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# Viscosinamide, a new cyclic depsipeptide with surfactant and antifungal properties produced by *Pseudomonas fluorescens* DR54

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T.H. NIELSEN, C. CHRISTOPHERSEN, I. ANTHONI AND J. SØRENSEN. 1999. *Pseudomonas fluorescens* DR54 showed antagonistic properties against plant pathogenic *Pythium ultimum* and *Rhizoctonia solani* both *in vitro* and *in planta*. Antifungal activity was extractable from spent growth media, and fractionation by semi-preparative HPLC resulted in isolation of an active compound, which was identified as a new bacterial cyclic lipodepsipeptide, viscosinamide, using 1D and 2D $^{1}$H-, $^{13}$C-NMR and mass spectrometry. The new antibiotic has biosurfactant properties but differs from the known biosurfactant, viscosin, by containing glutamine rather than glutamate at the amino acid position 2 (AA2). No viscosin production was observed, however, when *Ps. fluorescens* DR54 was cultured in media enriched with glutamate. *In vitro* tests showed that purified viscosinamide also reduced fungal growth and aerial mycelium development of both *P. ultimum* and *R. solani*. Viscosinamide production by *Ps. fluorescens* DR54 was tightly coupled to cell proliferation in the batch cultures, as the viscosinamide produced per cell mass unit approached a constant value. In batch cultures with variable initial C, N or P nutrient levels, there were no indications of elevated viscosinamide production during starvation or maintenance of the cultures in stationary phase. Analysis of cellular fractions and spent growth media showed that a major fraction of the viscosinamide produced remained bound to the cell membrane of *Ps. fluorescens* DR54. The isolation, determination of structure and production characteristics of the new compound with both biosurfactant and antibiotic properties have promising perspectives for the application of *Ps. fluorescens* DR54 in biological control.

## INTRODUCTION

Fluorescent pseudomonads often show antagonistic activity against fungal root pathogens (Weller 1988). However, screening for strains to be used for biocontrol has usually been carried out using *in vitro* agar plate tests. It is known that this protocol may not always detect strains which will antagonize the pathogens *in situ* (Keel *et al*. 1992). A possible way to increase the recovery of useful isolates may be to select for strains showing medium-independent *in vitro* antagonism (Nielsen *et al*. 1998b). Three *Pseudomonas fluorescens* strains isolated by such a protocol showed antagonistic activity against two root pathogenic fungi, *Pythium ultimum* and *Rhizoctonia solani*, both in *in vitro* and *in planta* experiments with sugar beet seedlings (Nielsen *et al*. 1998b). Subsequent tests suggested that the antagonistic activity by the highly active isolate, *Ps. fluorescens* strain DR54, was at least partially caused by production of an unknown antibiotic (Nielsen *et al*. 1998b).

Several *Pseudomonas* antibiotics are known (Dowling and O'Gara 1994) and their antagonistic activity against various pathogenic fungi has been well documented (Weller 1988).

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As suggested by Shanahan *et al*. (1992b), antibiotics against pathogenic fungi may be grouped into phenazines (Gurusiddaiah *et al*. 1986; Thomashow *et al*. 1990), pyo compounds (Takeda 1958), pyrrols (Howell and Stipanovic 1979), and other compounds such as phloroglucinols (Keel *et al*. 1992; Nowak-Thompson *et al*. 1994). Antibiotics with surfactant properties (Asaka and Shoda 1996; Stanghellini and Miller 1997) have also been found.

Various parameters influence antibiotic production in *Pseudomonas* spp. e.g. pH (Shanahan *et al*. 1992b), temperature (Bolton and Elliot 1989; Shanahan *et al*. 1992b), oxygen concentration (Bencini *et al.* 1983; Messenger and Turner 1983; Shanahan *et al*. 1992b), surface attachment (Shanahan *et al*. 1992b), substrate composition and concentration (Bencini *et al*. 1983; Messenger and Turner 1983; James and Gutterson 1986; Shanahan *et al*. 1992b; Stephens *et al.* 1993), trace element concentrations (Gross 1985; Slininger and Jackson 1992) and antibiotic precursors (Gorman and Lively 1967).

In batch cultures, the pseudomonads may show growth-phase dependent antibiotic production (Shanahan *et al*. 1992a). In a previous study, *Ps. fluorescens* strain DR54 was shown to produce variable amounts of an unknown antibiotic in cultures with different carbon sources (Nielsen *et al*. 1998a). It was also found that the purified antibiotic exhibited antifungal activity and specific stress responses in *Pythium ultimum* and *Rhizoctonia solani* (Thrane *et al*. in press). By vital fluorescent staining of fungal mycelium, it was thus evident that the antibiotic resulted in increased branching, swelling and septation of hyphae, and reduced intracellular activity. The changes were suggested to be caused by altered intracellular ion ($H^+$ and $Ca^{2+}$) contents, possible due to channel formation in the cell membrane (Thrane *et al*. in press). In the present study, the chemical identification of viscosinamide, which is a new cyclic lipodepsipeptide with both biosurfactant and antibiotic properties, is reported. The viscosinamide production in *Ps. fluorescens* DR54 as affected by different growth stages in glucose minimal medium, and the importance of C, N and P limitations in this medium, is also reported.

## MATERIALS AND METHODS

### Purification and structural analysis of a new *Pseudomonas* antibiotic

*Pseudomonas fluorescens* strain DR54 was isolated from the sugar beet rhizosphere (Nielsen *et al*. 1998b) and identified to *Ps. fluorescens* biovar I according to Palleroni (1984). Strain DR54 was initially found to show antibiotic production on Potato Dextrose Agar (PDA; Difco) (Nielsen *et al*. 1998b). To determine the chemical structure, a large aliquot of active compound was obtained after growth (7 d, 20 °C) of *Ps. fluorescens* DR54 on 200 PDA agar plates. The agar was homogenized and extracted twice with 600 ml of a mixture of ethyl acetate containing 6 ml formic acid. The solvent was subsequently evaporated by vacuum centrifugation and the residue was dissolved in 4 ml methanol. The active compound was purified by semi-preparative HPLC with automatic fraction collection, using a LiChroCART 250–10 HPLC-Cartridge with LiChrosphere 100 RP-18 (10 $\mu$m) (Merck) (20 °C) as a preparative column. Samples of 200 $\mu$l were injected and eluted in a gradient from 80% methanol in Milli-Q water (Millipore, MA, USA) increasing to 100% methanol over a 20 min period (flow rate 3 ml $min^{-1}$). Approximately 450 $\mu$mol antibiotic could be purified from the 200 PDA agar plates.

