The biocontrol strain *Pseudomonas fluorescens* F113 produces the *Rhizobium small* bacteriocin, *N*-(3-hydroxy-7-*cis*-tetradecenoyl)homoserine lactone, via HdtS, a putative novel *N*-acylhomoserine lactone synthase

Bridget E. Laue,¹ Yan Jiang,¹ Siri Ram Chhabra,¹ Sinead Jacob,² Gordon S. A. B. Stewart,^{1†} Andrea Hardman,¹ J. Allan Downie,³ Fergal O’Gara² and Paul Williams¹

Author for correspondence: Paul Williams. Tel: +44 115 9515047. Fax: +44 115 8466296.
e-mail: paul.williams@nottingham.ac.uk

¹ School of Pharmaceutical Sciences, University of Nottingham, Nottingham NG7 2RD, UK

² Biomerit Research Centre, Department of Microbiology, National University of Ireland, Cork, Ireland

³ John Innes Centre, Colney, Norwich NR4 7UH, UK

Several different species of *Pseudomonas* produce *N*-acylhomoserine lactones (AHLs), quorum-sensing signal molecules which are involved in the cell-density-dependent control of secondary metabolite and virulence gene expression. When *Pseudomonas fluorescens* F113 was cross-streaked against AHL biosensors capable of sensitively detecting either short (C₄–C₈) or long (C₁₀–C₁₄) acyl chain AHLs, no activity was detectable. However, by extracting cell-free stationary-phase culture supernatants with dichloromethane followed by reverse-phase HPLC, three distinct fractions were obtained capable of activating the AHL biosensors. Three AHLs were subsequently characterized using high-resolution MS and chemical synthesis. These were (i) *N*-(3-hydroxy-7-*cis*-tetradecenoyl)homoserine lactone (3OH,C_{14:1}-HSL), a molecule previously known as the *Rhizobium leguminosarum small* bacteriocin as a consequence of its growth inhibitory properties, (ii) *N*-decanoylhomoserine lactone (C₁₀-HSL) and (iii) *N*-hexanoylhomoserine lactone (C₆-HSL). A gene (*hdtS*) capable of directing synthesis of all three *P. fluorescens* AHLs in *Escherichia coli* was cloned and sequenced. *In vitro* transcription/translation of *hdtS* yielded a protein of approximately 33 kDa capable of directing the synthesis of 3OH,C_{14:1}-HSL, C₁₀-HSL and C₆-HSL in *E. coli*. HdtS does not belong to either of the known AHL synthase families (LuxI or LuxM) and is related to the lysophosphatidic acid acyltransferase family. HdtS may therefore constitute a member of a third protein family capable of AHL biosynthesis.

Keywords: *Pseudomonas fluorescens*, *N*-acylhomoserine lactone synthase, *Rhizobium*, *N*-(3-hydroxy-7-*cis*-tetradecenoyl)homoserine lactone, acyltransferase

INTRODUCTION

Apart from direct cell–cell contact, the production of small, diffusible signal molecules constitutes one of the

Abbreviations: AHL, *N*-acylhomoserine lactone; DCM, dichloromethane; C₆-HSL, *N*-hexanoylhomoserine lactone; C₁₀-HSL, *N*-decanoylhomoserine lactone; 3OH,C_{14:1}-HSL, *N*-(3-hydroxy-7-*cis*-tetradecenoyl)homoserine lactone; PDA, photodiode array.

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† Deceased.

few mechanisms through which bacteria can communicate with each other. The perception of such signal molecules enables an individual bacterial cell to sense ‘self’ or others, and so to adapt to environmental conditions in concert with population density. This cell–cell communication mechanism is now generally referred to as ‘quorum sensing’ and regulates a variety of physiological processes, including bioluminescence, swarming, swimming and twitching motility, antibiotic biosynthesis, biofilm differentiation, conjugation and the production of virulence determinants in plant and animal pathogens (for reviews, see Swift *et al.*,

1996; Dunny & Winans, 1999; England *et al.*, 1999; Williams *et al.*, 2000).

Quorum sensing relies upon the interaction of a diffusible signal molecule with either a sensor or a transcriptional regulator protein to couple gene expression with cell population density. Several chemically distinct families of quorum-sensing signal molecules have now been identified, amongst which the *N*-acylhomoserine lactones (AHLs) have probably been the most intensively investigated (Swift *et al.*, 1996, 1999; Fuqua *et al.*, 1996). AHLs are produced by bacteria belonging to many different Gram-negative genera and vary mainly with respect to the length (4–14 carbons) and substituent (H, O or OH) at the 3-position of their *N*-acyl side chains. Accumulation of the AHL molecule above a threshold concentration, through the activity of a signal generator protein (usually a member of the LuxI family; Moré *et al.*, 1996; Schaefer *et al.*, 1996; Jiang *et al.*, 1998) renders the population quorate. Appropriate target gene(s) are then activated via the action of a member of the LuxR family of transcriptional activators in concert with the AHL signal molecule (Swift *et al.*, 1996; Fuqua *et al.*, 1996).

