Appl Microbiol Biotechnol (2014) 98:7199–7209
DOI 10.1007/s00253-014-5904-3

METHODS AND PROTOCOLS

A new assay for rhamnolipid detection—important virulence factors of *Pseudomonas aeruginosa*

Maisem Laabei · William D. Jamieson · Simon E. Lewis · Stephen P. Diggle · A. Tobias A. Jenkins

Received: 5 February 2014 / Revised: 17 June 2014 / Accepted: 18 June 2014 / Published online: 29 June 2014

Abstract Rhamnolipids (RLs) are heterogeneous glycolipid molecules that are composed of one or two L-rhamnose sugars and one or two β-hydroxy fatty acids, which can vary in their length and branch size. They are biosurfactants, predominantly produced by *Pseudomonas aeruginosa* and are important virulence factors, playing a major role in *P. aeruginosa* pathogenesis. Therefore, a fast, accurate and high-throughput method of detecting such molecules is of real importance. Here, we illustrate the ability to detect RL-producing *P. aeruginosa* strains with high sensitivity, based on an assay involving phospholipid vesicles encapsulated with a fluorescent dye. This vesicle-lysis assay is confirmed to be solely sensitive to RLs. We illustrate a half maximum concentration for vesicle lysis (EC_{50}) of 40 μM (23.2 μg/mL) using pure commercial RLs and highlight the ability to semi-quantify RLs directly from the culture supernatant, requiring no extra extraction or processing steps or technical expertise. We show that this method is consistent with results from thin-layer chromatography detection and dry weight analysis of RLs but find that the widely used orcinol colorimetric test significantly underestimated RL quantity. Finally, we apply this methodology to compare RL production among strains isolated from either chronic or acute infections. We confirm a positive association between RL production and acute infection isolates (p=0.0008), highlighting the role of RLs in certain infections.

M. Laabei · W. D. Jamieson · S. E. Lewis · A. T. A. Jenkins (✉)
Department of Chemistry, University of Bath, Claverton Down, Bath BA2 7AY, UK
e-mail: a.t.a.jenkins@bath.ac.uk

M. Laabei
Department of Biology and Biochemistry, University of Bath, Claverton Down, Bath BA2 7AY, UK

S. P. Diggle
School of Life Sciences, University of Nottingham, University Park, Nottingham NG7 2RD, UK

Keywords Rhamnolipids · *Pseudomonas aeruginosa* · Lipid vesicles · Detection

Introduction

Pseudomonas aeruginosa is an opportunistic human pathogen, ubiquitous in the environment and capable of causing a multitude of infections in the immunocompromised host (Bodey et al. 1983; Driscoll et al. 2007). The ability to cause such a wide array of infections is dependent on the expression of virulence factors (Strateva and Mitov 2011). Virulence factor regulation in *P. aeruginosa* is achieved through a density-dependent cell-to-cell communication network, involving three main quorum-sensing systems; the *las*, *rhl* (Schuster et al. 2013; Smith and Iglewski 2003) and *Pseudomonas* quinolone signal system (Dubern and Diggle 2008; Pesci et al. 1999). The *las* and *rhl* systems are LuxRI homologues, where *lasI* and *rhlI* direct synthesis of *N*-3-oxododecanoylhomoserine lactone (3-oxo-C12-HSL) and *N*-butanoylhomoserine lactone (C4-HSL), respectively; these are diffusible signalling molecules which activate their respective DNA binding response regulators, LasR and RhlR, which, in turn, induces the expression of a wide range of genes, approximately 6 % of the genome (Schuster and Greenberg 2006; Schuster et al. 2013; Wagner et al. 2004; Williams et al. 2007). Another cell-to-cell signalling system responds to the quinolone compound 2-heptyl-3-hydroxy-quinolone (the *Pseudomonas* quinolone signal (PQS)), acting with the transcriptional activator, PqsR (Diggle et al. 2006; Pesci et al. 1999). An elegant hierarchy system predominates in this global regulatory network, with the *las* system positively regulating both the *rhl* and quinolone signalling systems (Pesci et al. 1999; Pesci et al. 1997; Williams and Camara 2009).

One of the most interesting extracellular factors produced by *P. aeruginosa* is rhamnolipids (RLs). RLs are surfactant-acting molecules, composed of a hydrophilic head of one or

two rhamnose molecules and a hydrophobic tail portion of one or two fatty acids. The amphiphilic nature of RLs allows these biosurfactans to partition into biological and artificial membranes altering their biophysical properties, previously shown in model membranes using 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) vesicles and purified RLs (Aranda et al. 2007; Sanchez et al. 2010). RLs are also very important virulence factors for *P. aeruginosa*, central in immune cell and erythrocyte destruction (Fujita et al. 1988; Jensen et al. 2007), swarming and twitching motility (Caiazza et al. 2005; Deziel et al. 2003) and biofilm formation and protection (Alhede et al. 2009; Pamp and Tolker-Nielsen 2007). Furthermore, they have been implicated in the deterioration of patients with ventilator-associated pneumonia (Kohler et al. 2010) and disruption and permeablisation of epithelial cells, a prerequisite to *P. aeruginosa* invasion (Zulianello et al. 2006).

Clearly, an accurate and rapid method to assess RL production is important. Current methods can be qualitative; the cetyltrimethylammonium bromide (CTAB) methylene blue (MB) agar test (Pinzon and Ju 2009) signals RL expression through the formation of blue halos due to the complexation of the anionic RL and cationic CTAB and MB. Although this method is quick, it suffers from a time delay due to incubation for 48 h for optimal results and also through distortion of halo formation due to fluorescent pigments produced naturally by certain *P. aeruginosa* strains. Other methods measure the tensioactive properties of the surfactant (Morikawa et al. 2000); however, these methods employ sensitive instruments and are laborious, not amenable to high throughput. Quantitative methods consist of spectrophotometric analysis, using the orcinol test (Koch et al. 1991)), chromatographic methods including thin-layer chromatography (TLC) high-performance liquid chromatography (HPLC) (Schenk et al. 1995), infrared spectroscopy (IR) (Leitermann et al. 2008) and dry weight analysis of rhamnolipids following solvent extraction (Gunther et al. 2005). Here, we present a new methodology in detecting and quantifying RLs using carboxylfluorescein-encapsulated phospholipid vesicles. We have previously shown that these vesicles are stable under various conditions (Marshall et al. 2013) and are susceptible to a suite of *Staphylococcus aureus* toxins that have surfactant-like properties (Laabei et al. 2014). In this report, we confirm that RLs can be detected and semi-quantified directly from pure overnight culture supernatants of *P. aeruginosa* using the vesicle-lysis assay, decreasing preparation time, hazardous extraction techniques or expert analysis.

Materials and methods

Bacterial strains and growth conditions

P. aeruginosa strains that were used in this study are given in Table 1. These strains were routinely stored at −80 °C in 15 % glycerol/broth stocks until required. These strains were maintained in Luria-Bertani (LB) medium (Fluka). A single colony was inoculated in 5 mL of LB broth contained within a 25-mL glass universal reaction tube for 20 h at 37 °C and shaking at 180 rpm. These cultures were subsequently sub-cultured 1:1,000 in LB broth and grown for 18 h to generate supernatants used in this study. Bacterial growth and vesicle fluorescence were measured using absorbance optical density at 600 nm (OD_{600}) and excitation and emission wavelengths of 485–520 nm, respectively. Bacterial cultures and vesicles were co-incubated at 37 °C with shaking at 300 rpm in a 96-well round-bottom plate using a FLUOROstar fluorimeter (BMG LABTECH). Antibiotics were added to the media at the following concentrations for maintaining particular mutant strains: PAO1Δ*rhlI* (tetracycline 200 μg/mL) and strains PAO1Δ*lasR* and PAO1Δ*lasI* (gentamicin 100 μg/mL).

