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Construction and analysis of *luxCDABE*-based plasmid sensors for investigating *N*-acyl homoserine lactone-mediated quorum sensing

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Abstract

Plasmid reporter vectors have been constructed which respond to activation of LuxR and its homologues LasR and RhlR (VsmR) by *N*-acyl homoserine lactones (AHLs). The expression of *luxCDABE* from transcriptional fusions to P_{luxI}, P_{lasI} and P_{rhlI} respectively, occurs in the presence of activating AHLs. A profile of structure/activity relationships is seen where the natural ligand is most potent. The characterisation of individual LuxR homologue/AHL combinations allows a comprehensive evaluation of quorum sensing signals from a test organism.

Keywords: Bioluminescence; *lux*; Quorum sensing; *N*-Acyl homoserine lactone

1. Introduction

The generic term 'quorum sensing' describes the phenomenon whereby the accumulation of a low molecular mass signal enables individual cells to sense when the minimal population unit or 'quorum' of bacteria has been achieved for a concerted population response to be initiated [1]. In Gram-negative bacteria *N*-acyl homoserine lactones (AHLs) are a common diffusible signal, controlling the expression of characteristics associated with virulence and secondary metabolism [1]. AHL-mediated control of gene expression in quorum sensing occurs through the activity of proteins with homology to LuxI and LuxR, which control bioluminescence in the marine bacterium *Vibrio* (*Photobacterium*) *fischeri*. The AHLs synthesised via LuxI homologues interact with a LuxR homologue to activate gene expression. Structural variations in the acyl side chain of indi-

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vidual AHLs have a profound effect upon the activation response of given LuxR homologues [2–4]. *N*-(3-Oxohexanoyl)-L-homoserine lactone (OHHL, Table 1) is the cognate molecule for LuxR [3]. Variations in acyl side chain length and in the substitution at C-3 are illustrated by the two quorum sensing systems operating within the regulatory hierarchy controlling virulence and secondary metabolism in *Pseudomonas aeruginosa*. *N*-3-Oxododecanoyl-L-homoserine lactone (OdDHL, Table 1) is the cognate activator molecule for LasR [4] and *N*-butanoyl-L-homoserine lactone (BHL, Table 1) is the cognate activator molecule for RhlR (VsmR) [5]. In these cases, although different AHLs can function as agonists, the natural ligand is the most effective [3,4]. Hence, reporter constructs able to detect different AHLs require different LuxR homologue effectors.

The use of bioluminescent sensors based on the LuxRI regulatory circuit has proven effective in the identification and analysis of quorum sensing in Gram-negative bacteria [6–10]. The lack of nucleotide sequence similarity between the genes encoding the LuxR and LuxI homologues from different bacterial species has limited the identification of these genes in other bacteria using DNA hybridisation and PCR-based methodologies. However, functional analysis using biosensor technology has proven particularly useful for identifying LuxI homologues. Reporter constructs using LacZ have provided important information in the study of quorum sensing in *Agrobacterium tumefaciens* [11] and *P. aeruginosa* [4,12]. In addition, the extensive range of AHLs activating the TraR-based reporter [11] has facilitated its use in a thin layer chromatography (TLC) based method for the detection and preliminary identification of AHL molecules produced by a given organism [13].

The use of bacterial luciferases as non-destructive, real-time reporters of gene expression is particularly suited to the study of phenomena such as quorum sensing where the gene expression exhibits temporal or cell density dependence. Promoter probe plasmids based on the *Photorhabdus luminescens luxCDABE* have been constructed using a number of compatible replicons and antibiotic markers [14]. In this work plasmid vectors using *lasRI′*, *luxRI′* and *rhlRI′::luxCDABE* reporter gene fusions have been constructed to aid the study of cell density-dependent characteristics, and have been utilised to determine the range of activating AHL signal molecules.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Bacteria used in this study are *Escherichia coli* JM109 (*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1* Δ(*lac-proAB*) *mcrA* [F′ *traD36 proAB lacI*^{q} *lacZ* ΔM15]) [15]; *E. coli* CC118 λpir (Δ(*ara-leu*) *araD* Δ*lacX74 galEgalK phoA20 thi-1 rpsE rpoB argE*(Am) *recA1* λ-pir lysogen) [16] and *E. coli* S17-1 λ-pir (*thi pro hsdR*$^{-}$ *hsd*M^{+} *recA* RP4 2-Tc::Mu-Km::Tn*7*(Tp^{R} Sm^{R}) λ-pir lysogen) [17]. Growth was on LB broth, Lennox (Difco) containing the appropriate antibiotics. Agar plates contained 1.5% (w/v) No. 1 agar (Oxoid).

