Regulation of the biosynthesis of cyclic lipopeptides from Pseudomonas putida PCL1445

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Chapter 3

The *ppuI-rsaL-ppuR* quorum sensing system regulates biofilm formation of *Pseudomonas putida* PCL1445 by controlling biosynthesis of the cyclic lipopeptides putisolvins I and II

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Abstract

Pseudomonas putida strain PCL1445 produces two cyclic lipopeptides, putisolvin I and putisolvin II, which possess surface tension-reducing abilities, are able to inhibit biofilm formation and to break down existing biofilms of several *Pseudomonas* sp. including *P. aeruginosa*. Putisolvins are secreted in the culture medium during growth at late exponential phase, indicating that production is possibly under regulation of quorum sensing. In the present study, we identified a quorum sensing system in PCL1445 composed of *ppuI*, *rsaL* and *ppuR* that shows very high similarity with gene clusters of *P. putida* strains IsoF and WCS358. Mutants in *ppuI* and *ppuR* showed a severe reduction of putisolvin production. Expression analysis of the putisolvin biosynthetic gene in a *ppuI* background showed decreased expression, which could be complemented by the addition of synthetic 3-oxo-C_{10}-AHL or 3-oxo-C_{12}-AHL to the medium.
A *rsaL* mutant overproduces AHLs and production of putisolvins is induced early during growth. Analysis of biofilm formation on polyvinylchloride (PVC) showed that *ppuI* and *ppuR* mutants produce a denser biofilm than PCL1445 which correlated with a decreased production of putisolvins, whereas a *rsaL* mutant shows a delay in biofilm production, which correlates with an early production of putisolvins. The results demonstrate that quorum sensing signals induce the production of cyclic lipopeptides putisolvins I and II and consequently controls biofilm formation by *Pseudomonas putida*.

Introduction

Bacteria can form multicellular aggregates on biotic and abiotic surfaces generally referred to as biofilms. Such communities are ubiquitous in natural environments but can also be found in industrial and clinical settings, for example on artificial surfaces of medical devices, thereby highly contributing to infections (Stewart *et al.*, 2001).

P. putida PCL1445 is capable of forming biofilms on roots and on polyvinylchloride in a commonly used biofilm assay (Kuiper *et al.*, 2004). We have shown that *P. putida* PCL1445 produces two novel lipodepsipeptides, putisolvins I and II, consisting of a C6 lipid moiety and a 12 amino acids peptide, which are produced via a putisolvin synthetase gene designated as *psoA* (Kuiper *et al.*, 2004). A mutant impaired in putisolvin biosynthesis was shown to form a thicker biofilm

than the wild type strain. In addition, purified putisolvins I and II inhibit biofilm formation and break down existing biofilms of various *Pseudomonas* spp. including the opportunistic human pathogen *P. aeruginosa* (Kuiper *et al.*, 2004).

The production of putisolvins occurs at the end of the exponential growth phase (Kuiper *et al.*, 2004), which may indicate that the production is mediated through a quorum sensing mechanism. The term quorum sensing describes an environmental sensing system, which allows bacteria to monitor their own population density. Quorum sensing in Gram-negative bacteria relies on the interaction of small diffusible signal molecules belonging to the class of *N*-acyl homoserine lactones (AHLs). They are synthetized via the LuxI protein, whereas the transcriptional activator protein LuxR couples cell population density to gene expression (Fuqua *et al.*, 2001; Swift *et al.*, 2001). These signal molecules can traffic in and out of the bacterial cell. Once a certain intracellular threshold concentration has been reached, the signals induce transcription of a set of target genes (Fuqua *et al.*, 1994). AHLs play a role in regulating different bacterial functions such as antibiotic biosynthesis, production of virulence factors, bacterial swarming, and transition to the stationary growth phase.

In this chapter we describe (i) the identification and characterization of the regulatory quorum sensing genes affecting cyclic lipopeptides putisolvins I and II in PCL1445, (ii) the involvement of the quorum sensing system in the regulation of biofilm formation of PCL1445 and, (iii) the direct relationship between production of quorum sensing signals, production of cyclic lipopeptides and reduction of the size of the biofilm formed by *P. putida* PCL1445.

Materials and methods

Bacterial strains, and growth conditions

Bacterial strains used in this study are listed in Table 1. *Pseudomonas* strains were grown in King's medium B (King *et al.*, 1954) or in a defined BM medium (Lugtenberg *et al.*, 1999) supplemented with 2.0 % of glycerol (BDH Laboratory Supplies Pool, England) at 28°C. *E. coli* strains were grown in Luria-Bertani medium (Sambrook and Russel, 2001) at 37°C. Media were solidified with 1.8 % agar (Select Agar; Invitrogen, Life Technologies, Paisley, United Kingdom). The antibiotics kanamycin, tetracyclin, gentamycin or carbenicillin, were added when necessary to final concentrations of 50, 40, 2 and 100 μg ml^{-1}, respectively.

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
Pseudomonas		
PCL1445	Wild-type *Pseudomonas putida*; colonizes grass roots and produces biosurfactants	Kuiper et al. (2001)
PCL1633	Tn5*luxAB* derivative of PCL1445; mutated in a *psoA*, a lipopeptide synthetase homologue	This study
PCL1636	PCL1445 derivative mutated in the *ppuI* homologue; constructed by single homologous recombination	This study
PCL1637	PCL1445 derivative mutated in the *ppuR* homologue; constructed by single homologous recombination	This study
PCL1638	PCL1445 derivative mutated in the *rsaL* homologue; constructed by single homologous recombination	This study
PCL1639	PCL1633 derivative mutated in the *ppuI* homologue; constructed by single homologous recombination	This study
E.coli		
DH5α	*EndA1 gyrSA96 hrdR17 (rK-mK-) supE44 recA1*; general purpose host strain used for transformation and propagation of plasmids	Hanahan et al. (1983)
Plasmids		
pBluescript	General-purpose cloning vector, Cb^r	Stratagene, La Jolla, CA
pME6010	Cloning vector which is maintained in *Pseudomonas* strains without selection pressure, Tc^r	Heeb et al. (2000)
pME3049	Cloning vector, used for homologous recombination, Tc^r, Hg^r	Ditta et al. (1980)
pRL1063a	Plasmid harbouring a promotorless Tn5*luxAB* transposon, Km^r	Wolk et al. (1991)
pRK2013	Helper plasmid for tri-parental mating, Km^r	Schnider et al. (1995)
pMP5285	pME3049 derivative, missing the Hg^r gene, used for single homologous recombination, Km^r	Kuiper et al. (2001)
pMP5548	pBluescript containing a 2.2-kb chromosomal fragment of strain PCL1445 with the *ppuI* and *rsaL* genes, and the first part of *ppuR* gene, Cb^r	This study

