Regulation of the biosynthesis of cyclic lipopeptides from Pseudomonas putida PCL1445

Dubern, J.F.

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Regulation of the biosynthesis of Novel cyclic lipopeptides from *Pseudomonas putida* strain PCL1445

Jean-Frédéric Dubern

Cover: Surface tension: Rain drop on a leaf just after a late afternoon shower, by Jeff Schneiderman.
In cooperation with Peter Hock.

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Regulation of the biosynthesis of Novel cyclic lipopeptides from *Pseudomonas putida* strain PCL1445

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Jean-Frédéric Bertrand Dubern
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Promotor:	Prof. Dr. E. J. J. Lugtenberg
Co-promotor:	Dr. G. V. Bloemberg
Referent:	Dr. J. M. Raaijmakers (WU)
Overige leden:	Prof. Dr. P. J. J. Hooykaas
	Prof. Dr. H. P. Spaink
	Prof. Dr. C. A. M. J. J. van den Hondel
	Prof. Dr. J. A. van Veen (NIOO-KNAW)

"Regulation of the biosynthesis of Novel cyclic lipopeptides from *Pseudomonas putida* strain PCL1445" by Jean-Frédéric Dubern

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Pour mes parents Jean et Annette

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List of abbreviations

AHL	*N*-acylhomolserine lactone
C_4-AHL	*N*-butanoyl-L-homoserine lactone
C_6-AHL	*N*-hexanoyl-L-homoserine lactone
C_8-AHL	*N*-octanoyl-L-homoserine lactone
C_{10}-AHL	*N*-decanoyl-L-homoserine lactone
C_{12}-AHL	*N*-dodecanoyl-L-homoserine lactone
C_{14}-AHL	*N*-tetradecanoyl-L-homoserine lactone
3-oxo-C_6-AHL	*N*-(3-oxo-hexanoyl)-L-homoserine lactone
3-oxo-C_8-AHL	*N*-(3-oxo-octanoyl)-L-homoserine lactone
3-oxo-C_{10}-AHL	*N*-(3-oxo-decanoy)-L-homoserine lactone
3-oxo-C_{12}-AHL	*N*-(3-oxo-dodecanoyl)-L-homoserine lactone
3-oxo-C_{14}-AHL	*N*-(3-oxo-tetradecanoyl)-L-homoserine lactone
CLP	Cyclic lipopeptide
CMC	Critical micelle concentration
DAPG	2, 4-diacetylphloroglucinol
EPS	Extracellular polysaccharide
GFP	Green fluorescent protein
HCN	Hydrocyanide
HPLC	High-performance liquid chromatography
NRPS	Non-ribosomal peptide synthetase
ORF	Open reading frame
PAH	Polyaromatic hydrocarbon
RBS	Ribosomal-binding site
TCA	Tricarboxylic acid
TLC	Thin-layer chromatography

Chapter 1

General Introduction

1. Microbial biofilms

1.1. Introduction

Biofilms are defined as bacterial cells that attach to and proliferate on a surface, often surrounded by an extracellular matrix (partially) consisting of exopolysaccharides (EPS) Biofilms are formed on a diverse range of biotic and abiotic surfaces.

Fundamental scientific interest in the process of bacterial biofilm formation has grown exponentially during recent years and studies of the regulation of biofilm formation have begun to reveal molecular mechanisms that are involved in the transition of the planktonic to the biofilm state of living.

Biofilm formation is an important aspect of bacterial infection and disease, including tooth decay, endocarditis and chronic lung infection in cystic fibrosis patients. Furthermore, biofilms formed on abiotic surfaces are an important source for infections, such as biofilms formed on medical devices and implants (Donlan and Costerton, 2002). The 10 to 1000-fold increased resistance of bacterial cells in biofilms to antibiotics as compared to planktonic cells , and their high resistance to phagocytosis, make biofilms extremely difficult to eradicate (Lewis *et al.*, 2003).

Colonization and biofilm formation by rhizobacteria play an important role in plant pathogenesis and beneficial interactions (Bloemberg *et al.*, 2004). Plant growth-promoting rhizobacteria can be classified as (i) biofertilizers which fix nitrogen (ii) phytostimulators which promote plant growth directly by production of hormones, and (iii) biocontrol agents which protect plants from infection by phytopathogenic organisms (Bloemberg *et al.*, 2004). Efficient rhizobacterial biofilm formers should be able to (i) attach to the root surface, (ii) survive in the rhizosphere, (iii) make use of nutrients exuded by the plant root, (iv) proliferate and form microcolonies, (v) efficiently colonize the entire root system, and (vi) compete with indigenous microorganisms (Bloemberg *et al.*, 2004).

Biofilms have an enormous impact in industrial, medical and agricultural settings, exhibiting both harmful and beneficial activities. The relevance of bacterial biofilms has highly stimulated the elucidation of the regulatory mechanisms involved in their formation, maturation and dissolution.

1.2. Biofilm formation

The process of biofilm formation can be divided in distinct developmental steps (Fig, 1), which are similar in many bacterial species. The model of biofilm development includes (i) initial reversible and (ii) irreversible attachment to a surface followed by (iii) the formation of microcolonies, either by aggregation of already attached cells, by recruitment of planktonic cells, or by cell division, (iv) the formation of macrocolonies, and finally (v) the maturation of macrocolonies, forming a “mushroom shape” or a “carpet-like” biofilm depending on the environmental conditions.

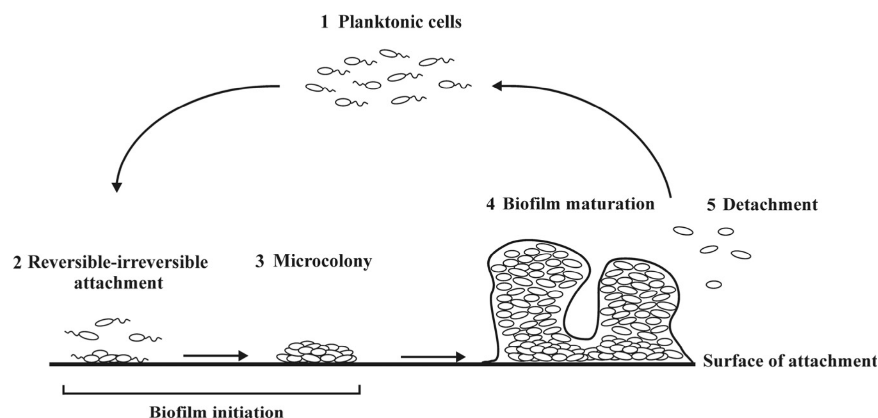

Fig. 1. Schematic representation of the distinct steps in biofilm development as typically observed for gram-negative bacteria (adapted from Toutain et al., 2003).

1.3. Ecological advantage and relevance of biofilms

1.3.1. Defense

Cells in a biofilm are usually surrounded by an extrapolymeric substance matrix, which forms a key component in the increased resistance and protection of a biofilm to environmental stress factors. This matrix is composed of a mixture of components, such as extracellular polysaccharides (EPS), proteins, nucleic acids, and other substances. Among these components, EPS is the best studied. It was reported to provide protection against a variety of environmental stresses, such as UV radiation, pH shifts, osmotic shock, and desiccation (Flemming, 1993). EPS has been shown to adsorb dissolved organic compounds, such as diclofop-methyl (an

herbicide) and other xenobiotics, providing a mechanism by which the bacterial community can concentrate essential nutrients and growth components (Wolfaardt *et al.*, 1998). The EPS matrix may also be involved in tolerance of biofilms to antimicrobial agents by restricting diffusion of compounds from the surrounding environment into the biofilm (Gilbert *et al.*, 1997). In addition, EPS was reported to sequester metals, cations, and toxins (Flemming, 1993).

1. 3. 2. Nutrient availability and metabolic cooperation

Biofilms can provide an environment for the establishment of syntrophic relationships. In a syntrophic association, two metabolically distinct bacteria depend on each other to utilize certain substrates for growth. Synthrophic associations have been well studied with regard to methanogenic degradation (Schink *et al.*, 1997). Recently, Kuiper et al. (2001) reported that *P.putida* strain PCL1445, which was isolated during a selection procedure together with strain PCL1444 from a grass plant heavily polluted by PAHs, does not grow on naphthalene in a pure culture but only in the presence of PCL1444. Thus, it was suggested that naphthalene degradation intermediates produced by PCL1444 can be used by PCL1445 in the rhizosphere, resulting in a symbiotic relationship (Kuiper *et al.*, 2001). On the plant root these two bacteria were found in close association, only in the presence of naphatalene (Kuiper I., Ph.D thesis).

1.3.3. Colonization

Biofilm formation can provide a mechanism for organisms to establish and maintain themselves in a favorable environment. Rhizobacteria not only benefit from nutrients secreted by the plant root but also influence the plant in a direct or indirect way. Bacteria from the genus *Pseudomonas*, which are ubiquitously present in the environment, are frequently found in association with plants either as mutualists, saprophytes or pathogens, and therefore have a strong impact on agriculture. The plant pathogen *P syringae* survives on aerial parts of host-plants (Hirano and Upper, 2000). *P. putida* is commonly found in rhizosphere (the root surface and surrounding soil area) and can play a beneficial role in protecting plants from the attack of pathogenic microorganisms, indirectly via the induction of systemic resistance in the plant, or directly by affecting the survival, or activity of the pathogen. The latter mechanism includes the production of antibiotics (for example phenazines and HCN), and/or competition for certain nutrients, in

particular Fe^{3+} via the synthesis of siderophores and subsequent uptake of Fe^{3+}-siderophore complexes (Bloemberg and Lugtenberg, 2001). Several *P. putida* strains have the capability to metabolize toxic aromatic compounds which, in combination with efficient rhizosphere colonization, define rhizoremediation (reviewed by Kuiper *et al.*, 2004).

1.3.4. Acquisition of new genetic traits

Plant-associated bacterial populations are hotspots for horizontal gene transfer due to the close proximity of biofilm cells (Trevors *et al.*, 1989; Dekkers *et al.*, 2000). Biofilms offer an ideal environment for horizontal exchange of genetic material, the rapid spread of phages, conjugation and uptake of plasmid DNA by competent bacteria. Plasmids and phages have developed mechanisms to induce the transition to the biofilm mode of growth in their host by promoting cell-cell interactions (Ghigo *et al.*, 2001). Interestingly, transfer functions are regulated by quorum sensing in plant-associated bacteria-like *Rhizobium* and *Agrobacterium* (He *et al.*, 2003; Piper *et al.*, 1993).

1.4. Regulation of biofilm formation

A summary of factors involved in the different stages of biofilm development is presented in Table 1.

1.4.1. Initiation of biofilm formation

The initiation of biofilm formation was suggested to start when bacteria sense certain environmental factors, which induce the transition from planktonic growth to life on a surface (Davey *et al.*, 2000; Stanley *et al.*, 2004).

The signals that regulate surface attachment and microcolony formation differ between bacterial species and strongly reflect the natural habitat of the bacterial species (e. g. a high-osmolarity environment in the case of *Staphylococcus epidermidis* and *S. aureus* and a low-osmolarity environment in the case of *Escherichia coli*).

Many environmental signals were indicated to influence initial attachment such as osmolarity, pH, iron availability, oxygen tension, and temperature (Fletcher *et al.*, 1996; Nyvad *et al.*, 1990; O'Toole *et al.*, 1998; Pratt *et al.*, 1998). Inorganic phosphate (Pi) may be a key environmental factor required for biofilm formation by pseudomonads (e.g. for *P. aureofaciens* and *P. fluorescens*) by modulating the Pho

regulon, which is formed by the PhoR/PhoB two-component regulatory system. Two-component regulatory systems are used by bacteria to sense and respond to environmental conditions. Another regulatory system is the *gac* system, which is involved in biofilm formation and is highly conserved in pseudomonads and other gram-negative bacteria (Laville *et al.*, 1992). A recent study showed that a *P. aeruginosa gacA* mutant attaches to the substratum but does not aggregate and does not form microcolonies (Parkins *et al.*, 2001).

The EnvZ/OmpR signaling system of *E. coli* is activated under conditions of moderate increase of osmolarity (Pratt and Silhavy, 1995), suggesting that osmolarity would stimulate stable cell-surface interactions. However, under high osmolarity, when bacteria would be in a non-favorable environment, the cells would remain in the planktonic phase and free to relocate to more environmentally favorable conditions.

In *P. aeruginosa*, the global carbon metabolism regulator Crc, regulates expression of *pilA* and *pilB*, which encode the main structural protein of type IV pili (O'Toole *et al.*, 2000). The Crc protein is activated by tricarboxylic acid (TCA) cycle intermediates, ensuring biofilm formation in environments that contain the preferred carbon source of *P. aeruginosa* (O'Toole and Kolter, 1998). Flagella and pili were also reported to be involved in the initiation of the early attachment processes of *E. coli* (Genevaux *et al.*, 1996; Pratt *et al.*, 1998). Attachment by an *E.coli* non-flagellated mutant is not completely eliminated and the formed biofilm consists of separate microcolonies (Pratt *et al.*, 1998). Thus, the role of flagella appears to be different in *E. coli* and in *P. aeruginosa*.

In *Bacillus subtilis*, the initiation of biofilm formation involves a complex regulatory system in response to a number of environmental stress factors (Wise and Price, 1995). The response regulator Spo0A is active under starvation and high cell density (Sonenshein, 2000), indicating that these conditions may reflect the environmental conditions under which there is a physiological advantage for *B. subtilis* to form a biofilm.

Finally, the chemical nature of the bacterial surface may have a dramatic effect on the surface attachment, which is governed by electrostatic interactions, and by the hydrophobicity of a bacterial cell due to its LPS composition (De Weger *et al.*, 1989). For example, Dekkers et al. (1998) showed that the presence of the O-antigen is necessary for colonization of plant roots by *P. fluorescens*.

1.4.2. Maturation of the biofilm

The process of biofilm maturation, which involves the controlling of the thickness and the architecture of the biofilm, is regulated by signals which are conserved between bacterial species. This is often associated with the production of EPS. Alginate produced by *P. aeruginosa* has been implicated to function as an EPS in biofilm development (Govan *et al.*, 1996). Matured biofilms can be thick, homogenenous, or they can consist of complex structures composed of pillars with water channels that have been proposed to allow for nutrient influx and waste efflux (Davey and O'Toole, 2000). Biofilm maturation was shown to be controlled by the availability of nutrients and quorum sensing. In *P. aeruginosa*, the depth of the mature biofilm is reduced by the transcriptional factor RpoS (Whiteley et al., 2001). RpoS production is regulated in Gram-negative bacteria in response to different stress conditions including nutrient limitation (Venturi, 2003). Thus, activation of RpoS would signal that nutrients are limiting in *P.aeruginosa* biofilm. In contrast, RpoS is required for biofilm initiation in *E. coli* (Adams and McLean, 1999), suggesting a role of RpoS in *E. coli* more analogous to the role of Spo0A in biofilm formation by *B. subtilis*. As a biofilm becomes larger and ages, cells in the centre would have reduced access to nutrients, resulting in a starvation signal, which in turn would activate RpoS in *P. aeruginosa* to reduce the biofilm thickness. Interestingly, in *Vibrio cholerae* the thickness of the mature biofilm was shown to be regulated by quorum sensing (Zhu and Mekalanos, 2003).

1.4.3. Regulation of the biofilm architectural structure

Surfactant production in *B. subtilis* and *P. aeruginosa* is required for the architectural structure of biofilms by reducing the surface tension (Branda *et al.*, 2001; Davey *et al.*, 2003). In *B. subtilis*, lipopeptide production is required to form the spore-containing fruiting bodies found at the surface of the biofilm (Branda *et al.*, 2001). In *P. aeruginosa*, rhamnolipid surfactant production is required for the maintenance of the pillar structures and water channel structures (Davey *et al.*, 2003). In both cases, surfactant production is regulated by quorum sensing, in *B. subtilis* by the ComX pheromone and the ComP sensor kinase (Lazazzera *et al.*, 1999), and in *P. aeruginosa* by the *lasI-lasR* quorum sensing system (Pearson *et al.*, 1997). Both the formation of fruiting bodies in *B. subtilis,* which results in the dissemination of spores in a new environment, and the formation of water channels in *P. aeruginosa* will finally result in the acquisition of nutrients.

Table 1. Summary of factors involved in biofilm formation [a].

Step of biofilm development	Factors involved	Organism
Initial attachment to a surface	Nutrient availability	*B. subtilis*
	Stress factors (osmolarity, iron availability, temperature, pH, O_2 tension)	*S. epidermidis, S. aureus, E. coli*
	Iron availability	*S. epidermidis*
	Inorganic phosphate	*P. aureofaciens, P. fluorescens*
	Hydrophobicity/hydrophilicity	*P. fluorescens*
	Flagella and swimming motility	*P. aeruginosa, E. coli*
	Secreted DNA, proteins	*S. epidermidis*
	Pili and twitching	*E. coli*
	Catabolite repression control protein (Crc)	*P. aeruginosa*
Microcolony formation	Virulence factor regulator (Vfr)	*P. aeruginosa*
	Two-component regulatory system (*gac*)	*P. aeruginosa*
Macrocolony formation	Exopolysaccharide production, alginate	*P. aeruginosa*
	Quorum sensing	*P. aeruginosa*
Maturation of biofilm	Surfactants	*P. aeruginosa*
	Quorum sensing	*P. aeruginosa, V. cholerae*
	Pheromones	*B. subtilis*
	RpoS	*P. aeruginosa*
Detachment	Nutrient limitation	*P. aeruginosa*
	Surfactants	*P.aeruginosa, B. subtilis*

[a] Adapted from Tourain et al. (2004).

2. Biosurfactants

2.1. Biosurfactant activity

Surfactants are amphipathic molecules with a hydrophilic and a hydrophobic moiety, that localize preferentially at the interface between fluid phases with different degrees of polarity such as oil/water or air/water interfaces. These properties make surfactants capable of reducing surface tension and capable of

forming emulsions in which a hydrophobic phase solubilizes in the water phase or in which the water phase solubilizes in the hydrophobic phase. Biosurfactants form a structurally diverse group of surface active molecules and are commonly synthesized by microorganisms.

Quick and reliable methods for screening biosurfactant-producing microbes have contributed to recent advances in the field of studying the role of microbial surfactants. Development of simple methods include: (i) a rapid drop-collapsing test (Jain *et al.*, 1991), in which a drop of cell suspension is placed on a hydrophobic surface such as parafilm, and drops containing biosurfactant collapse whereas non-surfactant-containing drops remain roundly shaped; and (ii) a direct thin-layer chromatographic technique for rapid characterization of biosurfactants-producing bacterial colonies (Matsuyama *et al.*, 1991).

Biosurfactant activity is commonly determined by measuring the changes in the surface tension of liquids. Surface tension at the air/water and oil/water interfaces can easily be measured with a tensiometer. The surface tension correlates with the concentration of the surface-active compound until the critical micelle concentration (CMC) is reached. The CMC is defined as the minimum concentration necessary to initiate micelle formation (Becher, 1965). Efficient surfactants have a low critical micelle concentration (i.e. less surfactant is necessary to decrease the surface tension).

2.2. Biosurfactants classification

Biosurfactant-producing microbes are found among a wide range of genera (Table 2). The structure of biosurfactants includes a hydrophilic moiety consisting of amino acids or peptides, anions or cations, mono-, di- or polysaccharides, and a hydrophobic moiety consisting of fatty acids. Biosurfactants have been commonly classified as (i) low-molecular-weight molecules, which decrease surface tension efficiently and (ii) high-molecular-weight polymers which bind to surfaces (Rosenberg and Ron, 1997).

In general, low-molecular-weight biosurfactants belong to the classes of glycolipids or lipopeptides. The best studied glycolipid is rhamnolipid, which is produced by several *Pseudomonas* species (Jarvis and Johnson, 1949; Tuleva *et al.*, 2002). Several lipopeptide antibiotics have surface-active properties such as surfactin produced by *B. subtilis* (Peypoux *et al.*, 1999).

High-molecular-weight polymers are extracellular polymeric surfactants composed of polysaccharides, proteins, lipopolysaccharides, lipoproteins or a complex mixture of these molecules. The best studied is emulsan, which is produced by different *Acinetobacter* species (Rosenberg and Ron, 1997).

Finally, several bacteria, such as *Acinetobacter* sp *T. thiooxidans* and *R. erythropolis*, produce a large quantity of fatty acid and phospholipids surfactants when grown on *n*-alkane (Kappeli *et al.*, 1979; Beeba *et al.*, 1971; Kretschneser *et al.*, 1982). A summary of some known biosurfactants, their origin, and properties is presented in Table 2.

Table 2. Microbial source and properties of major classes of biosurfactants.

Biosurfactant	Organisms	Surface tension (mN/m)	Reference(s)
Glycolipids			
Rhamnolipids	*P. aeruginosa*	29	Guerra-Santos et al
	Pseudomonas sp.	25-30	(1986)
Trehalolipids	*R. erythropolis*	32-36	Hisatsuka et al (1971)
	N. erythropolis	30	Raap et al. (1979)
	Mycobacterium sp.	38	Margaritis et al (1980)
Sophorolipids	*T. bombicola*	33	Cooper et al (1989)
	T. apicola	30	Cooper et al (1989)
	T. petrophilum		Hommel et al (1987)
Cellobiolipids	*U. zeae, U. maydis*		Cooper et al (1983)
			Boothroyd et al (1956)
Lipopeptides and lipoproteins			
Peptide-lipid	*B. licheniformis*	27	Javaheri et al (1985)
Serrawettin	*S. marcescens*	28-33	Matsuyama et al (1991)
Viscosin	*P. fluorescens*	26.5	Neu et al (1990)
Putisolvin I/II	*P. putida*	32	Kuiper et al (2004)
Amphisin	*Pseudomonas* sp. DSS73	30	Sørensen et al (2001)
Surfactin	*B. subtilis*	27-32	Arima et al (1968)
Subtilisin	*B. subtilis*		Bernheimer et al (1970)
Gramicidins	*B. brevis*		Marahiel et al (1977)
Polymyxins	*B. polymyxa*		Suzuki et al (1965)

Fatty acids, neutral lipids, and phospholipids			
Fatty acid	*C. lepus*	30	Cooper et al (1989)
Neutral lipids	*N. erythropolis*	32	MacDonald et al (1981)
Phospholipids	*T. thiooxidans*		Beeba et al (1971)
Polymeric surfactants			
Emulsan	*A. calcoaceticus*		Rosenberg et al (1979)
Biodispersan	*A. calcoaceticus*		Rosenberg et al (1988)
Mannan-lipid-protein	*C. tropicalis*		Kappeli et al (1984)
Liposan	*C. lipolytica*		Cirigliano et al (1984)
Carbohydrate-protein	*P. fluorescens*	27	Desai et al (1988)

2.3. Biological role and relevance of biosurfactants

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). One of the reasons which is hampering the widespread use of biosurfactants is their production costs. Since surfactants are produced by a large variety of microorganisms and have very different structures and surface properties, different groups of surfactants may have different biological roles in the functioning of the surfactant producing bacteria. These biological roles have been extensively reviewed (Ron *et al.*, 2002; Mulligan *et al.*, 2005; Cameotra and Makkar, 2004). Ron et al. (2002) have discussed the various roles of bioemulsifiers, some of which are unique to the physiology and ecology of the producing microorganisms, including increasing surface area of hydrophobic water insoluble substrates, binding of heavy metal, in antimicrobial activity, pathogenesis and in regulating attachment-detachment of microorganisms to and from surfaces. Mulligan et al. (2004) focussed in a recent review on the role of biosurfactants in the bioremediation of contaminated land sites. The author reports the role of rhamnolipids in oil-contaminated water, and in metals removal due to the anionic nature of rhamnolipids. Thus, biosurfactants seem to enhance biodegradation by influencing the bioavailability of the contaminant.

Intensively studied organisms and their 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).

One of the major reasons for the prolonged stability of hydrophobic compounds is their low water solubility, which limits their availability to biodegrading microorganisms. Uptake of hydrophobic compounds by bacteria is described to proceed via the water phase and is therefore dependent upon their solubility in water (Bouwer and Zehnder, 1993). Surfactants have been described to make the xenobiotic more soluble, which can result in spreading of the pollutant and making it available as nutrient. In a recent review, Maier and Soberon-Chavez (2000) indicated that addition of rhamnolipids can enhance biodegradation of hexadecane, octadecane, and phenanthrene. Rhaman et al. (2002) showed that addition of rhamnolipids produced by *Pseudomonas* sp. DS10-129 enhanced bioremediation of gasoline-contaminated soil. Due to the anionic structure of rhamnolipids, they are able to form complexes with and remove heavy metals from soil such as cadmium, copper, lead, and zinc (Herman *et al.,* 1995).

In recent years, the role and applications of biosurfactants (mainly glycolipids and lipopeptides) have been investigated from medicinal and therapeutic perspectives. In a review of Cameotra and Makkar (2004) biosurfactants are reported to possess a number of interesting properties, since they function as antimicrobial agents, immunoregulators, and in adhesion and desorption processes (important in surgical procedures). Immunoassays with sera from patients with Lyme disease showed specific antibody reactivity to glycolipids of *Borrelia burgdorferi* (the causal agent of Lyme disease), suggesting a possible role for glycolipids as promising candidates for diagnosis of Lyme disease (Hossain *et al.,* 2001). Biosurfactants of *Lactobacillus* were reported to prevent surgical implant infections by *S. aureus*, showing the potential for development of anti-adhesion biological coatings for catheter materials by delaying the initiation of biofilm growth (Millsap *et al.*, 1997). A number of cyclic lipopeptides were shown to play a role as antimicrobial agents. The cyclic lipopeptide viscosinamide produced be a *Pseudomonas* spp. isolated from the sugar beet rhizosphere has antibiotic properties towards root-pathogenic fungi (Nielsen *et al.,* 2003). Testing for the production of cyclic lipopeptides by *Pseudomonas* sp. provides a new important tool for the selection of biological control agents, as a single strain or in a consortium of strains to maximize the synergistic effect of multiple antagonistic traits. Cyclic lipopeptides of *Bacillus* sp. (i. e. lychenysin, surfactins, and iturins) were reported to play a role in membrane permeabilization, resulting in pore formation (Grangemard *et al.,*

2001). The loss of membrane integrity makes surfactins and lychenysin to potential commercial antibiotics. Finally, the cyclic lipopeptide amphisin produced by *Pseudomonas sp.* DSS73 was reported to have antagonistic activity towards the root-pathogenic organisms *Pythium ultimum* and *Rhizoctonia solani* due to both biosurfactant and antifungal properties (Sørensen *et al.*, 2001).

