RESEARCH LETTER

Bioactive lipopeptides of ice-nucleating snow bacterium *Pseudomonas syringae* strain 31R1

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Abstract

The production of secondary metabolite lipopeptides by ice-nucleating *Pseudomonas syringae* strain 31R1 was investigated. *Pseudomonas syringae* strain 31R1 is a rifampicin-resistant derivative of *P. syringae* no. 31 used for the commercial production of snow. It is shown that *P. syringae* strain 31R1 produces antifungal lipodepsipeptides, syringomycins E and G, and, in addition, a novel and unique lipopeptide, peptin31. Spectroscopic and spectrometric analyses revealed that peptin31 is a linear undecalipopeptide with sequence identities to N- and C-terminal portions but lacking 11 amino acids of known lipodepsipeptide syringopeptin SPPhv. Peptin31 displayed antifungal activities against *Rhodotorula pilimanae*, *Rhizoctonia solani*, and *Trichoderma harzianum* and also hemolytic and antibacterial activities. Extracts of *P. syringae* strain 31R1 grown in medium with chloride were fungicidal, but not when grown without chloride. The latter extracts lacked peptin 31 and contained des-chloro forms of syringomycins E and G with low antifungal activities. Thus, the three lipopeptides account for the fungicidal properties of *P. syringae* 31R1 extracts. The occurrence of these bioactive metabolites should be considered when *P. syringae* no. 31 and its derivatives are used in products for making artificial snow.

Introduction

Many strains of the plant-associated bacterium *Pseudomonas syringae* produce two types of lipodepsipeptides. These are: (1) lipodepsinonapeptides (Segre *et al.*, 1989; Ballio *et al.*, 1990; Fukuchi *et al.*, 1992) and (2) large lipodepsipeptide syringopeptins made of 22 or 25 amino acids (SP22 and SP25, respectively) (Ballio *et al.*, 1991; Isogai *et al.*, 1995; Grgurina *et al.*, 2002). Individual *P. syringae* strains produce both kinds simultaneously. The former are fungicidal, and the latter are phytotoxic, antifungal, and antibacterial; both are hemolytic. These metabolites play roles in the interactions with host plants that include virulence and microbial antagonism (Scholz-Schroeder *et al.*, 2001). Both lipodepsipeptide families act by forming pores in membranes (Feigin *et al.*, 1996; Hutchison & Gross, 1997; Dalla Serra *et al.*, 1999). The fungicidal action of the small lipodepsinonapeptide syringomycin E (SRE) against yeast is promoted by sphingolipids and sterols (Cliften *et al.*, 1996; Stock *et al.*, 2000). Similarly, bactericidal action by the large lipodepsipeptide syringopeptin SP25A against Gram-positive bacteria is promoted by interaction with teichoic acids (Bensaci & Takemoto, 2007). The *P. syringae* lipodepsipeptides are synthesized nonribosomally by large multimodular peptide synthetases (Grgurina & Benincasa, 1994; Guenzi *et al.*, 1998; Scholz-Schroeder *et al.*, 2003).

Certain biological properties of *P. syringae* have been exploited for practical applications. A prominent example is the use of lyophilized preparations of *P. syringae* no. 31 (Arny *et al.*, 1976; Lindow *et al.*, 1982) to make artificial snow. This application exploits the bacterium's ice-nucleating capabilities promoted by outer membrane-associated protein InaZ (Wolber *et al.*, 1986). Artificial snow production based on *P. syringae* no. 31 is practiced worldwide at

winter recreational venues. Environmental concerns related to its use for this purpose have been discussed for more than 20 years (Goodnow *et al.*, 1990; Rixen *et al.*, 2003). Earlier studies showed that *P. syringae* no. 31 is unable to survive in soil and water environments that mimic snow-making sites (Goodnow *et al.*, 1990). However, the occurrence of bioactive metabolites in *P. syringae* no. 31 preparations such as the lipodepsipeptides and the impacts of the environmental accumulation of these compounds have not been addressed.

In this work, we analyzed the occurrence of bioactive and fungicidal lipodepsipeptides in extracts of *P. syringae* strain 31R1 – a spontaneous rifampicin-resistant derivative of *P. syringae* no. 31 (Haefele & Lindow, 1987). We found that the prevalent fungicidal lipodepsinonapeptides SRE and syringomycin G (SRG) are present in these extracts. In addition, a novel linear lipopeptide (peptin31) was discovered, its structure was determined, and its antimicrobial and hemolytic properties were evaluated.

