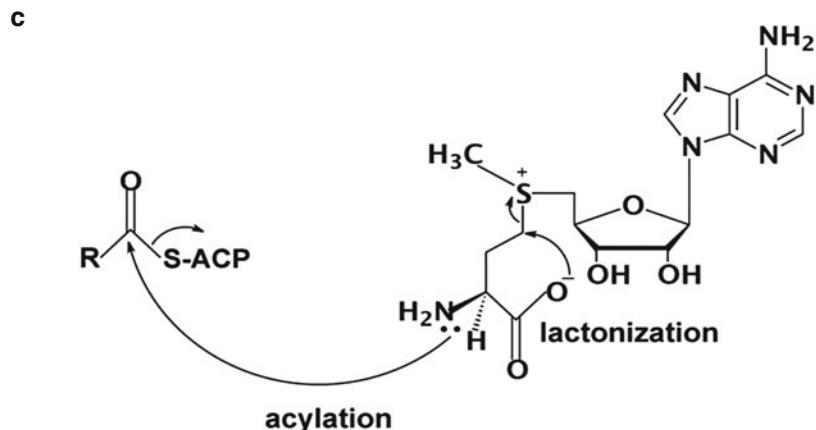
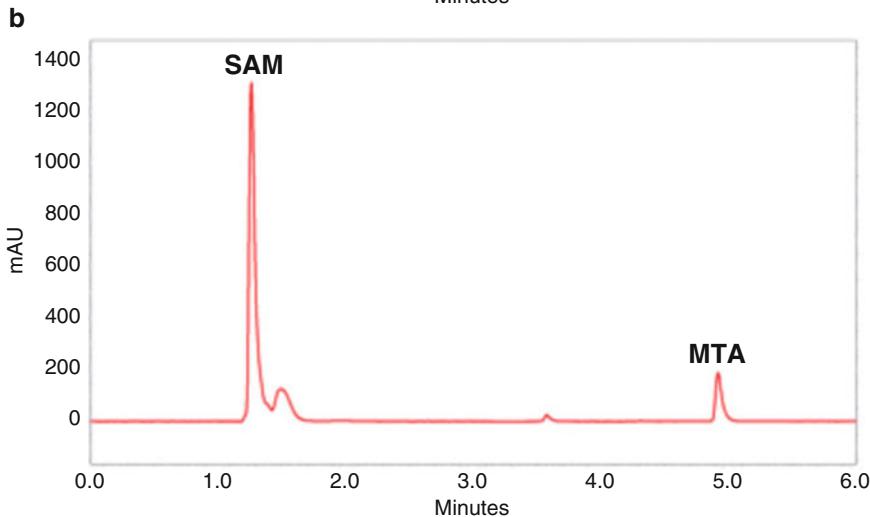
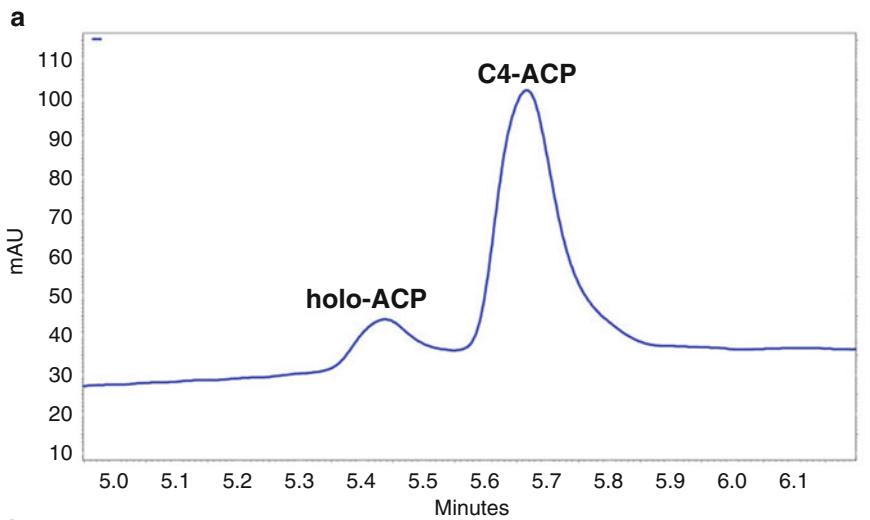


Fig. 5 HPLC assay. (a) Chromatogram for acylation half-reaction. The concentrations of C4-ACP, SAM, and RhII are, respectively, 20 μ M, 300 μ M, and 0.3 μ M. The chromatogram represents a snapshot of relative proportions of acyl-ACP and holo-ACP assay component present in the assay mixture that was quenched 4 min after enzyme addition. (b) Chromatogram for lactonization half-reaction. The chromatogram is a 6-min quenched reaction that contained 27 μ M C4-ACP, 300 μ M SAM, and 0.3 μ M RhII enzyme. (c) Acylation and lactonization chemical steps in AHL synthesis

3 Methods

3.1 Dichloro-pheno-lindophenol (DCPIP) Colorimetric Assay

3.1.1 Preparation of Stock Solutions

1. Prepare the following stock solutions: 10× DCPIP stock solution (300–500 μM) in nanopure water, 10× SAM stock solution (5–10 mM) in nanopure water, 10× HEPES buffer (1 M, pH 7.3), and several acyl-CoA stocks (5–20×) in nanopure water to cover a range of 1–200 μM final acyl-CoA concentrations in the assay (*see Note 4*).
2. Dilute acyl-ACP and enzyme freezer stocks using 10 mM MES and 20% glycerol storage buffer to make 5–20× stock for the assay (*see Note 5*).
3. Use the following extinction coefficients to determine stock concentrations:
 - (a) DCPIP $\epsilon_{600} = 21,000 \text{ M}^{-1} \text{ cm}^{-1}$
 - (b) SAM $\epsilon_{260} = 15,400 \text{ M}^{-1} \text{ cm}^{-1}$
 - (c) Acyl-CoA $\epsilon_{260} = 13,500 \text{ M}^{-1} \text{ cm}^{-1}$
 - (d) Acyl-ACP $\epsilon_{280} = 1490 \text{ M}^{-1} \text{ cm}^{-1}$
4. Store both enzyme and substrate solutions in ice throughout the experiment.

3.1.2 Determination of Background Rates

1. Set up the cuvette as mentioned in Table 1. Add all components except AHL synthase enzyme. The total cuvette volume in the assay is 100 μl, which can be scaled up or down depending on the type of cuvette/microplate accessory.
2. Instead of enzyme, add 10 μl nanopure water to the cuvette and record progress curve at 600 nm for 15–20 min.
3. Keep SAM concentration between 200 and 500 μM and vary the acyl-ACP/acyl-CoA concentrations over a wide range, e.g., 10 μM (low), 50 μM (medium), and 100–200 μM (high).

Table 1
DCPIP assay setup

Component	Volume (μl)
10× HEPES buffer	10
Water	60 – x
10× DCPIP	10
10× SAM	10
Acyl-CoA/acyl-ACP	x
AHL synthase	10
Total	100

Determine the time it takes for the change in A_{600} to level off. This should be the minimum incubation time necessary to reduce nonspecific, background reactions in the enzyme assay. Record the progress curve for an additional 5 min, and calculate the slope to determine background rate (Fig. 2).

4. Repeat the experiment with acyl-ACP/acyl-CoA as the fixed substrate. Maintain acyl-substrate concentration between 100 and 200 μM and vary SAM over a broad range, between 10 and 500 μM . After the change in absorbance levels off, continue to take measurements for an additional 5 min (Fig. 2). The slope in the last 5 min of the progress curve is the background rate in $\Delta\text{Abs}/\text{s}$.
5. Convert the slope to $\mu\text{M}/\text{min}$ using the following relation (*see Note 6*):

$$\frac{(\text{rate})\Delta\text{Abs}}{\text{s}} \times \frac{M\text{cm}}{21,000} \times \frac{1}{1\text{cm}} \times \frac{10^6\mu\text{M}}{\text{M}} \times \frac{60\text{s}}{1\text{min}} \times 2 = (\text{rate})\frac{\mu\text{M}}{\text{min}}$$

6. The rate calculated in **step 3** must be the appropriate background rate in the enzyme assay if SAM is maintained at a fixed concentration while the acyl-substrate concentration is varied. On the other hand, if acyl-substrate is the fixed substrate in the experiment, then the rate determined in **step 4** should be the background rate for the enzyme reaction (*see Note 6*).

3.1.3 Determination of Optimum Enzyme Concentration

1. Set up the enzyme assay as referred to in Table 1. Determine the initial rates for at least four different enzyme concentrations from 0.1 to 2 μM .
2. The enzyme rate at the lowest acyl-ACP/acyl-CoA concentration must be at least threefold higher than the background rate. The lowest substrate concentration in a substrate-velocity curve must be at least two- to threefold lower than the K_m of substrate used in the experiment.
3. Choose the lowest enzyme concentration that meets the condition outlined in **step 2**.

3.1.4 Determination of k_{cat} and K_m

1. Add 10× buffer, 10× SAM, nanopure water, DCPIP, and acyl-ACP/acyl-CoA to the cuvette (refer to Table 1) and let the mixture incubate for the time determined in Subheading 3.1.2. Add 10 μl enzyme and collect the absorbance at 600 nm for 5 min (Fig. 2).
2. Calculate the linear slope between 0 and 200 s to determine the initial rate for each enzyme reaction. Please note that the data points in the first 30 s are usually excluded in the slope calculation to account for sample mixing errors.

3. Convert the slope in Abs/s to $\mu\text{M}/\text{min}$ as mentioned in Subheading 3.1.2. Subtract the background rate from this rate to calculate the net enzymatic rate. Use this rate for fitting the data.
4. Repeat the assay for each substrate concentration in triplicate and use the entire dataset for data fitting.
5. Using an appropriate graphing program, fit the initial rate vs. substrate concentration data to Michaelis-Menten or substrate inhibition equation to determine kinetic constants (*see Note 7*).

3.2 C-S Cleavage Spectrophotometric Assay

1. Prepare stock solutions as described for the DCPIP assay.
2. Use Table 2 to add appropriate volumes of buffer, nanopure water, 10× SAM, and 10–200 μM acyl-CoA to a total volume of 90 μl . Add nanopure water instead of enzyme and record the change in the absorbance at 232 nm for 10–15 min. Conduct at least three assays covering a range of acyl-CoA concentrations between 10 and 200 μM .
3. Follow the change in absorbance at 232 nm to determine the incubation time, as outlined in Subheading 3.1.2 (Fig. 3).
4. Add 10 μl water to the cuvette and continue to follow change in absorbance at 232 nm for 5 min.
5. Calculate the background rate in $\Delta\text{Abs}/\text{s}$ from the slope of the initial, linear portion of each progress curve after water addition.
6. Convert the slope to $\mu\text{M}/\text{min}$ using the following relation (*see Note 8*):

$$\frac{(\text{rate})\Delta\text{Abs}}{\text{s}} \times \frac{M\text{cm}}{4000} \times \frac{1}{1\text{cm}} \times \frac{10^6\mu\text{M}}{\text{M}} \times \frac{60\text{s}}{1\text{min}} = (\text{rate})\frac{\mu\text{M}}{\text{min}}$$

7. Average the rates to estimate background rates.
8. Determine the optimum enzyme concentration as described for the DCPIP assay in Subheading 3.1.3.
9. Add 10× HEPES buffer, 10× SAM, water, and acyl-CoA to the cuvette (refer to Table 2) and let the mixture incubate for the time determined in Subheading 3.1.2. Add 10 μl enzyme and collect the absorbance at 232 nm for 5 min (Fig. 3).
10. Calculate the slope of the progress curve to determine initial rate for each enzyme reaction.
11. Subtract the background rate from this rate to calculate the net enzyme rate.
12. Repeat the assay for each data point (substrate concentration) in triplicate.
13. Fit the entire rate data to Michaelis-Menten or substrate inhibition equation to determine kinetic constants (*see Note 7*).

Table 2
C-S assay setup

Component	Volume (μl)
10× HEPES buffer	10
Water	70 – x
10× SAM	10
Acyl-CoA/acyl-ACP	x
AHL synthase	10
Total	100

3.3 Methylthioadenosine Nucleosidase-Xanthine Oxidase-Coupled Spectrophotometric Assay

3.3.1 Determination of Background Rates

- Calculate the volume of xanthine oxidase needed to achieve 2 units/ml in a 100 μl solution (see Note 9).
- To a cuvette, add 10 μl of 0.5 M KCl, 10 μl of 1 M HEPES buffer pH 7.3, 10 μl of 5 μM MTAN, 10 μl of 20 units/ml xanthine oxidase, and 40 μl of nanopure water.
- Incubate the solution in cuvette for 10 min.
- Initiate the reaction with 10 μl of 10× SAM and follow the reaction for 300 s at 305 nm (Fig. 4).
- Calculate the steady-state slope at 50–150-s time interval (steady-state portion of progress curve) and convert the slope to $\mu\text{M}/\text{min}$ using the following relation:

$$\frac{(\text{rate})\Delta\text{Abs}}{\text{s}} \times \frac{M \text{ cm}}{15,400} \times \frac{1}{1 \text{ cm}} \times \frac{10^6 \mu\text{M}}{\text{M}} \times \frac{60 \text{ s}}{1 \text{ min}} = (\text{rate})\frac{\mu\text{M}}{\text{min}}$$

3.3.2 Determination of Kinetic Constants

- Prepare 10× solutions for several concentrations of acyl-CoA/acyl-ACP.
- To a cuvette, add 10 μl of acyl-CoA/acyl-ACP stock solution, 10 μl of 0.5 M KCl, 10 μl of 1 M HEPES buffer pH 7.3, 10 μl of 5 μM MTAN, 10 μl of 20 units/ml xanthine oxidase, 10 μl of AHL synthase (10×), and 30 μl of nanopure water (Table 3).
- Incubate the assay solution for 10 min.
- Initiate the reaction with 10 μl of 10× SAM and follow the reaction for 300 s at 305 nm (Fig. 4).
- Calculate the enzyme reaction rate in the 50–150-s time interval using $\Delta\varepsilon = 15,400 \text{ M}^{-1} \text{ cm}^{-1}$.
- Subtract the background rate from the enzyme reaction rate to calculate the net rate.
- Fit the net rate vs. substrate concentration using Michaelis-Menten equation or substrate inhibition equation to determine kinetic constants (see Note 7).

Table 3
Methylthioadenosine nucleosidase assay setup

Component	Volume (μ l)
10× HEPES buffer	10
Water	40 – x
10× KCl	10
5 μ M MTAN	10
20 units/ml Xanthine oxidase	10
AHL synthase	10
Acyl-CoA/acyl-ACP	x
10× SAM	10
Total	100

Table 4
Column clean HPLC method

Time (min)	A% ^a	B% ^b	Flow rate (μ l/min)
0.0	50.0	50.0	250.0
8.0	0.0	100.0	250.0
10.0	0.0	100.0	250.0

^aA = $H_2O + 0.1\%$ trifluoroacetic acid (TFA)

^bB = methanol

Table 5
Acylation HPLC method^a

Time (min)	A% ^b	B% ^c	Flow rate (μ l/min)
0.0	75.0	25.0	600.0
10.0	25.0	75.0	600.0

^aObserve at 220 nm

^bA = $H_2O + 0.1\%$ trifluoroacetic acid (TFA)

^cB = acetonitrile + 0.1% TFA

3.4 HPLC Assay

3.4.1 Column Wash

1. Run the column clean method (Table 4).
2. After the column wash, equilibrate the column for 3–5 min with the initial conditions for the acylation (Table 5) or lactonization method (Table 6).
3. After the sample run is complete, repeat the above steps before loading a new sample (*see Note 10*).

Table 6
Lactonization HPLC method^a

Time (min)	A% ^b	B% ^c	Flow rate ($\mu\text{l}/\text{min}$)
0.0	100.0	0.0	500.0
10.0	70.0	30.0	500.0

^aObserve at 260 nm

^bA = $\text{H}_2\text{O} + 0.1\%$ trifluoroacetic acid (TFA)

^cB = acetonitrile + 0.1% TFA

3.4.2 Standard Curve for Lactonization Half-Reaction Analytes

1. Prepare 1 mM MTA stock solution in nanopure water (see Note 11). Determine the concentration using MTA extinction coefficient at 275 nm, $\Delta\epsilon_{275} = 14,900 \text{ M}^{-1} \text{ cm}^{-1}$.
2. Prepare two-fold serial dilutions of this sample, i.e., 500, 250, 128, 64, 32, and 16 μM .
3. Prepare standard solutions by adding 10 μl 6 M HCl (to simulate quenched reactions) to 260 μl water and 30 μM MTA stock solution.
4. Inject the sample on to HPLC and run the lactonization HPLC method (Table 6). Repeat this exercise at least three times ($3 \times 100 \mu\text{l}$).
5. Determine peak area for MTA peak using an appropriate software for the LC instrument (Fig. 5).
6. Plot peak area vs. MTA concentration and calculate the slope to determine the conversion factor for converting peak area to analyte concentration (see Note 12).

3.4.3 Standard Curve for Acylation Half-Reaction Analytes

1. Prepare 2–3 mM stock solutions for apo-ACP, holo-ACP, and acyl-ACP in water (or buffer). Determine the carrier protein concentration using the extinction coefficient at 280 nm, $\Delta\epsilon_{280} = 1490 \text{ M}^{-1} \text{ cm}^{-1}$.
2. Dilute this sample two-fold, at least six times as described in Subheading 3.4.2, step 2.
3. Prepare standard solutions by mixing 260 μl water, 30 μl carrier protein, and 10 μl 2 M acetate buffer pH 4 (to simulate quenched reactions). Please note that acyl carrier proteins tend to precipitate out in HCl.
4. Run the sample using the acylation HPLC method (Table 5) and determine the peak area for acyl-ACP and holo-ACP using an appropriate software that supports the LC instrument (Fig. 5).
5. Plot peak area vs. acyl carrier protein concentration to generate the standard curve. The peak area must vary linearly with analyte concentration. The slope of this line is the conversion factor for determining concentration from peak areas in the chromatogram (see Note 12).

3.4.4 Quench Test

1. Add 170 μ l water, 30 μ l 1 M HEPES buffer pH 7.3 (10 \times), 30 μ l 10 \times acyl-ACP/acyl-CoA stock, 30 μ l 10 \times AHL synthase, 30 μ l 10 \times SAM, and 10 μ l quench solution.
2. Run the sample immediately using acylation (for ACP) or lactonization (for SAM, MTA) HPLC methods.
3. Run the sample again after 15 and 30 min.
4. If the quench is effective, the difference in the analyte peak areas between 0, 15, and 30 min runs must be <0.5%.

3.4.5 Kinetic Studies: Lactonization Half-Reaction

1. MTA is a common contaminant in commercial SAM samples. Add 10 μ l quench to 260 μ l water and 30 μ l 10 \times SAM stock solution (see Notes 1 and 13).
2. Analyze the sample using lactonization method to determine basal MTA contaminant concentration in SAM stock.
3. Add 30 μ l 1 M HEPES buffer (pH 7.3), 30 μ l 10 \times acyl-ACP/acyl-CoA stock, and 30 μ l 10 \times SAM to 170 μ l water in a reaction vessel. Let the reaction mixture sit at room temperature for 5 min.
4. After 5 min, add 30 μ l 10 \times AHL synthase stock.
5. Quench the reaction using 10 μ l 6 M HCl after 2, 4, and 6 min (see Note 14).
6. Subtract MTA background peak area in each of these samples.
7. Use the conversion factor from the standard curve to convert sample peak area to concentration units (see Note 12).
8. Plot concentration over time and calculate the slope of this line to determine reaction rate. If a single time point is taken, divide the concentration over time to calculate rate.
9. Each data point must be repeated thrice to check for reproducibility.

3.4.6 Kinetic Studies: Acylation Half-Reaction

1. Add 30 μ l 1 M HEPES buffer pH 7.3, 30 μ l 10 \times acyl-ACP stock, and 30 μ l 10 \times SAM to 170 μ l water in a reaction vessel. Let the reaction mixture sit at room temperature for 5 min.
2. After 5 min, add 30 μ l 10 \times AHL synthase stock.
3. Quench the reaction using 10 μ l 2 M sodium acetate buffer pH 4 after 2, 4, and 6 min (see Note 14).
4. Use the conversion factor from the standard curve to convert sample peak area to concentration units (see Note 12).
5. Plot concentration over time and calculate the slope of this line to determine reaction rate.
6. Each data point must be repeated thrice to check for reproducibility.

4 Notes

1. *S*-adenosyl-L-methionine *p*-toluenesulfonate salt may also be used in place of chloride salt. The tosylate salt usually is more pure and thus has a lower background rate. However, we observed that the initial rates for AHL synthase with SAM-tosylate are lower than with SAM-chloride, possibly due to nonspecific enzyme inhibition by *p*-toluenesulfonate. If possible, SAM should be synthesized in the laboratory using SAM synthase to achieve the highest purity. The enzymatically synthesized SAM should be stored at pH <3 to minimize sample degradation. The pH of this sample must be raised to neutral pH prior to use in the enzyme assay.
2. Acyl-ACP and AHL synthases were purified according to protocols well documented in literature [6, 8–20].
3. It is preferable to use nonbinding plates for colorimetric and UV-transparent plates for coupled assay.
4. The volume of acyl-substrate used will depend on the stock concentration. For instance, in a 100 µl total reaction volume, 5 µl of a 20× stock is required to achieve 1× substrate concentration in the assay.
5. The enzyme stocks are typically stored in 10 mM MES and 20% glycerol.
6. The path length for the light passing through the cuvette is assumed to be 1 cm. Each DCPIP reduces two CoA or holo-ACP thiols. After the incubation period, the background rate is usually low around 0.1 µM/min, which does not fluctuate with substrate concentration. Occasionally, depending on the purity of substrate used, the background could be >0.2 µM/min that could increase with higher substrate concentrations. If the variation in background rate between low and high substrate concentrations is <20%, then the rates can be averaged to estimate the background. However, if this rate varied more than 20%, then the true background rate at each substrate concentration must be determined.
7. Michaelis-Menten (top) and substrate inhibition (bottom) equation:

$$v_0 = \frac{k_{\text{cat}}[E_t][S]}{K_m + [S]}$$

$$v_0 = \frac{k_{\text{cat}}[E_t][S]}{K_m + [S] \left(1 + \frac{[S]}{K_i}\right)}$$

v_0 is initial rate, k_{cat} is turnover number, K_m is Michaelis constant, and K_i is inhibition constant.

8. The change in extinction coefficient between intact and cleaved C-S bond is $4000 \text{ M}^{-1} \text{ cm}^{-1}$.
9. For instance, in a 20 units/ml xanthine oxidase stock, 10 μl of this stock in a 100 μl reaction volume would give 2 units/ml.
10. Multiple samples can be sequentially loaded using a sequence method. The column wash and equilibration steps must be included in this sequence method.
11. In addition to MTA, SAM can also be monitored as an analyte to follow the progress in lactonization half-reaction.
12. Analyte concentration = peak area/slope of standard curve.
13. The concentration of SAM stock should be tenfold higher than the SAM concentration in the enzyme assay. Depending on the desired SAM concentration of the assay, stock concentration could vary between 300 and 500 μM . For instance, if 400 μM SAM is required in the enzyme assay, use 4 mM SAM stock for this run.
14. If a mutant enzyme or a poor substrate is investigated, enzyme activity will be low. In those instances, extend the time range over a longer period until sufficient product has accumulated for detection by HPLC. If product linearity is confirmed over the time range of the enzyme assay, a single time point may be sufficient to calculate reaction rates.

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Chapter 14

Global Expression Analysis of Quorum Sensing-Controlled Genes by RNAseq

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Abstract

RNA sequencing (RNAseq) enables transcriptional profiling of many organisms. This chapter describes the use of RNAseq in prokaryotes to identify quorum sensing (QS)-controlled transcripts by comparing samples from QS-induced and -uninduced conditions. Briefly, each RNA sample is converted to ds-cDNA in a method that limits amplification of ribosomal RNA species. The ds-cDNA contains adapters that enable sequencing and quantification by next-generation sequencing (NGS).

Key words RNA sequencing (RNAseq), Next-generation sequencing (NGS), Quorum sensing, Not-so-random (NSR) primers, Transcriptomics

1 Introduction

RNA sequencing (RNAseq) uses next-generation sequencing (NGS) to provide information on the presence and quantity of RNA in a biological sample. For many purposes, RNAseq technology is replacing microarray technology to analyze transcript profiles. The first step in obtaining RNAseq data is preparing high-quality RNA samples. The RNA in the sample is then converted to ds-cDNA, and the ds-cDNA fragments are sequenced on a next-generation sequencer, such as the Illumina platform. During sequencing, information on the identity and quantity of each transcript is determined as the machine records the sequence and abundance of each molecule. The transcriptional profile of each sample can then be compared with another sample, in the same way that microarray analysis can compare transcriptional profiles. Advantages of RNAseq include: no requirement for a pre-synthesized microarray chip, analysis can be done on sequenced and unsequenced organisms (as there is potential to use NGS to sequence your organism of interest), data is generated for small and/or previously unidentified RNA species (including noncoding RNAs), and RNAseq provides detailed transcript information (such as the transcriptional start and stop sites). That being

said, microarrays are still useful in the identification of differentially regulated genes—depending on one’s experimental design, the goals in data analysis, and resources available. The use of microarrays to identify QS-controlled genes is described in a previous methods chapter [1].

This chapter focuses on the use of RNAseq to identify quorum sensing (QS)-regulated genes in prokaryotes. The main technical challenge to consider with RNAseq is that each RNA sample will contain large amounts of ribosomal RNA (rRNA), approximately 95% rRNA. This is problematic because one doesn’t want to perform RNAseq on rRNA! The goal is to perform RNAseq on the unique transcripts in each sample, such as messenger RNAs (mRNAs) or other RNA species. There are two main approaches to enrich a sample for unique RNAs. In the first approach, rRNA is removed prior to cDNA synthesis. This can be done with commercially available kits that capture rRNA molecules, leaving behind unique RNAs. These kits typically involve the use of affinity columns or magnetic beads. The rRNA-depleted RNA sample can then be used for cDNA synthesis with random hexamers. In the second approach, one can avoid the rRNA capture step by using not-so-random (NSR) hexamers for cDNA synthesis [2]. NSR primer sets are depleted for hexamers that anneal to the rRNA sequences, yet still contain hexamers that anneal to the rest of the genome. Thus, NSR primer sets limit the amplification of rRNA sequences. This chapter focuses on RNAseq libraries made with NSR primers.

To identify QS-controlled genes one must compare a condition in which QS is induced (“QS-ON”) to one in which QS is uninduced (“QS-OFF”). Many bacteria use QS to adapt to conditions of high cell density. During *in vitro* growth, QS regulons are often induced as bacteria leave the exponential phase of growth and enter stationary phase. For this reason, RNAseq sample preparation from post-exponential phase-grown cells may be a good starting point. However, the conditions of QS induction for each organism should be carefully considered. When selecting a sampling time for RNA-seq, it may be prudent to first identify a QS-controlled gene or factor and monitor it for induction.

Once the growth conditions of QS induction are established, the appropriate control must be chosen. This can be done by generating “QS-OFF” mutants or through signal interference (*see* Fig. 1 for overview). To generate “QS-OFF” mutants, inactivating mutations may be made in the gene(s) that produce or respond to the QS signal(s). Comparing the transcriptomes of the wild type (WT) with the signal-negative mutant or the receptor-negative mutant then identifies genes regulated by QS. Transcripts identified as being differentially regulated in the comparison of the WT to the signal-negative mutant and also in the comparison of the WT to the receptor-negative mutant are strong candidates for targets of QS regulation.

Conditions to Compare to Identify QS-Controlled Factors/Genes		
QS uninduced, "QS OFF"	vs.	QS induced, "QS ON"
QS signal-negative mutant		WT
QS receptor-negative mutant		WT
QS signal-negative mutant		QS signal-negative mutant w/ exogenous signal added back
WT w/ exogenous quorum quencher (ex.purified AiiA enzyme)		WT
WT w/ the quorum quencher gene (ex. WT + <i>aiaA</i>)		WT

Fig. 1 Overview of conditions to compare to identify QS-controlled factors or genes

Another approach to identify QS-controlled genes is performing “signal add-back” experiments. In this method, samples from two conditions are compared: (1) for the reference condition, the signal-negative strain is grown, and (2) for the experimental condition, the signal-negative mutant is grown in the presence of exogenous signal to induce QS. This approach is advantageous when dealing with an organism that has multiple QS systems, and you want to examine each QS system individually without making a slew of individual mutants. Please confirm that you are adding the correct signal at an appropriate concentration [see [3, 4] for specific examples of experimental design using “signal add-back” experiments].

Finally, QS regulons may be identified with an alternative approach by using QS signal interference, also called quorum quenching. This method is useful for organisms that are not genetically tractable or for situations in which one does not want to generate QS mutants. QS signal interference has been described for a handful of organisms and must be evaluated on a case-by-case basis. For example, AHL-mediated QS systems are candidates for QS quenching by an AHL-degrading enzyme AiiA [5, 6], and *Staphylococcus* species that use Agr-mediated QS are candidates for QS interference by using non-cognate signals [7, 8]. QS interference can be achieved in two ways: (1) by adding purified QS quencher to the growth medium, or (2) by genetically engineering your organism to produce the QS quencher. This approach has limitations. Specifically, a quorum quencher may not be available for your organism, and/or addition of the quorum quencher may produce artifacts and identify factors that are not truly QS-controlled.

This chapter focuses on RNAseq using NSR primers. This protocol was used to identify QS-controlled genes in three related *Burkholderia* species, *B. thailandensis*, *B. pseudomallei*, and *B. mallei* [4, 9], *Pseudomonas aeruginosa* [10], and *Rhodopseudomonas palustris* [11]. It is beyond the scope of this chapter to address the details of data analysis. Simply put, large amounts of raw data are generated for each sample following an RNAseq run. This data must be analyzed for quality, sorted if barcodes were used, aligned to a reference genome, and normalized prior to transcript analysis. This can be done in a number of ways. It is best to consult with the sequencing facility that will run the RNAseq samples for suggested pipelines of data analysis. There are an increasing number of commercially available software packages for NGS applications. When choosing a software package, take note of the availability of webinars, product manuals, and technical support services. These features can be invaluable when processing data.

2 Materials

2.1 RNA Preparation

1. RNAProtect Cell Reagent (Qiagen).
2. Vortexer.
3. Beadbeater for cell lysis (though not required if other methods of cell lysis are used).
4. 0.1 mm silica beads (for use with beadbeater).
5. 2 ml screw cap tubes with gasket (for use with beadbeater).
6. Ice.
7. Sterile, RNase-free tips.
8. Tabletop microfuge.
9. 1.5 and 2 ml microfuge tubes.
10. miRNeasy Mini Kit (Qiagen).
11. RNeasy MinElute Kit (Qiagen).
12. 2-Mercaptoethanol (β ME).
13. Ethanol.
14. NorthernMax-Gly Gel Prep/Running Buffer (Ambion, dilute 10 \times stock with DEPC treated RNase-free water).
15. 10 \times TURBO DNase Buffer (Ambion).
16. 2 Units (U)/ μ l TURBO DNase (Ambion).
17. RNase-free water.
18. Spectrophotometer for determining DNA and RNA concentration.
19. RNAase AWAY Decontamination Reagent (ThermoFisher).

20. Agarose.
21. Electrophoresis apparatus.
22. PCR machine and tubes.
23. A primer set (Forward and Reverse) for a gene in your organism.
24. PCR reagents.

2.2 RNAseq Library Construction

1. Sterile, RNase-free tips.
2. Microfuge and microfuge tubes.
3. 200 U/ μ l SuperScript[®] III Reverse Transcriptase (Invitrogen).
4. 10 U/ μ l DNA Polymerase I (Invitrogen).
5. 10 U/ μ l *E. coli* DNA Ligase (Invitrogen).
6. 2 U/ μ l RNase H (Invitrogen).
7. Invitrogen Second-Strand Buffer.
8. 0.2 M ethylenediaminetetraacetic acid (EDTA).
9. QIAquick PCR Purification Kit (Qiagen).
10. Quick Blunting[™] (New England Biolabs).
11. Quick Ligation[™] (New England Biolabs).
12. Deoxynucleotide Solution Mix (New England Biolabs).
13. 5 U/ μ l Taq DNA Polymerase with Standard Taq Buffer (New England Biolabs).
14. Ethanol.
15. Agencourt AMPure XP Kit (Fisher Scientific).
16. Magnetic bead stand (such as a MagneSphere, Promega).
17. Setup for DNA electrophoresis: casting tray, comb, agarose, running buffer, loading dye, ethidium bromide (EtBr).
18. PCR Primers (HPLC purified):
 - (a) Forward (Fwd) Primer (AATGATAACGGCGACCACCGA)
 - (b) Reverse (Rev) Primer (CAAGCAGAACGGCATACG)
19. Adapters (NON-Barcoded) (HPLC purified):
 - (a) FwdAD (AGATCGGAAGAGCGTAATGATAACGGCGA
CCACCGACACTCTTCCCTACACGACGCTCTTC
GATCT)
 - (b) RevAD (AGATCGGAAGAGCTCCAAGCAGAAC
GGCATACGAGCTCTCCGATCT)
20. Adapters (Barcoded) (HPLC purified):
 - (a) FwdAD-AGT (ACTAGATCGGAAGAGCGTAATGATA
CGGCGACCACCGACACTCTTCCCTACACGACGC
TCTTCCGATCTAGT)

- (b) FwdAD-AAC (GTTAGATCGGAAGAGCGTAATGATA CGGCGACCACCGACACTCTTCCCTACACGACGC CTCTCCGATCTAAC)
- (c) FwdAD-CGA (TCGAGATCGGAAGAGCGTAATGATA CGGCGACCACCGACACTCTTCCCTACACGACGC TCTTCCGATCTCGA)
- (d) FwdAD-CTG (CAGAGATCGGAAGAGCGTAATGATA CGGCGACCACCGACACTCTTCCCTACACGACGC TCTTCCGATCTCTG)
- (e) FwdAD-GTC (GACAGATCGGAAGAGCGTAATGATA CGGCGACCACCGACACTCTTCCCTACACGACGC TCTTCCGATCTGTC)
- (f) FwdAD-GCG (CGCAGATCGGAAGAGCGTAATGATA CGGCGACCACCGACACTCTTCCCTACACGACGC TCTTCCGATCTGCG)
- (g) FwdAD-TAA (TTAAGATCGGAAGAGCGTAATGATA CGGCGACCACCGACACTCTTCCCTACACGACGC TCTTCCGATCTTAA)
- (h) FwdAD-TCT (AGAAGATCGGAAGAGCGTAATGATA CGGCGACCACCGACACTCTTCCCTACACGACGC TCTTCCGATCTTCT)

3 Methods

3.1 RNA Preparation Using the miRNeasy Mini Kit (See Note 1)

1. Harvest the bacterial cells at the desired time by adding 2 volumes RNAProtect Bacteria Reagent (Qiagen) to 1 volume bacterial culture. Mix immediately by vortexing for 5 s, and then incubate at room temperature for 5 min. Next, centrifuge the cell mixture for 10 min at $5000 \times g$. After centrifugation, decant the supernatant, gently blot the inverted tube on a paper towel or Kimwipe, and leave inverted for 10 s to remove as much residual supernatant as possible (see Note 2). The pellets can then be stored at -15 to -30 °C for up to 2 weeks or at -80 °C for up to 4 weeks.
2. Thaw pellets at room temperature, immediately resuspend each pellet in 1 ml RLT (+ β ME) (see Note 3) and pipet into a 2 ml screw cap tube containing 0.1 mm silica beads (see Note 4), and place on wet ice. Once all samples are prepared and chilled, beat each sample in beadbeater at maximum rpm for 1 min. Immediately chill the tube on wet ice for 5 min. Repeat as needed; often another round of bead-beating is needed (see Note 5).
3. Spin at 4 °C for 15 min.
4. Taking care to leave the beads behind, remove 750 μ l of the supernatant and place in a fresh 1.5 ml microfuge tube

containing 50 μ l RLT + β ME. Centrifuge the tube at max speed for 30 s. Then, transfer 750 μ l to a fresh 2 ml microfuge tube.

5. Add 1.125 ml (1.5 \times volume) of 100% ethanol, vortex, and apply 700 μ l of the sample to the RNeasy column. Centrifuge for 15 s at 14,000 rpm ($\geq 17,000 \times g$) at room temperature, discard the flow-through, and repeat using the remainder of the sample in 700 μ l increments.
6. Add 700 μ l Buffer RWT to the RNeasy column, centrifuge for 30 s at 14,000 rpm ($\geq 17,000 \times g$), and discard the flow-through. Transfer the RNeasy Mini column into a new 2 ml collection tube and add 500 μ l Buffer RPE to the RNeasy Mini column. Centrifuge for 30 s at 14,000 rpm ($\geq 17,000 \times g$), and discard the flow-through. Add another 500 μ l Buffer RPE to the RNeasy Mini column and centrifuge for 2 min at 14,000 rpm ($\geq 17,000 \times g$). Transfer the RNeasy Mini column into new 1.5 ml microfuge tube and centrifuge for 1 min at 14,000 rpm ($\geq 17,000 \times g$). Next, transfer the RNeasy Mini column into another new 1.5 ml microfuge tube. Open the lid of the RNeasy Mini column and let sit on your bench for 2.5 min to remove any residual ethanol.
7. Pipet 30 μ l RNase-free water directly onto the Mini column silica-gel membrane, let the column stand for 1 min, and centrifuge for 1 min at 14,000 rpm ($\geq 17,000 \times g$). Pipet another 30 μ l RNase-free water onto the silica-gel membrane, let the column stand for 1 min, and centrifuge for 1 min at 14,000 rpm ($\geq 17,000 \times g$). The RNA should be stored at -80°C .
8. Determine the RNA concentration using a spectrophotometer.
9. Confirm that the RNA is not degraded. One option is to run the RNA on an agarose gel. To prepare an RNA gel, wipe all electrophoresis parts and the gel comb with RNAase AWAY Decontamination Reagent. In a clean beaker, prepare a 1.5% (wt/vol) agarose gel by melting 1.5 g agarose in 100 ml 1 \times NorthernMax-Gly Gel Prep Running buffer. Let gel solidify, remove comb, place in gel box, and submerge in 1 \times NorthernMax-Gly Gel Prep Running buffer. To prepare the RNA for electrophoresis, pipet ≈ 1 μ g of RNA sample in RNase-free 0.2 ml tube, add RNase-free water to bring volume to 6 μ l, add 6 μ l of Glyoxal Sample Loading Dye (already contains ethidium bromide), and heat the sample at 50 $^{\circ}\text{C}$ for 30 min. Allow sample to cool to room temperature and load 12 μ l of sample per well. Run electrophoresis at 50–100 V and observe gel by UV light to confirm the presence of two distinct bands of RNA (23S, 16S) and potentially minor species (including 5S). Check that your sample has limited degradation by ensuring that the bands are crisp and without smears.

10. Digest away the DNA in the RNA sample by mixing in a tube on ice: 10 µg RNA, 10 µl 10× TURBO DNase Buffer, 2 µl TURBO DNase, and RNase-free water to bring the total volume up to 100 µl. Incubate the tube in a 37 °C water bath for 30 min. Give the tube a quick spin to collect condensation, and add another 2 µl of TURBO DNase, mix by inverting tube, give it another quick spin, and incubate the tube in 37 °C water bath for another 30 min.
11. To clean the RNA again, pipet 350 µl Buffer RLT (+1:100 βME) into the DNase treated tube (total volume now at 450 µl) and mix thoroughly by pipetting. Add 675 µl of 100% ethanol to the sample and mix thoroughly by pipetting. Apply 700 µl of the sample to the RNeasy MinElute column (columns are stored at 4 °C upon arrival), centrifuge for 15 s at 14,000 rpm ($\geq 17,000 \times g$), and discard the flow-through. Repeat using the remainder of the sample in 700 µl increments. Transfer the RNeasy MinElute column into new 2 ml collection tube. Add 500 µl Buffer RPE to the RNeasy MinElute column, centrifuge for 15 s at 14,000 rpm ($\geq 17,000 \times g$), and discard the flow-through. Add 500 µl of 80% ethanol to the RNeasy MinElute column and centrifuge for 2 min at 14,000 rpm ($\geq 17,000 \times g$). Transfer the RNeasy MinElute column into new 2 ml microfuge tube, open the lid of the RNeasy MinElute column, and centrifuge for 5 min at 14,000 rpm ($\geq 17,000 \times g$) (don't forget to label the tube body!). Transfer the RNeasy MinElute column into new 1.5 ml tube and open the lid of the RNeasy MinElute column for 2.5 min to remove any residual ethanol. Pipet 12–20 µl RNase-free water to obtain $\approx 1 \mu\text{g}/\mu\text{l}$ RNA directly onto the MinElute column membrane, let the column stand for 1 min, and centrifuge for 1 min at $\geq 17,000 \times g$. Immediately place RNA on ice and store at –80 °C.
12. Again, determine the RNA concentration, run the RNA on an agarose gel for quality (*see steps 8 and 9*), and verify that the RNA is DNA-free by using PCR. Set up a PCR reaction with a primer set to a region in the genome of your organism. Use the appropriate PCR conditions for your primer set, use 500 ng RNA, and perform the PCR amplification step for 30–35 cycles. Take care to use a positive control (pure gDNA) and a negative control (no template). Run each sample on an agarose gel to confirm that the RNA sample and the negative control have no DNA (no PCR product), and that the positive control contains the desired DNA PCR product (*see Note 6*).

3.2 RNAseq Library Construction (See Fig. 2 for Overview)

1. Obtain a primer set for your organism that will limit amplification of ribosomal RNA (*see Note 7*).
2. To generate the barcoded adapters, combine 12.5 µl of 100 µM RevAD with 12.5 µl of 100 µM Fwd Adapter in a 1.5 ml microfuge tube. Add 75 µl of water and mix well. This working

stock can be stored indefinitely at -20°C and does not need to be remade with each library construction.

3. For First Strand Synthesis, prepare the following mix in a sterile, RNase-free PCR tube: 500 ng DNA-free RNA, 1 μl of 100 μM of your primer mix, and RNase-free water to reach a total volume of 5 μl . Heat at 65°C for 5 min and then snap chill to 4°C . At room temperature, prepare a premix (scaled up as needed) with each sample requiring 5 μl per reaction and consisting of: 2 μl 5 \times First Strand Buffer, 2 μl 10 mM dNTPs, 0.5 μl 100 mM dithiothreitol (DTT), and 0.5 μl Super-script III RT. Once prepared, add 5 μl premix to 5 μl RNA/primer mix. Heat at 40°C for 90 min, cool to 4°C (Fig. 2a).
4. For Second Strand Synthesis, prepare a premix on ice (scaled up as needed) with each sample requiring 65 μl per reaction and consisting of: 15 μl 5 \times Second Strand Buffer, 1.5 μl 10 mM dNTPs, 0.5 μl 10 U/ μl *E. coli* DNA ligase, 2 μl 10 U/ μl *E. coli* DNA Pol I, 0.5 μl 2 U/ μl *E. coli* RNase H, and 45 μl H₂O. Once prepared, add 65 μl of the ice-cold premix to 10 μl of the First Strand Synthesis reaction. Incubate at 16°C for 2 h, and then add 25 μl 0.2 M EDTA (Fig. 2b).
5. To purify the newly synthesized DNA, use the miniElute PCR purification kit. This purification step allows buffer exchange, concentrates the sample, and removes short products and unincorporated primers. Add 500 μl PB to 100 μl Second Strand Synthesis reaction, apply to the provided column, spin at 17,000 $\times g$ (13,000 rpm in conventional tabletop microcentrifuge) for 1 min, and decant the flow-through. Add 750 μl PE to the column, spin again, and decant flow-through. Place column back in collection tube, and spin again for 2 min to remove residual PE. Place the column in a fresh collection tube, open the column's cap, and air-dry for 2 min with cap open before elution. Add 12 μl EB to the center of the column membrane, wait 2 min, and spin for 1.5 min to collect eluent.
6. End-repair the DNA fragments by using the Quick Blunting Kit. Prepare a premix on ice (scaled up as needed) with each sample requiring 3 μl per reaction and consisting of: 1.25 μl 10 \times Quick Blunting Buffer, 1.25 μl 1 mM dNTPs, and 0.5 μl Blunting Enzyme Mix. Once prepared, add 3 μl of the premix to 10 μl of ds-cDNA in a PCR tube. Incubate at 23°C for 30 min, 70°C for 10 min, and then at 4°C (Fig. 2c).
7. To ligate the adapters use the Quick Ligation Kit. Prepare a premix on ice (scaled up as needed) with each sample requiring 16.5 μl per reaction and consisting of: 15 μl 2 \times Quick Ligase Buffer and 1.5 μl Quick Ligase. For each sample, add 1 μl of each 12.5 μM Fwd (from step 2) and Rev adapter mix to 13 μl of the end-repaired sample. Add 16.5 μl of the Quick Ligation

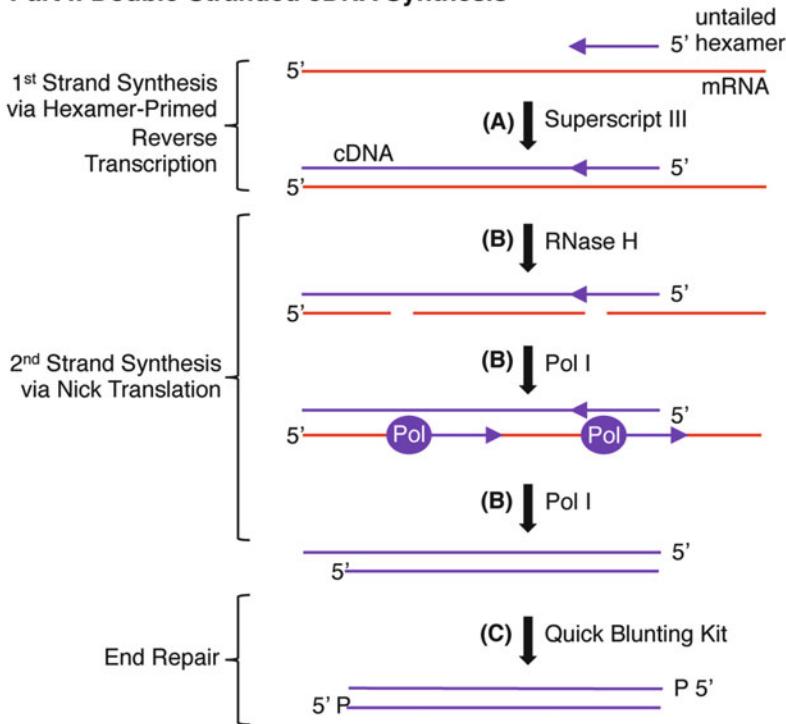
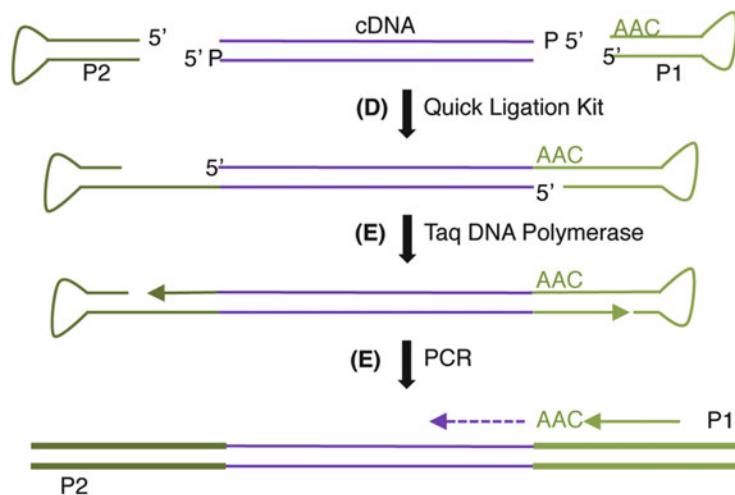
Part I: Double-Stranded cDNA Synthesis**Part II: Adaptor Ligation and PCR**

Fig. 2 Overview of RNAseq library synthesis. **(a)** For First Strand Synthesis, untailed hexamers (NSR Primers) and SuperScript III Reverse Transcriptase synthesize cDNA from the RNA. The products of this reaction are RNA:DNA heteroduplex molecules. **(b)** For Second Strand Synthesis, the first strand cDNA product is converted to dsDNA by RNase H-mediated nick translation. RNase H introduces breaks in the RNA template strand, and then Pol I synthesizes DNA from these sites by using the RNA as a primer. The 5'-3' exonuclease activity of Pol I removes RNA during nascent strand extension. DNA ligase then patches any gaps initiated from the different priming sites. The products of this reaction are dsDNA fragments that contain short 3' overhangs. **(c)** End

premix to the sample/adapter mix, and incubate at 23 °C for 15 min (*see Note 8*) (Fig. 2d).

8. To fill-in and PCR amplify each ligation, use Taq DNA Polymerase. Prepare a premix on ice (scaled up as needed) with each sample requiring 90 µl per reaction and consisting of: 39 µl H₂O, 10 µl 10× Reaction Buffer (+Mg), 10 µl 25 mM MgCl₂, 5 µl dimethyl sulfoxide (DMSO), 10 µl 10 µM Fwd primer, 10 µl 10 µM Rev primer, 5 µl 10 mM dNTPs, and 1 µl Taq DNA Polymerase. Once prepared, add 90 µl of premix to 10 µl of ligated sample and subject the mixture to the following thermal cycling conditions: 72 °C for 2 min; 95 °C for 2 min; 20 cycles of (95 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min); 72 °C for 5 min; and hold at 4 °C (Fig. 2e).
9. Purify the DNA by using AMPure XP Magnetic Beads, as bead purification results in a higher molecular weight cut off than Qiagen columns. This step will remove any adapter dimer artifacts that may have been produced during ligation. First, aliquot 150 µl of bead slurry into a clean 1.5 ml microfuge tube per sample. Allow solution to equilibrate to room temperature for 20 min. Add 100 µl of each PCR reaction to the beads and mix. Incubate at room temperature for 5 min. Place tubes on magnet stand for 2 min to capture beads. Remove supernatant with a pipet and decant. Remove each tube from the stand and resuspend beads in 200 µl 70% EtOH. Capture beads again for 2 min and decant supernatant, as before. Remove each tube from the stand and wash again with 200 µl 70% EtOH. Capture beads again for 2 min and decant supernatant. To remove the remaining EtOH, spin briefly (~30 s) in tabletop microfuge at maximum speed and carefully remove residual EtOH with a small pipet. Add 20 µl of water to the beads and mix well with the pipette. Let the bead and water mixture sit 1 min, capture beads on the stand for 2 min, and carefully transfer eluate containing your RNAseq library to a new labeled tube.
10. Once the RNAseq library is made, quantify the yield by measuring 2 µl of each sample (the total yield should be >1.5 µg). Also, analyze the products on a 2% (wt/vol) agarose gel. Library fragments should appear as a smear ranging from 120

Fig. 2 (continued) Repair using Quick Blunting Kit removes overhangs and adds 5' phosphate groups to the second strand synthesis products. (d) In Adapter Ligation, the stem-loop adapters attach the Illumina P1 and P2 sequences to the blunt ds-cDNA fragments. The P1/P2 sequences are used as universal amplification sites for PCR and bridge amplification during cluster generation. When multiplexing is desired, use the barcoded adapters. (e) The adapter ligation step results in a covalent bond at one end of each insert strand and a nick at the other end. This nick is repaired with a brief elongation step before PCR to generate a mature sequencing template for PCR amplification

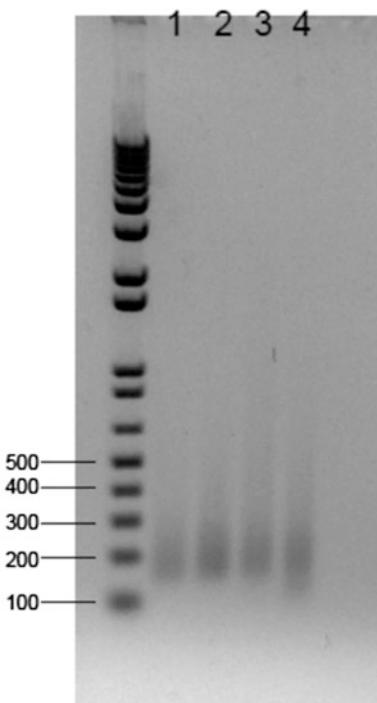


Fig. 3 2% (wt/vol) agarose gel of four representative RNAseq library preparations. Base pair size is indicated to the *left* of the gel

to 1000 base pairs with most falling below 500 base pairs. Figure 3 shows four representative samples.

11. If pooling samples, bring each barcoded sample to 500 ng in 4 μ l of water. Then mix at an equal ratio by adding 1 μ l each to a tube and mix well. Consult the sequencing center of your choice for the desired final concentration and submit your sample(s) for sequencing on an Illumina Genome Analyzer (*see Note 9*).

4 Notes

1. The miRNeasy Mini Kit from Qiagen is recommended for RNA isolation. This kit allows for the isolation of total RNA, including microRNAs. If you plan to analyze microRNAs, please take this into account when mapping your sequenced reads and establishing cutoff parameters in data analysis. It is recommended that each condition be represented in at least two biological replicates.
2. Dabbing the tube on a paper towel or Kimwipe ensures that the RNAProtect Bacteria Reagent is removed. If too much reagent

is left behind, it will interfere with downstream processes (such as RNA isolation and gDNA digestion of the RNA sample).

3. To prepare the RLT (+ β ME) mixture, add a 1:100 dilution of β ME (10 μ l β ME per 1 ml RLT). If you do not use all of the RLT + β ME mixture, it can be stored at room temperature for up to 1 month. Additionally, the pellet at this stage may not be visible, yet it is important to thoroughly resuspend all the material.
4. Prior to RNA isolation, place the appropriate amount of silica beads into the bottom portion of a screw cap microfuge tube. I recommend filling the tube $\frac{1}{4}$ of the way full with beads. In a separate autoclavable container, place the corresponding lid. This can be done in bulk ahead of time by using an autoclavable microfuge rack to hold the open screw cap microfuge tubes containing the beads, and then covering the open tubes/rack with foil. Autoclave the tubes/rack and the container with the lids. After autoclaving, wear gloves and carefully screw each sterile lid on each sterile tube containing beads. They are now ready for future use.
5. Depending on your organism, the extent of mechanical disruption required to lyse the cells may vary. It is recommended to optimize this procedure by determining the number of repetitions of bead-beating required for lysis. Initially, it is a good idea to bead-beat similar samples (from same strain, growth condition, and number of cells) for increasing amounts of repetitions and then carry each sample through RNA isolation. At the end, compare the samples for RNA yield and quality to determine which amount of bead-beating results in the best yield of undegraded RNA. Mechanical disruption can cause the samples to heat up. Thus, please ensure that you properly chill each sample on wet ice if you increase the amount of bead-beating. Also note that other methods of cell lysis are available, such as enzymatic disruption. Whichever lysis method is used, please take care to optimize it for each organism and sampling time (as sometimes stationary phase samples are harder to lyse). Note that enzymatic lysis often requires extended incubation periods at 37 °C; this is not optimal for RNA isolation as RNA molecules have short half-lives and may degrade. Thus, mechanical disruption is recommended, if available.
6. If there is gDNA contamination in your RNA sample, you will observe a PCR product. Even a faint band means that there is gDNA contamination, and the sample cannot be used. If this occurs, alter the conditions for the TURBO DNase Digestion (*see Subheading 3.1, step 9*) by using half the amount of starting RNA material. Never repeat digest the same RNA.

