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# Quorum-sensing cross talk: isolation and chemical characterization of cyclic dipeptides from *Pseudomonas aeruginosa* and other Gram-negative bacteria

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## Summary

**In cell-free *Pseudomonas aeruginosa* culture supernatants, we identified two compounds capable of activating an *N*-acylhomoserine lactone (AHL) biosensor. Mass spectrometry and NMR spectroscopy revealed that these compounds were not AHLs but the diketopiperazines (DKPs), cyclo(ΔAla-L-Val) and cyclo(L-Pro-L-Tyr) respectively. These compounds were also found in cell-free supernatants from *Proteus mirabilis*, *Citrobacter freundii* and *Enterobacter agglomerans* [cyclo(ΔAla-L-Val) only]. Although both DKPs were absent from *Pseudomonas fluorescens* and *Pseudomonas alcaligenes*, we isolated, from both pseudomonads, a third DKP, which was chemically characterized as cyclo(L-Phe-L-Pro). Dose–response curves using a LuxR-based AHL biosensor indicated that cyclo(ΔAla-L-Val), cyclo(L-Pro-L-Tyr) and cyclo(L-Phe-L-Pro) activate the biosensor in a concentration-dependent manner, albeit at much higher concentrations than the natural activator *N*-(3-oxohexanoyl)-L-homoserine lactone (3-oxo-C6-HSL). Competition studies showed that cyclo(ΔAla-L-Val), cyclo(L-Pro-L-Tyr) and cyclo(L-Phe-L-Pro) antagonize the 3-oxo-C6-HSL-mediated induction of bioluminescence, suggesting that these DKPs may compete for the same LuxR-binding site. Similarly, DKPs were found to be capable of activating or antagonizing other LuxR-based quorum-sensing systems, such as the *N*-butanoylhomoserine lactone-dependent swarming motility of *Serratia liquefaciens*. Although the physiological role of these DKPs has yet to be established, their activity suggests the existence of cross talk among bacterial signalling systems.**

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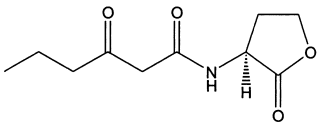
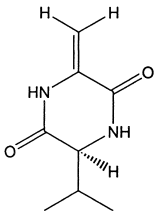
## Introduction

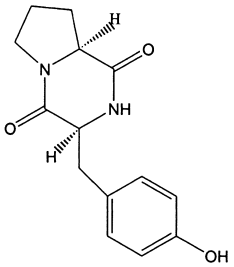
In recent years, it has become clear that bacterial cells can communicate with each other via small diffusible signal molecules, a process commonly termed 'quorum sensing'. By far the most intensively investigated family of intercellular signal molecules are the *N*-acylhomoserine lactones (AHLs), which differ in the length and degree of saturation of the acyl chain (4–14 carbons; presence or absence of a double bond) and substituent at the 3-position of their *N*-acyl side chains (no substituent, keto or hydroxy) (for reviews, see Salmond *et al*., 1995; Fuqua *et al*., 1996; Swift *et al*., 1996; Gray, 1997; Hardman *et al*., 1998). For many years, the AHLs were exclusively associated with the regulation of bioluminescence in a few marine vibrios, such as *Vibrio* (*Photobacterium*) *fischeri* and *Vibrio harveyi*. *N*-(3-oxohexanoyl)-L-homoserine lactone (3-oxo-C6-HSL; Fig. 1) and *N*-(3-hydroxybutanoyl)-L-homoserine lactone (3-hydroxy-C4-HSL) are, respectively, the primary *V. fischeri* and *V. harveyi* signal molecules. However, Bainton *et al*. (1992a; b) discovered that 3-oxo-C6-HSL regulates the biosynthesis of 1-carbapen-2-em-3-carboxylic acid, a β-lactam antibiotic produced by the terrestrial plant pathogenic bacterium *Erwinia carotovora*. They also provided evidence that a wide range of Gram-negative bacteria including *Pseudomonas aeruginosa*, *Serratia marcescens*, *Erwinia herbicola*, *Citrobacter freundii*, *Enterobacter agglomerans* and *Proteus mirabilis* produce 3-oxo-C6-HSL and/or structurally related AHLs. Following these initial studies, the list of AHL-producing Gram-negative bacteria and the number of AHL analogues identified



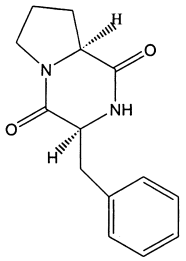
(i) *N*-(3-oxohexanoyl)-L-homoserine lactone



(ii) cyclo(ΔAla-L-Val)



(iii) cyclo(L-Pro-L-Tyr)



(iv) cyclo(L-Phe-L-Pro)

**Fig. 1.** Structures of (i) *N*-(3-oxohexanoyl)-L-homoserine lactone (3-oxo-C6-HSL); (ii) cyclo(ΔAla-L-Val); (iii) cyclo(L-Pro-L-Tyr); and (iv) cyclo(L-Phe-L-Pro).

has expanded rapidly. AHL-mediated cell–cell signalling is now known to play a role in regulating plasmid conjugal transfer (Piper *et al*., 1993; Fuqua and Winans, 1994), swarming (Eberl *et al*., 1996a), cessation of cell growth (Gray *et al*., 1996; Schripsema *et al*., 1996), stationary-phase survival (Thorne and Williams, 1999), capsular polysaccharide synthesis (Beck von Bodman and Farrand, 1995; Beck von Bodman *et al*., 1998), protein secretion (Chapon-Hervé *et al*., 1997), phenazine antibiotic biosynthesis (Wood and Pierson, 1996), and in the production of exoenzyme virulence determinants and cytotoxins in human and plant pathogens (Jones *et al*., 1993; Passador *et al*., 1993; 1996; Pearson *et al*., 1994; 1995; Latifi *et al*., 1995; 1996; Winson *et al*., 1995; Flavier *et al*., 1997a; Nasser *et al*., 1998; Reverchon *et al*., 1998; Lewenza *et al*., 1999).

AHL-dependent signalling enables a bacterium to control gene expression in response to bacterial cell population density. Accumulation of the AHL molecule above a threshold concentration, through the activity of a signal synthase protein (usually a member of the LuxI family; Moré *et al*., 1996; Swift *et al*., 1996), 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 (for reviews, see Salmond *et al*., 1995; Fuqua *et al*., 1996; Swift *et al*., 1996). The archetypal LuxR protein was first identified in *V. fischeri* and consists of at least two domains; an N-terminal domain, which contains the AHL-binding site, and a C-terminal domain, which contains a helix-turn-helix DNA-binding motif (Choi and Greenberg, 1992; Hanzelka and Greenberg, 1995).