Materials and methods

Organisms and culture conditions

Pseudomonas syringae strain 31R1 (obtained from S. Lindow, University of California, Berkeley) was maintained and propagated on King's B medium plus rifampicin ($100\,\mu g\,mL^{-1}$) (Haefele & Lindow, 1987). For lipopeptide production, cells were grown in 1-L Roux bottles containing 150 mL of IMM medium (medium A) (Surico *et al.*, 1988) or IMM medium with histidine monohydrochloride and calcium chloride replaced by equimolar amounts of histidine base (Merck) and calcium nitrate (Sigma) (medium B) (Grgurina *et al.*, 1994). The cultures were incubated at 25 °C for 9 days, with manual agitation once daily. The ice-nucleation property of *P. syringae* strain 31R1 was confirmed using the constant temperature drop freeze method (Lindow *et al.*, 1982). *Rhodotorula pilimanae* Hedrick et Burke (ATCC 26423) was grown and maintained as described previously (Zhang & Takemoto, 1987). *Rhizoctonia solani* strain 1556 (Culture Collection) and *Bacillus megaterium* strain 51S (Culture Collection, Department of Food Science, University Federico II, Naples, Italy) were grown and maintained as described previously (Grgurina *et al.*, 2002). *Trichoderma harzianum* strain T22 (from RooT Shield) was grown and maintained as described previously (Vinale *et al.*, 2006).

Lipopeptide extracts and purification

Cultures were treated with 0.4% (v/v) HCl in acetone and cell debris was removed by centrifugation (7000 ***g*** for 15 min, at 4 °C). The supernatant fractions were designated as extracts and directly assayed for activity or subjected to chromatography with Amberlite XAD-7 resin using previously described methods (Bidwai *et al.*, 1987). Final purification of lipopeptides was achieved by HPLC on a Jupiter 5 μ C_{18} 300A (250 × 10 mm) column (Phenomenex) using a Beckman System Gold 126 system as described previously (Segre *et al.*, 1989). SP22A and SRE were purified as described previously (Segre *et al.*, 1989; Ballio *et al.*, 1991) and stored at − 20 °C.

Antimicrobial activity

The inhibitory activities of *P. syringae* strain 31R1 extracts and purified lipopeptides were tested by spot diffusion assays against *R. pilimanae* Hedrick et Burke (ATCC 26423), *R. solani* strain 1556, and *T. harzianum* strain T22 on potato dextrose agar medium (Iacobellis *et al.*, 1992) and against *B. megaterium* strain 51S on Luria–Bertani agar medium (Bensaci & Takemoto, 2007). Activity is reported as zone of inhibition diameter (mm).

Hemolysis

Hemolytic activities of purified peptides were determined turbidimetrically at 650 nm as described previously (Dalla Serra *et al.*, 1999). Hemolytic activities were reported as $1/C_{50}$ values (concentrations causing 50% lysis of erythrocytes).

Amino acid composition

Amino acid composition analysis was performed using an LKB 4151 Alpha plus automatic analyzer on samples hydrolyzed with 6 N HCl at 110 °C, for 24 h, *in vacuo*.

MS

Pseudomonas syringae strain 31R1 extracts were analyzed by MS using a Kompact matrix-assisted laser desorption/ionization time-of-flight (MALDI) instrument (Kratos Analytical, Shimadzu Group Co., Japan), equipped with a N_2 laser emitting light at 337 nm with a pulse width of 3 ns. Positive ions were recorded in a linear mode. For calibration, insulin was used as an external standard. The matrix consisted of sinapinic acid. Ions were accelerated by acceleration voltages of 55 kV. Saturated solutions ($10\,mg\,mL^{-1}$) of matrix were prepared in acetonitrile/0.1% trifluoroacetic acid (TFA) in water, 2 : 3 or 1 : 1 (v/v). Purified fractions were analyzed by MS using an API-100 single quadrupole mass spectrometer (PerkinElmer Sciex Instruments, Canada) equipped with an electrospray interface. The samples were directly injected into the ion source at a flow rate of $6\,\mu L\,min^{-1}$. A probe voltage of 4.7 kV and a declustering potential of 40 V were used. Data acquisition was performed in positive polarity, using a dwell time of 1 ms and a step size of 0.5 *m/z*. Each scan was acquired from 500 to 2500 *m/z*. The instrument was calibrated with the ionic ammonium adducts of polypropylene glycol. Tandem MS experiments were performed

