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JOURNAL OF BACTERIOLOGY, Apr. 2006, p. 2898–2906 Vol. 188, No. 8
0021-9193/06/$08.00+0 doi:10.1128/JB.188.8.2898–2906.2006

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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Received 7 November 2005/Accepted 1 February 2006

Pseudomonas putida **strain PCL1445 produces two cyclic lipopeptides, putisolvin I and putisolvin II, which possess surface tension-reducing abilities and are able to inhibit biofilm formation and to break down existing biofilms of several *Pseudomonas* spp., including *P. aeruginosa*. Putisolvins are secreted in the culture medium during growth at late exponential phase, indicating that production is possibly regulated by quorum sensing. In the present study, we identified a quorum-sensing system in PCL1445 that is composed of *ppuI*, *rsaL*, and *ppuR* and shows very high similarity with gene clusters of *P. putida* strains IsoF and WCS358. Strains with mutations 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}-*N*-acyl homoserine lactone (3-oxo-C_{10}-AHL) or 3-oxo-C_{12}-AHL to the medium. An *rsaL* mutant overproduces AHLs, and production of putisolvins is induced early during growth. Analysis of biofilm formation on polyvinylchloride showed that *ppuI* and *ppuR* mutants produce a denser biofilm than PCL1445, which correlates with decreased production of putisolvins, whereas an *rsaL* mutant shows a delay in biofilm production, which correlates with early production of putisolvins. The results demonstrate that quorum-sensing signals induce the production of cyclic lipopeptides putisolvin I and II and consequently control biofilm formation by *Pseudomonas putida*.**

Bacteria can form multicellular aggregates generally referred to as biofilms on biotic and abiotic surfaces. 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 greatly contributing to infections (34).

Pseudomonas putida strain PCL1445 is capable of forming biofilms on roots and on polyvinylchloride (PVC) in a commonly used biofilm assay (21). We have shown that *P. putida* PCL1445 produces two novel lipodepsipeptides, putisolvin I and II, consisting of a C_6 lipid moiety and a 12-amino-acid peptide, which are produced by a putisolvin synthetase gene designated as *psoA* (21). A mutant with impaired 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* (21). The production of putisolvins occurs at the end of the exponential growth phase (21), 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 (12, 35). 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 (11). 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 paper 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 (17) or in a defined BM medium (25) supplemented with 2% of glycerol (BDH Laboratory Supplies Pool, England) at 28°C. *Escherichia coli* strains were grown in Luria-Bertani medium (30) at 37°C. Media were solidified with 1.8% agar (Select Agar; Invitrogen Life Technologies, Paisley, United Kingdom). The antibiotics kanamycin, tetracycline, gentamicin, and carbenicillin were added, when necessary, to final concentrations of 50, 40, 2, and 100 μg ml^{-1}, respectively.

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 a vacuum (27). Supernatant extracts were redissolved in 100 μl of ethyl acetate, and 10 μl was fractionated on a C_{18} reverse-phase thin-layer chromatography (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 (22) or pSB1075 (37) were grown in LB medium supplemented with 20 μg of chloramphenicol per ml for 10 h. TLC plates were overlaid with 0.8% LB top agar containing 50 μl of the pAK211 or pSB1075 strains per ml and then incubated at 28°C for 16 h. Autoinducer activity was then detected by the

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
Pseudomonas strains		
PCL1445	Wild-type *Pseudomonas putida*; colonizes grass roots and produces biosurfactants	19
PCL1633	Tn*5luxAB* derivative of PCL1445; mutated in *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
Escherichia coli strain		
DH5α	*endA1 gyrSA96 hrdR17*(r_K^- m_K^-) *supE44 recA1*; general-purpose host strain used for transformation and propagation of plasmids	13
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	14
pME3049	Cloning vector used for homologous recombination; Tc^r Hg^r	8
pRL1063a	Plasmid harboring a promotorless Tn*5luxAB* transposon; Km^r	38
pRK2013	Helper plasmid for triparental mating; Km^r	31
pMP5285	pME3049 derivative, missing the Hg^r gene, used for single homologous recombination; Km^r	20
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 harboring 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 EcoRI-EcoRI PCR fragment of the central part of the *ppuI* gene of PCL1445; Km^r	This study
pMP7571	pMP5285 containing a 0.55-kb EcoRI-EcoRI PCR fragment of the central part of *ppuR* gene of PCL1445; Km^r	This study
pMP7575	pMP5285 containing a 0.21-kb KpnI-SalI PCR fragment of the central part of the *rsaL* gene of PCL1445; Km^r	This study
pMP7583	pGEM-T vector containing a 0.6-kb PCR fragment of the *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 harboring the *rsaL* functional gene of strain PCL1445; Tc^r	This study
pAK211	Autoinducer reporter construct based upon the *Vibrio fischeri* bioluminescence (*lux*) system; Cm^r	22
pSB1075	Bioluminescent AHL sensor plasmid containing a fusion of *lasRI'*::*luxCDABE* in pUC18; used for the detection of long-chain AHLs; Ap^r	37

emission of light after Fuji medical X-ray film (Fuji Photo Film Co., Ltd., Tokyo, Japan) was applied to the TLC plates.