The cell density-dependent onset of bioluminescence in *V. fischeri* therefore serves as a useful paradigm for quorum-sensing-dependent gene regulation (for reviews, see Meighen, 1994; Salmond *et al*., 1995; Fuqua *et al*., 1996). At high cell densities such as those achieved within the light organs of certain marine animals, *V. fischeri* is highly bioluminescent. When free living, at low cell densities, cultures of *V. fischeri* emit little light. As the cell density increases, the level of 3-oxo-C6-HSL accumulates until it reaches a critical threshold concentration, at which point transcription of the luminescence (*luxICDABE*) operon is triggered. Because *luxI*, the 'autoinducer synthase', is included with this operon, this leads to increased levels of LuxI and hence more 3-oxo-C6-HSL, thus setting up an 'amplification loop'. The DNA-binding site for LuxR (termed the '*lux*-box') has been shown by *in vivo* and *in vitro* experiments to be a 20 bp inverted repeat located between the *luxR* and *luxICDABE* operons and centred around −40 bp from the transcriptional start site (Devine *et al*., 1989; Stevens *et al*., 1994; Egland and Greenberg, 1999). Because 3-oxo-C6-HSL freely diffuses across the cell membranes (Kaplan and Greenberg, 1985), the induction of one cell leads directly to the induction of the surrounding cells, creating a positive feedback circuit that can generate a rapid response to a small initial stimulus. LuxR, LuxI and 3-oxo-C6-HSL therefore constitute a quorum-sensing circuit that enables *V. fischeri* to sense its own population density.

Although AHL-mediated quorum sensing has been the most intensively investigated bacterial intercellular signalling mechanism, it is clear that not all bacteria communicate using AHLs. *Xanthomonas campestris*, for example, uses low molecular mass diffusible factors unrelated to AHLs to regulate expression of extracellular enzymes and polysaccharide virulence determinants (Barber *et al*., 1997; Chun *et al*., 1997; Poplawsky *et al*., 1998). Uniquely, the Gram-negative plant pathogen *Ralstonia* (*Pseudomonas*) *solanacearum* has been shown to utilize a non-AHL signal, a 3-hydroxypalmitic acid methyl ester in conjunction with AHLs to modulate pathogenicity. (Clough *et al*., 1997; Flavier *et al*., 1997b). Both classes of molecules

act as signals in a hierarchical regulatory cascade that controls the production of exopolysaccharide and extracellular enzymes. In Gram-positive bacteria, many processes (e.g. induction of genetic competence, production of antimicrobial peptides and regulation of virulence) are known to be regulated in a cell density-dependent manner (for reviews, see Kleerebezem *et al*., 1997; Lazazzera and Grossman, 1998). Many of the diffusible signal molecules identified have been found to be post-translationally modified peptides that function as input signals for specific sensor components of two-component histidine protein kinase response regulatory circuits. In *Streptomyces*, diffusible regulatory factors related to AHLs, i.e. the γ-buytrolactones, have been described which control antibiotic production, sporulation and morphogenesis (Horinouchi and Beppu, 1992; 1994).

In *Pseudomonas aeruginosa*, two major AHLs, *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL) and *N*-butanoyl-L-homoserine lactone (C4-HSL), and two minor AHLs, 3-oxo-C6-HSL and *N*-hexanoyl-L-homoserine lactone (C6-HSL), have been fully chemically characterized (Bainton *et al*., 1992a; Pearson *et al*., 1994; 1995; Winson *et al*., 1995). In our laboratory, purification of these AHL signal molecules from cell-free supernatants has largely depended on the use of solvent extraction followed by reverse-phase preparative HPLC. Fractions eluting from the column are collected and tested for the presence of AHLs using recombinant *Escherichia coli* AHL biosensors. These biosensors, which emit light in response to the addition of exogenous AHLs, are based on the *luxR* and *lux* promoter regions from *V. fischeri* coupled to either the *luxAB* (the luciferase subunits) of *V. fischeri* or to the entire *lux* structural operon (comprising *luxCDABE*) of *Photorhabdus luminescens* (Bainton *et al*., 1992a; b; Swift *et al*., 1993; Throup *et al*., 1995; Winson *et al*., 1995, Winson *et al*., 1998). The latter construct termed pSB401, unlike the former, does not require the addition of exogenous aldehyde and functions efficiently at 37°C.

In the present paper, we describe the purification, structural identification and chemical synthesis of a family of diketopiperazines (DKPs) from cell-free supernatants of *Pseudomonas aeruginosa* and other Gram-negative bacteria. The biological significance of these cyclic dipeptides as putative signal molecules is discussed, with evidence presented to show that these compounds are capable of activating or antagonizing *lux*-based AHL biosensors and AHL-dependent swarming in *Serratia liquefaciens*.

## Results

### *Detection of compounds in* P. aeruginosa *PA01 culture supernatants capable of activating* E. coli *(pSB401)*

Ethyl acetate extracts of *P. aeruginosa* PAO1 cell-free culture supernatants were subjected to HPLC on a $C_8$ reverse-phase semipreparative column and the fractions collected and assayed for their ability to stimulate light emission from *E. coli* (pSB401). Using a mobile phase of 20% v/v methanol in water, three active fractions were identified with peak retention times of 11.89, 13.37 and 14.81 min respectively (data not shown). These compounds were absent from extracts of fresh uninoculated minimal (M9) medium. The first of these compounds is 3-oxo-C6-HSL, a compound previously identified in *P. aeruginosa* culture supernatants by Bainton *et al*. (1992a). Neither of the two remaining compounds (termed F13 and F14 respectively) eluted with retention times similar to those of C4-HSL, C6-HSL or 3-oxo-C12-HSL, AHLs known to be produced by *P. aeruginosa* (Pearson *et al*., 1994; 1995; Winson *et al*., 1995). Because F13 and F14 both activated the AHL biosensor, at this stage we expected that they represented novel AHLs.

### *Elucidation of the structure of F13 and F14*

F13 was initially analysed using high-resolution electron impact (EI) mass spectrometry (MS). Accurate mass measurement on the molecular ion peak revealed an observed mass of 168.00911, which corresponds to the molecular formula $C_8H_{12}N_2O_2$. EI-MS gave the following data: *m/z* (%) 168.0911 (15; $M^+$, $C_8H_{12}N_2O_2$ requires *m/z* 168.0899), 126 (100), 97 (72), 69 (15), 57 (44), 41 (76). High-resolution $^1$H-NMR spectrum of F13 was measured in [$^2$H]-chloroform at 400 MHz (chemical shifts in p.p.m. from tetramethylsilane) and gave the following data: $\delta_H$ 0.96 (3 H, d, *J* 6.84 Hz, $CH_3$), 1.06 (3 H, d, *J* 7.11 Hz, $CH_3$), 2.42 (1 H, 10 lines, $CH(CH_3)_2$), 4.04 (1 H, t, *J* 2.8 Hz, Val α-H), 4.84 (1 H, t, *J* 1.1 Hz, alkene H), 5.61 (1 H, d, *J* 1.0 Hz, alkene H), 6.10 (1 H, bs, NH), 7.88 (1 H, bs, NH). The high-field proton NMR showed two doublets ($J = 7.0$ Hz) at δ 0.96 and 1.06, each corresponding to three protons. These combined with one proton multiplet at δ 2.42 are indicative of the presence of a $(CH_3)_2CH$ unit in the molecule. The connectivities between these protons were further confirmed by $^1$H,$^1$H correlation spectroscopy (COSY). The latter also revealed that the resonances at δ 4.84 and 5.61 were also connected. Their chemical shift and smaller *J*-value of 1.0 Hz suggested that they were olefinic protons with geminal coupling. The presence of 2 × NH was evident from the two broad resonances at δ 6.10 and 7.88. The chemical shift for the signal at δ 4.04 is typical of α-H in amino acids. Based on these observations, F13 was tentatively assigned the structure cyclo(ΔAla-L-Val), in which ΔAla is dehydroalanine (Fig. 1). This cyclic dipeptide structure is consistent with the major fragments observed in the EI-MS. Final confirmation of the structure as cyclo(ΔAla-L-Val) was obtained by chemical synthesis.

