Natural functions of lipopeptides from *Bacillus* and *Pseudomonas*: more than surfactants and antibiotics

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**Abstract**

Lipopeptides constitute a structurally diverse group of metabolites produced by various bacterial and fungal genera. In the past decades, research on lipopeptides has been fueled by their antimicrobial, antitumour, immunosuppressant and surfactant activities. However, the natural functions of lipopeptides in the lifestyles of the producing microorganisms have received considerably less attention. The substantial structural diversity of lipopeptides suggests that these metabolites have different natural roles, some of which may be unique to the biology of the producing organism. This review gives a detailed overview of the versatile functions of lipopeptides in the biology of *Pseudomonas* and *Bacillus* species, and highlights their role in competitive interactions with coexisting organisms, including bacteria, fungi, oomycetes, protozoa, nematodes and plants. Their functions in cell motility, leading to colonization of novel habitats, and in the formation and development of highly structured biofilms are discussed in detail. Finally, this review provides an update on lipopeptide detection and discovery as well as on novel regulatory mechanisms and genes involved in lipopeptide biosynthesis in these two bacterial genera.

**Introduction**

A wide range of structurally different biosurfactants have been identified to date, including glycolipids, lipopeptides, polysaccharides, proteins and lipoproteins, or mixtures thereof (Neu, 1996; Banat et al., 2000; Ron & Rosenberg, 2001; Maier, 2003; Mulligan, 2005; Muthusamy et al., 2008). Lipopeptide biosurfactants (LPs) are composed of a lipid tail linked to a short linear or cyclic oligopeptide (Fig. 1). They are produced by fungi, including *Aspergillus*, and various bacterial genera such as *Streptomyces*, *Pseudomonas* and *Bacillus*. LPs have received considerable attention for their antimicrobial, cytotoxic, antitumour, immunosuppressant and surfactant properties (Cameotra & Makkar, 2004; Donadio et al., 2007; Gross & Loper, 2009; Pirri et al., 2009). The proposed primary mode of action of LPs is pore formation in membranes, leading to an imbalance in transmembrane ion fluxes and cell death (Bender et al., 1999; Baltz, 2009). Hence, there is a growing interest in the discovery, combinatorial synthesis and exploitation of ‘new’ and ‘old’ LPs for diverse environmental and pharmaceutical applications (Pirri et al., 2009). Among the bacterial LP producers, *Pseudomonas* and *Bacillus* have received the most attention. Both genera are predominant in various natural habitats, they harbour pathogenic and beneficial species, and exhibit diverse lifestyles. For *Pseudomonas* and *Bacillus* species, there is a vast body of information on the structural diversity of LPs, their biosynthesis and broad-spectrum antimicrobial activities (Bender et al., 1999; Nybroe & Sørensen, 2004; Raaijmakers et al., 2006; Ongena & Jacques, 2008; Gross & Loper, 2009). In contrast, the natural roles of LPs for the producing bacteria have received considerable less attention.

This review brings together information for *Pseudomonas* and *Bacillus* LPs and gives a detailed overview of the versatile functions of LPs in the biology of these bacteria. We specifically address the roles of LPs in interactions with coexisting organisms, including bacteria, fungi, oomycetes, protozoa, nematodes and plants. We also provide an update on LP detection and discovery as well as on novel regulatory
mechanisms and genes involved in LP biosynthesis in Pseudomonas and Bacillus. Whenever possible, comparisons are made with related or structurally different surfactants produced by other bacterial genera.

**Structural diversity**

Among the LPs produced by Bacillus and Pseudomonas species, there is considerable structural diversity due to differences in the length and composition of the lipid moiety as well as in the type, number and configuration of the amino acids in the peptide chain (Fig. 1) (Nybroe & Sørensen, 2004; Stein, 2005; Raaijmakers et al., 2006; Onkena & Jacques, 2008; Gross & Loper, 2009). Based on these structural characteristics, the cyclic LPs produced by Pseudomonas were initially classified into four major groups: viscosin, amphisin, tolaasin and syringomycin (Nybroe & Sørensen, 2004). The viscosin group consists of LPs with nine amino acids (AA), whereas members of the amphisin group harbour 11 AA in the peptide moiety. For both LP groups, the lipid tail is in most cases 3-hydroxydecanoic acid. LPs in the tolaasin group are more diverse due to variations in the length of the peptide chain (19–25 AA) and the lipid tail (3-hydroxydecanoic acid or 3-hydroxyoctanoic acid). Furthermore, LPs of the tolaasin group harbour several unusual amino acids, such as 2,3-dehydro-2-amino-butyric acid and homoserine. Also the 9 AA LPs of the syringomycin group contain unusual amino acids such as 2,4-diaminobutyric acid and a C-terminal chlorinated threonine residue (Grgrurina et al., 1994; Bender et al., 1999; Bender & Scholz-Schroeder, 2004; Gross & Loper, 2009). In recent years, a number of structurally new LPs have been identified for Pseudomonas, including arthrolactin of Pseudomonas (formerly Arthrobacter) sp. strain MIS38 (Roongsawang et al., 2003), putisolvins I and II of Pseudomonas putida (Kuiper et al., 2004; Krujit et al., 2009), orfamide of Pseudomonas fluorescens strain Pf-5 (Paulsen et al., 2005; Gross et al., 2007) and pseudodesmins A and B of a Pseudomonas strain isolated from salamander skin (Sinnaeve et al., 2009). Several of these LPs have structural features that are different from the four main cyclic LP groups and make the initial classification more diffuse. For example, the putisolvins have a hexanoic lipid tail, a peptide moiety of 12 AA and cyclization occurs between the C-terminal carboxyl group and the ninth amino acid residue (Kuiper et al., 2004), instead of the first or third amino acid in other Pseudomonas LPs (Fig. 1). Furthermore, a number of linear LPs were recently discovered from Pseudomonas, including syringofactins of Pseudomonas syringae pv. tomato strain DC3000 (Berti et al., 2007) and peptin31, a linear derivative of syringopeptin produced by P. syringae strain 31R1 (Fiore et al., 2008).

LPs from Bacillus are classified in three families of cyclic compounds: surfactin, iturin and fengycin (Fig. 1). Each family contains variants with the same peptide length but with different residues at specific positions. Moreover, each variant can have several homologues of different length and isometry of the fatty acid chain, leading to a remarkable structural heterogeneity (Ongena & Jacques, 2008). The surfactin family encompasses the heptapeptide variants of the esperin, lichenysin, pumilacidin and surfactin groups. The peptide moiety is linked to a β-hydroxyl fatty acid.
have been identified for addition to these three main LP families, various other LPs from *Bacillus subtilis* and *Bacillus thuringiensis* with two-dimensional NMR (Volpon et al., 2000, 2007; Tsan et al., 2007) and neutron reflectometry (Shen et al., 2009). In addition to these three main LP families, various other LPs have been identified for *Bacillus* species, including kurstakin from *Bacillus thuringiensis* (a heptapeptide with the residues Thr-Gly-Ala-Ser-His-Gln-Gln; Hathout et al., 2000), the 12- AA-containing maltacines from *Bacillus subtilis* (Hagelin et al., 2007), polymyxins from *Bacillus polymyxa* with high content in diamino-butyric acid (Storm et al., 1977), and the surfactin-like bamylocin A from *Bacillus amyloliquefaciens* (Lee et al., 2007).

### Detection and identification of LPs

Several qualitative tests are available to detect LPs and other biosurfactants, including the drop collapse assay, tensiometric analysis, haemolysis assays and spectrophotometric analysis. Although these methods are useful for high-throughput screening of large collections of isolates, the obtained results should be interpreted with caution, as a positive outcome of these assays is not necessarily linked to LP or biosurfactant production. Therefore, the identity of the LPs requires confirmation by analytical–chemical techniques, such as thin-layer chromatography, HPLC, MS, chiral GC, crystallography, Fourier transform IR spectroscopy and/or nuclear magnetic resonance (NMR) (reviewed in Raaijmakers et al., 2006). The analytical power of these technologies was demonstrated in recent studies where the entire spectrum of *Bacillus* LPs and their biosynthetic intermediates were detected and identified in culture filtrates but also in swarming colonies and at the cellular level (Debois et al., 2008; Nihorimbere et al., 2009; Vater et al., 2009).