7. This protocol was developed for use with not-so-random (NSR) primers. NSR primers can be designed for your organism(s) of interest by starting with a random hexamer pool and doing an in silico analysis to identify primers predicted to anneal to the 5S, 16S, and 23S ribosomal RNA sequences in your genome(s) of interest. This is done by computationally aligning all possible hexamers (4096) to the desired 23S, 16S, and 5S rRNA sequences. Hexamers with a perfect match should be excluded from the NSR primer collection, as described originally by Armour et al. [2]. Individual NSR primer sets have been created for bacteria including: related *Burkholderia* species [4, 9], *Pseudomonas aeruginosa* [10], and *Rhodopseudomonas palustris* [11]. If one plans to generate de novo an NSR primer set, the primers should be ordered individually (likely in a 96 multi-well format) and pooled as desired. Though every primer set will vary, do not be alarmed if the NSR primer set comprises vastly fewer primers than the starting random hexamer pool. For *Burkholderia*, the NSR primer set included 949 primers. An NSR primer set can also be designed for a group of related organisms, as the rRNA sequences will be similar. For example, one primer set was developed for use with three *Burkholderia* species: *B. thailandensis*, *B. pseudomallei*, and *B. mallei*. Creating a new NSR primer set is costly and time-intensive, so it may be a good idea to investigate if an NSR primer set has already been developed for an organism moderately related to one's organism of choice. For example, it is reasonable to assume the *Burkholderia* NSR primer set could be used on other more distantly related *Burkholderia* species. Additionally, the NSR primer set used for *R. palustris* was successfully used for RNAseq on *Bradyrhizobium japonicum* (Nathan Ahlgren, Caroline Harwood, and E. Pete Greenberg, personal communication). A final alternative for NSR priming is to purchase a commercially available primer set. There are now “universal” NSR primer sets that work very well for groups of related organisms (a product line is available through NuGEN). Regardless of the approach taken, it is prudent to perform a test RNAseq run to ensure that the NSR primer set efficiently limits rRNA amplification, while retaining good coverage for the rest of the genome. The *Burkholderia* NSR primer set achieved an average coverage of 87–106 hits per gene, depending on the species.

If you choose not to use NSR primers, you will need to deplete rRNA from your total RNA sample. The protocol could then be adapted to use random hexamers for cDNA synthesis.

8. If multiplexing (running more than one sample per lane in the Illumina Sequencer) is desired, it is at this stage that a barcode is introduced. Only the forward adapters (FwdAD) are

barcoded. The reverse adapter (RevAD) is never barcoded. The stem-loop adapters are engineered each with a 3-base barcode tag to allow sample multiplexing during sequencing. Barcodes will appear at the beginning of the sequence reads (positions 1–3) and must be trimmed off prior to sample alignment. The codes were selected as a group to balance base composition. For this reason, libraries must be pooled prior to sequencing such that each of the eight code sequences is present in equal proportions. Said another way, samples can only be sequenced in lots of 8, if multiplexing is desired. However, if you choose to sequence only one sample per lane, libraries MUST be constructed with the NON-barcoded adapters. If you multiplex, make sure to document which sample is tagged with which barcode.

9. This chapter focuses on experimental design and library preparation for QS transcriptomics. RNAseq generates a large amount of data and there are a number of approaches for processing this data, depending on your goals. The raw data must be extracted. If barcodes are used, the sample must be sorted by barcode, the barcode removed, and then aligned to a genome. It is a good idea to remove the remaining sequences aligning to the ribosomal sequences. Once the data is aligned to a genome, the transcripts for each sample are processed and quantified. There are many approaches at this step in sample analysis. A good starting point to identify differentially regulated genes for biological replicates is to use the DESeq package (with a false discovery rate [FDR] cutoff of 0.05) and proceed with genes showing twofold or more regulation relative to the reference condition. Data analysis to compare gene expression between two conditions can be done with multiple approaches; if available, the Avadis NGS software package is recommended.

Acknowledgements

A number of people contributed to the methods described in this chapter. Christopher Armour developed the NSR primer approach and methods for library construction [2] and also supported the development of this method for prokaryotic species. Sudha Chugani and Yasuhiro Oda contributed to other aspects of method development, including RNA isolation and library synthesis and analysis. Additionally, this protocol utilizes a number of commercially available kits. Many of the procedures are taken directly from the kit protocols or handbooks.

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Chapter 15

Identification of AHL- and BDSF-Controlled Proteins in *Burkholderia cenocepacia* by Proteomics

Yilei Liu, Gabriella Pessi, Katharina Riedel, and Leo Eberl

Abstract

We used comparative proteome analysis to determine the target genes of the two quorum sensing (QS) circuits in the opportunistic pathogen *Burkholderia cenocepacia*: the *N*-acyl homoserine lactone (AHL)-based CepIR system and the BDSF (*Burkholderia* diffusible signal factor, *cis*-2-dodecenoic acid)-based RpfFR system. In this book chapter, we focus on the description of the practical procedure we currently use in the laboratory to perform a sensitive GeLC-MS/MS shotgun proteomics experiment; we also briefly describe the downstream bioinformatic data analysis.

Key words Quorum sensing, Cell fractionation, SDS-PAGE, In-gel tryptic digestion, Mass spectrometry

1 Introduction

B. cenocepacia is an opportunistic pathogen that, together with other 19 closely related bacterial species, belongs to the *Burkholderia cepacia* complex (Bcc) [1, 2]. All Bcc strains contain a highly conserved AHL-dependent LuxIR type QS system, known as CepIR [3, 4], with CepI encoding the AHL synthase, responsible for the production of the *N*-octanoyl homoserine lactone (C8-HSL) and, in minor amounts of the *N*-hexanoyl homoserine lactone (C6-HSL), and CepR acting as a transcriptional regulator. Certain Bcc members harbor additional AHL-based QS systems, such as BviIR in *B. vietnamiensis* [5] and CciIR in *B. cenocepacia* strains that both belong to the epidemic ET12 lineage [6]. The presence of yet another QS system in *B. cenocepacia*, which uses the fatty acid *cis*-2-dodecenoic acid (also known as *Burkholderia* diffusible signal factor or BDSF) as signal molecule, was reported in 2008 [7]. The key enzyme for BDSF biosynthesis is RpfF, an enzyme that has both dehydratase and thioesterase activities [8]. The BDSF receptor protein RpfR has recently been identified and was shown to modulate the intracellular c-di-GMP level [9]. The BDSF-dependent RpfFR system is highly

conserved throughout the Bcc [10, 11] and has been shown to interact with the AHL-dependent circuitries to regulate *B. cenocepacia* virulence [12]. Our group has been a pioneer in the utilization of comparative proteome analysis to identify QS-regulated proteins [13, 14]. In 2003, an approach including pre-fractionation (intracellular, extracellular, and surface proteins) followed by a two-dimensional gel electrophoresis (2-D PAGE) was used to identify 55 proteins with differential expression in a *cepI* mutant strain [13]. Later, gel-free proteomics using Isobaric Tags for Relative and Absolute Quantitation (iTRAQ) analysis was utilized to detect 1257 proteins in *B. cenocepacia*, 82 of which were differentially expressed in a *cepR* mutant [15]. More recently, we employed a highly sensitive GeLC-MS/MS shotgun proteomics analysis to elucidate the BDSF stimulon and detected close to 2700 *B. cenocepacia* proteins which account for 35% of the proteins annotated for *B. cenocepacia* strain H111 [16]. Among the detected proteins, 116 displayed significant regulation in the *rpfF* mutant [12].

In this review, we present a detailed practical procedure that our lab routinely uses to carry out GeLC-MS/MS shotgun proteomics experiments (Fig. 1), using *B. cenocepacia* strain H111 as a model organism. First, a subcellular fractionation is performed (extracellular, intracellular, and membrane fractions) to reduce the complexity of the sample and to determine the predominant subcellular localization of a protein of interest, which may provide important information about its function [17, 18]. Subsequently, protein samples are separated by 1D SDS-PAGE [19, 20], which has the advantage

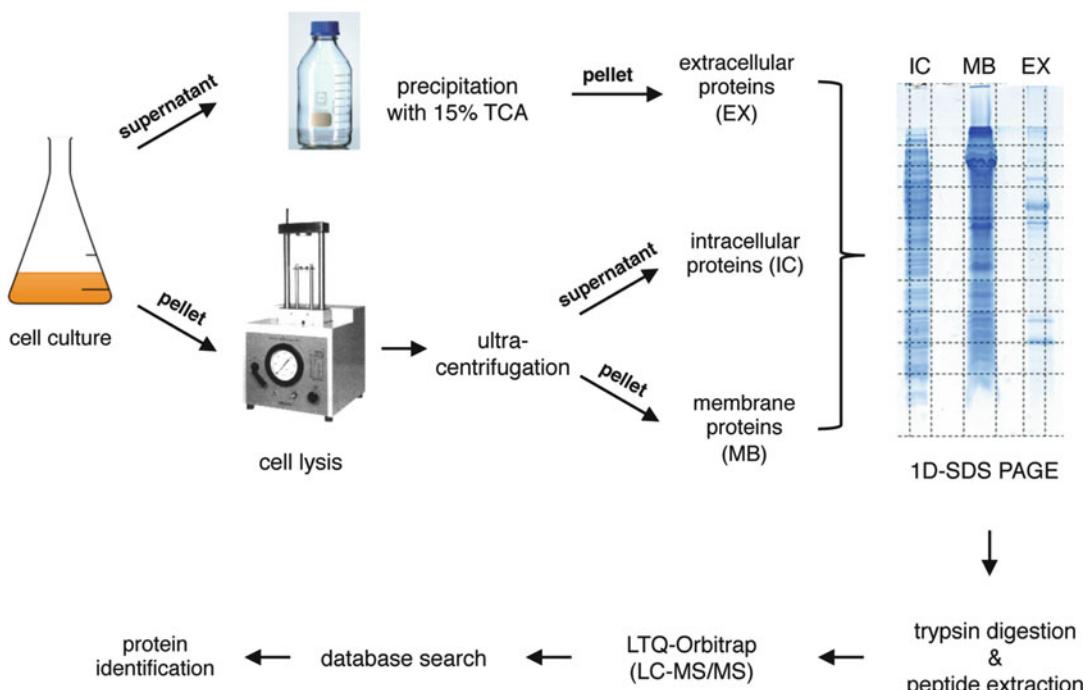


Fig. 1 The overall workflow of the GeLC-MS/MS shotgun proteomics experiment

that membrane proteins are not underrepresented. After cutting the individual lanes into at least ten gel slices, the proteins in these slices are then subjected to in-gel tryptic digestion. Next, the extracted peptides are separated by Reverse-Phase chromatography (RP-HPLC) and analyzed by a mass spectrometer interfaced with a nanoelectrospray source [21]. Fragment ion mass spectra are searched against the *B. cenocepacia* strain H111 protein database and further analyzed for differential expression using an in-house pipeline developed by Ahrens and coworkers [22] or commercial software solutions.

2 Materials

2.1 Bacterial Strains and Culturing

1. *B. cenocepacia* wild-type strain H111 [23].
2. *B. cenocepacia* mutant strain H111-rpfF_{Bc} [12].
3. 10 µM BDSF (*see Note 1*).
4. LB liquid medium: 10 g/l Bacto Tryptone, 5 g/l Bacto yeast extract, 4 g/l NaCl in distilled water. Adjust pH 7.4 and autoclave.
5. LB agar: LB liquid medium plus 12–15 g/l agar. Autoclave.

2.2 Protein Extraction

1. Trichloroacetic acid (TCA) (*see Note 2*).
2. Acetone, HPLC grade.
3. Tris–SDS buffer: 50 mM Tris/HCl pH 7.5 with 1% (wt/vol) sodium dodecyl sulfate (SDS).
4. Bradford protein assay.
5. Tris–PI buffer: 50 mM Tris/HCl pH 7.5 supplemented with protease inhibitor.
6. Sonicator: sonicate samples on ice.
7. French Press Cell: pre-chill the French Press Cell body before loading the sample in order to process the samples in cold.
8. 100 mM Tris–HCl, pH 7.5 with 2% SDS.

2.3 SDS-PAGE

1. Colloidal blue staining buffer: 100 g/l ammonium sulfate, 20 g/l phosphoric acid (85%), 25% (vol/vol) methanol, 0.625 g/l Coomassie Brilliant Blue G250 in distilled water.

2.4 In-Gel Digestion

1. Destaining solution: 50/50 (vol/vol) methanol/50 mM (NH₄)HCO₃, (*see Note 3*).
2. Acetonitrile.
3. Dithiothreitol (DTT) solution: 10 mM DTT in 50 mM (NH₄)HCO₃ (*see Note 4*).

4. Iodoacetamide (IAA) solution: 50 mM IAA in 50 mM (NH₄)HCO₃ (*see Note 5*).
5. 80% Acetonitrile.
6. Trypsin buffer: 20 mM Tris–HCl pH 8.5, 0.1 mM CaCl₂.
7. Trypsin solution: 10 µg/ml sequencing grade trypsin dissolved in trypsin buffer (*see Note 6*).
8. 10% Formic acid in H₂O.

2.5 Peptide Extraction

1. Wetting solution: 80% acetonitrile.
2. Washing solution: 5% acetonitrile, 0.1% formic acid.
3. Elution solution: 60% acetonitrile, 0.1% formic acid.

2.6 Prepare the Samples for MS Analysis

1. Buffer A: 3% acetonitrile, 0.1% formic acid.

3 Methods

Perform all procedures at room temperature, unless stated otherwise.

3.1 Culture Conditions

1. Streak *B. cenocepacia* H111 wild-type and mutant strains on LB agar plates with respective antibiotics and incubate the plates at 37 °C overnight.
2. Pick a single colony of each strain to inoculate 3 ml LB pre-culture with antibiotics. Incubate the pre-cultures with vigorous shaking at 37 °C overnight.
3. Inoculate 500 ml LB medium (*see Note 7*) in 3 l Erlenmeyer flasks with 3 ml overnight pre-culture. As a control, grow the H111-rpfF_{Bc} mutant in LB medium supplemented with 10 µM BDSF. Grow the culture with agitation at 37 °C until early stationary phase (i.e., an OD₆₀₀ of about 2.0).

3.2 Protein Extraction

3.2.1 Extracellular Protein Preparation

1. Filter the individual 500 ml cultures with 0.45 µm sterile filters (*see Note 8*).
2. Precipitate the soluble proteins in the filtered liquid with 12% (wt/vol) TCA. To this end, add the 500 ml filtered liquid into a bottle containing 60 g TCA. Incubate the mixture with agitation at 4 °C overnight [24].
3. Centrifuge the mixture at 12,500 × *g* for 1 h at 4 °C (*see Note 9*).
4. Wash the pellet with 250 ml 100% ice-cold acetone (*see Note 10*) and centrifuge again with the same condition to harvest the precipitated proteins.
5. Decant the acetone and air-dry the pellet in the fume hood (*see Note 11*).

6. Resolve the pellet in 50 mM Tris–SDS buffer. Measure the protein concentrations using the Bradford assay according to the manufacturer’s protocol.

3.2.2 Intracellular Protein Preparation

1. Centrifuge the individual 500 ml culture at $7000 \times g$ for 30 min at 4 °C (*see Note 12*).
2. Remove the supernatant. Resuspend the cell pellet in ice-cold 10 ml Tris–PI buffer.
3. Lyse the cells by sonication first and then pass them through French Press Cell twice (*see Note 13*). Check the lysate under the microscope to ensure the complete lysis.
4. Remove the cell debris by centrifugation at $4000 \times g$ for 15 min at 4 °C.
5. Separate the cell membrane proteins from soluble intracellular proteins by ultracentrifugation at $80,000 \times g$ for 1 h at 4 °C. *See Subheading 3.2.3* to continue with the membrane protein (the pellet after ultracentrifugation) extraction.
6. Carefully take out the supernatant and add 6 volumes of pre-chilled acetone to precipitate the intracellular proteins. Mix and incubate the solution at –20 °C overnight.
7. Centrifuge ($20,000 \times g$, 30 min, 4 °C) to collect precipitated proteins.
8. Discard the acetone supernatant. Air-dry the pellet. Dissolve the pellet in Tris–SDS buffer and determine the protein concentration by Bradford assay.

3.2.3 Cell Membrane Protein Preparation

1. After ultracentrifugation (*see Subheading 3.2.2, step 5*), gently wash the pellet with Tris–PI buffer to remove the remaining intracellular proteins.
2. Pellet the protein again with ultracentrifugation (the same setting as Subheading 3.2.2, step 5).
3. Carefully remove the supernatant. Dissolve the proteins in 100 mM Tris–HCl, pH 7.5 with 2% (wt/vol) SDS. Determine the protein concentration.

3.3 SDS-PAGE and Gel Staining

1. Load 2 µg of protein of each sample on 15% SDS-PAGE gel (*see Note 14*). Run the electrophoresis according to the standard protocol [25] until the front dye just migrates off the gel.
2. After electrophoresis, carefully open the gel cassette and take out the running/resolving gel. Gently rinse the gel with deionized water. Stain the gel with colloidal blue staining buffer with very gentle horizontal agitation for 2–12 h [26].
3. To remove the excessive stain, soak the gel in deionized water with gentle shaking. Change water every 30 min for a few times until distinct protein bands appear [26].

3.4 In-Gel Digestion

1. Excise individual sample lanes; cut the entire lane into ten gel bands, namely ten fractions (Fig. 1). Transfer each gel fraction into a Protein LoBind tube (*see Note 15*).
2. Destain each gel fraction in destaining solution at 37 °C for 30 min (*see Note 16*). Change the destaining solution and incubate another 15 min. Repeat this step a couple of times until the gel slices lose the blue color.
3. Remove the destaining solution. Shortly wash each gel fraction once with acetonitrile and discard the solvent.
4. Incubate each gel fraction in acetonitrile for 5 min. Then remove and discard the solvent.
5. Add DTT solution to soak the gel slices and incubate the tubes at 60 °C for 1 h. Remove DTT solution.
6. Wash the gel fractions again with acetonitrile.
7. Add IAA solution and incubate the tubes for 30 min in the dark (*see Note 17*). Then remove IAA solution.
8. Wash the gel fractions twice with 50 mM (NH₄)HCO₃.
9. Add 80% acetonitrile and incubate the tubes at 37 °C for 1 h (*see Note 18*). Discard the acetonitrile solution after incubation.
10. Dry the gel slices completely by incubating at 50 °C or using SpeedVac Concentrator.
11. Add 100 µl trypsin solution (1 µg) per gel fraction/per tube and incubate at 37 °C for 20 min (*see Note 19*). Then top up each gel fraction with 200 µl trypsin buffer and incubate at 37 °C at least 4 h or overnight.
12. Add 300 µl H₂O and 65 µl 10% formic acid to each digest (*see Note 20*).

3.5 Peptide Extraction Using ZipTip® C18 Resin Pipette Tips

Treat each sample fraction/each tube with the following procedure.

1. Use one 10 µl pipette to take one ZipTip. Aspirate 10 µl wetting solution and dispense to the waste. Repeat once.
2. Wash the tip with 10 µl washing solution twice. Aspirate 10 µl washing solution and dispense to the waste.
3. Load the resin with peptides by pipetting up and down many times in the sample fraction digest.
4. Wash the loaded resin three times with the washing solution.
5. Take a new Protein LoBind tube and add 50 µl elution solution.
6. Elute the peptides in the 50 µl elution solution by pipetting up and down at least ten times.

3.6 Prepare the Samples for MS Analysis

- When all the sample fractions are prepared, SpeedVac the eluted peptides to complete dryness. Store at -20°C until the day of MS analysis.
- Dissolve samples in 10 μl Buffer A prior to RP-HPLC-MS analysis.
- Load each sample into the RP-HPLC that is of an Eksigent 2D nano LC system using a 80 mm fused silica emitter column of 75 μm inner diameter (BGB Analytik) packed in-house with Magic C18 AQ 3 μm resin (Michrom BioResources). Peptides are separated at a flow rate of 250 nL/min, against a gradient from 0.8% to 96.8% acetonitrile in 94 min.
- Analyze the peptides on a LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher Scientific) interfaced with a nanoelectrospray source (*see Note 21*) (or an alternative mass spectrometer with similar specifications regarding mass accuracy and sensitivity) by using the following parameters. Record survey scans recorded in m/z range from 300 to 1700 with a resolution of 30,000 at m/z 400. Perform MS/MS experiments for the 20 most intensive precursor ions as determined in the survey scan excluding unassigned charge states or singly charged ions from the MS/MS experiments in the linear ion trap.

3.7 Differential Data Analysis

Several commercial software solutions are available for the analysis of proteomics data [27]. We used an in-house pipeline for proteomics searches and differential expression analysis developed by our collaborators [22]; it is based on the extraction of fragment ion mass spectra from Thermo RAW files using msconvert (ProteoWizard, version 3.0.3831), a search for *B. cenocepacia* H111 matching peptides with the powerful search engine MS-GF+ [22, 28], classification of all identified peptides and filtering of only those that uniquely identify one protein [29], and differential expression analysis with DESeq [30] or similar software packages. To call a protein identified, we applied the following criteria in Scaffold (peptide identification probability 95%; protein identification probability 99%; at least two peptides per protein) and the in-house pipeline (three PSMs for peptides that unambiguously identify one protein). Differentially expressed proteins were visualized in MA (“M” stands for log ratio and “A” for mean average) plots, and proteins of particular interest were independently validated with independent RT-PCR experiments, knock-out mutants, or additional functional assays.

4 Notes

- BDSF can be purchased from Sigma-Aldrich.
- TCA is a strongly corrosive acid, which must be handled with care.

3. Destaining solution shall be freshly prepared just before use.
4. Freshly make the DTT solution from DTT powder.
5. Freshly make the IAA solution from IAA powder.
6. Always prepare the trypsin solution freshly from lyophilized powder just before use.
7. Grow the main cultures without antibiotics.
8. The filtered liquid can be stored in a -80 °C freezer to continue the following procedure at a later time point.
9. Carefully discard the TCA containing supernatant into the appropriate liquid waste container.
10. This acetone wash step is to remove the remaining TCA and H₂O, and repeat this step may increase the purity of the precipitated proteins.
11. Dried pellet looks sticky.
12. The cell pellet can be stored at -80 °C freezer for long.
13. After this step, if the lysate still has sticky or snot-like lumps, additional sonication may apply to break down the DNA in the lysate.
14. We found 2 µg protein is enough for the following process and MS analysis.
15. Cutting each gel band further into few smaller pieces can increase the efficiency of the following process. If not continue the protocol right away, store the gel slices at -20 °C. We routinely use Protein LoBind 1.5 ml tubes (Eppendorf).
16. In this step and later procedures, unless stated otherwise, we use 1 ml as the solution volume for each gel fraction/each tube.
17. IAA is light sensitive.
18. Acetonitrile dehydrates the gel slices. The gel slices shrink and become white/opaque.
19. We add concentrated trypsin solution to the dried gel slices so that the trypsin can be absorbed into the gel to increase the digestion efficiency.
20. The final volume of each gel fraction/each tube is 665 µl. The final concentration of formic acid is 1%.
21. We usually use an LTQ-Orbitrap Velos mass spectrometer at the Functional Genomic Centre Zürich (FGCZ).

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Chapter 16

Imaging *N*-Acy Homoserine Lactone Quorum Sensing In Vivo

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Abstract

In order to study *N*-acyl homoserine lactone (AHL)-based quorum sensing in vivo, we present a protocol using an *Escherichia coli* strain equipped with a *luxR*-based monitor system, which in the presence of exogenous AHL molecules expresses a green fluorescent protein (GFP). Lungs from mice challenged intratracheally with alginate beads containing both a *Pseudomonas aeruginosa* strain together with the *E. coli* monitor strain can be investigated at different time points postinfection. Epifluorescent or confocal scanning laser microscopy (CSLM) is used to detect the GFP-expressing *E. coli* monitor strain in the lung tissues, indicating production and excretion of AHLs in vivo by the infecting *P. aeruginosa*.

Key words Quorum sensing, *Pseudomonas aeruginosa*, Pulmonary infection model, Epifluorescence microscopy, CSLM, In vivo

1 Introduction

The biofilm mode of growth is known to protect *Pseudomonas aeruginosa* against the host defense and antibiotics [1–6]. Due to the high density of cells in biofilms, *P. aeruginosa* is able to make use of its cell–cell communication (quorum sensing (QS)) systems. *P. aeruginosa* employs two *N*-acyl-L-homoserine lactone (AHL) signal molecule based QS sensors, encoded by the *las* and *rhl* systems. Both systems are organized with a LuxR-type transcriptional regulator, LasR and RhlR, and a cognate LuxI-type synthetase, LasI and RhlI, that mainly synthesize the signal molecules *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C₁₂-HSL) and *N*-butanoyl-L-homoserine lactone (C₄-HSL), respectively [7, 8]. In addition to the AHL signal molecule based QS sensors a third QS system, the *Pseudomonas* quinolone signal (PQS) based system, has been identified [9].

In simple terms, AHL regulation comprises an *I* gene encoding the AHL synthetase and an *R* gene encoding the receptor

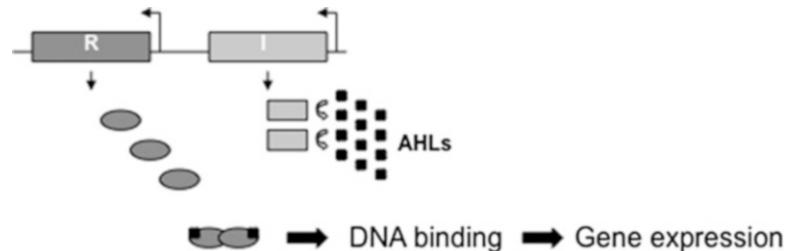


Fig. 1 The basics of AHL regulation. The AHL regulatory components are encoded by an *R* gene and an *I* gene. The gene products of these two genes are the regulator (R protein) and the signal synthetase (I protein), respectively

(see Fig. 1). During growth of the bacteria the AHLs, produced by the I synthetase, accumulate in the medium until a critical threshold concentration is reached. At this concentration the R protein forms a multimeric complex with the AHLs. This complex exhibits DNA binding properties and is believed to interact with specific recognition sequences positioned in the promoter regions of QS regulated target genes. Binding can either (dependent on the genes) upregulate or downregulate transcription initiation (see review [10]).

The ability to monitor the production of AHLs by pathogenic Gram-negative bacteria during infection can contribute to the understanding of the host-pathogen interaction. In principle, the presence of exogenous AHLs can be detected by a reporter gene fused to any QS target gene maintained in a bacterial background devoid of the signal generator (*I*). We have previously designed a sensitive green fluorescent protein (GFP) reporter, which enables visualization of cell-cell communication at the single-cell level and it has been used to detect *in vitro* and *in vivo* production of AHLs by *P. aeruginosa* [11–16].

2 Materials

2.1 Bacterial Strains and Growth Media

1. Any AHL producing *P. aeruginosa* strain can be used, we use *P. aeruginosa* (PAO1) originally obtained from Professor Barbara Igleswski (University of Rochester Medical Center, NY, USA). This strain is well tolerated by the mice, it is QS-proficient, however it features a reduced production of C₄-HSL [17], resulting in lowered rhamnolipid production, which we have shown to be extremely toxic for the mice [18]. After choosing a bacterial strain, a dose-response experiment must be conducted to optimize the inoculum since the virulence of bacterial strains significantly affects the load of bacteria that can get instilled in the lungs without leading to mortality. It must be emphasized that our end point of the mouse model is not death but visualization of QS or gradual eradication of the bacteria.

A dose–response experiment should also be conducted for the *E. coli* AHL monitor strain.

2. AHL monitor strain: The unstable GFP derivative GFP(ASV), containing alanine (A), serine (S), and valine (V) at the C-terminus, is used as a reporter [15]. The GFP-based monitor plasmid pJBA132, which carries the *Vibrio fischeri* region encoding *luxR* and a fusion between *PluxI* and *gfp* (*PluxI-gfp* (ASV)), has been cloned into the stable broad-host-range vector pME6031 (pVS1 replicon, accession no. AF118811). The *E. coli* monitor strain MT102 is a restriction-minus derivative of MC1000 (*araD139* (Δ *ara-leu*)7697 Δ *lac*, *thi*, *hsdR*) (for further details see [12, 14]). The JB525-*gfp*(ASV) is *E. coli* MT102 containing the plasmid pJBA132.
3. Luria Bertani (LB) liquid medium: 1% (wt/vol) bacto-tryptone, 0.5% (wt/vol) yeast extract, 1% (wt/vol) sodium chloride in distilled water. Autoclave.
4. LB agar medium: LB liquid medium plus 1.5% (wt/vol) agar. Autoclave.
5. Blue agar plates.
6. 250 ml conical flasks.
7. Shaking incubator at 37 °C.
8. 15 and 50 ml plastic tubes.
9. Centrifuge capable of 5000 $\times g$.

2.2 Alginic Beads

1. Protanal: Seaweed alginate with approximately 60% guluronic acid content (10/60) is dissolved in 0.9% NaCl to a concentration of 11 mg/ml and autoclaved. Store at 4 °C until use. Can be stored for several months.
2. TRIS-buffer: 0.1 M Tris Base ($C_4H_{11}NO_3$), adjust the pH to 7.0 by adding 25% HCl and autoclave. Store at 4 °C until use. Can be stored for several months.
3. TRIS-buffer with 0.1 M CaCl₂: mix 100 ml TRIS-buffer with 1.47 g (0.1 M) CaCl₂ on a magnetic stirrer.
4. Bead making: the viscous suspension of bacteria and protanal is placed in a cylindrical reservoir and forced through an 18-G cannular by compressed air (0.4 bar), while another jet of air is forced coaxially along the needle to blow off the alginate droplets (1.0 bar) [19] (for a figure see in [20]). Clean the equipment in 70% (vol/vol) ethanol.

2.3 Mice

1. Female BALB/c mice at 9–11 weeks of age. BALB/c mice have been shown to have a Th₂ immune response as seen in chronically infected cystic fibrosis patients and are susceptible to infection with *P. aeruginosa* [21]. The mice should be maintained on standard mouse chow and water ad libitum for a

minimum 1 week before challenge. All animal studies should be performed in accordance with relevant guidelines and regulations.

2.4 Analgesia, Anesthesia and Euthanasia

1. Anesthetic: mix one part Hypnorm® (0.315 mg/ml fentanyl citrate and 10 mg/ml fluanisone) with two parts of sterile water. Pour them together gently. Then add one part 5 mg/ml midazolam. Pour together gently! A fresh solution should be made every time. Store at room temperature until use.
2. Analgesia: 5 mg/ml bupivacaine. Store at room temperature.
3. Euthanasia: 200 mg/ml pentobarbital with 20 mg/ml lidocain hydrochloride. Store at room temperature.

2.5 Infection Procedure

1. A bead tipped needle is used when installing the alginate beads in the mouse lung.
2. Sutur: ethicon Vicryl, 3-0, FS-2.
3. Surgical equipment can be chosen as desired. We use Nasal scissors, Durogrip needleholder, and tweezers.
4. Heating plates: LP reptiles vivarium heating mat 1200 × 280 mm.

2.6 Freeze Microtomy

1. Tissue-Tek Cryomold standard (25 mm × 20 mm × 5 mm). Disposable vinyl specimen molds for placement of lungs.
2. Tissue-tek O.C.T.™ gel for embedment of lungs before freezing.
3. Tissue-Tek Cryo 2000 for sectioning of the lungs.
4. Hexane.
5. Dry ice.

2.7 Epifluorescence Microscopy

1. Use an epifluorescence microscope with a camera to visualize GFP of the AHL monitor strains. Images can be obtained using objectives in the range 40–100×.

2.8 Confocal Scanning Laser Microscopy (CSLM)

1. Image acquisitions of bacteria in the lungs can be performed with confocal scanning laser microscopy (CSLM) equipped with a detector and a filter set for monitoring GFP. In addition, a reflection detector for acquiring bright-field images is installed. Images can be obtained using objectives in the range 40–100×, we prefer oil-based objectives. Image scanning should be carried out with the 488 nm laser line from an Ar/Kr laser. Imaris software package can be used to generate images of the bacteria.

3 Methods

The GFP from the jellyfish *Aequorea victoria* was chosen as the reporter since it only requires a trace amount of oxygen to mature and thereby no external compounds needs to be added in order to detect green fluorescence [22]. However, since the GFP is extremely stable (derived from *gfpmut3* [23]) different *gfp* genes with varying half-lives ranging from 40 min to a few hours was constructed [15]. One of these variants is the unstable version *gfp* (ASV). The construct of a live bacterial AHL sensor using the *luxR* gene from *V. fischeri* as the quorum sensor acts in the presence of exogenous AHLs by LuxR, and positively affects the expression of the *luxI* promoter (*luxR-P_{luxI}*), which in turn induces the expression of the *gfp*-reporter [14]. The sensitivity of this *gfp*-reporter enables the visualization of AHLs produced by *P. aeruginosa* in vivo. In practice this means that no signal should be observed in mice infected with alginate beads containing either the *E. coli* monitor strain or a *P. aeruginosa* strain alone. However, when a mixture of the two strains are used to inoculate the lungs of mice, one should be able to detect a GFP signal produced by the *E. coli* monitor strain in response to AHLs produced by *P. aeruginosa*.

Other AHL monitor strains have been constructed to study the ability of anti-pathogenic drugs to block QS. One example is the *P. aeruginosa* strain constitutively expressing a red fluorescent protein, which is advantageous for easy identification of the bacteria in the tissue samples. Moreover the same strain contains a *lasR-PlasB_{gfp}* (ASV) reporter fusion in order to detect an activated QS system. In practice this means that the strain always fluorescence red, and when QS is activated it will produce a GFP signal in addition. When QS is blocked by the presence of exogenous QSI compounds, the GFP signal will disappear. For further details see [13]. The different monitor stains can also be used in in vitro continuous-culture flow cell experiments (see details in Chapter 21) where both the AHL production from different strains and QSIs can be investigated.

In the present method section the protocol for in vivo detection of AHLs produced by *P. aeruginosa* is described. When isolating lungs for ex vivo inspection, lungs should also be isolated, at the same time, for quantitative bacteriology to verify that bacteria are present and that the monitor strain is functional.

3.1 Preparation of Bacteria in Alginate Beads

1. Plate bacteria from a freezer stock onto a blue agar plate, which are selective for Gram-negative bacilli [24], and incubate the plate at 37 °C overnight. Use your choice of *P. aeruginosa* strain and the *E. coli* monitor strain JB525-*gfp*(ASV).
2. Use one bacterial colony from the plate to inoculate an overnight culture in 100 ml LB liquid medium in a 250 ml conical

flask. Allow the culture to grow for 18–20 h at 37 °C on an orbital shaker at 180 rpm.

3. Transfer the bacterial overnight culture to two 50 ml centrifuge tubes and centrifuge for 10 min at 5000 × *g*. Discard the supernatant and re-suspended the bacterial pellet in 4.5 ml LB medium.
4. Mix 0.5 ml re-suspended bacterial pellet with 4.5 ml sterile protanal for production of beads.
5. When making the beads (*see* Subheading 2.2, item 4) the droplets are directed into a cross-linking solution of 0.1 M CaCl₂ in TRIS-HCL buffer (0.1 M, pH 7.0). Allow the beads to cure for 1 h in the calcium bath with continuous stirring at minimum rotation.
6. Transfer the beads to two 50 ml centrifuge tubes and centrifuge for 10 min at 600 × *g*.
7. Discard the supernatant and wash the beads in 0.9% (wt/vol) NaCl. Centrifuge for 10 min at 600 × *g*. Do this twice.
8. Re-suspend the beads in 7.5 ml 0.9% (wt/vol) NaCl using a 5 ml pipette. Homogenize the beads gently using a 5 ml pipette, then a 1 ml pipette, and last a 200 µl pipette to avoid clumps. DO NOT USE A VORTEX FOR MIXING. Make two tenfold serial dilutions using two different pipette tips.
9. Plate appropriate dilutions using sterile glass spreaders on blue plates. Plate each dilution double. Store the beads at 4 °C until next day and incubate the plates at 37 °C.

3.2 Infection Procedure

1. Count the plates and calculate the colony forming units (CFU)/ml.
2. Right before challenge, adjust the beads containing *P. aeruginosa* and the beads containing the *E. coli* monitor strain to the appropriate concentration in 0.9% (wt/vol) NaCl (*see Note 1*).
3. The bead solution containing both bacteria is mixed at ratio 1:2 (*P. aeruginosa:E. coli*).
4. Use epifluorescence or CSLM to verify that the beads are not fluorescing green and thus insure that the monitor is not turned on before infection.
5. There should be three groups of mice in an experiment using a *P. aeruginosa* strain and one AHL monitor strain. One group of mice is infected with *P. aeruginosa* alone, one group of mice is infected with the AHL monitor strain alone, and one group of mice is infected with the mixture of *P. aeruginosa* and the AHL monitor strain (*see Note 2*).
6. Sedate the mice using 10 ml/kg body weight Hypnorm®/midazolam (*see* Subheading 2.4, item 1) injected subcutaneously (s.c.) in the groin area (*see Note 3*).

7. Fixate each mouse on its back. Use a rubber band fastened between two needles to hold the head of the mouse fixated. We use a tray off styrofoam (29 cm × 39 cm) as the operation area.
8. Sterilize the throat of the mouse with 70% (vol/vol) ethanol.
9. Make an incision in the skin above the trachea with a pair of scissors and expand the incision using the pair of scissors by making it larger between the cutting area.
10. Move the muscles to the sides to locate the trachea using the pair of scissors. Again do this by making the cutting area larger. Do not cut the muscles.
11. Use the scissors to make an incision in the membrane surrounding the trachea.
12. Make a way into the trachea using an 18-G needle.
13. Use a bead tipped needle to install 0.04 ml of the bead suspension in the left lung, 11 mm from the tracheal penetration site.
14. Suture the incision with silk.
15. Sterilize the surgical equipment between each mouse using 70% (vol/vol) ethanol.
16. Euthanize four mice right after challenge and isolate the lungs (*see Subheading 3.3*). The lungs are used to estimate the infection inoculum.
17. Alternative: make two tenfold serial dilutions of the bead suspension and plate the appropriate dilutions on blue plates.
18. Use an 18-G needle to apply one drop of bupivacaine on the incision site for postoperative pain.
19. Inject the mice s.c. with 0.75 ml of 0.9% (wt/vol) NaCl in the neck-skin area to avoid dehydration.
20. Place the mice on a row on top of bedding in the cage and cover them with a paper napkin for recovering after surgery. Place the part of the cage where the mice are placed on top of the heating pad. There should also be an area of the cage, which is free from heating. Heating should be no more than 28 °C.
21. Check the mice after 4–6 h and thereafter minimum once a day (*see Note 4*).

3.3 Quantitative Bacteriology

1. Injecting pentobarbital (10.0 ml/kg body weight) intraperitoneal (i.p.) to euthanize the mice. Ensure that local animal care regulations are followed.
2. Fixate each dead mouse on its back and apply 70% (vol/vol) ethanol on the chest and stomach to avoid contamination.
3. To isolate the lungs, first remove the skin with a pair of scissors. Then open the thoracic cavity without damaging the lungs.

Take hold of the arteries below the lungs using tweezers and cut the arteries with a pair of scissors. Lift the lungs upright and cut the arteries next to the heart. The lungs should now be isolated with the tweezers.

4. Place the isolated lungs in 5 ml of 0.9% (wt/vol) NaCl in a 15 ml centrifuge tube and keep it on ice until homogenization.
5. Sterilize the surgical equipment between each mouse using 70% (vol/vol) ethanol.
6. Homogenize the lungs using a homogenizer. The crusher is washed in 35 ml 70% (vol/vol) ethanol for 5 s, for 20 s in 35 ml 70% (vol/vol) ethanol, for 5 s in 35 ml 0.9% (wt/vol) NaCl, and for 5 s in 30 ml 0.9% (wt/vol) NaCl. Use 50 ml centrifuge tubes for the washing. All the tubes are changed after 5–8 samples. Between groups the homogenizer must be taken apart, cleaned, and sterilized. Please notice that the homogenizer must only be used when placed in fluid.
7. Make tenfold serial dilutions of each lung homogenate and plate all the dilutions onto blue agar plates. Incubate the plates at 37 °C overnight.
8. Count the plates and calculate the CFU/lung (*see Notes 5 and 6*).

3.4 Freeze Microtomy

1. After a chosen period of time (*see Note 7*), the mice are euthanized using pentobarbital (10.0 ml/kg body weight) injected intraperitoneal (i.p.). Ensure that local animal care regulations are followed.
2. Fixate each dead mouse on its back and apply 70% (vol/vol) ethanol on the chest and stomach to avoid contamination.
3. Isolate the lungs by first removing the skin with a scissor and tweezer. Then open the thoracic cavity without damaging the lungs. Take hold of the arteries below the lungs using a tweezers and cut the arteries with the scissor. Lift the lungs upright and cut the arteries next to the heart. The lungs should now be isolated with the tweezers.
4. Whole lungs are placed in the Tissue-Tek mold and covered with Tissue-Tek O.C.T.™ gel. Hexane is cooled down using dry ice (*see Note 8*) and the embedded lungs are placed in the cooled hexane for 5–10 min. Keep it on dry ice afterwards. This should happen as soon as possible after isolation of the lungs.
5. Cool the Tissue-Tek Cryo 2000 to operating temperature and make different sections of the lung tissue. Frozen sections should be 10–50 µM thick.
6. Place the sections on cover glasses for microscopy inspection.
7. Study the lung sections with epifluorescence or CSLM (*see Note 9*).

4 Notes

1. With respect to the Iglewski PAO1, we adjust the beads to a concentration of 1.5×10^8 CFU/ml.
2. A power analysis should be made to estimate the group sizes.
3. This is the concentration we use, but it has to be adjusted depending on the mouse strain. Please consult other references regarding this subject.
4. Usually the mice appear sick within the first 24 h after infection. We believe that this is due to the fact that they have been given pneumonia and anesthetics.
5. In case of the Iglewski PAO1, homogenates from most of the mice will display CFUs when diluted to 10^{-3} . If no CFU can be counted the mice were inoculated with too little beads.
6. Bacteria from lung tissues containing mixed inoculates of *P. aeruginosa* and *E. coli* can be discriminated based on the colony color on the blue agar plates combined with the ability of *E. coli* colonies to express GFP in the presence of exogenous AHLs. Therefore the plates can be supplemented with 10 nM 3-oxo-C₆-HSL.
7. The experiments should not run for longer than 3 days since it is not possible to detect the *E. coli* monitor stain.
8. Make sure that the hexane is cold enough.
9. Ensure that what are observed under the microscope are in fact bacteria. This can be verified by the morphology and by looking at the bacteria under normal light.

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Chapter 17

Assessing *Pseudomonas aeruginosa* Autoinducer Effects on Mammalian Epithelial Cells

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Abstract

The human mucosal environment in the gut is rich with interactions between microbiota and mammalian epithelia. Microbes such as the Gram-negative bacterium *Pseudomonas aeruginosa* may use quorum sensing to communicate with other microorganisms and mammalian cells to alter gene expression. Here, we present methodologies to evaluate the effects of *P. aeruginosa* N-(3-oxo-dodecanoyl)-L-homoserine lactone (3O-C₁₂-HSL) on Caco-2 cell monolayers. First, we describe a method for assessing barrier function and permeability of epithelial cells when exposed to 3O-C₁₂-HSL by measuring transepithelial electrical resistance (TER) and paracellular flow using fluorescently labeled dextran. Secondly, we detail methods to investigate the effect of 3O-C₁₂-HSL on protein–protein interactions of epithelial junction proteins. Lastly, we will detail imaging techniques to visualize Caco-2 barrier disruption following exposure to 3O-C₁₂-HSL through the use of confocal laser scanning microscopy (CLSM) and a super resolution technique, stimulated emission depletion (STED) microscopy, to achieve nanoscale visualization of Caco-2 monolayers.

Key words Host pathogen interaction, *Pseudomonas aeruginosa*, Quorum sensing, Acyl homoserine lactone, Transepithelial electrical resistance, Paracellular permeability, Cell junction, IQGAP1, Imaging

1 Introduction

Quorum sensing is a cell signaling system whereby microbes secrete and detect small molecule signals to orchestrate gene expression based on population density. This system allows Gram-negative bacteria to use these signals, termed autoinducers, to modulate growth, virulence, motility, and biofilm formation in a coordinated fashion. *Pseudomonas aeruginosa* is a model Gram-negative opportunistic pathogen that utilizes two *N*-acyl homoserine lactones as autoinducers, *N*-(3-oxo-dodecanoyl)-L-homoserine lactone (3O-C₁₂-HSL) and *N*-butyryl-L-homoserine lactone (C₄-HSL), to coordinate these responses. 3O-C₁₂-HSL and C₄-HSL bind to

the transcriptional regulators LasR and RhlR, respectively [1]; in turn, these regulators can alter gene expression of as much as 10% of the entire *P. aeruginosa* genome [2, 3]. Such extensive control of gene expression via quorum sensing gives *P. aeruginosa* the means to coordinate multi-faceted and aggressive infections once the cell population has achieved a significant density.

The secretion of these autoinducers into the environment affords the opportunity for microbes to coordinate an orchestrated attack; however, these chemical signals are also sensed by host cells. Mammalian cells detect and respond to molecules produced by microorganisms, such as *P. aeruginosa* 3O-C₁₂-HSL, in a process known as inter-kingdom signaling. There are numerous microbe–host interactions in the human gastrointestinal tract, including those between the gut microbiota and intestinal epithelial cells that can impact host health and the development of disease [1, 4]. However, the downstream consequences on host cells that incur as a result of inter-kingdom signaling are still poorly understood.

Given the plethora of host–microbe activity in the gut, it comes as no surprise that intestinal epithelial cells can be disrupted by a variety of exogenous agents, including those of bacterial origin. 3O-C₁₂-HSL has been shown to affect intestinal epithelial cells by disrupting junctional proteins, which regulate epithelial barrier integrity and the permeability of small molecules [5–10]. Human Caco-2 cells, which are derived from a human epithelial colorectal adenocarcinoma, are routinely employed to evaluate aspects of epithelial barrier integrity such as absorption and permeability [11]. These cells functionally and morphologically mimic epithelial cells of the small intestine when cultured under specific conditions, making them an excellent model to study various aspects of epithelial barrier activities [12, 13]. 3O-C₁₂-HSL can disrupt not only the barrier integrity of Caco-2 cells by altering tight junction and adherens junction proteins but can also perturb cell migration and Rac1- and Cdc42-dependent reorganization of the actin cytoskeleton [7], which can have significant implications on bacterial translocation.

Tight junctions compose a complex of proteins on the apical membrane of epithelial cells and function to maintain cell–cell adhesion and barrier permeability. Similarly, at the basolateral membrane of epithelial cells, adherens junctions also play a role in barrier integrity in addition to maintaining the structure of tight junctions. The *P. aeruginosa* quorum sensing molecule 3O-C₁₂-HSL interacts with and targets the IQ-motif-containing GTPase-activating protein (IQGAP1) in human epithelial cells. Interaction with scaffold IQGAP1 triggers changes in the cytoskeleton network and signaling cascades [7]. Exposure to 3O-C₁₂-HSL increases intracellular free calcium in Caco-2 cells and alters the phosphorylation status of tight and adherens junction protein complexes [8]. As a result, 3O-C₁₂-HSL downregulates expression of

these proteins and activates p38 and p42/44 MAPK cascades, causing the dissociation of junction complexes and compromising barrier integrity [8–10]. Additionally, different concentrations of 3O-C₁₂-HSL, such as those found in biofilms, modulate epithelial Caco-2 cell migration [7].

Communication between the microbiota and mammalian cells of the mucosa is valuable to better understand both microbe–host interactions and its relevance to human health and disease. By investigating the effects of the *P. aeruginosa* autoinducer 3O-C₁₂-HSL on Caco-2 cell monolayers, we can better appreciate the complex relationship between bacteria and host cells within the gastrointestinal environment.

2 Materials

1. *N*-3-Oxo-dodecanoyl-L-homoserine (3O-C₁₂-HSL) (C₁₆H₂₇-NO₄, MW 297.4) is synthesized by Peter Konradsson and Lan Bui (Department of Organic Chemistry, Linköping University, Sweden) as described by Chhabra et al. [14]. These synthetic analogues are structurally and functionally identical to those obtained from *P. aeruginosa* cultures. Synthesized 3O-C₁₂-HSL is also commercially available (see Note 1). The identity and purity of the synthesized 3O-C₁₂-HSL should be verified by HPLC and its activity as a quorum sensing molecule can be confirmed using the lux-based *E. coli* JM109 pSB1075 biosensor assay described in detail by Surette et al. [15]. 3O-C₁₂-HSL is prepared as a stock solution in 100% dimethylsulfoxide (DMSO) and diluted with aqueous buffer of choice dictated by each independent experiment.
2. Cell lysis buffer: RIPA Buffer (1% NP-40, 1% deoxycholic acid sodium salt, 0.1% SDS, 150 mM NaCl, 10 mM Tris pH 7.4, 10 mM EDTA pH 8.0 dissolved in 1× phosphate-buffered saline solution (PBS)) containing 25 U nulcease, 1 mM phenyl-methyl-sulfonyl-fluoride, 1 mM Na₃VaO₄, 25 mM NaF, and other protease inhibitors.
3. Blocking buffer (immunofluorescence imaging): 1× PBS supplemented with 1% bovine serum albumin (BSA) and 10 mM glycine.
4. Blocking buffer (immunoblotting): 5% nonfat milk in 1× PBS pH 7.6, supplemented with 0.18% Tween 20.
5. Primary Antibodies (Abs) for Fluorescence Staining: anti-ZO-3 (Thermo Fisher Scientific, Rockford, IL, USA); anti-JAM-A (Thermo Fisher Scientific, Rockford, IL, USA); anti-IQGAP1

(Millipore, Temecula, CA, USA). All primary Abs are diluted in blocking buffer.

6. Secondary Abs for Fluorescence Staining: Alexa Fluor 568-conjugated goat anti-mouse (Life Technologies, Grand Island, NY, USA); Alexa Fluor 488-conjugated goat anti-rabbit (Life Technologies, Grand Island, NY, USA); Atto 647 N (fluorophore for STED) goat anti-mouse Abs (Active Motif, Carlsbad, CA, USA). All secondary Abs are diluted in blocking buffer.
7. Primary Abs for Immunoblot Analysis: anti-ZO-3 (Thermo Fisher Scientific, Rockford, IL, USA); anti-JAM-A (Thermo Fisher Scientific, Rockford, IL, USA). All primary Abs are diluted in blocking buffer. Recommended dilution for primary Abs is 1:1000 in blocking buffer.
8. Secondary Abs for Immunoblot Analysis: IRDye 800CW (LI-COR Biosciences, Cambridge, UK); IRDye 680CW (LI-COR Biosciences, Cambridge, UK). All secondary Abs are diluted in blocking buffer: 5% nonfat milk in 1× PBS pH 7.6, supplemented with 0.18% Tween 20. Recommended dilution for secondary Abs is 1:10,000.
9. Krebs-Ringer glucose phosphate buffer (KRG): 120 mM NaCl, 4.9 mM KCl, 1.2 mM MgSO₄, 8.3 mM KH₂PO₄, 1 mM CaCl₂, 10 mM glucose.
10. For Immunoblot analysis, immunoreactive bands are visualized by using a capable imaging system and the density ratios of the bands are quantified by image analysis software.
11. Fetal bovine serum is heat treated at 55 °C for 30 min, aliquoted, and then stored at –20 °C.
12. Dulbecco's Modified Eagle's Medium (DMEM) is supplemented with 10% heat-inactivated fetal bovine serum as well as 100 U/ml penicillin, 100 µg/ml streptomycin, 1% nonessential amino acids, and 2 mM L-glutamine. For the remainder of this protocol, this standard culture medium will be referred to as "growth medium" (GM).
13. Human epithelial colorectal adenocarcinoma Caco-2 cells (#86010202, obtained directly from Sigma Aldrich, St. Louis, MO, USA) are used for the experiments described here throughout this protocol. Caco-2 cells are grown in GM at 37 °C in 5% CO₂ and passaged weekly upon reaching 80% confluence for a total of 84–95 passages.
14. For experiments, Caco-2 cells are grown in GM at 37 °C in 5% CO₂ for 7–10 days until the cells become matured and differentiated, establishing polarized epithelial monolayers on the specified growth surface.

3 Methods

3.1 Transepithelial Electrical Resistance (TER) and Paracellular Flow

1. TER and paracellular flux is evaluated using a two-compartment cell culture model.
2. Caco-2 cells are seeded at a density of 10^5 cells/cm² onto Costar Transwell permeable culture inserts (pore size 3 µm, Corning Inc., Corning, NY, USA) in standard 24-well plates.
3. Cells are cultivated in GM at 37 °C in 5% CO₂ and maintained for 10–12 days until a tight monolayer is formed and a stable TER is attained. TER of the cell monolayers is measured using an epithelial volt-ohm meter with a stick-type electrode as per the manufacturer's instructions (Fig. 1). Only cell monolayers with a stable TER of 500–700 Ω × cm² and above should be used.
4. TER value is calculated via the following equation: TER [Ω × cm²] = (measured resistance for membrane with cells [Ω] – measured black resistance for membrane without cells [Ω]) × area of the membrane (0.33 cm²)

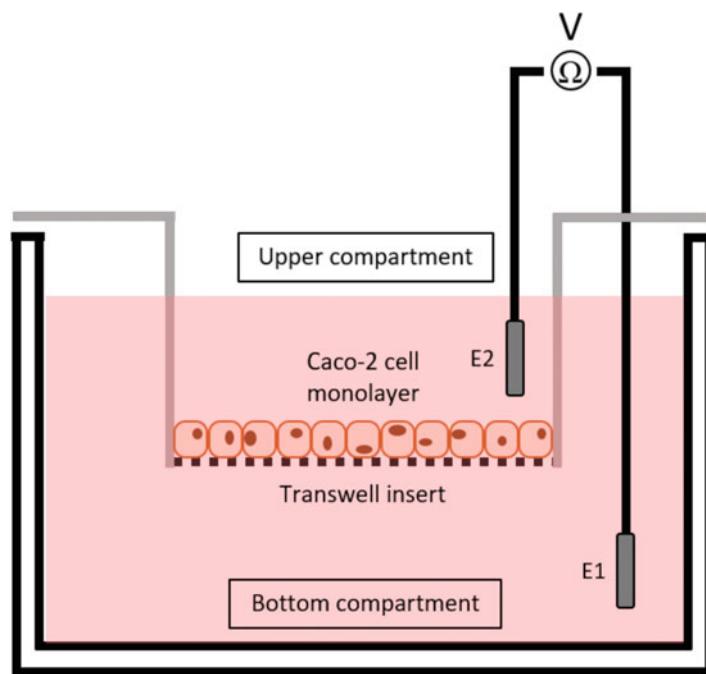


Fig. 1 Transepithelial electrical resistance (TER) setup. Caco-2 cell monolayers are grown in 24-well plates on Costar Transwell permeable culture inserts (3 µm pore size). TER resistance of Caco-2 cell monolayers is measured using an epithelial volt-ohm meter with stick-type electrodes (E1 and E2). TER [Ω × cm²] = (measured resistance for membrane with cells [Ω] – measured black resistance for membrane without cells [Ω]) × area of the membrane (0.33 cm²)

$[\Omega]) \times$ area of the membrane (0.33 cm^2) as described by Katouli et al. [16].