The structure of F14 was elucidated using the same

approach. EI-MS gave an observed mass of 260.1110840, which corresponds to the molecular formula $C_{14}H_{16}N_2O_3$ [*m/z* (%) 260.1111 (16; $M^+$, $C_{14}H_{16}N_2O_3$ requires *m/z* 260.1161), 154 (100), 125 (10), 107 (94), 77 (15), 70 (75), 41 (25)]. High-field proton NMR gave the following data: $\delta_H$ 1.90–2.03 (2 H, m, Pro 4-$H_2$), 2.34 (1 H, m, Pro 3-H), 2.73 (1 H, m, Pro 3-H), 3. 56 (4 H, m, Pro 5-$H_2$ and Tyr $CH_2$), 4.09 (1 H, dt, Pro α-H), 4.22 (1 H, m, Tyr α-H), 5.39 (1 H, bs, OH), 5.64 (1 H, bs, NH), 6.80 (2 H, d, *J* 8.5 Hz, Tyr 3,5-$H_2$), 7.08 (2 H, d, *J* 8.5 Hz, Tyr 2,6-$H_2$). From these observations, it appeared that F14, like F13, is a diketopiperazine, but in this case consisting of L-Tyr and L-Pro (Fig. 1). Final confirmation of the structure of F14 was obtained by chemical synthesis.

### *Production of DKPs by other Gram-negative bacteria*

To determine whether cyclo(ΔAla-L-Val) and cyclo(L-Pro-L-Tyr) were produced by other bacteria, cell-free culture supernatants from *P. fluorescens*, *P. alcaligenes, E. agglomerans, Pr. mirabilis* and *C. freundii* grown in M9 medium were extracted with ethyl acetate or dichloromethane and the residue obtained was subjected to HPLC as described above. Compounds capable of activating *E. coli* (pSB401) and with the same retention time as cyclo(ΔAla-L-Val) were detected in *Pr. mirabilis*, *C. freundii* and *E. agglomerans* cultures. For each of these organisms, the identity of cyclo(ΔAla-L-Val) was confirmed by MS (data not shown). In addition, cyclo(L-Pro-L-Tyr) was present in extracts from both *P. mirabilis* and *C. freundii*. Although we failed to detect either cyclo(ΔAla-L-Val) or cyclo(L-Pro-L-Tyr) in *P. fluorescens* and *P. alcaligenes* supernatants, when crude supernatants were applied to TLC plates overlaid with *E. coli* (pSB401) a spot which migrated with a similar $R_F$ value to the *P. aeruginosa* AHL C4-HSL (Winson *et al.*, 1995) was identified ($R_F$ values 0.67 and 0.64 respectively; Fig. 2). When subjected to HPLC, this compound (termed F35) eluted in 35% v/v methanol with a retention time of 18.9 min or 6.9 min in 35% v/v acetonitrile. Because C4-HSL elutes with a retention time of 5.1 min in 35% v/v acetonitrile, this suggested that F35 is a different compound. We therefore purified sufficient F35 to facilitate full chemical characterization.

### *Elucidation of the structure of F35*

EI-MS revealed a molecular ion of *m/z* 244.1190, indicative of a molecular formula of $C_{14}H_{16}N_2O_2$. Apart from the aromatic region, the proton NMR was similar to the F14 spectrum, thus indicating the DKP nature of the product. The spectrum was measured in [$^2$H]-chloroform at 400 MHz (chemical shift in p.p.m. from tetramethylsilane) and gave the following data: [$\delta_H$ 1.89–2.18 (2 H, m, Pro 4-$H_2$), 2.35 (1 H, m, Pro 3-H), 2.78 (1 H, m, Pro 3-H), 3.62 (4 H, m, Pro 5-$H_2$ and Phe $CH_2$), 4.09 (1 H, dt, Pro 2H), 4.28 (1 H, m, Phe α-H), 5.57 (1 H, bs, NH), 7.00–7.39 (5 H, m, Ph)]. Electron impact mass spectrometry gave the following data: *m/z* (%) 244.1191 (24; $M^+$, $C_{14}H_{16}N_2O_2$ requires *m/z* 244.1212), 153 (32), 125 (100), 91 (57), 70 (62), 43 (10). These data suggested that F35 was cyclo(L-Phe-L-Pro) (Fig. 1). Confirmation that this is indeed the case was obtained by chemical synthesis of the predicted product.

### *Quantitative responses of* E. coli *(pSB401) to DKPs*

Having identified the molecules by their ability to activate the LuxR-based AHL biosensor *E. coli* JM109 (pSB401), we sought to quantify the potency of these molecules

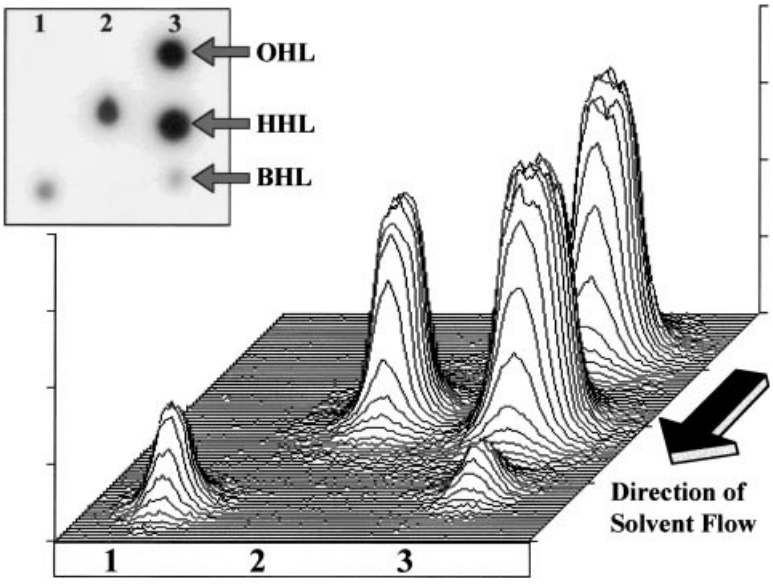



**Fig. 2.** TLC plate showing the presence of a novel compound in *P. alcaligenes* culture supernatant capable of activating *E. coli* (pSB401). AHL standards and *P. alcaligenes* culture supernatant extract were chromatographed on a $C_{18}$ reverse-phase TLC plate and overlaid with a lawn of *E. coli* (pSB401). Light output was recorded using a Berthold LB 980 imaging system. The figure illustrated is a 3D image showing the relative intensities of the light detected on the TLC plate (raw image top left). Lane 1, *P. alcaligenes* culture supernatant extract; lane 2, *N*-(3-oxooctanoyl)-L-homoserine lactone (3-oxo-C8-HSL) loaded as $1\times10^{-11}$ mol; lane 3, *N*-butanoyl-L-homoserine lactone (C4-HSL; bottom), *N*-hexanoyl-L-homoserine lactone (C6-HSL; middle) and *N*-octanoyl-L-homoserine lactone (C8-HSL; top) loaded as $6\times10^{-8}$ mol; $1\times10^{-10}$ mol and $2\times10^{-8}$ mol respectively.