Isolation and identification of quorum-sensing gene homologues. 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 EcoRI into pBluescript (Stratagene, La Jolla, CA). The resulting fragment library was introduced into an *E. coli* strain harboring the *lux* reporter plasmid pAK211 (22). After overnight growth on LB agar plates, clones that induced the luciferase reporter were identified using photographic film. To remove pAK211 (22) from the *E. coli* reporter strain, the total plasmid was isolated and reintroduced into DH5α cells by standard transformation protocols (31) followed by carbenicillin selection, whereas chloramphenicol selection was omitted. The DNA sequence of the chromosomal fragment inserted in the selected plasmid pMP5548 was determined using universal primers, including M13-20 and M13 reverse primers flanking the multiple cloning sites 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 an EcoRI-EcoRI insert in the pMP5285 suicide plasmid (20) derived from pME3049 (8), resulting in pMP7568. pMP7568 was transferred to *Pseudomonas* PCL1445 by triparental mating using pRK2013 as a helper plasmid (31) and using selection on KB agar medium (17) 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 gentamicin resistance cassette cloned as a SalI-SalI 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 by using a similar mutagenesis strategy. The *ppuR* fragment for the construction of the pMP5285-based suicide plasmid pMP7571 resulted from a PCR using primers oMP905 (5′-AATTCTTCGAAGAAGCCGCCG-3′) and oMP906 (5′-TTGCTGGATGG CTTTGAGCACC-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 pMP7575 suicide plasmid based on pMP5285 obtained after cloning a 0.21-kb KpnI-SalI PCR fragment of the central part of the *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 (14) in which a 1.4-kb fragment containing *ppuI* and *rsaL* of strain PCL1445 was inserted. This insert was obtained by EcoRI digestion from pMP5548. pMP7565 was transferred to strain PCL1636 by triparental mating as described above, and transformants were selected on KB agar medium supplemented with tetracycline (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 the *rsaL* gene of strain PCL1445 was obtained using primers oMP1011 (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, in which the reduction of the water surface tension can be observed in the collapse of a round droplet placed on a hydrophobic surface, as described previously (16).

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 HPLC analysis of putisolvins. To quantify the production of putisolvins in BM culture medium, 10 ml of a BM culture supernatant was extracted with 1 volume of ethyl acetate (Fluka Chemie, Zwijndrecht, The Netherlands) as described previously (21). Ethyl acetate extracts were evaporated under a vacuum to dryness and dissolved in 55% acetonitrile (Labscan Ltd., Dublin, Ireland). The dried pellet obtained from the 10-ml culture was resuspended in 500 μl of 50/50 acetonitrile/water (vol/vol) and filtered using a 0.45-μm-pore-size SpinX centrifuge tube filter (Corning Costar Corporation, Cambridge, MA). A 500-μl volume of the samples was separated by use of a high-performance liquid chromatography (HPLC) system (Jasco International Co. Ltd., Japan) with a reverse-phase C_8 5-μm Econosphere column (Alltech, Deerfield, IL), a PU-980 pump system (B&L Systems, Boechout, Belgium), an LG-980-02 gradient unit (Jasco), and an MD 910 detector (Jasco). Separation was performed using a linear gradient, starting at 35/65 acetonitrile/water (vol/vol) and ending at 20/80 after 50 min at a flow rate of 1 ml min^{-1}. Chromatograms were analyzed in the wavelength range between 195 nm and 420 nm. Fractions that corresponded to the retention times 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 putisolvin produced was quantified as the peak area in microabsorbance units at 206 nm.

