# Regulation of the biosynthesis of cyclic lipopeptides from Pseudomonas putida PCL1445

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# Chapter 4

## Genetic characterization of the regulatory region of the putisolvin biosynthetic gene, *psoA*, in *Pseudomonas putida* PCL1445

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*in preparation*

**Abstract**

The rhizobacterium *Pseudomonas putida* PCL1445 secretes two surfactants putisolvins I and II, the production of which is determined by the lipopeptide synthetase gene *psoA*. Putisolvins play an important role in swarming, biofilm formation and biofilm degradation. Previously, we have shown that expression of *psoA* is controlled by several interacting regulatory systems including GacA/GacS, DnaK/DnaJ/GrpE and the PpuI/PpuR quorum sensing system. In this study, sequence analysis upstream of *psoA* revealed the presence of a *luxR* homologous gene named *psoR*. Analysis of the PsoR protein sequence showed that it contains a predicted helix-turn-helix DNA-binding motif at its C-terminus. A *psoR* mutant fails to produce detectable amounts of putisolvin and showed a lack of *psoA* expression. Transcriptional fusions of the *psoR* with the *luxAB* genes were constructed to quantify *psoR* expression and to determine its relationship with other previously identified regulatory genes in PCL1445. The results showed that expression of *psoR* required functional *gacA*, *gacS* and *dnaK* genes. Mutation in *gacA*, *gacS*, *dnaK*, or *psoR* did not affect production of *N*-acylhomoserine lactones (AHLs) in PCL1445. These results demonstrate that *psoR* is located downstream of GacA/GacS and DnaK in the regulatory hierarchy controlling putisolvin production in *P. putida* PCL1445 and indicate that the *ppuI*/*ppuR* quorum sensing system constitutes a separate regulatory pathway of putisolvin production.

**Introduction**

*Pseudomonas putida* strain PCL1445 produces two cyclic lipopeptides, putisolvins I and II, that inhibit biofilm formation and degrade existing biofilms from several *Pseudomonas* spp. among which the opportunistic human pathogen *P. aeruginosa*. Both molecules function as biosurfactants and have a similar structure (differing in one amino acid) consisting of a 12 amino acids polar peptide head N-terminally attached to a hexanoic lipid chain (Kuiper *et al.*, 2004). Putisolvins I and II are synthesized via a lipopeptide synthetase gene designated as *psoA*. Biosynthetic mutants defective in putisolvins I and II synthesis form a thicker biofilm than the wild type strain, demonstrating that these biosurfactants are major determinants of the regulation of biofilm formed by *P. putida.*

Regulation of putisolvin production in *P. putida* PCL1445 is complex, in which the *gacA*/*gacS* regulon plays a critical role (Dubern *et al.*, 2005; this Thesis,

Chapter 2). The *gacS* and *gacA* genes, encode a sensor and a response regulator of a two-component signal transduction system, respectively. The GacA/GacS system controls putisolvin production and consequently the biofilm structure of PCL1445. The transmembrane protein GacS functions as a histidine autokinase that undergoes phosphorylation, supposedly in response to a so far unidentified environmental stimulus (Hrabak and Willis, 1992). GacA is a cognate response regulator that contains a receiver domain (phosphorylation) at its N terminus and a helix-turn-helix (HTH) DNA-binding motif at its C-terminus. GacS/GacA homologs are widely conserved in fluorescent pseudomonads and form a global regulatory system that controls the expression of many cellular functions such as the production of proteases, *N*-acylhomoserine lactones and various antimicrobial metabolites (Kitten *et al.*, 1998; Pierson *et al.*, 1998; Chin-A-Woeng *et al.*, 2001). The *dnaK* gene of strain PCL1445 was recently characterized as a member of the *gacS*/*gacA* dependent regulatory cascade controlling putisolvin production (Dubern *et al.*, 2005; this Thesis, Chapter 2). It was shown that DnaK is part of a complex heat-shock chaperone system, which together with DnaJ and GrpE, acts positively on the expression of *psoA*. Recently, it was shown that also the quorum sensing system *ppuI-rsaL-ppuR* of PCL1445 takes part in the regulation of biofilm formation by controlling the production of putisolvins.

Despite evidence that these three regulatory systems, GacA/GacS, DnaK-DnaJ-GrpE, and PpuI-RsaL-PpuR, are involved in regulating putisolvin production, details about the regulatory cascade (or cascades) are largely unexplored. In the present study, we describe that a regulatory gene, named *psoR*, is present in the region upstream of *psoA* and that it controls the expression of *psoA*. In addition, the effects of mutations in *gacA*, *gacS* and *dnaK* on *psoR* expression and on production of AHLs are analyzed. Evidence is presented that the *psoR* gene positively regulates the expression of *psoA* and thereby putisolvin biosynthesis.

## Materials and Methods

*Bacterial strains and growth conditions*

All bacterial strains used are listed in Table 1. *Pseudomonas* strains were grown at 28°C in King's medium B (King *et al.*, 1954) or in the defined BM medium (Lugtenberg *et al.*, 1999) supplemented with 2.0 % glycerol (BDH Laboratory Supplies, Pool, England). *E. coli* strains were grown in Luria-Bertani medium

(Sambrook *et al.*, 2001) at 37°C. Media were solidified with 1.8 % agar (Select Agar; Invitrogen, Life Technologies, Paisley, United Kingdom). The antibiotics kanamycin, tetracyclin, gentamycin and carbenicillin were added, when necessary, to final concentrations of 50, 40, 2 and 100 µg $ml^{-1}$, respectively.

