Regulation of the biosynthesis of cyclic lipopeptides from Pseudomonas putida PCL1445

Dubern, J.F.

Chapter 5

Influence of environmental conditions on putisolvin I and II production by *Pseudomonas putida* PCL1445

Jean-Frédéric Dubern and Guido V. Bloemberg

submitted

Abstract

Pseudomonas putida PCL1445 produces the cyclic lipopeptides putisolvin I and II, which are biosurfactants affecting environmentally important functions including swarming motility, solubility of nutrients and influencing biofilm formation and maintenance. In this study the effect of relevant nutritional and environmental factors on putisolvin production by PCL1445 was analyzed. Growth and levels of putisolvin I and II biosynthesis were significantly influenced by various carbon sources, ions (phosphate and iron), and by the nature of amino acid supplemented to the culture medium. Increase in the salt concentration of BM medium as well as growth at 1 % oxygen significantly increased putisolvin production. In addition, we recently demonstrated that growth at low temperature has a positive effect on putisolvin production. Analysis of the expression of *gacA* and *gacS* in Tn5*luxAB* mutant strains at low temperature or under high salt concentration suggests that biosynthesis of putisolvins is modulated through the GacA/GacS two-component regulatory system in PCL1445 under the tested conditions.

Introduction

Putisolvins I and II are cyclic lipopeptides produced by the rhizobacterium *Pseudomonas putida* strain PCL1445 (Kuiper *et al.*, 2004). Due to their amphipatic character putisolvins are biosurfacrants, i. e., compounds reducing the surface tension at interfaces (for example oil/water or air/water). Putisolvins are involved in multiple traits which are of great relevance for the survival and spreading of bacterial cells in their living environment. They increase surface motility, stimulate dispersal of naphthalene and phenanthrene crystals and reduce bacterial biofilms (Kuiper *et al.*, 2004). Putisolvins I and II have a similar structure that consists of a 12 amino acids polar peptide head linked to a fatty acid moiety (Kuiper *et al.*, 2004). The difference between both structures resides in the nature of the eleventh residue that is Val in the case of putisolvin I, and Leu or Ileu in the case of putisolvin II (Kuiper *et al.*, 2004).

Cyclic lipopeptides offer commercial applications, by enhancing the solubility of polyaromatic hydrocarbons (PAHs) or other hydrophobic xenobiotics (Desai and Banat, 1997). The ability of putisolvins to disrupt biofilms of several *Pseudomonas* sp. including those of the opportunistic pathogen *P. aeruginosa*

(Kuiper *et al.*, 2004) suggest a second potential application in the inhibition of the formation or breaking down of hazardous biofilms and infectious bacterial biofilms.

The GacA/GacS two-component system is present in many Gram degative bacteria, and plays a role as global regulators of secondary metabolism (Barta *et al.,* 1992; Liao *et al.,* 1997; Koch *et al.,* 2002; Pierson *et al.*, 1998; Chin-A-Woeng *et al.*, 2001; Dubern *et al.*, 2005; this Thesis, Chapter 2). Production of putisolvin is dependent on GacA/GacS system (Dubern *et al.*, 2005; this Thesis, Chapter 2). GacS functions as a histidine kinase that undergoes phosphorylation in response to (so far unidentified) environmental stimuli (Hrabak and Willis, 1992). GacA is a cognate response regulator that contains a receiver domain (phosphorylation) site at its N-terminus (Hrabak and Willis, 1992). Recently, we reported that growth at temperatures lower than 21°C results in an increasing putisolvin production (Dubern *et al.*, 2005; this Thesis, Chapter 2).

In this study we investigate the influence of nutrients, e.g. carbon sources and amino acids, the concentration of NaCl, iron, and phosphate, and the oxygen tension on the biosynthesis of putisolvin I and II. Furthermore, we present evidence that low temperature and high NaCl concentration have a positive effect on *gacA* and *gacS* expression in *P. putida* PCL1445.