Quantification of bioluminescent Tn*5luxAB* reporter strains. Expression of Tn*5luxAB* genes was determined by quantification of bioluminescence during culturing. Cells from overnight cultures were washed with fresh medium and diluted to an optical density at 620 nm (OD_{620}) of 0.1. Cultures were grown in BM medium in a 10-ml volume with vigorous shaking. During growth, 100-μl samples were taken in triplicate to quantify luminescence. A 100-μl volume of a 0.2% *n*-decyl-aldehyde substrate solution (Sigma, St. Louis, MO) in a 2.0% bovine serum albumin solution was added, and luminescence was 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 Tn*5luxAB* 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 of synthetic AHL or 25 μl of a 1,000-fold-concentrated crude extract of spent culture supernatant dissolved in 100% acetonitrile.

Biofilm assay. Biofilm formation on PVC was conducted as previously described by O'Toole and Kolter (28) and adapted for strain PCL1445 as previously described by Kuiper et al. (21). When the effect of AHLs on biofilm formation was tested, the culture medium and planktonic cells were removed after 4 h. Subsequently, 100 μl of M63 medium containing 1 μl 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 numbers. 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 under accession numbers DQ151886 and DQ151887, respectively.

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 with an OD_{620} of 1 was tested for induction of an *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 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 were detected (Fig. 1A and B). Furthermore, when the standard molecules were mixed with PCL1445 dichloromethane extracts, the four detected compounds comigrated with the standard AHLs (Fig. 1A and B, lanes 3). Dichloromethane extracts of the putisolvin biosynthetic mutant PCL1633 showed the same profile as the wild-type strain (data not shown).

Identification of quorum-sensing genes of *P. putida* PCL1445. To isolate a chromosomal fragment of strain PCL1445 containing *luxI* and *luxR* homologues, an EcoRI chromosomal library of PCL1445 was introduced into *E. coli* DH5α containing pAK211, a reporter strain for AHLs based on the *lux* system of *Vibrio fischeri* (22). 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 open reading frames 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 (33) and WCS358 (1), 57% identity with *lasI* of *P. aeruginosa* (29), and 51% identity with *mupI* of *P. fluorescens* (10) (Fig. 2A). The sequence of the gene located upstream of *ppuI* showed 100% identity with *rsaL* gene in *P. putida* IsoF (33) and WCS358 (1), and 60% identity with *rsaL* of *P. aeruginosa* (6). The *rsaL* gene was described first 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 open reading frame located downstream of *ppuI* showed 91% identity with the *suhB* of *P. putida* IsoF (33) and 78% with *suhB* of *P. aeruginosa*. The latter gene was suggested to possess inositol monophosphatase activity in *E. coli* (26).

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 R_f values similar to those of 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 identical to *ppuI* and *ppuA lux* box elements found in *P. putida* strains IsoF (33) and WCS358 (1) were found. A 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* (33), *P. aeruginosa* (36), *P. chlorora-*

