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Identification and Characterization of an *N*-Acylhomoserine Lactone-Dependent Quorum-Sensing System in *Pseudomonas putida* Strain IsoF

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Recent reports have shown that several strains of *Pseudomonas putida* produce *N*-acylhomoserine lactones (AHLs). These signal molecules enable bacteria to coordinately express certain phenotypic traits in a density-dependent manner in a process referred to as quorum sensing. In this study we have cloned a genomic region of the plant growth-promoting *P. putida* strain IsoF that, when present in *trans*, provoked induction of a bioluminescent AHL reporter plasmid. Sequence analysis identified a gene cluster consisting of four genes: *ppuI* and *ppuR*, whose predicted amino acid sequences are highly similar to proteins of the LuxI-LuxR family, an open reading frame (ORF) located in the intergenic region between *ppuI* and *ppuR* with significant homology to *rsaL* from *Pseudomonas aeruginosa*, and a gene, designated *ppuA,* present upstream of *ppuR*, the deduced amino acid sequence of which shows similarity to long-chain fatty acid coenzyme A ligases from various organisms. Using a transcriptional *ppuA*::*luxAB* fusion we demonstrate that expression of *ppuA* is AHL dependent. Furthermore, transcription of the AHL synthase *ppuI* is shown to be subject to quorum-sensing regulation, creating a positive feedback loop. Sequencing of the DNA regions flanking the *ppu* gene cluster indicated that the four genes form an island in the *suhB*-PA3819 intergenic region of the currently sequenced *P. putida* strain KT2440. Moreover, we provide evidence that the *ppu* genes are not present in other AHL-producing *P. putida* strains, indicating that this gene cluster is so far unique for strain IsoF. While the wild-type strain formed very homogenous biofilms, both a *ppuI* and a *ppuA* mutant formed structured biofilms with characteristic microcolonies and water-filled channels. These results suggest that the quorum-sensing system influences biofilm structural development.

Evidence that has accumulated over the past few years has established that numerous bacteria employ cell-cell communication systems that rely on small signal molecules to express certain phenotypic traits in a density-dependent manner. This regulatory principle is now generally referred to as quorum sensing (for reviews, see references 13, 17, and 49). Most quorum-sensing systems thus far identified in gram-negative bacteria utilize *N*-acylhomoserine lactone (AHL) signal molecules, which are usually synthesized by a member of the LuxI protein family. As the density of the population increases, AHL molecules accumulate in the growth medium and upon reaching a critical threshold concentration bind to their cognate receptors (a member of the LuxR protein family). The receptor/AHL complex then activates or represses target gene expression.

AHL-dependent quorum-sensing systems have been identified in more than 50 species, which inhabit various ecosystems. Many of the AHL-producing bacteria, however, are associated with plants. In fact, Cha et al. (6) demonstrated that the majority of plant-associated bacteria produce AHL signal molecules. Specifically, they showed that almost all tested isolates of the genera *Agrobacterium*, *Pantoea*, and *Rhizobium* and about half of the erwinias and pseudomonads tested synthesized detectable levels of AHLs. By contrast, only a few AHL producers could be identified among *Xanthomonas* sp. isolates. More recently, Elasri et al. (14) screened 137 soilborne and plant-associated strains belonging to different *Pseudomonas* species. Of all the strains tested, 54 (39%) were positive for AHL production. In the same vein Pierson et al. (37), who screened 700 isolates of wheat root-associated bacteria by the aid of different AHL biosensors, showed that about 8% of the strains were able to activate at least one of the sensors. Given that a large proportion of the bacteria colonizing the roots of plants produces AHL signals, it is not too surprising that these pheromones not only serve as population density sensors of one species but also for communication between cells of different species (37, 45). These interactions appear to be important for coordinating the functions of the different populations within the rhizosphere community.

Many root-colonizing fluorescent pseudomonads can promote plant growth and exhibit biocontrol potential against certain pathogens (48, 38). A well-known example is *Pseudomonas aureofaciens* strain 30-84, which is used as a biocontrol agent to protect wheat from take-all disease caused by *Gaeumannomyces graminis* var. *tritici.* When this strain is present in the rhizosphere of wheat, the severity of the disease is strongly reduced due to the production of phenazine antibiotics, which

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description	Source or reference
Bacterial strains		
E. coli		
CC118 λ *pir*	Δ(*ara-leu*) *araD* Δ*lacX74 galE galK phoA20 thi-1 rpsE rpoB argE*(Am) *recA1* λ *pir* lysogen	20
HB101	*recA thi pro leu hsd M*$^+$ Sm^r	24
MT102	*araD139* (*ara-leu*)7697 Δ*lac thi hsdR*	Laboratory collection
XL1Blue	*recA1 endA1 gyrA*96 *thi-1 hsdR17 supE44 relA1 lac* [*F′ proAB lacI*^{q} ZΔ*M15* Tn*10* (Tet^r)]	Stratagene
P. putida		
IsoF	Wild-type isolate from tomato roots	45
F117	Km^r *ppuI*::*npt* mutant of IsoF	This study
IsoF-D4	*ppuA*::*luxAB* mutant of IsoF	This study
F117-D1	Km^r *ppuI*::*npt ppuA*::*luxAB* double mutant of IsoF	This study
Plasmids		
pAS-L22	pGA-L14 containing a P_{ppuI}-*luxAB* transcriptional fusion	This study
pBBR1MCS-2	Broad-host-range plasmid, *lac*Zα; Kan^r	27
pBBR1MCS-5	Broad-host-range plasmid, *lac*Zα; Gm^r	27
pBK-miniTn*7*-gfp3	pUC19-based delivery plasmid for miniTn*7*-gfp3; Km^r Ap^r Cm^r Sm^r *mob*$^+$	25
pEX18Gm	Gm^r *oriT*$^+$ *sacB*$^+$, gene replacement vector with multiple-cloning site from pUC18	21
pEXF1	Gm^r Km^r, pEX18Gm derivative for inactivation of *ppuI*	This study
pEXF2	Gm^r, pEX18Gm derivative for inactivation of *ppuA*	This study
pGA-L14	Broad-host-range *luxAB*-based promoter probe vector	Laboratory collection
pGEM-3Zf(+)	*lac*Zα, cloning vector; Ap^r	Promega, Madison, Wis.
pGF1	2.9-kb *Sal*I chromosomal fragment containing *ppuI-rsaL-ppuR* of IsoF cloned into pGEM-3Zf(+)	This study
pGF3	1.4-kb *Eco*RI chromosomal fragment containing *ppuI* of IsoF cloned into pBBR1MCS-5	This study
pGF4	Km^r, 13-kb *Sac*I chromosomal fragment containing the *ppu* locus of F117 plus flanking regions cloned into pGEM-3Zf(+)	This study
pKR-C12	AHL sensor plasmid; pBBR1MCS-5 carrying P_{lasB}-*gfp*(ASV)-P_{lac}-*lasR*; Gm^r	39
pPPUR1	pGEM-3Zf(+) containing the *ppuR* gene of IsoF	This study
pRK600	ColE1 RK2-Mob$^+$ RK2-Tra$^+$; helper plasmid; Cm^r	12
pSB403	Bioluminescent AHL sensor plasmid; Tet^r	53
pUX-BF13	R6K replicon-based helper plasmid, providing the Tn*7* transposition function in *trans,* Ap^r *mob*$^+$	2

are active against the ascomycete fungus. Synthesis of these antibiotic compounds is regulated by the PhzR-PhzI quorum-sensing system that utilizes the AHL molecule *N*-hexanoyl-L-homoserine lactone (C6-HSL) (54). Recently, a second quorum-sensing system, which was designated CsaR-CsaI, was identified in the strain (55). This system is not involved in the regulation of phenazine production but controls biosynthesis of cell surface components and plays an important role in the rhizosphere competence of the strain. *Pseudomonas fluorescens* F113 is capable of protecting sugar beet seedlings from damping-off disease caused by *Pythium ultimum* (16, 43). The biocontrol capability of the strain resides, at least in part, in the production of the antifungal agent 2,4-diacetylphloroglucinol. Evidence has been provided that *P. fluorescens* F113 produces at least three different AHLs with the aid of a novel AHL synthase, HdtS, which is not related to members of the LuxI protein family (29). Although no definite phenotype has yet been linked to the quorum-sensing system operating in this strain, it has been speculated that AHLs may be involved in regulating the biocontrol properties of the organism.