pMP7565	pME6010 containing a chromosomal fragment of 1.4 kb harbouring the *ppuI* gene of pMP5548, Tc^r	This study
pMP7566	pME6010 containing a PCR fragment of 1.1 kb with the *ppuR* gene of strain PCL1445, Tc^r	This study
pMP7568	pMP5285 containing a 0.5-kb *EcoR*I-*EcoR*I PCR fragment of the central part of *ppuI* gene of PCL1445, Km^r	This study
pMP7571	pMP5285 containing a 0.55-kb *EcoR*I-*EcoR*I PCR fragment of the central part of *ppuR* gene of PCL1445, Km^r	This study
pMP7575	pMP5285 containing a 0.21-kb *Kpn*I-*sal*I PCR fragment of the central part of *rsaL* gene of PCL1445, Km^r	This study
pMP7583	pGEM-T vector containing a 0.6-kb PCR fragment of *ppuI* gene of PCL1445 and a blunted Gm^r box; Cb^r, Gm^r	This study
pMP7587	pME6010 containing a PCR fragment of 1.6 kb harbouring the *rsaL* functional gene of strain PCL1445, Tc^r	This study
pAK211	Autoinducer reporter construct based upon the *Vibrio fisheri* bioluminescence (*lux*) system; Cm^r	Kuo et al. (1994)
pSB1075	Bioluminescent AHL sensor plasmid containing a fusion of *lasRI*::*luxCDABE* in pUC18; used for the detection of long-chain AHLs; Cb^r.	Winson et al. (1998)

Extraction and detection of AHLs autoinducers from spent culture medium

To isolate autoinducer activity, 3 volumes of dichloromethane were added to 7 volumes of supernatant of a 50 ml BM bacterial culture and shaken for 1 h at 120 rpm. The organic phase was removed and dried by evaporation under vacuum to dryness (Mc Clean *et al.*, 1997). Supernatant extracts were redissolved in 100 µl of ethyl acetate and 10 µl fractionated on a C18 reverse-phase TLC plate (Merck, Darmstadt, Germany), developed in methanol-water (60:40; vol/vol).

To detect autoinducer activity, overnight cultures of *E.coli* DH5α containing pAK211 (Kuo *et al.*, 1994) or pSB1075 (Winson *et al.*, 1998) were grown in LB medium supplemented with 20 µg of chloramphenicol or carbenicillin per ml, respectively, for 10 h. TLC plates were overlaid with 0.8 % LB top agar layer containing 50 µl of the pAK211 or pSB1075 strains per ml, followed by incubation

at 28°C for 16 h. Autoinducer activity was then detected by the emission of light after applying a Fuji medical X-Ray film (Fuji Photo Film CO., Ltd., Tokyo, Japan) on the TLC plates.

Isolation and identification of quorum sensing gene homologs

A plasmid library of chromosomal fragments of strain PCL1445 was constructed by cloning 1.5 to 3.0-kb fragments of chromosomal DNA digested with *EcoR*I into pBluescript (Stratagene, La Jolla, CA). The resulting fragment library was introduced into an *E. coli* strain harbouring the *lux* reporter plasmid pAK211 (Kuo *et al.*, 1994). After overnight growth on LB agar plates, clones that induced the luciferase reporter were identified using photographic film. To remove pAK211 (Kuo *et al.*, 1994) from the *E. coli* reporter strain, total plasmid was isolated and reintroduced into DH5α cells by standard transformation protocols (Schnider *et al.*, 1995) followed by carbenicillin selection, whereas chloramphenicol selection was omitted. The nucleotide sequence of the chromosomal fragment inserted in the selected plasmid pMP5548 was determined using universal primers - 40 reverse primer flanking the multiple cloning site of pBluescript.

Construction of ppuI*,* ppuR *and* rsaL *mutant strains*

ppuI mutant derivatives of strains PCL1445 and PCL1633 were constructed by homologous recombination. A 0.5-kb internal fragment of *ppuI* of strain PCL1445 was obtained by PCR using primers oMP902 (5'-ATGCATAAACTTCGGGCA-3') and oMP903 (5'-CATTTTCTCGACCCCCAC-3'), cloned into the pGEM-T Easy Vector System I (Promega Corporation, Madison, WI) and ligated as a *EcoR*I-*EcoR*I insert in the pMP5285 suicide plasmid (Kuiper *et al.*, 2001) derived from pME3049 (Ditta *et al.*, 1980) resulting in pMP7568. pMP7568 was transferred to PCL1445 by tri-parental mating using pRK2013 as a helper plasmid (Schnider *et al.*, 1995) and using selection on KB agar medium supplemented with kanamycin (50 µg ml^{-1}). Strain PCL1636 was obtained as a kanamycin resistant colony resulting from single homologous recombination. The insertion of the suicide construct was confirmed by sequence analysis.

To construct a PCL1633 *ppuI* mutant, the pGEM-T vector containing the 0.5-kb fragment of *ppuI* and a gentamycin resistance cassette cloned as a *Sal*I-*Sal*I fragment resulting in pMP7583, was used as a suicide plasmid. Single homologous recombination in PCL1633 carried out using pMP7583 resulted in PCL1639. A *P.*

putida PCL1445 *ppuR* mutant was constructed using a similar mutagenesis strategy. The *ppuR* fragment for the construction of the pMP5285 based suicide plasmid pMP7571 resulted from a PCR reaction using primers oMP905 (5'-AATTCTTCGAAGAAGCCGCCG-3') and oMP906 (5'-TTGCTGGATGGCTTTGAGCACC-3') and chromosomal DNA of strain PCL1445 as a template. Single homologous recombination in *ppuR* of PCL1445 resulted in strain PCL1637.

The *P. putida* PCL1445 *rsaL* mutant was constructed using the pMP5285 suicid plasmid based pMP7575 obtained after cloning a 0.21-kb *Kpn*I-*Sal*I PCR fragment of the central part of *rsaL* gene of PCL1445 obtained using primers oMP897 (3'-TACCTCAGCTGTGCGCGAGGT-5') and oMP898 (3'-GGTGGGCCAGGTCGCTTTCCT-5'). Single homologous recombination in *rsaL* of PCL1445 resulted in strain PCL1638.

Complementation of ppuI*,* ppuR*, and* rsaL *mutants of PCL1445*

Complementation of strain PCL1636 (*ppuI*) was carried out using pMP7565, a shuttle vector derived from pME6010 (Heeb *et al.*, 2000) in which a 1.4-kb fragment containing *ppuI* and *rsaL* of strain PCL1445 was inserted. This insert was obtained by *EcoR*I digestion from pMP5548. pMP7565 was transferred to strain PCL1636 by tri-parental mating as described above and transformants were selected on KB agar medium supplemented with tetracyclin (40 µg ml^{-1}). To complement the *ppuR* insertion in PCL1637, a 1.1-kb PCR fragment containing the *ppuR* gene of strain PCL1445 was obtained using primers oMP883 (3'-TGTATATCCTGCTGCGCCTTTA-5') and oMP884 (3'-CATGTGCATCGTGGTGCTGCCT-5'), and cloned into pME6010, resulting in pMP7566.

