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Characterization of two *Pseudomonas putida* lipopeptide biosurfactants, putisolvin I and II, which inhibit biofilm formation and break down existing biofilms

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Summary

Pseudomonas putida **strain PCL1445 was isolated from roots of plants, grown on a site polluted with polycyclic aromatic hydrocarbons. PCL1445 produces biosurfactant activity at the end of the exponential growth phase. High-performance liquid chromatography (HPLC) analysis of supernatant extracts of PCL1445 showed two peaks with surface-tension reducing activity, tentatively assigned as biosurfactants putisolvin I and putisolvin II and was followed by structural analyses. A transposon mutant of PCL1445, strain PCL1436, which lacks the two surface-active peaks appeared to be mutated in an open reading frame (ORF) with amino acid homology to various lipopeptide synthetases. Structural analyses of the two biosurfactants of PCL1445 revealed that both are novel cyclic lipodepsipeptides with a hexanoic lipid chain connected to the N-terminus of a 12-amino-acid peptide moiety, in which the C-terminal carboxylic acid group forms an ester with the hydroxyl side-chain of Ser9. The difference between the two structures is located in the second amino acid from the C-terminus, being valine for putisolvin I, and leucine/isoleucine for putisolvin II. We show that these novel compounds lower the surface tension and influence the biofilm development on polyvinyl chloride (PVC). Biofilm formation of the biosynthetic mutant PCL1436 was strongly increased containing more cells, which formed aggregates earlier as compared with wild-type PCL1445 biofilms. Using purified putisolvin I and II it was shown that biofilm formation of different** ***Pseudomonas*** **strains was inhibited and most interestingly, that both putisolvins are also able to break down existing** ***Pseudomonas*** **biofilms.**

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Introduction

Microbial-derived surfactants or biosurfactants are produced by a wide variety of microbes and are amphipathic molecules with a hydrophilic and a hydrophobic domain. Because of these traits, biosurfactants accumulate at interfaces, can form micelles, lower the surface tension and thereby enhance the solubility of poorly soluble compounds in water (Rosenberg, 1993; Zhang and Miller, 1994; Shreve *et al*., 1995; Grimberg *et al*., 1996; Schippers *et al*., 2000).

Biosurfactants are best known for enhancing the bioavailability of hydrophobic soil pollutants such as polycyclic aromatic hydrocarbons (PAHs). By reducing the surface tension between water and hydrophobic surfaces the formation of emulsions of hydrophobic xenobiotics in water is enhanced, thereby increasing the growth surfaces for bacteria. Some biosurfactants can also stimulate the attachment to and/or detachment of bacteria from the surface of xenobiotics by influencing the hydrophobicity of the bacterial cell surface or the surface of the xenobiotic (Rosenberg, 1993; Al-Tahhan *et al*., 2000; Garcia-Junco *et al*., 2001).

Although biosurfactants have in common their amphipathic character, their primary structures show a wide diversity. An intensely studied group of biosurfactants is that of the glycolipids, with rhamnolipids produced by *Pseudomonas* bacteria as a major representative (Zhang and Miller, 1994; Providenti *et al*., 1995; Shreve *et al*., 1995; Deziel *et al*., 1996; Sim *et al*., 1997; Stanghellini and Miller, 1997). Lipopeptides form another important group of biosurfactants, which are produced by members

of the genera *Bacillus*, *Lactobacillus, Streptococcus* and *Pseudomonas*. They are non-ribosomally produced by peptide synthetases, which are encoded by large gene clusters. Lipopeptides produced by *Pseudomonas* have been reported as agents for the biocontrol of phytopathogenic fungi (Stanghellini and Miller, 1997; Nielsen *et al*., 1999; Hendriksen *et al*., 2000; Thrane *et al*., 2000), or as phytotoxins (Hutchison and Gross, 1997). Lipopeptides produced by Gram-positive *Bacillus*, *Lactobacillus* and *Streptococcus* strains were reported to play a role in the attachment of bacteria to surfaces (Neu, 1996; Busscher *et al*., 1997; Velraeds *et al*., 2000; Meylheuc *et al*., 2001; Mireles *et al*., 2001). Attachment is an essential part of bacterial biofilm development and maintenance (O'Toole *et al*., 2000). The formation of hazardous bacterial biofilms on medical and technical equipment is an important problem, especially as the bacteria within such biofilms are highly resistant to antibiotics (Donlan and Costerton, 2002). Blocking hazardous biofilms would be a useful aspect of biosurfactants.

Here we describe purification and structural analyses of two novel biosurfactants produced by *Pseudomonas putida* strain PCL1445, referred to as putisolvin I and putisolvin II, and show that they belong to the class of lipopeptides. They consist of 12 amino acids, which are cyclic by virtue of an ester bond formed between the side-chain of the ninth amino acid and the C-terminal carboxyl group and have a hexanoic acid chain in amide linkage to the N-terminus. We show that putisolvin I and putisolvin II influence the development of the biofilm and can inhibit the formation of *Pseudomonas* biofilms on polyvinyl chloride (PVC). Moreover they can break down biofilms of various *Pseudomonas* spp. which have been formed on PVC.

Results

Isolation and characterization of P. putida *strain PCL1445*

Strain PCL1445 was isolated together with the naphthalene degrading *P. putida* strain PCL1444 after a selection procedure for efficient root colonization and growth on naphthalene as the sole carbon source (Table 1; Kuiper *et al*., 2001). To identify isolate PCL1445 phylogenetically, a PCR reaction amplifying the 16S ribosomal DNA was performed as described by Kuiper *et al*. (2001). PCL1445 was designated as *P. putida* as sequence analysis of the resulting 1.4 kb PCR fragment showed highest homology (99%) with the 16S rDNA of *P. putida* strain ATCC 17494 (GenBank AF094740) and *P. putida* KT2440 (Franklin *et al*., 1981; Nelson *et al*., 2003).

It appeared that *P. putida* PCL1445 secreted compounds that were able to lower the surface tension of basic medium (BM) and KB culture media. The surface tension of KB culture supernatant was measured using the Du Nouy ring method. Figure 1A shows that in supernatant of PCL1445 the surface tension is reduced by 25 mN m^{-1} when compared with sterile KB medium. KB culture supernatant of rhizosphere isolates *P. putida* PCL1444 and *P. fluorescens* WCS365 (Geels and Schippers, 1983) showed no reduction of the surface tension when compared with sterile KB (Fig. 1A). As predicted also no activity in the drop-collapsing assay was detected for *P. fluorescens* strain WCS365 and *P. putida* strain PCL1444, whereas supernatant of PCL1445 does result in a collapsed droplet (Fig. 1B). To determine the emulsifying activity of KB supernatants of strains PCL1445, PCL1444 and WCS365, culture supernatants were mixed with toluene and allowed to separate for 1 h. The resulting phases were stable and showed a clear difference

Table 1. Bacterial strains and plasmids.

Strains and plasmids	Relevant characteristics	Reference
Pseudomonas		
WCS365	Wild-type *P. fluorescens* strain, excellent root colonizer	Geels and Schippers (1983), Simons *et al*. (1996)
PCL1444	Wild-type *P. putida* strain, naphthalene degrader and efficient grass root colonizer	Kuiper *et al*. (2001)
PCL1445	Wild-type *P. putida* strain, colonizes grass roots and produces biosurfactants	This study
PCL1436	Tn*5luxAB* derivative of PCL1445, mutated in a lipopeptide synthetase homologue	This study
UCBPP-PA14	*P. aeruginosa*, opportunistic pathogen of plants and animals	Rahme *et al*. (1995)
Escherichia coli		
DH5α	*endA1 gyrSA96 hrdR17(rK-mK-) supE44 recA1*; general purpose host strain used for transformation and propagation of plasmids	Boyer and Roulland-Dussoix (1969)
Plasmids		
pRL1063a	Plasmid harbouring a promoterless Tn*5luxAB* transposon, Km^r	Wolk *et al*. (1991)
pRK2013	Helper plasmid for tri-parental mating	Ditta *et al*. (1980)
pMP5459	pRL1063a-based plasmid recovered from chromosomal DNA of PCL1436 after digestion with *Eco*RI with the Tn*5luxAB* inserted in a lipopeptide synthetase homologue, Km^r	This study

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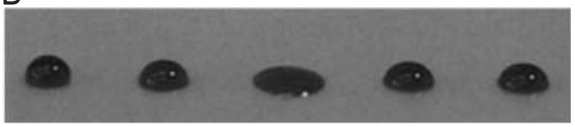
A

Surface tension in mN/m ± SD				
WCS365	PCL1444	PCL1445	PCL1436	KB
54 ± 1	55 ± 1	30 ± 0.5	54 ±1	55 ± 1

B



C

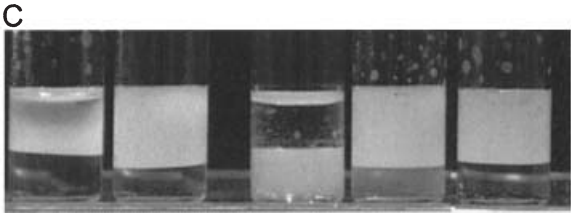


Fig. 1. Biosurfactant activity of culture supernatant of *Pseudomonas* strains. The surface tension of King's medium B (KB) supernatants of overnight cell cultures of *P. putida* strain PCL1445, its mutant derivative PCL1436, *P. fluorescens* strain WCS365 and *P. putida* strain PCL1444 was determined in three experiments. Sterile KB medium was used as a control.
A. The surface tension quantified with the Du Nouy ring method. Results are the mean of three measurements.
B. Twenty-five microlitre droplets of each culture supernatant on a hydrophobic (parafilm) underground. Methylene blue was added to stain the supernatants for photographic purposes and had no influence on the shape of the droplets.
C. The emulsifying activity of the culture supernatants. An equal volume of toluene was added to supernatant and mixed in glass vials. The layers represent the stabilized emulsions after 1 h of incubation.

between PCL1445 and PCL1444 or WCS365 (Fig. 1C). In strain PCL1445, the toluene layer was clear, but the water layer was turbid. This indicates the presence of toluene droplets in the water phase, resulting in a dense water/toluene emulsion. The phases in supernatants of strains PCL1444 and WCS365 show a turbid toluene layer, indicating a dense toluene/water emulsion. The water layer remained clear.