Some *Pseudomonas putida* strains not only synthesize secondary metabolites, which exhibit antagonistic activities against plant pathogens (48), but also have the ability to promote plant growth and to degrade toxic organic compounds. Thus, *P. putida* is a highly attractive candidate for agricultural and environmental uses. Recent work has shown that some *P. putida* strains produce AHL signal molecules (3, 14, 26, 42), suggesting that they are capable of triggering expression of certain functions in a population density-dependent manner. However, neither have the genes encoding the components of the *P. putida* quorum-sensing system been identified nor has a definite AHL-regulated gene or phenotype been described. In this study, we report the cloning and molecular characterization of the *ppu* quorum-sensing locus of *P. putida* IsoF.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains used in this study are listed in Table 1. Unless otherwise stated, all strains were grown aerobically in modified Luria-Bertani (LB) medium (4) containing 4 g of NaCl/liter instead of 10 g of NaCl/liter at 30°C (*P. putida*) or 37°C (*Escherichia coli*). Solid media contained 15 g of agar/liter. For selection of pseudomonads, cells were grown on *Pseudomonas* isolation agar (PIA) (Difco). The following antibiotics were added as required at the indicated final concentrations: tetracycline, 10 mg/liter; gentamicin, 25 mg/liter for *E. coli* and 50 mg/liter for *P. putida*; kanamycin, 50 mg/liter for *E. coli* and 100 mg/liter for *P. putida*; chloramphen-

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icol, 10 mg/liter; streptomycin, 50 mg/liter; and ampicillin, 100 mg/liter. Growth of liquid cultures was monitored spectrophotometrically by an Ultraspec Plus spectrophotometer (Pharmacia) by measurement of optical density at 600 nm (OD_{600}).

Conjugative plasmid transfer. Plasmids were delivered to *P. putida* by triparental mating as described previously (7). Briefly, donor and recipient strains as well as helper strain *E. coli* HB101(pRK600) were grown overnight in 5 ml of LB medium supplied with the appropriate antibiotics. Following subculturing to an OD_{600} of 0.9, the cells from 2 ml of culture were harvested by centrifugation, washed, and resuspended in 200 μl of LB medium. Donor and helper cells (100 μl each) were carefully mixed and incubated for 20 min at room temperature. Then, 200 μl of the recipient cells was added, and the mixture was spot-inoculated onto the surface of prewarmed LB plates. After overnight incubation at 30°C, the cells were scraped off and resuspended in 1 ml of 0.9% NaCl. Serial dilutions were plated on PIA containing the appropriate antibiotic for selection of transconjugants.

DNA manipulation and nucleotide sequencing. Cloning, restriction enzyme analysis, and transformation of *E. coli* were performed essentially as described previously (41). PCR was performed using Herculase (Stratagene, La Jolla, Calif.). Plasmid DNA was isolated with the QIAprep Spin Miniprep kit, and chromosomal DNA from *P. putida* was purified with the DNeasy tissue kit. DNA fragments were purified from agarose gels using the QIAquick gel extraction kit (all kits from QIAGEN, Hilden, Germany). DNA probes were labeled with digoxigenin and detected using the DNA labeling and detection kit (Roche, Mannheim, Germany).

For flow-chamber experiments, the strains were tagged with the green fluorescent protein (GFP). This was accomplished by the insertion of a miniTn*7*-$P_{A1/04/03}$-*gfp*-T_0T_1 transposon cassette into the chromosomes of target strains using the suicide construct pBK-miniTn*7*-gfp3 (25). Plasmid pBK-miniTn*7*-gfp3 was delivered to target strains by four-parental mating using *E. coli* HB101(pRK600) and *E. coli* XL1Blue(pUX-BF13) as helper strains.

Cloning of *ppuI* and *ppuR*. The AHL synthase of *P. putida* IsoF was cloned by complementation of an AHL biosensor strain as described previously (47). Briefly, a chromosomal bank of *P. putida* IsoF was constructed in plasmid pGEM-3Zf(+) using the restriction enzyme *Sal*I. The resulting plasmids were electroporated into *E. coli* MT102 harboring the AHL sensor plasmid pSB403 (53). This sensor plasmid contains the *Vibrio fischeri luxR* gene together with a transcriptional fusion of the *luxI* promoter to the promoterless bioluminescence gene cluster *luxCDABE* of *Photorhabdus luminescens.* In the presence of a recombinant plasmid expressing a *luxI*-homologous gene, light emission of plasmid pSB403 will be induced due to the synthesis of AHLs.

Bioluminescent clones were detected using a highly sensitive photon-counting camera (C2400-40; Hamamatsu Photonics, Herrsching, Germany). A recombinant plasmid, designated pGF1, conferring a bioluminescent phenotype when present in *E. coli* MT102(pSB403), was isolated. DNA sequencing of the plasmid, which contained a 2.9-kb chromosomal *Sal*I DNA fragment, was performed using the M13 universal sequencing primers and specifically designed primers as sequence information became available. DNA sequences were compared to other sequences in GenBank using the on-line BLAST search engine (1) at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/).

Construction of a defined *ppuI* mutant. A defined *ppuI* mutant was constructed by the gene replacement method described by Hoang et al. (21). The mutant allele was constructed from pGF1 by blunt end ligation of the *npt* gene from transposon Tn*903* (32) into a unique *Apa*I restriction site at bp 225 of *ppuI*. Next, the *ppuI*::*npt* cassette was cloned into the *Sal*I site of pEX18Gm. The resulting plasmid, pEXF1, was mobilized into IsoF by triparental mating, and gene replacement mutants were selected on PIA medium containing kanamycin (100 mg/liter) and 5% sucrose. Insertion of the kanamycin cassette into the *ppuI* gene was confirmed by Southern blot hybridization using a 1.4-kb *Eco*RI fragment of pGF1 containing *ppuI* as a probe. This 1.4-kb fragment was also cloned into the *Eco*RI site of pBBR1MCS-5, and the resulting plasmid, pGF3, was used for complementation purposes.

Cloning and chromosomal inactivation of *ppuA*. A gene library of *P. putida* F117, the *ppuI* null mutant, was constructed by ligating *Sac*I-digested chromosomal DNA into the compatible sites of pGEM-3Zf(+). Following electroporation of *E. coli* XL1Blue, kanamycin-resistant clones were selected. Restriction analysis of one transformant, named pGF4, revealed the presence of a 13-kb chromosomal fragment containing *ppuI*::*npt*. Using sequence information from pGF1, the flanking regions of *ppuI*::*npt* and *ppuR* were sequenced by primer walking. A defined *ppuA* mutant was constructed as follows. Two DNA fragments were amplified: a 1.1-kb *Eco*RI-*Sac*I fragment containing part of the *ppuA* gene, using primers PPUAII-V (5′-CGGAATTCCCAAGCCGGTCTAGCG-3′) and PPUAII-R (5′-CGGAGCTCGACCAGTTGCGCGCC-3′), and a 1.3-kb *Sal*I-*Hin*dIII fragment containing the *ppuA* start codon and the complete *ppuR* gene, using primers PPUAR-V (5′-ACATGTCGACGCTAGTAAATAGGCTG CG-3′) and PPUAR-R (5′-AACTAAGCTTCGGCATCAGCCAGGCC-3′). Using the restriction sites introduced by the PCR primers (respective sites are underlined) these two DNA fragments were successively inserted into the compatible sites of pEX18Gm. Next, the promoterless *luxAB* cassette from pJMS10 (28) was cloned as a 2.5-kb *Xba*I-*Sal*I fragment into the vector cut with the same enzymes. The resulting construct was transferred to both IsoF and F117 by triparental mating, and integrants were selected on PIA medium containing gentamicin. Merodiploids were resolved by plating on LB medium containing 5% sucrose. The genetic structures of the resulting strains, which were designated IsoF-D4 (*ppuA*::*luxAB*) and F117-D1 (*ppuI*::*npt*, *ppuA*::*luxAB*), were confirmed by Southern blot analysis.

Analysis of *ppuA* promoter activity. As strain F117-D1 bears a promoterless *luxAB* cassette within the *ppuA* gene, transcriptional regulation of *ppuA* can be followed by light measurements. Cells from 1 ml of overnight culture were harvested by centrifugation, washed in LB medium, and inoculated into 100 ml of fresh LB medium to a final OD_{600} of 0.05. The cultures were incubated at 30°C under constant agitation, and samples were taken at regular time intervals. Following addition of 1 μl of *N*-decanal (Sigma, Deisenhofen, Germany) to 10 μl of sample, bioluminescence was measured using the EG&G Berthold MiniLumat LB9506 (Berthold, Bundoora, Australia). Specific luminescence units were defined as relative bioluminescence per unit of OD_{600}. Stimulation of *ppuA* promoter activity by different AHL molecules was analyzed by measuring bioluminescence of F117-D1 after addition of synthetic AHL standards, which either were purchased from Fluka Chemie AG (Buchs, Switzerland) or were kindly provided by P. Williams (School of Pharmaceutical Sciences, University of Nottingham, United Kingdom). To this end, cells of an overnight culture of F117-D1 were diluted five times in fresh LB medium and incubated 1 h at 30°C, and then 1.5-ml aliquots were added to 1.5 ml of LB medium containing 2 μM concentrations of 3-oxo-C14-HSL, 3-oxo-C12-HSL, 3-oxo-C10-HSL, 3-oxo-C8-HSL, 3-oxo-C6-HSL, C12-HSL, C10-HSL, C8-HSL, C6-HSL, and C4-HSL.

