Microbiology (1997), **143**, 3703–3711 Printed in Great Britain

Quorum sensing and *Chromobacterium violaceum*: exploitation of violacein production and inhibition for the detection of *N*-acylhomoserine lactones

Kay H. McClean,[1,2] Michael K. Winson,[1,2]† Leigh Fish,[1,2] Adrian Taylor,[1,2] Siri Ram Chhabra,[2] Miguel Camara,[2] Mavis Daykin,[1,2] John H. Lamb,[3] Simon Swift,[1,2] Barrie W. Bycroft,[2] Gordon S. A. B. Stewart[1] and Paul Williams[2]

Author for correspondence: Paul Williams. Tel: +44 115 951 5047. Fax: +44 115 951 5110. e-mail: Paul.Williams@nottingham.ac.uk

[1] Department of Applied Biochemistry and Food Science, University of Nottingham, Sutton Bonington Campus, Loughborough LE12 5RD, UK

[2] Department of Pharmaceutical Sciences, University of Nottingham, University Park, Nottingham NG7 2RD, UK

[3] MRC Toxicology Unit, University of Leicester, Leicester LE1 9HN, UK

Quorum sensing relies upon the interaction of a diffusible signal molecule with a transcriptional activator protein to couple gene expression with cell population density. In Gram-negative bacteria, such signal molecules are usually *N*-acylhomoserine lactones (AHLs) which differ in the structure of their *N*-acyl side chains. *Chromobacterium violaceum*, a Gram-negative bacterium commonly found in soil and water, produces the characteristic purple pigment violacein. Previously the authors described a violacein-negative, mini-Tn*5* mutant of *C. violaceum* (CV026) in which pigment production can be restored by incubation with supernatants from the wild-type strain. To develop this mutant as a general biosensor for AHLs, the natural *C. violaceum* AHL molecule was first chemically characterized. By using solvent extraction, HPLC and mass spectrometry, a single AHL, *N*-hexanoyl-L-homoserine lactone (HHL), was identified in wild-type *C. violaceum* culture supernatants which was absent from CV026. Since the production of violacein constitutes a simple assay for the detection of AHLs, we explored the ability of CV026 to respond to a series of synthetic AHL and *N*-acylhomocysteine thiolactone (AHT) analogues. In CV026, violacein is inducible by all the AHL and AHT compounds evaluated with *N*-acyl side chains from C_4 to C_8 in length, with varying degrees of sensitivity. Although AHL compounds with *N*-acyl side chains from C_{10} to C_{14} are unable to induce violacein production, if an activating AHL (e.g. HHL) is incorporated into the agar, these long-chain AHLs can be detected by their ability to inhibit violacein production. The versatility of CV026 in facilitating detection of AHL mixtures extracted from culture supernatants and separated by thin-layer chromatography is also demonstrated. These simple bioassays employing CV026 thus greatly extend the ability to detect a wide spectrum of AHL signal molecules.

Keywords: quorum sensing, *N*-acylhomoserine lactones, violacein, *Chromobacterium violaceum*, bioassay

INTRODUCTION

N-Acyl homoserine lactones (AHLs) were first identified in Gram-negative marine bacteria, where they were discovered to play a central role in the regulation of bioluminescence in bacteria such as *Photobacterium* (*Vibrio*) *fischeri* and *Vibrio harveyi* (for reviews see Fuqua *et al.*, 1996; Salmond *et al.*, 1995; Swift *et al.*,

Abbreviations: AHL, *N*-acylhomoserine lactone; AHT, *N*-acylhomocysteine thiolactone (abbreviations for specific AHLs and AHTs are listed in Table 1).

†**Present address:** Institute of Biological Sciences, University of Wales, Aberystwyth, Ceredigion SY23 3DA, UK.

1996; Williams *et al.*, 1992). In *P. fischeri*, the accumulation of AHLs enables the organism to monitor its cell population density and regulate bioluminescence (*lux*) gene expression accordingly (Meighen, 1991). *N*-(3-Oxohexanoyl)-L-homoserine lactone (synthesized via LuxI, activates the *P. fischeri lux* operon via the AHL-responsive transcriptional activator protein, LuxR (Fuqua *et al.*, 1996; Meighen, 1991; Salmond *et al.*, 1995). Analogous regulatory systems which control gene expression in concert with cell population density and essentially function as cell–cell communication devices have now been discovered in a variety of different Gram-negative bacteria including *Erwinia carotovora*, *Erwinia stewartii*, *Enterobacter agglomerans*, *Serratia liquefaciens*, *Yersinia enterocolitica*, *Agrobacterium tumefaciens*, *Pseudomonas aeruginosa*, *Pseudomonas aureofaciens* and *Rhizobium leguminosarum* (for reviews see Fuqua *et al.*, 1996; Salmond *et al.*, 1995; Swift *et al.*, 1996). In these organisms, AHL-mediated cell–cell signalling plays a role in regulating carbapenem (Bainton *et al.*, 1992a, b) and phenazine antibiotic biosynthesis (Wood & Pierson, 1996), plasmid conjugal transfer (Fuqua & Winans, 1994; Piper *et al.*, 1993), swarming (Eberl *et al.*, 1996), cessation of cell growth (Gray *et al.*, 1996; Schripsema *et al.*, 1996) and capsular polysaccharide synthesis (Beck von Bodman & Farrand, 1995), and in the production of exoenzyme virulence determinants and cytotoxins in human and plant pathogens (Jones *et al.*, 1993; Latifi *et al.*, 1995, 1996; Passador *et al.*, 1993, 1996; Pearson *et al.*, 1994, 1995; Winson *et al.*, 1995).