Analysis of the surface tension of spent culture supernatant of PCL1445 grown in KB medium showed that the biosurfactant activity was first detected at the end of the exponential phase (Fig. 2).

Generation and characterization of a mutant of P. putida *strain PCL1445, impaired in biosurfactant production*

To elucidate the genetic basis for biosurfactant production by PCL1445, transposon mutagenesis was performed with PCL1445 in order to obtain a mutant impaired in biosurfactant production. Four hundred Tn*5*::*luxAB* transposants were screened for loss of biosurfactant activity in the drop-collapsing assay using cells derived from a single colony. One mutant lacking activity was selected and named PCL1436. After overnight growth in KB medium, the supernatant of this mutant was tested in the drop-collapsing assay. Results show that the supernatant of PCL1436 did not spread on a hydrophobic support, in contrast to supernatant derived from the wild-type strain (Fig. 1B). The culture supernatant from mutant derivative PCL1436 showed a surface tension similar to that of the control strains PCL1444 and WCS365 and to sterile KB medium (Fig. 1A). In the assay to measure the emulsifying activity of supernatants, the resulting phases of mutant PCL1436 were similar to those of control supernatants of strains PCL1444 and WCS365 showing a turbid toluene layer (Fig. 1C). PCL1436 did not differ in growth rate as compared with its wild-type PCL1445 (Fig. 2). Measuring the surface tension of spent culture medium of PCL1436 during growth showed the absence of biosurfactant activity production (Fig. 2).

The swarming motilities of wild-type strain PCL1445 and mutant derivative PCL1436 were compared on 0.3% agar plates. After spot-inoculation of strain PCL1445 a thin 'film', presumably caused by a change in the hydrophobicity of the agar, spread in minutes to hours over the surface of the agar plate. This was not observed with strain PCL1436. After incubation for 16 h at 28°C the diameter of the spot of PCL1445 was 25 mm, whereas the diameter of the spot of mutant PCL1436 was 9 mm. The thin film produced by the wild type was still visible and partly overgrown by the cells of the inoculation spot.

To identify the sequences adjacent to the transposon, total chromosomal DNA from mutant PCL1436 was isolated and digested with *Eco*RI. Subsequent circularization resulted in plasmid pMP5459. Sequence analysis of 9.2 kb of the chromosomal parts of PCL1436 flanking the

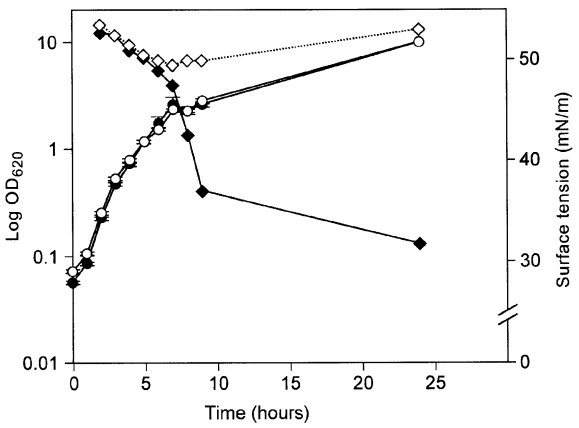


Fig. 2. Growth and culture supernatant surface tension of *P. putida* PCL1445 and its mutant PCL1436. *Pseudomonas putida* PCL1445 (●) and its mutant strain PCL1436 (○) were grown in liquid King's medium B. Surface tension of the culture media of PCL1445 (◆) and PCL1436 (◇) were quantified after removal of cells using the Du Nouy ring method. The experiment was performed in triplicate.

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Tn*5luxAB* insertion showed that the transposon is inserted in an open reading frame (ORF) of which the start and end was not present on the 9.2 kb sequence and showed highest homology (57%) at the amino acid level with *syrE* of *P. syringae* pv. *syringae* (Guenzi *et al*., 1998). These sequence data have been submitted to the GenBank database under Accession number AY256972. *syrE* is 28.4 kb in size and encodes a syringomycin synthetase involved in the production of syringomycin, a cyclic lipodepsipeptide toxin (Hutchison and Gross, 1997). The inserted ORF of PCL1436 showed also high levels of homology with other lipopeptide synthetases, such as the surfactin synthetase of *Bacillus* spp. (33% identity, 50% positives; Yakimov *et al*., 1995).

Structural identification of the biosurfactants of PCL1445

In order to purify and isolate the compounds with surface activity produced by PCL1445, supernatants of cultures of PCL1445 and PCL1436, grown in BM medium with glycerol as the sole carbon source, were extracted and fractionated using C8 reversed-phase high-performance liquid chromatography (HPLC). When the HPLC chromatograms of the extracts of wild-type PCL1445 and mutant strain PCL1436 were compared in the wavelength range between 200 and 400 nm, two peaks were observed at 206 nm in the chromatogram obtained from the wild-type extract (Fig. 3A), both of which were absent in that of the mutant extract (Fig. 3B). The collected HPLC fractions (collected each minute) from wild-type strain PCL1445 were tested for surface activity in the drop-collapsing assay, and only those fractions corresponding to the retention times of the two peaks present exclusively in the wild type chromatogram (Fig. 3, fractions 38/39 and 41/42) appeared to be active. The peak eluting at 38/39 min was named putisolvin I, the peak eluting at 41/42 min was named putisolvin II. The HPLC fractions from mutant strain PCL1436, corresponding to the retention times of the peaks in the chromatogram of PCL1445 extract, showed no activity in the drop-collapsing assay. The active fractions obtained thus from wild-type PCL1445 and the fractions derived from the mutant strain with similar retention times were submitted to mass spectrometric analysis, amino acid analysis and NMR analysis.

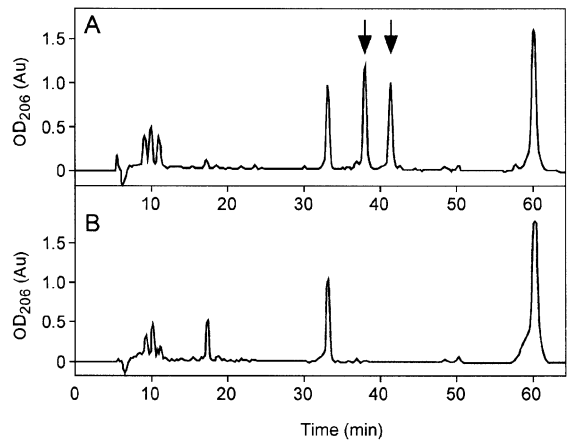

Fig. 3. C8-RP HPLC analysis of the surface-active compound(s) of *P. putida* PCL1445. C8-RP high-performance liquid chromatography (HPLC) analysis of ethyl acetate fractions derived from BM supernatants of PCL1445 (A) and PCL1436 (B). Chromatograms are obtained through sample analysis at a wavelength of 206 nm. After elution, fractions of 1 ml were collected and tested for biosurfactant activity. Peaks representing fractions with biosurfactant activity are indicated with an arrow.

Mass spectrometric analysis of the lipopeptides

Diode array UV of the HPLC fractions showed no evidence for the presence of aromatic amino acids (that absorb at 254 nm). Electrospray mass spectrometric analysis was used to determine the molecular mass of the species present in the fractions corresponding to surfactant activity (Fig. 3). The spectra obtained from the fractions which had surfactant activity showed that the earlier-eluting compound, putisolvin I, gave rise to an ion of $[M+H]^+$ at *m/z* 1380 and that the later-eluting compound, putisolvin II, yielded an $[M+H]^+$ ion at *m/z* 1394. $[M+Na]^+$ ions were also observed. A difference of 14 Da between the two species is consistent with the later eluting species containing an extra CH2 group. This would also explain the separation between the two on RP-HPLC analysis. The observed ions at *m/z* 1380 and *m/z* 1394 were absent from the mass spectra obtained from the corresponding HPLC fractions obtained from the extract of culture supernatant of mutant PCL1436.

Q-oToF collision-induced dissociation (CID) tandem mass spectra of the $[M+H]^+$ ions for the two compounds were obtained, using a hybrid quadrupole-orthogonal time-of-flight instrument (Micromass), to produce structurally diagnostic fragment ions (Fig. 4B). Both b and y sequence ions were obtained, although neither series provided complete sequence coverage. The C-terminal region of the lipopeptide failed to yield intense fragment ions that could be used to determine amino acid sequence. Comparison of the tandem mass spectra obtained from the two protonated molecules allowed the additional CH_2 group to be located within this C-terminal region. It was postulated that there was no fragmentation in this C-terminal region due to cyclization of the amino acid backbone via an ester bond, as is described for many lipopeptides.