**Table 1.** Bacterial strains and plasmids

| Strain or plasmid | Relevant characteristics | Reference or source |
|---|---|---|
| *Pseudomonas* | | |
| PCL1445 | Wild-type *P. putida*; colonizes grass roots and produces biosurfactants | Kuiper et al. (2004) |
| PCL1622 | Tn5*luxAB* derivative of PCL1445; mutated in a *gacA* homolog | Dubern et al. (2005) |
| PCL1623 | Tn5*luxAB* derivative of PCL1445; mutated in a *gacS* homolog | Dubern et al. (2005) |
| PCL1626 | PCL1445 derivative mutated in the *psoR* homologue; constructed by single homologous recombination using pMP7570 | This study |
| PCL1627 | Tn5*luxAB* derivative of PCL1445; mutated in a *dnaK* homolog | Dubern et al. (2005) |
| PCL1633 | Tn5*luxAB* derivative of PCL1445; mutated in a *psoA*, a lipopeptide synthetase homologue | Dubern et al. (2005) |
| PCL1636 | PCL1445 derivative mutated in the *ppuI* homologue; constructed by single homologous recombination | Dubern et al. (2005) |
| PCL1644 | PCL1445 derivative mutated in the orf2 (*oprM* homolog); constructed by single homologous recombination using pMP7590. | This study |
| *E.coli* | | |
| DH5α | *EndA1 gyrSA96 hrdR17(rK-mK-) supE44 recA1*; general purpose host strain used for transformation and propagation of plasmids | Hanahan et al. (1983) |
| Plasmids | | |
| pRL1063a | Plasmid harbouring a promotorless Tn5*luxAB* transposon, $Km^r$ | Wolk et al. (1991) |
| pRK2013 | Helper plasmid for tri-parental mating, $Km^r$ | Schnider et al. (1995) |

| | | |
|---|---|---|
| pME6010 | Cloning vector which is maintained in *Pseudomonas* strains without selection pressure, $Tc^r$ | Heeb et al. (2000) |
| pME6031 | Cloning vector which is maintained in *Pseudomonas* strains without selection pressure, containing a terminator beside the MCS, $Tc^r$ | Heeb et al. (2000) |
| pME3049 | Cloning vector, used for homologous recombination, $Tc^r$, $Hg^r$ | Ditta et al. (1980) |
| pMP5285 | pME3049 derivative, missing the $Hg^r$ gene, used for single homologous recombination, $Km^r$ | Kuiper et al. (2001) |
| pMP5512 | pMP6010 containing a PCR fragment of 1.3 kb containing *gacA* gene of PCL1445, $Tc^r$ | Dubern et al. (2005) |
| pMP5539 | pMP6031 based plasmid harboring a *psoA*::*gfp* transcriptional fusion and a P*tac*DsRed, $Gm^r$, $Tc^r$ | Dubern et al. (2005) |
| pMP5540 | pME6031 based control plasmid harboring a transcriptionally inactive *psoA*::*gfp* and a Ptac DsRed, $Gm^r$, $Tc^r$ | Dubern et al. (2005) |
| pMP7570 | pMP5285 plasmid containing a PCR fragment of 0.58 kb of *psoR* gene of PCL1445 | This study |
| pMP7579 | pME6031 containing the *psoR*::*luxAB* promoter in transcriptionally active orientation, $Tc^r$ | This study |
| pMP7582 | pME6031 containing the *psoR*::*luxAB* promoter in transcriptionally active orientation, $Tc^r$ | This study |
| pMP7589 | pME6010 containing a PCR fragment of 3.35 kb with P*tac*-*psoR*-*oprM* of PCL1445, $Tc^r$ | This study |
| pMP7590 | pMP5285 plasmid containing a PCR fragment of 0.6 kb of *oprM* of PCL1445, $Km^r$ | This study |
| pAK211 | Autoinducer reporter construct based upon the *Vibrio fisheri* bioluminescence (*lux*) system; $Cm^r$ | Kuo et al. (1994) |

*Quantification of biosurfactant production*

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) as described previously (Kuiper *et al.*, 2004).

*Construction of* psoR *and* oprM *mutants of PCL1445*

The *P. putida* PCL1445 *psoR* mutant, PCL1626 was constructed by single homologous recombination. A 0.58-kb internal fragment of the *psoR*-homologous gene of strain PCL1445 was obtained by PCR using primers oMP872 (5' ACCTCAGTGAATGGACCCTTG 3') and oMP873 (5' GAGCTGTTTTTCACGTTCAGC 3'), cloned into the pGEM-T Easy Vector System I (Promega Corporation, Madison, WI, USA) and transferred as a *EcoR*I-*EcoR*I insert to pMP5285 (Kuiper *et al.*, 2001) resulting in pMP7570. pMP7570 was conjugated to *P. putida* PCL1445 by tri-parental mating using *E. coli* containing pRK2013 as a helper strain (Schnider et al., 1995). Strain PCL1626 was obtained as a resistant colony resulting from single homologous recombination on KB agar medium supplemented with kanamycin (50 µg $ml^{-1}$). The insertion of the suicide construct in *psoR* was confirmed by sequence analysis of the suicide plasmid that was recovered from the genomic DNA of PCL1626 using *Cla*I.

A *P. putida* PCL1445 *oprM* mutant was constructed by single homologous recombination as described above. A 0.6-kb internal fragment of the *oprM* homologous gene of strain PCL1445, that was obtained by PCR using primers oMP1060 (5' GCCGAGCTGTTGCCCAAGGT 3') and oMP1061 (5' ACCGCGTCGTGCACGCCGCAA 3'), was cloned into pMP5285, resulting in pMP7590. Plasmid pMP7590 was transferred to strain PCL1445 by tri-parental mating and transformants were selected on KB agar medium supplemented with kanamycin (50 µg $ml^{-1}$). Strain PCL1644 was obtained as a resistant colony resulting from single homologous recombination.

*Complementation of* psoR *mutant of PCL1445*

Complementation of strain PCL1626 (*psoR*) was carried out using pMP7589, a cloning vector derived from pME6010 (Heeb *et al.*, 2000) in which a 3.35-kb fragment containing *psoR* and *oprM* was inserted. This insert was obtained by PCR using primers oMP885 (5' GGGGAATTCTTGACAATTAATCATCGGCTCGTATAATGTG TGGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGCTAAATGAAGCTGACCGA CAGCCTC 3') and oMP1016 (5' GGGTTACCAGCAAACGCTTGA 3'). Plasmid pMP7589 was transferred to strain PCL1626 by tri-parental mating and transformants were selected on KB agar medium supplemented with tetracyclin (40 µg $ml^{-1}$).