Analysis of *ppuI* promoter activity. The intergenic region between *rsaL* and *ppuI* was PCR amplified using the primers PPUI_out (5′-CGGGATCCATGTC ATTGATCAGCGG-3′) and RSAL_out (5′-CGGGATCCGAACCTTGACCA GAATTCG-3′). The 360-bp amplicon was digested with *Bam*HI (restriction sites are underlined) and cloned into the promoter probe vector pGA-L14 cut with the same enzyme. The resulting plasmid, pAS-L22, containing the insert in the orientation that P_{ppuI} drives transcription of *luxAB* was transferred to *P. putida* IsoF. Bioluminescence of the resulting strain, IsoF(pAS-L22), was monitored along the growth curve.

Detection and characterization of AHLs. AHLs were extracted twice with dichloromethane (250:100, supernatant-dichloromethane) from 250 ml of sterile-filtered supernatants of cultures grown in LB medium at 30°C to an OD_{600} of 1.0. The combined extracts were dried over anhydrous magnesium sulfate, filtered, and evaporated to dryness. Residues were dissolved in 250 μl of ethyl acetate. Ten-microliter samples were then applied to C_{18} reversed-phase thin-layer chromatography (TLC) plates (catalog no. 1.15389; Merck, Darmstadt, Germany) and dried with a stream of cold air. Samples were separated by using methanol (60%, vol/vol) in water as the mobile phase. For detection of AHLs the TLC plate was overlaid with a thin film of 0.8% (wt/vol) LB agar (143 ml) seeded with 7 ml of an exponentially grown AHL biosensor and was then incubated at 30°C for 24 h. In order to detect AHL molecules we used two AHL monitor strains, each one capable of responding to a certain range of AHL molecules. The bioluminescent monitor strain *E. coli* MT102(pSB403) (see above) exhibits the highest sensitivity for 3-oxo-C6-HSL and related molecules. Bioluminescent spots were visualized by exposure of the TLC plate to an X-ray film (X-Ray-90; AGFA-Gevaert, Munich, Germany). To detect the long-chain AHL molecules, the GFP-based monitor strain *P. putida* F117(pKR-C12) was used (45). Plasmid pKR-C12 contains a P_{lasB}-*gfp*(ASV) translational fusion together with the *lasR* gene placed under control of P_{lac}. This sensor strain is most sensitive for 3-oxo-C12-HSL and 3-oxo-C10-HSL. Green fluorescent spots were visualized by illuminating the TLC plates with blue light using an HQ 480/80 filter (F44-001; AHF-Analysentechnik, Tübingen, Germany) in combination with a halogen lamp (Intralux 5000-1; Volpi, Schlieren, Switzerland) as a light source. Illumination took place in a dark box equipped with the C2400-40 camera connected to a Pentax closed-circuit television camera lens and an HQ 535/20 filter (F42-001; AHF-Analysentechnik). The tentative identification of AHLs present in spent culture supernatant extracts was achieved by comparison of mobilities (R_f values) relative to those for the synthetic AHL standards. AHL concentrations were estimated by comparing the sizes of TLC spots with those from a dilution series of the respective AHL reference substance.

For quantification of AHL concentrations in liquid cultures, 100 μl of filter-

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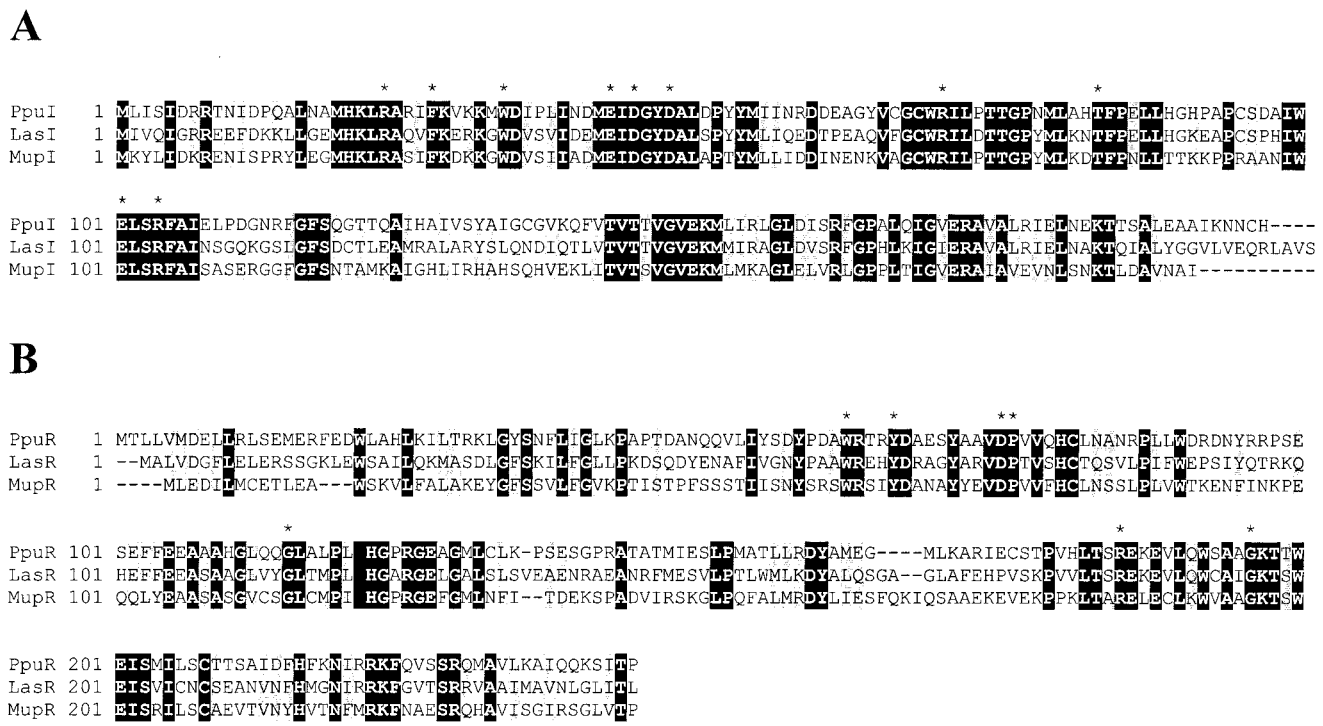


FIG. 1. (A) Protein sequence alignments of PpuI (*P. putida* IsoF), LasI (*P. aeruginosa* PAO1), and MupI (*P. fluorescens* NCIMB 10586). Shown on a black background are identical amino acids, and shown on a grey background are similar amino acids; the asterisks indicate the 10 amino acids that are highly conserved among all members of the LuxI family. (B) Protein sequence alignments of PpuR (*P. putida* IsoF), LasR (*P. aeruginosa* PAO1), and MupR (*P. fluorescens* NCIMB 10586). Asterisks indicate the seven amino acids that are identical in all members of the LuxR family.

sterilized culture supernatants was incubated with 100 μl of logarithmic culture of the AHL monitor strain F117(pKR-C12) in a microtiter plate (all samples were measured in triplicate). Following 6 h of incubation at 30°C, the green fluorescence was measured using the microtiter plate reader Lambda Fluoro 320 Plus (MWG Biotech, Ebersberg, Germany) with an excitation wavelength of 474 nm and emission detection at 515 nm. Data were processed with the KC4 software (Bio-Tek Instruments, Winooski, Vt.). Background fluorescence of the AHL monitor strains was determined by incubation of the sensors with LB medium as a negative control. Specific fluorescence units are defined as relative fluorescence units corrected for background fluorescence per unit of OD_{600}.

Monitoring biofilm formation by confocal laser scanning microscopy (CLSM) and image analysis. Biofilms were grown in artificial flow cells supplied with AB medium (8) containing 0.1 mM citrate. The flow system was assembled and prepared as described previously (7). The substratum consisted of a microscope glass coverslip (Knittel Gläser, Braunschweig, Germany). Overnight cultures in LB medium were subcultured to an OD_{600} of 0.7 before dilution in 0.9% NaCl to an OD_{600} of 0.1. Aliquots (250 μl) of these dilutions were used to inoculate the flow channels. Medium flow was kept at a constant rate of 0.2 mm s^{-1} by a Watson-Marlow 205S peristaltic pump. Incubation temperature was 30°C.