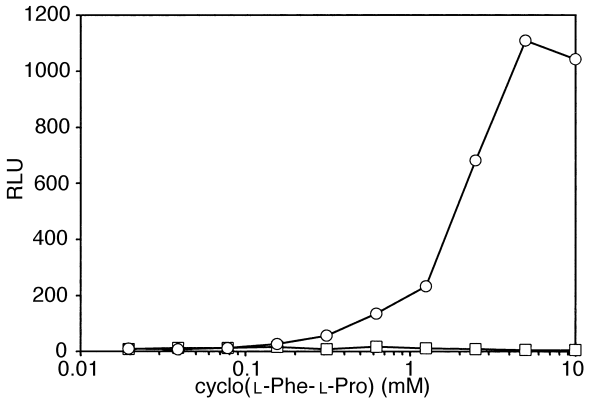



**Fig. 3.** Activation of the *E. coli* (pSB401) AHL biosensor by cyclo(L-Phe-L-Pro). Induction of bioluminescence in *E. coli* (pSB401): dose–response curve for synthetic cyclo(L-Phe-L-Pro). A range of concentrations (from 0 to 10 mM) of cyclo(L-Phe-L-Pro) (○) in acetonitrile/water was added to wells in a microtitre plate and made up to 100 μl with LB. Equivalent dilutions of acetonitrile in water as negative controls were also made up to 100 μl with LB (□). To each well, 100 μl of a 1:10 dilution of an overnight culture of *E. coli* (pSB401) in LB (containing tetracycline at 10 μg ml$^{-1}$) was added. The plate was then incubated at 30°C for 4 h. Bioluminescence was detected and quantified using an Anthos Lucy 1 luminometer.

compared with AHLs. Figure 3 shows the dose–response curve for the induction of light in the *E. coli* (pSB401) biosensor by cyclo(L-Phe-L-Pro). From the figure, it can be seen that the detection threshold for cyclo(L-Phe-L-Pro) is ≈0.3 mM, above which there is an increase in the amount of light emitted as the concentration of the dipeptide increases up to around 5 mM. Similar results were obtained for cyclo(ΔAla-L-Val) and cyclo(L-Pro-L-Tyr) (data not shown). The activity of these is several orders of magnitude lower than 3-oxo-C6-HSL (induction threshold 1 nM), the natural ligand for activation of *lux* via the LuxR protein (Eberhard *et al*., 1981; Engebrecht *et al*., 1983; Engebrecht and Silverman, 1984; Winson *et al*., 1998). To determine whether the diketopiperazines required LuxR for their activity, we examined their effect on an *E. coli* strain constitutively expressing *lux* genes (data not shown). The addition of the diketopiperazines had no effect on light output, suggesting that they do not induce light production by altering the physiology of the cells.

### *Competition between 3-oxo-C6-HSL and DKPs for LuxR*

Having observed the dose-dependent activation of JM109 biosensor, we speculated that the molecules were exerting their effect via an interaction with LuxR transcriptional activator. To test this further, we carried out a series of competition experiments to see whether cyclo(ΔAla-L-Val), cyclo(L-Pro-L-Tyr) and cyclo(L-Phe-L-Pro) could compete with 3-oxo-C6-HSL for LuxR and antagonize the light output from *E. coli* (pSB401). All three of the compounds elicited a dose-dependent reduction in light output from *E. coli* (pSB401), activated by its cognate AHL 3-oxo-C6-HSL (data not shown). The doses inhibiting 3-oxo-C6-HSL-mediated light output by 50%, i.e. the i.d.$_{50}$, for cyclo(-ΔAla-L-Val), cyclo(L-Pro-L-Tyr) and cyclo(L-Phe-L-Pro) were calculated to be 0.8 mM, 0.9 mM and 1 mM, respectively, at a 3-oxo-C6-HSL concentration of 1.56 nM. These data suggest that cyclo(ΔAla-L-Val), cyclo(L-Pro-L-Tyr) and cyclo(L-Phe-L-Pro) are weak competitive inhibitors of the 3-oxo-C6-HSL-mediated activation of LuxR.

### *Modulation of quorum-sensing-dependent phenotypes by DKPs*

The DKPs were identified by their ability to activate a LuxR-based AHL biosensor. To determine whether all DKPs were active in the biosensor based on their structural characteristics, four additional DKPs [cyclo(L-Ala-L-Pro), cyclo(L-Leu-L-Pro), cyclo(L-Met-L-Pro) and cyclo(L-Pro-L-Val)] were synthesized and assayed for their activity in the *E. coli* (pSB401) biosensor. Each of these DKPs has previously been identified in bacterial culture supernatants (Jayatilake *et al*., 1996; Stierle *et al*., 1988a). Cyclo(L-Ala-L-Pro), cyclo(L-Leu-L-Pro) and cyclo(L-Pro-L-Val) were prepared from the cyclization of the corresponding linear dipeptides in hot phenol (Kopple and Ghazarian, 1968). Cyclo(L-Met-L-Pro) was prepared by an *N,N′*-dicyclohexylcarbodiimide-mediated coupling of *N*-(tert-butyloxycarbonyl)-L-proline with L-methionine methyl ester followed by treatment with formic acid and a mixture of toluene and butan-2-ol (Jayatilake *et al*., 1996).

With the exception of cyclo(L-Met-L-Pro), the additional DKPs failed to induce light in the *E. coli* (pSB401) biosensor. When these DKPs were used in the *E. coli* (pSB401) inhibition assay, each compound antagonized bioluminescence to varying degrees. The i.d.$_{50}$ values calculated for cyclo(L-Ala-L-Pro), cyclo(L-Leu-L-Pro), cyclo(L-Met-L-Pro) and cyclo(L-Pro-L-Val) were 2 mM, 0.7 mM, 3 mM and 0.4 mM respectively. These data clearly show that DKP antagonist activity varies with structure, cyclo(L-Pro-L-Val) being the most potent compound (Fig. 4A) inhibiting the 3-oxo-C6-HSL-induced bioluminescence from *E. coli* (pSB401) to a greater extent than cyclo(ΔAla-L-Val), cyclo(L-Pro-L-Tyr) and cyclo(L-Phe-L-Pro) (data not shown). Interestingly, although the inhibition data suggests that this compound is the most effective in antagonizing the action of 3-oxo-C6-HSL, it does not induce luminescence. Conversely, cyclo(L-Met-L-Pro) is not as effective in antagonizing the effects of 3-oxo-C6-HSL, but is a more active agonist. Of the DKPs tested, this is the most potent activator of the *E. coli* (pSB401) biosensor. This is clearly illustrated in Fig. 4B in which, at low concentrations of 3-oxo-C6-HSL, bioluminescence above background is induced by concentrations of the DKP above 0.25 mM.