*Construction of* psoR::luxAB *reporter fusions*

Plasmid pME6031, derived from pME6010 in which the constitutive promoter of the kanamycin resistance gene was removed and a terminator inserted near the multicloning site (Heeb *et al.*, 2000), was used to create a *psoR*::*luxAB* reporter plasmid. The *luxAB* reporter genes were obtained from pRL1063a (Wolk *et al.*, 1991) and cloned as a 3.5-kb *sac*I-*pst*I fragment into pME6031, resulting in pMP7579. The promoter region upstream of *psoR* was amplified from PCL1445 by PCR using primer oMP912 (5' GGTACCAGGTCCTTCTGATTGATCCG 3') and primer oMP913 (5' GAGCTCCATATCATTGTCTTCCTTGATTC 3'). The 0.5-kb PCR product was cloned as a *kpn*I-*Sac*I fragment into pMP7579, resulting in pMP7582 containing *psoR*::*luxAB* in which a terminator is located upstream of the *psoR* promoter. Plasmid pMP7582 was transferred into PCL1445 and in its derivatives PCL1622, PCL1623, PCL1626, PCL1627, and PCL1636 by tri-parental mating. Ex-conjugants were selected on KB agar medium supplemented with tetracyclin (40 µg $ml^{-1}$). The activity of the *psoR* transcriptional fusions was assayed by determining their luminescence activity (expressed in Luminescence Counts per Second). Aliquots (100 µl) were removed from cultures diluted to a proper $OD_{620nm}$ and analyzed for bioluminescence activity by the method described as below.

*Quantification of bioluminescence in* luxAB *reporter strains*

Expression of *luxAB* genes was determined by quantification of bioluminescence during culturing. Cells from overnight cultures were washed with fresh medium and diluted to an $OD_{620}$ of 0.1. Cultures were grown in KB or in BM medium in a volume of 20 ml under vigorous shaking. During growth, 100 µl samples were taken in triplicate to quantify luminescence. A volume of 100 µl of an 0.2 % *n*-decyl-aldehyde substrate solution (Sigma, St. Louis, MO, USA) in a 2.0 % bovine serum albumin solution was added and luminescence was determined with a MicroBeta 1450 TriLux luminescence counter (Wallac, Turku, Finland), and normalized to luminescence per $OD_{620}$ units.

*Quantification of fluorescence in* gfp *reporter strains*

Green fluorescent protein (GFP) was quantified using a HTS7000 Bio Assay Reader (Perkin & Elmer Life Sciences, Oosterhout, The Netherlands). Bacterial strains were grown to an optical density at 620 nm of 2.0 and diluted to $OD_{620nm}$ 0.6. Fluorescence of the diluted cultures was quantified using a white 96-well

microtiter plate containing 200 µl culture aliquots. Fluorescence of the cultures was determined at an excitation wavelength of 485 nm and an emission wavelength of 520 nm.

*High-Performance Liquid Chromatography (HPLC) analysis of putisolvins*

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

*Extraction and detection of AHLs autoinducers from spent culture medium*

Autoinducer activity was isolated by adding 3 volumes of dichloromethane to 7 volumes of supernatant fluid of a 50 ml KB or BM bacterial culture. After shaking for 1 h at 120 rpm, the organic phase was removed by evaporation under vacuum to dryness (Mc Clean *et al.*, 1997). Supernatant extracts were redissolved in 100 µl of ethyl acetate and the content of 5 µl was applied on a $C_{18}$ reverse-phase TLC plate (Merck, Darmstadt, Germany), which was developed with methanol-water (60:40; vol/vol).

The autoinducer production was analyzed by adding overnight cultures of *E.coli* DH5α containing pAK211 (Kuo *et al.*, 1994) grown for 10 h in LB medium supplemented with 20 µg of chloramphenicol per ml. TLC plates were overlaid with

0.8 % LB top agar layer containing 50 µl. $ml^{-1}$ of the pAK211 or pSB1075 harbouring strain, followed by incubation at 28°C for 16h. Autoinducer activity was detected by the emission of light after applying a Fuji medical X-Ray film (Fuji Photo Film CO., Ltd., Tokyo, Japan) on the TLC plates.

## Results

*Sequence analysis of the region upstream of* psoA

Previously it was shown that putisolvin synthesis is governed by *psoA*, which shows homology to lipopeptide synthetase genes (Kuiper *et al.*, 2004). PsoA shows highest similarity to *syrE* encoded syringomycin synthetase of *P. syringae* pv. *syringae* (Guenzi *et al.*, 1998). Due to the large size of lipopeptide synthetase genes only part of *psoA,* which lacks the promoter region, was identified (Kuiper *et al.*, 2004).

Identification of the upstream region of *psoA* resulted from further sequence analysis of mutant PCL1436, which was obtained from a screening of four hundred Tn5::*luxAB* transposants of *P. putida* PCL1445 and selected for loss of biosurfactant activity as judged by the drop collapsing assay (Kuiper *et al.*, 2004). Digestion of the total chromosomal DNA of mutant PCL1436 with *EcoR*I followed by subsequent re-circularization of the fragments resulted in plasmid pMP5459, which containes a 12 kb-chromosomomal insert flanking the Tn5*luxAB*. Sequence analysis identified the ATG start codon of *psoA* and the presence of an *orf* (*orf1*) transcribed in the opposite direction of *psoA* (Fig. 1A). The predicted protein encoded by *orf1* showed 60% homology at the amino acid level with a still uncharacterized transcriptional regulator from the LuxR family in *P. syringae* pv. *syringae* B728a (Feil *et al.*, 2005) and 30 % homology with the putative DNA-binding protein SalA characterized in *P. syringae* pv. *syringae* B301D (Kitten *et al.*, 1998). A conserved ribosome-binding site (RBS) was identified 10 to 15 bp (GAAGG) upstream of the start codon of *psoA*.