Lipid vesicle development

Vesicles were developed as described previously (Laabei et al. 2012). Briefly, vesicle suspensions were prepared using the molar ratio of 25 % 10,12-tricosadiynoic acid (Sigma), 53 % 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 2 % 1,2-dipalmitoyl-*sn*-glycerol-3-phosphoethanolamine (DPPE) and 20 % cholesterol. Lipid films were rehydrated in 50 mM 5(6)-carboxyfluorescein (Sigma) in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer solution (pH 7.4), freeze-thawed three times using liquid nitrogen and extruded through a 100-nm membrane (LiposoFast). Vesicles were further purified through a NAP-25 column (GE Healthcare) kept at 4 °C overnight and cross linked using a UV cross linker (UVP CL1000). The size distribution and concentration of vesicles were measured via dynamic light scattering (Malvern) and nanosight tracking analysis (NanoSight Ltd), respectively, highlighting a vesicle size distribution of 90–110 nm and concentration of 1×10^8 vesicles/μL.

Vesicle-lysis assay

The parameter for measuring the fluorescence intensity of lysed vesicles was set at excitation and emission wavelengths of 485–520 nm, respectively, and a gain of 550 using a FLUOROstar fluorimeter (BMG LABTECH). Two types of experiments were designed, one including whole bacterial cells and one with harvested supernatant. In the former method, bacterial starting inoculums were normalised to specific concentrations of 1×10^6, 1×10^5 and 1×10^4 CFU/mL. The bacterial culture (200 μL) was added to the vesicle solution (50 μL) in triplicate, and the fluorescence intensity was measured at 5-min intervals for 18 h. For the supernatant–vesicle assay, bacterial supernatants were harvested from 1 mL of 18-h culture after centrifugation for 10 min at 14,000×*g* using

Table 1 List of strains used in this study

Strain	Description	Reference
PAO1	Nottingham collection wild-type *P. aeruginosa* strain	Klockgether et al. (2010)
PAOlΔ*rhlR*	*rlhlR* mutant of PAO1	Diggle et al. (2002)
PAOlΔ*rhlI*	PAO1 derivative with a Tc cassette insertion into the *rhlI* gene, Tc	Beatson et al. (2002)
PAOlΔ*lasR*	PAO1 mutant with a Gm cassette insertion into the *lasR* gene, Gm	Popat et al. (2012)
PAOlΔ*lasI*	PAO1 mutant with a Gm cassette insertion into the *lasI* gene, Gm	Beatson et al. (2002)
PAOlΔ*plcH*	*plcH* mutant of PAO1	Wargo et al. (2011)
PAOlΔ*pqsA*	*pqsA* mutant of PAO1	Aendekerk et al. (2005)
PAO1Δ*pqsE*	*pqsE* mutant of PAO1	Diggle et al. (2007)
PAO1Δ*pqsH*	*pqsH* mutant of PAO1	Diggle et al. (2007)
PAO1Δ*rhlA*	*rhlA* mutant of PAO1	Heurlier et al. (2004)
Chronic Isolates	*n*=48 isolated from patients with cystic fibrosis	Gift from M.C. Enright
Acute isolates	*n*=30 isolated from patients with acute injury	Gift from Southmead Hospital, Bristol

Tc tetracycline, *Gm* gentamicin

a bench top centrifuge. Bacterial supernatant (50 μL) and vesicle solution (50 μL) were incubated for 30 min using the same parameters as above. Normalised fluorescence was achieved using the equation (Ft−F0)/(Fm/F0) where Ft is the average fluorescence value at a specific time point, F0 is the minimum and Fm is the maximum fluorescence value in that particular experiment, determined using the negative and positive controls of HEPES buffer and 0.1 % Triton X-100, respectively. In order to understand which exoprotein(s) was causing vesicle lysis, PAO1 bacterial supernatant was heated to 95 °C for 1 h and its lytic potential assessed using the above parameters. All experiments were done in triplicate three times, and the error represents the 95 % confidence interval.

Thin-layer chromatography

Rhamnolipids were extracted from filtered culture supernatants using ethyl acetate in a 1:1 (*v*/*v*) ratio. Samples were mixed by vortexing with subsequent phase separation by centrifuging for 1 min at 14,000×*g*. The upper, rhamnolipid-containing phase was transferred to a new Eppendorf tube and the procedure repeated three times. The organic solvent was removed by evaporation using a vacuum centrifuge. For the detection of rhamnolipids, this dried pellet was dissolved in 10 μL of ethanol. This solution (5 μL) was spotted on silica 60 TLC plates (Fisher). In addition, 0.1 % rhamnolipid solution (5 μL) containing mono- and di-rhamnolipid (R-95 Sigma) was used as a standard. TLC was performed using chloroform/methanol/acetic acid in a ratio of 65:15:2 as a developing solvent. For visualisation, the dried plate was briefly submerged in a detection agent composed of 0.15 g orcinol, 8.2 mL sulphuric acid (60 %, *v*/*v*) and 42 mL deionised H_2O. The plate was left to dry at room temperature, and then, the sugar moieties were stained by incubating the plates at 110 °C for 10 min.

Orcinol assay

Culture supernatants were obtained by centrifugation, and 300 μL of supernatant was extracted twice with 1 mL of diethyl ether. The samples were pooled and evaporated to dryness using a vacuum centrifuge, and then, 0.5 mL of sterile H_2O was added. To each 100-μL sample, 900 μL of a solution containing 0.19 % orcinol (in 53 % H_2SO_4) was added. This solution was heated to 80 °C for 30 min, after which the samples were cooled at room temperature for 15 min. The absorbance of the samples was measured at 421 nm (BMG LABTECH) and the concentration of rhamnolipids compared to those generated using a standard.

Dry weight analysis

Dry weight analysis was based on the procedure by Gunther et al. (2005). Briefly, the supernatant was separated from an overnight culture (100 mL) by centrifugation at 7,000×*g*. The supernatant was then acidified to pH 2.0 by the dropwise addition of 12 M hydrochloric acid. This solution was then centrifuged at 13,000×*g*, and the RL-containing precipitate was extracted three times with a chloroform–ethanol (2:1) mixture. This was then evaporated away leaving the characteristic honey-like appearance. This oily residue was dissolved in methanol and transferred to a previously weighted container, where the methanol was evaporated giving the total rhamnolipid yield.

