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Identification of *N*-3-hydroxyoctanoyl-homoserine lactone production in *Pseudomonas fluorescens* 5064, pathogenic to broccoli, and controlling biosurfactant production by quorum sensing

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Abstract

Quorum sensing controls a number of key processes in growth, reproduction and virulence of many gram-negative bacteria via signalling molecules or autoinducers. It can control, for instance, the production of pectic enzymes which are virulence factors in disease. *Pseudomonas fluorescens* 5064 produces biosurfactants which are important for bacterial establishment on the plant surface prior to causing disease in broccoli. The aim of this paper was to determine if biosurfactant production in this bacterium is controlled via quorum sensing. To do this, 35 surfactant-minus Tn*5* mutants of *P. fluorescens* 5064 were screened for their abilities to produce a quorum sensing signal. Six of these biosurfactant-deficient mutants showed a large reduction in quorum sensing signal production and varied in their abilities to cause disease. In one mutant, 6423, which contains a single Tn*5* insertion, the production of the signal was almost eliminated. Synthetic quorum sensing signal and quorum sensing signal extracted from wild type *P. fluorescens* 5064 restored biosurfactant production by addition to the culture in mutant 6423. The quorum sensing signal in wild type *P. fluorescens* 5064 was identified by high pressure liquid chromatography and mass spectrometry as *N*-3-hydroxyoctanoyl-homoserine lactone.

Abbreviations: AHL – acyl-homoserine lactone; HPLC – high pressure liquid chromatography; HSL – homoserine lactone; MS – mass spectrometry; QS – quorum sensing; QSS – quorum sensing signal; RLU – relative luminescence unit.

Introduction

Quorum sensing (QS) is a communication mechanism by which bacteria regulate the expression of specialized gene sets in response to their cell densities. This type of cell density-dependent gene regulation was first described in the marine bacterium *Vibrio fischeri* (Nealson et al., 1970; Eberhard et al., 1981). The bacteria gauge the size of their population by sensing the concentration of a small signal molecule (quorum sensing signal, QSS) that they themselves produce. In many gram-negative bacteria, the QSS is a member of the *N*-acyl homoserine lactone (AHL) family (Salmond et al., 1995; Fuqua et al., 1996; Swift et al., 1996). Accumulation of QSS above a threshold concentration, through the activity of a signal generator protein, indicates that the population has reached a critical level, and the appropriate target gene(s) is switched on via the action of a transcriptional activator (usually a member of the LuxR family) (Swift et al., 1997). In general, the signal generator protein that is responsible for AHL synthesis belongs to the LuxI family. Both

LuxI and LuxR family were originally identified within the *V. fischeri* lux operon (Eberhard et al., 1981; Engebrecht et al., 1983; Devine et al., 1989).

AHL-dependent QS controls a number of bacterial physiological processes, including extracellular compound production (e.g. antibiotics, siderophores), virulence factor production (e.g. pectic enzymes), secondary metabolite production, bioluminescence, swarming, plasmid transfer, and so on (Eberhard et al., 1981; Gambello et al., 1993; Zhang et al., 1993; Pierson et al., 1994; Hwang et al., 1995; Dunphy et al., 1997; Swift et al., 1997).

The bacterium *Pseudomonas fluorescens* 5064 is an opportunistic soft rot pathogen of broccoli (*Brassica oleracea* var *italica*). It produces an extracellular lipopeptide biosurfactant, viscosin, which has been suggested to play a major role in disease incidence (Hildebrand, 1989). Viscosin gene expression and regulation have not been previously studied.

A number of investigators have demonstrated an over-production of biosurfactants in *P. fluorescens* when the cultures reach the stationary phase of growth (Guerra-Santos et al., 1986; Persson et al., 1988; Mulligan and Gibbs, 1989; Ramana and Karanth, 1989). A previous study in our laboratary has also shown that *P. fluorescens* 5064, isolated from infected broccoli, will only commence biosurfactant production after 30 h incubation when the culture has reached a late exponential growth phase (Darling, 1998). As QS dependent gene expression is always associated with high cell densities in the late exponential or stationary phase, we hypothesised that biosurfactant production in *P. fluorescens* 5064 might be controlled by QS. The main objective of this study was to provide evidence for this hypothesis.

Several types of QSS have been reported from different *P. fluorescens* strains. Laue et al. (2000) found that *P. fluorescens* F113 produces at least three different AHLs. These are (1) *N*-(3-hydroxy-7-*cis*-tetradecenoyl) homoserine lactone (3-OH, $C_{14:1}$-HSL); (2) *N*-decanoyl HSL (C_{10}-HSL) and (3) *N*-hexanoyl HSL (C_6-HSL). None of these three AHLs have yet been identified in other *P. fluorescens* strains. Shaw et al. (1997) have reported up to five different AHLs in *P. fluorescens* 2–79 by thin layer chromatography (TLC) and mass spectrometry (MS) analysis, three of which were identified as *N*-(3-hydroxyhexanoyl)-HSL, *N*-(3-hydroxyoctanoyl)-HSL, and *N*-(3-hydroxydecanoyl)-HSL. Later on, *N*-octanoyl-HSL and *N*-hexanoyl-HSL were identified in this strain by Cha et al. (1998). However, no AHL molecules were detected in *P. fluorescens* 1855.344 (Cha et al., 1998), *P. fluorescens* pf 7–14 (Dumenyo et al., 1998), and *P. fluorescens* NCIMB 10586 (Holden et al., 1999). Given the differences in AHL profiles from the *P. fluorescens* strains examined to date, it would clearly be of future interest to determine whether there is any association between AHL profile and strain habitat.