5. Prior to beginning the experiment, cell monolayers are incubated overnight at 37°C in 5% CO_2 in serum- and antibiotics-free GM.
6. Cell monolayers with stable TER are either untreated, treated with the desired concentration(s) of *P. aeruginosa* 3O-C₁₂-HSL (e.g., 100, 200, or 300 μM 3O-C₁₂-HSL), or exposed to 0.6% DMSO as a diluent control (see Notes 2 and 3). 3O-C₁₂-HSL or DMSO is added to both the top and bottom compartments.
7. At desired time points, record the TER of the cell monolayers using an epithelial volt-ohmmeter as per the manufacturer's instructions (Fig. 1).
8. To evaluate paracellular permeability in the *apical to basolateral direction*, cell monolayers grown on permeable Transwell inserts are incubated under desired experimental conditions (as described above) in the presence of a paracellular flux tracer: 4 kDa or 10 kDa fluorescein isothiocyanate (FITC)-dextrans (see Note 1). Dextran tracers of varying molecular weight and fluorophores are also available.
9. FITC-dextrans (4 kDa or 10 kDa) are dissolved in pre-warmed (37°C) Krebs-Ringer glucose phosphate buffer (KRG), pH 7.3 to a concentration of 10 mg/ml.
10. To start the experiment, transfer the Transwell insert into a new 24-well plate containing 1 ml pre-warmed KRG buffer per each well.
11. 200 μl of the FITC-dextran tracer solution is added to the apical surface of the cell monolayer (i.e., the top compartment) and the cells are incubated at 37°C in 5% CO_2 for the desired period of time.
12. At desired time points, samples from the apical and basal sides (i.e., top and bottom compartments) are transferred to a black 96-well plate and fluorescence is measured using any appropriate plate reader. The excitation and emission wavelengths for FITC-dextran fluorescence measurements should be calibrated to 485 nm and 538 nm, respectively.

3.2 Preparation of Whole Cell Lysates

1. Caco-2 cells grown to confluence (5–8 days) on tissue culture plates or flasks are treated with the desired concentration(s) of *P. aeruginosa* 3O-C₁₂-HSL or 0.6% DMSO as a diluent control in GM at 37°C in 5% CO_2 for the determined amount of time (see Notes 2 and 3).
2. Adherent cells in plates or flasks are placed on ice, washed twice with 1× PBS pH 7.6 (in the same dish or flask), and

then immediately lysed with ice-cold cell lysis buffer (*see Subheading 2*). 1 ml cell lysis buffer per 10^7 cells in a 150 cm^2 flask, or 200–300 μl cell lysis buffer per well in a 6-well plate is acceptable.

3. While still on ice, scrape cells in lysis buffer off the growing surface using a rubber scraper and transfer to a centrifuge tube. The cell suspensions are homogenized through a 21-gauge needle and then centrifuged at $14,000 \times g$ for 30 min at 4°C . Resulting supernatant is collected, aliquoted into fresh centrifuge tubes, and stored at -70°C until ready to be assessed.
4. Protein concentrations of the cell lysates is determined using a detergent compatible colorimetric assay (*see Note 4*); average protein concentration has previously been determined to be around 5000–7000 $\mu\text{g}/\text{ml}$.
5. Whole cell lysates are diluted in Laemmli's sample buffer at equal protein concentrations, heated at 95°C for 5 min, and then subjected to SDS-PAGE.
6. Alternative: whole cell lysates are immunoprecipitated prior to loading on a gel.

3.3 Immuno-precipitation

1. Prepare Protein G Sepharose 4 Fast Flow beads (GE Healthcare, Uppsala, Sweden) by washing the beads twice in $1\times$ PBS and then restoring to a 50% slurry in $1\times$ PBS. Take care to avoid disruption of the beads.
2. Samples are pre-cleared by combining 100 μl of 4 Fast Flow beads (50% bead slurry) with 1 ml of whole cell lysates and incubating for 30–60 min at 4°C on a rocker/orbital shaker. Pre-clearing samples will aid in reducing nonspecific binding of proteins to the beads in succeeding steps. Remove 4 Fast Flow beads by centrifugation at $14,000 \times g$ for 10 min as per the manufacturer's instructions. Transfer the resulting supernatant to a new centrifuge tube.
3. The protein concentration of the cell lysates should be predetermined as described in Preparation of Cell Lysates methods. Dilute the whole cell lysates to approximately 1 $\mu\text{g}/\mu\text{l}$ total protein in $1\times$ PBS. If necessary, a more concentrated cell concentrate (e.g., 10 $\mu\text{g}/\mu\text{l}$) may be required to immunoprecipitate found at low concentrations.
4. Samples with equivalent protein concentrations are immunoprecipitated by combining 500 μl of cell lysate with primary Abs and incubating overnight at 4°C with gentle rocking on a rocker/orbital shaker. As an example, we have used anti-ZO-3 and anti-JAM-A Abs (Fig. 2a). Appropriate dilutions for primary antibodies should be empirically determined.

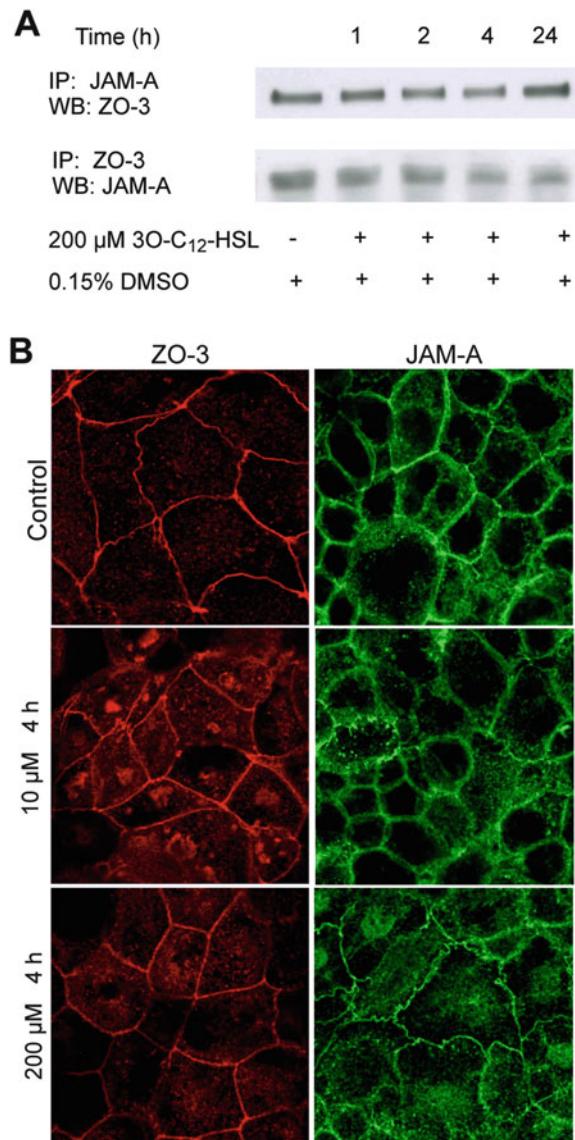


Fig. 2 Epithelial tight junctions in 3O-C₁₂-HSL-treated human cells. **(a)** Expression of ZO-3-JAM-A protein complexes in epithelial Caco-2 monolayers. Cells were treated with 200 μ M 3O-C₁₂-HSL or diluent (0.6% DMSO) as a control, for 1, 2, 4, or 24 h. Total cell extracts were immunoprecipitated for JAM-A or ZO-3 and further immunoblotted for ZO-3 and JAM-A, respectively, using specific antibodies. **(b)** Visualization of ZO-3 and JAM-A distribution in epithelial Caco-2 monolayers. Cells were treated with 10 or 200 μ M 3O-C₁₂-HSL or diluents (0.6% DMSO) as a control, for 4 h. Samples were fixed and stained with antibodies against ZO-3, JAM-A and Alexa Fluor 594 and 488 secondary Abs and analyzed by confocal laser scanning microscopy (CLSM). Image size is 67.6 \times 67.6 μ m and pixel size is 0.13 μ m

5. Immunocomplexes are captured by adding 100 μ l 4 Fast Flow beads and incubating overnight at 4 °C with gentle rocking on a rocker/orbital shaker. 4 Fast Flow beads are collected by gentle pulse centrifugation (5 s at 14,000 \times g). Discard the supernatant and wash the beads three times in ice-cold 1× PBS pH 7.6 with centrifugation at 14,000 \times g for 20 s.
6. Following the final wash, 4 Fast Flow beads are resuspended in Laemmli sample buffer, boiled for 5 min at 95 °C, and then collected by centrifugation at 14,000 \times g for 20 s. The resulting supernatant is then loaded on polyacrylamide (e.g., 4–12% or 8–16%) for SDS-PAGE. Samples can be stored frozen in fresh centrifuge tubes at –20 °C but must be re-boiled for 5 min immediately prior to loading onto a gel.

3.4 SDS-PAGE and Immunoblotting

1. Protein concentration of whole cell lysates should be predetermined as stated in Preparation of Cell Lysates methods.
2. Samples are diluted in Laemmli's sample buffer at equal protein concentrations, heated at 95 °C for 5 min, and then loaded on 4–12% or 8–16% SDS-polyacrylamide gels and run at standard voltage. Following electrophoresis, gels can proceed to immunoblotting or staining with Coomassie Blue for further in-gel digestion and mass spectrometric characterization of proteins/proteomes [17].
3. For immunoblotting, separated proteins are electrophoretically transferred to a PVDF membrane and quality of the transfer is monitored by Ponceau S staining.
4. PVDF membrane is washed twice with distilled water (or 1× PBS) and then incubated for 1 h at room temperature in blocking buffer (5% nonfat milk in 1× PBS pH 7.6, containing 0.18% Tween 20) to prevent nonspecific binding.
5. Desired primary Abs (*see* Subheading 2) are diluted in blocking buffer, added to the membrane, and incubated overnight at 4 °C with gentle rocking on a rocker/orbital shaker. As an example, we have used anti-ZO-3 and anti-JAM-A antibodies diluted 1:1000 in blocking buffer (Fig. 2a). Appropriate dilutions for primary antibodies should be empirically determined. Routinely, anti-GAPDH Abs should be included as a loading control.
6. Following overnight incubation, wash the PVDF membrane in 1× PBS pH 7.6 with 0.18% Tween 20 and then incubate with IRDye 800CW or IRDye 680CW secondary Abs diluted 1:10,000 for 1 h at room temperature. If using other detection Abs, the appropriate dilutions should be empirically determined.

3.5 Fluorescence Staining, Confocal Laser Scanning Microscopy (CLSM), and Stimulated Emission Depletion (STED) Super-Resolution Microscopy

7. Repeat wash procedure in 1× PBS containing 0.18% Tween 20 at least 3–5 times and then visualize the immunoreactive bands.
8. A capable imaging system and dedicated image analysis software can be employed to detect the signals and quantify the density ratios of immunoreactive bands.
1. Caco-2 cell monolayers are allowed to age 7–10 days on glass coverslips (13 mm diameter with a thickness of 0.17 ± 0.01 mm) and then exposed to the desired concentration of 3O-C₁₂-HSL or 0.6% DMSO as a diluent control for the desired period of time at 37 °C.
2. Following exposure to quorum sensing molecule(s) or diluent control, rinse coverslips twice with 1× PBS pH 7.3 and fix cells with 3% paraformaldehyde in 1× PBS for 20 min at room temperature. Again, rinse coverslips in 1× PBS following fixation and then permeabilize the cells in 0.18% Triton X-100 in 1× PBS for 5 min at room temperature. Rinse cells on coverslip with 1× PBS when completed.
3. Block nonspecific staining with blocking buffer (1× PBS supplemented with 1% BSA and 10 mM glycine) for 1 h at room temperature. Wash cells on coverslip with 1× PBS when completed.
4. Dilute the desired primary Abs in blocking buffer (working dilutions should be empirically determined for each respective Ab) and then apply to coverslips and incubate for 1 h at room temperature (or overnight at 4 °C) in a humid chamber. As an example, we have used anti-ZO-3 Abs, anti-JAM-A antibodies, or anti-IQGAP1 Abs (Figs. 2b and 3).
5. Following primary Ab(s) incubation, coverslips are rinsed with 1× PBS and the Alexa Fluor 488-conjugated goat anti-rabbit Abs, Alexa Fluor 568-conjugated goat anti-mouse Abs, or Atto 647 N (fluorophore for STED)-conjugated goat anti-mouse secondary Abs are added to the coverslips and incubated in humid chamber for 1 h at room temperature in the dark. Appropriate dilutions of detection Abs should be empirically determined.
6. If desired, cell nuclei can be stained with DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride).
7. Finally, after secondary Ab(s) incubation, coverslips are rinsed with 1× PBS a final time and then mounted onto glass slides with Prolong® Gold Antifade Reagent (Life Technologies, Eugene, OR).

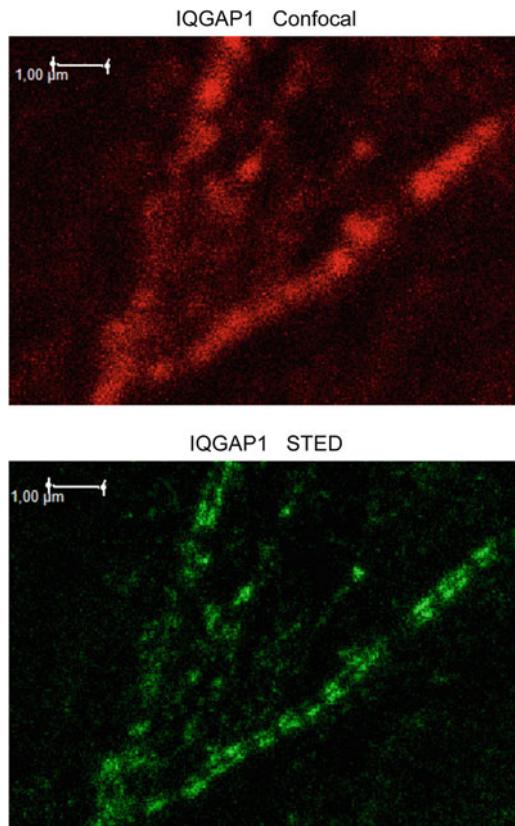


Fig. 3 High resolution imaging of scaffold IQGAP1 in 30-C₁₂-HSL-treated human epithelial Caco-2 cells. Cell monolayers were stimulated with 12 μ M 30-C₁₂-HSL for 20 min. Cells were fixed and stained with mouse anti-IQGAP1 and Atto 647 N goat anti-mouse antibodies and visualized by confocal microscopy (*upper image, in red*) and STED nanoscopy (*bottom image, in green*). Bar: 1 μ m

8. For CLSM, specimens can be examined using any appropriate confocal laser scanning microscope (Fig. 2b) and fluorescence staining intensity can be quantified using the Image J software (NIH).
9. For nanoscale visualization, STED microscopy can achieve resolutions of 5–10 times higher than that which is capable by CLSM (approximately 20–40 nm), allowing the capture of very fine structural details (Fig. 3). For high resolution imaging, the samples can be examined on a STED nanoscope system in conjunction with a 100 \times oil immersion objective. This advanced imaging technique is based on CLSM and achieved by switching fluorophores on and off in time [18]. Analysis and quantification of STED microscopy images can be performed using the appropriate image analysis software.

4 Notes

1. Synthetic 3O-C₁₂-HSL and 4 kDa or 10 kDa fluorescein isothiocyanate (FITC)-dextrans can be purchased from Sigma Aldrich (St. Louis, MO, USA).
2. If assessing the contribution of the phosphorylation state of junction proteins on epithelial barrier integrity, cell monolayers can be pre-treated with protein phosphatase and kinase inhibitors prior to incubation with the desired quorum sensing molecule, e.g., cell monolayers can be pre-treated with 25 µM genistein (protein kinase inhibitor), 0.05 µM okadaic acid (protein phosphatase inhibitor), 0.05 µM staurosporine (protein kinase inhibitor), or with all three inhibitors combined for 1 h followed with desired concentration of 3O-C₁₂-HSL, or with 0.6% DMSO as a diluent control being added to both compartments.
3. If assessing the contribution of calcium signaling on epithelial barrier integrity, cell monolayers can be pre-treated with calcium blockers prior to incubation with the desired quorum sensing molecule, e.g., cell monolayers can be pre-treated with 0.5 µM thapsigargin (sarco/endoplasmic reticulum Ca²⁺ inhibitor), 0.1 µM xestospongin C (inositol 1,4,5-trisphosphate (IP(3))-induced Ca²⁺ inhibitor), and 5 µM dantrolene (sarcoplasmic reticulum Ca²⁺ inhibitor) for 1 h followed with the desired concentration of 3O-C₁₂-HSL, or with 0.6% DMSO as a diluent control being added to both compartments.
4. We routinely use the Bio-Rad Detergent Compatible (DC) protein assay (Bio-Rad Laboratories, Hercules, CA) for protein quantification.

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Chapter 18

Animal Models for *Pseudomonas aeruginosa* Quorum Sensing Studies

Damien Maura, Arunava Bandyopadhyaya, and Laurence G. Rahme

Abstract

Quorum sensing (QS) systems play global regulatory roles in bacterial virulence. They synchronize the expression of multiple virulence factors and they control and modulate bacterial antibiotic tolerance systems and host defense mechanisms. Therefore, it is important to obtain knowledge about QS modes of action and to test putative therapeutics that may interrupt QS actions in the context of infections. This chapter describes methods to study bacterial pathogenesis in murine acute and persistent/relapsing infection models, using the Gram-negative bacterial pathogen *Pseudomonas aeruginosa* as an example. These infection models can be used to probe bacterial virulence functions and in mechanistic studies, as well as for the assessment of the therapeutic potential of antibacterials, including anti-virulence agents.

Key words Lung infection, Abdominal burn infection, Open wound infection, Back burn infection, Antibiotic tolerance, Persistence, *Pseudomonas aeruginosa*

1 Introduction

Pseudomonas aeruginosa is a widespread opportunistic human pathogen responsible for acute and chronic/persistent infections, primarily in patients with immuno-compromising conditions (e.g., HIV infection, cancer, and diabetes), cystic fibrosis (CF), and trauma [1, 2]. Furthermore, *P. aeruginosa* infections have been exhibiting increasing recalcitrance to all available antibiotics [3].

Clinically relevant model systems that allow the assessment of virulence factors, their actions, and new therapeutics are needed. Notably, there are two distinct clinical syndromes of *P. aeruginosa* lung infection that require different models. In ventilated patients, *P. aeruginosa* infection causes an acute pneumonia with a high mortality rate [4]. Meanwhile, in patients with CF, *P. aeruginosa* infection is the primary cause of chronic inflammation, which is a key factor in the progression of CF lung disease [5]. In addition,

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serious burn injuries result in immunosuppression that predisposes affected patients to opportunistic nosocomial infections. In this context, *P. aeruginosa* infection, in particular, is feared due to its high mortality and pervasiveness worldwide [6–8]. Indeed, most deaths in severely burn-injured patients are due to burn wound sepsis. Immunocompromised patients, including burn patients, are also at risk for developing sepsis secondary to pneumonia and catheter-related infections [9].

P. aeruginosa infections are facilitated by a wide array of virulence factors that impact various stages of the infection process, host defenses, and host metabolic systems. Many of these factors are regulated by three major quorum sensing (QS) regulators, namely LasR, RhlR, and MvfR [10–13]. Accordingly, QS has been the focus of extensive mechanistic and therapeutic studies over the past 20 years [10, 14–16]. Several animal models have been developed and used in order to evaluate these findings *in vivo* in the context of mammalian infections [17, 18].

In this chapter, we describe five clinically relevant murine infection models that are used to assess the role of biological pathways in acute or persistent *P. aeruginosa* infections. These models provide a means of evaluating antibacterial, anti-virulence, or anti-persister drugs *in vivo*, a prerequisite to move forward in the discovery of drugs for the treatment of multidrug resistant *P. aeruginosa* infections, which are currently lacking.

The first model simulates a soft-tissue invasive wound infection [19]. It consists of an abdominal full-skin thickness burn generated with heated brass plugs, wherein the underlying rectus abdominus muscle is left uninjured, followed by local *P. aeruginosa* inoculation at the burn eschar site. This kind of burn injury disrupts the skin barrier and skin vascularization, dampens re-epithelialization of the basal dermal tissue, and promotes systemic disturbances that lead to immune suppression [20, 21]. The risk of subsequent burn wound infection and systemic infection may correlate with the size of the burn injury [22, 23]. This full-skin thickness burn injury model has been used extensively to assess the role of the MvfR QS system in *P. aeruginosa* virulence as well as the therapeutic potential of anti-QS inhibitors [11, 24–26].

Recently, we adapted the aforementioned abdominal burn and infection model for studies of bacterial antibiotic tolerance and persistence [25]. Antibiotic tolerance—defined as the ability of a fraction of an antibiotic-susceptible bacterial population to survive exposure to normally lethal concentrations of bactericidal antibiotics—was demonstrated to be regulated by QS [12, 25, 27, 28]. The clinical importance of bacterial antibiotic tolerance is reflected by many cases in which antibiotics fail to clear infections despite the absence of resistant bacteria. Furthermore, clinical reports suggest that the contribution of bacterium tolerance to treatment failure and mortality in some patients with infections

can be as significant as that of antibiotic resistance. The murine persistent/relapsing full-skin thickness burn injury model utilizes a short-term antibiotic treatment postinfection to allow assessment of antibiotic tolerant cells that survive antibiotic killing, repopulate the infected tissues, and thus resume infection following antibiotic cessation. Recently, this model was used to examine the therapeutic potential of *P. aeruginosa* antibiotic tolerance inhibitors [25].

The third model simulates an invasive infection of large-area burn wounds [30% total body surface area, (TBSA)]. In this model, animals receive a burn injury on the back followed by intradermal injection of the bacterial inoculum. This model has been used widely in burn infection studies, including bacterial translocation [29], gene therapy, and antibiotic efficacy studies [9].

The fourth model discussed herein models acute lung infection in a manner considered to be clinically relevant to pneumonia and potentially CF. Typically, pathophysiological changes in the lung due to *P. aeruginosa* infection include micro-abscesses with focal hemorrhage and the formation of bacteria filled necrotic foci throughout the lung parenchyma [30, 31]. In this lung infection mouse model, bacterial inoculum is administrated via a simple-to-administer intranasal route. Consequently, it has been used extensively in studies of acute pneumonia examining the biological pathways of various pathogens, as well as the therapeutic potential of antibacterial agents [25, 32–34].

Finally, the fifth model mimics *P. aeruginosa* open wound infection. It is highly clinically important given that *P. aeruginosa* can be found in about half of all human chronic wounds [35]. In these wounds, pathogens persist in adhesive, polymeric matrix biofilm communities, which induce chronic inflammation that delays healing and increases antimicrobial tolerance [36]. In this model, mice receive a full thickness excisional wound on the back, into the center of which bacterial cells are applied. The wound is then covered with Tegaderm film to prevent secondary infections. This model is used to investigate the virulence and QS properties of pathogens as well as the efficacy of combined topical and systemic antibiotic prophylaxis in experimental wound infection studies [17, 37–39].

2 Materials

2.1 Bacterial Inoculum Preparation

1. LB-Lennox liquid medium: 10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl. Sterilize by filtration.
2. LB-Lennox agar plates: LB-Lennox liquid medium plus 15 g/l agar. Sterilize by autoclaving.
3. Incubator set at 37 °C for incubation of agar plates.

4. Shaker incubator set at 37 °C 200 rpm for bacterial culture growth.
5. 1.5 and 15 ml bacterial culture glass tubes.
6. Spectrophotometer to measure OD_{600nm} of bacterial cultures.
7. Centrifuge with a rotor for 1.5 ml tubes.
8. Filter-sterilized 10 mM MgSO₄ solution.

2.2 Animal Infection

2.2.1 Core Material

Required for Every Infection Model

1. 6-week-old CD1 male mice weighing 22–25 g (Charles River Laboratories).
2. Anesthesia solution: 6.25 mg/ml ketamine and 0.625 mg/ml xylazine in sterile saline.
3. 0.5 ml insulin syringes.
4. Standard dissection tools and sterile alcohol pads.
5. 5 ml plastic tubes for tissue collection.
6. Tissue homogenizer (Brinkman Polytron PT3000).
7. 5 ml round bottom polystyrene tubes.
8. LB-Lennox agar plates containing 100 µg/ml rifampicin.

2.2.2 Additional Material

Required for the Burn-Infection Model

1. Electric hair clipper.
2. Hair remover cream.
3. Heating plate, metal bowl, and brass plugs (*see Fig. 1a, c*).
4. Device to lift mouse abdominal skin (*see Fig. 1b, h*).

2.2.3 Additional Material

Required for the Antibiotic Tolerance/Relapsing Model

1. Ciprofloxacin solution, 10 mg/ml.
2. Ciprofloxacin E-test.

2.2.4 Additional Material

Required for the Back Burn Infection Model

1. Electric hair clipper.
2. Hair remover cream.
3. Heating plate and clear water container (*Fig. 2g*).
4. Foam template (*Fig. 2d*).

2.2.5 Additional Material

Required for the Lung Infection Model

1. Metal rack (*Fig. 3b*).

2.2.6 Additional Material

Required for the Open Wound Infection Model

1. Electric hair clipper.
2. Hair remover cream.
3. Betadine Antiseptic Pads (*Fig. 4c*).
4. Acu-Punch 12 mm skin biopsy punch (*Fig. 4a*).
5. Skin covering film (Tegaderm™) (*Fig. 4b*).

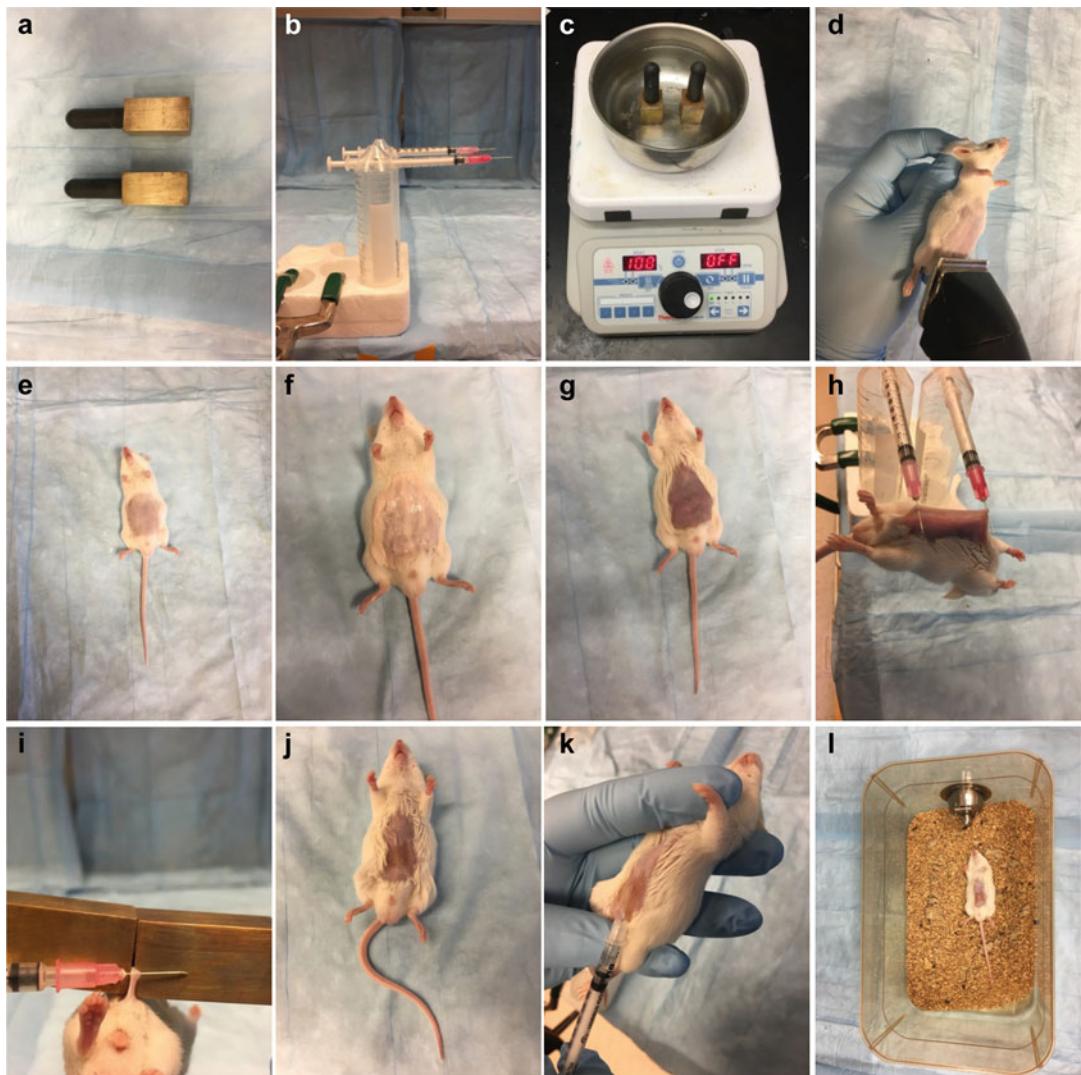


Fig. 1 Burn and infection model. Materials used in this model (a–c). Mice are shaved (d, e), depilated (f, g), given a full thickness skin burn injury (h–j), infected (k), and put back in their cages (l)

3 Methods

3.1 Burn and Infection Model

3.1.1 Bacterial Inoculum Preparation

1. Streak *P. aeruginosa* PA14 cells from a -80°C stock onto LB agar plate and incubate the plates at 37°C overnight.
2. The next day, inoculate one bacterial culture tube containing 5 ml of LB-Lennox liquid medium with one isolated colony and incubate at 37°C , rotating at 200 rpm, until an $\text{OD}_{600\text{nm}} = 1.0$ is reached.

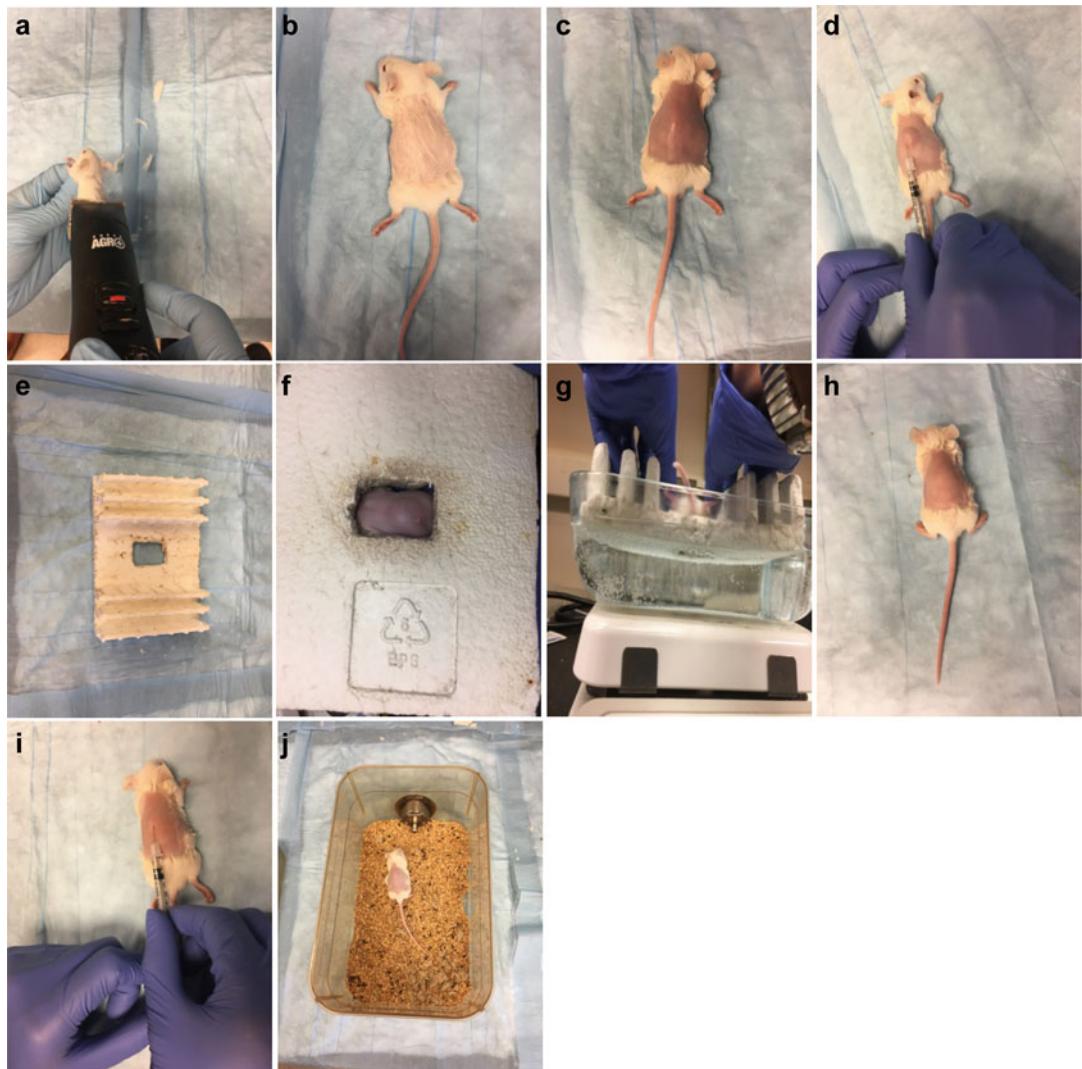


Fig. 2 Back burn and infection model. Mice are shaved (**a**), depilated (**b**, **c**), injected with saline (**d**), burned (**e–h**), infected (**i**), and put back in their cages (**j**)

3. Dilute the bacterial culture 1/1000 in LB-Lennox, then dilute 1/3 serially 20 times in 5 ml LB-Lennox and incubate the tubes at 37 °C, rotating at 200 rpm, overnight.
4. The next morning, select a culture tube containing cells at exactly OD_{600nm} = 3.0 (see Note 1).
5. Spin down 1 ml of bacterial culture solution for 5 min at 12,000 × g, remove the supernatant, and resuspend the pellet in 10 mM MgSO₄.
6. Dilute cells 1/20,000 in 10 mM MgSO₄ to obtain 2.5 × 10⁴ colony forming units (CFU) in 100 µl (see Note 2).
7. Keep the prepared bacterial cells on ice until they are used for animal infection (see Note 3).

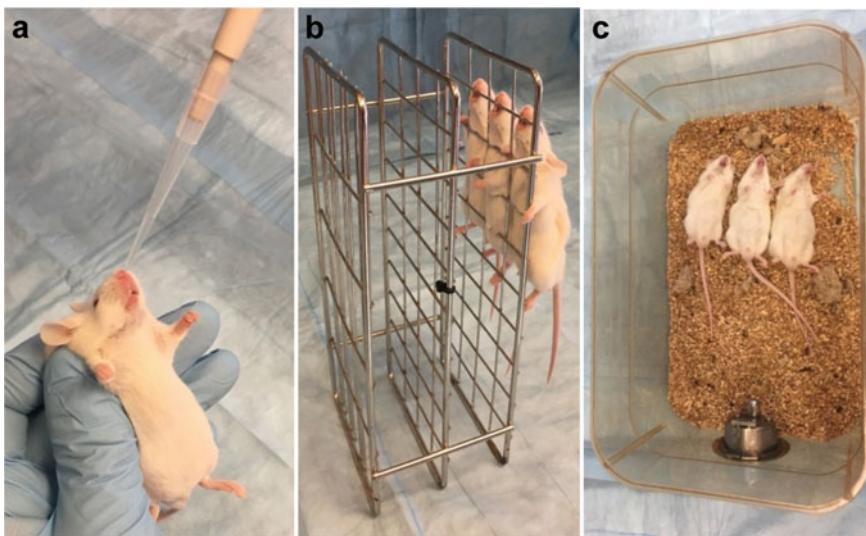


Fig. 3 Lung infection model. After intranasal infection (**a**), place mice upright for a few minutes to facilitate drainage from the nostrils into the lower respiratory system (**b**), and then put them back in their cages (**c**)

3.1.2 Animal Infection

1. Perform intraperitoneal (i.p.) injection of 500 μ l of a 6.25 mg/ml ketamine and 0.625 mg/ml xylazine anesthesia solution per animal (final concentration: 125 mg/kg ketamine 12.5 mg/kg xylazine). Keep anesthetized animals on a heating pad.
2. Shave each animal's abdomen with an electric hair clipper (Fig. 1d, e).
3. Eliminate remaining fur from the abdomen by applying a coat of hair remover cream for 1 min (Fig. 1f); wipe the abdomen clean with soft tissue paper, removing any trace of the cream (Fig. 1g; *see Note 4*).
4. Place brass plugs in water and bring the water to a boil (Fig. 1c).
5. Lift the mouse abdominal skin and hang the animal by the skin by two syringe needles mounted parallel to the table surface (Fig. 1h; *see Note 5*).
6. Apply the brass plugs for 10 s on the mouse's abdominal skin to produce a full thickness burn (5–8% TBSA) (Fig. 1i, j).
7. Following burn injury, deliver a 500 μ l i.p. injection of saline for animal resuscitation.
8. Inject 100 μ l of bacterial suspension intradermally in the burn eschar. The fold of the eschar will provide easy access (Fig. 1k).
9. Place the animals back in their cages lying in a supine position until they recover from the anesthesia (Fig. 1l).
10. For inoculum CFU assessment, serially dilute the bacterial suspension in LB and plate 100 μ l of each dilution on LB

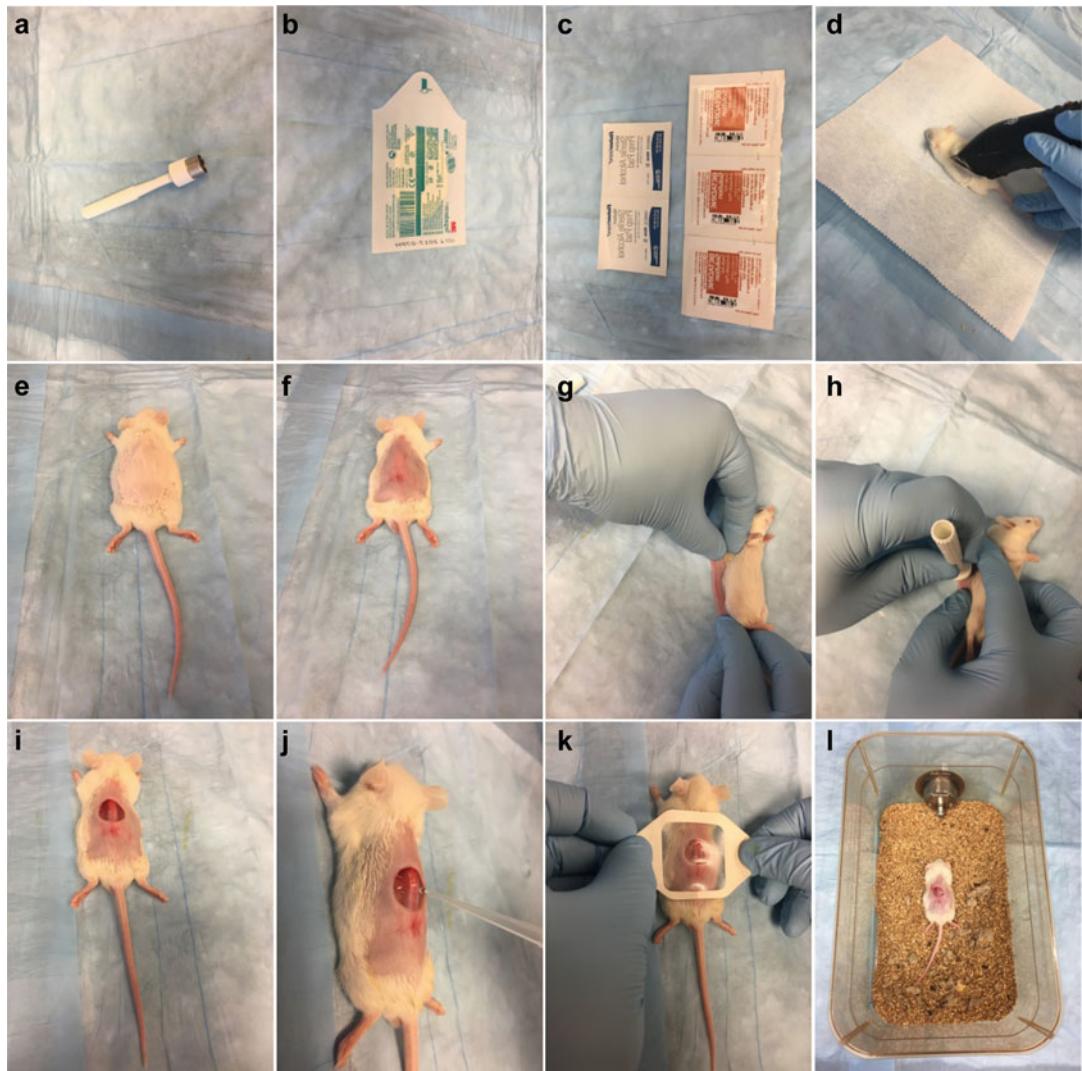


Fig. 4 Open wound and infection model. Materials used in this model (a–c). Mice are shaved (d), depilated (e, f), wounded (g–i), infected (j), covered (k), and put back in their cages (l)

agar plates. Count the CFUs after an overnight incubation at 37 °C to determine the bacterial inoculum concentration.

11. Assess animal survival over time (*see Note 6*).
12. At the experimentally determined assessment time points, sacrifice subgroups of animals. Dissect the rectus abdominis and pectoralis major muscles and place muscle samples into 5 ml plastic tubes for bacterial load quantification (*see Note 7*).
13. Blood samples by submandibular or cardiac puncture can be used to assess bacterial systemic dissemination serially or at the time of euthanasia. Kidney, liver, or spleen samples can be also obtained to assess systemic dissemination.

14. Cut the tissue samples into small pieces with dissection scissors to facilitate homogenization.
15. Weigh the tissue samples and add 1 ml of sterile phosphate buffered saline pH 7.4 to each sample. Keep samples in 5 ml round bottom polystyrene tubes. For blood samples, move directly to **step 16**.
16. Homogenize each sample using a Polytron blender at maximum speed for 10–15 s or until all tissue is homogenized and no tissue pieces are present in the tip of the blade.
17. Serially dilute the sample homogenates in phosphate buffered saline and plate 100 μ l of the dilutions on LB agar plates containing 100 μ g/ml rifampicin. Count the CFUs after an overnight incubation at 37 °C to determine the bacterial concentration in each sample (*see Notes 8 and 9*).

3.1.3 Adaptation of the Burn and Infection Model for Assessment of Antibiotic Tolerance

1. Infect animals with a bacterial inoculum of 8×10^3 CFUs (rather than 2.5×10^4 CFUs) to avoid animal mortality and permit long-term infection assessment following the procedures described in Subheadings 3.1.1 and 3.1.2. A 1/62,500 dilution of bacterial cells is required instead of 1/20,000 as described in Subheading 3.1.1, **step 6**.
2. Separate infected mice into two groups: one in which mice are given 10 mg/kg ciprofloxacin twice a day, and the other to serve as an untreated control group. These groups can be subdivided to assess the efficacy of antibiotic tolerance inhibitors [25].
3. At 6, 24, and 32 h postinfection, inject 10 mg/kg (50 μ l of 5 mg/ml) ciprofloxacin into the tail vein.
4. At 48 h postinfection, sacrifice five animals per group and collect rectus abdominis and pectoralis major muscle samples.
5. Process the samples as described in Subheading 3.1.2, **steps 11–16**.
6. At 48 and 56 h postinfection, inject 10 mg/kg ciprofloxacin as above.
7. Continue ciprofloxacin injections twice a day and muscle sampling once a day to assess CFU as described above until no bacterial cells are detected in the muscle samples (*see Note 10*).
8. Stop antibiotic treatment as soon as PA14 cells are no longer detectable in the muscle samples.
9. 48 h post-ciprofloxacin treatment arrest and every 2 days thereafter, collect muscle samples and quantitate PA14 cells as described above.
10. Check the ciprofloxacin minimal antibiotic concentration (MIC) on bacterial colonies that emerge after ciprofloxacin

treatment arrest using E-test to confirm that cells are antibiotic tolerant (unchanged MIC) rather than antibiotic resistant (increased MIC).

11. A representative example of PA14 cells killing by ciprofloxacin and relapsing infection caused by antibiotic tolerant PA14 cells can be seen in [25].

3.2 Back Burn Infection Model

3.2.1 Bacterial Inoculum Preparation

3.2.2 Animal Infection

1. For the back burn infection model, prepare the bacterial inoculum using the procedure described in Subheading 3.1.1, except with a dose of 2.5×10^5 CFUs per 100 μl . Accordingly, a 1/2500 dilution of the $\text{OD}_{600\text{nm}} = 3$ bacterial cells will be used.
2. Anesthetize the animals as described in Subheading 3.1.2, step 1.
3. Administer the analgesic buprenorphine via i.p. injection (0.05–0.10 mg/kg) while the animals are anesthetized.
4. Shave an area of skin on each mouse's back with mouse fur clippers (Fig. 2a).
5. Depilate with depilatory cream for 1 min (Fig. 2b), then wipe the abdomen clean with soft tissue paper to remove any trace of cream (Fig. 2c).
6. Calculate TBSA using Meehs formula ($A = k \times W(2/3)$, where A = surface area in cm^2 ; k = proportionality constant 12.3; W = weight in gram).
7. Inject 0.5 ml of saline [0.9% (wt/vol) NaCl in distilled water] subcutaneously beside the designated burn site before inflicting the back burn to facilitate resuscitation and protect the spinal cord from burn damage (Fig. 2d).
8. Lay each animal in a supine position on a foam template of an appropriate size, such that the skin on the animal's back and sides that protrudes from the template equals 30% TBSA based on the calculation (Fig. 2e, f).
9. Immerse the back of the animal in hot water (90 °C) for 8 s while being careful not to expose its head or limbs to the hot water; then dry the burned area gently with paper towels (Fig. 2g; see Note 11).
10. Resuscitate each mouse with another 0.5 ml of saline, injected i.p. while the animal is still under anesthesia.
11. Inject 100 μl of bacterial suspension intradermally immediately underneath the burn eschar (Fig. 2i).
12. Place the animals back into their cages, making sure to lay them on their abdomens, while they recover from the anesthesia (Fig. 2j).

12. Assess the bacterial inoculum concentration as described in Subheading 3.1.2, step 8.
13. Record animal survival over time (*see Note 12*).
14. At the experimentally determined time points, sacrifice subgroups of animals and collect muscle from the wound for bacterial load quantification.
15. Process the animal tissue samples as described in Subheading 3.1.2, steps 11–16.

3.3 Lung Infection Model

3.3.1 Bacterial Inoculum Preparation

3.3.2 Animal Infection

1. For the lung infection model, prepare the bacterial inoculum according to the procedure in Subheading 3.1.1, except with an inoculum dose of 5×10^6 CFUs per 20 μl . Accordingly, a 1/20 dilution of the $\text{OD}_{600\text{nm}} = 3$ bacterial cells is required.

1. Anesthetize the animals as described in Subheading 3.1.2, step 1.
2. Administer 20 μl of bacterial suspension slowly into the mouse nose with a micropipette, ~10 μl in each nostril (Fig. 3a; *see Note 13*).
3. Hold the animals upright for 2 min by placing them vertically on a metal rack to allow the inoculum to pass the sinuses and reach the lungs (Fig. 3b).
4. Place the animals back into their cages, lying on their backs, and allow them to recover from the anesthesia (Fig. 3c).
5. Assess the bacterial inoculum concentration as described in Subheading 3.1.2, step 8.
6. Record animal survival over time (*see Note 14*).
7. At experimentally determined time points, sacrifice subgroups of animals. Collect the lungs, and any other tissues of interest, for bacterial load quantification.
8. Process the animal tissue samples as described in Subheading 3.1.2, steps 11–16. Both lungs from each animal should be pooled and homogenized together.

3.4 Open Wound Infection Model

3.4.1 Bacterial Inoculum Preparation

3.4.2 Animal Infection

1. Prepare the bacterial inoculum according to the procedure in Subheading 3.1.1, except with a dose of 2.5×10^6 CFUs per 10 μl . Use a 1/20 dilution of bacterial cells for the open wound infection model.

1. Anesthetize the animals as described in Subheading 3.1.2, step 1.
2. Shave target area of skin on the back with mouse fur clippers (Fig. 4d).

3. Depilate with depilatory cream for 1 min (Fig. 4e), then wipe the abdomen clean with soft tissue paper to remove any trace of cream (Fig. 4f).
4. Administer 0.1 mg/kg buprenorphine by i.p. injection right before the wounding procedure and once daily for up to 3 days.
5. Sterilize the skin with iodine and alcohol swabs.
6. Lay the mouse on its side and lift skin from the back, pushing it down onto the working surface (Fig. 4g).
7. Make an excision on the mouse's back with a skin biopsy punch (12 mm diameter) on top of a sterile sheet (Fig. 4h, i; *see Note 15*).
8. Inoculate 10 μ l of bacterial suspension into the center of the wound (Fig. 4j).
9. Allow the suspension to be absorbed and cover the wound with a 4 cm \times 4 cm square Tegaderm film (Fig. 4k).
10. Place the animals back in their cages, making sure to lay them on their abdomens, and allow them to recover from the anesthesia (Fig. 4l).
11. Assess the bacterial inoculum concentration as described in Subheading 3.1.2, step 8.
12. Record animal survival over time (*see Note 16*).
13. At experimentally determined time points, sacrifice subgroups of animals and collect muscle tissue from the wound site, blood through cardiac puncture, and, if indicated experimentally, other tissues for bacterial load quantification.
14. Process the animal tissue samples as described in Subheading 3.1.2, steps 11–16.

4 Notes

1. The OD_{600nm} of the bacterial culture used to prepare the infection inoculum is critical because it ensures that an appropriate number of bacterial cells are present at a defined cell metabolic stage, which is optimal for the infection process. For these models, it is very important that cultures with an OD_{600nm} = 3.0 are used. Assess the OD_{600nm} by diluting culture (usually 1:10) to accurately measure cell density within the spectrophotometer's reading limit.
2. For the PA14 strain, an OD_{600nm} = 3.0 culture grown in LB-Lennox at 37 °C rotating at 200 rpm contains 5×10^9 CFU/ml. For other strains, the CFU quantity at OD_{600nm} = 3.0 may be slightly different and needs to be taken into consideration in the inoculum preparation.

3. Perform all animal infections within 2 h of cell preparation.
4. Make sure to wipe each mouse's abdomen thoroughly first with a soft dry tissue and then with a wet one to remove any trace of cream, which can interfere with the burn step and inflict a chemical burn.
5. When lifting the skin, be sure to lift only the skin to ensure that the underlying muscle and internal organs are not punctured by the needles or burned in the following step.
6. In the burn infection model, an inoculum of 2.5×10^4 CFU induces ~50% mortality. Most animals succumb to infection between 32 and 56 h. Moribund animals should be euthanized. The moribundity is defined as when the animal shows (1) difficulty with ambulation or reluctant to move when given stimuli, or at least three of the following five symptoms known to be associated with infection morbidities: (2) abnormal posture, (3) ruffled hair coat, (4) head-tucked into abdomen, (5) exudate around eyes or closed eyes, and (6) abnormal breathing.
7. Samples obtained from the rectus abdominis muscle area underlying the burn site allow assessment of bacterial invasion at the most proximal site of infection, whereas samples from the pectoralis major muscle adjacent to the infection site enable assessment of bacterial dissemination from the inoculation site via the bloodstream to other tissues, including kidney, spleen, and liver.
8. LB agar plates containing antibiotic (i.e., rifampicin for PA14) are required to determine the exact number of PA14 cells present in each sample and to prevent the growth of other bacteria. The particular antibiotic used should be selected based on the strain used. *Pseudomonas* isolation agar can be used instead of LB agar plates containing antibiotic.
9. Express bacterial cell concentrations in CFU per gram of tissue to take into account the variation in the amount of tissue in each sample.
10. Usually, it takes 4–5 days to clear antibiotic sensitive bacterial cells from the animals.
11. Apply EMLA cream to the margin of burn wounds in burn-infection model animals at least once after the procedure.
12. In the back burn infection model, an inoculum of 2.5×10^5 CFU induces ~50% mortality. Most animals succumb to the infection within 24–72 h. Moribund animals should be euthanized.
13. Make sure to administer the bacterial suspension very slowly and gingerly to avoid coughing or suffocation of the animal. Note that this is a very delicate step.

14. In this lung acute infection model, an inoculum of 5×10^6 CFU induces ~50% mortality. Most animals start succumbing infection by 24 h postinfection and continue up to 48 h. Moribund animals should be euthanized.
15. When inflicting the wound, take care to avoid injuring the panniculus carnosus. The excision will measure roughly 1 cm². Give each mouse a single wound.
16. In the open wound infection model, an inoculum of 2.5×10^6 CFU induces ~50% mortality. Most animals succumb the infection between 48 and 96 h. Moribund animals should be euthanized.

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Chapter 19

Methods to Study Quorum Sensing-Dependent Virulence and Movement of Phytopathogens In Planta

Shulamit Manulis-Sasson and Laura Chalupowicz

Abstract

Cell-to-cell communication mediated by the diffusible signal factor (DSF) is a common form of gene regulation and plays an important role in virulence of many plant pathogenic bacteria including *Xanthomonas* spp. Here we describe several approaches to study the involvement of DSF-dependent QS system of the plant pathogenic bacteria *Xanthomonas campestris* pv. *pelargonii* (*Xhp*) as an example of the *Xanthomonas* spp. The methods described include detection and measurement of DSF, movement in planta, colonization, and aggregate formation.

Key words DSF, *Xanthomonas campestris*, GFP, Quorum sensing, Plant pathogen, *rpfF*, *rpfC*

1 Introduction

The Quorum Sensing (QS) system plays a significant role in virulence of plant pathogenic bacteria. Two major classes of QS-signals used by Gram-negative bacteria are acylated homoserine lactones (AHLs) and diffusible signal factor (DSF) composed of α , β -unsaturated fatty acids [1–3]. DSFs act as the main QS signal in *Xanthomonas* spp. and in other genera of the Xanthomonadaceae family like *Xylella fastidiosa* and *Stenotrophomonas* spp., and are found together with AHLs in bacteria belonging to other families such as *Burkholderia cenocepacia* or *Pseudomonas aeruginosa* [4]. Several species and pathovars of *Xanthomonas* contain the cluster of pathogenicity-associated genes designated as *rpf* (regulation of pathogenicity factors) [4] (Fig. 1). In *Xanthomonas campestris* pv. *campestris* DSF, which is cis-11-methyl-2-dodecenoic acid (Fig. 2) [5], is synthetized by RpfF [1, 6]. The DSF sensing mechanism involves RpfC, which encodes the histidine sensor kinase of the two-component system RpFC/RpfG.

Genetic and gene expression analyses showed that the DSF QS-signaling pathway regulates diverse biological functions related to virulence, including biosynthesis of polysaccharides (xanthan),

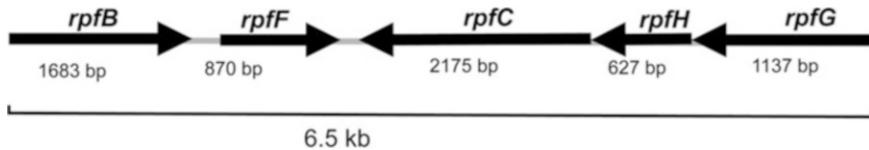


Fig. 1 A 6.5 kb DNA fragment containing the QS-related genes in *Xanthomonas hortorum* pv. *pelargonii* (*Xhp*) strain 305

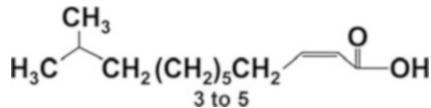


Fig. 2 Chemical structure of the QS Diffusible Signal Factor molecule

extracellular enzymes, dispersal of biofilm and other determinants involved in ecological competence [7, 8].

Here, we describe methods used to study the involvement of DSF-dependent QS system in virulence of plant pathogenic bacteria. These include detection and measurement of DSF, movement in planta, colonization, and aggregate formation. The plant pathogenic bacteria *Xanthomonas hortorum* pv. *pelargonii* (*Xhp*) [9] is used as an example of the *Xanthomonas* spp. or other genera of the Xanthomonadaceae family.

