Regulation of the biosynthesis of cyclic lipopeptides from Pseudomonas putida PCL1445

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Citation

Dubern, J. F. (2006, June 19). *Regulation of the biosynthesis of cyclic lipopeptides from Pseudomonas putida PCL1445*. Retrieved from https://hdl.handle.net/1887/4408

Version:	Not Applicable (or Unknown)
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Note: To cite this publication please use the final published version (if applicable).

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.

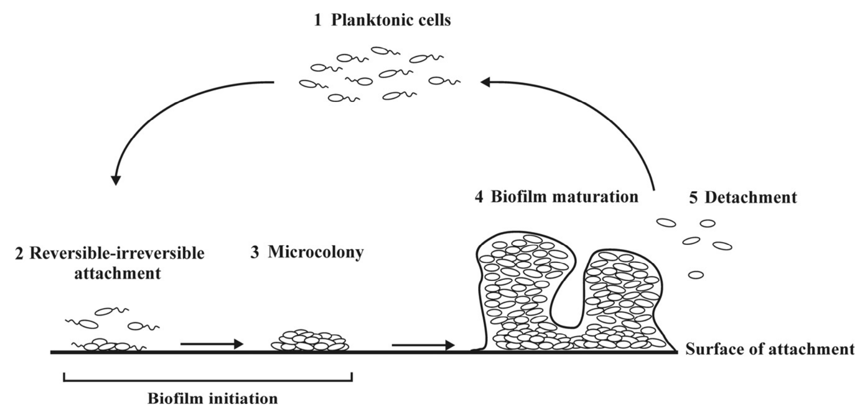

Fig. 1. Schematic representation of the distinct steps in biofilm development as typically observed for gram-negative bacteria (adapted from Toutain et al., 2003).

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).

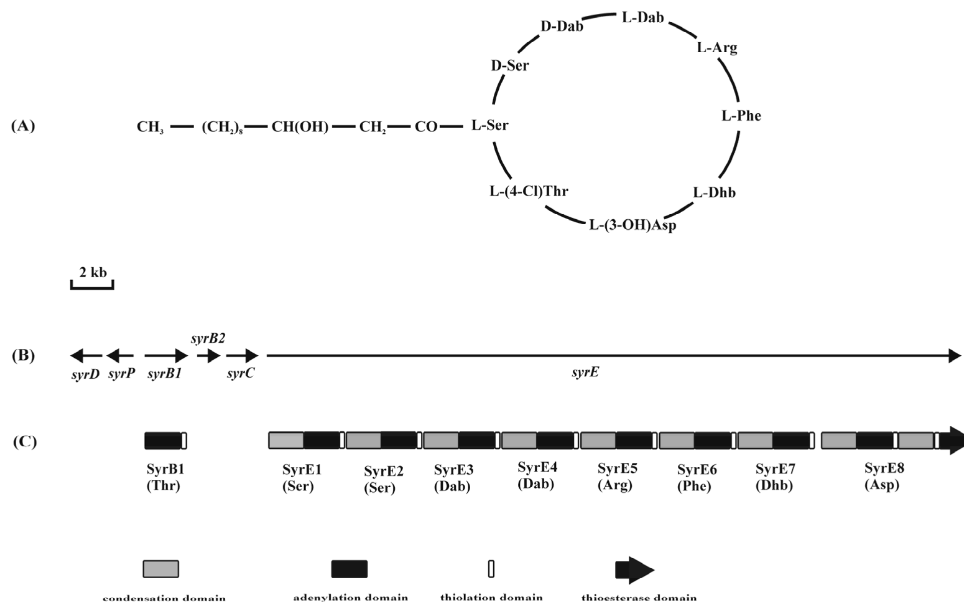

Fig. 2. Biosynthesis of the peptide moiety of the cyclic lipopeptide syringomycin. (A) Structure of syringomycin. (B) Genetic map of the syringomycin biosynthetic gene cluster in *P. syringae* pv. *syringae*. (C) The enzymes are composed of modules that can be subdivided into domains (see section 2.4). 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.

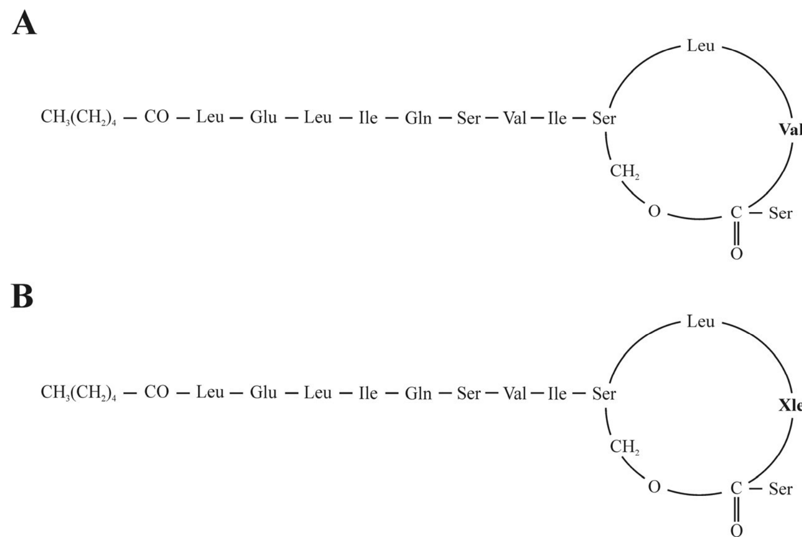

Fig. 3. Structure of putisolvin, adapted from Kuiper et al. (2004). **A:** putisolvin I; **B:** putisolvin II. Xle represents Leu or Ile.