Among the species belonging to the genus *Pseudomonas*, *Pseudomonas aeruginosa*, *Pseudomonas aureofaciens* (now *Pseudomonas chlororaphis*), *Pseudomonas fluorescens* and *Pseudomonas syringae* have all been reported to produce AHLs (Bainton *et al.*, 1992; Pearson *et al.*, 1994, 1995; Winson *et al.*, 1995; Shaw *et al.*, 1997; Wood *et al.*, 1997). *P. aeruginosa*, for example, possesses a sophisticated regulatory hierarchy consisting of two separate but linked quorum-sensing circuits (Latifi *et al.*, 1995, 1996; Pesci *et al.*, 1997). These are termed the *las* and *rhl* circuits, and each possesses a LuxI homologue (LasI or RhlI) and a LuxR homologue (LasR or RhlR). The AHLs that signal within the *las* and *rhl* systems are *N*-(3-oxododecanoyl)-L-homoserine lactone (3O,C₁₂-HSL) and *N*-butanoyl-L-homoserine lactone (C₄-HSL), respectively. Together, the two systems constitute a hierarchical cascade that co-ordinates the production of virulence factors (Latifi *et al.*, 1995, 1996; Pesci *et al.*, 1997), the *xcp* general secretion apparatus (Chapon-Hervé *et al.*, 1997), twitching motility (Glessner *et al.*, 1999) and stationary-phase genes (via the alternative sigma factor, RpoS; Latifi *et al.*, 1996). Although C₄-HSL and 3O,C₁₂-HSL have not been found in other pseudomonads, AHLs including *N*-hexanoyl-homoserine lactone (C₆-HSL, *P. aureofaciens*; Wood *et al.*, 1997), *N*-(3-oxohexanoyl)-L-homoserine lactone (3O,C₆-HSL, *P. syringae* group; Shaw *et al.*, 1997; Cha *et al.*, 1998) and the 3-hydroxy-forms of *N*-hexanoyl-, *N*-octanoyl- and *N*-decanoyl-homoserine-L-lactones (*P. fluorescens*; Shaw *et al.*, 1997) have been identified as the dominant AHLs produced in some, but not all, strains of each *Pseudomonas* species tested.

As many root-colonizing fluorescent pseudomonads can efficiently control diseases caused by soil-borne pathogens, there is considerable interest in exploiting their potential as crop protectants (Rainey, 1999). Their biocontrol capabilities reside largely in their capacity to

produce a range of antifungal secondary metabolites, including hydrogen cyanide, phenazines, pyoluteorin and 2,4-diacetylphloroglucinol. *P. aureofaciens* 3-84, for example, produces three phenazine antibiotics which suppress take-all disease of wheat by inhibiting the causative agent, *Gaeumannomyces graminis* var. *tritici* (Wood *et al.*, 1997). Phenazine synthesis is controlled, in part, via the LuxR homologue PhzR and C₆-HSL such that mutation of the *luxI* homologue *phzI* leads to loss of phenazine production and hence biocontrol activity (Wood *et al.*, 1997). Similarly, 2,4-diacetylphloroglucinol production by *P. fluorescens* F113 inhibits growth of *Pythium ultimum* *in vitro* and protects sugar beet seedlings from damping-off disease caused by *P. ultimum* in soil microcosms (Fenton *et al.*, 1992; Shanahan *et al.*, 1992). Since 2,4-diacetylphloroglucinol is produced at high cell densities, it is possible that synthesis of this potent antifungal may be under quorum-sensing-dependent control (Delany *et al.*, 2000). However, although a gene cluster involved in phloroglucinol synthesis and regulation has been cloned and sequenced from *P. fluorescens* F113, no *luxI* or *luxR* homologues have been identified (Delany *et al.*, 2000).

To begin to explore the possibility that AHLs may be involved in regulating the biocontrol properties of *P. fluorescens* F113, we sought to (i) identify and chemically characterize any AHLs produced and (ii) clone and sequence any *P. fluorescens* genes involved in AHL biosynthesis. In this paper, we (a) show that F113 makes at least three different AHLs including *N*-(3-hydroxy-7-*cis*-tetradecenoyl) homoserine-lactone 3OH,C_{14:1}-HSL, an AHL previously associated exclusively with growth inhibition in *Rhizobium leguminosarum*, and (b) identify a gene (*hdtS*) which does not belong to the LuxI or LuxM family of AHL synthases but which, nevertheless, when introduced into *Escherichia coli*, is capable of directing the synthesis of the *P. fluorescens* AHLs.

METHODS

Bacterial strains, plasmids and growth media. Bacterial strains and plasmids used in this study are listed in Table 1. *P. fluorescens* F113 or the 2,4 diacetylphloroglucinol-negative Tn5 derivative *P. fluorescens* F113G22 (Fenton *et al.*, 1992) was grown with shaking at 28 °C in sucrose asparagine (SA) medium (Scher & Baker, 1982) supplemented with 100 µM FeCl₃. *E. coli* JM109 was grown at 37 °C in LB broth or on LB agar containing, where required, ampicillin (100 µg ml⁻¹). For blue/white screening of recombinants, IPTG and X-Gal were added to a final concentration of 0.1 mM and 40 µg ml⁻¹, respectively.

AHL reporter plate assays. To detect AHL production on agar plates, in spent culture supernatants or in HPLC fractions, biosensors (Table 1) which respond to exogenous AHLs by either producing the purple pigment violacein (*Chromobacterium violaceum* CV026; McLean *et al.*, 1997) or by emitting light [*E. coli*(pSB401) or *E. coli*(pSB1075)]; Winson *et al.*, 1998] were employed. *C. violaceum* CV026 responds most sensitively to AHLs with 4–6 carbon acyl side chains irrespective of the substituent at the C-3 position of the acyl chain; *E. coli*(pSB401) detects compounds with 6–8 carbon