2 Materials

2.1 Bacterial Strains and Growth Media

1. The DSF biosensor strain used in this study is *Xanthomonas campestris* pv. *campestris* 8523 (rifampicin resistant), which is a mutant in *rpfF* [10]. It carries the plasmid pKLN55 (spectinomycin resistance gene) harboring the DSF-inducible *eng:gfP* reporter gene.
2. *Xanthomonas hortorum* pv. *pelargonii* 305 (*Xhp305*) (rifampicin resistant) wild type and isogenic mutants in *rpfF* and *rpfC* genes with kanamycin resistance (see Note 1).
3. Luria-Bertani (LB) broth: 10 g/l NaCl, 10 g/l tryptone, 5 g/l yeast extract.
4. LB agar plates: LB broth plus 15 g/l agar.
5. Antibiotics: rifampicin (100 µg/ml), spectinomycin (100 µg/ml), kanamycin (50 µg/ml), chloramphenicol (10 µg/ml). Antibiotics are added to the autoclaved and cooled growth media.
6. Peptone Glycerol (PG) solution for storage of cultures: glycerol (40 ml), peptone (2 g), and K₂HPO₄ (0.15 g) in distilled water (to final volume of 100 ml). Overnight LB liquid cultures are mixed 1:1 with sterile PG before storing at 80 °C.

2.2 Detection and Measurement of DSF

1. The DSF biosensor strain *Xanthomonas campestris* pv. *campestris* 8523.
2. Strains to be tested for DSF production.
3. Glass tubes containing 3 ml LB broth supplemented with the appropriate antibiotic(s).
4. Erlenmeyer flasks (250 ml) containing 50 ml LB broth.
5. Sterile pipette tips or toothpicks.
6. Temperature-controlled shaker.
7. Spectrophotometer.
8. Autoclaved Whatman paper discs (10 mm diameter).
9. Fluorescent binocular microscope.
10. Plastic loops (2 mm diameter).
11. 1.5 ml tubes.
12. 96-well black microtiter plate.
13. Fluorescence microplate reader.

2.3 Pathogenicity and Colonization Tests

1. Young plants (3-week-old) of *Pelargonium x hortorum* growing in individual pots (see Note 2).
2. Pots filled with a mixture of 2/3 peat and 1/3 Tuff (crushed volcanic rocks).
3. Bacterial suspension of strains to be tested at a concentration of 10^8 cells/ml.
4. Disposable needles, size 21 G.
5. Sterile plastic (low density polyethylene) bags (0.16 cm × 0.1 cm).
6. Hammer and crusher for plant maceration.
7. Pipettes and sterile tips (5–50, 40–200 µl).
8. Sterile scissor.
9. Sterile 1.5 ml tubes.
10. Sterile water.
11. LB agar plates.
12. Incubator.
13. Greenhouse for maintaining the plants with temperature of 25–28 °C and drip irrigation.

2.4 Confocal Microscopy

1. *Xhp* (or other plant pathogenic strains) labeled with GFP (see Note 3).
2. Confocal laser scanning microscope.
3. Young *Pelargonium* plants growing in pots.
4. Syringe-driven membrane filters 0.22 µm.

2.5 Quantitation of Aggregate Formation by Crystal Violet Assay

5. Sterile scissor.
6. Sterile razor.
7. Sterile glass microscope slides.
8. Petri dishes.
9. Paraffin film.
10. Fresh plant extract (*see* Subheading 3.6).

1. Cultures of the wild type *Xhp* and its QS mutants.
2. LB broth (*see* Subheading 2.1, item 3).
3. Minimal M9 medium [11]: 6 g/l Na₂HPO₄, 3 g/l KH₂PO₄, 1 g/l NH₄Cl, 0.5 g/l NaCl, 1 mM MgSO₄, 0.01 mM CaCl₂, 200 mg/l methionine, 200 mg/l thiamine, 20 mg/l nicotinic acid, 2 g/l glucose, 8 g/l agar.
4. Fresh plant extract (*see* Subheading 3.6).
5. Spectrophotometer.
6. Incubator.
7. 24-well multidishes.
8. Crystal violet (0.1%) solution in water.
9. Ethanol 95%.

3 Methods

3.1 Bioassay for Detection of DSF Production

1. Scrub frozen bacterial culture from the glycerol stocks of *Xanthomonas campestris* pv. *campestris* 8523 (biosensor strain) and the strains to be tested for DSF production (in this case *Xhp305* and its QS mutants) with a sterile pipette tip or toothpick and inoculate glass tubes containing 3 ml LB broth supplemented with the appropriated antibiotics. Grow the bacterial cultures overnight at 28 °C with shaking at 150 rpm.
2. On the following day, transfer 100 µl of each of the overnight cultures into 250 ml Erlenmeyer flasks containing 50 ml LB broth. Incubate at 28 °C with shaking at 150–180 rpm (*see Note 4*). Grow the cultures for approximately 5 h and then monitor the optical densities (OD) using spectrophotometer.
3. When the OD, measured at 595 nm, reaches 0.25–0.3 (early exponential phase of the growth), transfer 100 µl of the biosensor culture and spread uniformly over surface of LB agar plates. Allow the plates to dry for 15 min by placing them uncovered in a biological hood.
4. After drying the plates, carefully lay the Whatman paper discs on the agar surface (Fig. 3a) (*see Note 5*).

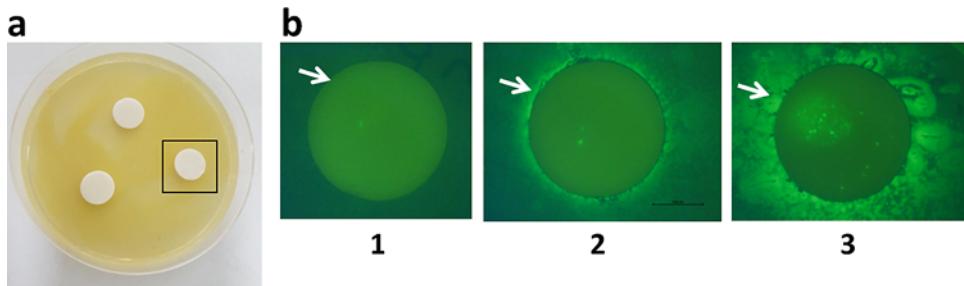


Fig. 3 Bioassay for DSF production. (a) LB agar plate spread with the reporter strain *Xcc* 8523 containing paper discs with the tested strains. The *square in black* denotes the area to examine fluorescence with fluorescent binocular microscope. (b) Fluorescent binocular images: (1) control water disc, no fluorescence; (2) and (3) discs with low and high DSF-producing *Xanthomonas* strains, respectively. Arrows point to the detection area surrounding the discs

5. Transfer 50 μ l of the *Xhp* culture ($OD_{595} = 0.25\text{--}0.3$) and slowly pipette the suspension on each disc allowing the suspension to be completely absorbed. For negative control, add 50 μ l of sterile water to the discs.
6. Incubate the plates at 28 °C for 48 h.
7. Examine the fluorescence emission in the area surrounding the discs using a fluorescent binocular microscope. Detection of green fluorescence indicates production of DSF that diffuses from the tested strain to the reporter (Fig. 3b).

3.2 Measurement of DSF Production

1. Collect with a plastic loop the cells of the reporter culture surrounding the paper disc which contains the tested strain or water.
2. Suspend each harvested culture in 1.5 ml tubes containing 500 μ l of sterile water.
3. Transfer 100 μ l from each suspension and adjust OD_{595} to 0.5 by diluting with sterile water.
4. Transfer each standardized culture to wells of a black microtiter plate (see Note 6). Load each sample by triplicate into wells.
5. Measure fluorescence with a fluorescence microplate reader using an excitation of 485 nm and emission of 528 nm.
6. Fluorescence is recorded as Fluorescence Units (FU) of each well normalized by subtracting the background fluorescence value of the control wells containing only the reporter. Calculate the average reading of 3 wells.

3.3 Pathogenicity Test

1. Puncture the plant stem three times with a needle, at approximately 2 cm above the soil. The punctures should be close.
2. Place at the wounds 10 μ l of the tested bacterial suspension (10^8 cells/ml) or sterilized distilled water as control. For each

treatment use 20 plants and the experiments should be repeated three times.

3. Transfer the plants to the greenhouse and inspect the plants weekly (up to 3 weeks) for appearance of symptoms.
4. Determine the degree of disease severity and disease incidence. In the case of *Xhp* a four-grade scale for disease severity was defined (*see Note 7*).
5. For disease incidence determine the percentage of symptomatic plants in each treatment at the end of the experiments (in the case of *Xhp* 21 days post inoculation).

3.4 Plant Colonization

1. After 15–21 days post inoculation, cut pieces of 1 cm with a sterile scissor along the plant stem: at the inoculation site, 1.5 cm above the inoculation site, and at the apex (*see Note 8*).
2. Weight each stem sections and transfer them to sterile plastic bags.
3. Macerate manually the plant samples with a hammer and a crusher until the plant tissue is completely disintegrated.
4. Add 1 ml of sterile water to the sample bag and shake it gently at an orbital shaker for 5 min.
5. Remove with a pipette, 0.9 ml of suspension and place in a sterile 1.5 ml tube.
6. Prepare tenfold serial dilutions of these suspensions with sterile water.
7. Spread 100 µl of each dilution on three LB agar plates amended with the appropriate antibiotics.
8. Incubate the plates at 28 °C for 48 h and calculate the number of CFU (colony forming units) per gram of plant fresh weight.

3.5 Movement of GFP-Labeled Pathogen In Planta

To study the effect of QS on virulence it is important to determine the movement of the pathogen in its host as compared to its QS mutants in different tissues and time after inoculation.

1. Inoculate plants with the wild type and its QS-mutant strains labeled with GFP as described in Subheading 3.3.
2. At the defined time point after inoculation, excise with a sterile scissor symptomatic tissues (root, stem, leaves, fruit, and flower).
3. Prepare plant samples by cutting thin transversal and longitudinal sections with a razor.
4. Place the samples on a glass microscope, add 1–2 drops of water, and cover with a cover glass.
5. Proceed to confocal microscopy examination by placing the sample with the cover glass facing down.
6. Examine several tissue samples (at least 4) taken from each plant.

3.6 Determination of Aggregate Formation by Confocal Microscopy

Another approach to study the effect of QS on virulence is to determine its effect on aggregate formation. In *Xanthomonas* spp. the reversible transition from biofilm to planktonic forms is mediated by DSF. It affects the attachment to the plant cells, migration, and colonization within the host.

1. Grow overnight cultures of GFP-labeled strains (wild type and its QS mutants) in LB broth until stationary phase (in case of *Xhp* after 48 h).
2. Centrifuge the bacterial cultures for 1 min.
3. Discard the supernatant and wash twice the remained pellets with sterile water.
4. Prepare plant extracts as follows: cut 2 g plant stem sections (2-week-old pelargonium plants), place in plastic bags, and crush until plant tissue is completely disintegrated. Add 5 ml of distilled water and shake with a rotary shaker at 80 rpm for 5 min. Filter the plant extract through a syringe-driven membrane filter.
5. Resuspend the pellet obtained in step 3 by adding 3 ml of filtered plant extract. Vortex for approximately 1 min until the pellet is completely dissolved.
6. Load 200 μ l of the bacterial suspension on the surface of a sterile glass microscope slide and place it inside a Petri dish containing a wet filter paper.
7. Seal the Petri dish with paraffin film to avoid water evaporation and incubate at 28 °C.
8. After 3–7 days examine biofilm formation with a confocal laser scanning microscope.

3.7 Measurement of Aggregate Formation by Crystal Violet Assay

Crystal violet assay has extensively been used to examine aggregate formation developed by phytopathogenic bacteria [12–14]. Here we present an example for quantitation of aggregate formation when grown in plant extracts or minimal medium, which mimics the apoplast (plant intercellular space) environment, in comparison to rich media.

1. Inoculate *Xhp* and its QS mutants from frozen stocks in 3 ml of LB broth and incubate at 28 °C for 48 h.
2. Dilute culture 1:1000 in fresh LB medium and grow until reaching an $OD_{595} = 0.5$.
3. Centrifuge the cells, discard the supernatant, wash once the remained pellet with sterile distilled water, and then resuspend in 3 ml with sterile water.
4. Pipette in individual wells of multidish, 150 μ l of LB, minimal M9 media or fresh pelargonium extracts.

5. Pipette 50 µl of the resuspended culture strains into wells containing the different media. Prepare three to five replicates for each strain and media.
6. Cover the plate with the lid and seal with paraffin to avoid excessive evaporation.
7. Incubate the plates in incubator at optimal growth temperature (*see Note 9*).
8. Remove carefully the supernatants with a pipette. Avoid disruption of attached cells at the base of the wells.
9. Gently wash twice the wells with 200 µl of sterile water.
10. Allow fixation of the surface-attached bacteria by placing the plates at 60 °C for 20 min.
11. Add 120 µl of 0.1% crystal violet solution in water to each well of the multidish.
12. Incubate the plates at room temperature for 1 h.
13. Gently rinse the wells three times with 200 µl distilled water.
14. Let the plates to air-dry for at least 1 h.
15. Solubilize the crystal violet in each well by adding 1 ml of 95% ethanol.
16. Briefly pipet the content of each well and transfer to 1.5 ml tubes.
17. Dilute 1:10 with sterile water.
18. Transfer the solutions to cuvette and measure the absorbance with spectrophotometer at 595 nm.

4 Notes

1. To investigate the role of QS system in virulence and movement of plant pathogenic bacteria knockout mutants in *rpfF* and *rpfC* should be generated. Any knockout or insertional mutagenesis protocol can be used [15].
2. Pathogenicity and colonization tests depend on the pathogen and its host. For example, in the case of vascular pathogens, as presented here with *Xhp*, the inoculation will be performed by puncturing the stem. In foliar diseases, bacterial inoculations will be performed by spraying the leaves with the pathogen [16]. It is recommended to do the tests with young plants which are more sensitive to the pathogens.
3. To study movement in planta, label the strains with a plasmid carrying the *gfp* gene. In the case of *Xhp*, the vector pKT-Cm [9] carrying the constitutively expressed *gfp* gene is used. Any other plasmids driving *gfp* expression can be used. In that case,

we recommend performing preliminary tests to examine the stability of the *gfp*-plasmid and fluorescence emission of the tested bacterial strains in vitro and in planta.

4. It is important to provide good aeration conditions to assure fast growing of the bacterial cultures.
5. We recommend using no more than three discs per plate to avoid diffusion of the DSF among discs.
6. Black microtiter plates reduce background and cross-reactivity between samples.
7. Here we present an example to determine disease severity in pelargonium plants based on a four-grade scale: (1) no symptoms, plants look healthy as mock-inoculated plants; (2) low severity, small necrotic areas are observed on one or two leaves close to the inoculation site; (3) moderate severity, more than three leaves develop necrotic areas; and (4) high severity, wilting, and necrosis symptoms are observed on most leaves or the plant died.
8. Depend on the pathogen-host system colonization can be determined at different time intervals after inoculation and samples can be taken from different plant tissues.
9. Aggregate formation is variable depending on the bacteria and growth conditions, therefore it is recommended to perform this assay at different time of incubation.

Acknowledgement

We are grateful to Steven Lindow for providing strain *Xanthomonas campestris* pv. *campestris* 8523 (pKLN55). Contribution from the Agricultural Research Organization, The Volcani Center, Bet Dagan, Israel.

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Chapter 20

Differential Equations Models to Study Quorum Sensing

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Abstract

Mathematical models to study quorum sensing (QS) have become an important tool to explore all aspects of this type of bacterial communication. A wide spectrum of mathematical tools and methods such as dynamical systems, stochastics, and spatial models can be employed. In this chapter, we focus on giving an overview of models consisting of differential equations (DE), which can be used to describe changing quantities, for example, the dynamics of one or more signaling molecule in time and space, often in conjunction with bacterial growth dynamics. The chapter is divided into two sections: ordinary differential equations (ODE) and partial differential equations (PDE) models of QS. Rates of change are represented mathematically by derivatives, i.e., in terms of DE. ODE models allow describing changes in one independent variable, for example, time. PDE models can be used to follow changes in more than one independent variable, for example, time and space. Both types of models often consist of systems (i.e., more than one equation) of equations, such as equations for bacterial growth and autoinducer concentration dynamics. Almost from the onset, mathematical modeling of QS using differential equations has been an interdisciplinary endeavor and many of the works we revised here will be placed into their biological context.

Key words Quorum sensing, Differential equations, Derivatives, Ordinary differential equations, Partial differential equations, Mathematical models

1 Introduction

In this section, we will introduce some basic terminology and concepts concerning mathematical modeling and differential equations. We will try, as much as possible, to place these concepts directly into the context of QS applications.

1.1 Mathematical Models

A mathematical model is a representation of a system using mathematical language. A model can be used to describe interactions between components of the system, for example, biological interactions. Mathematical models are often simplified representations of a real system, which allow us to understand its essential features.

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Models can be used to test a hypothesis of how a system works, to try to estimate how a certain event could affect the system (for example, the introduction of a therapy) or in general to deduce the consequences of the interactions depicted by the model. Studying and analyzing such models is usually more time and resource effective than constructing real-life systems for the same purpose.

Typically, a model is composed of independent variables, e.g., time t , and dependent (on the independent variable(s)) variables, for example, autoinducer concentration depending on time $A(t)$. An independent variable causes a change in a dependent variable. A dependent variable can depend on one or more independent variables, for example, time and space. Models often also contain parameters, which are fixed values (e.g., gravitational constant) or can be varied under experimental conditions (e.g., growth rate of the bacteria strain under consideration or diffusion rate of a molecule). Different parameter values can lead to qualitative changes in the system behavior.

The mathematical modeling process often starts with a real-life problem and consists of transforming it to a mathematical problem, which can be solved using mathematical methods. The solution should then be interpreted in terms of the original problem so that it can provide answers and allow to make predictions (Fig. 1).

Note that for reasons of simplicity we will refer to all types of QS signal molecules with the generic name autoinducers (AIs). Some of the mathematical models presented have been developed for a specific type such as homoserine lactones, but we will not make a difference in these cases.

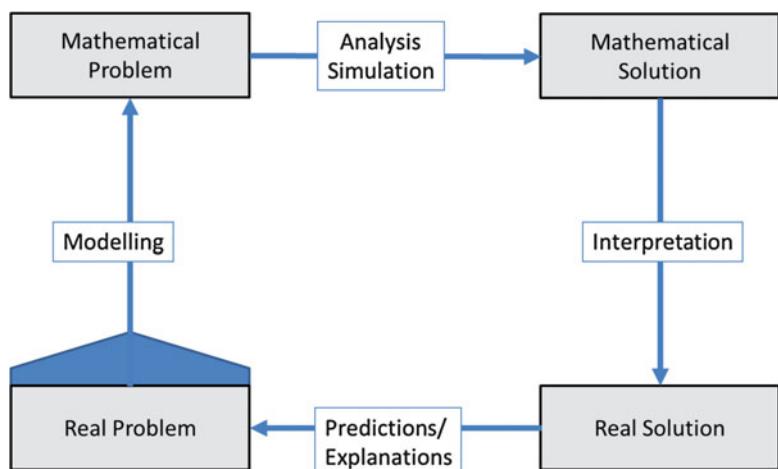


Fig. 1 The process of mathematical modeling; the starting point is a real-life problem

1.2 Differential Equations

Differential equations (DE) are mathematical tools to study changes. While an equation in general involves an unknown, usually a number, a DE contains an unknown which is a function. We use DE as many important principles in science are rules for the way variables change, i.e., we usually have some information, given by the laws of science, about the way things change. In the real world, one usually does not have a formula. The formula, in fact, is what one would like to have: the formula is the unknown.

If the unknown function in a DE depends on one independent variable, say time $x(t)$, the differential equation is called ordinary (ODE), otherwise is called partial (PDE). More formally, a differential equation involves an expression in terms of the function and some of its derivatives. Differential equations are continuous mathematical models; i.e., the independent variables are continuous.

Note that whereas the differential equation describes the rate of change of a variable, the solution of a differential equation describes the amount or size of a variable as a function of its independent variable (e.g., time). Example: If Eq. 1 in Table 1 is for the rate at which the numbers of individuals in a population changes, its solution $y(t)$ is the number of individuals in a population at time t .

In the following, we will discuss some existing mathematical model of QS, which use DE. We have organized this chapter into two sections: ODE and PDE models of QS. Our aim is to give an overview of the modeling process of QS using DE, so we only present selected models; however, we have elaborated a table (ordered chronologically, by publication year) to give the reader a broad overview of existing mathematical models of QS, when possible placing them in our classification (Table 2).

Table 1
An ordinary differential equation

Rates of change are represented mathematically by derivatives, i.e., in terms of differential equations:

$$\frac{dy}{dt} = f(y, t) \quad (1)$$

the Δ in dy and dt stands for delta or a change in that variable. Indeed, the amount of change of y , dy divided over a time interval dt in which it occurs represents the rate of change of y . In the context of QS, the rate at which the numbers of cells in a bacterial population or AIs concentration changes with time are examples of phenomena involving rates of change.

Table 2
Brief classification of some existing mathematical models of QS

Year	Author	Bacteria	Method
2000	James et al.	<i>Vibrio fischeri</i>	ODE
2001	Nilsson et al.	Generic	ODE
2001	Dockery and Keener et al.	<i>Pseudomonas aeruginosa</i>	ODE
2001	Ward et al.	<i>V. fischeri</i>	ODE
2002	Koerber et al.	<i>Pseudomonas aeruginosa</i>	PDE
2002	Ward et al.	Generic	PDE
2002	Chopp et al.	<i>Pseudomonas aeruginosa</i>	PDE
2003	Chopp et al.	<i>Pseudomonas aeruginosa</i>	PDE
2003	Fagerlind et al.	<i>Pseudomonas aeruginosa</i>	ODE
2004	Ward et al.	<i>Pseudomonas aeruginosa</i> and <i>Agrobacterium tumefaciens</i>	ODE
2004	Kuznetsov et al.	Generic	ODE
2004	Viretta et al.	<i>Pseudomonas aeruginosa</i>	ODE
2004	You et al.	<i>E. coli</i>	ODE
2004	Gustafsson et al.	<i>Staphylococcus aureus</i>	ODE
2004	Garcia et al.	<i>E. coli</i>	ODE
2004	Anguige et al.	<i>Pseudomonas aeruginosa</i>	ODE
2006	Goraychev et al.	Generic	ODE
2005	Fagerlind et al.	<i>Pseudomonas aeruginosa</i>	ODE
2005	Anguige et al.	<i>Pseudomonas aeruginosa</i>	PDE
2006	Muller et al.	Generic	PDE
2006	Anguige et al.	<i>Pseudomonas aeruginosa</i>	PDE
2007	Karlsson et al.	<i>Streptococcus pneumoniae</i>	ODE
2007	Hense et al.	Generic	ODE
2008	Williams et al.	Generic	ODE
2008	Kuttler	<i>Vibrio fischeri</i>	ODE
2008	Müller et al.	Generic	ODE

(continued)

Table 2
(continued)

Year	Author	Bacteria	Method
2009	Janakiraman et al.	<i>Pseudomonas aeruginosa</i>	PDE
2010	Fekete et al.	<i>Pseudomonas putida</i>	ODE
2010	Frederick et al.	Generic	PDE
2010	Jabbari et al.	<i>Staphylococcus aureus</i>	ODE
2010	Vaughan et al.	<i>Pseudomonas aeruginosa</i>	PDE
2010	Barbarossa et al.	<i>Pseudomonas putida</i>	ODE
2011	Frederick et al.	Generic	PDE
2012	Müller et al.	Generic	ODE
2012	Ward et al.	Generic	PDE
2012	Liu et al.	<i>Vibrio harveyi</i>	ODE
2012	Meyer et al.	<i>Pseudomonas putida</i>	ODE
2012	Hense et al.	Generic	PDE
2013	Hunter et al.	<i>Vibrio harveyi</i> and <i>Vibrio cholerae</i>	ODE
2013	Fujimoto and Sawai	Generic	ODE
2013	Anand et al.	<i>Vibrio fischeri</i>	ODE
2013	Brown	Generic	ODE
2014	Gölgeli et al.	Generic	PDE
2014	Langebrake et al.	<i>Aliivibrio fischeri</i>	PDE
2015	Emerenini et al.	Generic	PDE
2016	Mund et al.	Generic	ODE
2016	Barbarossa et al.	<i>Pseudomonas putida</i>	ODE
2016	Kumberger et al.	Generic	ODE

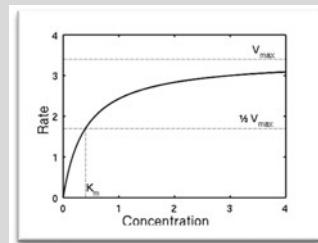
As many of the models described here use the Michaelis-Menten model, which accounts for the kinetic properties of many enzymes, we give a brief explanation of this principle in Box 1.

Box 1 Michaelis-Menten Model:

Biochemical reactions in living cells are often catalyzed by enzymes. These enzymes are proteins that bind and subsequently react specifically with other molecules (substrates). In enzyme kinetics, the phenomenon of saturation plays an important role: even for very high substrate concentrations, one does not consider metabolic rates per se but a maximum rate V_{\max} . Let us consider an enzymatic reaction: The enzyme (E) forms a complex with the substrate. This complex can again de-couple or the substrate is converted into a product P and the enzyme can cleave again, we can then write an equation describing the rate of the enzymatic reaction, by relating the reaction rate v to $[S]$, the concentration of the substrate S:

$$v = \frac{d[P]}{dt} = \frac{V_{\max}[S]}{K_M + [S]}$$

where P is the product formed, V_{\max} represents the maximum rate achieved by the system, at saturating substrate concentration, K_M is the substrate concentration at which the reaction rate is half of V_{\max} .



This means: complex formation of the enzyme and substrate quickly goes into its equilibrium (in this case substrate hardly changes and therefore neither the product). Subsequently, the slow dynamics determines the behavior: substrate is transformed until there is no left.

2 Ordinary Differential Equations (ODE) Models

The first ODEs models of QS were the almost parallel works of four groups: James and coworkers [1] who developed a model for the QS system of *Vibrio fischeri*, Nilsson and coworkers [2] who did not concentrate in a particular QS system, Dockery and Keener [3] who examined the QS of *Pseudomonas aeruginosa*, and Ward and

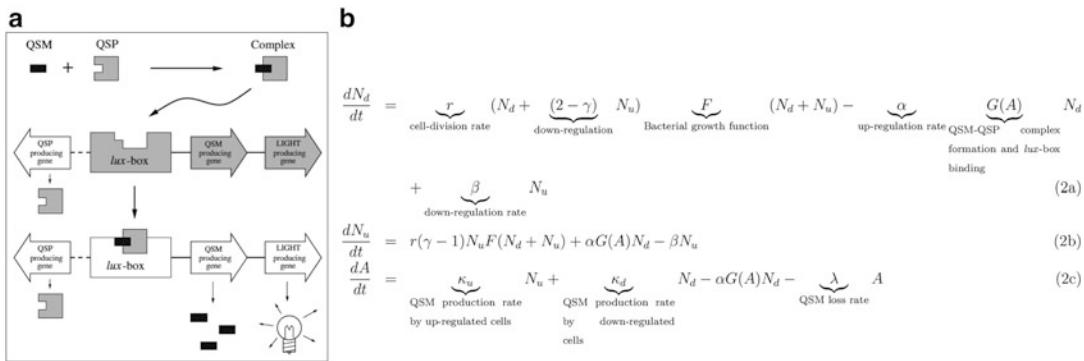


Fig. 2 (a) Schematic diagram of *V. fischeri* QS system. (b) Model equations from [4]. Reproduced with permission

colleagues [4] who also focused in *V. fischeri*. In this section, we will present a deeper review of three ODE models of QS which portray many important features that have become customary to model QS, with many existing models based on the ideas contained in these models. They basically represent two ways to mathematically model QS: (1) the population is divided into QS active and QS inactive population, respectively, and each is represented by an equation; (2) explicit equations to describe bacterial dynamics plus AIs dynamics. To exemplify the first category, we revised the model for *V. fischeri* QS system [4]. To the second category belong two models from our group developed to describe the QS of *V. fischeri* [5] and *P. putida* [6], respectively, which we will also discuss in this section. Models described in [1], [2], and [3] all belong to the second category, which in fact became the most common approach to model QS. For a review, see [7].

Ward and colleagues [4] developed a model of the QS system of *V. fischeri*, see Fig. 2, which consists of the schematic diagram they considered (Fig. 2a) and the mathematical model they created (Fig. 2b). Their model examines bacterial population growth and AIs (which they denoted by QSMs) dynamics, in view of down-regulated (N_d) and up-regulated (N_u) cells, corresponding to cells with an empty *lux-box* or a complex-bound (formed by AIs and protein, which in diagram they term QSM and QSP, respectively), respectively. The binding of QSM-QSP complex to the *lux-box* induces QS activation from a down-regulated state (shown in grey in Fig. 2a) to an up-regulated state (shown in white in Fig. 2b). The switch is assumed to be regulated with increasing AIs (external) concentration A . In the up-regulated (induced) state, genes involved in bioluminescence, but also in production of AIs are expressed on higher level.

To write a mathematical model from the interactions depicted in the diagram developed in [4], three dynamic (i.e., whose value changes with time) quantities were defined: down-regulated (N_d),

up-regulated (N_u) cells and AIs concentration (A). We describe here how to obtain the first equation (Eq. 2a) which appears in Fig. 2b: the down-regulated population divides with a cell-division rate r , the specific growth law they follow is given by function F (which may depend on the data available). Cell division of up-regulated cells produces on average γ up-regulated and 2γ down-regulated cells, assuming that only a proportion of replicated chromosomes contain occupied *lux*-boxes. The portion of the population which goes onto becoming active is then accounted for by the second term (after the minus), it is assumed that up-regulation happens at a rate α . Up-regulated population can down-regulate at a rate β . They included a linear function G to describe the complex formation and *lux*-box binding process.

Experiments were specifically designed to estimate the model parameters, see Fig. 3a. The rapid switch observed in experiments from a population of down-regulated cells to an up-regulated state is captured by their model, see the rapid increase in the numerical simulation, Fig. 3b. They, however, questioned whether there is in fact a critical concentration of QSM prompting this switch, their model solutions showed that the behavior is observed without imposing a switch explicitly.

The basic model described in [4] was extended several times, for example, in [8].

Kuttler and Hense developed an ODE model for the two main QS systems (*lux* and *ain*) of *Vibrio fischeri* [5] (see in Fig. 4a the diagram considered). They followed the modeling approach described by Müller and colleagues [9]—see next section—for the *lux* system. One of their aims was to check the plausibility of the modeled pathway. They did this by comparing the qualitative behavior of the model with some experimental results for the strains ES114 [10] and MJ1 [11], including different mutants of both strains. The main AIs being considered here are 3OC6HSL, but since the model is for two QS systems, the C8HSL-producing enzyme AinS, also forms part of the model. The dynamical behavior of the model fitted qualitatively well to the experimental findings which showed that the behavior of several strains can be described by the same model system, just by modifying parameters concerning the binding preferences of the AHL-LuxR polymers to the *lux* box, respectively, the activation of *luxI* transcription.

For an illustration, we present three of their equations (the full system contains 19 in total), in Fig. 4b. We explain how to derive Eq. 3a: the AIs in the cytoplasm is produced (by the AIs-producing enzyme (I)) at a rate α_1 , it is also degraded at a rate γ_c and it can be lost (from the cytoplasm) due to diffusion out of the cell, at a rate \tilde{d}_1 and it can increase due to diffusion into the cell (\tilde{d}_2). The equation also considers complex disassociations. Note that there is a natural limitation of AinS production, so they used a Michaelis-

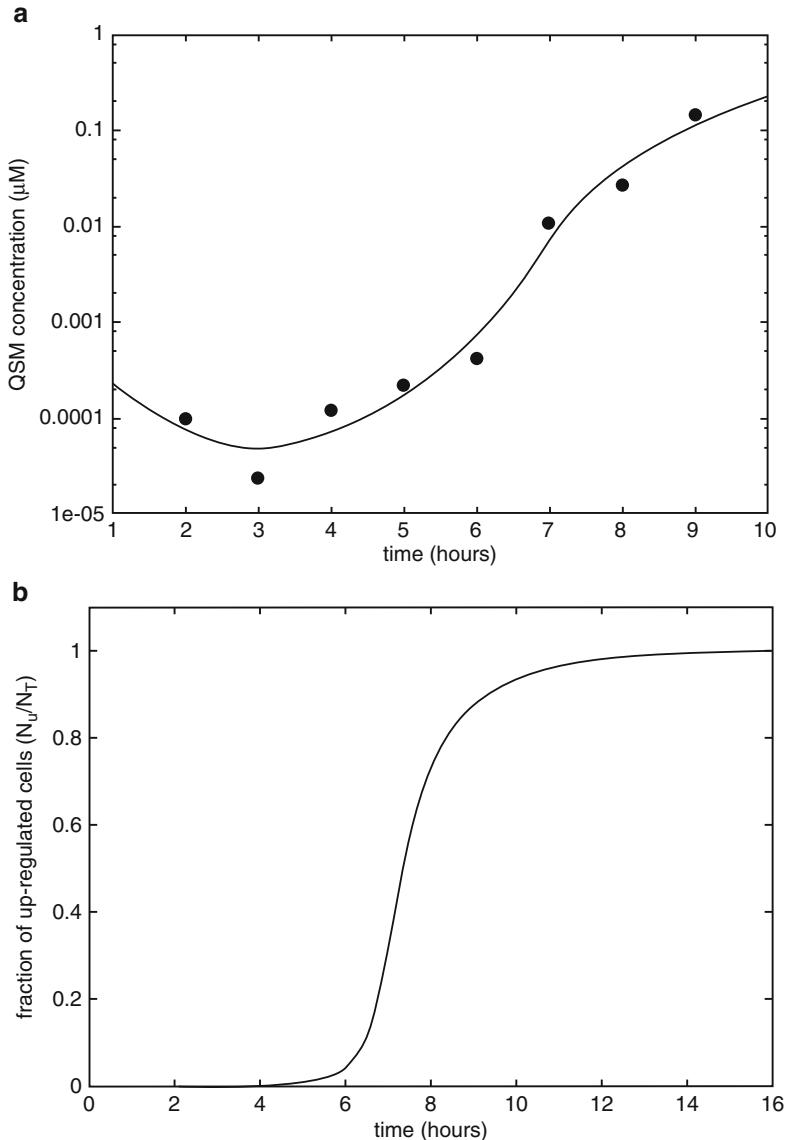
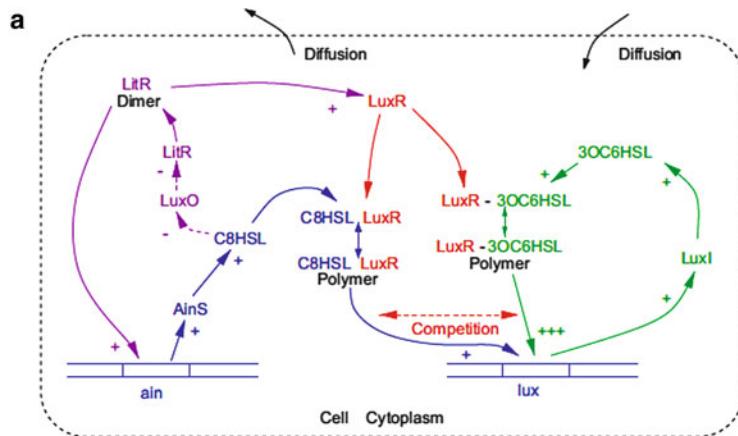


Fig. 3 (a) Numerical results from [4], fitting model to the experimental data. (b) Predicted evolution of the up-regulated cell fraction (up-regulated cells divided by total number of cells N_u/N_T). Reproduced with permission

Menten approach (see Eq. 3c, for s and Box 1). The equations for the intracellular and the extracellular C8HSL are formed similar to those for 3OC6HSL. They found that the system may possess up to 21 different stationary levels; however, the actual number of stationary levels depends on the given external AIs concentrations; in any case many of these stationary states will be unstable and therefore not experimentally observable.

The last model we will present in this section is a model for the one QS system of *Pseudomonas putida* IsoF. The QS signal

**b**

$$\frac{dx_c}{dt} = \underbrace{\alpha_1}_{\text{Production rate of } 3\text{OC6HSL by LuxI}} l - \underbrace{\gamma_c}_{\text{Degradation rate AHL in the cytoplasm}} x_c - \underbrace{\tilde{d}_1}_{\text{Diffusion rate of } 3\text{OC6HSL out of the cell}} x_c + \underbrace{\tilde{d}_2}_{\text{Diffusion rate of } 3\text{OC6HSL into the cell}} x_e - \underbrace{\pi_1^-}_{\text{Rate of } 3\text{OC6HSL-LuxR complex dissociation}} rx_c + \pi_1^- y_1 \quad (3a)$$

$$\frac{dx_e}{dt} = \tilde{d}_1 x_c - \tilde{d}_2 x_e - \underbrace{\gamma_e}_{\text{Degradation rate of AHL outside of the cell}} x_e \quad (3b)$$

$$\frac{ds}{dt} = \underbrace{\alpha_6}_{\text{Background production rate of AinS}} - \underbrace{\gamma_5}_{\text{Degradation rate of AinS}} s + \underbrace{\beta}_{\text{Slope of increase of AinS-production (low LitR concentration)}} \frac{\tau_2}{1 + (\beta_3/\underbrace{\kappa_3}_{\text{Asymptotics of increase of AinS-production (high LitR dimer concentration)}}) \tau_2} \quad (3c)$$

Fig. 4 (a) Schematic diagram of the two QS systems, ain and lux [5]. The dotted line signifies a bacterium cell, i.e., the processes shown inside this line take place intracellularly. Reproduced with permission. **(b)** Equations for the concentration of extracellular AIs (in this case 3OC6HSL) (x_e), within the cytoplasm (x_c) and the C8HSL-producing enzyme, AinS (s). LuxI, AinS: synthases of the AIs 3OC6HSL resp C8HSL; LuxO, LitR: parts of the regulation pathway of C8HSL. LuxR: 3OC6HSL receptor. Note that LuxR also binds (competitively) to C8HSL. +: promotion, +++: strong promotion, -: inhibition. LitR activates gene expression as a dimer, LuxR-C8HSL resp. LuxR-3OC6HSL activate gene expression as polymers. Both AIs freely diffuse inside and outside the cells, i.e., are assumed to be homogeneously mixed intra- and extracellularly

molecule in this case is an *N*-acyl-homoserine lactone (AHL). The work of Fekete and colleagues [6] is mentioned here as it produced, to our knowledge, first quantitative information regarding the QS processes by fitting experimental data, for example, the rate of production of the signaling molecules or the AIs threshold concentration to achieve activation. This type of quantitative information is often used in mathematical models of QS but it is seldom computed from real data. Secondly, because of this quantitative information, the key role of an AHL-regulated enzyme which degrades AHL in *P. putida* IsoF was identified.

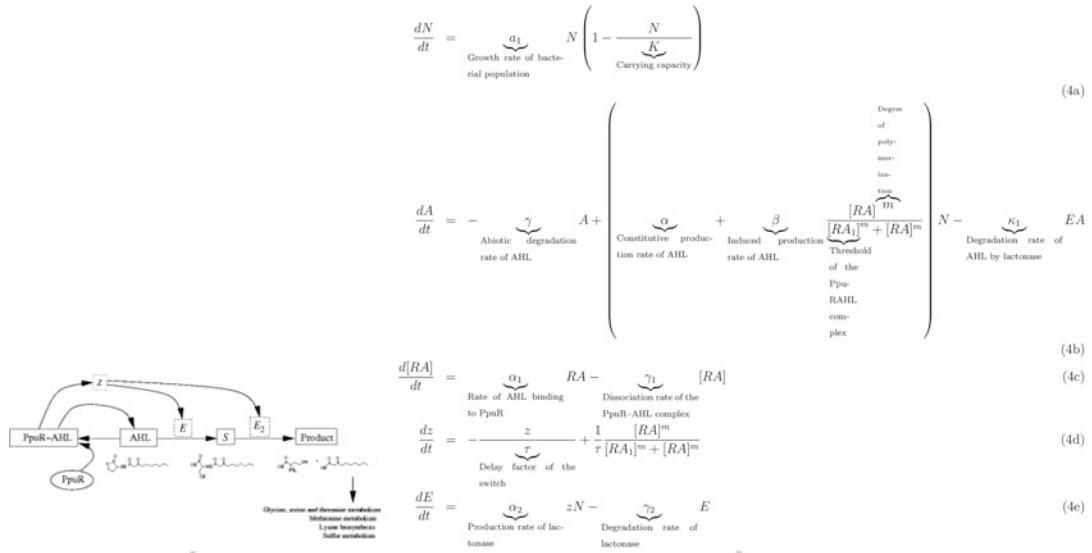


Fig. 5 (a) Schematic diagram of the QS regulation pathway in *Pseudomonas putida*. Reproduced with permission. **(b)** Mathematical model considered by [6]

See in Fig. 5a the schematic considered, the main variables are in square boxes (AIs, AIS complex, homoserines), hypothetical components are shown in dashed boxes (E , putative lactonase; E_2 , putative homoserine-degrading enzyme), and the switch variable z in a dotted box. The basic mathematical model is presented in Fig. 5b; it consisted of an equation describing AIs (here denoted as A , corresponding in this case to 3-oxo-C10-HSL) net production (involving a Hill-type function, i.e., Michaelis-Menten dynamics, see Box 1), which contains a background production of AIs (α), a positive feedback loop leading to an increased production rate of AIs (β), influenced by the actual AIs concentration A , especially if exceeding a certain threshold A_{thresh} , and an abiotic degradation term γ . They further have equations for bacterial population density (N), concentration of AIs-degrading enzyme (E), concentration of first AI-degradation product homoserine HS (S), complexes $[RA]$, concentration of the HS-degrading enzyme (E_2), and enzyme production (z). The model possesses bistability (stable resting state and stable active) with the possibility of hysteresis. They investigated how the homoserines and the homoserine-degrading enzymes E and E_2 interact. They further describe the complete AIs-controlling circuit (five equations, Fig. 5b) suggesting that AHL degradation is an integral part of the whole AI circuit of *P. putida* IsoF.

See in Fig. 6 possible outcomes of AHL time dynamics assuming possible AIs degradation, under a high abiotic degradation rate the AIs dynamics can be affected to the point of almost no AIs present (dotted line). However, this is not what is observed experimentally. Accumulation (dashed line) is also not observed.

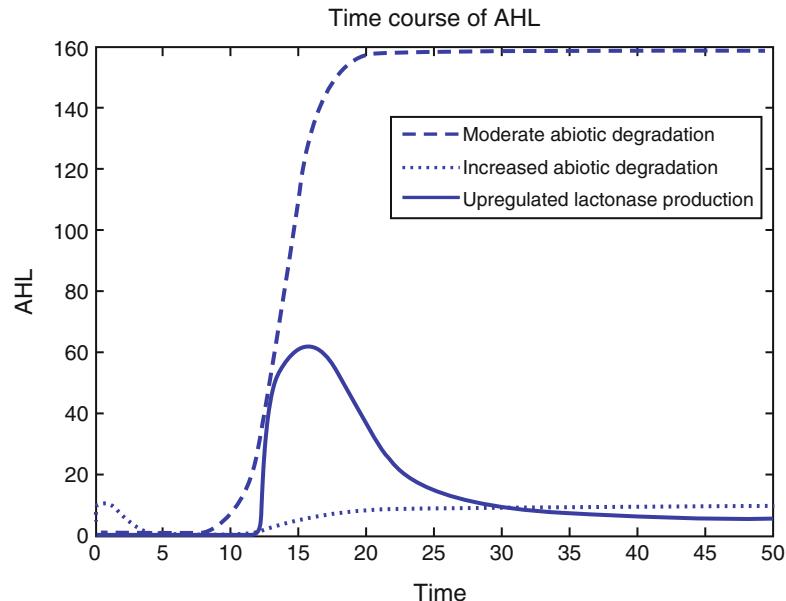


Fig. 6 A model-predicted time course of AIs, from [6]. Reproduced with permission

Therefore, a middle ground of abiotic degradation must be present. If we also assume there is AIs degradation through lactonase (an enzyme actively degrading AIs) we obtained a time course more similar to the one observed experimentally (solid line). *P. putida* QS system has further been studied for continuous cultures [12]. Surprisingly, the mathematical analysis showed constant and similar values to those reported for batch cultures, indicating the stability of the system under different environmental conditions.

3 Partial Differential Equations Models of QS

Koeber and coworkers developed a PDE model for the early stages of an infection caused by *Pseudomonas aeruginosa* in burn wounds [13]. They modeled only the primary QS system in *P. aeruginosa*, i.e., AIs are 3-oxo-C12-HSL. Their equations involve the concentration of AIs (3-oxo-C12-HSL) in time and space, $A(x, t)$, the density of up-regulated cells $N_u(x, t)$ and down-regulated cells $N_d(x, t)$. They basically used a previously described modeling framework [4] to include space. Population growth is modeled by a logistic expression. They assume that bacteria are in a zone of colonization within the wound (see Fig. 7a). Note how their equation describes changes in space and time through the partial derivative sign ∂ , instead of a d (like in previous section), this sign means

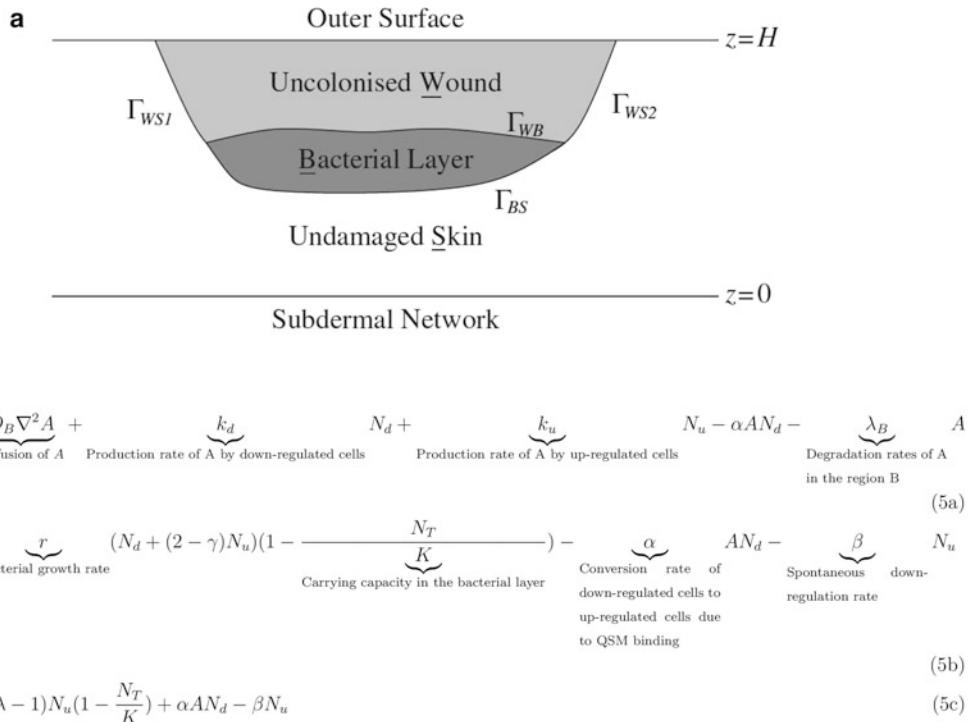


Fig. 7 (a) Schematic of the general wound geometry, reproduced with permission. (b) Bacterial region equations from [13]. Region $z = 0$ means subdermal network and $z = H$ means outer surface

that one can describe changes in either of the two variables, time or space; depending which of the variables is next to the sign, in the case of the equations shown, the changes describe changes in time.

The model addresses the effect of space on QS and the infection process. The model described in [13] could be used to compare treatments, for example, topical versus blood-delivered agents. They divide the wound into two regions: the bacterial zone and the uncolonized wound zone (see Fig. 7a). QS molecules are produced in the bacterial zone and then diffuse into the surrounding areas. The equations describing the QS molecules concentration in the wound will be different in each region, for illustration purposes we only show the equations related to the bacterial region (see Fig. 7b). Note how model in Fig. 7b is very similar to that of Fig. 3b, that is, the model described in [4] was extended to include time and space. In Fig. 8, the evolution of (a) the AIs concentration and (b) the up-regulated cell fraction of cells in one dimension found between $z = 0.4$ and $z = 0.6$ (bacterial layer location) are shown. The total wound width is $2L$, and the wound depth (uncolonized wound plus bacterial layer) is $1 - z_1$. Figure 8 is meant to show how the model is able to display the rapid rise in A and N_u (at $t = 16$), which corresponds to quorum being reached. They used a PDE (spatial) system as they wanted to investigate whether the

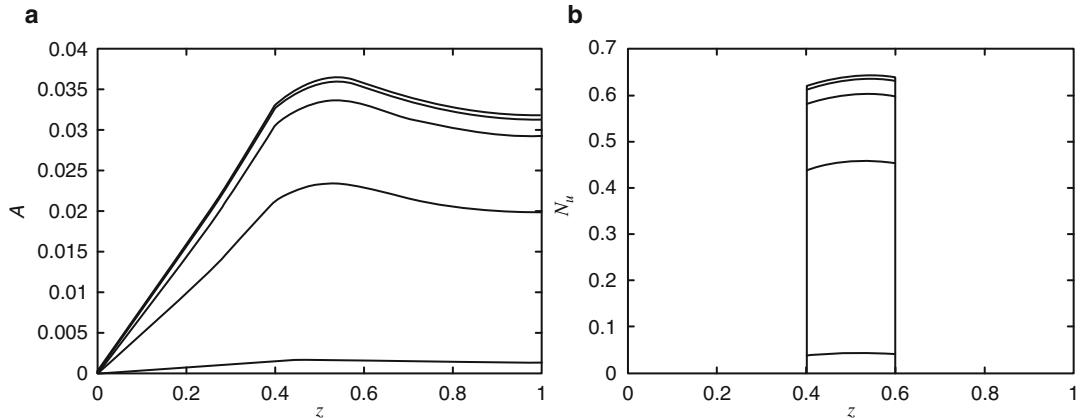


Fig. 8 Numerical simulations from [13]. The evolution of (a) Alc concentration and (b) the up-regulated cell fraction in one dimension. The sequence of curves is evenly spaced in time starting from zero and increasing to steady state. In (b) up-regulated cells are only found between $z = 0.4$ and $z = 0.6$ and no bacteria are present outside this layer. Reproduced with permission

presence of a subdermal plexus in partial thickness burn wounds has a significant effect on the dissemination of AIs in the wound environment. They concluded that, because QS depends upon diffusible signaling molecules, the wound environment plays a critical role in how the initial stages of infection develop.

Müller and colleagues [9] introduced a multi-scale model of QS, i.e., a model considering the QS dynamics within the cell and how this reflects at the population level once a cell is QS activated. Their modeling approach starts describing the QS dynamics of a single-cell to later consider a spatially structured model for a cell population. Their model is based on the *V. fischeri* QS system but can be adopted to other systems (as done in [6] for *P. putida*) and contains two parts: within the cell (basically the regulatory pathway, see Fig. 9a) and among cells (the communication). For within the cell, they considered that there is a certain background amount of LuxI and AHL present in the cytoplasm; that AHL diffuses in and out of the cell and that a complex with the receptor molecule LuxR is formed in the cytoplasm. They obtained a basic model for the mass of AIs outside of the cell x_c and mass of AIs within the cytoplasm x_{c_i} . See Fig. 9b for the model equations of the single cell model, where the QS term has the familiar form:

$$f(x_c) = \left(\alpha + \frac{\beta x_c^n}{x_{\text{thresh}}^n + x_c^n} \right) - \gamma x_c$$

which contains a background production of AHL (α), a positive feedback loop leading to an increased production rate of AHL (β),

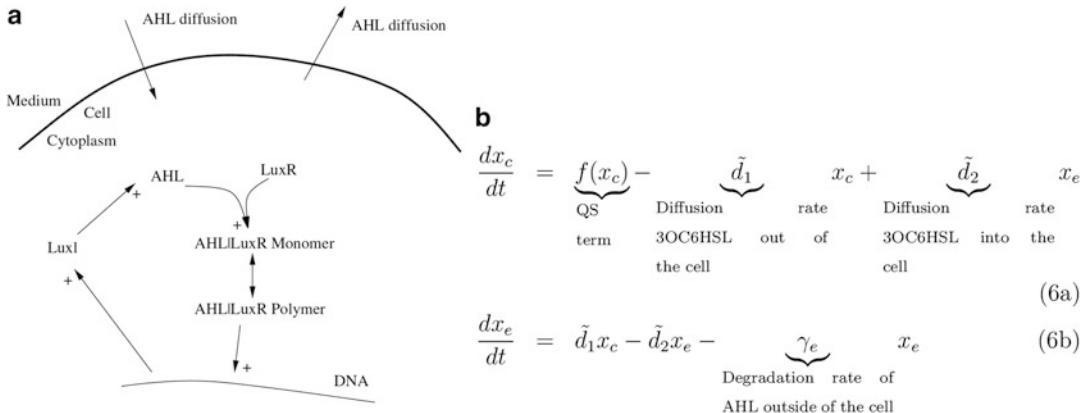


Fig. 9 (a) Scheme of the regulatory pathway considered in [9]. Reproduced with permission. (b) Single cell model

influenced by the actual AHL concentration, especially if exceeding a certain threshold x_{thresh} , n denotes the degree of polymerization and also there is an abiotic degradation term γ . See equations in Fig. 5b.

They later combined the two parts (single cell and population level) which results in a model with spatial structure, i.e., they combine the ODE model (Fig. 9b) with spatial structure by considering the influx/efflux of AIs through the cell membrane. They also include diffusion of AIs in the medium outside the cell.

Note, despite equations in Fig. 9b are ODE, the complete model is a spatial model which includes PDE, which we do not include as its complexity reaches far beyond the scope of this chapter. We mention this model as it offers an innovative approach to include several scales (temporal and spatial) involved in QS.

See in Fig. 10 an example of their results. Circles denote the measured location of producers (they are black if the model predicts activation while grey dots represent resting producer cells). Plus signs mark the measured location of resting, cross signs that of active detector cells. The equipotential lines denote the density of AHL concentration predicted by the model. Their aim was to develop a model to analyze spatial data about individual cells. Even if the model contains a lot of simplifications, their results are able to show the possible loss of the ability of the detector cells to become activated and the importance of boundary effects. Their model can potentially be used to reveal information about communication distances and intercellular variability.

Hense and colleagues studied how AIs regulation may generate spatially heterogeneous behavior Hense et al [14]. The motivation of this model was the fact that in biofilms or colonies, spatial gradients of AIs may emerge which can result in an inhomogeneous AIs induction. They developed a 3D model of AIs regulation in

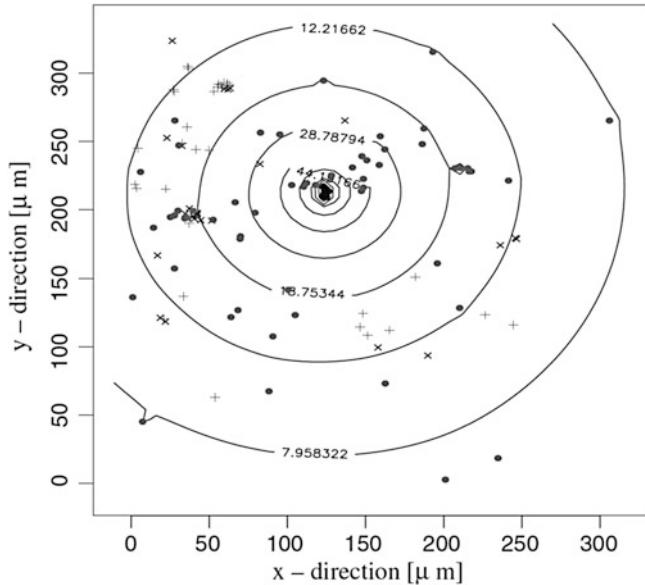


Fig. 10 Simulation results of [9]. Reproduced with permission

attached microcolonies. They focus on the influence of nutrients on an AI system. This is a PDE model containing an equation for a generic nutrient $N(x,t)$, the AHL concentration $A(x,t)$ and involving implicitly the cell concentration.

