REVIEW

Cyclic Lipopeptide Production by Plant-Associated *Pseudomonas* spp.: Diversity, Activity, Biosynthesis, and Regulation

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Cyclic lipopeptides (CLPs) are versatile molecules produced by a variety of bacterial genera, including plant-associated *Pseudomonas* spp. CLPs are composed of a fatty acid tail linked to a short oligopeptide, which is cyclized to form a lactone ring between two amino acids in the peptide chain. CLPs are very diverse both structurally and in terms of their biological activity. The structural diversity is due to differences in the length and composition of the fatty acid tail and to variations in the number, type, and configuration of the amino acids in the peptide moiety. CLPs have received considerable attention for their antimicrobial, cytotoxic, and surfactant properties. For plant-pathogenic *Pseudomonas* spp., CLPs constitute important virulence factors, and pore formation, followed by cell lysis, is their main mode of action. For the antagonistic *Pseudomonas* spp., CLPs play a key role in antimicrobial activity, motility, and biofilm formation. CLPs are produced via nonribosomal synthesis on large, multifunctional peptide synthetases. Both the structural organization of the CLP synthetic templates and the presence of specific domains and signature sequences within peptide synthetase genes will be described for both pathogenic and antagonistic *Pseudomonas* spp. Finally, the role of various genes and regulatory mechanisms in CLP production by *Pseudomonas* spp., including two-component regulation and quorum sensing, will be discussed in detail.

Additional keywords: *Bacillus*, nonribosomal peptide synthesis.

The genus *Pseudomonas* not only harbors plant- and human-pathogenic species, but also accommodates species that promote plant growth, degrade xenobiotic compounds, antagonize plant pathogenic fungi, or induce resistance in plants (Compant et al. 2005; Haas and Défago 2005; Pieterse et al. 2002; Ryu et al. 2003). The diverse life styles of *Pseudomonas* spp. and the complexity of their interactions with multiple hosts have been the subject of numerous molecular, biochemical, and ecological studies (Ramos 2004). Recently, whole genome sequences of multiple *Pseudomonas* strains representing various species have become available and allow a more in-depth study of metabolic pathways and regulatory networks as well as the exploration and exploitation of yet-unknown genes and gene products. The extensive interest in *Pseudomonas* spp. is due, in part, to the production of a wide variety of metabolites, including enzymes, volatiles, bacteriocins, toxins, antibiotics,

and cyclic lipopeptides (Haas and Défago 2005; Raaijmakers et al. 2002).

Cyclic lipopeptides (CLPs) are versatile molecules with antimicrobial, cytotoxic, and surfactant properties. CLPs are produced by several plant-associated *Pseudomonas* spp., including pathogenic *Pseudomonas syringae*, *P. tolaasii*, *P. fuscovaginae*, *P. corrugata*, and *P. fluorescens* (Bender et al. 1999), and by multiple strains classified as antagonistic *P. fluorescens* and *P. putida* (Nielsen et al. 2002; Nybroe and Sørensen 2004). In a recent review by Nybroe and Sørensen (2004), several chemical and biological aspects of CLP production in fluorescent pseudomonads were described. Also, CLP production by plant-pathogenic *P. syringae* has been the subject of several reviews (Bender et al. 1999; Bender and Scholz-Schroeder 2004; Willis and Kinscherf 2004). The purpose of this review is to provide an up-to-date overview of the current knowledge of the structural diversity and activity of CLPs produced by plant-associated *Pseudomonas* spp., in particular the antagonistic *Pseudomonas* spp. A detailed description of genes involved in the regulation and biosynthesis of CLPs will be presented, with emphasis on specific domains and signature sequences within CLP biosynthetic templates. These analyses provide insights into the specificity-conferring codes in peptide synthetase genes of *Pseudomonas* spp. and allow a prediction of the final structure of the CLP molecule based on sequence data. Whenever necessary, comparisons will be made with CLPs of other bacteria and, in particular, with surfactin produced by *Bacillus* spp.

Structural diversity.

A variety of methods have been used to elucidate the primary and secondary structures of CLPs produced by bacteria (Ballio et al. 1996; Bare et al. 1999; De Souza et al. 2003; Henriksen et al. 2000; Koumoutsis et al. 2004; Monti et al. 2001; Nielsen et al. 1999, 2002; Sørensen et al. 2001). CLPs produced by *Pseudomonas* spp. are composed of a fatty acid tail linked to a short oligopeptide, which is cyclized to form a lactone ring between two amino acids in the peptide chain. Based on the length and composition of the fatty acid tail as well as the number, type, and configuration of the amino acids in the peptide moiety, CLPs of *Pseudomonas* spp. were classified into four major groups (i.e., the viscosin, amphisin, tolaasin, and syringomycin groups) (Table 1). In brief, the viscosin group harbors CLPs with 9 amino acids linked at the N-terminus to, in most cases, 3-hydroxy decanoic acid (3-HDA). *Pseudomonas* spp. producing CLPs in this group are from a variety of origins, including soil, rhizosphere, phyllosphere, and marine environments. For example, viscosin production has been de-

scribed for pectolytic strains of *P. fluorescens* causing head rot of broccoli (Hildebrand et al. 1998) and for a *Pseudomonas* isolate obtained from a tube worm collected from a marine environment (Gerard et al. 1997). Similarly, massetolide A production originally was described for a yet-unidentified *Pseudomonas* sp. isolated from the surface of leafy red algae collected at -15 m in the sea near the coast of British Columbia (Gerard et al. 1997), and later was described for a *P. fluorescens* strain isolated from the rhizosphere of wheat grown in a soil suppressive to take-all disease of wheat (De Souza et al. 2003). In a comprehensive survey, Nielsen and associates (2002) showed that, among the *Pseudomonas* spp. isolated from the rhizosphere of sugar beet, viscosin-like CLPs were produced primarily by strains belonging to *P. fluorescens* biovar I. Whether members of specific CLP groups are produced predominantly by specific biovars of fluorescent *Pseudomonas* spp. remains to be further investigated using large collections of isolates from different environmental origins.

CLPs in the amphisin group consist of 11 amino acids in the peptide part coupled to 3-HDA (Table 1). For several members of this group, including amphisin and tensin, the crystal structure has been resolved (Henriksen et al. 2000; Sørensen et al. 2001). For both tensin and amphisin, the structures are mainly helical, with the cyclic peptide wrapping around a water molecule. Based on the distance between two negatively charged residues of the molecule, Henriksen and associates (2000) suggested that tensin most likely cannot form complexes with Ca^{2+} , as was shown for surfactin produced by *Bacillus* spp. (Peypoux et al. 1999). Crystallography further showed that, in amphisin, the 3-HDA fatty acid tail protrudes from the hydro-

phobic side of the molecule (Nybroe and Sørensen 2004). For the *P. fluorescens* strains producing amphisin-like CLPs, Nielsen and associates (2002) identified several other interesting characteristics, including the production of chitinases and the volatile hydrogen cyanide (HCN), two traits that have been associated with biological control of plant-pathogenic fungi (Haas and Défago 2005; Whipps 2001). These traits and, in particular, HCN production were not common among the *P. fluorescens* strains producing viscosin-like CLPs (Nielsen et al. 2002).

Compared with the viscosin and amphisin groups, CLPs in the tolaasin group are much more diverse due to multiple variations in both the composition and length of the peptide chain (19 to 25 amino acids) and the lipid tail (3-HDA or 3-hydroxyoctanoic acid [3-HOA]). The peptide part of the CLPs in this group contains several unusual amino acids, including 2,3-dihydro-2-aminobutyric acid (Dhb) and homoserine (Hse), the first always being in front of the *allo*-Thr residue (Table 1). The cyclic part of the peptide moiety contains five to eight amino acids and the lactone ring is formed between the C-terminal amino acid and the *allo*-Thr residue. Several tolaasin-like CLPs are produced by plant-pathogenic strains of *Pseudomonas* and constitute important virulence factors.