2.2. DNA manipulation

Plasmid DNAs were isolated by alkaline lysis [18] and further purified using a caesium chloride gradient [18] or Qiagen plasmid preparation columns. Restriction enzyme digests and DNA ligations were performed according to the manufacturers' instructions (Promega, Boehringer Mannheim, or Pharmacia). PCR amplifications were performed according to a standard protocol [18] and the sequence of PCR-derived DNA was checked for polymerase errors. Oligonucleotides were synthesised by the Biopolymer Synthesis and Analysis Unit, University of Nottingham. DNA sequencing was performed by the University of Nottingham Automated Sequencing Facility.

2.3. Conjugation

The pRK415 replicon [19] can be mobilised by conjugation from *E. coli* S17-1 λpir into the target species of interest. For conjugation, the donor and recipient strains were grown overnight in 5 ml LB at the appropriate temperature with antibiotic selection and the cells were harvested by centrifugation. Harvested cells were gently resuspended and centrifuged twice in LB without antibiotic before finally being resuspended in 1/10th of the original culture volume.

100-μl volumes of donor and recipient cells were gently mixed and spot-inoculated onto the surface of LB agar plates. The mating plates were incubated at 30°C for 4–8 h before the cells were scraped from the surface and resuspended in 1 ml of LB. The cells were washed once with 1 ml LB and dilutions spread onto selective agar plates and incubated for 48 h at 30°C. Transconjugants containing the pRK415 replicon were isolated by selection for the tetracycline resistance of the recipient. Transconjugants were purified by subculturing twice on selective medium.

2.4. Luminometry

Induction of bioluminescence in bacteria carrying bioluminescence reporter plasmids was detected using a Hamamatsu Argus 100 Vim3 (Hamamatsu Photonics U.K. Ltd., Enfield, UK) or a Berthold LB980 photon video camera (E.G. and G. Berthold U.K. Ltd., Milton Keynes, UK). Microtitre plate assays used to assess AHL activation of *E. coli* plasmid-borne *lux* sensors were performed as follows: sensor strains were grown in LB at 30°C for 18 h (with antibiotic selection) and diluted 1:10 in fresh LB. Dilutions of the AHL being assayed were made in acetonitrile (far UV grade) and dried overnight. 200-μl volumes of diluted *E. coli lux* sensor cells were added to the AHL dilutions and the assay plates were incubated at 30°C, on a rocking platform, for 4 h before bioluminescence was measured using an Amerlite luminometer (Kodak Ltd).

2.5. N-Acylhomoserine lactones (AHLs)

The general method described by Chhabra et al. [2] was used to synthesise AHLs. Each compound was purified to homogeneity by semi-preparative HPLC and its structure confirmed by mass spectrometry and proton NMR spectroscopy [2,5].

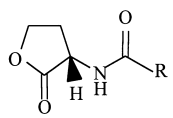
3. Results

3.1. Construction of quorum sensing luxCDABE reporters

To reflect the structural variations of AHL activation profiles LuxR-, LasR- and RhlR-based reporters were constructed featuring the quorum sensing response regulator cloned with the promoter of the *luxI* homologue which it positively activates in gene fusion with the *luxCDABE* from *P. luminescens*. Each of these constructs respond to AHLs via activation of the LuxR homologue to initiate transcription of the reporter (Fig. 1).