Gene regulation through quorum sensing is thus emerging as a generic phenomenon. The development of biological assays dependent on either *lux* or *lacZ* reporter fusions for the detection of AHLs has greatly facilitated screening of micro-organisms for new AHL molecules and accelerated the identification of the LuxI family of proteins responsible for AHL synthesis (Bainton *et al.*, 1992a; Hwang *et al.*, 1995; Pearson *et al.*, 1994, 1995; Swift *et al.*, 1993). Since there is often a genetic linkage between *luxI* and *luxR* homologues, both genes are frequently cloned together (Salmond *et al.*, 1995; Swift *et al.*, 1996), affording a more rapid insight into the study of quorum sensing in a given bacterium. The availability of a simple and rapid screen for a broad spectrum of AHLs would greatly facilitate extension of our understanding of the generic nature of quorum sensing. Previously we have described several recombinant *lux*-based AHL sensors (Bainton *et al.*, 1992a; Swift *et al.*, 1993; Throup *et al.*, 1995a) such as pSB401, in which the *luxR* and *lux* promoter region from *Photobacterium fischeri* is coupled to the entire *lux* structural operon (comprising *luxCDABE*) from *Photorhabdus luminescens*. This construct, when expressed in *E. coli*, responds to a limited range of AHLs. Using this bioassay, we noted that stationary-phase culture supernatants from the Gram-negative bacterium *Chromobacterium violaceum* were capable of inducing bioluminescence, suggesting that this bacterium produces an AHL(s)(Throup *et al.*, 1995b). *C. violaceum* characteristically produces violacein, a water-insoluble purple pigment with antibacterial activity (Ballantine *et al.*, 1958; Lichstein & van de Sand, 1945). By subjecting *C. violaceum* to mini-Tn5 transposon mutagenesis we obtained a double Tn5 insertion, violacein-negative, white mutant (CV026) defective in the production of the factor(s) capable of inducing bioluminescence in the recombinant *Escherichia coli* AHL biosensor (Latifi *et al.*, 1995; Throup *et al.*, 1995b). The transposon insertion sites have been mapped to a putative repressor locus and to a *luxI* homologue (*cviI*) respectively (M. K. Winson, A. Taylor, G. S. A. B. Stewart & P. Williams, unpublished data). When cross-streaked against the parental *C. violaceum* strain, CV026 responds by producing violacein (Throup *et al.*, 1995b). This finding suggested that pigment production in *C. violaceum* is regulated via quorum sensing and indicated the potential of CV026 as a simple biosensor for the detection of AHLs.

In the present paper, we identify the major *C. violaceum* AHL as *N*-hexanoyl-L-homoserine lactone (HHL). By evaluating the activity of a range of AHL and *N*-acylhomocysteine thiolactone (AHT) analogues, we show that stimulation or inhibition of violacein production in CV026 can be usefully employed in both plate bioassays and thin-layer chromatography overlays to detect a structurally diverse series of natural and synthetic AHL and AHT molecules.

METHODS

Bacterial strains and growth media. The *Chromobacterium violaceum* wild-type strain ATCC 31532 was obtained from the American Type Culture Collection (Rockville, MD, USA); *C. violaceum* CV026 is a mini-Tn5 mutant of ATCC 31532 (Throup *et al.*, 1995b; Latifi *et al.*, 1995; Winson *et al.*, 1995). *Yersinia enterocolitica* strain W828 was provided by J. Throup (University of Nottingham; Throup *et al.*, 1995a) and *Serratia liquefaciens* strain MG1 by M. Giskov (The Technical University of Denmark; Eberl *et al.*, 1996). Bacteria were routinely grown with shaking in LB broth at 30 °C. For the purification of AHLs, bacteria were grown in M9 minimal medium (1 l) supplemented as follows: for *C. violaceum*, M9 was supplemented with succinate (0·4 %, w/v) plus Casamino acids (0·2 %, w/v); for *Y. enterocolitica* M9 was supplemented with glucose (0·4 %, w/v), Casamino acids (0·2 %, w/v), proline 0·001 % (w/v) and methionine (0·1 μg ml^{-1}); for *S. liquefaciens* M9 was supplemented as for *Y. enterocolitica* plus thiamin (1 μg ml^{-1}). Where required, antibiotics were incorporated into growth media at the following concentrations: ampicillin (50 μg ml^{-1}), kanamycin (50 μg ml^{-1}), and chloramphenicol (30 μg ml^{-1}).

AHL reporter plate bioassays. Five-millilitre volumes of molten semi-solid LB agar (0·3 %, w/v) were seeded with 50 μl of an overnight LB culture of *C. violaceum* CV026 and poured immediately over the surface of prewarmed LB agar plates prepared in square Petri dishes (100 mm × 100 mm). When the overlaid agar had solidified, wells were punched in the agar with a sterile cork borer (diameter 6 mm). The wells were filled with the preparation to be assayed [solvent extracts, HPLC fractions or synthetic AHLs (see below)]. Where necessary the final volume per well was adjusted to 50 μl by

addition of sterile LB broth. Positive [*N*-(hexanoyl)-L-homoserine lactone: HHL] and negative (sterile LB broth) controls were included in each assay plate. The Petri dishes were incubated in the upright position overnight (30 °C), then examined for the stimulation of violacein synthesis (indicated by blue/purple pigmentation of the bacterial lawn around wells). A modification of this basic assay was also developed to detect molecules which antagonize AHL stimulation of violacein synthesis. In this modified assay, HHL or *N*-(3-oxohexanoyl)-L-homoserine lactone (OHHL) together with CV026 was added to the semisolid agar (final concentration, 5 μM) and the assay procedure carried out as before. Inhibition of violacein synthesis was defined by the presence of white haloes in a purple background. The limits of detection of activity in the two assays were determined by applying serial dilutions of the compounds (using LB broth as the diluent) to assay plates as described above. End-points were estimated as the lowest quantity of compound giving discernible activation or inhibition of violacein synthesis respectively.

Isolation, purification and chemical characterization of the *C. violaceum* AHL. Spent supernatants (4 l) from stationary-phase cultures of *C. violaceum* ATCC 31532 grown in minimal medium were extracted with dichloromethane (700:300 supernatant/dichloromethane). Dichloromethane was removed by rotary evaporation and the residue reconstituted in 1·0 ml acetonitrile. This was then applied to a C_8 reverse-phase preparative HPLC column [Kromasil KR100-5C8 (250 mm × 8 mm) column; Hichrom] eluted with an isocratic mobile phase of 70% (v/v) acetonitrile in water at a flow rate of 2 ml min^{-1} and monitored at 210 nm. Fractions showing activity in the CV026 violacein induction bioassay were pooled and re-chromatographed using 35% (v/v) acetonitrile in water. Active fractions were analysed by mass spectrometry (MS). Mass spectra were obtained on a VG 70-SEQ instrument of EBqQ geometry (Fisons Instruments, VG Analytical). Samples were ionized by positive-ion fast-atom bombardment (FAB). The pseudo-molecular ion $(M+H)^+$ peaks recorded by FAB-MS were further analysed by tandem mass spectrometry (MS-MS) and shown to be identical to the MS-MS spectra of the authentic synthetic AHL.

Thin-layer chromatography (TLC). This was performed on C_{18} reversed-phase plates using a solvent system of methanol/water (60:40, v/v) essentially as described by Shaw *et al.* (1997) but using the *C. violaceum* mutant CV026 rather than *A. tumefaciens* as the indicator organism. Synthetic AHLs made up as 10 mM solutions in acetonitrile or extracts of culture supernatants (from *Y. enterocolitica* or *S. liquefaciens*, extracted with dichloromethane as described for *C. violaceum*) were spotted (2–30 μl) onto glass-backed RP18 reverse-phase TLC plates (BDH) and dried in a stream of cold air. Samples were separated using methanol (60%, v/v) in water as the solvent. Once the solvent front had migrated to within 2 cm of the top of the chromatogram, the plate was removed from the chromatography tank, dried in air and overlaid with a thin film of CV026 seeded in 0·3% (w/v) LB agar without or with OHHL (final concentration 1 μM). After overnight incubation at 30 °C, AHLs were located either as purple spots on a white background or as white spots in a purple background respectively.