Biosurfactants play an important role in pathogenesis. Rhamnolipid is considered to be one of the virulence exoproducts of *P. aeruginosa*. Its production correlates with production of other virulence factors and contributes to the maintenance of biofilm formation of *P. aeruginosa* (Davey *et al.*, 2003). Recently, it was reported that biosurfactants of *P. aeruginosa* are also involved in the solubility and bioactivity of quinolone, one of the signal molecules involved in the complex quorum sensing mechanism of *P. aeruginosa* and which is important for bacterial adaptation to the lung environment during infection (Calfee *et al.,* 2005). The lipopeptide syringomycin was shown to contribute to the pathogenicity of the plant pathogen *P. syringae* pv. *syringae* strain B301D (Bender *et al.,* 1999; Scholz-Schroeder *et al.*, 2001).

2.4. Lipopeptides biosynthesis

Lipopeptides form an important group of biosurfactants which are produced by a large variety of bacteria from different genera such as *Bacillus*, *Lactobacillus*, *Streptococcus*, *Serratia*, *Burkholderia*, and *Pseudomonas* (Velraeds *et al.*, 2000; Busscher *et al.*, 1997; Mireles *et al.*, 2001; Lindum *et al.*, 1998; Huber *et al.*, 2002; Bender *et al.*, 1999). Several chemical and biological aspects of CLP production in fluorescent Pseudomonads has been discussed by Nybroe and Sørensen (2004). In a recent review, Raaijmakers et al. (*in press*) have highlighted the structural diversity and activity of CLPs produced by plant-associated *Pseudomonas* spp. The authors have presented a detailed description of the genes involved in biosynthesis and regulation of CLPs as well as an update of the signature sequences within CLP biosynthetic gene cluster in Pseudomonas species.

Lipopeptides are composed of a peptide moiety that can be cyclized to form a lactone ring between two amino acids in the pepetide chain 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 including antifungal, phyto-toxicity and regulation of biofilm formation (Nielsen *et al.*, 1999; Hutchinson *et al.*, 1995; Huber *et al.*, 2002).

Based on the length and composition of the fatty acid chain as well as the peptide chain, CLPs of Pseudomonas species were classified into four major groups, i.e. the viscosin, amphisin, tolaasin, and syringomycin groups (Raaijmakers *et al.*, *in press*). The viscosin class harbours CLPs with 9 amino acids and Pseudomonas sp. producing this class of CLPs originate from diverse environmental niches including soil, rhizosphere, phyllosphere, as well as marine environments (Raaijmakers *et al.*, *in press*). CLPs from the amphisin class, consisting of tensin and amphisin (Henriksen *et al.*, 2000; Sørensen *et al.*, 2001) contain 11 amino acids in the peptide moiety. CLPs from the tolaasin class vary in length of the peptide moiety (19 to 25 amino acids), in the lipid tail and contains several unusual amino acids including 2,3-dihydro-2-aminobutyric acid (Dhb), homoserine (Hse), and *allo*-Thr. Several cyclic lipopeptides from the tolaasin class are virulence factors produced by plant pathogenic *Pseudomonas* sp. The CLPs from the syringomycin class show structural similarity with viscosin group but contain unusual amino acids including Dhb, or 2, 4-diamino butyric acid (Dab) and the lactone ring is formed between the N-terminal and the C-terminal amino acids whereas the ring is formed between the the C-terminal amino acid and the 3rd amino acid in the peptide moiety for viscosin (Fig. 2). Another recently studied cyclic lipopeptides structures include arthrofactin produced by Pseudomonas strain MIS38 (Morikawa *et al.*, 1993), which contains a 11-amino acid peptide moiety linked to a β-hydroxydecanoyl fatty acid chain and putisolvin I and II (Kuiper *et al.*, 2004), which consists of a 12-amino acid peptide chain bound to an hexanoic lipid chain. The cyclization of the putisolvins is different from other lipopeptides since the lacone ring is formed between the C-terminal and the 9th amino acid residues instead of the 1st or 3rd amino acid as described for other lipopeptides. Recently, Paulsen et al. (2005) has identified a cluster encoding a cyclic lipodecapeptide by analyzing the entire genome sequence of *P.fluorescens* Pf-5. This finding showed that whole genome sequence allows the identification of unknown genes and traits with interesting biological activity in antagonistic *Pseudomonas* sp.

Lipopeptide biosynthesis occurs non-ribosomally via multifunctional proteins which are encoded by large gene clusters, homologs of which were first described for peptide antibiotics produced by *Bacillus* and *Streptomyces* (Kleinkauf *et al.*, 1995). The biosynthesis of lipopeptides synthetases has been intensively investigated and reviewed (Kleinkauf *et al.*, 1996, Marahiel *et al.*, 1997; Stachelhaus *et al.*, 1995). The genes encoding the multifunctional peptide synthetase possess very conserved modular structures. The order and number of the modules of the

non-ribosomal peptide synthetase (NRPS) are frequently found to be colinear to the amino acid sequence of the peptide moiety of the CLP molecule. Typically, all multi-enzymatic systems are composed of a reaction sequence which includes (i) carboxyl activation of the substrate amino acid by adenylation, (ii) acylation of the enzyme-attached pantothenoyl-thiols, and (iii) directed transfer to the next acyl intermediate and condensation. The linear peptide is released from the enzyme complex by a thioesterase domain which results in cyclization, amidation, or hydrolysis of the CLP molecule. Additional domains in the NRPS may include an epimerization domain, responsible for the conversion of the L- or D-configuration of an amino acid.

Among Cyclic lipopeptide producing Pseudomonas species, arthrofactin (Roongsawang *et al.*, 2003) and syringomycin biosynthetic clusters (Bender *et al.*, 1999) are the best characterized. Arthrofactin biosynthetic gene cluster is composed of three ORFs *arfA*, *arfB*, and *arfC* which codes together for the 11 modules required for arthrofactin biosynthesis and obeys to the colinearity rule. In contrast, syringomycin biosynthetic gene cluster does not respect the colinearity rule since the *syrB1* gene, responsible for the incorporation of the 9th amino acid in the peptide moiety, is located upstream of the *syrE* incorporate the first 8 amino acids residues (Fig. 2). Together with syringopeptin biosynthetic gene cluster, syringomycin forms the large *syr-syp* genomic island in *P. syringae* pv. *syringae* (Scholz-Schroeder *et al.*, 2001). The *syrC* gene located next to syrB1-2 was proposed be involved in the transfer of the 3-hydroxy fatty acid chain to the first amino group (Ser) bound to SerE1 module (Fig. 2). The *syrD* gene located upstream of *syrB1-2* showed to codes for an outer-membrane protein of the ABC transporter family and was proposed to be involved in the secretion of the phytotoxins syringomycin and syringopeptin (Scholz-Schroeder *et al.*, 2001). Recently, Kang and Gross have reported the presence of another ABC transporter system, PseABC, located at the left border of the *syr-syp* cluster of *P.syringae* pv. *syringae* strain B301D. Such genetic organization involving the CLP biosynthetic gene cluster and efflux genes was also found for lipodecapeptide of *P.fluorescens strain* Pf5-5 (Paulsen *et al.*, 2005).

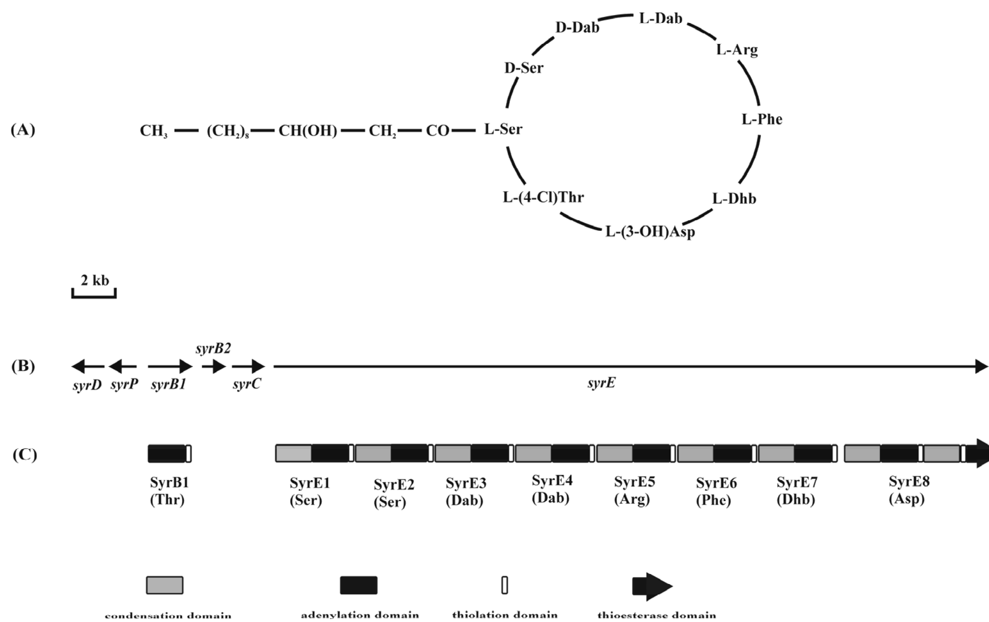

Fig. 2. Biosynthesis of the peptide moiety of the cyclic lipopeptide syringomycin. (A) Structure of syringomycin. (B) Genetic map of the syringomycin biosynthetic gene cluster in *P. syringae* pv. *syringae*. (C) The enzymes are composed of modules that can be subdivided into domains (see section 2.4). A thioesterase-like domain is believed to act as a cyclase. Adapted from Bender et al. (1999).

2.5. Characterization of the lipopeptide biosurfactants putisolvins I and II of *Pseudomonas putida*

P. putida strain PCL1445 was selected from a rhizobacterial population from grass like vegetation (Barmultra), grown on soil heavily contaminated with polycyclic aromatic hydrocarbons (PAHs) (Kuiper *et al.,* 2001). PCL1445 is an efficient root colonizer and is proliferating on degradation products of naphtalene and phenantrene. It is able to form a biofilm on the plant root surface and on PVC (Kuiper *et al.,* 2001). PCL1445 produces two cyclic lipodepsipeptides, named putisolvins I and II, each with a hexanoic lipid chain connected to the N-terminus of a 12-amino-acid peptide moiety, in which the C-terminal carboxylic acid group forms an ester with the hydroxyl side-chain of Ser-9 (Fig. 3) (Kuiper *et al.,* 2004). The difference between the two structures is located in the second amino acid from the C-terminus, which is a Val for putisolvin I and a Leu/Ile for putisolvin II.

Biosurfactant production by PCL1445 is inititated at the end of the exponential phase, suggesting that the production is regulated by a quorum sensing like-system. A Tn5 mutant of PCL1445, strain PCL1436, which does not produce putisolvins I and II, is mutated in an open reading frame (ORF) which has homology with several lipopeptide synthetases. The putisolvin synthetase gene of PCL1445 was named *psoA* (Dubern *et al.*, 2005, this Ph.D Thesis, Chapter 2).

Putisolvins I and II represent structurally novel cyclic lipopeptides (Kuiper *et al.,* 2004). The surface tension reducing ability of putisolvins I and II appears to improve emulsification of toluene and resulted in an enhanced dispersion of naphthalene and phenantrene (Kuiper *et al.,* 2004). These properties show that putisolvin I and II can play an important role in increasing the availability of hydrophobic compounds (Rosenberg *et al.,* 1993).

In addition, it was shown that secretion of putisolvins I and II stimulates the swarming motility of cells presumably by altering the cell surface hydrophobicity. Moreover, it inhibits biofilm formation, and degrades existing biofilms of its own wild type and of various pseudomonads, including of the opportunistic human pathogen *P. aeruginosa* (Kuiper *et al.,* 2004). In this Ph.D Thesis, I describe some of the mechanisms of the regulation of putisolvins in PCL1445, and therefore the work contributes to the understanding of the ecological advantage of biosurfactant production for rhizosphere *Pseudomonas* species that proliferate in an environment such as polluted soil. Moreover, studies on the mechanism of dissolving biofilms could result in application of a biosurfactant producing strain in combination with pollutant degraders to improve biodegradation of hydrophobic pollutants in soils.

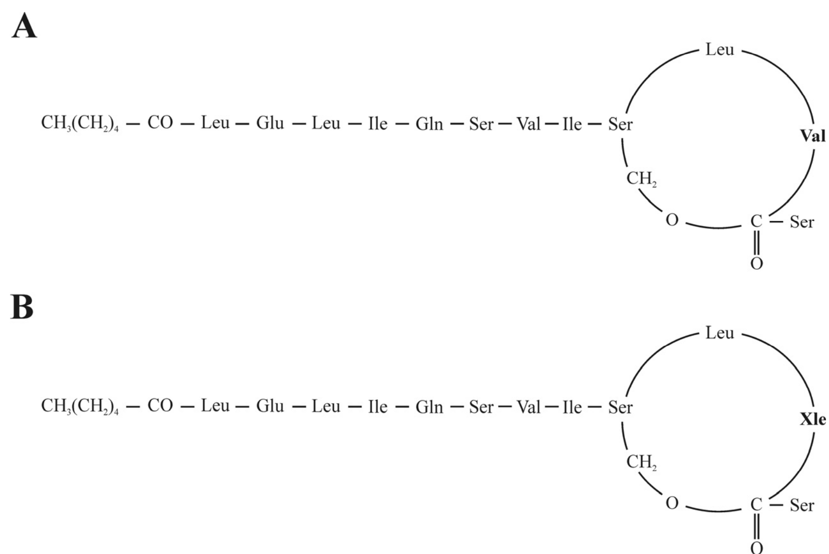

Fig. 3. Structure of putisolvin, adapted from Kuiper et al. (2004). **A:** putisolvin I; **B:** putisolvin II. Xle represents Leu or Ile.

3. Regulation of the synthesis of secondary metabolites and cyclic lipopeptides

3.1. Quorum sensing system

Many gram-negative bacteria regulate the expression of specific sets of genes that are involved in secondary metabolism when growth is restricted because of nutrient limitation at high cell density, which functions as a strategy to remain competitive in their environment (Vining, 1990). The regulation of gene expression in response to a high cell density is known as quorum sensing. Quorum sensing is an intercellular communication mechanism enabling bacteria to sense high cell density via the production and the sensing of signal molecules (also refered to as quorum sensing signals or autoinducers), which in Gram-negative bacteria belong to the class of acyl homoserine lactones (AHLs). The detection of a minimal threshold concentration of an autoinducer leads to the activation of a regulator and subsequently to the autoinduction of AHL production and induction of expression of other genes. Many bacteria use quorum sensing to regulate a diverse array of

physiological activities. Functions regulated by these signal molecules include bioluminescence in *Vibrio fischeri* (Stevens and Greenberg, 1997), production of rhamnolipid biosurfactants for example in *Pseudomonas aeruginosa* (Passador *et al.,* 1993), production of a carbapenem antibiotic in *Erwinia carotovora* (Bainton *et al.,* 1992), conjugative plasmid transfer in *Agrobacterium tumefaciens* (Piper *et al.,* 1993), phenazine-1-carboxamide production in *P. chlororaphis* (Chin-A-Woeng *et al.,* 2001) and swarming motility in *Serratia liquefaciens* (Eberl *et al.,* 1996). Recent studies have started to integrate AHL quorum sensing into global regulatory networks and establish its role in development and maintenance of the structure of bacterial communities (Fuqua *et al.,* 2001; Kjelleberg and Molin, 2002). Quorum sensing mechanisms were reported to be involved in biofilm development in *P. aeruginosa* (Davies *et al.*, 1998), in *Serratia liquefaciens* MG1 (Labbate *et al.,* 2004), in *V. cholerae* (Hammer and Bassler, 2003), and in *P. putida* IsoF (Steidle *et al.*, 2002). Cell density plays an important a important role in the production of cyclic lipopeptides production in *Pseudomonas*. In this context, the role of quorum sensing in CLPs regulation has been investigated in numerous *Pseudomonas* sp. Recent studies by Cui et al. (2005) showed that *N*-AHL-mediated quorum sensing plays a role in viscosin synthesis in the phytopathogen *P. fluorescens* strain 5064. In *P. putida* strain PCL1445, the *ppuI-rsaL-ppuR* quorum sensing system regulates the biosynthesis of putisolvins I and II and biofilm formation (Dubern *et al.*, 2006; this Thesis, Chapter 3). In contrast, *N*-AHL-mediated quorum sensing does not appear to play a role in amphisin and syringomycin production (Andersen *et al.*, 2003; Kinscherf and Willis 1999).

Quorum sensing was first described in the marine symbiont *Vibrio fischeri* (Nealson *et al.*, 1970) (Fig.4). AHLs were first identified in *V. fisheri* and *V. harveyi* where quorum sensing plays a central role in the regulation of bioluminescence production (Eberhard *et al.,* 1981). AHLs are synthetized via the LuxI protein, whereas the transcriptional activator protein LuxR is believed to interact with the signal molecules to couple cell population density to gene expression (Fuqua *et al.* 2001) (Fig. 4). LuxR homologues show weak similarity (around 20-35%) among each other. The C-terminal harbors a helix-turn-helix DNA-binding domain (Fuqua *et al.,* 1994). The N-terminal domain contains the AHL binding domain (Swift *et al.,* 1996). The LuxR proteins were suggested to bind as dimers since promoters that are thought to function as binding sites for LuxR-like proteins contain dyad sequence symmetry (Choi and Greenberg, 1992).

Gram-negative bacteria synthesize AHLs from S-adenosyl methionine (SAM), the source for the homoserine moiety, which is linked to acyl chains (Fuqua *et al.,* 1994). These signal molecules can traffic in and out of the bacterial cell using an active transport system for long chain AHLs (Pearson *et al.,* 1999), or by simple diffusion through the cell membrane (Kaplan and Greenberg, 1985). Once a certain intracellular threshold concentration has been reached, the signals induce transcription of a set of target genes (Fuqua *et al*., 1994). In *V. fisheri*, the genes encoding the AHL-synthetase LuxI, and the response regulator LuxR are divergently transcribed. The operator region of the *luxI* gene contains a specific inverted repeat sequence of 20 nucleotides, referred to as *lux* box, which is believed to be the binding site for LuxR (Fuqua *et al*., 1994).

The detection of AHLs activity in Gram-negative bacteria has been greatly facilitated by the development of sensitive *lux*-based reporter assays that allow fast screening of microorganisms for diffusible signal molecules that can activate the *lux* system. These *lux*-based reporters plasmids lack a functional *luxI* homolog and *E. coli* cells transformed with such constructs do not produce light unless supplied with an the corresponding exogenous AHL (Swift *et al.,* 1993). Lux-based biosensors differ in their sensitivity. In this study two reporters are used. Firstly pSB1075, in which *luxR* and the *luxR* promoter region are replaced by the *P. aeruginosa luxR* homolog and which is more specific for long chains *N*-(3-oxo)-AHLs. Secondly pAK211 which harbors *luxR* and the *luxI* promoter of *V. fisheri*, and which detects specifically shorter chains *N*-(3-oxo)-AHLs.

In addition, certain LuxI homologues have been shown to be involved in the production of more than one type of AHL molecules. In *P. chlororaphis* PCL1391, PhzI is involved in the production of *N*-octanoyl-L-homoserine lactone (C_8-AHL), *N*-hexanoyl-L-homoserine lactone (C_6-AHL), and *N*-butanoyl-L-homoserine lactone (C_4-AHL) (Chin-A-Woeng *et al*., 2001). In *P. putida*, *ppuI* is involved in the production of four AHL molecules *N*-(3-oxo-hexanoyl)-L-homoserine lactone (3-oxo-C_6-AHL), *N*-(3-oxo-octanoyl)-L-homoserine lactone (3-oxo-C_8-AHL), *N*-(3-oxo-decanoyl)-L-homoserine lactone (3-oxo-C_{10}-AHL), and *N*-(3-oxo-dodecanoyl)-L-homoserine lactone (3-oxo-C_{12}-AHL) (Steidle *et al.,* 2002; Bertani *et al.,* 2004; Dubern *et al*., 2006; this ph.D Thesis, Chapter 3).

Finally, investigations have been focused on the role of cross-talk in which autoinducer molecules produced by one bacterium may initiate a response in another organism, for example in plants (Mathesius *et al.,* 2003), in the stimulation

of cytokine production in mammalian cells (Dimango *et al.,* 1995), or in the induction of morphological changes in fungi (Hogan *et al*, 2004).

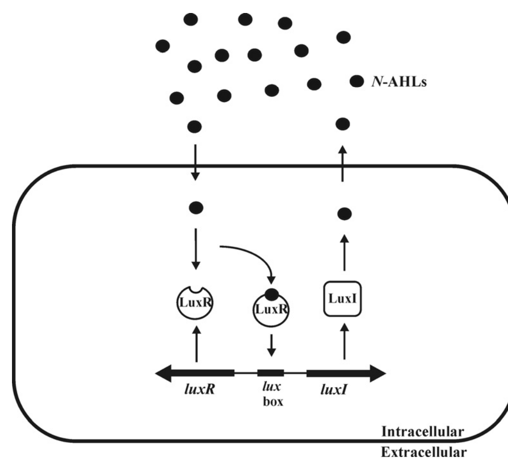

Fig. 4. Model for quorum sensing in the marine symbiont *V. fisheri*.

3.2. Global regulatory systems and other systems of regulation of secondary metabolites and CLPs

Two-component signaling systems, usually consisting of a sensor kinase and a response regulator, have been subject to intensive investigations during the past years due to their global role in the adaptation of microorganisms to different growth conditions and in colonization of specific ecological niches in reponse to environmental signals. The GacA/GacS system was reported to control the production of many secondary metabolites and extracellular enzymes involved in pathogenicity e.g. by regulating the production of tabtoxin in *P. syringae* (Barta *et al.,* 1992), of pyoverdin in *P. marginalis* (Liao *et al.,* 1997), and of the cyclic lipopeptide syringomycin in *P. syringae* (Kitten *et al.,* 1998). GacA/GacS was also shown to be involved in biocontrol of soilborne diseases by regulating the production of phenazine and exoproteases in *P. aureafaciens* (Chancey *et al.,* 1999) and *P. cholororaphis* (Chin-A-Woeng *et al.*, 2000), amphisin in *Pseudomonas* sp. DSS73 (Koch *et al.,* 2002), and ecological fitness by controlling phase variation (van den Broek *et al.,* 2003). In *P. putida* strain PCL1445, the GacA/GacS two-component system controls the biosynthesis of putisolvins I and II (Dubern *et al.*,

2005; this Thesis, Chapter 2). Several other regulators acting downstream of the GacA/GacS system were described to be involved in CLP production. Kitten et al. (1998) identified *salA* as a member of the *gacS-gacA* regulatory regulon in *P. syringae* pv. *syringae* that was demonstrated to be involved in the production of the phytotoxin syringomycin by inducing expression of *syrB1* and of *syrF*, another regulator of the LuxR family. SalA showed similarity with other response regulators by the presence of an H-T-H DNA binding motif (Lu *et al.*, 2002). Another regulator of syringomycin biosynthesis, SyrP, located in the *syr* cluster between *syrB* and *syrD* genes (Fig. 2), was shown to function in a phosphorelay signal transduction pathway (Zhang *et al.,* 1997). Recently Lu et al. (2005) showed using an oligonuscleotide microarray that genes involved in synthesis, secretion and regulation of syringomycin amd syringopeptin were upregulated by *salA*.

GacS is a transmembrane protein which functions as a histidine protein kinase that undergoes phoshorylation in response to environmental stimuli (Hrabak *et al.,* 1992) (Fig. 5). GacA is a response regulator protein that is phosphorylated by GacS (Rich *et al.,* 1994). GacS-GacA homologues are highly conserved in fluorescent pseudomonads (Heeb *et al.,* 2001; de Souza *et al.,* 2003). Although GacA/GacS is frequently found at the top of the regulatory cascade controlling secondary metabolite production (Chin-A-Woeng *et al.*, 2001; Chatterjee *et al.,* 2003; Kitten *et al.*, 1998), very little is still known about the signals that serve as a trigger. Koch et al. (2002) showed that exudates of sugar beet seeds induce the production of cyclic lipopeptide amphisin and that the signal transmission requires a functional GacS protein.

Recent detailed studies of the GacS/GacA signal transduction cascade revealed the involvement of small regulatory RNAs. Examples of these are RsmZ and RsmY in *P. fluorescens* (Aarons *et al.,* 2000, Heeb *et al.,* 2001), which play a role in the post-transcriptional activities of the biosynthetic *hcn* (hydrogencyanide production) and *phl* (encoding 2,4-diacetylphloroglucinol or DAPG production) operons by titrating translational repressors like RsmA and RsmE, thereby making the ribosome binding site of target mRNAs accessible for translation (Fig. 5).