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).

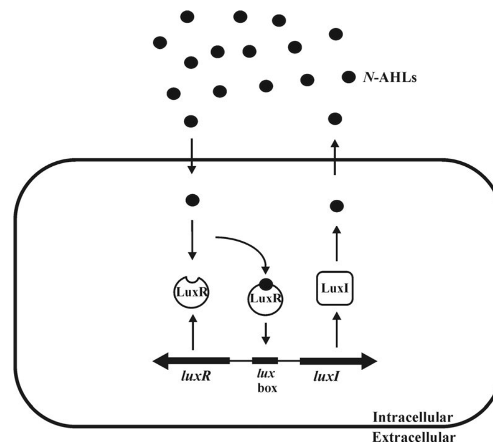

Fig. 4. Model for quorum sensing in the marine symbiont *V. fisheri.*

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. chololoraphis* (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).

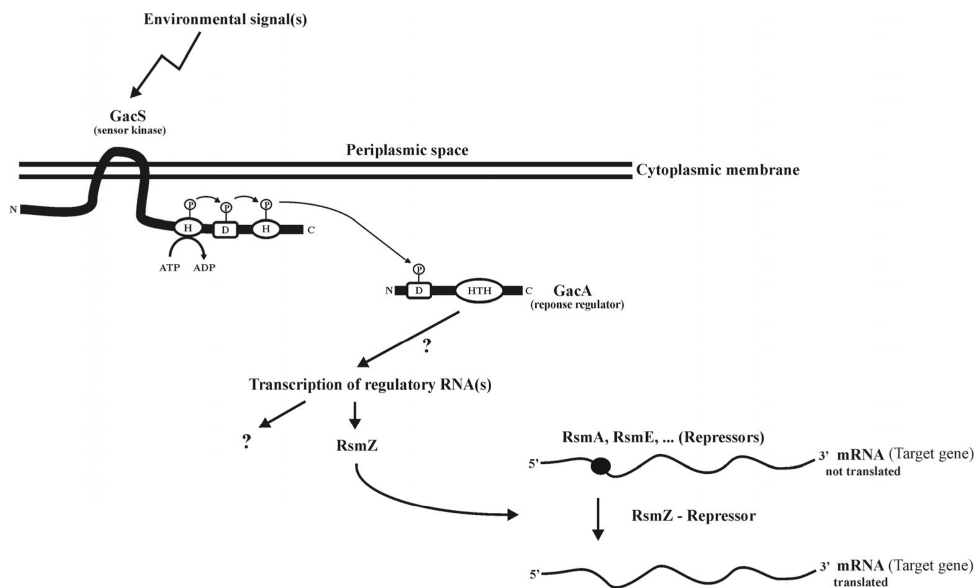

Fig. 5. Current model for the regulation of secondary metabolites involving the GacS/GacA two-component system (adapted from Haas et al., 2003).

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).

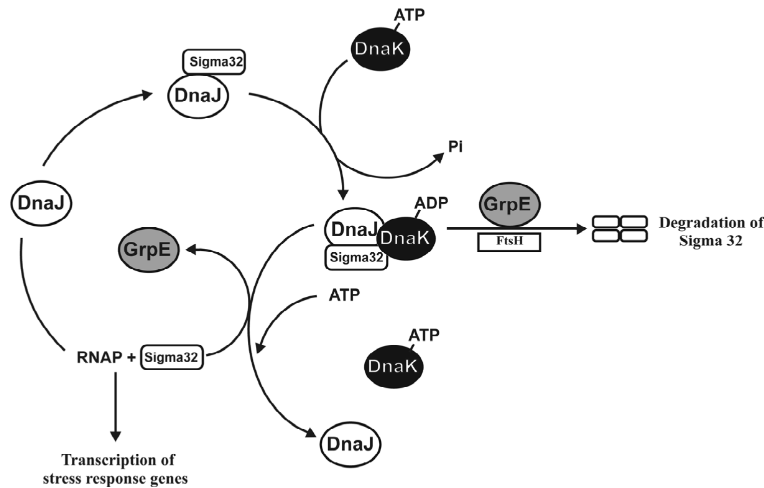

Fig. 6. Model for the regulation of the heat-shock reponse in *Escherichia coli* (Adapted from Hughes and Mathee, 1998).

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**).