See in Fig. 11 their model, note how their equations also describe changes in space and time through the partial derivative sign ∂ . They based their assumptions on data of the lux AI system in *Vibrio fischeri*. The cells they considered possess an AI system of lux-type. $N(x; t)$ is a generic nutrient which is 100% available. The nutrient follows a Michaelis-Menten dynamics (see Eq. 7a in Fig. 11 and Box 1) and diffuses, generating nutrients gradients. The symbol ΔA denotes the second derivative of A with respect to space and it is used in modeling to denote diffusion of A . As this symbol appears in both Eqs. 7a and b, it means that both the nutrient and the AI are assumed to be diffusing. It normally is accompanied by a rate of diffusion (D_A in Eq. 7b) which denotes how fast does A diffuse. Following Hense et al. usual modeling approach, they use a constant production rate to indicate AHL basic production, and a Hill function that corresponds to the increased production rate in the induced state (see term involving α and β in Eq. 7b, similar to [9], and Box 1).

In order to understand the effect of nutrient availability on AHL production, they simulate the system with and without influence of nutrient on the AHL production. They simulated the mathematical model with ($f(N) = \frac{N_1 N_{N,1}^n}{N_{\tau,1}^{2nN,1} + N^{2nN,1}} + \frac{N_2 N_{N,2}^n}{N_{\tau,2}^{2nN,2} + N^{2nN,2}}$) and without ($f(N) = 1$) influence of nutrient on AI production

$$\frac{\partial N}{\partial t} = \underbrace{D_N \Delta N}_{\substack{\text{This terms denotes} \\ \text{that the nutrient dif-} \\ \text{fuses and at a rate} \\ D_N}} - \underbrace{\rho(x)}_{\substack{\text{fraction of volume oc-} \\ \text{cupied by cells}}} \frac{\overbrace{N + \frac{K_{cat,N}}{K_{m,n}}}^{\substack{\text{nutrient consumption} \\ \text{nutrient consumption}}} N}{\underbrace{N + \frac{K_{cat,N}}{K_{m,n}}}_\text{nutrient consumption}}$$

(7a)

$$\frac{\partial A}{\partial t} = \underbrace{D_A \Delta A}_{\substack{\text{This terms denotes} \\ \text{that AHL diffuses and} \\ \text{at a rate } D_A}} + \rho(x) \underbrace{f(N)}_{\substack{\text{modulation of the sig-} \\ \text{nalling system by nu-} \\ \text{trient availability}}} \left[\alpha + \beta \frac{A^n}{A^n + A_\tau^n} \right]$$

(7b)

Fig. 11 Model described in [14]; $K_{cat,N}$ and $K_{m,n}$: Michaelis-Menten parameters of nutrient consumption

(see Fig. 12). They found that, depending on colony size, the maximum activation may be at the colony center, somewhere between colony center and colony boundary, or at the colony boundary. Other models of AI regulation without influence of additional factors such as nutrients usually predict highest activity only in the center.

4 Concluding Remarks

Mathematical modeling of QS has proved to be a valuable tool to explore specific aspects of this type of bacterial communication [7]. As many of the events involved in activation are dynamic (change with time), DE are an ad hoc instrument to help describe interactions between the many players involved. Closely tracking AIs concentration changes has led to a better understanding of the regulation mechanism, in species with many signaling molecules the possibilities are many and DE can help discerned distinct contributions. We remark, however, that DE represent one mathematical approach, namely deterministic, where the outcome is determined through the relationships given to the variables involved, without any room for random variation. In contrast, stochastic models use ranges of values for variables in the form of probability distributions. This approach has also been successfully used to model certain aspects of QS.

Numerical simulations and mathematical analysis have shown that there are a series of common characteristics when it comes to

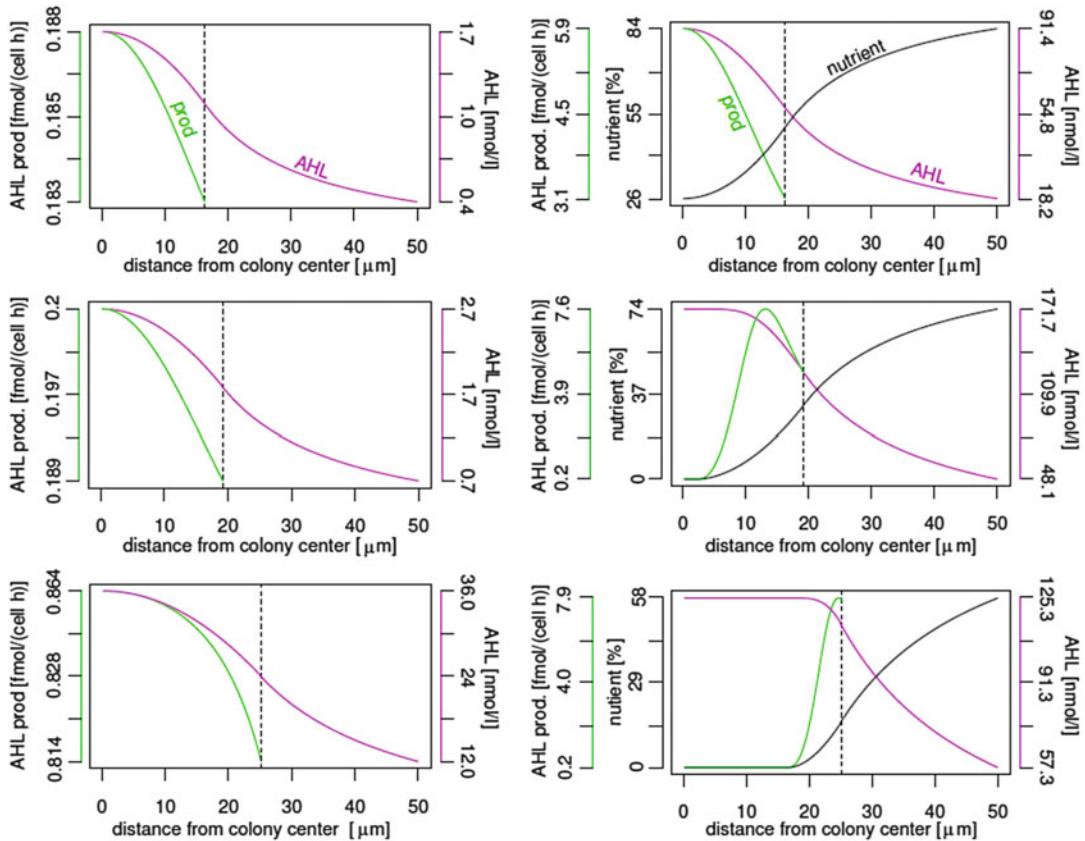


Fig. 12 Simulations from [14]. Profiles of Als production (green curve), concentration (magenta curve), and nutrient concentration (black curve) in colonies of different sizes (vertical line); left: without nutrient influence, right: with nutrient influence. Reproduced with permission

QS. From the modeling viewpoint, Fujimoto and Sawai argued that AI circuits typically found in bacteria generally originate from two distinct forms of bistability [15]. Brown, however, discussed that some models fail to satisfy physical constraints and presented canonical models for both Gram-negative and Gram-positive QS systems, which can be applied in well-mixed and biofilm populations [16]. Hense and Schuster described properties common to all AI systems [17], allowing for a deeper understanding of their ecological and evolutionary functions. Overall, the original idea of AIs systems as a cell density-dependent process giving rise to synchronous behavior is, considering the current state of knowledge about QS, far from adequate. The presented models are instances that mathematical modeling is contributing towards improving our comprehension of this complex regulation system. As theoretical biologists, we are hopeful that this continues to be the case.

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Part III

Identification and Characterization of Anti-Quorum Sensing Agents

Chapter 21

Qualitative and Quantitative Determination of Quorum Sensing Inhibition In Vitro

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Thomas Bjarnsholt, and Michael Givskov

Abstract

The formation of biofilms in conjunction with quorum sensing (QS) regulated expression of virulence by opportunistic pathogens contributes significantly to immune evasion and tolerance to a variety of antimicrobial treatments. The present protocol describes methods to determine the in vitro efficacy of potential QS inhibitors (QSI). Work on *Pseudomonas aeruginosa* has shown that chemical blockage of QS is a promising new antimicrobial strategy. Several live bacterial reporter systems have been developed to screen extracts and pure compounds for QSI activity. Here we describe the usage of reporter strains consisting of a *lasB-gfp* or *rhlA-gfp* fusion in *P. aeruginosa* for qualitative and quantitative evaluation of the inhibition of two of the major QS pathways, monitored as reduced expression of green fluorescence. By the use of an in vitro flow cell system it is possible to study the QSI activity by monitoring its ability to interfere with the protective functions of bacterial biofilm. For evaluation of the global effects of QSI compounds, we present a protocol for the DNA microarray based transcriptomics. Using these in vitro methods it is possible to evaluate the potential of various QSI compounds.

Key words Quorum sensing inhibitor, Halogenated furanones, QSI monitor screen, DNA microarray, In vitro continuous-culture biofilm flow cell system, Confocal scanning laser microscopy

1 Introduction

Treatment of infectious diseases is becoming increasingly more difficult as bacterial resistance to antibiotics evolves with an alarming rate. Several opportunistic pathogens rely on biofilm formation and quorum sensing (QS) controlled expression of virulence factors in the process of establishing persistent infections in humans and animals. Both processes help the bacteria against the host defense and otherwise detrimental effects of antimicrobial drug treatments [1–4]. However, research has shown that administration of QS inhibitors (QSI) can block QS controlled phenotypes including production of virulence factors [5, 6], thereby functioning as antimicrobials [6, 7].

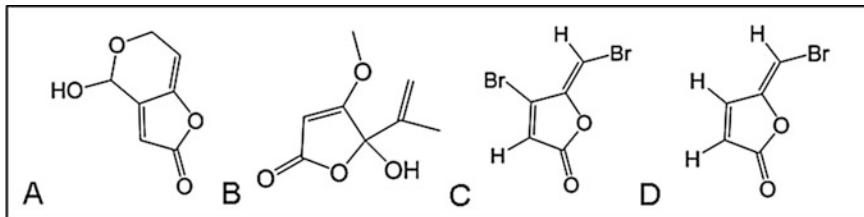


Fig. 1 Chemical structures of QS inhibitors produced by *Penicillium* species (**a, b**) and synthetic halogenated furanones (**c, d**). (**a**) Patulin, (**b**) penicillic acid, (**c**) furonone C30, and (**d**) furonone C56

The first discovery of environmental QSI s came from the Australian red macro algae, *Delisea pulchra* which was found to produce an antifouling cocktail of halogenated furanone compounds of which some were able to inhibit QS [8]. To increase the effect against the opportunistic human pathogen *P. aeruginosa*, synthetic derivatives were synthesized. Two such halogenated furanone compounds, C30 [6] and C56 [5, 7] showed significant activity against QS in *P. aeruginosa*. The discovery of the ability of halogenated furanones to inhibit QS gave rise to the idea that other compounds with similar capability could be found in a variety of ecological niches. Our further search revealed that QSI s can be extracted from many different natural sources including fungi [9], herbs [10–13], corals, and sponges [14] (Fig. 1).

A multitude of synthetic QSI compounds have been described in following articles [15–21].

This chapter describes methods we have invented and routinely use to identify and test the potency of QSI s for their ability to attenuate transcription of the bulk of QS controlled genes in *P. aeruginosa*. We present simple, quantitative LasR and RhlR controlled monitor systems [5], as well as a qualitative method to identify interference or disruption of important biofilm phenotypes. Finally, the chapter contains a description of how to evaluate the target specificity of QSI compounds using DNA microarray based transcriptomics.

2 Materials

2.1 QS Monitor Screens

1. Bacterial monitor strains: LasB-GFP, *P. aeruginosa* (PAO1-ATCC) harboring a *lasB-gfp*(ASV) fusion together with P_{lac} -*lasR*-mini-Tn5 inserted upstream to enhance the sensitivity [5], and RhlA-GFP, *P. aeruginosa* (PAO1-ATCC) harboring a *rhlA-gfp*(ASV) fusion together with P_{lac} -*lasR*-mini-Tn5 inserted upstream to enhance the sensitivity [22].
2. Black 96 wells microtiter plates. Black microtiter plates are used to hinder interference between the wells.

3. To measure fluorescence and growth of monitor strains, an automated multi label plate reader is used. Growth is measured at an optical density at 450 nm. Fluorescence is measured as excitation and emission wavelength at 485 nm and 535 nm, respectively.
4. AB minimal growth medium: 1 mM MgCl₂, 0.1 mM CaCl₂, and 0.01 mM FeCl₃ in Milli-Q water; sterilize the solution by autoclaving. Thereafter, add 10% (vol/vol) of A10 (see description below). Store at 4 °C.
5. A10: 20 g/l of (NH₄)₂SO₄, 60 g/l of Na₂HPO₄, 30 g/l of KH₂PO₄, and 30 g/l of NaCl in Milli-Q water. Adjust pH to 6.4 and sterilize the solution by autoclaving. Store at 4 °C for several months.
6. Glucose.
7. Bacto™ Casamino Acid.

2.2 In Vitro Continuous-Culture Biofilm Flow Cell System

1. Bacterial strains: *P. aeruginosa* (PAO1-ATCC) obtained from the *Pseudomonas* Genetic Stock Center (www.pseudomonas.med.ecu.edu, strain PAO0001), and *P. aeruginosa* (PAO1-ATCC) constitutively expressing a stable GFP [2].
2. AB trace minimal growth medium (ABT): 1 mM MgCl₂, 0.1 mM CaCl₂, and 0.01% (vol/vol) trace metals solution (see description below) in Milli-Q water. Sterilize the solution by autoclaving. Thereafter add 10% (vol/vol) A10 (see Sub-heading 2.1, item 5). Store at 4 °C.
3. Trace metal solution: 200 mg/l CaSO₄ · 2 H₂O, 200 mg/l FeSO₄ · 7 H₂O, 20 mg/l MnSO₄ · H₂O, 20 mg/l CuSO₄ · 5 H₂O, 20 mg/l ZnSO₄ · 7 H₂O, 10 mg/l CoSO₄ · 7 H₂O, 12 mg/l Na₂MoO₄ · H₂O, and 5 mg/l H₃BO₃. Can be stored at room temperature for several months. Remember to mix before use.
4. 0.3 mM glucose.
5. Bacto™ Casamino Acid.
6. 16-channel peristaltic pump.
7. Equipment required to assemble the system:
 - (a) Silicon tubing, int. diameter: 2 mm, ext. diameter: 4 mm (labeled “A” in Fig. 2).
 - (b) Silicon tubing, int. diameter: 1 mm, ext. diameter: 3 mm (labeled “B” in Fig. 2).
 - (c) Straight connectors 1/8" (labeled “1” in Fig. 2).
 - (d) T-connectors 1/8" (labeled “2” in Fig. 2).
 - (e) Reducing connectors 1/8" × 1/16" (labeled “3” in Fig. 2).
 - (f) Barrel tip cap orange (labeled “4” in Fig. 2).

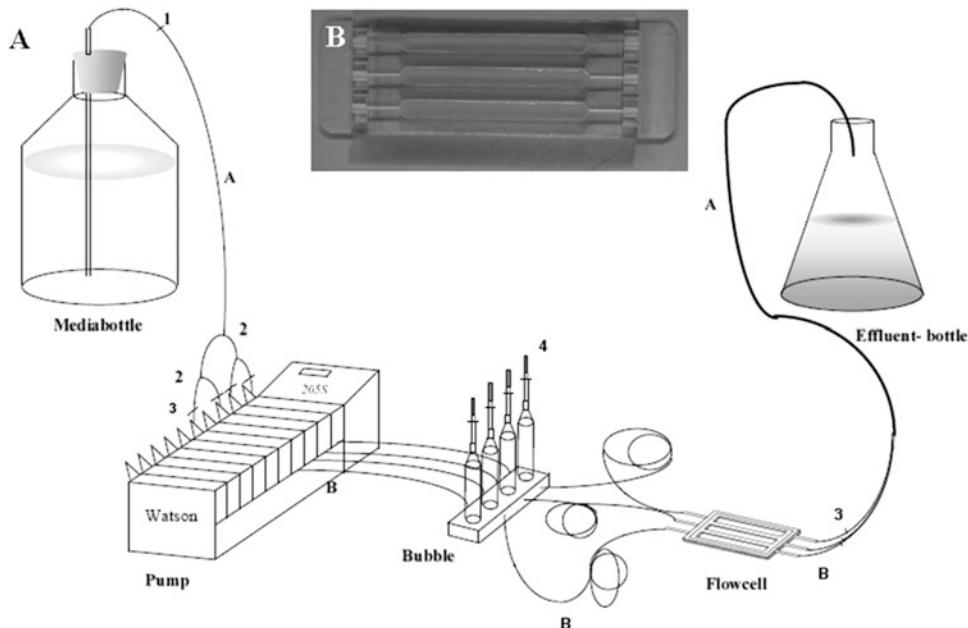


Fig. 2 (a) In vitro continuous-culture biofilm flow cell system setup. The *numbers* and *letters* refer to the designation in Subheading 2 of the different connectors and tubes. **(b)** Three channel flow cell, in which the biofilms are cultivated

8. Silicone to assemble the cover slips with the flow chambers (*see Note 1*).
9. Surface-attached biofilms are cultivated in flow chambers with channel dimensions of 1 by 4 by 40 mm.
10. The substratum consists of a 24 mm by 50 mm microscope cover slip.
11. In order to sterilize biofilm system, use 13% sodium hypochlorite (NaClO) solution (bleach) and dilute it in water to 0.5%. Be aware that NaClO is an alkaline that may cause skin irritation; therefore wear protective clothes, gloves, and safety glasses.
12. Image acquisitions of biofilms are performed using a Confocal Scanning Laser Microscope (CSLM) equipped with a detector and a filter set for monitoring GFP and propidium iodide (PI). In addition, a reflection detector for acquiring bright-field images is installed. We recommend images to be obtained using a 40–100× oil objective. Image scanning is carried out with the 488 nm laser line from an Ar/Kr laser. Imaris software package (Bitplane, AG) can be used to generate pictures of the biofilm.

2.3 Culture Preparation for DNA Microarray

1. *Bacterial strain:* *P. aeruginosa* (PAO1-ATCC) obtained from the *Pseudomonas* genetic stock center (www.pseudomonas.med.ecu.edu, strain PAO0001).
2. AB minimal growth medium (*see* Subheading 2.1, items 4 and 5).
3. BactoTM Casamino Acid.
4. RNAlater (Ambion) is used to stabilize and protect RNA.

3 Methods

We have developed a simple assay [5], which is able to detect the inhibition of either the *las* or the *rhl* encoded QS systems in *P. aeruginosa*. The monitor systems are constructed by fusing an unstable version of green fluorescence protein (GFP) [23] to the QS controlled *lasB* and *rhlA* promoters in a wild-type background of *P. aeruginosa*. These monitors switch on expression of GFP in QS dependent manner in batch cultures of *P. aeruginosa*, typical in late exponential or early stationary phases of growth. Hence, administration of a QSI compound to the growth medium will result in reduced expression of green fluorescence compared with the untreated batch culture. However, compounds inhibiting or reducing growth of the monitor strains will also affect the fluorescence. Therefore, in order to omit scoring false positives, growth should be measured simultaneously. We calculate the specific activity of GFP expression as change in GFP expression per time unit divided by change in OD₄₅₀ per unit time. Reduction in specific GFP expression and unaffected growth rate indicates the presence of a functional QSI compound. These screens are based on transcription of only two QS regulated genes. There is a minimum of 170 QS controlled genes. Consequently, only DNA microarray or “deep sequencing” technologies give the opportunity to monitor changes in transcription of the entire bacterial genome and thereby gain a more specific knowledge about the target specificity. Furthermore, it is important to test the efficacy of a possible QSI in regard to bacteria living in a biofilm. The in vitro continuous-culture flow cell system [24] makes it possible to follow and investigate biofilm development receiving fresh nutrients continually. The continuous-culture biofilm flow cell system is perfect for visual inspection of formation, disruption, and killing of biofilms using CSLM. The biofilm is monitored using either GFP-tagged bacterial cells or Syto9 as a stain. The killing of the bacteria is monitored using PI which will stain the DNA of cells with impaired membrane, i.e., dead cells.

3.1 QS Monitor Assays

1. Inoculate the two monitor strains (LasB-GFP or RhlA-GFP) in 2×10 ml ABT media supplemented with 0.5% (wt/vol) glucose and 0.5% (wt/vol) casamino acids.
2. Incubate cultures overnight at 30 °C with shaking at 180 rpm.
3. Add 150 µl AB-medium containing 0.5% (wt/vol) glucose and 0.5% (wt/vol) casamino acid to all wells in a black 96 wells microtiter dish.
4. Add 150 µl of test sample (possible QSI) to the first row of microtiter dish (*see Note 2*).
5. Make a twofold serial dilution from row 1–11. No test sample/QSI are added to row number 12, which works as a reference in order to confirm the growth of the monitor strain and compare the fluorescence.
6. The overnight culture (monitor strain) is diluted in 0.9% (wt/vol) NaCl to an optical density at 450 nm (OD₄₅₀) of 0.2.
7. Add 150 µl of diluted overnight culture to all wells in order to make a total volume of 300 µl in each well.
8. The microtiter dish is placed in the multi label plate reader and measurements are started. The fluorescence (GFP-expression) is measured with an excitation and emission wavelength at 485 nm and 535 nm, respectively, and growth of the bacteria is determined by measuring OD₄₅₀. Both GFP expression and growth are measured every 15 min for 15 h. The temperature is set at 34 °C.

3.2 In Vitro Continuous-Culture Flow Cell System

1. The flow cells are assembled minimum 24 h prior to use by gluing (use silicone) a 24 × 50 mm glass cover slip onto the top of the flow cell (Fig. 2b).
2. Assemble the rest of the system as shown in Fig. 2a.
3. The following description is based on a 16 channel pump. Sterilize the whole system using 1 l of a 0.5% (vol/vol) NaClO solution in sterilized Milli-Q water. The pump is set at 90 rpm to fill the whole system. When the bubble traps are filled, the lids are put on and the pump is set at 12 rpm. Sterilize for approximately 2 h. Make sure that nothing is leaking, otherwise use silicone to stop it. Remember to wear gloves and glasses when working with NaClO (*see Note 3*).
4. Empty the system at 90 rpm.
5. Wash the system with 2×1 l of sterilized Milli-Q water the same way as with the NaClO. Set the pump at 50 rpm when the system is filled. The system is emptied after 1 l and filled up again with the new flask (*see Note 3*).
6. After the last wash, the system is emptied and filled up with ABT minimal medium. Place the system at 37 °C overnight (*see Note 3*).

7. Use minimum two medium flasks. Hook, for example, channels 1–8 up to the medium flask which contains the test QSI compound and hook the growth medium flask up to channels 9–16 as the controls (*see Note 4*). The medium flasks (not the connecting tubing's) can be kept on ice in order to avoid turn over (chemical instability) of the test compound, and flasks with freshly prepared contents can be prepared each day.
8. Grow an overnight culture with *P. aeruginosa* (PAO1) or a GFP-tagged PAO1.
9. Before inoculation with the overnight culture, the flow is stopped and the tubes are clamped off between the flow channels and the bubble traps.
10. Inoculate the flow chambers using 250 µl of the overnight culture diluted to an OD₆₀₀ of 0.1 in 0.9% (wt/vol) NaCl. Inject the diluted culture in the flow channels by using a syringe needle which is inserted in the tubing next to the flow channel inlet. Close the injection hole with a thin layer of silicone.
11. The flow of media is arrested for 1 h to allow efficient colonization of the glass surface. Flip the flow cell upside down placing it on the glass surface.
12. Flip the flow cells back in “upright” position (glass slides are facing upwards) and start the flow of media. Remove the clamps and let the medium perfuse the flow chambers at a constant rate of approximately 3.3 ml/h using the peristaltic pump.
13. We usually allow the biofilm to develop and mature in the flow chambers for 3 days.
14. At day 3 the growth medium is changed to fresh growth medium containing antibiotic and antibiotic with QSI. The medium is changed by:
 - (a) Stopping the flow.
 - (b) Clamp the tube of between the flow cells and bubble traps.
 - (c) Empty the bubble traps by pulling the syringe off and quickly again.
 - (d) Remove the barrel tip cap and fill the bubble traps with fresh growth medium containing, for example, antibiotics, e.g., at 90 rpm.
 - (e) When the bubble traps are filled up, stop the system and remove the clamps.
 - (f) Start the system again on 1.75 rpm (approximately 3.3 ml/h).

15. The biofilm is investigated after approximately 24 h.
16. For examination of the biofilm with CSLM, a viability staining kit is used:
 - (a) If a non-GFP-tagged *P. aeruginosa* strain is used, LIVE/DEAD viability staining kit can be applied. Syto 9 (live; Invitrogen) and PI (dead) are added at a concentration of 0.005 mM and 0.01 mM, respectively, 15 min before examination of the flow cells by injecting it the same way as the bacterial culture explained in step 10 (see Note 5).
 - (b) If a GFP-tagged *P. aeruginosa* strain is used, only PI is added to the flow chambers (see Note 6).
17. Biofilm formation in flow chambers is examined by CSLM. Figure 3 is an example of 3-day-old biofilms of *P. aeruginosa* (PAO1) grown in flow chambers treated or untreated with tobramycin and garlic extract, which we have shown to inhibit QS in *P. aeruginosa* [13].

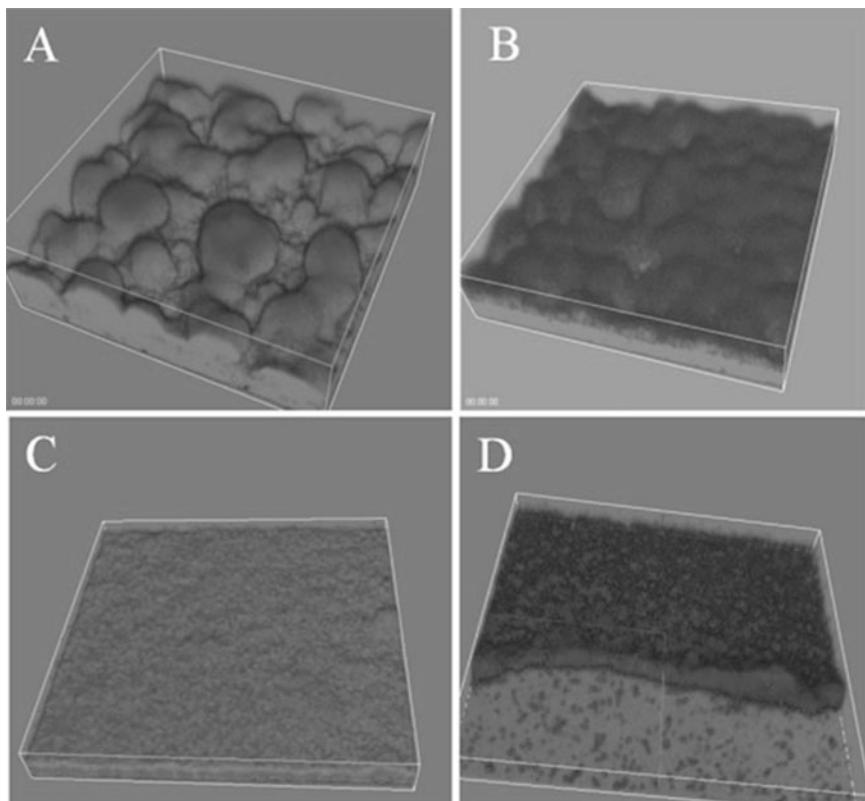


Fig. 3 Images of 3-day-old biofilms grown in flow chambers. Biofilm shown in picture (a-d) are grown with the following; (a) untreated, (b) tobramycin for 24 h, (c) garlic extract, (d) tobramycin for 24 h + garlic extract. Live bacterial cells are appearing as *light gray* whereas dead cells are *dark gray*. Reproduced from [13] with permission from Journal of Bacteriology

3.3 Culture Preparation for DNA Microarray

1. Inoculate *P. aeruginosa* (PAO1) in AB medium supplemented with 0.5% (wt/vol) casamino acids.
2. Grow culture at 180 rpm at 37 °C to an OD₆₀₀ of approx. 0.5.
3. Dilute culture to an OD₆₀₀ of 0.05 in 200 ml fresh medium.
4. Culture in a 1 l conical flask at 180 rpm at 37 °C.
5. At OD₆₀₀ of 0.5 the culture is split into two 100 ml cultures in 500 ml flasks (*see Note 7*).
6. From a 100–1000-fold concentrated stock solution (in the appropriate solvent), add QSI to one culture flask (the concentration must not affect growth rate of *P. aeruginosa*). A similar volume of the solvent used for preparing the stock solution is added to the other culture flask, which then serves as a reference (or no treatment) culture.
7. Grow cultures with shaking (180 rpm) at 37 °C.
8. Retrieve samples at OD₆₀₀ of 2.0 and immediately add RNA later (1:2) (*see Note 8*).
9. Store samples at –20 °C if not used the same day.
10. Isolate RNA by using the “RNeasy Mini Purification Kit” (Qiagen) (*see Note 9*).

4 Notes

1. Flow chambers and bubble traps can be purchased from DTU BIOENGINEERING, Department of Biotechnology and Biomedicine, Technical University of Denmark.
2. If the added test compound (QSI) is diluted in solvents that effect growth of the monitor strain, dilute the solution in 0.9% (wt/vol) NaCl to a concentration that is not affecting growth of the monitor strain.
3. It is important that there are no bubbles in the flow chambers. If bubbles are present, try to remove them by gently knocking the flow chamber on the inlet side to the table. Remember to check for bubbles in the flow chamber during the entire experimental period, but after the flow cells are inoculated with bacteria removal of bubbles should be avoided. If larger bubbles are generated, the biofilm development can be affected resulting in unusable results. Smaller bubbles can be displaced by the bacteria and will therefore not affect the result.
4. The amount of growth media used is dependent on the number of flow channels used and therefore must be calculated before initiating the experiment to make sure that the system does not exhaust the media.

5. Syto 9 and PI are light sensitive and have to be covered with aluminum foil. Remember to also cover the flow cells with aluminum foil during the 15 min of staining.
6. It is possible to add PI to the medium from the beginning of the experiment or when the media is changed to contain antibiotics and QSI. If this procedure is used, the final concentration of PI in the media has to be 0.0015 mM. Remember to cover every media containing parts with aluminum foil.
7. The flask has to be warmed in a 37 °C incubator before use.
8. It is important that the growth of the treated and untreated culture is similar and they reach the decided density ($OD_{600} = 2.0$) at the same time.
9. Detailed description of RNA isolation for transcriptomic analysis can be found in Chapter 14 of this book.

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Chapter 22

A Coculture-Based Approach for Screening Campaigns Aimed at Identifying Novel *Pseudomonas aeruginosa* Quorum Sensing Inhibitors

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and Livia Leoni

Abstract

Quorum sensing (QS) is recognized as a promising target for the identification of anti-virulence drugs hampering *Pseudomonas aeruginosa* adaptability to the host environment and pathogenicity. Consequently, a number of studies in the last decade focused on the identification of small molecules or proteins with anti-QS activity, mainly targeting the *las* QS system, which is based on *N*-3-oxododecanoyl-homoserine lactone (3OC₁₂-HSL) as signal molecule. Different experimental approaches have been successfully used to identify QS blockers interfering with the activity/stability of the 3OC₁₂-HSL receptor LasR, with the functionality of the 3OC₁₂-HSL synthase LasI, or with the stability/bioavailability of the 3OC₁₂-HSL signal molecule itself.

Here we describe the use of a high-throughput screening system for the identification of novel *las* QS inhibitors based on the cocultivation of *P. aeruginosa* wild type and the *P. aeruginosa*-derived biosensor strain PA14-R3, in which light emission relies on the ability of the wild type strain to synthesize 3OC₁₂-HSL and of the biosensor strain to perceive this signal molecule. With respect to other screening systems, this method has the advantage of being cost-effective and allowing the identification of compounds targeting, besides 3OC₁₂-HSL reception, any cellular process critical for the functionality of the *las* QS system, including 3OC₁₂-HSL synthesis and secretion.

Key words Quorum sensing inhibitors, Screening, Whole-cell biosensors, Anti-virulence drugs, Niclosamide, *Pseudomonas aeruginosa*, *lasR*, *lasI*

1 Introduction

The introduction of antibiotics into clinical practice at the middle of the twentieth century is a milestone in the history of medicine. However, the original expectation that all bacterial infections could be defeated by antibiotics has been soon disregarded by the emergence of antibiotic-resistant strains. As traditional antibiotic research appears to be helpless in coping with the emergence of

antibiotic-resistant strains, novel approaches should be undertaken in order to identify new drugs [1, 2].

An innovative strategy to combat bacterial infections relies on specific inhibition of bacterial virulence, hence the ability to cause disease rather than bacterial growth. The use of anti-virulence drugs is expected to reduce bacterial adaptability to the host environment, facilitating the host immune system to resolve the infection, and to diminish the strong selective pressure exerted by conventional antibiotics, although this is not yet supported by direct clinical evidence [3–5].

Since in many bacteria pathogenicity is controlled and coordinated by quorum sensing (QS), this communication system is considered one of the most promising targets for anti-virulence therapies [5, 6].

The Gram-negative bacterium *Pseudomonas aeruginosa* is one of the most dreaded opportunistic pathogens, and represents a prototype of multidrug resistant bug for which effective therapeutic options are limited. The ability of *P. aeruginosa* to cause a wide range of both community- and hospital-acquired infections in humans is linked to its capacity to produce a large repertoire of virulence factors, form antibiotic-tolerant biofilms and, ultimately, respond and adapt to environmental fluctuations, including host immune responses and antibiotic treatments. For these reasons, *P. aeruginosa* infections are generally characterized by high morbidity and mortality rates [7, 8].

The pathogenic potential of *P. aeruginosa* relies on the coordinated expression of a large array of virulence factors, the majority of which are positively controlled by QS [9, 10]. The *P. aeruginosa* QS network consists of at least three different QS systems, *las*, *rhl*, and *pqs*, based on the production and perception of the signal molecules *N*-3-oxododecanoyl-homoserine lactone (3OC₁₂-HSL), *N*-butanoyl-homoserine lactone (C₄-HSL), and 2-heptyl-3-hydroxy-4-quinolone (PQS), respectively. *P. aeruginosa* QS is hierarchically organized, since the *las* QS system is required for optimal activation of the *rhl* and *pqs* QS systems. Overall, QS controls the expression of nearly 10% of the *P. aeruginosa* genome, including genes for secreted virulence factors, biofilm formation, and immune-modulatory and pro-inflammatory agents [10, 11].

QS signal molecules can be detected in clinical samples, proving that QS is active during *P. aeruginosa* infections. Moreover, QS-defective mutants show strongly impaired virulence in several animal models of infection, corroborating the importance of QS for *P. aeruginosa* pathogenicity and its suitability as a target for the development of anti-*Pseudomonas* drugs [12, 13].

On these bases, a number of studies focused on the identification of small molecules or proteins with anti-QS activity, mainly targeting the *P. aeruginosa las* QS system. Different experimental approaches have been successfully used to identify small molecules

interfering with the activity/stability of the 3OC₁₂-HSL receptor LasR or with the functionality of the 3OC₁₂-HSL synthase LasI. Also the identification of enzymes that inactivate 3OC₁₂-HSL and the development of antibodies that limit the bioavailability of this signal molecule have been reported [14, 15].

Here we describe a convenient strategy for the identification of compounds affecting the *P. aeruginosa* *las* QS system at multiple levels: (1) expression/activity of the signal receptor LasR; (2) activity/availability of the signal molecule 3OC₁₂-HSL; (3) expression/activity of the signal synthase LasI [16]. This strategy is defined by a primary assay, suitable for high-throughput screening of chemical compounds, and by secondary assays, used to confirm the specific activity of the hit compounds selected in the primary assay. This approach has been successfully employed for the identification of a novel *las* QS inhibitor, the FDA-approved anthelmintic drug niclosamide [17].

As a general remark, it should be pointed out that, while we chose to illustrate a screening strategy linked to the activity of the *las* QS system in *P. aeruginosa*, some of the techniques presented here can be applied to a variety of different biological systems. For instance, reporter-based assays modelled on the one described here can be devised for the *rhl* and *pqs* QS systems of *P. aeruginosa*, as well as for different QS systems in other Gram-negatives or in Gram-positive bacteria.

2 Materials

1. Bacterial strains: *P. aeruginosa* PA14 [18] and PA14-R3 bio-sensor [16].
2. Growth media: Luria-Bertani broth (LB: 10 g/l NaCl, 10 g/l tryptone, 5 g/l yeast extract); Luria-Bertani agar (LA, as LB plus 15 g/l agar).
3. 1 M MOPS Buffer: 83.7 g/l 3-(*N*-morpholino) propanesulfonic acid (MOPS), 13.6 g/l sodium acetate trihydrate, 3.7 g/l ethylenediaminetetraacetic acid (EDTA) disodium salt, pH 7.0.
4. Protease Buffer: 100 mM Tris, 1 mM CaCl₂, pH 7.5.
5. Synthetic *N*-3-oxododecanoyl-homoserine lactone (3OC₁₂-HSL).
6. Elastin-Congo Red.
7. Chloroform.
8. 0.2 N HCl.
9. Black clear-bottom 96-wells microtiter plates.

10. Automated luminometer-spectrometer plate reader.
11. UV/Vis spectrophotometer.
12. Plastic microcuvettes for UV/Vis spectrophotometer.

3 Methods

3.1 Rationale of the Primary Screening

The screening system for inhibitors of *P. aeruginosa* QS is based on a biosensor strain (PA14-R3) able to detect the QS signal molecule 3OC₁₂-HSL [16]. The PA14-R3 biosensor is available from the authors upon request.

The PA14-R3 strain carries a nonfunctional allele of the *lasI* gene, and is thus unable to synthesize 3OC₁₂-HSL; however, it can respond to exogenous 3OC₁₂-HSL provided either through supply of the purified molecule or by cocultivation with a wild type *P. aeruginosa* proficient in 3OC₁₂-HSL production, such as the PA14 strain. The screening system is detailed in Fig. 1. The 3OC₁₂-HSL signal synthesized by the wild type PA14 diffuses into the PA14-R3 biosensor and induces bioluminescence emission. The addition of a molecule with inhibitory activity towards any process related to the 3OC₁₂-HSL-dependent QS system, namely, 3OC₁₂-HSL synthesis, transport, and perception, will reduce light emission by the biosensor with respect to a control coculture grown in the absence of any chemical compound.

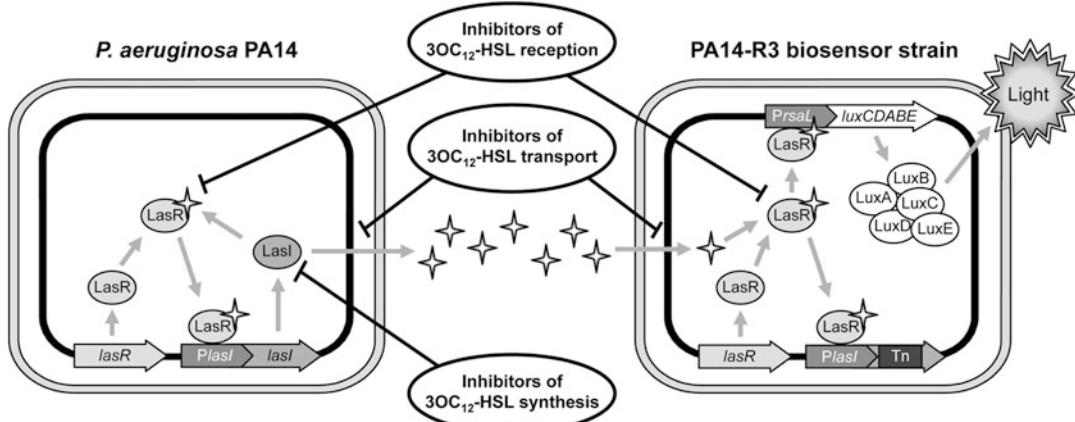


Fig. 1 Schematic representation of the PA14/PA14-R3 cocultivation screening system. The wild type PA14 produces 3OC₁₂-HSL signal molecules that induce bioluminescence emission in the biosensor strain PA14-R3. PA14-R3 is a PA14 derivative in which a transcriptional fusion between the LasR-dependent *rslA* promoter (*P_{rslA}*) and the *luxCDABE* operon was integrated at the *attB* neutral site of the chromosome. In addition, in order to avoid self-activation of the reporter strain, the *lasI* gene, encoding the 3OC₁₂-HSL synthase LasI, was inactivated by transposon insertion (Tn). Molecules interfering with different steps of the *las* QS system are expected to cause a reduction in bioluminescence in comparison to the untreated control. Modified from [16]

3.2 Primary Screening Procedure

1. Grow *P. aeruginosa* PA14 wild type and the 3OC₁₂-HSL reporter strain PA14-R3 (see Note 1) overnight at 37 °C on LA plates.
2. Scrape bacteria from LA plate surfaces and dilute in 1 ml of LB supplemented with 100 mM MOPS Buffer to an absorbance at 600 nm wavelength (A_{600}) of 0.09 and 0.03 for PA14-R3 and PA14, respectively (3:1 reporter:wild type ratio). Mix isovolumines of the PA14-R3 and PA14 diluted cultures, to obtain a coculture in which the A_{600} of PA14-R3 and PA14 is 0.045 and 0.015, respectively.
3. Aliquot 100 µl per well of the coculture in a 96-well microtiter black plates with clear bottom.
4. As untreated control, add 100 µl of LB in six wells containing the coculture.
5. Set up serial dilutions of the compounds to 2× the final concentrations to be tested. For example: chemical compounds to be used in the screening assays are dissolved in 10 mM DMSO and then diluted to 400 µM, 40 µM, and 4 µM in LB medium (to obtain 200 µM, 20 µM, and 2 µM final concentrations in the assay, respectively). Aliquot 100 µl of each compound in the microtiter wells containing the coculture.
6. Incubate the microtiter plate at 37 °C for 4 h with gentle shaking.
7. Measure A_{600} and light counts per second (LCPS), simultaneously with an automated luminometer-spectrometer plate reader (see Note 2).
8. Average the A_{600} and LCPS measurements of the untreated control samples. For all samples, normalize the LCPS to the A_{600} to obtain PA14-R3 reporter activity. Compare PA14-R3 reporter activity of the treated samples to one of the untreated controls (see Note 3).

3.3 Rational of the Secondary Assays: 3OC₁₂-HSL, Elastase, and Pyocyanin Production

As previously described, compounds reducing light emission in the primary screening could hamper the *las* QS system at different levels, including 3OC₁₂-HSL synthesis, transport, and perception. Inhibition of any of these steps would limit 3OC₁₂-HSL synthesis in PA14 wild type, as well as the production of 3OC₁₂-HSL-dependent virulence factors such as elastase and pyocyanin.

Elastase is a *P. aeruginosa* secreted protease that mainly targets mammalian elastin and plays a key role in virulence [19]. Transcription of the elastase gene, *lasB*, is strongly activated by the *las* QS system [20].

Pyocyanin is a redox-active phenazine responsible for the blue-green color characteristic of *P. aeruginosa* cultures. Besides its role in virulence, pyocyanin has been also recognized as a signalling

molecule, as an electron shuttle for bacterial respiration, and as an antibacterial and antifungal agent [21, 22].

Therefore, a convenient method to validate the *las* inhibitory activity of a hit compound is to measure its effect on 3OC₁₂-HSL, elastase, and pyocyanin production, in comparison with an untreated control.

The assay for 3OC₁₂-HSL production by PA14 wild type is based on the use of the same PA14-R3 biosensor strain used in the primary screening [16].

The elastase assay is based on the Elastin-Congo Red reagent, a water-insoluble powder in which elastin is bound to the Congo Red dye. Hydrolysis of elastin causes the release of the dye in the aqueous phase. The amount of released (soluble) dye is proportional to the level of hydrolyzed elastin and, ultimately, to the level of elastase present in a *P. aeruginosa* culture supernatant. The method here described is modified from ref. 23.

Pyocyanin can be easily quantified in chloroform extracts of *P. aeruginosa* culture supernatants by spectrophotometric analysis. The method here described is modified from ref. 24.

3.4 Quantification of 3OC₁₂-HSL

1. Inoculate *P. aeruginosa* PA14 in 5 ml of LB and grow the culture overnight at 37 °C with 200 rpm shaking.
2. Refresh the overnight culture to an *A*₆₀₀ of 0.015 in 30 ml of LB supplemented with 50 mM MOPS Buffer, in the presence of increasing concentrations of the test compound. Incubate at 37 °C with 200 rpm shaking.
3. Withdraw 7 ml of bacterial cultures every 3 h, up to 9 h. Measure *A*₆₀₀ of the samples, harvest the cells by centrifugation, and recover the culture supernatants (see Notes 4 and 5).
4. Scrape bacteria from the surfaces of an LA plate of the biosensor strain PA14-R3 and dilute in 2 ml of LB. Measure the *A*₆₀₀ of this bacterial suspension and use it to prepare an inoculum of the biosensor strain in LB supplemented with 50 mM MOPS Buffer to an *A*₆₀₀ of 0.045.
5. Aliquot 195 µl per well of the biosensor culture in a 96-well microtiter black plates with clear bottom.
6. Add 5 µl of each culture supernatant from step 3 in three wells containing the reporter culture. As untreated control, add 5 µl of LB in six wells containing the reporter culture.
7. For the calibration curve, set up 1:3 serial dilutions of synthetic 3OC₁₂-HSL in LB, from a maximal concentration of 120 µM to a minimum concentration of ~18 nM. Add 5 µl of each diluted 3OC₁₂-HSL sample in three wells containing the reporter culture.
8. Incubate the microtiter plate at 37 °C for 4 h with gentle shaking.

9. Measure A_{600} and LCPS, simultaneously (*see Note 2*). Average the A_{600} and LCPS measurements of the replicates. Normalize the averaged LCPS to the averaged A_{600} to obtain PA14-R3 reporter activity. Extrapolate 3OC₁₂-HSL concentration in the treated and untreated supernatants based on the values obtained for the calibration curve (*see Note 6*).

3.5 Elastase Assay Procedure

1. Set up 1.5 ml tubes each one containing 20 mg of Elastin-Congo Red and 1 ml of Protease Buffer (*see Note 7*).
2. Add 100 μ l of culture supernatant collected in Subheading 3.4, step 3 (*see Notes 5 and 8*) to the tube containing the Elastin-Congo Red suspension. Prepare a control sample (blank) by adding 100 μ l of sterile LB instead of the culture supernatant.
3. Incubate 2 h with gentle shacking at 37 °C.
4. Centrifuge for 5 min at 11,000 $\times g$ at room temperature.
5. Measure absorbance at 495 nm wavelength (A_{495}) of the clear supernatants in plastic microcuvettes, using as blank the control sample (see above). Normalize with respect to the A_{600} of the corresponding culture measured in Subheading 3.4, step 3.

3.6 Pyocyanin Assay Procedure

1. Add 3 ml of chloroform to 15 ml conical tubes containing 5 ml of the supernatants collected in Subheading 3.4, step 3 (*see Notes 5 and 8*). Mix vigorously by vortexing for 10 s. As control sample (blank), use 5 ml of sterile LB in place of the bacterial supernatant.
2. Centrifuge the tubes at 3,000 $\times g$ for 5 min.
3. Transfer 2 ml of the lower organic phase in clean 15 ml conical tubes (the lower organic phase is blue if pyocyanin is present) and add 1 ml of 0.2 N HCl. Mix vigorously by vortexing for 10 s.
4. Centrifuge the tubes at 3,000 $\times g$ for 5 min.
5. Transfer 800 μ l of the upper aqueous phase in plastic microcuvettes (the upper aqueous phase is pink if pyocyanin is present).
6. Measure the absorbance at 520 nm wavelength (A_{520}), using as blank the control sample (see above). Normalize with respect to the A_{600} of the corresponding culture measured in Subheading 3.4, step 3.

4 Notes

1. By using bacterial biosensors in which light emission is induced by exogenous C₄-HSL, PQS, or other QS molecules different from 3OC₁₂-HSL, similar coculture-based approaches can be designed to identify inhibitors of other QS systems. Please

consider that growth of biosensor strains different from PA14-R3 may require LB supplementation with antibiotics for plasmid selection. Moreover, the use of other biosensor strains may require preliminary optimization of experimental parameters, including wild type/biosensor ratio, A_{600} of the coculture at t_0 , and incubation time of the coculture at 37 °C.

2. We routinely use a Wallac 1420 Victor^{3V} multiplate reader (Perkin-Elmer) as automated luminometer-spectrometer plate reader. For the Wallac 1420 Victor^{3V} multiplate reader relevant parameters for bioluminescence measurement are: emission aperture, large; counting time, 1 s. Relevant parameters for absorbance measurement are: filter 595/60; excitation aperture, normal; reading time, 0.1 s.
3. The criteria used for the selection of hit compounds in [17] were: (a) ≥50% inhibition of PA14-R3 reporter activity; (b) ≤10% reduction of growth with respect to the untreated controls. The latter criterion was aimed at avoiding any unspecific effect of impaired growth on the QS response.
4. This step is just for harvesting cells, so speed and times for centrifugation can vary. Our standard conditions are $6,300 \times g$ for 5 min at room temperature.
5. For the 3OC₁₂-HSL quantification assay, the supernatants can be stored at -20 °C. Conversely, for elastase and pyocyanin assays it is recommended to process supernatants as soon as possible.
6. The same method can be used to quantify the amount of 3OC₁₂-HSL produced by different strains of *P. aeruginosa* (e.g., to compare a wild type and a mutant) or of *P. aeruginosa* grown in different media. Also quantification of 3OC₁₂-HSL in *P. aeruginosa* clinical isolates has been described [16].
7. Avoid the preparation of a stock suspension of Elastin-Congo Red. This powder is highly insoluble and aliquots of a suspension could contain different amounts of the reagent. We have observed that aliquoting the powder in each sample tube enhances the assay reliability.
8. Under these conditions, *P. aeruginosa* usually starts to produce detectable levels of elastase and pyocyanin at around $A_{600} \approx 3.0$, therefore consider using only supernatants collected after 6 and 9 h incubation in Subheading 3.4, step 3.

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Chapter 23

A Culture-Dependent Method for the Identification of Quorum Quenching Enzymes of Microbial Origin

Kaihao Tang and Xiao-Hua Zhang

Abstract

Although it has been more than a decade since the first discovery of AHL lactonase AiiA in *Bacillus* sp. 240B1, we are only beginning to understand the diversity of quorum quenching (QQ) enzymes. Most of the previously identified QQ enzymes are derived from nonmarine microorganisms. A novel marine-derived secretory AHL lactonase, MomL, was found in *Muricauda olearia* in our previous work and represents a novel type of AHL lactonase widespread in the ocean. Herein, we describe a culture-dependent method for the identification of microbial QQ enzymes, especially the high-throughput method for screening QQ bacteria from cultivable bacterial strains. This method should be capable of efficiently identifying QQ enzymes from various microbial origins. The discovery of more QQ enzymes will help us to understand their ecological roles and may provide potential as therapeutic agents.

Key words Quorum sensing, Quorum quenching, QQ enzyme, Lactonase, *Agrobacterium tumefaciens* A136 (pCF218)(pCF372)

1 Introduction

Quorum sensing (QS) refers to a population-dependent intercellular communication in microorganisms, involved in important behaviors such as biofilm formation [1], bioluminescence [2, 3], and secretion of virulence factors [4]. Gram-negative bacteria commonly use *N*-acyl homoserine lactones (AHLs) as QS signaling molecules and AHL-dependent QS is related with virulence in many bacterial pathogens [5, 6]. In the first few decades, researchers focused mostly on the study of various QS circuits, and it was not until 2000 that the AHL-degrading ability was firstly discovered in *Bacillus* sp. 240B1 [7]. Since then, a number of AHL-degrading enzymes have been found in mammals, plants, fungi, archaea, and bacteria [4, 5]. This enzymatic degradation of QS signaling molecules is termed quorum quenching (QQ). QQ has been recognized as a promising antivirulence therapy because it can attenuate virulence without killing the bacteria, and thereby

weaken the selective pressure imposed on the pathogens and reduce the potential for evolution of drug resistance [8]. To date, three types of AHL-degrading enzymes have been found: AHL lactonases (lactone hydrolysis), AHL acylases (amidohydrolysis), and AHL oxidases and reductases (oxidoreduction).

Most of the identified AHL-degrading enzymes are derived from nonmarine bacteria. However, the diversity of AHL-degrading enzymes may be more than we expected. A novel marine-derived AHL lactonase, MomL, was found in *Muricauda olearia* in our previous work [9, 10]. Further study revealed that it represents a novel type of secretory AHL lactonase widespread in marine bacteria of the family *Flavobacteriaceae*. Moreover, our results showed that AHL acylases seem more common than lactonases in the ocean. In other words, we may be only beginning to understand the AHL-degrading enzyme diversity, especially in the ocean.

A new high-throughput method was developed for identifying QQ bacteria and 14 novel QQ bacterial species were found in our previous work [9]. In this method, the biosensor *Agrobacterium tumefaciens* A136 responds to a broad range of AHLs by expressing β -galactosidase (encoded by the report gene *lacZ*) [11]. By using the β -galactosidase substance 5-bromo-4-chloro-3-indolyl β -D-galactopyranoside (X-gal), the β -galactosidase activity can be quantified by spectrophotometric measurement of the final blue products 5,5'-dibromo-4,4'-dichloro-indigo (indigo) at 630 nm wavelength (OD_{630}) [12]. However, only measurement of OD_{630} is not accurate enough in a whole-cell assay because the absorbance at 630 nm is actually a combination of absorption and light scattering by indigo and *A. tumefaciens* A136 biosensor cells. In the mixture, according to the Beer-Lambert law [13], absorbance of each component can be expressed as: $OD_{630\ cell} = a \times OD_{492\ cell}$; $OD_{492\ indigo} = b \times OD_{630\ indigo}$; $OD_{630} = OD_{630\ indigo} + a \times OD_{492\ cell}$; $OD_{492} = b \times OD_{630\ indigo} + OD_{492\ cell}$ (wavelengths were selected according to the absorbance spectra of indigo) [9]. The correction factors a and b are constants that can be easily obtained by measuring the values of OD_{492} and OD_{630} of a dilution series of biosensor *A. tumefaciens* A136 or indigo [9]. Therefore, the β -galactosidase activity is normalized to the cell amount of biosensor:

$$\frac{OD_{630\ indigo}}{OD_{492\ cell}} = \frac{0.653 \times OD_{492} - OD_{630}}{0.267 \times OD_{630} - OD_{492}}$$

This improvement allows simultaneous measurement of large numbers of samples with a minimum of hands-on time, requiring merely standard equipment available in any research laboratory.

Herein, we describe the culture-dependent method for the identification of microbial QQ enzymes, including high-throughput screening, primary characterization of enzymatic AHL

degradation, and prediction of QQ enzymes from whole genome sequencing data. The discovery of more QQ enzymes will contribute to our knowledge about their diversity and may provide potential as therapeutic agents.