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference
Strains		
<i>P. fluorescens</i> F113	Wild-type; 2,4-diacetylphloroglucinol producer	Shanahan <i>et al.</i> (1992)
<i>P. fluorescens</i> F113G22	2,4-diacetylphloroglucinol-negative Tn5 mutant derived from F113	Fenton <i>et al.</i> (1992)
<i>C. violaceum</i> CV026	AHL biosensor; violacein-negative, double mini-Tn5 mutant from <i>C. violaceum</i> ATCC 31532	McClellan <i>et al.</i> (1997)
<i>E. coli</i> JM109	<i>recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi Δ(lac-proAB)</i> F'[traD36 proAB ⁺ lacI ^q lacZΔM15]	Sambrook <i>et al.</i> (1989)
Plasmids		
pSB401	AHL biosensor based on <i>luxR::luxI'</i> luxCDABE	Winson <i>et al.</i> (1998)
pSB1075	AHL biosensor based on <i>lasR::lasI'</i> luxCDABE	Winson <i>et al.</i> (1998)
pBluescript II SK(+)	<i>E. coli</i> cloning and expression vector	Stratagene
pBL59	2.4 kb <i>Bam</i> H1– <i>Pst</i> I fragment of <i>P. fluorescens</i> F113 DNA in pBluescript II SK(+)	This study
pBL70	1.5 kb <i>Sac</i> II– <i>Pst</i> I fragment derived from pBL59 containing the complete <i>hdtS</i> gene	This study
pBL71	1.8 kb <i>Bam</i> H1– <i>Apa</i> I fragment derived from pBL59 in which ORF4 and 145 bp of the 3' region of <i>hdtS</i> have been deleted	This study
pT7Blue-2	<i>E. coli</i> cloning and expression vector	Novagen
pBLYJ3.1	782 bp <i>hdtS</i> gene cloned in pT7Blue-2	This study

side chains and *E. coli*(pSB1075) is preferentially activated by AHLs with long (10–14 carbon) side chains. Cross-streaks of *P. fluorescens* or recombinant *E. coli* against the AHL biosensors were carried out on LB agar as described by Latifi *et al.* (1995) and Swift *et al.* (1997). For analysis of spent culture supernatants and HPLC fractions, 3 ml vols molten semi-solid LB agar (0.5%, w/v) were seeded with 100 µl of an overnight culture of the appropriate AHL biosensor and poured immediately over the surface of pre-warmed LB agar plates. Wells were punched in the agar and filled with either spent culture supernatant or HPLC fractions. Synthetic AHL standards were used as positive controls and sterile LB broth as a negative control. After overnight incubation, plates were examined for zones of purple pigmentation (*C. violaceum* CV026) or for light output [*E. coli*(pSB401) or *E. coli*(pSB1075)] using a Berthold LB980 photon video camera.

Rhizobium growth inhibition assay. Bioassay plates were prepared by overlaying TY agar plates with 3 ml TY soft agar (0.65%, w/v, agar) containing 100 µl of a stationary-phase culture of *R. leguminosarum* A34 (Rodelas *et al.*, 1999). HPLC fractions or synthetic AHLs were added to wells punched into the agar. Synthetic 3OH,C_{14:1}-HSL (1 µg ml⁻¹) was used as a positive control. Plates were incubated at 28 °C for 48 h and examined for zones of growth-inhibitory activity around the wells.

Purification and chemical characterization of AHLs produced by *P. fluorescens* F113. Cell-free stationary-phase culture supernatants were extracted with dichloromethane (DCM) using 70:30 supernatant/DCM as described previously (McClellan *et al.*, 1997; Cámara *et al.*, 1998). DCM was removed by rotary evaporation and the residue reconstituted in acetonitrile for fractionation by HPLC using a C₈ reverse-phase preparative HPLC column (Kromasil KR100-5C8; 250 mm × 8 mm). Fractions were eluted with a linear gradient of acetonitrile in water (20–95%, v/v) over a 30 min period at a flow rate of 2 ml min⁻¹ and monitored at 210 nm. Six fractions (F1–F6) were collected, each covering a 5 min

interval, and assayed for activity using the AHL assays described above. Active fractions were pooled and, for short-chain AHLs, rechromatographed using 35% acetonitrile in water, then analysed by TLC on reverse-phase RP-18 F_{254s} plates (BDH) with a solvent system of methanol/water (60:40, v/v), essentially as described previously using *C. violaceum* CV026 as the indicator organism (McClellan *et al.*, 1997; Shaw *et al.*, 1997). For long-chain AHL molecules, active fractions were rechromatographed using 60% acetonitrile in water and then analysed by TLC on normal-phase silica gel 60 F₂₅₄ plates (BDH) with a solvent system of acetone/hexane (55:45, v/v). These plates were overlaid with *E. coli*(pSB1075) as the AHL indicator organism. After overnight incubation of TLC plates at 30 °C, AHLs were located as purple spots on a white background (for *C. violaceum* CV026) or as bright spots on a dark background (for the *E. coli* lux-based AHL biosensors) when viewed with a Berthold LB980 photon video camera.

Active samples were also analysed on an analytical HPLC column attached to a photodiode array (PDA) system (Waters 996 PDA system operating with a Millennium 2010 Chromatography Manager), and both retention time and PDA spectral profiles were compared with those of synthetic AHL standards. Following preparative HPLC, the final active sub-fractions were analysed by HPLC-MS (Micromass Instruments). This technique couples the resolving power of C₈ reverse-phase HPLC directly with MS such that the mass of the molecular ion [M + H] and its major component fragments can be determined for a compound with a given retention time. Samples eluting from the HPLC column were ionized by positive ion electrospray (ES)-MS and the spectra obtained were compared with those of the synthetic AHL standard subjected to the same HPLC-MS conditions.

AHL synthesis. N-Hexanoylhomoserine-L-lactone (C₆-HSL), N-(3-oxohexanoyl)-L-homoserine lactone (3O,C₆-HSL), N-octanoylhomoserine-L-lactone (C₈-HSL), N-decanoyl-L-homoserine lactone (C₁₀-HSL), N-(3-oxotetradecanoyl)-L-homoserine lactone (3O,C₁₄-HSL) and N-(3-hydroxy-7-cis-tetradecenoyl)-L-homoserine lactone (3OH,C_{14:1}-HSL), were

synthesized essentially as previously described by Chhabra *et al.* (1993). Each synthetic AHL was purified to homogeneity by preparative HPLC and its structure confirmed by MS and proton NMR spectroscopy.