Results

Bacterial-mediated lysis of vesicles occurs during the early stationary phase of growth

In order to investigate at what stage of growth *P. aeruginosa* PAO1 wild-type lysed lipid vesicles, different starting inoculums were used to initiate growth in a mix of nutrient-rich broth and lipid vesicles (Fig. 1a). Expression of specific genes is essential at different stages of growth, particularly in the expression of virulence factors (Winzer and Williams 2001), capable of lysing lipid vesicles. Therefore, we investigated the ability of *P. aeruginosa* to break down vesicles and correlated this with the respective growth rate with and without vesicles within the media (Fig. 1b). Differences in optical density (OD_{600}) observed between the two experiments are most probably due to the vesicles impacting on light scattering, resulting in higher absorbance values. Nonetheless, vesicles did not have a negative impact on bacterial growth (Fig. 1b). By using different starting inocula of 1×10^4–1×10^6 CFU/mL, we illustrate that a time delay exists in lysis of vesicles with respect to a smaller starting inoculum. With a starting inoculum of 1×10^6 CFU/mL, bacteria reach late exponential/early stationary phase of growth after approximately 400 min (Fig. 1b) (a blue line in both Fig. 1a, b represents a 400-min time point). With a starting inoculum of 1×10^6 CFU/mL, this is the approximate time point when vesicles begin to break down followed by increasing fluorescence release over the next 150 min (Fig. 1a). This signifies that bacterial-mediated breakdown of vesicles occurs during the early stationary phase, when the local concentrations of bacteria reach a critical threshold, leading to the expression of quorum-sensing (QS) regulatory genes known to be involved in the expression of virulence factors capable of causing membrane damage.

Identification of rhamnolipids as the vesicle-lysing agent

The production of toxins/enzymes in *P. aeruginosa* is governed by a hierarchical cell-to-cell QS system, which when activated, leads to the production of a whole suite of virulence factors, some of which are excreted into the extracellular environment. Therefore, we investigated the ability of QS mutants to lyse lipid vesicles (Fig. 2a), in order to determine factor(s) involved.

The quinolone signalling system contains the *pqsABCDE* operon, in which the *pqsABCD* section is required for the synthesis of the hydrophobic quinolone signal which interacts with a LysR-like regulator PqsR, modulating the expression of genes known to function in virulence (Diggle et al. 2007; McKnight et al. 2000). Therefore, we assayed the culture supernatant of a *pqsA* mutant to identify any deleterious effects on lipid vesicle lysis; however, no difference between wild type (WT) and mutant strain was evident (Fig. 2a). It has been shown previously that a *pqsE* mutant, although not impacting on the production of the PQS signal, negatively affects PQS-controlled virulence factors (Diggle et al. 2003; Gallagher et al. 2002) and has been shown to enhance the *rhl* system (Farrow et al. 2008). However, no difference was seen in the lysis of lipid vesicles with culture supernatants derived from the *pqsE* mutant (Fig. 2a). Unlike the signalling molecules of the *las* and, to an extent, *rhl* systems, the PQS is very hydrophobic, inhibiting free diffusion between bacterial communities (Mashburn-Warren et al. 2009). To circumvent this, these PQS signals are encapsulated within membrane vesicles (MVs), derived from the outer membrane of the bacterial cell envelope (Mashburn and Whiteley 2005). Interestingly, PQS has been shown to induce membrane curvature in erythrocytes, leading to haemolysis (Schertzer and Whiteley 2012). The formation of membrane vesicles requires the expression

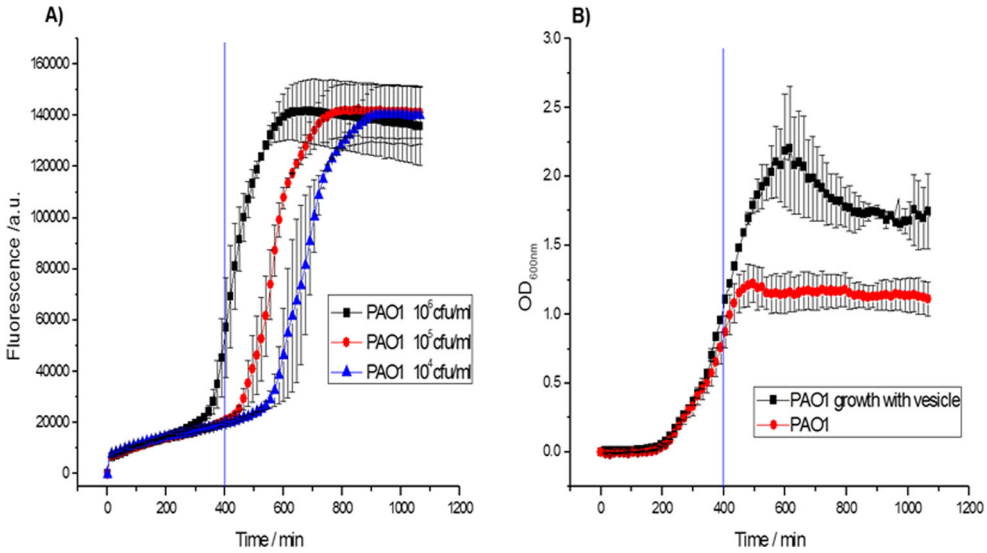

Fig. 1 Lysis of vesicles occurs during the early stationary phase of growth of *Pseudomonas aeruginosa*. **a** The breakdown of vesicles by different starting inoculums (10^6–10^4 CFU/mL) was measured over 18 h of growth, shown here by the detection of carboxyfluorescein released from lysed vesicles. **b** The growth curves of *P. aeruginosa*, with and without vesicles added to the medium, are illustrated over 18 h from a starting inoculum of 10^6 CFU/mL