Materials and Methods

Bacterial strains and growth conditions

The bacterial strains used in this study are listed in Table 1. *Pseudomonas* strains were grown in King's medium B (King *et al.*, 1954) or in a defined BM medium (Lugtenberg *et al.*, 1999) supplemented with 2.0 % of glycerol (BDH Laboratory Supplies Poole, England) at 28°C. The defined BM medium contains K_2HPO_4 (7.6 g l^{-1}), KH_2PO_4 (3 g l^{-1}), $(NH_4)SO_4$ (1 g l^{-1}), MgSO4 (20 g l^{-1}), NaFeEDTA(III) (78 µM), biotine (0.1 µg l^{-1}), thiamine (0.1 µg l^{-1}), H_3BO_3 (51 µM), $MnSo_4$ (6.8 µM), $ZnSO_4.7H_2O$ (0.85 µM), $Na_2MoO_4.2H_2O$ (4.1 µM), and $CuSO_4.5H_2O$ (0.35 µM). Strains were cultured in BM medium supplemented with 200 mM glycerol or KB medium for monitoring growth, putisolvin production and expression of *psoA*::Tn5*luxAB*, *gacA*::Tn5*luxAB*, or *gacS*::Tn5*luxAB*.

Table 1. Bacterial strains and plasmids.

Bacterial strains and plasmids	Relevant characteristics	Reference or source
Pseudomonas		
PCL1445	Wild-type *P. putida*; colonizes grass roots and produces biosurfactants	Kuiper et al. (2001)
PCL1633	Tn5*luxAB* derivative of PCL1445; mutated in *psoA*, a lipopeptide synthetase homolog; contains Tn5*luxAB* in a transcriptionally active orientation; Km^r	Dubern et al. (2005)
PCL1622	Tn5*luxAB* derivative of PCL1445; mutated in a *gacA* homolog; contains Tn5*luxAB* in a transcriptionally active orientation; Km^r	Dubern et al. (2005)
PCL1623	Tn5*luxAB* derivative of PCL1445; mutated in a *gacS* homolog; contains Tn5*luxAB* in a transcriptionally active orientation; Km^r	Dubern et al. (2005)
Plasmids		
pRL1063a	Plasmid harbouring a promotorless Tn5*luxAB* transposon, Km^r	Wolk et al. (1991)

To test the influence of different carbon sources on the production of putisolvins, each carbon source was added to BM in equal amounts depending on its number of carbon atoms per molecule and used at the following concentrations: citric acid (100 mM), fructose (100 mM), fumaric acid (150 mM), glucose (100 mM), glycerol (200 mM), ribose (120 mM), succinic acid (150 mM), and sucrose (50 mM).

The effect of iron on the production of putisolvins and expression of *psoA* was analyzed by adding $FeCl_3$ instead of NaFeEDTA(III) to BM at concentrations of 0.01 mM, 0.04 mM and 0.08 mM. When BM-glycerol was used to analyze the influence of the phosphate on putisolvin biosynthesis, phosphate concentrations of 10, 30, 50 and 100 mM were used by varying the total concentration of phosphate buffer. When the influence of the phosphate on putisolvin biosynthesis was analyzed, pH of the stationary phase culture medium was determined using a pH Meter (Mettler-Toledo GmbH, Schwerzenbach, Germany).

The effect of the amino acids L-threonine, L-valine, L-leucine, L-isoleucine and L-serine was investigated by adding them as a supplement to BM-glycerol medium at 3 mM.

The effect of salt on putisolvin biosynthesis was tested by supplementing BM-glycerol with KCl or NaCl at a concentration of 1 M.

The influence of aeration and temperature on putisolvin production and *psoA* expression was tested using KB medium. The effect of aeration was analyzed by using various oxygen concentrations (1 % or 21 %) as described previsouly (Camacho Carvajal *et al.*, 2002). For this purpose, cells were cultured in 40 ml KB medium amended with 0.005 % silicon antifoam agant (BDH Limited, Poole, UK). Gas mixtures of oxygen and nitrogen were controlled by a gas mixer (Brook Instruments B. V. Veenendaal, The Netherlands) with a gas flow rate of 0.25 l min^{-1}.

Media were solidified with 1.8 % agar (Select Agar, Invitrogen, Life Technologies, Paisley, UK). The antibiotic kanamycin was added, when necessary, to a final concentration of 50 µg ml^{-1}.

Quantification of biosurfactant production

Putisolvin (biosurfactant) production in culture medium was quantified by measuring the decrease of surface tension between culture medium and air using a Du Nouy ring (K6 Krüss, GmbH, Hamburg, Germany) as previously described (Kuiper *et al.*, 2004).