In terms of shear numbers of amino acids in the peptide moiety, CLPs in the syringomycin group are structurally similar to the CLPs in the viscosin group (Table 1). However, syringomycin-like CLPs harbor unusual amino acids, including Dhb, 2,4-diamino butyric acid (Dab), and the C-terminal 4-chlorothreonine (Thr[4-Cl]), the latter being important for the antifungal activity of syringomycin (Grgurina et al. 1994). Furthermore, the lactone ring is formed between the N-terminal Ser

Table 1. Simplified primary structures of cyclic lipopeptides (CLPs) produced by plant-associated *Pseudomonas* spp.^a

<u>Viscosin Group</u>																										
Viscosin	C ₁₀ HO acid	1	2	3	4	5	6	7	8	9																
Viscosin-amide	C ₁₀ HO acid	- L-Leu-	D-Glu-	D-aThr-	D--Val-	L-Leu-	D-Ser-	L-Leu-	D-Ser-	L-Ile																
Massetolide A	C ₁₀ HO acid	- L-Leu-	D-Gln-	D-aThr-	D--Val-	L-Leu-	D-Ser-	L-Leu-	D-Ser-	L-Ile																
Massetolide D	C ₁₀ HO acid	- L-Leu-	D-Glu-	D-aThr-	D-aIle-	L-Leu-	D-Ser-	L-Leu-	D-Ser-	L-Ile																
WLIP	C ₁₀ HO acid	- L-Leu-	D-Glu-	D-aThr-	D--Val-	D-Leu-	D-Ser-	L-Leu-	D-Ser-	L-Ile																
Pseudophomin A	C ₁₀ HO acid	- L-Leu-	D-Glu-	D-aThr-	D--Ile-	D-Leu-	D-Ser-	L-Leu-	D-Ser-	L-Ile																
Pseudophomin B	C ₁₂ HO acid	- L-Leu-	D-Glu-	D-aThr-	D--Ile-	D-Leu-	D-Ser-	L-Leu-	D-Ser-	L-Ile																
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<u>Amphisin Group</u>																										
Amphisin	C ₁₀ HO acid	1	2	3	4	5	6	7	8	9	10	11														
Tensin	C ₁₀ HO acid	- D-Leu-	D-Asp-	D-aThr-	D-Leu-	D-Leu-	D-Ser-	L-Leu-	D-Gln-	L-Leu-	L-Ile-	L-Asp														
Pholipeptin A	C ₁₀ HO acid	- D-Leu-	D-Asp-	D-aThr-	D-Leu-	D-Leu-	D-Ser-	L-Leu-	D-Gln-	L-Leu-	L-Ile-	L-Glu														
Lokisin	C ₁₀ HO acid	- D-Leu-	L-Asp-	L--Thr-	D-Leu-	D-Leu-	D-Ser-	D-Leu-	D-Ser-	D-Leu-	L-Ile-	D-Asp														
Arthrofactin	C ₁₀ HO acid	- D-Leu-	D-Asp-	D-aThr-	D-Leu-	D-Leu-	D-Ser-	D-Leu-	D-Ser-	D-Leu-	L-Ile-	D-Asp														
Arthrofactin	C ₁₀ HO acid	- D-Leu-	D-Asp-	D--Thr-	D-Leu-	D-Leu-	D-Ser-	L-Leu-	D-Ser-	L-Ile-	L-Ile-	L-Asp														
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<u>Tolaasin Group</u>																										
Tolaasin	C ₈ HO acid	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22			
FP-B	C ₈ HO acid	- Dhb-	Pro-	Ser-	Leu-	Val-	Ser-	Leu-	Val-	Val-	Gln-	Leu-	-	-	-	-	-	Val-	Dhb-	aThr-	Ile-	Hse-	Dab-	Lys		
Corpeptin A	C ₁₀ HO acid	- Dhb-	Pro-	Leu-	Ala-	Ala-	Ala-	Ala-	Val-	Gly-	Ala-	Val-	Ala-	-	-	-	-	Val-	Dhb-	aThr-	Ala-	Dab-	Dab-	Phe		
Corpeptin A	C ₁₀ HO acid	- Dhb-	Pro-	Ala-	Ala-	Ala-	Val-	Dhb-	Hse-	Val-	aIle-	Dhb-	Ala-	Ala-	Ala-	Ala-	Val-	Dhb-	aThr-	Ala-	Dab-	Dab-	Ser-	Ile		
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SP22	CH ₃ (CH) ₂ -8CH(OH)CH ₂ CO-	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
SP25A	CH ₃ (CH) ₂ -8CH(OH)CH ₂ CO-	Dhb-Pro-Val-Val-Ala-Ala-Val-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
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<u>Syringomycin Group</u>																										
Syringomycin	3-hydroxy fatty acyl	1	2	3	4	5	6	7	8	9																
Syringostatins	3-hydroxy fatty acyl	- L-Ser-	D-Ser-	D-Dab-	L-Dab-	L-Arg-	L-Phe-	-	Dhb-	L-Asp(3-OH)-	L-Thr(4-Cl)															
Syringotoxin	3-hydroxy fatty acyl	- L-Ser-	D-Dab-	L-Dab-	D-Hse-	L-Orn-	L-aThr-	-	Dhb-	L-Asp(3-OH)-	L-Thr(4-Cl)															
Pseudomycin A	3-hydroxy fatty acyl	- L-Ser-	D-Dab-	L-Asp-	L-Lys-	L-Dab-	L-aThr-	-	Dhb-	L-Asp(3-OH)-	L-Thr(4-Cl)															
Cormycin A	3-hydroxy fatty acyl	- L-Ser-	D-Dab-	L-Asp-	L-Lys-	L-Dab-	L-aThr-	-	Dhb-	L-Asp(3-OH)-	L-Thr(4-Cl)															
Cormycin A	3-hydroxy fatty acyl	- L-Ser-	D-Orn-	L-Asn-	D-Hse-	L-His-	L-aThr-	-	Dhb-	L-Asp(3-OH)-	L-Thr(4-Cl)															
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<u>other</u>																										
Putisolvin I	CH ₃ (CH ₂) ₄ -CO-	1	2	3	4	5	6	7	8	9	10	11	12													
Putisolvin II	CH ₃ (CH ₂) ₄ -CO-	Leu- Glu- Leu- Ile- Gln- Ser- Val- Ile- Ser- Leu- Val- Ser																								
Putisolvin II	CH ₃ (CH ₂) ₄ -CO-	Leu- Glu- Leu- Ile- Gln- Ser- Val- Ile- Ser- Leu- Xle- Ser																								
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PFL2145/46/47	C ₇₇ HO acid	1	2	3	4	5	6	7	8	9	10															
PFL2145/46/47	C ₇₇ HO acid	- Leu- Glx- aThr- Ile- Leu- Ser- Leu- Leu- Ser- Val																								

^a Adapted from Ballio et al. 1991; Ballio et al. 1995; Coiro et al. 1998; Gerard et al. 1997; Kuiper et al. 2004; Morikawa et al. 2000; Nybroe and Sørensen, 2004; Paulsen et al. 2006; Quail et al. 2002; and Scaloni et al. 2004. The lines represent the lactone ring of the cyclic lipopeptide. ⊗: isomer not identified; Asp(3-OH): 3-hydroxyaspartic acid; Dab: 2,4-diaminobutyric acid; Dhb, 2,3-dihydroxy-2-aminobutyric acid; Glx: glutamic acid or glutamine; Hse: homoserine; *alle*: *allo*-isoleucine; Orn: ornithine; Thr(4-Cl): 4-chlorothreonine; *aThr*: *allo*-threonine; Xle: isoleucine or leucine; PFL2145/46/47 refers to the predicted lipodecapeptide of *P. fluorescens* strain Pf-5.

and the C-terminal Thr(4-Cl); whereas, for members of the viscosin group, the ring usually is formed between the C-terminal amino acid and the D-*allo*-Thr at the third amino acid position in the peptide chain (Table 1). The fatty acid tail of CLPs in the syringomycin group may consist of a 3-hydroxy or 3,4-dihydroxy fatty acid composed of 10 to 14 carbon atoms. For example, three forms of syringomycin have been described which differ only in the length of the fatty acid moiety, being either decanoic, dodecanoic, or tetradecanoic acid (Bender et al. 1999; Bender and Scholz-Schroeder 2004).