To determine whether the two surfactants are indeed cyclized via an ester bond, the two compounds were treated with mild base (1:1 v/v ammonia/methanol), under conditions known to cleave ester bonds, and the products analysed by mass spectrometry. The major ions in the

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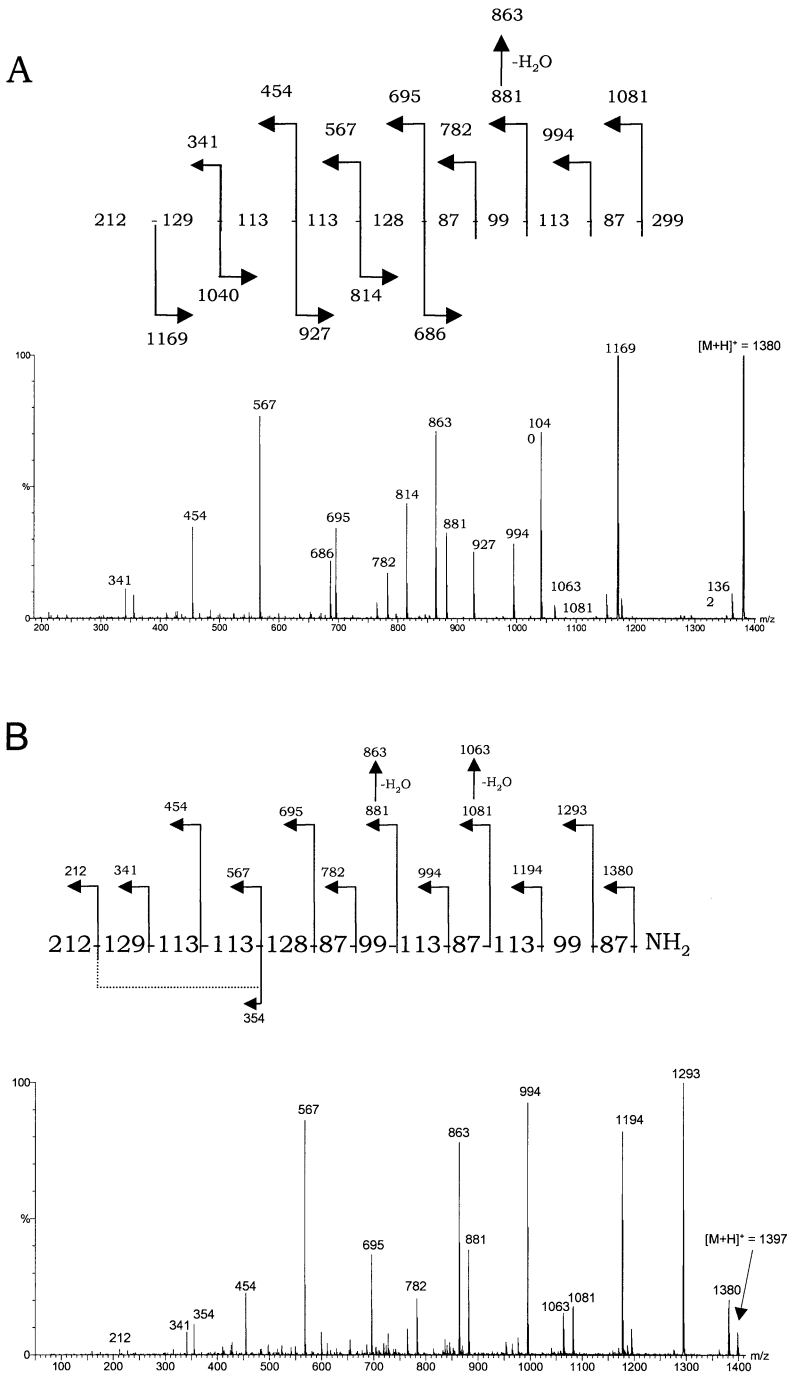

Fig. 4. Collision-induced dissociation (CID) tandem mass spectrum and fragmentation scheme of m/z 1380 species (A) and of m/z 1397 (1380 + 17) species (B). Spectra were obtained from HPLC fractions 38/39 (A) and 41/42 (B) after extraction of BM culture supernatant of *P. putida* PCL1445 and mild base treatment of this fraction (B).

mass spectra obtained after mild base treatment were observed 17 Th higher than before. This increase in mass corresponds to ring-opening by the addition of ammonia across the ester bond. Q-oToF CID tandem mass spectra of the base-treated species were obtained (Fig. 4B).

The linear, ring-opened, lipopeptides yielded more complete fragment ion series – b ions covering the complete sequence were obtained, allowing a complete sequence to be proposed, and supporting our initial proposal that cyclization prevented facile and useful fragmentation. The extra CH_2 group in the larger homologue was found to be located in the second amino acid from the C-terminus. The difference of 14 Da here could correspond to the difference between valine and leucine/isoleucine.

Specific chemical reactions involving the addition of a small organic moiety to the lipopeptide were performed to determine the identity of the functional groups present and their positions. This was necessary because the non-

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ribosomal synthesis of these species means that unusual amino acids can be present and so the residue cannot be identified purely on the basis of its mass. Chemistry was performed on the linearized species, as these yielded more informative tandem mass spectra.

Acetylation was performed, using acetic anhydride in methanol, on the smaller homologue putisolvin I. The conditions used acetylate both hydroxyl and amino functional groups. Each acetyl group introduced results in a mass increase of 42 Th. The mass spectrum of the acetylated product contained singly charged ions corresponding to the addition of both one and two acetyl groups. Sodiated forms were again observed. The presence of more than one acetylated product indicated that the reaction did not reach completion. CID tandem mass spectra of the two products were obtained, in order to identify the sites of acetyl incorporation. Interpretation of the product ion spectra showed that the two acetyl groups were distributed between the three amino acid residues having a residue mass of 87 Th. Several isomeric forms corresponding to different combinations of acetylation positions were thus isolated during the tandem mass spectrometric experiment and fragmented together. These results added evidence for the assignment of these residues as serine; the residues were shown to have a residue mass and functionality consistent with them being serine.

Methyl esterification involves the specific conversion of acidic hydroxyl groups to their methyl esters. This was carried out on an RP-HPLC fraction containing a mixture of the two lipopeptides after ring-opening, by the addition of 1 M methanolic HCl for 30 min at room temperature. The mass spectrum of the products showed ions at *m/z* 1411 and *m/z* 1425, corresponding to the addition of 14 Th to both analytes. CID tandem mass spectra were obtained to allow the position of methyl esterification to be elucidated. The fragment previously observed at m/z 341 increased by 14 Th to m/z 355 after methyl esterification. It was concluded that the amino acid of mass 129 had an acidic functionality present and was proposed to be glutamic acid, or an isomer. The sample was also treated with methanolic HCl at room temperature for 10 h. The mass spectrum indicated that the additional reaction time had allowed a second modification to take place. This modification introduced a second mass increase, but of 15 Th rather than 14 Th. CID tandem mass spectra were obtained to determine the site of the additional modification. The ions observed in the tandem mass spectrum indicated that the residue of mass 128 had been modified by the addition of 15 Th. This result is consistent with the amino acid being glutamine, if the amide is hydrolysed to the carboxylic acid in the acidic medium and then esterified. Despite sharing the same nominal mass as glutamine, lysine would not be methyl esterified under these conditions and can thus be excluded.

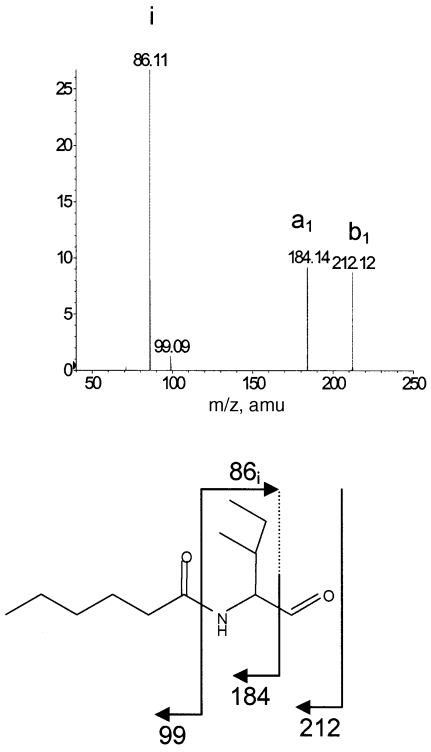

Fig. 5. Collision-induced dissociation (CID) tandem mass spectrum of the in-source generated fragment ion at m/z 212. The spectrum was obtained from the in-source generated fragment ion at *m/z* 212 as observed in the fragmentation scheme of species 1380 and 1397 (see Fig. 3).

Further tandem mass spectrometric information on the structure of the species giving rise to the fragment ion at m/z 212 was difficult to obtain. This fragment was expected to correspond to the lipid-containing portion of the molecule. The problem was solved by colliding and fragmenting the electrospray-generated ions in the source region of the mass spectrometer (QStar, Applied Biosystems). In-source fragmentation produced the b_1 ion at *m/z* 212, which had also been observed in the CID tandem mass spectra. This in-source generated ion was then isolated for tandem mass spectrometry; this experimental arrangement allowed MS^3 to be performed (Fig. 5).