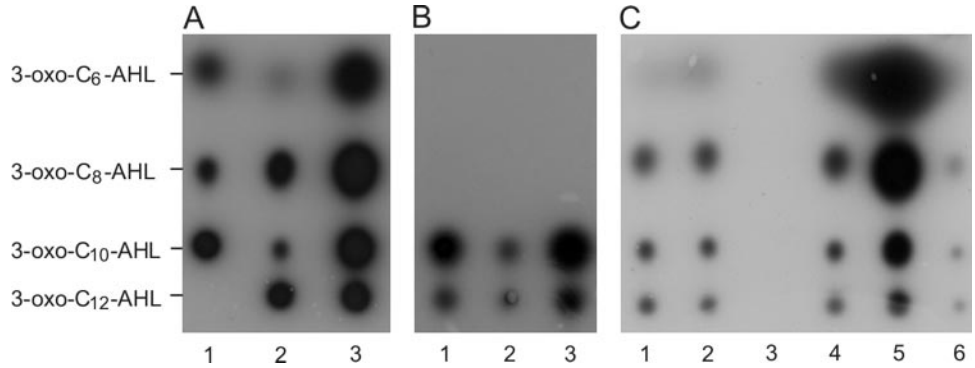

FIG. 1. C_{18} reverse-phase thin-layer chromatography analysis of *N*-acyl-L-homoserine lactones produced by *P. putida* PCL1445 and its mutant derivatives. Cells of strain *P. putida* PCL1445 and its derivatives, the *ppuI* mutant PCL1636, PCL1636 harboring plasmid pMP5548 (*ppuI*), the *rsaL* mutant PCL1638, and PCL1638 harboring plasmid pMP7587 (*rsaL*) were grown in BM-glycerol to an OD_{620} of 0.7 and centrifuged. The supernatant fluids were extracted with dichloromethane, and the organic fractions were analyzed using TLC. The chromatograms were overlaid with *E. coli* reporter strains for the detection of AHLs. (A) The *E. coli* biosensor strain harboring pAK211 was used to visualize AHLs produced by PCL1445. Lanes: 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; 2, culture supernatant extract of PCL1445; 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. (B) The *E. coli* biosensor strain harboring pSB1075 was used to visualize long-chain AHLs produced by PCL1445. Lanes: 1, 50 ng of 3-oxo-C_{10}-AHL and 50 ng of 3-oxo-C_{12}-AHL were mixed; 2, culture supernatant extract of PCL1445 cells; 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. (C) The *E. coli* biosensor strain harboring pAK211 was used to visualize AHLs present in culture supernatant extracts of cells of strains PCL1445, PCL1636 (*ppuI*), and PCL1638 (*rsaL*). Lanes: 1, PCL1445; 2, PCL1445 harboring the cloning vector pME6010; 3, PCL1636 (*ppuI*); 4, PCL1637 harboring pMP5548 (*ppuI*); 5, PCL1638 (*rsaL*); 6, PCL1638 harboring pMP7587 (*rsaL*).

phis (2), and *V. fischeri* (7), is present 92 bp upstream of the *psoA* gene start codon (Fig. 2B) (9). These palindromes might constitute a binding site for the LuxR response regulator.

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 *psoA*, *ppuI* was mutated in strain PCL1633 (*psoA*::Tn*5luxAB*), 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*) than the transcriptional activity detected in PCL1633 (*psoA*) (Table 2). The transcriptional activity of the *psoA* promoter in

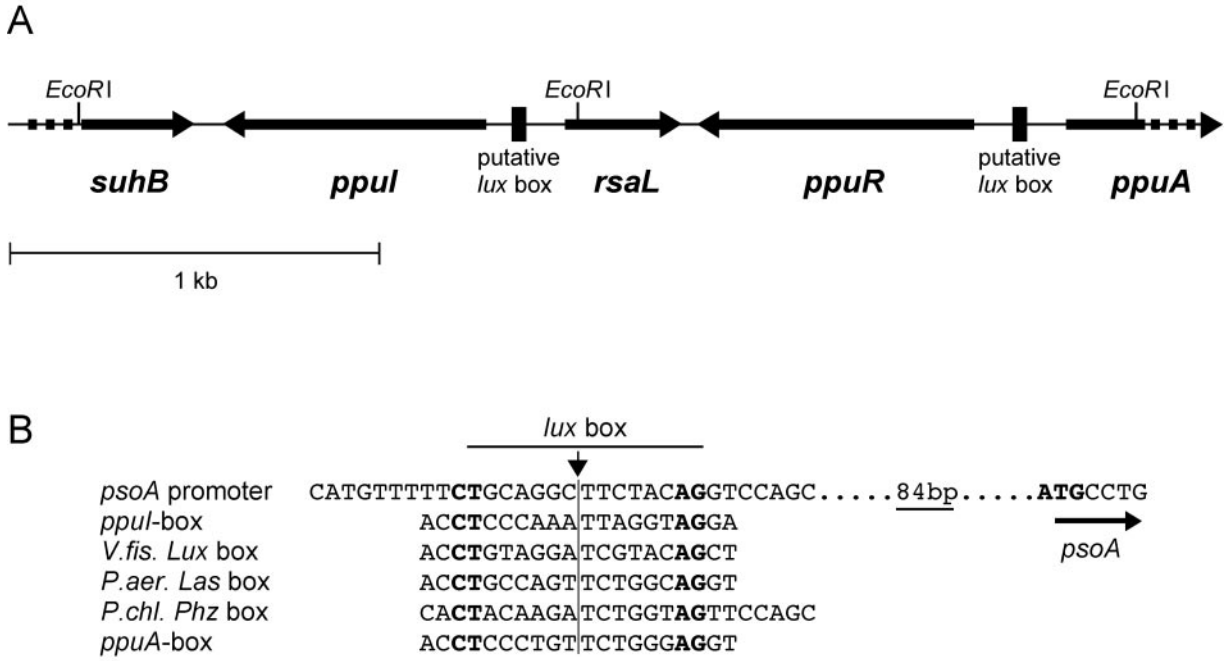

FIG. 2. Chromosomal organization of the *ppu* locus and analysis of the *lux* box in the upstream region of putisolvin biosynthetic gene *psoA* of *P. putida* PCL1445. (A) The *ppu* locus of strain PCL1445. Putative *lux* boxes are present in the intergenic regions of *ppuI-rsaL* and *ppuR-ppuA*, respectively. Dotted lines indicate nondetermined sequences. (B) Comparison of a *lux* box homologous sequence in the region upstream of the *psoA* gene of *P. putida* PCL1445 with similar sequences. Abbreviations: *V.fis.*, *V. fischeri*; *P.aer.*, *P. aeruginosa*; *P.chl.*, *P. chlororaphis*.