Interestingly, a nucleotide sequence similar to $\sigma^{70}$-dependent promoters (referred to as P2) was identified 418 bp upstream of the *psoA* start codon and overlaps the start codon of *orf1* (Fig. 1B). A conserved ribosome-binding site (RBS) was identified 7 to 11 bp (GGAGG) upstream of the start codon of *orf1*.

Two nucleotide sequences (referred to as P1 and P1') are proposed to regulate the transcription of *orf1*. The predicted promoter region P1 showed similarity with a TyrR regulating promoter (Yang *et al.*, 2004). In *E. coli*, TyrR

protein can act both as a repressor and as an activator of transcription. The regulation of transcription is determined by the position and nature of the recognition sites (TyrR boxes) associated with each of the promoters (Pittard *et al.*, 2005; Yang *et al*, 2002). P1 was found to overlap the putative *lux* box located 83 bp upstream of *psoA* start codon. The second nucleotide sequence (P1') located 12 to 38 bp upstream of the *orf1* transcriptional start codon showed similarity with an Integration Host Factor (IHF) binding site (Mc Leod *et al.*, 2001) (Fig. 1B).

Two additional ORFs were identified downstream of *orf1* (Fig. 1B). The predicted ORF2 protein was most similar to outer membrane proteins associated with secretion systems in gram-negative bacteria. The OprM protein of *P. aeruginosa* (Nakajima *et al.*, 2000) shares, with 63 % identity, the highest degree of similarity to ORF2. The start codon of *orf2* was identified 17 bp downstream of the *orf1* stop codon. In addition, no consensus promoter-like sequence was detectable upstream of *orf2*, suggesting that transcription of *orf1* and *orf2* may be coupled. Downstream of *orf2*, *orf3* was identified, which shows 86 % identity with *uspA* of *P. putida* KT2440 (Nystrom and Neidhardt, 1992), which encodes the Universal Stress Protein A. A ribosomal-binding site (RBS) was identified 7 to 11 bp (GGAGG) upstream of the start codon of *uspA* (Fig. 1B). Conserved -10 and -35 regions that are characteristic of a $\sigma^{70}$-dependent promoter were identified upstream of the transcriptional start site of the *uspA* gene (Fig. 1B), but no rho-independent or rho-dependent transcriptional terminator region was observed at the 3' end of *orf2.* For the rest of the work described in this chapter *orf1* will be referred to as *psoR* and *orf2* as *oprM.*

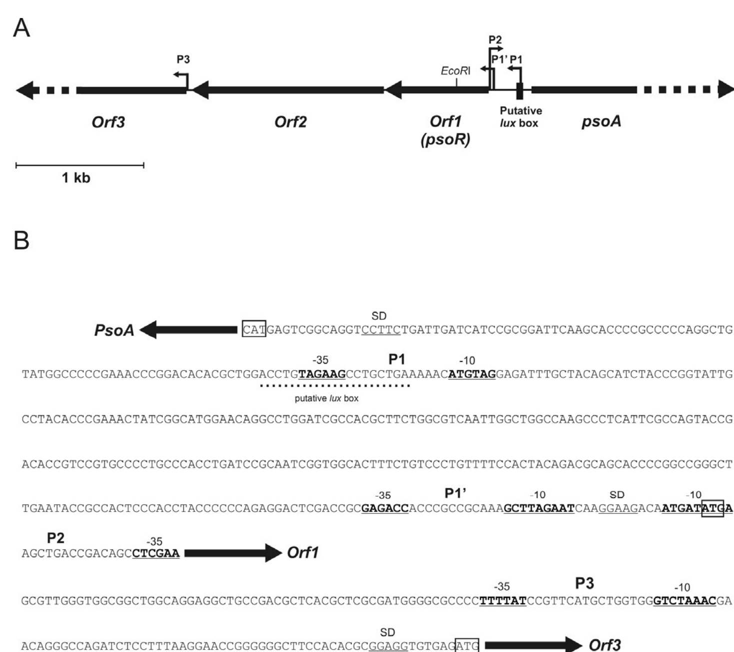


**Fig. 1.** Sequence analysis of the upstream region of the putisolvin biosynthetic *psoA* gene. **Panel A.** Genetic organization of the region upstream of putisolvin biosynthetic gene cluster (*psoA*). The putative promoter elements P1 and P1' of *psoR*, P2 of *psoA*, P3 of *orf3* are indicated. **Panel B.** Sequence of the *psoA* – *orf1* intergenic region. Features of the putative promoters P1 and P1' of *orf1*, P2 of *psoA*, P3 of *orf3* are indicated. Nucleotide sequence of the putative - 10 and - 35 boxes are shown in bold. The putative ribosome-binding site (SD) is underlined. The putative *lux* box in the region upstream of *psoA* is underlined with dots.

*Effects of insertional mutagenesis of* orf1 *(*psoR*) and* orf2 *(*oprM*) on putisolvin production*

To investigate whether *psoR* and *oprM* are involved in putisolvin production, insertion mutants of PCL1445 were constructed by single homologous recombination using suicide plasmids pMP7570 and pMP7590 (for construction see Materials and Methods section), resulting in strains PCL1626 and PCL1644, respectively. The proper integration of pMP7570 and pMP7590 was confirmed by sequence analysis (data not shown).

Biosurfactant production by PCL1626 (*psoR*) and PCL1644 (*oprM*) grown in KB medium under standard conditions until stationary phase was reached, was

quantified by the Du Nouy ring assay. In contrast to culture supernatant of the wild type that decreases the surface tension between culture medium and air, culture supernatant of PCL1626 (*psoR*) was not able to decrease the surface tension (53 mN $m^{-1}$), indicating a lack of putisolvin production (Fig. 2A). Culture supernatant of strain PCL1644 (*oprM*) showed a delayed decrease of surface tension during growth when compared to the wild type strain, and eventually reached the same value as that of the wild type strain during stationary phase (32 mN $m^{-1}$) (data not shown).