To complement the *rsaL* insertion in PCL1638, a 1.6-kb PCR fragment containing *rsaL* gene of strain PCL1445 was obtained using primers oMP1011 and oMP1012 (3'-TTGTCAAGCAGTGCCACTGGTTCTAGAAAA-5') and oMP1012 (3'-ATCAGCGACATCTAGTCGTGGGAGCTCAAA-5'), and cloned into pME6010, resulting in pMP7587.

Biosurfactant production

The production of biosurfactant activity was detected using the drop collapsing assay as described previously (Jain *et al.*, 1991), in which the reduction of the water surface tension can be observed as the collapse of a round droplet placed on a hydrophobic surface (Jain *et al.*, 1991).

To quantify the biosurfactant production in culture medium, the decrease of surface tension between culture medium and air was determined using a Du Nouy ring (K6 Krüss, GmbH, Hamburg, Germany).

Extraction and High-Performance Liquid Chromatography (HPLC) analysis of putisolvins

To quantify the production of putisolvins in BM culture medium, 10 ml of a BM culture supernatant was extracted with one volume of ethyl acetate (Fluka Chemie, Zwijndrecht, The Netherlands) as described previously (Kuiper *et al.*, 2004). Ethyl acetate extracts were evaporated under vacuum to dryness and dissolved in 55 % acetonitrile (Labscan Ltd, Dublin, Ireland). The dried pellet obtained from 10 ml culture was resuspended in 500 µl of 50/50 acetonitrile/water (v/v) and filtered using a spinX centrifuge tube filter of 0.45 µm pore size (Corning Costar Corporation, Cambridge, MA). A volume of 500 µl of the samples was separated by HPLC (Jasco International CO. Lt., Japan), using a reverse phase C8 5 µm Econosphere column (Alltech, Deerfield, IL), a PU-980 pump system (Jasco, B&L systems, Boechout, Belgium), a LG-980-02 gradient unit (Jasco) and a MD 910 detector (Jasco). Separation was performed using a linear gradient, starting at 35/65 acetonitrile/water (v/v) and ending at 20/80 after 50 min at a flow rate of 1ml min^{-1}. Chromatograms were analyzed in the wavelength range between 195 nm and 420 nm. Fractions that corresponded to the retention time of 20 min for putisolvin I and 21 min for putisolvin II were collected and tested for activity in the drop collapsing assay. The amount of putisolvins produced was quantified as the peak area in micro absorbance units (µAU) at 206 nm.

*Quantification of bioluminescent Tn5*luxAB *reporter strains*

Expression of Tn5*luxAB* genes was determined by quantification of bioluminescence during culturing. Cells from overnight cultures were washed with fresh medium and diluted to an OD_{620} of 0.1. Cultures were grown in BM medium in a volume of 10 ml under vigorous shaking. During growth 100 µl samples were taken in triplicate to quantify luminescence. A volume of 100 µl of an 0.2 % *n*-decyl-aldehyde substrate solution (Sigma, St. Louis, MO) in a 2.0 % bovine serum albumin solution was added and luminescence determined with a MicroBeta 1450 TriLux luminescence counter (Wallac, Turku, Finland), which was normalized to the luminescence per OD_{620} unit. The synthetic AHL molecules *N*-hexanoyl-L-

homoserine lactone (C_6-AHL) (Fluka, Zwijndrecht, The Netherlands), *N*-octanoyl-L-homoserine lactone (C_8-AHL) (Fluka), *N*-decanoyl-L-homoserine lactone (C_{10}-AHL) (Fluka), *N*-dodecanoyl-L-homoserine lactone (C_{12}-AHL), *N*-(3-oxo-hexanoyl)-L-homoserine lactone (3-oxo-C_6-AHL), *N*-(3-oxo-octanoyl)-L-homoserine lactone (3-oxo-C_8-AHL), *N*-(3-oxo-decanoyl)-L-homoserine lactone (3-oxo-C_{10}-AHL), *N*-(3-oxo-dodecanoyl)-L-homoserine lactone (3-oxo-C_{12}-AHL), and *N*-(3-oxo-tetradecanoyl)-L-homoserine lactone (3-oxo-C_{14}-AHL) were tested for the ability to induce the Tn5*luxAB* reporter strains. Briefly, cells were grown in BM – 2 % glycerol medium for 48 h, washed, and resuspended to an OD_{620} of 0.1 in fresh medium supplemented with either 5 µM synthetic AHL or 25 µl of a 1000-fold concentrated crude extracts of spent culture supernatant dissolved in 100 % acetonitrile.

Biofilm assay

Biofilm formation on polyvinylchloride (PVC) was conducted as described by O'Toole and Kolter (1998) and adapted for strain PCL1445 as described by Kuiper *et al.* (2004). When the effect of AHLs on biofilm formation was tested, the culture medium and planktonic cells was removed after 4 h. Subsequently, 100 µl of M63 medium containing 1µl of 5 µM synthetic 3-oxo-C_{12}-AHL dissolved in 100% acetonitrile was added to the wells. An equal volume of acetonitrile was added to control wells. All conditions were tested in triplicate.

Nucleotide sequence accession number

The nucleotide sequences of the *P. putida* PCL1445 *ppuI-rsaL-ppuR* DNA region and putisolvin synthetase promoter region reported in this paper have been deposited in the GenBank database, respectively, under accession numbers DQ151886 and DQ151887.

Results

Production of AHLs by P. putida PCL1445

To test the possible production and secretion of AHLs, a crude dichloromethane extract of the spent BM-glycerol medium of a culture of OD_{620} 1 was tested for induction of *E. coli* reporter strain based on the *lux* quorum sensing system of *Vibrio fischeri* (Fig. 1A) and the *las* system of *P. aeruginosa* (Fig. 1B). After separation on C_{18} reverse phase TLC, four compounds were detected with R_f values

similar to those of 3-oxo-C_{12}-, 3-oxo-C_{10}-, 3-oxo-C_8-, and 3-oxo-C_6-AHLs (Fig.1A and B). Furthermore, when the standard molecules were mixed with PCL1445 dichloromethane extracts, the four detected compounds co-migrated with the standard AHLs (Fig. 1A, lane 3 and Fig. 1B, lane 3). Dichloromethane extracts of the putisolvin biosynthetic mutant PCL1633 showed the same profile as the wild type strain (data not shown).