For the LuxR based sensor a 1-kb *Eco*RI fragment containing *V. fischeri luxRI′* from pSB237 [7] was cloned into the *Eco*RI sites of pSB384, pSB377

Table 1
Structures of *N*-acyl homoserine lactone molecules used in bioluminescent reporter assays



R	Chemical name	Abbreviation
$CH_3CH_2CH_2$	*N*-Butanoyl-L-homoserine lactone	BHL
$CH_3(CH_2)_4$	*N*-Hexanoyl-L-homoserine lactone	HHL
$CH_3(CH_2)_6$	*N*-Octanoyl-L-homoserine lactone	OHL
$CH_3(CH_2)_8$	*N*-Decanoyl-L-homoserine lactone	DHL
$CH_3(CH_2)_{10}$	*N*-Dodecanoyl-L-homoserine lactone	dDHL
CH_3COCH_2	*N*-3-(Oxobutanoyl)-L-homoserine lactone	OBHL
$CH_3(CH_2)_2COCH_2$	*N*-3-(Oxohexanoyl)-L-homoserine lactone	OHHL
$CH_3(CH_2)_4COCH_2$	*N*-3-(Oxooctanoyl)-L-homoserine lactone	OOHL
$CH_3(CH_2)_6COCH_2$	*N*-3-(Oxodecanoyl)-L-homoserine lactone	ODHL
$CH_3(CH_2)_8COCH_2$	*N*-3-(Oxododecanoyl)-L-homoserine lactone	OdDHL
$CH_3(CH_2)_{10}COCH_2$	*N*-3-(Oxotetradecanoyl)-L-homoserine lactone	OtDHL

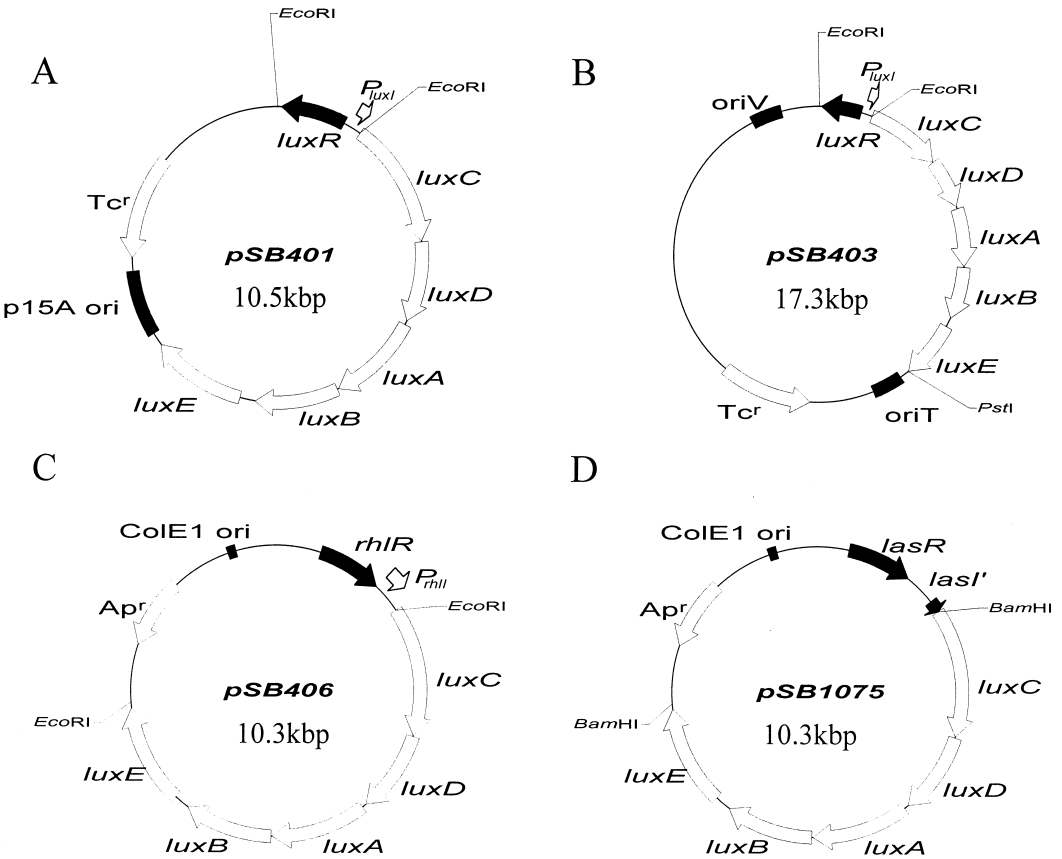


Fig. 1. *N*-Acyl homoserine lactone sensor plasmids. pSB401 (A) contains a fusion of *luxRI′*::*luxCDABE* on a pACYC184 plasmid backbone; pSB403 (B) contains contains a fusion of *luxRI′*::*luxCDABE* on a pRK415 plasmid backbone; pSB406 (C) contains a fusion of *rhlRI′*::*luxCDABE* on a pUC18 plasmid backbone; pSB1075 (D) contains a fusion of *lasRI′*::*luxCDABE* on a pUC18 plasmid backbone. These plasmids confer a bioluminescent phenotype in the presence of AHLs activating the LuxR homologue.