Putisolvin extraction and analysis

To quantify the production of putisolvins in KB or in BM culture medium, 7 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 and dissolved in 55 % acetonitrile (Labscan Ltd., Dublin, Ireland). Dry material obtained from 7 ml culture supernatant was resuspended in 500 µl of 50/50 acetonitrile/water (v/v) mixture and purified over a spinX centrifuge tube filter with a 0.45 µm pore size (Corning Costar Corporation, Cambridge, MA). Material corresponding to a volume of 500 µl of the samples was separated by HPLC (Jasco International CO. Ltd., Japan), using a reverse phase C8 5 µm Econosphere column (Alltech, Deerfield, IL), a PU-980 pump system (Jasco, B&L Systems, Boechout, Belgium), a LG-980-02 gradient unit (Jasco) and a MD 910 detector (Jasco). Separation was performed using a linear gradient, starting at 35/65 acetonitrile/water (v/v) and ending at 20/80 after 50 min at a flow rate of 1 ml min^{-1}. Chromatograms were analyzed in the wavelength range between 195 nm and 420

nm. The amount of putisolvins produced was quantified as the peak area detected in micro absorbance unit (µAU) at 206 nm.

*Quantification of luciferase activity of Tn5*luxAB *reporter strains*

Expression of *luxAB* reporter genes was determined by quantification of bioluminescence during culturing. Cells from overnight cultures were washed with fresh medium and diluted to an OD_{620nm} of 0.1. Cultures were grown in KB or 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*-dodecyl-aldehyde substrate solution (Sigma, St. Louis, MO) in a 2.0 % bovine serum albumin solution was added and luminescence was determined with a MicroBeta 1450 TriLux luminescence counter (Wallac, Turku, Finland), and was normalized to luminescence per OD_{620} unit.

Results

Effect of the nature of carbon sources on putisolvin production

To evaluate the influence of different carbon sources on the production of putisolvins, PCL1445 cells were cultured in BM medium supplemented with various carbon sources in equal concentrations with respect to the number of carbon atoms. *P. putida* PCL1445 was isolated from Barmultra grass roots (Kuiper *et al.*, 2001). Previous analysis of the exudates of Barmultra grass showed that in all exudates, succinic acid was by far the most predominant organic acid followed by citric acid (Kuiper *et al.*, 2001). Glucose and fructose are the most predominant sugar in root exudates of Barmultra grass (Kuiper *et al.*, 2001). These analyses were partially taken as a basis for the choice of carbon sources to be tested.

The generation time, optical density at stationary phase, and surface tension of culture supernatant of cells grown in BM with various carbon sources were determined (Table 2). The shortest generation times were observed for PCL1445 in presence of succinic acid, fumaric acid and glucose (69, 71, and 72 min, respectively) and the longest generation times were observed in presence of glycerol, citric acid, sucrose, fructose and ribose (167, 112, 108, 327 and 534 min, respectively). Stationary phase was reached at high optical density (OD_{620}) when the carbon source was succinic acid, glucose, or glycerol (3.8, 4.5 and 4.7, respectively)

and at low OD_{620} when the carbon source was ribose, fumaric acid, fructose, sucrose, or citric acid (1.0, 2.2, 1.3, 1.7, 3.3 respectively).

Determination of the surface tension of stationary phase cultures indicated that the highest production of biosurfactant and the highest OD_{620} occurred when BM was supplemented with glycerol. Interestingly, the data indicated that glucose or succinic acid causes a high growth rate, resulting in a high OD_{620} at stationary phase but not in a high production of putisolvins as observed with glycerol (Table 2).

Table 2. Influence of various carbon sources on growth and production of putisolvins on *P. putida* PCL1445 in BM medium.

Carbon source (molarity)	**Generation time (min)**	**Optical density at stationary pahse[a] (OD_{620nm})**	**Surface tension[a] (mN m^{-1})**
Citric acid (100 mM)[b]	112 ± 8.6	3.3 ± 0.3	53 ± 1
Fructose (100 mM)	327 ± 19.0	1.3 ± 0.2	54 ± 1
Fumaric acid (150 mM)	71 ± 5.2	2.2 ± 0.1	54 ± 1
Glucose (100 mM)	72 ± 2.3	4.5 ± 0.2	35 ± 1
Glycerol (200 mM)	167 ± 13.0	4.7 ± 0.3	29 ± 1
Succinic acid (150 mM)	69 ± 5.4	3.8 ± 0.2	43 ± 1
Sucrose (50 mM)	108 ± 8.3	1.7 ± 0.1	48 ± 1
Ribose (120 mM)	534 ± 21	1.0 ± 0.2	53 ± 1

[a]Optical density and surface tension were quantified from cultures having reached the stationary growth phase.