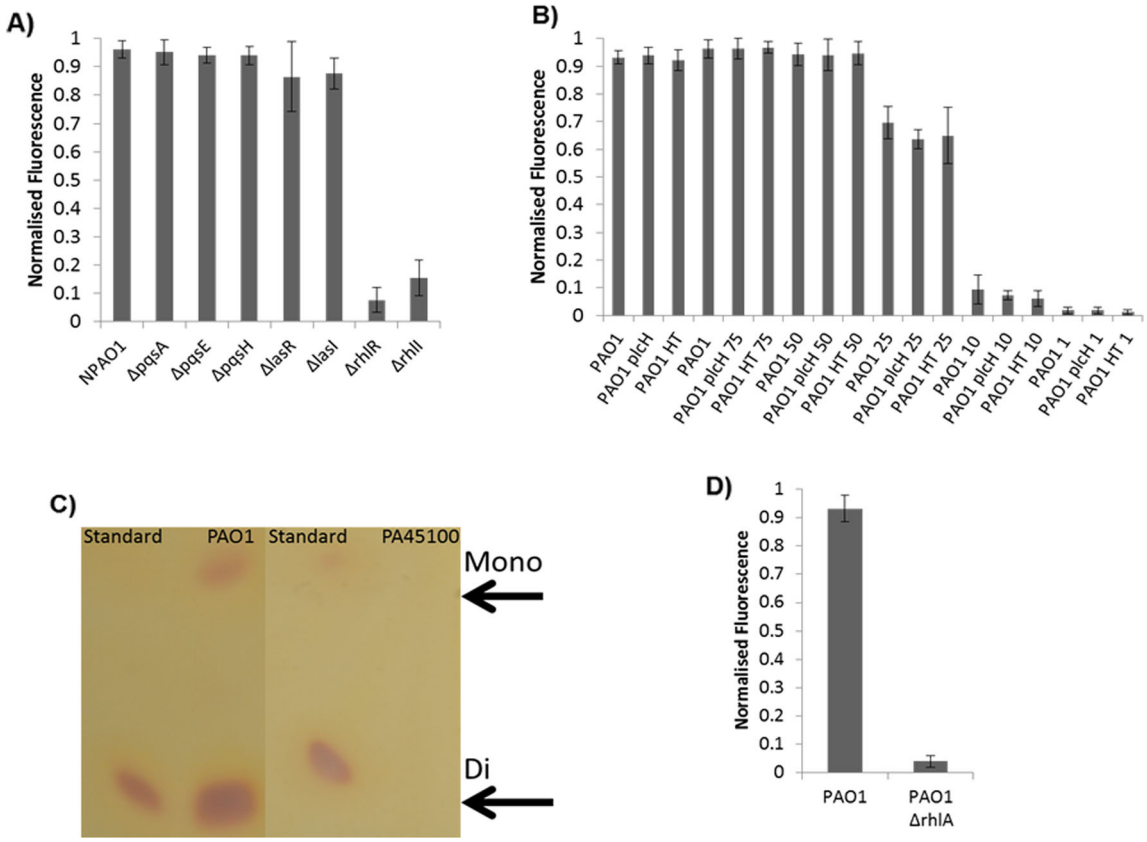

Fig. 2 Identification of vesicle-lysing agent. **a** Culture supernatants of several quorum-sensing mutants (Δ*pqsA*, Δ*pqsE*, Δ*pqsH*, Δ*lasR*, Δ*lasI*, Δ*rhlR* and Δ*rhlI*) were tested against lipid vesicles, highlighting the important role of the *rhlRI* QS system in vesicle lysis. **b** Culture supernatants of wild-type (WT) PAO1, a lipase mutant PAO1Δ*plcH* and heat-treated (HT) PAO1 culture supernatant at various dilutions (neat, 75, 50, 25, 10 and 1) were tested against lipid vesicles, with no significant differences observed. **c** TLC was used to detect the presence of rhamnolipid from two strains, PAO1 (vesicle-lysis positive) and PA45100 (vesicle-lysis negative) **d** Comparison of WT PAO1 and the isogenic *rhlA* mutant.

of PqsH, which is a monooxgenase, essential for the conversion of 2-heptyl-4-quinolone (HHQ) to PQS (Gallagher et al. 2002). Therefore, in a *pqsH* mutant, there is a reduced capacity to form membrane vesicles due to reduced quinolone formation to induce membrane blebbing and structural perturbations. However, no difference was seen in vesicle lysis with culture supernatants derived from this mutant and WT, suggesting that the quinolone signalling system did not play a role in the lysis of this specific vesicle type (Fig. 2a).

The *lasRI* and *rhlRI* regulatory systems are the most well-characterised QS systems in *P. aeruginosa*. Therefore, in order to investigate what secreted factor caused lysis, we examined culture supernatants from deletion mutants of the signal synthase (*lasI* and *rhlI*) and the response regulators (*lasR* and *rhlR*) (Fig. 2 a). The results demonstrated that an active *rhlRI* system was required for vesicle lysis. *P. aeruginosa* expresses three quorum-regulated phospholipases C (PLC) enzymes: a haemolytic PLC (PlcH), a non-haemolytic PLC (PlcN) (Ostroff et al. 1990) and a PLC involved in phospholipid chemotaxis (Barker et al. 2004) (PlcB). PlcH causes cytolysis, with a preferred substrate affinity for phosphatidylcholine (PC) and sphingomyelin (Ostroff et al. 1990); importantly, PC is the most abundant lipid in our vesicle. For this reason, we examined the effect of a *plcH* deletion mutant on the capacity to lyse the vesicle (Fig. 2b). By using serial dilutions of supernatants, it was evident that PlcH did not have any effect on lysis. Certain proteins are inactivated at high temperatures; therefore, to understand what was causing vesicle breakdown, supernatants were heat-treated and compared to non-treated supernatants (Fig. 2b); however, no difference in vesicle lysis ability was observed. This suggested that a heat-resistant glycolipid could be involved in overall vesicle lysis.

RLs are biosurfactant glycolipids, in which the synthesis of these molecules is under the control of the *rhlABC* operon, where the *rhlA* gene is itself directly regulated by the *rhl* QS system (Pearson et al. 1997). We performed TLC on solvent extracts in order to detect RLs from *P. aeruginosa* strains that were either vesicle-lysis positive or negative (Fig. 2c and Table 2), resulting in those strains rupturing vesicles being RL positive and those being lysis negative having no detectable RLs present. In order to understand conclusively what caused *P. aeruginosa*-mediated breakdown of lipid vesicles,

Table 2 Detection and quantification of rhamnolipids

Strain (acute)	Orcinol test[a]	Lipid vesicle test[a]	TLC	Strain (chronic)	Orcinol test[a]	Lipid vesicle test[a]	TLC
44883	21.7	125.4	+	PA00856	22.2	85.5	+
44981	8.6	25.6	+	PA00887	20.83	30.9	+
45197	16.7	86.9	+	PA00889	21.7	91.1	+
45445	32.5	150.2	+	PA00935	16.2	90.1	+
45076	2.3	N/A	−	PA00261	3.2	N/A	−
45100	15.2	N/A	−	PA00848	3.9	N/A	−
45122	1.88	N/A	−	PA00918	3.4	N/A	−
45394	2.83	N/A	−	PA00929	3.7	N/A	−

N/A not applicable

[a] Quantification of rhamnolipids derived from these techniques, μg/mL

culture supernatants from an *rhlA* mutant were used, and no vesicle lysis was observed (Fig. 2d). This *rhlA* gene is critical in the formation of RL precursors (Deziel et al. 2003), and thus, without this, neither mono- nor di-RLs are formed.

Estimation of secreted rhamnolipid concentration from *P. aeruginosa*

To investigate whether this vesicle-lysis assay could be used to quantify RLs secreted from culture supernatants, we created a standard curve (Fig. 3a) incubating vesicles and purified R-95 rhamnolipids, a mixture of the highly abundant mono- and di-rhamnolipids congeners from *P. aeruginosa*. This graph illustrates the EC_{50}, the concentration of purified rhamnolipids to cause 50 % lysis of vesicles, as 23.2 μg/mL (40 μM). Using this value, the quantity of RLs from culture supernatants can be estimated (Fig. 3b), by graphing the respective normalised fluorescence obtained from serial dilutions of culture supernatants, using a sigmoidal curve fit, generating the best fit line, giving the dilution constant required for EC_{50} and multiplying to give an estimation of RLs in the original culture (92.8 μg/mL for PAO1). This PAO1 RL value generated from the vesicle-lysis assay was relatively consistent with that of our dry weight analysis of PAO1 RLs (172.5±56.4 μg/mL). It is important to note that the quantity and structure of RLs is dependent on many factors, including carbon source, reaction vessel and conditions, temperature and strain-specific details, and that very high concentrations of RLs have been recorded using optimised growth medium and bioreactors (Perfumo et al. 2013; Rikalovic et al. 2012). The growth conditions and reaction conditions that we have employed are not conducive to high levels of RL production, as this was not the aim of this study.