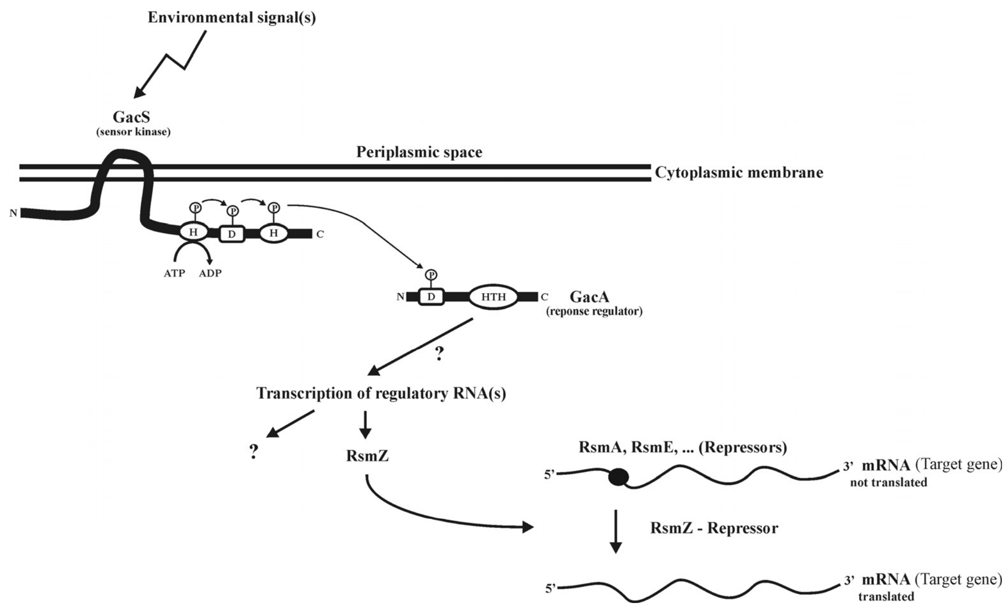

Fig. 5. Current model for the regulation of secondary metabolites involving the GacS/GacA two-component system (adapted from Haas et al., 2003).

In *E. coli*, the general stress response is mediated by the alternate σ^S factor, which is encoded by *rpoS* (Loewen *et al.,* 1994). RpoS is known for its crucial role in gene regulation during entry into stationary phase (Hengge-Aronis *et al.,* 2002; Lange *et al.,* 1991). It was recently discovered that RpoS has a major function in the general stress response in *P. aeruginosa*, *P. putida*, and *P. fluorescens* in which it regulates the production of virulence factors and secondary metabolites (Schuster *et al.,* 2004; Venturi *et al.*, 2003). Environmental stress factors inducing RpoS include low temperature (Sledjeski *et al.,* 1996), high osmolarity (Hengge-Aronis *et al.,* 1993), and acid stress (Lee *et al.,* 1995). It was shown that the two-component regulators GacS and GacA influence the accumulation of the stationary-phase sigma factor σ^S and the stress response in *P. fluorescens* Pf-5 (Whistler *et al.,* 1998). Recently, it was shown that *psrA* plays an important role in the transcription of *rpoS* during late-exponential phase and stationary growth phase in *Pseudomonas* sp. (Bertani *et al.*, 2003). Girard et al. (2005) have demonstrated the role of *psrA* in the regulation of phenazine production in *P. chlororaphis* strain PCL1391.

The heat-shock gene *dnaK* is another target of the GacA/GacS two-component system (Dubern *et al.*, 2005; this Thesis, Chapter 2). The DnaK protein,

which has been characterized as an anti-sigma factor due to its central role in the turnover of alternate σ^{32} factor under heat-shock condition (Fig. 6), has been reported to be implicated in *rpoS* translation. In stationary phase, a *dnaK* mutant showed reduced σ^{S} levels and exhibited a pleiotropic phenotype similar to that of an *rpoS* mutant (Rockabrand *et al.,* 1995; Rockabrand *et al.,* 1998). It was later suggested that the DnaK system could play a role in controlling stabilization of σ^{S}, and in the degradation of σ^{32} (Fig. 6) (Hengge-Aronis, 2002).

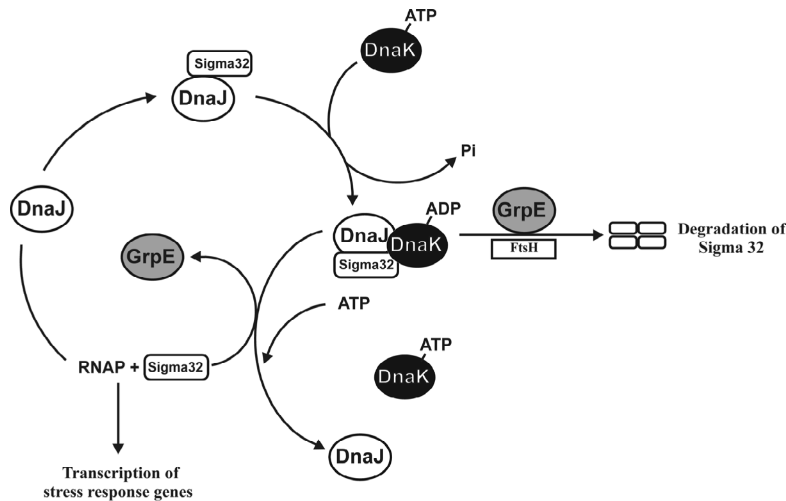

Fig. 6. Model for the regulation of the heat-shock reponse in *Escherichia coli* (Adapted from Hughes and Mathee, 1998).

Under normal growth conditions and in presence of ATP, DnaJ-σ^{32}-DnaK-ADP forms a stable complex after ATP autohydrolysis by DnaK. GrpE directs this complex to proteases (FtsH) for degradation of σ^{32} and complex dissociation. After heat-shock, the DnaK-DnaJ-GrpE complex acts as chaperone of cell proteins, including the house-keeping sigma factor, inactivated as result of heat-shock. σ^{32} is unaffected by heat shock and is available to interact with core RNA polymerase, resulting in the transcription of σ^{32} dependent operons. In *P.putida*, *dnaJ* and *grpE* are involved together with *dnaK* in the regulation of cyclic lipopeptide synthesis at low temperatures (Dubern *et al.*, 2005; this Thesis, Chapter 2).

4. Aims of this thesis

The significance of lipopeptide biosurfactants for growth, survival and functioning of rhizobacteria remains largely unknown. Putisolvins produced by *P. putida* PCL1445 were shown to be involved in important processes for the functioning of bacterial cells including swarming, and therefore may play a role in nutrient availability and bacterial biofilm formation (Kuiper *et al.*, 2004). The aim of the work described in this thesis was to investigate the regulatory mechanisms of putisolvin synthesis by *P. putida* PCL1445.

The first experimental approach to study the regulation of putisolvin synthesis was to generate and analyze Tn5*luxAB* mutants of PCL1445, which are impaired in putisolvin production, but which are not mutated in the putisolvin synthase gene *psoA* (**Chapter 2**). The production of putisolvins I and II starts at the end of the exponential growth phase, which suggests that the production is mediated through a quorum sensing mechanism. The aims of **Chapter 3** were to investigate the presence of quorum sensing system(s) in PCL1445, its role in the biosynthesis of putisolvin and consequently on biofilm formation.

In **Chapter 4**, the regulatory region of the putisolvin biosynthetic gene *psoA* was characterized. Putisolvins I and II have very similar structures and were indicated to be produced by a single gene since production of both was abolished by a mutation in a single ORF. Putisolvins are produced via a non-ribosomal peptide synthetase, which was preliminary named putisolvin synthetase (*psoA*). Putisolvins are the first lipopeptides identified, which consist of a 12 amino acid peptide moiety linked to a hexanoic lipid chain. Other known *Pseudomonas* lipopeptides, such as viscosinamide, syringomycin, amphysin and tensin have shorter amino acid moieties and the fatty acid chains are longer. Cyclization also appears to be variable.

In order to gain knowledge on the role of putisolvins in the functioning of PCL1445 and to possibly increase lipopeptide production of PCL1445, the regulation of putisolvins by (various) environmental factors was tested (**Chapter 5**).

Chapter 2

The heat-shock genes *dnaK*, *dnaJ*, and *grpE* are involved in the regulation of putisolvin biosynthesis in *Pseudomonas putida* PCL1445

Jean-Frédéric Dubern, Ellen L. Lagendijk, Ben J. J. Lugtenberg,
and Guido V. Bloemberg

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Abstract

Pseudomonas putida PCL1445 produces two cyclic lipopeptides, putisolvins I and II, which possess surfactant activity and play an important role in biofilm formation and degradation. In order to identify genes and traits that are involved in the regulation of putisolvin production of PCL1445, a Tn5*luxAB* library was generated and mutants were selected for the lack of biosurfactant production using a drop-collapsing assay. Sequence analysis of the Tn5*luxAB* flanking region of one biosurfactant mutant, strain PCL1627, showed that the transposon had inserted in a *dnaK* homologue which is located downstream of *grpE*, and upstream of *dnaJ*. Analysis of putisolvin production and expression studies indicate that *dnaK*, together with the *dnaJ* and *grpE* heat-shock genes, take part in the positive regulation (directly or indirectly) of putisolvin biosynthesis at the transcriptional level. Growth of PCL1445 at low temperature resulted in an increased level of putisolvins, and mutant analyses showed that this requires *dnaK* and *dnaJ* but not *grpE*. In addition, putisolvin biosynthesis of PCL1445 was found to be dependent on the GacA/GacS two-component signaling system. Expression analysis indicated that *dnaK* is positively regulated by GacA/GacS.

Introduction

Lipopeptides are produced by members of the genera *Bacillus*, *Serratia*, *Burkholderia*, and *Pseudomonas*. Lipopeptides are non-ribosomally synthesized via multifunctional proteins, which are encoded by large gene clusters (Bender *et al.*, 2003; Stachelhaus *et al.*, 1999, von Döhren *et al.*, 1997). Lipopeptides produced by *Pseudomonas* have been reported as agents for biocontrol of phytopathogenic fungi (Nielsen *et al.*, 1999), or as phytotoxins (Hutchinson *et al.*, 1995). Lipopeptides produced by Gram-positive *Bacillus* play a role in bacterial attachment to surfaces (Neu *et al.*, 1996). Lipopeptides produced by *Serratia* (Lindum *et al.*, 1998) and *Burkholderia* (Huber *et al.*, 2002) were shown to be essential for the stimulation of swarming motility and thus could contribute to the regulation of biofilm formation (Huber *et al.*, 2002).

Lipopeptides function as biosurfactants (Desai *et al.*, 1997) by stimulating swarming motility (Lindum *et al*, 1998; Kuiper *et al.*, 2004), facilitating bacterial growth on water-insoluble carbon sources (Ron *et al.*, 2001), or by altering the cell surface hydrophobicity and therefore influencing the interaction between the

individual cells (Ron *et al.*, 2001). However, the significance of lipopeptides for growth and survival of rhizobateria remains unkown. The regulation of lipopeptides in soil *Pseudomonas* is poorly understood. The GacA/GacS two-component regulatory system was shown to control regulation of lipopeptides syringomycin (Hrabak *et al.*, 1992), and lipopeptides of *Pseudomonas* DSS73 (Koch *et al.*, 2002). Whether *gac* system controls directly the lipopeptide biosynthesis remains to be investigated as, to our knowledge, no intermediate involved in this regulation has been identified.

Pseudomonas putida PCL1445 was isolated from soil heavily polluted with polyaromatic hydrocarbons (PAHs) (Kuiper *et al.*, 2001) and produces two surface-active compounds, putisolvin I and putisolvin II, which have been identified as cyclic lipopeptides (Kuiper *et al.*, 2004). They represent a new class of lipodepsipeptides consisting of 12 amino acids linked to a hexanoic lipid chain. Strain PCL1445 produces putisolvins I and II via a putisolvin synthetase (Kuiper *et al.*, 2004), later designated as *psoA*.

Putisolvins I and II have important functions for PCL1445 as they were shown (i) to reduce the surface tension of the medium, (ii) to increase the formation of an emulsion with toluene, (iii) to stimulate swarming motility, (iv) to inhibit biofilm formation, and to degrade existing biofilms (Kuiper *et al.*, 2004).

Putisolvins are not constitutively produced. Surfactant activity appeared in the culture medium at the end of the exponential growth phase (Kuiper *et al.*, 2004). The aim of the present work is to identify and characterize genes that are involved in the regulation of lipopeptide production and to investigate their function. To this end we generated a Tn5*luxAB* library of PCL1445 and screened for mutants defective in biosurfactant production using a drop-collapsing assay. We analyzed one biosurfactant mutant in detail. Its transposon appeared to be integrated in a *dnaK* homolog, encoding a Heat-Shock protein. DnaK, DnaJ and GrpE chaperones have been described to form the central regulatory system of the heat-shock response in *Escherichia coli* (Ellis *et al.*, 1989; Hughes *et al.*, 1998; Paek *et al.*, 1987). In this chapter we describe the analysis of the function of *dnaK*, *dnaJ* and *grpE* in putisolvin biosynthesis, as well as their roles at different temperatures.

Materials and methods

Bacterial strains, and growth conditions

All bacterial strains used are listed in Table 1. *Pseudomonas* strains were grown in King's medium B (King *et al.*, 1954) at 28°C under vigorous shaking (190 rpm). *E. coli* strains were grown in Luria-Bertani medium (Sambrook and Russel, 2001) at 37°C under vigorous shaking. Media were solidified with 1.8 % agar (Select Agar, Invitrogen, Life technologies, Paisley, UK). The antibiotics kanamycin, tetracyclin, gentamycin or carbenicillin was 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., (2001)
PCL1436	Tn5*luxAB* derivative of PCL1445; mutated in *psoA,* a lipopeptide synthetase homologue	Kuiper et al., (2004)
PCL1622	Tn5*luxAB* derivative of PCL1445; mutated in a *gacA* homologue	This study
PCL1623	Tn5*luxAB* derivative of PCL1445; mutated in a *gacS* homologue	This study
PCL1627	Tn5*luxAB* derivative of PCL1445; mutated in a *dnaK* homologue	This study
PCL1628	PCL1445 derivative mutated in the *dnaJ* homologue; constructed by single homologous recombination	This study
PCL1629	PCL1445 derivative mutated in the *grpE* homologue; constructed by single homologous recombination	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)
pMP5505	pRL1063a-based plasmid recovered from chromosomal DNA of PCL1627 after digestion with *Eco*RI with Tn5*luxAB*, Km^r	This study
pME6010	Cloning vector which is maintained in *Pseudomonas* strains without selection pressure, Tc^r	Heeb et al. (2000)
pME3049	Cloning vector, used for homologous recombination, Tc^r, Hg^r	Ditta et al. (1980)
pML103	pML10 derivative *lac*-fusion broad-host-range vector for Gram-negative bacteria, Gm^r	Labes et al. (1990)
pJBA89	pUC18 *Not* – *luxR* – P_{luxI} PBSII – *gfp* (ASV) – To – T1, Ap^r	Andersen et al. (2001)
pBBR1MCS-5	broad-host-range cloning vector for Gram-negative bacteria, Gm^r	Kovach et al. (1995)
pMP4669	pME6010 derivative harboring P_{tac} DsRed, Tc^r	Bloemberg et al. (2000)
pMP6562	pME6010 containing *gacS* gene of PCL1171, used for complementation, Tc^r	van den Broek et al. (2003)
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 with *gacA* gene of PCL1445, Tc^r	This study
pMP5518	pME6010 containing a PCR fragment of 3.5 kb with the *dnaK* and *dnaJ* genes from pMP5505, used for complementation, Tc^r	This study
pMP5519	pMP5518 derivative containing *dnaK* gene and the 5'-366bp of *dnaJ* gene, Tc^r	This study
pMP5530	pMP5518 containing the 3'-520bp of *dnaK* and *dnaJ* gene Tc^r	This study
pMP5534	pME6010 containing a PCR fragment of 1.1 kb with the *grpE* gene from pMP5505, Tc^r	This study
pMP5524	pMP5285 containing a 0.6 kb *EcoR*I-*EcoR*I PCR fragment of the central part of *dnaJ* gene from pMP5505, Km^r	This study

pMP5535	pML103 containing the *dnaK*::*lacZ* promoter in transcriptionally active orientation, Gm^r	This study
pMP5536	pML103 containing the *dnaK*::*lacZ* promoter in transcriptionally inactive orientation, Gm^r	This study
pMP5537	pMP6516 derivative with *phzA* promoter replaced by *psoA* promoter in transcriptionally active orientation, Gm^r	This study
pMP5538	pMP6516 derivative with *phzA* promoter replaced by *psoA* promoter in transcriptionally inactive orientation, Gm^r	This study
pMP5539	pMP5537 derivative harboring *psoA*::*gfp* transcriptionally active fused to pMP4669 harboring P_{tac} DsRed, Gm^r, Tc^r	This study
pMP5540	pMP5538 derivative harboring *psoA*::*gfp* transcriptionally inactive fused to pMP4669 harboring P_{tac} DsRed, Gm^r, Tc^r	This study
pMP7551	pGemT cloning vector containing an amplified cDNA fragment of 0.75 kb with the beginning part of *dnaJ*, Cb^r	This study

Generation, selection and characterization of mutants defective in biosurfactant production

Transposon mutants were generated by tri-parental mating using pRL1063a that harbors a Tn5 transposon carrying the promoterless *luxAB* reporter genes (Wolk *et al.*, 1991), and the helper plasmid pRK2013 (Schnider *et al.*, 1995). Transposants were initially screened for the decreased ability to flatten a droplet of water on parafilm using cells of a single colony as described below. Culture supernatants of the selected mutants, obtained after growth overnight in KB medium, were analyzed for the presence of surfactant production using the drop collapsing assay.

To isolate the DNA region flanking the Tn5, total genomic DNA was isolated and digested with *EcoR*I, which does not cut pRL1063a. Digested genomic DNA fragments were recirculated and selected for kanamycin resistance, resulting in plasmids containing genomic DNA regions flanking the Tn5*luxAB*. All DNA techniques were performed as described by Sambrook and Russel (2001). Sequencing of the plasmids was performed by BaseClear (Leiden, The Netherlands).

DNA sequences were analyzed with the software packages provided by the NCBI (National Center for Biotechnology Information) BLAST network server. Biolog SF-N microplates (Biolog, Hayward, CA) were used according to the protocol provided by the manufacturer. The plates were read after 24 hours of incubation at 28°C using a micro-plate reader model 3550 (Bio-Rad Laboratories, Hercules, CA) at OD_{595nm}.

Construction of dnaJ *and* grpE *mutants*

A *Pseudomonas putida* PCL1445 *dnaJ* mutant was constructed by homologous recombination. A 0.6-kb internal fragment of the *dnaJ*-homologous gene of strain PCL1445 was obtained by PCR using primers oMP862 (5' CAGTTCAAGGAGGCCAACGAG 3') and oMP863 (5' CGGGCCACCATGGGTACC 3'), cloned into the pGEM-T Easy Vector System I (Promega Corporation, Madison, WI, USA) and ligated as a *EcoR*I-*EcoR*I insert with the pMP5285 (Kuiper *et al.*, 2001) suicide plasmid derived from pME3049 (Ditta *et al.*, 1980) resulting in pMP5524. pMP5524 was transferred to *P. putida* PCL1445 by tri-parental mating using pRK2013 as a helper plasmid (Schnider *et al.*, 1995) and using selection on KB agar medium supplemented with kanamycin (50 µg ml^{-1}). Strain PCL1628 was obtained as a resistant colony resulting from single homologous recombination. The insertion of the suicide construct was confirmed by Southern hybridization. A *P. putida* PCL1445 *grpE* mutant was constructed using a similar mutagenesis strategy. The *grpE* fragment for the construction of the suicide plasmid pMP5532 resulted from a PCR reaction using primers oMP874 (5' GAAGAGACTGGTGCAGCAGAT 3') and oMP875 (5' CATTGATCGAAGGCTGAGCGG 3') and chromosomal DNA of strain PCL1445 as a template. Single homologous recombination in *grpE* resulted in strain PCL1629.

Complementation of dnaK, dnaJ*, and* grpE *mutants of PCL1445*

To complement *dnaK*, *dnaJ*, and *grpE* mutants, several plasmids were constructed. pMP5518 containing *dnaK* and *dnaJ*, pMP5519 containing *dnaK,* pMP5530 containing *dnaJ* and pMP5532 containing *grpE*. Complementation of strain PCL1627 (*dnaK*) and mutant PCL1628 (*dnaJ*) was carried out using pMP5518, a shuttle vector derived from pME6010 (Heeb *et al.*, 2000) in which a 3.5-kb fragment containing *dnaK* and *dnaJ* of strain PCL1445 was inserted. This insert was obtained by PCR reaction using primers oMP918 (5' TGCTCAAGGTGTTCCAGAAGG 3') and oMP919 (5' GCGCCCATTACCGCAATA 3').

pMP5518 was transferred to strains PCL1627 and PCL1628 by tri-parental mating as described above and transformants were selected on KB agar medium supplemented with tetracyclin (40 µg ml^{-1}). To complement the *dnaK* insertion in PCL1627 with only *dnaK*, pMP5518 was digested with *Sph*I to create a deletion removing the second part of the *dnaJ* gene resulting in pMP5519. In order to be able to complement the mutation in the *dnaJ* gene of PCL1628 with only *dnaJ*, digestion of pMP5518 with *Sca*I was carried out to delete the first part of the *dnaK* gene, resulting in pMP5530. To complement the mutation in *grpE* of PCL1629, a 1.1-kb PCR fragment containing the *grpE* gene of strain PCL1445 was obtained using primers oMP876 (5' GAGGGCGTCAAGCATGATCGA 3') and oMP877 (5' TGGTCCCCAAGTCGATACCGA 3'), and cloned into pME6010, resulting in pMP5534.

Complementation of gacA *and* gacS *mutants of PCL1445*

Complementation of the *gacA* mutant was carried out, as described in the above section, using pMP5512 derived from plasmid pME6010 in which a 1.3-kb insert containing *gacA* of strain PCL1445 was inserted. This insert was obtained by PCR reaction using primers oMP1047 (5' AGCGGACTACTTGTCGCGTG 3') oMP1048 (5' GCAGTGCTTCGGTTTCATTGG 3'). Complementation of the mutation in *gacS* of PCL1623 was carried out using pMP6562 derived from pME6010 and harboring the functional *gacS* gene of *Pseudomonas* sp. strain PCL1171 (van den Broek *et al.*, 2003).

Rapid Amplification of cDNA Ends (5' RACE)

A 5' RACE system, second generation (Roche Diagnostics GmbH, Penzberg, Germany), was used to determine the length of the *dnaJ* messenger RNA. Briefly, total RNA (1 µg) isolated from log-phase PCL1445 cells by RNeasy silica gel membrane column (Qiagen GmbH, Hilden, Germany) purification was reverse transcribed into cDNA with the 3' *dnaJ* primer oMP899 (5' GGATCTTCAGCTTCACCCGGCCAT 3'). The purified cDNA was subjected to PCR using the *dnaJ* gene-specific nested primer oMP900 (5' TGTAGCTGATCGGCACTTCGCAGTA 3') and the 5' RACE anchor primer containing 3' sequence complementary to the homopolymeric poly (dC) tail. The resulting PCR product was reamplified using primer oMP901 (5' AGATCTCGTGCTCACGCACGTTGAT 3') and the 5' RACE primer complementary to

the homopolymeric poly(dC) tail. The length of the product was estimated by gel electrophoresis.

Biosurfactant production

The production of biosurfactant activity was detected using the drop collapsing assay as described previously (Jain *et al.*, 1991), in which the reduction of the water surface tension can be observed as the collapse of a round droplet placed on a hydrophobic surface (Jain *et al.*, 1991).

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) (Kuiper *et al.*, 2004).

Extraction and High-Performance Liquid Chromatography (HPLC) analysis of putisolvins

To quantify the production of putisolvins in KB 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 and dissolved in 55 % acetonitrile (Labscan Ltd., Dublin, Ireland). Dry material obtained from 5 ml culture was resuspended in 500 µl of 50/50 acetonitrile/water (v/v) and purified on a spinX centrifuge tube filter of 0.45 µm pore size (Corning Costar Corporation, Cambridge, MA). 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. Fractions that corresponded to the retention time of 34 min for putisolvin I and 36 min for 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 the wavelength of 206 nm.

Construction of psoA*::*gfp *transcriptional fusions*

A 1.2-kb *Hind*III fragment containing the *luxI* promoter and the gene encoding green fluorescent protein *gfp* from pJBA89 (Andersen *et al.*, 2001) was cloned into the broad host range vector pBBR1MCS-5 (Kovach *et al.*, 1995), resulting in pMP4670. Subsequently the *Sph*I fragment containing *lac*, *luxR* and *luxI* promoters was removed, resulting in pMP4683. Removal of one *Hind*III site at the end of the *gfp* gene in pMP4683 resulted in pMP4689. The N-terminal ASV tag from pMP4689 was removed using *Stu*I and *Sma*I digestion followed by religation, which resulted in pMP6516. To construct a *psoA*::*gfp* transcriptional fusion, a 0.75-kb PCR fragment containing the *psoA* promoter of strain PCL1445 was obtained using primers oMP907 (5' GCATGCAAGCGATGAAAGCAGATGACCCAG 3') and oMP908 (5' GCATGCGTCGGCAGGTCCTTCTGATTGATC 3') in which *Sph*I sites were incorporated (see underlined nucleotides). The *psoA* promoter was cloned into pMP6516 as a *Sph*I fragment resulting in pMP5537, containing *psoA*::*gfp* in the transcriptionally active orientation and into pMP5538, containing *psoA*::*gfp* in the transcriptionally inactive orientation, by cloning the fragment in the reverse orientation. The constructs pMP5537 and pMP5538 were fused as *BamH*I fragments to *Bgl*II digested pMP4669 harbouring P_{tac}DsRed resulting in rhizosphere stable plasmids pMP5539 and pMP5540, respectively. The constructs were transferred to PCL1445 and PCL1627 by tri-parental mating as described previously and transformants were selected with gentamycin (2 µg ml^{-1}) and tetracyclin (40 µg ml^{-1}). Expression of *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 620nm 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.