with either electrospray or MALDI mass spectrometers. For electrospray, collision-induced dissociation (CID) experiments were performed using an LCQ Deca Xp Plus mass spectrometer (ThermoFinnigan). Spectra were acquired in the range *m/z* 200–2000 by a manual control using a capillary voltage and temperature of 40 V and 190 °C, respectively. The mass isolation window and collision energy were set to 3 *m/z* and 35%, respectively. For MALDI, postsource decay (PSD) experiments were performed using a Voyager-DE PRO mass spectrometer (Applied Biosystems). Samples were loaded onto the instrument target using the dried droplet technique and α-cyano-4-hydroxycinnamic acid as the matrix. Spectra were acquired either in a reflectron or a linear mode with delayed extraction. PSD fragment ion spectra were acquired after isolation of the appropriate precursor using timed ion selection. All precursor ion segments were acquired at low laser power (variable attenuator = 1950) for < 200 laser pulses. Laser power was increased by 200 U for all the remaining segments. Typically, 300 laser pulses were acquired for each fragment–ion segment.

Nuclear magnetic resonance (NMR) spectroscopy

Samples for the NMR study were prepared by dissolving *c.* 1 mg of freeze-dried peptide in 700 μL of CF_3CD_2OD/H_2O (5 : 1, v/v). NMR spectra were measured on a Bruker AVANCE AQS600 instrument operating at 600.13 MHz with *z*-gradient selection at 300 K. ^{1}H NMR experiments were performed as described (Braun *et al.*, 1998) ^{1}H–^{1}H TOCSY and NOESY experiments (Braun *et al.*, 1998) were acquired in the phase-sensitive mode with the time proportional phase increment(TPPI) method using WATERGATE water suppression (Piotto *et al.*, 1992). The heteronuclear single quantum correlation experiment was performed using the echo/antiecho detection method with a soft presaturation to suppress the water signal. All the 2D NMR experimental results were acquired with a time domain of 1024 data points in the F2 dimension, 512 data points in the F1 dimension, and a recycle delay of 2–3 s. ^{1}H–^{1}H TOCSY was acquired with a spin–lock duration of 90 ms. ^{1}H–^{1}H NOESY was acquired with a mixing time of 200 ms. Multiple scans were performed to optimize signal to noise ratios.

Results

Identification of antifungal lipopeptides in *P. syringae* strain 31R1 extracts

Extracts of *P. syringae* strain 31R1 cultures showed strong growth-inhibitory activities against *R. pilimanae* Hedrick et Burke, similar to those obtained with other lipodepsipeptide-producing *P. syringae* strains. HPLC profiles of the extracts showed three major components eluting at 16.1, 19.1, and 21.1 min. Each was collected separately, and all three components showed fungicidal activities. Mass spectral peaks of the species eluting at 16.1 and 19.1 min corresponded to the well-known SRE (MH^+ *m/z* 1225.3–1227.3) and SRG (MH^+ *m/z* 1253.3–1255.3), respectively (Segre *et al.*, 1989). No HPLC peaks with retention times typical of syringopeptins (30–35 min) (Ballio *et al.*, 1991) were observed. The component eluting at 21.1 min showed an MH^+ signal at *m/z* 1268.5, which did not match with any previously observed metabolite from *P. syringae*. Moreover, the absence of a typical isotopic pattern of Cl–Thr characteristic of the *P. syringae* lipodepsinonapeptides and an amino acid composition of Pro (1), Val (1), Ala (3), Tyr (1), Dab (2), and Leu (1) suggested the occurrence of a novel peptide. Because mild base hydrolysis (6.5% aqueous triethylamine, pH 9, at 25 °C) did not yield a product with an MH^+ value compatible with the lactone