NMR spectra were recorded on a Varian 400 FT-NMR spectrometer (Varian NMR Instruments, Palo Alto, California, USA) at 400·0 and 100·6 MHz for $^1$H- and $^{13}$C-NMR spectra, respectively, using samples in hexadeuteriodimethylsulfoxide (DMSO-$d_6$). $^1$H,$^1$H correlated spectroscopy (COSY) was performed using pulse gradient multiquantum-filtered, phase-sensitive analysis. Rotating frame NOESY (ROESY) data were determined using a mixing time of 200 ms. Heteronuclear multiple quantum coherence (HMQC) data were recorded with pulse gradients and reverse detection and optimized for $J_{CH}$ 140 Hz (single bond) and 7 Hz (multiple bond), respectively. FAB-MS spectra were determined on a JEOL JMS-HX/HX110 A tandem mass spectrometer in a *meta*-nitrobenzylic alcohol(NBA)-glycerol-thioglycerol 1 : 1: 2 matrix containing 1% trifluoroacetic acid. Circular dichroism (CD) spectra were measured on a JASCO J-70 spectropolarimeter (JASCO International Co. Ltd, Tokyo, Japan).

A Hofmann degradation (Fieser *et al*. 1981) was performed by addition of a solution of iodobenzene bis(trifluoroacetate) (1·3 $\mu$mol, 560 $\mu$g) in acetonitrile/water 1 : 1 (70 $\mu$l) to a solution of viscosinamide (1·3 $\mu$mol, 1·45 mg) in acetonitrile/water 1 : 1 (300 $\mu$l). The mixture was then left for 5 h at room temperature protected against light. The reaction mixture was lyophilized after addition of 0·01 M HCl (1·8 ml) and the reaction product subjected to FAB-MS.

### Antifungal properties of purified antibiotic

The plant pathogenic Basidiomycete *Rhizoctonia solani* Kühn AG-4 was isolated from a diseased sugar beet root (Danisco Seed, Holeby, Denmark) and maintained as described by Thrane *et al*. (in press). The antifungal activity of viscosinamide was tested against the fungus using *in vitro* tests. A sample of 100 $\mu$g purified compound in methanol was applied to a sterile glass fibre filter (5 mm diameter). The methanol solvent was evaporated in the hood for 15 min and the filter was placed 1·5 cm from *Rhizoctonia solani* mycelium inocu-

lated on a Potato Dextrose Agar plate (PDA; Difco) (Nielsen *et al.* 1998b). A filter treated with pure methanol (HPLC grade) was used as control. After incubation at 27 °C for 30 h, the fungal growth was photographed with a Nikon F70 camera with a Sigma zoom, 28–80, Macro using a Fujicolour 100 ISO film.

### Production of antibiotic in liquid media

Prior to all growth experiments in liquid media, the strain was pre-cultured in Davis Minimal Broth (DMB; 30 mmol $l^{-1}$ $K_2HPO_4$, 14 mmol $l^{-1}$ $KH_2PO_4$, 0·4 mmol $l^{-1}$ $MgSO_4$, 7·6 mmol $l^{-1}$ $(NH_4)_2SO_4$, 60 mmol $l^{-1}$ C (glucose), and 1 ml $l^{-1}$ trace element solution, pH 7·3. The trace element solution contained 20 mg $CoCl_2.6H_20$, 30 mg $H_3BO_3$, 10 mg $ZnSO_4.7H_20$, 1 mg $CuCl_2.2H_2O$, 2 mg $NiCl_2.6H_2O$, 3 mg $NaMoO_4.2H_2O$, 10 mg $FeSO_4.7H_2O$ and 2·6 mg $MnSO_4.H_2O$ dissolved in 1 litre Milli-Q water. Unless specified, all cultures were grown in 50 ml Capsenberg glass flasks on a rotary shaker (150 rev $min^{-1}$) at 20 °C for 2 d, before cells were harvested by centrifugation (7000 *g*, 10 min, 4 °C), washed twice and dissolved in phosphate-buffered saline (PBS; 25 mmol $l^{-1}$ phosphate buffer, 125 mmol $l^{-1}$ NaCl, pH 7·4). In the following experiments, cells from the washed pre-culture were inoculated at an optical idensity (O.D.) of 0·1 at 600 nm.

To determine the antibiotic production during different growth stages in the batch cultures, *Ps. fluorescens* DR54 was inoculated in fresh DMB medium with 60 mmol $l^{-1}$ C (glucose) in a 140 ml Erlenmeyer flask with glass invaginations, designed to facilitate high aeration of the culture. The cultures were incubated at 20 °C under shaking and 9 ml sub-samples were collected at defined time intervals. A 6 ml aliquot of the sub-sample was transferred into a 9 ml polyethylene vial for subsequent extraction and analysis (described below). The remaining 3 ml sub-sample was used to determine optical density (O.D.).