Construction of dnaK*::*lacZ *transcriptional fusions*

Plasmid pML103 (Labes *et al.*, 1990), which contains a promoterless *lacZ* gene downstream of a multicloning site, was used to create a *dnaK*::*lacZ* transcriptional fusion. The region upstream of *dnaK* was amplified from PCL1445 by PCR using primer oMP870 (5' TCAAGCGCTACAACCTCGAGG 3') and primer oMP871 (5' GCATGCCATGTTAACTCTCCCGAAAC 3') in which *Sph*I sites were incorporated (see underlined nucleotides). The 0.35-kb PCR product was cloned as a *Sph*I

fragment into pML103, resulting in pMP5535 containing *dnaK*::*lacZ* in the transcriptionally active orientation and pMP5536 containing *dnaK*::*lacZ* in the transcriptionally inactive orientation (reverse orientation of the fragment). Plasmids pMP5535 and pMP5536 were transformed into PCL1445 and its derivatives PCL1622 and PCL1623 by tri-parental mating. Transformants were selected on KB agar medium supplemented with gentamycin (2 μg ml^{-1}) and X-Gal (40 μg ml^{-1}) (Ophaero Q, Biosolve B.V., The Netherlands). The activity of *dnaK* transcriptional fusions was assayed by determining β-galactosidase activity (expressed in Miller Units). Aliquots (200 μl) were removed from cultures diluted to OD_{620nm} 0.6 and analyzed for β-galactosidase activity by a standard method (Miller *et al.*, 1972).

Nucleotide sequence accession number

The nucleotide sequences of the *P. putida* PCL1445 *grpE-dnaK-dnaJ* DNA region reported in this paper have been deposited in the GenBank database under accession number AY823737. The nucleotide sequences of the *P. putida* PCL1445 *gacS* and *gacA* DNA regions have been deposited in the GenBank database under accession numbers AY920315 and AY920316 respectively.

Results

Isolation and characterization of the biosurfactant mutant PCL1627

In order to identify genes involved in putisolvin biosynthesis of *P. putida* PCL1445, two thousand Tn5*luxAB* transposants were screened for loss of surfactant activity as judged by the drop-collapsing assay, using cells derived from a single colony. Strain PCL1627 was isolated together with two other mutants PCL1622 and PCL1623. After overnight growth in liquid KB, medium supernatant of strain PCL1627 was not able to decrease the surface tension between culture medium and air (54 mN m^{-1}) when compared to PCL1445 (32 mN m^{-1}).

Sequence analysis of the chromosomal regions flanking the Tn5*luxAB* showed that the transposon is inserted in an open reading frame (ORF) with 93% similarity at the amino acid level with the *dnaK* gene of *P.putida* KT2440 and 85% with the *dnaK* gene of *P.aeruginosa* PAO1 (Fig. 1A). *dnaK* codes for a molecular chaperone belonging to the Hsp70 protein family, which is part of the heat shock response system (Hughes *et al.*, 1998; Keith *et al.*, 1999; Strauss *et al.*, 1990). In *P. putida* PCL1445 a sequence similar to those of σ^{32}-dependent promoters was

identified 78 bp upstream of the *dnaK* translational start (Figure 1B). The *dnaK* promoter recognized by σ^{32} is located 121 bp upstream of the *dnaK* translational start in *E. coli* (Cowing *et al.*, 1985) and 86 bp upstream of the *dnaK* translational start in *P. syringae* pv. *glycinea* PG4180 (Keith *et al.*, 1999). The *E. coli* σ^{32} consensus sequence is TCTC-CCCTTGAA (-35) and CCCCAT-TA (-10). In *E. coli*, these two regions are separated by 13 to 17 bp. In *P.syringae* pv. *glycinea* and in *P. putida* PCL1445 the two putative -35 and -10 regions are separated by 14 bp (Table 2).

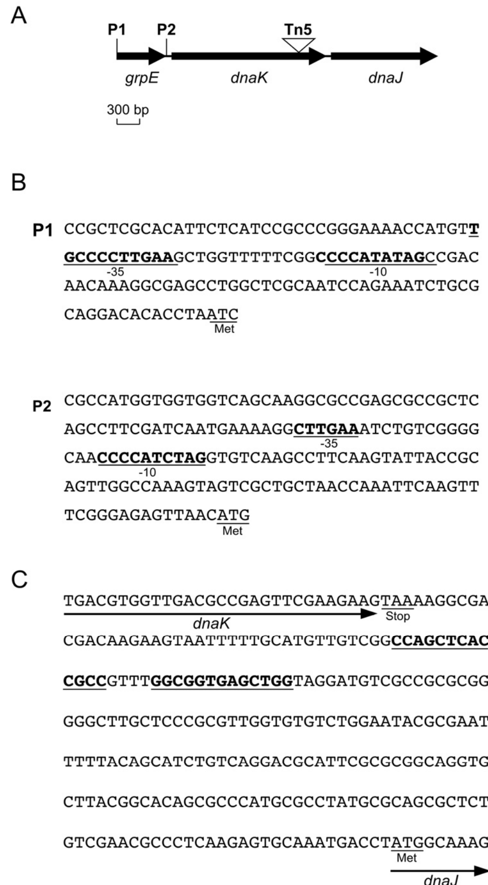

Fig. 1. Panel A. Schematic representation of the *grpE-dnaK-dnaJ* chromosomal region of *Pseudomonas putida* PCL1445 showing the location of the transposon insertion in *dnaK* of mutant strain PCL1627. **Panel B.** Sequence of the 5' upstream region of *grpE* and the adjacent *dnaK* gene. Features of the putative promoters P1 and P2 are indicated. **Panel C.** Sequence of the *dnaK-dnaJ* intergenic region. Features of the putative terminator stem loop are indicated (dG = - 21 Kcal mol^{-1}). Nucleotides forming the stem are indicated bold and underline.

Downstream of *dnaK* a DNA sequence is present that contains complementary nucleotides, which can form a hairpin structure with 13 bp in the stem and 3 bp in the loop, resulting in a hairpin with dG of - 21 kcal mol^{-1} (Fig. 1C).

No consensus terminator sequence was found. Further downstream of *dnaK* the presence of an ORF was found (Fig. 1A) with an amino acid homology of 95 % with the *dnaJ* gene product of *P. putida* KT2440 and 85 % with *dnaJ* of *P. aeruginosa* PAO1, which encodes another molecular chaperone (Hughes *et al.*, 1998). The region upstream of *dnaK* revealed an ORF that showed 85 % homology with *grpE* of *P. putida* and 73 % with *grpE* of *P. aeruginosa* PAO1 at the amino acid level (Hughes *et al.*, 1998). Upstream of this *grpE* homologue, a similar conserved nucleotide sequence as in the promoter region of the *dnaK* homologue, corresponding to the binding site for σ^{32} sub-unit, was found. This suggests that *grpE* is also heat-shock regulated in PCL1445 (Table 2). Comparison of the order of these genes in strain PCL1445 with those of *P. aeruginosa* (gene bank website: www.pseudomonas.bit.uq.edu.au), *P. syringae* pv. *tomato* DC3000, and *P. putida* KT2440 showed the same gene arrangement. Two results suggest that *dnaK* and *dnaJ* are not co-transcribed in PCL1445. Firstly, a putative terminator stem loop was identified in the region upstream of *dnaJ* (Fig. 1C). Secondly, the intergenic region between *dnaK* and *dnaJ* (213 bp) was found to be longer than in other *Pseudomonas* sp. (varying between 115 bp in *P. aeruginosa* PAO1 and 198 bp in *P. putida* KT2440). However, no typical heat-shock promoter consensus was found in front of the *dnaJ* gene.

dnaJ-containing mRNA was amplified by PCR using a 3'-*dnaJ* specific primer, which resulted in a 750-bp *dnaJ*-containing PCR product (data not shown). Thus, this indicates that *dnaJ* is transcribed as a single gene in PCL1445.

Table 2. Comparison of the putative *P. putida* PCL1445 *grpE* and *dnaK* heat-shock promoter sequences with promoters from *E. coli*, *P. syringae* pv. *glycinea*, and *C. crescentus*.

Promoter	- 35 region	Spacing (bp)	- 10 region
E. coli σ^{32} consensus	TCTC-CCCTTGAA	13-15	CCCCATTTA
P. syringae pv. *glycinea dnaK*	GAGCAGGCTTGAA	13	CCCCATTTA
Caulobacter crescentus dnaK P1	TTATGGCCTTGCG	13	CCCCATATC
P. putida PCL1445			
grpE (P1)	TGCCCCTTGAA	14	CCCCATATA
dnaK (P2)	AAAGGCTTGAA	14	CCCCATCTA

*Temperature tolerance of PCL1627 (*dnaK*), PCL1628 (*dnaJ*), and PCL1629 (*grpE*)*

To test the tolerance to a temperature shift from low to high incubation temperature of cells from strains PCL1627 (*dnaK*), PCL1628 (*dnaJ*), PCL1629 (*grpE*), and its wild type PCL1445, the cells were precultured overnight in KB medium at 18ºC under vigorous aeration. These cells were subsequently diluted to OD_{620nm} 0.1 in fresh KB medium and incubated at 28ºC (the optimal growth temperature for *Pseudomonas*) (Fig. 2A) or at 35ºC to follow cell growth in time (Fig. 2B). A temperature shift from 18ºC to 28ºC did not affect the growth rate of PCL1627 (*dnaK*), PCL1628 (*dnaJ*), and PCL1629 (*grpE*) as compared with the wild type (Fig. 2A). However, when the incubation temperature was shifted to 35ºC, mutants PCL1627 (*dnaK*), PCL1628 (*dnaJ*), and PCL1629 (*grpE*) had a higher generation time (110.3 ± 1.8 min) than PCL1445 (74.6 ± 1.4 min) and the optical density of PCL1627 (*dnaK*), PCL1628 (*dnaJ*), and PCL1629 (*grpE*) never reached the same value as that of the wild type (Fig. 2B). The determination of the number of C.F.U. (Colony Forming Unit) during growth at 35ºC for PCL1627 (*dnaK*) (Fig. 2C) strongly correlated with the cell density at 35ºC (Fig. 2B). The growth phenotype of PCL1627 (*dnaK*) could be restored only by introduction of a plasmid carrying functional *dnaK* and *dnaJ* but not with *dnaK* alone (Fig. 2C). This result suggests that at high incubation temperatures, *dnaK* and *dnaJ* regulation depends on a single heat-shock promoter.

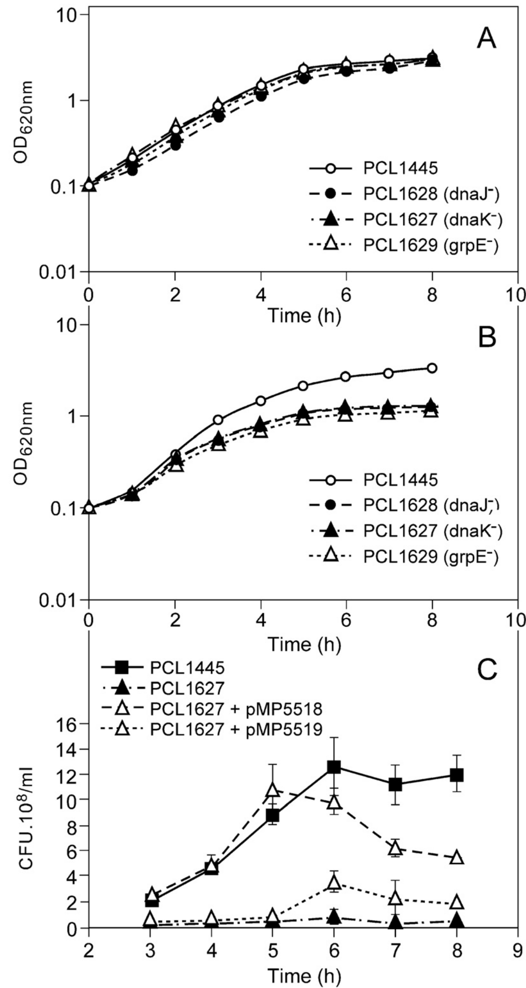

Fig. 2. Growth of *P. putida* PCL1445 and its mutants PCL1436 (*psoA*), PCL1627 (*dnaK*), PCL1628 (*dnaJ*), and PCL1629 (*grpE*). Cells were precultured overnight at 18°C in KB medium, adjusted to OD_{620nm} 0.1, and then grown at 28°C (**Panel A**) or 35 °C (**Panel B**) with vigorous aeration (190 rpm). **Panel C** represent the number of C.F.U.ml^{-1} during growth at 35°C of PCL1627 (*dnaK*), PCL1627 (*dnaK*) harboring pMP5518 containing dnaK followed by *dnaJ*, and PCL1627 (*dnaK*) harboring pMP5519 containing *dnaK* followed by part of *dnaJ.* Samples were taken at regular time points to determine the optical density or C.F.U. ml^{-1}. Standard deviations are based on the mean values of two parallel cultures.

Construction of a dnaJ *mutant and a* grpE *mutant, and complementation analyses of mutants for the production of lipopeptides*

To investigate whether *dnaJ* and *grpE* (Fig. 3E and 3G) are also involved in putisolvin production, insertion mutants were constructed by single homologous recombination using suicide plasmids pMP5524 and pMP5532 (see Material & Methods section), resulting in PCL1628 and PCL1629, respectively. The integration of pMP5524 and pMP5532 was confirmed by Southern hybridization (data not shown).

Biosurfactant production of PCL1628 (*dnaJ*) and PCL1629 (*grpE*) grown in KB medium under standard conditions until the stationary phase was reached (28°C and vigorous aeration) was quantified by the Du Nouy ring method. Culture supernatant of PCL1628 (*dnaJ*) was not able to decrease the surface tension between culture medium and air (54 mN m^{-1}), indicating a lack of biosurfactant production. Culture supernatant of PCL1629 (*grpE*) caused a slight decrease of the surface tension (48 mN m^{-1}). In comparison the surface tension of PCL1445 was decreased to the value of 32 mN m^{-1}.

Complementation analyses were conducted using the constructs pMP5519 (*dnaK*), pMP5530 (*dnaJ*) and pMP5534 (*grpE*). The production of putisolvins by PCL1445, PCL1627 (*dnaK*), PCL1628 (*dnaJ*), and PCL1629 (*grpE*) was tested by HPLC analysis (Fig. 3). Putisolvins were extracted from overnight KB culture supernatant and production was quantified by determination of the area of the peaks with surfactant activity as tested by the drop collapsing assay. Putisolvins I and II were eluted at 34 and 36 minutes, respectively (Fig. 3). Mutant PCL1627 (*dnaK*) showed a significant reduction (90%) of putisolvin production (Fig. 3). Introduction of pMP5519, harbouring a functional *dnaK,* restored putisolvin production in PCL1627 (*dnaK*) (Fig. 3D). This result shows that the insertion of the transposon in *dnaK* is responsible for the decrease of lipopeptide production and that this decrease is not due to a downstream effect on *dnaJ* (Fig. 3). Production of putisolvins by mutant PCL1628 (*dnaJ*) was almost completely abolished (Fig. 3E) while mutant PCL1629 (*grpE*) showed a 50% reduction of putisolvin production (Fig. 3G). Introduction of pMP5530 containing the 3'-*dnaK* end and *dnaJ* into PCL1628 (*dnaJ*) strain restored biosurfactant activity and putisolvin production (Fig. 3F).

Finally, introduction of pMP5534 carrying *grpE* into PCL1629 (*grpE*) complemented for the reduced putisolvin production (Fig. 3H).

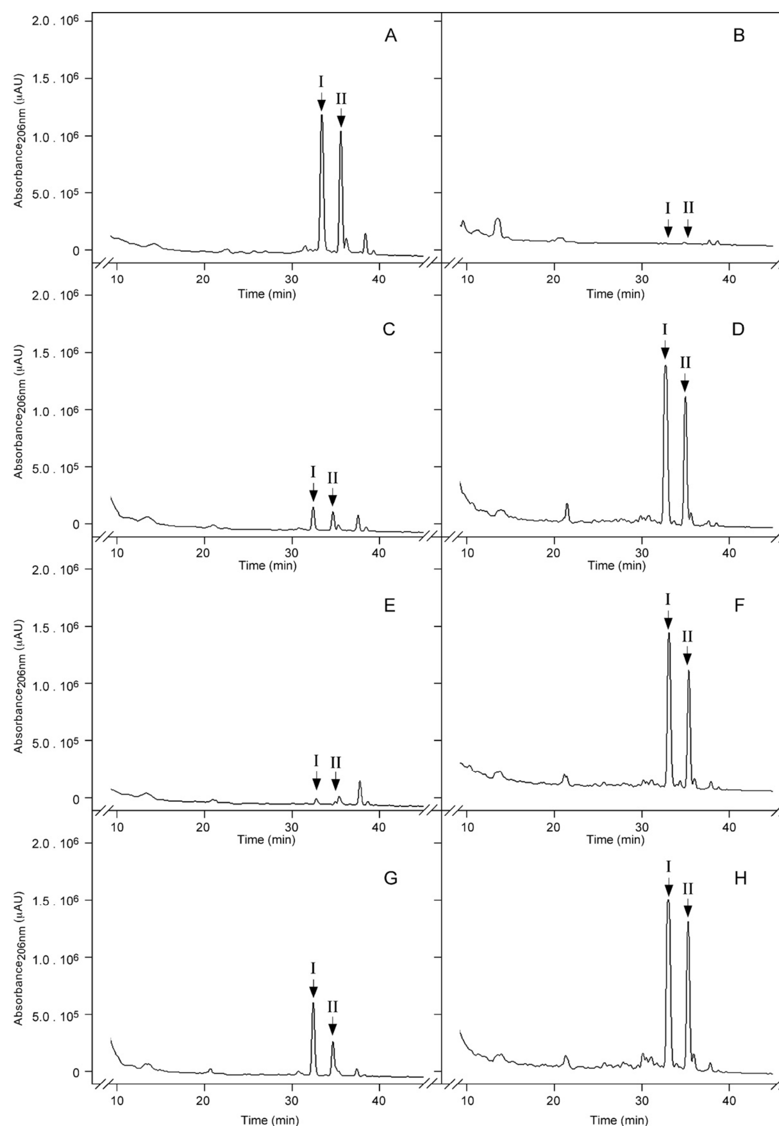

Fig. 3. C8-Reverse Phase HPLC analysis of putisolvin production by *P. putida* PCL1445 and its mutants, PCL1436 (*psoA*), PCL1627 (*dnaK*), PCL1628 (*dnaJ*), and PCL1629 (*grpE*). The panels depict: PCL1445 (**A**), PCL1436 (**B**), PCL1627 (**C**), PCL1628 (**E**), PCL1629 (**G**), PCL1627 harbouring pMP5519 containing *dnaK* of PCL1445 (**D**), PCL1628 harbouring pMP5530 containing the last part of *dnaK* followed by *dnaJ* of PCL1445 (**F**), and PCL1629 harbouring pMP5534 containing *grpE* of PCL1445 (**H**). Cells were grown to the stationary phase in 5 ml KB medium at 28ºC under vigorous aeration. Compounds from the ethyl acetate extracted culture supernatant were separated and analyzed at a wavelength of 206 nm. HPLC fractions of 1 ml were collected and tested for surfactant activity using the drop collapsing assay.

5' RACE of dnaJ

Reverse transcription was used to test whether *dnaJ* is expressed as a single transcript in PCL1445 at 28°C. One primer for the reverse transcriptase reaction and two different nested primers for the PCR specific to the 3'-*dnaJ* gene were chosen to test if *dnaJ* is transcribed continuously (see Materials and Methods). To exclude contamination of genomic DNA in the RT-reaction or the following PCR, the 3'-*dnaJ* specific primers were used in a negative control reaction with RNA after DNase digestion (Fig. 4; lane 2). The PCR gave a product of 800 bp which sequence was homologous to the *dnaJ* gene (Fig. 4; lane 1). This result supports complementation analysis of *dnaK* and *dnaJ* mutations indicating that *dnaK* and *dnaJ* are independently transcribed at 28°C.

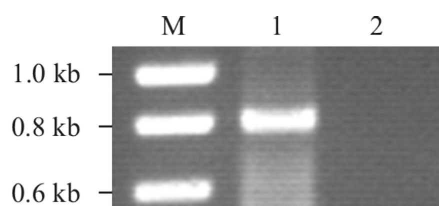

Fig. 4. Determination of the length of the *dnaJ* messenger RNA by gel electrophoresis. **M:** marker Smart Ladder. **Lane 1:** *dnaJ* cDNA end amplified by PCR. **Lane 2:** messenger RNA of PCL1445 amplified by PCR.

Effect of temperature on production of putisolvins I and II

Low incubation temperature had hardly any effect on the growth of the three mutants (data not shown). The effect of temperature (32°C, 28°C, 21°C, 16°C, and 11°C) on the production of putisolvins was analyzed in stationary phase liquid cultures of PCL1445, PCL1627 (*dnaK*), PCL1628 (*dnaJ*), and PCL1629 (*grpE*) (Fig. 5). HPLC analysis showed that the level of putisolvin production decreases with increasing growth temperature. Moreover, a mutation in *dnaK* (PCL1627) decreased putisolvin production at 21°C and 16°C and practically abolished putisolvin production at higher and lower temperatures (Fig. 5A). Production of putisolvins by mutant PCL1627 (*dnaK*) at low and high temperatures could be restored by introduction of pMP5519 carrying the functional *dnaK* gene. Analysis of the *dnaJ* mutant for the production of putisolvins at the same range of temperatures showed that DnaJ has a similar effect as DnaK (Fig. 5B). The mutation in *dnaJ* was complemented for the production of putisolvins using pMP5530 carrying 3'-520 bp of *dnaK* and *dnaJ* gene. Although a mutation in *grpE* had a significant effect on putisolvin production at temperatures higher than 21°C, the level of putisolvins was

comparable to that of PCL1445 at lower temperatures (Fig. 5C). Thus, this results show that i) putisolvin production is up-regulated at low temperatures and that ii) DnaK and DnaJ are required for the production of putisolvins at low temperatures.

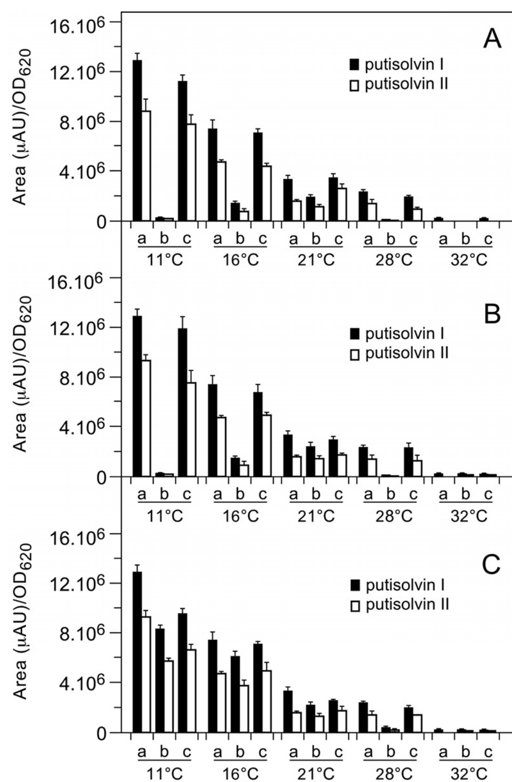

Fig. 5. Effect of growth temperature on the production of putisolvins I and II by *Pseudomonas putida* PCL1445 and its mutant derivatives. PCL1445, PCL1627 (*dnaK*), PCL1627 (*dnaK*) with pMP5530 (*dnaK*), PCL1628 (*dnaJ*), PCL1628 (*dnaJ*) with pMP5530 (*dnaJ*), PCL1629 (*grpE*), PCL1629 (*grpE*) with pMP5532 (*grpE*) were cultured at 11, 16, 21, 28, and 32ºC to stationary phase. The production of putisolvins was quantified by C8-Reverse Phase HPLC analysis. Values depicted represent the area of the peaks over cell density ($\mu AU/OD_{620nm}$). **Panel A:** PCL1445 (column a), PCL1627 (*dnaK*) (column b), and PCL1627 (*dnaK*) complemented using pMP5519 (*dnaK*) (column c). **Panel B:** PCL1445 (column a), PCL1628 (*dnaJ*) (column b), and PCL1628 (*dnaJ*) complemented using pMP5530 (*dnaJ*) (column c). **Panel C:** PCL1445 (column a), PCL1629 (*grpE*) (column b) and PCL1629 (*grpE*) complemented using pMP5532 (*grpE*) (column c).

Effect of a dnaK, dnaJ*, or* grpE *mutation on* psoA *expression*

To analyze a possible effect of DnaK on the expression of *psoA*, a *psoA*::*gfp* transcriptional fusion was constructed. Transcriptional activity of the putisolvin promoter was analyzed in strains PCL1445, PCL1627 (*dnaK*), PCL1628 (*dnaJ*), and PCL1629 (*grpE*) in liquid culture at different temperatures (28ºC, 21ºC, 16ºC, and 11ºC) (Fig. 6). Our data showed a strong correlation between *psoA*::*gfp* transcriptional activity and the production of putisolvins in culture medium. The absence of transcriptional activity in PCL1627 (*dnaK*) and PCL1628 (*dnaJ*) at 28ºC, and 11ºC and its reduction at 16ºC indicates that DnaK and DnaJ regulate (directly or indirectly) putisolvin synthesis at the transcriptional level. Furthermore, a mutation in *grpE* had hardly any effect on the expression of *psoA*::*gfp* at 11ºC (Fig. 6). This result, which supports HPLC analysis (Fig. 5), shows that *grpE* does not take part in the regulation of putisolvins at low temperature.

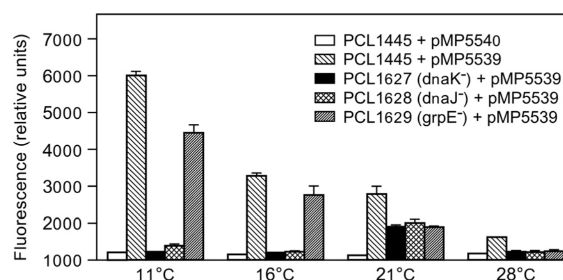

Fig. 6. Expression of *psoA* in *Pseudomonas putida* PCL1445, PCL1627 (*dnaK*), PCL1628 (*dnaJ*), and PCL1629 (*grpE*). Expression was determined by measuring fluorescence from cells containing the putisolvin synthetase promoter fused to *egfp* (pMP5539). Plasmid pMP5540 in which the *psoA* promoter was cloned in the reverse orientation was used as a control vector. Strains were grown at 11, 16, 21, and 28 ºC in KB medium. Mean values of duplicate cultures are given.