The product ions allowed the structure to be determined. An ion corresponding to the immonium ion of isoleucine/leucine was observed at *m/z* 86. A fragment corresponding to the loss of carbon monoxide was observed, which is consistent with the a_1 ion generated from the b_1 precursor ion. The fragment at *m/z* 99 was assigned as the acylium ion of the C6 saturated alkyl chain – $CH3(CH2)_4CO^+$. Taken together, the mass spectrometric results allowed structures to be postulated for putisolvins I and II (Fig. 6A and B), although it was not possible from the mass spectra data alone to discriminate between Leu and Ile residues, nor to exclude the possi-

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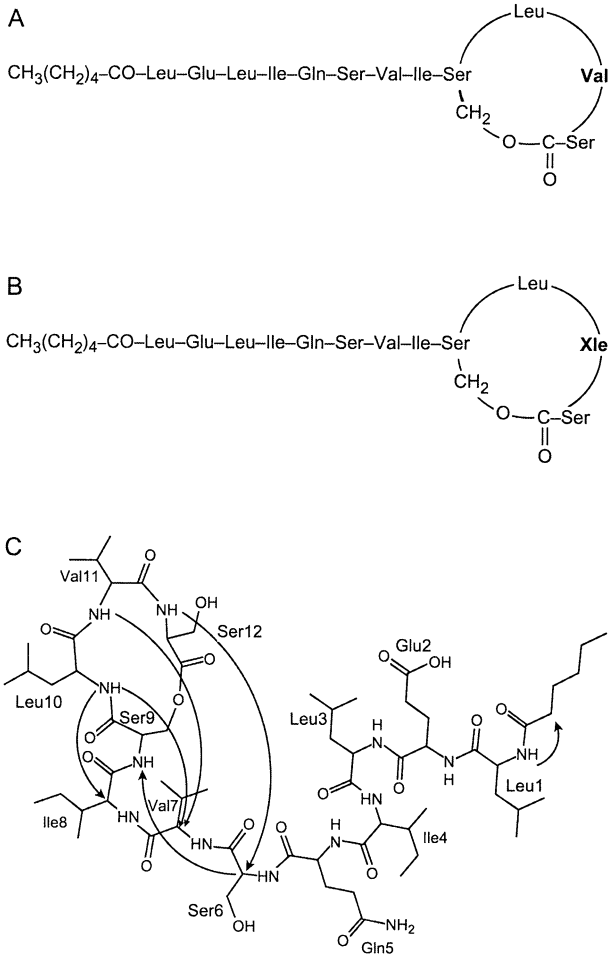

Fig. 6. Structures of putisolvin I and putisolvin II. The structure for putisolvin I based on mass spectrometric and amino acid analyses is depicted in (A), the structure for putisolvin II in (B). The result of the NMR analysis of putisolvin I is shown in panel (C). This structure shows an ester bond between Ser9 and Ser12 which was consistent with the long range NOEs indicated. Ser: serine; Glu: glutamic acid; Gln: glutamine; Val: valine; Ile: isoleucine; Leu: leucine; Xle: isoleucine or leucine.

bility that some of the amino acids might be present as unusual isomers. Amino acid analysis and NMR were used (see below) to exclude the possibility of unusual isomeric amino acids and to discriminate between leucine and isoleucine residues and thus to be able to assign the final structures shown in Fig. 6.

Results of amino acid analysis

HPLC fractions containing the two species of the lipopeptide were subjected to amino acid analysis. The results are summarized in Table 2. The molar ratio of each amino acid obtained by amino acid analysis is consistent with those expected from the structure proposed on the basis of the results of the ES-CID tandem mass spectrometry and NMR spectroscopy (see below), except for the value obtained for serine, which was slightly lower than might be expected, possibly due to oxidative loss. The molar ratios of Glu+Gln and Val were both consistent with the mass spectrometric data. The molar ratios of Ile and Leu are presented together as they cannot be discriminated by the mass spectrometric techniques employed to date. The molar ratio of Leu/Ile obtained by amino acid analysis was consistent with the mass spectrometric results. NMR allows discrimination between the Leu/Ile isomers, but NMR data were only obtained for one of the two homologues and so the molar ratios for both species together cannot be calculated.

Analysis of putisolvin I by ^{1}H NMR

The results of the mass spectrometric and amino acid analyses show that putisolvin I is a 12 residue cyclic peptide with a hexanoic acid moiety at the N terminus. In order to confirm these observations and obtain further details on the structure of putisolvin I, including assigning Leu and Ile, which are not discriminated by mass spectrometry, the sample was analysed by two-dimensional ^{1}H, ^{1}H NMR. The COSY spectrum showed 12 CαH-NH cross-peaks, consistent with the number of residues determined by mass spectrometry. Analysis of the spin systems from the TOCSY spectrum identified 3 Ser, 2 Val, 2 Glu/Gln, 3 Leu and 2 Ile residues. Assignments were completed, including those of the remaining Leu/Ile residues, from consecutive CαH(i)-NH(i + 1) and NH(i)-NH(i + 1) NOEs, obtained from ROESY spectra. These results were confirmed by NOEs from amide protons to side-chain protons. A structural model for putisolvin I, consistent with the NMR data, is shown in Fig. 6C. Several long range NOEs were identified which provided further evidence for cyclization of the peptide. Although it is not possible to draw any firm conclusions regarding the site of ester formation from the NMR data, the mass spectrometric data exclude the possibility of ester formation between Ser6 and the C-terminal carboxyl group and instead are clearly consistent with a Ser9-C-terminal ester. Signals were identified in the aliphatic region of the TOCSY spectrum that were consistent with the presence of hexanoic acid linked to Leu1 (2.35, 2.26, 1.65, 1.36,

Table 2. Data obtained from amino acid analysis of the purified lipopeptides.

Amino acid	Experimental molar ratio	Molar ratio predicted from proposed structure
Ser	5.3	6
Glu + Gln	4	4
Val	3	3
Ile + leu	11	11

Ser: serine; Glu: glutamic acid; Gln: glutamine; Val: valine; Ile: isoleucine; Leu: leucine.

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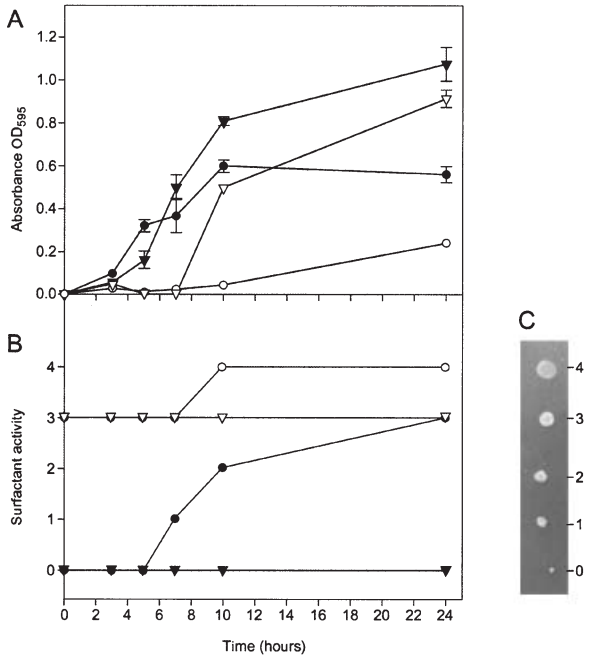

Fig. 7. Influence of purified putisolvin I on biofilm formation of *P. putida* PCL1445 and its mutant PCL1436 during time. Cells of *P. putida* PCL1445 and its mutant derivative PCL1436 were incubated in microtitre plates in M63 medium with or without purified putisolvin I and analysed for biofilm formation during time. After incubation, 25 μl of the culture was pipetted as a droplet on parafilm and allowed to dry to analyse production of biosurfactant activity in the well. Cells attached to the wells were stained with crystal violet and dissolved in ethanol after which the OD_{595} was measured. Experiments were performed in triplicate.
A. Biofilm formation of PCL1445 with (○) and without (●) 18 μM purified putisolvin I and PCL1436 with (▽) and without (▼) 18 μM purified putisolvin I.
B. Biosurfactant activity determined according the biosurfactant index as presented in (C) of bacterial cultures presented in (A). PCL1445 with (○) and without (●) 18 μM purified putisolvin I and PCL1436 with (▽) and without (▼) 18 μM purified putisolvin I.
C. Index 0–4 used for the detection of biosurfactant production by bacterial cells in the biofilm assay. Shown are dried droplets of 25 μl culture supernatant with increasing diameter due to decreased surface tension caused by increased biosurfactant activity.

0.93 p.p.m.). This hypothesis was confirmed by the observation of two NOEs from the amide proton of Leu1 to protons in hexanoic acid (2.35, 2.26 p.p.m., C2).

The effect of putisolvin I and II on Pseudomonas *spp. biofilms*

Biofilm formation on PVC by *Pseudomonas* strains was measured according to the procedure described by O'Toole and Kolter (1998). PCL1445 efficiently formed biofilms (Fig. 7A), similar to *P. fluorescens* WCS365 (O'Toole and Kolter, 1998). PCL1445 biofilms formed a clear ring in the well just below the interface of air and liquid (Fig. 8A). Electron microscopy analysis of PCL1445 biofilms formed after 10 h showed a regular pattern of cells in the biofilm (Fig. 8B). Cells of the mutant strain PCL1436 formed an increased biofilm after 5 h of incubation as compared with its wild type (Fig. 7A). Microscopy studies showed a broader biofilm for PCL1436 (Fig. 8A) and the increase of bacterial cells in the biofilm, which formed aggregates and were not as regularly distributed as compared with PCL1445 (Fig. 8B). Such aggregates were also observed for the wild-type PCL1445 but only after 24 h of incubation (data not shown). Biosurfactant activity produced by PCL1445 was detected after 5 h of incubation in the microtitre wells (Fig. 7B). No biosurfactant activity was detected in wells containing PCL1436 (Fig. 7B). To test whether the biosurfactants of PCL1445 influence biofilm formation, HPLC-purified putisolvin I was added to the growth medium prior to incubation in the wells. The results showed that biofilm formation of PCL1445 is severely reduced and inhibited by addition of putisolvin I (Figs 7A and 8A). This inhibitory effect is concentration dependent as a reduction of 52% was observed at a concentration of 1 μM, whereas a reduction of 88% was observed at 18 μM after 8 h of incubation (data not shown). Surfactant activity in the wells increased after 5 h of incubation (Fig. 7B). Addition of putisolvin I also resulted in a significantly delayed and reduced biofilm formation of PCL1436 (Figs 7A and 8A), but the formed biofilm contained more cells as compared with PCL1445. Electron microcopy studies showed that only a small number of PCL1445 cells had attached after 10 h, whereas PCL1436 had formed a clear biofilm (Fig. 8A); however, this biofilm contained less cells as compared with the situation when no putisolvin I had been added (Fig. 8B).