Immunological assays were also proposed as a sensitive approach to detect and quantify LPs *in situ*, especially in plant-associated environments where relatively low amounts of LPs are produced and where plant-derived compounds may interfere with chemical detection. Immunological detection of LPs is still in its infancy, but it has been successfully adopted to monitor *in situ* production of syringopeptins by *P. syringae* pv. *lachrymans*, the causal agent of angular leaf spot. In that study, a competitive ELISA assay appeared to be approximately 100 times more sensitive than HPLC analysis and did not require extraction of plant material with organic solvents (Fogliano et al., 1999). The potential of immunological techniques is exemplified by *in situ* detection of coronatine, another phytotoxin produced by *P. syringae* (Zhao et al., 2001). The monoclonal antibodies allowed detection of coronatine in infected leaf extracts with a sensitivity of around 1 pg. Subsequent immunofluorescence microscopy and immuno-gold TEM revealed that coronatine was specifically associated with chloroplasts and vacuolar proteins in plant cells, and that the toxin could be transported away from the infected part of the plant. Hence, specific antibodies will be highly instrumental as a supplementary approach to study the localization of LPs in complex environments or to monitor their fate and stability after application to plant tissues or other habitats.

For molecular detection of LP producers, PCR-based methods were developed for syringomycin-producing *P. syringae* (Quigley & Gross, 1994; Sørensen et al., 1998; Bultreys & Gheysen, 1999) and for tolaasin-producing *Pseudomonas tolaasii* (Lee et al., 2002). For *Bacillus*, PCR primers were not always specific due to the generation of new sequence diversity in the recent past. For syringomycin-producing *P. syringae*, the initially developed *syrD* primers were not always specific (Sørensen et al., 1998), possibly due to the fact that *syrD* encodes an ATP-binding cassette (ABC) transporter with orthologues in non-syringomycin producers (Quigley et al., 1993). Subsequent use of primers specific for *syrB*, one of the syringomycin biosynthesis genes, resulted in more specific detection of syringomycin producers with a positive result for 27 of 39 strains tested. However, some of these 27 *P. syringae* isolates did not produce syringomycin, but the structurally related siringostatin or syringotoxin (Sørensen et al., 1998), suggesting that the high level of sequence similarity between different LP biosynthesis genes makes it more difficult to design specific primers for detection of strains producing a particular LP.
**Biosynthesis and regulation**

**LP biosynthesis**

For *Pseudomonas* species, the biosynthesis genes for syringomycin, syringopeptin, syringofactins, arthrofactin, viscosin, orfamide, massetolides, putisolvins and entolysins are fully sequenced (Feil *et al.*, 2005; Paulsen *et al.*, 2005; Berti *et al.*, 2007; De Bruijn *et al.*, 2007, 2008; Gross *et al.*, 2007; Dubern *et al.*, 2008; Gross & Loper, 2009; Vallet-Gely *et al.*, 2010). Also for *Bacillus*, several biosynthetic templates have been resolved, including those for surfactin, fengycin, iturin, mycosubtilin and bacillomycin (Menkhaus *et al.*, 1993; Duitman *et al.*, 1999; Steller *et al.*, 1999; Tsuge *et al.*, 2001; Moyne *et al.*, 2004; Wu *et al.*, 2007). For both genera, most LPs are synthesized by large nonribosomal peptide synthetases (NRPSs) via a thiotemplate process. However, for members of the iturin family, polyketide synthases (PKS) as well as fatty acid synthetases are involved, yielding a hybrid PKS/NRPS biosynthetic template (Duitman *et al.*, 1999; Tsuge *et al.*, 2001; Hansen *et al.*, 2007).

NRPSs possess a modular structure and each module is a building block for the stepwise incorporation of an amino acid in the LP peptide moiety (Finking & Marahiel, 2004; Fischbach & Walsh, 2006; Gross & Loper, 2009; Marahiel & Essen, 2009). In most cases, the order and number of the NRPS modules are colinear to the amino acid sequence of the peptide (colinearity rule). The modules can be subdivided in initiation and elongation modules. Initiation modules typically consist of an adenylation (A) domain, responsible for amino acid selection and activation, and a thiolation (T or PCP) domain responsible for thioesterification of the activated amino acid. In LP biosynthesis, however, the first module also contains a condensation (C) domain. This C1 domain catalyzes N-acylation of the first amino acid of the LP molecule, thereby linking the lipid moiety to the oligopeptide (Konz *et al.*, 1999; Roongsawang *et al.*, 2005). Elongation modules contain A, T and C domains, in which the C domain catalyzes peptide bond formation between 2 AA. Collectively, these domains generate a lipopeptide, which is cleaved at the end of the assembly line by a thioesterase (TE). In most cases, the TE domain catalyzes the cyclization of the mature peptide product rather than its hydrolytic cleavage, resulting in the release of a cyclic LP (cLP) (Schwarzer *et al.*, 2001; Traeger *et al.*, 2001; Samel *et al.*, 2006). As the corresponding linearized forms of cLPS are in many cases biologically less active, it was postulated that cyclization reduces conformational freedom and provides stabilization of the compound, which is necessary for interaction with the biological target (Sieber & Marahiel, 2003). A second type of TE acting as repair enzyme is also necessary for the functioning of these NRPS systems. This type II external TE interacts with T domains, where it ensures the regeneration of phosphopantetheine cofactors in its functional form (Koglin *et al.*, 2008).

NRPSs may harbour epimerization (E) domains that determine the configuration (L or D) of the incorporated amino acid. For example, the surfactin biosynthetic template in *Bacillus* contains two E domains that are responsible for the incorporation of two D-Leu residues (Peypoux *et al.*, 1999; Sieber & Marahiel, 2005). In contrast to *Bacillus*, no E domains have been found so far in LP biosynthetic templates of *Pseudomonas* species. Roongsawang *et al.* (2003) initially postulated that external racemases may be responsible for the D-configuration of the amino acids in arthrofactin produced by *Pseudomonas* and that sequence differences downstream of a conserved core motif [FFELGGHSLA(V/M)] in the T domains might reflect the recognition by these external racemases. For massetolides of *P. fluorescens* strain SS101, however, no relationship could be established between this sequence motif and the amino acid configuration (De Bruijn *et al.*, 2008). Studies on arthrofactin biosynthesis by Balibar *et al.* (2005) indicated that in *Pseudomonas* the D-configuration of the amino acids in the LPs can be generated by C domains that have dual catalytic activities, i.e. condensation and epimerization. They showed that this subclass of C domains, referred to as C/E domains, is involved in epimerization of the amino acid that is loaded onto the T domain of the preceding module. Alignment of the primary sequences showed that this was consistent for most of the individual C domains of the massetolide, arthrofactin, syringomycin and syringopeptin biosynthesis genes (Balibar *et al.*, 2005; De Bruijn *et al.*, 2008). For two C domains (C2, C6) of the massetolide biosynthesis cluster, however, the predicted dual catalytic activity did not match the configuration of the amino acid (De Bruijn *et al.*, 2008). Also Balibar *et al.* (2005) found three exceptions for syringopeptin (C5, C13, C22) and suggested that these C/E domains could also function as dual condensation/dehydration domains with or without prior epimerization.

Analyses of the metabolite profiles of *Pseudomonas* and *Bacillus* species show that single strains can simultaneously produce representatives of various LP families, but also multiple structural analogues of one particular LP. For example, *B. subtilis* can produce 12 surfactin analogs differing in the nature of the peptide residues and/or in the length and branching of the fatty acid chain (Kowall *et al.*, 1998). Also *P. fluorescens* strain SS101 produces at least eight structural analogs of massetolide A (De Bruijn *et al.*, 2008). The observations that LP-biosynthesis mutants do not produce the main LP or any of the LP derivatives indicate that these analogues are the result of the flexibility in amino acid selection and activation by the A domains. Substrate flexibility of A domains is a common phenomenon in nonribosomal peptide synthesis and, instead of being considered a ‘mistake’ of the A domains, this may have
biological functions for the producing strain. This was notably shown for massetolides and surfactins where small structural changes in the peptide or lipid tail not only affected physicochemical properties such as micellization, oil displacement or reduction of surface tension, but also their interaction with phospholipid bilayers and antimicrobial activities (Gerard et al., 1997; Peypoux et al., 1999; Bonmatin et al., 2003; De Bruijn et al., 2007, 2008). However, such flexibility of the A domains is not always the rule as exemplified for iturins: iturin A, mycosubtilin and bacillomycins produced by different strains of *B. subtilis* differ in their amino acid composition but, for certain strains, peptidic variants appear to be lacking (Bonmatin et al., 2003).