and pSB395 [14] to create pSB401 (Fig. 1A), pSB402 and pSB403 (Fig. 1B) respectively. To construct the *P. aeruginosa rhlRI′*::*luxCDABE* sensor pSB404, an *Eco*RI fragment containing *rhlRI′* from pMW47.3 [6] was cloned into the *Eco*RI site of pSB384. To construct the *rhlRI′*::*luxCDABE* sensor pSB406 (Fig. 1C), an *Eco*RI-flanked *luxCDABE* cassette from pSB390 [14] was cloned into the unique *Eco*RI site downstream of *rhlRI′* in pMW47.1 [6]. For pSB407 a *Pst*I fragment containing *rhlRI′*::*luxCDABE* DNA was excised from pSB406 and ligated into the *Pst*I site of pUCP18 [20] with transcription of the *lux* genes convergent to vector *lac* promoter transcription.

To construct the *P. aeruginosa lasRI′*::*luxCDABE* sensor pSB1075, we first cloned the *lasRI′* regulatory region. A PCR product comprising *lasRI* DNA from bases 78 to 1620 (GenBank accession number M59425) was amplified with the primers 5′-GCGTGGCGATGGGCCGACAGTG-3′ and 5′-CACTAACGTCCCAGCCTTTGCGC-3′ and cloned into pUC57/T (MBI Fermentas) to create pSB1074. The *luxCDABE Bam*HI cassette from pSB417 was cloned into pSB1074 to give the reporter pSB1075 (Fig. 1D).

3.2. Characterisation of quorum sensing luxCDABE reporters

To analyse the range of AHL molecules (Table 1)