In order to determine the effect on existing biofilms, PCL1445 or PCL1436 cells were allowed to form biofilms for 7 h. Subsequently the culture medium containing the planktonic cells was removed and replaced by fresh medium containing purified putisovin I. The results show that PCL1445 and PCL1436 biofilms were reduced after the addition of putisolvin I (Fig. 9A and B). The effect was clearly visualized by microscopic analyses of PCL1445 biofilms showing that upon addition of putisolvin I the structure of the biofilms was altered within 15 min and subsequently broken down after 90 min (Fig. 10A and B). Interestingly the structure of the PCL1445 biofilm in the control well, which did not contain putisolvin I, was less regularly organized (Fig. 10B), resembling more the biofilm produced by PCL1436 at 10 h as shown in Fig. 8B. The latter can be explained by the fact that removal of the 7 h culture supernatant also removed biosurfactants already secreted in the medium and planktonic cells producing biosurfactants.

Similar effects on biofilms were obtained for purified putisolvin II (data not shown).

In addition to PCL1445 and its mutant derivative

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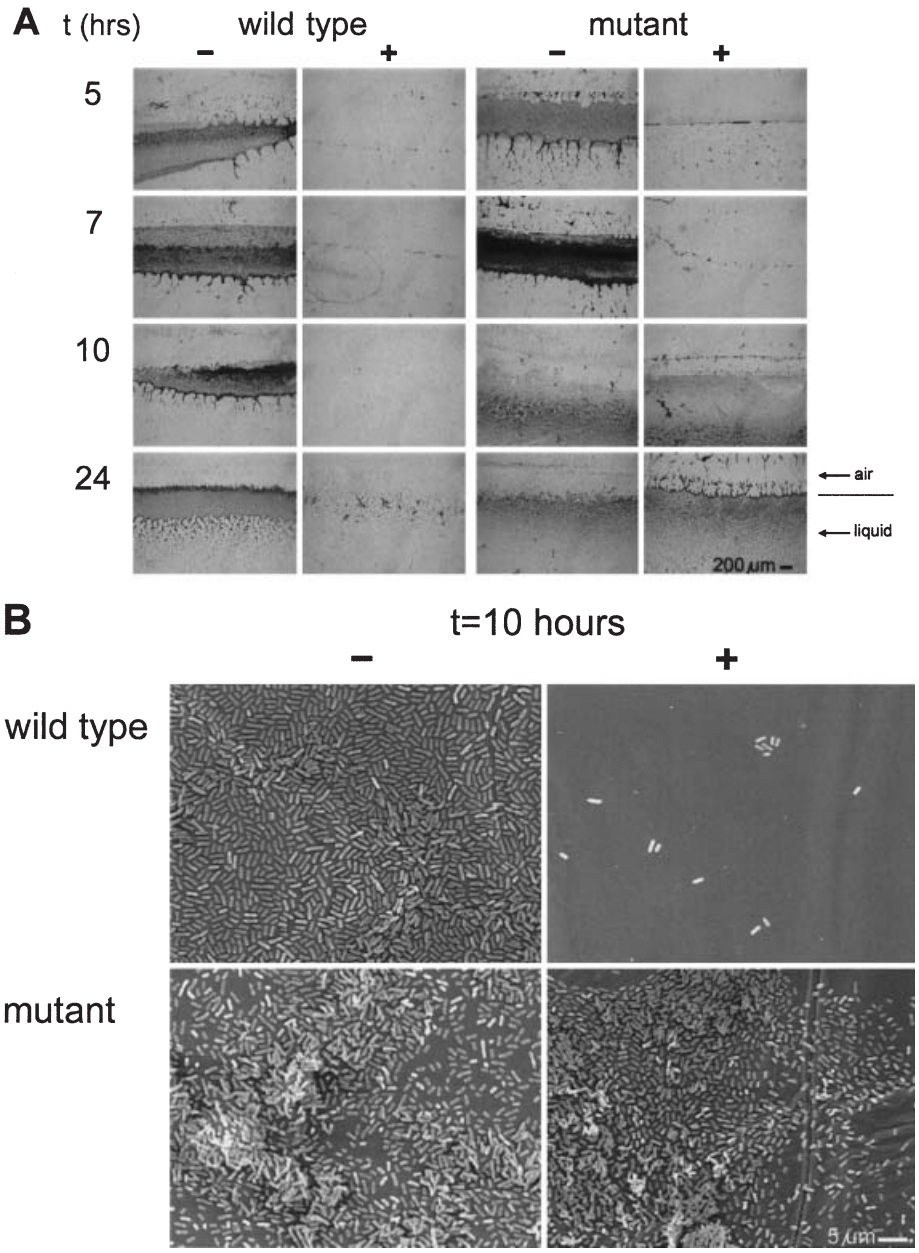

Fig. 8. Microscopic analyses of biofilm formation and inhibition by putisolvin I on biofilm formation of *P. putida* PCL1445 and its mutant PCL1436. Cells of *P. putida* PCL1445 (indicated as wild type) and its mutant derivative PCL1436 (indicated as mutant) were incubated in microtitre plates in M63 medium with (+) or without (–) purified putisolvin I (18 μM) and analysed for biofilm formation after 5, 7, 10 and 24 h using light microscopy (A) and at 10 h using scanning electron microscopy (SEM) (B). For light microscopy, cells were stained with crystal violet. The area of the biofilm ring, as typically observed in the microtitre assay, was visualized over the full width.

PCL1436, we also tested whether biofilms of other *Pseudomonas* strains could be affected by putisolvins. We used *P. fluorescens* strain WCS365, which had been described to form biofilms (O'Toole and Kolter, 1998) and *P. auruginosa* strain UCBPP-PA14. Both strains formed biofilms under the conditions used for PCL1445 (Fig. 11A). When crude culture extracts of PCL1445, putisolvin I (18 μM) or putisolvin II (18 μM) were added to the medium at the start of the incubation WCS365 and PA14 were strongly reduced in biofilm formation as was monitored after 10 h (Fig. 11A).

To test if biofilms of WCS365 and UCBPP-PA14 could also be broken down, cells were incubated for 7 h. Subsequent application of fresh medium containing crude culture extract of PCL1445 (estimated 2 μM putisolvins), putisolvin I (28 μM) or putisolvin II (28 μM) resulted in break down of the formed biofilms as was observed after 1.5 h (Fig. 11B). The extract was slightly more active than

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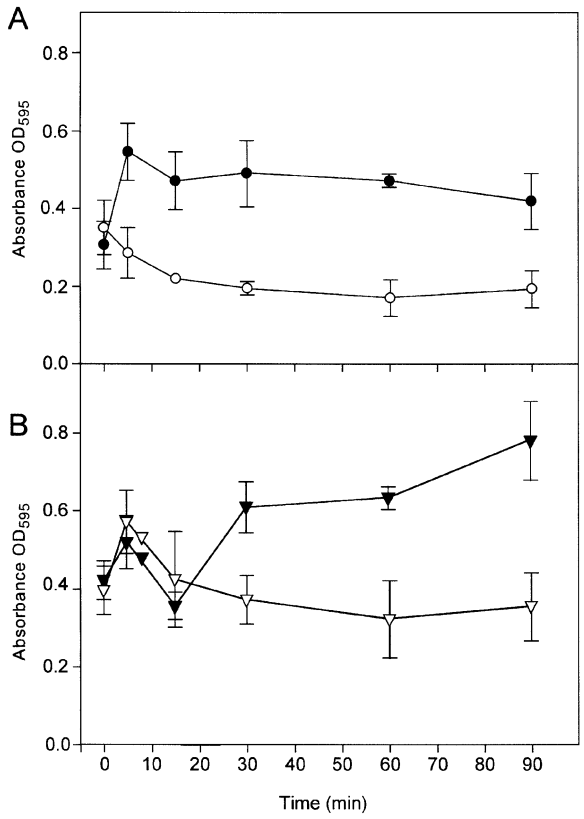

Fig. 9. Influence of purified putisolvin I on formed biofilms of *P. putida* PCL1445 and its mutant PCL1436. Biofilms of *P. putida* PCL1445 and its mutant PCL 1436 were allowed to establish for 7 h in microtitre wells. Subsequently the culture medium (including planktonic cells) was removed and replaced by fresh M63 medium with and without purified putisolvin I (18 μM) and biofilms were analysed during 1.5 h after the application. T = 0 in the figure represent the time point of putisolvin I application. Biofilms were quantified by crystal violet staining.
A. PCL1445 with (○) and without (●) 18 μM purified putisolvin I.
B. PCL1436 with (▽) and without (▼) 18 μM purified putisolvin I. The experiment was performed in triplicate.

the purified putisolvins. We did not observe an effect on the growth of bacterial cells after addition of putisolvins (data not shown).

Discussion

Cells and culture supernatant of *P. putida* strain PCL1445 appeared to decrease the surface tension between the hydrophobic surface of parafilm and a droplet of water (Fig. 1). Because surface-active compounds can influence interactions of bacterial cells with various surfaces (Neu, 1996), we characterized the components responsible for the surface tension-reducing ability of strain PCL1445.

The presence of putisolvin I and II in culture supernatant was shown to (i) reduce the surface tension of the medium by approximately 40% (Fig. 1), (ii) increase the formation of emulsions with toluene (Fig. 1) and (iii) enhance swarming motility on agar plates. The role of biosurfactants in swarming motility is known for *Serratia* (Lindum *et al*., 1998) and we can hypothesize that the biosurfactants produced by PCL1445 stimulate not only motility on agar plates, but presumably also on other surfaces, by altering the cell surface hydrophobicity. Monitoring the surface tension during growth it was shown that surfactant activity is produced at the end of the exponential phase (Fig. 2), which could indicate its regulation by a quorum sensing-like system.