2 Materials

2.1 High-Throughput Screening for QQ Bacteria

1. *A. tumefaciens* A136 (pCF218) (pCF372) [11] (see Note 1).
2. Marine Broth 2216 (MB; Becton Dickinson).
3. Luria-Bertani (LB) agar: 10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl, 15 g/l agar. Sterilize by autoclaving.
4. AT minimal glucose medium:
 - (a) 20× AT salts: 40 g/l $(\text{NH}_4)_2\text{SO}_4$, 3.2 g/l $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 200 mg/l CaCl_2 , 100 mg/l $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, and 40 mg/l $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$ in distilled water, autoclave-sterilized, and stored at 4 °C.
 - (b) 20× AT buffer: 214 g/l KH_2PO_4 in distilled water, adjusted with KOH to pH 6.7, autoclave-sterilized, and stored at 4 °C.
 - (c) 50% (wt/vol) glucose: filter-sterilized and stored at –20 °C.
 - (d) AT minimal glucose medium (1 l): 50 ml of 20× AT salts, 50 ml of 20× AT buffer, and 10 ml of 50% (wt/vol) glucose combined with sterile deionized H_2O to a total volume of 1 l.
5. 5-Bromo-4-chloro-3-indolyl β -D-galactopyranoside (X-gal) stock solution: 20 mg/ml X-gal dissolved in dimethylformamide (DMF), stored at –20 °C.
6. 1,4-Piperazinediethanesulfonic acid (PIPES) stock solution: 1 M PIPES dissolved in distilled water, pH 6.7, filter-sterilized, and stored at –20 °C.
7. N-Hexanoyl-L-homoserine lactone (C6-HSL) stock solution: 1 mM C6-HSL dissolved in dimethyl sulfoxide (DMSO), stored at –20 °C (see Note 2).
8. Antibiotic stock solutions: 50 mg/ml spectinomycin and 4.5 mg/ml tetracycline dissolved in distilled water, filter-sterilized, and stored at –20 °C.
9. Sterile 96-well plates.
10. Sterile 0.22 μm filter.
11. Microplate absorbance reader.

2.2 Further Characterization of QQ Activities of Selected Bacteria

1. Sonicator equipped with a microtip.
2. Water bath.
3. 20 mg/ml proteinase K, filter-sterilized, and stored at -20 °C.
4. 6 M HCl, filter-sterilized, and stored at 4 °C.

2.3 Identification of QQ Enzymes from Selected Bacteria

1. Kit for genomic DNA extraction.
2. Protein sequences of identified QQ enzymes collected from literatures.
3. BLAST software: ncbi-blast-2.2.31 + win32.exe (download from <ftp://ftp.ncbi.nlm.nih.gov/blast/executables/blast+/>).

3 Methods

3.1 High-Throughput Screening for QQ Bacteria

The protocol of high-throughput screening for QQ bacteria is shown in Fig. 1.

1. Inoculate marine bacterial strains in 5 ml of Marine Broth 2216 (MB), and incubate the cultures at 28 °C, 170 rpm for 24 h. Run at least two technical replicates (see Note 3).
2. Transfer 178 µl of each bacterial culture to a clean 1.5-ml tube.
3. Add 2 µl of C6-HSL stock solution and 20 µl of PIPES stock solution to the tube. Mix gently by pipetting and incubate the reaction mixtures at 28 °C for 24 h under static condition. Use MB supplemented with C6-HSL as a negative control and MB supplemented with DMSO as a blank control (see Note 4).
4. During the incubation of mixtures, inoculate the biosensor strain *A. tumefaciens* A136 in 5 ml of AT minimal glucose medium supplemented with 50 µg/ml spectinomycin and

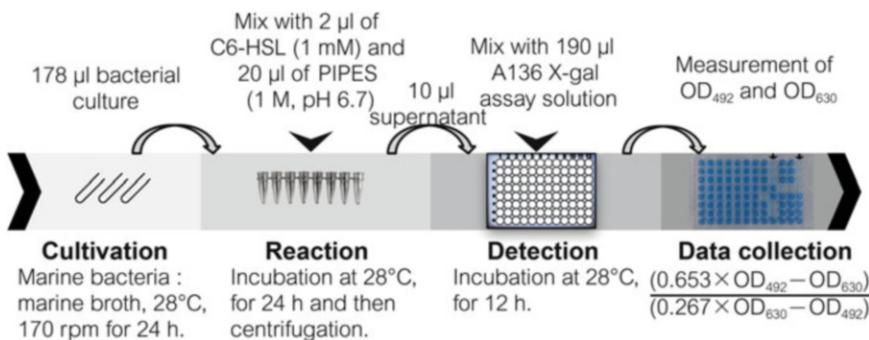


Fig. 1 The high-throughput method for identifying quorum quenching bacteria. The reaction step was carried out in eight PCR tubes for easy subsequent centrifugation. 96-well plates were used in the detection step to facilitate high-throughput measurements. Reproduced with permission from Copyright © 2013, Rights Managed by Nature Publishing Group [9]

4.5 µg/ml tetracycline and incubate at 28 °C, 170 rpm for 12 h (*see Note 5*).

5. Prepare the A136 X-gal assay solution by inoculating the culture of A136 ($OD_{600} \approx 2$) in AT minimal glucose medium (supplemented with 50 µg/ml spectinomycin and 4.5 µg/ml tetracycline) with an inoculum size of 1% and mixed with X-gal to a final concentration of 250 µg/ml. Generally, 20 ml of the A136 X-gal assay solution are sufficient for assay in a whole 96-well plate (*see Note 5*).
6. After incubation (**step 3**), centrifuge the reaction mixture at $13,000 \times g$ at 4 °C for 10 min and transfer 100 µl of supernatant to a clean 1.5-ml tube. Incubate the supernatant at 95 °C for 3 min (*see Note 6*). Centrifuge the mixture at $13,000 \times g$ for 10 min. Transfer 10 µl of supernatant to a well of sterile 96-well plate, being careful not to disturb the cell pellet. Add 190 µl of the A136 X-gal assay solution and mix gently by pipetting. Incubate the mixtures at 28 °C for 12 h under static condition.
7. Detect the absorbance at the wavelengths of 492 and 630 nm with microplate absorbance reader. Calculate the values of OD_{492} and OD_{630} by subtracting original values of blank control from that of samples.
8. Calculate the normalized β-galactosidase activity using the formula: $\frac{0.653 \times OD_{492} - OD_{630}}{0.267 \times OD_{630} - OD_{492}}$
9. Compare samples with negative control. Select samples that cause more than 40% reduction in the normalized β-galactosidase activities for further tests (*see Note 7*). The normalized β-galactosidase activities of 25 QQ bacterial strains in our previous work are shown in Fig. 2.

3.2 Further Characterization of QQ Activities of Selected Bacteria

3.2.1 Collect Whole Culture, Supernatant, and Cell Content of *M. olearia* Th120

1. Inoculate Th120 in 5 ml of MB, and incubate the culture at 28 °C, 170 rpm for 24 h. Run at least two technical replicates.
2. Transfer 1.5 ml of bacterial culture into two distinct 2-ml tubes. Keep the remaining 2 ml of the bacterial culture on ice.
3. Centrifuge the culture at $5000 \times g$ for 10 min at 4 °C.
4. Collect the cell-free supernatant of Th120 by filtration through a 0.22 µm filter. Keep it on ice.
5. Wash the harvested cells three times with ice-cold MB, and resuspend in 3 ml of ice-cold MB. Keep it on ice until sonication using a sonicator equipped with a microtip. Before

The marine bacterium *M. olearia* Th120 is used as an example in the subsequent Sections [10].

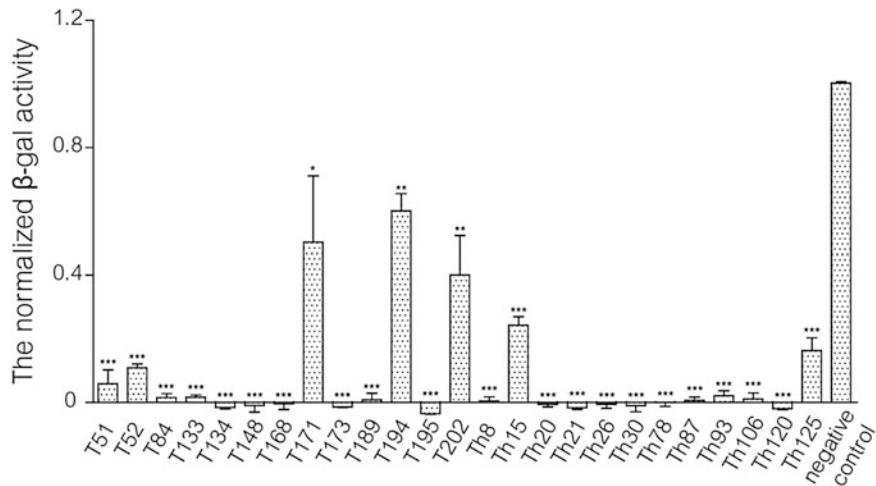


Fig. 2 The normalized β -galactosidase activities of the 25 QQ bacterial strains. Each strain was compared with negative control using unpaired *t*-test ($n = 3$; two-tailed p value; *** $p < 0.0001$, ** $p < 0.001$, * $p = 0.0143$). Reproduced with permission from Copyright © 2013, Rights Managed by Nature Publishing Group [9]

sonication, make sure the microtip is surface sterilized with 70% (vol/vol) ethanol.

6. Sonicate the cell suspension in ice 20 times with 10 s bursts at 200 W, with a 10 s cooling interval. Sterilize the surface of microtip with 70% (vol/vol) ethanol before sonicating another bacterial sample (*see Note 8*).
7. Centrifuge the clear cell lysate at $13,000 \times g$ for 10 min at 4°C . Transfer the supernatant to a clean 1.5-ml tube, being careful not to disturb the cell debris.
8. Collect cell content of Th120 by filtering the supernatant through a 0.22 μm filter. Keep it on ice (*see Note 9*).

3.2.2 Determine Whether the QQ Activity of Th120 is Due to Lactonase Activity

1. Transfer 250 μl of whole culture, supernatant, and cell content of Th120 to clean 1.5-ml tubes, respectively.
2. Prepare heat-treated samples by boiling samples in water bath capable of reaching temperatures of 100°C for 5 min.
3. Transfer another 250 μl of whole culture, supernatant, and cell content of Th120 to clean 1.5-ml tubes, respectively.
4. Prepare proteinase K-treated samples by adding 2.5 μl of 20 mg/ml proteinase K (final concentration of approximate 200 $\mu\text{g}/\text{ml}$), and incubate samples at 37°C for 3 h. Run a control without adding proteinase K but incubating the sample at 37°C for 3 h to exclude possible effects of temperature on the reduction of QQ activity.
5. In order to determine whether QQ activity is due to enzymatic degradation, add 178 μl of each sample into a clean 1.5-ml

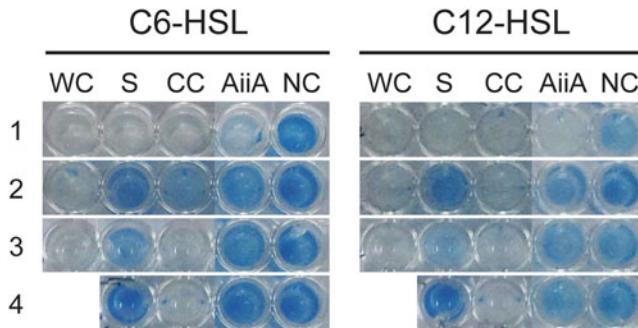


Fig. 3 Characterization of the AHL degradative activity of *M. olearia* Th120. AHL degradative activity of Th120 was analyzed by the A136 liquid X-gal assay. AiiA and Marine Broth medium were used as the positive and negative control, respectively. *WC* whole culture, *S* supernatant, *CC* cell content, *NC* negative control. In consideration of incomplete degradation of proteins, no proteinase K-treated WC was included. Four Arabic numbers represent different pretreatments of each sample: (1) untreated, (2) recovery of AHLs by acidification, (3) heat-treated, and (4) proteinase K-treated. Reproduced with permission from Copyright © 2015, American Society for Microbiology, Appl Environ Microbiol, 81, 2015:774–782. doi:10.1128/AEM.02805-14 [10]

tube. Add 2 μ l of C6-HSL and 20 μ l of PIPES stock solutions to the tube. Mix gently by pipetting and incubate the mixtures at 28 °C for 24 h under static condition. Detect the QQ activity according to the method described in Subheading 3.1, steps 4–8 (see Note 10).

- Determine whether QQ activity is due to AHL lactonase. After transferring 10 μ l of untreated whole culture, supernatant, and cell content of Th120 to a 96-well plate in the previous step, boil the rest of the reaction mixtures (190 μ l) for 5 min. Add 2–3 μ l of 6 M HCl. Mix gently by pipetting and incubate the mixtures at 28 °C for 24 h under static condition. Detect the QQ activity according to the method described in Subheading 3.1, steps 4–8 (see Note 11). The QQ activity of each component of Th120 is shown in Fig. 3.

3.3 Identification of QQ Enzymes from Selected Bacteria

Many methods can be used to identify the responsible QQ enzymes in bacteria, such as purification of QQ enzyme [14] and construction of a genomic library [15]. Here, we only describe searching putative QQ enzymes through local BLASTP in a computer with Windows operating system. Detailed methods for heterologous overexpression, purification, and in vitro characterization of putative QQ enzymes are described elsewhere [16].

- Inoculate Th120 in MB, and incubate the cultures at 28 °C, 170 rpm for 24 h.
- Collect cell pellets by centrifuging the culture at 13,000 $\times g$ for 10 min at 4 °C.
- Extract genomic DNA of Th120 by using a commercial kit for genomic DNA extraction.

4. Sequence the whole genome of Th120 in an available company or institute providing whole genome sequencing service. Generally, you will get several files including a file containing predicted genes and a file containing predicted proteins. Rename the protein sequence file with “Th120.fasta.”
5. Collect amino acid sequences of all identified AHL enzyme from literatures [17, 18] in FASTA form and rename the file with “QQenzymes.fasta.”
6. The FASTA format is a text-based format and the first line starts with a “>” and sequence name or other identifiers. Check the sequence of the AHL lactonase of Th120 in NCBI database: <https://www.ncbi.nlm.nih.gov/protein/725542033?report=fasta>
7. Download BLAST software ncbi-blast-2.2.31 + win32.exe from [ftp://ftp.ncbi.nlm.nih.gov/blast/executables/blast+/?](ftp://ftp.ncbi.nlm.nih.gov/blast/executables/blast+/)
8. Install it in the default Downloads folder. This creates the directory Downloads\blast-2.2.31 + \.
9. Copy files of Th120.fasta and QQenzymes.fasta to Downloads\blast-2.2.31 + \bin folder.
10. Click on “Start” menu and Type in “cmd” and press “Enter.” This will bring up the Command Prompt window.
11. Go into the BLAST folder you just created by entering:

```
cd Downloads\blast-2.2.31 + \bin
```
12. Building a BLAST database with protein sequences of Th120 by entering (*see Note 12*):

```
makeblastdb -in Th120.fasta -dbtype prot -out Th120DB
```
13. BLASTP searching putative QQ enzymes using identified QQ enzymes by entering (*see Note 13*):

```
blastp -query QQenzymes.fasta -db Th120DB -out QQTh120.txt -evalue 1e-5 -outfmt 6 -max_target_seqs 3
```
14. The QQTh120.txt file will show the three best hits in Th120 with expect value lower than 1×10^{-5} for each identified QQ enzyme (Table 1).
15. Further confirmation of QQ activities of the four putative QQ enzymes can be carried out by heterologous overexpression [16]. In our research, we only found murol 181 and murol 1831 possessing AHL degradative activities, and the AHL lactonase MomL (murol 1831) was studied in detail [10] (*see Note 14*).

Table 1
Selected results of BLASTP searching showing four putative QQ enzymes from Th120

qseqid	sseqid	pident	length	mismatch	gapopen	qstart	qend	sstart	send	evalue	bitscore
QuIP	murol1354	22.1	810	452	33	42	812	19	688	1.00E-20	93.6
PvdQ	murol1354	19.87	780	513	26	10	749	1	708	1.00E-18	87
AiiC	murol1354	22.88	612	342	21	59	641	31	541	4.00E-20	92.4
HacB	murol1354	25.17	592	351	21	25	588	14	541	4.00E-22	98.6
QuIP	murol181	25.58	825	516	23	41	835	31	787	3.00E-68	241
PvdQ	murol181	18.77	810	498	28	32	750	45	785	1.00E-11	64.7
AiiC	murol181	26.63	830	517	22	46	847	31	796	1.00E-89	301
HacB	murol181	37.18	780	480	5	15	789	18	792	0	550
YtnP	murol1676	32.56	258	158	6	14	259	6	259	2.00E-36	130
AidC	murol1676	28.87	194	104	7	87	260	35	214	7.00E-12	62
AiiA	murol1676	25.95	131	71	4	30	144	38	158	4.00E-08	50.1
AiiB	murol1676	24.26	136	91	4	5	138	3	128	4.00E-06	43.9
4-Pyridoxolactonase	murol1831	21.21	264	181	5	3	258	37	281	5.00E-09	53.1
AhID	murol1831	25.57	219	143	8	3	217	35	237	9.00E-12	60.8
AhIK	murol1831	25.95	262	180	6	2	259	36	287	1.00E-20	86.3
AhIS	murol1831	22.91	227	161	6	41	266	68	281	1.00E-09	55.1
AiiA	murol1831	28.85	260	163	7	5	250	39	290	1.00E-21	89
AiiB	murol1831	20.39	255	177	7	20	267	48	283	1.00E-07	48.5
AttM	murol1831	26.25	240	157	4	21	254	64	289	3.00E-22	90.5
QlcA	murol1831	23.47	196	132	8	28	217	107	290	2.00E-06	43.9

The specifiers of the first line mean: query seq-id, subject seq-id, percent of identical matches, alignment length, number of mismatches, total number of gaps, start of alignment in query, end of alignment in query, start of alignment in subject, end of alignment in subject, expect value, and bit score.

4 Notes

1. The biosensor *A. tumefaciens* A136 (pCF218) (pCF372) lacks the Ti plasmid and provides AHL response system, the AHL-responsive transcription factor TraR (pCF218) and the TraR-regulated *traI-lacZ* fusion genes (pCF372). The biosensor A136 can detect a broad range of AHLs (C6 to C14-HSL). A136 was routinely maintained on LB agar supplemented with 50 µg/ml spectinomycin and 4.5 µg/ml tetracycline at 28 °C, and was grown in AT minimal glucose medium for liquid X-gal assays.
2. We routinely purchase C6-HSL from Cayman Chemical Company or Sigma-Aldrich. C6-HSL can be alternatively prepared in methanol. However, it is difficult to precisely pipette C6-HSL dissolved in methanol even pre-wetting the tip several times with the solvent before actual pipetting. Because solvents with high vapor pressure (e.g., chloroform and methanol) can easily drip outside during pipetting due to the evaporation of the solvent. Furthermore, DMSO did not show effect on biosensor A136 responding to AHLs. Therefore, we prepared C6-HSL in DMSO.
3. Please use the optimal media and incubation conditions for growth of candidate bacteria. Generally, the mentioned media and incubation conditions are suitable for most of the cultivable marine bacteria.
4. C6-HSL can be substituted by other AHLs (3-oxo-C6-HSL, C8-HSL, 3-oxo-C8-HSL, C10-HSL, 3-oxo-C10-HSL, C12-HSL, 3-oxo-C12-HSL, C14-HSL and 3-oxo-C14-HSL). Compared to C6-HSL (1 mM), the recommended concentration was 2 mM for C10-HSL, C12-HSL, and C14-HSL, and 200 µM for the rest of the AHLs. As mentioned previously, AHL lactonase generally has a broad substrate specificity, while AHL acylase has a preference for long-chain AHLs. Additionally, we found AHL acylases seem more common than lactonases in the ocean. Therefore, C6-HSL and C12-HSL are recommended to be used in the primary screening for QQ bacteria from marine bacterial isolates.
5. Make sure that the biosensor A136 is freshly streaked on LB agar plate before inoculating in AT minimal glucose medium. If the colonies on LB plate are too old, the value of OD₆₀₀ of A136 after 12 h incubation will not reach 2 (Subheading 3.1, step 5). In this situation, incubation time can be extended up to 24 h. Sometimes, biosensor A136 with this state may affect the blue color development in 96-well plate. In this situation, incubation time can be extended up to approximate 18 h (Subheading 3.1, step 6). It should be noticed that excessive

incubation time might decrease the difference of the normalized β -galactosidase activities between candidate bacteria and negative control.

6. Possible β -galactosidases from candidate bacteria can be effectively abolished by incubating the supernatant at 95 °C for 3 min without significant effect on the stability of C6-HSL.
7. Many samples can cause significant reduction in the normalized β -galactosidase activities if only Student *t*-test was used to compare the results. However, some of the obtained positive samples did not show significant QQ activity in further tests. It is inferred that the reduction of the normalized β -galactosidase activity in some samples may be caused by unknown metabolites of candidate bacteria. Therefore, we usually select samples that caused more than 40% reduction in the normalized β -galactosidase activities for further study.
8. The cell suspension is clear if cells are completely lysed after sonication. Most of Gram-negative bacteria can be completely lysed after this sonication treatment. If cell suspension is not clear, more times of sonication treatment are required.
9. Cell contents are generally prepared in phosphate buffer in order to avoid changes in pH caused by numerous enzymes in the presence of high organic substrate (prepared in Marine Broth). In this assay, PIPES is used to maintain a slightly acid pH of mixture. Therefore, cell contents prepared in Marine Broth is appropriate for subsequent tests. Just in case, pH of each of the mixture should be checked after incubation to avoid alkaline hydrolysis of AHL.
10. It can be easily inferred that whether QQ activity is due to enzymatic degradation of candidate bacteria from the results of these steps (Fig. 3). An identified AHL lactonase should be included as a positive control.
11. AHL lactonases hydrolyze the lactone ring of AHL, yielding the corresponding *N*-acyl-homoserine. This hydrolysis may also occur spontaneously at alkaline pH, and can be reversed at lower pH [19]. Therefore, lactonase activity can be inferred from the results of acidification.

In this step, enzymes in the samples can be abolished by boiling treatment before acidification. The pH of mixture can be monitored by pH-indicator strips to approximate pH 2. The recovery of AHLs indicates AHL lactonase of candidate bacteria.

It must be noticed that unrecovery of AHLs after acidification does not mean no lactonase activity. As shown in Fig. 3, AHLs degraded by whole culture of Th120 are not recovered even lactonase activity exists in the supernatant. This may be due to further metabolism of ring-opened AHLs by unknown

enzymes. In addition, HPLC-MS should be used to identify the lactonase activity of subsequently expressed proteins [7].

12. Description of the command-line:

- in Th120.fasta means inputting the Th120.fasta file;
- dbtype prot means this file contains protein not DNA sequence;
- out Th120DB means creating BLAST database Th12DB.

13. Description of the command-line:

- query QQenzymes.fasta means inputting the QQenzymes.fasta file;
- db Th120DB means using the BLAST database Th120DB in previous step;
- out QQTh120.txt means creating the QQTh120.txt for saving hits;
- evalue 1e-5 means saving hits with expectation value lower than $1 \times e^{-5}$;
- outfmt 6 means results are shown in tabular format;
- max_target_seqs 3 means up to three aligned sequences to keep.

14. Many expression vectors with different characteristics can be used. Actually, expression vectors pET24a (+) (Novagen) and pTWIN1 (New England Biolabs) were used in our initial work. Afterwards, we prefer to use pTWIN1 because this vector can result with recombinant protein without vector derived amino acid residues and the CBD tag (chitin binding domain tag) can be easily removed by altering the pH of elution buffer.

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Chapter 24

Directed Evolution of Quorum-Quenching Enzymes: A Method for the Construction of a Directed Evolution Platform and Characterization of a Quorum-Quenching Lactonase from *Geobacillus kaustophilus*

Maybelle Kho Go, Jeng Yeong Chow, and Wen Shan Yew

Abstract

A thermostable quorum-quenching lactonase from *Geobacillus kaustophilus* (GKL) was used as a template for in vitro directed evolution experiments. Here we describe the overexpression and purification of wild-type GKL, the construction of a quorum-quenching directed evolution platform using bioluminescence as a reporter, and the in vitro kinetic assay for the determination of kinetic parameters of wild-type GKL and its mutants.

Key words *N*-Acyl-homoserine lactones, *N*-Acyl-homoserine lactonases, Directed evolution, Bioluminescence

1 Introduction

The amidohydrolase superfamily of enzymes consists of members bearing a conserved mononuclear or binuclear metal center within a $(\beta/\alpha)_8$ -barrel structural scaffold [1]. This superfamily was first reported by Holm and Sander in 1997 [2] and has since expanded to cover more than 30 reactions involving a diverse range of substrates [3, 4], including quorum-sensing *N*-acyl-homoserine lactones (AHLs) [5].

Quorum sensing is an integral part of microbial interaction. It is responsible for the virulence and pathogenicity of disease-causing bacteria [6]. Current studies show that modulation and perturbation of a quorum-sensing pathway is an effective anti-microbial strategy [7]. The disruption of pathways does not present the selective pressure that always results in the development of resistance in microbes. This suggests the possible use of quorum-quenching enzymes, such as lactonases, as attractive therapeutic biomolecules.

A thermostable lactonase from *Geobacillus kaustophilus* (GKL) was used as the template for the in vitro evolution of enhanced quorum-quenching activity. GKL was chosen as the template for in vitro evolution because of its high solubility and expression and high thermostability. However, it was found to only catalyze a limited range of medium to long-chain AHLs. Thus, this study created a robust and tunable directed evolution platform to screen for enhanced quorum-quenching activity against a broad range of AHLs. This will allow the identification of mutants that will have better or broader range of lactonase activity compared to the wild-type GKL.

2 Materials

Prepare all solutions using distilled and deionized water and analytical grade reagents. Prepare and store all reagents at room temperature (unless indicated otherwise). Diligently follow all waste disposal regulations when disposing waste materials.

1. 1× TAE buffer [tris(hydroxymethyl)aminomethane (Tris)-Acetate-Ethylenediaminetetraacetic acid (EDTA)]: dissolve 4.84 g Tris and 0.37 g EDTA disodium salt in 500 ml H₂O while stirring, add 57.1 ml glacial acetic acid and bring the volume to 1 l.
2. 1% (wt/vol) agarose gel: dissolve 0.5 g of agarose in 50 ml 1× TAE buffer. Heat the solution until the agarose is fully dissolved. Add 5 µl of a 10 mg/ml ethidium bromide solution. Swirl the solution until the ethidium bromide is fully dissolved. Pour the hot solution carefully onto a tray of a DNA electrophoresis apparatus and place an appropriate gel comb. Cool the molten gel at room temperature until it is solid.
3. Antibiotic stock solutions: 100 mg/ml ampicillin in H₂O; 30 mg/ml chloramphenicol in ethanol; 10 mg/ml gentamycin in H₂O. Filter the stock solutions using 0.22 µm membrane. Store the solutions at -20 °C. The stock solutions are stable for at least 1 year. Upon addition of the antibiotics in LB media or LB agar, the media or plates are stable for 2 months at 4 °C.
4. Luria-Bertani (LB) broth: 10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl. Shake the solution on a magnetic stirrer to prevent clumps. Autoclave the medium at 121 °C for 20 min. Cool the solution to room temperature prior to use. If required add antibiotics: 100 µg/ml ampicillin; 30 µg/ml chloramphenicol; 10 µg/ml gentamycin.
5. LB/antibiotic agar plates: 10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl, 15 g/l agar. Shake the solution on a magnetic stirrer to prevent clumps. Autoclave the medium at 121 °C for

- 20 min. Cool the molten agar solution to 60 °C before adding the appropriate amount of antibiotic (100 µg/ml ampicillin; 30 µg/ml chloramphenicol; 10 µg/ml gentamycin). Transfer 15 ml of the molten LB/antibiotic agar into sterile Petri dishes. Let the agar solidify in sterile conditions at room temperature.
6. Binding buffer: dissolve 0.34 g imidazole, 29.22 g NaCl, and 2.42 g Tris in 1 l deionized water while stirring. Adjust the pH to 8.0 with 12 M HCl. Store the buffer at 4 °C.
 7. Wash buffer: dissolve 6.99 g imidazole, 29.22 g NaCl, and 2.42 g Tris in 1 l deionized water while stirring. Adjust the pH to 8.0 with 12 M HCl. Store the buffer at 4 °C.
 8. Histidine-elute buffer: dissolve 15.51 g L-histidine, 29.22 g NaCl, and 2.42 g Tris in 1 l deionized water while stirring. Filter the solution using 0.22 µm membrane. Store the buffer at 4 °C.
 9. Bipyridyl dialysis buffer: dissolve 15.62 mg 2,2-bipyridyl, 1.17 g NaCl, and 2.42 g Tris in 1 l deionized water while stirring. Store the buffer at 4 °C.
 10. Storage buffer: dissolve 1.17 g NaCl and 2.42 g Tris in 1 l deionized water while stirring. Store the buffer at 4 °C.
 11. ZnCl₂ dialysis buffer: dissolve 13.63 mg ZnCl₂, 1.17 g NaCl, and 2.42 g Tris in 1 l deionized water while stirring. Store the buffer at 4 °C.
 12. Bicine buffer: dissolve 40.79 g bicine in 1 l deionized water while stirring. Adjust the pH to 8.0 using 6 M HCl. Store the buffer at 4 °C.
 13. Cresol purple: dissolve 2.0 mg *m*-cresol sodium salt in 1 ml deionized water. Store the indicator at 4 °C.
 14. 2% (wt/vol) arabinose: dissolve 0.2 g L-arabinose in 10 ml deionized water. Filter the solution using 0.22 µm membrane. Store the solution at 4 °C.
 15. 1 M isopropyl-β-D-thiogalactoside (IPTG): dissolve 0.24 g in 1 ml deionized water. Filter the solution using 0.22 µm membrane. Store the solution at -20 °C.
 16. Dimethyl sulfoxide (DMSO).
 17. Quartz cuvette.
 18. Spectrophotometer.
 19. Kit for DNA extraction from agarose gel.
 20. PCR reagents.
 21. Oligonucleotides:
 - (a) GKL forward primer: 5'-GAAAGGGGTGAAATTAA-TATGGCGGAGATGGTAGAACGG-3'.

- (b) GKL reverse primer: 5'-CCGACCTTACAAGGATCCT-CAAGCCGAGAACAGCGCC-3'.
 - (c) T7 Pro primer: 5'-CGAAATTAAATACGACTCACTA-TAGG-3'.
 - (d) T7 Term primer: 5'-GCTAGTTATTGCT-CAGCGGTGGCAGC-3'.
 - (e) Lux forward primer: 5'-GAGAGACTCGAGT-TAATTTTAAAGTATGGGC-3'.
 - (f) Lux reverse primer: 5'-CTCTCTGGTACCTCAACTAT-CAAACGCTTCGG-3'.
 - (g) Gm-up primer: 5'-TGGAGCAGCAACGATGTTAC-3'.
 - (h) Gm-down primer: 5'-TGTTAGGTGGCGGTACTTGG-3'.
22. Thermocycler.
23. DNA ladder.
24. Electrophoresis apparatus for DNA agarose gel.
25. Electrophoresis apparatus for SDS-PAGE analysis.
26. DNA and protein ladders.
27. Enzymes (with appropriate buffers): T4 DNA Ligase, NdeI, BamHI, XhoI, KpnI, Mutazyme II (Agilent).
28. Plasmids: pET15b plasmid (Novagen); pUC18R6K-mini-Tn7T-Gm^R plasmid [1]; pTNS2 plasmid [1]; pBAD33 [8].
29. *E. coli* BL21 (DE3) cells (Novagen).
30. *E. coli* XL1 Blue cells (Stratagene).
31. Refrigerated centrifuge.
32. Shaking incubator.
33. 200 µl, 1.5 ml, and 50 ml tubes.
34. 2 l conic flasks.
35. Electroporation cuvettes and apparatus.

3 Methods

3.1 Amplification and Cloning of the *gkl* Lactonase Gene

This method describes amplification and cloning of the *gkl* lactonase gene from the genomic DNA isolated from *G. kaustophilus* HTA426 (GI: 56420041).

1. Prepare a standard PCR reaction mixture (50 µl) containing 800 nM of the oligonucleotides GKL forward primer and GKL reverse primer, and 10 ng of genomic DNA.
2. Amplify the gene in a thermocycler with the following parameters: 98 °C for 2 min followed by 30 cycles of 98 °C for 10 s,

55 °C for 15 s, and 72 °C for 30 s, and a final extension of 72 °C for 10 min.

3. Prepare a 1% (wt/vol) agarose gel while waiting for the PCR amplification to complete (*see Subheading 2, item 2*).
4. After PCR amplification, add 10 µl 6× gel-loading dye to the PCR sample. Load the solution into the solid agarose gel. In a separate well load an appropriate DNA ladder. Run the gel in a DNA electrophoresis apparatus at 120 V for 35 min.
5. After electrophoresis, view the gel using a UV illuminator. Correct PCR amplification should lead to a DNA band of 978 bp.
6. If the molecular weight of the PCR band is correct, excise the band containing the amplified gene from the agarose gel by using a commercial kit for DNA extraction. Elute the DNA in distilled water and quantify by spectrophotometric analysis at 260 nm wavelength with a quartz cuvette.
7. Add the following into the tube containing approximately 500 ng of the purified PCR product purified in **step 6**: 6 µl 10× appropriate restriction buffer, 3 µl NdeI, 3 µl BamHI, distilled water to 60 µl. Mix well and incubate the reaction for 2.5 h at 37 °C.
8. Prepare the pET15b vector (Novagen) as well for cloning (*see Note 1*). Add the following into the tube containing approximately 500 ng of vector: 6 µl 10× appropriate restriction buffer, 3 µl NdeI, 3 µl BamHI, distilled water to 60 µl. Mix well and incubate the reaction for 2.5 h at 37 °C.
9. After incubation, add 12 µl of 6× gel-loading dye to both the gene and the vector tubes. Purify both using a 1% (wt/vol) agarose gel. Extract the gene and vector from the agarose gel by using a commercial kit.
10. Ligate the gene into the pET15b vector using T4 DNA ligase. Mix 100 ng of digested *gkl* gene, 20 ng of digested pET15b vector, 2 µl of 10× T4 DNA ligase buffer, 1 µl T4 DNA ligase, and distilled water to 20 µl. Incubate the reaction for 16 h at 15 °C.
11. Add the ligation reaction into 50 µl of chemically competent *E. coli* XL1 Blue cells (Stratagene) (*see Note 2*). Incubate the cells on ice for 30 min. Transform the cells using heat shock treatment. Heat the cells containing the plasmid at 42 °C for 30 s. Incubate the cells on ice for 2 min. Add 200 µl of LB broth into the transformed cells. Incubate and shake the cells at 37 °C for 1 h. Plate 100 µl of the transformed cells onto selective LB agar plates supplemented with 100 µg/ml ampicillin. Incubate the plates at 37 °C for 16–24 h.

12. Pick five to ten colonies that grew on the selective plate and screen them for the presence of the gene by PCR. Prepare 10 µl PCR reaction mixtures containing 200 nM of the oligonucleotides T7 Pro primer and T7 Term primer. Using sterile pipet tips or toothpicks, pick the single colony, streak a small amount onto a clean LB agar plate supplemented with 100 µg/ml ampicillin, and dip the tips into the PCR reaction mixtures. Incubate the plates at 37 °C for 18–24 h and amplify the gene in a thermocycler using the following parameters: 98 °C for 2 min followed by 30 cycles of 98 °C for 10 s, 55 °C for 15 s, and 72 °C for 2 min, and a final extension of 72 °C for 10 min.
13. After PCR amplification, add 2 µl of 6× gel-loading dye to the PCR reaction mixtures. Load the solutions into the solid 1% (wt/vol) agarose gel. In a separate well load an appropriate DNA ladder. Run the gel in a DNA electrophoresis apparatus at 120 V for 35 min.
14. After electrophoresis, view the gel using a UV illuminator. Correct PCR amplification should lead to a DNA band of approximately 1000 bp.
15. If the molecular weight of the PCR band is correct, inoculate the corresponding colony from the plate created in **step 12** into 5 ml of LB broth supplemented with 100 µg/ml ampicillin. Incubate overnight at 37 °C with shaking.
16. Harvest the cells by centrifugation at $4000 \times g$ for 10 min at 4 °C. Extract the plasmid from the resulting cell pellet by using a commercial kit for plasmid DNA extraction from bacterial cells.
17. Sequence the plasmid with the oligonucleotides described in **step 12** to confirm the presence of the *gkl* gene.

3.2 Overexpression and Purification of the GKL Lactonase

1. Add 5 µl of purified plasmid (prepared in Subheading **3.1, step 16**) into 50 µl of chemically competent *E. coli* BL21 (DE3) cells (Novagen). Incubate the cells on ice for 30 min. Transform the cells using heat shock treatment. Heat the cells containing the plasmid at 42 °C for 30 s. Incubate the cells on ice for 2 min. Add 200 µl of LB broth into the transformed cells. Incubate and shake the cells at 37 °C for 1 h. Plate 100 µl of the transformed cells onto selective LB agar plates supplemented with 100 µg/ml ampicillin. Incubate the plates at 37 °C for 16–24 h.
2. Pick one colony that grew on the selective plate. Using a sterile tip or inoculation loop, scrape cells from the plate and dip it into 5 ml of LB broth supplemented with 100 µg/ml ampicillin. Close the tube and let it shake overnight at 37 °C.

3. Add 1 ml of the culture grown in **step 2** in 2-l conical flasks containing 1 l of LB broth supplemented with 100 µg/ml ampicillin. Let the culture grow for 6–8 h with shaking at 37 °C.
4. When the OD at 600 nm reaches 0.4–0.8, add 0.1 mM IPTG. Continue shaking the culture at 37 °C for 16 h.
5. Harvest the cells by centrifugation at 4000 × g for 10 min at 4 °C.
6. Resuspend the cells in 40 ml of binding buffer at 4 °C.
7. Break the cells using a sonicator. Sonicate for 8 min in ice at 20 Hz, with 5 s intervals “on” and 10 s intervals “off.”
8. Separate the cell debris from the cell supernatant by centrifugation at 40,000 × g for 20 min at 4 °C.
9. Load the cell supernatant into a Ni²⁺ chelating sepharose column (10 ml column volume). Wash the column with the following buffers in sequence: 40 ml binding buffer, 40 ml wash buffer, and 40 ml histidine-elute buffer. Collect 2 ml fractions of the histidine-elute buffer flow through.
10. Check the histidine-elute fractions for the presence of GKL by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). The expected molecular weight of the purified protein is 36.4 kDa.
11. Pool the fractions containing the protein together and concentrate the solution by using a 10 kDa centrifugal filter.
12. Dialyze the concentrated protein fraction 1:200 at 4 °C against the following buffers in sequence: bipyridyl dialysis buffer, storage buffer, ZnCl₂ dialysis buffer, and storage buffer.
13. Aliquot 100 µl of the dialyzed protein in separate tubes and store at –80 °C.

3.3 Kinetic Assays of Lactonase Activity

1. Equilibrate a UV-Vis spectrophotometer to 37 °C. Set the detector to measure the absorbance at 577 nm.
2. In a 1 ml quartz cuvette, add 10 µl of bicine buffer, 8 µl of cresol purple (577 nm, $\epsilon = 12,500 \text{ M}^{-1} \text{ cm}^{-1}$), 1 µl of ZnCl₂ dialysis buffer, 25–50 µM homoserine lactone (*see Note 3*), and 1–10 µM GKL (*see Note 4*). Add enough DMSO to make the final concentration of DMSO in the cuvette 1% (vol/vol) (*see Note 5*). Add enough H₂O to bring the solution to 1 ml.
3. Monitor the change of absorbance at 577 nm for 5–10 min (*see Note 6*).
4. Determine the kinetic parameters by generating a Michaelis-Menten curve (*see Note 7*).

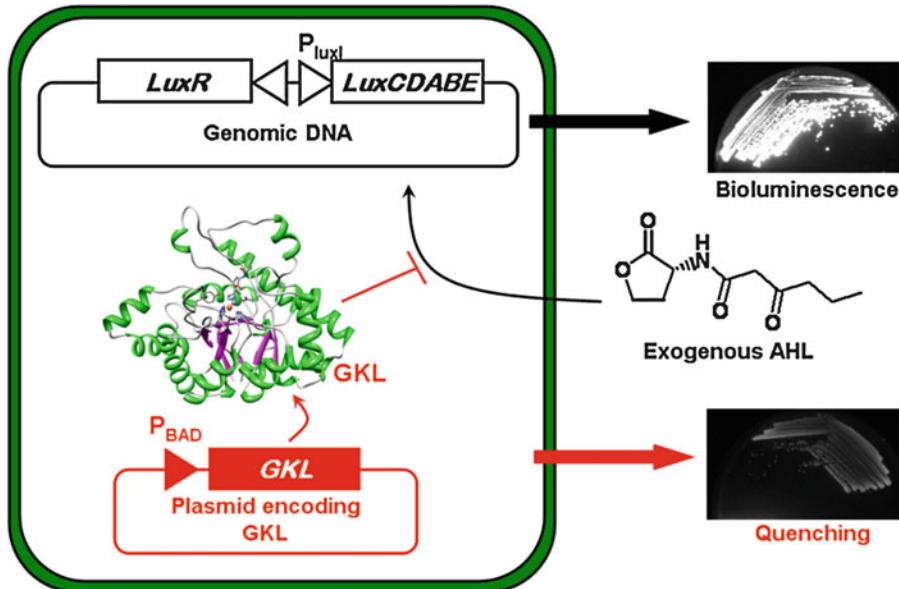


Fig. 1 Schematic representation of the quorum-quenching molecular circuit

3.4 Construction of a Quorum-Quenching Directed Evolution Platform (Fig. 1)

3.4.1 Amplification and Cloning of the luxR-luxCDABE Cassette

1. Amplify the *luxR-luxCDABE* cassette from the pAL103 vector [8] by PCR using the oligonucleotides Lux forward primer and Lux reverse primer.
2. Amplify the gene using the following parameters: 98 °C for 2 min followed by 30 cycles of 98 °C for 10 s, 55 °C for 15 s, and 72 °C for 15 s, and a final extension of 72 °C for 10 min.
3. Check gene amplification by agarose gel analysis as indicated in Subheading 3.1, steps 3–6. In this case, the expected amplicon is of approximately 6800 bp.
4. Purify the band from agarose gel by using a commercial kit.
5. Digest the eluted PCR product and the pUC18R6K-mini-Tn7T-Gm^R vector [1] by using the XhoI and KpnI restriction enzymes. Follow the same procedure for DNA digestion, purification, ligation, and *E. coli* transformation as in Subheading 3.1, steps 7–11.
6. In this case, verify the presence of the *luxR-luxCDABE* cassette inside the pUC18R6K-mini-Tn7T-Gm^R vector by restriction analysis performed with the XhoI and KpnI restriction enzymes on plasmids extracted by the transformed *E. coli* cells by means of a commercial kit.

3.4.2 Integration of the luxR-luxCDABE Cassette into *E. coli* JLD271 Cells

Co-transform the plasmid prepared in Subheading 3.4.1 and the helper plasmid pTNS2 [1] in *E. coli* JLD271 cells by electroporation.

1. Add 50 ng of the LuxR-LuxCDABE/pUC18R6K-mini-Tn7T-Gm^R plasmid and 50 ng of the pTNS2 plasmid into 100 µl of *E. coli* JLD271 electrocompetent cells (*see Note 8*) and transfer the cell suspension into a 0.2 cm gap-width electroporation cuvette.
2. Electroporate at 2.5 kV and immediately add 1 ml of LB broth. Incubate with shaking for 1 h at 37 °C.
3. Plate 100 µl of the cell suspension onto a selective LB agar plate supplemented with 10 µg/ml gentamycin. Incubate at 37 °C overnight.
4. Pick five to ten colonies that grew on the selective plate and screen for the insertion of the *luxR-luxCDABE* cassette at the *att*Tn7 by PCR using 300 nM Gm-up primer and 300 nM Gm-down primer. Use the following thermocycler parameters: 95 °C for 5 min followed by 30 cycles of 95 °C for 45 s, 59 °C for 30 s, and 72 °C for 20 s, and a final extension of 72 °C for 10 min.
5. Check gene amplification by agarose gel analysis as indicated in Subheading 3.1, steps 3–6. In this case, the expected amplicon is of 6800 bp.
6. For positive clones, extract the genomic DNA by using a commercial kit and verify chromosomal insertion of the *luxR-luxCDABE* cassette by sequencing.

3.4.3 Subcloning of the *gkl* Gene into a Modified pBAD33 Plasmid

1. Digest the GKL/pET15b plasmid prepared in Subheading 3.1, step 16, and modified pBAD33 plasmid with the restriction enzymes NdeI and BamHI. Follow the same procedure for DNA digestion, purification, ligation, and *E. coli* transformation as in Subheading 3.1, steps 7–11.
2. In this case, verify the presence of the *gkl* gene inside the modified pBAD33 vector by restriction analysis performed with the NdeI and BamHI restriction enzymes on plasmids extracted by the transformed *E. coli* cells by means of a commercial kit.

3.4.4 Generation of the GKL Quorum-Quenching Molecular Circuit

1. Transform the GKL/pBAD33 plasmid into chemically competent *E. coli* JLD271 reporter cells (generated in Subheading 3.4.2), as described in Subheading 3.3, step 1. In this case, use LB agar plates supplemented with 30 µg/ml chloramphenicol as selective plates. Incubate the plates at 37 °C for 16–24 h.
2. Pick five to ten colonies grown on the selective plates and streak them each onto LB agar plates supplemented with 30 µg/ml chloramphenicol, 0.02% (wt/vol) arabinose, and an AHL substrate (*see Note 4*). Incubate the plates overnight at 37 °C.
3. Detect bioluminescence using a luminescent image analysis system. Set exposure time ranging from 5 s to 1 min.

3.5 Directed Evolution of GKL

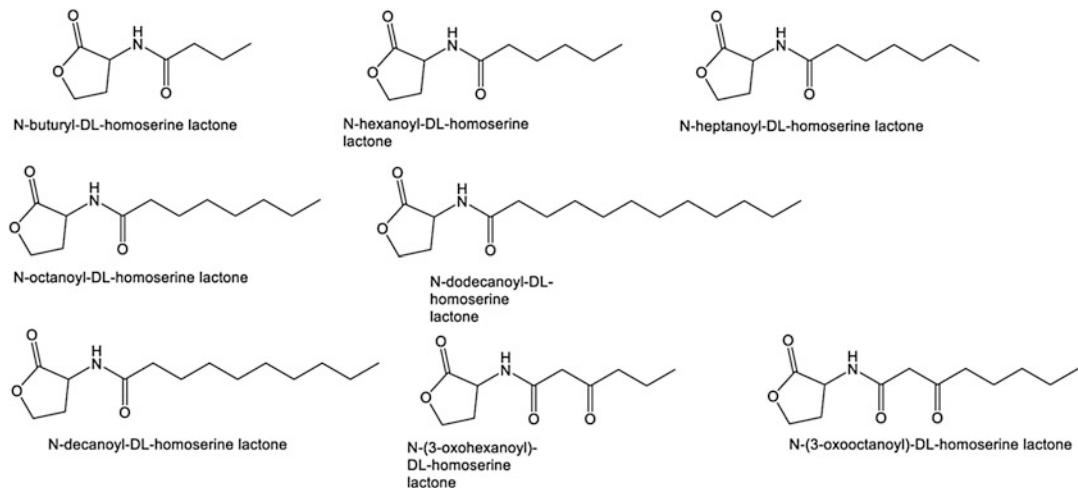
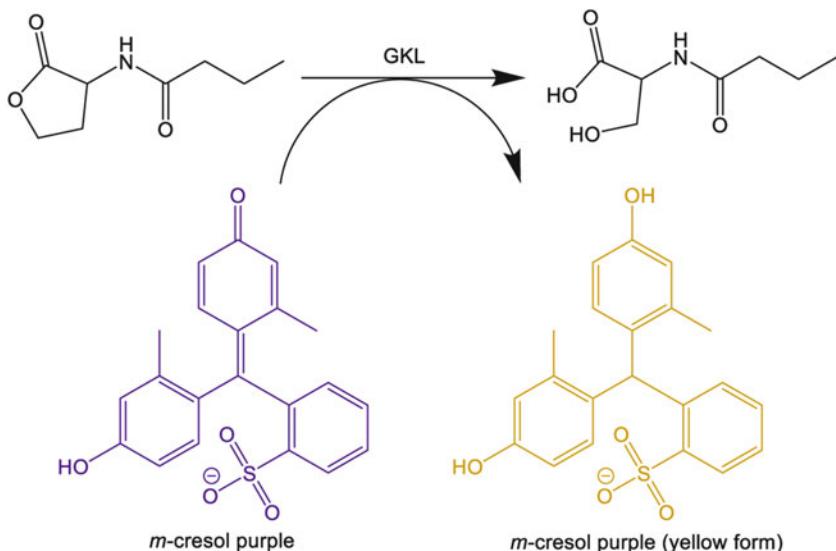
The generation of *gkl* mutated genes is obtained by PCR amplification with a blend of two error-prone DNA polymerases, Mutazyme II (Agilent).

1. In a 200 μ l PCR tube, add the following: 41.5 μ l H₂O, 5 μ l of 10× Mutazyme II reaction buffer, 1 μ l of 40 mM dNTP mix, 0.25 μ l of 250 ng/ μ l GKL forward primer, 0.25 μ l of 250 ng/ μ l GKL reverse primer, 1 μ l of 2.5 U/ μ l Mutazyme II DNA polymerase, 1 μ l of 450 ng/ μ l PCR product encompassing wild-type *gkl* from Subheading 3.1, step 2 (see Note 9).
2. Spin-down the samples in a benchtop microcentrifuge.
3. Amplify the samples in a thermocycler with the following parameters: 95 °C for 2 min followed by 30 cycles of 95 °C for 30 s, 64 °C for 30 s, and 72 °C for 1 min, and a final extension of 72 °C for 10 min.
4. Quantitate the PCR yield by spectrophotometric analysis at 260 nm with quartz cuvettes.
5. Clone the PCR products into pBAD33 via NdeI-BamBI restriction as previously indicated for wild-type *gkl*.
6. Transform the mutant plasmid library into electrocompetent *E. coli* XL1 Blue cells (Stratagene). Add the cell suspension into a 0.2 cm gap-width electroporation cuvette and electroporate at 1.8 kV.
7. Immediately add 1 ml of LB medium and incubate with shaking for 1 h at 37 °C.
8. Plate 100 μ l of the cell suspension onto an LB agar plate supplemented with 30 μ g/ml chloramphenicol. Incubate at 37 °C overnight.
9. Resuspend the resulting colonies in 5 ml LB broth supplemented with 30 μ g/ml chloramphenicol. Grow overnight at 37 °C with shaking, harvest the cells by centrifugation, and purify the plasmids using a commercial kit for plasmid DNA extraction from bacterial cells.
10. Transform the library of mutant plasmids into 50 μ l of chemically competent *E. coli* JLD271 reporter cells, as described in Subheading 3.3, step 1. In this case, use LB agar plates supplemented with 30 μ g/ml chloramphenicol as selective plates. Incubate the plates at 37 °C for 16–24 h.
11. Streak the resulting colonies onto LB agar plates supplemented with 30 μ g/ml chloramphenicol, 0.02% (wt/vol) arabinose, and an AHL substrate (see Note 4). Incubate the plates overnight at 37 °C.
12. Detect for enhanced quorum-quenching activity by bioluminescence (see Subheading 3.4.4, step 3).

13. Pick colonies that have enhanced quorum-quenching activity relative to the *E. coli* JLD271 reporter cells expressing wild-type GKL.
14. Grow selected colonies overnight at 37 °C in 5 ml of LB broth supplemented with 30 µg/ml chloramphenicol.
15. Harvest the cells by centrifugation and extract the plasmid by using a commercial kit for plasmid DNA extraction from bacterial cells.
16. Sequence the plasmids to obtain the DNA sequence of the *gkl* mutant genes with increased lactonase activity.

4 Notes

1. Transform 1 µl of the commercial pET15b vector into 50 µl of chemically competent *E. coli* XL1 Blue cells (Stratagene). Pick a colony and grow in 5 ml LB broth supplemented with 100 µg/ml ampicillin. Prepare a frozen stock of the cells by adding 850 µl of the culture to 250 µl of sterile glycerol. Store the stock in –80 °C. The stock will be stable for at least 3 years. You may prepare other vectors in the same manner.
2. You may prepare a batch of chemically competent cells instead of using the commercially prepared cells. Grow overnight at 37 °C a 5 ml LB culture of the *E. coli* cells that you need to use (for XL1 Blue, grow in LB supplemented with 10 µg/ml tetracycline). Inoculate a 100 ml LB culture the next day with 200 µl of the starter culture. Grow the culture at 37 °C with shaking until the OD at 600 nm reaches 0.4–0.8. Harvest the cells by centrifugation at 6000 × g at 4 °C for 10 min. Gently resuspend the cells in ice-cold water with 10% (vol/vol) glycerol and 0.1 M CaCl₂. Do not vortex. Repeat this step three times. Resuspend the cells in 500 µl of ice-cold water with 10% (vol/vol) glycerol and aliquot 60 µl of the cells in different tubes. Freeze the tubes at –80 °C. The cells will be stable for at least 3 years. Thaw cells on ice prior to use.
3. Homoserine lactones have different solubility in DMSO and H₂O. Their solubility ranges from 100 to 500 mM in DMSO and from 0.2 to 5 mM in H₂O. You need to check test the solubility of the homoserine lactone that you want to use first before proceeding with the experiment. The homoserine lactones used in this project are shown in Fig. 2.
4. Different homoserine lactones have different activity with GKL. You need to test their activities with GKL first before proceeding with the experiment.
5. DMSO will inactivate the enzyme at high concentrations. Add a maximum of 1% (vol/vol) DMSO in the reaction. This will

**Fig. 2** Chemical structures of different AHLs**Fig. 3** Schematic representation of the reactions carried out by the GKL lactonase. The assay used to quantify GKL activity is based on the GKL-dependent conversion of *m*-cresol purple to the yellow form (maximum absorbance at 577 nm)

limit the amount of homoserine lactone that you may use in the assay.

6. The kinetic assay uses the change in absorbance at 577 nm of *m*-cresol purple (Fig. 3).
7. You may use graphing programs such as Prism or Enzfitter to determine the kinetic parameters.

8. You may prepare a batch of electrocompetent cells instead of using the commercially prepared cells. Grow overnight at 37 °C a 5 ml LB culture of the *E. coli* cells that you need to use (for XL1 Blue, grow in LB supplemented with 10 µg/ml tetracycline). Inoculate a 100 ml LB culture the next day with 200 µl of the starter culture. Grow the culture at 37 °C with shaking until the OD at 600 nm reaches 0.4–0.8. Harvest the cells by centrifugation at 6000 × g at 4 °C for 10 min. Gently resuspend the cells in ice-cold water with 10% (vol/vol) glycerol. Do not vortex to resuspend the cells. Repeat this step three times. Resuspend the cells in 500 µl ice-cold water with 10% (vol/vol) glycerol and aliquot 100 µl in separate tubes. Freeze the tubes at –80 °C. The cells will be stable for at least 3 years. Thaw cells on ice prior to use.
9. The mutation frequency of the protocol used for directed evolution is three mutations per kb. You may increase or decrease the mutation rate by varying the amount of template. Please refer to the GeneMorph II Random Mutagenesis Kit (Agilent) for more information.