In this paper, we report the identification of an AHL, 3-hydroxyoctanoyl-HSL, in *P. fluorescens* 5064 and also present evidence for the regulation of biosurfactant production via this QSS in *P. fluorescens* 5064.

Materials and methods

Bacterial strains and growth conditions

Wild type *P. fluorescens* 5064, isolated at Scottish Agricultural College from infected broccoli collected in SE Scotland (Robertson et al., 1993), was grown at 26 °C in LB or KB medium. Tn*5* mutants of *P. fluorescens* 5064, deficient in biosurfactant production and designated surf $^-$(Darling 1998), were grown at 26 °C in LB or KB medium with kanamycin at 50 μg/ml. Biosensors used for AHL detection and their growth conditions are listed in Table 1.

Biosurfactant extraction and assay

The biosurfactant extraction method was based on Laycock et al. (1991). Bacteria (*P. fluorescens* 5064 or its surf $^-$ mutants) were grown in 200 ml KB broth flasks for about 30 h (or as indicated for specific experiments). Cells were removed by centrifugation at $20{,}000 \times g$ for 30 min. The cell free supernatant was acidified to pH 2 with 2 N HCI and allowed to stand at 2 °C for at least 1 h. The white precipitate that formed upon acidification was centrifuged at $20{,}000 \times g$ for 15 min, the supernatant was decanted and the pellets were extracted with 60 ml of 95% ethanol; the residue was discarded and the ethanolic extract was concentrated on a centrifugal evaporator (Jouan, RC 10-22, France) to 3–5 ml (level 0, no heating).

Table 1. Biosensor strains used for quorum sensing signal detection

Biosensor strains	Growth conditions	Characteristics/genotype	Acyl-homoserine lactone (AHL) detecting profile	Source/reference
Chromobacterium violaceum CV026	30 °C in LB medium	Violacein-negative, double mini-Tn5 mutant; Km^r	Sensitive to AHL with 4–6 carbon side chains	Latifi et al. (1995)
Escherischia coli (pSB401)	37 °C in LB medium	*E. coli* JM109 carrying plasmid: luxR $luxI^-$ :: lux CDABE; Tc^r	Sensitive to AHL with 6–8 carbon side chains	Winson et al. (1995)
Escherischia coli (pSB1075)	37 °C in LB medium	*E. coli* JM109 carrying plasmid: lasR $lasI^-$ :: lux CDABE; Am^r	Sensitive to AHL with 10–14 carbon side chains	Winson et al. (1995)

The biosurfactant assay method is based on the Water Drop Test (Hildebrand, 1989). After 30 h incubation, a loopful of bacteria from agar or 5 µl of biosurfactant extract (as described above) was transferred to a 50 µl water droplet situated on a plastic Petri dish. If biosurfactant was present, the surface tension of the water droplet was immediately reduced giving a corresponding collapse or spreading of the water droplet. Three replicates were carried out for each test.

Crude QSS extraction

Cell-free supernatants from *P. fluorescens* 5064 or its Tn*5* $surf^-$ mutants were prepared from cultures (1–3 l of LB broth) grown for 50–55 h at 26 °C (optical density of 2.1 at 500 nm). The supernatants were extracted twice with equal volumes of dichloromethane. The dichloromethane organic phases were combined and taken to dryness by rotary vacuum evaporation at 37 °C. The resulting residue was redissolved in 100 µl of 95% ethanol (for further AHL bioassay) or 500 µl of dichloromethane (for QSS chemical identification).

AHL bioassay using the AHL-dependent biosensors

QSS extracts from bacteria were detected by different bacterial biosensors. Positive assays were judged as induction of purple pigment in *Chromobacterium violaceum* CV026 or the induction of bioluminescence in *E. coli* (pSB401) and *E. coli* (pSB1075). The AHL bioassay method was based on McClean et al. (1997) with some modifications: 5 ml of pre-warmed semi-LB agar [0.5% (w/v)], heated up in a microwave until melted and then cooled down to about 50 °C) was seeded with 50 µl of appropriate bacterial biosensor from an overnight LB culture. In addition, 30 µl of QSS extract (substance to be detected) was also added. The mixture was mixed well and immediately overlaid on the surface of a 35 mm Petri dish containing LB agar. The plates were left on the bench for about 20 min until the overlaid semi-LB agar had solidified and were then subjected to the first (zero time) bioluminescence detection (except for the CV026 plates). The data were recorded as background luminescence. The plates were incubated at 37 °C (30°C for CV026) in an upright position for 4 h and were then subjected to the second luminescence detection, or examined for the stimulation of purple pigment (for CV026 treatments). Instead of the QSS extracts, 20 µl of synthetic AHL signal solution [3-oxohexanoyl-homoserine lactone (OHHL), Sigma Chem Co. or *N*-hexanoyl-homoserine lactone, Fluka Chem. Co.] was dissolved in 95% ethanol at a concentration of 0.1 mM and added to the semi-LB agar as positive control; 20 µl of 95% ethanol served as negative control.