**Regulation in Pseudomonas**

In *Pseudomonas*, the GacA/GacS two-component regulatory system functions as a master switch: a mutation in either one of the two encoding genes results in loss of LP production (Kitten et al., 1998; Koch et al., 2002; Dubern et al., 2005; De Bruijn et al., 2007, 2008). Although the GacA/GacS system has been the subject of numerous studies (Heeb & Haas, 2001; De Souza et al., 2003a, b; Haas & Defago, 2005), little is known about the signals that serve as a trigger for LP biosynthesis. Work by Gross and colleagues on plant signals that trigger the production of syringomycin in pathogenic *P. syringae* pv. *syringae* showed that specific phenolic β-glycosides (e.g. arbutin) and specific sugars common to plant tissues enhanced expression of the *srB* gene and led to an increased production of syringomycin (Mo & Gross, 1991; Mo et al., 1995). Recent studies by Wang et al. (2006) showed that syringopeptin production is also activated by these plant signal molecules and that the sensor kinase GacS and the transcriptional regulators SalA and SyrF mediate transmission of the signal molecules to the *syr-syp* biosynthesis cluster. Studies by Koch et al. (2002) with *Pseudomonas* strain DSS73 showed that exudates from sugar beet seeds contain triggers for amphisin production and that the signal transmission requires a functional GacS protein. As the active compound(s) in the seed exudates were heat stable and could be removed by dialysis, Koch et al. (2002) suggested that small organic molecules may be involved, yet their results do not exclude a role for inorganic compounds such as micronutrients.

The role of a second well-known regulatory system, referred to as quorum sensing, has also been investigated for a number of LP-producing species and strains (Table 1). In many *Pseudomonas* species, quorum sensing is directed by N-acyl homoserine lactones (N-AHLs) encoded by luxI-type genes. When a certain threshold of these signal molecules is reached, they bind to the cognate LuxR regulatory protein, which in turn activates transcription of target genes (Venturi, 2006; Williams & Camara, 2009). Quorum-sensing regulated metabolites are generally detected in the late exponential or stationary growth phase of the producing strains. For plant pathogenic *P. fluorescens* strain 5064 and saprophytic *P. putida* strain PCL1445, N-AHL-based quorum sensing was shown to be involved in viscosin and putisolvin biosynthesis, respectively (Cui et al., 2005; Dubern et al., 2006). In strain 5064, the quorum-sensing signal was identified as N-3-acyl-hydroxyoctanoyl-HSL and addition of culture extracts or the synthetic signal molecules restored viscosin biosynthesis in the mutants (Cui et al., 2005). For *P. putida* strain PCL1445, four N-AHLS were found to be associated with regulation of putisolvin biosynthesis (Dubern et al., 2006). For various other *P. fluorescens* strains, however, no indications were found for a role of N-AHL-based quorum sensing in LP biosynthesis (Dumenyo et al., 1998; Kinscherf & Willis, 1999; Andersen et al., 2003; De Bruijn et al., 2007, 2008), emphasizing that the molecular and biochemical basis of cell-density dependent regulation of LP biosynthesis may differ considerably among species and strains within a given species.

Next to global regulatory mechanisms, specific attention has been given to the LuxR-type transcriptional regulators positioned up- and downstream of the LP biosynthesis genes. These LuxR-type regulators were shown to be involved in the biosynthesis of syringomycin, syringopeptin, syringofactins, putisolvin, viscosin and massetolides (Lu et al., 2002; Wang et al., 2006; Berti et al., 2007; Dubern et al., 2008; De Bruijn & Raaijmakers, 2009a). Based on sequence analysis, the LuxR-type regulators contain the typical DNA-binding helix-turn-helix motif in the C-terminal region, but do not harbour the autoinducer-binding domain found for the quorum-sensing-associated LuxR regulators (Wang et al., 2006; De Bruijn & Raaijmakers, 2009a, b). Instead, these genes appear to belong to a separate LuxR-type regulator subfamily (Wang et al., 2006; De Bruijn & Raaijmakers, 2009a).

Via genome-wide random mutagenesis, a number of other genes were recently identified that regulate LP biosynthesis in *Pseudomonas*. One of these regulatory genes is dnaK in *P. putida* (Dubern et al., 2005). As DnaK is a member of the Hsp70 heat shock protein family, the effect of temperature on putisolvin production was further investigated. The results showed that putisolvin production is upregulated at low temperatures and that *dnaK* is required for putisolvin production at low temperatures. Sequencing both up- and downstream of the *dnaK* gene further led to the identification of *dnaJ* and *grpE*, two other genes that adversely affected putisolvin biosynthesis. It was postulated that these heat shock proteins may be required for proper folding or activity of other regulators of the putisolvin biosynthesis gene *psoA*, or that the DnaK complex is necessary for proper assembly of the putisolvin peptide.
synthetases (Dubern et al., 2005). In *P. fluorescens*, random mutagenesis led to the identification of *clpP* as a regulator of massetolide biosynthesis (De Bruijn & Raaijmakers, 2009b). ClpP is a serine protease that is highly conserved in bacteria and eukaryotes. Together with other proteases, ClpP plays a crucial role in intracellular refolding and degradation of proteins. At the transcriptional level, ClpP-mediated regulation of massetolide biosynthesis appeared to operate independently of regulation by the Gac system. Based on these findings, a tentative model was proposed where ClpP regulates, alone or together with a chaperone other than ClpX, massetolide biosynthesis via degradation of putative transcriptional repressors of *massAR* or via modulation of the citric acid cycle and amino acid metabolism (De Bruijn & Raaijmakers, 2009b).

**Regulation in Bacillus**

Also in *Bacillus*, two-component systems and quorum sensing play important roles in the regulation of LP biosynthesis (Table 1; Sullivan, 1998; Duitman et al., 2007). For example, the expression of the surfactin genes is associated with increased cell densities and occurs especially in the transition from exponential to stationary growth phase, whereas the biosynthesis of fengycins and iturins usually occurs later in the stationary phase. The regulation of the surfactin genes is the best known so far, probably because the genetic determinants responsible for competence are linked to the *srf* operon. The main components of the regulatory network for surfactin biosynthesis comprise the two-component system ComA/ComP, the cell-density dependent pheromone ComX and the phosphatase RapC. Upon perception of ComX, the membrane histidine-kinase ComP activates the response regulator ComA, which in turn binds to the promoter region of *srfA* in its phosphorylated form. RapC performs the opposite reaction by dephosphorylating ComA, and the activity of RapC in turn depends on intracellular concentrations of the pentapeptide PhrC, which in turn stimulates expression of the *srf* genes, whereas high concentrations of PhrC repress surfactin biosynthesis. The intracellular concentration of PhrC is dependent on a range of other components, including the permease SpoOK, which transports PhrC across the membrane. The complexity of the regulatory network is further exemplified by the observation that expression of the *srf* genes is also modulated by other transcription factors such as DegU (Mader et al., 2002) or the *H₂O₂* stress-responsive PerR (Hayashi et al., 1998), both acting as positive regulators. Even repressors play a role, as shown for AbrB and, in certain cases, the GTP sensor CodY (Serror & Sonenshein, 1996; Duitman et al., 2007). Furthermore, post-transcriptional activation of the Sfr synthetases depends on the Sfp transferase encoded by the *sfp* gene located downstream of *srfABCD* (Steller et al., 2004).

Expression of the mycosubtilin genes is also under the influence of AbrB, one of the main transition state regulators in *B. subtilis*. However, in strain ATCC 6633, an *abrB* mutant still shows growth-phase-dependent induction of the mycosubtilin (*myc*) operon, so there may be additional regulators involved (Duitman et al., 2007). Greater insight into the regulatory network controlling the synthesis of members of the iturin family was obtained from the study.

<table>
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<th>Species</th>
<th>Strain</th>
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*HSL, homoserine lactone.*
on bacillomycin D produced by *B. amyloliquefaciens* strain FZB42. The transcriptional start site of the bacillomycin (*bmy*) operon was identified and this $\sigma_A$-dependent promoter region was activated in the early stationary growth phase by interaction with DegU (Koumoutsi et al., 2007). By analogy with the ComA/ComP regulon, DegU is a two-component response regulator that controls many cellular processes in *Bacillus* (Dahl et al., 1992; Steil et al., 2003). An additional pleiotropic regulatory protein DegQ positively affects bmy expression, but only indirectly by interacting with DegU in a yet unidentified manner. The expression of the degQ gene seems to be influenced by ComA, which appears to be required for full expression of the bacillomycin genes (Koumoutsi et al., 2007). At the post-transcriptional level, bacillomycin production in strain FZB42 is also influenced by the membrane protein YczE. To our knowledge, little information is available about the regulation of fengycin biosynthesis, although it was demonstrated that expression of the plipastatin operon *ppsABCDE* requires degQ (Tsuge et al., 2007).