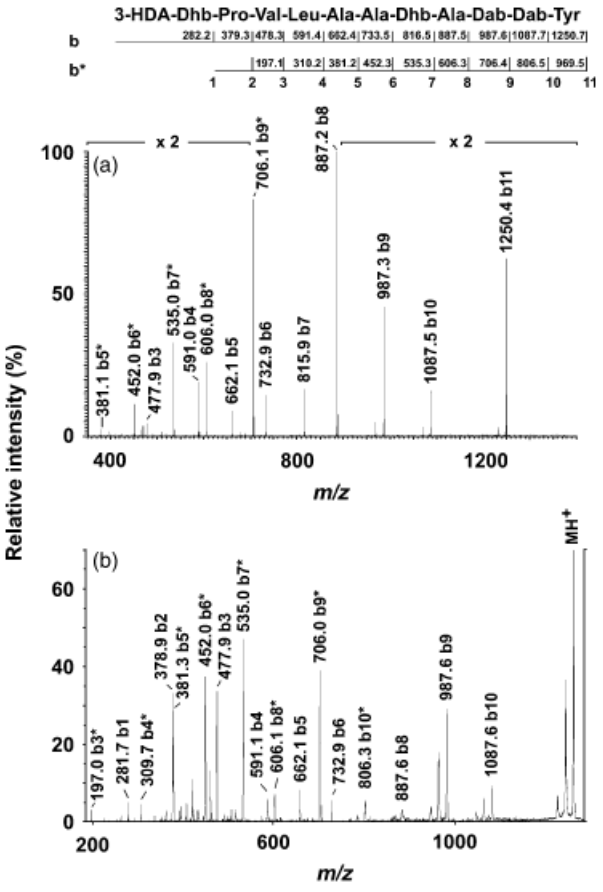

Fig 1. Tandem MS analyses of the compound that elutes at 21.1 min in HPLC as performed using ESI–CID (a) and MALDI–PSD (b). Peak numbers are values relative to fragmentation of the MH^+ ion. The deduced peptide sequence is shown (top) together with the values of the theoretical fragment ions. HDA, 3-hydroxydodecanoate; Dhb, dehydroaminobutanoate; Dab, diaminobutyrate.

Table 1. ^{1}H and ^{13}C NMR assignments of peptin31 in CF_3CD_2OD/H_2O 5 : 1 by volume at 300 K

		Chemical shift (p.p.m.)						
Alkyl chain length	Atom	C2	C3	C4	C5–C9	C10	C11	C12
	^{1}H	2.60, 2.55	4.12	1.58	1.31–1.34	1.31	1.32	0.90
	^{13}C	44.0	70.8	38.0	31.2–30.4	33.1	23.9	14.3
		Chemical shift (p.p.m.)						
Amino acid	Atom	NH	Cα	Cβ, β′		Cγ, γ′	Cδ	
Dhb1	^{1}H	8.898		5.806		1.788		
	^{13}C			125.1		12.5		
Pro2	^{1}H		4.443	2.345		1.995, 1.958	3.66	
	^{13}C		63.3	35.6		26.4	51.5	
Val3	^{1}H	7.941	3.888	2.368		1.038, 1.009		
	^{13}C			30.7		20.4, 19.8		
Leu4	^{1}H	7.929	4.224	1.767, 1.618		1.614	0.962, 0.919	
	^{13}C		55.8	41.2		26.3	23.6, 21.5	
Ala5	^{1}H	8.028	4.258	1.478				
	^{13}C		52.4	17.3				
Ala6	^{1}H	7.708	4.298	1.557				
	^{13}C		52.6	17.4				
Dhb7	^{1}H	9.068		6.689		1.842		
	^{13}C					12.2		
Ala8	^{1}H	7.839	4.328	1.533				
	^{13}C		52.6	17.4				
Dab9	^{1}H	7.951	4.391	2.279, 2.160		3.126		
	^{13}C		53.5	30.6		38.8		
Dab10	^{1}H	7.974	4.460	2.188, 2.071		3.048		
	^{13}C		53.1	31.0		38.6		
Tyr11	^{1}H	7.706	4.658	3.235, 2.972		(o) 7.149	(m) 6.857	
	^{13}C		56.5	37.9		(o) 132.5	(m) 117.1	

ring opening of a lipodepsipeptide (Segre *et al.*, 1989), a linear structure was hypothesized. The combined data were consistent with the occurrence of a novel fungicidal linear peptide in *P. syringae* 31R1 extracts named here as peptin31.