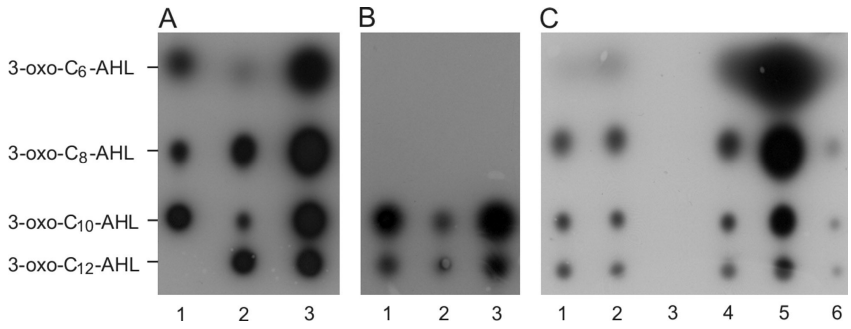

Fig. 1. C18-reverse phase thin-layer chromatography analysis of *N*-acyl-L-homoserine lactones produced by *P. putida* PCL1445 and its mutant derivatives. Cells of strain *P. putida* PCL1445 and its derivatives *ppuI* mutant PCL1636 strain, PCL1636 harbouring plasmid pMP5548 (*ppuI*), the *rsaL* mutant PCL1638, and PCL1638 harbouring plasmid pMP7587 (*rsaL*) were grown in BM-glycerol to OD_{620} 0.7 and centrifuged. The supernatant fluids were extracted with dichloromethane and the organic fractions were analyzed using TLC. The chromatograms were overlayed with *E. coli* reporter strains for the detection of AHLs. **Panel A.** The biosensor *E. coli* harbouring pAK211 was used to visualize AHLs produced by PCL1445. Lane 1: 16 ng of 3-oxo-C_6-AHL, 20 ng of 3-oxo-C_8-AHL, and 50 ng of 3-oxo-C_{10}-AHL were mixed. Lane 2: culture supernatant extract of PCL1445. Lane 3: culture supernatant of PCL1445, 16 ng of 3-oxo-C_6-AHL, 20 ng of 3-oxo-C_8-AHL, and 50 ng of 3-oxo-C_{10}-AHL were mixed. **Panel B.** The biosensor *E. coli* harbouring pSB1075 was used to visualize long chains AHLs produced by PCL1445. Lane 1: 50 ng of *N*-(3-oxo-decanoyl)-L-homoserine lactone (3-oxo-C_{10}-AHL) and 50 ng of *N*-(3-oxo-dodecanoyl)-L-homoserine lactone (3-oxo-C_{12}-AHL) were mixed. Lane 2: culture supernatant extract of PCL1445 cells. Lane 3: culture supernatant extract of PCL1445 cells, 50 ng of 3-oxo-C_{10}-AHL, and 50 ng of 3-oxo-C_{12}-AHL were mixed. **Panel C.** The biosensor *E. coli* harboring pAK211 was used to visualize AHLs present in culture supernatant extracts of cells of strains PCL1445, PCL1636 (*ppuI*), and PCL1638 (*rsaL*). Lane 1: PCL1445, Lane 2: PCL1445 harbouring the cloning vector pME6010. Lane 3: PCL1636 (*ppuI*). Lane 4: PCL1637 harbouring pMP5548 (*ppuI*). Lane 5: PCL1638 (*rsaL*). Lane 6: PCL1638 harbouring pMP7587 (*rsaL*).

Identification of quorum sensing genes of P. putida PCL1445

To isolate a chromosomal fragment of strain PCL1445 containing *luxI* and *luxR* homologues, an *Eco*RI chromosomal library of PCL1445 was introduced into *E. coli* DH5α containing pAK211, a reporter strain for AHLs based on the *lux* system of *Vibrio fisheri* (Kuo *et al.*, 1994). The plasmid of one luminescent transformant, pMP5548, was isolated for analysis. Nucleotide sequence analysis of the 2.2-kb genomic fragment present in pMP5548 revealed the presence of several ORF's, which show homologies to *suhB*, *ppuI*, *rsaL*, and *ppuR* of *P. putida*. The identified sequences of the genes showed 99 % identity with the *ppu* locus characterized in *P. putida* strains IsoF (Steidle *et al.,* 2002) and WCS358 (Bertani *et al.*, 2004), 57 % identity with *lasI* of *P. aeruginosa* (Pearson *et al.*, 1994), and 51 % identity with *mupI* of *P. fluorescens* (El-Sayed *et al.*, 2001) (Fig. 2A). The sequence of the gene located upstream of *ppuI* showed 100 % identity with *rsaL* gene in *P. putida* IsoF (Steidle *et al.*, 2002) and WCS358 (Bertani *et al.*, 2004), and 60 % identity with *rsaL* of *P. aeruginosa* (de Kievit *et al.*, 1999). The *rsaL* gene was first described as a repressor of virulence genes in *P. aeruginosa* and later as a repressor of the *ppuI* gene in *P. putida* strains IsoF and WCS358. The ORF located downstream of *ppuI* showed 91 % identity with the *suhB* of *P. putida* IsoF (Steidle *et al.*, 2002) and 78 % with *suhB* of *P. aeruginosa*. The latter gene was suggested to possess inositol monophosphatase activity in *E. coli* (Matsuhisa *et al.*, 1995).

To test whether the *ppuI* gene present in pMP5548 was responsible for the production of C_{10}-, 3-oxo-C_{10}-, C_{12}-, and 3-oxo-C_{12}-AHLs, dichloromethane extracts of the DH5α reporter containing pAK211 with or without pMP5548 were subjected to TLC analysis. The results showed the presence of the four AHLs detected in PCL1445 crude extracts with similar Rf-values to C_{10}-, 3-oxo-C_{10}-, C_{12}-, and 3-oxo-C_{12}-AHLs (data not shown).

In the region upstream of *ppuI* and *ppuA* nucleotide sequences were found which were identical to *ppuI* and *ppuA lux* box elements found in *P. putida* strains IsoF (Steidle *et al.*, 2002) and WCS358 (Bertani *et al.*, 2004).

An 16-bp palindromic sequence with high similarity to the *lux* box elements, which are located in the promoter region of quorum sensing regulated genes of *P. putida* (Steidle *et al.*, 2002), *P. aeruginosa* (Whiteley *et al.*, 2000), *P. chlororaphis* (Chin-A-Woeng *et al.*, 2001), and *V. fischeri* (Devine et al., 1989), is present 92 bp

upstream of the *psoA* gene start codon (Fig. 2B) (Dubern *et al.*, 2005). These palindromes might constitute a binding site for the LuxR response regulator.