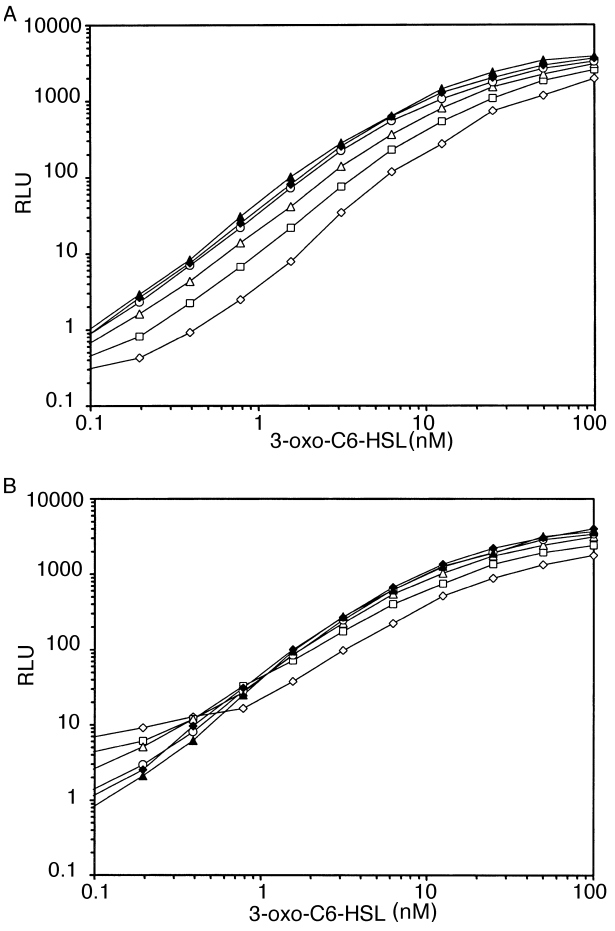



**Fig. 4.** Cyclo(L-Pro-L-Val) and cyclo(L-Met-L-Pro) antagonize 3-oxo-C6-HSL induction of *E. coli* (pSB401). Induction of bioluminescence in *E. coli* (pSB401): competition between 3-oxo-C6-HSL and (A) cyclo(L-Pro-L-Val) and (B) cyclo(L-Met-L-Pro). The assays were set up and carried out as follows. A range of concentrations (from 0 to 2 mM) of either cyclo(L-Pro-L-Val) or cyclo(L-Met-L-Pro) in acetonitrile/water were added to wells in a microtitre plate. The plate was then left to air dry for 20 min. For each concentration of DKP, a range of concentrations of 3-oxo-C6-HSL (0–100 nM) was added and the contents of each well made up to 100 μl with LB. The concentrations of cyclo(L-Phe-L-Pro) used were 2 mM (◇), 1 mM (□), 0.5 mM (△), 0.25 mM (○), 0.125 mM (◆) and 0 mM (▲). To each well, 100 μl of a 1:10 dilution of an overnight culture of *E. coli* (pSB401) in LB (containing tetracycline at 10 μg ml$^{-1}$) was added. The plate was then incubated at 30°C for 4 h. Bioluminescence was detected and quantified using an Anthos Lucy 1 luminometer.

In addition, we undertook preliminary experiments to determine whether DKPs activated AHL biosensors based on the *P. aeruginosa* LuxR homologues LasR and RhlR (Gambello and Iglewski, 1991; Latifi *et al*., 1995). The LasR-based biosensor [*E. coli* (pSB1075); Winson *et al*., 1998] but not the RhlR-based biosensor (pSB406; Winson *et al*., 1995; 1998) responded to cyclo(L-Met-L-Pro) and cyclo(ΔAla-L-Val) at concentrations above 1 mM (data not shown).

To determine whether the DKPs play a biologically significant role in modulating quorum sensing, they were screened against a range of AHL-dependent assays. These included swarming by *Serratia liquefaciens*, violacein pigment production by *Chromobacterium violaceum* and antibiotic and protease production by *E. carotovora* (Bainton *et al*., 1992b; Jones *et al*., 1993; Eberl *et al*., 1996a; McClean *et al*., 1997). The molecules were also tested for their ability to activate an *Agrobacterium tumefaciens* AHL biosensor (Shaw *et al*., 1997).

DKPs were tested at a range of concentrations (15 nM to 15 μM) for their ability to stimulate swarming motility in the *S. liquefaciens swrI* mutant strain MG44 by incorporating the compounds into the swarming medium and monitoring the colony expansion. On soft agar plates, *S. liquefaciens* MG44 is unable to form a swarming colony without the exogenous supply of *N*-butanoylhomoserine lactone (C4-HSL) or a closely related AHL (Eberl *et al*., 1996a). None of the DKPs stimulated swarming motility at any of the concentrations tested (data not shown). The DKPs were then examined for their ability to inhibit swarming motility in the wild-type *S. liquefaciens* strain MG1. A range of concentrations (15 nM to 15 μM) of each DKP were incorporated into the swarming medium and colony expansion was monitored (Table 1). At 15 μM, cyclo(L-Pro-L-Tyr) and cyclo(ΔAla-L-Val) both reduced colony expansion by 27% (Fig. 5A) and 21% of the control (data not shown), respectively, after 19 h incubation. Furthermore, at 15 μM cyclo(L-Pro-L-Tyr) inhibited colony swarming when *S. liquefaciens* MG44 was incubated with exogenously supplied C4-HSL (150 nM) (Fig. 5B).

The ability of DKPs to restore carbapenem antibiotic and protease production in an *E. carotovora carI* mutant (Swift *et al*., 1993) was evaluated; no exoproduct restoration was obtained over a wide concentration range (from 100 μM to1 mM). No inhibition of exoproduct production was observed when DKPs were added to the wild-type *Erwinia* strain. Cyclo(L-Pro-L-Val), however, activated violacein pigment production in the *C. violaceum* AHL reporter strain CV026 (100 μM) (Table 1; McClean *et al*., 1997). Furthermore, three of the seven DKPs synthesized in this study activated the *Agrobacterium tumefaciens* AHL biosensor strain NT1(pDCI41E33) at concentrations ranging from 50 μM to 100 μM (Table 1; Shaw *et al*., 1997; Cha *et al*., 1998).

## Discussion

Diverse Gram-negative bacteria are now known to use small diffusible signal molecules to co-ordinate gene expression in a bacterial population through cell–cell communication (reviewed by Salmond *et al*., 1995; Fuqua *et al*., 1996; Swift *et al*., 1996; Hardman *et al*., 1998). Most of the signal molecules so far characterized are AHLs, the detection of which has been greatly facilitated by the

**Table 1.** Influence of DKPs on quorum sensing dependent phenotypes

| | *E. coli* JM109(pSB401) | | *C. violaceum* CV026 | | *S. liquefaciens* MG1 | *A. tumefaciens* NT1(pDCI41E33) |
|---|---|---|---|---|---|---|
| DKP | Activation | Inhibition | Activation | Inhibition | Inhibition | Activation |
| Cyclo(ΔAla-L-Val) | + | + | NA | NA | + | NA |
| Cyclo(L-Pro-L-Tyr) | + | + | NA | NA | + | + |
| Cyclo(L-Phe-L-Pro) | + | + | NA | NA | NA | NA |
| Cyclo(L-Ala-L-Pro) | NA | + | NA | NA | NA | NA |
| Cyclo(L-Leu-L-Pro) | NA | + | NA | NA | NA | + |
| Cyclo(L-Met-L-Pro) | + | + | NA | NA | NA | + |
| Cyclo(L-Pro-L-Val) | NA | + | + | + | NA | NA |