**Habitats and in situ production**

**Habitats**

At present, little is known about the distribution and frequency of LP-producing *Pseudomonas* and *Bacillus* species in natural habitats. The most detailed information comes from studies performed in soil- and plant-associated environments. One of the first studies on the frequency of LP-producing *Pseudomonas* in natural environments was the work by Nielsen et al. (2002): they showed that 6% vs. 60% of the fluorescent *Pseudomonas* isolates obtained from the rhizosphere of sugar beet seedlings grown in Danish sandy loam and loamy sand soils, respectively, produced LPs. The pH and soil texture were significantly different between the two Danish soils, but the actual factors contributing to this difference in frequency were not identified (Nielsen et al., 2002). Slightly lower estimates of biosurfactant-producing rhizosphere pseudomonads were obtained by De Souza et al. (2003a, b) for Dutch agricultural soils: on average 1–5% of the pseudomonads isolated from the wheat rhizosphere produced a biosurfactant. A similar percentage of biosurfactant producers was found among the *Pseudomonas* population obtained from the rhizosphere of black pepper plants (Tran et al., 2008). In terms of structural diversity, Nielsen et al. (2002) showed that about half of the tested 155 Danish soil isolates produced amphisin-like LPs, whereas the rest produced viscosin-like LPs. In contrast, genotypically diverse populations of biosurfactant-producing *P. putida* from the rhizosphere of black pepper were shown to produce the same biosurfactants, which were recently identified as putisolvin-like LPs (Tran et al., 2008; Kruijt et al., 2009). LP-producing pseudomonads were also found in other habitats, including oil spills (Roongsawang et al., 2003), drinking water reservoirs (Ivanova et al., 2002), hydroponic plant cultivation systems (Hultberg et al., 2008; Saini et al., 2008), fungal hyphae (Munsch & Alatossava, 2002; Lo Cantore et al., 2006), marine tube worms and red algae (Gerard et al., 1997), and salamander skin (Sinnaeve et al., 2009).

Comparable surveys are not available for phytopathogenic *Pseudomonas* species. Instead, collections of strains from different plant species and geographical locations have been compared for the production of phytotoxic LPs belonging to the syringomycin and tolaasin groups. LP production was reported for *P. syringae* strains belonging to the pathovars *syringae*, *lachrymans* and *aptata*, whereas strains of the pathovars *corrugata* and *japonica* were negative, as were other pv. *syringae* strains (Monti et al., 2001). The studies by Vollsch & Weingart (1998), Bultreys & Gheysern (1999) and Hwang et al. (2005) further documented production of phytotoxic LPs by *P. syringae* strains of pathovars *syringae*, *atrofaciens* and *aptata* as well as by *Pseudomonas* *fusovaginiae*. The LP-producing strains were isolated from a number of different plant sources and each strain appeared to have an individual LP profile composed of different syringomycin-group and/or tolaasin-group compounds. Other LPs of the syringomycin group, ecomycin, pseudomycins and cormycin A, are known to be produced by the plant-associated *Pseudomonas* species *viridiflava*, *syringae* and *corrugata* (Harrison et al., 1991; Miller et al., 1998; Scaloni et al., 2004). These results indicate that LP production is not uncommon among saprophytic and pathogenic *Pseudomonas* isolates and occurs in diverse habitats. This is confirmed by detailed analyses of the genome sequences of a variety of different *Pseudomonas* species (reviewed in Gross & Loper, 2009).

LP-producing *Bacillus* species can be readily isolated from soil- and plant-associated environments, but are also found in other ecological niches such as deep-sea sediments (Trischman et al., 1994), injection brine (Jenneman et al., 1983), fermented food (Mukherjee & Das, 2005) and the human gastrointestinal tract (Hong et al., 2009). Biosynthesis of some LPs, such as the iturins, seems to be limited to some *Bacillus* species (*B. subtilis, B. amyloliquefaciens, Bacillus pumilus*), whereas surfactin and fengycin production are more widespread among multiple species, including *Bacillus coagulans* (Huszcz & Burczyk, 2006), *B. pumilus* (Kalinovskaya et al., 2002), *Bacillus licheniformis* (Li et al., 2008), *Bacillus cereus* (Tsuge et al., 1999), *B. thuringiensis* (Kim et al., 2004), *Bacillus mojavensis* (Snook et al., 2009), *Bacillus megaterium* (Pueyo et al., 2009), *Bacillus polyfermenticus* (Kim et al., 2009) and *Brevibacillus brevis* (Haddad et al., 2008). In a comparative study of > 50 *B. subtilis* and *B. licheniformis* strains isolated from seven diverse and
extreme habitats ranging from glacier to desert, Price et al. (2007) described a correlation between the LP production profile and geographic origin of the strains. This led the authors to speculate that the specificity in LP production allows the Bacillus populations to compete better with the microbial communities indigenous to that particular habitat. From a more general ecological perspective, the ability of Bacillus and Pseudomonas to produce structurally different LPs may allow an adequate response to changes in abiotic and biotic conditions in their natural habitats (discussed further below).

**In situ production and persistence**

Despite the conceptual and technological advances made during the past decade, in situ production of antibiotic compounds by Pseudomonas and Bacillus has only been studied for a limited number of metabolites. In complex environments such as soil and rhizosphere, detection and quantification of LPs produced in situ can be extremely difficult, as these amphiphilic compounds can adsorb to soil particles or irreversibly embed into the lipid structures present on the surface of target and nontarget organisms coexisting in the same niche. There are also several inherent technical difficulties in detecting LPs in complex environments due to the interference of other compounds. For example, HPLC analysis of extracts of tomato leaves did not allow quantification of in situ production levels of massetolides by P. fluorescens due to interference of leaf-derived compounds (Tran et al., 2007). However, in some other systems in situ production levels of LPs were successfully determined, including viscosinamide production by P. fluorescens strain DR54, and amphisin and tensin production by Pseudomonas strains DDS73 and 96.578 (Nielsen & Sørensen, 2003). In bulk soil, neither of the latter LPs was determined, including viscosinamide production by Bacillus subtilis inoculated with Bacillus strains DSS73 and 96.578 (Nielsen & Sørensen, 2003). In the following sections, the stability and persistence of LPs in natural environments and to identify the microorganisms and underlying mechanisms involved in LP degradation.

**Natural functions of LPs**

The substantial structural diversity of LPs produced by Bacillus, Pseudomonas and other bacterial genera suggests that these metabolites have different natural roles, some of which may be unique to the biology of the producing bacterium. The three main natural functions of LPs described to date are their role in antagonism towards other (micro)organisms, motility and attachment to surfaces (Nybroe & Sørensen, 2004; Raaijmakers et al., 2006). For Bacillus, LPs were also shown to function as signal molecules for coordinated growth and differentiation (Marahiel et al., 1997; Lopez et al., 2009a, b). In the following sections, the natural functions of LPs will be addressed and, whenever possible, details on structure–function relationships will be presented.

**Role in antagonism**

In natural habitats, LPs have been proposed to confer a competitive advantage in interactions with other microorganisms. When tested *in vitro*, LPs of Pseudomonas and Bacillus species indeed exhibit lytic and growth-inhibitory activities against a broad range of microorganisms, including viruses, mycoplasmas, bacteria, fungi and oomycetes. The antiviral activity of LPs was already reported in 1951 by Groupé and colleagues (reviewed in Nybroe & Sørensen, 2004) for viscosin against enveloped viruses. In addition,
surfactin was shown to inactivate various enveloped viruses by acting directly on the lipid envelope, leading to disintegration of the virus particles (Vollenbroich et al., 1997b; Huang et al., 2006). Disintegration of membranes was also shown to be the primary activity of surfactin against several human and animal pathogenic mycoplasmas (Vollenbroich et al., 1997a). These studies significantly contribute to a better understanding of the importance of specific structural features of LPs for their activity, such as the fatty acid chain length (Kracht et al., 1999) and the charge of the peptide moiety (Bonmatin et al., 2003). However, it seems unlikely that in their natural habitats, LP-producing bacteria are exposed to, and adversely affected by, enveloped viruses and mycoplasmas. In this context, LP production may be more relevant in competitive interactions with other coexisting bacteria, fungi and oomycetes as well as during interactions with nematodes and protozoan predators.