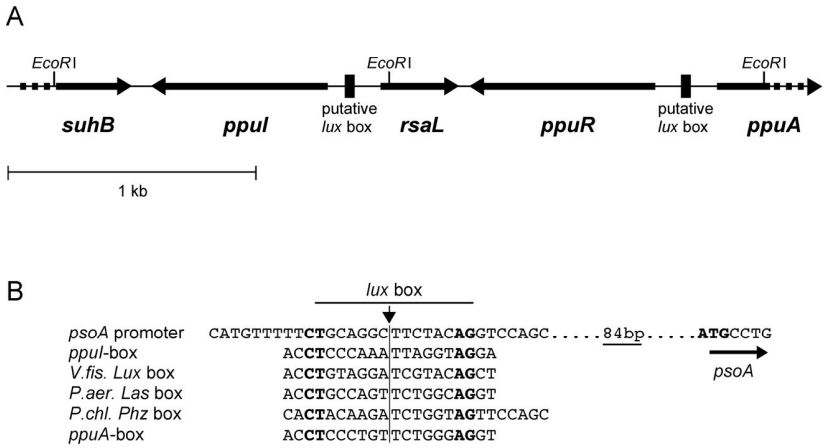

Fig. 2. *ppu* locus and analysis of the *lux* box in the upstream region of putisolvin biosynthetic gene *psoA* of *P.putida* PCL1445. **Panel A.** *ppu* locus of strain PCL1445. Putative *lux* boxes are present in the intergenic regions of *ppuI-rsaL* and of *ppuR-ppuA*, respectively. Dotted lines indicate non-determined sequence. **Panel B.** Comparison of a *lux* box homologous sequence in the region upstream of *psoA* gene of *P. putida* PCL1445 with similar sequences.

Expression of the putisolvin biosynthetic gene psoA *is stimulated by AHLs*

To analyze the effect of a mutation in the AHL biosynthetic gene *ppuI* on the expression of the *psoA*, *ppuI* was mutated in strain PCL1633 (*psoA*::Tn5*luxAB)*, resulting in strain PCL1639 (*psoA*/*ppuI*)*,* in which *psoA* expression was quantified by measuring luminescence. The *psoA* expression appeared to be 10-fold lower in strain PCL1639 (*ppuI*/*psoA*) when compared to the transcriptional activity detected in PCL1633 (*psoA*) (Table 2). Transcriptional activity of the *psoA* promoter was analyzed in strain PCL1639 (*ppuI*/*psoA*) in liquid culture at OD_{620} 1.5 after addition of crude 1000-fold concentrated dichloromethane extracts of the wild type strain culture supernatant, or of the *ppuI* mutant, to early-log phase culture (OD_{620} 0.2) of PCL1639. PCL1445 dichloromethane extract but not *ppuI* mutant extract, was able to complement part of the *psoA* promoter activity in PCL1639 (Table 2).

The response to C_4-, C_6-, 3-oxo-C_6-, C_8-, C_{10}-, 3-oxo-C_{10}-, C_{12}-, and 3-oxo-C_{12}-AHLs signals, added at a concentration of 5 µM to early-log phase culture (OD_{620} 0.2) of PCL1639, on *psoA* transcriptional activity was quantified at OD_{620} 1.5.

The addition of AHLs without a 3-oxo-group or with short acyl chains (C_4, C_6 and C_8) did not significantly affect *psoA*::*luxAB* expression (Table 2). However, the *psoA* promoter activity was stimulated by addition of 3-oxo-C_{10}-AHLs and even more by 3-oxo-C_{12}-AHLs (Table 2).

Table 2. Transcriptional activity of *psoA* of *P. putida* PCL1445 in response to synthetic AHLs.

Strain[a]	***N*-acyl homoserine lactone (AHL) (5 µM)**	**Supernatant**		**Bioluminescence/Cell density (x 10^3 LCPS/OD_{620nm})**
		PCL1445	**PCL1636 (*ppuI*)**	
PCL1445	None	-	-	0.06 ± 0.01
PCL1633 (*psoA*)	None	-	-	3.48 ± 0.07
PCL1639 (*psoA*/*ppuI*)	None	-	-	0.34 ± 0.03
	None	-	+	0.33 ± 0.03
	None	+	-	2.71 ± 0.11
	C_4-AHL	-	-	0.23 ± 0.03
	C_6-AHL	-	-	0.22 ± 0.01
	3-oxo-C_6-AHL	-	-	0.25 ± 0.02
	C_8-AHL	-	-	0.31 ± 0.01
	3-oxo-C_8-AHL	-	-	0.26 ± 0.02
	C_{10}-AHL	-	-	0.35 ± 0.03
	3-oxo-C_{10}-AHL	-	-	2.10 ± 0.14
	C_{12}-AHL	-	-	0.32 ± 0.05
	3-oxo-C_{12}-AHL	-	-	3.11 ± 0.13
	3-oxo-C_{14}-AHL	-	-	2.15 ± 0.20

[a]Expression of the putisolvin biosynthetic gene *psoA* was determined by measuring bioluminescence from cells cultures of the double mutant PCL1639 (*psoA*::Tn5*luxAB*/*ppuI*) grown to OD_{620} 1.5 in BM-glycerol medium. Crude 1000-fold concentrated dichloromethane extracts of the wild type strain culture supernatant, or of the *ppuI* mutant, or AHLs molecules were added to early-log phase culture (OD_{620} 0.2). Standard deviations are based on the mean values of three parallel cultures.

Construction and characterization of ppuI*,* ppuR*, and* rsaL *mutants*

To investigate whether *ppuI*, *ppuR*, and *rsaL* are involved in putisolvin production, insertion mutants were constructed by single homologous recombination using suicide plasmids pMP7568, pMP7571 and pMP7575, respectively (see Materials and Methods section), resulting in strains PCL1636, PCL1637 and PCL1638, respectively. The proper integration of plasmids pMP7568, pMP7571 and pMP7575 by homologous recombination into the chromosome was confirmed by sequencing the region flanking the suicide plasmids after isolation of the chromosomal DNA recombinants.

Putisolvin production by mutant strains PCL1636 (*ppuI*), PCL1637 (*ppuR*), and PCL1638 (*rsaL*) was investigated by two different approaches. Firstly, biosurfactant production by strains PCL1626 (*ppuI*), PCL1637 (*ppuR*), and PCL1638 (*rsaL*) was quantified during growth until the stationary phase was reached by the Du Nouy ring method (Fig. 3A). Secondly, the production of putisolvins I and II by strains PCL1445, PCL1636 (*ppuI*), PCL1637 (*ppuR*) and PCL1638 (*rsaL*) was tested by HPLC analysis (Fig. 3B and 3C).

Culture supernatants of PCL1636 (*ppuI*) and PCL1637 (*ppuR*) were not able to decrease the surface tension between culture medium and air when compared to the wild type, indicating a lack of biosurfactant production (Fig. 3A). Culture supernatant of strain PCL1638 (*rsaL*) caused a decrease of surface tension during the early exponential phase (to 32 mN m^{-1} at OD 1), indicating an earlier production of biosurfactant than by the wild type strain (48 mN m^{-1} at OD 1) (Fig. 3A).