Determination of the structure of peptin31

The amino acid sequence of peptin31 was determined by tandem MS, using two independent approaches, and confirmed by 2D NMR spectroscopy. Fragmentation experiments of the peptin31 MH^+ using CID and PSD techniques produced ion spectra that alone permitted determination of the complete structure (Fig. 1). Only b- and b*-type ions were observed, with the latter originating from peptide cleavage between Dhb and Pro consistent with the occurrence of the parent ion with *m/z* 987.1 in the spectrum of intact peptin31. The presence of a 3-hydroxydodecanoyl moiety at the N-terminus was inferred from the values of the b-type fragments. Comparisons between the MS/MS data (Fig. 1) and those reported for SP_{22}Phv A and B and SP(SC)-1 and -2 (Grgurina *et al.*, 2002) allowed determination of the N-terminus structure of peptin31. The b*-type ion spectra up to *m/z* 452.3 (fragment b_6^*) were common to

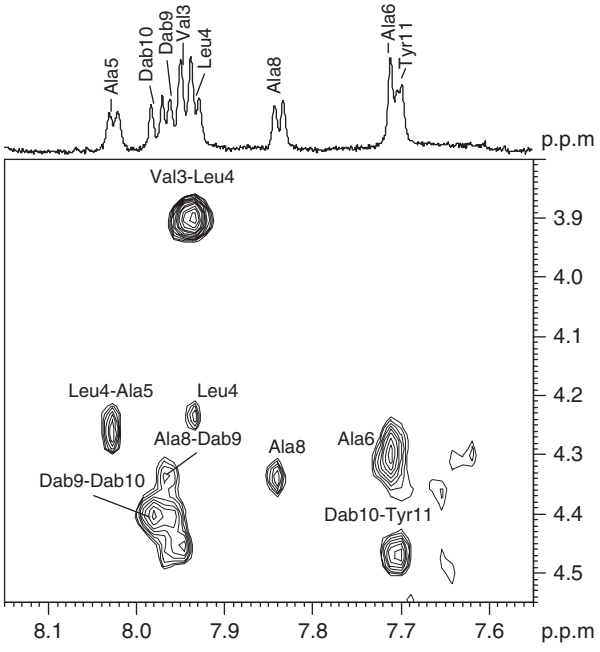

Fig. 2. Slice of ^{1}H-^{1}H NOESY map of peptin31 in CF_3CD_2OD/H_2O (5 : 1, v/v) determined at 300 K.

Table 2. Bioactivities of peptin31, SRE and SP22A

	Antimicrobial activity*				Hemolytic activity
	ZI (mm)†				
	Bacillus megaterium strain 51S	*Rhodotorula pilimanae* Hedrick et Burke	*Rhizoctonia solani* strain 1556	*Trichoderma harzianum* strain T22	IC_{50} (μM)‡
Peptin31	8 ± 2	13§ ± 2	4.5 ± 1	6 ± 1	5.7
SRE	15 ± 3	20§ ± 4	19 ± 3	24 ± 3	0.48
SP22A	20 ± 3	15§ ± 3	ND¶	3 ± 1	2.5

*Spot diffusion assays with application of 10 μL lipopeptide solutions at concentrations of 0.5 mg mL^{-1} unless indicated otherwise.
†ZI is the zone of inhibition diameter in spot diffusion assays. SD was calculated from three independent experiments.
‡IC_{50} is the lipopeptide concentration causing 50% hemolysis of human erythrocytes.
§Peptide concentration was 0.2 mg mL^{-1}.
¶ND, not determined.

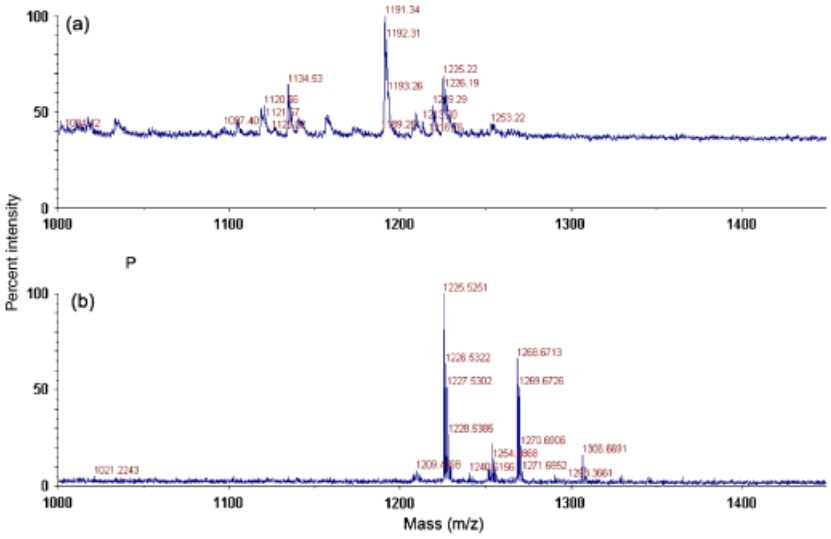

Fig. 3. MALDI–TOF mass spectra of extracts of *Pseudomonas syringae* strain 31R1 grown on medium B (a) and medium A (b).

