Chapter 5

Influence of environmental conditions on
putisolvin I and II production by

*Pseudomonas putida* PCL1445

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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 Tn5luxAB 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 biosurfactants, 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 negative 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$_2$HPO$_4$ (7.6 g l$^{-1}$), KH$_2$PO$_4$ (3 g l$^{-1}$), (NH$_4$)$_2$SO$_4$ (1 g l$^{-1}$), MgSO$_4$ (20 g l$^{-1}$), NaFeEDTA[III] (78 µM), biotine (0.1 µg l$^{-1}$), thiamine (0.1 µg l$^{-1}$), H$_3$BO$_3$ (51 µM), MnSO$_4$ (6.8 µM), ZnSO$_4$.7H$_2$O (0.85 µM), Na$_2$MoO$_4$.2H$_2$O (4.1 µM), and CuSO$_4$.5H$_2$O (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::Tn5luxAB, gacA::Tn5luxAB, or gacS::Tn5luxAB.
To test the influence of different carbon sources on the production of putisolvin, 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 putisolvin and expression of psoA was analyzed by adding FeCl₃ 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 previously (Camacho Carvajal et al., 2002). For this purpose, cells were cultured in 40 ml KB medium amended with 0.005 % silicon antifoam agent (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 Tn5luxAB 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 $O_D_{620nm}$ 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 $O_D_{620}$ 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 ($O_D_{620}$) 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>
<thead>
<tr>
<th>Carbon source</th>
<th>Generation time (min)</th>
<th>Optical density at stationary phase$^a$ (OD$_{620nm}$)</th>
<th>Surface tension$^a$ (mN m$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citric acid (100 mM)$^b$</td>
<td>112 ± 8.6</td>
<td>3.3 ± 0.3</td>
<td>53 ± 1</td>
</tr>
<tr>
<td>Fructose (100 mM)</td>
<td>327 ± 19.0</td>
<td>1.3 ± 0.2</td>
<td>54 ± 1</td>
</tr>
<tr>
<td>Fumaric acid (150 mM)</td>
<td>71 ± 5.2</td>
<td>2.2 ± 0.1</td>
<td>54 ± 1</td>
</tr>
<tr>
<td>Glucose (100 mM)</td>
<td>72 ± 2.3</td>
<td>4.5 ± 0.2</td>
<td>35 ± 1</td>
</tr>
<tr>
<td>Glycerol (200 mM)</td>
<td>167 ± 13.0</td>
<td>4.7 ± 0.3</td>
<td>29 ± 1</td>
</tr>
<tr>
<td>Succinic acid (150 mM)</td>
<td>69 ± 5.4</td>
<td>3.8 ± 0.2</td>
<td>43 ± 1</td>
</tr>
<tr>
<td>Sucrose (50 mM)</td>
<td>108 ± 8.3</td>
<td>1.7 ± 0.1</td>
<td>48 ± 1</td>
</tr>
<tr>
<td>Ribose (120 mM)</td>
<td>534 ± 21</td>
<td>1.0 ± 0.2</td>
<td>53 ± 1</td>
</tr>
</tbody>
</table>

$^a$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
Environmental regulation 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.

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

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 FeCl₃. Omitting NaFeEDTA (III) or FeCl₃ from BM-glycerol resulted in loss of growth (data not shown). However, FeCl₃ concentrations of 0.04 or 0.08 mM did not change the growth rate during the exponential phase (data not shown). A FeCl₃ 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 \textit{psoA} gene (Fig. 1C) and consequently of putisolvin production (Fig. 1D) were found to be dependent on the concentration of FeCl$_3$.

\textbf{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 (\textit{psoA}) and putisolvin production in \textit{P. putida} PCL1445. \textbf{Panel A.} Quantification of bioluminescence in mutant strain PCL1633 (\textit{psoA}::Tn5\textit{luxAB}). Cells were cultured in BM-glycerol containing different concentrations of PO$_4^{3-}$. \textbf{Panel B.} Determination of surface tension of culture supernatant of strain PCL1445 grown in BM-glycerol amended with different concentrations of PO$_4^{3-}$. \textbf{Panel C.} Quantification of bioluminescence in mutant strain PCL1633 (\textit{psoA}::Tn5\textit{luxAB}) grown in BM-glycerol containing different concentrations of FeCl$_3$. \textbf{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.

\textit{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 \%
Environmental regulation of putisolvin

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::Tn5luxAB. 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::Tn5luxAB) and PCL1623 (gacS::Tn5luxAB), 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 \textit{gacA} and \textit{gacS} genes. In these experiments we measured bioluminescence from PCL1622 (\textit{gacA}:\textit{Tn5}lux\textit{AB}) and PCL1623 (\textit{gacS}:\textit{Tn5}lux\textit{AB}).

Expression of \textit{gacA} and \textit{gacS} in KB medium and in BM medium were highest at the mid-exponential growth phase (Fig. 3). Transcriptional activity of \textit{gacA} and \textit{gacS} in strains PCL1622 (\textit{gacA}:\textit{Tn5}lux\textit{AB}) and PCL1623 (\textit{gacS}:\textit{Tn5}lux\textit{AB}) 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 \textit{gacA} and \textit{gacS} at 32°C was strongly repressed (Fig. 3A and 3B, respectively).

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

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig3}
\caption{Effect of temperature and NaCl concentration on the expression of \textit{gacA} and \textit{gacS} of \textit{Pseudomonas putida} strain PCL1445. Quantification of bioluminescence produced by cells of \textbf{(panel A)} strain PCL1622 (\textit{gacA}:\textit{Tn5}lux\textit{AB}), and \textbf{(panel B)} strain PCL1623 (\textit{gacS}:\textit{Tn5}lux\textit{AB}) cultured in KB medium at different temperatures. Quantification of bioluminescence in cells of \textbf{(panel C)} strain PCL1622 (\textit{gacA}:\textit{Tn5}lux\textit{AB}) and \textbf{(panel D)} strain PCL1623 (\textit{gacS}:\textit{Tn5}lux\textit{AB}) cultured in BM-glycerol amended with 1 M NaCl. Standard deviations are based on the mean values of two parallel cultures.}
\end{figure}


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 putisolvin 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 putisolvin production is 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 utilisable 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 \textit{psoA} expression and of putisolvin production in \textit{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 \textit{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 \textit{psoA} expression and putisolvin production demonstrates that diverse environmental factors influence the expression of genes involved in putisolvin production in \textit{P. putida} PCL1445. Little is known about the genetic network responsible for the perception and transduction of these signals to the \textit{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 \textit{gacA}/\textit{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 \textit{gacA} and \textit{gacS} in strains PCL1622 (\textit{gacA}:\textit{Tn5luxAB}) and PCL1623 (\textit{gacS}:\textit{Tn5luxAB}) 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 \textit{psoA} expression and putisolvin production.

These results suggest that \textit{gacA} and \textit{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 \textit{P. putida} PCL1445 in its environment.

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