Mutants PCL1636 (*ppuI*) and PCL1637 (*ppuR*) showed a significant reduction (85 %) of putisolvin production (Fig. 3B, bars c and e, respectively). Introduction of pMP7565, harbouring the genomic fragment from pMP5548 with *ppuI* and pMP7566 harbouring *ppuR* restored putisolvin production to wild type levels in both strains PCL1636 (*ppuI*) and PCL1637 (*ppuR*) (Fig. 3B, bars d and f, respectively). The production of putisolvins by PCL1445 and mutant PCL1638 (*rsaL*) was compared by HPLC analysis at different stages of bacterial growth (Fig. 3C). Mutant PCL1638 (*rsaL*) shows a significantly increased putisolvin production during the early exponential phase when compared to the wild type (4-fold at OD_{620} 0.6). This difference in production tends to decrease when the cells reach the stationary phase (2-fold at OD_{620} 1.1, and hardly any difference at OD_{620} 2). Introduction of pMP7587 harbouring *rsaL* into PCL1638 (*rsaL*) significantly decreased putisolvin

production during exponential phase resulting in lower value than for the wild type strain (2-fold lower at OD_{620} 0.6), which could be explained by the multiple copy effect of the plasmid used for complementation of *rsaL* mutation (Fig. 3C).

To investigate the involvement of *ppuI* in AHLs biosynthesis in PCL1445 and *rsaL* in regulation of AHLs biosynthesis in PCL1445, AHLs production by strains PCL1445, PCL1636 (*ppuI*), PCL1636 harbouring pMP7565 (*ppuI*), PCL1638 (*rsaL*), and PCL1638 harbouring pMP7587 (*rsaL*) was examined by TLC analysis. PCL1445 harbouring the control vector pME6010 did not have any influence on the AHL production when compared to the wild type without pME6010 (Fig. 1C, lane 2). Mutant PCL1636 (*ppuI*) showed a total absence of AHL production (Fig. 1C, lane 3). AHL production of PCL1636 (*ppuI*) was restored by introduction of pMP7565 harbouring a functional *ppuI* gene (Fig. 1C, lane 4). Finally, mutating *rsaL* (PCL1638) had a strong positive effect on the production of AHLs of PCL1445 (Fig. 1C, lane 5). The AHL production of PCL1628 (*rsaL*) decreased dramatically by introduction of pMP7587 harbouring a functional *rsaL* gene when compared to the wild type (Fig 1C, lane 6).

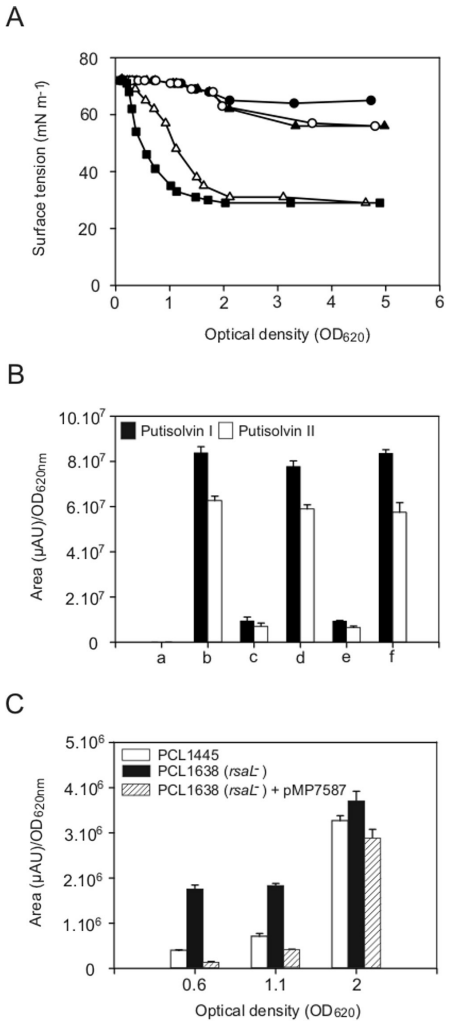

Fig. 3. Effects of mutations in *ppuI*, *ppuR*, and *rsaL* on the production of putisolvins of *P. putida* PCL1445. **Panel A**. Quantification of surface tension decrease by culture supernatants of *P. putida* strain PCL1445 (Δ), PCL1633 (*psoA*) (●), PCL1636 (*ppuI*) (○), PCL1637 (*ppuR*) (▲), and PCL1638 (*rsaL*) (■) grown to the stationary phase in BM-glycerol medium. **Panel B**. C8-Reverse Phase HPLC analysis of putisolvin production by *P. putida* strain PCL1445 and its mutants PCL1636 (*ppuI*) and PCL1637 (*ppuR*). a. Mutant strain PCL1633 (*psoA*). b. PCL1445. c. PCL1636 (*ppuI*). d. PCL1636 harboring pMP5548 (*ppuI*). e. PCL1637 (*ppuR*). f. PCL1637 harboring pMP7566 (*ppuR*). Cells were grown to the stationary phase in 5 ml BM-glycerol medium at 28°C under vigorous aeration. Ethyl acetate extracts of culture supernatants were separated and the peak areas of putisolvin I and II were quantified at a wavelength of 206 nm. **Panel C**. C8-Reverse Phase HPLC analysis of putisolvin production by PCL1445, mutant

PCL1638 (*rsaL*), and PCL1638 harbouring pMP7587 (*rsaL*). Compounds from the ethyl acetate extracted culture supernatant of cultures grown to OD 0.6, 1.1, and 2 in BM-glycerol were separated and analyzed by HPLC as described under panel B.

Effect of ppu *quorum sensing system on biofilm formation of PCL1445*

Biofilm formation on PVC titer wells by PCL1445 and its mutants PCL1633 (*psoA*), PCL1636 (*ppuI*), PCL1637 (*ppuR*), and PCL1638 (*rsaL*) was measured at various times after inoculation (Fig. 4A). The size of the biofilms formed by mutants PCL1636 (*ppuI*) and PCL1637 (*ppuR*) was comparable to that of the putisolvin-deficient mutant (PCL1633) and considerably thicker than that of the wild type (Fig. 4A). To monitor the surfactant activity produced by the bacterial cells in the titer wells, culture samples were analyzed by the drop collapsing assay. Indexes from 0 to 4 were used to quantify biosurfactant production by bacterial cell in the biofilm assay (Fig. 4E). PCL1636 (*ppuI*) and PCL1637 (*ppuR*) did not produce any detectable biosurfactant activity (Fig. 4B). Analysis of PCL1638 (*rsaL*) showed that biofilm formation decreased 1.5 fold as compared with the wild type strain (Fig. 4A), which correlates with an earlier appearance of biosurfactant activity (visible after 6 h) than observed for PCL1445 (visible after 10 h) (Fig. 4B).