Isolation and characterization of biosurfactants mutants PCL1622 and PCL1623

In the screening of 2,000 Tn5*luxAB* PCL1445 transconjugants on agar plate and in liquid cultures, three mutants altered in putisolvin production as judged by their non-surface active ability were identified, of which the transposon was not inserted in a non-ribosomal peptide synthetase gene. One of these mutants was described to have a transposon insertion in a *dnaK* gene. The region flanking the

Tn5 insertion in the two remaining mutants PCL1622 and PCL1623 were recovered in the plasmids pMP5501 and pMP5502, respectively. Nucleotide sequence analyses of the flanking regions revealed that the transposons were inserted in homologues of the genes *gacA* and *gacS*, respectively.

Ethyl acetate extracts of the culture supernatants of strains PCL1622 (*gacA*::Tn5*luxAB*) and PCL1623 (*gacS*::Tn5*luxAB*) did not contain putisolvins I and II when tested by HPLC (data not shown).

The part of the nucleotide sequence flanking the transposon in strain PCL1622 had 93 % identity at amino acid level with the sequence of the *gacA* gene of *P. fluorescens* (Zhang *et al.*, 2001). The nucleotide sequence flanking the transposon in strain PCL1623 showed 57 % identity with the sequence of the *gacS* (formerly known as *lemA*) gene of *P. fluorescens* (Whistler *et al.*, 1998) and 58 % identity with the *gacS* gene of *P. chlororaphis* (Pierson *et al.*, 2001).

The *gacA* and *gacS* mutants were complemented for production of putisolvins I and II after introduction of pMP5512 harbouring the *gacA* gene of strain PCL1445 and pMP6562 harbouring the *gacS* gene of *Pseudomonas* sp. strain PCL1171 (van den Broek *et al.*, 2003), respectively (data not shown).

Effect of a gacA *or* gacS *mutation on* dnaK *expression*

Strains PCL1622 (*gacA*) and PCL1623 (*gacS*) did not produce detectable amounts of putisolvins (data not shown). Mutations in *gacA* and *gacS* genes could be complemented for the production of putisolvins using plasmids pMP5512 (*gacA*) and pMP6562 (*gacS*), respectively (data not shown).

Since putisolvin production is induced during late exponential phase and since DnaK appears to regulate transcriptional activity of putisolvin synthetase gene promoter (Fig. 5), we tested whether *dnaK* is regulated by the GacA/GacS two-component system. To test this hypothesis, a *dnaK*::*lacZ* transcriptional fusion was constructed and its expression was analyzed in both PCL1622 (*gacA*) and PCL1623 (*gacS*) (Table 3). Strains PCL1445, PCL1622 (*gacA*), and PCL1623 (*gacS*) harbouring *dnaK*::*lacZ* were cultured at 28°C in liquid KB medium to stationary phase. The expression of β-galactosidase activity was reduced in the *gac* mutants PCL1622 (*gacA*) and PCL1623 (*gacS*) and was restored to wild type levels in the complemented derivatives (Table 3). This result shows that the GacA/GacS two component regulatory system has a positive effect on DnaK in PCL1445.

Table 3. Expression of the *dnaK* promoter fused to *lacZ* in *P. putida* PCL1445, PCL1622 (*gacA*) and PCL1623 (*gacS*).

Strain[a]	**Relative β-galactosidase activity (Miller Units)**
Wild type strain	
PCL1445 (empty vector)	9.68 ± 0.93
PCL1445-pMP5536 (P_{dnaK} *lacZ* ⁻)[b]	24.71 ± 2.83
PCL1445-pMP5535 (P_{dnaK} *lacZ* ⁺)[c]	75.43 ± 1.36
PCL1622 (*gacA*)	
PCL1622-pMP5535 (P_{dnaK} *lacZ* ⁺)	32.18 ± 1.79
PCL1622-*gacA* ⁺-pMP5535 (P_{dnaK} *lacZ* ⁺)	70.80 ± 3.11
PCL1623 (*gacS*)	
PCL1623-pMP5535 (P_{dnaK} *lacZ* ⁺)	30.94 ± 2.44
PCL1623-*gacS* ⁺-pMP5535 (P_{dnaK} *lacZ* ⁺)	69.55 ± 3.46

[a] pML103 (empty reporter vector) and pMP5536 containing the *dnaK* promoter cloned in the reverse orientation were used as control vectors. Cells were grown in KB medium to stationary phase under the normal growth condition (28^{0}C).

[b] *lacZ* mutant, *lacZ* reporter gene in transcriptionally active orientation.

[C] *lacZ*⁺, *lacZ* reporter gene in transcriptionally active orientation.

Discussion

The aim of this work was to identify and characterize genes involved in regulating the production of the cyclic lipopeptides putisolvins I and II by *Pseudomonas putida* PCL1445. Putisolvins are biosurfactants that are required for swarming motility and are able to inhibit biofilm formation and degrade existing biofilms (Kuiper *et al.*, 2003). We have screened a Tn5 library for mutants defective in lipopeptides production. One of the mutants, PCL1627, carried the transposon in a *dnaK* homolog. DnaK is a member of the Hsp70 heat-shock protein family. In *E.coli*, the *rpoH* gene product, σ^{32}, positively regulates heat shock genes by directing the core RNA polymerase to the *dnaK* promoter (Cowing *et al.*, 1985, Hughes *et al.*, 1998). A sequence similar to the *E.coli* consensus σ^{32}-dependent promoters was identified in the *dnaK* promoter region of *P. putida* PCL1445 indicating that *dnaK* is also regulated by σ^{32} in PCL1445.

Sequencing of the region downstream of *dnaK* revealed *dnaJ*, an organization that is conserved in *Staphylococcus aureus* (Ohta *et al.*, 1994), *Xanthomonas campestris* (Weng *et al.*, 2001), *Neisseria gonorrhoeae* (Laskos *et al.,*

2004), or *Clostridium acetobutylicum* (Naberhaus *et al.*, 1992). In many organisms, *dnaK* and *dnaJ* are organized as an operon and the gene products are part of an equimolar protein complex which is formed with the co-chaperone GrpE. Sequencing of the region upstream of *dnaK* localized a *grpE* homolog (Fig. 1A).

Deletion of *grpE* in *E. coli* (Ang *et al.,* 1989) and *dnaK* or *grpE* in *P. syringae* pv. *glycinea* (Keith *et al.*, 1999) results in a loss of viability due to a severely compromised physiological function. In contrast, our results show that mutation in any of these three genes does not affect growth of PCL1445 at 28°C (Fig. 2A). Southern blot analysis of PCL1445 wild type and its *dnaK* mutant, using a *dnaK* probe did not indicate the presence of a second *dnaK* homolog in the genome (data not shown). This suggests that under the used growth conditions at 28°C *dnaK* is not important or that the loss of *dnaK* can be compensated by the production of other heat-shock proteins such as GroEL-GroES (Muffler *et al.*, 1997). Growth of *dnaK*, *dnaJ*, and *grpE* mutants is reduced at 35°C (Fig. 2B) indicating that functioning of *dnaK*, *dnaJ*, and *grpE* becomes important for PCL1445 at high temperature and is (at least) not completly compensated by the production of other chaperones. Furthermore, the growth deficiency of a *dnaK* mutant can be restored only by introduction of both *dnaK* and *dnaJ* and not with *dnaK* alone indicating that *dnaK* and *dnaJ* are co-regulated at high temperature (Fig. 2C). The results on the growth of *dnaK*, *dnaJ*, and *grpE* mutants demonstrate that DnaK, DnaJ and GrpE are not essential for growth of PCL1445.

Mutants of *dnaK*, *dnaJ*, or *grpE* were analyzed for putisolvin production to assess the significance of the three heat-shock genes for putisolvin production. Putisolvin production was almost eliminated in *dnaK* and *dnaJ* mutant strains while production was decreased in a *grpE* mutant (Fig. 3). This implicates that DnaK-DnaJ-GrpE act as a complex in the regulation of putisolvin production. In addition, expression analysis of the putisolvin synthetase gene *psoA*, tested by a *psoA*::*gfp* transcriptional fusion in the wild type and in *dnaK*, *dnaJ* and *grpE* mutants, showed that transcriptional activity (Fig. 6) correlated with putisolvin production as determined by HPLC (Fig. 5). Finally, we also showed that the GacA/GacS two-component regulatory system is important for putisolvin production and interestingly that expression of *dnaK* was also regulated by the *gac* system (Table 3). This provides genetic evidence that DnaK could play a role in temperature sensing via GacA/GacS two-component regulatory system in PCL1445.

We do not know yet how the DnaK, DnaJ, and GrpE complex is involved in transcription of *psoA* and if GacA/GacS directly or indirectly regulates *dnaK*.

However, the heat-shock response does not seem to take part in this regulation. Although complementation of phenotypic growth at high temperature (35°C) shows that *dnaK-dnaJ* may function as an operon (Fig. 2C), two results i) complementation of *dnaK* and *dnaJ* mutations for the production of putisolvins at 28°C (Fig. 3) ii) and transcriptional analysis using 5' RACE (Fig. 4), strongly suggest that *dnaK* and *dnaJ* are transcribed separately at lower temperatures. This is in accordance with two previous studies, which showed that in *Pseudomonas syringae* pv. *glycinea dnaK* and *dnaJ* are not organized as an operon (Keith *et al.*, 1999) and in *Neisseria gonorrhoeae* a promoter is present in front of *dnaJ* (Laskos *et al.*, 2004).

A number of possible mechanisms involving DnaK complex in the regulation of putisolvins can be predicted. DnaK DnaJ and GrpE may be required for the proper folding or activity of an unknown positive regulator of *psoA*. One particularly appealing possibility is that GacA/GacS positively regulate *psoA*. In that case, DnaK, DnaJ, and or GrpE may regulate proper folding of some known sRNA mediators regulated by *gac* system such as RsmZ and RsmY, and which have been shown to control biosynthesis of antibiotics of *P. fluorescens* (Haas *et al.*, 2003). Another possible target for the DnaK complex is σ^{S}, which is encoded by *rpoS* and which plays a crucial role in gene regulation during entry into stationary phase and was suggested to be regulated by DnaK in previous study (Hengge-Aronis, 2002; Muffler *et al.*, 1997; Rockabrand *et al.*, 1998). Alternatively, DnaK-DnaJ-GrpE may be required for the proper assembly of the large lipopeptide synthase complex. Finally, the effect on lipopeptide synthesis may be an indirect consequence of other cellular changes in *dnaK*, *dnaJ*, and *grpE* mutant strains.

In this chapter we have demonstrated that the synthesis of the surfactants putisolvins at low temperatures requires DnaK chaperone complex in *P. putida* (Fig. 5) and that consequently the putisolvin synthetase gene *psoA* is up-regulated (Fig. 6).

It is still unknown how the DnaK chaperone complex controls transcription of the *psoA* gene at low temperatures. However, GrpE does not take part in the regulation indicating that the functioning of the DnaK complex differs at 11°C and at 28°C. Performance of *dnaK*::*lacZ* expression analysis in PCL1445 indicated in accordance with a study in *E. coli* (Zhou *et al.*, 1988) that *dnaK* expression decreases gradually at lower temperatures, with respective values of 67.38 ± 1.4 Miller Units at 28°C, 28.89 ± 0.48 Miller Units at 21°C, 7.421 ± 0.50 Miller Units at 16°C, and 2.88 ± 0.08 Miller Units at 11°C. Although the expression of *dnaK*

decreases at lower temperatures, the presence of a functional DnaK is required since mutation results in loss of putisolvin production. This hypothesis is supported by the results in *E. coli* indicating that DnaK is not only involved in the regulation of heat-shock response, but could also take part in the regulation of environmental stress response such as temperature and stationary phase (Hengge-Aronis, 2002; Rockabrand *et al.*, 1995; Rockabrand *et al.*, 1998).

Temperature as well as heat-shock proteins have been reported to play an important role in the modulation of virulence in phytopathogenic bacteria, for example for tumor induction by *Agrobacterium tumefaciens* (Braun *et al.*, 1947) and for phytotoxin production by *P. syringae* pv. *glycinea* (Keith *et al.*, 1999). Low temperature restricts growth of *P. putida* PCL1445 and positively regulates putisolvin production during late exponential phase via DnaK stress response system. Low temperature could constitute a challenge for the dissemination of *Pseudomonas putida* due to for instance a reduction of metabolic functions, a reduction of nutrients availability such as root exudates or intermediates of polyaromatic hydrocarbons degradation process (Kuiper *et al.*, 2001). Production of biosurfactants could confer an ecological advantage for bacteria at low temperature. Their specific activity could be involved in important functions such as i) creating a protective micro-environment by reducing the surface tension, ii) taking part in the solubilization of nutrient (hydrophobic carbon sources), iii) forming an emulsion as a result of reduction of the interfacial tension between water and oil at low temperatures, which in turn could increase the available surface for growth or iv) taking part in swarming motility in order to colonize a more favorable environment.

Acknowledgments

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Chapter 3

The *ppuI-rsaL-ppuR* quorum sensing system regulates biofilm formation of *Pseudomonas putida* PCL1445 by controlling biosynthesis of the cyclic lipopeptides putisolvins I and II

Jean-Frédéric Dubern, Ben J. J. Lugtenberg, and Guido V. Bloemberg

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Abstract

Pseudomonas putida strain PCL1445 produces two cyclic lipopeptides, putisolvin I and putisolvin II, which possess surface tension-reducing abilities, are able to inhibit biofilm formation and to break down existing biofilms of several *Pseudomonas* sp. including *P. aeruginosa*. Putisolvins are secreted in the culture medium during growth at late exponential phase, indicating that production is possibly under regulation of quorum sensing. In the present study, we identified a quorum sensing system in PCL1445 composed of *ppuI*, *rsaL* and *ppuR* that shows very high similarity with gene clusters of *P. putida* strains IsoF and WCS358. Mutants in *ppuI* and *ppuR* showed a severe reduction of putisolvin production. Expression analysis of the putisolvin biosynthetic gene in a *ppuI* background showed decreased expression, which could be complemented by the addition of synthetic 3-oxo-C_{10}-AHL or 3-oxo-C_{12}-AHL to the medium.
A *rsaL* mutant overproduces AHLs and production of putisolvins is induced early during growth. Analysis of biofilm formation on polyvinylchloride (PVC) showed that *ppuI* and *ppuR* mutants produce a denser biofilm than PCL1445 which correlated with a decreased production of putisolvins, whereas a *rsaL* mutant shows a delay in biofilm production, which correlates with an early production of putisolvins. The results demonstrate that quorum sensing signals induce the production of cyclic lipopeptides putisolvins I and II and consequently controls biofilm formation by *Pseudomonas putida*.

Introduction

Bacteria can form multicellular aggregates on biotic and abiotic surfaces generally referred to as biofilms. Such communities are ubiquitous in natural environments but can also be found in industrial and clinical settings, for example on artificial surfaces of medical devices, thereby highly contributing to infections (Stewart *et al.*, 2001).

P. putida PCL1445 is capable of forming biofilms on roots and on polyvinylchloride in a commonly used biofilm assay (Kuiper *et al.*, 2004). We have shown that *P. putida* PCL1445 produces two novel lipodepsipeptides, putisolvins I and II, consisting of a C6 lipid moiety and a 12 amino acids peptide, which are produced via a putisolvin synthetase gene designated as *psoA* (Kuiper *et al.*, 2004). A mutant impaired in putisolvin biosynthesis was shown to form a thicker biofilm

than the wild type strain. In addition, purified putisolvins I and II inhibit biofilm formation and break down existing biofilms of various *Pseudomonas* spp. including the opportunistic human pathogen *P. aeruginosa* (Kuiper *et al.*, 2004).

The production of putisolvins occurs at the end of the exponential growth phase (Kuiper *et al.*, 2004), which may indicate that the production is mediated through a quorum sensing mechanism. The term quorum sensing describes an environmental sensing system, which allows bacteria to monitor their own population density. Quorum sensing in Gram-negative bacteria relies on the interaction of small diffusible signal molecules belonging to the class of *N*-acyl homoserine lactones (AHLs). They are synthetized via the LuxI protein, whereas the transcriptional activator protein LuxR couples cell population density to gene expression (Fuqua *et al.*, 2001; Swift *et al.*, 2001). These signal molecules can traffic in and out of the bacterial cell. Once a certain intracellular threshold concentration has been reached, the signals induce transcription of a set of target genes (Fuqua *et al.*, 1994). AHLs play a role in regulating different bacterial functions such as antibiotic biosynthesis, production of virulence factors, bacterial swarming, and transition to the stationary growth phase.

In this chapter we describe (i) the identification and characterization of the regulatory quorum sensing genes affecting cyclic lipopeptides putisolvins I and II in PCL1445, (ii) the involvement of the quorum sensing system in the regulation of biofilm formation of PCL1445 and, (iii) the direct relationship between production of quorum sensing signals, production of cyclic lipopeptides and reduction of the size of the biofilm formed by *P. putida* PCL1445.

Materials and methods

Bacterial strains, and growth conditions

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 Pool, England) at 28°C. *E. coli* strains were grown in Luria-Bertani medium (Sambrook and Russel, 2001) at 37°C. Media were solidified with 1.8 % agar (Select Agar; Invitrogen, Life Technologies, Paisley, United Kingdom). The antibiotics kanamycin, tetracyclin, gentamycin or carbenicillin, were added when necessary to final concentrations of 50, 40, 2 and 100 µg ml^{-1}, respectively.

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
Pseudomonas		
PCL1445	Wild-type *Pseudomonas putida*; colonizes grass roots and produces biosurfactants	Kuiper et al. (2001)
PCL1633	Tn5*luxAB* derivative of PCL1445; mutated in a *psoA*, a lipopeptide synthetase homologue	This study
PCL1636	PCL1445 derivative mutated in the *ppuI* homologue; constructed by single homologous recombination	This study
PCL1637	PCL1445 derivative mutated in the *ppuR* homologue; constructed by single homologous recombination	This study
PCL1638	PCL1445 derivative mutated in the *rsaL* homologue; constructed by single homologous recombination	This study
PCL1639	PCL1633 derivative mutated in the *ppuI* homologue; constructed by single homologous recombination	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		
pBluescript	General-purpose cloning vector, Cb^r	Stratagene, La Jolla, CA
pME6010	Cloning vector which is maintained in *Pseudomonas* strains without selection pressure, Tc^r	Heeb et al. (2000)
pME3049	Cloning vector, used for homologous recombination, Tc^r, Hg^r	Ditta et al. (1980)
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)
pMP5285	pME3049 derivative, missing the Hg^r gene, used for single homologous recombination, Km^r	Kuiper et al. (2001)
pMP5548	pBluescript containing a 2.2-kb chromosomal fragment of strain PCL1445 with the *ppuI* and *rsaL* genes, and the first part of *ppuR* gene, Cb^r	This study

pMP7565	pME6010 containing a chromosomal fragment of 1.4 kb harbouring the *ppuI* gene of pMP5548, Tc^r	This study
pMP7566	pME6010 containing a PCR fragment of 1.1 kb with the *ppuR* gene of strain PCL1445, Tc^r	This study
pMP7568	pMP5285 containing a 0.5-kb *EcoR*I-*EcoR*I PCR fragment of the central part of *ppuI* gene of PCL1445, Km^r	This study
pMP7571	pMP5285 containing a 0.55-kb *EcoR*I-*EcoR*I PCR fragment of the central part of *ppuR* gene of PCL1445, Km^r	This study
pMP7575	pMP5285 containing a 0.21-kb *Kpn*I-*sal*I PCR fragment of the central part of *rsaL* gene of PCL1445, Km^r	This study
pMP7583	pGEM-T vector containing a 0.6-kb PCR fragment of *ppuI* gene of PCL1445 and a blunted Gm^r box; Cb^r, Gm^r	This study
pMP7587	pME6010 containing a PCR fragment of 1.6 kb harbouring the *rsaL* functional gene of strain PCL1445, Tc^r	This study
pAK211	Autoinducer reporter construct based upon the *Vibrio fisheri* bioluminescence (*lux*) system; Cm^r	Kuo et al. (1994)
pSB1075	Bioluminescent AHL sensor plasmid containing a fusion of *lasRI*::*luxCDABE* in pUC18; used for the detection of long-chain AHLs; Cb^r.	Winson et al. (1998)

Extraction and detection of AHLs autoinducers from spent culture medium

To isolate autoinducer activity, 3 volumes of dichloromethane were added to 7 volumes of supernatant of a 50 ml BM bacterial culture and shaken for 1 h at 120 rpm. The organic phase was removed and dried by evaporation under vacuum to dryness (Mc Clean *et al.*, 1997). Supernatant extracts were redissolved in 100 µl of ethyl acetate and 10 µl fractionated on a C18 reverse-phase TLC plate (Merck, Darmstadt, Germany), developed in methanol-water (60:40; vol/vol).

To detect autoinducer activity, overnight cultures of *E.coli* DH5α containing pAK211 (Kuo *et al.*, 1994) or pSB1075 (Winson *et al.*, 1998) were grown in LB medium supplemented with 20 µg of chloramphenicol or carbenicillin per ml, respectively, for 10 h. TLC plates were overlaid with 0.8 % LB top agar layer containing 50 µl of the pAK211 or pSB1075 strains per ml, followed by incubation

at 28°C for 16 h. Autoinducer activity was then 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.

Isolation and identification of quorum sensing gene homologs

A plasmid library of chromosomal fragments of strain PCL1445 was constructed by cloning 1.5 to 3.0-kb fragments of chromosomal DNA digested with *EcoR*I into pBluescript (Stratagene, La Jolla, CA). The resulting fragment library was introduced into an *E. coli* strain harbouring the *lux* reporter plasmid pAK211 (Kuo *et al.*, 1994). After overnight growth on LB agar plates, clones that induced the luciferase reporter were identified using photographic film. To remove pAK211 (Kuo *et al.*, 1994) from the *E. coli* reporter strain, total plasmid was isolated and reintroduced into DH5α cells by standard transformation protocols (Schnider *et al.*, 1995) followed by carbenicillin selection, whereas chloramphenicol selection was omitted. The nucleotide sequence of the chromosomal fragment inserted in the selected plasmid pMP5548 was determined using universal primers - 40 reverse primer flanking the multiple cloning site of pBluescript.

Construction of ppuI*,* ppuR *and* rsaL *mutant strains*

ppuI mutant derivatives of strains PCL1445 and PCL1633 were constructed by homologous recombination. A 0.5-kb internal fragment of *ppuI* of strain PCL1445 was obtained by PCR using primers oMP902 (5'-ATGCATAAACTTCGGGCA-3') and oMP903 (5'-CATTTTCTCGACCCCCAC-3'), cloned into the pGEM-T Easy Vector System I (Promega Corporation, Madison, WI) and ligated as a *EcoR*I-*EcoR*I insert in the pMP5285 suicide plasmid (Kuiper *et al.*, 2001) derived from pME3049 (Ditta *et al.*, 1980) resulting in pMP7568. pMP7568 was transferred to PCL1445 by tri-parental mating using pRK2013 as a helper plasmid (Schnider *et al.*, 1995) and using selection on KB agar medium supplemented with kanamycin (50 μg ml^{-1}). Strain PCL1636 was obtained as a kanamycin resistant colony resulting from single homologous recombination. The insertion of the suicide construct was confirmed by sequence analysis.

To construct a PCL1633 *ppuI* mutant, the pGEM-T vector containing the 0.5-kb fragment of *ppuI* and a gentamycin resistance cassette cloned as a *Sal*I-*Sal*I fragment resulting in pMP7583, was used as a suicide plasmid. Single homologous recombination in PCL1633 carried out using pMP7583 resulted in PCL1639. A *P.*

putida PCL1445 *ppuR* mutant was constructed using a similar mutagenesis strategy. The *ppuR* fragment for the construction of the pMP5285 based suicide plasmid pMP7571 resulted from a PCR reaction using primers oMP905 (5'-AATTCTTCGAAGAAGCCGCCG-3') and oMP906 (5'-TTGCTGGATGGCTTTGAGCACC-3') and chromosomal DNA of strain PCL1445 as a template. Single homologous recombination in *ppuR* of PCL1445 resulted in strain PCL1637.

The *P. putida* PCL1445 *rsaL* mutant was constructed using the pMP5285 suicid plasmid based pMP7575 obtained after cloning a 0.21-kb *Kpn*I-*Sal*I PCR fragment of the central part of *rsaL* gene of PCL1445 obtained using primers oMP897 (3'-TACCTCAGCTGTGCGCGAGGT-5') and oMP898 (3'-GGTGGGCCAGGTCGCTTTCCT-5'). Single homologous recombination in *rsaL* of PCL1445 resulted in strain PCL1638.

Complementation of ppuI*,* ppuR*, and* rsaL *mutants of PCL1445*

Complementation of strain PCL1636 (*ppuI*) was carried out using pMP7565, a shuttle vector derived from pME6010 (Heeb *et al.*, 2000) in which a 1.4-kb fragment containing *ppuI* and *rsaL* of strain PCL1445 was inserted. This insert was obtained by *EcoR*I digestion from pMP5548. pMP7565 was transferred to strain PCL1636 by tri-parental mating as described above and transformants were selected on KB agar medium supplemented with tetracyclin (40 μg ml^{-1}). To complement the *ppuR* insertion in PCL1637, a 1.1-kb PCR fragment containing the *ppuR* gene of strain PCL1445 was obtained using primers oMP883 (3'-TGTATATCCTGCTGCGCCTTTA-5') and oMP884 (3'-CATGTGCATCGTGGTGCTGCCT-5'), and cloned into pME6010, resulting in pMP7566.