DNA manipulation and sequencing. DNA was manipulated by standard methods (Sambrook *et al.*, 1989). Restriction enzymes (Promega) were used according to the manufacturer's instructions. Agarose gel electrophoresis and Southern blot transfer were performed essentially as described by Sambrook *et al.* (1989). DNA probes were labelled with digoxigenin and detected using the DIG Luminescent Detection kit supplied by Boehringer Mannheim. Oligonucleotide synthesis and DNA sequencing were performed at the Biopolymer Synthesis and Analysis Unit, University of Nottingham, Queen's Medical Centre, Nottingham. Automated non-radioactive sequencing reactions were carried out using the BigDye terminator cycle sequencing kit in conjunction with a 373A automated sequencer (Perkin Elmer Applied Biosystems).

Screening of a *P. fluorescens* genomic library for AHL synthases. The method described by Swift *et al.* (1993) and by Throup *et al.* (1995) for cloning *luxI* homologues by complementation of AHL biosensor strains was used. A genomic *P. fluorescens* library of random *Bam*HI/*Pst*I fragments was constructed in pBluescript II SK(+) (Stratagene) and introduced by electroporation into *E. coli* JM109(pSB401). The resulting clones were examined for light output using the Berthold LB980 photon video camera.

Identification of the *hdtS* gene product. The *hdtS* gene was amplified with *Taq* DNA polymerase (Promega) using pBL59 as a template with the primers 5'-GATGTCGATATTGC-AGGCCATC and 5'-CTCAAACAGCGAGCTGGTCCG. The amplified DNA fragment (782 bp) was cloned into the blunt-ended vector pT7Blue-2 as described by the supplier (Novagen). The recombinant pT7Blue-2 plasmid containing *hdtS* was termed pBLYJ3.1. *E. coli* JM109 was transformed by electroporation and the transformants were grown and screened on X-Gal/IPTG indicator plates containing ampicillin. To identify the *hdtS* gene product, the STP3-Biotin non-radioactive transcription/translation kit (Novagen) was used. Prior to performing an *in vitro* transcription/translation assay, a DNA template incorporating the T7 promoter and the *hdtS* insert with primers R-20mer and U-19mer (Novagen) was generated from pBLYJ3.1 by PCR. The *in-vitro*-generated biotinylated translation products were analysed by SDS-PAGE and Western blotting. Western blots were developed with a Streptavidin AP LumiBlot kit (Novagen).

DNA and protein sequence analysis. The GenBank database (release 116) was searched using the BLAST suite of programs (Altschul *et al.*, 1997). Sequences were extracted from GenBank using the ACNUC retrieval software (Gouy *et al.*, 1985). Sequences were aligned and compared using CLUSTAL W (Thompson *et al.*, 1994).

RESULTS

Chemical characterization of AHLs produced by *P. fluorescens* F113: C₆-HSL and C₁₀-HSL

When *P. fluorescens* F113 was cross-streaked on agar plates against either *C. violaceum* CV026 or the *lux*-based *E. coli* AHL biosensors, neither stimulation of violacein production nor bioluminescence was observed. Similarly, no activity was observed when spent culture

Fig. 1. Structures of (a) *N*-hexanoyl-L-homoserine lactone (C₆-HSL), (b) *N*-decanoyl-L-homoserine lactone (C₁₀-HSL), (c) *N*-(3-oxotetradecanoyl)-L-homoserine lactone (3O,C₁₄-HSL), and (d) *N*-(3-hydroxytetradecenoyl)-L-homoserine lactone (3OH,C_{14:1}-HSL).

supernatants were added to wells cut into agar and overlaid with an AHL biosensor (data not shown). Since this may have been due to the production of secondary metabolites such as 2,4-diacetylphloroglucinol, we also examined the response of the AHL biosensors to *P. fluorescens* F113G22, which carries a Tn5 insertion in genes determining 2,4-diacetylphloroglucinol biosynthesis (Fenton *et al.*, 1992). Again, no stimulation of any of the AHL biosensors was observed. Since this may have been due to other secondary metabolites which are toxic for the biosensors, we sought to separate any AHLs produced by DCM extraction of cell-free culture supernatants followed by HPLC.

The crude F113G22 DCM extract was separated into six fractions (F1–F6) by preparative reverse-phase HPLC using a linear gradient of acetonitrile in water as described in Methods and by Cámara *et al.* (1998). When assayed by TLC overlaid with *C. violaceum* CV026, fraction F3 stimulated violacein production and contained a compound with an *R_F* value similar to that of the synthetic C₆-HSL standard. Further sub-fractionation of F3 using an isocratic mobile phase of 35% acetonitrile in water yielded a compound which eluted with the same retention time (17 min) and PDA spectrum as synthetic C₆-HSL [Fig. 1(a) and data not shown]. To unequivocally confirm the identity of this AHL, F3 was subjected to HPLC-MS. The ES-MS spectrum obtained revealed the presence of a molecular ion [M+H] of *m/z* 200 and a profile of breakdown products including the [M+H] 102 fragment (characteristic of the HSL moiety), indistinguishable from the synthetic C₆-HSL standard (data not shown).