Microscopic inspection and image acquisition were performed on a CLSM (LSM510; Zeiss, Oberkochen, Germany) equipped with a 40× (1.3 numerical aperture) oil objective. Image scanning was carried out with the 488-nm laser line of an Ar-Kr laser. Captured images were visualized using the IMARIS software package (Bitplane AG, Zürich, Switzerland) running on an Indigo 2 workstation (Silicon Graphics, Mountain View, Calif.).

Nucleotide sequence accession number. The nucleotide sequence of the *ppu* locus and flanking region has been deposited in GenBank under accession number AY115588

RESULTS

Cloning and sequencing of the *P. putida luxI* and *luxR* homologs *ppuI* and *ppuR*. The plant-associated *P. putida* strain IsoF produces a wide spectrum of AHL signal molecules, including 3-oxo-C6-HSL, 3-oxo-C8-HSL, 3-oxo-C10-HSL, and 3-oxo-C12-HSL (45). In order to clone the gene(s) directing synthesis of these signal molecules we employed a previously described strategy for the isolation of *luxI* homologs which is based on complementation in *trans* of an AHL biosensor (47). For this purpose *E. coli* MT102 harboring the *lux*-based AHL sensor plasmid pSB403 (53) was transformed with a plasmid library of the *P. putida* IsoF genome. Bioluminescent colonies were picked and were found to contain a 2.9-kb *Sal*I chromosomal fragment. DNA sequencing revealed the presence of two open reading frames (ORFs) with homology to *luxI* and *luxR* genes. One ORF, designated *ppuI*, spans 591 bases and is predicted to encode a 22.0-kDa protein that is homologous to members of the LuxI family of AHL synthases (Fig. 1A). PpuI has 56% identity (74% similarity) with *P. aeruginosa* LasI (34, 35) and 51% identity (69% similarity) with *P. fluorescens* NCIMB 10586 MupI (15). PpuI contains each of the 10 amino acids that are completely conserved among members of the LuxI family (33). The *ppuI* promoter region contains putative −10 and −35 *E. coli* consensus sequences. In addition, adjacent to the −35 site is a 20-bp imperfect palindrome with high similarity to *lux* box-like elements, in particular with operator sequences present upstream of quorum-sensing-regulated genes in *P. aeruginosa* (50) (Fig. 2). These inverted repeats are thought to represent binding sites of LuxR homologs.

The second ORF, designated *ppuR*, is located upstream of *ppuI* and is transcribed in the same direction (Fig. 3). The *ppuR* gene spans 714 bases and encodes a protein with a predicted

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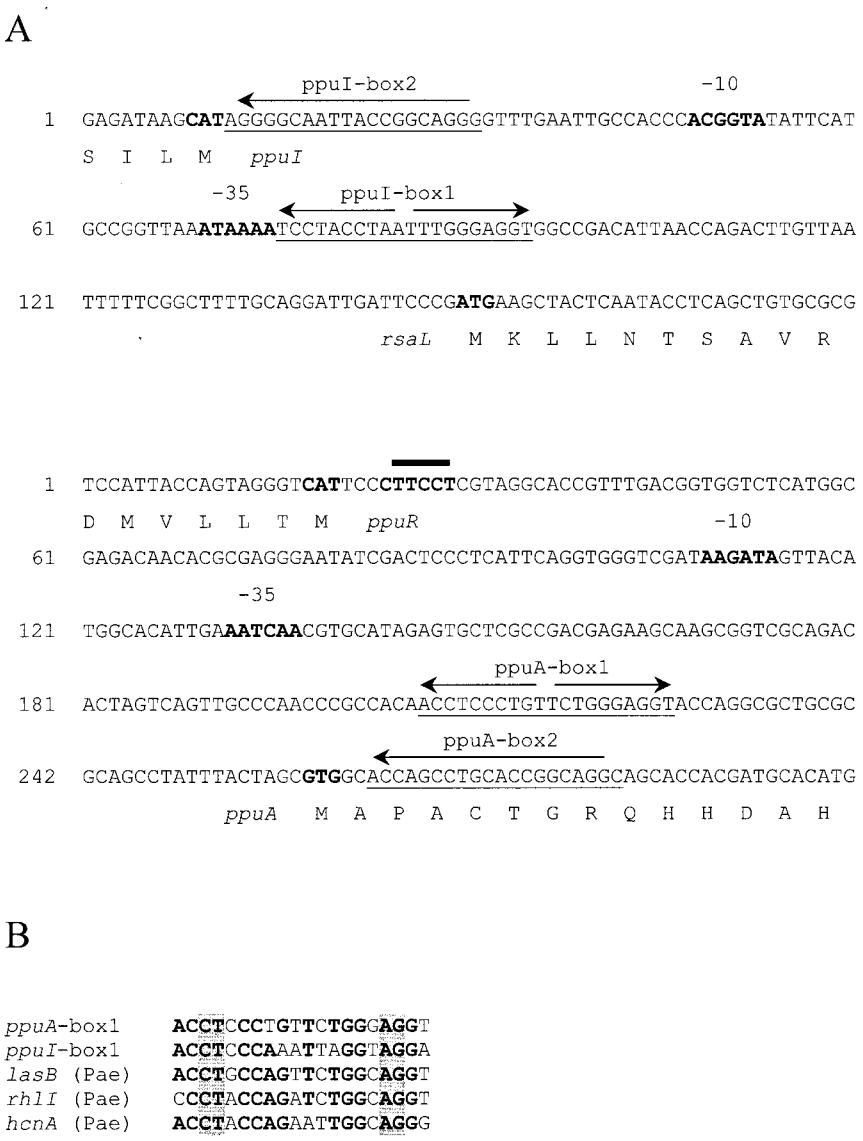


FIG. 2. (A) Nucleotide sequence analysis of the intergenic regions of *ppuI-rsaL* and *ppuR-ppuA*, respectively. Putative −35 and −10 promoter sequences are shown in boldface type. A putative ribosome binding site upstream of *ppuR* is indicated by a line above the sequence. No apparent promoter sequences are present upstream of *ppuA* and *rsaL*. Two inverted repeats exhibiting similarity to *lux* boxes were identified: One of the repeats, denoted ppuI-box1, is located in the *ppuI-rsaL* intergenic region; the second repeat, denoted ppuA-box1, is present in the *ppuR-ppuA* intergenic region. Two additional nonpalindromic sequences, denoted ppuI-box2 and ppuA-box2, were identified in the 5′ regions of *ppuI* and *ppuA*, respectively. (B) Nucleotide sequence comparison of *lux* box-like sequences of the *ppu* locus and previously identified sequences involved in population density-dependent regulation of *lasB*, *rhlI*, and *hcnA* in *P. aeruginosa* (Pae). Nucleotides in boldface type are identical in at least five of the six sequences. The nucleotides shown on a grey background represent the minimal consensus sequence defined for operators of quorum sensing-controlled (*qsc*) genes in *P. aeruginosa* (50).

molecular mass of 27.0 kDa. Over the entire protein, PpuR has 40% identity (62% similarity) with *P. fluorescens* NCIMB 10586 MupR (15) and 42% identity (62% similarity) with *P. aeruginosa* LasR (18) (Fig. 1B). PpuR has all seven invariant amino acids typical of the LuxR family (17).

A further ORF, tentatively designated *rsaL*, which potentially encodes a 76-amino-acid protein of 8.6 kDa, was identified in the intergenic region between *ppuI* and *ppuR*; it is divergently oriented to *ppuI* and separated from it by 138 nucleotides (Fig. 3). When translated, this ORF was 36% identical (66% similar) to the regulatory protein RsaL of *P. aeruginosa*, which functions as a negative regulator of the *P. aeruginosa* quorum-sensing cascade (10).

Furthermore, the 2.9-kb *Sal*I fragment contains a partial ORF downstream of *ppuI* and oriented in the opposite direction with high homology to *suhB* genes from various bacteria (see below).