Synthesis of AHLs and AHTs. A series of AHLs and AHTs were synthesized as previously described (Table 1; Chhabra *et al.*, 1993). Representative compounds from each series varied in *N*-acyl chain length from C_4 [*N*-(3-oxobutanoyl)-L-homoserine lactone (OBHL) and *N*-butanoyl-L-homoserine lactone (BHL)], to C_{14} [*N*-tetradecanoyl-L-homoserine lactone (tDHL)] in the case of the *N*-alkanoyl-L-homoserine lactones. In addition, the D-isomer of *N*-(3-oxohexanoyl)-L-homoserine lactone (D-OHHL), the homocysteine thiolactones *N*-(3-oxohexanoyl)-L-homocysteine thiolactone (OHHT) and *N*-hexanoyl-L-homocysteine thiolactone (HHT) were also prepared together with *N*-benzoylacetyl-L-homoserine lactone (BAHL). The chemical formulae for the compounds used in this paper are given in Table 1. In addition, to facilitate unambiguous identification of the individual compounds a uniform method of abbreviation has been adopted (Table 1; Swift *et al.*, 1996; Winson *et al.*, 1995). Each compound was purified to homogeneity by preparative HPLC and its structure confirmed by MS and proton NMR spectroscopy. Stock solutions (final concentration of compound, 5 mM), were prepared in acetonitrile and stored at −20 °C.

RESULTS AND DISCUSSION

Purification and identification of the *C. violaceum* AHL

Stationary-phase cell-free supernatants from the *C. violaceum* wild-type were extracted with dichloromethane and evaporated to dryness as described in Methods. A fraction capable of stimulating violacein production by CV026 was located to a single peak on

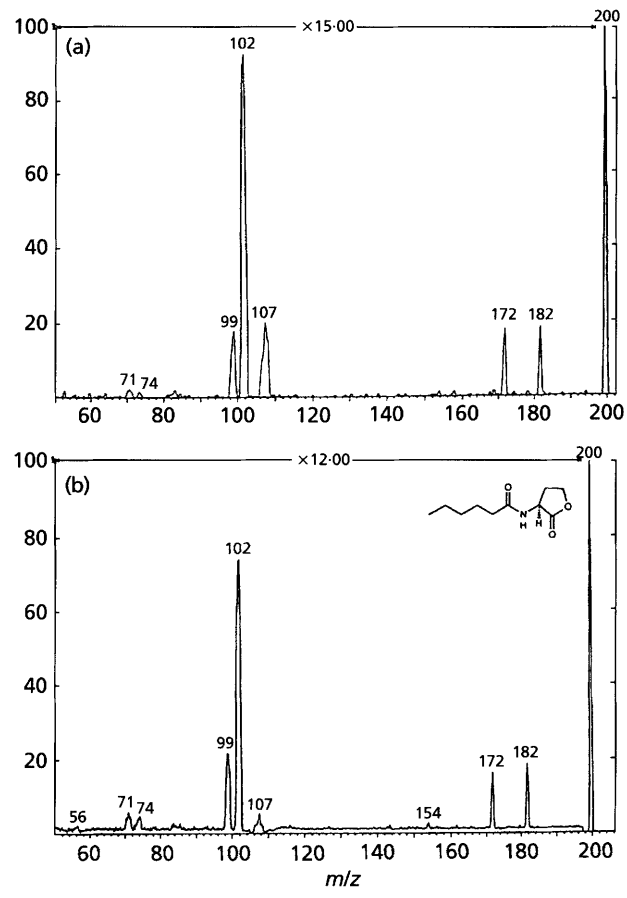
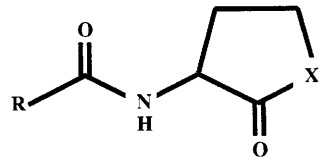


Fig. 1. Tandem mass spectrometry (MS-MS) of the active compound from an extract of *C. violaceum* culture supernatant. The compound was isolated and purified by preparative HPLC as described in Methods. The MS-MS spectrum obtained for the natural product (a) is indistinguishable from synthetic HHL (b).

Table 1. Structures and relative activities of AHL and AHT compounds in the *C. violaceum* CV026 agar plate bioassays



R	X	Chemical name	Abbreviation	Amount applied (nmol)* Activation	Amount applied (nmol)* Inhibition
$CH_3CH_2CH_2$	O	*N*-Butanoyl-L-homoserine lactone	BHL	0·9	NA
$CH_3CH_2CH_2$	S	*N*-Butanoyl-L-homocysteine thiolactone	BHT	1·7	NA
CH_3COCH_2	O	*N*-(3-Oxobutanoyl)-L-homoserine lactone	OBHL	3·5	NA
$PhCH_2COCH_2$	O	*N*-Benzoylacetyl-L-homoserine lactone	BAHL	1·53	NA
$CH_3(CH_2)_4$	O	*N*-Hexanoyl-L-homoserine lactone	HHL	0·03	NA
$CH_3(CH_2)_4$	S	*N*-Hexanoyl-L-homocysteine thiolactone	HHT	0·05	NA
$CH_3(CH_2)_2COCH_2$	O	*N*-(3-Oxohexanoyl)-L-homoserine lactone	OHHL	0·19	NA
$CH_3(CH_2)_2COCH_2$	O	*N*-(3-Oxohexanoyl)-D-homoserine lactone	(D)OHHL	12·0	NA
$CH_3(CH_2)_2COCH_2$	S	*N*-(3-Oxohexanoyl)-L-homocysteine thiolactone	OHHT	0·69	NA
$CH_3(CH_2)_6$	O	*N*-Octanoyl-L-homoserine lactone	OHL	0·22	NA
$CH_3(CH_2)_4COCH_2$	O	*N*-(3-Oxo-octanoyl)-L-homoserine lactone	OOHL	0·33	NA
$CH_3(CH_2)_8$	O	*N*-Decanoyl-L-homoserine lactone	DHL	NA	0·49
$CH_3(CH_2)_6COCH_2$	O	*N*-(3-Oxodecanoyl)-L-homoserine lactone	ODHL	NA	4·6
$CH_3(CH_2)_{10}$	O	*N*-Dodecanoyl-L-homoserine lactone	dDHL	NA	4·4
$CH_3(CH_2)_8COCH_2$	O	*N*-(3-Oxododecanoyl)-L-homoserine lactone	OdDHL	NA	4·2
$CH_3(CH_2)_{12}$	O	*N*-Tetradecanoyl-L-homoserine lactone	tDHL	NA	4·0
$CH_3(CH_2)_{10}COCH_2$	O	*N*-(3-Oxotetradecanoyl)-L-homoserine lactone	OtDHL	NA	3·8

*Amount of a given compound added to a well cut in the agar to either induce violacein production (activation) or inhibit HHL-mediated induction of violacein production (inhibition) in CV026. NA, No activity i.e. the compound is unable to either induce or to antagonize HHL-mediated induction of violacein in CV026.