Comparison of vesicle assay with orcinol and TLC assays

A comparison was made between the vesicle-lysis assay and the orcinol and TLC methods of RL detection and quantification. We selected 16 strains, 8 derived from acute strains and 8 derived from chronic strains, and measured their respective RL content using the above methods (Table 2). The orcinol assay was chosen as this colorimetric assay is widely used. However, this method provided a significant underestimation of RL values compared to our results, perhaps due to loss of RL during the extraction procedure, which were produced at small quantities initially. This was not the first study which has highlighted erroneous results using the orcinol assay (Perfumo et al. 2013). The orcinol method can also suffer from contamination from the growth media and also other components of the cellular envelope which has rhamnose as a component in their structure, namely lipopolysaccharides. The TLC method was used qualitatively and complemented the results of our vesicle-lysis assay, where vesicle-lysis positive strains were also shown to secrete RLs (Table 2). The CTAB method can suffer from distortion of RL complexation circles due to pigment production (Pinzon and Ju 2009), and we were not able to generate consistent results using this method. The RL values of clinical isolates examined here (Table 2) are consistent with values shown by other groups (Rikalovic et al. 2012). Although the vesicle-lysis assay is dependent on fluorescence detection from lysed vesicles, this is not influenced by fluorescent molecules being expressed by *P. aeruginosa* (data not shown): Pyoverdine is typically excited by low-wavelength light (UV), whilst carboxyfluorescein is excited by blue (490 nm) light (Meyer and Abdallah 1978).

Rhamnolipid expression is associated with acute infections

It has been shown previously that RLs are important virulence factors as they have been implicated in cell death and essential in correct biofilm construction and protection. We wanted to explore the use of this assay to determine rhamnolipid expression among clinical isolates. We performed vesicle–supernatant experiments on a range of clinical strains (n=78), from a chronic (n=48) and an acute (n=30) infection background to gain an understanding of the clinical importance of RLs in these two classes of infection (Fig. 4). There was a positive association between RL expression and acute infection

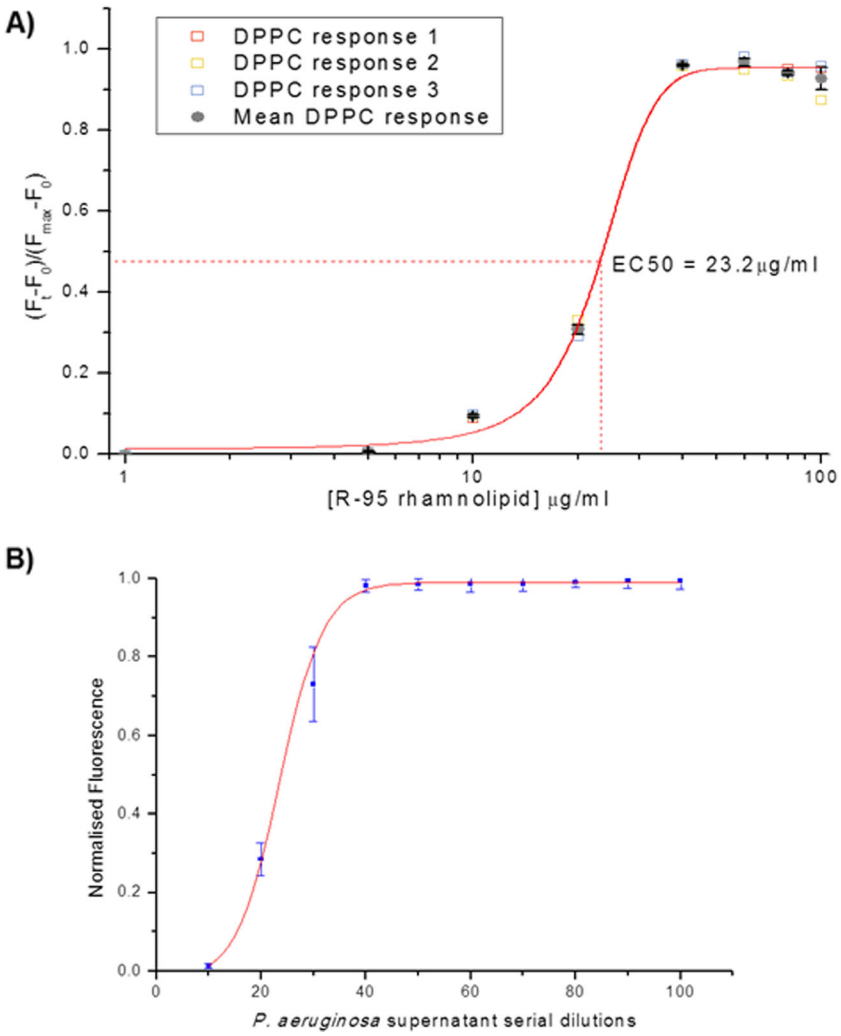

Fig. 3 Estimation of rhamnolipids in culture supernatants. **a** Vesicle–purified rhamnolipid interaction illustrating the EC_{50}, the amount of purified rhamnolipid required to cause 50 % of vesicle lysis as measured by fluorescence release. **b** Estimation of rhamnolipid concentration via serial dilutions of PAO1 culture supernatant exposed to vesicles, generating different fluorescence values. EC_{50} value obtained using 25 % supernatant, thus EC_{50} times 4 giving the estimated starting RL quantity (23.2 µg/mL×4= 92.8 µg/mL)

isolates (Fisher test p=0.0008), which suggests that RLs play an active role during acute infections.

Discussion

The vesicle-lysis assay is responsive to QS-regulated factors, which are expressed during the transition from late exponential to early stationary phase of growth (Fig. 1). *P. aeruginosa* expresses a wide spectrum of exofactors, some of which are important in membrane damage. Using isogenic mutants in key regulatory QS genes and virulence determinants, we investigated which factor(s) was important in the observed lysis. Due to their hydrophobic nature, PQS molecules induce the formation of MVs through an interaction with lipopolysaccharides on the bacterial outer membrane facilitating cell-to-cell communication, dependent on the expression of the *pqs* operon (Mashburn and Whiteley 2005). Interestingly, it has also been reported that exogenously added PQS molecules can also induce membrane curvature in erythrocytes, lacking any of the receptors important for MV formation, leading to haemolysis in a concentration-dependent manner (Schertzer and Whiteley 2012). Additionally, after the blebbing of MVs from the bacterial cell envelope, these structures are then able to fuse with recipient cells, transferring their cargo in an elegant transport mechanism (Schertzer and Whiteley 2012). Considering these observations, we investigated whether the PQS signalling system was influencing the lysis of vesicles used in this study. Genetic inactivation of *pqsA*, *pqsE* or *pqsH* conferred no reduction in vesicle lysis (Fig. 2a), suggesting no role for the PQS system in the disruption of these vesicles.