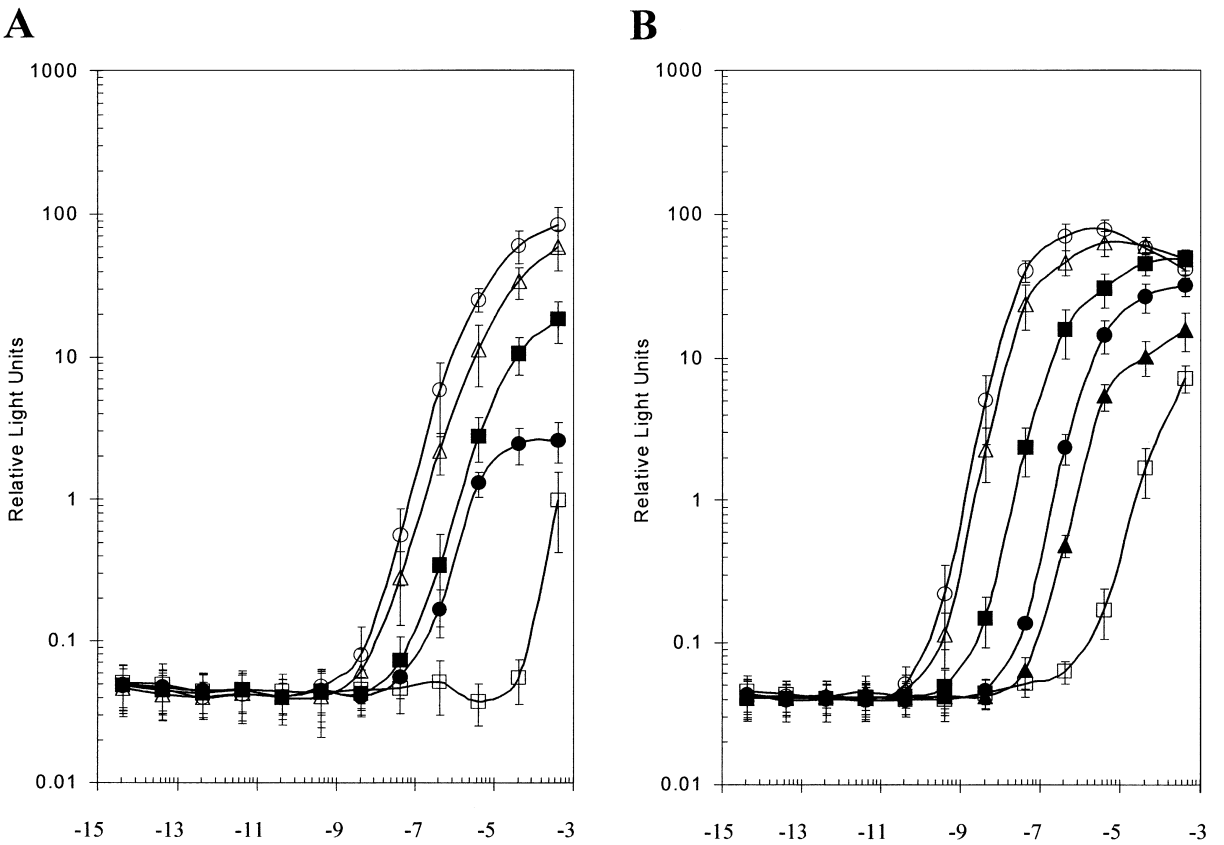


Fig. 2. Effect of acyl chain length and substitution of AHL molecules on the response of bioluminescent quorum sensing reporters in *E. coli*. The *E. coli* bioluminescent reporters based on *luxRI′* (pSB401) (A, B), *rhlRI′* (pSB406) (C, D; see p. 190) and *lasRI′* (pSB1075) (E, F; see p. 191) were used to investigate the effect of the *N*-acyl-L-homoserine lactones (A, C, E) and *N*-(3-oxoacyl)-L-homoserine lactones (B, D, F). In each case acyl side chains of C-4 (□), C-6 (○), C-8 (△), C-10 (■), C-12 (●) or C-14 (▲) (B, D, F only) are designated. The level of bioluminescence in each case corresponds to transcription from the respective *luxI* homologue promoter mediated by the AHL-activated LuxR homologue.

detected by each reporter, bioluminescence induction assays were performed. The response of LuxR (Fig. 2A,B), LasR (Fig. 2E,F) and RhlR (Fig. 2C,D) to AHL molecules lacking a C-3 substituent of the acyl side chain (chain length of 4, 6, 8, 10 and 12 carbons) and with a 3-oxo substitution (4, 6, 8, 10, 12 and 14 carbons) is shown. For each LuxR homologue it is clear that the cognate molecule is most active, followed by its closest analogues. The high dynamic range of the LuxR-based (3 log) and the LasR-based (1.5 log) biosensors allows for the quantitative assay of specified AHLs over a linear concentration range. To improve upon the lower dynamic range of the RhlR-based biosensor pSB406, the *rhlRI′::luxCDABE* fusion in the lower copy number plasmid pSB404 was assayed. A reduction in background bioluminescence was observed of about 10 times at all AHL concentrations (data not shown).

These AHL-biosensor constructs, or their parents, have proven useful in the identification of the widespread nature of the AHL signal using cross streaking and conditioned medium assays [5–10]. Furthermore, they have been used to demonstrate the density-dependent production of the AHL signal in a range of organisms and to screen recombinant gene libraries in *E. coli* for *luxI* homologues [5–10].