To complement the *rsaL* insertion in PCL1638, a 1.6-kb PCR fragment containing *rsaL* gene of strain PCL1445 was obtained using primers oMP1011 and oMP1012 (3'-TTGTCAAGCAGTGCCACTGGTTCTAGAAAA-5') and oMP1012 (3'-ATCAGCGACATCTAGTCGTGGGAGCTCAAA-5'), and cloned into pME6010, resulting in pMP7587.

Biosurfactant production

The production of biosurfactant activity was detected using the drop collapsing assay as described previously (Jain *et al.*, 1991), in which the reduction of the water surface tension can be observed as the collapse of a round droplet placed on a hydrophobic surface (Jain *et al.*, 1991).

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).

Extraction and High-Performance Liquid Chromatography (HPLC) analysis of putisolvins

To quantify the production of putisolvins in BM culture medium, 10 ml of a BM 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). The dried pellet obtained from 10 ml culture was resuspended in 500 µl of 50/50 acetonitrile/water (v/v) and filtered using a spinX centrifuge tube filter of 0.45 µm pore size (Corning Costar Corporation, Cambridge, MA). A volume of 500 µl of the samples was separated by HPLC (Jasco International CO. Lt., 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 1ml min^{-1}. Chromatograms were analyzed in the wavelength range between 195 nm and 420 nm. Fractions that corresponded to the retention time of 20 min for putisolvin I and 21 min for putisolvin II were collected and tested for activity in the drop collapsing assay. The amount of putisolvins produced was quantified as the peak area in micro absorbance units (µAU) at 206 nm.

*Quantification of bioluminescent Tn5*luxAB *reporter strains*

Expression of Tn5*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 BM medium in a volume of 10 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) in a 2.0 % bovine serum albumin solution was added and luminescence determined with a MicroBeta 1450 TriLux luminescence counter (Wallac, Turku, Finland), which was normalized to the luminescence per OD_{620} unit. The synthetic AHL molecules *N*-hexanoyl-L-

homoserine lactone (C_6-AHL) (Fluka, Zwijndrecht, The Netherlands), *N*-octanoyl-L-homoserine lactone (C_8-AHL) (Fluka), *N*-decanoyl-L-homoserine lactone (C_{10}-AHL) (Fluka), *N*-dodecanoyl-L-homoserine lactone (C_{12}-AHL), *N*-(3-oxo-hexanoyl)-L-homoserine lactone (3-oxo-C_6-AHL), *N*-(3-oxo-octanoyl)-L-homoserine lactone (3-oxo-C_8-AHL), *N*-(3-oxo-decanoyl)-L-homoserine lactone (3-oxo-C_{10}-AHL), *N*-(3-oxo-dodecanoyl)-L-homoserine lactone (3-oxo-C_{12}-AHL), and *N*-(3-oxo-tetradecanoyl)-L-homoserine lactone (3-oxo-C_{14}-AHL) were tested for the ability to induce the Tn5*luxAB* reporter strains. Briefly, cells were grown in BM – 2 % glycerol medium for 48 h, washed, and resuspended to an OD_{620} of 0.1 in fresh medium supplemented with either 5 µM synthetic AHL or 25 µl of a 1000-fold concentrated crude extracts of spent culture supernatant dissolved in 100 % acetonitrile.

Biofilm assay

Biofilm formation on polyvinylchloride (PVC) was conducted as described by O'Toole and Kolter (1998) and adapted for strain PCL1445 as described by Kuiper *et al.* (2004). When the effect of AHLs on biofilm formation was tested, the culture medium and planktonic cells was removed after 4 h. Subsequently, 100 µl of M63 medium containing 1µl of 5 µM synthetic 3-oxo-C_{12}-AHL dissolved in 100% acetonitrile was added to the wells. An equal volume of acetonitrile was added to control wells. All conditions were tested in triplicate.

Nucleotide sequence accession number

The nucleotide sequences of the *P. putida* PCL1445 *ppuI-rsaL-ppuR* DNA region and putisolvin synthetase promoter region reported in this paper have been deposited in the GenBank database, respectively, under accession numbers DQ151886 and DQ151887.

Results

Production of AHLs by P. putida PCL1445

To test the possible production and secretion of AHLs, a crude dichloromethane extract of the spent BM-glycerol medium of a culture of OD_{620} 1 was tested for induction of *E. coli* reporter strain based on the *lux* quorum sensing system of *Vibrio fischeri* (Fig. 1A) and the *las* system of *P. aeruginosa* (Fig. 1B). After separation on C_{18} reverse phase TLC, four compounds were detected with R_f values

similar to those of 3-oxo-C_{12}-, 3-oxo-C_{10}-, 3-oxo-C_8-, and 3-oxo-C_6-AHLs (Fig.1A and B). Furthermore, when the standard molecules were mixed with PCL1445 dichloromethane extracts, the four detected compounds co-migrated with the standard AHLs (Fig. 1A, lane 3 and Fig. 1B, lane 3). Dichloromethane extracts of the putisolvin biosynthetic mutant PCL1633 showed the same profile as the wild type strain (data not shown).

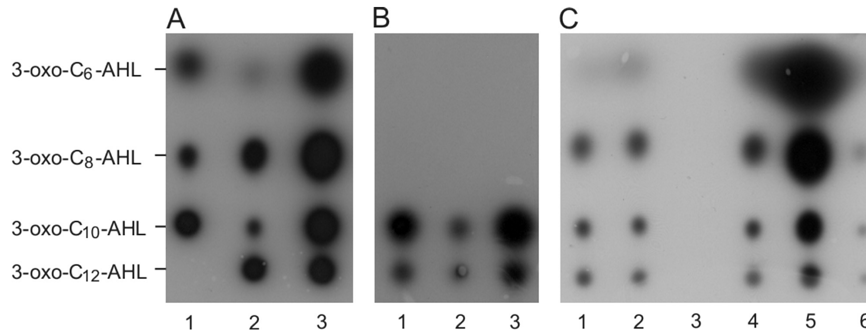

Fig. 1. C18-reverse phase thin-layer chromatography analysis of *N*-acyl-L-homoserine lactones produced by *P. putida* PCL1445 and its mutant derivatives. Cells of strain *P. putida* PCL1445 and its derivatives *ppuI* mutant PCL1636 strain, PCL1636 harbouring plasmid pMP5548 (*ppuI*), the *rsaL* mutant PCL1638, and PCL1638 harbouring plasmid pMP7587 (*rsaL*) were grown in BM-glycerol to OD_{620} 0.7 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. **Panel A.** The biosensor *E. coli* harbouring pAK211 was used to visualize AHLs produced by PCL1445. Lane 1: 16 ng of 3-oxo-C_6-AHL, 20 ng of 3-oxo-C_8-AHL, and 50 ng of 3-oxo-C_{10}-AHL were mixed. Lane 2: culture supernatant extract of PCL1445. Lane 3: culture supernatant of PCL1445, 16 ng of 3-oxo-C_6-AHL, 20 ng of 3-oxo-C_8-AHL, and 50 ng of 3-oxo-C_{10}-AHL were mixed. **Panel B.** The biosensor *E. coli* harbouring pSB1075 was used to visualize long chains AHLs produced by PCL1445. Lane 1: 50 ng of *N*-(3-oxo-decanoyl)-L-homoserine lactone (3-oxo-C_{10}-AHL) and 50 ng of *N*-(3-oxo-dodecanoyl)-L-homoserine lactone (3-oxo-C_{12}-AHL) were mixed. Lane 2: culture supernatant extract of PCL1445 cells. Lane 3: culture supernatant extract of PCL1445 cells, 50 ng of 3-oxo-C_{10}-AHL, and 50 ng of 3-oxo-C_{12}-AHL were mixed. **Panel C.** The biosensor *E. coli* harboring pAK211 was used to visualize AHLs present in culture supernatant extracts of cells of strains PCL1445, PCL1636 (*ppuI*), and PCL1638 (*rsaL*). Lane 1: PCL1445, Lane 2: PCL1445 harbouring the cloning vector pME6010. Lane 3: PCL1636 (*ppuI*). Lane 4: PCL1637 harbouring pMP5548 (*ppuI*). Lane 5: PCL1638 (*rsaL*). Lane 6: PCL1638 harbouring pMP7587 (*rsaL*).

Identification of quorum sensing genes of P. putida PCL1445

To isolate a chromosomal fragment of strain PCL1445 containing *luxI* and *luxR* homologues, an *EcoR*I chromosomal library of PCL1445 was introduced into *E. coli* DH5α containing pAK211, a reporter strain for AHLs based on the *lux* system of *Vibrio fisheri* (Kuo *et al.*, 1994). The plasmid of one luminescent transformant, pMP5548, was isolated for analysis. Nucleotide sequence analysis of the 2.2-kb genomic fragment present in pMP5548 revealed the presence of several ORF's, which show homologies to *suhB*, *ppuI*, *rsaL*, and *ppuR* of *P. putida*. The identified sequences of the genes showed 99 % identity with the *ppu* locus characterized in *P. putida* strains IsoF (Steidle *et al.,* 2002) and WCS358 (Bertani *et al.*, 2004), 57 % identity with *lasI* of *P. aeruginosa* (Pearson *et al.*, 1994), and 51 % identity with *mupI* of *P. fluorescens* (El-Sayed *et al.*, 2001) (Fig. 2A). The sequence of the gene located upstream of *ppuI* showed 100 % identity with *rsaL* gene in *P. putida* IsoF (Steidle *et al.*, 2002) and WCS358 (Bertani *et al.*, 2004), and 60 % identity with *rsaL* of *P. aeruginosa* (de Kievit *et al.,* 1999). The *rsaL* gene was first described as a repressor of virulence genes in *P. aeruginosa* and later as a repressor of the *ppuI* gene in *P. putida* strains IsoF and WCS358. The ORF located downstream of *ppuI* showed 91 % identity with the *suhB* of *P. putida* IsoF (Steidle *et al.*, 2002) and 78 % with *suhB* of *P. aeruginosa*. The latter gene was suggested to possess inositol monophosphatase activity in *E. coli* (Matsuhisa *et al.*, 1995).

To test whether the *ppuI* gene present in pMP5548 was responsible for the production of C_{10}-, 3-oxo-C_{10}-, C_{12}-, and 3-oxo-C_{12}-AHLs, dichloromethane extracts of the DH5α reporter containing pAK211 with or without pMP5548 were subjected to TLC analysis. The results showed the presence of the four AHLs detected in PCL1445 crude extracts with similar Rf-values to C_{10}-, 3-oxo-C_{10}-, C_{12}-, and 3-oxo-C_{12}-AHLs (data not shown).

In the region upstream of *ppuI* and *ppuA* nucleotide sequences were found which were identical to *ppuI* and *ppuA lux* box elements found in *P. putida* strains IsoF (Steidle *et al.*, 2002) and WCS358 (Bertani *et al.*, 2004).

An 16-bp palindromic sequence with high similarity to the *lux* box elements, which are located in the promoter region of quorum sensing regulated genes of *P. putida* (Steidle *et al.*, 2002), *P. aeruginosa* (Whiteley *et al.*, 2000), *P. chlororaphis* (Chin-A-Woeng *et al.*, 2001), and *V. fischeri* (Devine et al., 1989), is present 92 bp

upstream of the *psoA* gene start codon (Fig. 2B) (Dubern *et al.*, 2005). These palindromes might constitute a binding site for the LuxR response regulator.

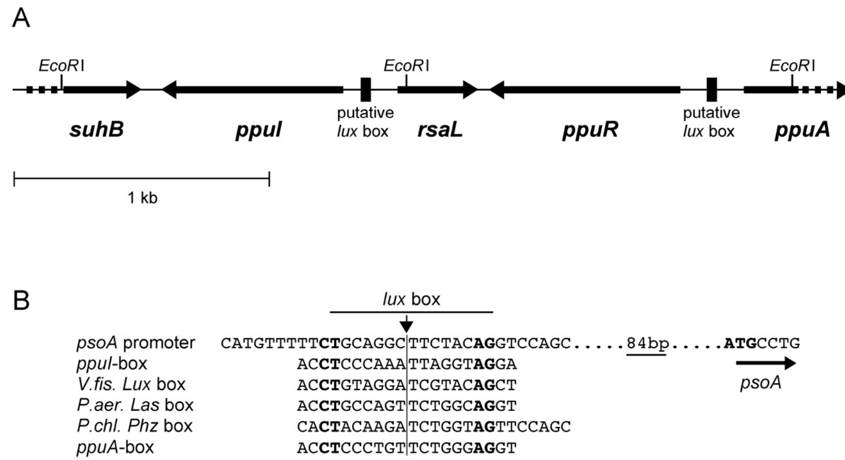

Fig. 2. *ppu* locus and analysis of the *lux* box in the upstream region of putisolvin biosynthetic gene *psoA* of *P.putida* PCL1445. **Panel A.** *ppu* locus of strain PCL1445. Putative *lux* boxes are present in the intergenic regions of *ppuI-rsaL* and of *ppuR-ppuA*, respectively. Dotted lines indicate non-determined sequence. **Panel B.** Comparison of a *lux* box homologous sequence in the region upstream of *psoA* gene of *P. putida* PCL1445 with similar sequences.

Expression of the putisolvin biosynthetic gene psoA *is stimulated by AHLs*

To analyze the effect of a mutation in the AHL biosynthetic gene *ppuI* on the expression of the *psoA*, *ppuI* was mutated in strain PCL1633 (*psoA*::Tn5*luxAB)*, resulting in strain PCL1639 (*psoA*/*ppuI*), in which *psoA* expression was quantified by measuring luminescence. The *psoA* expression appeared to be 10-fold lower in strain PCL1639 (*ppuI*/*psoA*) when compared to the transcriptional activity detected in PCL1633 (*psoA*) (Table 2). Transcriptional activity of the *psoA* promoter was analyzed in strain PCL1639 (*ppuI*/*psoA*) in liquid culture at OD_{620} 1.5 after addition of crude 1000-fold concentrated dichloromethane extracts of the wild type strain culture supernatant, or of the *ppuI* mutant, to early-log phase culture (OD_{620} 0.2) of PCL1639. PCL1445 dichloromethane extract but not *ppuI* mutant extract, was able to complement part of the *psoA* promoter activity in PCL1639 (Table 2).

The response to C_4-, C_6-, 3-oxo-C_6-, C_8-, C_{10}-, 3-oxo-C_{10}-, C_{12}-, and 3-oxo-C_{12}-AHLs signals, added at a concentration of 5 µM to early-log phase culture (OD_{620} 0.2) of PCL1639, on *psoA* transcriptional activity was quantified at OD_{620} 1.5.

The addition of AHLs without a 3-oxo-group or with short acyl chains (C_4, C_6 and C_8) did not significantly affect *psoA*::*luxAB* expression (Table 2). However, the *psoA* promoter activity was stimulated by addition of 3-oxo-C_{10}-AHLs and even more by 3-oxo-C_{12}-AHLs (Table 2).

Table 2. Transcriptional activity of *psoA* of *P. putida* PCL1445 in response to synthetic AHLs.

Strain[a]	*N*-acyl homoserine lactone (AHL) (5 µM)	Supernatant		Bioluminescence/Cell density (x 10^3 LCPS/OD_{620nm})
		PCL1445	PCL1636 (*ppuI*)	
PCL1445	None	-	-	0.06 ± 0.01
PCL1633 (*psoA*)	None	-	-	3.48 ± 0.07
PCL1639 (*psoA*/*ppuI*)	None	-	-	0.34 ± 0.03
	None	-	+	0.33 ± 0.03
	None	+	-	2.71 ± 0.11
	C_4-AHL	-	-	0.23 ± 0.03
	C_6-AHL	-	-	0.22 ± 0.01
	3-oxo-C_6-AHL	-	-	0.25 ± 0.02
	C_8-AHL	-	-	0.31 ± 0.01
	3-oxo-C_8-AHL	-	-	0.26 ± 0.02
	C_{10}-AHL	-	-	0.35 ± 0.03
	3-oxo-C_{10}-AHL	-	-	2.10 ± 0.14
	C_{12}-AHL	-	-	0.32 ± 0.05
	3-oxo-C_{12}-AHL	-	-	3.11 ± 0.13
	3-oxo-C_{14}-AHL	-	-	2.15 ± 0.20

[a]Expression of the putisolvin biosynthetic gene *psoA* was determined by measuring bioluminescence from cells cultures of the double mutant PCL1639 (*psoA*::Tn5*luxAB*/*ppuI*) grown to OD_{620} 1.5 in BM-glycerol medium. Crude 1000-fold concentrated dichloromethane extracts of the wild type strain culture supernatant, or of the *ppuI* mutant, or AHLs molecules were added to early-log phase culture (OD_{620} 0.2). Standard deviations are based on the mean values of three parallel cultures.

Construction and characterization of ppuI*,* ppuR*, and* rsaL *mutants*

To investigate whether *ppuI*, *ppuR*, and *rsaL* are involved in putisolvin production, insertion mutants were constructed by single homologous recombination using suicide plasmids pMP7568, pMP7571 and pMP7575, respectively (see Materials and Methods section), resulting in strains PCL1636, PCL1637 and PCL1638, respectively. The proper integration of plasmids pMP7568, pMP7571 and pMP7575 by homologous recombination into the chromosome was confirmed by sequencing the region flanking the suicide plasmids after isolation of the chromosomal DNA recombinants.

Putisolvin production by mutant strains PCL1636 (*ppuI*), PCL1637 (*ppuR*), and PCL1638 (*rsaL*) was investigated by two different approaches. Firstly, biosurfactant production by strains PCL1626 (*ppuI*), PCL1637 (*ppuR*), and PCL1638 (*rsaL*) was quantified during growth until the stationary phase was reached by the Du Nouy ring method (Fig. 3A). Secondly, the production of putisolvins I and II by strains PCL1445, PCL1636 (*ppuI*), PCL1637 (*ppuR*) and PCL1638 (*rsaL*) was tested by HPLC analysis (Fig. 3B and 3C).

Culture supernatants of PCL1636 (*ppuI*) and PCL1637 (*ppuR*) were not able to decrease the surface tension between culture medium and air when compared to the wild type, indicating a lack of biosurfactant production (Fig. 3A). Culture supernatant of strain PCL1638 (*rsaL*) caused a decrease of surface tension during the early exponential phase (to 32 mN m^{-1} at OD 1), indicating an earlier production of biosurfactant than by the wild type strain (48 mN m^{-1} at OD 1) (Fig. 3A).

Mutants PCL1636 (*ppuI*) and PCL1637 (*ppuR*) showed a significant reduction (85 %) of putisolvin production (Fig. 3B, bars c and e, respectively). Introduction of pMP7565, harbouring the genomic fragment from pMP5548 with *ppuI* and pMP7566 harbouring *ppuR* restored putisolvin production to wild type levels in both strains PCL1636 (*ppuI*) and PCL1637 (*ppuR*) (Fig. 3B, bars d and f, respectively). The production of putisolvins by PCL1445 and mutant PCL1638 (*rsaL*) was compared by HPLC analysis at different stages of bacterial growth (Fig. 3C). Mutant PCL1638 (*rsaL*) shows a significantly increased putisolvin production during the early exponential phase when compared to the wild type (4-fold at OD_{620} 0.6). This difference in production tends to decrease when the cells reach the stationary phase (2-fold at OD_{620} 1.1, and hardly any difference at OD_{620} 2). Introduction of pMP7587 harbouring *rsaL* into PCL1638 (*rsaL*) significantly decreased putisolvin

production during exponential phase resulting in lower value than for the wild type strain (2-fold lower at OD_{620} 0.6), which could be explained by the multiple copy effect of the plasmid used for complementation of *rsaL* mutation (Fig. 3C).

To investigate the involvement of *ppuI* in AHLs biosynthesis in PCL1445 and *rsaL* in regulation of AHLs biosynthesis in PCL1445, AHLs production by strains PCL1445, PCL1636 (*ppuI*), PCL1636 harbouring pMP7565 (*ppuI*), PCL1638 (*rsaL*), and PCL1638 harbouring pMP7587 (*rsaL*) was examined by TLC analysis. PCL1445 harbouring the control vector pME6010 did not have any influence on the AHL production when compared to the wild type without pME6010 (Fig. 1C, lane 2). Mutant PCL1636 (*ppuI*) showed a total absence of AHL production (Fig. 1C, lane 3). AHL production of PCL1636 (*ppuI*) was restored by introduction of pMP7565 harbouring a functional *ppuI* gene (Fig. 1C, lane 4). Finally, mutating *rsaL* (PCL1638) had a strong positive effect on the production of AHLs of PCL1445 (Fig. 1C, lane 5). The AHL production of PCL1628 (*rsaL*) decreased dramatically by introduction of pMP7587 harbouring a functional *rsaL* gene when compared to the wild type (Fig 1C, lane 6).

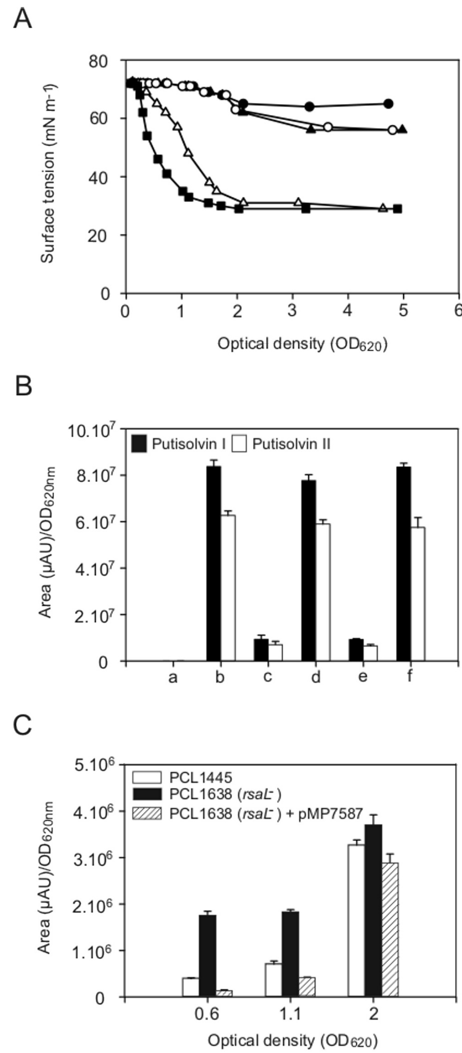

Fig. 3. Effects of mutations in *ppuI*, *ppuR*, and *rsaL* on the production of putisolvins of *P. putida* PCL1445. **Panel A**. Quantification of surface tension decrease by culture supernatants of *P. putida* strain PCL1445 (∆), PCL1633 (*psoA*) (●), PCL1636 (*ppuI*) (○), PCL1637 (*ppuR*) (▲), and PCL1638 (*rsaL*) (■) grown to the stationary phase in BM-glycerol medium. **Panel B**. C8-Reverse Phase HPLC analysis of putisolvin production by *P. putida* strain PCL1445 and its mutants PCL1636 (*ppuI*) and PCL1637 (*ppuR*). a. Mutant strain PCL1633 (*psoA*). b. PCL1445. c. PCL1636 (*ppuI*). d. PCL1636 harboring pMP5548 (*ppuI*). e. PCL1637 (*ppuR*). f. PCL1637 harboring pMP7566 (*ppuR*). Cells were grown to the stationary phase in 5 ml BM-glycerol medium at 28°C under vigorous aeration. Ethyl acetate extracts of culture supernatants were separated and the peak areas of putisolvin I and II were quantified at a wavelength of 206 nm. **Panel C**. C8-Reverse Phase HPLC analysis of putisolvin production by PCL1445, mutant

PCL1638 (*rsaL*), and PCL1638 harbouring pMP7587 (*rsaL*). Compounds from the ethyl acetate extracted culture supernatant of cultures grown to OD 0.6, 1.1, and 2 in BM-glycerol were separated and analyzed by HPLC as described under panel B.

Effect of ppu *quorum sensing system on biofilm formation of PCL1445*

Biofilm formation on PVC titer wells by PCL1445 and its mutants PCL1633 (*psoA*), PCL1636 (*ppuI*), PCL1637 (*ppuR*), and PCL1638 (*rsaL*) was measured at various times after inoculation (Fig. 4A). The size of the biofilms formed by mutants PCL1636 (*ppuI*) and PCL1637 (*ppuR*) was comparable to that of the putisolvin-deficient mutant (PCL1633) and considerably thicker than that of the wild type (Fig. 4A). To monitor the surfactant activity produced by the bacterial cells in the titer wells, culture samples were analyzed by the drop collapsing assay. Indexes from 0 to 4 were used to quantify biosurfactant production by bacterial cell in the biofilm assay (Fig. 4E). PCL1636 (*ppuI*) and PCL1637 (*ppuR*) did not produce any detectable biosurfactant activity (Fig. 4B). Analysis of PCL1638 (*rsaL*) showed that biofilm formation decreased 1.5 fold as compared with the wild type strain (Fig. 4A), which correlates with an earlier appearance of biosurfactant activity (visible after 6 h) than observed for PCL1445 (visible after 10 h) (Fig. 4B).

The effect of AHLs produced by PCL1445 on its biofilm forming ability and consequently on the production of biosurfactants was analyzed in two different ways: (i) mutant PCL1636 (*ppuI*) was transformed with pMP5548 harbouring *ppuI* and (ii) exogenous 3-oxo-AHL (5 µM) was added to the medium. Biofilms were assayed after 24 h of incubation (Fig. 4C). Mutants PCL1636 (*ppuI*) (Fig. 4C, bar e) and PCL1633 (*psoA*) (Fig. 4C, bar d) form thicker biofilms than the wild type (Fig. 4C, bar b). Introduction of pMP5548 into PCL1636 (*ppuI*) restored biosurfactant production (Fig. 4D, lane f) and decreased biofilm formation to the same level as reached by PCL1445 (Fig. 4C, bar f). Exogenous 3-oxo-C_{12}-AHL signaling molecules appeared also to be able to stimulate production of biosurfactant by *ppuI* mutants (Fig. 4D, lane j), and to reduce the thickness of the biofilm (Fig. 4C, bar j).