HPLC with a retention time, in 35 % (v/v) acetonitrile in water, of 10·1 min (data not shown). By comparison with the known retention time of synthetic standards, this suggested that the compound was likely to be *N*-hexanoyl-L-homoserine lactone (HHL). To confirm the identity of the *C. violaceum* AHL, sufficient material was purified by preparative HPLC and subjected to spectrometry. In the FAB-MS spectrum, a pseudomolecular ion of m/z 200 $(M+H)^+$ was clearly identified in the sample (data not shown). To unequivocally assign the m/z 200 peak, it was subjected to tandem mass spectrometry. Fig. 1 shows that the product-ion spectrum (MS-MS) derived from the m/z 200 peak was identical in all respects to the MS-MS data obtained with synthetic HHL. No other AHLs were detected in *C. violaceum* culture supernatants and this AHL was absent from the spent cell-free culture supernatants of *C. violaceum* CV026 (data not shown).

HHL is, however, not unique to *Chromobacterium*, having previously been isolated from spent culture supernatants of *Agrobacterium tumefaciens* (Zhang *et al.*, 1993), *Yersinia enterocolitica* (Throup *et al.*, 1995a), *Serratia liquefaciens* (Eberl *et al.*, 1996) *Pseudomonas aeruginosa* (Winson *et al.*, 1995) and *Photobacterium fischeri* (Kuo *et al.*, 1994). For both *P. aeruginosa* and *S. liquefaciens*, HHL is produced only as a minor component, with BHL as the primary AHL synthesized via the LuxI homologues RhlI (VsmI; Winson *et al.*, 1995) and SwrI (Eberl *et al.*, 1996) respectively. The *P. fischeri* LuxI protein also directs the synthesis of HHL as a minor component alongside OHHL (Kuo *et al.*, 1994; Schaefer *et al.*, 1996b) whereas YenI from *Y. enterocolitica* directs the synthesis of HHL and OHHL in a 50:50 ratio in both the homologous and heterologous (*E. coli*) genetic backgrounds (Throup *et al.*, 1995a). Since HHL in *C. violaceum* is synthesized via a LuxI homologue (M. K. Winson, P. Williams & G. S. A. B. Stewart, unpublished data), this LuxI homologue differs from those of *Agrobacterium*, *Yersinia*, *Pseudomonas*, *Serratia* and *Photobacterium* in that HHL is the predominant AHL synthesized.

Utility of *C. violaceum* mutant CV026 as an AHL sensor

Stimulation of violacein synthesis. To evaluate the potential of CV026 as a generic biosensor for AHLs, a series of AHLs and AHTs were synthesized and their

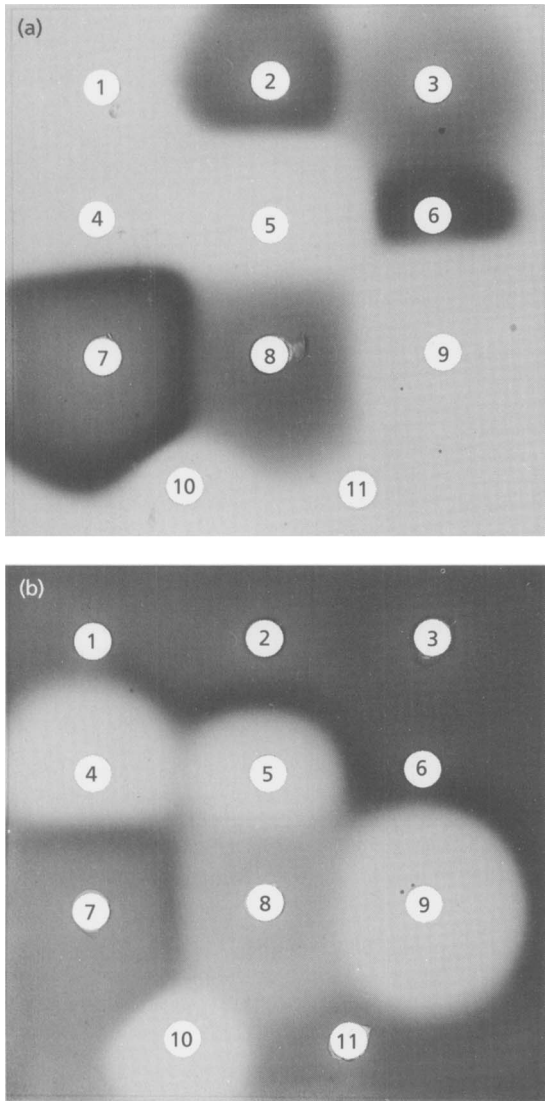


Fig. 2. Induction (a) and inhibition (b) of violacein synthesis in *C. violaceum* CV026 by synthetic AHLs. Five microlitres of each compound (5 mM stock solution in acetonitrile) was diluted to 50 µl with LB and added to wells cut into the LB agar seeded with either (a) CV026 or (b) CV026 plus HHL (5 µM) as described in Methods. 1, OBHL; 2, OHHL; 3, OOHL; 4, ODHL; 5, OdDHL; 6, BHL; 7, HHL; 8, OHL; 9, DHL; 10, dDHL; 11, control (5 µl acetonitrile diluted to 50 µl with LB). In (a), a purple halo around the well is indicative of violacein production. Although OBHL (well 1) can weakly activate violacein production, no pigment is observed in the figure shown. This is due to a combination of the concentration applied and its proximity to an inhibitory AHL (ODHL) in an adjacent well (4). In (b), a white halo around the well in a violet background is indicative of the presence of an antagonistic AHL.

ability to induce violacein production in CV026 examined in an agar plate assay (Fig. 2a, Table 1). The most active agonist in this assay was HHL, the natural *C. violaceum* AHL. Replacement of the HHL lactone ring oxygen with sulfur to give HHT had little effect on activity. However, OHHL, the 3-oxo derivative, was about sixfold less active than HHL. Furthermore, the D-isomer of OHHL was some 60-fold less active than the L-isomer, indicating the importance of the chiral centre. Shortening the HHL acyl side chain from C_6 to C_4, to give BHL, reduced the comparative activity by around 30-fold. Whilst the thiolactone BHT had similar activity to BHL, the 3-oxo derivative, OBHL, was very much weaker (Table 1). However, the addition of a hydrophobic phenyl group to give *N*-benzoylacetyl-L-homoserine lactone (BAHL) generated a compound with similar agonist activity to BHL. Whilst the C_8 acyl chain OHL and OOHL were both able to induce violacein synthesis in CV026, none of the longer C_{10} to C_{14} compounds synthesized was able to do so (Fig. 2a, Table 1).