To determine the antibiotic production during C, N or P nutrient limitation in the cultures, *Ps. fluorescens* DR54 was pre-cultured as described above, washed twice and dissolved in DMB without C, N or P. The effect of C limitation was subsequently studied by inoculation of *Ps. fluorescens* DR54 in 50 ml vials containing 20 ml DMB with 15, 30, 45, 60, 90, 120 or 150 mmol $l^{-1}$ C (glucose) and 15 mmol $l^{-1}$ N ($(NH_4)_2SO_4$). Similarly, the influence of N limitation was determined in DMB containing 60 mmol $l^{-1}$ C (glucose) with 0·1, 0·5, 1, 2·5, 5, 7·5 or 15 mmol $l^{-1}$ N ($(NH_4)_2SO_4$). Finally, P limitation was studied in DMB prepared with 10 mmol $l^{-1}$ Hepes buffer (pH 7·3) rather than phosphate buffer. This medium contained 60 mmol $l^{-1}$ C (glucose), 15 mmol $l^{-1}$ N ($(NH_4)_2SO_4$) and 0·5, 1, 2, 3, 4, 5 or 10 mmol $l^{-1}$ P ($K_2HPO_4$). All cultures were incubated under shaking (150 rev $min^{-1}$) at 20 °C for 2 d and subsequently sub-sampled for O.D. determinations and extractions of viscosinamide from the cells and supernatant fluid as described below. Concentrations of antibiotic accumulated in both the supernatant fluid and cells were expressed in $\mu$mol $l^{-1}$ of culture. All experiments were performed in triplicate.

### Extraction and analysis of antibiotic in cell and supernatant fractions

Cell and supernatant fractions of the cultures were separated by centrifugation at 7000 *g* for 10 min at 4 °C. Antibiotic was extracted from the cell fraction (pellet) with 1 ml acetone, which was subsequently evaporated under an $N_2$ flow, and the extract was dissolved in methanol for HPLC analysis. The supernatant fraction was extracted twice using a 1·5 volume of ethyl acetate containing 0·1% formic acid in the first extraction. After evaporation of the solvent, the extract was dissolved in methanol. Prior to HPLC analysis, impurities were precipitated by centrifugation at 15 000 *g* for 10 min at 4 °C. The amount of antibiotic produced per millilitre of original culture was calculated from the HPLC analysis of extractant from supernatant and cell fractions.

The efficacy of the extraction procedure was tested by adding purified antibiotic dissolved in 300 $\mu$l methanol directly into 7 ml of DMB media, spent culture supernatant fluid or *Ps. fluorescens* DR54 culture. These additions increased the antibiotic concentrations by 17·9 $\mu$mol $l^{-1}$ and 37·6 $\mu$mol $l^{-1}$. All samples were vortexed and left for 1 h before they were extracted as described above. Before the culture-amended sample was extracted, cells and supernatant fractions were separated by centrifugation and extracted as described above. To determine whether the methanol solvent influenced the extraction efficacy, samples amended with 300 $\mu$l HPLC-grade methanol were included for comparison.

To determine the cellular localization of antibiotic in *Ps. fluorescens* DR54, cells were washed twice and concentrated to a higher cell density. Sub-samples of 3 ml cell suspension were first sonicated for 1 min interval on ice (Sanyo MSE 150 W ultra sonic disintegrator, probe diameter 9·5 mm, 10 $\mu$m amplitude, Kontram, Denmark) during a total of 10 min. Unbroken cells were removed by centrifugation at 7000 *g* for 10 min at 4 °C, and the supernatant fluid containing both membranes and cytoplasm fractions were transferred into Corex tubes (Du Pont Company, Delaware, USA) before all samples were centrifuged at 50 000 *g* for 10 min at 4 °C. The supernatant fluid from each sample was subsequently removed and extracted twice with a mixture of 9 ml ethyl acetate and 0·1 ml formic acid. The membrane pellet was extracted with 1 ml acetone, which was evaporated before the pellet material was re-dissolved in 0·2 ml methanol for HPLC analysis as described below.

Analysis of antibiotic in the extracts was performed using a Hewlett Packard 1100 Series HPLC with a LiChroCART 250–4 HPLC-Cartridge containing LiChrosphere 100 RP-18 Merck (5 $\mu$m) column kept at 40 °C. The samples were analysed in a gradient of 80% acetonitrile and 20% 0·1% o-phosphoric acid (0–5 min), and 80–99% acetonitrile (5–15 min), 99% acetonitrile (15–18·5 min) and 99–80% (18·5–20 min), respectively, run at 1 ml min$^{-1}$. Standards of purified antibiotic were included. A diode array detector measured absorption at 210 $\pm$ 8 nM. Chromatograms were analysed using the Hewlett-Packard HPLC 3D ChemStation software Hewlett-Packard GmbH, Waldbronn, Germany.

## RESULTS

### Structural analysis and detection of a new antibiotic, viscosinamide

Figure 1 shows the structure of the new *Pseudomonas* antibiotic as inferred from the FAB-MS pseudomolecular ions at $m/z$ 1125·69 $(M + H)^+$ and 1147·68 $(M + Na)^+$. The molecular composition, $C_{54}H_{96}N_{10}O_{15}$, represents the amide of the known *Pseudomonas* surfactant, viscosin (Hiramoto *et al*. 1970). Viscosinamide has a glutamine residue at amino acid position 2 (AA2), where a glutamic acid residue is found in viscosin. In routine HPLC analysis, viscosin and viscosinamide both showed absorption at 210 nm and could therefore not be distinguished by UV-VIS spectra. However, the new viscosinamide compound could be chromatographically separated from viscosin owing to the slightly longer retention time (data not shown). The FAB-MS data further displayed signals from fragment ions representing loss of a hydroxydecanoyl moiety, acylated Leu, acylated Gln-Leu, acylated and dehydrated Thr-Gln-Leu, and acylated Val-dehydrated Thr-Gln-Leu as depicted in Fig. 1(b). This finding is analogous to the recorded fragmentation of the *Pseudomonas* surfactant, massetolide F (Gerard *et al*. 1997), and represents fragmentation of an acylated linear peptide formed by a ring opening between the protonated *C*-terminal Ile and the Thr leaving the unsaturated dehydrated Thr in the peptide chain.