Although F5 did not stimulate *C. violaceum* CV026 to produce pigment, it was able to stimulate light emission from *E. coli*(pSB1075). This AHL biosensor preferentially responds to AHLs with acyl side chains of 10–14 carbons in length. Thus the results suggest that *P. fluorescens* F113 makes AHLs of short and long chain lengths. Subfractionation of F5 yielded a compound which eluted in 70% acetonitrile in water with a

Fig. 2. HPLC-mass spectra of the natural C_{10} -HSL isolated from *P. fluorescens* F113G22 supernatants (a) and synthetic C_{10} -HSL (b) recorded under positive ion mode atmospheric pressure ionization using (above) low cone voltage (~ 15 kV) showing m/z at 256 for $[M+H]^+$ and (below) high cone voltage (~ 25 kV) showing the expected fragmentation products, for example m/z 155 (C_{10} acyl side chain) and 102 (HSL-moiety).

retention time of 8.5 min and possessed a PDA spectrum identical to that of C_{10} -HSL. The HPLC-mass spectrum of the compound present in F5 revealed a molecular ion $[M+H]^+$ of m/z 256 (Fig. 2a) and is identical to that of synthetic C_{10} -HSL (Figs 2b, 1b).

P. fluorescens F113 produces $3OH, C_{14:1}$ -HSL, the small bacteriocin of *R. leguminosarum*

HPLC subfractionation of fraction F5 using different acetonitrile gradients also yielded a peak of activity with a different retention time and PDA spectrum from that of C_{10} -HSL. Using a mobile phase of 60% acetonitrile in water, this compound eluted with a retention time of approximately 13 min. The HPLC-mass spectrum of this compound revealed a molecular ion $[M+H]^+$ of m/z 326 and the characteristic fragmentation products of m/z 308, 225 and 102 (Fig. 3). This pattern of fragmentation is consistent with the presence of a homo-

serine lactone ring (m/z 102) and a C_{14} acyl chain (m/z 225), suggesting that this compound could be either *N*-(3-oxotetradecanoyl)-L-homoserine lactone ($3O, C_{14}$ -HSL; Fig. 1c) or *N*-(3-hydroxytetradecenoyl)-L-homoserine lactone ($3OH, C_{14:1}$ -HSL; Fig. 1d). The $[M+H]^+$ 308 fragment, however, indicates that it is likely to be the latter compound since hydroxylated AHLs characteristically lose water during MS analysis (Shaw *et al.*, 1997). These data therefore suggested that this *P. fluorescens* AHL is related to *N*-(3-hydroxy-7-*cis*-tetradecenoyl)homoserine lactone ($3OH, C_{14:1}$ -HSL), the small bacteriocin of *R. leguminosarum* (Gray *et al.*, 1996, Schripsema *et al.*, 1996). To determine whether this *P. fluorescens* AHL was indeed the *Rhizobium* small bacteriocin, we synthesized both $3OH, C_{14:1}$ -HSL and $3O, C_{14}$ -HSL. Both the natural and synthetic $3OH, C_{14:1}$ -HSL compounds, but not $3O, C_{14}$ -HSL (or C_{10} -HSL or C_6 -HSL), inhibited the growth of the small-sensitive *R. leguminosarum* strain A34, which harbours the *sym*

Fig. 3. HPLC-mass spectra of the natural 3OH,C_{14:1}-HSL isolated from *P. fluorescens* F113G22 supernatants (a) and synthetic 3OH,C_{14:1}-HSL (b) recorded under positive ion mode atmospheric pressure ionization using (above) low cone voltage (~15 kV) showing *m/z* at 325.8 for [M+H] and (below) high cone voltage (~25 kV) showing the expected fragmentation products, for example *m/z* 308 [M-H₂O], 225 [14-C acyl side chain], 207 [side chain-H₂O] and 102 [HSL moiety].

plasmid pRL1JI (Rodelas *et al.*, 1999). In addition, the chromatographic properties and both the PDA spectrum (data not shown) and HPLC-mass spectrum (Fig. 3b) of the molecule isolated from *P. fluorescens* were indistinguishable from that of the synthetic 3OH,C_{14:1}-HSL. Based on these properties, the position and stereochemistry of the double bond is tentatively assigned to be 7Z, i.e. the *P. fluorescens* molecule has a 7-8 *cis* double bond, thus validating that the *P. fluorescens* molecule is N-(3-hydroxy-7-*cis*-tetradecenoyl)homoserine lactone (Fig. 1d).

Identification of a novel genetic locus responsible for AHL production in *P. fluorescens*

To identify the gene(s) responsible for AHL production in *P. fluorescens* F113, we used a previously described strategy for the isolation of genes, the products of which

belong to the LuxI family of AHL synthases. (Swift *et al.*, 1993; Throup *et al.*, 1995). The basis of selection is the *in trans* complementation of an *E. coli* AHL biosensor deleted for *luxI* such as pSB401, which is based on the *Vibrio (Photobacterium) fischeri luxR::luxI'luxCDABE* (Throup *et al.*, 1995; Winson *et al.*, 1998). A *Bam*HI/*Pst*I digest of *P. fluorescens* F113 chromosomal DNA was cloned into similarly digested pBluescript II SK(+). The ligation was used to transform *E. coli* JM109(pSB401). A number of bioluminescent colonies were obtained and the brightest was selected for further analysis. This clone, designated pBL59, contained a 2.4 kb DNA insert. DCM extracts of spent culture supernatants and HPLC fractions F3 and F5 of *E. coli*(pBL59) were subjected to both reverse-phase (RP₁₈) and normal-phase (silica) TLC as described in Methods and overlaid with either *C. violaceum* or *E. coli*(pSB1075), respectively. The results obtained