+ indicates activity, NA indicates no activity. The assays used were: the induction (activation) of bioluminescence in the *E. coli* (pSB401) or the antagonism (inhibition) of 3-oxo-C6-HSL-induced bioluminescence in *E. coli* (pSB401); *C. violaceum* activation assay using the CV026 mutant strain, in which restoration of the purple violacein pigment indicates activation and antagonism of 3-oxo-C6-HSL-induced pigment production indicates inhibition; *S. liquefaciens* wild-type strain MG1 swarming assay, in which activity corresponds to inhibition of motility and the *A. tumefaciens* sensor NT1(pDCI41E33) in which activity corresponds to induction of the *traG*::*lacZ* fusion.

construction of *lux*- or *lacZ*-based reporter fusions driven by specific LuxR homologues (Bainton *et al*., 1992a; Swift *et al*., 1993; Pearson *et al*., 1994; Hwang *et al*., 1995; Shaw *et al*., 1997; Winson *et al*., 1998). However, we have demonstrated that molecules which activate such biosensors need not be AHLs, underlining the importance of chemically characterizing the molecules identified using such bioassays.

The recombinant LuxR-based AHL biosensor used in this paper (pSB401) responds most sensitively to 3-oxo-C6-HSL (Winson *et al*., 1998). However, it can also be activated in a concentration-dependent manner by AHLs with *N*-acyl chains from four to 10 carbons in length with or without a keto group at the C-3 position (Throup *et al*., 1995; Milton *et al*., 1997; Winson *et al*., 1998). On this basis, we assumed that the compounds identified in *P. aeruginosa* supernatants which activated *E. coli* (pSB401) were likely to be novel AHLs, especially given their similar physicochemical properties. Subsequent chemical characterization revealed that the compounds were in fact the DKPs, cyclo(ΔAla-L-Val) and cyclo(L-Pro-L-Tyr). These compounds were also found to be produced by other Gram-negative bacteria including *E. agglomerans, Pr. mirabilis* and *C. freundii*. A third peptide, cyclo(L-Phe-L-Pro) was also identified in *P. fluorescens* and *P. alcaligenes*.

A variety of DKPs have been isolated from protein hydrolysates and fermentation broths from yeast, lichen and fungi (Prasad, 1995). Their presence has also been detected in food and drink products, such as cheese and beer (Roudot-Algaron *et al*., 1993; Gautschi *et al*., 1997). Their origin in foods is unclear (i.e. whether they are produced *in situ* by biological or chemical processes) because DKPs can be generated via the non-enzymatic cyclization of linear dipeptides at extremes of temperature and pH (Skwierczynski and Connors, 1993). As we first isolated cyclo(ΔAla-L-Val) and cyclo(L-Pro-L-Tyr) from *P. aeruginosa* grown in Luria–Bertani (LB) medium, we were concerned that they may have been generated by heat sterilization of this complex medium. However, when sterile LB medium was extracted with solvent and assayed with *E. coli* (pSB401), no activity was observed (unpublished data). Furthermore, the compounds were isolated and purified from bacteria cultured in M9 minimal medium containing a single carbon source and no preformed amino acids. This provides evidence that these DKPs are either synthesized *de novo* from the products of primary metabolism or generated via the metabolism of bacterially generated peptides.

Although cyclo(ΔAla-L-Val) has not previously been identified as a natural product, two of the compounds identified in this study, cyclo(L-Phe-L-Pro) and cyclo(L-Pro-L-Tyr), have recently been isolated during a search for novel antibacterial agents from an Antarctic sponge-associated *P. aeruginosa* strain (Jayatilake *et al*., 1996). Furthermore, they are also produced by the phytopathogenic fungus *Alternaria alternata*, along with a series of proline-containing DKPs (Stierle *et al*., 1988b). Both cyclo(L-Phe-L-Pro) and cyclo(L-Pro-L-Tyr) have been shown to possess significant phytotoxic activity on spotted knapweed (*Centaurea maculosa*) (Stierle *et al*., 1988b; Bobylev *et al*., 1996). Additionally, a derivative of cyclo(L-Phe-L-Pro), cyclo[L-(4-hydroxyprolinyl)-L-Phe], has been shown to act as plant growth promoter (Cronan *et al*., 1998). Although *P. aeruginosa* can be pathogenic for plants, *P. fluorescens* is more commonly associated with the biocontrol of fungal plant pathogens such as *Gaeumannomyces graminis* var. *tritici*, which causes take-all in wheat (Keel *et al*., 1992; Rahme *et al*., 1995). The contribution, if any, of these DKPs to pseudomonad–plant interactions remains to be established.

A number of DKPs have also been shown to elicit pharmacological effects in humans and other mammals. However, only cyclo(L-His-L-Pro) has been unequivocally identified in mammalian tissues. This DKP and cyclo(L-Phe-L-Pro) and cyclo(L-Pro-L-Tyr) have been shown to act on the central nervous system, where they modulate

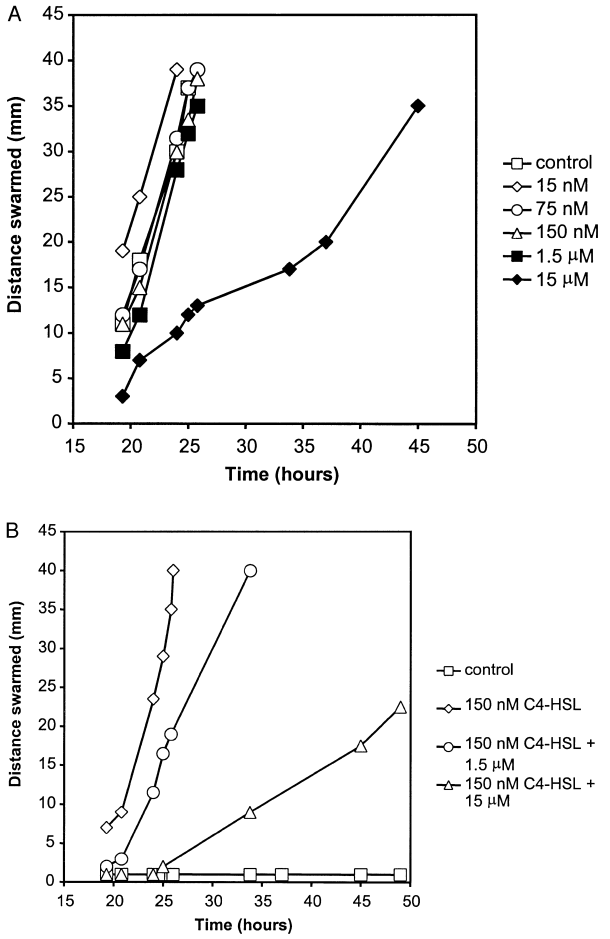



**Fig. 5.** The effects of increasing concentrations (15–15000 nM) of cyclo(L-Pro-L-Tyr) on (A) swarming of wild-type *Serratia liquefaciens* and (B) swarming of *Serratia liquefaciens swrI*$^-$ on plates supplemented with 150 nM C4-HSL. Compounds were incorporated into the agar, and the plates air dried before stab inoculation at the central point of the plate. The distance swarming (mm) was measured over a 50 h period.

a remarkable range of behaviours (Prasad, 1995). This is perhaps not that surprising considering the structural similarity shared between some DKPs and endogenous signalling peptides such as thyrotropin-releasing hormone (Prasad, 1995). Therefore, it is also possible that bacterially derived DKPs may influence eukaryotic host–bacteria interactions.