Luminescence measurements

Luminescence produced by the biosensors was measured by an ID-20/20 Luminometer (DOP Solutions LTD, England). Images of plates showing induced luminescence were taken by a ChemiImager 4400 camera (Alpha Innotech Coporation, USA).

Chemical identification of the QSS(s) produced by P. fluorescens *5064*

The QSS extraction from 3 l LB medium as described above was re-dissolved in 500 µl of dichloromethane for HPLC analysis. HPLC was

performed on a Hewlett Packard 1100 series LC system. The sample was applied to a C_{18}-reverse-phase semi-preparative HPLC column [Lichros 5RP18 (250 × 8 mm, 5 μm), Technicol Ltd., UK] eluted with a mobile phase of acetonitrile (HPLC grade) in purified water at a flow rate of 2 ml/min and monitored at 210 nm. The gradient of acetonitrile was as follows: 15% from 0 to 8 min, 35% from 10 to 20 min, 60% from 22 to 30 min. Fractions showing luminescence induction in the *E. coli* (pSB401) biosensor were pooled and rechromatographed using an analytical PLRP-S reversed-phase column (250 × 4.6 mm, 100 Å, 5 μm, Polymer Labs). The column was eluted with a mobile phase of acetonitrile (changing from 0% to 35% in 20 min and remaining at 35% for another 2 min) in water at a flow rate of 1 ml/min and monitored at 210 nm. Active fractions were subject to mass spectrometry (MS) performed on a Micromass Platform II quadrupole mass spectrometer equipped with an electrospray ion source (collision energy 25 eV). The average molecular mass was determined using the MaxEnt algorithm of MassLynx software.

Excised head pathogenicity test

Excised broccoli heads were inoculated to determine the pathogenicity of the biosurfactant mutants to broccoli. The test method was as described in Darling et al. (2000). Greenhouse-grown heads were used to reduce saprophyte levels. To assess the disease, a five point scoring scale was used: 0 = no symptoms, 1 = water soaking (loss of waxy bloom from florets), 2 = water soaking and tissue browning but no rot, 3 = tissue browning and softening (with rot), 4 = extensive black soft rot. Data were subjected to percentile angular transformation using arc sine (Schefler, 1969), thus the above categories 0–4 became 0% (0), 33% (1), 50% (2), 67% (3) and 100% (4) respectively.

Results

P. fluorescens *5064 QSS bioassay and dynamics*

QSS production in *P. fluorescens* 5064 supernatant extracts was assayed by the AHL-dependent biosensors *E. coli* (pSB401), *E. coli* (pSB1075) and *C. violaceum* CV026. CV026 is a violacein-negative strain that responds to exogenous AHLs by the production of the purple pigment violacein. Both of the other two biosensors are *E. coli* strains carrying bioluminescence reporter plasmids that will respond to exogenous AHL by emitting light. QSS extracted from *P. fluorescens* 5064 was found to induce light in both pSB401 (Figure 1) and pSB1075 (data not shown), but it could not induce any purple pigment formation in biosensor CV026 (data not shown). To determine the light emission at different growth phases of *P. fluorescens* 5064, QSS extracts at different incubation times (5, 11, 19, 25 and 30 h) were assessed for luminescence induction with biosensor *E. coli* (pSB401). The results showed that *P. fluorescens* 5064 exhibited a lag in AHL production during early- and mid-exponential growth phases, followed by a rapid increase during the late exponential phase and reached maximum production at early stationary growth phase (Figure 2).

Screening of Tn5 surf$^-$ mutants for QSS production

To determine the potential relationship between biosurfactant production and QSS production in *P. fluorescens* 5064, 35 *P. fluorescens* 5064 biosurfactant-deficient mutants, generated by Tn*5* mutagenesis (Darling, 1998), were screened for their abilities to produce QSS. Culture filtrates from each mutant were assayed for QSS using the *E. coli* (pSB401) biosensor. According to the screening results, the 35 mutants were divided into three groups on the basis of their QSS producing abilities (Table 2). Twenty-four mutants (group 1) showed strong luminescence induction, i.e. were similar to wild type in production of QSS. Six mutants (group 2) showed greatly reduced luminescence induction. The luminescence induction of mutant 6423 was almost 100 times lower than the wild type *P. fluorescens* 5064 (Figure 3). No luminescence could be detected in five of the mutants in group 3 (Table 2). Among the mutants in group 2 that showed reduced QSS production, mutants 6423, 6421, and 6429 are more interesting due to their lack of additional transposon insertions (Darling, 1998). A single Tn*5* insertion into genomic DNA makes further gene analysis easier. Mutant 6423 (m6423), in particular, warrants further genetic analysis to determine the nature of the mutation because its QSS production was almost eliminated.