Thus the length of the *N*-linked acyl chain is a key structural feature determining the relative agonist activities of the series of AHL and AHT compounds evaluated in the CV026 violacein induction assay. In *C. violaceum*, the natural ligand for the *Chromobacterium* LuxR homologue, CviR (M. K. Winson, P. Williams & G. S. A. B. Stewart, unpublished data), is HHL; this is, not surprisingly, the most active compound in the violacein induction assay. Conservative changes to the acyl chain or lactone ring of HHL are tolerated and compounds such as OHHL and HHT have significant violacein-inducing activity. Similar structure–activity data have been obtained in bacteria such as *P. fischeri* (Eberhard *et al.*, 1986; Schaefer *et al.*, 1996a) and *E. carotovora* (Chhabra *et al.*, 1993), which both employ OHHL, to regulate bioluminescence and carbapenem synthesis respectively. However, as for the induction of violacein, AHLs with acyl chains longer than C_8 have little or no agonist activity (Eberhard *et al.*, 1986; Schaefer *et al.*, 1996a). Conversely, compounds with C_4 or C_6 acyl chains are unable to activate the *P. aeruginosa* LuxR homologue LasR, the natural ligand for which is the C_{12} AHL, OdDHL (Passador *et al.*, 1996).

Inhibition of AHL-mediated violacein synthesis. Although AHL compounds with *N*-acyl side chains longer than C_8 failed to stimulate violacein production even at high concentrations (Fig. 2a, Table 1), we noted that if placed in an adjacent well to short-chain (C_4 or C_6 acyl chain) AHLs, compounds such as *N*-decanoyl-L-homoserine lactone (DHL) antagonized violacein production (Fig. 2a; DHL in well 9 is clearly inhibiting violacein induction by BHL and OHL in adjacent wells 6 and 8 respectively). To explore this further, we seeded OHHL or HHL and CV026 into the overlay agar and placed DHL in the wells. After overnight incubation, we observed white haloes surrounding the well in a purple background (data not shown). These data suggested that inhibition of violacein production could be exploited as an assay for the detection of longer chain AHLs. Fig. 2(b) illustrates that compounds with C_{10}, C_{12} or C_{14} acyl chains, with or without an oxygen at the 3 position, could be detected by inhibition of HHL-mediated activation of violacein production. Whilst none of the shorter-chain C_4 or C_6 acyl chain compounds were active in this assay, it is clear that the C_8 compound OHL does inhibit HHL-mediated activation of violacein

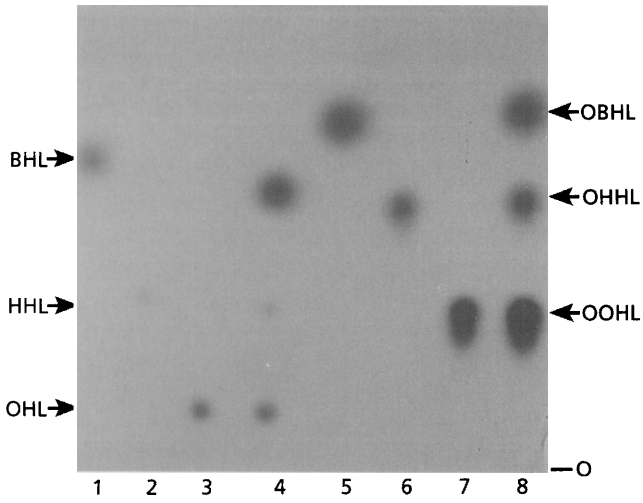


Fig. 3. Thin-layer chromatogram illustrating the CV026 overlay technique for AHL detection. Synthetic AHLs, either as single compounds or as mixtures, were dissolved in acetonitrile and spotted onto a reversed-phase RP18 TLC plate. The chromatogram was developed with 60% (v/v) methanol in water and then overlaid with CV026 as described in Methods. For each AHL, the amount applied to the TLC plate corresponds to the minimum detectable amount. Lane 1, BHL ($1{\cdot}8 \times 10^{-9}$ mol); lane 2, HHL (1×10^{-11} mol); lane 3, OHL ($4{\cdot}4 \times 10^{-10}$ mol); lane 4, a mixture of BHL, HHL and OHL; lane 5, OBHL ($7{\cdot}3 \times 10^{-8}$ mol); lane 6, OHHL ($1{\cdot}4 \times 10^{-10}$ mol); lane 7, OOHL ($8{\cdot}3 \times 10^{-10}$ mol); lane 8, a mixture of OBHL, OHHL and OOHL. A purple spot is indicative of AHL-mediated activation of violacein production. O marks the position of the origin.

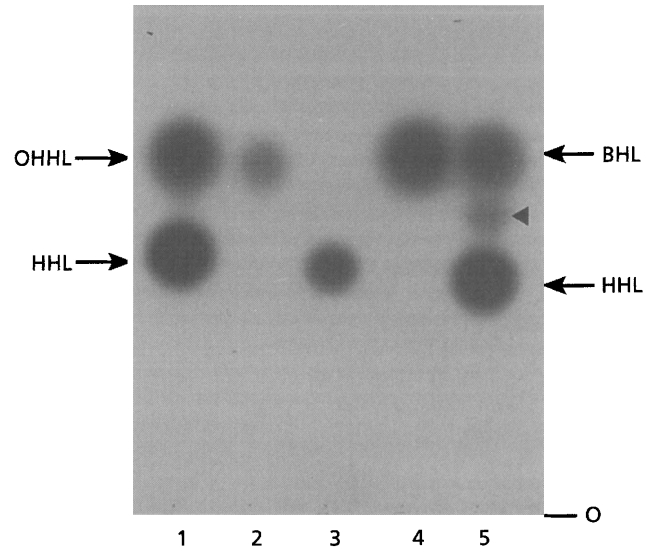


Fig. 4. Thin-layer chromatogram of the AHLs present in cell-free supernatants of *Y. enterocolitica* and *S. liquefaciens* detected using a CV026 overlay. Cell free supernatants from each micro-organism were extracted with dichloromethane and chromatogaphed as described in Methods. Lane 1, *Y. enterocolitica* supernatant extract; lane 2, OHHL standard; lane 3, HHL standard; lane 4, BHL standard; lane 5, *S. liquefaciens* extract. For the OHHL, HHL and BHL standards, $1{\cdot}8 \times 10^{-9}$, 1×10^{-11} and $8{\cdot}3 \times 10^{-10}$ mol respectively were applied to the chromatogram. O marks the position of the origin. The filled arrowhead (lane 5) marks the position of the novel *S. liquefaciens* AHL.

to some extent (Fig. 2b, well 8). Although the 3-oxo derivative of OHL, OOHL, does not show any inhibition at the concentration tested in Fig. 2(b), some weak inhibition can be observed in plates containing lower concentrations of the activating AHL.