Surprisingly, the phospholipase C enzyme (PlcH), a key component in the degradation of lipids due to their affinity for phosphatidylcholine head groups, did not play a role in lipid vesicle lysis as no reduction in fluorescence was observed in the *plcH* mutant or heat-treated supernatants (Fig. 2b). Work on phospholipase 2 (PLA) has shown that the degree of saturation and, most importantly, the acyl chain length of the phospholipids impact on the ability of this lipase to lyse lipid bilayers (Haimi et al. 2010). The structure of the vesicles in

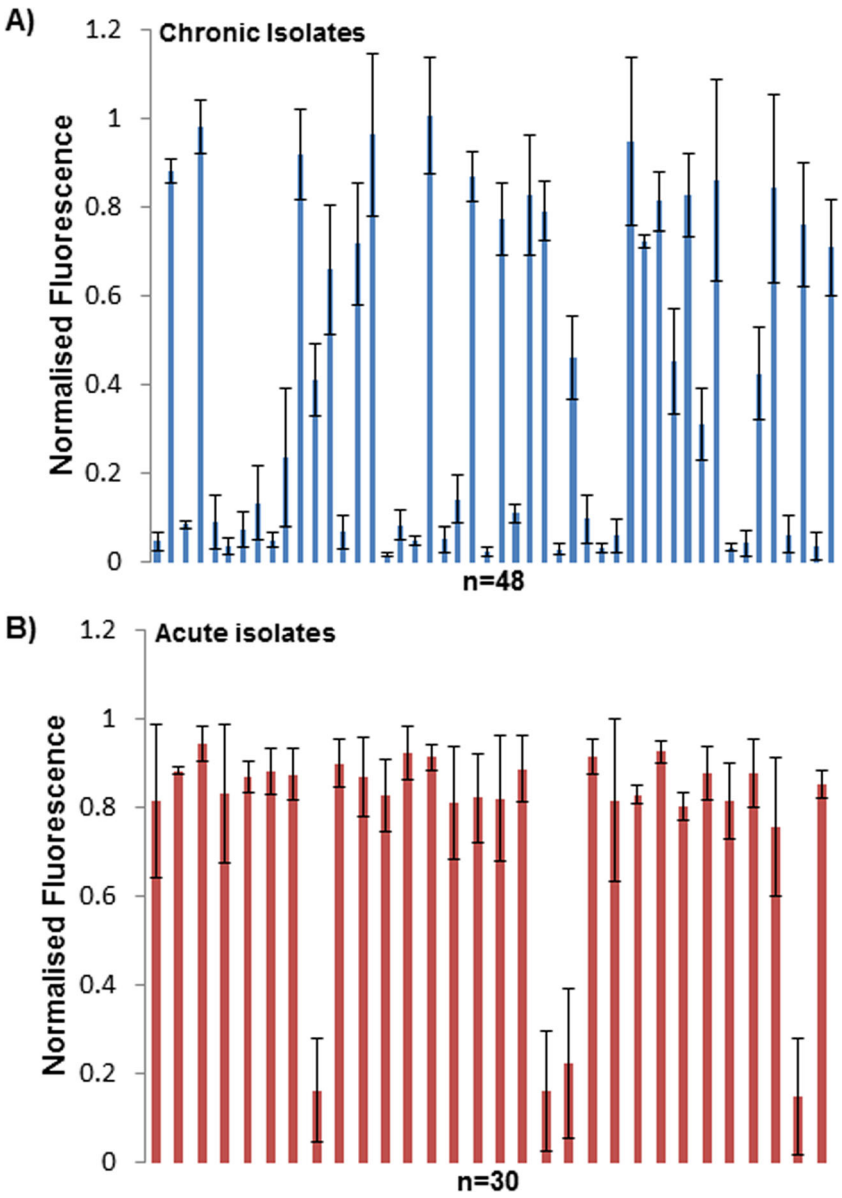

Fig. 4 Positive association between rhamnolipid expression and isolates from acute infections. Vesicle–supernatant assay exploring the rhamnolipid-producing capacity of clinical *P. aeruginosa* isolates derived from either a **a** chronic (n=48) or **b** acute infection (n=30). A positive association between rhamnolipid production and isolates from acute infections was observed, using an exact Fisher test, p=0.0008

this study is composed of DPPC and DPPE, which are saturated phospholipids containing fatty acids with a 16 carbon chain length which may not be efficient substrates for PLC activity.

Inactivation of the *rhl* QS system leads to a reduction in vesicle lysis (Fig. 2a). RL expression is under the control of the *rhlABC* operon, where *rhlA*, a gene which encodes a rhamnosyltransferase which catalyses the transfer of L-rhamnose to 3-(3-hydroxyalkanoyloxy) alkonic acid (HAA) and is required for subsequent RL formation, is regulated by the *rhl* system. RLs are also heat-resistant molecules, and our results show that heat treatment was insufficient to inhibit the lytic function of bacterial supernatants derived from *P. aeruginosa* strain PAO1 (Fig. 2b). Finally, deletion of the *rhlA* gene confers a non-lytic phenotype, consistent with TLC results showing no RL production in strains which do not lyse vesicles (Fig. 2c, d), confirming RL as the sole vesicle lytic agent.

As a result of these experiments, we sought to develop a new method to detect RLs based on the lysis of carboxyfluorescein-encapsulated phospholipid vesicles. Here, we illustrate that this assay is more rapid, sensitive and easier to perform than current methods. The vesicle-lysis assay requires no extraction procedure and therefore is not susceptible to contamination or sample loss. We have shown that this assay is semi-quantitative and can estimate the amount of RLs present in culture supernatants (Fig. 3). We observe that our results are in contrast to the values generated using the orcinol assay but are consistent with TLC and dry weight analysis. However, the orcinol test relies on the extraction of RLs from the supernatant, the measurement of the rhamnose content by use of orcinol and concentrated

sulphuric acid and applying these absorbance values to a standard curve (Koch et al. 1991). This procedure can be susceptible to contamination from other rhamnose-containing molecules such as lipopolysaccharides or from unextracted media components and can also give erroneous results due to differences in incubation temperature and sample loss (Perfumo et al. 2013).

RL production also had a positive association with isolates derived from acute infections. It is known that genetic changes occur during chronic infections, which can lead to the down-regulation of extracellular virulence factors (Van Delden and Iglewski 1998) with a loss of function mutations in the central regulator *lasR* being most frequent in chronic infections, but other QS mutants involving the *rhl* system are evident (Hoffman et al. 2009; Smith et al. 2006; Wilder et al. 2009). Since *las* and *rhl* systems are intricately linked, with the *las* system controlling the *rhl* system at a transcriptional and post-translational level, it was conceivable that mutations in *lasR* were causing this RL negative phenotype in certain clinical strains. However, in PAO1 we did not see any statistically significant difference in lysis potential between the *lasR* mutant and WT strains. This leads us to believe that mutations in either the *rhl* system or the *rhlABC* operon are the most likely reasons for RL negative phenotype. Novel point mutations in quorum regulator genes and mutations in the multitude of quorum regulators may also be responsible for the RL negative phenotype and work into elucidating this in ongoing.

Following from these observations, we envisage that this vesicle-lysis test may be applied as a rapid phenotypic assay, useful in screening large numbers of clinical strains in an effort to determine novel mutations that may affect the expression of this important virulence factor. Given the specificity of this assay, it could be an important tool in analysing the highly complex and interconnected QS systems of *P. aeruginosa*. The effects of mutation on candidate putative regulatory genes or single base pair mutations of known regulators could be assayed quickly in order to determine their effect on key QS-regulated virulence factors.