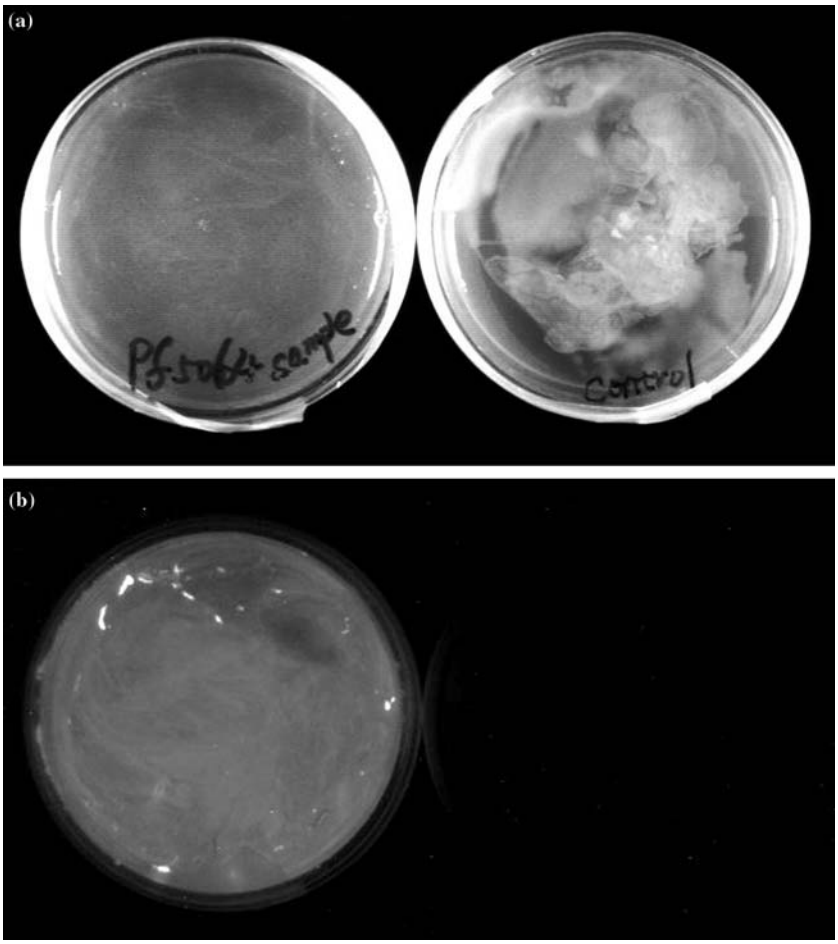

Figure 1. Bioassay of quorum sensing signal production by *P. fluorescens* 5064. Quorum sensing signal was extracted from culture filtrates of *P. fluorescens* 5064, added to semi-LB agar seeded with biosensor *E. coli* pSB401, and overlaid on LB agar plates. The biosensor produces light in the presence of quorum sensing signal. (a): Photographed in white light. Left: pSB401 biosensor strain was exposed to quorum sensing signal extracted from *P. fluorescens* 5064. Right: Biosensor strain only as negative control. (b): Photographed in darkness. Light emitted by the biosensor strain confirms production of quorum sensing signal by *P. fluorescens* 5064.

Biosurfactant restoration by exogenous AHL in m6423

m6423 was incubated in 200 ml KB broth (without antibiotic) in shaking flasks (200 rpm) at 26 °C for 6 h. The exogenous AHLs, either *N*-hexanoyl-homoserine lactone, 3-oxohexanoyl-homoserine lactone (OHHL), or QSS extract from wild type *P. fluorescens* 5064 were then added to the medium in the flasks to give a final concentration at 12.5 mM. The flasks were then further incubated for another 24 h followed by biosurfactant extraction and bioassay as described in Materials and methods. Biosurfactant restoration was observed when either synthetic OHHL or QSS extracts of wild type *P. fluorescens* 5064 were added to m6423 (Figure 4). This provides strong evidence for the regulation of biosurfactant production by the quorum sensing system in *P. fluorescens* 5064.

Chemical identification of the QSS produced by P. fluorescens *5064*

The fact that *P. fluorescens* 5064 QSS extract was able to induce light in the AHL-dependent biosensors and that exogenous AHL molecules (OHHL) could restore biosurfactant production in

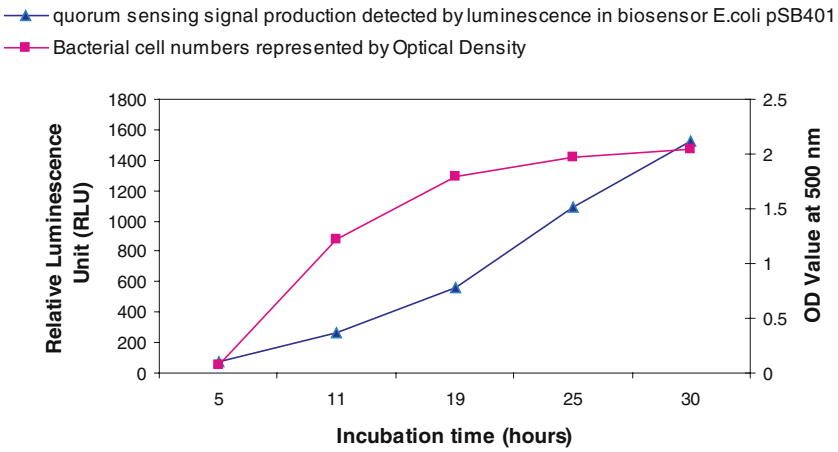

Figure 2. Dynamics of quorum sensing signal production in *P. fluorescens* 5064. Quorum sensing signal production showed a lag during early- and mid- exponential growth phases, followed by a rapid increase during the late exponential and early stationary phases.