Thus the length of the acyl side chain is again a major structural feature determining the sensitivity of detection of AHLs in this modified assay. This inhibition assay should prove useful for detecting compounds with acyl chains ranging from C_{10} to C_{14}. Using the violacein inhibition assay, we observed inhibitory activity in culture supernatants from the fish pathogen *Vibrio* (*Listonella*) *anguillarum* (Milton *et al.*, 1997). Subsequently chemical analysis led to the identification of the C_{10} acyl chain compound ODHL as the major *V. anguillarum* quorum-sensing signal molecule (Milton *et al.*, 1997). Furthermore the development of both the direct and modified *C. violaceum* assays should facilitate the assay of culture supernatants which contain mixtures of long- and short-chain AHLs. The versatility of the *C. violaceum* assays should therefore prove extremely valuable for the identification of novel AHLs. However, since many bacteria produce antibacterial substances, the inhibition assay should be interpreted with care. The white zone of antagonism observed should be opaque and not transparent, i.e. antibacterial agents will inhibit the growth of CV026, leading to a clear zone of inhibition around the well, whereas a long-chain antagonist will permit growth of the biosensor.

TLC analysis. Since HPLC can be both expensive and time consuming, other techniques such as TLC may offer an inexpensive and rapid route to the tentative identification of AHL signal molecules. Shaw *et al.* (1997) have recently demonstrated the utility of TLC in 60% (v/v) methanol/water to effectively separate AHLs with acyl chains from C_4 to C_{12} in length. We therefore examined the utility of the CV026 bioassay strain to detect these molecules on TLC plates as described in Methods. Fig. 3 illustrates the results obtained when a series of AHLs with C_4, C_6 or C_8 side chains are separated on a C_{18} reversed-phase TLC plate and, after chromatography, overlaid with agar containing CV026. Members of both the *N*-alkanoyl-L-homoserine lactones series and the *N*-(3-oxoalkanoyl)-L-homoserine lactones are well separated using 60% (v/v) methanol as the solvent (Shaw *et al.*, 1997) such that tentative identification of a novel AHL can be made by comparison of the R_F value of an unknown with that of a synthetic standard. Furthermore, full chemical characterization can be made by running samples streaked across the entire baseline such that a strip can be removed and overlaid with CV026 to aid location of the putative AHL. Once located on the chromatogram, the area containing the putative AHL can be scraped off,

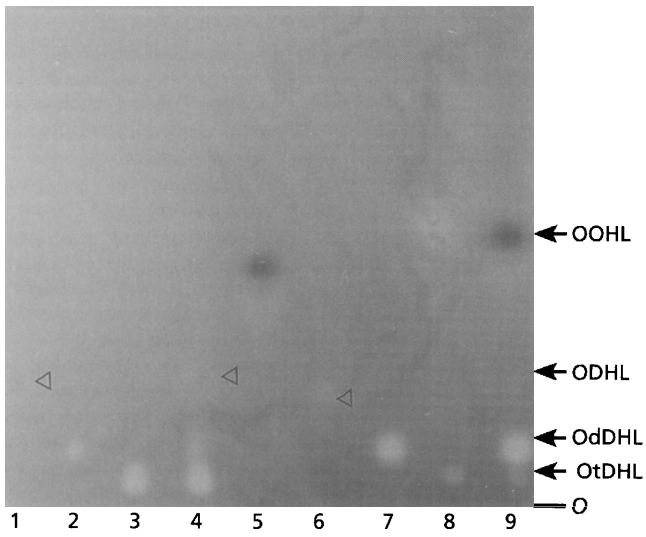


Fig. 5. Thin-layer chromatogram illustrating the inhibition of OHHL-mediated violacein synthesis in CV026 by AHLs with acyl chains from C_8 to C_{14}. AHLs, either as single compounds or as mixtures, were dissolved in acetonitrile and spotted onto a reverse-phase RP18 TLC plate. The chromatogram was developed with 60% (v/v) methanol in water and then overlaid with CV026 plus OHHL (1 μM) as described in Methods. For each AHL, the amount applied to the TLC plate corresponds to the minimum detectable amount. Lane 1, OHL ($4{\cdot}4 \times 10^{-10}$ mol); lane 2, DHL ($2{\cdot}4 \times 10^{-9}$ mol); lane 3, dDHL ($1{\cdot}1 \times 10^{-7}$ mol); lane 4, a mixture of OHL, DHL and dDHL; lane 5, OOHL ($8{\cdot}3 \times 10^{-10}$ mol); lane 6, ODHL (4×10^{-8} mol); lane 7, OdDHL (4×10^{-8} mol); lane 8, OtDHL ($1{\cdot}3 \times 10^{-7}$ mol); lane 9, a mixture of OOHL, ODHL, OdDHL and OtDHL. Active compounds in this assay give white spots in a purple background. O marks the position of the origin. The positions of OOHL, ODHL, OdDHL and OtDHL in lane 9 are marked with open arrowheads on the right. OOHL in lanes 5 and 9 is observed as the more intense purple spot with a slight tail of inhibition. Open arrowheads in lanes 1 and 4 mark the positions of the OHL spots; the arrowhead in lane 6 marks the position of the ODHL spot.

extracted with acetone and the material obtained subjected directly to MS and/or NMR spectroscopy. Using synthetic BHL, HHL and OHHL, we have found that a spot containing as little as 0·1 μg of the respective AHL is sufficient for detection by MS.

To demonstrate the utility of this assay for the preliminary identification of natural AHLs, we extracted spent culture supernatants from *Y. enterocolitica* and *S. liquefaciens*, bacteria we have previously shown to make OHHL plus HHL (Throup *et al.*, 1995a) and BHL plus HHL (Eberl *et al.*, 1996) respectively. Fig. 4 shows that the CV026 TLC overlay clearly detects each of these AHLs, which can be tentatively identified by comparison with AHL standards after extracting as little as 5 ml culture supernatant. Intriguingly, in the *S. liquefaciens* extract, there is a third positive spot, which runs between BHL and HHL (Fig. 4, lane 5). This compound does not migrate with an R_F value corresponding to any of the AHLs available in our compound library and may represent a novel *S. liquefaciens* AHL.