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Chapter 25

Generation of High-Sensitivity Monoclonal Antibodies Specific for Homoserine Lactones

Soumya Palliyil

Abstract

A number of bacteria use a class of chemical compounds called acyl-homoserine lactones (AHLs) as quorum sensing (QS) signals to coordinate their behavior at the population level, including pathogens like *Pseudomonas aeruginosa*. Blocking QS using antibodies is an attractive strategy for infection control as this process takes a central role in *P. aeruginosa* infections. Here the methods involved in the generation of high sensitivity anti-QS monoclonal antibodies from an immunized sheep phage display antibody library are described. A panel of AHL compounds conjugated to carrier proteins are used for sheep immunization and a phage display antibody library is constructed using the immune repertoire of sheep as a source of antibody genes. High sensitivity single chain antibody fragments (scFv) are isolated from the library using “smart selection strategies” and reformatted into single chain antibodies (scAbs). The resultant monoclonal antibodies: (1) recognize HSL compounds at low nanomolar concentrations; (2) have the potential to reduce virulence gene expression in *P. aeruginosa*; and (3) offer protection in a nematode model of infection.

Key words *Pseudomonas aeruginosa*, Quorum sensing, Acyl-homoserine lactones, Monoclonal antibodies, Anti-infective antibodies

1 Introduction

Many Gram-negative bacteria rely on acyl-homoserine lactones (AHLs) as QS signal molecules [1, 2]. The human pathogen *Pseudomonas aeruginosa* has two AHL-dependent QS systems: the *las* and the *rhl* systems, relying on *N*-3-oxododecanoyl-homoserine lactone (3OC₁₂-HSL) and *N*-butyryl-homoserine lactone (C₄-HSL) as signal molecules, respectively [3]. These molecules positively control the expression of extracellular virulence factors associated with *P. aeruginosa* infection and biofilm formation [3]. The extracellular distribution of QS signals like AHLs make them ideal targets for anti-infective therapy since the evolutionary pressure on bacteria to develop resistance will be limited [2]. Monoclonal antibody mediated inhibition of the QS process may offer exquisite

target specificity and less off-target cytotoxicity [2]. Quorum quenching using antibodies would not only block the signalling cascade and virulence factors production, but also neutralize 3OC₁₂-HSL, a molecule that beyond its action as QS signal has immunomodulatory effects important for *P. aeruginosa* infections [4].

Displaying antibody binding sites (proteins or peptides) on the surface of bacteriophage by fusing the antibody gene to one of the phage coat proteins and its selection based on the antigen binding of individual clones is generalized as phage display. Phage display antibody libraries are constructed by PCR-based cloning of the antibody heavy chain variable domain (VH) and light chain variable domain (VL) repertoires by random pairing into a phage or phagemid vector system and displayed on the surface of bacteriophage to facilitate the combinatorial selection of the best antibody binding site. This procedure has been widely used in the antibody engineering field as a technique to mimic B cells, which in the body act as self-replicating systems containing antibody genes encoding a specific antibody displayed on its surface [5]. A key feature of the phage display approach is the similar linking of genotype (phagemid vector) and phenotype (phage coat protein-antibody fragment), which allows immediate access to the corresponding gene sequences of selected antibodies. The sequences encoding the epitope binding sites can be easily subcloned into various antibody formats based on downstream applications. Phage display technologies have been successfully used for the development of therapeutic antibody candidates over the last 20 years with more and more products now also entering the diagnostic and research markets [6].

This chapter describes the methods involved in the construction of an immunized sheep phage display library for the generation of antibodies with high affinity and sensitivity towards AHL signal molecules and for the characterization of their binding properties using ELISA based techniques. The overall procedure used to generate the anti-AHL antibodies is schematized in Fig. 1. In brief, sheep are immunized with AHLs conjugated to carrier proteins and antibody genes derived from the immunized sheep are used to generate a phage display antibody library. High sensitivity single chain antibody fragments (scFv) are isolated from the library using “smart selection strategies” and reformatted into single chain antibodies (scAbs).

The neutralization potential and protective effect of anti-quorum sensing antibodies is validated by monitoring the inhibition of a main QS-dependent virulence factor of *P. aeruginosa* (i.e., elastase) and of *P. aeruginosa* virulence in a simple *in vivo* infection model (i.e., slow killing of the nematode *Caenorhabditis elegans*).

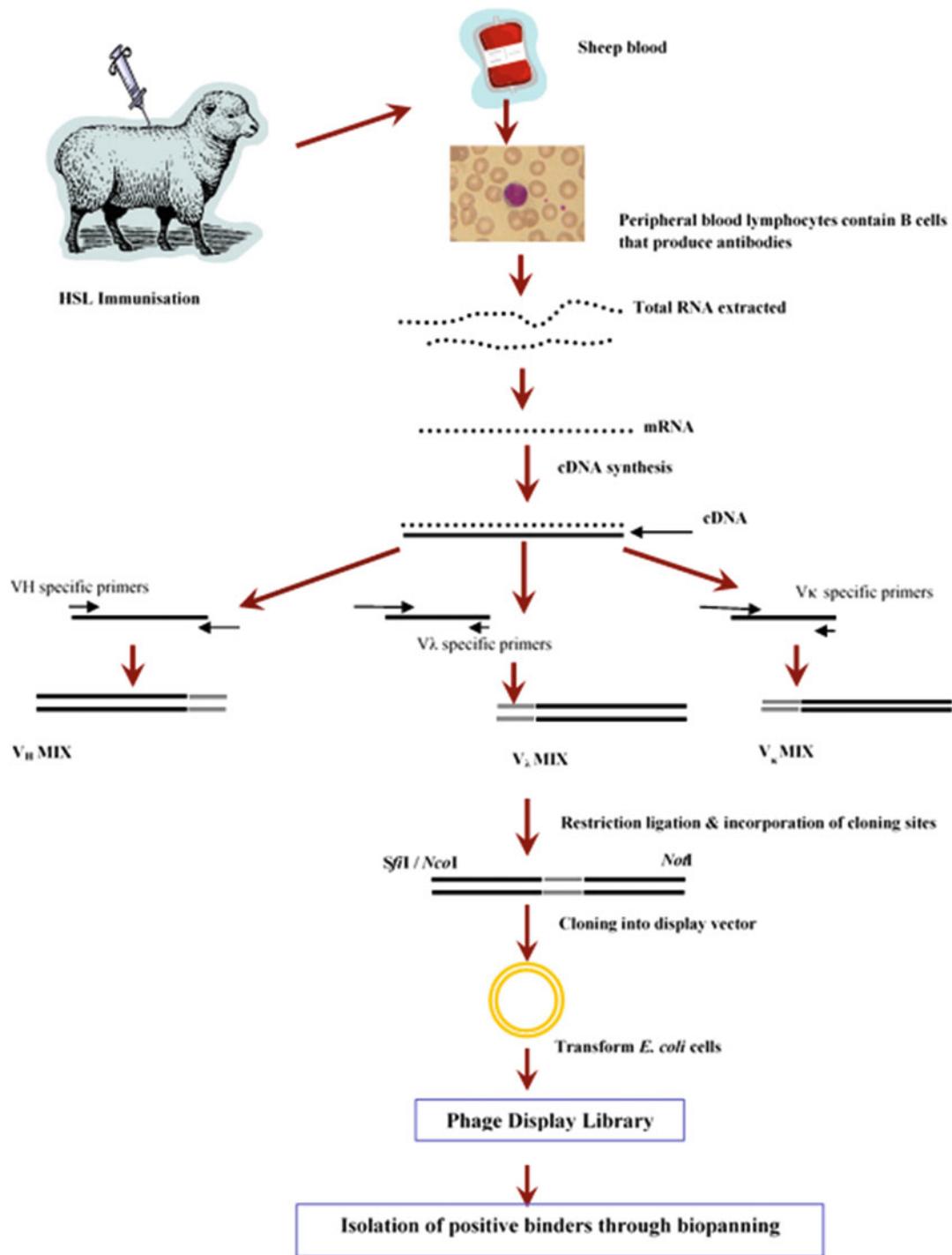


Fig. 1 Diagrammatic representation of various steps involved in the construction of an immunized library

2 Materials

All solutions and media should be sterilized by autoclaving and prepared using water purified to 18.2 MΩ/cm.

2.1 Buffers and Solutions

1. Tris Acetate Ethylenediaminetetraacetic acid (EDTA) buffer (TAE) stock solution (50×): dissolve 242 g Tris-base and 18.5 g EDTA disodium salt in 500 mL H₂O while stirring; add 57.1 mL glacial acetic acid and bring the volume to 1 L with H₂O.
2. 10× DNA loading dye: 30% (vol/vol) glycerol in TAE buffer 1×. Add 0.3 mg/mL Xylenol Orange dye, mix well, and sterilize by filtration.
3. 0.1 M sodium phosphate buffer, pH 7.0: mix 57.7 mL of Na₂HPO₄ and 42.3 mL of NaH₂PO₄ and bring the volume to 1 L with H₂O.
4. Elastin-Congo Red suspension: 2 g/mL of Elastin-Congo red dissolved in 10 mM sodium phosphate buffer, pH 7.0
5. M9 buffer: 15.1 g/L Na₂HPO₄, 3 g/L KH₂PO₄, 5 g/L NaCl, 0.25 g/L MgSO₄·7H₂O.
6. Phosphate-buffered saline (PBS): dissolve 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na₂HPO₄, and 0.24 g of KH₂PO₄ in 800 mL of H₂O. Adjust pH to 7.4 with HCl. Add H₂O to 1 L. Sterilize by autoclaving.
7. MPBS: PBS containing 2% (wt/vol) skim milk powder.
8. ELISA stop solution: 1 M H₂SO₄.
9. Fractionation buffer: 200 mM Tris-HCl, 20% (wt/vol) sucrose, 1 mM EDTA, and 0.05 mg/mL lysozyme.
10. Ampicillin (Ap) stock solution: 50 mg/mL Ap dissolved in H₂O. Sterilize by filtration and store at -20 °C.
11. Kanamycin (Km) stock solution: 25 mg/mL Km dissolved in H₂O. Sterilize by filtration and store at -20 °C.
12. Glucose stock solution: 25% (wt/vol) glucose dissolved in H₂O. Sterilize by filtration and store at +4 °C.
13. PEG-NaCl solution: 20% (wt/vol) PEG 6000, 2.5 M NaCl.
14. Potassium phosphate stock solution for Terrific Broth (TB) medium: 23.1 g/L KH₂PO₄, 125.4 g/L K₂HPO₄. Sterilize by filtration.
15. Cholesterol stock solution: 5 mg/mL cholesterol dissolved in ethanol. Sterilize by filtration.
16. Nematode bleaching solution: add 200 μL of bleach and 25 μL of 4 M NaOH or KOH to 775 μL of H₂O.

2.2 Culture Media

1. 2× Tryptone-Yeast (TY) medium: 16 g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl. Add 2% (wt/vol) glucose and Ap 100 µg/mL after autoclaving.
2. Tryptone-Yeast extract (TYE) agar: 10 g/L tryptone, 5 g/L yeast extract, 8 g/L NaCl, 20 g/L agar. Add 2% (wt/vol) glucose and 100 µg/mL Ap after autoclaving.
3. Luria-Bertani (LB) broth: 10 g/L NaCl, 10 g/L tryptone, 5 g/L yeast extract. Sterilize by autoclaving.
4. Luria-Bertani (LB) agar: LB plus 15 g/L agar. Sterilize by autoclaving.
5. Terrific Broth (TB) medium: dissolve 12 g tryptone, 24 g yeast extract, and 4 mL glycerol in 900 mL of H₂O. After autoclaving, add aseptically 100 mL of potassium phosphate stock solution for TB medium.
6. Nematode Growth Medium (NGM) agar: dissolve 2.4 g of NaCl, 2 g of peptone, and 13.6 g of agar in 777 mL of H₂O. After autoclaving cool to 50 °C and add pre-sterilized 0.8 mL cholesterol stock solution, 0.8 mL 1 M CaCl₂, 0.8 mL 1 M MgSO₄, and 20 mL 1 M KH₂PO₄ pH 6.0.
7. Nematode Growth Medium (NGM) extra-peptone (EP) agar: dissolve 4 g of NaCl, 6 g of peptone, 1.2 g of yeast extract, and 16 g of agar in 777 mL of H₂O and autoclave. After autoclaving cool to 50 °C and add pre-sterilized 0.8 mL cholesterol stock solution, 0.8 mL 1 M CaCl₂, 0.8 mL 1 M MgSO₄, and 20 mL 1 M KH₂PO₄ pH 6.0.

2.3 Bacteria, Phages, and Nematodes

1. For construction of an immunized phage display library, electrocompetent suppressor *Escherichia coli* TGI cells {*SupE thi-1* Δ(*lac-proAB*)Δ(*mcrB-hsdSM*) 5 (*rk-* *mk-*) [F' *traD36 proAB lacIq ZΔM15*] } are used.
2. For the expression of recombinant proteins, recombinant-deficient strain *E. coli* XL-1 Blue cells {*endA1 supE44 thi-1 hsdR17 recA1 gyrA96 relA1 lac* [F' *proAB lacIq ZΔM15 Tn10 (Tetr)*] } are used.
3. *P. aeruginosa* strains PAO1 and PA14, and the clinical isolate strain 04.232058R (referred as PA058 hereafter) used for *Cae-norhabditis elegans* slow killing assay, were kindly donated by Prof. Tim Mitchell, University of Glasgow, UK.
4. The helper phage M13KO7 carries a modified gene II from M13mp1 (G changed to T at position 6125, giving a Met to Ile change at codon 40 of the gene II protein) and a plasmid origin of replication (*ori*) derived from p15A. The mutated gene II products interact less efficiently with its own phage *ori* than

with the phagemid *ori* of replication. It also carries a gene for Km resistance to aid selection of infected bacteria.

5. Wild-type *Caenorhabditis elegans* strain Bristol N2.

2.4 Antibodies

1. Anti-M13 monoclonal HRP conjugate antibody (GE Health-care, 27-9421-01).
2. Donkey anti-sheep IgG (whole molecule) antibody HRP (Sigma, A3415).
3. Donkey anti-sheep IgG (whole molecule) antibody (Sigma, S2763).
4. Goat anti-human C κ (bound and free) antibody (Sigma, K-3502).
5. Goat anti-human C κ light chain (bound and free) peroxidase antibody (Sigma, A7164).
6. Human whole IgG standard (κ isotype) (Sigma, I2511).
7. Sheep IgG standard (Sigma, I5131).

2.5 Antigen Conjugates and Free Antigens

1. *N*-Dodecanoyl-homoserine lactone (C₁₂-HSL), *N*-3-oxododecanoyl-homoserine lactone (3OC₁₂-HSL), and *N*-3-hydroxydodecanoyl-homoserine lactone (3OHC₁₂-HSL) are commercially available or can be custom synthesized by different companies.
2. Synthetic analogues of C₁₂-HSL, 3OC₁₂-HSL, and 3OHC₁₂-HSL with a carboxylic group at the end of the acyl side-chain were custom synthesized (see Note 1) and coupled to bovine serum albumin (BSA) or bovine thyroglobulin (TG) via the carboxylic group [7].

2.6 Plasmids

1. Phagemid vector pHEN2a can be used for phage display library construction. This is a pUC119-based phagemid vector derived from pHEN1 with origins of replication from ColE1 and M13 to facilitate the growth in *E. coli* and packaging in filamentous phage, respectively [8]. Restriction sites SfiI/NcoI and NotI permit cloning of antibody fragments fused in between the *pelB* leader sequence and the phagemid gene III protein sequence. A hexa-histidine tag and c-myc tag are present after the NotI cloning site, followed by an amber stop codon introduced by site-directed mutagenesis. When expressed in a non-suppressor strain of *E. coli*, the amber codon is read as a stop and the expressed single chain Fv can be easily purified and detected using the hexa-histidine tag or the c-myc tag.
2. The expression vector pIMS147 allows improving the solubility of single chain antibody fragments by co-expressing the *E. coli* Skp chaperone [9]. This dicistronic vector is inducible with

isopropyl β -D-thio galactosidase (IPTG) and contains a human C κ domain downstream of the cloned antibody fragment that facilitates immunodetection and purification of the recombinant protein, and also improves its solubility.

2.7 Oligonucleotides

2.7.1 Routine PCR Reactions and Sequencing

Degenerate nucleotide code (M = A/C, R = A/G, W = A/T, S = G/C, Y = C/T, K = G/T).

1. AH18 REV: 5'-AAA TAC CTA TTG CCT ACG GCA GCC GCT GG-3'.
2. GIII FOR: 5'-GAA TTT TCT GTA TGA GGT TTT GC-3'.
3. HuC κ forward: 5'-GAA GAT GAA GAC AGA TGG TGC-3'.

1. Ovine heavy chain constant region 3' primer.
 (a) OvCHFOR: 5'-GAC TTT CGG GGC TGT GGT GGA GGC-3'.
2. Ovine kappa chain constant region 3' primer.
 (a) OvCKFOR: 5'-GA TGG TTT GAA GAG GGA GAC GGA TGG CTG AGC-3'.
3. Ovine lambda chain constant region 3' primer.
 (a) OvCLFOR: 5'-A CAG GGT GAC CGA GGG TGC GGA CTT GG-3'.
4. Ovine heavy chain variable region 5' primers.
 (a) OvVH1BACK: 5'-CAG GTK CRR CTG CAG GRG TCG GG-3'.
 (b) OvVH2BACK: 5'-CAG GTK CAG YTK CAG GAG TCG GG-3'.
 (c) MuVH1BACK: 5'-SAG GTS MAR CTG CAG SAG TCW GG-3'.
 (d) Hu4aBACK: 5'-CAG GTG CAG CTG CAG GAG TCG GG-3'.
5. Ovine heavy chain variable region 3' primers.
 (a) OvJH1LINKFOR: 5'-CTC AGA AGG CGC GCC AGA AGA TTT ACC TTC TGA GGA GAC GGT GAC CAG GAG TCC-3'.
 (b) OvJH2LINKFOR: 5'-CTC AGA AGG CGC GCC AGA AGA TTT ACC TTC TGA GGA GRC GGW GAY YAG KAG TCC-3'.
 (c) OvJH3LINKFOR: 5'-CTC AGA AGG CGC GCC AGA AGA TTT ACC TTC TGA GGA GAY RGT RAS CAG GAS TCC-3'.
 (d) OvJH4LINKFOR: 5'-CTC AGA AGG CGC GCC AGA AGA TTT ACC TTC TGA AAG AAC GCT GAT CAG GAG-3'.

6. Ovine lambda chain variable region 5' primers.

- (a) OvVL1LINKBACK: 5'-AG TCA AAC GCG TCT GGC GAG TCT AAA GTG GAT GAC CAG GCT GTG CTG ACT CAG CCG-3'.
- (b) OvVL2LINKBACK: 5'-AG TCA AAC GCG TCT GGC GAG TCT AAA GTG GAT GAC CAR GCT GTG CTG ACY CAR CYG-3'.
- (c) OvVL3LINKBACK: 5'-AG TCA AAC GCG TCT GGC GAG TCT AAA GTG GAT GAC CAG GCY STG STG ACT CAG CCR-3'.
- (d) OvVL4LINKBACK: 5'-AG TCA AAC GCG TCT GGC GAG TCT AAA GTG GAT GAC MRG GTC RTG CKG ACT CAR CCG-3'.
- (e) OvVL5LINKBACK: 5'-AG TCA AAC GCG TCT GGC GAG TCT AAA GTG GAT GAC CAG KCT GYS CTG ACT CAG CCK-3'.

7. Ovine lambda chain variable region 3' primers.

- (a) OvJL1FOR: 5'-ACC CAG GAC GGT CAG CCT GGT CC-3'.
- (b) OvJL2FOR: 5'-ACC CAG GAC GGT CAG YCK RGW CC-3'.

8. Ovine kappa chain variable region 5' primers.

- (a) OvVK1LINKBACK: 5'-AG TCA AAC GCG TCT GGC GAG TCT AAA GTG GAT GAC GAC ATC CAG GTG ACC CAG TCT CCA-3'.
- (b) OvVK2LINKBACK: 5'-AG TCA AAC GCG TCT GGC GAG TCT AAA GTG GAT GAC GAC ATC CAG CTC ACC CAG TCT CCA-3'.

9. Ovine kappa chain variable region 3' primers.

- (a) OvJK1FOR: 5'-CCG TTT GAT TTC CAC GTT GGT CC-3'.
- (b) OvJK2FOR: 5'-CCT TTT GAT CTC TAG TTT GGT TCC-3'.
- (c) OvJK3FOR: 5'-CCT TTT GAT CTC TAC CTT GGT TCC-3'.

10. Cloning primers (restriction sites underlined).

- (a) OvVH1BACK-*Sf*: 5'-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC CAG GTK CRR CTG CAG GRG TCG GG-3'.
- (b) OvVH2BACK-*Sf*: 5'-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC CAG GTK CAG YTK CAG GAG TCG GG-3'.

- (c) MuVH1BACK-*Sf*: 5'-CAT GCC ATG ACT GCG GCC
CAG CCG GCC ATG GCC SAG GTS MAR CTG CAG
SAG TCW GG-3'.
- (d) Hu4aBACK-*Sf*: 5'-GTC CTC GCA ACT GCG GCC
CAG CCG GCC ATG GCC CAG GTG CAG CTG
CAG GAG TCG GG-3'.
- (e) OvJL1FOR-*Not*: 5'-GAG TCA TTC TCG ACT TGC
GGC CGC ACC CAG GAC GGT CAG CCT GGT
CC-3'.
- (f) OvJL2FOR-*Not*: 5'-GAG TCA TTC TCG ACT TGC
GGC CGC ACC CAG GAC GGT CAG YCK RGW
CC-3'.
- (g) OvJK1FOR-*Not*: 5'-GAG TCA TTC TCG ACT TGC
GGC CGC CCG TTT GAT TTC CAC GTT GGT
CCC-3'.
- (h) OvJK2FOR-*Not*: 5'-GAG TCA TTC TCG ACT TGC
GGC CGC CCT TTT GAT CTC TAG TTT GGT
TCC-3'.
- (i) OvJK3FOR-*Not*: 5'-GAG TCA TTC TCG ACT TGC
GGC CGC CCT TTT GAT CTC TAC CTT GGT
TCC-3'.

3 Methods

3.1 Production and ELISA Validation of Anti-QS Polyclonal Antisera

In order to construct an immunized antibody phage display library, two Welsh breed sheep are hyper-immunized with AHLs conjugated to thyroglobulin (TG) (*see Note 1*) as described below.

1. A mixture of three AHL-TG conjugates, i.e., C₁₂-HSL-TG, 3OC₁₂-HSL-TG, and 3OHC₁₂-HSL-TG are injected into two sheep. For primary immunization, 500 µg of AHL-conjugate mixture is made up to a 2 mL volume in PBS and administered per sheep. For subsequent re-boots (after 4, 8, 12, 16, 20, and 24 weeks from the primary immunization), 250 µg of AHL-conjugate mixture in 2 mL of PBS is used per sheep.
2. Serum samples are taken at weeks 6, 10, 14, 18, and 22 for preliminary analyses, while the production bleed is collected 3–5 days after the final boost at week 24.
3. Purification of sheep polyclonal sera are performed by means of commercial resins that allow removing nonrelevant proteins often present in high abundance in the serum, according to manufacturer's instructions (*see Note 2*). After purification, antibodies are dialyzed 1:200 against PBS to remove any remaining trace of purification buffer and stored at 4 °C until needed.

4. Quantification of sheep polyclonal IgG concentration by Capture ELISA. A 96-well flat bottomed ELISA plate is incubated with 100 µL per well of 1 µg/mL anti-sheep IgG antibody and incubated at 37 °C for 1 h. After washing the plates three times with 200 µL of PBS containing 0.1% (vol/vol) Tween 20, and three times with 200 µL of PBS, the wells are blocked with 200 µL of MPBS and incubated at 37 °C for 1 h.
5. Purified polyclonal sheep serum from **step 3** is diluted 1:1000 in PBS, and 200 µL of this solution are added to desired wells (in duplicate) and double diluted across the plate.
6. In order to produce a standard curve, sheep IgG standard with a starting concentration of 4 µg/mL is double diluted in PBS across the plate.
7. The plate is incubated at room temperature for 1 h and washed as described in **step 4**.
8. Dilute anti-sheep IgG antibody HRP conjugate 1:1000 in PBS and add 100 µL of this dilution per well.
9. Incubate for 1 h at room temperature, wash the plate as described in **step 4**.
10. The plate is developed by adding 100 µL per well of 1-Step Ultra TMB-ELISA substrate solution (Thermo Fisher Scientific). The reaction is stopped by adding 50 µL per well of 1 M H₂SO₄ and the optical density of the wells is measured at 450 nm wavelength with a multiplate spectrophotometer. The polyclonal IgG concentration is calculated based on the standard curve plotted from the absorbance values of Sheep IgG at known concentrations.
11. Polyclonal sheep sera Binding ELISA. 100 µL aliquots of PBS containing 1 µg/mL AHL-TG conjugates are dispensed in a high-binding affinity polystyrene 96-well flat bottomed ELISA plate. The plate is incubated at 37 °C for 1 h (or at 4 °C overnight). A control plate incubated with 1 µg/mL BSA and TG is included to check the nonspecific binding of the polyclonal sera. The plates are washed as described in **step 4**, blocked with 200 µL of MPBS per well and incubated at 37 °C for 1 h. The washing step is repeated as before and then 200 µL of crude or purified sheep polyclonal serum (obtained in **step 3**) are added to designated wells, double diluted in PBS across the plate, and incubated at room temperature for 1 h. The plates are washed as described in **step 4**, and 100 µL of anti-sheep IgG antibody HRP conjugate diluted 1:1000 in PBS is added to the wells as secondary antibody. The plate is incubated for 1 h at room temperature. Afterwards the wells are washed as indicated in **step 4** and developed as described in **step 10**.

12. Indirect competition ELISA. 100 μ L aliquots of PBS containing 1 μ g/mL AHL-TG conjugates are dispensed in a polystyrene 96-well flat bottomed ELISA plate. The plate is incubated at 37 °C for 1 h (or at 4 °C overnight). The plate is washed twice with 250 μ L PBST and twice with 250 μ L PBS, blocked with 200 μ L of MPBS per well, and incubated at 37 °C for 1 h. Twofold sequential dilutions of free AHL compound solution (free antigen) in PBS (125 μ L) are prepared in microfuge tubes and mixed with an equal volume of a sub-saturating concentration of the polyclonal sera purified in **step 3** as determined from binding profiles. PBS is used in place of free antigen to give a 100% signal (non-competitive binding). The reactions are incubated for 1 h at room temperature. The wells are washed twice with 250 μ L PBST and twice with 250 μ L PBS. Then, 100 μ L of each sample containing free HSL compound and polyclonal antiserum or the control corresponding to PBS and polyclonal antiserum control is added to replicate wells and incubated for 1 h at room temperature. The plate is washed twice with 250 μ L PBST and twice with 250 μ L PBS. Then, 100 μ L anti-sheep IgG antibody HRP conjugate diluted 1:1000 in PBS are added to the wells as the secondary antibody and incubated for 1 h at room temperature. The wells are washed as described in **step 4** and developed as described in **step 10**. The absorbance value (A_{450}) obtained for polyclonal sera incubated with PBS represents 100% binding. This value is used to determine the % binding for polyclonal serum in the presence of free antigen. The sensitivity of polyclonal sera against free antigen can be represented as IC₅₀ and IC₂₀ values. The IC₅₀ value is defined as the concentration of free antigen required to reduce the binding of the antibody to HSL conjugate by 50%, and IC₂₀ as the free antigen concentration of free antigen required to reduce the binding of the antibody to HSL conjugate by 20%.

3.2 Anti-Virulence Activity of the Anti-QS Polyclonal Sera

3.2.1 Elastase Assay Using Anti-QS Polyclonal Sera

Elastase production can be measured using the published method [10] with some modifications.

1. An overnight culture of *P. aeruginosa* PA14 grown in LB broth at 37 °C with shaking at 200 rpm is diluted to an absorbance at 600 nm wavelength (A_{600}) of 0.05 in two 15 mL tubes each containing 2 mL of LB broth. Culture of *E. coli* OP50 (non-elastase producing strain) are prepared in a similar manner to be used as a negative control.
2. Purified anti-AHL polyclonal sera prepared in Subheading 3.1, **step 3**, are added to *P. aeruginosa* and *E. coli* cultures at final IgG concentration of 600 nM. Pre-immune and nonspecific antigen immunized polyclonal serum (600 nM) are added to different *P. aeruginosa* and *E. coli* cultures as negative controls.

3. Grow the bacterial cultures at 37 °C with shaking at 200 rpm until an $A_{600} \cong 2.0$.
4. Record the A_{600} of the bacterial cultures and recover culture supernatants by centrifugation at $17,000 \times g$ for 10 min.
5. Mix 1 mL of culture supernatants with 2 mL of Elastin-Congo Red suspension and incubate at 37 °C for 16–18 h with shaking at 250 rpm.
6. Centrifuge at $17,000 \times g$ for 10 min, transfer the supernatants to disposable cuvettes, and measure the absorbance at 495 nm wavelength (A_{495}). If the purified anti-AHL polyclonal sera are functional in repressing QS, A_{495} values are expected to be lower in the samples derived from the *P. aeruginosa* cultures treated with the anti-AHL sera with respect to samples derived from the *P. aeruginosa* cultures treated with pre-immune and nonspecific antigen immunized polyclonal serum.

3.2.2 Nematode Killing Assay Using Anti-QS Polyclonal Sera

This assay can be performed using any wild-type *Caenorhabditis elegans* strain. We routinely use the wild-type Bristol N2 strain and follow the protocol described in [11].

1. *C. elegans* is maintained routinely on NGM agar plates seeded with lawns of *E. coli* OP50 for 3–4 weeks or longer. When healthy mixed population of adults and larval stages are required, small “agar chunks” with worms are transferred onto new 90 mm NGM agar plates containing fresh lawns of *E. coli* OP50. The “chunked” plates are kept upside down in a plastic box with slightly opened lid to prevent build-up of condensation and incubated at 15 °C for 5 days.
2. The worms from agar plates are resuspended in 5 mL of M9 and centrifuged for 2 min at $600 \times g$ in 15 mL polypropylene tubes.
3. The supernatant is removed carefully without disturbing the worm pellet and 1 mL of nematode bleaching solution is added to the tubes and incubated for no longer than 5 min at room temperature with continuous shaking.
4. The tubes are centrifuged at $600 \times g$ for 2 min, and the supernatant containing bleach solution is removed.
5. The worm pellet is washed by adding 1 mL M9 buffer. The suspension is centrifuged at $600 \times g$ for 2 min.
6. The worm pellet is finally suspended in 100 µL M9 buffer and transferred to fresh sterile NGM agar plates and left overnight at 25 °C. Bleaching kills all the adult worms and therefore triggers a synchronized population of newly hatched *C. elegans* at the L1 larval stage that the following day will be ready for the slow killing assay.

7. The plates containing young worms are washed with M9 buffer, transferred onto NGM agar plates containing fresh lawns of *E. coli* OP50, and incubated at 25 °C for the next 3 days.
8. Grow *P. aeruginosa* PA14 and *E. coli* OP50 cultures in 5 mL of LB at 37 °C overnight, with shaking. Dilute the *P. aeruginosa* PA14 overnight culture 1:100 in 5 mL of LB containing 600 nM anti-QS polyclonal sera or pre-immune and non-specific antigen immunized polyclonal sera (negative control) in duplicate. Dilute the *E. coli* OP50 overnight culture 1:100 in 5 mL LB without any antibody. An additional negative control consisting of *P. aeruginosa* PA14 culture supplemented with PBS can also be included.
9. Grow bacterial cultures by shaking at 37 °C until $A_{600} \approx 0.4$ is reached. 100 µL of anti-QS polyclonal sera or control sera made up to 600 nM in PBS are added to the center of 3.5 cm NGM-EP plates in duplicates and allowed to dry.
10. Ten microliters of *P. aeruginosa* PA14 or *E. coli* OP50 cultures grown up to log phase are spotted onto NGM-EP plates and incubated at 37 °C overnight.
11. The following day, another 100 µL of 600 nM anti-QS polyclonal sera or control sera are added on top of PA14/OP50 cultures on NGM-EP plates and incubated at 37 °C for 8 h.
12. The process is repeated and the plates are incubated at 25 °C overnight.
13. The plates were treated with antibodies as before and synchronized adult worms suspended in a drop of M9 were added onto the center of each of the PA14 plates containing anti-QS polyclonal sera or control sera. As a positive control, worms were added onto NGM-EP plates containing *E. coli* OP50.
14. The number of worms in each plate is counted and plates are incubated at 25 °C for ~24 h.
15. Worm count is repeated every 12 h and dead worms are removed with a wire pick.
16. The plates are treated with 600 nM polyclonal sera / control antibodies at 12 h time intervals as before. Overall, the worms are monitored for 80 h.

3.3 Preparation of Monoclonal Antibodies

1. To separate peripheral blood lymphocytes (PBLs) from sheep blood, PBLs are prepared from sheep blood collected 4 days after antigen-boost and processed within 18 h using Accuspin system Histopaque 1077 columns (Sigma). About 250 mL of production bleed in 3.2% (wt/vol) sodium citrate solution are used for the procedure.

2. Approximately 30 mL sheep blood is carefully poured onto each Histopaque-1077 column and spun at $2000 \times g$ for 15 min at room temperature. After centrifugation, the second layer from the top (buffy layer), which consists of a thin layer of lymphocytes, is recovered using a plastic Pasteur pipette by circular motion.
3. The recovered cells are pooled and topped up with sterile PBS and centrifuged at $250 \times g$ for 30 min at room temperature. After centrifugation, the supernatant is removed and the cell pellet washed in fresh sterile PBS and centrifuged at $250 \times g$ for 15 min at room temperature.
4. Total cell number is determined by hemocytometer analysis after staining with Trypan Blue. The cells are resuspended in an appropriate volume of RNA stabilization buffer to make up the cell density to 5×10^7 cells/mL and stored at -80°C .
5. Total RNA is extracted from 2×10^8 lymphocytes using a commercial kit for midi-RNA extraction, following manufacturer's instructions. Total RNA is then treated with DNase to remove genomic DNA contamination, as indicated by the manufacturer.
6. For cDNA synthesis, the DNase reaction mix is heated at 70°C for 10 min to denature both DNaseI and RNA, and chilled on ice. DNase-treated total RNA is used as template for cDNA synthesis using Superscript III RNase H-Reverse Transcriptase (Invitrogen). To 25 μL (50–500 ng) of total RNA, 1 μL each of (25 pmoles) forward primers specific for the ovine antibody heavy and light chain constant region of interest (OvCHFOR, OvCkFOR, OvC λ FOR) are added, and heated at 70°C for 10 min, cooled immediately on ice, and contents collected by brief centrifugation. Twelve sets of reactions are set up.
7. Add to the reaction mix, 8 μL of 5× first strand buffer, 4 μL of 0.1 M dithiothreitol (DTT), and 1 μL of 10 mM dNTP mix (2.5 mM each of dATP, dGTP, dTTP, dCTP). Incubate at 42°C for 2 min before the addition of 1 μL 200 U/ μL Superscript reverse transcriptase and then incubate at 42°C for 50 min.
8. Mix the reaction gently with a pipette and incubate for additional 50 min at 42°C . Stop the reaction by heating the tube at 70°C for 15 min. Store the cDNA at -20°C until required.
9. To amplify the ovine antibody variable heavy chain, set up gradient PCR reactions, by using the following primer pairs:
 - (a) OvVH1BACK and mixture of OvJH1LINKFOR, OvJH2-LINKFOR, OvJH3LINKFOR, OvJH4LINKFOR.
 - (b) OvVH2BACK and mixture of OvJH1LINKFOR, OvJH2-LINKFOR, OvJH3LINKFOR, OvJH4LINKFOR.

- (c) MuVH1BACK and mixture of OvJH1LINKFOR, OvJH2-LINKFOR, OvJH3LINKFOR, OvJH4LINKFOR.
 - (d) Hu4aBACK and mixture of OvJH1LINKFOR, OvJH2-LINKFOR, OvJH3LINKFOR, OvJH4LINKFOR.
10. To amplify ovine variable lambda and kappa chains, set up gradient PCR reactions using the following primer pairs:
- (a) OvVL1LINKBACK and mixture of OvJL1FOR and OvJL2FOR.
 - (b) OvVL2LINKBACK and mixture of OvJL1FOR and OvJL2FOR.
 - (c) OvVL3LINKBACK and mixture of OvJL1FOR and OvJL2FOR.
 - (d) OvVL4LINKBACK and mixture of OvJL1FOR and OvJL2FOR.
 - (e) OvVL5LINKBACK and mixture of OvJL1FOR and OvJL2FOR.
 - (f) OvVK1LINKBACK and mixture of OvJK1FOR, OvJK2-FOR and OvJK3FOR.
 - (g) OvVK2LINKBACK and mixture of OvJK1FOR, OvJK2-FOR and OvJK3FOR.
11. Each PCR reaction contains 1 μ L (25 pmoles) of each primer/primer mixture, 1 μ L cDNA from **step 8**, 25 μ L 2 \times Phusion master mix, and 22 μ L of H₂O. Heat up to 98 °C for 30 s and then start a 30 cycle temperature gradient program of denaturation at 98 °C for 10 s, annealing at gradient temperatures of 52–72 °C for 30 s and polymerization at 72 °C for 30 s followed by a final extension of 72 °C for 10 min.
12. Check the PCR products by agarose gel electrophoresis. Multiple reactions (about 12 \times 50 μ L) should be performed for each primer pair using the optimum annealing temperature, in order to obtain a suitable quantity of DNA.

3.4 Construction of Single Chain Variable Fragment (scFv) Phage Display Library

1. For small scale plasmid preparation, plasmid DNA is harvested from 3 mL of bacterial culture grown in Ampicillin (100 μ g/mL) containing medium using appropriate commercial kits following manufacturer's instructions. For large scale plasmid preparation, DNA is extracted from 500 mL of bacterial culture grown under same conditions and using appropriate commercial kits following manufacturer's instructions.
2. Gel extraction and purification of DNA. Plasmid DNA is electrophoresed on a 1% (wt/vol) agarose TAE gel containing ethidium bromide. A commercial DNA Molecular Weight Marker can be used for comparing the size of DNA bands. All samples are mixed (9:1 ratio) with 10 \times DNA loading dye and

electrophoresed at approximately 1 V/cm², using TAE buffer, until the dye front reach three-fourth of the agarose gel. The DNA bands of interest are then excised from the gel using a clean scalpel blade and cleaned up using a commercial kit following manufacturer's instructions.

3. The PCR products obtained in Subheading 3.3, step 12, are cleaned up using a commercial PCR purification kit following the manufacture's guidelines.
4. Both plasmid and PCR DNA concentration are determined by spectrophotometric analysis at A₂₆₀.
5. Cloning of antibodies heavy and light chains genes. The primers OvJH(1-4)LINKFOR, OvVL(1-5)LINKBACK, and OvVK(1-2)LINKBACK, used for amplification of variable heavy (VH) and light chains (Vλ or Vκ) from cDNA (see Subheading 3.3, steps 9 and 10), are designed to incorporate restriction sites AscI for heavy chain and MluI for light chain at the linker region. Equal quantities of purified VH PCR products are digested with 10 U of AscI per μg of DNA for 3 h at 37 °C. Equal quantities of purified Vλ or Vκ PCR products are digested with 10 U of MluI per μg of DNA for 3 h at 37 °C.
6. After digestion, the DNA is purified by gel electrophoresis.
7. Equal quantities of purified VH DNA are independently ligated to Vλ or to Vκ DNA by adding 150 U T4 ligase enzyme per μg of DNA and incubating the reaction mix at 37 °C in a water bath for 4 h. Into the reaction mix, 10 U each of AscI and MluI is added per μg of DNA to avoid the formation of VH-VH, Vλ-Vλ, and Vκ-Vκ products as successful ligation of VH-Vλ and VH-Vκ removes AscI and MluI sites from the linker region.
8. After a 4 h ligation, 5 μL of reaction product are run on a 1.5% (wt/vol) agarose gel and checked for successful ligation by a shift in band size from ~350 base pair to ~750 base pair. Successfully ligated DNA is purified by gel electrophoresis.
9. In order to incorporate cloning sites SfiI and NotI into VH-Vλ or VH-Vκ DNA fragments originated in the step 8, set up PCR reactions as follows. Using 1 μL of purified VH-Vλ PCR product as template add 1 μL (25 pmol) of primer mixture OvVH1BACK-Sfi, OvVH2BACK-Sfi, MuVH1BACK-Sfi, Hu4aBACK-Sfi and 1 μL (25 pmol) of primer mixture OvJL1-FOR-Not, OvJL2FOR-Not for VH-Vλ template. For VH-Vκ template add 1 μL (25 pmol) of primer mixture OvVH1BACK-Sfi, OvVH2BACK-Sfi, MuVH1BACK-Sfi, Hu4aBACK-Sfi and 1 μL (25 pmol) of primer mixture OvJK1FOR-Not, OvJK2-FOR-Not, OvJK3FOR-Not. To each reaction mix add 25 μL Phusion mix and 22 μL of H₂O. The reaction mix is heated up

to 98 °C for 30 s and subjected to two cycles of annealing and polymerization using 65 °C as the annealing temperature for VH-V λ and 64.4 °C as the annealing temperature for VH-V κ , a further extension at 72 °C for 10 min follows. After 2 cycles of initial amplification, the reaction mixes are subjected to a further 25 cycles of PCR reactions with 72 °C as the annealing temperature for both VH-V λ and VH-V κ . PCR products are then purified using gel electrophoresis and extraction.

10. For construction of the scFv phage display library, 10 µg of the phagemid vector pHEN 2a are digested with SfiI enzyme by adding 20 U per µg of DNA and incubating at 50 °C for 4 h. Afterwards, NotI enzyme is added to the reaction mix (20 U per µg of DNA) along with a conversion buffer which increases the NaCl concentration in the buffer to 50 mM and Tris-HCl to 40 mM, which is optimum for the NotI reaction. The reaction mix is incubated at 37 °C for further 4 h. The phagemid vector backbone is then purified by running the restriction products on a 1% (wt/vol) agarose TAE gel, excising the DNA band corresponding to ~3500 base pairs and extracting the DNA by means of a commercial kit.
11. The VH-V λ and VH-V κ DNA with cloning sites incorporated as described in **step 9** are digested with SfiI and NotI and purified by gel electrophoresis. The VH-V λ and VH-V κ fragments (1 µg of DNA each) are ligated into phagemid vector backbone using T4 Ligase enzyme (400 U) with a vector:insert ratio of 1:3. The ligation mixture is incubated at 16 °C for 16 h. Independent ligation reactions are set up for VH-V λ and VH-V κ inserts. Ligated DNA is extracted with phenol/chloroform, cleaned up using ethanol precipitation, and resuspended in 20 µL of H₂O.
12. The ligated DNA from **step 11** is transformed by electroporation into *E. coli* TG1 cells by standard electroporation procedures. Multiple electroporations are performed separately for VH-V λ and VH-V κ ligation reactions and collected into two distinct pools (one for VH-V λ and one for VH-V κ ligation mixture) after recovery. 100 µL of cells from each pool are serially diluted tenfold in LB medium and plated onto TYE plates containing 100 µg/mL Ampicillin and 1% (wt/vol) glucose. The plates are incubated at 30 °C overnight and the size of the phage display library is calculated from the titer obtained from serial dilution plates. The rest of the cells are plated onto large square TYE plates containing 100 µg/mL Ampicillin and 1% (wt/vol) glucose and incubated overnight at 30 °C. After overnight incubation, the cells are scraped from the bioassay dishes using 2 × TY medium and stored at -80 °C as 1 mL aliquots.

13. Single colonies from **step 12** are picked from serial dilution plates of VH-V λ and VH-V κ libraries and used as source of template DNA in colony PCR to check the presence of the scFv inserts. Set up a PCR reaction mix containing 25 pmol of AH18REV primer and 25 pmol of GIIIFOR primer, 1 μ L 100 mM dNTPmix (final 25 mM each of dATP, dTTP, dCTP, dGTP), 2.5 μ L 50 mM MgCl₂, 5 μ L 10 \times NH₄ Buffer, 1 μ L (1 U) Taq Polymerase, and 38.5 μ L water to make up the volume to 50 μ L. The reactions are heated up to 95 °C for 3 min and then subjected to a 30 cycle program of 95 °C for 30 s, 57 °C for 30 s, and 72 °C for 1 min, followed by a final incubation at 72 °C for 5 min. The PCR products (5 μ L) are run on a 1.5% (wt/vol) agarose gel alongside with a DNA molecular weight marker to analyze the presence of the scFv inserts (~750 bp).

3.5 Propagation of Helper Phage in Large Quantity

1. M13KO7 helper phage stock is serially diluted as tenfold dilutions in 2 \times TY medium. Ten microliter aliquots of diluted helper phage from 10 $^{-3}$ to 10 $^{-10}$ are added into 200 μ L of *E. coli* ER2738 cells grown in 2 \times TY medium supplemented with 12.5 μ g/mL tetracycline to an A₆₀₀ of 0.4–0.5, and incubated at 37 °C for 30 min to facilitate infection with phage particles.
2. After infection, ER2738 cells are added to 5 mL warm H-top agar, poured onto TYE agar plates supplemented with 12.5 μ g/mL tetracycline and incubated at 37 °C overnight.
3. Fresh ER2738 cells are grown in 2 \times TY medium containing 12.5 μ g/mL tetracycline up to A₆₀₀ of 0.5, and 3 mL cells are infected with a small plaque picked up from overnight grown H-top agar plates, by incubating for 1 h at 37 °C with shaking.
4. Culture from **step 3** is mixed with 500 mL 2 \times TY medium in a 2 L conical flask and grown at 37 °C with 250 rpm shaking for 1 h.
5. Add 50 μ g/mL of kanamycin to the culture from **step 4** and incubate for additional 16 h at 30 °C with shaking at 250 rpm.
6. The culture from **step 5** is centrifuged at 10,800 \times g for 15 min in sterile 50 mL polypropylene tubes. The supernatant containing M13KO7 helper phage is carefully poured out, filter sterilized using 0.45 μ m filter, and stored at 4 °C.
7. The helper phage titer is determined by performing tenfold dilutions of the phage-containing supernatant in 2 \times TY medium and infecting log phase ER2738 cells as described above.
8. After infection, plate 100 μ L of infected cells onto TYE plates supplemented with 50 μ g/mL kanamycin and 12.5 μ g/mL tetracycline.

9. After overnight incubation at 30 °C, the number of plaques on each dilution plate is counted and helper phage titer calculated as plaque forming units (PFU)/mL by taking into consideration the dilution factor and the volume of cells plated.

3.6 Immunized Sheep Antibody Phage Display Library Rescue and Panning

The following protocol for library rescue and panning has been adapted from MRC Centre for Protein Engineering Tomlinson (Tomlinson & Winter) and Griffin1 library protocols.

1. Independently inoculate VH-V λ and VH-V κ phagemid library glycerol stocks obtained in TG1 cells in Subheading [3.4, step 12](#) in 200 mL of pre-warmed 2× TY medium containing 100 µg/mL ampicillin and 1% (wt/vol) glucose. In this way the starting culture should have 10–100 copies of each antibody clone from the library. The number of cells is estimated by measuring the initial optical density of the culture postulating that for an $A_{600} \approx 0.1$, the number of cells in the culture is $\sim 8 \times 10^7$ cells/mL. Cells are grown at 37 °C with shaking at 250 rpm until $A_{600} \approx 0.5$.
2. M13KO7 helper phage is added to the cultures to a 1:20 ratio (cell:helper phage) and cells are infected by incubating at 37 °C without shaking followed by 1 h at 37 °C with shaking at 250 rpm.
3. To determine the helper phage rescue rate of the library, 10 µL of the infected culture is set aside and serially diluted in 2× TY medium, plated onto TYE agar plates containing 100 µg/mL ampicillin and 50 µg/mL kanamycin, and incubated at 37 °C overnight. The infection rate is determined by counting bacterial colonies on the agar plates.
4. The two 200 mL cultures infected overnight with M13KO7 are then centrifuged at $2000 \times g$ for 10 min at 4 °C.
5. Cell pellets from [step 4](#) are suspended in 100 mL of 2× TY medium supplemented with 100 µg/mL ampicillin and 50 µg/mL kanamycin, and incubated overnight at 30 °C with shaking at 250 rpm.
6. For the precipitation of rescued phage, overnight cultures from [step 5](#) are centrifuged at $4000 \times g$ for 30 min and the supernatants containing phage particles are transferred to sterile tubes. Add 20 mL of PEG-NaCl solution to the phage supernatants, mix well, and incubate on ice for 2 h. Centrifuge for 30 min at $4000 \times g$ at 4 °C and discard the supernatants.
7. Wash the phage pellets with 40 mL of H₂O and centrifuge at $4000 \times g$ at 4 °C for 20 min. The supernatants containing the phage are precipitated by mixing with 8 mL of PEG-NaCl and 30 min incubation on ice. Centrifuge at $4000 \times g$ at 4 °C for 10 min, discard supernatant and resuspend phage pellets in

2 mL PBS, spin for 10 min at 17,000 $\times g$ on a tabletop centrifuge to remove any bacterial debris. The phage obtained after double PEG-NaCl precipitation can be stored at 4 °C for immediate use or as a glycerol stock (15% (wt/vol) glycerol) at –80 °C for long-term storage.

8. To determine the titer of PEG-NaCl precipitated rescued phage, precipitated phage is titrated by performing tenfold serial dilutions of phage stock in PBS and infecting 900 μ L of log-phase TG1 cells with 100 μ L of each dilution.
9. After incubation at 37 °C for 30 min, 100 μ L of infected cells are plated onto TYE plates supplemented with 100 μ g/mL ampicillin and 1% (wt/vol) glucose, and incubated at 30 °C overnight.
10. Determine the phage titer by counting the number of plaques present on the agar plates.
11. Panning rescued phage against AHL-conjugates is aimed at selecting phages that bound to the conjugate of interest (i.e., AHL-BSA or AHL-TG conjugates). The panning strategy is designed to drive the selection towards enrichment of cross-reactive clones as shown schematically in Fig. 2. Coat Nunc Maxisorp Immuno tubes (Thermo Fisher Scientific) by adding 200 μ g of HSL-conjugate dissolved in 4 mL of PBS and incubating overnight at 4 °C. The immunotube is then washed three times with PBS, blocked with MPBS for 2 h at 37°C, and further washed three times with PBS.
12. To select for specific binding, 1 mL of rescued phage mixed with 3 mL of MPBS is added to the immunotube and incubated for 30 min at room temperature on a rolling tumbler.
13. The tube is then allowed to stand for 90 min at room temperature. Following incubation, the unbound phage is discarded by washing the tube ten times with PBS containing 0.1% (vol/vol) Tween 20, and then ten times with PBS.
14. For triethylamine elution of AHL-binding phages, following an initial PBS wash, the bound phage is eluted from the immunotube by adding 1 mL of 100 mM triethylamine and rotating for exactly 10 min at room temperature. Triethylamine is alkaline and increasing the pH of the immunotube causes phage release. The solution is then transferred to another tube and neutralized by adding 500 μ L of 1 M Tris-HCl (pH 7.4). Eluted phage can be stored at 4 °C or as glycerol stock at –80°C by adding 15% (vol/vol) glycerol.
15. Free antigen elution of AHL-binding phages. This additional elution strategy is included from the second and subsequent rounds of selection to increase the chance of recovering “free AHL” (non-conjugated) binding phage. After phage binding

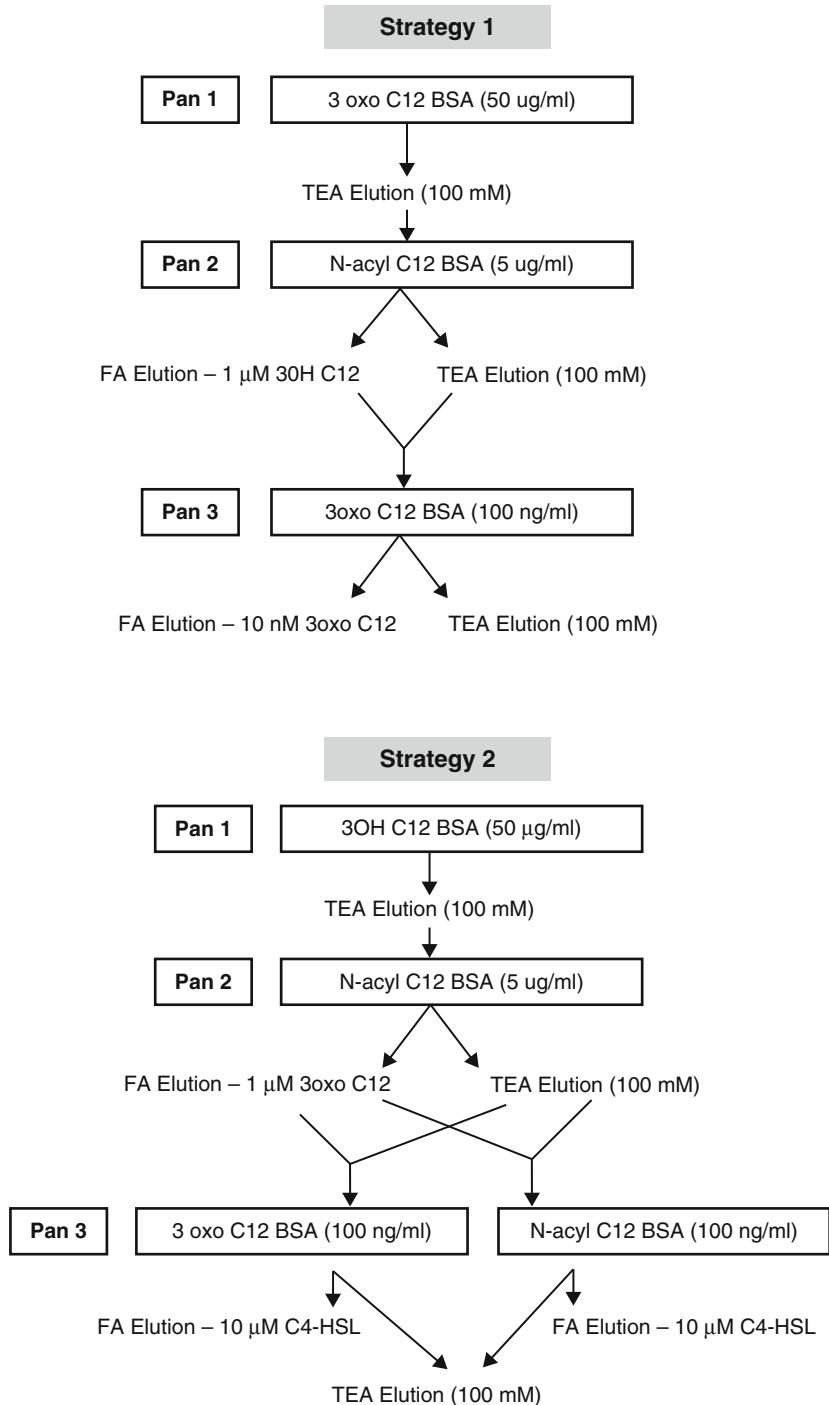


Fig. 2 Outline of the panning strategy adopted for the selection of cross-reactive anti-HSL clones

to the AHL-conjugate in the immunotube and washing as described in Subheading **3.6**, step 13, 4 mL of free AHL at a desired concentration (10 nM–10 μ M; *see* Fig. 2) is added to the immunotube and incubated at room temperature for 2 h on rolling tumbler.