To date, however, LPs produced by Pseudomonas and Bacillus species have not been tested extensively for activity against other saprophytic bacteria, but mostly for activity against human pathogenic bacteria. Antibacterial activity was shown for corpeptins, syringopeptins and tolaasin against B. megaterium (Lavernicocca et al., 1997; Emanuele et al., 1998; Soler-Rivas et al., 1999), for massetolides, viscosin, syringopeptin and syringomycins against Mycobacterium tuberculosis, Mycobacterium avium-intercellulare and Mycobacterium smegmatis (Gerard et al., 1997; El Sayed et al., 2000; Buber et al., 2002), and for surfactin against B. cereus (Huang et al., 2007), and the plant pathogens Xanthomonas campestris (Etchegaray et al., 2008) and P. syringae (Bais et al., 2004). In the latter study, surfactin production on roots of Arabidopsis correlated well with a reduction in root infection by P. syringae. For putisolvin produced by P. putida strain PCL1445, no antibacterial effects were observed against P. fluorescens and Pseudomonas aeruginosa (Kuiper et al., 2004). The apparent lack of activity of many LPs against Gram-negative bacteria has been ascribed to protective effects of the outer membrane (Nybroe & Sørensen, 2004), although the underlying mechanisms of resistance/tolerance to many LPs in target organisms remain elusive.

Antifungal activities have been reported for many different LPs. For example, fungicidal activities have against Fusarium graminearum (Wang et al., 2007b), Botrytis cinerea (Touré et al., 2004) and Podosphaera fusca (Romero et al., 2007), and iturins against Colletotrichum dematium (Hiradate et al., 2002), Penicillium roqueforti (Chitarra et al., 2003), Aspergillus flavus (Moyne et al., 2001), Rhizoctonia solani (Yu et al., 2002), wood-staining fungi (Velmurugan et al., 2009) and nematophagous fungi (Li et al., 2007). Intriguingly, no marked fungitoxic effects have been reported for surfactin except in the study by Tendulkar et al. (2007), who showed adverse effects of surfactin from B. licheniformis on the fungal rice pathogen Magnaporthe grisea. Most of the studies on antifungal effects of LPs focus primarily on the spectrum of activity and the minimal concentrations required to inhibit fungal growth (Raaijmakers et al., 2006; Ongena & Jacques, 2008). For a few LPs, more detailed investigations were conducted that shed light on their effects on fungal cell morphology and physiology. It was shown that low doses (~1 µM) of surfactin caused hyphal swellings and growth inhibition of the rice pathogen M. grisea (Tendulkar et al., 2007). Studies of LP effects on the fungus R. solani demonstrated similar changes in fungal growth and morphology. When growing on an agar surface supplemented with the LP tensin, R. solani mycelium showed retarded growth accompanied by increased branching and rosette formation as well as hyphal swelling (Nielsen et al., 2000). When tested in a comparable set-up, viscosinamide was able to inhibit hyphal growth and the development of aerial hyphae (Nielsen et al., 1999; Thrane et al., 1999). This growth inhibitory effect was again paralleled by increased branching and swelling of the hyphae (Fig. 2a), but also by decreased activities of esterases and mitochondria, changed organization of mitochondria, decreased intracellular pH and decreased hydrophobicity of the hyphae (Thrane et al., 1999; Hansen et al., 2000). Many of these effects have been confirmed in more complex soil microcosm studies using purified viscosinamide (Thrane et al., 1999).

LPs from Pseudomonas have significant impacts on oomycetes such as Phytophthora and Pythium species, the most conspicuous being their ability to lyse zoospores (Fig. 2b). At present, the impacts on zoospores have been well characterized for the viscosin-group compounds viscosin, viscosinamide and massetolide A, for the putisolvins and for orfamide A. At low concentrations, massetolide A (5 µg mL⁻¹) and viscosinamide (5–10 µg mL⁻¹) induced encystment of zoospores of Phytophthora infestans and Phytophthora species P11, respectively (Thrane et al., 2000; Van de Mortel et al., 2009). Further, low concentrations of massetolide A negatively impacted zoospore autoaggregation while their chemotactic response was not affected (Van de Mortel et al., 2009). At higher concentrations (20–25 µg mL⁻¹), massetolide A, putisolvin and orfamide immobilize zoospores from different oomycetes and cause lysis of entire zoospore populations within 1 min (De Souza et al., 2003a, b; Gross et al., 2007; Tran et al., 2008; Krujút et al., 2009; Van de Mortel et al., 2009). Viscosin only inhibited zoospore motility at this concentration, and 50 µg mL⁻¹ was required to obtain zoospore lysis within 1 min (De Bruijn et al., 2007). Hence, although the general effects of LPs on zoospores are comparable, there appear to be minor differences in their potency. The effects of LPs on zoospores resemble the effects caused by rhamnolipids produced by P. aeruginosa. For comparison, this class of
biosurfactants inhibits motility and causes lysis of *Phytophthora capsici* zoospores at concentrations above 10 μg mL⁻¹ (Kim et al., 2000; Yoo et al., 2005).

The *Pseudomonas* LPs viscosinamide, orfamide and the putisolvins have little or no effect on the growth of oomycete mycelium, even at relatively high concentrations (Thrane et al., 1999; Gross et al., 2007; Kruijt et al., 2009). However, this need not indicate an absence of effects on mycelial morphology and physiology as viscosinamide induced increased hyphal branching and swelling in *Pythium* as well as reduction of nuclear size, decreased mitochondrial activity and reduced intracellular pH (Thrane et al., 1999). Furthermore, viscosinamide decreased the formation of oospores by this oomycete pathogen (Thrane et al., 2000). Studies of massetolide impacts on *Phytophthora* confirmed that zoospores are more sensitive to LPs compared with oomycete mycelium or cysts (Van de Mortel et al., 2009). However, in addition to the effects on zoospores, this LP actually inhibited mycelial growth of the oomycete, besides causing increased branching of hyphae and hyphal swellings along with a decreased formation of sporangia (Van de Mortel et al., 2009).

The genetic and physiological mechanisms underlying the cellular responses of oomycetes and fungi to LPs remain largely unknown. It has been hypothesized that some of the effects are caused by increased Ca²⁺ and H⁺ influx in target cells that may or may not be associated with the ability of LPs to form pores in the cell membrane (Thrane et al., 1999). Additional work with fluorescent vitality stains is required to shed light on such initial changes induced by LPs. Using a molecular-based approach, Van de Mortel et al. (2009) showed that the responses of *Phytophthora* to massetolide A are, in part, dependent on G-proteins, key regulators in many eukaryote signalling pathways. A loss-of-function transformant of *P. infestans* lacking the G-protein α-subunit was more sensitive to massetolide A, whereas a gain-of-function transformant required higher massetolide A concentrations to interfere with zoospore aggregation. Also for mammalian cells, LPs can interfere with canonical signalling pathways by inhibiting the activity of a G-protein subunit (Takasaki et al., 2004; Taniguchi et al., 2004). Their work showed that an LP of *Chromobacterium* acts as a selective inhibitor of Gαq/11, one of the several mammalian Gα-subunits, and that the target of this LP is the exchange of GDP for GTP in Gαq/11 activation. Whether a similar function could be assigned to the effect of massetolides on *P. infestans* is as yet unknown and requires more in-depth biochemical analyses of a variety of G-protein mutants.
Current and future studies on changes in global gene expression in response to LPs are ongoing and may reveal more insights into the biochemical changes and the underlying signalling pathways induced by LPs. Preliminary results of a pilot experiment performed with a limited edition of an Affymetrix GeneChip containing > 15,000 _P. infestans_ unigenes showed that approximately 300 genes in _P. infestans_ were differentially expressed after a 20-min exposure to massetolides produced by _P. fluorescens_ (J.M. Raaijmakers, H. Meijer, L. Sibbel & F. Govers, unpublished data). At least 12 of these encode membrane transporters, including major facilitator superfamily and ABC transporters, which may act as a first line of defence against the deleterious effects of LPs. We also discovered that massetolides significantly downregulated the transcription of a _P. infestans_ gene that encodes a putative suppressor of necrosis of infected plant tissue. Whether transcriptional repression of this RxLR-like effector by massetolides affects infection or subsequent outgrowth of _P. infestans_ in plant tissue is currently being investigated.