By incorporating an AHL such as HHL or OHHL into agar seeded with CV026, we have discovered that it is possible to detect AHLs with acyl chains between C_{10} and C_{14} by their ability to inhibit short-chain AHL-mediated activation of violacein. Fig. 5 shows that inhibition of violacein production can also be employed to detect long-chain AHLs on TLC as white spots in a purple background. Certain compounds, notably DHL, dDHL and OdDHL, are more effectively detected than others, although the sensitivity generally depends on the amount of AHL loaded onto the plate, the nature and concentration of the violacein-inducing AHL incorporated in the agar overlay and the thickness of the overlay. Interestingly, the C_8 compounds OHL and OOHL, which can induce violacein production, also exhibit some antagonistic activity; OHL for example appears as a white halo around a weak purple spot (see Fig. 5, lane 4). Furthermore, ODHL, which in the agar plate bioassay gives a strong inhibition of violacein activity, is very much weaker in the TLC assay. Similarly, OtDHL, which has a C_{14} chain, is only weakly antagonistic, implying that efficient competition with OHHL for the *C. violaceum* LuxR homologue CviR (M. K. Winson, P. Williams & G. S. A. B. Stewart, unpublished data) is best achieved with AHLs with C_{10} to C_{12} acyl chains. However, it is also possible that given its hydrophobic characteristics, the C_{14} compound OtDHL penetrates into but is unable to cross the *C. violaceum* outer and/or inner membrane.

Conclusions

This study adds *C. violaceum* to the growing family of Gram-negative bacteria which produce AHLs. In *C. violaceum*, it is clear that in addition to violacein synthesis, HHL is involved in the regulation of exoprotease, chitinase and cyanide production (Throup *et al.*, 1995b; M. K. Winson, P. Williams & G. S. A. B. Stewart, unpublished data). However, it is the induction or antagonism of AHL-mediated violacein synthesis in HHL-negative mutants such as CV026 which is likely to prove most useful since it offers a convenient tool for the biological assay of a broad range of AHL and AHT analogues. Furthermore, because supernatants from *E. coli* do not induce violacein activation or inhibition (unpublished data), *E. coli* is a very useful cloning host for LuxI homologues, since clones can easily be screened using the *Chromobacterium* assays. In addition, the ease of the end-point detection and the flexibility of the TLC assays underlines the versatility of this technique, which does not require expensive reagents, HPLC equipment or the use of film or luminometry.

ACKNOWLEDGEMENTS

We thank Nigel Bainton for his contribution to the preliminary experiments to isolate the *C. violaceum* AHL. We also thank Stephen Farrand and Paul Shaw for their helpful comments on the manuscript and for providing us with pre-publication data. This work was supported by grants and a studentship (for A.T.) from the Biotechnology and Biological Sciences

Research Council (to P.W., G.S.A.B.S. and B.W.B.) and a Wellcome Trust Prize studentship for L.F. (041310/Z/94).

REFERENCES

Bainton, N. J., Bycroft, B. W., Chhabra, S. R., Stead, P., Gledhill, L., Hill, P. H., Rees, C. E. D., Winson, M. K., Salmond, G. P. C., Stewart, G. S. A. B. & Williams, P. (1992a). A general role for the *lux* autoinducer in bacterial cell signalling: control of antibiotic biosynthesis in *Erwinia*. *Gene* **116**, 87–91.

Bainton, N. J., Stead, P., Chhabra, S. R., Bycroft, B. W., Salmond, G. P. C., Stewart, G. S. A. B. & Williams, P. (1992b). *N*-(3-oxohexanoyl)-L-homoserine lactone regulates carbapenem antibiotic production in *Erwinia carotovora*. *Biochem J* **288**, 997–1004.

Ballantine, J. A., Beer, R. J. S., Crutchley, D. J., Dodd, G. M. & Palmer, D. R. (1958). The synthesis of violacein and related compounds. *Proc Chem Soc* 232–233.

Beck von Bodman, S. & Farrand, S. K. (1995). Capsular polysaccharide biosynthesis and pathogenicity in *Erwinia stewartii* require induction by an *N*-acylhomoserine lactone autoinducer. *J Bacteriol* **177**, 5000–5008.

Chhabra, S. R., Stead, P., Bainton, N. J., Salmond, G. P. C., Stewart, G. S. A. B., Williams, P. & Bycroft, B. W. (1993). Autoregulation of carbapenem biosynthesis in *Erwinia carotovora* ATCC 39048 by analogues of *N*-(3-oxohexanoyl)-L-homoserine lactone. *J Antibiot* **46**, 441–454.

Eberhard, A., Widrig, C. A., McBath, P. & Schineller, J. B. (1986). Analogs of the autoinducer of bioluminescence in *Vibrio fischeri*. *Arch Microbiol* **146**, 35–40.

Eberl, L., Winson, M. K., Sternberg, C., Stewart, G. S. A. B., Christiansen, G., Chhabra, S. R., Bycroft, B. W., Williams, P., Molin, S. & Giskov, M. (1996). Involvement of *N*-acyl-L-homoserine lactone autoinducers in controlling the multicellular behaviour of *Serratia liquefaciens*. *Mol Microbiol* **20**, 127–136.

Fuqua, W. C. & Winans, S. C. (1994). A LuxR–LuxI type regulatory system activates *Agrobacterium* Ti plasmid conjugal transfer in the presence of a plant tumor metabolite. *J Bacteriol* **176**, 2796–2806.

Fuqua, W. C., Winans, S. C. & Greenberg, E. P. (1996). Census and consensus in bacterial ecosystems: the LuxR–LuxI family of quorum sensing transcriptional regulators. *Annu Rev Microbiol* **50**, 727–751.

Gray, K. M., Pearson, J. P., Downie, J. A., Boboye, B. B. & Greenberg, E. P. (1996). Cell-to-cell signaling in the symbiotic nitrogen-fixing bacterium *Rhizobium leguminosarum*: autoinduction of stationary phase and rhizosphere-expressed genes. *J Bacteriol* **178**, 372–376.

Hwang, I., Li, P.-L., Zhang, L., Piper, K. R., Cook, D. M., Tate, M. E. & Farrand, S. K. (1995). TraI, a LuxI homologue, is responsible for production of conjugation factor, the Ti plasmid *N*-acylhomoserine lactone autoinducer. *Proc Natl Acad Sci USA* **91**, 4639–4643.

Jones, S., Yu, B., Bainton, N. J., Birdsall, M., Bycroft, B. W., Chhabra, S. R., Cox, A. J. R., Golby, P., Reeves, P. J., Stephens, S., Winson, M. K., Salmond, G. P. C., Stewart, G. S. A. B. & Williams, P. (1993). The *lux* autoinducer regulates the production of exoenzyme virulence determinants in *Erwinia carotovora* and *Pseudomonas aeruginosa*. *EMBO J* **12**, 2477–2482.

Kuo, A., Blough, N. V. & Dunlap, P. V. (1994). Multiple *N*-acyl-L-homoserine lactone autoinducers of luminescence in the marine symbiotic bacterium *Vibrio fischeri*. *J Bacteriol* **178**, 971–976.

Latifi, A., Winson, M. K., Foglino, M., Bycroft, B. W., Stewart, G. S. A. B., Lazdunski, A. & Williams, P. (1995). Multiple homologues of LuxR and LuxI control expression of virulence determinants and secondary metabolites through quorum sensing in *Pseudomonas aeruginosa* PAO1. *Mol Microbiol* **17**, 333–343.