Fig. 4. Thin layer chromatograms of the AHLs purified from spent culture supernatants by HPLC as described in Methods from *E. coli* JM109(pBL59) and *E. coli* JM109(pBLYJ3.1). In (a), the silica TLC plate was overlaid after chromatography with the long-chain AHL biosensor *E. coli*(pSB1075) and in (b) a reverse-phase RP18 plate was overlaid after chromatography with the short-chain AHL biosensor *C. violaceum* CV026. In (a) bioluminescence was detected using a Berthold photon video imaging camera and in (b) a purple spot is indicative of the AHL-mediated activation of violacein production. (a) Lanes: 1, C_{10} -HSL; 2, $3OH,C_{14:1}$ -HSL; 3, crude DCM extract from *E. coli* JM109(pBL59); 4, HPLC fraction F5 from JM109(pBL59); 5, crude DCM extract from *E. coli* JM109(pBLYJ3.1); 6, HPLC fraction F5 from JM109(pBLYJ3.1). (b) Lanes: 1, $3O,C_6$ -HSL plus C_8 -HSL; 2, C_6 -HSL; 3, HPLC fraction F3 from JM109(pBL59); 4, HPLC fraction F3 from *E. coli* JM109(pBLYJ3.1). The direction of solvent migration is marked with an arrow.

showed that pBL59 directs the synthesis of C_6 -HSL and a compound(s) migrating with a similar R_F value to that of synthetic C_{10} -HSL and $3OH,C_{14:1}$ -HSL standards (Fig. 4). These data confirm that pBL59 contains a gene capable of directing AHL synthesis.

DNA sequence analysis of the pBL59 2.4 kb insert revealed two complete and two partial ORFs (Fig. 5a). ORF1, which is incomplete at the 5' end, is a homologue of *glyS*, which encodes glycine tRNA synthetase β -subunit and is 43% identical to the *E. coli* K-12 GlyS protein (GenBank accession no. AE000433). Database sequence comparisons of ORF4, which is also 5'-incomplete, revealed that the gene product exhibits some similarity to the histidine kinase sensor proteins of two-component response regulator systems. However, since both ORF1 and ORF4 are incomplete, the AHL synthase was presumed to be either ORF2 or ORF3. Surprisingly, database sequence analysis indicated that neither ORF was a member of the established LuxI

Fig. 5. (a) Map of the 2.4 kb *P. fluorescens* F113 DNA insert in pBL59. The complete (ORF2 and ORF3) and partial (ORF1 and ORF4) ORFs corresponding to the *glyS* homologue (ORF1), *hisB* homologue (ORF2), *hdtS* (ORF3) and the sensor kinase homologue (ORF4) are shown as open arrows. The sizes of the two complete genes are shown above the arrows. The dotted arrows indicate the positions of the regions deleted to construct pBL70 (deletion 1, which removed partial ORF1 and 277 bp of the 5' end of ORF2) and pBL71 (deletion 2, which removed 145 bp of the 3' end of ORF3 and partial ORF4). (b) Light emission from *E. coli*(pSB401) transformed with pBL59 (1), pBL70 (2), pBluescript II SK(+) (3) or pBL71 (4) and patch-streaked onto L agar plates. Bioluminescence was recorded after overnight growth using a Berthold LB980 photon video camera. Only *E. coli*(pSB401)(pBL59) and *E. coli*(pSB401)(pBL70) are strongly bioluminescent.

or LuxM families of AHL synthases. The product of ORF2 is related to the imidazoleglycerol-phosphate dehydratase/histidinol-phosphatases, as encoded by *hisB* genes found in *Synechocystis* (GenBank accession no. D64004), *Haemophilus influenzae* (U32744) and *E. coli* (*hisB*, AE000293 and *yaeD*, AE000129). Database analysis of ORF3 indicated that the product of this gene exhibits similarity to numerous entries indicated to encode lysophosphatidic acid acyltransferases (or 1-acyl-sn-glycerol-3-phosphate acyltransferases). ORF3 is 32% identical to the *Neisseria meningitidis* *nlaB* gene product and 34% identical to the translated *Rickettsia prowazekii* gene *plsC*. The *plsC* name has been allocated to the *R. prowazekii* gene via the genome project on the basis of its similarity to the *E. coli* *plsC*, but since the similarity to *nlaB* is higher, the *R. prowazekii* gene is probably not the orthologue of *plsC*. Thus, the *P. fluorescens* ORF3 shares only about 20% identity with the *E. coli* *plsC* gene product.

To determine which of these ORFs was involved in

Fig. 6. Western blot analysis of the *hdtS* gene product by *in vitro* transcription/translation as described in Methods. Lanes: 1, positive control template, β -galactosidase (119 kDa); 2 and 3, negative control (no DNA template); 4 and 5, pBLYJ3.1 as the DNA template. The ~ 33 kDa *hdtS* product is clearly visible in lanes 4 and 5. Lane 6 shows the molecular mass markers.

directing AHL biosynthesis, two deletion mutations were constructed in pBL59 (Fig. 5a). In the first, 277 bp of the 5' end of ORF2 was deleted together with partial ORF1. This plasmid (termed pBL70) when transformed into *E. coli*(pSB401) retained its ability to induce light emission (Fig. 5b). In contrast, a deletion of the 3' end of the 2.4 kb insert which removed 145 bp of ORF3 together with partial ORF4 (plasmid pBL71; Fig. 5a) disrupted light output, i.e. no bioluminescence was observed when pBL71 was transformed into *E. coli*(pSB401) (Fig. 5b). These data implied that the AHL synthase activity resided within ORF3, which we termed *hdtS* (see Discussion).

HdtS directs the synthesis of C_6 -HSL, C_{10} -HSL and 3OH, $C_{14:1}$ -HSL in *E. coli*

To identify the *hdtS* gene product and to determine whether HdtS could direct the synthesis of the *P. fluorescens* AHLs, the gene was amplified from pBL59 by PCR and cloned into the expression plasmid pT7Blue-2, to give pBLYJ3.1. Using a non-radioactive transcription/translation assay, the HdtS protein was identified after SDS-PAGE and Western blotting as a protein of ~ 33 kDa (Fig. 6), which is in good agreement with the predicted size of the encoded protein.