PpuI directs the synthesis of multiple AHLs. TLC in combination with AHL biosensors provides a simple and rapid technique to characterize and quantify the AHL species produced by a given organism (44). Using the bioluminescent monitor strain *E. coli* MT102(pSB403), we identified four

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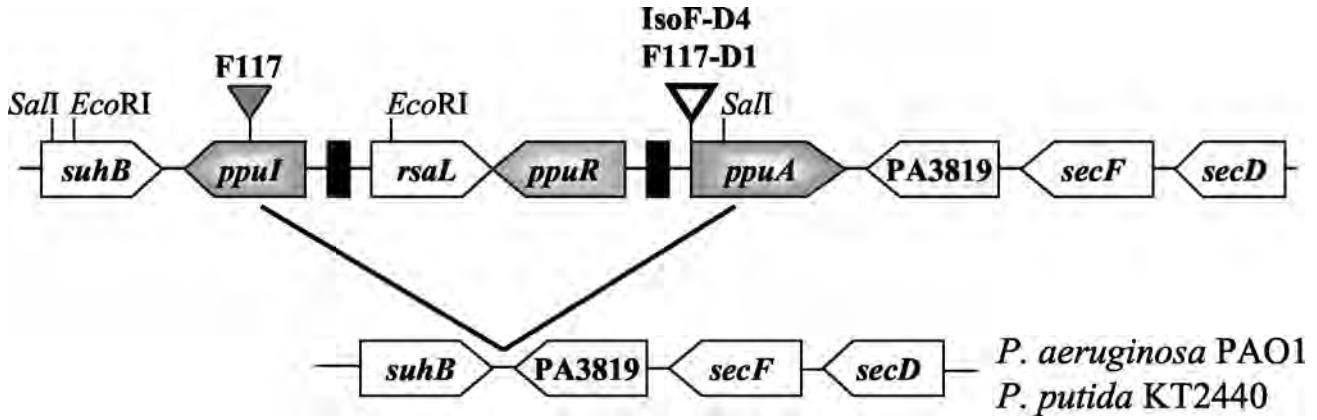


FIG. 3. Genetic organization of the *ppu* locus in *P. putida* IsoF. The identified ORFs are represented by arrows indicating the direction of transcription. Two *lux* box-like sequences in the intergenic regions of *ppuI-rsaL* and *ppuR-ppuA* are indicated as black boxes. Relevant restriction sites are shown. The position of the *npt* cassette in the *ppuI* mutant F117 is indicated by a grey triangle. The position of the *luxAB* cassettes in the *ppuA* mutant IsoF-D4 and the *ppuI ppuA* double mutant F117-D1 is indicated by an open triangle. The panel below the *ppu* locus shows the chromosomal regions of *P. aeruginosa* PAO1 and *P. putida* KT2440 containing *suhB*, PA3819, *secF*, and *secD*. The *ppu* gene cluster of IsoF appears to form an island in a region that is highly conserved in *P. putida* KT2440 and *P. aeruginosa* PAO1.

AHL species with mobility properties similar to those of 3-oxo-C12-, 3-oxo-C10-, 3-oxo-C8-, and 3-oxo-C6-HSL in spent culture supernatants of *P. putida* IsoF (Fig. 4A). As the *E. coli* MT102(pSB403) sensor is not very sensitive for molecules with acyl side chains longer than C_8 (53), we also determined the AHL profile of the wild-type strain IsoF with the aid of the GFP-based monitor strain *P. putida* F117(pKR-C12). As shown in Fig. 4B this sensor allowed the visualization of 3-oxo-C10- and 3-oxo-C12-HSL, while 3-oxo-C8- and 3-oxo-C6-HSL could not be detected due to the insensitivity of the sensor for these molecules. We estimated that the concentrations of 3-oxo-C10- and 3-oxo-C12-HSL in the IsoF culture supernatant are in the range of 1 to 5 μM, while 3-oxo-C8-HSL was produced at a concentration below 100 nM and 3-oxo-C6-HSL was present only in trace amounts (below 1 nM).

The AHL profiles of *E. coli* strains harboring a plasmid with an intact *ppuI* gene, e.g., pGF1 or pGF3, resembled closely the profile of the *P. putida* wild type (Fig. 4A). Inactivation of *ppuI* by insertion of an antibiotic marker gene into the *Apa*I

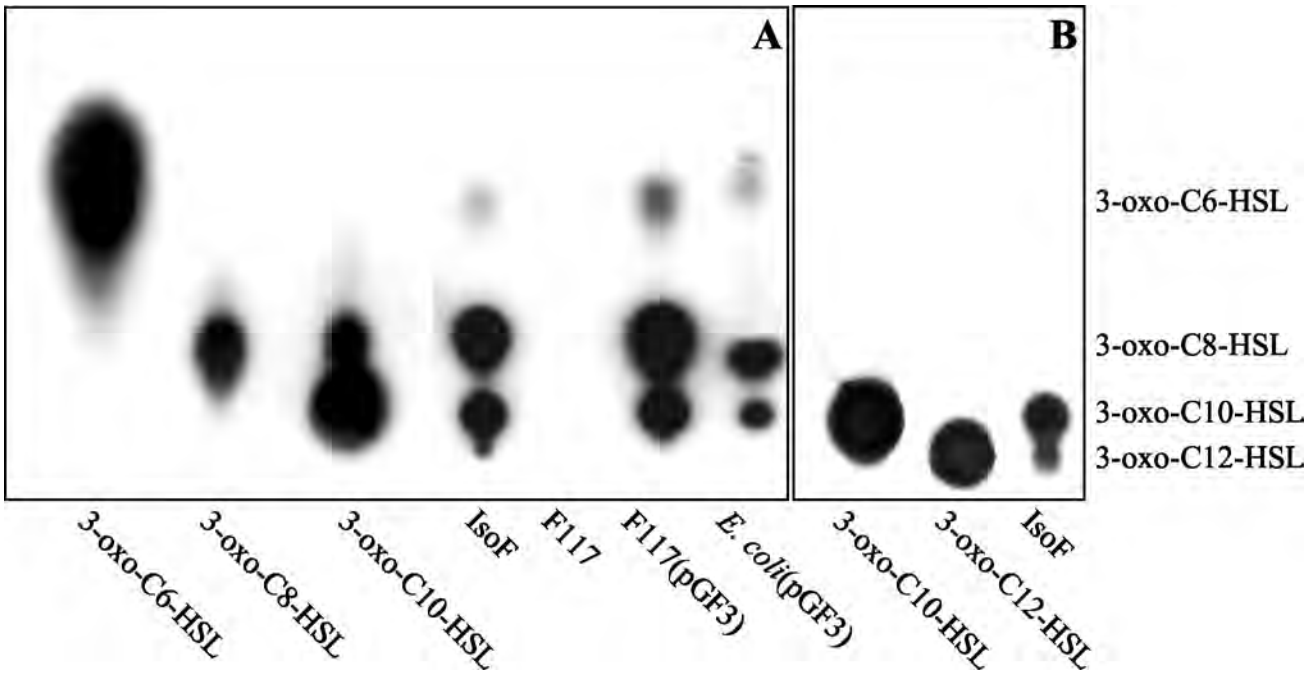


FIG. 4. (A) TLC analysis of AHLs produced by the wild-type IsoF, the *ppuI* mutant F117, F117 harboring plasmid pGF3 (*ppuI*$^+$), and *E. coli* XL1Blue harboring pGF3 (*ppuI*$^+$). The AHL biosensor *E. coli* MT102(pSB403) was used for detection of the spots. Synthetic AHLs were included as reference compounds, as indicated. (B) TLC analysis of AHLs produced by strain IsoF using the long-chain AHL monitor strain F117(pKR-C12).

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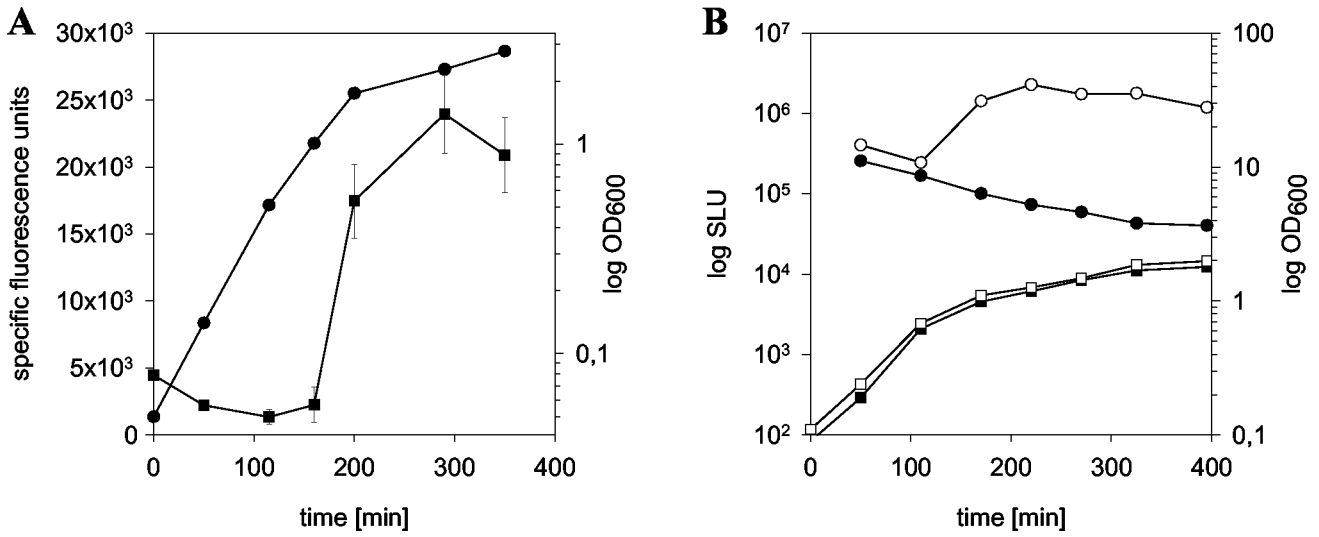


FIG. 5. AHL production of *P. putida* IsoF is growth phase dependent. (A) Samples of spent culture supernatants were taken at regular time intervals throughout the growth curve, and AHLs were quantified by the aid of the GFP-based AHL monitor strain *P. putida* F117(pKR-C12). Specific fluorescence units (■) were defined as relative fluorescence units per unit of OD_{600}. Mean values of three replicates are shown with standard deviations (error bars). Growth (●) was measured spectrophotometrically at 600 nm. (B) Transcription of *ppuI* is induced in the late exponential phase. IsoF harboring plasmid pAS-L22 (open symbols), which contains a transcriptional *ppuI*::*luxAB* fusion, and IsoF harboring the promoter probe vector pGA-L14 (closed symbols) were grown in LB medium, and bioluminescence (○, ●) and optical density (□, ■) were measured at regular time intervals. SLU, specific luminescence units.

site of pGF1 abolished AHL production in *E. coli* (data not shown).