Table 2. Quorum sensing signal production in 35 biosurfactant-negative mutants of *P. fluorescens* 5064

Phenotypic Group	Quorum sensing signal production detected by biosensor *E. coli* pSB401	*P. fluorescens* 5064 mutants	Other phenotypic characteristics [1]
1	Same as wild type *P. fluorescens* 5064	6401, 6402, 6403, 6404, 6405, 6406, 6408, 6409, 6410, 6411, 6413, 6414, 6420, 6422, 6424, 6425, 6426, 6428, 6430, 6431, 6432, 6433, 6434, 6435	None
2	Quorum sensing signal production was greatly reduced compared to wild type *P. fluorescens* 5064	6412, 6415, 6421, 6423, 6427, 6429	None
3	No quorum sensing signal production could be detected because culture filtrates from these mutants killed the biosensor strains	6407, 6416, 6417, 6418, 6419	No extracellular product expression (i.e. protease, pectinase, siderophore production). Powerful unknown antibiotic compound produced

[1] Darling (1998).

a surf $^-$ mutant gave an indication that the *P. fluorescens* 5064 QSS may also belong to the AHL family.

A fraction capable of stimulating luminescence in *E. coli* (pSB401) was obtained from stationary-phase cell-free supernatants of wild type *P. fluorescens* 5064 after preparative HPLC and followed by analytical HPLC (Figure 5). MS analysis of the fraction yielded a parent peak of M + H ions at *m/z* 244 (Figure 5, arrowed), which matched the formula *N*-(3-hydroxyoctanoyl)-HSL and a peak at *m/z* 103 indicating the existence of a homoserine lactone moiety (Eberhard et al., 1981). The other molecular ions that displayed on the resulting MS graph were showing losses of the C_2H_4 (*m/z* 29), C_3H_7 (*m/z* 41–43), C_4H_6O (*m/z* 70) and $C_6H_9O_2$ (*m/z* 113) substitutes from the molecule. The presence of the parent peak at *m/z* 244 and the *m/z* 103 peak corresponding to the homoserine lactone moiety, taken together with the intense fragments of *m/z* 144 for the 3-hydroxyoctanoyl moiety, confirmed that QSS isolated from *P. fluorescens* 5064 had the structure of *N*-3-hydroxyoctanoyl-HSL (Figure 5). The prominent peak of *m/z* 125 indicates a loss of water from the C_8-HSL side chain, which characteristically occurs during MS analysis of compounds with hydroxyl groups (Shaw et al., 1997).

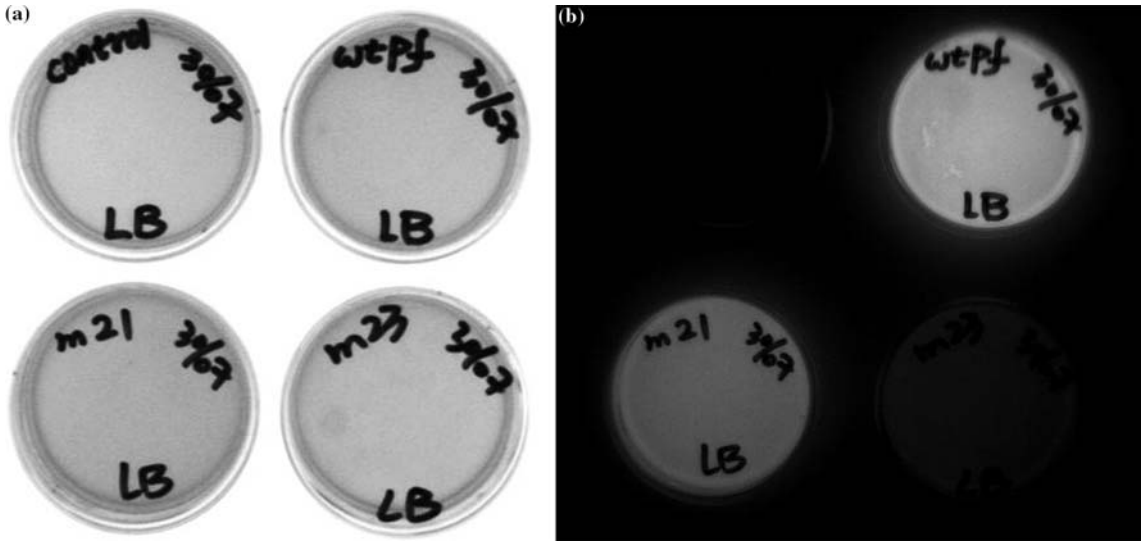

Figure 3. Quorum sensing signal production in biosurfactant-negative mutants using *E. coli* (pSB401) biosensor assay. Quorum sensing signal was extracted from culture filtrates of mutants, added to semi-LB agar seeded with biosensor *E. coli* pSB401, and overlaid on LB agar plates. (a) Plates photographed under white light. (b) Plates photographed in darkness. Row 1 left: Water as negative control (Relative Luminescence Unit (RLU) = 11). Row 1 right: *P. fluorescens* 5064 wild type as positive control (RLU = 5600). Row 2 left: mutant 6421 (RLU = 618). Row 2 right: mutant 6423 (RLU = 62). Quorum sensing signal produced in mutant 6421 was less than in wild type, and almost eliminated in mutant 6423.