[b] Equimolar of C-atoms.

Influence of amino acids on putisolvin I and II biosynthesis

The peptide moiety of putisolvins I and II consists of 12 amino acids (Kuiper *et al.*, 2004). The growth medium was supplemented with various amino acids to analyze their effect on putisolvin production and to determine the ratio between putisolvins I and II when the amino acids in which putisolvins I and II differ were added in the medium. Supplementation of the BM-glycerol medium with 3 mM L-serine resulted in a significant increase of putisolvin I and II production when compared to BM-glycerol without amino acid supplementation (Table 3). Threonine which is not present in putisolvin structure did not cause significant increase of

putisolvin production when compared to BM-glycerol without supplementation with amino acid (Table 3). Interestingly, the ratio between putisolvins I and II was significantly changed when L-valine, L-leucine, or L-isoleucine, were added to BM medium at 3 mM. Supplementation with L-valine resulted in an increase of putisolvin I production but not of putisolvin II. Addition of L-leucine of L-isoleucine resulted in a higher level of putisolvin II and a reduced level of putisolvin I (Table 3).

Table 3. Effect of amino acids supplemented to BM-glycerol medium on putisolvin production by *Pseudomonas putida* PCL1445.

Supplemented amino acid (3 mM)	**Putisolvin I (peak area in µAU 10^6)**	**Putisolvin II (peak area in µAU 10^6)**	**Ratio Putisolvin I/II**	**Total amount of putisolvin (peak area in µAU 10^6)**
None	8.54 ± 1.23	5.55 ± 1.69	1.54	14.09 ± 2.95
L-Threonine	11.92 ± 1.80	8.41 ± 0.67	1.42	20.33 ± 3.87
L-Valine	19.05 ± 1.52	5.62 ± 0.64	3.39	24.67 ± 2.16
L-Leucine	12.76 ± 1.08	21.07 ± 1.16	0.6	33.84 ± 3.25
L-Isoleucine	5.20 ± 0.49	16.63 ± 0.67	0.31	21.82 ± 1.16
L-Serine	23.02 ± 0.98	17.95 ± 0.72	1.28	40.97 ± 1.70

Effect of phosphate and iron levels on putisolvin biosynthesis

Phosphate and iron are ions present in soil and important for the functioning of the bacterial cell. When the phosphate concentration of the BM medium was changed to higher (100 mM) or lower concentrations (10 or 30 mM) than the standard concentration (50 mM), the expression of the putisolvin biosynthetic gene *psoA* (Fig. 1A) and putisolvin activity (Fig, 1B) were reduced.

The effect of iron concentrations on *psoA* expression and putisolvin production was tested by replacing 0.075 mM NaFeEDTA (III) of the BM-glycerol medium by concentrations of 0.01, 0.04 or 0.08 mM of $FeCl_3$. Omitting NaFeEDTA (III) or $FeCl_3$ from BM-glycerol resulted in loss of growth (data not shown). However, $FeCl_3$ concentrations of 0.04 or 0.08 mM did not change the growth rate during the exponential phase (data not shown). A $FeCl_3$ concentration of 0.01 mM resulted in a low optical density at stationary phase (OD620nm = 1.4) and a reduction of the expression of the *psoA* gene and of putisolvin production. The induction of the

expression of the *psoA* gene (Fig. 1C) and consequently of putisolvin production (Fig. 1D) were found to be dependent on the concentration of $FeCl_3$.