To determine which AHL(s) was produced by *E. coli* JM109(pBLYJ3.1), stationary-phase cell-free culture supernatants were extracted with DCM, subjected to either reverse-phase or normal-phase TLC, and then overlaid with *C. violaceum* CV026 or *E. coli*(pSB1075), respectively, as described in Methods. Fig. 4 shows that HdtS can direct the synthesis of both short-chain and long-chain AHLs. A compound migrating with the same R_F value as C_6 -HSL could be clearly observed on the

CV026 TLC overlay (Fig. 4b). On normal-phase silica TLC plates, at least one active spot capable of activating pSB1075 is clearly apparent (Fig. 4a). We therefore subjected *E. coli* JM109(pBLYJ3.1) supernatants to DCM extraction, HPLC, HPLC-MS and the *Rhizobium small* bacteriocin assay. Three compounds with molecular ions $[M+H]$ of m/z 200, 256 and 326, corresponding, respectively, to C_6 -HSL, C_{10} -HSL and 3OH, $C_{14:1}$ -HSL, were clearly identified, indicating that HdtS directed synthesis of each of the three *P. fluorescens* AHLs. None of these compounds was present in spent supernatants from *E. coli* JM109(pT7Blue-2). Furthermore, the compound identified as 3OH, $C_{14:1}$ -HSL inhibited the growth of the *small*-bacteriocin-sensitive strain of *R. leguminosarum* A34, but did not inhibit the growth of a derivative of A34 known to be resistant to *small* bacteriocin, as would be expected for growth inhibition by 3OH, $C_{14:1}$ -HSL (data not shown).

DISCUSSION

In this study, we have shown that *P. fluorescens* F113 produces at least three different AHLs. Although two of these compounds, namely C_6 -HSL and 3OH, $C_{14:1}$ -HSL, are known to be produced by other Gram-negative bacteria, C_{10} -HSL has not previously been found as a naturally occurring AHL. AHLs with a C_{10} acyl side chain, namely *N*-(3-hydroxydecanoyl)homoserine lactone (3OH, C_{10} -HSL) and *N*-(3-oxodecanoyl)homoserine lactone (3O, C_{10} -HSL), have been unequivocally chemically characterized in *P. fluorescens* 2-79 (Shaw *et al.*, 1997) and in the fish pathogen *Vibrio* (now *Listonella*) *anguillarum* (Milton *et al.*, 1997), respectively. A compound migrating on TLC plates with a similar R_F value to that of 3O, C_{10} -HSL has also been reported to occur in the *Agrobacterium tumefaciens* strain Bo542 (Cha *et al.*, 1998). In contrast, C_6 -HSL is produced by a number of different Gram-negative genera, including *Aeromonas* (Swift *et al.*, 1997), *Chromobacterium* (McClellan *et al.*, 1997), *Pseudomonas* (Winston *et al.*, 1995), *Rhizobium* (Rodenas *et al.*, 1999), *Serratia* (Eberl *et al.*, 1996), *Vibrio* (Kuo *et al.*, 1994) and *Yersinia* (Throup *et al.*, 1995; Atkinson *et al.*, 1999). Interestingly, none of the three AHLs made by *P. fluorescens* F113 and described in the present paper have yet been identified in other *P. fluorescens* strains. Shaw *et al.* (1997) reported that *P. fluorescens* 2-79 produced up to five putative AHL signal molecules, three of which were identified as *N*-(3-hydroxyhexanoyl)homoserine lactone (3OH, C_6 -HSL), *N*-(3-hydroxyoctanoyl)homoserine lactone (3OH, C_8 -HSL) and 3OH, C_{10} -HSL respectively. However, no AHLs were detected in the culture supernatants of either *P. fluorescens* 1855.344 (Cha *et al.*, 1998) or *P. fluorescens* NCIMB 10586 (Holden *et al.*, 1999). Given the differences in AHL profiles of the *P. fluorescens* strains examined to date, it will clearly be of interest to determine whether there is any correlation between AHL profile and strain habitat. In this context, the discovery that *P. fluorescens* F113 synthesizes 3OH, $C_{14:1}$ -HSL, a molecule which, prior to its chemical characterization, was known as the

Rhizobium small bacteriocin (Hirsch, 1979; Wijffelman *et al.*, 1983) is particularly interesting. *Small* is produced by most strains of *R. leguminosarum* and was considered to be a bacteriocin because it produces zones of growth inhibition when assayed using a sensitive strain of *R. leguminosarum* as a lawn in an agar plate (Hirsch, 1979; Wijffelman *et al.*, 1983).

AHL-mediated inhibition of bacterial growth is unusual and Gray *et al.* (1996) concluded that in *R. leguminosarum*, 3OH,C_{14:1}-HSL converted exponentially growing cells into stationary-phase cells, arresting further growth even though the cell density remained low. Subsequently, Thorne & Williams (1999) showed that *R. leguminosarum* cells which enter stationary phase at low cell density (e.g. due to carbon limitation) do not have the same prolonged survival characteristics as those that enter stationary phase at high cell density. However, addition of exogenous 3OH,C_{14:1}-HSL induced long-term survival characteristics in cells of *R. leguminosarum* that entered stationary phase at a low cell density (Thorne & Williams, 1999). The addition of AHL-containing spent culture supernatants from the 2,4-diacetylphloroglucinol-negative mutant F113G22 to the wild-type F113 strain had no effect on growth or on 2,4-diacetylphloroglucinol production (data not shown). Whether 3OH,C_{14:1}-HSL produced by *P. fluorescens* is employed to control growth of *Rhizobium* (or other bacteria) within the soil or rhizosphere environment is not yet known. Interestingly, the sensitivity of *R. leguminosarum* to 3OH,C_{14:1}-HSL, but not its synthesis, is mediated by a genetic locus on Sym plasmids such as pRL1JI since strains cured of the plasmid are not susceptible to growth inhibition (Hirsch 1979; Wijffelman *et al.*, 1983; Gray *et al.*, 1996).