all five compounds, while the b-type ion spectra up to *m/z* 733.5 (fragment b_6) were common to peptin31, SP_{22}Phv B, and SP(SC)-2 and differed in +28 a.m.u. from those of SP_{22}Phv A and SP(SC)-1. The latter difference accounted for the relative occurrence of 3-hydroxydodecanoyl (for SP_{22}Phv A and SP(SC)-1) vs. 3-hydroxydecanoyl moieties blocking the peptide N-terminus. The remaining peptide region was deduced from the b-type fragments and corresponded to the C-terminal sequence of SP_{22} A and B (Ballio *et al.*, 1991), SP_{22}Phv A and B (Grgurina *et al.*, 2002), and SP(SC)-1 and -2 (Isogai *et al.*, 1995).

The above peptin31 structure predictions were consistent with results from 2D high-field NMR spectroscopy. The olefinic spectral region showed two quartets that were assigned to the CH protons of two Dhb residues (Sklenar *et al.*, 1993). The presence of the hydroxyl group in the β position of the fatty acid chain was confirmed by TOCSY. The length of the fatty acid moiety was determined by integration of the fatty acid chain resonances. All resonance assignments in the ^{1}H-NMR spectrum of peptin31 in CF_3CD_2OD/H_2O are shown in Table 1. NOESY spectra show crosspeaks due to dipolar connectivities (Fig. 2). In particular, analysis of the CαH*i*/NH(*i*+1) crosspeaks confirmed the proposed amino acid sequence from MS data (Fig. 1). Also, the *z*-configuration of all the Dhb residues was assigned on the basis of NOE crosspeaks between CβH-Dhb*i* and NH(*i*+1).

Bioactivities of the *P. syringae* 31R1 lipopeptides

SRE and peptin31 were more strongly inhibitory to fungi than SP22A, with SP22A and peptin31 displaying relatively

lower activities against *R. solani* and *T. harzianum*. As expected, SRE had strong fungicidal activities against *R. pilimanae*, *R. solani*, and *T. harzianum*. Peptin31 was slightly less hemolytic than SP22A. All three lipopeptides showed antibacterial activity against *B. megaterium* (Table 2).

Contributions of the lipopeptides to fungicidal activity

Fungicidal activity was detected in extracts of *P. syringae* strain 31R1 cells grown in medium A (with chloride), but no or very low activities were measured in extracts of cells grown in medium B (no chloride). When grown in medium B, the extracts contained the des-chloro forms of SRE and SRG: H-SRE and H-SRG (MH^+ at *m/z* 1191 and 1219, respectively) as observed previously with *P. syringae* pv. syringae strain B359 (Grgurina *et al.*, 1994). However, no MH^+ *m/z* 1268.5 signal associated with peptin31 was evident. HPLC fractions from medium A-grown cells that contained SRE, SRG, and peptin31 showed strong fungicidal activities (Fig. 4b). But H-SRE and H-SRG from medium B-grown cells had lower fungicidal activities (Fig. 4b), consistent with previous findings (Grgurina *et al.*, 1994). These results show that chloride in the growth medium is essential for the production of peptin31 as well as SRE and SRG, and that the fungicidal activity of *P. syringae* 31R1 is due to the combined activities of these three compounds.

Discussion

The current work reveals that ice-nucleating *P. syringae* strain 31R1 produces lipopeptide secondary metabolites. Two well-known small lipodepsinonapeptide homologs that differ in the length of their lipid moieties, SRE and SRG, but no SP22 or SP25 or other forms of the syringopeptins, were detected. Instead, a compound with a mass differing from all known *P. syringae* lipodepsipeptides was observed, namely peptin31, and its chemical structure was elucidated (Fig. 3). Peptin31 is a structural variant of *P. syringae* lipodepsipeptides. It is a novel linear undecapeptide with an N-terminal amino acid that is N-acylated by a 3-hydroxydodecanoyl chain. Other linear lipopeptides produced by *P. syringae* have been reported (Berti *et al.*, 2007), but (to the best of our knowledge) not simultaneously with the production of lipodepsipeptides.