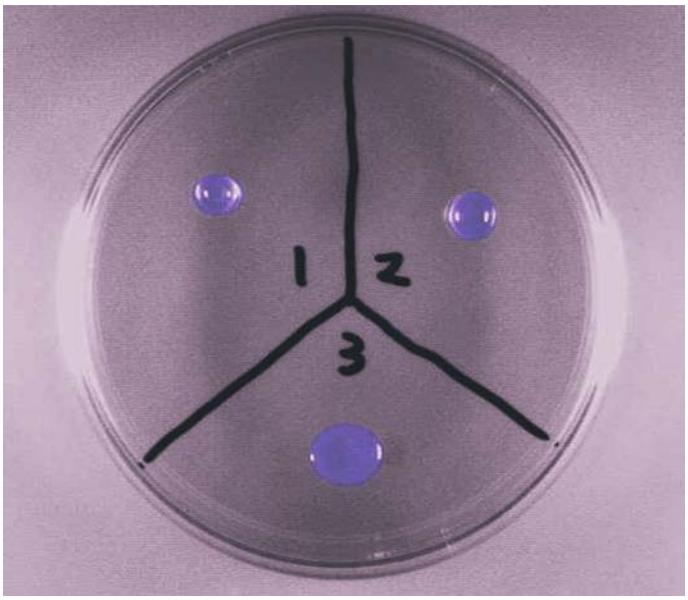

Figure 4. Restoration of biosurfactant production in *P. fluorescens* mutant 6423 in response to exogenous quorum sensing signal using the water droplet test. (1): Water droplet alone on plastic Petri dish. (2): Water droplet containing extracted biosurfactant from mutant 6423 grown without exogenous QSS. 3: Water droplet containing extracted biosurfactant from mutant 6423 grown in presence of exogenous QSS. Loss of surface tension in droplet in (3) shows that biosurfactant production was restored in mutant 6423 by addition of QSS [either extracted from wild type *P. fluorescens* 5064, or synthetic 3-oxohexanoyl-homoserine lactone (OHHL)].