Determining whether an isolate is a strong- or weak-RL producer may be important considering that a specific concentration of RLs is required to lyse important immune cells and disrupt the cellular barrier which prevent invasion and dissemination (Jensen et al. 2007; Zulianello et al. 2006). We anticipate that this vesicle-lysis assay may be used to semi-quantify RL production from a set of clinical strains isolated from different infections in order to assess whether RL is an important virulence factor for that specific infection, with our preliminary results suggesting that RL production is more important in acute rather than chronic infections. One limitation of this assay is that it is susceptible to other microbial surfactants, notably the phenol-soluble modulin (PSM) peptide toxins of *S. aureus* (Laabei et al. 2014). Therefore, isolation of pure *P. aeruginosa* isolates is required before this assay can be used to detect and semi-quantify RL production.

Acknowledgment We would like to thank Prof. Mark C. Enright (University of Bath) and Southmead Hospital (Bristol, UK) for clinical bacterial strains, Dr. Stephan Heeb (University of Nottingham, UK) for the PAO1 quorum and *rhlA* mutant strains and Dr. Mathew J. Wargo (University of Vermont, USA) for the PAO1Δ*plcH* mutant strain. We also acknowledge the European Commission's Seventh Framework Programme for funding via the EC-FP7 consortium project no.245500 *Bacteriosafe* and the Royal Society and NERC grant ref: NE/J007064/1.

Conflict of interest The authors declare that they have no conflict of interest.

References

Alhede M, Bjarnsholt T, Jensen PO, Phipps RK, Moser C, Christophersen L, Christensen LD, van Gennip M, Parsek M, Hoiby N, Rasmussen TB, Givskov M (2009) *Pseudomonas aeruginosa* recognizes and responds aggressively to the presence of polymorphonuclear leukocytes. Microbiology 155(Pt 11):3500–8. doi:10.1099/mic.0.031443-0

Aranda FJ, Espuny MJ, Marques A, Teruel JA, Manresa A, Ortiz A (2007) Thermodynamics of the interaction of a dirhamnolipid biosurfactant secreted by *Pseudomonas aeruginosa* with phospholipid membranes. Langmuir 23(5):2700–5. doi:10.1021/la061464z

Barker AP, Vasil AI, Filloux A, Ball G, Wilderman PJ, Vasil ML (2004) A novel extracellular phospholipase C of *Pseudomonas aeruginosa* is required for phospholipid chemotaxis. Mol Microbiol 53(4):1089–98. doi:10.1111/j.1365-2958.2004.04189.x

Bodey GP, Bolivar R, Fainstein V, Jadeja L (1983) Infections caused by *Pseudomonas aeruginosa*. Rev Infect Dis 5(2):279–313

Caiazza NC, Shanks RM, O'Toole GA (2005) Rhamnolipids modulate swarming motility patterns of *Pseudomonas aeruginosa*. J Bacteriol 187(21):7351–61. doi:10.1128/JB.187.21.7351-7361.2005

Deziel E, Lepine F, Milot S, Villemur R (2003) *rhlA* is required for the production of a novel biosurfactant promoting swarming motility in *Pseudomonas aeruginosa*: 3-(3-hydroxyalkanoyloxy)alkanoic acids (HAAs), the precursors of rhamnolipids. Microbiology 149(Pt 8):2005–13

Diggle SP, Winzer K, Chhabra SR, Worrall KE, Camara M, Williams P (2003) The *Pseudomonas aeruginosa* quinolone signal molecule overcomes the cell density-dependency of the quorum sensing hierarchy, regulates rhl-dependent genes at the onset of stationary phase and can be produced in the absence of LasR. Mol Microbiol 50(1):29–43

Diggle SP, Cornelis P, Williams P, Camara M (2006) 4-quinolone signalling in *Pseudomonas aeruginosa*: old molecules, new perspectives. Int J Med Microbiol: IJMM 296(2–3):83–91. doi:10.1016/j.ijmm.2006.01.038

Diggle SP, Matthijs S, Wright VJ, Fletcher MP, Chhabra SR, Lamont IL, Kong X, Hider RC, Cornelis P, Camara M, Williams P (2007) The *Pseudomonas aeruginosa* 4-quinolone signal molecules HHQ and PQS play multifunctional roles in quorum sensing and iron entrapment. Chem Biol 14(1):87–96. doi:10.1016/j.chembiol.2006.11.014

Driscoll JA, Brody SL, Kollef MH (2007) The epidemiology, pathogenesis and treatment of *Pseudomonas aeruginosa* infections. Drugs 67(3):351–68

Dubern JF, Diggle SP (2008) Quorum sensing by 2-alkyl-4-quinolones in *Pseudomonas aeruginosa* and other bacterial species. Mol BioSyst 4(9):882–8. doi:10.1039/b803796p

Farrow JM 3rd, Sund ZM, Ellison ML, Wade DS, Coleman JP, Pesci EC (2008) PqsE functions independently of PqsR-Pseudomonas quinolone signal and enhances the rhl quorum-sensing system. J Bacteriol 190(21):7043–51. doi:10.1128/JB.00753-08

Fujita K, Akino T, Yoshioka H (1988) Characteristics of heat-stable extracellular hemolysin from *Pseudomonas aeruginosa*. Infect Immun 56(5):1385–7

Gallagher LA, McKnight SL, Kuznetsova MS, Pesci EC, Manoil C (2002) Functions required for extracellular quinolone signaling by *Pseudomonas aeruginosa*. J Bacteriol 184(23):6472–80

Gunther NW, Nunez A, Fett W, Solaiman DK (2005) Production of rhamnolipids by *Pseudomonas chlororaphis*, a nonpathogenic bacterium. Appl Environ Microbiol 71(5):2288–93. doi:10.1128/AEM.71.5.2288-2293.2005

Haimi P, Hermansson M, Batchu KC, Virtanen JA, Somerharju P (2010) Substrate efflux propensity plays a key role in the specificity of secretory A-type phospholipases. J Biol Chem 285(1):751–60. doi:10.1074/jbc.M109.061218

Hoffman LR, Kulasekara HD, Emerson J, Houston LS, Burns JL, Ramsey BW, Miller SI (2009) *Pseudomonas aeruginosa lasR* mutants are associated with cystic fibrosis lung disease progression. J Cyst Fibros 8(1):66–70. doi:10.1016/j.jcf.2008.09.006

Jensen PO, Bjarnsholt T, Phipps R, Rasmussen TB, Calum H, Christoffersen L, Moser C, Williams P, Pressler T, Givskov M, Hoiby N (2007) Rapid necrotic killing of polymorphonuclear leukocytes is caused by quorum-sensing-controlled production of rhamnolipid by *Pseudomonas aeruginosa*. Microbiology 153(Pt 5):1329–38. doi:10.1099/mic.0.2006/003863-0

Koch AK, Kappeli O, Fiechter A, Reiser J (1991) Hydrocarbon assimilation and biosurfactant production in *Pseudomonas aeruginosa* mutants. J Bacteriol 173(13):4212–9

Kohler T, Guanella R, Carlet J, van Delden C (2010) Quorum sensing-dependent virulence during *Pseudomonas aeruginosa* colonisation and pneumonia in mechanically ventilated patients. Thorax 65(8):703–10. doi:10.1136/thx.2009.133082

Laabei M, Young A, Jenkins TA (2012) In vitro studies of toxic shock toxin-1-secreting *Staphylococcus aureus* and implications for burn care in children. Pediatr Infect Dis J 31(5):e73–7. doi:10.1097/INF.0b013e3182493b21