The $^1$H- and $^{13}$C-NMR data were compatible with this structure. A pivotal point in the structure elucidation of viscosinamide was the appearance in comparison with viscosin, a perturbed glutamine residue with the $\gamma$-carbonyl group at $\delta_C$ 173·9, and two broadened singlets at $\delta_H$ 6·78 and 7·24, respectively. These signals showed a weak coupling in the COSY spectrum and a weak cross peak in a ROESY experiment. The signal at $\delta_H$ 7·24 exhibited two bond coupling to the $\delta_C$ signal at 173·9 and the one at $\delta_H$ 6·78 to the same carbonyl and to the Gln-$\gamma$ methylene carbon at $\delta_C$ 31·4 bearing the protons appearing at $\delta_H$ 2·09 (dd, $J$ 14·5 and 6·5 Hz). As a result of extensive overlap in proton signals, it was not entirely possible to assign the individual signals. The nine $\alpha$-carbon atoms and the attached protons appear at $\delta_C$ 51·5 and 53·0 ($\delta_H$ 4·20), 56·4, 55·1, 51·2, 51·1 (4·30), 55·3 (4·33), 56·4 (Thr, 4·45, dd, 14·5, 6·5 Hz) and 59·1 (Val, 3·99, dd). The 3-hydroxydodecanoyl moiety appear at $\delta_C$ 43·6 (C-2, $\delta_H$ 2·24, ddd), 67·6 (C-3, 3·81, dt), 37·1 (C-4, 1·35) and $\delta_H$ 4·61 (OH, d, $J$ 5·5 Hz). Except for the signals mentioned above, the following signals are diagnostic because of their position in otherwise unoccupied parts of the spectrum: Gln residue $C_\beta$ 35·9 (1·88, 1·74), Thr $C_\beta$ 69·7 (5·00 dq), $C_\gamma$ 16·5 (1·05, d) and Ser OH 4·82, dd, 13·7, 5·5 Hz.

Confirmation of the exact structure was achieved from the fact that viscosinamide could be transformed to the corresponding depsipeptide containing 2,4-diaminobutyric acid instead of glutamine. The transformation was effected by treatment of viscosinamide with bis(trifluoroacetoxy) iodobenzene, giving rise to a Hofmann rearrangement resulting in elimination of the acid carbonyl. The product was characterized by FAB-MS displaying the pseudomolecular ion at $m/z$ 1097·64 $(M + H)^+$. The calculated exact molecular mass of the Hofmann degradation product, $C_{53}H_{96}N_{10}O_{14}$, is 1096·71. The absolute stereochemistry was deemed identical to the one determined for viscosin based on the almost identical positive Cotton effect CD curves for the two compounds. Viscosin CD [EtOH, c = 0·196] nm ($\Delta\varepsilon$) 210 (24·7), 196 (−78·8) compared with viscosinamide [EtOH, c = 0·300]nm ($\Delta\varepsilon$) 210 (27·7), 196 (−92·2).

In view of the structural similarity between viscosin and viscosinamide, experiments were carried out to test whether *Ps. fluorescens* DR54 was able to produce viscosin when cultured in DMB media supplemented with 250 mg l$^{-1}$ L-glutamic acid. It was found, however, that the strain was unable to incorporate L-glutamic acid into the cyclic depsipeptide at the AA2 position and was unable to produce viscosin (data not shown).

### Extraction and antifungal properties of viscosinamide

The extraction protocol for viscosinamide in liquid bacterial cultures was tested by adding purified viscosinamide (dissolved in methanol) at two concentration levels. The addition was made to both fresh DMB medium, spent DMB medium and a stationary phase bacterial culture, which was later separated into cell and supernatant fractions. In Table 1, the total extractable pool of viscosinamide is shown, together with percentage recoveries for these treatments. The recovery of added viscosinamide was always very high and addition of the methanol solvent did not change the amount of extractable viscosinamide. Hence, it was anticipated that the extraction protocol was valid for all the produced or added viscosinamide pools in the bacterial cultures.