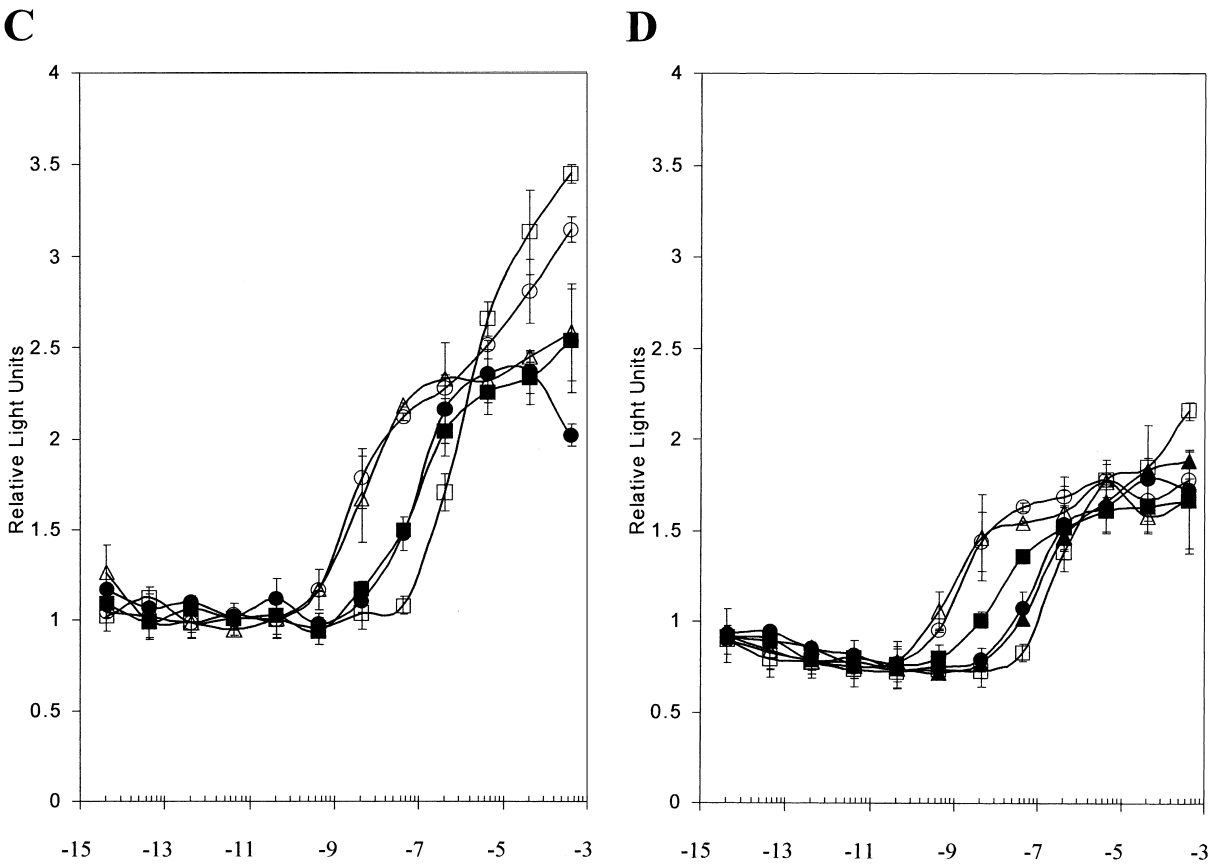


Fig. 2. (*continued*)

4. Discussion

The use of biosensors for detection of AHLs has been instrumental in the recent and rapid discovery of the widespread nature of AHL-mediated quorum sensing in a number of Gram-negative bacteria. The realisation that structurally different AHLs exist, having variation in the acyl side chain and having different activation profiles for given LuxR homologues, has prompted the construction of the reporters detailed in this study which respond to overlapping subsets of AHL structures. These sensors can be used in a series of experiments to characterise the AHL signal(s) involved in quorum sensing for a given organism. Simple T-streaks or assays of conditioned media can be used to determine the production of activating molecules by a target organism. False-negative results in these assays occasionally occur because of the bactericidal or bacteriostatic effects of compounds produced by the micro-organism under investigation upon the biosensor. The extraction of AHLs from spent culture medium [2,5] using organic solvents (e.g. dichloromethane) can overcome this problem and also be used for concentrating any AHL present. The transformation of target organisms with biosensor plasmids, where possible, can also circumvent the problem of antimicrobial activity [7,9,10]. Furthermore, the assay of target gene expression throughout growth in these transformed strains has allowed the determination of a cell density-dependent production of AHL by the bacterium under study [7,9,10]. The broad host range vectors pSB403 and pSB407 are best suited to this type of study. The use of *luxCDABE* as the reporter genes allows for the non-destructive measurement of promoter activity in a single sample