The production of putisolvins I and II by strains PCL1445, PCL1626 (*psoR*), PCL1644 (*oprM*) was analyzed by HPLC analysis (Fig. 2B). Putisolvins were extracted from stationary phase KB culture supernatants and production was quantified by determination of the area of the putisolvin I and II peaks showing surfactant activity as tested by the drop collapsing assay. Production of putisolvins by PCL1626 (*psoR*) was not detectable (Fig. 2B). Production of putisolvins by PCL1644 (*oprM*) did not show a significant reduction when compared to the wild type (data not shown). Introduction of pMP7589 harbouring *psoR* gene restored putisolvin production to wild type levels in strain PCL1626 (*psoR*) (Fig. 2B).

Production of putisolvins by mutant PCL1622 (*gacA*) is abolished and severly decreased in mutant PCL1627 (*dnaK*) (Dubern *et al.*, 2005; this Thesis, Chapter 2). To investigate whether PsoR is able to complement a mutation in *gacA* or *dnaK*, pMP7589 harboring the constitutively expressed *psoR* gene was introduced into PCL1622 (*gacA*) and PCL1627 (*dnaK*) and production of putisolvins by the resulting strains was analyzed by HPLC analysis. Introduction of pMP7589 restored putisolvin production to the level comparable to the wild type in strain PCL1622 (*gacA*) and restored putisolvin production partially in strain PCL1627 (*dnaK*) (Fig. 2B).

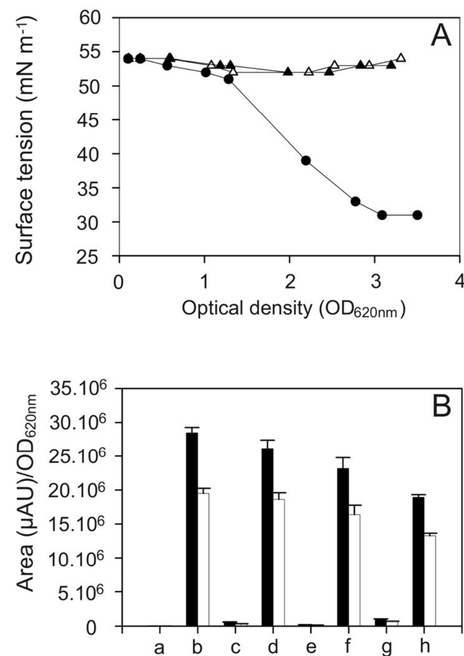


**Fig. 2.** Biosurfactant production in *psoR* mutant of strain PCL1445. **Panel A.** Quantification of surface tension of culture supernatants of *P. putida* strain PCL1445 (wild type) (●), PCL1633 (*psoA*) (∆), PCL1626 (*psoR*) (▲), all grown to the stationary phase in KB medium. **Panel B.** Quantification of production of putisolvins I and II by HPLC analysis of culture supernatants after ethyl acetate axtraction. Bar a: PCL1633 (*psoA*); bar b: PCL1445; bar c: PCL1626 (*psoR*); bar d: PCL1626 (*psoR*) harbouring pMP7589 (*psoR*); bar e: PCL1622 (*gacA*); bar f: PCL1622 (*gacA*) harbouring pMP7589 (*psoR*); bar g: PCL1627 (*dnaK*); bar h: PCL1627 (*dnaK*) harbouring pMP7589 (*psoR*).

*The PsoR (ORF1) regulatory protein is a member of the LuxR family*

PsoR protein of strain PCL1445, which is predicted to be 260 amino acids in length, revealed homology to prokaryotic regulatory proteins. The HTH DNA-binding domain, frequently observed in prokaryotic regulatory proteins, was identified at the C terminus of PsoR (Fig. 3). Database searches with BlastP demonstrated that approximately 60 amino acids in the C-terminal region of PsoR shared significant similarity to similar regions of known regulatory proteins. The C terminus of PsoR exhibited 35 % identity to GerE (Cutting and Mandelstam, 1986), 34 % identity to

DctR (Hamblin *et al.*, 1993), and 32 % identity to FixJ (Anhhamatten and Hennecke, 1991), which are members of the LuxR family (Fig. 3).

Further analysis of the C-terminal regions of PsoR identified a three-element fingerprint that provides a signature for the HTH DNA-binding motif of LuxR bacterial regulatory proteins (Bairoch *et al.*, 1993). Moreover, three highly conserved residues in the amino terminal regions of members of the response regulator subfamily corresponding to Asp206, Glu207, and Lys244 in FixJ (Parkinson and Kofoid, 1992) were detected in PsoR (Fig. 3). However, 5 highly conserved amino acids in the amino-terminal regions of members of an autoinducer-binding subfamily (Fuqua *et al.*, 1996) corresponding to Trp59, Tyr69, Asp79, Pro80, and Gly121 of LuxR were not detected in PsoR.