All these abilities are absent from the mutant strain PCL1436, which was selected for its loss of activity in the drop-collapsing assay and did not show an altered growth rate (Fig. 2). The sequence obtained from the mutated ORF in PCL1436 shows high homology to the *syrE* gene, coding for a lipopeptide synthetase (Guenzi *et al*., 1998), which is required for synthesis of syringomycin in *P. syringae*. Syringomycin is a phytotoxin with biosurfactant activity (Hutchison and Gross, 1997). Because inoculation of seeds or seedlings with cells of PCL1445 has never resulted in negative effects on grass and tomato plants (results not shown), this indicates that the components produced by PCL1445 are not phytotoxic. When in homology studies we focused only on the functional domains of the PCL1445 synthetase, high homology was observed with other lipopeptide synthetases, such as the surfactin synthetase of *Bacillus* sp. (33% identity, 50% similarity; (Yakimov *et al*., 1995).

Structural identification of the biosurfactants produced by PCL1445 showed that the two surfactant active compounds referred to as putisolvin I and II, respectively, appeared to be very similar and only have a mass difference of 14, with Mrs of 1379 and 1393 (Figs 4 and 5). Both compounds are cyclic lipopeptides with an ester linkage between the ninth serine residue and the C-terminal carboxyl group (Fig. 6). The only difference between putisolvin I and II is that the Val11 in putisolvin I is replaced by Ile or Leu in putisolvin II, which accounts for the mass difference of 14 Da.

Putisolvin I and putisolvin II are, to our knowledge, the first lipopeptides consisting of 12 amino acids linked to a hexanoic lipid chain. Other known *Pseudomonas* lipopeptides, such as viscosinamide (Nielsen *et al*., 1999), syringomycin (Hutchison and Gross, 1997), amphisin (Sørensen *et al*., 2001) and tensin (Hendriksen *et al*., 2000) have a shorter amino acid moiety and the fatty acid chain is longer. Cyclization also appears to be different. For the previously described lipopeptides an ester linkage between the C-terminal carboxyl group and the side-chain of the first or third amino acid is formed, while this linkage involves the ninth amino acid in the case of both putisolvin I and II. The lipopeptides produced by *P. syringae* and *P. tolaasii* (Nutkins *et al*., 1991) also have a longer fatty acid

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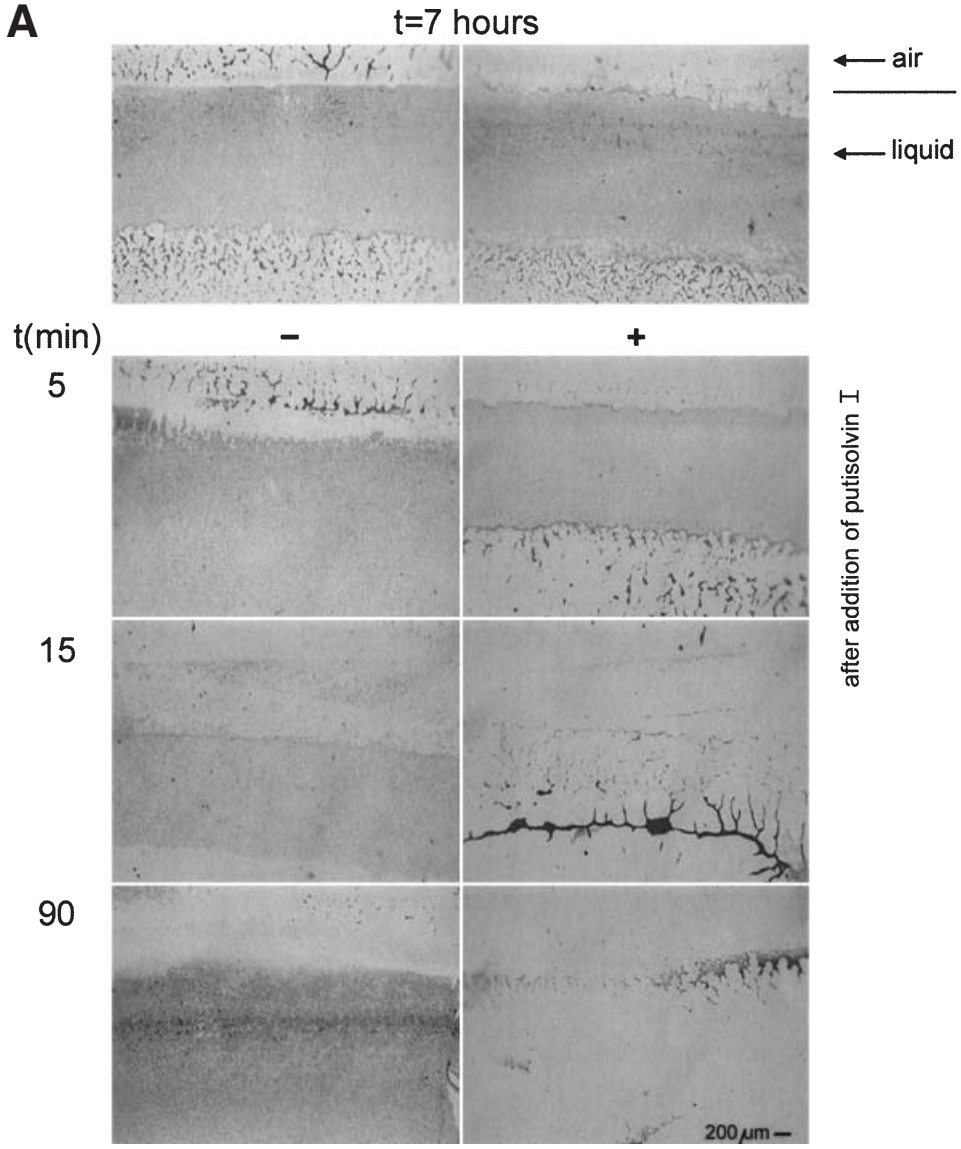

Fig. 10. Microscopic analyses of the break down of formed biofilms of *P. putida* PCL1445 by purified Putisolvin I. Biofilms of *P. putida* PCL1445 were allowed to establish for 7 h in microtitre wells. Subsequently, the culture medium (including planktonic cells) was removed and replaced by fresh M63 medium with (+) and without (–) purified putisolvin I (18 µM) and biofilms were visualized during 1.5 h after the application using light microscopy (A; 5, 15 and 90 min) or scanning electron microscopy (B; 15 and 90 min). For light microscopy, cells were stained with crystal violet. The area of the biofilm ring, as typically observed in the microtitre assay, was visualized over the full width.

chain than putisolvin I and II. In addition, syringopeptin and tolaasin have many more amino acids in the peptide moiety. Structural differences could account for the different properties of the different lipopeptides such as antifungal activity, phyto-toxicity and biofilm formation inhibition.

The ability of surfactants to inhibit biofilm formation is described for the rhamnolipid surfactant of *P. aeruginosa* PAO1 (Davey *et al.*, 2003) and for lipopeptides produced by the Gram-positive bacteria *Lactobacillus*, *Bacillus* and *Streptococcus* (Busscher *et al.*, 1997; Velraeds *et al.*, 2000; Mireles *et al.*, 2001). These lipopeptides consist of seven amino acids and have a fatty acid chain, which can vary in length. We tested whether putisolvin I and II could influence the formation of *Pseudomonas* biofilms on polyvinyl chloride. This was tested in a biofilm formation assay described by O'Toole and Kolter (1998). Their influence was indicated by the fact that the mutant PCL1436 showed an increased biofilm formation as compared with its wild type (Fig. 7), and by the fact that the start of the observed difference coincided with the detection of biosurfactant production in the wells of PCL1445 (Fig. 7). Interestingly after 10 h of incubation, biofilms of PCL1436 contain cell aggregates whereas PCL1445 biofilms are

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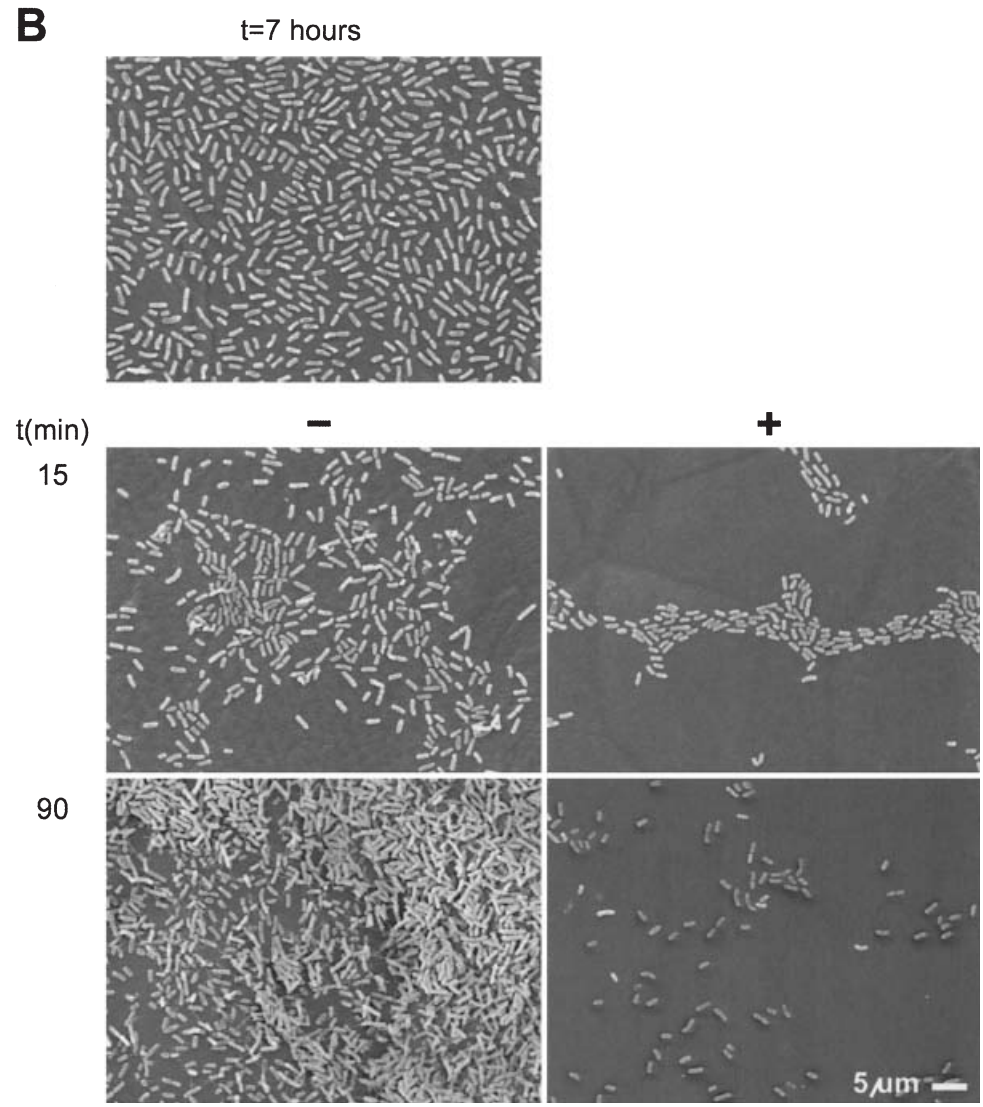