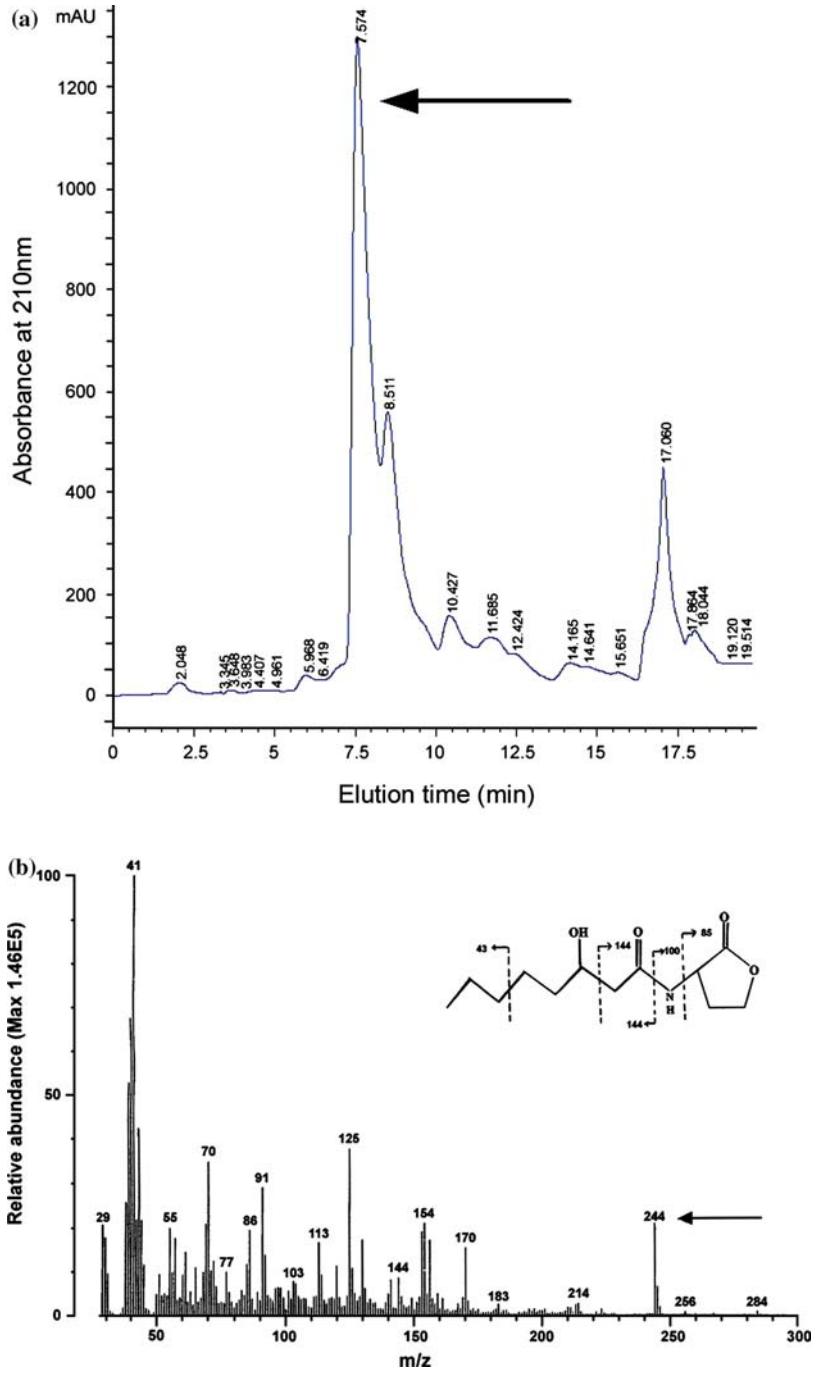

Figure 5. Chemical identification of the quorum sensing signal in *P. fluorescens* 5064 by high pressure liquid chromatography (a) and mass spectrometry (b). The arrow in (a) shows the fraction with bioluminescence inducing activity in biosensor *E. coli* pSB401. The arrow in (b) shows the parent peak with m/z of 244.

Table 3. Pathogenicity of *P. fluorescens* 5064 biosurfactant-negative mutants showing reduced quorum sensing signal production

Mutant	Disease score (%)	Intensity of quorum sensing signal[2]
6412	87	814
6415	31[1]	319
6421	81	1200
6423	100	601
6427	25[1]	1841
6429	50[1]	2187
5064 wild type	100	8806
	39.4 LSD ($P = 0.05$)	40 Background level

[1] Significantly different to wild type level of disease, $P = 0.05$.
[2] Measured as relative luminescence units (RLU), the intensity of light emitted by the biosensor *E. coli* pSB401, in response to quorum sensing signal produced by the mutants.

Excised head pathogenicity test

The group 2 mutants 6412, 6415, 6421,6423, 6427 and 6429 produced no biosurfactant and less QSS. The pathogenicity test results showed that mutants 6415, 6427 and 6429 were much less aggressive than their wild type. Mutants 6412, 6421 and 6423 were not significantly different to the wild type (Table 3).

Discussion

AHL-based quorum sensing has been identified in a wide range of gram-negative bacteria (Salmond et al., 1995; Fuqua et al., 1996; Hardman et al., 1998). The detection of AHL molecules has been greatly facilitated by the construction of lux- or lacZ-based reporter fusions driven by specific LuxR homologues (Bainton et al., 1992; Swift et al., 1993; Hwang et al., 1995; Shaw et al., 1997; Winson et al., 1998). Three AHL-dependent reporters have been used in this study to detect the quorum sensing signal(s) in *P. fluorescens* 5064. The first reporter strain we tested was *Chromobacterium violaceum* CV026. *P. fluorescens* 5064 QSS extract from culture supernatant did not induce any pigment production in the CV026 biosensor even after 2 days' incubation, whilst pigment induction was detected in the positive control using synthetic OHHL. The other two biosensors that we employed here were *E. coli* JM109 strains carrying either bioluminescence reporter plasmids pSB401 or pSB1075. Though *P. fluorescens* 5064 AHL extract from culture supernatant activated both *E. coli* (pSB401) and *E. coli* (pSB1075), the light output induced by pSB401 was much stronger than that induced by pSB1075 under the same incubation conditions. This indicated that pSB401 was more sensitive than pSB1075 in responding to *P. fluorescens* 5064 AHL production. Since CV026 responds most sensitively to AHLs with 4–6 carbon acyl side chains, pSB401 prefers 6–8 carbon side chains, and pSB1075 detects compounds with 10–14 carbon side chains, the QSS produced by *P. fluorescens* 5064 was hypothesised to be 8–10 carbons in side chain length. Our work using HPLC and MS identified the QSS produced by *P. fluorescens* 5064 as an 8-C length AHL (acyl-hydroxyoctanoyl-homoserine lactone). Cha et al. (1998) point out that the CV026 biosensor could not detect any of the 3-hydroxy-derivatives of AHL, which could explain the reason that *P. fluorescens* 5064 AHL (which is a 3-hydroxy-derivative) did not induce any purple pigment production.