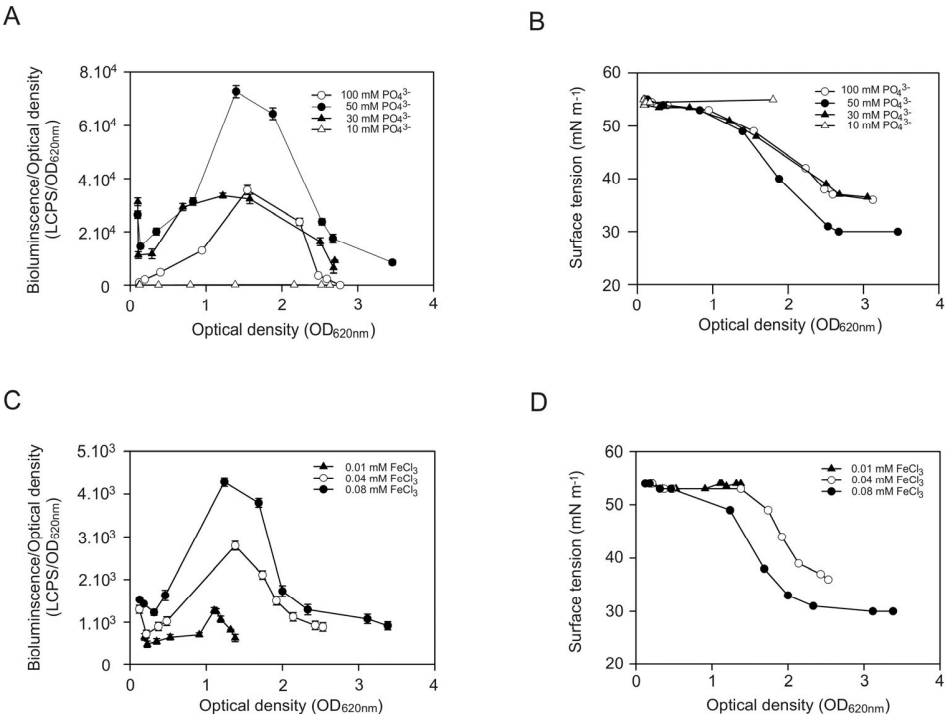

Fig. 1. Effect of phosphate concentration (PO_4^{3-}) and ferric iron ion concentration ($FeCl_3$) in BM-glycerol medium on expression of putisolvin synthetase gene (*psoA*) and putisolvin production in *P. putida* PCL1445. **Panel A.** Quantification of bioluminescence in mutant strain PCL1633 (*psoA*::Tn5*luxAB)*. Cells were cultured in BM-glycerol containing different concentrations of PO_4^{3-}. **Panel B.** Determination of surface tension of culture supernatant of strain PCL1445 grown in BM-glycerol amended with different concentrations of PO_4^{3-}. **Panel C.** Quantification of bioluminescence in mutant strain PCL1633 (*psoA*::Tn5*luxAB)* grown in BM-glycerol containing different concentrations of $FeCl_3$. **Panel D.** Determination of the surface tension of culture supernatant of strain PCL1445 grown in BM-glycerol containing different concentrations of $FeCl_3$. The experiments were performed in triplicate.

Effect of oxygen concentration and salt stress on putisolvin biosynthesis

The influence of oxygen depletion on putisolvin production was analyzed by aerating cells of PCL1445 cultured in KB medium with gas-mixtures containing 1 %

or 21 % oxygen (system described in “Materials and Methods”). Aeration with 1 % oxygen resulted in a reduced growth rate (two-fold lower than with 21 % oxygen; data not shown) accompanied by an initiation of *psoA* expression at a lower OD_{620} (Fig. 2A). Consequently, putisolvin production was initiated at a lower optical density and was found to be slightly higher than with aeration of 21 % oxygen (Fig. 2B).

The effect of salt stress on *psoA* transcriptional activity and putisolvin production was tested by adding NaCl or KCl to BM-glycerol medium. A concentration of 1 M NaCl or KCl did not affect the PCL1445 growth rate nor the optical density reached at stationary phase (data not shown). Under these conditions the transcriptional activity of *psoA* (Fig. 2C) and the production of putisolvin (Fig. 2D) highly increased when compared with BM-glycerol without addition of salt.