During the recovery experiments, it was noted that when

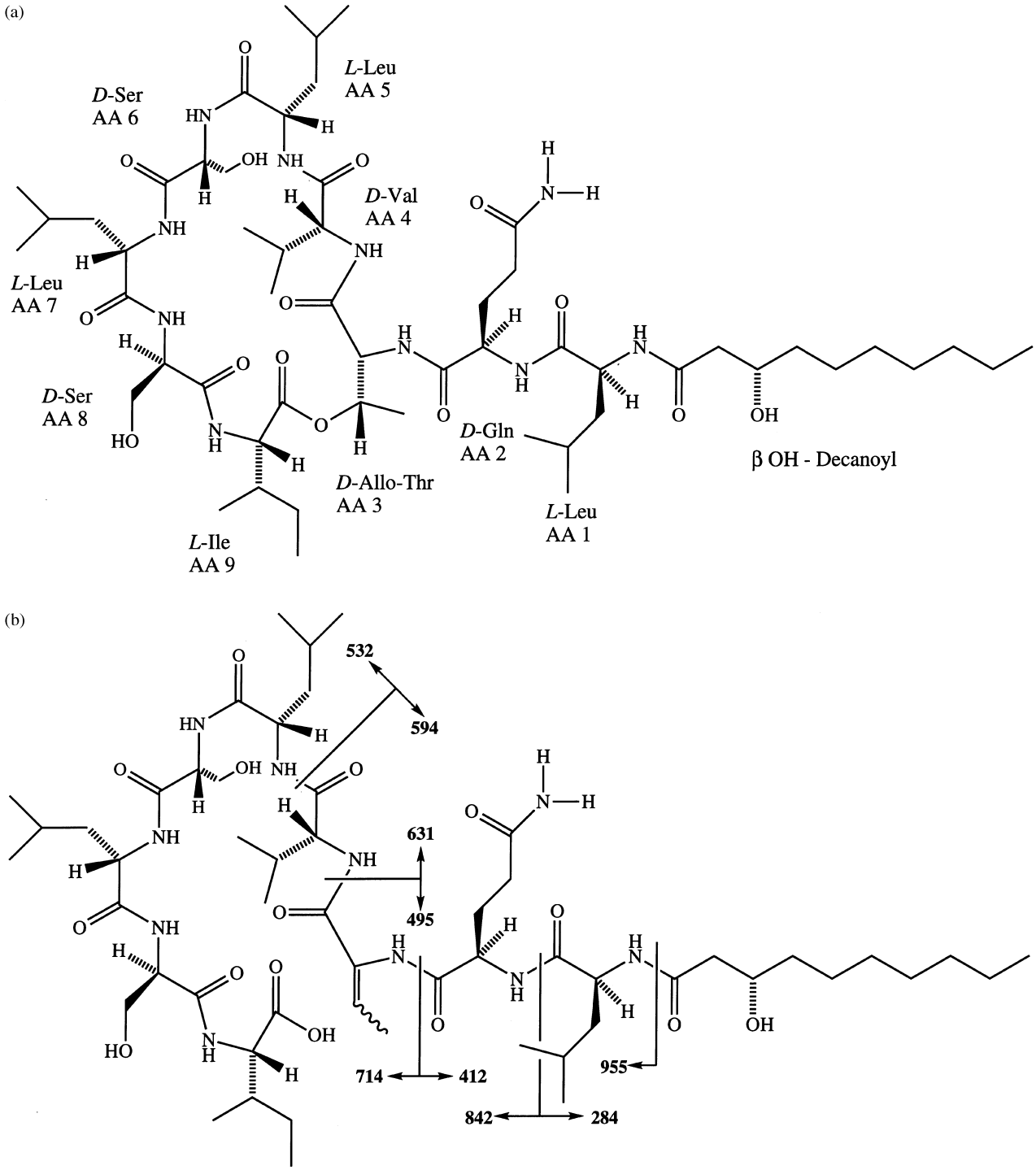


**Fig. 1** Chemical structure (a) and FAB-MS fragmentation (b) of the new compound viscosinamide

viscosinamide was added to a culture of *Ps. fluorescens* DR54, most of the compound was subsequently extracted from the cell fraction, while the supernatant fluid concentration increased only slightly (Table 1). To determine whether the produced viscosinamide was bound to specific cell fractions, *Ps. fluorescens* DR54 was cultured to stationary phase in DMB medium with 60 mmol $l^{-1}$ C (glucose), and cells were separated from the supernatant fluid. Figure 2 shows that 76%

**Table 1** Ectraction efficiency for viscosinamide from control medium, medium with methanol and medium with added 17·9 or 37·6 $\mu$mol $l^{-1}$, viscosinamide in methanol

| Fraction | Control* ($\mu$mol $l^{-1}$) | +Methanol ($\mu$mol $l^{-1}$) | +Methanol +20·1 $\mu$mol $l^{-1}$ viscosinamide ($\mu$mol $l^{-1}$)† | +Methanol +42·3 $\mu$mol $l^{-1}$ viscosinamide ($\mu$mol $l^{-1}$)† |
|---|---|---|---|---|
| Fresh medium | 0 | 0 | 18·0 ± 0·8 (101) | 39·0 ± 1·6 (104) |
| Supernatant fluid | 4·1 ± 1·0 | 4·2 ± 0·1 | 21·5 ± 1·1 (97) | 39·9 ± 1·5 (95) |
| Stationary phase culture‡ | 65·1 ± 0·2 | 65·4 ± 0·9 | 82·4 ± 0·3 (96) | 102·9 ± 3·8 (100) |
| Cells from stationary phase culture | 60·8 ± 0·8 | 60·2 ± 0·8 | 76·0 ± 1·0 (88) | 96·3 ± 3·6 (96) |
| Supernatant fluid from stationary phase culture | 4·4 ± 0·6 | 5·2 ± 0·1 | 6·4 ± 0·9 (7) | 6·6 ± 0·4 (4) |

Viscosinamide extraction was tested in fresh and spent DMB media, and in stationary phase culture which was separated into cells and supernatant fluid before extraction. Viscosinamide recovery was calculated using the medium with added methanol as reference.
* Values are means ($n = 3$) ± standard deviation.
† Viscosinamide recovery (%) is given in parantheses.
‡ Results are calculated by adding results from cells and supernatant fluid.

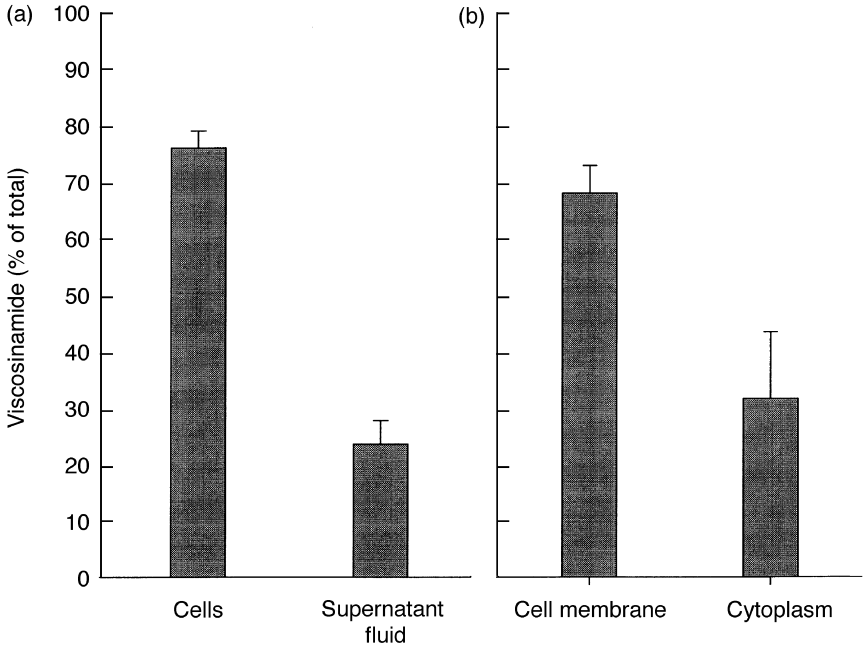


**Fig. 2** Localization of viscosinamide in culture (a) and cell fractions (b) in stationary phase cultures of *Pseudomonas fluorescens* DR54 grown in DMB medium with 10 mmol $l^{-1}$ glucose. ($n = 3$ ± S.D.)

of the viscosinamide in the culture was located in the cell fraction and the remaining 24% was extracted from the spent growth medium. The cellular localization of viscosinamide produced in *Ps. fluorescens* DR54 was further confirmed by sonicating the cells and separating the cell membrane and cytoplasm sub-fractions by centrifugation. Following this treatment, 68% of the detected viscosinamide was located in the precipitating cell membranes, while 32% was in the cytoplasm (Fig. 2).