Fig. 10. *cont.*

more homogenously structured (Fig. 8). Similar cell aggregates were observed for PCL1445 after 24 h of incubation indicating a regulatory role of putisolvins in biofilm development (data no shown). Interestingly, it was recently shown that the rhamnolipid surfactant of *P. aeruginosa* PAO1 is involved in maintaining open channels in biofilm forms in a flow cell setup, by affecting cell–cell interactions and the attachment of bacterial cells to surfaces (Davey *et al*., 2003). Therefore, it would be interesting to compare biofilms of PCL1445 and PCL1436 in such a flow cell setup. Using purified putisolvin I, we showed that biofilm formation of PCL1445 and PCL1436 was inhibited (Fig. 7) by putisolvins. A stronger effect in these experiments was observed for PCL1445, which can be explained by the fact that PCL1445 starts to produce putisolvins during growth and thereby increasing the putisolvin concentration, which was shown by an increased surfactant activity in the well (Fig. 7B). Moreover, the biofilm-forming ability of *P. fluorescens* strain WCS365 and *P. aeruginosa* strain UCBPP-PA14 was strongly reduced in the presence of purified putisolvin I and II (Fig. 11) indicating the potential of putisolvins in reducing bacterial biofilm formation.

A possible mode of action of putisolvin I and II during the inhibition of biofilm formation could be by binding to the cell surface or to components of the cell surface, thereby influencing the outer membrane hydrophobicity, as was reported for other biosurfactants (Neu, 1996). The change in cell surface hydrophobicity could be the reason for the altered behaviour of the cells of PCL1436 (and not only supernatant) in the drop-collapsing assay. In addition, the change in cell surface hydrophobicity might also influence the interaction between the individual cells in a biofilm, making the formation of a dense and stable biofilm

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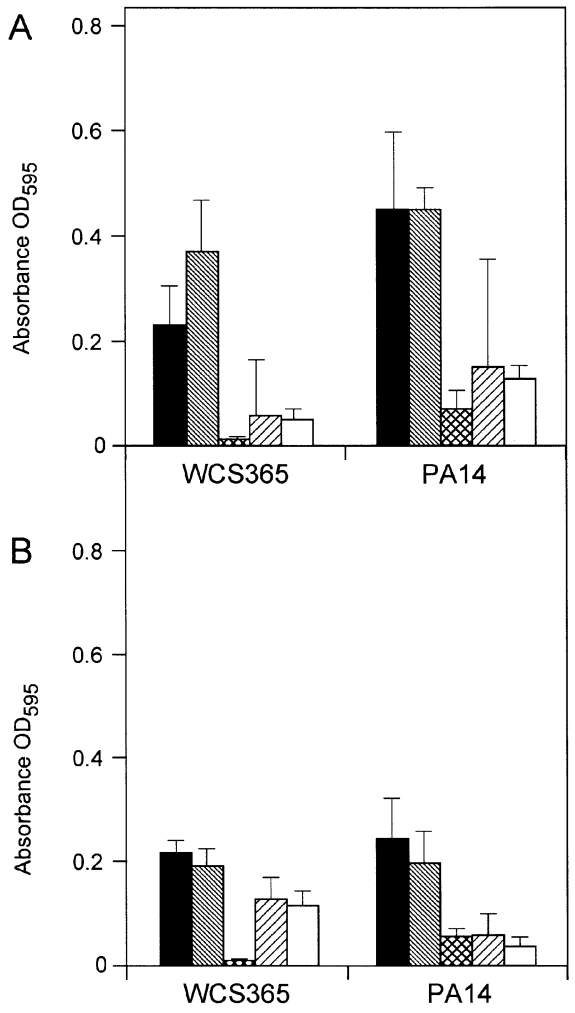

Fig. 11. Influence of putisolvins on biofilm formation and formed biofilms of *Pseudomonas fluorescens* WCS365 and *P. aeruginosa* PA14. Cells of *P. fluorescens* WCS365 and *P. aeruginosa* PA14 were incubated in microtitre plates in M63 medium with the following additions (0.5 μl): water (black bars), water and methanol/acetonitrile, 1:1, v/v (grey bars), with PCL1445 culture supernatant extracts (blocked bars), with putisolvin I (arced bars) or putisolvin II (white bars) dissolved in methanol/acetonitrile, 1:1.
A. Biofilms were analysed after 10 h of incubation with the additions (18 μM putisolvin) applied at time 0.
B. Biofilms were allowed to form for 7 h without additions after which the medium was replaced by fresh M63 medium with additions (28 μM putisolvin). Biofilms were analysed 1.5 h after the application. The experiment was performed in triplicate.

more difficult. Another explanation for the role of putisolvin I and II in biofilm formation could be their influence on swarming motility. Mutant PCL1436 is severely inhibited in its swarming abilities but enhanced in biofilm formation (Fig. 7). Interestingly Huber *et al*. (2001) indicated for *Burkholderia cepacia* that swarming motility is not essential for biofilm formation on PVC. Another possibility for the mode of action of putisolvins is an antibacterial effect. The almost identical growth curves of PCL1445 and PCL1436 indicate that putisolvins do not have such an effect; however, a resistance mechanism could be present in PCL1445. An antibacterial effect on WCS365 and PA14 has not been observed when these strains were grown together on an agar plate (data not shown). However, more detailed studies have to be performed.

In addition to the inhibition of biofilm formation, we also observed that existing PCL1445 biofilms were altered in structure and subsequently broken down within 1.5 h after addition of purified putisolvin I (Figs 9 and 10). The observed effect was stronger for the wild-type PCL1445 than its mutant PCL1436, which is presumably due to the additive production of putisolvins by the PCL1445 cells in the biofilm. Addition of the lipopeptides to other *Pseudomonas* wild-type strains, e.g. WCS365 and PA14, also resulted in break down of existing biofilms (Fig. 11), showing that the interaction of the lipopeptides with cells within a biofilm is not strain specific. The results showed that the supernatant extract was more active than the purified putisolvins (Fig. 11), whereas the estimated concentration of putisolvins in the extract is lower. The latter could be explained by the fact that during purification the biosurfactants have been pelleted which might influence their activity after dissolving as the putisolvins have an amphipatic character. Alternatively other factors which enhance solubility of the putisolvins in water or inhibit biofilm formation could have been lost during purification. Mixing of purified Putisolvin I and II did not result in an enhanced activity (data not shown).

Because biofilm formation by hazardous bacteria on artificial surfaces is a serious and sometimes life-threatening problem (Stewart and Costerton, 2001; Donlan and Costerton, 2002), the biosurfactants of PCL1445 have a very interesting potential to reduce the hazardous effects of biofilms.

To our knowledge putisolvin I and II are the first structurally characterized lipopeptides produced by a *Pseudomonas* strain with 12 amino acids and a hexanoic acid lipid. We conclude that the novel biosurfactants we describe have intriguing properties of inhibiting the formation of hazardous biofilms, e.g. on medical or technical equipment, and of breaking down existing biofilms.

Experimental procedures

Bacterial strains and growth conditions

All bacterial strains used are listed in Table 1. *Pseudomonas* strains were grown at 28°C in liquid King's medium B (KB; King *et al*., 1954) under vigorous shaking. *Escherichia coli* strains were grown at 37°C in liquid Luria–Bertani medium (LB, Sambrook *et al*., 1989) under vigorous shaking. Media were solidified with 1.8% agar (Bacto Agar, Difco Laboratories). The antibiotics kanamycin or carbenicillin were added, when appropriate, to final concentrations of 50 and 100 μg ml^{-1} respectively.

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For growth in a defined medium, BM (Lugtenberg *et al*., 1999) was used. When appropriate, the standard carbon source succinic acid was replaced by the carbon sources naphthalene or glycerol (2%), both purchased from Sigma Chemical Co. Naphthalene was added as crystals in liquid medium or placed in the lid of a Petri disc.

In order to test swarming motility on agar medium, strains were spot-inoculated on 20-fold diluted KB medium, solidified with 0.3% agar and subsequently incubated at 28°C.