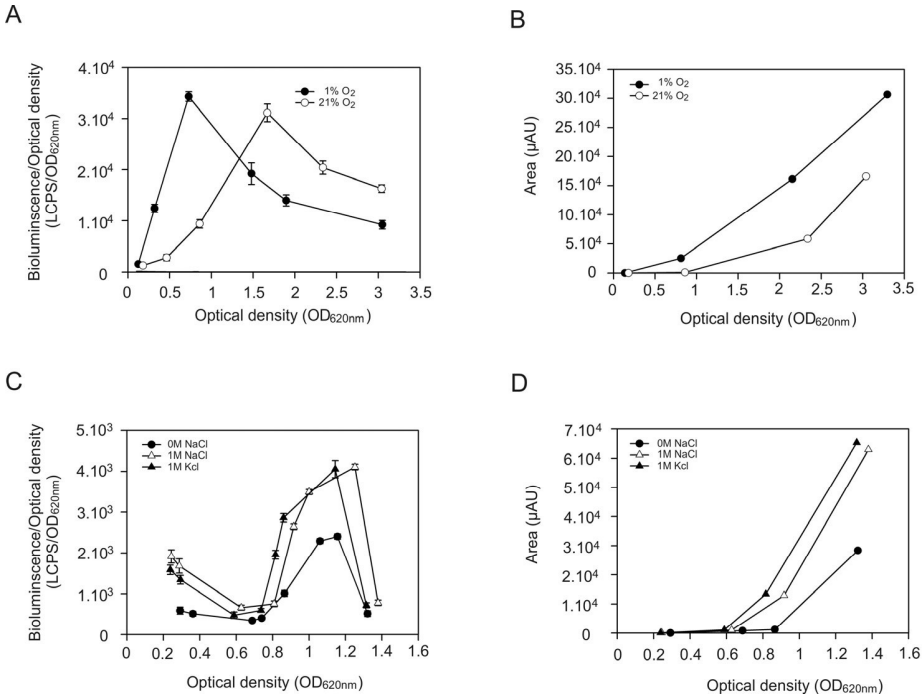

Fig. 2. Influence of different concentrations of oxygen and salt (NaCl or KCl) on expression of the putisolvin synthetase gene (*psoA*) and putisolvin production in *P. putida* PCL1445. **Panel A.** Bioluminescence in mutant strain PCL1633 (*psoA*::Tn5*luxAB)*. Cells were cultured in KB medium with different concentrations of oxygen. **Panel B.** Amount of putisolvin quantified by HPLC analysis of strain PCL1445 grown in KB medium with different concentrations of oxygen. **Panel C.** Bioluminescence in mutant strain PCL1633 harboring *psoA*::Tn5*luxAB*. Cells were cultured in BM-Glycerol amended with 1 M NaCl or 1 M KCl. **Panel D.** Determination of the amount of putisolvin by HPLC analysis of strain PCL1445 grown in BM-glycerol amended with 1 M NaCl or 1 M KCl. Mean values of duplicate cultures are given.

Influence of temperature and salt concentration on gacA and gacS expression

In addition to the strong positive effect of salt stress, it was recently demonstrated that growth at low temperatures highly increases putisolvin production (Dubern *et al.*, 2005, this Thesis, Chapter 2). Therefore, these conditions were chosen to analyze their influence on the expression of GacA/GacS two-component regulatory system. *P. putida* PCL1445 mutant strains PCL1622 (*gacA*::Tn5*luxAB*) and PCL1623 (*gacS*::Tn5*luxAB*), in which the *luxAB* genes are under control of the P_{gacA} and P_{gacS} respectively, were exploited to study how

temperature and salt stress influence expression of *gacA* and *gacS* genes. In these experiments we measured bioluminescence from PCL1622 (*gacA*::Tn5*luxAB*) and PCL1623 (*gacS*::Tn5*luxAB*).

Expression of *gacA* and *gacS* in KB medium and in BM medium were highest at the mid-exponential growth phase (Fig. 3). Transcriptional activity of *gacA* and *gacS* in strains PCL1622 (*gacA*::Tn5*luxAB*) and PCL1623 (*gacS*::Tn5*luxAB*) increased (approximately two fold) at 11°C when compared to their expression values at 28°C (Fig. 3A and 3B, respectively). In contrast, expression of *gacA* and *gacS* at 32°C was strongly repressed (Fig. 3A and 3B, respectively).

When strains PCL1622 (*gacA*::Tn5 *luxAB*) and PCL1623 (*gacS*::Tn5 *luxAB*) were grown in BM-glycerol supplemented with 1 M NaCl, the expression of *gacA* and *gacS* was higher than in BM-glycerol (Fig. 3C and 3D, respectively).