Using a gene replacement method, we constructed a *P. putida* IsoF mutant strain, designated F117, in which the chromosomal *ppuI* gene is interrupted by the insertion of a kanamycin resistance cassette (see Materials and Methods and Fig. 3). Even in highly concentrated dichloromethane extracts of spent culture supernatants from *P. putida* F117, no AHL molecules were detected. Quantification of AHL concentrations of spent culture supernatants using the AHL monitor strain F117(pKR-C12) revealed that the presence of plasmid pGF3 (*ppuI*$^+$) in F117 restored AHL production of the mutant to the level of the wild type (data not shown). In conclusion, these data show that a single polypeptide encoded by the *ppuI* gene is sufficient for the production of AHL signal molecules from precursors present in both *P. putida* and *E. coli*.

Expression of *ppuI* is subject to positive feedback regulation. The finding that a *lux* box-like element is present in the promoter region of *ppuI* (Fig. 2) prompted us to investigate whether AHL synthesis in *P. putida* IsoF is autoregulated. In a first step, we determined the AHL concentrations of spent culture supernatants prepared at various time points during growth of a culture of the wild-type strain. In support of the view that expression of *ppuI* is induced in a density-dependent fashion, we observed that the AHL concentrations increased dramatically (almost 20-fold) between OD_{600} of 1.0 and 2.0 (Fig. 5A). We next constructed a transcriptional fusion of the *ppuI* promoter region to the promoterless *luxAB* genes on vector pGA-L14. The resulting plasmid, pAS-L22, was transferred to *P. putida* IsoF, and the bioluminescence of the recombinant strain was determined along the growth curve. In agreement with our AHL measurements, transcription of *ppuI* was specifically induced (approximately ninefold) at the transition between exponential growth and stationary phase (Fig. 5B). The approach taken to investigate the role of PpuR for transcriptional activation of *ppuI* was to complement the P_{ppuI}-*luxAB* promoter fusion on pAS-L22 present in *E. coli* MT102 with plasmid pPPUR1, which contains the *ppuR* gene under control of the P_{lac} promoter of pGEM-3Zf(+). We observed that bioluminescence increased 10-fold upon addition of 1 μM 3-oxo-C12-HSL to the medium, indicating that transcription of *ppuI* is positively regulated by the PpuR/AHL complex (data not shown).

Sequence analysis of the DNA regions flanking *ppuI* and *ppuR*. We noticed the presence of an additional *lux* box-like element upstream of *ppuR* close to the end of the 2.9-kb chromosomal *Sal*I fragment. This sequence is strikingly similar to the inverted repeat identified in the promoter region of *ppuI* (Fig. 2). This observation led us to speculate that a quorum sensing-regulated gene might be present upstream of *ppuR*. To address this issue we decided to determine the DNA sequences flanking *ppuR* and *ppuI*. For this purpose we digested chromosomal DNA of F117 with *Sac*I and cloned the resulting fragments into pGEM-3Zf(+). Following transformation of *E. coli* XL1Blue, a Km^r clone, designated pGF4, containing a 13-kb chromosomal DNA fragment was isolated. Sequence analysis of the *ppuR* upstream region revealed an ORF, denoted *ppuA*, the predicted product of which has 509 amino acids and a molecular mass of 55 kDa. PpuA showed significant similarity to various long chain fatty acid coenzyme A (CoA) ligases and to nonribosomal peptide synthetases. The best identity was 24% to the AttG CoA ligase of *Rhodococcus fascians* (41% similarity). This protein is predicted to be involved in the synthesis of an autoregulatory compound, which is essential for full virulence on tobacco (31). PpuA contains a putative AMP-binding domain (amino acids 176 to 187) and a sequence motif

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exhibiting similarity to the fatty acyl-CoA synthetase signature motif. This 25-amino-acid consensus sequence, D**GW**LHTG DIGXWXPXGXLKII**D**R**KK**, is thought to compose part of the fatty acid binding site and was shown to be essential for catalytic activity of the enzyme (5). However, PpuA contains only five (underlined residues) of nine of the amino acid residues described to be invariant or highly conserved (residues shown in boldface type).

The DNA sequence downstream of *ppuA* shows striking similarity to chromosomal regions of *P. putida* KT2440 (89% identity over a DNA stretch of 1.8 kb) and *P. aeruginosa* PAO1. In *P. aeruginosa*, this region encodes the conserved hypothetical protein PA3819, SecF, and SecD. SecF and SecD are highly conserved proteins involved in protein export, PA3819 is a putative membrane protein of unknown function. The *ppuI* flanking region was highly homologous to the regions present downstream of PA3819 in *P. putida* KT2440 (93% identity over a stretch of 5.5 kb) and *P. aeruginosa* PAO1. The genetic organization of the corresponding chromosomal regions in *P. aeruginosa* PAO1, *P. putida* KT2440, and *P. putida* IsoF is depicted in Fig. 3. The ORF downstream of *ppuI* showed 82% identity (91% similarity) to SuhB of *P. aeruginosa* and 96% identity (98% similarity) to SuhB of *P. putida* KT2440. SuhB of *E. coli* possesses inositol monophosphatase activity and was suggested to participate in the posttranscriptional control of gene expression by modulating mRNA decay (23).

On the basis of these data we suggest that the *ppu* locus of *P. putida* IsoF comprises four genes, *ppuI*, *rsaL*, *ppuR*, and *ppuA*, which form an island in a chromosomal region that is highly conserved between *P. putida* KT2440 and *P. aeruginosa* PAO1.

Expression of *ppuA* is AHL dependent. To analyze regulation of *ppuA* expression we constructed a *ppuA*::*luxAB* transcriptional fusion in the chromosome of the *ppuI* mutant F117. This was accomplished by allelic exchange of the wild-type *ppuA* gene with the recombinant *ppuA*::*luxAB* allele present on the suicide plasmid pEXF2 (see Materials and Methods for details). When the resulting mutant, which was denoted F117-D1, was grown in LB medium, light levels were low throughout the growth curve (Fig. 6). However, upon addition of 1 μM 3-oxo-C10-HSL, *luxAB* activity increased dramatically (56-fold) within 90 min.

We also determined the response of F117-D1 to different AHL molecules. To this end, early-log-phase cells of F117-D1 were supplemented with 1 μM AHL (Fig. 7) and bioluminescence was measured after 4 h of incubation at 30°C. As expected, *ppuA* promoter activity was highest when 3-oxo-C10-HSL or 3-oxo-C12-HSL, the two most abundant AHL molecules produced by the organism, was added to the cultures. A response was also observed with 3-oxo-C14-HSL, while 3-oxo-C8-HSL or 3-oxo-C6-HSL had no effect. Molecules without a 3-oxo group were also unable to stimulate *ppuA* expression at a concentration of 1 μM. The minimal concentration of 3-oxo-C10-HSL and 3-oxo-C12-HSL required for activation of bioluminescence in F117-D1 was below 10 nM (data not shown). These results demonstrate that expression of *ppuA* is quorum sensing regulated.