When the fungus *Rhizoctonia solani* was challenged with viscosinamide, hyphal growth was reduced near the viscosinamide-containing filter but not the control filter (Fig. 3). Aerial mycelium was lacking in a 5–6 mm zone around the viscosinamide source.

### Viscosinamide production in liquid cultures of *Ps. fluorescens* DR54

In DMB medium with 10 mmol $l^{-1}$ glucose, *Ps. fluorescens* DR54 exhibited exponential growth during the initial 15–

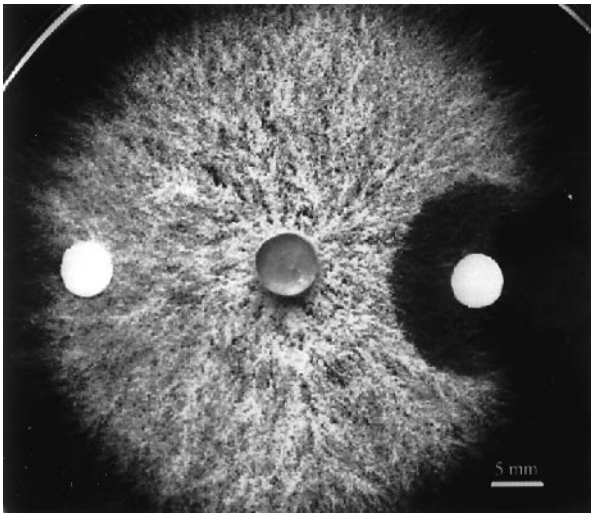


**Fig. 3** *In vitro* test of *Rhizoctonia solani* growth on Potato Dextrose agar plate containing filters with 100 $\mu$g viscosinamide (right of fungal mycelium) and methanol solvent control (left of fungal mycelium)

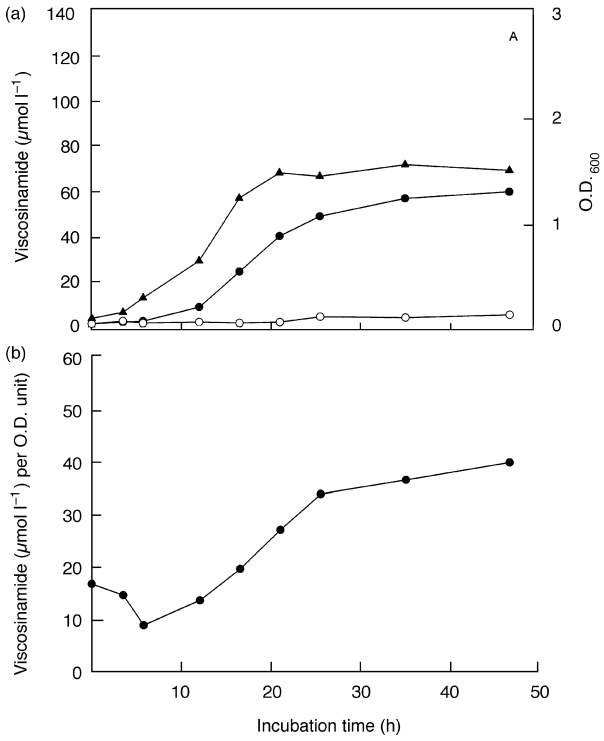


**Fig. 4** Growth curve and viscosinamide production in batch cultures of *Pseudomonas fluorescens* DR54 grown in DMB medium with 10 mmol $l^{-1}$ glucose (a). Production of viscosinamide is expressed as O.D. (optical density) units of cell mass throughout an experiment. (●), Cells; (○), supernatant fluid; (▲), O.D.

20 h, followed by a stationary phase after 20 h. As shown in Fig. 4(a), the viscosinamide accumulation in the cell fraction followed the cell production (O.D.), indicating that viscosinamide production was primarily associated with the logarithmic growth phase rather than the stationary phase (Fig. 4b). The viscosinamide concentration of 4–6 $\mu$mol $l^{-1}$ in the supernatant fraction was much smaller than in the cell-bound fraction. Furthermore, the supernatant fraction remained almost constant during the logarithmic growth phase, and increased only slightly as the culture entered stationary phase.

When *Ps. fluorescens* DR54 was cultured at variable initial C levels in the DMB medium, it was evident that C was limiting growth when less than 100 mmol $l^{-1}$ C were added. Hence, Fig. 5(a) shows that increasing glucose concentrations up to 100 mmol $l^{-1}$ C resulted in parallel increases of cells (O.D.) and viscosinamide. Again, the large majority of viscosinamide produced was associated with the cell fraction, contrary to the low (approximately 7 $\mu$mol $l^{-1}$) and constant level in the supernatant fraction. Figure 5(b) shows that the viscosinamide produced per cell mass unit (O.D.) was increasing slightly with initial C levels up to 100 mmol $l^{-1}$ C.