The DKPs produced by *P. aeruginosa* and other Gram-negative bacteria were detected using the *E. coli* (pSB401) AHL biosensor, which is based on the transcriptional activator LuxR. The data presented here show that these DKPs are capable of mimicking the action of AHLs, presumably by interacting with LuxR. The N-terminal domain of LuxR is considered to contain the 3-oxo-C6-HSL-binding site, which when charged with ligand induces a conformational change thus facilitating the interaction of the C-terminal LuxR DNA-binding region with the target DNA (Choi and Greenberg, 1992; Hanzelka and Greenberg, 1995). Because the DKPs do not influence bioluminescence from *E. coli* transformed with a plasmid carrying constitutively expressed *lux* genes (data not shown), it is likely that they interact at, or near, the 3-oxo-C6-HSL-binding site of LuxR. Evidence to support this hypothesis was obtained from competitive binding assays that showed DKPs have agonist-like activity in *E. coli* (pSB401). Previously, Givskov *et al*. (1996) showed that light emission from AHL-mediated activation of *E. coli* (pSB401) could be abolished by furanones isolated from the macroalga *Delisea pulchra*, although the structures of these natural marine products are distinct from DKPs.

Significantly, when evaluating a range of DKPs, it is apparent that certain DKPs can interfere with the AHL-mediated swarming motility in wild-type *S. liquefaciens*. Previous genetic analysis demonstrated the importance of a functional *swrI* gene for swarming motility (Eberl *et al*., 1996a; b). In a *swrI*$^-$ mutant, the formation of a swarming colony is abolished, but can be restored by the addition of exogenous C4-HSL. When DKPs were added together with C4-HSL, a reduction in swarming was observed. This provides further evidence that DKPs can modulate quorum-dependent phenotypes by antagonizing the action of AHLs. Notably, the concentrations at which the DKPs were active in this assay were significantly lower than those observed with *E. coli* (pSB401) biosensor.

DKPs were also shown to modulate LuxR-dependent quorum-sensing systems in other Gram-negative bacteria. For example, cyclo(L-Pro-L-Val) but not cyclo(L-Ala-L-Pro) activates violacein production in *C. violaceum* even though both contain similar aliphatic amino acids (Table 1). This suggests that there is differential recognition of DKPs by different LuxR homologues. The observation that some DKPs can activate AHL biosensors strains [*C. violaceum* CV026, *A. tumefaciens* NT1(pDCI41E33) and *E. coli* (pSB401)] that are routinely used to detect the presence of AHL quorum-sensing molecules highlights the limitations of these bioassays. During the course of this work, it became apparent that DKPs have similar physicochemical properties to short-chain AHLs. Therefore, when characterizing the AHL molecules produced by a given organism, reporter assays alone cannot be relied upon; it is essential that chemical structures are established unequivocally using MS and NMR techniques.

Although the data presented in the present paper demonstrate that DKPs can modulate the activity of LuxR-based quorum-sensing systems, the concentrations required are much greater than those of the cognate AHL. The detection of these molecules using LuxR-based AHL biosensors probably constitutes fortuitous cross-talk between distinct signalling systems. It has also been demonstrated that

DKPs influence the transcription of specific stationary-phase-regulated genes in *E. coli* (de Nys *et al*., 1999). Experiments are currently under way to investigate the role of DKPs in regulating gene expression in *Pseudomonas*. Whether these molecules modulate AHL-mediated quorum sensing in the producer organism, or in other organisms occupying the same ecological niche or indeed whether they function as diffusible signal molecules *per se* remains to be established.

## Experimental procedures

### *Bacterial strains and growth conditions*

*E. coli* strain JM109 containing the recombinant plasmids pSB401, pSB406 or pSB1075 (Throup *et al*., 1995; Winson *et al*., 1998), *C. violaceum* CV026 (McClean *et al*., 1997) and *A. tumefaciens* NT1(pDCI41E33) (Shaw *et al*., 1997) that use different LuxR homologues were used as AHL biosensors. The *S. liquefaciens* strains MG1 and MG44 were used to investigate the influence of the DKPs on AHL-mediated swarming (Givskov *et al*., 1988; Eberl *et al*., 1996b). *P. aeruginosa* PA01, *P. fluorescens* NCIMB 10586, *E. carotovora* ssp. *carotovora* GS101, *S. marcescens* CP1 and *E. agglomerans* G1 strains were from the School of Pharmaceutical Sciences, University of Nottingham culture collection. The *C. freundii* and *P. mirabilis* strains used in this study were also from the same culture collection and have been described before (Bainton *et al*., 1992a; Swift *et al*., 1993). *P. alcaligenes* Ps537 was kindly provided by W. Quax (Genencor International, Rijswijk, The Netherlands). Where appropriate, tetracycline was used at a concentration of 10 $\mu g\,ml^{-1}$. Bacteria were grown with aeration in LB medium, or in M9 minimal medium, at 30°C or 37°C.

### *Solvent extraction of culture supernatants*

For routine detection of biological activity, organic solvent extracts of cell-free culture supernatants were prepared from 1 l of bacterial culture grown overnight to stationary phase. For the elucidation of chemical structure, extracts were prepared from large volume cultures, typically from 10 l cultures. Bacteria were removed from the growth medium by centrifugation and the resulting spent supernatant was extracted twice with either ethyl acetate or dichloromethane (1000:700 supernatant/solvent). To aid efficient separation of organic and aqueous phases, the emulsified mixture was centrifuged before separation. The combined organic phases were dried over anhydrous magnesium sulphate and filtered before being rotary evaporated to dryness at 37°C. The residue was then resuspended in 2 ml of dichloromethane, transferred to a smaller vessel and dried under a nitrogen stream. Before use, the residue was dissolved in acetonitrile (typically 200 $\mu$l per litre of original culture).

### *Purification and chemical characterization of DKPs*

DKPs were purified from the extracted spent culture supernatants as previously described for AHLs (Bainton *et al*., 1992b; Throup *et al*., 1995). Extracts were applied to a $C_8$ reverse-phase semipreparative HPLC column (Kromasil KR100–5C8 column; Hichrom). Fractions were eluted with methanol (20% v/v or 35% v/v in water) or a linear gradient of acetonitrile (20–95% v/v) over 30 min at a flow rate of $2\,ml\,min^{-1}$ and monitored at 210 nm. Eluted fractions were dried in a rotary evaporator and the residue resuspended in an appropriate volume of acetonitrile. The resulting fractions were tested for activity using the *E. coli* (pSB401) bioassay, the active fractions were rechromatographed when necessary using an isocratic mobile phase of 35% v/v acetonitrile in water. Single active peaks were rechromatographed on an analytical HPLC attached to a photodiode array system (Waters 996 PDA system operating with a Millenium 2010 Chromatography Manager) and both retention times and spectral properties were compared with those obtained for AHL standards. Active fractions were pooled after preparative HPLC and analysed by mass spectrometry (MS) and NMR spectroscopy.