The structures of a number of other CLPs from *Pseudomonas* spp. have been elucidated in the past years. These include arthrfactin produced by *Pseudomonas* sp. strain MIS38 (Morikawa et al. 1993; Roongsawang et al. 2003) and putisolvin I and II produced by *P. putida* PCL1445 (Kuiper et al. 2004). Arthrfactin contains an 11-amino acid peptide moiety linked to a β -hydroxydecanoyl tail. Based on its chemical structure, arthrfactin falls within the amphisin group and is most similar to lokisin (Table 1). Putisolvin I and II harbor some unique features, including a peptide moiety of 12 amino acids and a hexanoic lipid tail. The cyclization in the two putisolvins also is different from other CLPs because the linkage occurs between the C-terminal carboxyl group and the ninth amino acid residue (serine) instead of the first or third amino acid as described previously for many of the other CLPs (Table 1). Recently, in silico analysis of the whole genome sequence of the biocontrol strain *P. fluorescens* Pf-5 has resulted in the identification of a gene cluster that most likely encodes a cyclic lipodecapeptide (Paulsen et al. 2005). Although the amino acid composition of the peptide moiety of the predicted CLP of strain Pf-5 (Table 1) as well as the identity of the fatty acid tail needs to be confirmed by chemical analyses, this approach elegantly illustrates that knowledge of whole genome sequences allows the discovery of yet-unknown genes and traits which may be biologically relevant for the activity of antagonistic *Pseudomonas* spp.

Activity.

Given the structural diversity of CLPs produced by *Pseudomonas* spp. and other bacterial genera, Ron and Rosenberg (2001) postulated that CLPs and, more generally, microbial surface-active compounds (biosurfactants) also have different natural roles, some of which may be unique to the physiology and ecology of the producing microorganism. Several natural roles for CLPs and other biosurfactants were proposed (Ron and Rosenberg 2001), including their function in i) pathogenicity, ii) antimicrobial activity, iii) regulation of attachment and detachment to and from surfaces, and iv) motility. Another natural role, possibly limited to spore-forming bacteria, is that CLPs may function as signal molecules for coordinated growth and differentiation (Marahiel et al. 1997). To date, most of the hypotheses for the natural roles of CLPs and other biosurfactants have been derived mainly from their physical and chemical properties. In recent years, mutants deficient in CLP production have been obtained for several *Pseudomonas* strains and currently are being used to further investigate the natural roles of CLPs. In this section, several of the potential functions of CLPs produced by plant-associated *Pseudomonas* spp. will be discussed. Whenever possible, details on structure-function relationships will be included.

Role in plant pathogenesis. CLPs produced by plant-pathogenic *P. syringae* and *P. fluorescens* function as virulence factors and, as such, are not solely required for pathogenicity (Bender et al. 1999). For example, the production of syringomycin and syringopeptin by *P. syringae* pv. *syringae* substantially increases disease severity, although disease may still occur in their absence (Scholz-Schroeder et al. 2001a). Similarly,

a viscosin-deficient mutant of a pectolytic strain of *P. fluorescens* still caused decay of wounded broccoli florets, but the decay failed to spread to adjacent nonwounded florets (Hildebrand et al. 1998). One of the main modes of action of CLPs produced by plant-pathogenic *Pseudomonas* spp., including syringomycin, syringopeptin, and tolaasin, involves the formation of ion channels in the host plasma membrane leading to cytolysis (Dalla Serra et al. 1999; Hutchison and Gross 1997; Hutchison et al. 1995; Mott and Takemoto 1989; Rainey et al. 1991). Pore formation results in the alkalization of the intercellular fluid and in the release of multiple cellular compounds, conditions that are favorable for further multiplication and increased CLP production by the invading bacteria. At high concentrations (well above the critical micelle concentration [CMC value]), CLPs can directly solubilize plasma membranes. In several studies (Bender et al. 1999), solubilization of membranes also was demonstrated for erythrocytes. Interestingly, erythrocyte hemolysis by tolaasin can be inhibited by addition of divalent metal ions such as Zn^{2+} , Ca^{2+} , and Mg^{2+} (Rainey et al. 1991). Given that pre-incubation of tolaasin with Zn^{2+} did not affect its ability to lyse erythrocytes and that cells pretreated with Zn^{2+} were no less susceptible to tolaasin-induced lysis than untreated cells, Rainey and associates (1991) proposed that the inhibitory effect of divalent metal ions operates by binding to negatively charged groups on the extracellular side of the plasma membrane close to the site of pore formation. Recent studies with surfactin (Dufour et al. 2005) further indicated that the cyclic character of the peptide moiety is important for erythrocyte hemolysis because linear products of surfactin failed to cause lysis.

In addition to the direct toxic effects of CLPs from pathogenic *Pseudomonas* spp. on plant cells, their surfactant properties play an important indirect role in virulence by facilitating colonization of plant tissue and enhancing physical access of cell-wall-degrading enzymes (CWDE) to the plant surface (Hildebrand et al. 1998; Hutchison and Johnstone 1993; Lindow and Brandl 2003). Although the order in which CLPs and CWDE operate is subject to discussion (Fogliano et al. 2002), studies on the indirect and direct effects of CLPs on plant cells, including their impact on physiological processes and signaling pathways (Bender et al. 1999), clearly exemplify the versatile functions of CLPs in pathogenicity. From a more applied perspective, knowledge of the detrimental effects of CLPs on plant cells has led to an increased interest in the potential use of these CLPs or the producing *Pseudomonas* spp. as microbial herbicides (Li et al. 2003; Pedras et al. 2003).

Antimicrobial activity. CLPs produced by *Pseudomonas* and *Bacillus* spp. also have received considerable attention for their activity against a range of other microorganisms, including viruses, mycoplasmas, trypanosomes, bacteria, fungi, and oomycetes. Studies on the antiviral activity of CLPs produced by *Pseudomonas* spp. date back to work by Groupé and colleagues in 1951 (Nybroe and Sørensen 2004), in which viscosin was shown to have activity against enveloped human-pathogenic viruses, including the infectious bronchitis virus. Similarly, surfactin was shown to inactivate multiple enveloped viruses, including herpes viruses and retroviruses (Vollenbroich et al. 1997a). Electron microscopy indicated that surfactin acts directly on the lipid envelope, leading to disintegration of the virus particles (Vollenbroich et al. 1997a). Additional studies revealed that surfactins with fatty acid chains of 13 carbon atoms showed low antiviral activity compared with C14 and C15 isoforms, indicating that the hydrophobicity is an important determinant of the antiviral activity of surfactin (Kracht et al. 1999). Disintegration of membranes also was shown to be the primary activity of surfactin against several mycoplasmas (Vollenbroich et al. 1997b). Surfactin was active only at concentrations above

the CMC value, suggesting that disruption of mycoplasma membranes is due to nonspecific detergent activity.

In studies on the human pathogen *Trypanosoma cruzi*, the causal agent of Chagas's disease, Mercado and Colon-Whitt (1982) observed lysis of trypomastigotes isolated from the blood of infected mice. Further analysis of the parasite samples led to the isolation of a *P. fluorescens* isolate that produces viscosin, which itself also causes trypanosomal lysis (Burke et al. 1999). An interesting microscopic observation was that, in direct interactions, the *P. fluorescens* isolate approached a single flagellate of *T. cruzi* at the posterior end, the site of the flagellar origin, resulting in immobilization (Mercado and Colonwhitt 1982). Observations further revealed rounding up of the parasite with the flagellum coiled around the cell body, followed by disintegration. Similar stages were observed in the disintegration process of zoospores of oomycete pathogens exposed to massetolide A and viscosin produced by antagonistic strains of *P. fluorescens* (De Souza et al. 2003; J. M. Raaijmakers, *personal communication*).