Biosurfactant production

To test whether bacterial strains were able to decrease the surface tension between water and hydrophobic surfaces, the ability to collapse a droplet of water was tested as described by Jain *et al*. (1991) with some alterations. Some cells of a single colony were transferred by a toothpick and suspended into a droplet of 25 μl sterile water on parafilm 'M' laboratory film (American National Can). When culture supernatant was tested for biosurfactant activity in this assay, 25 μl was pipetted as a droplet onto parafilm. The flattening of the droplet and the spreading of the droplet on the parafilm surface upon addition of cells or culture supernatant was followed over seconds or minutes. Subsequently, methylene blue (which had no influence on the shape of the droplets) was added to stain the water and supernatants for photographic purposes. To analyse the biosurfactant activity during biofilm formation in the PVC well, 25 μl of a culture was pipetted as a droplet onto parafilm. The spreading of the droplet on the parafilm was observed over hours, the droplet was allowed to dry and the diameter of the dried droplet was recorded.

To determine the surface tension between culture medium and air, bacterial strains were grown overnight in KB medium, cells were pelleted and culture supernatants were collected for analysis. Surface tension was measured by the ring method using a tensiometer (type K6, Krüss) equipped with a Du Nouy (platinum) ring. Briefly, the ring was placed just below the surface of 5 ml of the liquid. Subsequently, the force to move this ring from the liquid phase to the air phase was determined.

KB supernatant of strains was tested for emulsifying activity by mixing an equal volume of toluene (Sigma-Aldrich) through the supernatant. Subsequently the mixture was incubated at room temperature for 1 h to allow the phases to separate and the layers formed were visually inspected for turbidity.

Generation, selection and genetic characterization of a mutant defective in biosurfactant production

Transposon mutants were generated by tri-parental mating using plasmid pRL1063a, harbouring a Tn*5luxAB* transposon (Wolk *et al*., 1991), helper plasmid pRK2013 (Ditta *et al*., 1980) and strain PCL1445. Transposants were initially screened for decreased ability to flatten a droplet of water using cells of a single colony, as described above. Subsequently, the supernatants of the selected mutants, obtained after overnight growth in liquid KB medium, were tested using the drop-collapsing assay.

To isolate the Tn*5luxAB* and the flanking chromosomal regions, total DNA of the mutant strain was isolated and digested with *Eco*RI, which does not cut plasmid pRL1063a. Subsequent circularization of the digested chromosomal fragments and selection for kanamycin resistance resulted in plasmid pMP5459 containing the Tn*5luxAB* flanking DNA regions. All DNA techniques were performed as described by Sambrook *et al*. (1989) and sequencing was performed by BaseClear. DNA sequence comparisons with GenBank were performed using the BLAST program (Altschul *et al*., 1997).

Isolation, purification and characterization of the surface-active compounds

To purify surface-active compounds, strains were grown for 2 days in 600 ml BM medium with glycerol as the sole carbon source. Cells were removed by centrifugation and culture supernatants were extracted with 1 volume of ethyl acetate (Fluka Chemie). Ethyl acetate extracts were evaporated under vacuum to dryness and the dry material was dissolved in 6 ml acetonitrile (Labscan Ltd). Subsequently, 25 μl amounts of these samples were mixed with 75 μl acetonitrile/water (50:50, v/v) and small particles were removed by filtration using a spinX centrifuge tube filter of 0.45 μm pore size (Corning Costar Corporation). Next, 100 μl of these samples was separated using high-pressure liquid chromatography (HPLC; Jasco International Co. Ltd), equipped with a reverse phase C8 Econosphere, 5 μm, 250 mm × 4.6 mm column (Alltech), a PU-980 pump system (Jasco International Co. Ltd), a LG-980-02 gradient unit (Jasco International Co. Ltd) and a MD-910 diode array detector (Jasco International Co.). Separation was carried out using a linear gradient, starting at 50/50 (acetonitrile + 0.05% TFA/water + 0.05% TFA: v/v) which subsequently changed to finally 80/20 in 70 min, at a flow rate of 1 ml min^{-1}. Chromatograms were analysed in a wavelength range between 200 and 400 nm and the eluens were collected in 1 ml fractions. After evaporation of acetonitrile, collected samples were tested for biosurfactant activity using the drop-collapsing assay. For large-scale purification, preparative reverse phase HPLC was performed using a semi preparative C8 Econosphere, 5 μm, 250 mm × 10 mm column (Alltech) with the same mobile phase and gradient as above at a flow of 2 ml min^{-1}.

Amino acid analysis was performed using Ansynth BV by fractionating a 6 M HCl hydrolysis reaction sample. The HPLC fractions, active in the drop-collapsing assay were analysed using diode array UV to detect aromatic amino acids, which absorb at 254 nm.

HPLC fractions were dried into glass vials. Each fraction was initially split by being redissolved in 100 μl methanol and then 10 μl aliquots of this solution were taken into eppendorf tubes and dried. Mass spectrometric analysis and derivatizations were performed on these dried portions.

Mass spectrometric analysis

The surface active wild-type HPLC fractions and corresponding HPLC fractions of the biosynthetic mutant were dissolved in 20 μl acetonitrile:1% aqueous formic acid (1:1, v/v) for mass spectrometric analysis. The redissolved analytes were introduced into the Q-ToF (Micromass) at 1 μl min^{-1} using a

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syringe driver. The capillary was maintained at 3000 V and the cone at 47 V. Tandem mass spectra were obtained at a range of collision offsets, typically around 40 V. Specta were collected typically between 200 and 2000 Th.

Alternatively, the redissolved analytes were introduced into the Q-Star (Applied Biosystems) at 1 μl min^{-1} using the built-in syringe driver. The capillary was maintained at 5800 V, and the cone at 65 V. Tandem mass spectra were obtained at a range of collision offsets, typically around 60 V. In-source fragmentation was performed by increasing the cone to 120 V. Spectra were collected between 50 and 2000 Th.

Next, 50 μl ammonia/methanol (1:1, v/v) was added to the dried fractions. The solution was vortexed and then left at room temperature for 18 h. The reaction mixture was then reduced to dryness.

One droplet of water was added to the dried fractions to redissolve them. Then, 400 μl acetic anhydride/methanol (3:1, v/v) was added and the reaction allowed to take place at room temperature for 3 h. The products were reduced to dryness.

A few droplets of 1 M methanolic HCl were added to the dried fractions. The mixture was vortexed and one sample left for 30 min and then reduced to dryness. The other sample was left for 8 h and then reduced to dryness.

^{1}H NMR analysis

NMR spectra were recorded on a Bruker 600 MHz NMR spectrometer (Bruker BioSpin Limited), using samples in deuterated methanol (CD_3OH). Rotating frame NOESY (ROESY) spectra were recorded with a mixing time of 200 ms and TOCSY spectra with a mixing time of 60 ms.

Biofilm formation assays

Biofilm formation on polyvinylchloride (PVC) was tested as described by O'Toole and Kolter (1998). Briefly, strains were grown overnight in LB medium. PVC microtitre plates (Falcon 3911 Microtest III flexible assay plate, Becton Dickinson Labware) containing 100 μl M63 medium (O'Toole and Kolter, 1998) in each well were inoculated using 100-fold dilutions of these cultures and incubated at 28°C. Subsequently, the medium was removed from the microtitre plates, which were rinsed with water and filled with 125 μl 1% crystal violet solution to stain the attached bacterial cells. After 15 min, the unbound crystal violet solution was removed and plates were rinsed with water. Subsequently, the bacterial rings were dissolved in 200 μl 96% ethanol and transferred to flat-bottom microtitre plates in order to measure the absorbance at 595 nm. When ethyl acetate extracts of culture supernatants were tested in the biofilm assay, 10 μl of the 500-fold concentrated extract dissolved in methanol/acetonitrile (1:1, v/v) (obtained as described under 'Isolation, purification, and characterization of the biosurfactants') was added to 5 ml of M63 medium to an estimated concentration of 2 μM putisolvins (based on HPLC analysis). Subsequently, 100 μl of this solution was used per well for the biofilm formation assay, as described previously. C8 HPLC purified lipopeptides were added dissolved in methanol/ acetonitrile (1:1, v/v) to the M63 medium. An equal volume of methanol/acetonitrile (1:1, v/v) was added in the control wells.

When the lipopeptides were tested for the ability to disrupt existing biofilms, the protocol for biofilm formation was slightly adapted. The incubation time to form a biofilm was reduced from 10 to 7 h, followed by the removal of the medium. Subsequently, 100 μl M63 medium, containing 10 μl of the 500-fold concentrated extract dissolved in methanol/acetonitrile (1:1, v/v) or the respective lipopeptides in a final concentration of 28 μM, was added to the wells. An equal volume of methanol/acetonitrile (1:1, v/v) was added to control wells. After incubation, microtitre plates were treated as described above. All conditions were tested (at least) in triplicate, using three independent wells.

Microscopic analysis

The attachment of bacterial cells to PVC and the mode of biofilm formation (assayed as described above), were studied by light and scanning electron microscopy. For light microscopy, bacterial cells attached to the PVC wells were stained with 1% crystal violet. After 15 min, the wells were rinsed with water and air-dried. Pieces of the PVC wells were examined with a Zeiss Axioplan 2.

For scanning electron microscopy, unstained cells were used after incubation in the PVC well. The culture medium was removed from the well and the bacterial cells attached to the PVC were fixed *in situ* with 200 μl 2% glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.4 for 2 h at room temperature. After rinsing with 0.1 M sodium cacodylate buffer (pH 7.4), the samples were dehydrated in a series of ethanol-water solutions (70%, 80%, 90%, 96% and 100% ethanol), critical point drying was performed and PVC parts were mounted on metal stubs. Samples were sputter coated with gold and examined in a JSM6400 scanning electron microscope (JEOL).

All digital images obtained were processed in Adobe Photoshop 7.0 (Adobe).

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