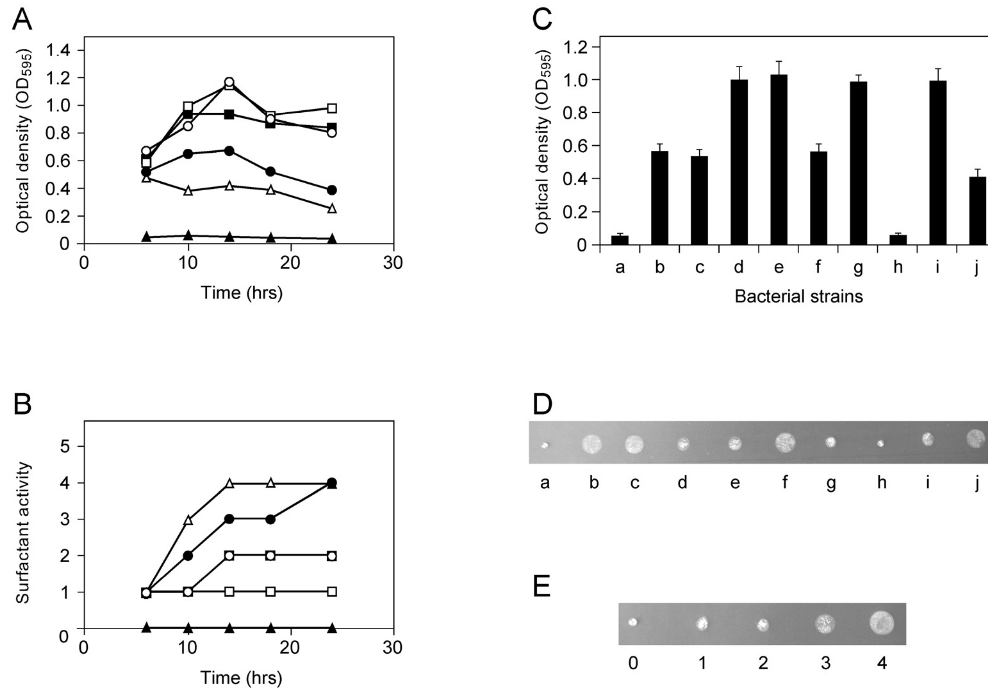

Fig. 4. Influence of quorum sensing on biofilm formation of *P. putida* PCL1445 in PVC micro titer wells. Cells of PCL1445 and its quorum sensing mutant derivatives were incubated in microtiter plates in M63 medium and their biofilm formation quantified over time using the crystal violet-staining procedure. Cells attached to the microtiter wells were stained with crystal violet, washed and the crystal violet in the biofilm was dissolved in ethanol after which the OD_{595} was measured. To determine surface tension reducing activity in the well, 25 µl of culture was pipetted as a droplet on parafilm and allowed to dry. The diameter of the dried droplet correlates with surface tension reduction. A surfactant activity index based on the droplet diameter ranging from 0 to 4 was used to quantify surface tension reduction. All experiments were performed in triplicate. **Panel A**. Time course of biofilm formation of PCL1445 (●), PCL1633 (*psoA*) (□), PCL1637 (*ppuI*) (■), PCL1638 (*ppuR*) (○), and PCL1639 (*rsaL*) (Δ). As a negative control, uninoculated M63 medium was used (▲). **Panel B**. Biosurfactant activity present in the titer well during biofilm formation of bacterial cultures presented in panel A as determined by the drop collapsing assay. **Panel C**. Biofilm formation of PCL1445 and PCL1636 (*ppuI*) measured after 24 hours of incubation. Bar a, M63 medium without bacteria; bar b, PCL1445; bar c, PCL1445 containing pME6010; bar d, PCL1436 (*psoA*); bar e, PCL1636 (*ppuI*); bar f, PCL1636 (*ppuI* $^{-}$) harboring pMP5548 (*ppuI*); bar g PCL1639 (*psoA*/*ppuI*); bar h, M63 + 3-oxo-C12-AHL (5 µM); bar i PCL1636 (*ppuI*) + pure acetonitrile (control); bar j PCL1636 (*ppuI*) + 3-oxo-C12-AHL (5 µM). Standard deviations are based on the mean values of triplicate cultures. **Panel D**. Biosurfactant activity shown by the drop collapsing assay of

bacterial cultures in the biofilm assay in panel C; lane a. M63 medium without bacteria; lane b. PCL1445. lane c. PCL1445 containing pME6010. lane d. PCL1436 (*psoA*). lane e. PCL1636 (*ppuI*). Lane f. PCL1636 (*ppuI*) harbouring pMP5548 (*ppuI*). lane g. PCL1639 (*psoA*/*ppuI*). lane h. M63 + 3-oxo-C12-AHL (5 µM). Lane i. PCL1636 (*ppuI*) + pure acetonitrile (control). Lane j. PCL1636 (*ppuI*) + 3-oxo-C12-AHL (5 µM). **Panel E.** Index 0-4 used for the detection of biosurfactants production by bacterial cell in the biofilm assay. Shown are dried droplets of 25 µl culture supernatant with increased diameter due to decreased surface tension caused by increased biosurfactants activity.

Discussion

Pseudomonas putida strain PCL1445 produces two cyclic lipopeptides biosurfactants, putisolvins I and II, which inhibit biofilm formation and degrade existing *Pseudomonas* biofilms (Kuiper *et al.*, 2004). Initiation of putisolvin production starts at the onset of stationary phase (Kuiper *et al.*, 2004) suggesting that putisolvin biosynthesis might be population density regulated, which would imply that putisolvins are regulating the formation and thickness of the biofilm after the initial formation steps of the biofilm at high bacterial cell density. The aim of this work was to determine whether quorum sensing is regulating the production of the cyclic lipopeptides putisolvins I and II by *P. putida* PCL1445 and, consequently, biofilm formation.

Using several bacterial reporter strains for the detection of AHLs we showed that PCL1445 produces at least four different inducing compounds, which are migrating at the same positions as 3-oxo-C_6-, 3-oxo-C_8-, 3-oxo-C_{10}-, and 3-oxo-C_{12}-AHL on TLC (Fig. 1). Two of these compounds, 3-oxo-C_{10}- and 3-oxo-C_{12}-AHLs, were shown to restore *psoA* promoter activity in double mutant PCL1639 (*ppuI*/*psoA*) (Table 2). The AHLs lacking the 3-oxo-group did not stimulate the *psoA* promoter (Table 2). Furthermore, we detected a palindromic sequence in the promoter region of *psoA* similar to the regulatory *lux* box (Fig. 2B), the presence of which is typical for genes under control of quorum sensing.

Regulation via quorum sensing involves a LuxI (homologous) protein, which directs the synthesis of signaling molecules, and the cognate transcriptional regulator LuxR, which binds to the operator of the target regulated gene. In strain PCL1445, a *luxI* homologous gene was identified as *ppuI* and a *luxR* homologous gene as *ppuR* (Fig. 2A). The *ppuI* and *ppuR* genes are transcribed in the same direction and separated by *rsaL*, which is transcribed in the opposite direction (Fig.

2A). RsaL was reported to play a role in the repression of *lasI* of *P. aeruginosa* (de Kievit *et al.*, 1999) and of *ppuI* of *P. putida* WCS358 (Bertani *et al.*, 2004). A highly conserved palindromic sequence (*lux* box) was identified in the promoter regions of the *ppuI* and *ppuR* genes (Fig 2A). Such a regulatory element is thought to represent the binding site for the LuxR homolog after activation by binding the appropriate AHL. The genetic organization of the *ppu-rsaL-ppuR* locus of PCL1445 is identical to the loci identified in *P. putida* IsoF (Steidle *et al.*, 2002) and *P. putida* WCS358 (Bertani *et al.*, 2004). Although the *ppuI-rsaL-ppuR* locus was reported to be involved in biofilm formation by *P. putida* IsoF, the molecular mechanism could not be explained (Steidle *et al.*, 2002). Members of the *luxI* and *luxR* families usually show weak homologies. The *ppuI/ppuR* quorum sensing system is not widespread among *P. putida* members but seems to be evolutionary well conserved and might regulate similar genes (Steidle *et al.*, 2002).

More detailed studies showed that a mutation in *ppuI* of PCL1445 abolishes the production of all four detected AHL compounds (Fig. 1C), indicating that *ppuI* is responsible for the production of AHLs. Mutation of *ppuI* and *ppuR* abolishes putisolvin production almost completely (Fig. 3B). Transcriptional analysis of the *psoA* promoter in a *ppuI* mutant background showed clearly that at least one of the quorum sensing signals present in the medium (3-oxo-C_{12}-AHL), which can be synthesized via *ppuI*, is able to induce putisolvin biosynthesis (Table 2). Our results show that *ppuI* and *ppuR* are responsible for production of AHLs and regulate putisolvin expression in PCL1445. Mutation of *rsaL* resulted in an increased AHL production (Fig. 1C), suggesting that *rsaL* is involved in repressing *ppuI* and/or *ppuR*. Mutating *rsaL* had a positive effect on putisolvin production during the lag phase (Fig. 3C), which can be explained by its repressive effect on AHL synthesis.

Biofilm formation in PVC titer wells indicated that *ppuI* and *ppuR* mutants, in which putisolvin production is strongly reduced (Fig. 4B), exhibit the same phenotype as a putisolvin biosynthetic mutant by forming a thicker biofilm (Fig 4A), while a *rsaL* mutant forms even less biofilm than the wild type (Fig. 4A) and produced putisolvins at an earlier stage of biofilm formation (Fig. 4B). Most interestingly, when AHL signal molecules were added to the medium, the *ppuI* mutant started to produce biosurfactant activity and lost the ability to form a dense biofilm with a thickness comparable to a putisolvin biosynthetic mutant (Fig. 4C and 4D). These results show that the biofilm formation in PCL1445 is regulated by the production of putisolvins in a cell density dependent manner.

P. aeruginosa possesses two quorum sensing systems, *lasI*/*lasR* and *rhlI*/*rhlR*, both of which are involved in the regulation of rhamnolipid surfactant production (Davies *et al.*, 1998). In a recent study by Davey et al. (2003) (Davey *et al.*, 2003) it was indicated that *rhlI* influences biofilm development. Rhamnolipids were shown to be involved in the maintenance of the *P. aeruginosa* biofilm architecture, by keeping the fluid-water channels of the biofilm opened (Davey *et al.*, 2003). The observation that chemically unrelated molecules such as rhamnolipids and the cyclic lipopeptides putisolvins I and II, all of which have biosurfactant activity, are regulated by quorum sensing and are involved in the regulation of biofilm formation and structure suggests that biosurfactants play an important role in biofilm structure and development.

The synthesis of the biosurfactant viscosin in *P. fluorescens* 5064 (Cui *et al.*, 2005), as well as biosurfactants serrawettin W2 in *S. liquefaciens* (Lindum *et al.*, 1998) and lipopeptide of unknown structure in *Burkholderia cepacia* (Huber *et al.*, 2002) were also reported to be regulated by AHLs. The production of biosurfactants was shown to be essential for swarming motility of *S. liquefaciens* (Lindum *et al.*, 1998), *P. aeruginosa* (Kohler *et al.*, 2000) and *B. cepacia* (Huber *et al.*, 2002). Previously we have shown that putisolvins stimulate swarming motility (Kuiper *et al.*, 2004), which could provide an explanation for the reducing effect of putisolvins on biofilm size or when added to a formed biofilm resulting in a break down of biofilm (Kuiper *et al.*, 2004).

A role for AHL-mediated quorum sensing in biofilm formation was shown for *B. cepacia* (Huber *et al.*, 2002), *S. liquefaciens* MG1 (Labatte *et al.*, 2004), and *P. putida* IsoF (Steidle *et al.*, 2002). For *B. cepacia* (Huber *et al.*, 2002) and *S. liquefaciens* MG1 (Labatte *et al.*, 2004) it was demonstrated that expression of quorum sensing system-controlled genes is crucial at a specific stage for the development and maturation of the biofilm. In contrast, *P. putida* IsoF wild type produces a very homogeneous biofilm while a quorum sensing mutant appears to form a dense and structured biofilm with characteristic microcolonies and water-filled channels (Steidle *et al.*, 2004).

The present study clearly links quorum sensing in *P. putida* PCL1445 with the synthesis of the cyclic lipopeptides putisolvins I and II and thereby with biofilm formation. Putisolvins seem to function when the bacterial population reaches a high-cell density. The high cell density could form a signal for the release of *Pseudomonas putida* cells. Such a release from the biofilm could be favorable when the nutrient level in the biofilm environment becomes limiting. Moreover, starvation-

mediated stress could play an important role in cell detachment from biofilms since it has been shown for several *Pseudomonas* spp. that the stationary phase sigma factor RpoS influences AHL production (Bertani *et al.*, 2004; Schuster *et al.*, 2004). The production of biosurfactants could stimulate part of the bacteria to colonize other, more favorable, niches, therefore enhancing competitiveness (fitness), pollutant degradation capabilities, or even rhizosphere colonization.

Acknowledgments

We thank P. Williams of the University of Nottingham, UK, for kindly providing the synthetic autoinducers *N*-dodecanoyl-L-homoserine lactone (C_{12}-AHL), *N*-(3-oxo-hexanoyl)-L-homoserine lactone (3-oxo-C_6-AHL), *N*-(3-oxo-octanoyl)-L-homoserine lactone (3-oxo-C_8-AHL), *N*-(3-oxo-decanoyl)-L-homoserine lactone (3-oxo-C_{10}-AHL), *N*-(3-oxo-dodecanoyl)-L-homoserine lactone (3-oxo-C_{12}-AHL), and *N*-(3-oxo-tetradecanoyl)-L-homoserine lactone (3-oxo-C_{14}-AHL). We thank E. Lagendijk for technical assistance and P. Hock for processing the images and the graphs. 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).

Chapter 4

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

Jean-Frédéric Dubern and Guido V. Bloemberg

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 TGGAATTGTGAGCGGATAACAATTTTCACACAGGAAACAGCTAAATGAAGCTGACCGA 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 σ^{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 σ^{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*.

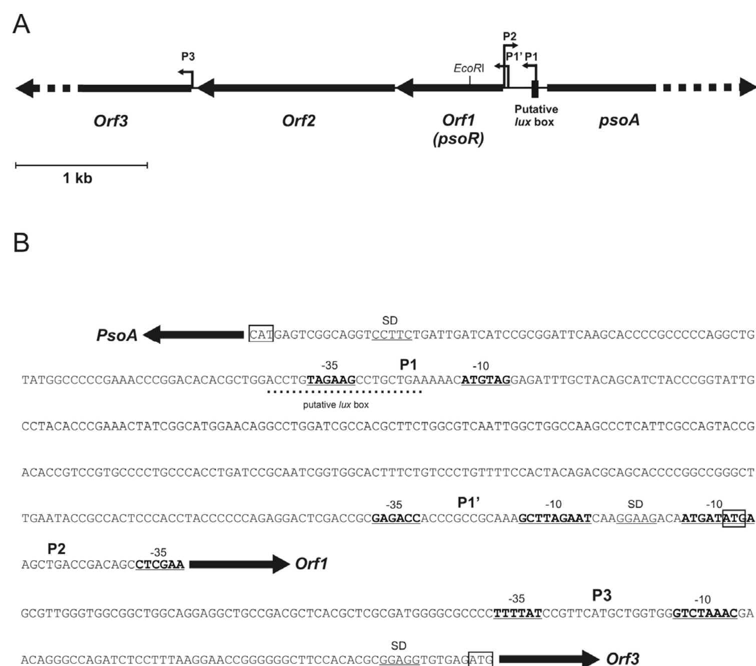

Fig. 1. Sequence analysis of the upstream region of the putisolvin biosynthetic *psoA* gene.
Panel A. Genetic organization of the region upstream of putisolvin biosynthetic gene cluster (*psoA*). The putative promoter elements P1 and P1' of *psoR*, P2 of *psoA*, P3 of *orf3* are indicated. **Panel B.** 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).

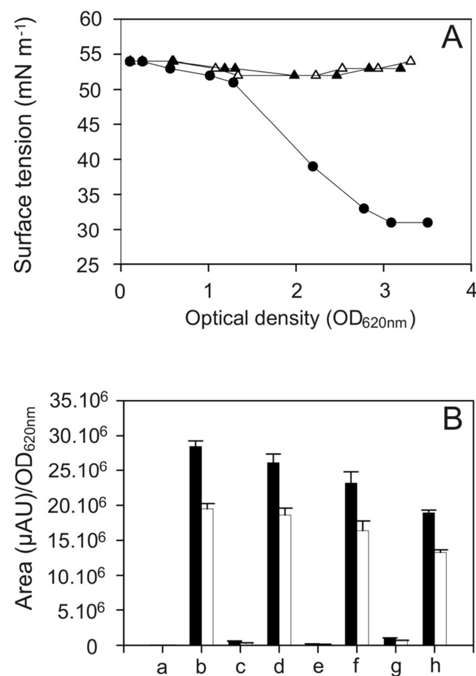

Fig. 2. Biosurfactant production in *psoR* mutant of strain PCL1445. **Panel A.** Quantification of surface tension of culture supernatants of *P. putida* strain PCL1445 (wild type) (●), PCL1633 (*psoA*) (Δ), PCL1626 (*psoR*) (▲), all grown to the stationary phase in KB medium. **Panel B.** Quantification 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.

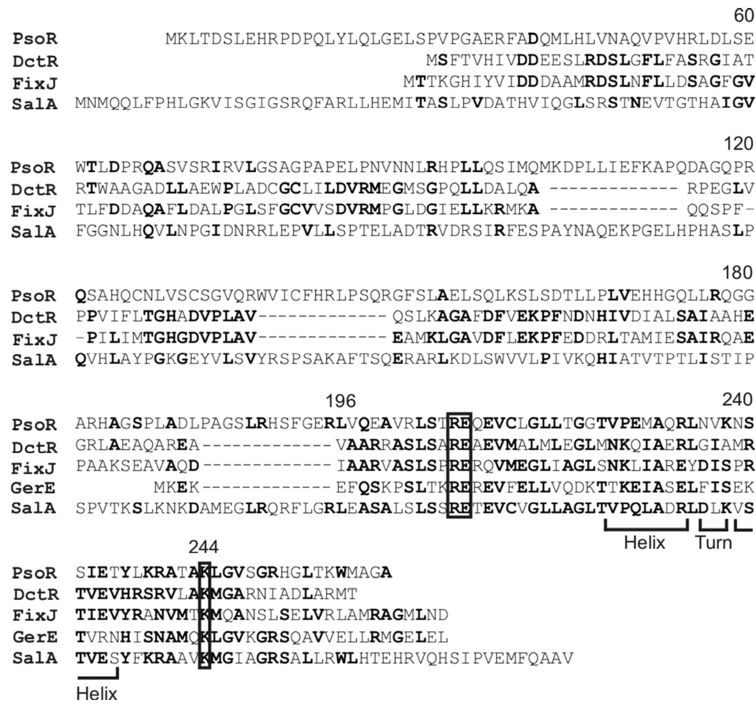

Fig. 3. Alignment of the predicted amino acid sequence of *psoR* with that of related proteins. Amino acids that are shared among two or more proteins are indicated in bold. Dots indicate gaps introduced to optimize alignments. The three conserved amino acid residues among regulatory proteins of the 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).

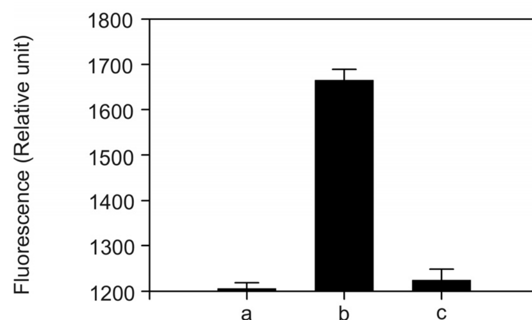

Fig. 4. Expression of *psoA* of *P. putida* PCL1445. Expression was determined using the *psoA*::*gfp* reporter in PCL1445, and PCL1626 (*psoR*) by measuring fluorescence from cells containing the *psoA* promoter fused to e*gfp* (pMP5539). pMP5540 containing the *psoA* promoter in the transcriptionally 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°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).

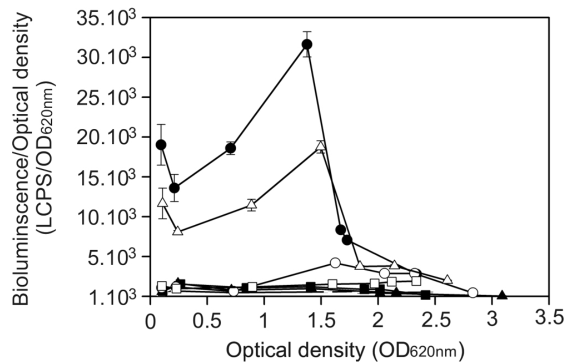

Fig. 5. Expression of *psoR* of *P. putida* PCL1445. Expression was determined using the *psoR*::*luxAB* reporter in PCL1445, PCL1623 (*gacS*), PCL1622 (*gacA*), PCL1627 (*dnaK*) by measuring luminescence from cells containing the *psoR* promoter fused to *luxAB* (pMP782). pMP7579 lacking the *psoR* 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.

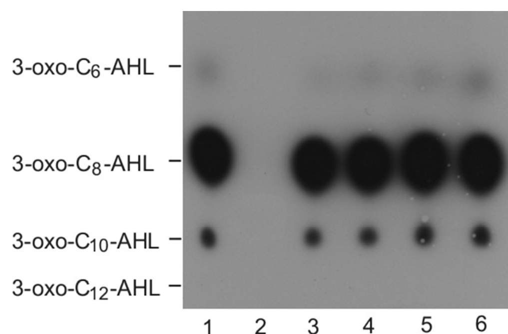

Fig. 6. C18-reverse phase thin-layer chromatography analysis of *N*-acyl-L-homoserine lactones produced by *P. putida* PCL1445 and its mutant derivatives.
Cells of strain *P. putida* PCL1445 and its derivatives mutants were grown in BM-glycerol to an OD_{620} value of 1.0 and centrifuged. The supernatant 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°). 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.

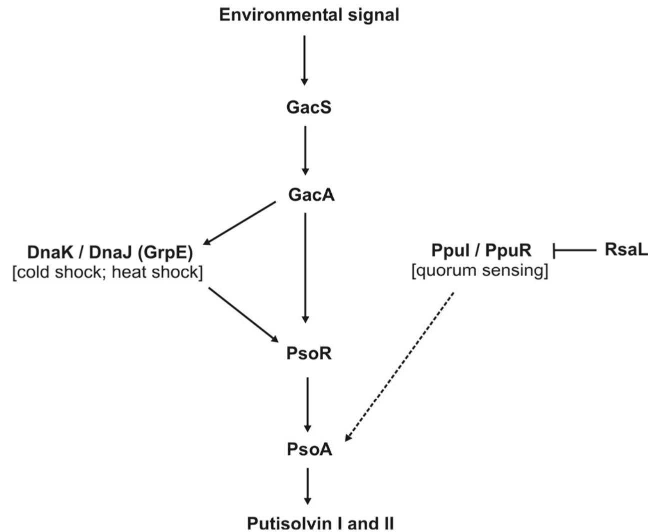

Fig. 7. Working model for the regulation of the putisolvin biosynthetic gene, *psoA*, in *P. putida* PCL1445. Regulatory systems and regulatory proteins that were reported to take part in putisolvin biosynthesis are GacA/GacS two-component system, DnaK-DnaJ-GrpE heat-shock system, PpuI-RsaL-PpuR quorum 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

We thank P. Williams of the University of Nottingham, UK, for kindly providing the synthetic autoinducers *N*-dodecanoyl-L-homoserine lactone (C_{12}-AHL), *N*-(3-oxo-hexanoyl)-L-homoserine lactone (3-oxo-C_6-AHL), *N*-(3-oxo-octanoyl)-L-homoserine lactone (3-oxo-C_8-AHL), *N*-(3-oxo-decanoyl)-L-homoserine lactone (3-oxo-C_{10}-AHL), *N*-(3-oxo-dodecanoyl)-L-homoserine lactone (3-oxo-C_{12}-AHL), and *N*-(3-oxo-tetradecanoyl)-L-homoserine lactone (3-oxo-C_{14}-AHL). 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).

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 $FeCl_3$.

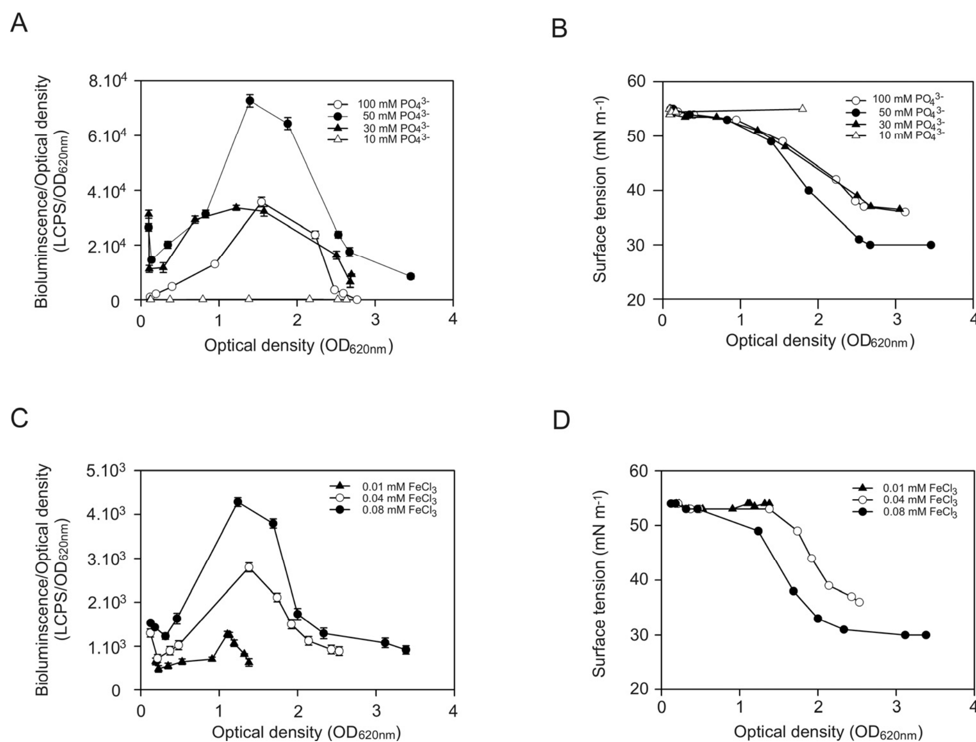

Fig. 1. Effect of phosphate concentration (PO_4^{3-}) and ferric iron ion concentration ($FeCl_3$) in BM-glycerol medium on expression of putisolvin synthetase gene (*psoA*) and putisolvin production in *P. putida* PCL1445. **Panel A.** Quantification of bioluminescence in mutant strain PCL1633 (*psoA*::Tn5*luxAB)*. 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.