Based on the knowledge of SRE (Guenzi *et al.*, 1998) and SP22 (Scholz-Schroeder *et al.*, 2003) biosyntheses, peptin31 is likely synthesized nonribosomally by a multimodular peptide synthetase. The architecture of the gene cluster for peptin31 biosynthesis remains to be elucidated. However, based on the deduced similarities of the C-terminal six residues of peptin31 and SP22, it can be hypothesized that a similarity exists in the peptin31 and SP22 synthetases involved in constructing this portion of the two peptides. It is unclear why peptin31 is not produced in the absence of chloride. Peptin31 does not possess Cl–Thr or any other chlorinated derivative, but its biosynthesis is apparently regulated by chloride.

Cell extracts of *P. syringae* strain 31R1 cultures have strong fungicidal activities that are attributed to the presence of SRE, SRG, and peptin31. Extracts from cells grown without chloride show no or negligible fungicidal activity and concomitantly lack all three of these metabolites (Fig. 4). Their fungicidal nature should be considered when assessing the ecological impacts of making snow based on

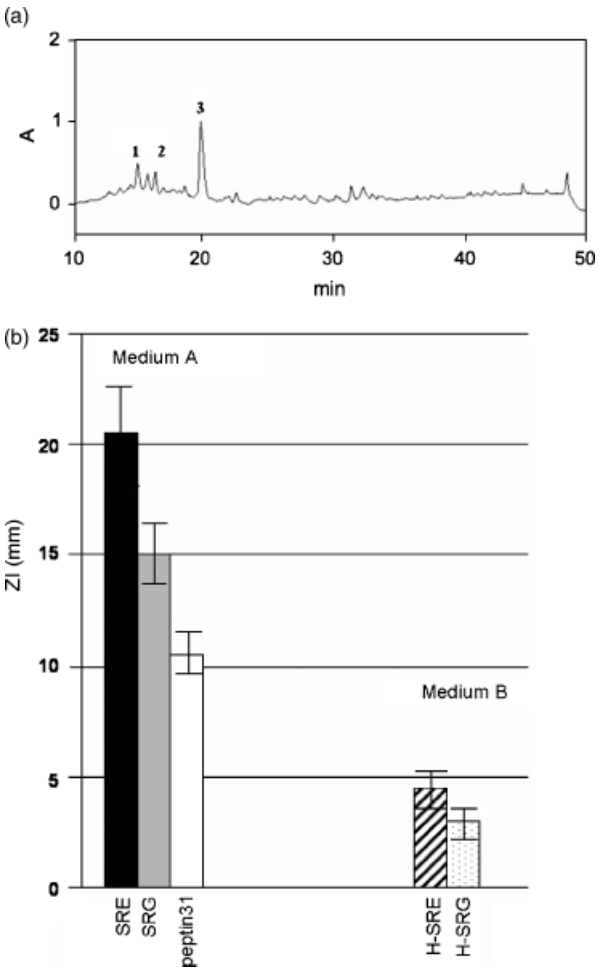

Fig. 4. Influence of chloride on *Pseudomonas syringae* 31R1 lipopeptide production (a) and fungicidal activities of recovered lipopeptides (b). Growth in medium B lacking chloride yielded SRE and SRG analogs, H-SRE (peak 2), and H-SRG (peak 3) (a). H-SRE and H-SRG activities were lower than SRE and SRG activities, respectively (b).

P. syringae 31R. Being physically stable (Segre *et al.*, 1989), the accumulation of these compounds in alpine soils and water could have the potential to alter fungal community structures.

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Supplementary material

The following supplementary material for this article is available online:

Fig. S1. Reversed-phase HPLC profile of a *P. syringae* 31R1 extract.

Fig. S2. Mass spectrum of the compound that elutes at 21.1 min in HPLC.

This material is available as part of the online article from: http://www.blackwell-synergy.com/doi/abs/10.1111/j.1574-6968.2008.01247.x (This link will take you to the article abstract.)

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