TABLE 2. Transcriptional activity of *psoA* of *P. putida* PCL1445 in response to synthetic AHLs[a]

Strain	AHL added (5 μM)	Supernatant		Bioluminescence/ cell density (10^3 LCPS/OD_{620})
		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 the bioluminescence in luminescence counts per second (LCP5) of cell cultures of the double mutant PCL1639 (*psoA*::Tn*5luxAB*/*ppuI*) grown to an OD_{620} of 1.5 in BM-glycerol medium. Crude 1,000-fold-concentrated dichloromethane extracts of the wild-type strain culture supernatant, of the *ppuI* mutant, or of AHL molecules were added to early-log-phase cultures (OD_{620}, 0.2). Standard deviations are based on the mean values for three parallel cultures.

strain PCL1639 (*ppuI*/*psoA*) was analyzed in liquid culture at an OD_{620} of 1.5 after addition of crude 1,000-fold-concentrated dichloromethane extracts of the wild-type strain culture supernatant, or of the *ppuI* mutant, to an early-log-phase culture (OD_{620} of 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 effect of C_4-, C_6-, 3-oxo-C_6-, C_8-, C_{10}-, 3-oxo-C_{10}-, C_{12}-, and 3-oxo-C_{12}-AHLs, added at a concentration of 5 μM to an early-log-phase culture (OD_{620}, 0.2) of PCL1639, on *psoA* transcriptional activity was quantified at an OD_{620} of 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 strongly stimulated by addition of 3-oxo-C_{12}-AHLs (Table 2).

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), 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 by the Du Nouy ring method during growth until the stationary phase was reached (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 compared to the wild type, indicating a lack of biosurfactant production (Fig. 3A). The culture supernatant of strain PCL1638 (*rsaL*) caused a dramatic decrease of surface tension during the early exponential phase (to 32 mN m^{-1} at an OD of 1), indicating an earlier production of biosurfactant than that of 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 harboring the genomic fragment from pMP5548 with *ppuI* and pMP7566 harboring *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 compared to the wild type (fourfold at an OD_{620} of 0.6). This difference in production tends to decrease when the cells reach the stationary phase (twofold at an OD_{620} of 1.1 and hardly any difference at an OD_{620} of 2). Introduction of pMP7587 harboring *rsaL* into PCL1638 (*rsaL*) significantly decreased putisolvin production during exponential phase more than for the wild-type strain (twofold lower at an OD_{620} of 0.6). This is possibly due to the multiple-copy effect of the plasmid used for complementation of *rsaL* mutation (Fig. 3C).

To investigate the involvement of *ppuI* in AHL biosynthesis in PCL1445 and of *rsaL* in regulation of AHL biosynthesis in PCL1445, AHL production by strains PCL1445, PCL1636 (*ppuI*), PCL1636 harboring pMP7565 (*ppuI*), PCL1638 (*rsaL*), and PCL1638 harboring pMP7587 (*rsaL*) was examined by TLC analysis. Control vector pME6010 did not influence the AHL production, which remained the same as that of 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 by PCL1636 (*ppuI*) was restored by introduction of pMP7565 harboring a functional *ppuI* gene (Fig. 1C, lane 4). Finally, introduction of a mutation into *rsaL* (PCL1638) had a strongly positive effect on the production of AHLs by PCL1445 (Fig. 1C, lane 5). The AHL production by PCL1628 (*rsaL*) decreased dramatically compared to that of the wild type following introduction of pMP7587 harboring a functional *rsaL* gene (Fig. 1C, lane 6).

Effect of *ppu* quorum-sensing system on biofilm formation by 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 of the bacterial cells in the titer wells, culture samples were analyzed by the drop-collapsing assay. An index ranging from 0 to 4 was used to quantify biosurfactant production by bacterial cells in the biofilm assay