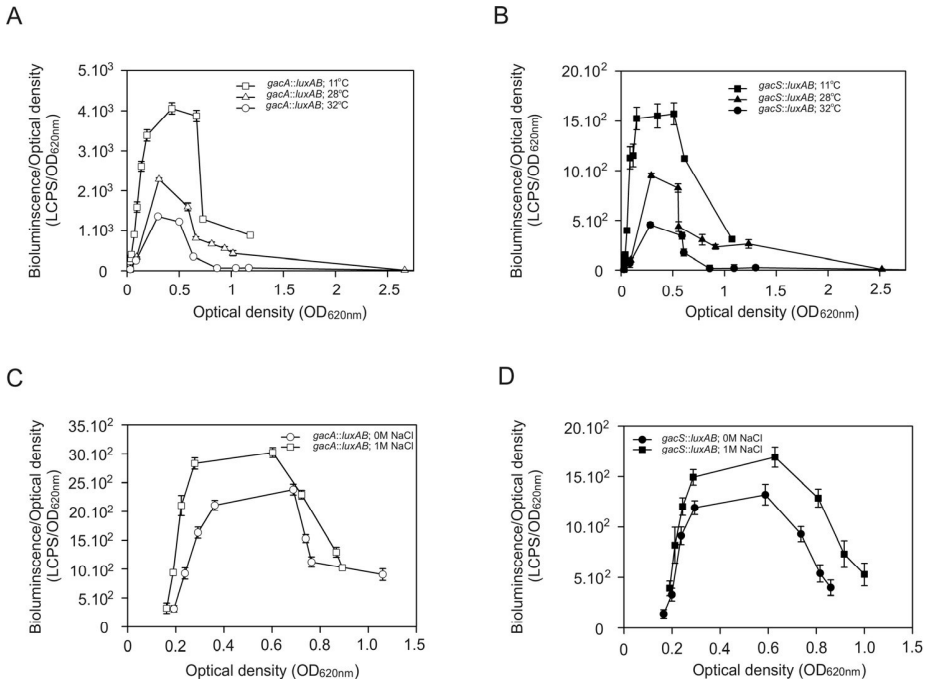

Fig. 3. Effect of temperature and NaCl concentration on the expression of *gacA* and *gacS* of *Pseudomonas putida* strain PCL1445. Quantification of bioluminescence produced by cells of (**panel A**) strain PCL1622 (*gacA*::Tn5*luxAB*), and (**panel B**) strain PCL1623 (*gacS*::Tn5*luxAB*) cultured in KB medium at different temperatures. Quantification of bioluminescence in cells of (**panel C**) strain PCL1622 (*gacA*::Tn5*luxAB*) and (**panel D**) strain PCL1623 (*gacS*::Tn5*luxAB*) cultured in BM-glycerol amended with 1 M NaCl. Standard deviations are based on the mean values of two parallel cultures.

Discussion

Pseudomonas putida strain PCL1445 was isolated from grass root (Kuiper *et al.*, 2001) and in its natural environment it will encounter different conditions, which are determined by the plant root and the soil. *P. putida* PCL1445 is of specific interest since it produces putisolvins I and II, which exhibit important functions with potential product application. The application of biosurfactants offers a number of advantages over chemical surfactants such as lower toxicity, higher biodegradability, environmental compatibility and activity under specific environmental conditions (for instance temperature, pH, and salinity) (Mulligan *et al.*, 2004).

Previous studies show that putisolvins are not constitutively produced (Kuiper *et al.*, 2004; Dubern *et al.*, 2005; Chapter 2). Putisolvin production is dependent on various regulatory genes, including the two component regulatory system GacA/GacS and the DnaK heat-shock system (Dubern *et al.*, 2005, this Thesis, Chapter 2). These studies also showed that temperature strongly influences the production. Recent reports have shown that cyclic lipopeptides play an important role for growth and survival of the producing bacteria, resulting in increasing interests in the significance of environmental signaling for the biosynthesis of lipopeptides of rhizosphere Pseudomonads (Koch *et al.*, 2002; Dubern *et al.*, 2005; this Thesis, Chapter 2; Gross *et al.*, 1991; Mo *et al.*, 1991). The present study was performed to evaluate the effects of relevant physiological and environmental factors on the expression of the putisolvin biosynthetic gene (*psoA*) and on the production of putisolvins.

Our studies showed that *psoA* expression and consequently the production of putisolvins is influenced by various physiological factors including carbon source, iron and phosphate concentrations, and environmental factors including salt stress and oxygen concentrations.

From the carbon sources tested, glycerol yielded the highest production of putisolvins in BM medium. Testing the most common carbon sources found in Barmultra grass root exudates (succinic acid and glucose) (Kuiper *et al.*, 2001) resulted in an increased putisolvin production as compared to other carbon sources tested and a decreased putisolvin production as compared with glycerol (Table 2). Glycerol had a negative influence on growth rate and a positive effect on both the optical density reached at stationary phase and putisolvin production (Table 2), suggesting that compounds that cause a slow growth rate and a increased cell

density at stationary phase could have a positive effect on putisolvin production. Interestingly, nutrient availability has been identified as part of the environmental signals that regulate the nature and structure of biofilm (Stanley *et al.*, 2004).