Studies on the antibacterial activity of CLPs have focused primarily on human-pathogenic bacteria and, in particular, the gram positives. In summary, activity was shown for corpeptins, syringopeptins, and tolaasin against *Bacillus megaterium* (Emanuele et al. 1998; Lavermicocca et al. 1997; Soler-Rivas et al. 1999), and for massetolides, viscosin, syringopeptin, and syringomycins against *Mycobacterium tuberculosis*, *M. avium-intercellulare*, and *M. smegmalis* (Buber et al. 2002; El Sayed et al. 2000; Gerard et al. 1997). A twofold difference between the activity of massetolide A and viscosin against *M. tuberculosis* and *M. avium-intercellulare* (Gerard et al. 1997) illustrated that a difference of only 1 amino acid residue in the peptide moiety (Table 1) may have a significant impact on the antibacterial activity. For massetolide A and viscosin, no activity was observed against a panel of other human-pathogenic bacteria, including *Escherichia coli* and *Staphylococcus aureus* (Gerard et al. 1997). Also, for putisolvin produced by *P. putida* PCL1445, no antibacterial effects against *P. fluorescens* and *P. aeruginosa* were observed when these strains were grown together on agar plates (Kuiper et al. 2004). The apparent lack of activity of several CLPs against gram-negative bacteria has been ascribed to protective effects of the outer membrane (Nybroe and Sørensen 2004). However, recent studies by Bais and associates (2004) indicate that this general observation does not apply to all CLPs; in their study, surfactin was shown to have bactericidal activity against *P. syringae* pv. *tomato*.

The antifungal activity has been studied for many different CLPs and for a wide variety of plant- and human-pathogenic fungi and yeasts, including *Rhizoctonia solani*, *Phoma lingam*, *Alternaria brassicae*, *Sclerotinia sclerotiorum*, *Geotrichum candidum*, *Botrytis cinerea*, *Ophiostoma ulmi*, *Aspergillus* and *Fusarium* spp., *Penicillium digitatum*, *Cryptococcus neoformans*, *Candida albicans*, and *C. glabrans* (Nybroe and Sørensen 2004). In particular, the studies with viscosinamide produced by antagonistic *Pseudomonas* sp. strain DR54 provide several lines of evidence that CLPs are important constituents in the biological control of plant-pathogenic fungi. In vitro studies showed that viscosinamide adversely affected mycelium of *R. solani* and the oomycete *Pythium ultimum*, causing reduced growth and intracellular activity, hyphal swellings, increased branching, and rosette formation (Hansen et al. 2000; Thrane et al. 1999, 2000a). Viscosinamide is produced by strain DR54 in situ (Nielsen and Sørensen 2003), and several of the adverse effects on *R. solani* and *P. ultimum* recorded in vitro, including reduced mycelium density and intracellular activity of *P. ultimum* and reduced sclerotia formation by *R. solani*, also were observed in situ (Thrane et al. 1999, 2000b, 2001). However, in these and several other studies with antagonistic CLP-producing *Pseudomonas* strains,

a comparison between the biocontrol activity of a wild type strain and CLP-deficient mutants was not included. Advances in the understanding of CLP biosynthesis have resulted in the construction of CLP-deficient mutants and also mutants overproducing CLPs. For example, a mutant of *Bacillus subtilis* 6051 defective in surfactin production was substantially less effective than the wild-type strain in controlling root infection of *Arabidopsis* by *Pseudomonas syringae* (Bais et al. 2004). Furthermore, a derivative of *B. subtilis* BBG100 that overproduces mycosubtilin showed an increased activity against *Pythium* spp. on tomato seedlings (Leclère et al. 2005). Studies with *Pseudomonas fluorescens* R1SS101 also showed reduced activity of massetolide A-deficient mutants against several plant-pathogenic fungi and oomycetes (A. Ficke and J. M. Raaijmakers, *personal communications*). The activity of massetolide A produced by strain R1SS101 against *Pythium* spp. has, so far, been attributed mainly to their zoosporicidal activity (De Souza et al. 2003). The zoosporicidal activity of massetolide A also may explain, at least in part, the biocontrol activity of strain R1SS101 against a range of other oomycete pathogens, including *Phytophthora* spp. However, the observation that strain R1SS101 and purified massetolide A also exert significant biocontrol activity against *Pythium* spp. that do not produce zoospores point to other modes of action (J. M. Raaijmakers, *personal communications*). A complicating factor in studying the role of CLPs in interactions between antagonistic *Pseudomonas* strains and plant pathogens is that CLPs have a dual function, as described previously for their role in pathogenesis. In addition to their direct effects on pathogen membranes, their surface activity also may enhance or even be essential for the delivery of and exposure of target pathogens to other antagonistic traits. In this context, Fogliano and associates (2002) demonstrated that, in combination, with CWDE of *Trichoderma atroviride*, CLPs produced by *Pseudomonas syringae* acted synergistically in antagonism toward several plant-pathogenic fungi.

Attachment and detachment. Attachment and detachment of bacteria to synthetic and natural surfaces has been the subject of numerous studies in the past and present, especially because of the importance of these processes in biofilm formation (Neu 1996; O'Toole et al. 2000). The role of CLPs in attachment or biofilm formation has been studied for several bacterial genera, including *Pseudomonas* and *Bacillus* spp. Arthrofactin produced by *Pseudomonas* MIS38 was shown to play an important role in developing mature biofilms (Roongsawang et al. 2003). An arthrofactin-deficient mutant formed unstable, but more, biofilms than the wild-type strain; furthermore, the structure of the biofilm was different, being more flat and fused for the mutant in contrast to the well-developed biofilms separated by channels for the wild-type strain. For *P. putida* PCL1445, Kuiper and associates (2004) showed that putisolvin I and II also influenced biofilm formation. The biofilm formed by a putisolvin-deficient mutant was strongly increased, containing more cells which formed aggregates that were not distributed as regularly as those of the parental strain. Addition of purified putisolvin I to the growth medium prior to incubation reduced biofilm formation by the wild-type strain in a concentration-dependent manner. Furthermore, putisolvin I and II also adversely affected biofilm formation of at least two other *Pseudomonas* strains and were shown to break down existing biofilms (Kuiper et al. 2004). In addition, surfactin inhibits biofilm formation of several human-pathogenic bacteria, including *Salmonella* spp. (Mireles et al. 2001). This capacity to destroy existing biofilms emphasizes the potential use of these CLPs in removal of biofilms formed by hazardous bacteria. Furthermore, surfactin was shown to play an essential role in adherence of *B. subtilis* 6051 to a synthetic surface and in biofilm formation on *Arabidopsis* roots (Bais et al. 2004).

They postulated that biofilm formation and CLP production may enable bacteria to efficiently colonize plant roots, thereby providing protection to their host. Recent studies by Nielsen and associates (2005) showed that the production of amphisin is an important trait for *Pseudomonas* sp. strain DSS73 to colonize sugar beet seed and barley straw residues. However, given that CLP production impairs biofilm formation for several *Pseudomonas* strains (discussed above), Nielsen and associates (2005) questioned the role of amphisin in biofilm formation during colonization of seed and straw by strain DSS73. Instead, they postulated that amphisin contributes to cell proliferation of strain DSS73 on the germinating seed and supports motility toward the plant material. Other putative roles of CLPs in colonization and proliferation suggested by Nielsen and associates (2005) included relieve of a nutrient limitation or a toxic effect of unknown seed components.