16. The eluted phage is mixed with 10 mL of log-phase TG1 cells and incubated at 37 °C for 30 min to allow infection. Any remaining phage bound to the immunotube is recovered by adding 4 mL of log-phase TG1 cells into the tube and incubating at 37 °C for 30 min.
17. 100 μ L of each infected culture are serially diluted tenfold in 2 \times TY medium and plated onto TYE agar plates containing 100 μ g/mL ampicillin and 1% (wt/vol) glucose and incubated at 30 °C overnight.
18. The library size after pan 1 or the number of cells infected by phage recovered from selection is estimated by counting the number of bacterial colonies on the plate. The remaining infected culture is centrifuged at 2000 \times g for 10 min, the cell pellet suspended in 1 mL of 2 \times TY medium and plated onto 140 mm Petri dish containing TYE supplemented with 100 μ g/mL ampicillin and 1% (wt/vol) glucose and incubated overnight at 37 °C.
19. For helper phage rescue and enrichment of AHL binders, bacterial colonies grown on 140 mm Petri plates from **step 18** are recovered by adding 2–3 mL of 2 \times TY medium containing 30% (vol/vol) glycerol and 10% (wt/vol) glucose.
20. The bacterial lawn is loosened using a disposable scraper and the resultant suspension is mixed thoroughly to remove any bacterial clump. The suspension is stored at –80 °C as a bacterial stock containing and representative of selected phage from the first round of selection. This is then used as the starting material for the next round of panning.
21. From the pan 1 glycerol stock, a small amount is inoculated into 100 mL of 2 \times TY containing 100 μ g/mL ampicillin and 1% (w/v) glucose, in order to reach an A_{600} between 0.08 and 0.1. The culture is then incubated at 37 °C with shaking at 250 rpm until an A_{600} of 0.4–0.5 is reached.
22. 50 mL of culture from **step 21** are infected with the M13KO7 helper phage and incubated at 37 °C for 30 min. The culture is centrifuged at 2000 \times g for 10 min and the pellet resuspended in 100 mL 2 \times TY supplemented with 100 μ g/mL ampicillin and 50 μ g/mL kanamycin, and grown overnight at 30 °C with 250 rpm shaking.
23. Phage rescue and precipitation is carried out as described in **steps 6–10**. Second round of selection is carried out on

immunotube coated with AHL-conjugate as described in **steps 11–17**. Subsequent rounds of rescue and selection are carried out and the stringency of panning is increased in each round by increasing the number of washing steps and also by adding 1 mg/mL BSA, 1 mg/mL TG, 2% (wt/vol) skim milk, 0.5% (vol/vol) Tween 20 along with PEG precipitated phage (Fig. 2).

3.7 Production of Anti-QS Antibodies

1. Screening of antibody fragments binding to AHL-conjugates by ELISA. A 96-well flat bottomed Immulon 4 ELISA plate (Dynatech Laboratories Ltd.) is coated with 100 µL per well of 10 µg/mL AHL-conjugates for 2 h at 37 °C. The plate is washed three times with PBST and three times with MPBS and incubated at 37 °C for 1 h.
2. The washing step is repeated and 50 µL of PEG-precipitated phage from pan 0 to pan 3 are added to distinct wells. 50 µL of 4% MPBS are added to the same wells and incubated at room temperature for 1 h.
3. After discarding the phage solution, the wells are washed with PBST and PBS as in **step 1**, and 100 µL of a 1:1000 dilution of anti-M13 monoclonal HRP conjugate is added to the wells.
4. The plate is incubated for 1 h at room temperature, washed, and the wells developed by adding 100 µL per well of HRP substrate solution. The reaction is stopped with 50 µL of 1 M H₂SO₄ per well. The absorbance of the wells is measured at 450 nm using a microplate reader.
5. Individual bacterial colonies are picked from Pan 2 and Pan 3 serial dilution plates and inoculated into 100 µL of 2× TY containing 100 µg/mL ampicillin and 1% (w/v) glucose in a sterile 96-well tissue culture plate and incubated overnight at 37 °C with shaking at 250 rpm. The following day, using a sterile 96-well transfer device, a small inoculum is transferred into a new 96-well plate containing 200 µL per well of fresh 2× TY containing 100 µg/mL ampicillin and 1% (w/v) glucose (*see Note 3*).
6. Incubate the refreshed 96-well plate at 37 °C with shaking for 2 h. Then add to each well 25 µL of 2× TY containing 100 µg/mL ampicillin, 1% (wt/vol) glucose and helper phage M13KO7 (10⁹ PFU per well) obtained as described in Sub-heading **3.5, step 6**.
7. The plate is incubated at 37 °C for 30 min without shaking followed by 1 h shaking at 37 °C. Cells are pelleted by centrifuging at 700 × g for 10 min and supernatants are carefully removed from the plate by pipetting.
8. Resuspend the pellets in 200 µL of 2× TY containing 100 µg/mL ampicillin and 50 µg/mL kanamycin and incubate

overnight at 30 °C with shaking. Centrifuge the plate at 700 × \mathcal{g} for 10 min and transfer the supernatant containing monoclonal phage to sterile 96-well plates.

9. Phage monoclonal binding ELISA. Coat 96-well Immulon 4 ELISA plates with 100 μL per well of 1 $\mu\text{g}/\text{mL}$ AHL conjugates (i.e., C₁₂-BSA, 3OC₁₂-BSA, 3OHC₁₂-BSA) and carrier protein BSA for 2 h at 37 °C (or overnight at 4 °C). Wash and block as explained in Subheading 3.1, step 4.
10. Add 50 μL of phage supernatant from step 8 and 50 μL of 4% MPBS and incubated at room temperature for 1 h. The following steps are repeated as detailed in Subheading 3.1, steps 7–10. Secondary antibody used here is 100 μL of a 1 in 1000 dilution anti-M13 monoclonal HRP conjugate per well.
11. Monoclonal phage Competition ELISA. Monoclonal phage clones specific for free AHLs are identified by Competition ELISA. ELISA plates coated with HSL blocked with MPBS and washed as described in Subheading 3.1, step 4.
12. After washing, varying concentrations of free AHL solutions (1 μM –100 nM) were prepared in 4% MPBS and added to the ELISA plate during incubation with the phage supernatant. The rest of the assay is carried out as explained in Subheading 3.1, step 12, except the secondary antibody used is 100 μL of a 1 in 1000 dilution anti-M13 monoclonal HRP conjugate per well and the clones which showed reduced binding (greater than 50% when compared with the control) against AHL-conjugates in the presence of free AHLs, are selected.
13. Conversion of positive AHL binders into a single chain antibody format (scAb). Extract plasmid DNA from phage clones recognizing free AHLs selected in the step above and sequence using AH18REV and GIIIFOR primers. After sequence analysis, the scFV region of unique positive clones is cloned into the soluble antibody expression vector pIMS147 using NcoI and NotI restriction sites following the general cloning procedures described in Subheading 3.4. Briefly, 40 μg of pIMS147 DNA and 2 μg of each plasmid DNA extracted by the clones selected in the step above are digested with 100 U of NcoI and 100 U of NotI for 16 h at 37 °C.
14. The restriction products are separated by gel electrophoresis on a 1% (wt/vol) agarose TAE gel and the correct sized DNA band (~5.5 kbp for the vector and ~750 bp for the insert) are purified by using a commercial kit.
15. Set up a ligation reaction using about a 1:7 (vector:insert) molar ratio (i.e., for each clone, use 100 ng of insert, 100 ng of vector, 200 U of T4 ligase and incubate at 16 °C overnight).

16. Dialyze the ligation mix on a 0.025 µm filter paper against sterile water for 30 min and transform into electrocompetent XL1-Blue cells.
17. Cells recovered from transformation are plated on LB agar plates containing 100 µg/mL ampicillin. Pick single colonies from transformation plates and plasmid DNA prepared by routine technique and digest with NcoI–NotI enzymes to check the presence of correct sized insert (~750 bp) by gel electrophoresis. Check the insert by sequencing using primers AH18REV and HuCkFOR.
18. Small scale prokaryotic expression of scAb fragments. Single colonies of positive clones selected in **step 17** are inoculated into 5 mL of LB medium containing 1% (wt/vol) glucose, 50 µg/mL ampicillin, and 12.5 µg/mL tetracycline, and incubated overnight at 37 °C with shaking.
19. The overnight cultures from **step 18** are centrifuged at 2000 × g for 10 min and the cell pellets resuspended in 5 mL of fresh LB containing 100 µg/mL ampicillin and 12.5 µg/mL tetracycline, and grown at 25 °C with shaking for 1 h.
20. Protein expression is induced by adding IPTG to a final concentration of 1 mM. After IPTG addition, cells are grown for further for 4 h and further processed as described in **step 25**.
21. Medium scale prokaryotic expression of scAb fragments. Inoculate 0.5 mL of overnight cultures of single positive clones selected in **step 17** in 50 mL of TB medium containing potassium phosphate buffer, 100 µg/mL ampicillin, 12.5 µg/mL tetracycline, and 1% (wt/vol) glucose in 250 mL conical flasks. Grow the cultures for 7–8 h at 37 °C with shaking.
22. Centrifuge the cultures from **step 21** at 2000 × g for 15 min. Resuspend the cell pellets in fresh TB medium as above and incubate overnight at 30 °C with shaking.
23. The following day, collect the pellets by centrifuging at 2000 × g for 20 min and resuspend in fresh TB medium containing potassium phosphate buffer and 100 µg/mL ampicillin by vortexing.
24. Recover cells for 1 h at 25 °C with shaking and then induce with IPTG as described in **step 20**.
25. Periplasmic release of expressed scAbs. After IPTG induction, cultures from **steps 20** and **24** are centrifuged at 4000 × g for 20 min, and the cell pellets resuspended in 1/10 of the original culture volume of Fractionation buffer. Incubate on ice for 15 min with gentle shaking. To this suspension, add 1/10 of the original culture volume of ice cold 5 mM MgSO₄ and incubate 15 min on ice. Centrifuge at 4000 × g for 20 min,

recover the supernatant containing released protein, and filter through a 0.30 µm filter.

26. For purification of scAbs, IMAC Sepharose 6 Fast Flow resin (Binding Capacity 5–10 mg/mL, GE Healthcare) is added to a poly-prep chromatography column and the resin supernatant is allowed to drain. After capping the bottom of the column, wash once with 5 volumes of PBS and twice with 5 volumes of sterile H₂O.
27. Charge the resin with nickel by adding 5 gel volumes of 0.1 M NiSO₄ and incubate on a rotator for 1 h at room temperature. Drain the column and wash with 5 gel volumes of PBS containing 10 mM imidazole. The activated Ni-sepharose column has a binding capacity of 5–10 mg of protein per mL of resin. The column thus prepared can be used immediately for the purification of antibody fragments or stored at 4 °C by leaving 2 gel volumes of PBS in the column until required.
28. Add scAb fragments obtained from **step 25** to the column prepared in **steps 26** and **27**. Mix gently and incubate overnight at 4 °C by gentle rotation. Drain the column, collect the flow-through and pass it again through the column two times. Wash with 5 mL of PBS and 5 mL of 20 mM imidazole to remove any nonspecific protein. Cap the column and gently resuspend the resin in 1 mL of 200 mM imidazole. Allow the resin to settle for 5 min and drain. Collect the eluent containing antibody fragments in a 1.5 mL microfuge tube. Repeat this elution step four times and collect separate fractions.
29. Dialyze separately each eluted fraction in 5 L of PBS for 6 h at 4 °C with gentle stirring. Change PBS and continue dialysis at 4 °C overnight. Collect dialyzed scAb fragments in 1.5 mL microfuge tubes and store at 4 °C.
30. The purified antibodies can be analyzed by SDS-polyacrylamide gel electrophoresis and western analysis. After blotting and hybridization, the presence of the purified antibodies is revealed with HRP-conjugated secondary antibody and developed with an appropriate luminescent substrate following manufacturer's instructions.
31. The HuC κ domain fused downstream of the scFv region in the expression vector pIMS147 facilitates quantification of antibody fragments by comparison with a human whole IgG standard using Capture ELISA. Coat an Immulon 4 96-well flat bottomed ELISA plate with 100 µL of a 1:1000 dilution of anti-human IgG (C-kappa specific) antibody (Sigma) and incubate at 37 °C for 1 h.
32. Wash three times with PBST and three times with PBS, add 200 µL of MPBS for blocking and incubate at 37 °C for 1 h. Repeat washing steps and add double dilutions of crude or

purified antibody fragments to the wells alongside double dilutions of human whole IgG (κ isotype) with starting concentration of 1.25 μ g/mL.

33. Incubate plates at room temperature for 1 h, wash as before, and add 100 μ L goat anti-human C-kappa light chain peroxidase conjugate diluted 1:1000 (Sigma). Incubate 1 h at room temperature, wash, and develop the plates as described in Subheading 3.1, step 10.
34. scAb Binding ELISA. Coat 96-well flat bottomed Immulon 4 plates with 100 μ L of 1 μ g/mL AHL-conjugates (i.e., C₁₂-BSA, 3OC₁₂-BSA, 3OHC₁₂-BSA) for 2 h at 37 °C or overnight at 4 °C. Include plates coated with 1 μ g/mL BSA as a control. Wash three times with 200 μ L of PBST and three times with 200 μ L of PBS. Add 200 μ L of MPBS for blocking and incubate at 37 °C for 1 h. Repeat the washing step and add 200 μ L of purified antibody samples of known concentration (as determined by Capture ELISA, steps 31–33) making double dilutions in PBS. Incubate at room temperature for 1 h. Wash plates as before and add 100 μ L of goat anti-HuC κ peroxidase conjugate diluted 1:1000 in PBS. Incubate 1 h at room temperature, wash, and develop the plates as described in Subheading 3.1, step 10.
35. scAb Competition ELISA. Coat 96-well flat bottomed Immulon 4 plates with 1 μ g/mL AHL-BSA conjugates and block with MPBS as in the step above. Set up double dilutions of free AHL solutions (i.e., C₁₂-HSL, 3OC₁₂-HSL, 3OHC₁₂-HSL, C₄-HSL) in 125 μ L of PBS and mix with an equal volume of a sub-saturating concentration of AHL scAbs as determined by binding ELISA (Subheading 3.7, step 34). For 100% binding control, use AHL scAb in PBS. The reactions are pre-incubated at room temperature for 1 h and 100 μ L of each reaction mix is added to replicate wells and incubated for a further 1 h at room temperature. Following normal washing steps, HRP-conjugated secondary antibody is added to the wells and incubated as described above. The plates are then washed and developed as described in Subheading 3.1, step 10.

4 Notes

1. We routinely purchase customized AHL-compounds from Salford Ultrafine Chemicals and Research Ltd., Manchester, UK (presently known as SAFC, a division of Sigma Aldrich Corporation).
2. In principle, the melon gel support bind to non-antibody serum proteins, such as albumin and transferrins, using

physiological pH and the antibody present in the flow-through is collected and used for downstream purposes without any need for neutralization. This system has been adopted because unlike Protein A and Protein G purification systems, no elution step is involved and thus the antibodies are not subjected to or altered by potential harsh elution conditions.

3. Add 15% (vol/vol) glycerol and 5% (wt/vol) glucose to each well of the first 96-well plate (the one corresponding to the overnight culture) and store at –80 °C as a bacterial stock.

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Chapter 26

Identification of AI-2 Quorum Sensing Inhibitors in *Vibrio harveyi* Through Structure-Based Virtual Screening

Tianyu Jiang, Peng Zhu, Lupei Du, and Minyong Li

Abstract

Quorum sensing (QS) is a cell-to-cell communication system that regulates gene expression as a result of the production and perception of signal molecules called autoinducers (AIs). AI-2 is a QS autoinducer produced by both Gram-negative and Gram-positive bacteria, in which it regulates intraspecies and interspecies communication. The identification of QS inhibitors is considered a promising strategy for the development of anti-virulence drugs with reduced selective pressure for resistance. Here we describe a high-throughput virtual screening approach to identify AI-2 quorum sensing inhibitors on the basis of *Vibrio harveyi* LuxPQ crystal structure. Seven potent inhibitors with IC₅₀ values in the micromolar range were selected with no effect or low effect on *V. harveyi* growth rate.

Key words AI-2, *Vibrio harveyi*, LuxPQ, Quorum sensing inhibitors, Virtual screening

1 Introduction

Quorum sensing (QS) is a mechanism of bacterial communication that governs gene expression in response to population density and fluctuation in the surrounding environment. QS is based on the production, secretion, and perception of chemical signaling molecules called autoinducers [1–3]. Bacterial quorum sensing plays a role in many biological functions, including biofilm formation, bacterial virulence, antimicrobial resistance, antibiotic production, competence, conjugation, swarming motility, and sporulation [4–9]. It is believed that QS is the suitable target for antimicrobial therapy, since QS inhibitors are likely to be applied as anti-virulence drugs with reduced selection for resistance [10]. The main QS AIs are acyl-homoserine lactones (AI-1), AI-2, AI-3, AI peptides, cholerae autoinducer-1 (CAI-1), and *Pseudomonas* quinolone signal (PQS) [11–17]. AI-2 is a QS signaling molecule produced by both Gram-negative and Gram-positive bacteria, in which it mediates intraspecies and interspecies communication [18–20]. Hence,

molecules able to interfere with AI-2 QS could lead to the development of next generation broad-spectrum antimicrobial agents [21].

As the predominant precursor for all AI-2 signals, 4,5-dihydroxy-2,3-pentandione (DPD) can undergo cyclization and additional reactions spontaneously to generate distinct AI-2 signal molecules. For example, S-THMF-borate, a boron-containing AI-2 molecule, is used as autoinducer in *V. harveyi*. In *V. harveyi*, two proteins, LuxP and LuxQ are involved in the response to AI-2. LuxP is a periplasmic-binding protein and LuxQ is a two-component hybrid sensor kinase [22]. LuxP binds to LuxQ as a complex named LuxPQ [23]. In 2002, the crystal structure of the receptor LuxP from *V. harveyi* with AI-2 was determined [22]. Moreover, the crystal structure of the AI-2-LuxPQ complex was resolved by the Hughson group in 2006 [24].

As shown in Fig. 1, when cell density is low, autophosphorylation of LuxQ leads to convey phosphate through LuxU to LuxO. Phosphorylated LuxO cooperates with σ^{54} and promotes the production of small regulatory RNAs. As a consequence, mRNA of the transcriptional regulators LuxR is destabilized, and LuxR-dependent genes are not transcribed. Conversely, at high cell density AI-2 binds to LuxP and induces the LuxPQ complex to initiate

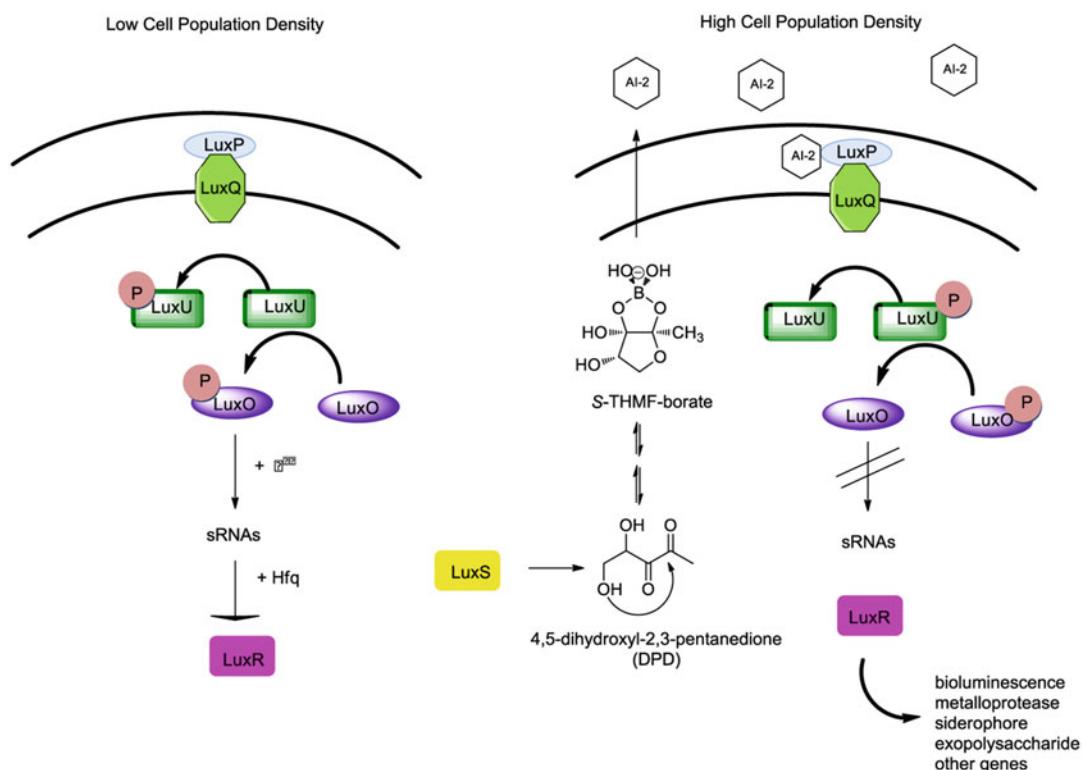


Fig. 1 Mechanism of AI-2 mediated quorum sensing in *V. harveyi*

a switch from kinase to phosphatase activity, which results in dephosphorylation of the downstream proteins LuxU and LuxO. Dephosphorylated LuxO does not promote the production of small regulatory RNAs, and this event leads to the LuxR-dependent transcription of target genes [21, 24].

Most of the AI-2 QS inhibitors reported primarily target the receptor protein LuxP. However, in most cases LuxP is still able to bind to LuxQ and AI-2 to form the LuxPQ complex. Hence, the LuxPQ complex is a more accurate target for AI-2 QS inhibitors with respect to the LuxP protein alone [25].

Herein, we describe a high-throughput virtual screening [26] to identify AI-2 QS inhibitors based on the crystal conformations of LuxPQ in complex with AI-2 (PDB entry: 2HJ9) published by the Bassler lab [24], by the use of the DOCK6 program and SPECS chemical database. The MM32 strain of *V. harveyi* is chosen for the anti-QS assay due to the absence of the LuxN receptor, needed to respond to AI-1, and of LuxS, needed to catalyze the biosynthesis of DPD [25]. The top 1000 compounds are selected by virtual screening. Then 42 hits that are chosen by using consensus scoring can be evaluated for their ability to inhibit the AI-2 QS system. We found that seven inhibitors, including compound 5 ($IC_{50} = 3.06 \pm 0.18 \mu M$), compound 12 ($IC_{50} = 40.1 \pm 14.7 \mu M$), compound 19 ($IC_{50} = 5.51 \pm 1.05 \mu M$), compound 23 ($IC_{50} = 3.42 \pm 0.63 \mu M$), compound 27 ($IC_{50} = 41.0 \pm 0.99 \mu M$), compound 31 ($IC_{50} = 19.8 \pm 1.48 \mu M$), and compound 33 ($IC_{50} = 14.8 \pm 1.77 \mu M$) (Fig. 2), were able to interfere with the AI-2-QS system of *V. harveyi* in the micromolar range. In particular, compounds 5, 19, and 23, exhibited IC_{50} values of 3.06, 5.51, and 3.42 μM , respectively, with low effect or no effect on *V. harveyi* growth rate.

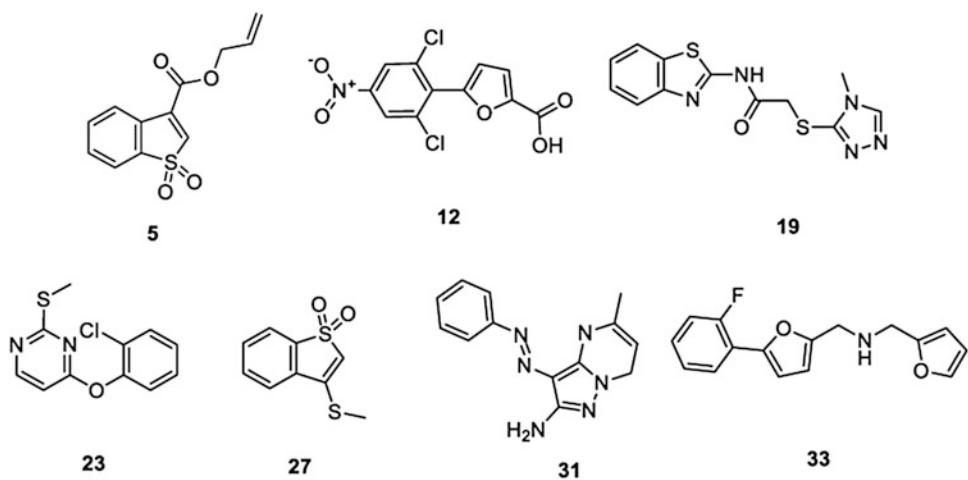


Fig. 2 Chemical structures of seven compounds as LuxPQ inhibitors

2 Materials

1. Virtual screening (DOCK 6) was performed using a Linux-based cluster.
2. Visualization (PyMOL 0.99, HBPLUS 3.06, and Ligplot 4.22) was conducted on a dual-Xeon Linux graphical workstation.
3. Consensus scoring (FRED 2.2.3) and reanalysis of virtual screening (FILTER 2.0.1 and IDEA 8.8) were performed on a Windows workstation.
4. Compounds information comes from SPECS chemical database.
5. The crystal structure information is based on conformation of LuxPQ in complex with AI-2 (PDB entry: 2HJ9).
6. *V. harveyi* MM32 (ATCC# BAA-1211).
7. Luria Marine (LM) plates: 1% (wt/vol) bacto-tryptone, 0.5% (wt/vol) yeast extract, 2% (wt/vol) sodium chloride, 1.5% (wt/vol) agar in distilled water. Sterilize by autoclaving.
8. Autoinducer Bioassay (AB) medium: Dissolve 2.0 g vitamin-free casamino acids, 12.3 g magnesium sulfate heptahydrate, and 17.5 g sodium chloride in 960 mL of distilled water. Adjust pH to 7.5 with potassium hydroxide. After autoclave sterilization (121 °C, 20 min), the medium is cooled to room temperature. Then the following sterile solutions are added to reach 1 L: 10 mL of 1 M potassium phosphate buffer pH 7.0, 10 mL of 100 mM L-arginine solution, 20 mL of 50% (v/v) glycerol (*see Note 1*).
9. 12 mM FeCl₃ in filter-sterilized water.
10. Antibiotic stock solutions: 50 mg/mL kanamycin in distilled water (filter-sterilized); 10 mg/mL chloramphenicol in ethanol.
11. Luminometer microplate reader.
12. 96-well microtiter plates.
13. Spectrophotometer.
14. 250 mL conical flasks.
15. Shaking incubator.
16. Freshly synthesized 20 µM DPD solution (pH = 7).
17. 4 mM boric acid in filter-sterilized water.
18. Compounds to be tested dissolved in AB medium.

3 Methods

3.1 Virtual Screening

1. The database including 0.5 million compounds from Specs Ltd. is first converted into 3D structures using the CONCORD program [27] and filtered using drug-like property criteria [28] by the FILTER 2.0.1 software [29].
2. The protein structure is added with hydrogen atoms. All atoms are assigned Kollman-all charges by the SYBYL 7.1 program (SYBYL 7.1, 2005 Tripos Inc., St. Louis, MS).
3. The 3D structures of the small molecules are added with hydrogen and assigned AM1-BCC partial charges.
4. The active site for the virtual screening is in the radius of 6 Å around the center of AI-2, includes residues Pro74, Gln77, Ser79, Asp80, Tyr81, Trp 82, Pro109, Asn159, His180, Phe206, Ile211, Arg215, Cys264, Ser265, Thr266, Asp267, Trp289, Gly290, Gly291, Glu295, and Arg310. In the LuxPQ *holo*-form crystal structure, the borate moiety of AI-2 generates several hydrogen bonds with the positively charged residues Arg215 and Arg310 for stabilizing the negative charge. Meanwhile, this borate group can also form hydrogen bonding with Ser79 and Thr266. Further, residues Trp82, Ser79, and Gln77 provide multiple hydrogen bonds with the two hydroxyl groups of AI-2 also have, and Asn159 and Arg215 has hydrogen bonds with the oxygen atom of furanoyl ring (Fig. 3).
5. The position and conformation of each compound are optimized first by the anchor fragment orientation and then by the torsion minimization method implemented in the DOCK6 program [30, 31].
6. Each compound is generated with 50 conformations and a maximum of 100 anchor orientations. By the way, all of the docked conformations are energy minimized by 100 iterations [31].
7. The docked molecules are ranked based on the sum of the *van der Waals* and electrostatic energies to get the top 1000 hits.
8. In order to identify a specific association between the ligands and LuxP, the resulting structures are analyzed using PyMOL 0.99, HBPLUS 3.06, and Ligplot+ 1.4.5.

3.2 Compounds Selection

1. Carry out a consensus scoring evaluation of the top 1000 hits from virtual screening results and select the top 10% compounds (see Note 2). Our consensus scoring efforts consisted of ChemScore, OEChemScore, PLP, ScreenScore, ChemGauss3, CGO, and ShapeGauss implemented in the FRED 2.2.3 software (FRED 2.2.3, 2007 OpenEye Scientific Software, Inc., Santa Fe, NM).

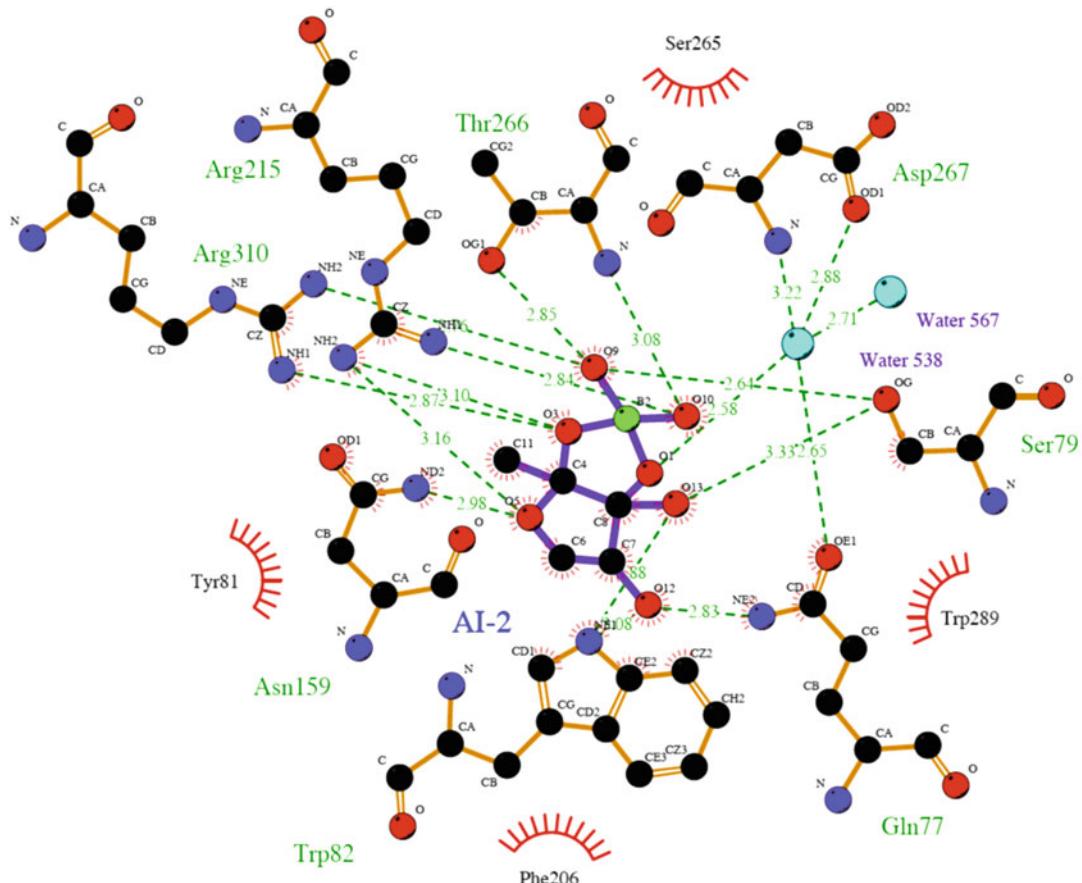


Fig. 3 A schematic illustration of the interactions of AI-2 with LuxPQ

2. Check the hydrogen bond and hydrophobic interaction profiles of the selected top 10% compounds by the IDEA 8.8 software (IDEA 8.8, 2007 Breadth Technology, Taipei, Taiwan) (*see Note 3*).
3. Carry out a manual binding orientation and conformation analysis by comparing with AI-2 to make the final 42 hits (Fig. 4).

3.3 Quorum Sensing Inhibition Assay

The anti-QS tests here described are performed according to previously reported procedures [32].

1. *V. harveyi* MM32 (ATCC# BAA-1211) is streak-seeded on a Luria Marine (LM) plate supplemented with 50 µg/mL kanamycin and 10 µg/mL chloramphenicol, and the plate is incubated overnight at 30 °C.
2. A single colony is picked from the LM plate and inoculated into 2 mL of Autoinducer Bioassay (AB) medium [33]

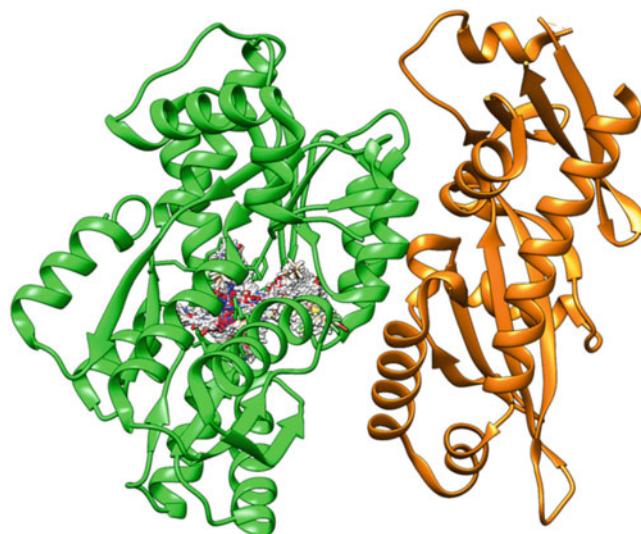


Fig. 4 Docking conformations of 42 hits and AI-2 within the binding site of *V. harveyi* LuxPQ (LuxP: green ribbons and LuxQ: orange ribbons)

supplemented with 50 µg/mL kanamycin and 10 µg/mL chloramphenicol. The culture is grown for 16 h with aeration (175 rpm) at 30 °C.

3. The culture from **step 2** is diluted to an optical density at 600 nm wavelength (OD_{600}) of 0.7 in 2 mL of AB medium supplemented with 1.2 mM $FeCl_3$, and incubated for 1–1.5 h at 30 °C with shaking (175 rpm) to an OD_{600} of 1.0–1.1 [29]. Then, the resulting culture is diluted 5000-fold in fresh AB medium.
4. 25 µL of the compounds to be tested freshly dissolved in AB medium to the appropriate concentration are dispensed into 96-well microtiter plate.
5. 25 µL of a freshly synthesized 20 µM DPD solution (pH = 7) and 25 µL of 4 mM boric acid are also added to the test compounds solutions into the 96-well microtiter plate (final concentration of DPD in the well = 5 µM; final concentration of boric acid in the well = 1 mM) (*see Note 4*).
6. 25 µL of the bacterial culture prepared in **step 3** are dispensed into the wells of the microtiter plate prepared in **steps 4** and **5**. The microtiter plate is covered with a nontoxic plate sealer and incubated at 30 °C with shaking for 4–6 h.
7. Light production is measured every 30 min by using a luminometer microplate reader (Fig. 5).

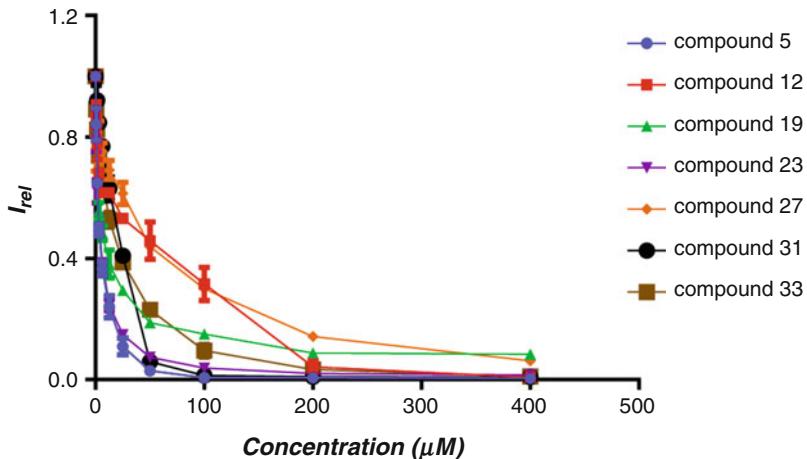


Fig. 5 The inhibitory curves of compounds against *V. harveyi* MM32 ($I_{\text{rel}} = I/I_0$)

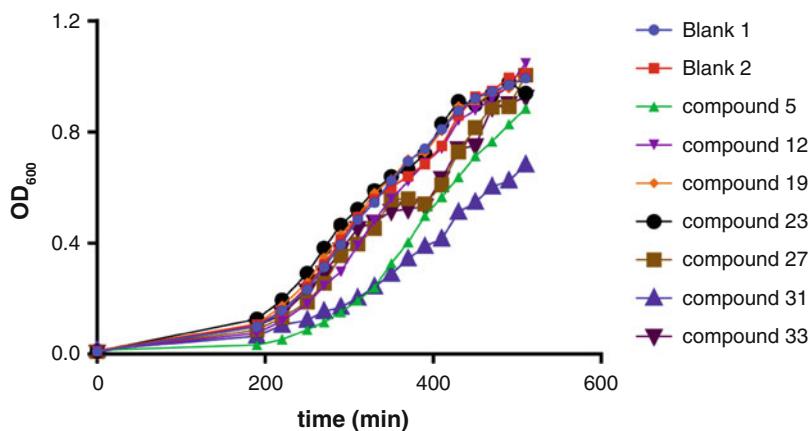


Fig. 6 *V. harveyi* MM32 growth curves

3.4 Effect of the Test Compounds on Bacterial Growth Rate

1. A single colony of *V. harveyi* MM32 is picked from the LM plate prepared in Subheading 3.2, step 1, and inoculated into 2 mL of AB medium supplemented with 50 $\mu\text{g}/\text{mL}$ kanamycin and 10 $\mu\text{g}/\text{mL}$ chloramphenicol. The culture is grown for 16 h with aeration (175 rpm) at 30 °C.
2. The culture from step 1 is diluted 100 fold in 198 mL of AB medium, in the absence and in the presence of the tested compounds at appropriate concentration, in a 250 mL conical flask, and incubated at 30 °C with aeration (175 rpm).
3. The OD_{600} value is measured every 20 min with a spectrophotometer. The doubling time is calculated according to the OD_{600} value (Fig. 6).

4 Notes

1. Never add glucose to the medium because glucose inhibits bioluminescence of *Vibrio harveyi*.
2. The chance of identifying true positive compounds can be improved if multiple scoring functions are combined. It means that (a) each of the individual scoring functions has relatively high performance and (b) the individual scoring functions are distinctive [34].
3. Make sure that they form at least one hydrogen bond and hydrophobic interaction with the LuxP protein.
4. DPD was synthesized following literature procedures before biological evaluation [35].

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Chapter 27

Identification of Staphylococcal Quorum Sensing Inhibitors by Quantification of δ -Hemolysin with High Performance Liquid Chromatography

Cassandra L. Quave and Alexander R. Horswill

Abstract

Quorum sensing plays a major role in regulation of virulence factor production by staphylococci. Chemical inhibitors that block this process and prevent the production of exotoxins and exoenzymes could have medical utility for infection prophylaxis and therapy. Here, we describe a high performance liquid chromatography method amenable to medium throughput screening for staphylococcal quorum sensing inhibitors by quantification of δ -hemolysin, a direct protein output of this system.

Key words Staphylococcal quorum sensing inhibitors, Accessory gene regulator, *Staphylococcus aureus*, High performance liquid chromatography, δ -Toxin

1 Introduction

Staphylococci perform cell-to-cell communication via the release of autoinducing peptide (AIP) molecules into the extracellular environment. When the cell density reaches a critical concentration, enough AIP has accumulated outside the cells to induce a regulatory cascade, a mechanism often called quorum sensing. The activation of this cascade induces the expression of accessory factors, including a large suite of exotoxins and exoenzymes, and hence this system was named the accessory gene regulator (*agr*) [1, 2]. The expression of many of these accessory factors is controlled by a transcript called RNAIII, and encoded within RNAIII is a low molecular weight toxin called δ -toxin or δ -hemolysin. The *agr* system plays a crucial role in the pathogenesis of *Staphylococcus aureus* and methicillin-resistant *S. aureus* (MRSA), and thus anti-virulence approaches to treating staphylococcal infections have focused on disruption of this system. A number of small molecule inhibitors of *agr* have already been identified from natural product

screening efforts [3–7]. δ -hemolysin is a direct output of the *agr* system and thus serves an ideal product to track for the discovery of effective inhibitors.

The aim of this experiment is to identify non-biocide inhibitors of virulence pathways in staphylococci. Here, we explain how a high performance liquid chromatography (HPLC) method developed for detection of δ -hemolysin in staphylococcal supernatants [8] is used to quantify δ -hemolysin in the supernatant for the purposes of identifying quorum sensing inhibitors. A major advantage of this method is that it allows for the direct injection of the bacterial supernatant into the HPLC system without any prior cleanup steps. When the HPLC is paired with an autosampler, the method is amenable to medium-throughput testing. This technique is useful for the phenotypic profiling of isolates, but it is also powerful for the identification of chemical agents with quorum sensing inhibitory activity.

2 Materials

Prepare all solutions with Type 1 (ultrapure) water and American Chemical Society (ACS) grade solvents or equivalent. HPLC mobile phases are prepared with HPLC grade reagents.

2.1 Chemical Matter

1. Chemical matter for testing, in powder form (e.g., natural product extracts or single compounds).
2. Vehicle: dimethyl sulfoxide (DMSO) or water, depending upon solubility of the chemical matter.

2.2 Bacterial Culture

1. *S. aureus* or *S. epidermidis* strains: High toxin producing clinical isolates are preferred for drug screening (e.g., *S. aureus* strains: LAC, NRS 225, NRS 232, NRS242, NRS249, NRS385; *S. epidermidis*: NRS101 obtained from the NARSA collection curated by BEI Resources, www.beiresources.org/).
2. Tryptic soy agar.
3. Tryptic soy broth.
4. Petri dishes.
5. 96-well plates.
6. 14 ml snap cap test tubes.
7. Microcentrifuge tubes.
8. Inoculating loop.

2.3 High Performance Liquid Chromatography

1. GE Healthcare Resource PHE 1-ml column.
2. Mobile Phase A: 0.1% (vol/vol) trifluoroacetic acid (TFA) in water.

3. Mobile Phase B: 0.1% (vol/vol) TFA in acetonitrile (ACN).
4. HPLC system.
5. 2 ml HPLC vials.

3 Methods

Carry out all experiments at room temperature unless otherwise noted.

3.1 Prepare Chemical Matter for Testing

1. Carefully weigh the dry test compound or natural product extract, collectively referred to as “drug” below. Create a stock solution at 10 mg/ml of extract (mixture of compounds) in vehicle (DMSO or water, depending on solubility), or 1 mg/ml suggested for individual compounds.
2. Vortex the stock solution for 1 min. Sonicate in a water bath for 15 min or more. Monitor to ensure that no solids are floating in the stock. Store at 4 °C until needed for assays.

3.2 Measure Growth Inhibitory Effects

1. Determine the Minimum Inhibitory Concentration (MIC) of your drug by broth microtiter dilution, following established Clinical & Laboratory Standards Institute (CLSI) guidelines (M100-S23) [9]. Briefly, overnight cultures of *S. aureus* should be standardized to reach a final inoculum density of 5×10^5 colony forming units per ml (CFU/ml) in Cation-adjusted Mueller-Hinton Broth (CAMHB), verified by colony plate counts. Your drug should be added to the top well on a 96-well plate and serial diluted down to the desired concentration test range. Following an 18 h period of incubation at 37 °C in a humidified chamber, the MIC can be determined by identifying the lowest concentration well with no visible growth (determined by eye or by a plate reader at an optical density of 600 nm).
2. Select a sub-MIC concentration for the starting point in serial dilutions of the chemical matter for δ -hemolysin quantification. In general, we recommend starting at concentrations at less than 50% growth inhibitory levels.

3.3 Grow Cultures and Harvest Supernatant

1. *Day 1:* Make a fresh 3-way streak plate of *S. aureus* on tryptic soy agar (TSA). Incubate at 37 °C overnight (18–24 h).
2. *Day 2:* Add 6 ml of tryptic soy broth to a 14 ml snap cap test tube. Using a sterile inoculating loop, inoculate the tube with a single colony from the overnight plate. Place in the incubator at a 45° angle; incubate at 37 °C with shaking (200 rpm) overnight (18–24 h).

3. *Day 3:* Standardize the inoculum to a final starting density of 5×10^5 colony forming units (CFU) per ml by diluting into fresh TSB. The final starting inoculum density (CFU/ml) should be checked by dilution plating and colony counts.
4. The freshly diluted inoculum should be placed in a test tube or flask at a volume-to-flask ratio of ~1:10 for optimal aeration with shaking. For example, 1.5 ml of inoculum is added to 14 ml snap cap test tubes. Use three untreated growth controls (no drug added) for establishing the baseline toxin levels for that particular strain. Vehicle controls should also be included in the study for comparison.
5. To test a single concentration of the drug, add an appropriate amount of the drug stock to achieve a sub-MIC concentration, preferably at a level eliciting less than 50% inhibition. Then, add the appropriate amount of inoculum created in **step 4**. Always vortex the stock solution well prior to use. If the drug exhibits issues with solubility, additional sonication prior to use may be helpful. Perform all tests with four replicates. To determine the volume of drug stock to add, use the following formula:

$$\frac{x \mu\text{g}}{z \text{ml}} = \frac{y \mu\text{g}}{\text{ml}}$$

where x = amount of drug required to achieve y desired final test concentration in z volume (*see Note 1*).

6. To investigate dose-response activity, test serial dilutions of the drug following the methods described in **step 5**, with the exception that you start with double the desired final volume in tube 1. Twofold serial dilutions can be made by vortexing tubes between dilution steps (*see Note 2*). Perform all tests with four replicates. To achieve twofold serial dilution with four final test concentrations, add 1.5 ml of inoculum from **step 3** to tubes 2–4. Place cap on tube 1 and vortex to mix the drug and inoculum. Transfer 1.5 ml of tube 1 to tube 2 and vortex with cap. Continue this process of transfer and vortexing from tube 2 to 3 and tube 3 to 4. Dispose of the last 1.5 ml from tube 4. The final concentration per tube in this example will be 32 µg/ml in tube 1, and then 16, 8, and 4 µg/ml in tubes 2–4, respectively.
7. Incubate the cultures at 37 °C for 15 h at 275 rpm for optimal growth. If using test tubes for this step, it is critical to place the tubes in the incubator rack at a 45°angle to achieve optimal aeration and toxin production.
8. Label microcentrifuge tubes and HPLC vials with the strain and drug information in preparation for Day 4 steps.

9. *Day 4:* Remove cultures from the incubator and immediately place tubes on ice. Take a 100 μ l aliquot of each culture and transfer to a 96-well plate for a plate read at an optical density (OD) of 600 nm. This information will be used to check for potential growth inhibitory activity of the drug and will also be used to normalize toxin production by OD.
10. Vortex test tubes and transfer the remaining volume into pre-labeled microcentrifuge tubes. Use a benchtop microcentrifuge to spin down the cell pellets at $8000 \times g$ for 5 min.
11. Using a 1 ml pipette, carefully transfer at least 0.7 ml of the supernatant to an HPLC vial without disturbing the cell pellet. Seal vials with lids and store at -20°C until ready for testing by HPLC. Do not thaw and refreeze prior to testing.

3.4 HPLC

Quantification of δ -Hemolysin

1. Prepare mobile phases A and B, mixing each solvent well, and if necessary filtering with 0.2 μm filter. Attach the Resource PHE 1-ml column to the HPLC. Flush the HPLC system, check the system for leaks, and flush the column with at least 10 column volumes (10 ml) of mobile phase at initial conditions until a stable system pressure is obtained. *See Note 3* for instrumentation details.
2. Thaw the supernatant samples in their HPLC vials at room temperature. Vortex each vial prior to loading in the HPLC autosampler.
3. Program the HPLC to the following parameters: 500 μl injection volume with one injection per vial; flow rate of 2 ml/min; column temperature at 25°C ; UV/Vis monitored at 214 nm. Use mobile phases (a) 0.1% (vol/vol) TFA in H_2O and (b) 0.1% (vol/vol) TFA in ACN with the linear gradient profile for HPLC analysis reported in Table 1.

Table 1
Linear gradient profile for HPLC analysis

Time (min)	% A	% B
0	90	10
3.00	90	10
10.50	10	90
10.51	0	100
12.00	0	100
12.01	90	10
15.50	90	10

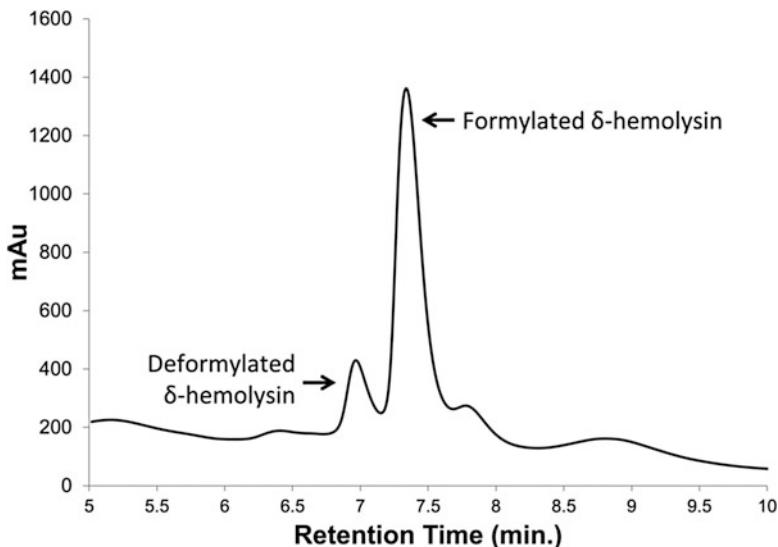


Fig. 1 HPLC chromatogram of δ -hemolysin, with deformylated and formylated peaks highlighted

4. The chromatogram will show two prominent peaks, one corresponding to the deformylated δ -hemolysin at a retention time of \sim 7.2 min, and the second corresponding to the formylated δ -hemolysin at \sim 7.5 min (Fig. 1). There can be a large strain to strain variation in total levels of δ -hemolysin produced (Fig. 2). For this reason, we recommend that high level producers be used for drug screening initiatives in order to better detect potential inhibitors.
5. Integrate the peaks corresponding to the deformylated (\sim 7.2 min) and formylated (\sim 7.5 min) forms of δ -hemolysin at 214 nm. Adjust the LC software to use a “perpendicular drop method” of peak detection. The parameters need to accurately detect the baseline under the peaks and drop vertical lines from the valley between the peaks to the extended baseline. For samples with moderate to high levels of δ -hemolysin production the software defined integration parameters usually do not require manipulation. Care needs to be taken with samples that have no or low levels of toxin production, verify that the correct peak is being identified and integrated.
6. Calculate the standard deviation of the deformylated and formylated δ -hemolysin peak integrations separately for each set of replicates.
7. In order to normalize the δ -hemolysin production, the area corresponding to the deformylated and formylated δ -

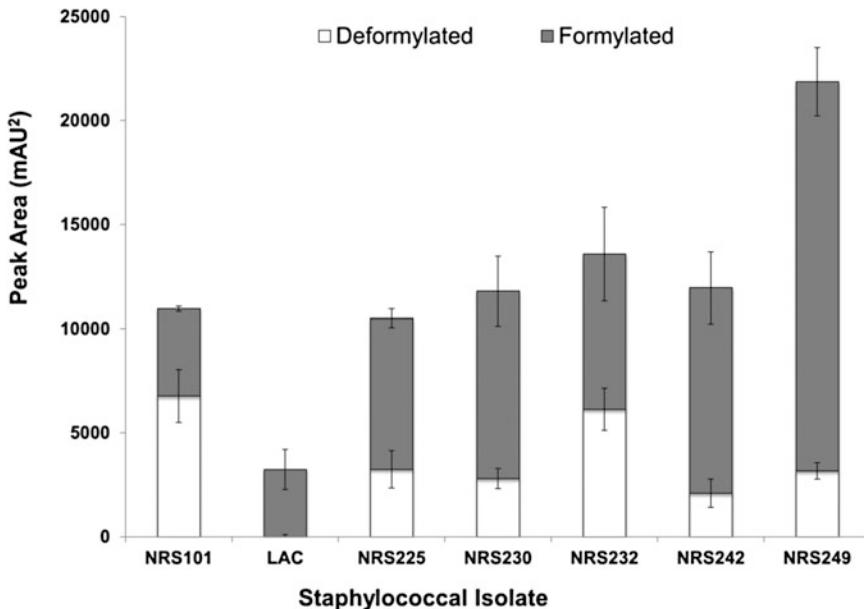


Fig. 2 Staphylococcal strains exhibit different capacities for δ -hemolysin production, illustrated here by total peak area of the deformylated and formylated peaks. All isolates are *S. aureus*, with exception of NRS101 (*S. epidermidis*)

hemolysin must be divided by the OD reading determined in Subheading 3.2, step 9

$$A_{\text{norm}} = \frac{A_{\text{raw}}}{\text{OD}_{600}}$$

- Once the integration data has been normalized, then it may be presented as separate deformylated and formylated δ -hemolysin values including standard deviation using a stacked histogram chart (Fig. 2).

4 Notes

- Example: To achieve a final test concentration of 32 $\mu\text{g}/\text{ml}$, using a drug stock concentration of 10 mg/ml and a final volume of 1.5 ml in the test tube:

$$\frac{x \ \mu\text{g}}{1.5 \ \text{ml}} = \frac{32 \ \mu\text{g}}{\text{ml}}; 1.5 \times 32; \text{then } x = 48 \ \mu\text{g} \text{ need to be added.}$$

If working with a drug stock concentration of 10 mg/ml , or 10 $\mu\text{g}/\mu\text{l}$, then $48 \ \mu\text{g} \times \frac{\mu\text{l}}{10 \ \mu\text{g}} = 4.8 \ \mu\text{l}$ should be added to achieve the desired final concentration in a total volume of 1.5 ml (1.4952 ml of inoculum + 4.8 μl drug stock).

2. Example: To achieve a starting concentration of 32 µg/ml, using a drug stock concentration of 10 mg/ml and a starting volume of 3 ml in the first test tube:

$$\frac{x \mu\text{g}}{3 \text{ ml}} = \frac{32 \mu\text{g}}{\text{ml}}; = 3 \times 32; \text{ then } x = 96 \mu\text{g} \text{ need to be added.}$$

If working with a drug stock concentration of 10 mg/ml, then $96 \mu\text{g} \times \frac{\mu\text{l}}{10 \mu\text{g}} = 9.6 \mu\text{l}$ should be added to achieve the desired final concentration in a total volume of 3 ml (2.9904 ml of inoculum + 9.6 µl drug stock).

3. The method was performed on an Agilent 1260 Infinity system equipped with a quaternary pump, automatic liquid sampler, thermostatted column compartment, and diode array detector. The system was controlled and data processed using OpenLab CDS ChemStation (Agilent Technologies, Santa Clara, CA, USA).

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Erratum to : Fluorescence Quenching Studies of γ -Butyrolactone-Binding Protein (CprB) from *Streptomyces coelicolor* A3(2)

Jessy Mariam and Ruchi Anand

Erratum to:

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The original version of this chapter was inadvertently published with incorrect figure (Fig. 3). The correct figure is updated in the current version.

The updated online version of this chapter can be found at
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