**Protection against predators**

Predation represents a major mortality factor for bacteria in natural habitats and many studies show that the grazing activity of protozoa has a significant impact on the dynamics, diversification and evolution of bacterial communities, for example in soil ecosystems (Rønn et al., 2002). To protect themselves against predation, bacteria have evolved a range of defence strategies that can be divided in preingestional and postingestional defences (Matz & Kjelleberg, 2005). While the preingestional defences typically involve changes in cell morphology, surface properties and motility, the postingestional resistance strategies include the production of toxic metabolites (Matz et al., 2004; Jousset et al., 2006). The work by Jousset et al. (2008) illustrated the general significance of attenuating predation by comparing the root-colonizing abilities of _P. fluorescens_ strain CHA0 and the _gacS_ regulatory mutant lacking toxic metabolite production. The bacteria were introduced into the rhizosphere in the presence or absence of the protozoan predator _Acanthamoeba castellanii_ and the calculated gain in fitness due to attenuation of predation was two to three times higher than the gain due to competition against the indigenous microbial community. Amoebae, flagellate and ciliate predators were able to proliferate on the _gacS_ regulatory mutant deficient in exoproduct formation, whereas the wild-type strain was toxic to the predators. Whether LPs and/or other specific metabolites account for these effects of CHA0 has not been addressed in detail yet. For _Pseudomonas_ species strain DSS73, Bjørnland et al. (2009) showed that _gac_-regulated traits decreased the food quality of this bacterium for the nematode _Caenorhabditis elegans_. However, the LP amphisin produced by strain DSS73 was not toxic to the nematode. Serrawettin W2 produced by _Serratia marcescens_ and also surfactin produced by _Bacillus_ induced avoidance in _C. elegans_ to feed on bacterial lawns (Pradel et al., 2007). Pradel et al. (2007) further showed that this LP-induced lawn-avoidance behaviour is mediated by two chemosensory neurons, probably through G-protein coupled receptors, and by the Toll-like receptor gene _tol-1_. The observation that in other avoidance assays, some surfactants did not show repellent activities led to the hypothesis that _C. elegans_ has an ‘exquisitely refined recognition’ of different food sources (Pradel et al., 2007).

Early evidence for impacts of LPs on soil protozoa was presented by Andersen & Winding (2004). These authors showed that _P. fluorescens_ strain DR54, which produces the cell-associated LP viscosinamide (Nielsen et al., 1999), had negative effects on soil amoebae as well as on natural assemblages of soil protozoa. Interestingly, the negative effect was also exerted by methanol extracts and could be correlated to the viscosinamide content of these extracts. However, due to the lack of a viscosinamide-deficient DR54 mutant the authors could not rule out that other bacterial metabolites in the extract could be responsible for the adverse effect on the protozoa. Recently, Mazzola and colleagues demonstrated that massetolide and viscosin are involved in protection of _P. fluorescens_ strains against predation by the amoeba _Naegleria americana_. The purified compounds caused lysis of the amoebae, and feeding by the amoebae on LP-producing wild-type strains was limited, whereas enhanced feeding occurred on LP-deficient mutants (Mazzola et al., 2009). These findings corresponded well with a superior persistence of the massetolide and viscosinamide produced wild-type strains as compared with their LP-deficient mutants, when confronted with the amoebae in soil and rhizosphere environments (Mazzola et al., 2009). Using reporter genes and quantitative reverse transcription PCR, they further demonstrated that the presence of the amoebae or their released signal molecules significantly induced expression of the massetolide and viscosin biosynthetic genes (Mazzola et al., 2009). Although the nature of the signals and the specificity of the induction of LP-gene expression have not been resolved yet, these novel findings highlight the functional versatility of LPs and document novel signalling mechanisms in the molecular dialogues between bacteria and their predators.

**Role in motility**

Movement of bacteria on surfaces has been extensively studied _in vitro_ and several distinct forms have been recognized, including swimming, swarming and twitching (Henrichsen, 1972). For swimming and swarming, the flagella coalesce into a bundle and, by the rotating flagellar...
motor, push the bacteria forward (Harshey, 2003). With swimming, the bacterial cells move individually, whereas with swarming, the cells move in groups forming distinct phenotypes on soft agar plates (Fig. 3). During swarming, vegetative cells of some bacterial species can differentiate into specialized swarmer cells that are hyperflagellated and generally longer (Harshey, 2003). The viscosity of the surface can regulate this cell differentiation: for example, when branched polymers such as Ficoll 400 or polyvinylpyrrolidone 360 are added to liquid medium, a viscous matrix is created and results in cell differentiation in Proteus mirabilis and Vibrio parahaemolyticus (McCarter & Silverman, 1990; Allison et al., 1993). Biosurfactants can change the viscosity of surfaces, thereby influencing cell differentiation and motility. In plant-associated environments, biosurfactants may act as wettability agents of the hydrophobic cuticle of leaves, which may promote not only cell motility but even solubilization and diffusion of substrates for growth (Lindow & Brandl, 2003). To address the role of LPs in the motility of Pseudomonas and Bacillus species, LP-deficient mutants were generated and their surface motility tested in vitro on semi-solid agar plates. In almost all cases, surface motility was lost in the LP-deficient mutants (Fig. 3, Table 2a). Alternatively, swarming was restored in B. subtilis strain 168 by introduction of the sfp gene to re-establish surfactin production (Julkowska et al., 2005). The LP is thought to be involved in aggregation of the cells into dendrites and in the coordination of their movement throughout the swarm front. Enhancement of motility was also observed for mycosubtilin overproducers (Leclère et al., 2006). The typical fingering swarming patterns (Fig. 3) are caused by liquid flows, also known as Marangoni flows, which are induced by a concentration gradient of the biosurfactants resulting in a surface tension gradient (Daniels et al., 2006). For several Pseudomonas and Bacillus mutants deficient in LP production, the reduced surface motility can be restored by addition of the purified LP to the medium (Andersen et al., 2003; Kearns & Losick, 2003; Kinsinger et al., 2003; De Bruijn et al., 2007). Addition of structurally related and unrelated biosurfactants produced by other bacterial strains and species could also restore the deficiency in motility (Andersen et al., 2003). For example, in mutants of S. marcescens swarming could be restored by its own biosurfactant serrawettin, but also by addition of surfactin and rhamnolipid produced by B. subtilis and P. aeruginosa, respectively (Matsuyama et al., 1995). Addition of structurally related and unrelated biosurfactants could also restore the deficiency in motility of Pseudomonas species DSS73 mutants, whereas the synthetic surfactants NP40 and Triton X-100 failed to do so (Andersen et al., 2003). This suggests that not only is the reduction of surface tension essential for motility but also the physical–chemical nature of the surfactant. The function of LPs in dispersal in natural habitats has not been studied in detail. Tran et al. (2007) showed that wild-type strain P. fluorescens SS101, when applied to tomato seeds, was more effective in colonization of the root system of tomato seedlings than its massetolide-deficient mutant. Similarly, a viscosin-deficient mutant of plant pathogenic P. fluorescens strain 5064 was unable to colonize the surface of intact broccoli florets (Hildebrand et al., 1998). Surfactin and amphisin produced by B. subtilis strain 6031 and Pseudomonas species DSS73, respectively, were also shown to be important traits in the colonization of Arabidopsis roots and sugar beet seeds (Bais et al., 2004; Nielsen et al., 2005). In this context, Nielsen et al. (2005) suggested

![Image](https://academic.oup.com/femsre/article/34/6/1037/592387)

**Fig. 3.** Role of LPs in motility of Pseudomonas and Bacillus on soft-agar plates. (a) Five soft-agar (0.6% w/v) plates inoculated in the centre with *Pseudomonas fluorescens* strain SS101 and one (right-most picture) inoculated with the LP-deficient mutant of strain SS101. Plates were incubated for 48 h. Under these conditions, the LP-deficient mutant has the same growth rate as its parental strain SS101, but is not motile. (b) Swarming phenotypes of *Bacillus* strains S499 and FZB42. Surfactin plays a crucial role in motility of *Bacillus amyloliquefaciens* strain FZB42 as evidenced by the swarming phenotypes of the mutants AK3 (surf⁺, feng⁺, itu⁺), CH1 (surf⁺/feng⁻/itu⁻) and CH2 (surf⁻/feng⁻/itu⁻) (adapted from Koumoutsi et al., 2004; strain FZB42 and mutants were kindly provided by Prof. R. Borriss, Center of Bacterial Genetics, University of Berlin, Germany).
that LPs help the producing bacteria to translocate more efficiently from an inoculum source to new and more nutrient-rich niches on the plant surface. However, in those studies, the LPs may have also provided, next to facilitating motility, protection against protozoan predation and competing microorganisms, as discussed above.