Motility. As described previously, CLPs facilitate the movement of plant-pathogenic *Pseudomonas* spp. on the phylloplane (Hildebrand et al. 1998; Lindow and Brandl 2003). Also, for antagonistic *Pseudomonas* strains, efficient colonization of the phytosphere (spermosphere, rhizosphere, and phyllosphere) as well as in situ production and delivery of the antagonistic traits at the right time and place are key aspects of their success to control plant pathogens (Lugtenberg et al. 2001; Whipps 2001). Several studies have shown that CLPs produced by antagonistic *Pseudomonas* strains and other bacterial genera are essential for surface motility (Andersen et al. 2003; Kuiper et al. 2004; Lindum et al. 1998; Nielsen et al. 2002; Roongsawang et al. 2003). For *Pseudomonas* sp. strain DSS73, amphisin production plays an important role in surface motility and in efficient containment of mycelial growth of root-infecting fungi, which most likely results from a combination of spatial limitation and delivery of antifungal compounds (Andersen et al. 2003). The reduced surface motility of amphisin-deficient mutants could be restored by addition of amphisin to the medium and also by addition of other CLPs, including tensin, viscosi-

namide, and serrawettin. Interestingly, several synthetic surfactants failed to complement the deficiency in surface motility of the mutants, suggesting that not only the reduction of surface tension is essential in motility but also the physical-chemical nature of the surfactant (Andersen et al. 2003).

Biosynthesis.

CLPs are produced nonribosomally on large, multifunctional peptide synthetases via a thiotemplate process. Nonribosomal peptide synthesis has been the subject of numerous biochemical and molecular studies. For a more detailed understanding of the structural and functional characteristics of the enzymes involved, we refer to several reviews (Challis and Naismith 2004; Challis et al. 2000; Finking and Marahiel 2004; Marahiel et al. 1997; Stachelhaus et al. 1999; von Dohren et al. 1999). As elegantly described by Gewolb (2002), nonribosomal peptide synthetases (NRPSs) are not bound by the restrictions of the "ribosome's rulebooks" and are involved not only in CLP production but also in the synthesis of an array of other unusual and potent peptides, including several clinically important drugs such as penicillin, vancomycin, and cyclosporin. NRPSs possess a modular structure and each module is a building block resulting in the stepwise incorporation and modification of one amino acid unit. Their substrates are not restricted to the usual proteinogenic amino acids but also can incorporate multiple nonproteinogenic D-amino acids, carboxy acids, or fatty acids (Gewolb 2002; Sieber and Marahiel 2005). The order and number of the modules of an NRPS protein are, in many cases, colinear to the amino acid sequence of the corresponding peptide moiety of the final CLP molecule ("colinearity rule"). A typical NRPS module consists of an adenylation (A) domain responsible for amino acid activation, a thiolation (T or, alternatively, PCP) domain for thioesterification of the activated amino acid, and a condensation (C) domain for peptide bond formation between two neighboring substrates to elongate the peptide chain (Fig. 1). These catalytic domains generate a linear

Fig. 1. Physical maps of the synthetic templates for arthrofactin (*arfABC*) of *Pseudomonas* sp. strain MIS38 (Roongsawang et al. 2003), the predicted lipodecapeptide (PFL2145/46/47) of *P. fluorescens* strain Pf-5 (Paulsen et al. 2005), and syringomycin (*syr*) and syringopeptin (*syp*) of *P. syringae* pv. *syringae* strain B301D (Bender and Scholz-Schroeder 2004). In the genome of *P. syringae* pv. *syringae*, the syringomycin and syringopeptin gene clusters are physically linked (indicated by a dotted line). The modular structure is depicted under each nonribosomal peptide synthetase (NRPS) gene. The number in each module indicates the position of the incorporated amino acid in the peptide moiety of the cyclic lipopeptide (CLP). Condensation (C) domains are responsible for peptide bond formation between neighboring amino acids. Adenylation (A) domains activate each of the respective amino acids and thiolation (T) domains are responsible for thioesterification. The thioesterase (TE) domains at the end of the synthetic templates are involved in cyclization of the CLP molecule. White arrows represent putative transporter genes and grey arrows indicate putative regulatory genes.

peptide which is cleaved at the end of the assembly line by a thioesterase (TE) domain, which results in the release of a linear product or a cyclic compound via an intramolecular cyclization reaction. In the latter case, TE domains also have been referred to as peptide cyclases (Sieber and Marahiel 2005). These cyclases display a high level of specificity by selecting a particular residue of the substrate for cyclization, which possibly explains the structural diversity in the peptide ring sizes described previously for the various CLP-groups of plant-associated *Pseudomonas* spp. (Table 1). Additional domains in nonribosomal peptide synthesis may include an epimerization (E) domain, responsible for the conversion of the L- or D-configuration of an amino acid. For example, in modules 3 and 6 of the surfactin synthetic template in *Bacillus* spp., there are two internal E domains that result in the incorporation of a D-Leu at the corresponding positions in the peptide moiety (Peypoux et al. 1999; Sieber and Marahiel 2005).

For CLP-producing *Pseudomonas* spp., a number of partial and complete sequences of NRPSs have been obtained over the past decade (Table 2). Two of the best-characterized bio-

synthetic templates are the synthetase clusters for arthrofactin (Roongsawang et al. 2003) and syringomycin (Bender et al. 1999; Guenzi et al. 1998a). Arthrofactin synthesis is governed by three NRPSs genes, designated *arfA*, *arfB*, and *arfC* (Fig. 1). Based on analogy with other NRPSs, ArfA, B, and C are composed of a total of 11 modules, which is consistent with the number of amino acids in the peptide moiety (Fig. 1; Table 1). Each module contains the typical features of NRPSs, including the A, C, and T domains. In this respect, the arthrofactin synthetase cluster is a classic example of the colinearity rule. Although seven of the 11 amino acids in the peptide moiety of arthrofactin have the D-configuration (Table 1), none of the 11 modules in the *arf* synthetic cluster harbor an internal E domain for the conversion from L to D form. Also, in the synthetic templates of syringopeptin (Scholz-Schroeder et al. 2003), the predicted lipodecapeptide of strain Pf-5 (Paulsen et al. 2005), and in the gene clusters for massetolide A and viscosin production by antagonistic *P. fluorescens* strains (M. J. D. de Kock and J. M. Raaijmakers, *personal communications*), internal E domains were not found. Based on additional ATP-

Table 2. Genes involved in the biosynthesis and regulation of cyclic lipopeptides (CLPs) produced by plant-associated *Pseudomonas* spp.^a

CLP group	CLP	Species/strain	Gene/protein information	GenBank accession no.	P/C*	References
<i>Synthesis (including efflux transporters)</i>						
Viscosin	Viscosin	<i>P. fluorescens</i> PfA7B	Nonribosomal peptide synthetases	No sequence available	–	Braun et al. 2001
	Massetolide A	<i>P. fluorescens</i> R1SS101	Nonribosomal peptide synthetases	AY303770; AY303771	P	De Souza et al. 2003
Amphisin	Amphisin	<i>Pseudomonas</i> sp. strain DSS73	<i>amsY</i> , peptide synthetase	AJ416154	P	Koch et al. 2002
	Arthrofactin	<i>Pseudomonas</i> sp. MIS38	<i>arfA</i> ; <i>arfB</i> ; <i>arfC</i> ; nonribosomal peptide synthetases	AB107223	C	Roongsawang et al. 2003
	Arthrofactin	<i>Pseudomonas</i> sp. MIS38	ORF5; putative outer membrane efflux protein	AB107223	C	Roongsawang et al. 2003
	Arthrofactin	<i>Pseudomonas</i> sp. MIS38	ORF6; putative ABC transporter protein	AB107223	P	Roongsawang et al. 2003
Tolaasin	Tolaasin	<i>P. tolaasii</i>	TL1, TL2, TL3; high-molecular weight protein	No sequence available	–	Rainey et al. 1993
	Tolaasin	<i>Pseudomonas</i> NZ17	Homology to syringomycin synthetase	No sequence available	–	Godfrey et al. 2001
	Syringopeptin	<i>P. syringae</i> pv. <i>syringae</i> B728a	Syringopeptin synthetase genes	CP000075	C	Feil et al. 2005
	Syringopeptin	<i>P. syringae</i> pv. <i>syringae</i> B301D	<i>sypA</i> , <i>sypB</i> , <i>sypC</i> ; syringopeptin synthetase	AF286216	C	Scholz-Schroeder et al. 2001a,b, 2003
	Syringopeptin	<i>P. syringae</i> pv. <i>syringae</i> B301D	<i>pseABC</i> ; tripartite resistance-nodulation-cell division transporter system	No sequence available	–	Kang and Gross 2005
Syringomycin	Syringomycin	<i>P. syringae</i> pv. <i>syringae</i> B728a	Syringomycin synthetase genes	CP000075	C	Feil et al. 2005
	Syringomycin	<i>P. syringae</i> pv. <i>syringae</i> B301D	<i>syrE</i> ; nonribosomal peptide synthetase	AF047828	C	Guenzi et al. 1998; Scholz-Schroeder et al. 2001a
	Syringomycin	<i>P. syringae</i> pv. <i>syringae</i> B301D	<i>syrB1</i> ; nonribosomal peptide synthetase	U25130	C	Zhang et al. 1995; Guenzi et al. 1998
	Syringomycin	<i>P. syringae</i> pv. <i>syringae</i> B301D	<i>syrC</i> ; thioesterase	U25130	C	Zhang et al. 1995; Guenzi et al. 1998
	Syringomycin	<i>P. syringae</i> pv. <i>syringae</i> B301D	<i>syrB2</i> ; nonheme Fe ^{II} halogenase	U25130	C	Vaillancourt et al. 2005
	Syringomycin	<i>P. syringae</i> pv. <i>syringae</i> B301D	<i>syrD</i> ; putative ABC transporter protein	M97223	C	Quigley et al. 1993
	Syringomycin	<i>P. syringae</i> pv. <i>syringae</i> B301D	<i>pseABC</i> ; tripartite resistance-nodulation-cell division transporter system	No sequence available	–	Kang and Gross 2005
	Syringomycin	<i>P. syringae</i> pv. <i>syringae</i> B301D	<i>pseABC</i> ; tripartite resistance-nodulation-cell division transporter system	No sequence available	–	Kang and Gross 2005
Other	Putisolvin	<i>P. putida</i> PCL1445	<i>psoA</i> ; putisolvin synthetase	DQ151887	P	Kuiper et al. 2004; Dubern et al. 2005
	Viscosin-like	<i>P. fluorescens</i> Pf-5	Nonribosomal peptide synthetases	CP000076 (PFL2145; PFL2146; PFL2147)	C	Paulsen et al. 2005