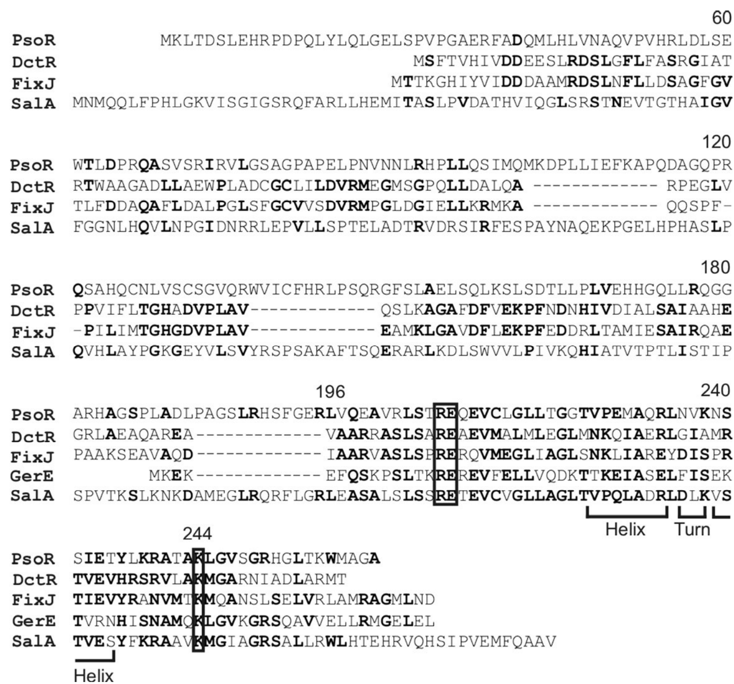


**Fig. 3.** Alignment of the predicted amino acid sequence of *psoR* with that of related proteins. Amino acids that are shared among two or more proteins are indicated in bold. Dots indicate gaps introduced to optimize alignments. The three conserved amino acid residues among regulatory proteins of the FixJ subfamily are boxed. When amino acids 204-265 of PsoR were used for a Blastp analysis, P-values for the alignments were 1.5 x $10^{-6}$ for DctR, 2.9 x $10^{-5}$ for FixJ and 4.0 x $10^{-5}$ for GerE. The percentage of identity for amino acids 196-244 of PsoR to the corresponding regions of the other three proteins was 34 % (DctR), 32 % (FixJ), 35 % (GerE), 36 % (SalA).

*Regulation of* psoR *and* psoA

To determine whether *psoR* transcriptionally regulates putisolvin expression in PCL1445, a *psoA*::*gfp* transcriptional fusion was introduced into PCL1626 (*psoR*). The expression of the *gfp* strongly decreased in the *psoR* mutant when compared to the wild type strain (Fig. 4).

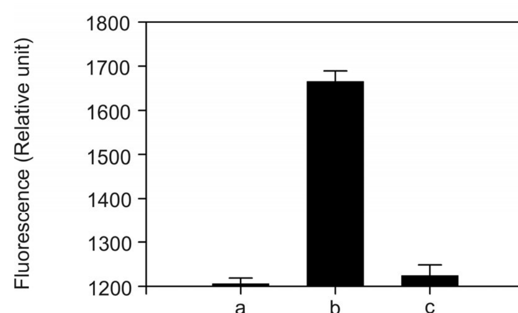


**Fig. 4.** Expression of *psoA* of *P. putida* PCL1445. Expression was determined using the *psoA*::*gfp* reporter in PCL1445, and PCL1626 (*psoR*) by measuring fluorescence from cells containing the *psoA* promoter fused to e*gfp* (pMP5539). pMP5540 containing the *psoA* promoter in the transcriptionally inactive orientation was used as a control vector. Bar a: PCL1445 harbouring the control vector pMP5540; bar b: PCL1445 harbouring pMP5539; bar c: PCL1626 (*psoR*) harbouring pMP5539. Mean values of duplicate cultures are given.

The biosynthesis of putisolvin was demonstrated to be regulated by the GacA/GacS two component regulatory system and by the DnaK/DnaJ/GrpE heat-shock chaperone system (Dubern *et al.*, 2005; this Thesis, Chapter 2). In addition, *dnaK* transcriptional activity was shown to require functional GacA/GacS (Dubern *et al.*, 2005; this Thesis, Chapter 2). Interestingly, complementation of a mutation in *gacA* by introduction of the *psoR* gene suggested that *psoR* requires *gacA* for its expression (Fig. 2B). To follow *psoR* expression during growth and to analyze the influence of the identified regulatory genes on *psoR* expression, a *psoR*::*luxAB* transcriptional fusion was constructed and its expression was analyzed during growth of PCL1445, PCL1622 (*gacA*), PCL1623 (*gacS*) and PCL1627 (*dnaK*). Strains PCL1445, PCL1622 (*gacA*), PCL1623 (*gacS*), and PCL1627 (*dnaK*) harbouring *psoR*::*luxAB* were cultured at 28$^{o}$C in liquid KB medium to the stationary phase. The expression of luciferase activity started during early exponential phase of

growth ($OD_{620}$ 0.3) and reached its maximum at the end of exponential phase ($OD_{620}$ 1.5) in the wild type strain (Fig. 5). The expression of luciferase activity appeared to be reduced in the *gac* mutants PCL1622 (*gacA*) and PCL1623 (*gacS*) and was partially reduced in PCL1627 (*dnaK*) when compared to the wild type, indicating that GacA/GacS two-component system as well as DnaK have a positive effect on PsoR synthesis in PCL1445 (Fig. 5). To test whether *psoR* is autoregulated, *psoR*::*luxAB* was introduced into PCL1626 (*psoR*). The transcriptional activity of *psoR*::*luxAB*, in PCL1626 was slightly reduced when compared to that of the wild type strain (Fig. 5).

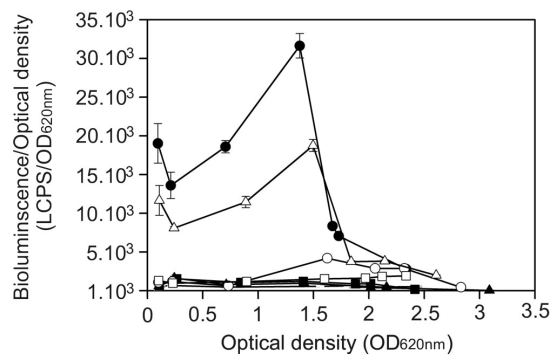


**Fig. 5.** Expression of *psoR* of *P. putida* PCL1445. Expression was determined using the *psoR*::*luxAB* reporter in PCL1445, PCL1623 (*gacS*), PCL1622 (*gacA*), PCL1627 (*dnaK*) by measuring luminescence from cells containing the *psoR* promoter fused to *luxAB* (pMP782). pMP7579 lacking the *psoR* promoter insertion was used as a control vector. Strains were grown at 28°C in KB medium. Luminescence of cell cultures was determined during growth of PCL1445 harbouring the control vector pMP7579 (■), PCL1445 harbouring pMP7582 (●), PCL1622 (*gacA*) harbouring pMP7582 (□), PCL1623 (*gacS*) harbouring pMP7582 (▲), PCL1627 (*dnaK*) harbouring pMP7582 (○), PCL1626 (*psoR*) harbouring pMP7582 (Δ). Mean values of duplicate cultures are given.