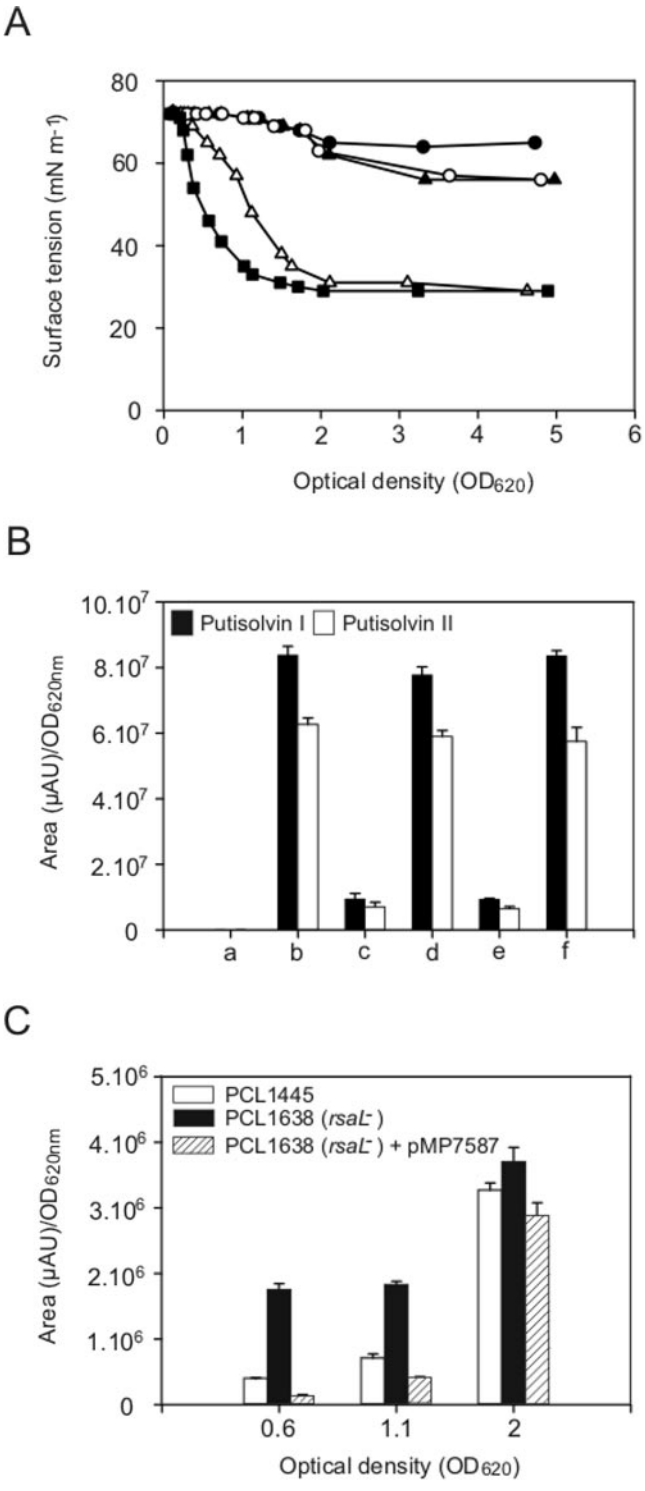

FIG. 3. Effects of mutations in *ppuI*, *ppuR*, and *rsaL* on production of putisolvins of *P. putida* PCL1445. (A) Quantification of surface tension decrease by culture supernatants of *P. putida* strain PCL1445 (Δ), PCL1633 (*psoA*) (●), PCL1636 (*ppuI*) (○), PCL1637 (*ppuR*) (▲), and PCL1638 (*rsaL*) (■) grown to the stationary phase in BM-glycerol medium. (B) C_8 reverse-phase HPLC analysis of putisolvin production by *P. putida* strain PCL1445 and its mutants PCL1636 (*ppuI*) and PCL1637 (*ppuR*). Bars: 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 with 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. (C) C_8 reverse-phase HPLC analysis of putisolvin production by PCL1445, mutant PCL1638 (*rsaL*), and PCL1638 harboring pMP7587 (*rsaL*). Compounds from the ethyl acetate-extracted supernatant of cultures grown to OD of 0.6, 1.1, and 2 in BM-glycerol were separated and analyzed by HPLC as described for panel B. µAU, microabsorbance units.

(Fig. 4E). PCL1636 (*ppuI*) and PCL1637 (*ppuR*) did not have any detectable biosurfactant activity (Fig. 4B). Analysis of PCL1638 (*rsaL*) showed that biofilm formation decreased 1.5-fold compared to the wild-type strain (Fig. 4A), which correlates with an earlier appearance of biosurfactant activity (visible after 6 h) than that 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 harboring *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 that reached by PCL1445 (Fig. 4C, bar f). Exogenous 3-oxo-C_{12}-AHL signaling molecules also appeared to be able to stimulate production of biosurfactant by *ppuI* mutants (Fig. 4D, lane j) and reduce the thickness of the biofilm (Fig. 4C, bar j).