Continued on following page

^a C = complete CDS and P = partial sequence.

PPi exchange assays with a total of 20 typical L amino acids, Roongsawang and associates (2003) showed that the D-Leu1 A domain of the arthrofactin synthetic template adopted only L-Leu as a substrate and not D-Leu, suggesting that an external racemase is responsible for the L to D conversion. This suggestion for an external racemase was further supported by specific sequence motifs in the T domains of the *arf* synthetic template. T domains that are associated with an internal E domain, as in the surfactin synthetic template in *Bacillus* spp., have the highly conserved motif (F[F/Y]XXGGDSIKA[I/L]Q), in which the aspartate residue in front of the serine residue is important in the interaction between the T and internal E domains (Linne et al. 2001). In the arthrofactin synthetic template, the T domains contain a different conserved sequence motif (FFELGGHSLLA[V/M]) and can be further classified into the T(L) and T(D) domains, responsible for transferring L or D amino acids, respectively, to the intermediate peptide moiety (Roongsawang et al. 2003). Roongsawang and associates (2003) suggested that these differences may reflect the different topologies between A and T domains or, alternatively, be important for the recognition by an external racemase that is necessary for D-amino acid incorporation.

The C-terminal regions of CLP synthetic templates in *Pseudomonas* spp., including SypC in *P. syringae* and PFL2147 of *P. fluorescens* Pf-5, usually harbor one or two TE domains (Fig. 1). Also, in the arthrofactin synthetic template, the C-terminal

region of ArfC contains two putative TE domains designated ArfCTe1 and ArfCTe2 (Fig. 1). Based on phylogenetic analyses and alignments of the amino acid sequences, it was postulated that ArfCTe1 and ArfCTe2 have a separate or, possibly, a coordinated function in hydrolysis and cyclization of the final product (Roongsawang et al. 2003). Further analysis of the regions downstream of *arfC* resulted in the identification of two other genes, designated *ORF5* and *ORF6* (Roongsawang et al. 2003) (Fig. 1). Based on the direction of transcription of these two open reading frames (ORFs) and their homology to ATP-dependent transport proteins, these two ORFs were postulated to play a role in the secretion of arthrofactin (Roongsawang et al. 2003). The physical link between CLP synthetic templates and putative efflux genes also is the case for the predicted lipodecapeptide of *P. fluorescens* Pf-5 (Paulsen et al. 2005) (Fig. 1) and for syringopeptin and syringomycin of *P. syringae* pv. *syringae* (Feil et al. 2005; Scholz-Schroeder et al. 2001b) (Fig. 1).

In the plant pathogen *P. syringae* pv. *syringae*, the gene cluster for syringomycin synthesis makes up a substantial portion of the total genome. Together with the syringopeptin cluster, it composes a region of 132 kb which accounts for approximately 2% of the genome (Feil et al. 2005; Scholz-Schroeder et al. 2001b, 2003; Wang et al. in press; D. C. Gross, *personal communication*). This cluster, referred to as the *syr-syp* genomic island (Scholz-Schroeder et al. 2001b), was not found in the genome of *P. syringae* pv. *tomato* DC3000 (Feil et al. 2005).

Table 2. Continued from preceding page

CLP group	CLP	Species/strain	Gene/protein information	GenBank accession no.	P/C*	References
<i>Regulation</i>						
Viscosin	Viscosin	<i>P. fluorescens</i> 5064	AHL biosynthesis	No sequence available	–	Cui et al. 2005
Amphisin	Amphisin	<i>Pseudomonas</i> sp. strain DSS73	<i>gacS</i> ; sensor kinase in two-component regulatory system	AJ416155	P	Koch et al. 2002
	Arthrofactin	<i>Pseudomonas</i> sp. MIS38	ORF1; putative DNA binding protein (luxR type)	AB107223	C	Roongsawang et al. 2003
Tolaasin	Tolaasin	<i>P. tolaasii</i>	<i>pheN</i> ; two-component regulatory protein (GacS)	U95300	C	Grewal et al. 1995
	Syringopeptin	<i>P. syringae</i> pv. <i>syringae</i> B728a	<i>gidA</i> ; initiation of chromosome replication	AF302083	C	Kinscherf and Willis 2002
	Syringopeptin	<i>P. syringae</i> pv. <i>syringae</i> B301D	<i>salA</i> , <i>syrG</i> , <i>syrF</i> ; putative DNA-binding regulatory proteins (luxR type)	AF372703	C	Lu et al. 2002
Syringomycin	Syringomycin	<i>P. syringae</i> pv. <i>syringae</i> B728a	<i>gidA</i> ; initiation of chromosome replication	AF302083	C	Kinscherf and Willis 2002
	Syringomycin	<i>P. syringae</i> pv. <i>syringae</i> B728a	<i>lemA</i> ; two-component regulatory protein (GacS)	M80477	C	Hrabak and Willis 1992; Kitten et al. 1998
	Syringomycin	<i>P. syringae</i> pv. <i>syringae</i> B728a	<i>gacA</i> ; response regulator in two-component regulatory system	CP000075	C	Rich et al. 1994; Feil et al. 2005
	Syringomycin	<i>P. syringae</i> pv. <i>syringae</i> B728a	<i>salA</i> ; putative DNA-binding protein (luxR type)	AF022808	C	Kitten et al. 1998
	Syringomycin	<i>P. syringae</i> pv. <i>syringae</i> B301D	<i>salA</i> , <i>syrG</i> , <i>syrF</i> ; putative DNA-binding proteins (luxR type)	AF372703	C	Lu et al. 2002
	Syringomycin	<i>P. syringae</i> pv. <i>syringae</i> B301D	<i>syrA</i> ; N-acetylglutamate synthase (arginine biosynthesis)	AY374326	C	Lu et al. 2003
	Syringomycin	<i>P. syringae</i> pv. <i>syringae</i> B301D	<i>syrP</i> ; histidine kinase in two-component regulatory system (phosphorelay)	U88574	C	Zhang et al. 1997
Other	Putisolvin	<i>P. putida</i> PCL1445	<i>gacA</i> ; response regulator in two-component regulatory system	No sequence available	–	Dubern et al. 2005
	Putisolvin	<i>P. putida</i> PCL1445	<i>gacS</i> ; sensor kinase in two-component regulatory system	No sequence available	–	Dubern et al. 2005
	Putisolvin	<i>P. putida</i> PCL1445	<i>dnaK</i> ; <i>dnaJ</i> , <i>grpE</i> ; heat-shock proteins	AY823737	C	Dubern et al. 2005