Using a functional complementation assay which has been successfully employed to clone members of the LuxI family of AHL synthases from many different Gram-negative bacteria (Atkinson *et al.*, 1999; Eberl *et al.*, 1996; Milton *et al.*, 1997; Swift *et al.*, 1993, 1997; Throup *et al.*, 1995), we identified a *P. fluorescens* F113 gene termed *hdtS* which, when introduced into *E. coli*, results in the synthesis of the same AHLs as those produced by *P. fluorescens*. HdtS is clearly neither a member of the LuxI family nor a member of the LuxM family of AHL synthases and was so called since it directs the synthesis of AHLs with acyl side chains of six (hexa-), ten (deca-) and fourteen (tetradeca-) carbons in length. Thus, in common with the LuxI protein family, HdtS is capable of directing the synthesis of more than one AHL. LuxI homologues, such as RhII from *P. aeruginosa* (Winson *et al.*, 1995) and Ahyl from *A. hydrophila* (Swift *et al.*, 1997), in both homologous and heterologous (*E. coli*) genetic backgrounds, produce both a major (N-butanoylhomoserine lactone; C₄-HSL) and a minor (C₆-HSL) AHL. Other homologues, such as YenI and YpsI from *Yersinia enterocolitica* (Throup *et al.*, 1995) and *Yersinia pseudotuberculosis* (Atkinson *et al.*, 1999), respectively, produce a 50:50 mixture of C₆-HSL and 3O,C₆-HSL, whilst RhII from *R. leguminosarum* makes four AHLs, two of which have been

identified as C₆-HSL and C₈-HSL (Rodelas *et al.*, 1999). Furthermore, 3OH,C_{14:1}-HSL in *R. leguminosarum* and the closely related N-(7-*cis*-tetradecenoyl)homoserine lactone (C_{14:1}-HSL) in *Rhodobacter sphaeroides* are also synthesized via LuxI homologues, in this case CinI (Lithgow *et al.*, 2000) and CerI (Puskas *et al.*, 1997), respectively.

Apart from the LuxI family of AHL synthases, of which there are now over 20 homologues in the sequence databases, a second type of AHL synthase has been identified in both *Vibrio harveyi* (Bassler *et al.*, 1993) and *V. fischeri* (Gilson *et al.*, 1995). These have been termed LuxLM and AinS, respectively, and are responsible for the synthesis of N-(3-hydroxybutanoyl)-homoserine lactone (3OH,C₄-HSL) and C₈-HSL, respectively. Interestingly, both AHL synthase families employ S-adenosylmethionine (SAM) as the source of the homoserine lactone moiety, whilst the acyl chain is supplied by either the appropriately charged acyl-acyl carrier protein (acyl-ACP) or acyl-coenzyme A (acyl-CoA) (Hanzelka & Greenberg, 1996; Jiang *et al.*, 1998; Moré *et al.*, 1996; Schaefer *et al.*, 1996; Val & Cronan, 1998). For RhII (Jiang *et al.*, 1998; Parsek *et al.*, 1999) and AinS (Hanzelka *et al.*, 1999), butanoyl-CoA and octanoyl-CoA, respectively, have also been shown to be capable of supplying the acyl side chain. For both AHL synthases, it is probable that SAM binds to the enzyme followed by the acyl-ACP (or acyl-CoA), such that an amide bond is formed between SAM and the acyl group, followed by cyclization of the acyl-SAM and then the release of the AHL and 5'-methylthioadenosine (Moré *et al.*, 1996; Hanzelka *et al.*, 1999; Parsek *et al.*, 1999).

In this study, we have described the identification and characterization of HdtS, a putative third class of AHL synthases. Database comparisons suggest that HdtS is most closely related to the lysophosphatidic acid (LPA) acyltransferase family, which includes NlaB from *N. meningitidis* (Shih *et al.*, 1999) and PlsC from *E. coli* (Rock *et al.*, 1996). However, neither of these Gram-negative bacteria have been shown to produce any AHLs (Swift *et al.*, 1999). In *E. coli*, the LPA acyltransferase, PlsC, catalyses the transfer of an acyl chain from either acyl-CoA or acyl-ACP onto LPA to produce phosphatidic acid, which, like LPA, is a critical phospholipid intermediate in cell membrane biosynthesis. In this respect, HdtS possesses two motifs, NHQS and PEGTR, which are highly conserved in prokaryotic and in eukaryotic LPA acyltransferases (West *et al.*, 1997). These may constitute an acyl-CoA/acyl-ACP binding site, although amino acid residues outside these motifs have been identified which alter the activity or specificity of these acyltransferases (Shih *et al.*, 1999). It is therefore possible that HdtS is an acyltransferase which transfers acyl chains onto a substrate such as SAM to generate AHLs. However, it is also possible that HdtS is involved in the synthesis of substrates required for AHL formation via another enzyme. Further *in vitro* work using the purified HdtS protein will therefore be required to define its enzymic function and substrate specificity.

In addition, the target *P. fluorescens* structural gene(s) whose expression is controlled via the AHLs identified in this paper, and which may also influence the biocontrol characteristics of *P. fluorescens* F113, await the construction of an *hdtS*-negative mutant.

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