Mutations in *gacS* and *gacA* are often reported to affect the ability of Gram Negative bacteria to produce acyl homoserine lactones (AHLs) involved in quorum sensing (Pierson and Pierson, 1996; Chin-A-Woeng *et al.*, 2001; Bertani *et al.*, 2004). The biosynthesis of putisolvin was shown to be controlled to quorum sensing regulation by the *ppuI-rsaL-ppuR* system when grown in BM-glycerol medium (Dubern *et al.*, 2006; this Thesis, Chapter 3). These data and the limited similarity

of the PsoR protein to members of the LuxR subfamily of quorum sensing regulators raised the question of whether any of the observed phenotypes of *psoR* mutations might be the result of effects on AHLs production. Accordingly, relevant strains from this study were tested for the production of AHLs by TLC analysis and were assayed using the *lux* indicator from *Vibrio fisheri*. The wild type strain PCL1445 produces four or more AHLs which are recognized by the bioreporter (Dubern *et al.*, 2006; this Thesis, Chapter 3). Surprisingly, the *gacA* and *gacS* genes as well as *dnaK* and *psoR* are apparently not necessary for the production of AHLs in PCL1445 (Fig. 6). The same results were obtained when strains were grown in BM-glycerol (Fig. 5) or in KB medium (data not shown) until stationary phase of growth was reached.

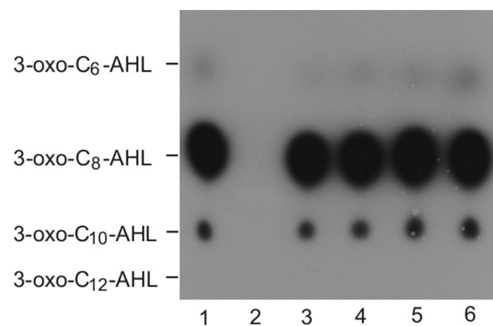


**Fig. 6.** C18-reverse phase thin-layer chromatography analysis of *N*-acyl-L-homoserine lactones produced by *P. putida* PCL1445 and its mutant derivatives.
Cells of strain *P. putida* PCL1445 and its derivatives mutants were grown in BM-glycerol to an $OD_{620}$ value of 1.0 and centrifuged. The supernatant fluids were extracted with dichloromethane and the organic fractions were analyzed using TLC. The chromatograms were overlayed with *E. coli* reporter strains for the detection of AHLs. The biosensor *E. coli* harbouring pAK211 was used to visualize AHLs produced by PCL1445 and mutants derivatives. Culture supernatant extracts of following strains were analyzed: PCL1445 (lane 1). PCL1936 (*ppuI*) (lane 2), PCL1623 (*gacS*) (lane 3), PCL1622 (*gacA*) (lane 4), PCL1627 (*dnaK*) (lane 5), PCL1626 (*psoR*) (lane 6).

## Discussion

The aim of this work was to identify the promoter and further upstream region of *psoA* relevant to the regulation of putisolvin biosynthesis. An ORF located upstream of *psoA* was identified as a member of the LuxR family of regulatory

proteins based on homology analysis and referred to as *psoR* (Fig. 1A). Sequence analysis revealed the presence of HTH DNA-binding motifs at the C-terminus of PsoR (Fig. 3). The HTH motif has been observed in many regulatory proteins (Pabo and Sauer, 1992) which are divided into more than 10 groups, including the LuxR, AraC, and MarR families. The PsoR protein appeared to be most closely related to members of the LuxR regulatory family, such as DctR (Hamblin *et al.*, 1993) and FixJ (Anthamatten and Hennecke, 1991). An approximately 60 amino acid residue region of the C-terminus containing the four helices and their turns, which is called a three-element fingerprint, provides the signature for the HTH motif of the LuxR family of bacterial regulatory proteins. The observation that the PsoR protein exhibits the highest similarity to DctR and FixJ and contains the three-element fingerprint suggests that it is a member of the LuxR family (Fig. 3). Despite this homology, PsoR protein lacks five highly conserved residues at the N-terminus characteristic for the LuxR subfamily which is composed of autoinducer-binding regulators activated by homoserine lactones (Fuqua *et al.*, 1996). Moreover, PsoR does not affect synthesis of homoserine lactones produced by PCL1445 (Fig. 6). In conclusion, it does not appear to belong to the autoinducer-binding regulator subfamily. The second major subfamily of transcriptional regulators is composed of the response regulators of two-component signal transduction systems, such as FixJ (Anthamatten and Hennecke 1991) and DctR (Hamblin *et al.*, 1993). Three highly conserved residues (Arg, Glu, Lys) characteristic of the reponse regulators were found in the PsoR sequence, suggesting that PsoR may be closely related to this subfamily of regulators (Fig. 3).

Interestingly, sequence analysis revealed almost immediately downstream (17 bp) of *psoR* the presence of another *orf* (*orf2*), which suggests that the translation of the *orf2* is coupled to that of *psoR*. ORF2 is a homologue of OprM, a component of a prokaryotic type I secretion system as shown for *P. aeruginosa* (Nakajima *et al.*, 2000). Mutation of ORF2 of strain PCL1445 resulted in a delayed putisolvin production (data not shown), suggesting that OprM might be involved in the (initial) secretion of putisolvins.