In comparable experiments with variable initial N levels in the DMB medium, *Ps. fluorescens* DR54 showed a nearly linear increase in cell production (O.D.) as the N level was increased to approximately 5 mmol $l^{-1}$. Hence, Fig. 5(c) shows that O.D. became constant above 5 mmol $l^{-1}$ N, indicating that N was no longer the limiting factor. Increasing N concentrations up to 5 mmol $l^{-1}$ also resulted in increased viscosinamide production, parallel to the increasing cell production; above this N level, the amount of viscosinamide produced was constant. Also, in the N-limited cultures, the final viscosinamide concentration in the supernatant fraction was very low (approximately 6 $\mu$mol $l^{-1}$) compared with that in the cell-bound fraction. Figure 5(d) shows that the viscosinamide produced per cell mass unit (O.D.) increased with initial N levels up to 5 mmol $l^{-1}$, which again corresponded well with the N level limiting the cell production in the cultures.

Finally, in experiments with variable initial P levels in the medium, *Ps. fluorescens* DR54 strain showed a steady increase in final cell production (O.D.) as the P level increased from 0·1 to 5 mmol $l^{-1}$ (Fig. 5e). The O.D. became constant at P concentrations above 5 mmol $l^{-1}$, indicating that P was no longer the limiting factor. The viscosinamide in the cellular fraction followed the increase in O.D. up to 5 mmol $l^{-1}$ P; above this level, the amount of viscosinamide produced was constant. As was the case in the C- or N-limited cultures described above, the viscosinamide concentration was relatively low (approximately 9 $\mu$mol $l^{-1}$) and nearly constant in the supernatant fraction, irrespective of the initial P amend-

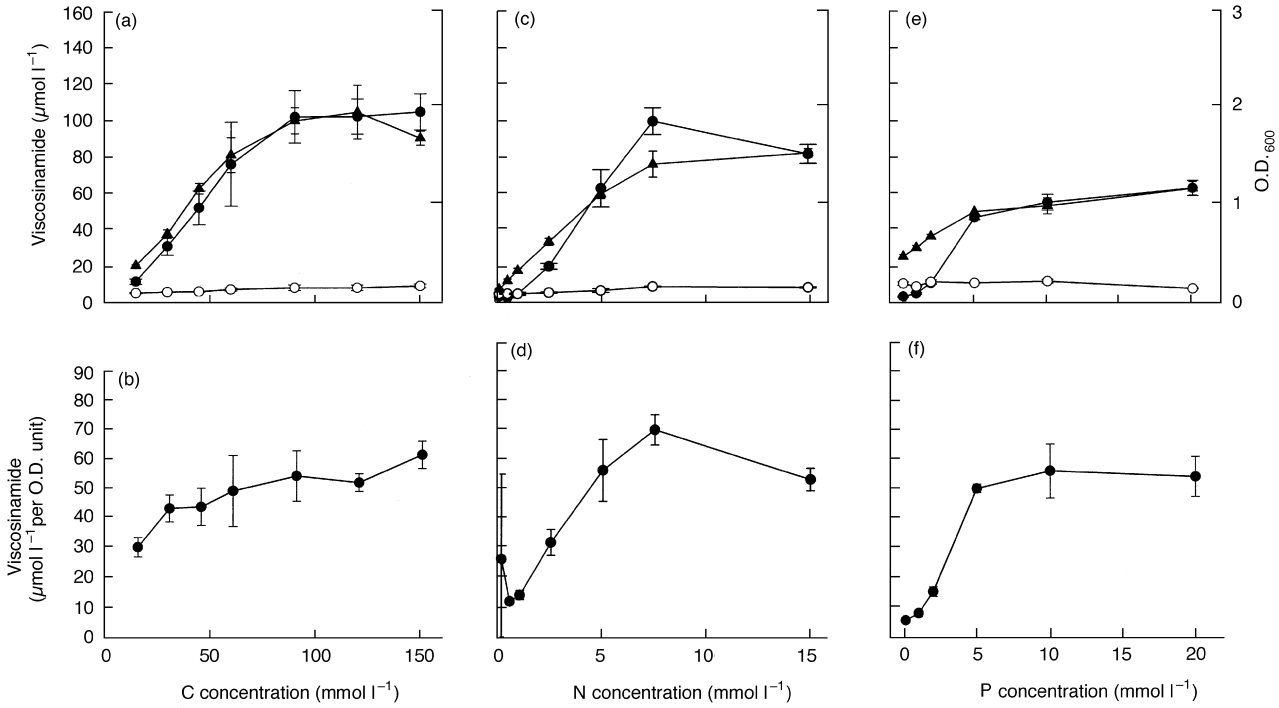


**Fig. 5** Viscosinamide production and O.D. (optical density) units of cell mass in batch cultures of *Pseudomonas fluorescens* DR54 grown in DMB medium with variable initial concentrations of carbon (a), nitrogen (c) and phosphorous (e) ($n = 3 \pm$ S.D.). Production of viscosinamide per O.D. unit is also shown for the variable initial concentrations of carbon (b), nitrogen (d) and phosphorous (f). (●), Cells; (○), supernatant fluid; (▲), O.D.

ment. Figure 5(f) demonstrates that, as for the other experiments, viscosinamide production per cell mass unit (O.D.) increased with initial P levels up to approximately 5 mmol $l^{-1}$, in accordance with the initial P level limiting both O.D. and viscosinamide production.

## DISCUSSION

### Viscosinamide structure

The new antibiotic and biosurfactant compound termed viscosinamide belongs to a family of closely related cyclic depsipeptides isolated from *Pseudomonas* spp. The structure resembles that of the antibiotic and biosurfactant, viscosin (Hiramoto *et al.* 1970), isolated from *Ps. viscosa* (Kochi *et al.* 1951) and *Ps. fluorescens* biovar II strains (Hildebrand 1989); the AA2 amino acid in the cyclic peptide is D-glutamic acid and D-glutamine in viscosin and viscosinamide, respectively. Other compounds with high similarity to viscosin have been isolated recently. One of these compounds, WLIP (white line inducing principle), isolated from *Ps. reactans* (Mortishire-Smith *et al.* 1991), has an antipodal AA-5 amino acid (L-Leu) compared with that (D-Leu) found in viscosin. Recently, a new series of compounds, termed massetolides, was isolated from an unidentified marine strain of *Pseudomonas* sp. (Gerard *et al.* 1997). These compounds also differ from viscosin either in one of the amino acid units, in the fatty acid moiety, or in both. The authors reported that the massetolide-producing strain was often able to incorporate amino acids artificially at various positions, thereby producing new types of massetolides. In some *Pseudomonas* sp. strains, it therefore appears that the peptide synthetases involved in biosynthesis of this class of cyclic depsipeptides (Marahiel *et al.* 1997; von Dören *et al.* 1997) may be partially unspecific in their assembly of amino acids in the cyclic depsipetides. Based on our experiments with excess glutamic acid in the growth medium, however, it seems that the peptide synthetases in *Ps. fluorescens* DR54 were unable to incorporate glutamic acid into the cyclic depsipeptide. Alternatively, the amidation of the glutamic acid to glutamine may also result in viscosinamide rather than viscosin production in *Ps. fluorescens* DR54.