DISCUSSION

P. putida PCL1445 produces two cyclic lipopeptide biosurfactants (putisolvin I and II), which inhibit biofilm formation and degrade existing *Pseudomonas* biofilms (21). Initiation of putisolvin production starts at the onset of stationary phase (21), suggesting that putisolvin biosynthesis might be regulated by population density, 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 production of the cyclic lipopeptides putisolvin 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 (homolo-

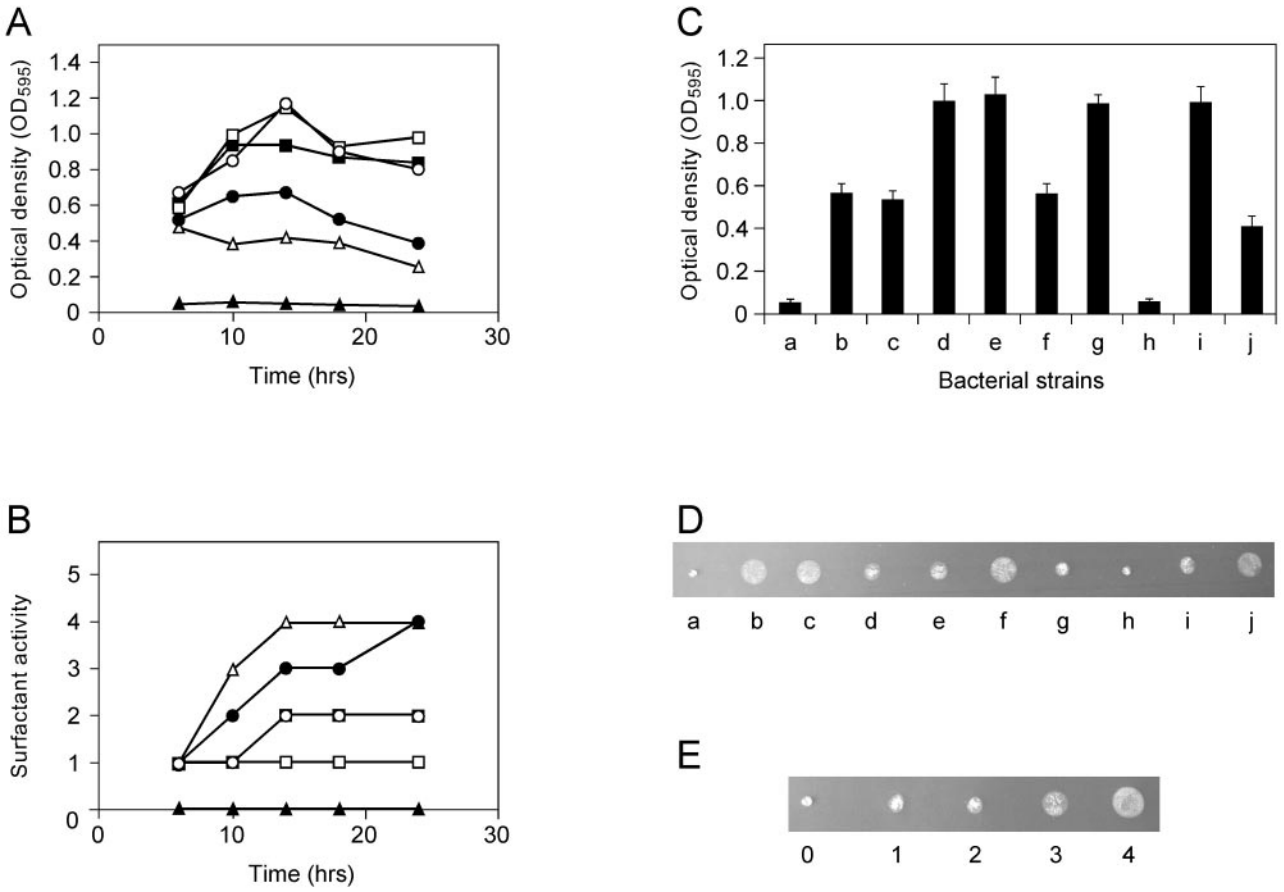

FIG. 4. Influence of quorum sensing on biofilm formation by *P. putida* PCL1445 in PVC microtiter wells. Cells of PCL1445 and its quorum-sensing mutant derivatives were incubated in microtiter plates in M63 medium, and their biofilm formation was quantified over time using the crystal violet staining procedure. Cells attached to the microtiter wells were stained with crystal violet and 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 and ranging from 0 to 4 was used to quantify surface tension reduction. All experiments were performed in triplicate. (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 (▲). (B) Biosurfactant activity present in the titer well during biofilm formation by bacterial cultures shown in panel A, as determined by the drop-collapsing assay. (C) Biofilm formation by PCL1445 and PCL1636 (*ppuI*) measured after 24 h of incubation. Bars: a, M63 medium without bacteria; b, PCL1445; c, PCL1445 containing pME6010; d, PCL1436 (*psoA*); e, PCL1636 (*ppuI*); f, PCL1636 (*ppuI*) harboring pMP5548 (*ppuI*); g, PCL1639 (*psoA*/*ppuI*); h, M63 plus 3-oxo-C12-AHL (5 μM); i, PCL1636 (*ppuI*) plus pure acetonitrile (control); j, PCL1636 (*ppuI*) plus 3-oxo-C12-AHL (5 μM). Standard deviations are based on the mean values of triplicate cultures. (D) Biosurfactant activity as determined by the drop-collapsing assay of bacterial cultures shown in the biofilm assay in panel C. Lanes: a, M63 medium without bacteria; b, PCL1445; c, PCL1445 containing pME6010; d, PCL1436 (*psoA*); e, PCL1636 (*ppuI*); f, PCL1636 (*ppuI*) harboring pMP5548 (*ppuI*); g, PCL1639 (*psoA*/*ppuI*); h, M63 plus 3-oxo-C12-AHL (5 μM); i, PCL1636 (*ppuI*) plus pure acetonitrile (control); j, PCL1636 (*ppuI*) plus 3-oxo-C12-AHL (5 μM). (E) An index ranging from 0 to 4 was used for the detection of biosurfactant production by bacterial cells in the biofilm assay. Shown are dried droplets of 25-μl culture supernatants with increased diameter due to decreased surface tension caused by increased biosurfactant activity.

gous) 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 was identified 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* (6) and of *ppuI* of *P. putida* WCS358 (1). 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 that of the loci identified in *P. putida* IsoF (33) and *P. putida* WCS358 (1). 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 (33). 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 evolutionarily well conserved and might regulate similar genes (33).