The GacS sensor kinase and its cognate GacA response regulator control the expression of a large number of secondary metabolites and extracellular enzymes involved in pathogenicity (Barta *et al.*, 1992; Liao *et al.*, 1997; Kitten *et al.*, 1998), in biocontrol of soilborne diseases (Chancey et al., 1999; Chin-A-Woeng et al., 2000; Koch *et al.*, 2002), and in ecological fitness (van den Broek *et al.*, 2003). The GacA/GacS two component system is frequently found at the top of the

regulatory hierarchy controlling secondary metabolite production (Chin-A-Woeng *et al.*, 2001; Chatterjee *et al.*, 2003; Kitten *et al.*, 1998) and quorum sensing activity is often reported to be *gacA*/*gacS*-dependent (Chin-A-Woeng *et al.*, 2001; Kitten *et al.*, 1998; Bertani *et al.*, 2004). Although there are reports on subordinate LuxR-like regulatory proteins involved in phytotoxin biosynthesis controlled by GacA/GacS (Kitten *et al.*, 1998), PsoR appears to belong to a different subgroup of regulatory proteins and therefore may fulfill a different function in the biosynthesis of putisolvin in *P. putida* strain PCL1445.

A mutation in *psoR* abolished putisolvin production in the wild type strain (Fig. 2), giving the first evidence of its regulatory role in putisolvin biosynthesis. The decrease of expression of the *psoA*::*gfp* fusion in *psoR* mutant when compared to the wild type (Fig. 4) suggests that the effect of *psoR* on putisolvin production can be accounted for by its effect on *psoA* transcriptional activity (Fig. 7), although PsoR may regulate other genes involved in putisolvin production as well.

The restoration of putisolvin production in a *gacA* mutant and in a *dnaK* mutant by the constitutively expressed *psoR* gene *in trans* (Fig. 2B), leads us to hypothesize that *psoR* is regulated by *gacA* and *dnaK* (Fig. 7). The reduced levels of transcriptional activity of *psoR*::*luxAB* fusion observed in *gacA*, *gacS* and *dnaK* backgrounds (Fig. 5) clearly supports this conclusion (Fig. 7).

Although our data show that *gacA*, *gacS*, and *dnaK* regulate *psoR* expression, it is not clear whether this regulation occurs directly of through intermediate factors. There are two interesting features observed in the *psoR* promoter region that may be related to its expression (Fig. 1B). One of these is the presence of a nucleotide consensus sequence similar to those involved in the regulation of response regulators such as TyrR (Yang *et al.*, 2004). Interestingly, this regulatory element overlaps the putative *lux box* of *psoA*, a specific inverted repeat sequence of 20 nucleotides that is believed to be the binding site for the quorum sensing regulator LuxR resulting in transcriptional activation (Fuqua *et al.*, 1994). Another interesting feature is the presence of a second regulatory element similar to the integration host factor (IHF) binding site, which was reported to modulate the activity of the promoter of the styrene catabolic operon *styA* in *P. fluorescens* ST under different growth conditions (Leoni *et al.*, 2005). IHF is a small heterodimeric protein that binds DNA and induces a sharp bend (>160$^{o}$). This bending is thought to facilitate the formation of a higher-order structure in processes such as recombination, transposition, replication, and transcription (Mc Leod *et al.*, 2001). The location and the nature of the regulatory elements in the *psoR*-*psoA* intergenic

region could have significant regulatory consequences for the expression of *psoA* from the point of view of transcriptional competition. At this stage, however, any role of the two identified regulatory elements in the *psoR* promoter region in any process involving *psoA* transcriptional activity remains hypothetical and requires biochemical analyses including the identification of the transcriptional start sites of *psoR* and *psoA*, and of the presence and affinity of binding sites for PsoR.

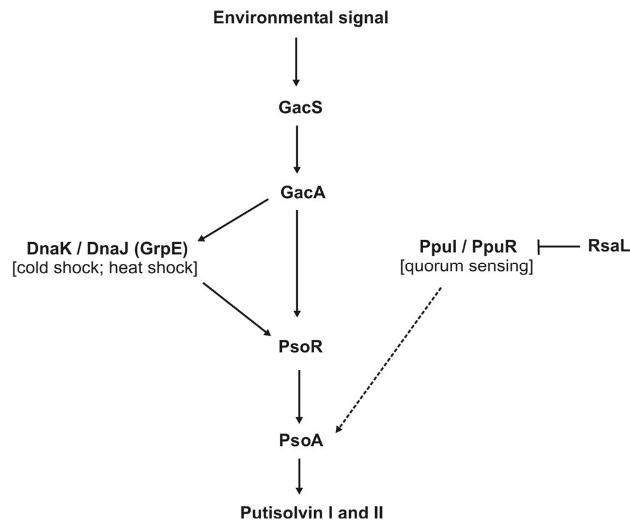


**Fig. 7.** Working model for the regulation of the putisolvin biosynthetic gene, *psoA*, in *P. putida* PCL1445. Regulatory systems and regulatory proteins that were reported to take part in putisolvin biosynthesis are GacA/GacS two-component system, DnaK-DnaJ-GrpE heat-shock system, PpuI-RsaL-PpuR quorum sensing system, and the PsoR transcriptional regulator. RsaL is a repressor of the *ppuI* quorum sensing gene transcription. For explanations, see Discussion section.

The status of the *ppuI-rsaL-ppuR* quorum sensing system in the hierarchy of the regulation of putisolvin production remains unclear. We recently showed the importance of the *ppuI* quorum sensing system for the regulation of biofilm formation by controlling the production of putisolvin (Dubern *et al.*, 2006; this Thesis, Chapter 3). The results presented in Fig. 6 indicate that in PCL1445 AHL synthesis is neither regulated by *gacA* and *gacS*, nor by *dnaK* and *psoR*. This

observation indicates that the quorum sensing system may constitute a separate branch of the regulatory network of putisolvin production in PCL1445 (Fig. 7). This hypothesis raises the question of whether putisolvins are regulated by different pathways depending on the environmental conditions.

The production of biosurfactants could confer an ecological advantage for bacteria when the bacterial population reaches a high-cell density and AHLs could provide a signal e.g. in biofilm formation for the release of *P. putida* cells. Alternatively, environmental stresses such as low temperature could constitute a challenge for the dissemination of *P. putida* due to, for instance a reduction of metabolic functions or a reduction of nutrient availability.

## Acknowledgment

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