### Role in biofilm formation and development

Single bacterial cells can attach to surfaces and, after cell division and proliferation, form dense aggregates commonly referred to as biofilms. The bacterial cells secrete polymers such as polysaccharides and proteins that form a hydrated gel-like slime that holds the biofilm together (Stewart & Franklin, 2008). The extracellular matrix of the biofilm protects bacteria against adverse environmental conditions: for example, biofilms are less susceptible to antibiotic treatment or shearing (Drenkard & Ausubel, 2002; Hall-Stoodley et al., 2004). Biofilms also provide protection against protozoan predation and are a niche for horizontal gene transfer (Danhorn & Fuqua, 2007). For *Bacillus* and *Pseudomonas*, LPs play an important role in surface attachment and biofilm formation, although the outcome may differ depending on the type of LP (Fig. 4a; Table 2b). For example, arthrofactin-producing *Pseudomonas* species MIS38 forms a biofilm on polypropylene microcentrifuge tubes, whereas arthrofactin-deficient mutants form more bulky, but unstable, biofilms (Roongsawang et al., 2003). Similar results were found for putisolvin-producing *P. putida* strains (Kuiper et al., 2004, 2008). For comparison, rhamnolipids produced by *P. aeruginosa* are essential for the initiation of biofilm formation, but also for migration of the subpopulation within the biofilm (Pamp & Tolker-Nielsen, 2007). In addition, Davey et al. (2003) and Klausen et al. (2009) proposed that these biosurfactants can maintain the liquid-filled channels in the biofilm and thereby facilitate the distribution of nutrients and oxygen. For *Bacillus*, Hofemeister et al. (2004) showed that surfactin was required for the formation of biofilms and pellicles by *B. subtilis* strain A1/3. The production of this LP appears important but not always sufficient for pellicle formation, as observed in a comparative study of five different *B. subtilis* strains (Chollet-Imbert et al., 2009). A crucial role for surfactin has also been demonstrated in the

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**Table 2a.** Involvement of lipopeptides and other biosurfactants in motility of *Pseudomonas*, *Bacillus* and other bacterial species

<table>
<thead>
<tr>
<th>Species</th>
<th>Biosurfactants</th>
<th>Motility in deficient mutant</th>
<th>References</th>
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<td>Lost</td>
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<td>Viscosin</td>
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</tr>
<tr>
<td><em>Salmonella enterica sv. typhimurium</em></td>
<td>Lipopolysaccharide</td>
<td>Lost</td>
<td>Toguchi et al. (2000)</td>
</tr>
<tr>
<td><em>Serratia liquefaciens</em></td>
<td>Serratwettin W2</td>
<td>Lost</td>
<td>Lindum et al. (1998), Eberl et al. (1999)</td>
</tr>
</tbody>
</table>

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**Table 2b.** Involvement of lipopeptides and other biosurfactants in biofilm formation by *Pseudomonas* and *Bacillus*

<table>
<thead>
<tr>
<th>Species</th>
<th>Biosurfactants</th>
<th>Biofilm in deficient mutant</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pseudomonas fluorescens</em></td>
<td>Massetolide A</td>
<td>Reduced</td>
<td>De Bruijn et al. (2008)</td>
</tr>
<tr>
<td></td>
<td>Viscosin</td>
<td>Reduced</td>
<td>De Bruijn et al. (2007)</td>
</tr>
<tr>
<td></td>
<td>Orfamide</td>
<td>No change</td>
<td>Gross et al. (2007)</td>
</tr>
<tr>
<td><em>Pseudomonas putida</em></td>
<td>Putisolvin</td>
<td>Increased</td>
<td>Kuiper et al. (2004), Krujit et al. (2008)</td>
</tr>
<tr>
<td><em>Pseudomonas</em> aeruginosa</td>
<td>Rhamnolipids</td>
<td>Reduced/different architecture</td>
<td>Boles et al. (2005), Davey et al. (2003)</td>
</tr>
<tr>
<td><em>Pseudomonas</em> species MIS38</td>
<td>Arthrofactin</td>
<td>Increased</td>
<td>Roongsawang et al. (2003)</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>Surfactin</td>
<td>Reduced</td>
<td>Hofemeister et al. (2004), Bais et al. (2004)</td>
</tr>
</tbody>
</table>
formation of aerial fruiting bodies that represent another type of structured bacterial communities (Branda et al., 2001).

The contrasting roles of LPs in biofilm formation could be partly attributed to differences in their physicochemical properties (Neu, 1996) and to the potential effects of LPs on hydrophobicity of the cell surface and/or the substratum. LPs may be oriented with the hydrophilic part facing the bacterial cell surface, thereby exposing the hydrophobic part of the LP to the outside and thus facilitating attachment to hydrophobic surfaces; when the orientation is reversed, i.e. when the hydrophobic part of the LP is anchored in the outer layers of the bacterial cell surface, the cells can attach...
to hydrophilic but not to hydrophobic surfaces (Neu, 1996). Given the diversity in structures and hydrophobicities of various LPs, it was postulated that depending on the cell surface charge of the producing strain and of the substratum as well as the charge and hydrophobicity of the LP produced, their role in biofilm formation may be different (De Bruijn et al., 2008). The ionic conditions and pH, which may create positive or negative charges, can also influence the interaction of LPs and other biosurfactants with a surface or interphase (Neu, 1996).

Recent studies by Kolter and colleagues provided intriguing new insights into the role of LPs in biofilm formation and development in B. subtilis. They showed that surfactin acts as a signalling molecule in triggering cannibalism and matrix formation in biofilms (Lopez et al., 2009a, b). Biofilm formation was stimulated by surfactin and also by other molecules that cause potassium leakage, whereas other compounds including iturin failed to induce multicellularity (Lopez et al., 2009a). They subsequently demonstrated that potassium leakage stimulates the activity of the histidine kinase KinC, which in turn governs the expression of epsA-O and ypqM-sipW-taxA, genes that are essential in matrix production. Lopez et al. (2009b) showed that the cannibal/matrix-producing subpopulation can also be generated in response to antimicrobial compounds produced by other microorganisms. Based on these findings, they postulated that other organisms, which produce compounds that mimic the action of surfactin, may trigger toxin production and biofilm formation in B. subtilis as a defence mechanism (Lopez et al., 2009b). Hence, it appears likely that both Bacillus and Pseudomonas LPs function as morphoregulatory molecules in multicellular behaviour.

LPs may also adversely influence the attachment to surfaces and biofilm formation by other microorganisms. For example, pretreatment of polypropylene and stainless steel surfaces with surfactin strongly reduced adhesion of food pathogens such as Listeria monocytogenes and Enterobacter sakazakii (Nitschke et al., 2009). The same LP produced by B. subtilis inhibited biofilm formation by Salmonella enterica sv. typhimurium (Mireles et al., 2001) and impaired the formation of aerial structures by Streptomyces coelicolor, thereby affecting developmental processes involved in colonization (Straight et al., 2006). Similarly, putisolvin produced by P. putida inhibited biofilm formation by P. aeruginosa PA14 and P. fluorescens WCS365 (Kuiper et al., 2004). Recent studies further showed that addition of viscosin and massetolide A from P. fluorescens significantly reduced microcolony formation by P. aeruginosa PAO1 (Fig. 4c; I. de Bruijn, J.M. Raaijmakers & O. Nybroe, unpublished data). When the microcolonies were first allowed to develop and subsequently placed on media containing viscosin or massetolide A, the impairment in microcolony formation was less obvious. These results suggest that LPs may adversely affect the early stages of biofilm formation and, in some cases, can break down existing biofilms of other microorganisms.

