Regulation of the biosynthesis of cyclic lipopeptides from Pseudomonas putida PCL1445

Dubern, J.F.

Chapter 6

General Discussion

Introduction

Biosurfactants are produced by both Gram-Negative and Gram-Positive bacteria. Biological functions of biosurfactant molecules include their involvement in swarming motilily, bacterial growth on water-insoluble carbon sources, the solubility of hydrophobic compounds (often important nutrients) and the regulatory effect on bacterial biofilms.

The commercial applications of biosurfactants attract much attention in the areas of environmental pollution and biomedicine. Intensively studied organisms producing biosurfactants include *Pseudomonas* sp. strains producing rhamnolipids (Lang and Wullbrandt 1999; Providenti *et al.*, 1995; Shreve *et al.*, 1995) and *Bacillus* sp. strains, producing surfactins (Fuma *et al.*, 1993; Yakimov *et al.,* 1995). Biosurfactants have a number of advantages over chemical surfactants such as lower toxicity, higher biodegradability, environmental compatibility, high selectivity, and specific activity under extreme environmental conditions (for instance temperature, pH, and salinity) (Ron *et al.*, 2002; Mulligan *et al.*, 2005; Cameotra and Makkar, 2004). One of the reasons which prevent the widespread use of biosurfactants is their production costs.

The strain used for the studies described in this thesis is *P. putida* PCL1445 (Kuiper *et al.*, 2001). Previously, it was shown that PCL1445 produces two cyclic lipopeptides, named putisolvins I and II (Kuiper *et al.*, 2001), which represent a novel class of biosurfactants. Putisolvins I and II reduce the surface tension between liquid and air, improve the emulsification of toluene, and enhance dispersion of naphthalene and phenanthrene (Kuiper *et al.*, 2004). These properties suggest a potential application in bioremediation by enhancing the solubility of polyaromatic hydrocarbons (PAHs) and other hydrophobic xenobiotics (Desai and Banat, 1997). In addition, the ability of putisolvins to disrupt already existing biofilms of several *Pseudomonas* sp. including those of the opportunistic pathogen *P. aeruginosa* (Kuiper *et al.*, 2004) suggest a second promising potential application of the use of biosurfactants for preventing or removing hazardous biofilms. Biofilms have a tremendous impact in industrial, medical and agricultural settings, exhibiting harmful and beneficial activities, which depends on the organism and setting.

Biofilm formation is a key component of processes such as pathogenicity and biological control of phytopathogens. The biological relevance of biosurfactants for rhizobacteria has higly stimulated the elucidation of their biosynthesis as well as

the regulatory mechanisms involved in their production. The aim of this thesis was to identify the regulatory systems involved in the biosynthesis of putisolvin, in order to evaluate their significance for the rhizobacterium *P. putida* PCL1445, and to possibly enhance putisolvin production for future potential applications.

Three approaches were used in order to identify the genes involved in putisolvin biosynthesis. Firstly, random transposon mutagenesis of PCL1445 resulted in the isolation and characterization of two regulatory systems, a heat-shock system and a two-component signaling system (Chapter 2). Secondly, genes predicted to play a role in putisolvin biosynthesis at the onset of stationary phase as well as in biofilm regulation were mutated in PCL1445. This resulted in the identification of a quorum sensing system in *P. putida* PCL1445. Its regulatory role in putisolvin biosynthesis and its effect on biofilm formation were investigated in Chapter 3. Thirdly, the surrounding region of the putisolvin biosynthetic gene *psoA* was analyzed for the presence of putative regulatory elements, resulting in the isolation and characterization of a novel transcriptional regulator for putisolvin biosynthesis (Chapter 4).

Finally, the effect of selected environmental factors and conditions on the production of putisolvins were investigated in order to explore if production of putisolvins I and II can be increased and to evaluate the environmental relevance of lipopeptides of *P. putida* (Chapter 5).

Molecular mechanisms regulating putisolvin biosynthesis in *P. putida* PCL1445

In Chapter 2 the identification and the role of the *dnaK*, *dnaJ*, and *grpE* heat shock genes in regulating putisolvin production are described. The transposon in mutant strain PCL1627, which was selected for its decreased putisolvin production, appeared to have inserted in a *dnaK* homolog, which forms an operon together with *dnaJ*. The DnaK-DnaJ-GrpE heat-shock chaperone system is well conserved among many bacterial species. By constructing *dnaJ* and *grpE* mutants, it was demonstrated that this heat-shock chaperone system takes part in the transcriptional regulation of putisolvin biosynthesis in *P. putida*. In addition, it was shown that putisolvin biosynthesis is strongly up-regulated by low temperatures and that the DnaK system is actively involved in this process. Interestingly, *dnaK* and *dnaJ* appeared to be independently transcribed in *P. putida* PCL1445. The product of these two heat-shock genes is frequently found to be part of an