Laabei M, Jamieson WD, Massey RC, Jenkins AT (2014) *Staphylococcus aureus* interaction with phospholipid vesicles–a new method to accurately determine accessory gene regulator (agr) activity. PLoS One 9(1):e87270. doi:10.1371/journal.pone.0087270

Leitermann F, Syldatk C, Hausmann R (2008) Fast quantitative determination of microbial rhamnolipids from cultivation broths by ATR-FTIR Spectroscopy. J Biol Eng 2:13. doi:10.1186/1754-1611-2-13

Marshall SE, Hong SH, Thet NT, Jenkins AT (2013) Effect of lipid and fatty acid composition of phospholipid vesicles on long-term stability and their response to *Staphylococcus aureus* and *Pseudomonas aeruginosa* supernatants. Langmuir 29(23):6989–95. doi:10.1021/la401679u

Mashburn LM, Whiteley M (2005) Membrane vesicles traffic signals and facilitate group activities in a prokaryote. Nature 437(7057):422–5. doi:10.1038/nature03925

Mashburn-Warren L, Howe J, Brandenburg K, Whiteley M (2009) Structural requirements of the Pseudomonas quinolone signal for membrane vesicle stimulation. J Bacteriol 191(10):3411–4. doi:10.1128/JB.00052-09

McKnight SL, Iglewski BH, Pesci EC (2000) The Pseudomonas quinolone signal regulates rhl quorum sensing in *Pseudomonas aeruginosa*. J Bacteriol 182(10):2702–8

Meyer JM, Abdallah MA (1978) The fluorescent pigment of *Pseudomonas fluorescens*: biosynthesis, purification and physicochemical properties. J Gen Microbiol 107:319–328

Morikawa M, Hirata Y, Imanaka T (2000) A study on the structure-function relationship of lipopeptide biosurfactants. Biochim Biophys Acta 1488(3):211–8

Ostroff RM, Vasil AI, Vasil ML (1990) Molecular comparison of a non-hemolytic and a hemolytic phospholipase C from *Pseudomonas aeruginosa*. J Bacteriol 172(10):5915–23

Pamp SJ, Tolker-Nielsen T (2007) Multiple roles of biosurfactants in structural biofilm development by *Pseudomonas aeruginosa*. J Bacteriol 189(6):2531–9. doi:10.1128/JB.01515-06

Pearson JP, Pesci EC, Iglewski BH (1997) Roles of *Pseudomonas aeruginosa* las and rhl quorum-sensing systems in control of elastase and rhamnolipid biosynthesis genes. J Bacteriol 179(18):5756–67

Perfumo A, Rudden M, Smyth TJ, Marchant R, Stevenson PS, Parry NJ, Banat IM (2013) Rhamnolipids are conserved biosurfactants molecules: implications for their biotechnological potential. Appl Microbiol Biotechnol 97(16):7297–306. doi:10.1007/s00253-013-4876-z

Pesci EC, Pearson JP, Seed PC, Iglewski BH (1997) Regulation of *las* and *rhl* quorum sensing in *Pseudomonas aeruginosa*. J Bacteriol 179(10):3127–32

Pesci EC, Milbank JB, Pearson JP, McKnight S, Kende AS, Greenberg EP, Iglewski BH (1999) Quinolone signaling in the cell-to-cell communication system of *Pseudomonas aeruginosa*. Proc Natl Acad Sci U S A 96(20):11229–34

Pinzon NM, Ju LK (2009) Improved detection of rhamnolipid production using agar plates containing methylene blue and cetyl trimethylammonium bromide. Biotechnol Lett 31(10):1583–8. doi:10.1007/s10529-009-0049-7

Rikalovic MG, Gojgic-Cvijovic G, Vrvic MM, Karadzic I (2012) Production and characterization of rhamnolipids from *Pseudomonas aeruginosa* san-ai. J Serbian Chem Soc 77(1):27–42

Sanchez M, Aranda FJ, Teruel JA, Espuny MJ, Marques A, Manresa A, Ortiz A (2010) Permeabilization of biological and artificial membranes by a bacterial dirhamnolipid produced by *Pseudomonas aeruginosa*. J Colloid Interface Sci 341(2):240–7. doi:10.1016/j.jcis.2009.09.042

Schenk T, Schuphan I, Schmidt B (1995) High-performance liquid chromatographic determination of the rhamnolipids produced by *Pseudomonas aeruginosa*. J Chromatogr A 693(1):7–13

Schertzer JW, Whiteley M (2012) A bilayer-couple model of bacterial outer membrane vesicle biogenesis. MBio 3(2) doi:10.1128/mBio.00297-11

Schuster M, Greenberg EP (2006) A network of networks: quorum-sensing gene regulation in *Pseudomonas aeruginosa*. Int J Med Microbiol 296(2–3):73–81. doi:10.1016/j.ijmm.2006.01.036

Schuster M, Sexton DJ, Diggle SP, Greenberg EP (2013) Acyl-homoserine lactone quorum sensing: from evolution to application. Annu Rev Microbiol 67:43–63. doi:10.1146/annurev-micro-092412-155635

Smith RS, Iglewski BH (2003) *P. aeruginosa* quorum-sensing systems and virulence. Curr Opin Microbiol 6(1):56–60

Smith EE, Buckley DG, Wu Z, Saenphimmachak C, Hoffman LR, D'Argenio DA, Miller SI, Ramsey BW, Speert DP, Moskowitz SM, Burns JL, Kaul R, Olson MV (2006) Genetic adaptation by *Pseudomonas aeruginosa* to the airways of cystic fibrosis patients. Proc Natl Acad Sci U S A 103(22):8487–92. doi:10.1073/pnas.0602138103

Van Delden C, Iglewski BH (1998) Cell-to-cell signaling and *Pseudomonas aeruginosa* infections. Emerg Infect Dis 4(4):551–60. doi:10.3201/eid0404.980405

Wagner VE, Gillis RJ, Iglewski BH (2004) Transcriptome analysis of quorum-sensing regulation and virulence factor expression in *Pseudomonas aeruginosa*. Vaccine 22(Suppl 1):S15–20. doi:10.1016/j.vaccine.2004.08.011

Wilder CN, Allada G, Schuster M (2009) Instantaneous within-patient diversity of *Pseudomonas aeruginosa* quorum-sensing populations from cystic fibrosis lung infections. Infect Immun 77(12):5631–9. doi:10.1128/IAI.00755-09

Williams P, Camara M (2009) Quorum sensing and environmental adaptation in *Pseudomonas aeruginosa*: a tale of regulatory networks and multifunctional signal molecules. Curr Opin Microbiol 12(2): 182–91. doi:10.1016/j.mib.2009.01.005

Williams P, Winzer K, Chan WC, Camara M (2007) Look who's talking: communication and quorum sensing in the bacterial world. Philos Trans R Soc Lond B Biol Sci 362(1483):1119–34. doi:10.1098/rstb.2007.2039

Winzer K, Williams P (2001) Quorum sensing and the regulation of virulence gene expression in pathogenic bacteria. Int J Med Microbiol 291(2):131–43. doi:10.1078/1438-4221-00110

Zulianello L, Canard C, Kohler T, Caille D, Lacroix JS, Meda P (2006) Rhamnolipids are virulence factors that promote early infiltration of primary human airway epithelia by *Pseudomonas aeruginosa*. Infect Immun 74(6):3134–47. doi:10.1128/IAI.01772-05