The *ppu* quorum-sensing system influences biofilm structural development. Previous work has provided evidence that AHL-dependent quorum-sensing systems are involved in the regulation of biofilm formation (9, 22, 30). These findings prompted us to investigate the role of quorum sensing in biofilm formation of *P. putida* IsoF. Biofilms were grown in artificial flow cells, which allow biofilm development to be monitored on a glass surface under highly defined conditions in real time (7). Moreover, by using a CLSM the three-dimensional structures of the biofilm can be reconstructed. For this analysis it was necessary to tag *P. putida* IsoF and the two mutants F117 and IsoF-D4 with GFP. This was accomplished by inserting a $P_{A1/04/03}$-*gfp*-T_0T_1 transposon cassette into the chromosome of

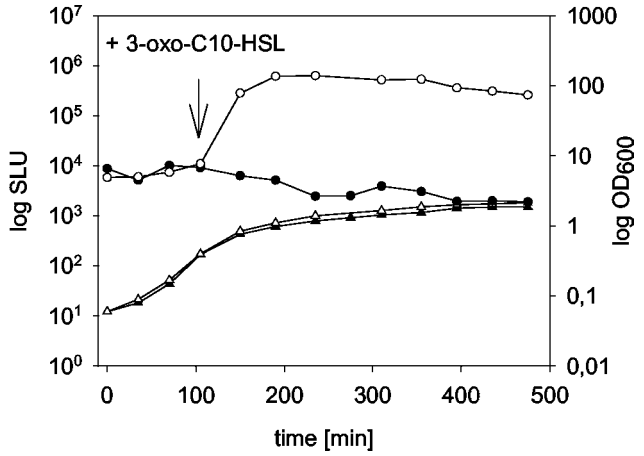


FIG. 6. Transcription of *ppuA* is quorum sensing regulated. Bioluminescence of strain F117-D1 (*ppuA*::*luxAB ppuI*), in which the promoterless *luxAB* genes are transcriptionally fused with the chromosomal *ppuA* gene, was monitored in the presence (open symbols) or absence (closed symbols) of 1 μM 3-oxo-C10-HSL. Parallel cultures of F117-D1 were grown in LB medium, and bioluminescence (●, ○) and OD_{600} (▲, △) were monitored along the growth curve. At the time point indicated by the arrow, one culture was supplemented with 1 μM 3-oxo-C10-HSL. SLU, specific luminescence units.

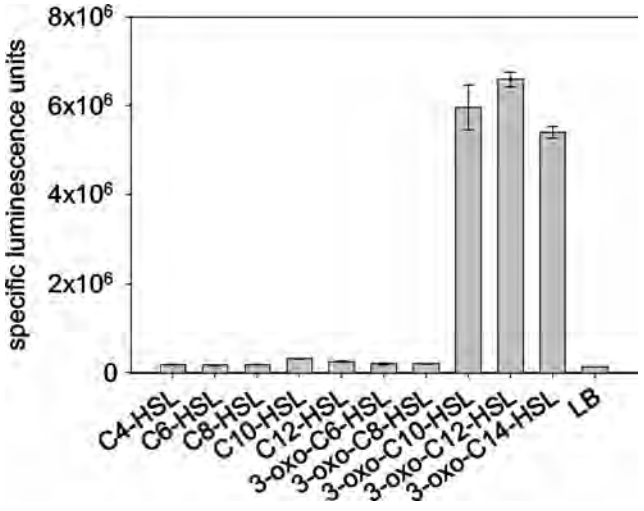


FIG. 7. Response of *P. putida* F117-D1 (*ppuA*::*luxAB ppuI*) to different AHL molecules. F117-D1 was grown in the presence of various synthetic AHL compounds, and bioluminescence of the cultures was determined at an OD_{600} of 1.0. Mean values of the specific luminescence units obtained from three independent experiments are shown with standard deviations (error bars).

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each of the three strains using the suicide plasmid pBK-miniTn*7*-gfp3. The tagged strains used for further investigations were carefully tested with respect to growth rates and were found to be indistinguishable from the parental strains (data not shown).

Parallel flow chambers were inoculated with each of the three GFP-tagged strains, and biofilm development was monitored for 8 days. Visual inspection of CLSM images revealed that the structures of the biofilms formed during the first 2 days showed only minor differences. Thereafter, however, the *ppuI* mutant developed into a highly structured biofilm with characteristic microcolonies, while cells of the wild type uniformly covered the surface, giving rise to unstructured biofilms (Fig. 8).

Addition of 3-oxo-C12-HSL to the growth medium of F117 gave rise to an unstructured biofilm which was virtually indistinguishable from the one formed by the wild type. We also analyzed the biofilm formed by the *ppuA* mutant IsoF-D4. This biofilm was strikingly similar to the one formed by F117, suggesting that the inability of the *ppuI* mutant to form biofilms with a wild-type structure may be due to a defect in *ppuA* expression.

DISCUSSION

Several reports have shown that some *P. putida* strains produce AHL signal molecules, indicating that they employ quorum-sensing systems to coordinate expression of certain phenotypic traits in a density-dependent manner (14, 26, 42). In this study, we cloned and sequenced the quorum-sensing locus of *P. putida* IsoF, a strain which has been isolated from the tomato rhizosphere (45). The IsoF quorum-sensing system, termed *ppu*, is most similar to the *las* system of *P. aeruginosa*. PpuI and PpuR share the greatest similarities with LasI and LasR, and both systems employ 3-oxo-C12-HSL as a signal molecule. In fact, when *ppuI* is expressed in a *P. aeruginosa lasI* mutant which, due to the genetic lesion, no longer produces extracellular proteolytic activity, the protease synthesis defect is fully restored (data not shown), indicating that LasI and PpuI are functionally interchangeable. Furthermore, an ORF with significant similarity to *rsaL*, which is present in the intergenic region between *lasI* and *lasR*, was identified in the region between *ppuI* and *ppuR*. In *P. aeruginosa* RsaL acts as a repressor of the *las* system, preventing premature activation of the quorum-sensing cascade at low cell densities. Further work will be needed to analyze the function of the *rsaL* homolog in *P. putida* IsoF.

The *las* system of *P. aeruginosa* is on top of a hierarchically arranged signaling cascade (36). The lower-level quorum-sensing circuit, the *rhl* system, consists of the transcriptional activator RhlR and the AHL synthase RhlI, which directs the synthesis of C4-HSL. This quorum-sensing cascade regulates expression of a battery of extracellular virulence factors such as exoenzymes (elastase and alkaline protease), secondary metabolites (pyocyanin, hydrogen cyanide, and pyoverdin), and toxins (exotoxin A) (for reviews, see references 11 and 51).

Using various AHL monitor strains with different specificities we were unable to detect AHL molecules in the spent culture supernatant of the *ppuI* mutant F117 (Fig. 4A; data not shown), indicating that the *ppu* system may be the only AHL-dependent quorum-sensing locus present in *P. putida* IsoF. Furthermore, when supernatants of *E. coli* cultures expressing *ppuI* were analyzed by TLC, the AHL pattern resembled closely the one of the IsoF wild type. Our data suggest that PpuI directs the synthesis of 3-oxo-C12- and 3-oxo-C10-HSL and, as minor products, 3-oxo-C8- and 3-oxo-C6-HSL. This result is reminiscent of the situation found with LasI from *P. aeruginosa*, which catalyzes the synthesis of primarily 3-oxo-C12-HSL together with smaller amounts of 3-oxo-C10-, 3-oxo-C8-, and 3-oxo-C6-HSL (19, 35, 52).

Two *lux* box-like sequences with striking similarity to operator sequences present in the promoter regions of quorum sensing-regulated genes in *P. aeruginosa* (50) were identified within the *ppu* locus. One of these palindromic sequences is present upstream of *ppuA*, and the second one is located in the promoter region of *ppuI*. As both genes are quorum sensing regulated, it appears likely that these sequences are essential for AHL-dependent gene expression. Interestingly, two additional sequences, which show limited homology to the *lux* box-like elements but are not inverted repeats, are present in the 5′ regions of *ppuA* and *ppuI* (Fig. 2). While the function of these sequences remains to be elucidated, it is interesting that similar arrangements of *lux* box-like sequences are also found in the promoter region of *lasB* in *P. aeruginosa* (40) and *mupA* in *P. fluorescens* NCIMB 10586 (15). In the case of *P. aeruginosa* both operator sequences are involved in AHL-dependent *lasB* expression (40).

Previous work has shown that the *las* quorum-sensing system of *P. aeruginosa* is involved in the regulation of biofilm formation (9). Compared with the wild type, a *lasI* mutant of *P. aeruginosa* only formed flat and undifferentiated biofilms, suggesting that the *las* system is required for the development of a typical biofilm structure. Importantly, the *lasI* mutant biofilm exhibited greater sensitivity to the biocide sodium dodecyl sulfate than did the wild-type biofilm. More recently, it was shown that AHL-dependent quorum sensing plays a role in biofilm development of *Burkholderia cepacia* and *Aeromonas hydrophila* (22, 30). In both cases AHL-negative mutants showed defects in the late stages of biofilm development and thus were unable to form biofilms with a typical wild-type structure.

P. putida IsoF formed very homogenous rather unstructured biofilms, while the *ppuI* mutant F117 formed structured biofilms with characteristic microcolonies and water-filled channels. Most interestingly, when AHL signal molecules were added to the medium the mutant biofilm lost its structure and converted into an unstructured biofilm that was similar to the one formed by the wild type.