equimolar protein complex involved in the heat-shock response during the exponential growth phase. Mutations in *dnaK* and *dnaJ* in PCL1445 do not have a lethal effect, which suggests that other heat-shock chaperones could fulfill the vital functions of DnaK-DnaJ during growth and that in *P. putida* PCL1445 the DnaK-DnaJ system could play a more specific role in the production of putisolvin during the transition between exponential phase and stationary phase. Supporting this hypothesis, we showed experimental evidence that mutation in the third member of the DnaK heat-shock complex, *grpE*, did not result in a significant reduction of lipopeptide synthesis at low temperatures when compared with the effect of mutations in *dnaK* or *dnaJ*. GrpE is known to play a direct role in the recognition of unfolded proteins by the FtsH proteases under heat-shock conditions (Hughes and Mathee, 1998). This may indicate a different and to our knowledge still unknown function for DnaK-DnaJ-GrpE at low temperatures, which merits further investigation.

Based on the results that putisolvin biosynthesis is initiated at the entry of stationary phase and that PCL1445 produces signal molecules which induce bioluminescence in a bioreporter strain harboring the LuxR quorum sensing transcriptional regulator of *Vibrio fisheri*, we investigated the involvement of quorum sensing in putisolvin biosynthesis in PCL1445 (Chapter 3). We constructed a plasmid library and screened for induction of bioluminescence using the reporter strain for *N*-acylhomoserine lactones (AHLs) based on the *lux* system of *Vibrio fisheri*. This resulted in the identification of the quorum sensing system of PCL1445 composed of PpuI, RsaL and PpuR. PpuI is involved in the synthesis of the *N*-acylhomoserine lactones O-C_{12}-AHL, O-C_{10}-AHL, O-C_8-AHL, and O-C_6-AHL, PpuR is a transcriptional regulator and RsaL is a repressor of the *ppuI* quorum sensing gene. The *ppu* quorum sensing system of *P. putida* shows high similarity with the *lasI*/*lasR* system of *P. aeruginosa* (Pearson *et al.*, 1994). We demonstrated that the PpuI-RsaL-PpuR regulates the transcriptional activity of *psoA* when PCL1445 is grown in BM-glycerol medium. Most interestingly, we observed in correlation with recent studies in *P. putida* (Steidler *et al.*, 2002) that quorum sensing mutants of PCL1445 form a denser biofilm than the wild type strain. Our studies clearly link quorum sensing in *P. putida* PCL1445 with the synthesis of the cyclic lipopeptides putisolvins I and II and thereby with biofilm formation. In agreement with these observations, it was reported that biosurfactants of *S. liquefaciens* (Lindum *et al.*, 1998), *P. aeruginosa* (Kholer *et al*, 1998) and *B. cepacia* (Huber *et al.*, 2002) are essential for swarming motility. In addition, biosurfactants are often found to be

regulated by quorum sensing (Huber *et al.*, 2002; Cui *et al.*, 2005; Lindum *et al.*, 1998). Therefore, we hypothesize that production of biosurfactants could confer a selective advantage when nutrients become limiting, resulting in a detachment of part of bacterial cell population from a biofilm in order to colonize a more favorable niche. We suggest that quorum sensing is one key component which modulates this process.

In Chapter 4, we describe the analysis of the flanking regions of the putisolvin biosynthetic gene (*psoA*) for putative regulatory elements. An *orf* located upstream of *psoA* was identified that encodes a protein, showing high homology to members of the LuxR family, which was tentatively named PsoR. Sequence analysis revealed the presence of HTH DNA-binding motifs on the C-terminus of PsoR and of three highly conserved amino acid residues characteristic to response regulators. Furthermore, the weak homology between PsoR and other LuxR-like regulatory proteins involved in phytotoxin biosynthesis (Kitten *et al.*, 1998), indicates that PsoR belongs to a different subfamily of regulatory proteins and therefore may fulfill a different regulatory role. Analysis of a *psoR* mutant showed that PsoR is required for *psoA* transcription. Directly downstream of *psoR*, an *orf* was identified, which showed high homology with *oprM*, a component of the prokaryotic type I secretion system. Mutation of the *oprM* homologue in PCL1445 resulted in a delayed putisolvin production, suggesting that OprM could be possibly involved in the (initial) secretion of putisolvin. Further investigations using a *psoR*::*luxAB* transcriptional fusion and complementation analysis positioned PsoR downstream of GacA/GacS and the DnaK-DnaJ-GrpE system in the hierarchy of the regulation of putisolvin production. Most interestingly, our results showed that AHL production is not affected in *gacA*, *gacS*, *dnaK* and *psoR* mutants of PCL1445. These data indicate that quorum sensing may form a separate pathway in the regulatory network modulating the production of putisolvins. In addition, two putative regulatory elements in the *psoR-psoA* intergenic region were identified, which requires further investigation. One of these is a short nucleotide sequence similar to those controlling the transcriptional activity of response regulatory genes (Yang *et al.*, 2004). It overlaps with the putative *lux* box of *psoA* that is believed to be the binding site for the quorum sensing regulator LuxR (Fuqua *et al.*, 1994). The second element is similar to integration host factor (IHF) binding sites which are known to modulate the activity of promoter genes in different growth conditions (Leoni *et al.*, 2005; McLeod *et al.*, 2001). Thus, it can be hypothesized that the presence, nature and position of these regulatory elements in the *psoA-psoR*

intergenic region significantly influence the expression of *psoA*, depending on the environmental and growth conditions. Further biochemical analyses are required to clearly identify the promoters of *psoA* and *psoR*, and the possible binding sites which could play a role in *psoA* and *psoR* transcriptional activation.