In contrast to the arthrfactin gene cluster discussed above, the syringomycin gene cluster has several different and unique features. First of all, the organization of the structural synthetase genes does not respect the colinearity rule because the *sydB1* gene, responsible for the incorporation of the ninth amino acid in the peptide moiety, is located upstream of *sydE*, which encodes the first eight amino acids (Fig. 1). Another interesting structural feature is the positioning of the single TE domain, which is not fused to the C-terminus of the last amino acid binding domain SydB1 but, instead, to the C-terminus of SydE-M8, the module required for the incorporation of the eighth amino acid (Bender and Scholz-Schroeder 2004; Guenzi et al. 1998a). The C-terminus of *sydE* does contain elements of a ninth module, including condensation and thiolation domains, but lacks an adenylation domain which is proposed to be provided by SydB1 (Bender et al. 1999; Bender and Scholz-Schroeder 2004). Recent studies have further indicated that SydB1 activates the amino acid L-Thr, after which SydB2-mediated chlorination takes place to yield the biologically active ninth amino acid residue 4-chlorothreonine (Vaillancourt et al. 2005). The fourth protein essential in syringomycin synthesis is SydC, which is proposed to hydrolyze 3-hydroxydodecanoyl-CoA, leading to subsequent transfer of the 3-hydroxy fatty acid to the amino group of serine bound to SydE-M1.

For the secretion of syringomycin, studies initially focused on SydD, a protein with homology to membrane proteins of the ABC transporter family (Quigley et al. 1993; Scholz-Schroeder et al. 2001b). A mutation in *sydD* resulted in a significant reduction in secretion of syringomycin and also syringopeptin and, concurrently, in a 70% reduction in virulence on cherry fruit. However, the *sydD* mutation did not lead to a complete loss of CLP secretion. Kang and Gross (2005) recently discovered an additional efflux system, designated PseABC, located at the left border of the *syd-syp* genomic island of *P. syringae* pv. *syringae* B301D (Fig. 1). This tripartite transporter system, with homology to the resistance-nodulation-cell division (RND) efflux system of *Ralstonia solanacearum*, was shown to be involved in syringomycin and syringopeptin secretion. Also, in *B. subtilis*, an “RND-like” gene, designated *yerP*, was shown to be at least partially involved in efflux of surfactin (Tsuge et al. 2001). Interestingly, expression of *yerP* was not induced by surfactin, but associated with the growth phase (late logarithmic) of the producing strain. Furthermore, *YerP* also appeared to play a role in resistance of *B. subtilis* to other antibiotics, including acriflavine (Tsuge et al. 2001). Although a role of PseABC of *P. syringae* pv. *syringae* in resistance to acriflavine could not be demonstrated, heterologous expression of the *pseABC* genes in a drug-supersensitive mutant of *E. coli* did result in a significant increase in resistance to acriflavine and other antibiotics, supporting their functional relationship with RND-type transporter genes (Kang and Gross 2005).

Based on the unusual and nonlinear genetic organization of the synthetic template of syringomycin and the templates of several other nonribosomal peptide antibiotics (Finking and Marahiel 2004), Guenzi and associates (1998a) postulated that, for the coordinated transcription of the enzyme subunits, individual modules can be dissociated without affecting CLP synthesis at both the qualitative and quantitative level. To further test this hypothesis, Guenzi and associates (1998b) designed two mutants of surfactin-producing *B. subtilis*. In one mutant, the operon structure of the surfactin synthetic template was altered by integration of a constitutive promoter; whereas, in the other mutant, the thioesterase domain was physically dissociated from the third amino acid binding domain and expressed independently. The results showed that both mutants produced surfactin at levels comparable with the wild-type strain (Guenzi et al. 1998b). These studies indicated that domain-domain inter-

actions rather than coordinated transcription and translation are essential for the correct assembly and activity of the NRPSs.

Substrate selection and selectivity-conferring elements of NRPSs. Comparative analysis of multiple NRPS sequences has shown that several of the modules harbor conserved motifs that allow the prediction of the sequence and configuration of the amino acids in the peptide moiety of the CLP molecule. A number of studies and reviews have addressed this issue in detail from a fundamental scientific perspective and also because it provides a means for targeted alteration of the structure and activity spectrum and, ultimately, enables the design of artificial nonribosomal peptide products (Challis et al. 2000; Gruenewald et al. 2004; Lautru and Challis 2004; Stachelhaus et al. 1999). One of the key questions in this context is how NRPSs select from the wide variety of precursors to be incorporated into the final CLP molecule (Lautru and Challis 2004). Most of the studies have focused on the selectivity-conferring codes within the adenylation (A) domains, because these domains select the amino acids and are regarded as the “gatekeeper” units of the repeated modules (Stachelhaus et al. 1999). Following the elucidation of the crystal structure of the phenylalanine-activating A domain (PheA) of gramicidin-S-synthetase from *B. brevis* (Conti et al. 1997), Stachelhaus and associates (1999) compared the amino acid residues of a large number of A domains from prokaryotic and eukaryotic origins with those lining the binding pocket of PheA. The *in silico* analyses provided a large number of signature sequences within A domains that allowed a prediction of the amino acid composition of the peptide moiety. This information, supplemented with new sequence data, has resulted in the design of web-based interfaces for analysis of NRPS sequences and polyketide (PKS) synthetase genes (Ansari et al. 2004; Challis et al. 2000). In addition to *in silico* analyses, Stachelhaus and associates (1999) provided experimental evidence for the specificity-conferring codes of A domains within NRPSs by introducing mutations in PheA, which led to the activation of L-Leu instead of phenylalanine. This and other studies also revealed that the same amino acid can be activated by A domains with different predicted selectivity pocket residues, which suggests some level of degeneracy in substrate recognition (Challis et al. 2000; Lautru and Challis 2004; Stachelhaus et al. 1999). Furthermore, certain signature sequences in the A domains contain so-called “wobble-like” positions which can lead to flexibility in amino acid usage (Stachelhaus et al. 1999). In other words, the signature sequences of A domains permit, to some degree, the incorporation of an “incorrect” amino acid, which may explain the presence of several less abundant “isoforms” of viscosin- or amphisin-like CLPs in extracts from cell cultures of antagonistic *P. fluorescens* strains (De Souza et al. 2003; Nielsen et al. 2002). Also C, E, and TE domains play an important role in substrate recognition (Finking and Marahiel 2004; Lautru and Challis 2004; Linne et al. 2004; Roongsawang et al. 2005; Stein et al. 2005), although specificity studies have not been conducted as extensively as for A domains. Based on the increased availability of NRPS gene sequences of *Pseudomonas* and other species, the list of signature sequences specific for the various A domains now can be further expanded and will provide a valuable tool in annotation of NRPS sequences and in the prediction of the CLP structures based on genome data. This information can be exploited further for targeted alteration of CLP structures and activities via modification or exchange of NRPS modules, and for combinatorial synthesis of artificial nonribosomal peptide products (Gruenewald et al. 2004; Lautru and Challis 2004).

Regulation.

Compared with our understanding of the biosynthesis of CLPs in *Pseudomonas* and other bacterial genera, relatively

little is known about the genetic network involved in the perception of external factors (abiotic and biotic) and the signal transduction pathways that drive transcription of the CLP biosynthetic genes. A number of studies have focused on the effects of abiotic conditions (e.g., temperature, pH, and oxygen), nutritional factors (e.g., carbon and nitrogen sources and trace elements), and plant signal molecules (glycosides and phenolics) on CLP production in *Pseudomonas* spp. (Bender et al. 1999; Bender and Scholz-Schroeder 2004; Nybroe and Sørensen 2004). This section focuses primarily on CLP regulatory genes and mechanisms identified so far for plant-associated *Pseudomonas* spp.