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 increased AHL production (Fig. 1C), suggesting that *rsaL* is involved in repressing *ppuI* and/or *ppuR*. Introducing a mutation into *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 produces 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 and lost the ability to form a dense biofilm with a thickness comparable to that of a putisolvin biosynthetic mutant (Fig. 4C and 4D). These results show that biofilm formation in PCL1445 is regulated by the production of putisolvins in a cell population-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 (5). In a recent study by Davey et al. (4) 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 water-filled channels of the biofilm opened (4). The observation that chemically unrelated molecules such as rhamnolipids and the cyclic lipopeptides putisolvin I and II, all of which have biosurfactant activity, are regulated by quorum sensing and are all 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 (3), as well as biosurfactants serrawettin W2 in *Serratia liquefaciens* (24) and a lipopeptide of unknown structure in *Burkholderia cepacia* (15), was also reported to be regulated by AHLs. The production of biosurfactants was shown to be essential for swarming motility of *S. liquefaciens* (24), *P. aeruginosa* (18), and *B. cepacia* (15). We have shown previously that putisolvins stimulate swarming motility (21), which could provide an explanation for the reducing effect of putisolvins on biofilm size or for the resultant breakdown of biofilm when they are added to a formed biofilm (21).

A role for AHL-mediated quorum sensing in biofilm formation was shown for *B. cepacia* (15), *S. liquefaciens* MG1 (23), and *P. putida* IsoF (33). For *B. cepacia* (15) and *S. liquefaciens* MG1 (23) 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, wild-type *P. putida* IsoF produces a very homogenous biofilm while a quorum-sensing mutant appears to form a dense and structured biofilm with characteristic microcolonies and water-filled channels (33).

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 (1, 32). The production of biosurfactants could stimulate some of the bacteria to colonize other, more favorable, niches, therefore enhancing competitiveness (fitness), pollutant degradation capabilities, or even rhizosphere colonization.

ACKNOWLEDGMENTS

We thank P. Williams of the University of Nottingham, United Kingdom, for kindly providing the synthetic autoinducers C_{12}-AHL, 3-oxo-C_6-AHL, 3-oxo-C_8-AHL, 3-oxo-C_{10}-AHL, 3-oxo-C_{12}-AHL, and 3-oxo-C_{14}-AHL. We thank E. Lagendijk for technical assistance and P. Hock for processing the images and the graphs.

This research was supported by grant number 700.50.015 from the Council for Chemical Sciences of The Netherlands Foundation for Scientific Research (NWO/CW).

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