Influence of environmental signals on the production of cyclic lipopeptides in *P. Putida* PCL1445

The aim of Chapter 5 was to evaluate the effect of selected environmental factors on the expression of *psoA* and consequently on putisolvin production in order to increase the production of putisolvins and to assess the significance of these environmental factors for putisolvin production by PCL1445. These studies led to the observation that nutrient availability is an important stimulatory factor for the production of putisolvins by PCL1445. Furthermore, we showed that specific environmental stimuli, including salt stress and low oxygen levels, play an essential role in activating *psoA* gene expression and putisolvin production. Interestingly, expression of *gacA* and *gacS* expression was upregulated at low temperatures and under salt stress, which correlates with the observed increased putisolvin production. These results clearly indicate that these environmental factors control the transcriptional activity of genes involved in the production of putisolvins in PCL1445 and stresses the importance of putisolvins for the environmental fitness of PCL1445. Additional studies are required in order to unravel the complex genetic network responsible for the perception and transduction of these signals to the *psoA* transcriptional apparatus.

Significance of lipopeptides biosynthesis for rhizobacteria

Lipopeptides have a wide range of biological roles in the functioning of bacteria. They are composed of a peptide moiety and a fatty acid chain at the N-terminal amino acid, both varying in length, which could account for different properties of the different lipopeptides such as antifungal activity, phytotoxicity and biofilm inhibitors. Secretion of putisolvins I and II stimulates swarming motility possibly by altering the cell surface hydrophobicity, inhibits biofilm formation, and degrades existing biofilms of its own wild type. This suggests that putisolvins could be involved in a new mechanism of biofilm regulation (Kuiper *et al.*, 2004). Interestingly, the components that regulate putisolvin production, such as the

DnaK-DnaJ-GrpE complex, are frequently found to be associated with vital functions in many bacterial species. In addition, environmental factors, which had a negative influence on the growth rate of PCL1445, had in general a positive effect on putisolvin production. The tested environmental conditions, which are likely to be encountered in the rhizosphere, might act as a selective pressure for survival and competition of *Pseudomonas* sp. It can be hypothesized that putisolvins among other lipopeptides could play a previously unknown role in protection against environmental stresses that constitute a challenge for the dissemination of *P. putida,* for instance a shortage of nutrients, a reduction of the growth, or a depletion of metabolic activity.

Concluding remarks

The regulation of putisolvin production is complex. In this thesis, novel mechanisms of the regulation of putisolvin biosynthesis in PCL1445 are described, which contribute to the understanding of the ecological advantage of cyclic lipopeptide production for rhizosphere species that proliferate in an environment such as polluted soil. The identification of numerous regulatory genes during recent years enables a better understanding of the regulatory network controlling CLPs biosynthesis. One specific question to be addressed in the near future would be whether the regulation of cyclic lipopeptides biosynthesis differs fundamentally between antagonistic and pathogenic *Pseudomonas* species. Further studies directed towards the analysis of whole genome sequences (http//v2.pseudomonas.com; http://pseudo.bham.ac.uk/) together with microarray analysis and proteomics would lead to a better understanding of the regulation of CLPs production in *Pseudomonas sp.*

Moreover, the application of a biosurfactant producing strain in combination with pollutant degraders could improve biodegradation of hydrophobic pollutants in soils. Studies on lipopeptides are relevant for biomedicine, since they function as antimicrobial agents, immunoregulators, and in adhesion and desorption processes important in surgical procedures (Cameotra and Makkar, 2004; Hossain *et al.,* 2001; Millsap *et al.*, 1997; Donlan and Costerton, 2002).

The fundamental advances of the past few years in the understanding of non-ribosomal peptide synthesis on the genetic and mechanistic level could certainly be applied for genetic engineering of lipopeptide synthetases. The isolation and analysis of the structure of putisolvin biosynthetic gene cluster of *P.putida*

strain PCL1445 could offer promising applications. Construction of the engineered strains through cloning and expression of the genes responsible for biosynthesis of putisolvins in efficient degrading strains could improve biodegradation of hydrophobic pollutants in soil. The non-ribosomal synthesis of lipopeptides is catalyzed through a protein template that contains the correct number and order of activating units (Kleinkauf and von Döhren, 1996). Ultimately, it should be possible to use a genetic approach to synthesize novel lipopeptides with improved biological properties.