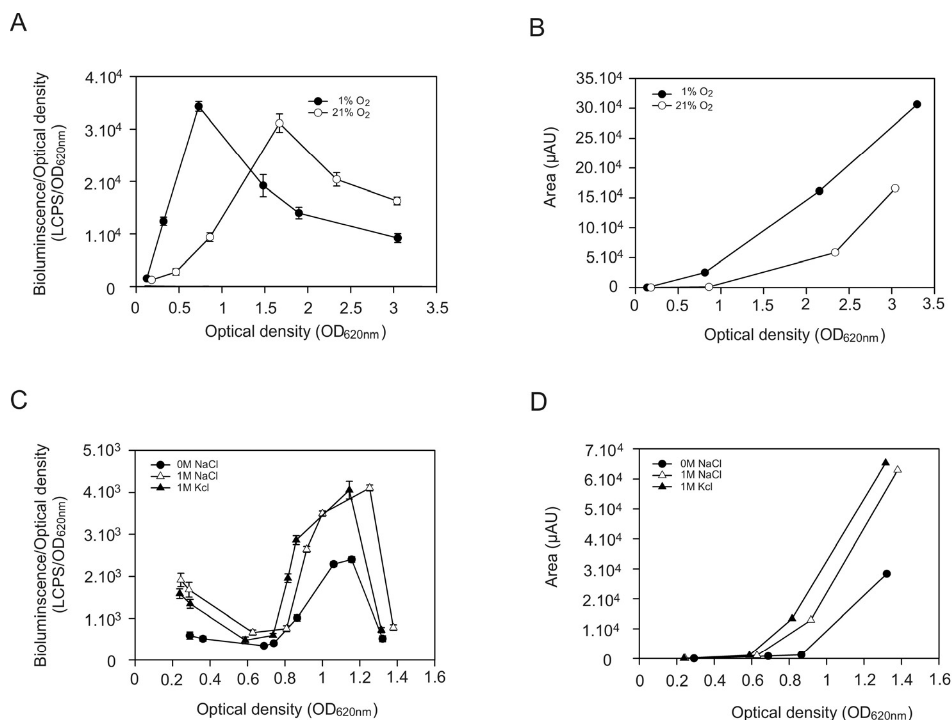

Fig. 2. Influence of different concentrations of oxygen and salt (NaCl or KCl) on expression of the putisolvin synthetase gene (*psoA*) and putisolvin production in *P. putida* PCL1445. **Panel A.** Bioluminescence in mutant strain PCL1633 (*psoA*::Tn5*luxAB*). Cells were cultured in KB medium with different 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 11ºC when compared to their expression values at 28ºC (Fig. 3A and 3B, respectively). In contrast, expression of *gacA* and *gacS* at 32ºC 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).

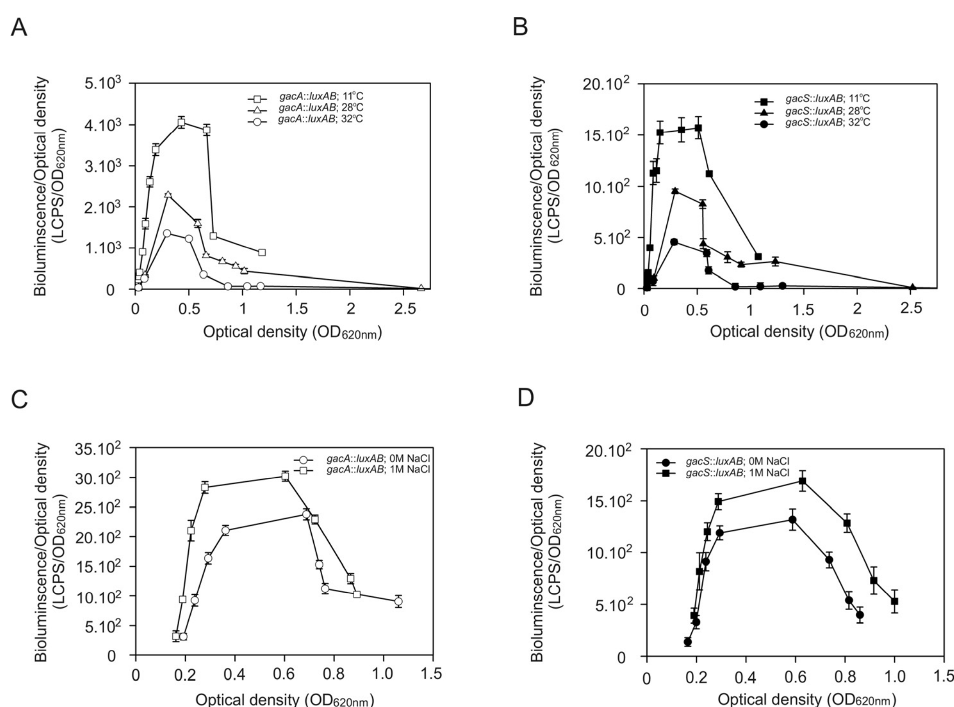

Fig. 3. Effect of temperature and NaCl concentration on the expression of *gacA* and *gacS* of *Pseudomonas putida* strain PCL1445. Quantification of bioluminescence produced by cells of (**panel A**) strain PCL1622 (*gacA*::Tn5*luxAB*), and (**panel B**) strain PCL1623 (*gacS*::Tn5*luxAB*) cultured 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

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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 hypothezised 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.

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Samenvatting

Een aantal structureel verschillende moleculen kunnen biosurfactant activiteit hebben. De bekendste van deze moleculen zijn lipopeptiden en glycolipiden. Biosurfactanten worden meestal geproduceerd door bacteriën. Ze zijn in staat om emulsies te vormen. De biosurfactant moleculen bevinden zich vooral in de interfase tussen fasen met verschillende polariteit zoals olie/water of lucht/water. Biosurfactanten kunnen interessant zijn voor toepassingen in zowel het milieu als in de geneeskunde. Een voorbeeld van het eerste is dat biosurfactanten de oplosbaarheid van vele xenobiotica verhogen (b.v. van olieproducten) waardoor ze sneller kunnen worden afgebroken. Een ander voorbeeld is dat biosurfactanten ionen van zware metalen kunnen binden (zoals die van cadmium, lood, koper en zink) waardoor deze beter uit verontreinigde grond kunnen worden verwijderd. Met betrekking tot medische en therapeutische toepassingen blijken biosurfactantia actief te kunnen zijn tegen zowel bacteriën, schimmels als virussen. Ze kunnen in principe zelfs worden toegepast voor vaccinatie en gentherapie.

Natuurlijke biosurfactanten hebben een aantal voordelen boven gesynthetiseerde surfactanten zoals een lage toxiciteit voor het milieu en voor de gezondheid van de mens, milieu-compatibiliteit en hoge selectiviteit (b.v. tegen één specifieke klasse van verontreiniging of tegen één klasse van pathogene micro-organismen) onder extreme milieu-omstandigheden (zoals hoge temperatuur, pH of zoutconcentratie). Hoge productiekosten belemmeren een brede toepassing van biosurfactanten. Daarom richt het onderzoek naar biosurfactanten zich momenteel voornamelijk op het ontdekken van nieuwe klassen biosurfactant moleculen, op hun rol in de natuur, en op de identificatie en regulatie van de genen die verantwoordelijk zijn voor de productie en regulatie van biosurfactanten.

De bacterie die gebruikt werd voor het onderzoek beschreven in dit proefschrift is *Pseudomonas putida* stam PCL1445. Eerder was aangetoond dat PCL1445 twee lipopeptide biosurfactanten produceert, genaamd putisolvin I en putisolvin II. Deze twee putisolvins vertegenwoordigen een nieuwe klasse van biosurfactant moleculen. Putisolvins vormen een nieuwe klasse van biosurfactant moleculen omdat ze de eerste lipopeptiden zijn die bestaan uit een 12 aminozuren lange peptideketen gekoppeld aan een zes C-atomen bevattende vetzuurketen. Deze nieuwe structuur geeft interessante eigenscappen aan putisolvins. Zo kunnen ze

emulsificering (mengen van olieachtige verbindingen met water) van toxische componenten zoals tolueen bevorderen. Ook kunnen ze de oplosbaarheid van bijvoorbeeld naftaleen en fenantreen, twee bekende milieuverontreinigingen, stimuleren. Deze eigenschappen maken putisolvins veelbelovend om toe te passen tegen milieuverontreinigingen.

In de natuur kunnen bacteriën een gemeenschap van cellen vormen op een bepaald oppervlak, zoals op de wortels van planten. Zulke biofilms hebben een enorme impact in medische en industriële settings en in de landbouw. Deze biofilms kunnen zowel een schadelijk als nuttig effect hebben. Een groot probleem is dat biofilms vaak ongevoelig zijn voor veel antibiotica. Putisolvins zijn in staat om reeds gevormde biofilms af te breken, zoals die gevormd door de mens-opportunistische pathogeen *Pseudomonas aeruginosa,* een veroorzaker van veelvoorkomende infecties in ziekenhuizen. Deze laatste eigenschap maakt putisolvins erg aantrekkelijk om te gebruiken in medische toepassingen tegen infecties. Al deze eigenschappen van biosurfactanten maken deze moleculen voor wetenschappers een erg gewild onderwerp om te bestuderen, met name de regulatie-mechanismen betrokken bij hun productie. Het doel van dit proefschrift is om de regulatie mechanismen betrokken bij de productie van putisolvins te identificeren, de functie van putisolvins voor de bacterie te begrijpen, en te proberen de putisolvin productie voor eventuele toepassingen in de toekomst te verhogen.

In dit proefschrift zijn drie verschillende benaderingen gebruikt om de genen the identificeren die betrokken zijn bij putisolvin productie. Ten eerste, willekeurige mutagenese. Deze benadering resulteerde in de identificatie van twee regulatie-systemen, nl. een heat shock systeem (de expressie van deze genen wordt gereguleerd door een plotselinge verandering van de omgevingstemperatuur) en een twee-componenten signaal systeem (waarbij een eiwit aan het bacterie-oppervlak een verandering in de omgeving, zoals stress, waarneemt en doorgeeft aan een tweede, binnen in de cel gelegen. eiwit hetgeen resulteert in expressie van specifieke genen) (**Hoofdstuk 2**). Als tweede benadering werden genen waarvan verwacht werd dat ze waarschijnlijk betrokken zijn bij putisolvin productie en/of bij biofilm vorming gemuteerd in PCL1445 door gerichte mutagenese. Dit resulteerde in de identificatie van een zgn “Quorum sensing” systeem. Zo’n systeem is betrokken bij het gedrag van en de communicatie tussen bacteriën door middel van kleine diffundeerbare moleculen. Het effect van deze moleculen op putisolvin productie en biofilm vorming is onderzocht en werd beschreven in **Hoofdstuk 3**. Ten derde werd de nucleotide volgorde van het DNA dat voorafgaat aan het putisolvin gen

bestudeerd op de aanwezigheid van regulatiegenen. Deze benadering resulteerde in de identificatie van nieuwe regulatie genen betrokken bij putisolvin productie (**Hoofdstuk 4**). Tenslotte is het effect van verschillende omgevingsfactoren en condities op het productie-nivo van putisolvin bestudeerd. In **Hoofdstuk 5** zijn pogingen beschreven om de productie van putisolvin te verhogen.

In **Hoofdstuk 2** wordt de identificatie en de functie van drie genen beschreven die worden geïnduceerd onder invloed van plotselinge temperatuurveranderingen. Deze drie genen, *dnaK*, *dnaJ* en *grpE,* spelen een cruciale rol bij het overleven van de bacterie onder extreme temperatuur variaties, vooral bij hoge temperaturen. Productie van putisolvins blijkt door deze genen te worden gereguleerd. Deze vinding resulteerde in de hypothese dat putisolvins van belang zijn voor de bacterie om te overleven in zijn eigen omgeving. Een tweede ontdekking was dat de putisolvin productie wordt gestimuleerd bij lage temperaturen en dat deze productie wordt gecontroleerd door de drie genoemde "heat shock" genen. Deze ontdekking resulteerde in het vermoeden dat PCL1445 zich goed voelt in een koude omgeving. Een derde ontdekking was dat een twee-componenten systeem betrokken is bij putisolvin productie. Dit systeem neemt een signaal uit de omgeving waar en vertaalt dit externe signaal naar het binnenste van de cel om de putisolvin productie te reguleren. Deze vinding laat zien dat de productie van putisolvin wordt bepaald door de leefomgeving van de bacterie. Dit versterkt ons vermoeden dat de functie van putisolvin is om het voor de bacterie makkelijk te maken zich aan de steeds veranderende omgeving aan te passen.

Bacteriën kunnen niet meer worden beschouwd als zelfstandige losse cellen met enig vermogen tot collectief gedrag. Bacteriecellen zijn in feite zeer communicatief en bezitten daardoor een buitengewone aanleg voor sociaal gedrag. Bacteriën coördineren hun activiteiten door het produceren en detecteren van kleine diffundeerbare moleculen die een bacteriegemeenschap in staat stelt als eenheid te functioneren. Deze vorm van samenwerking wordt "quorum sensing" genoemd en speelt een centrale rol in de levenscyclus van bacteriën. Door te begrijpen hoe quorum sensing bij bacteriën werkt kunnen we cel-tot-cel communicatie gebruiken om het gedrag van een totale leefgemeenschap, zoals een biofilm, te beinvloeden en daarmee het gedrag van een schadelijke of nuttige bacterie-gemeenschap te sturen. De bacterie *P. putida* PCL1445 is in staat tot bovengenoemd sociaal gedrag. In **Hoofdstuk 3** is beschreven dat PCL1445 kleine diffundeerbare moleculen, AHL's (*N*-acylhomoserine lactonen), produceert die productie van putisolvins reguleren en de biofilm vorming beïnvloeden. Wij speculeren dat de complete bacterie-populatie de

biosynthese van putisolvin coördineert. Wanneer voedingsstoffen limiterend worden of wanneer andere stress-omstandigheden heersen, zal een deel van de populatie de biofilm verlaten om een prettiger leefomgeving te zoeken en aldaar weer een biofilm te vormen.

In **Hoofdstuk 4** is een ander belangrijk gen beschreven dat de expressie van putisolvins controleert. Dit gen, dat dicht bij het structurele gen voor putisolvin *psoA* ligt, codeert voor een transcriptioneel regulator-eiwit. Dit eiwit herkent en bindt aan een korte DNA sekwentie stroomopwaarts van het *psoA* gen hetgeen resulteert in de inductie van het *psoA* gen.

In **Hoofdstuk 5** zijn studies beschreven over het effect van omgevingsfactoren op de productie van putisolvin. Deze studies laat zien dat specifieke omgevingsstimuli, zoals beschikbaarheid van voedingsstoffen, hoge zout-concentraties en lage zuurstof-beschikbaarheid een essentiële rol spelen bij putisolvin productie.

In dit proefschrift werden nieuwe mechanismen beschreven voor het reguleren van de biosynthese van biosurfactanten. De resultaten kunnen bijdragen aan het begrijpen van de functie van deze moleculen bij de proliferatie van bacteriën en van hun invloed op het milieu. Een vraag die overblijft is of deze mechanismen algemeen voorkomen onder bacteriën. Door onze kennis over biosurfactanten te vergroten maken we het mogelijk om de productie van biosurfactanten te manipuleren en kunnen we gebruik maken van hun eigenschappen voor toepassingen in geneeskunde, industrie en landbouw.

Résumé

Les biosurfactants regroupent une classe de molécules tensioactives, structuralement variées, et communément synthétisées par les micro-organismes. Les molécules de surfactant se localisent préférentiellement à l'interface entre deux milieux fluides ayant différents degrés de polarité. Cette propriété rend les surfactants capables de former des émulsions entre le milieu huileux et le milieu aqueux. Les biosurfactants offrent des applications variées aussi bien dans le domaine environnemental que le domaine médical. Dans le domaine environnemental, les biosurfactants sont capable de solubiliser les composés xénobiotiques (polluants issus des activités humaines) améliorant ainsi leur biodégradabilité et de chélater les métaux lourds (e.g. le cadmium, le cuivre, le plomb et le zinc) permettant ainsi leur élimination des sols contaminés. Récemment le rôle et les multiples applications des biosurfactants (principalement les lipopeptides et glycolipides) ont fait l'objet d'intenses recherches dans les domaines médical et thérapeutique. Certains lipopeptides et glycolipides sont d'excellent agents anti-bactériens, anti-fungiques et anti-viraux et font aussi l'objet de recherches approfondies dans le domaine de la thérapie génique.

Les biosurfactants, de par leur faible toxicité vis-à-vis de l'environnement et de la santé humaine, leur compatibilité, leur sélectivité pour une classe de polluant ou une espèce pathogène et pour des milieux extrêmes (e.g. variations élevées de températures, pH et salinité), possèdent de nombreux avantages par rapport aux surfactants synthétiques. Une des raisons limitant leur utilisation à grande échelle est leur coût de production. Par conséquent les recherches sur les biosurfactants se sont orientées vers la découverte de nouvelles molécules, l'étude de leur rôle naturel, et l'identification des gènes régulant leur biosynthèse.

La souche bactérienne décrite dans cette thèse est *Pseudomonas putida* PCL1445. *P. putida* PCL1445 produit deux biosurfactants formant une nouvelle classe de lipopeptides cycliques, putisolvin I et putisolvin II. Putisolvin solubilisent le naphtalène et le phénanthrène (deux composés hautement toxiques) et émulsifient le toluène. Ces propriétés rendent l'utilisation de putisolvin possible dans le traitement des sols contaminés par ces polluants.

Dans la nature, les bactéries forment des communautés de cellules adhérentes à une surface (e.g. sur la racine des plantes) et appelées biofilms. Les biofilms ont un énorme impact dans les milieux médical, industriel et agricole,

arborant des activités aussi bien bénéfiques que nuisibles. Les molécules de Putisolvin sont capables de solubiliser les biofilms incluant ceux, hautement résistants aux traitements par les antibiotiques, formés par le pathogène opportuniste *Pseudomonas aeruginosa* responsable d'infections graves acquises en milieu hospitalier (maladies nosocomiales). Cette propriété rend l'utilisation de biosurfactant putisolvin possible dans le domaine médical et notamment dans l'éradication des biofilms formés par les bactéries pathogènes pour l'homme. Le potentiel de ces biosurfactants stimule l'élucidation de leur biosynthèse et des mécanismes régulant leur production. Le but des travaux décrits dans cette thèse a donc été l'identification des systèmes de régulations jouant un rôle dans la biosynthèse de putisolvin, l'évaluation de leur rôle dans le fonctionnement de la bactérie, et l'amélioration de leur production pour de futures applications.

Trois approches ont été utilisées afin d'identifier les gènes contrôlant la production de putisolvin. Premièrement, la stratégie de la mutagenèse au hasard (ou « random mutagenesis ») de PCL1445 a conduit a l'identification de deux systèmes de régulation composés de gènes «heat-shock» (gènes dont l'expression est induite par une brusque variation de température dans l'environnement) et un système de transduction du signal (composé par une protéine sensorielle activée par un signal environnemental et une protéine transmettant le signal a travers une cascade de régulation conduisant à l'activation de gènes). (**Chapitre 2**). Deuxièmement, plusieurs gènes susceptibles de jouer un rôle dans la production de putisolvin et affectant la formation de biofilm ont été mutés dans PCL1445, ce qui a conduit a l'identification d'un système basé sur le « quorum sensing » contrôlant la communication intercellulaire et par conséquent le comportement de la communauté bactérienne toute entière. Les effets de ce système sur la production de putisolvin et sur la formation de biofilm par PCL1445 ont été étudiés dans le **Chapitre 3**. Troisièmement, la séquence d'ADN localisée en amont du gène biosynthétique dédié à la production de putisolvin a été analysée afin de détecter la présence de gènes régulateurs. Cette étude a conduit à l'identification d'un nouveau gène régulant la biosynthèse de putisolvin (**Chapitre 4**). Enfin les effets de facteurs environnementaux ont été analysés afin d'améliorer la production de putisolvin (**Chapitre 5**).

L'identification et le rôle de trois gènes dont l'expression est induite par une brusque variation de température et particulièrement par une brusque augmentation de celle-ci sont décrits dans le **Chapitre 2**. Ces trois gènes (*dnaK*, *dnaJ* et *grpE*) forment un système de réponse au stress et jouent un rôle crucial

dans la survie de la bactérie en contrôlant l'expression d'autres gènes qui protègent la bactérie contre les agressions extérieures à la cellule. Dans ce chapitre, nous avons démontré que la biosynthèse de putisolvin est régulée par ce système de réponse au stress. Cette découverte indique que le biosurfactant putisolvin joue un rôle central dans la survie de la bactérie dans son propre environnement. La seconde découverte est que la production de biosurfactant putisolvin augmente fortement à basse température et que cette production est sous le contrôle des gènes « heat-shock », indiquant que PCL1445 est adapté à une vie à basse température. L'identification d'un système de transduction du signal (appelé « two-component system ») jouant un rôle dans le contrôle de la production de putisolvin indique enfin que la production de ce biosurfactant est modulée par les conditions environnementales. Cette étude conforte l'hypothèse selon laquelle les biosurfactants jouent un rôle dans l'adaptation de la bactérie productrice dans des conditions environnementales changeantes.

Les bactéries ne sont pas des organismes unicellulaires autonomes au comportement collectif limité. Les cellules bactériennes sont en fait hautement communicatives et possèdent un comportement social. Les bactéries sont capables de coordonner leurs activités en produisant et en détectant des petites molécules signales diffusibles qui permettent à une population toute entière d'agir en groupe (formant ainsi un biofilm) plutôt que de manière individuelle. Un tel comportement social, appelé « quorum sensing », joue un rôle central dans le cycle de vie de la bactérie. En apprenant comment le système « quorum sensing » agit il est ainsi possible d'exploiter le système de communication intercellulaire bactérien et de découvrir le moyen de contrôler le comportement d'une communauté entière de cellules (le biofilm), et par conséquent le comportement nuisible ou bénéfique des micro-organismes. La bactérie *P. putida* PCL1445 est dotée d'un tel comportement social. Dans le **Chapitre 3**, nous avons découvert que PCL1445 produit des molécules signales (appelées *N*-acyl-homoserine lactones ou AHLs) contrôlant la production de putisolvin et participant à la formation de biofilm. Cette étude suggère que la communauté bactérienne coordonne la biosynthèse de biosurfactant lorsqu'une source nutritive devient limitante ou lors d'un stress environnemental conduisant ainsi grâce aux propriétés «tensioactives» de ces molécules au détachement d'une partie de la population bactérienne et ce afin de coloniser un autre milieux (ou niche écologique) plus favorable à leur établissement et à la formation de nouvelles communautés de bactéries

Le **Chapitre 4** décrit la découverte d'un autre gène important régulant l'expression du gène biosynthétique dédié à la production du biosurfactant putisolvin.

Dans le **Chapitre 5**, l'impact de différents facteurs environnementaux sur la production de putisolvin a été analysé. L'étude révèle que des stimuli spécifiques environnementaux, incluant la disponibilité de sources nutritives, une salinité élevée et une faible oxygénation du milieu jouent un rôle essentiel dans la biosynthèse de putisolvin en contrôlant l'expression des gènes *gacA* et *gacS* formant le système de transduction du signal.

En conclusion, de nouveaux mécanismes dédiés à la régulation de la biosynthèse de biosurfactants ont été décrits contribuant ainsi à la connaissance du rôle de ces molécules dans la prolifération bactérienne et de leur impact sur l'environnement. Cependant il reste encore à savoir si ces mécanismes de régulation sont répandus chez d'autres espèces bactériennes. Par conséquent l'accroissement des connaissances sur le fonctionnement des biosurfactants permettrait de manipuler leur production afin d'exploiter leur propriétés pour des applications dans les domaines médical, industriel ou agricole.

Curriculum vitae

Jean-Frédéric Bertrand Dubern was born on the 17th of May 1976 in Mulhouse, France. He attended high school at “Jean Monnet” in Montpellier, France. In 1997, he entered the “Université des Sciences et Techniques du Languedoc” in Montpellier. In February 2001, he joined the department of Molecular Microbiology at the Institute of Biology, Leiden, The Netherlands as an Erasmus student. At the IBL he was involved in the project “Isolation and Characterization of genes involved in phase variation in *Pseudomonas*”, under the supervision of Dr. T.F. Chin-A-Woeng and Dr. D. van den Broek. In June 2001, he received his “Maîtrise” of Molecular Cell Biochemistry. From October 2001 through January 2006, he was working on his Ph.D thesis “Regulation of the biosynthesis of novel cyclic lipopeptides from *Pseudomonas putida* strain PCL1445”, under the supervision of Prof. Dr. E. J. J. Lugtenberg and Dr. G. V. Bloemberg. Since the 1st of June 2006, he is working as a post-doc at the Laboratory of Phytopathology, Wageningen University, under the supervision of Prof. Dr. P. J. de Wit and Dr. J. M. Raaijmakers.