Role in plant pathogenesis and induction of systemic resistance

For plant pathogenic Pseudomonas species, LPs are important, although not essential, metabolites in the infection process. For example, the production of syringomycin and syringopeptin by P. syringae pv. syringae substantially increases disease severity, but disease may still occur in their absence (Scholz-Schroeder et al., 2001). For plant-pathogenic P. fluorescens 5064, the causal agent of broccoli crown rot, the LP viscosin does not appear to play a direct role in virulence but merely facilitates colonization of the host plant tissue: a viscosin-deficient mutant was still able to cause decay of the broccoli tissue, but was confined to the wounded florets only and did not cause disease on other parts (Hildebrand et al., 1998). Next to the phytotoxic effects of LPs on plants, recent studies have also shown that some LPs have beneficial effects on plants through the stimulation of the plant immune system. Several LPs produced by nonpathogenic Pseudomonas and Bacillus strains triggered defence responses in plants against pathogenic fungi and oomycetes. When tomato roots were treated with purified massetolide A of P. fluorescens, the leaves showed enhanced resistance to infection by P. infestans (Tran et al., 2007). Similarly, purified fengycins and surfactins, but not iturins, induced significant protection in bean and tomato leaves against the fungal pathogen B. cinerea (Ongena et al., 2007). The results of this latter study also showed that overexpression of the surfactin and fengycin biosynthetic genes in the low-producing B. subtilis strain 168 led to a significant increase in disease resistance. The macroscopic disease reduction induced by the Bacillus LPs was associated with defence-related metabolic changes. In tomato, two key enzyme activities of the oxylin pathway were stimulated upon treatment with LP-overproducing Bacillus isolates (Ongena et al., 2007). This metabolic route is initiated by the lipoxygenase enzyme and leads to the formation of a wide array of biologically active secondary metabolites (Blée, 2002). A clear accumulation of nonpolar antifungal compounds also occurred in the LP-treated plants, suggesting a phytoalexin-inducing activity of Bacillus LPs (Adam, 2008). In other systems, major changes in the defence response of plant cells, such as modifications in the pattern of phenolics, were observed upon treatment with Bacillus LPs (Ongena et al., 2005; Jourdan et al., 2009). Treatment of tobacco cell suspensions with low concentrations of surfactin induced several early plant defence-related events such as phosphorylation, Ca$^{2+}$-dependent extracellular alkalization and oxidative burst without causing any significant cell...
death or any marked toxicity (Jourdan et al., 2009). Compared with defence-related events triggered by elicitors from plant pathogens (so-called pathogen-associated molecular patterns), it is as yet unclear whether the induction of the systemic defence response by LPs requires specific receptors in the plant membrane (Jourdan et al., 2009). Optimum defence responses were observed by treating tobacco cells with surfactin derivatives with longer acyl chains (C\textsubscript{14}, C\textsubscript{15}) and also a cyclized and charged peptide moiety was necessary for maximum elicitation of the defence. As these structural traits contribute to the surfactant character and to the membrane-destabilizing potential of this molecule, Jourdan et al. (2009) postulated that some LPs may induce disturbance or transient channelling in the plasma membrane, which, in turn, activates a cascade of molecular events leading to enhanced defence. To decipher this cascade of molecular events, much can be learned from observations obtained in studies on the antitumour activities of surfactin and other LPs. Antitumour activity (leukaemia cells) was described for a novel cyclic lipoheptapeptide and proposed and other LPs. Antitumour activity (leukaemia cells) was obtained in studies on the antitumour activities of surfactin molecular events, much can be learned from observations disturbance or transient channelling in the plasma membrane, which, in turn, activates a cascade of molecular events leading to enhanced defence. To decipher this cascade of molecular events, much can be learned from observations obtained in studies on the antitumour activities of surfactin and other LPs. Antitumour activity (leukaemia cells) was described for a novel cyclic lipoheptapeptide and proposed to be based on the induction of apoptosis via cellular Ca\textsuperscript{2+} augmentation, induction of the mitogen-activated protein kinase and related up-/downregulation of proteins associated with apoptosis (Wang et al., 2007a).

Another in-depth study demonstrated that the antiproliferative activity of surfactin on human colon carcinoma cells was related to induction of apoptosis and cell cycle arrest (Kim et al., 2007). Collectively, these studies show that some LPs from nonpathogenic Pseudomonas and Bacillus species constitute a novel class of microbial-associated molecular patterns that can be perceived by plant and also animal cells to mount a defence response. To further exploit these potential beneficial effects, more knowledge is required about the key structural features and constituents of LPs involved in the induction of the defence responses.

### Chelation of metal ions and degradation of xenobiotics

Chelation of metal ions has been described for several biosurfactants, including LPs and rhamnolipids. The chelation capacity of LPs can be affected by minor structural changes. For example, the difference in the first amino acid between lichenysin and surfactin (glutaminyl instead of glutamic acid) resulted in a better chelation of Ca\textsuperscript{2+} by lichenysin (Grangemard et al., 2001). For surfactin, it was also shown that when the leucine at position 2 was substituted for isoleucine, a threefold increase in affinity for Ca\textsuperscript{2+} occurred, possibly by an increase in accessibility of the acidic side chains and carboxylate groups that constitute the calcium-binding site (Grangemard et al., 1997). Moreover, binding of surfactin to Ca\textsuperscript{2+} resulted in a conformational change of the peptide moiety allowing it to be incorporated deeper into a phospholipid bilayer (Maget-Dana & Ptak, 1995). Iturin and gramicidin S also have affinity for metal cations such as Na\textsuperscript{+}, Rb\textsuperscript{+} and K\textsuperscript{+}. For iturin, the order of cation selectivity was Na\textsuperscript{+} > K\textsuperscript{+} > Rb\textsuperscript{+}, indicating a size limitation in the interaction cavity or cavities (Rautenbach et al., 2000). Because of their metal chelation properties, biosurfactants, including some LPs, can be used for bioremediation of soils contaminated with heavy metals via detachment of the metal ion from soil particles and subsequent incorporation in the biosurfactant micelles (Mulligan et al., 2001). The metal–biosurfactant complex can then be washed from the contaminated soil, but also air can be applied and, due to the foaming properties of the biosurfactant, metal and soil can be separated (Banat et al., 2000; Mulligan, 2005). In addition to their use for bioremediation of heavy metal-contaminated soils, biosurfactants also have potential for degradation of aromatic compounds (Harvey et al., 1990; Mulligan, 2005). Aromatic compounds are aerobically degraded by oxidizing the aromatic ring, making them more susceptible to cleavage by dioxygenases (Phale et al., 2007). However, the limiting step in the degradation of partially insoluble aromatic compounds is bioavailability, which can be substantially improved by adding (bio)surfactants (Phale et al., 2007).

The actual functions of LP biosurfactants in relation to metal chelation and degradation of xenobiotics are not clear, and even the benefits to the producing bacterium need to be clarified. One may postulate that bacteria, via chelation, protect themselves against the toxic effects of certain metal ions. Alternatively, bacteria may use the biosurfactants for sequestering specific metal ions as micronutrients as was described for iron-chelating siderophores. With respect to the degradation of xenobiotics, LPs and other biosurfactants may provide access to persistent aromatic compounds with low solubility in water and utilize these compounds as carbon and/or nitrogen sources (Ron & Rosenberg, 2002). For example, some P. aeruginosa strains that did not produce a biosurfactant, showed slow growth on insoluble hydrocarbons, but when rhamnolipid was added the hydrocarbons could be utilized and growth was restored (Desai & Banat, 1997).

### Concluding remarks and future perspectives

LPs produced by Bacillus and Pseudomonas species are structurally diverse and exhibit an enormous functional versatility (Fig. 5). LP biosynthesis has been well studied in Bacillus and Pseudomonas, although there are still several questions to be answered. For example, the biosynthesis of the lipid moiety of the LP molecule has received little attention. Also our understanding of the regulatory networks involved in LP biosynthesis is limited and
The identification of genes and mechanisms underlying perception and signal transduction remains a major challenge. Furthermore, the resistance mechanisms in the producing microorganisms as well as in target organisms are largely unexplored. In this context, the studies performed with daptomycin (reviewed in Baltz, 2009) provide an excellent framework to investigate this for other LPs. These mechanistic studies may also shed light on the role of LPs in biotic interactions and further enlarge the amplitude of their natural functions.

With respect to the exploitation of LPs for (agro)industrial purposes or as new therapeutics, there are several constraints due to the intrinsic toxicity and low stability of some of these compounds. Hence, there is considerable interest in selecting highly active compounds with low cytotoxicity and increased stability. The relaxed substrate specificity of the biosynthetic apparatus can be exploited for precursor-directed biosynthesis of new variants via the supplementation of culture medium with appropriate amino acids. This approach requires optimization, but can be easily adapted in bioreactor-based processes, leading to the production of the compounds of interest at the pilot plant level. By coupling with efficient and cost-effective methods for recovery and purification, LP-based products are expected to reach more rapidly a broader variety of market niches. This strategy can be adopted simultaneously with combinatorial approaches to generate novel LP derivatives with different or enhanced activities (Gerard et al., 1997;
Wenzel & Müller, 2005; Sattely et al., 2008). Multiple synthetic linear and cLPS derived from molecules isolated from various origins were designed recently by combinatorial chemistry (Montesinos, 2007) and have shown promising and diverse activities (Badosa et al., 2007; Makovitzki et al., 2007, 2008; Brotman et al., 2009). Next to the combinatorial approaches, the tremendous increase in the availability of whole genome sequences has provided a wealth of resources to identify new LPs in *Pseudomonas*, *Bacillus* and other bacterial genera. Recent work by Bumpus et al. (2009) provided an elegant ‘protein-first’ strategy that may complement and accelerate these sequence-based approaches. Their MS-based proteomics analysis, referred to as PriSM, led to the identification of known NRPS and PKS compounds, and of an orphan NRPS cluster in *Bacillus* that generates a 7 AA LP. In support of these approaches, advances in the characterization of threedimensional structure and spatial organization of the NRPS modules (Tanovic et al., 2008) will provide crucial information for engineering these megaenzymes to obtain new derivatives with enhanced activities and optimal intrinsic properties.

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**Authors’ contribution**

J.M.R., I.d.B., O.N. and M.O. contributed equally to this work.

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Natural functions of lipopeptides