Upstream of *ppuR* we identified a gene, designated *ppuA*, which is expressed in an AHL-dependent manner. Our data indicate an involvement of *ppuA* in biofilm maturation, as the biofilm formed by IsoF-D4 (*ppuA*) was virtually indistinguishable from the one formed by F117 (*ppuI*). In a recent study, Zhang and Pierson (55) identified a second quorum-sensing system in *P. aureofaciens*, which consists of the LuxI/LuxR homologs CsaI/CsaR. This regulatory circuit does not interact with the *phz* quorum-sensing system for phenazine production but does interact with it for protease production. More important in the context of this study, however, was the finding that the *csa* system is involved in regulating biosynthesis of cell surface components. It was suggested that the *csaR* mutant had

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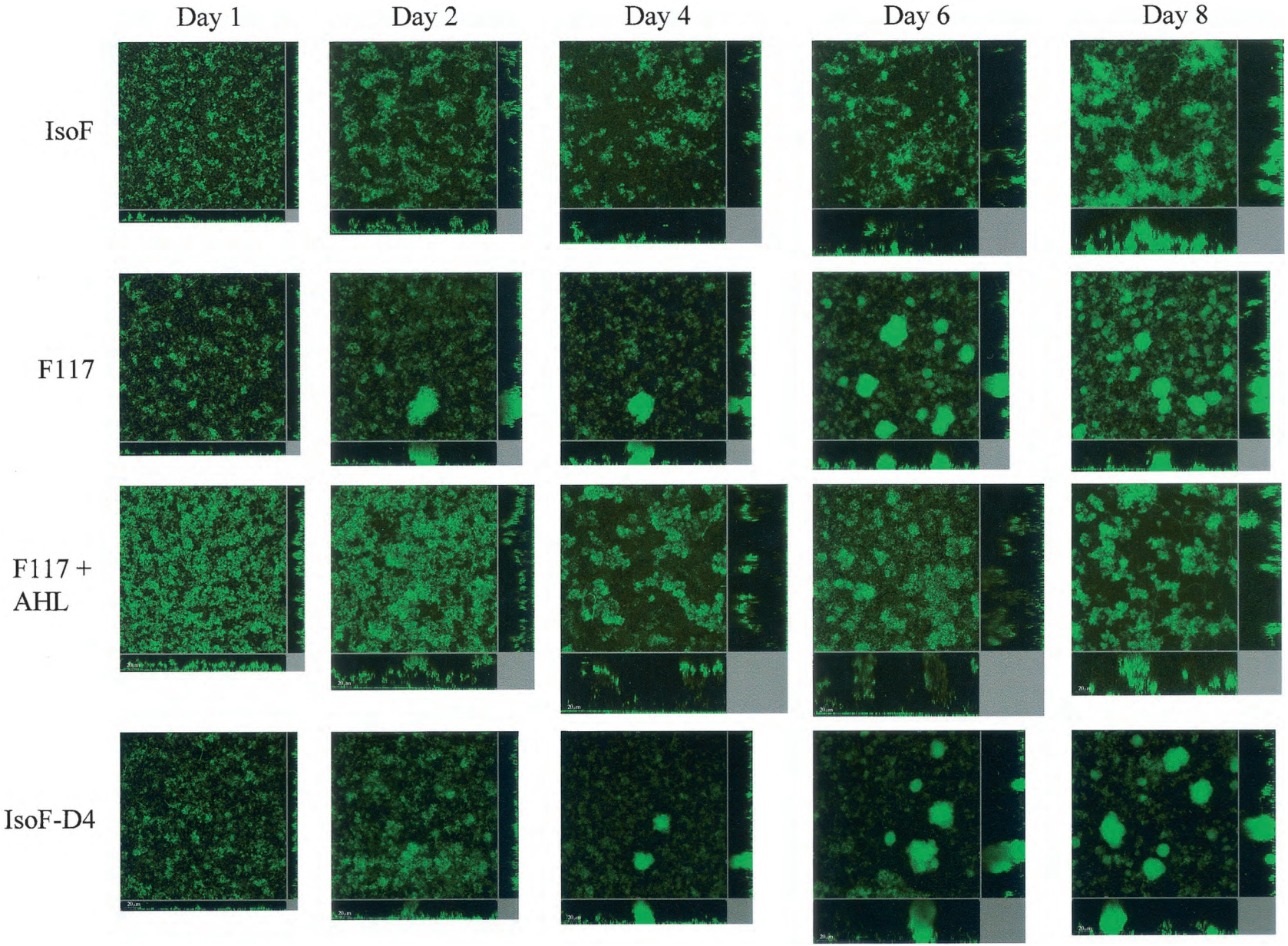


FIG. 8. Biofilm formation of *P. putida* IsoF is quorum sensing regulated. Flow chambers were inoculated with *gfp*-tagged derivatives of IsoF, the *ppuI* mutant F117 in the presence or absence of 2 μM 3-oxo-C12-HSL, and the *ppuA* mutant IsoF-D4. CLSM images were taken on days 1, 2, 4, 6, and 8 postinoculation. The larger central plots are simulated fluorescence projections. Shown in the right and lower frames of each panel are vertical sections through the biofilms.

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alterations in fatty acid composition or relative percentages of some fatty acids compared to the wild type. Given that the *ppuA* gene in *P. putida* IsoF is similar to long-chain fatty acid CoA ligases, we speculated that AHL-dependent expression of PpuA may in some way alter the fatty acid composition of the cell membrane. This could change the surface properties of the cells, which, in turn, would affect biofilm structures. However, a comparison of the whole-cell fatty acid profiles of the wild type and the *ppuI* mutant F117 did not reveal any significant differences (data not shown).

At present we cannot exclude the possibility that quorum sensing-regulated genes outside of the *ppu* locus exist in *P. putida* IsoF, which code for factors that affect biofilm structure. By employing proteomics Sauer and Camper (42) showed that 16 proteins are differentially expressed in the *P. putida* strain ATCC 39168 when the medium is supplemented with AHL signal molecules, indicating that the quorum-sensing system is a global regulator in this strain. As only one of the identified proteins, PotF, was found to be regulated by both adhesion to an abiotic surface and AHL addition, it was concluded that quorum sensing does not play a role in the initial attachment process but may play a role in the late stages of biofilm development. Work is currently under way to define the quorum-sensing regulon of *P. putida* IsoF and to identify target genes outside of the *ppu* gene cluster.

Comparison of the sequence retrieved from pGF4 with the unfinished *P. putida* KT2440 genome database (http://www.tigr.org) revealed that none of the four genes contained in the *ppu* locus is present in the KT2440 genome. A comparison with the *P. aeruginosa* PAO1 genome (46) showed that the *ppuR-rsaL-ppuI* region is highly homologous to the *lasR-rsaL-lasI* region of PAO1. However, no *ppuA* homolog was found in the PAO1 genome. Most interestingly, the regions flanking the *ppu* gene cluster of *P. putida* IsoF are virtually identical with the *suhB*-PA3819-*secD-secF* chromosomal regions of both *P. aeruginosa* and *P. putida* (Fig. 3). The gene order of this region is even conserved in *E. coli*. Hence, the *ppuR-rsaL-ppuI-ppuA* gene cluster of *P. putida* IsoF forms an island within a highly conserved genomic region. These data may indicate that the *ppu* locus was acquired via horizontal gene transfer and was subsequently integrated into the *suhB*-PA3819 intergenic region. We searched the sequence for the presence of IS elements using the IS database of the Centre National de la Recherche Scientifique (http://www-appli.biotoul.fr) but could not detect any significant homologies to IS elements available in this database. However, we noticed the presence of five copies of a short DNA sequence (5′-CCAAGCCGGDC-3′) within an approximately 200-bp DNA fragment spanning the region between the C-terminal ends of *ppuA* and PA3819 (data not shown). Two of these sequences form an inverted repeat, while the remaining three sequences are present as direct repeats. It is tempting to speculate that these repeats were involved in the acquisition of the *ppu* gene cluster.

In a recent survey, we screened more than 80 *P. putida* strains for their ability to produce AHLs. Of all the strains tested, 44% provoked a positive signal with at least one of the AHL biosensors used (3). Southern blot analyses of nine AHL-producing isolates using DNA fragments containing part of the *ppuI* or *ppuR* gene as probes failed to give positive hybridization signals, indicating that the investigated strains do not carry homologous genes. Moreover, using a PCR assay we were able to show that none of the AHL-positive strains contain an insertion in the *suhB*-PA3819 intergenic region. These results show that, although production of AHL signal molecules is widespread among different *P. putida* isolates, the *ppu* gene cluster is so far unique for *P. putida* IsoF.

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