EI-MS and accurate mass measurement of molecular ions were determined by using an AEIMS902 instrument. NMR spectra were recorded either on a Varian EM390 operating at 90 MHz or on a Bruker AR250 or AM400 spectrophotometer operating at 250 and 400 MHz respectively.

### *Chemical synthesis of AHLs*

The AHLs *N*-(3-oxohexanoyl)-L-homoserine lactone (3-oxo-C6-HSL), *N*-butanoyl-L-homoserine lactone (C4-HSL), *N*-hexanoyl-L-homoserine lactone (C6-HSL), *N*-octanoyl-L-homoserine lactone (C8-HSL) and *N*-(3-oxooctanoyl-L-homoserine lactone (3-oxo-C8-HSL) were synthesized as described previously (Chhabra *et al*., 1993). Each compound was purified to homogeneity by preparative HPLC and its structure confirmed by MS and proton NMR spectroscopy.

### *Chemical synthesis of DKPs*

The preparation of cyclo(ΔAla-L-Val) is described as an example for the synthesis of DKPs. For each compound, the structure of the synthetic material was confirmed by MS and proton NMR.

*Preparation of cyclo(ΔAla-L-Val)*. Water-soluble carbodiimide [1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC.HCl)]-mediated coupling of Boc-L-Val.OH and L-Ser.OMe gave the Boc-dipeptide ester as a crystalline material. Boc protection was removed with formic acid, followed by refluxing in a mixture of toluene and 2-butanol and yielded the DKP cyclo(L-Ser-L-Val). Dehydration by the method of Miller (1980) using EDC.HCl and a catalytic amount of cuprous chloride at room temperature yielded cyclo(ΔAla-L-Val).

### *Preparation of cyclo(L-Pro-L-Tyr)*

The linear dipeptide was prepared by the imidazole-mediated coupling of *N*-Boc-L-Pro *p*-nitrophenyl ester (Bodanszky and du Vigneau, 1959) with L-Tyr.OMe hydrochloride (Bodanszky and Bodanszky, 1984). The corresponding DKP [cyclo(L-Pro-L-Tyr)] was then prepared by the method of Nitecki *et al*. (1968),

which is essentially the same as that described above for cyclo(ΔAla-L-Val).

### *Preparation of cyclo(L-Phe-L-Pro)*

This diketopiperazine was prepared as described above for cyclo(L-Pro-L-Tyr) but using *N*-Boc-Pro-Phe.OMe as the starting linear dipeptide.

### *Detection using the* E. coli *AHL biosensors*

Biological activity was monitored using *E. coli* biosensor strains, each containing different sensor plasmids capable of detecting a range of AHL signalling molecules (Throup *et al*., 1995; Winson *et al*., 1995; 1998). The three plasmid constructs used were pSB401, pSB406 and pSB1075, each of which is based on the three different quorum-sensing systems *luxRI′*, *rhlRI′* and *lasRI′* respectively (Throup *et al*., 1995; Winson *et al*., 1995; 1998). Sample activity was measured in a microtitre plate format, with bioluminescence detected and quantified using either a Berthold LB980 imaging system (E.G. and G. Berthold), or an Anthos Lucy 1 luminometer (Anthos Labtech Instruments). In pSB401, *luxR* and the *luxI* promoter region from *P. fischeri* have been coupled to the entire *lux* structural operon (comprising *luxCDABE*) from *Photorhabdus luminescens*. This construct, when expressed in *E. coli*, responds to a range of AHLs and does not require the addition of exogenous aldehyde (Throup *et al*., 1995; Winson *et al*., 1998). Both the plasmids pSB406 and pSB1075 were constructed in a similar fashion, with the *rhlR* and *rhlI* promoter region, and *lasR* and *lasI* promoter region, coupled to the entire *lux* structural operon (Winson *et al*., 1995; 1998). For the detection of activating compounds in cell-free supernatants, assays were set up as follows. Solvent extracts, HPLC fractions, synthetic DKPs or 3-oxo-C6-HSL were made up to 100 μl volumes with LB in a microtitre plate. One hundred microlitres of a 1:10 dilution (in LB containing tetracycline at 10 μg ml$^{-1}$) of an overnight culture of the *E. coli* biosensor strain was then added and the plate incubated at 30°C. The amount of light produced by the assay increased with time for ≈7 h. Typically, readings were taken after 3–4 h, as this was when the ratio of induced to background light was at its highest. The amount of light measured was expressed in relative light units (RLU).

### *Competition assays using the* E. coli *(pSB401) biosensor*

The influence of cyclo(L-Phe-L-Pro) and cyclo(ΔAla-L-Val) on the induction of *E. coli* (pSB401) by 3-oxo-C6-HSL were determined in microtitre plates as described above, with the following modifications. A dilution series of 3-oxo-C6-HSL (doubling dilution with concentrations ranging from 0 to 100 nM) were set up in 100 μl of LB, or LB containing different concentrations (0–2 mM) of either DKP. Then, 100 μl of a 1:10 dilution (in LB containing tetracycline at 10 μg ml$^{-1}$) of an overnight culture of the *E. coli* (pSB401) was added and the plate incubated at 30°C. Light emission from the plate was measured as described previously.

### *Detection of DKPs by thin layer chromatography (TLC)*

Extracts (typically 1–6 μl) were applied to RP18 $C_{18}$ reverse-phase plates (20 cm × 20 cm; Whatman) 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, air dried and then overlaid with a thin film of soft agar containing the *E. coli* (pSB401) biosensor. The biosensor overlay was prepared from 10 ml of an overnight culture added to 100 ml of warm (45°C) LB medium containing 0.80 g of melted agar and tetracycline at 10 μg ml$^{-1}$. After the agar had set, the TLC plate was incubated at 30°C for at least 4 h. Active spots were located either using a Berthold LB980 imaging system or by autoradiography. In addition, the *A. tumefaciens* AHL biosensor strain NT1(pDCI41E33) and *C. violaceum* AHL biosensor strain CV026 were used to overlay TLC plates. The overlays were prepared as described by Shaw *et al*. (1997) and McClean *et al*. (1997). In the case of *C. violaceum* AHL biosensor, both activation and inhibition assays were performed as described by McClean *et al*. (1997).

### *Effect of DKPs on S. liquefaciens swarming motility*

Swarming assays with *S. liquefaciens* MG1 *wt* and a *S. liquefaciens* MG44 *swrI* were performed in duplicate on soft (0.7%) AM medium agar plates. AB medium was supplemented with 0.5% (w/v) casamino acids and 0.5% (w/v) glucose. DKPs and AHLs were incorporated into the agar during plate pouring with a thorough mixing in concentrations ranging from 15 nM to 15 μM. Plates were air dried before stab inoculation at the central point of the plate. As controls for the possible growth inhibition by DKPs or AHLs, strains were grown with and without DKPs or AHLs (15 μM) and optical density was monitored throughout growth.

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