Latifi, A., Foglino, M., Tanaka, T., Williams, P. & Lazdunski, A. (1996). A hierarchical quorum sensing cascade in *Pseudomonas aeruginosa* links the transcriptional activators LasR and VsmR to expression of the stationary phase sigma factor RpoS. *Mol Microbiol* **21**, 1137–1146.

Lichstein, H. C. & van de Sand, V. F. (1945). Violacein, an antibiotic pigment produced by *Chromobacterium violaceum*. *J Infect Dis* **76**, 47–51.

Meighen, E. A. (1991). Molecular biology of bacterial bioluminescence. *Microbiol Rev* **55**, 123–142.

Milton, D. L., Hardman, A., Camara, M., Chhabra, S. R., Bycroft, B. W., Stewart, G. S. A. B. & Williams, P. (1997). Quorum sensing in *Vibrio anguillarum*: characterization of the *vanI/R* locus and identification of the autoinducer *N*-(3-oxodecanoyl)-L-homoserine lactone. *J Bacteriol* **179**, 3004–3012.

Passador, L., Cook, J. M., Gambello, M. J., Rust, L. & Iglewski, B. H. (1993). Expression of *Pseudomonas aeruginosa* virulence genes requires cell-to-cell communication. *Science* **260**, 1127–1130.

Passador, L., Tucker, K. D., Guertin, K. R., Journet, M. P., Kende, A. S. & Iglewski, B. H. (1996). Functional analysis of the *Pseudomonas aeruginosa* autoinducer PAI. *J Bacteriol* **178**, 5995–6000.

Pearson, J. P., Gray, K. M., Passador, L., Tucker, K. D., Eberhard, A., Iglewski, B. H. & Greenberg, E. P. (1994). Structure of the autoinducer required for expression of *Pseudomonas aeruginosa* virulence genes. *Proc Natl Acad Sci USA* **91**, 197–201.

Pearson, J. P., Passador, L., Iglewski, B. H. & Greenberg, E. P. (1995). A second *N*-acylhomoserine lactone signal produced by *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA* **92**, 1490–1494.

Piper, K. R., Beck von Bodman, S. & Farrand, S. K. (1993). Conjugation factor of *Agrobacterium tumefaciens* regulates Ti plasmid transfer by autoinduction. *Nature* **362**, 448–450.

Salmond, G. P. C., Bycroft, B. W., Stewart, G. S. A. B. & Williams, P. (1995). The bacterial 'enigma': cracking the code of cell–cell communication. *Mol Microbiol* **16**, 615–624.

Schaefer, A. L., Hanzelka, B. L., Eberhard, A. & Greenberg, E. P. (1996a). Quorum sensing in *Vibrio fischeri*: probing autoinducer–LuxR interactions with autoinducer analogs. *J Bacteriol* **178**, 2897–2901.

Schaefer, A. L., Val, D. L., Hanzelka, B. L., Cronan, J. E., Jr & Greenberg, E. P. (1996b). Generation of cell-to-cell signals in quorum sensing: acyl homoserine lactone synthetase activity of a purified *Vibrio fischeri* LuxI protein. *Proc Natl Acad Sci USA* **93**, 9505–9509.

Schripsema, J., de Rudder, K. E. E., van Vliet, T. B., Lankhorst, P. P., de Vroom, E., Kijne, J. W. & van Brussel, A. A. (1996). Bacteriocin *small* of *Rhizobium leguminosarum* belongs to the class of *N*-acyl-L-homoserine lactone molecules known as autoinducers and as quorum sensing co-transcription factors. *J Bacteriol* **178**, 366–371.

Shaw, P. D., Ping, G., Daly, S., Cronan, J. E., Jr, Rinehart, K. & Farrand, S. K. (1997). Detecting and characterizing acyl-homoserine lactone signal molecules by thin layer chromatography. *Proc Natl Acad Sci USA* **94**, 6036–6041.

Swift, S., Winson, M. K., Chan, P. F., Bainton, N. J., Birdsall, M., Reeves, P. J., Rees, C. E. D., Chhabra, S. R., Hill, P. J., Throup, J. P., Bycroft, B. W., Salmond, G. P. C., Williams, P. & Stewart, G. S. A. B. (1993). A novel strategy for the isolation of *luxI* homologues: evidence for the widespread distribution of a LuxR:LuxI superfamily in enteric bacteria. *Mol Microbiol* **10**, 511–520.

Swift, S., Throup, J. P., Salmond, G. P. C., Williams, P. & Stewart, G. S. A. B. (1996). Quorum sensing: a population-density component in the determination of bacterial phenotype. *Trends Biochem Sci* **21**, 214–219.

Throup, J., Bainton, N. J., Briggs, G., Winson, M. K., Bycroft, B. W., Williams, P. & Stewart, G. S. A. B. (1995a). YenI: a LuxI homologue from *Yersinia enterocolitica* mediating the synthesis of the quorum sensor signal *N*-(3-oxohexanoyl) homoserine lactone. *Mol Microbiol* **17**, 345–356.

Throup, J., Winson, M. K., Bainton, N. J., Bycroft, B. W., Williams, P. & Stewart, G. S. A. B. (1995b). Signalling in bacteria beyond bioluminescence. In *Bioluminescence and Chemiluminescence: Fundamentals and Applied Aspects*, pp. 89–92. Edited by A. Campbell, L. Kricka & P. Stanley. Chichester: Wiley.

Williams, P., Bainton, N. J., Swift, S., Winson, M. K., Chhabra, S. R., Stewart, G. S. A. B., Salmond, G. P. C. & Bycroft, B. W. (1992). Small molecule mediated density dependent control of gene expression in prokaryotes: bioluminescence and the biosynthesis of carbapenem antibiotics. *FEMS Microbiol Lett* **100**, 161–168.

Winson, M. K., Camara, M., Latifi, A., Foglino, M., Chhabra, S. R., Daykin, M., Bally, M., Chapon, V., Salmond, G. P. C., Bycroft, B. W., Lazdunski, A., Stewart, G. S. A. B. & Williams, P. (1995). Multiple *N*-acyl-L-homoserine lactone signal molecules regulate production of virulence determinants and secondary metabolites in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA* **92**, 9427–9431.

Wood, D. W. & Pierson, L. S., III. (1996). The *phzI* gene of *Pseudomonas aureofaciens* 30-84 is responsible for the production of a diffusible signal required for phenazine antibiotic production. *Gene* **168**, 49–53.

Zhang, L., Murphy, P. J., Kerr, A. & Tate, M. E. (1993). *Agrobacterium* conjugation and gene regulation by *N*- acyl-L-homoserine lactones. *Nature* **362**, 446–448.

Received 24 June 1997; revised 28 August 1997; accepted 1 September 1997.