The effect of AHLs produced by PCL1445 on its biofilm forming ability and consequently on the production of biosurfactants was analyzed in two different ways: (i) mutant PCL1636 (*ppuI*) was transformed with pMP5548 harbouring *ppuI* and (ii) exogenous 3-oxo-AHL (5 µM) was added to the medium. Biofilms were assayed after 24 h of incubation (Fig. 4C). Mutants PCL1636 (*ppuI*) (Fig. 4C, bar e) and PCL1633 (*psoA*) (Fig. 4C, bar d) form thicker biofilms than the wild type (Fig. 4C, bar b). Introduction of pMP5548 into PCL1636 (*ppuI*) restored biosurfactant production (Fig. 4D, lane f) and decreased biofilm formation to the same level as reached by PCL1445 (Fig. 4C, bar f). Exogenous 3-oxo-C_{12}-AHL signaling molecules appeared also to be able to stimulate production of biosurfactant by *ppuI* mutants (Fig. 4D, lane j), and to reduce the thickness of the biofilm (Fig. 4C, bar j).

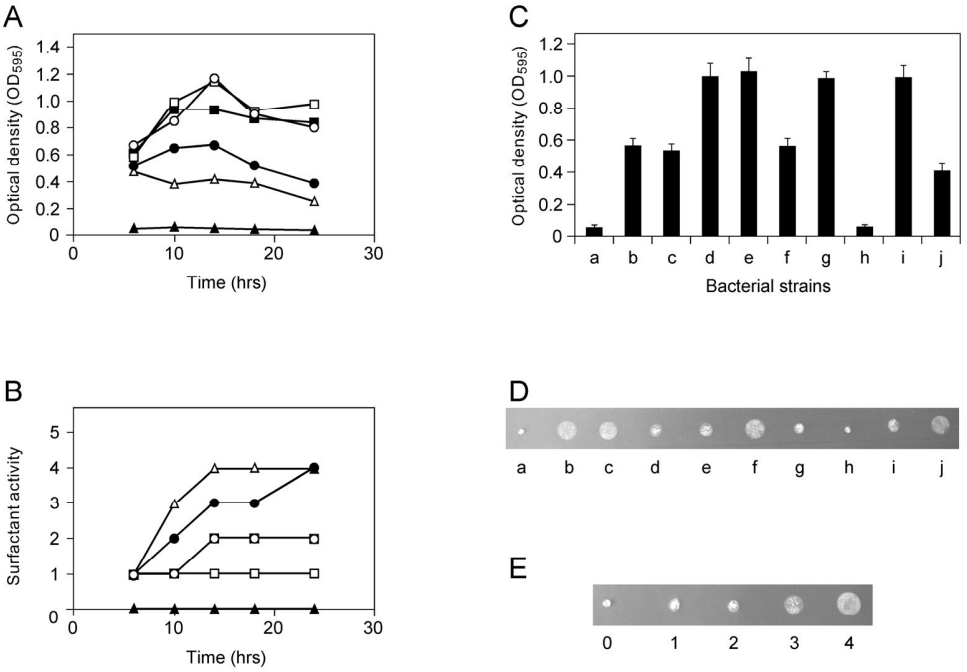

Fig. 4. Influence of quorum sensing on biofilm formation of *P. putida* PCL1445 in PVC micro titer wells. Cells of PCL1445 and its quorum sensing mutant derivatives were incubated in microtiter plates in M63 medium and their biofilm formation quantified over time using the crystal violet-staining procedure. Cells attached to the microtiter wells were stained with crystal violet, washed and the crystal violet in the biofilm was dissolved in ethanol after which the OD_{595} was measured. To determine surface tension reducing activity in the well, 25 µl of culture was pipetted as a droplet on parafilm and allowed to dry. The diameter of the dried droplet correlates with surface tension reduction. A surfactant activity index based on the droplet diameter ranging from 0 to 4 was used to quantify surface tension reduction. All experiments were performed in triplicate. **Panel A**. Time course of biofilm formation of PCL1445 (●), PCL1633 (*psoA*) (□), PCL1637 (*ppuI*) (■), PCL1638 (*ppuR*) (○), and PCL1639 (*rsaL*) (Δ). As a negative control, uninoculated M63 medium was used (▲). **Panel B**. Biosurfactant activity present in the titer well during biofilm formation of bacterial cultures presented in panel A as determined by the drop collapsing assay. **Panel C**. Biofilm formation of PCL1445 and PCL1636 (*ppuI*) measured after 24 hours of incubation. Bar a, M63 medium without bacteria; bar b, PCL1445; bar c, PCL1445 containing pME6010; bar d, PCL1436 (*psoA*); bar e, PCL1636 (*ppuI*); bar f, PCL1636 (*ppuI*$^-$) harboring pMP5548 (*ppuI*); bar g PCL1639 (*psoA*/*ppuI*); bar h, M63 + 3-oxo-C12-AHL (5 µM); bar i PCL1636 (*ppuI*) + pure acetonitrile (control); bar j PCL1636 (*ppuI*) + 3-oxo-C12-AHL (5 µM). Standard deviations are based on the mean values of triplicate cultures. **Panel D**. Biosurfactant activity shown by the drop collapsing assay of

bacterial cultures in the biofilm assay in panel C; lane a. M63 medium without bacteria; lane b. PCL1445. lane c. PCL1445 containing pME6010. lane d. PCL1436 (*psoA*). lane e. PCL1636 (*ppuI*). Lane f. PCL1636 (*ppuI*) harbouring pMP5548 (*ppuI*). lane g. PCL1639 (*psoA*/*ppuI*). lane h. M63 + 3-oxo-C12-AHL (5 µM). Lane i. PCL1636 (*ppuI*) + pure acetonitrile (control). Lane j. PCL1636 (*ppuI*) + 3-oxo-C12-AHL (5 µM). **Panel E.** Index 0-4 used for the detection of biosurfactants production by bacterial cell in the biofilm assay. Shown are dried droplets of 25 µl culture supernatant with increased diameter due to decreased surface tension caused by increased biosurfactants activity.

Discussion

Pseudomonas putida strain PCL1445 produces two cyclic lipopeptides biosurfactants, putisolvins I and II, which inhibit biofilm formation and degrade existing *Pseudomonas* biofilms (Kuiper *et al.*, 2004). Initiation of putisolvin production starts at the onset of stationary phase (Kuiper *et al.*, 2004) suggesting that putisolvin biosynthesis might be population density regulated, which would imply that putisolvins are regulating the formation and thickness of the biofilm after the initial formation steps of the biofilm at high bacterial cell density. The aim of this work was to determine whether quorum sensing is regulating the production of the cyclic lipopeptides putisolvins I and II by *P. putida* PCL1445 and, consequently, biofilm formation.

Using several bacterial reporter strains for the detection of AHLs we showed that PCL1445 produces at least four different inducing compounds, which are migrating at the same positions as 3-oxo-C_6-, 3-oxo-C_8-, 3-oxo-C_{10}-, and 3-oxo-C_{12}-AHL on TLC (Fig. 1). Two of these compounds, 3-oxo-C_{10}- and 3-oxo-C_{12}-AHLs, were shown to restore *psoA* promoter activity in double mutant PCL1639 (*ppuI*/*psoA*) (Table 2). The AHLs lacking the 3-oxo-group did not stimulate the *psoA* promoter (Table 2). Furthermore, we detected a palindromic sequence in the promoter region of *psoA* similar to the regulatory *lux* box (Fig. 2B), the presence of which is typical for genes under control of quorum sensing.