Addition of amino acids that are most present in the peptide moiety of putisolvins, to the growth medium had a strong effect on the ratio of putisolvins I and II (Table 3). Putisolvins I and II differ from each other in only one amino acid at position 11, which is Val in the case of putisolvin I, and Leu or Ileu in the case of putisolvin II (Kuiper *et al.*, 2004). The addition of valine strongly favors putisolvin I production (Table 3). Interestingly, valine also decreased the production of putisolvin II when compared with BM-glycerol. Previously, it was suggested that valine could cause a repressive effect on the biosynthesis of the phytotoxin coronatine by *Pseudomonas syringae* due to feedback inhibition in the branched-chain amino acid pathway (Mitchell and Frey, 1986), resulting in decreased levels of isoleucine, which could also explain the decreased production of putisolvin II. Addition of leucine or isoleucine strongly favors putisolvin II production (Table 3). Our result shows that addition of serine strongly increased production of putisolvins I and II, suggesting that serine production by PCL1445 or its presence in BM-glycerol medium is limiting (Table 3).

Iron exerts a positive regulatory effect on putisolvin production. Concentrations of ≥0.08 mM were required for expression of *psoA* gene and for maximum yields of putisolvins (Fig. 1A and 1B). The induction of putisolvin biosynthesis by iron resembles the effect of iron on syringomycin production in *P. syringae* pv. *syringae* B301D (Gross *et al.*, 1985) and coronatine production in *P. syringae* pv. *glycinea* (Palmer and Bender, 1993). This result suggests that ferric iron at concentrations of 0.08 mM is a limiting factor for putisolvin production, although not limiting for growth. In contrast, putisolvin production was significantly reduced by high phosphate levels (Fig. 1C and 1D). One explanation for the reduction in putisolvin biosynthesis at high phosphate concentration is that phosphate repression might play a role in the regulation of putisolvin production, which is consistent with the negative effect of easy utilizable phosphate source on the biosynthesis of antibiotics and other secondary metabolites at the level of transcription (Liras *et al.*, 1990).

Transcription of *psoA* and putisolvin synthesis increased under low aeration conditions and a depleted oxygen concentration (Fig. 2A and 2B). The latter result is of relevance since a decreasing oxygen concentration gradient occurs in biofilms (Stanley *et al.*, 2004) and in the rhizosphere (Højberg *et al.*, 1999).

The induction of *psoA* expression and of putisolvin production in *P. putida* PCL1445 by salt stress constitutes an interesting observation (Fig. 2C and 2D) that merits further investigations since to our knowledge a positive effect on lipopeptide production has never been reported. In contrast, a negative effect of salt stress has been reported on the production of syringomycin (Palmer *et al.*, 1993).

The observation that specific environmental signals including temperature, salt and to a lesser extent oxygen concentrations, play a significant role in activating *psoA* expression and putisolvin production demonstrates that diverse environmental factors influence the expression of genes involved in putisolvin production in *P. putida* PCL1445. Little is known about the genetic network responsible for the perception and transduction of these signals to the *psoA* transcriptional apparatus.

Previously, we have identified the two component regulatory system GacA/GacS as being required for putisolvin production. In order to test if expression of *gacA/gacS* correlates with the increased putisolvin production at low temperature (11°C) and in presence of high concentration of NaCl (1 M), expression of these genes was analyzed during growth at 11°C temperature and in the presence of 1 M NaCl. In this chapter, we have shown that expression of *gacA* and *gacS* in strains PCL1622 (*gacA*::Tn5*luxAB*) and PCL1623 (*gacS*::Tn5*luxAB*) is strongly induced at low temperatures and under high salt concentration (Fig. 3A and 3B), which is consistent with the positive effect of these two conditions on *psoA* expression and putisolvin production.

These results suggest that *gacA* and *gacS* expression responds to multiple signals which could originate from the extracellular environment. The identification of low temperature, high salt concentration, and low aeration as environmental signaling factors controlling putisolvin biosynthesis highlight their importance for the functioning of *P. putida* PCL1445 in its environment

Acknowledgements

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