Similar to regulatory mechanisms identified for CLP production in *Bacillus* spp. (Duitman 2003; Sullivan 1998), two-component systems and cell density also play a key role in the regulation of CLP production in *Pseudomonas* spp. (Table 2). For several CLPs, including syringomycin (Bender et al. 1999; Willis and Kinscherf 2004), amphisin (Koch et al. 2002), putisolvin I and II (Dubern et al. 2005), massetolide A, and viscosin (I. de Bruijn and J. M. Raaijmakers, *personal communication*), the GacS/GacA two-component regulatory system appears to serve as a master switch; mutants disrupted in either one of the two genes are deficient in CLP production. Although the GacA/GacS system has been the subject of numerous studies (Heeb and Haas 2001), very little still is known about signals that serve as a trigger. Studies by Koch and associates (2002) with antagonistic *Pseudomonas* DSS73 elegantly demonstrated that exudates of sugar beet seed contain triggers for amphisin production and that the putative signal transmission requires a functional GacS protein. The active trigger compound in the seed exudates was heat stable and could be removed by dialysis; therefore, Koch and associates (2002) suggested that small organic molecules are involved. To our knowledge, the identity of these small organic molecules has not yet been resolved.

Several CLP regulatory genes that act downstream of the GacA/GacS system have been identified. One of these genes is *salA* from *P. syringae* pv. *syringae* strains B301D and B728a. A *salA* mutant of strain B301D failed to produce syringomycin and was significantly reduced in virulence on cherry fruit (Lu et al. 2002). *SalA* is positively regulated by *gacA* and *gacS*, and overexpression of *salA* in a *gacS* mutant of strain B728a restored syringomycin production (Kitten et al. 1998). *SalA* contains a helix-turn-helix DNA binding motif and shares homology with members of the LuxR family of regulatory proteins (Lu et al. 2002). *SalA* activates the expression of *syrB1* (Kitten et al. 1998) and *syrF*, another LuxR-type regulatory gene involved in syringomycin synthesis (Lu et al. 2002). To further identify the members of the *SalA* regulon, Lu and associates (2005) designed an oligonucleotide microarray representing genes in the *syr-syp* genomic island as well as genes involved in the Hrp type III secretion system, phytohormone synthesis, siderophore and syringolin production, quorum sensing, and global regulation, including *gacS*, *gacA*, *rpoN*, and *rpoS*. Comparative analysis of the transcription profiles of a *salA* mutant and the wild-type strain B301D showed that genes involved in the synthesis, secretion, and regulation of syringomycin and syringopeptin were upregulated by the *salA* gene. No significant effects were observed for the genes located outside the *syr-syp* genomic island, except for the *sydD* gene, which is involved in syringolin synthesis (Lu et al. 2005). This “genome-wide” study serves as an excellent model to further elucidate regulatory elements and networks in CLP synthesis in other *Pseudomonas* spp.

DnaK is another gene that is positively regulated by the GacA/GacS system and involved in regulation of CLP synthesis in *P. putida* (Dubern et al. 2005). In a *dnaK* mutant of strain

PCL1445, production of putisolvin I and II was strongly reduced. Given that *DnaK* is a member of the Hsp70 heat shock protein family, Dubern and associates (2005) subsequently investigated the role of temperature on putisolvin production. Their 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 two other genes, *dnaJ* and *grpE*, that both adversely affected the synthesis of putisolvin I and II. Although the exact role of these three genes in regulation of putisolvin synthesis is not yet known, the authors speculated that they may be required for the proper folding or activity of other positive regulators of the putisolvin synthetic gene *psaA*. Two other possible mechanisms proposed were interaction of the *DnaK* complex with the *rpoS* encoded sigma factor σ^s , or the requirement of the *DnaK* complex for proper assembly of the peptide synthetase complex.

In addition to two-component regulation, cell density plays an important role in global regulation of CLP production in *Pseudomonas* spp. For many antagonistic *P. fluorescens* strains, CLP production occurs in the late exponential or stationary growth phase, but the underlying molecular mechanisms are far from being understood. In this context, Nybroe and Sørensen (2004) emphasized that, although CLP production is affected by the growth phase and nutritional conditions that lead to increased growth, the specific impact of these factors may differ considerably among strains. Quorum sensing via *N*-acyl homoserine lactones (*N*-AHL) does not appear to play a role in amphisin and syringomycin production (Andersen et al. 2003; Kinscherf and Willis 1999; Quinones et al. 2005). Studies with antagonistic *P. fluorescens* R1SS101 indicated that *N*-AHL-mediated quorum sensing does not seem to be involved in massetolide A production (I. de Bruijn and J. M. Raaijmakers, *personal communication*). For the plant-pathogenic *P. fluorescens* strain 5064, recent studies by Cui and associates (2005) provided, for the first time, evidence for *N*-AHL-mediated quorum sensing in viscosin synthesis. Screening of a mutant collection of strain 5064 resulted in the identification of six mutants affected in quorum sensing signal (QSS) production. The QSS was identified as *N*-3-acyl-hydroxyoctanoyl-HSL, and addition of QSS extracts or the synthetic signal molecule restored viscosin synthesis in the mutant. Cui and associates (2005) also observed that the mutation did not lead to a complete blockage in *N*-AHL expression and they speculated that there may be more than one quorum sensing system in strain 5064. Although the sequences of the genes involved in quorum sensing-mediated viscosin synthesis in strain 5064 were not described in this study (Cui et al. 2005), they may provide a useful tool to assess the presence (or absence) of homologues in other CLP-producing *Pseudomonas* strains.

In addition to two-component regulatory systems and cell density, Kinscherf and Willis (2002) showed that the *gidA* gene is involved in global regulation of syringomycin and syringopeptin synthesis in *P. syringae*. Next to the inability to produce CLPs, mutations in *gidA* (glucose-inhibited division) affected a variety of other phenotypes, including pyoverdine synthesis and surface motility on low-agar media. As was shown for *gacS* mutants of *P. syringae*, overexpression of *salA* restored syringomycin synthesis in *gidA* mutants, suggesting that *gidA* plays a role in *salA* regulation (Kinscherf and Willis 2002). Finally, analyses of regions physically linked to the CLP synthetic templates revealed a number of other interesting regulatory genes (Table 2). For syringomycin, these include the phosphate-regulated gene *syrP* (Fig. 1) and yet another LuxR-type gene *syrG*, although their exact roles remain to be determined (Bender and Scholz-Schroeder 2004).

Concluding remarks.

Based on this survey, we conclude that CLPs produced by plant-associated *Pseudomonas* spp. are enormously diverse, both structurally and in terms of their biological activity. Although a number of regulatory genes have been identified, the understanding of the regulatory networks involved in CLP synthesis by plant-associated *Pseudomonas* spp. is still in its infancy. Therefore, identification of genes and mechanisms involved in perception and signal transduction is a major challenge for the near future. Also, the synthesis of the fatty acid moiety of the CLP molecule has received little attention. At present, many other specific questions remain to be addressed. For example, which external racemases in *Pseudomonas* spp. play a role in epimerization of the amino acids in the CLP peptide moiety? Which other natural roles and modes of action can be assigned to CLPs? How can we modulate, both quantitatively and qualitatively, in situ production of CLPs by antagonistic and pathogenic *Pseudomonas* spp.? Is regulation of CLP synthesis fundamentally different between pathogenic and antagonistic *Pseudomonas* spp.? Significant advances in the analyses and mining of whole genome sequences, the design of microarrays, and developments in metabolomics and proteomics will enable a better understanding of the synthesis, regulation, and activity of CLPs produced by *Pseudomonas* and other bacterial genera.

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Pseudomonas Genome Database v2: v2.pseudomonas.com
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