In a previous study, we constructed a biosurfactant-minus library of *P. fluorescens* 5064 containing 35 mutants by Tn*5* mutagenesis (Darling 1998). In this paper we screened all 35 surf$^-$ mutants for QSS production using the *E. coli* (pSB401) biosensor. Most of these surf$^-$ mutants produced QSS at levels similar to the wild type *P. fluorescens* 5064 (Table 2). AHL extracts from five mutants showed very strong antibiotic activity: they killed not only the *E. coli* biosensors but also the wildtype *P. fluorescens* 5064 in a subsequent experiment (data not shown). A possible reason might be that the Tn*5* insertion had blocked a transcriptional repressor gene function, thereby restoring antibiotic production that is

normally repressed by this particular gene. Recently, Delany et al. (2000) identified a putative regulator gene (*phl*F) upstream and divergently transcribed from the phloroglucinol (antibiotic) biosynthetic genes in *P. fluorescens* F113. Introduction of *phl*F into *P. fluorescens* F113 in multiple copies resulted in repression of phloroglucinol production and inactivation of *phl*F resulted in depression of the antibiotic production in this strain. These facts indicate that *phl*F functions as a gene repressor. Whether the restored antibiotic production in mutant group 3 is due to a mutation of a repressor gene needs further investigation. As group 3 mutants killed the biosensors, it is not possible to draw any conclusion on their AHL producing abilities by the bioassay method described here.

A greatly reduced AHL production was observed within group 2 mutants, especially m6423, which suggested that the Tn*5* insertions might have disrupted a gene in the AHL synthesis pathway. None of these 35 surf $^-$ mutants showed a complete blockage in AHL expression. The lower and varying levels of luminescence in *E. coli* (pSB401) induced by group 2 mutants suggested that the AHL synthesis mechanism in *P. fluorescens* 5064 might be very complicated. Two quorum sensing systems have been identified in *Pseudomonas aeruginosa* including the LasR/LasI system, which produces QSS 3-oxo-C_{12}-HSL; and the RhlR/RhlI system, which produces C4-HSL as its cognate QSS. It is now apparent that both of these quorum sensing systems regulate the production of rhamnolipid (a biosurfactant produced by *P. aeruginosa*), though the LasR/LasI system also plays a role in expression of many other virulence genes. In this study, surf $^-$ mutant 6423 produced wildtype levels of protease, pectinase and siderophores (Darling, 1998). The production of these compounds are regulated in a cell-density-dependent manner in *P. aeruginosa* and also in a number of other bacteria including *P. fluorescens*. This implies that there may be more than one quorum sensing system in *P. fluorescens* 5064 and that these quorum sensing systems may interact with each other in controlling the expression of biosurfactants. This can possibly explain why the AHL production of m6423 was reduced greatly but not eliminated. In fact, a MupR/MupI quorum sensing system that regulates the mupirocin biosynthetic gene cluster has very recently been identified in *P. fluorescens* NCIMB 10586 (El-sayed et al., 2001). Both MupR and MupI genes have been cloned. It would be interesting to know if the MupR/MupI quorum sensing system affects biosurfactant synthesis in *P. fluorescens* 5064.

Biosurfactant restoration in m6423 was achieved by supplementing with either exogenous AHL or AHL purified from *P. fluorescens* 5064 supernatant. Instead of adding exogenous AHL to cultures as we described in 'Materials and methods', we had first tried to add synthetic AHL solution directly to m6423 clones growing on a KB agar plate (added the AHL solution around the bacterial clones evenly with a pipette tip), but no obvious biosurfactant restoration was observed. The reason for the failure in biosurfactant restoration by this means remains unknown, although it is a fact that exogenous AHL could be mixed better with bacterial cells in shaking liquid culture than on a solid agar plate.

Although the group 2 mutants produced no biosurfactant and less QSS, their abilities to cause head rot disease on broccoli varied. Three of the mutants showed reduced disease, whilst three mutants did not. Possible reasons for this are (1) residual bacteria present on the broccoli head (sterilisation of heads was not possible without tissue damage) were biosurfactant producers and facilitated the biosurfactant deficient mutants to cause disease (Hildebrand, 1989); (2) the residual bacteria were themselves QSS producers and thus were capable of restoring biosurfactant production in the mutants; (3) Pleiotrophic gene effects on other virulence genes as a result of Tn*5* mutagenesis resulted in less disease for some mutants (Darling, 1998).

The *N*-3-hydroxyoctanoyl-HSL which was identified in this study in *P. fluorescens* 5064, has been reported to be present, as well as four other different AHLs, in *P. fluorescens* 2–79 (a biocontrol agent isolated from wheat roots; Shaw et al., 1997). In our *P. fluorescens* 5064, although only one fraction after preparative HPLC induced luminescence in the pSB401 biosensor, it is possible that more than one QSS exists. These QSSs may not be sensitive enough or the quantity is too low to be detected by the biosensors we used.

A large number of pathogenic bacteria control the expression of many of their extracellular virulence factors by a QS system and QS plays an important role in bacterial disease development

and spread (Beck Von Bodman and Farrand, 1995; Flavier et al., 1997). A major objective of an invading bacterium is the evasion of its host's defence. The advantages of employing such a QS mechanism is to avoid premature gene expression, which could possibly alert the host and elicit a defensive response, and to make sure that the invading bacteria will only commence virulence gene expression when they have proliferated to a level where the coordinated production of virulence determinants may overwhelm the host (Pierson et al., 1999). By artificially blocking the cell-to-cell signalling mechanism, pathogenic organisms that use QS to control virulence could potentially be rendered avirulent (Beck Von Bodman and Farrand, 1995). The finding that *P. fluorescens* 5064 regulates biosurfactant production and, we speculate, other virulence factors by QS, opens the possibility for novel disease control methods.

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