### Viscosinamide production

Viscosinamide is produced by *Ps. fluorescens* DR54 in both complex media and defined media with different C sources (Nielsen *et al.* 1998a). In growth experiments with glucose as the C source, the viscosinamide production per unit of cell

mass increased throughout the logarithmic growth phase, but eventually stopped when the culture entered stationary phase. Hence, the growth experiment with glucose verified that viscosinamide production was tightly coupled to cell proliferation in the logarithmic growth phase. In similar experiments with sucrose as the sole C source, *Ps. fluorescens* DR54 also showed an increasing viscosinamide production parallel to the production of cell mass (Nielsen *et al*. 1998a). In a complex PDB medium (Difco), the viscosinamide produced per unit of cell mass also became constant during growth (Nielsen *et al*. 1998a). In all these experiments, viscosinamide production by *Ps. fluorescens* DR54 thus seemed to be associated with cell proliferation and appeared not to be linked to cessation of growth as the cultures entered stationary phase. However, the actual amount of viscosinamide accumulated per unit of cell mass depended strongly on the specific growth medium (Nielsen *et al*. 1998a).

The results obtained in the cultures with varying C, N or P additions gave no indication of elevated viscosinamide production in the growth phase when the cells experienced the stress conditions of nutrient limitation. This differs from the situation usually encountered in the production of metabolites such as biosurfactants (Desai and Banat 1997) and antibiotics (Shanahan *et al*. 1992a). Viscosinamide may thus be considered to be a primary rather than a secondary metabolite, as has also been observed for other biosurfactant molecules, where production may indeed be linked to cell proliferation and growth rather than starvation or stress conditions (Persson *et al*. 1988; Desai and Banat 1997).

### Viscosinamide characteristics and possible role in the environment

The small differences in the primary chemical structure of viscosinamide and several other cyclic depsipeptides led to a comparison of such properties as solubility, membrane affinity and biosurfactant properties, and antimicrobial action of the compounds. Viscosinamide displayed a low solubility in pure water (below 2 $\mu$mol $l^{-1}$, data not shown) compared with viscosin (9 $\mu$mol $l^{-1}$), but high solubility in methanol. The viscosinamide concentrations in the medium supernatant fluids (Fig. 4a,c,d) were thus higher than the solubility determined in pure water. A likely explanation for a high apparent solubility in the medium supernatant fluids resulted from earlier observations with viscosin, in which *Ps. fluorescens* strain SH10–3B was found to be able to accumulate viscosin in the surrounding medium at concentrations of 450 $\mu$mol $l^{-1}$, far exceeding the theoretical solubility. However, this was probably due to binding of viscosin to acidic polysaccharides excreted in the culture (Laycock *et al*. 1991).

To test the biosurfactant properties of viscosinamide, a simple drop assay (Hildebrand 1989) was used. It was found that both pure viscosinamide and cells of the viscosinamide-producing *Ps. fluorescens* DR54 were able to change the surface tension of sterile distilled water (data not shown). The results were similar, when the tests were carried out with pure viscosin and cells of the viscosin-producing *Ps. fluorescens* strains W44 (Kremer 1994) and SH10–3B (Hildebrand 1989).

Significant accumulations of viscosinamide were indeed found in the cell fraction of *Ps. fluorescens* DR54, and relatively small amounts of the compound were released to the supernatant fluid during the growth experiments. A similar cellular binding has been indicated for viscosin-like compounds (Persson *et al*. 1988) and other biosurfactants (Duvnjak *et al*. 1982). As viscosinamide accumulated in the cell membrane, it seems likely that one of its physiological roles is to act as a biosurfactant, aiding the organism in surface colonization and utilization of surface-bound substrates. It has been suggested that such amphilic properties of biosurfactants enable bacteria to condition both their cell surfaces and their substratum of growth (Neu and Poralla 1990), and it is therefore hypothesized that production of viscosinamide in *Ps. fluorescens* DR54 could be important during surface colonization of plant roots and surfaces of soil particles.

The characteristic features of a biosurfactant may also be involved in membrane binding and inhibition of growth of fungal pathogens (Stanghellini and Miller 1997). Macroscopic observations of viscosinamide-challenged *R. solani* clearly suggested that the plant pathogen was significantly inhibited by the compound (Fig. 3). Similar observations have been made with another plant pathogenic fungus *Pythium ultimum* (data not shown). Microscopic observations showed hyphal swellings when *R. solani* was challenged with either pure viscosinamide or whole cells of the viscosinamide-producing *Ps. fluorescens* DR54 (Thrane *et al*. in press). These observations were similar to those made for viscosin when challenging the plant pathogenic fungus, *Drechslera teres*, where it gave rise to swollen hyphae in germinating conidia (Kremer 1994). Using vital fluorescent stains, it is further proposed that viscosinamide affects the mycelium of the fungus by forming ion channels across the cell membrane (Thrane *et al*. in press). Our results thus demonstrate that viscosinamide exhibits a dual function as both a biosurfactant and an antibiotic, and whether both traits are involved in surface attachment and fungal antagonism on the plant root surface will be the focus of future studies.

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