Regulation via quorum sensing involves a LuxI (homologous) protein, which directs the synthesis of signaling molecules, and the cognate transcriptional regulator LuxR, which binds to the operator of the target regulated gene. In strain PCL1445, a *luxI* homologous gene was identified as *ppuI* and a *luxR* homologous gene as *ppuR* (Fig. 2A). The *ppuI* and *ppuR* genes are transcribed in the same direction and separated by *rsaL*, which is transcribed in the opposite direction (Fig.

2A). RsaL was reported to play a role in the repression of *lasI* of *P. aeruginosa* (de Kievit *et al.*, 1999) and of *ppuI* of *P. putida* WCS358 (Bertani *et al.*, 2004). A highly conserved palindromic sequence (*lux* box) was identified in the promoter regions of the *ppuI* and *ppuR* genes (Fig 2A). Such a regulatory element is thought to represent the binding site for the LuxR homolog after activation by binding the appropriate AHL. The genetic organization of the *ppu-rsaL-ppuR* locus of PCL1445 is identical to the loci identified in *P. putida* IsoF (Steidle *et al.*, 2002) and *P. putida* WCS358 (Bertani *et al.*, 2004). Although the *ppuI-rsaL-ppuR* locus was reported to be involved in biofilm formation by *P. putida* IsoF, the molecular mechanism could not be explained (Steidle *et al.*, 2002). Members of the *luxI* and *luxR* families usually show weak homologies. The *ppuI/ppuR* quorum sensing system is not widespread among *P. putida* members but seems to be evolutionary well conserved and might regulate similar genes (Steidle *et al.*, 2002).

More detailed studies showed that a mutation in *ppuI* of PCL1445 abolishes the production of all four detected AHL compounds (Fig. 1C), indicating that *ppuI* is responsible for the production of AHLs. Mutation of *ppuI* and *ppuR* abolishes putisolvin production almost completely (Fig. 3B). Transcriptional analysis of the *psoA* promoter in a *ppuI* mutant background showed clearly that at least one of the quorum sensing signals present in the medium (3-oxo-C_{12}-AHL), which can be synthesized via *ppuI*, is able to induce putisolvin biosynthesis (Table 2). Our results show that *ppuI* and *ppuR* are responsible for production of AHLs and regulate putisolvin expression in PCL1445. Mutation of *rsaL* resulted in an increased AHL production (Fig. 1C), suggesting that *rsaL* is involved in repressing *ppuI* and/or *ppuR*. Mutating *rsaL* had a positive effect on putisolvin production during the lag phase (Fig. 3C), which can be explained by its repressive effect on AHL synthesis.

Biofilm formation in PVC titer wells indicated that *ppuI* and *ppuR* mutants, in which putisolvin production is strongly reduced (Fig. 4B), exhibit the same phenotype as a putisolvin biosynthetic mutant by forming a thicker biofilm (Fig 4A), while a *rsaL* mutant forms even less biofilm than the wild type (Fig. 4A) and produced putisolvins at an earlier stage of biofilm formation (Fig. 4B). Most interestingly, when AHL signal molecules were added to the medium, the *ppuI* mutant started to produce biosurfactant activity and lost the ability to form a dense biofilm with a thickness comparable to a putisolvin biosynthetic mutant (Fig. 4C and 4D). These results show that the biofilm formation in PCL1445 is regulated by the production of putisolvins in a cell density dependent manner.

P. aeruginosa possesses two quorum sensing systems, *lasI/lasR* and *rhlI/rhlR*, both of which are involved in the regulation of rhamnolipid surfactant production (Davies *et al.*, 1998). In a recent study by Davey et al. (2003) (Davey *et al.*, 2003) it was indicated that *rhlI* influences biofilm development. Rhamnolipids were shown to be involved in the maintenance of the *P. aeruginosa* biofilm architecture, by keeping the fluid-water channels of the biofilm opened (Davey *et al.*, 2003). The observation that chemically unrelated molecules such as rhamnolipids and the cyclic lipopeptides putisolvins I and II, all of which have biosurfactant activity, are regulated by quorum sensing and are involved in the regulation of biofilm formation and structure suggests that biosurfactants play an important role in biofilm structure and development.

The synthesis of the biosurfactant viscosin in *P. fluorescens* 5064 (Cui *et al.*, 2005), as well as biosurfactants serrawettin W2 in *S. liquefaciens* (Lindum *et al.*, 1998) and lipopeptide of unknown structure in *Burkholderia cepacia* (Huber *et al.*, 2002) were also reported to be regulated by AHLs. The production of biosurfactants was shown to be essential for swarming motility of *S. liquefaciens* (Lindum *et al.*, 1998), *P. aeruginosa* (Kohler *et al.*, 2000) and *B. cepacia* (Huber *et al.*, 2002). Previously we have shown that putisolvins stimulate swarming motility (Kuiper *et al.*, 2004), which could provide an explanation for the reducing effect of putisolvins on biofilm size or when added to a formed biofilm resulting in a break down of biofilm (Kuiper *et al.*, 2004).

A role for AHL-mediated quorum sensing in biofilm formation was shown for *B. cepacia* (Huber *et al.*, 2002), *S. liquefaciens* MG1 (Labatte *et al.*, 2004), and *P. putida* IsoF (Steidle *et al.*, 2002). For *B. cepacia* (Huber *et al.*, 2002) and *S. liquefaciens* MG1 (Labatte *et al.*, 2004) it was demonstrated that expression of quorum sensing system-controlled genes is crucial at a specific stage for the development and maturation of the biofilm. In contrast, *P. putida* IsoF wild type produces a very homogeneous biofilm while a quorum sensing mutant appears to form a dense and structured biofilm with characteristic microcolonies and water-filled channels (Steidle *et al.*, 2004).

The present study clearly links quorum sensing in *P. putida* PCL1445 with the synthesis of the cyclic lipopeptides putisolvins I and II and thereby with biofilm formation. Putisolvins seem to function when the bacterial population reaches a high-cell density. The high cell density could form a signal for the release of *Pseudomonas putida* cells. Such a release from the biofilm could be favorable when the nutrient level in the biofilm environment becomes limiting. Moreover, starvation-

mediated stress could play an important role in cell detachment from biofilms since it has been shown for several *Pseudomonas* spp. that the stationary phase sigma factor RpoS influences AHL production (Bertani *et al.*, 2004; Schuster *et al.*, 2004). The production of biosurfactants could stimulate part of the bacteria to colonize other, more favorable, niches, therefore enhancing competitiveness (fitness), pollutant degradation capabilities, or even rhizosphere colonization.

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