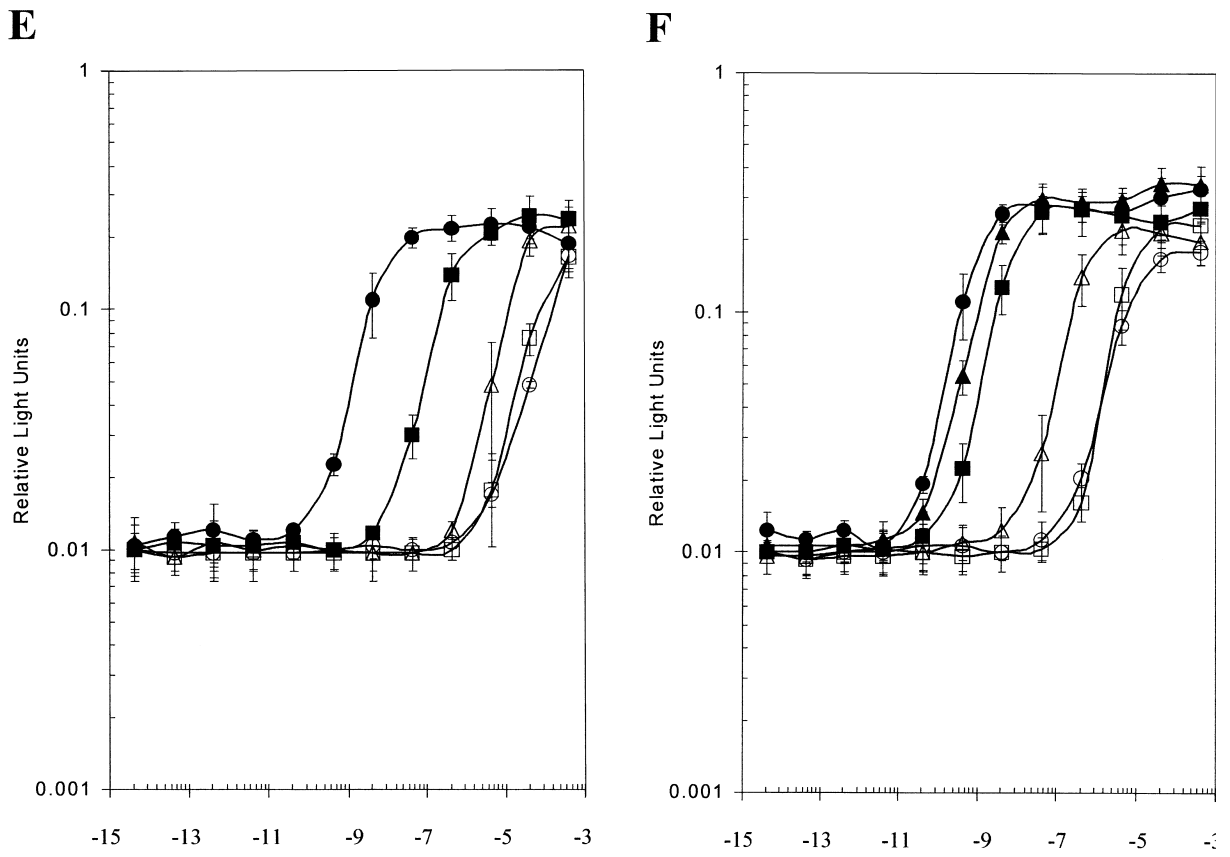


Fig. 2. (*continued*)

throughout growth. This is now particularly useful given the availability of automated systems for the measurement of bioluminescence and optical density [14].

To determine the chemical identity of the putative AHL, HPLC [5–10] or TLC [10,13] fractionation of concentrated organic extracts allows homogenous preparation of the signal AHL molecule. The biosensors are used to identify active fractions, which can then be subjected to mass spectrometric analysis and NMR [2,5].

Our results with reporters of LuxR, LasR and RhlR activation describe a subset of reporters with overlapping spheres of induction (Fig. 2). The findings for LuxR, LasR and RhlR are in broad agreement with the results from other studies [3,4] and show these *lux* sensors to be faithful reporters of the given AHL/LuxR homologue interaction.

The sensitivity to AHLs in pM and nM concentrations over a large linear range in real time is a significant advantage of using these *lux* sensors, being sufficient for detection of a wide range of AHL molecules at concentrations too low for analysis by HPLC. By combining the results from the different *lux* sensors the activity profiles can be compared with those of known standards to give a preliminary identification of the nature of the AHL under investigation. This information can then be used to aid the development of appropriate extraction and identification procedures.

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