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Structure, production characteristics and fungal antagonism of tensin – a new antifungal cyclic lipopeptide from *Pseudomonas fluorescens* strain 96.578

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Aim: To study the antagonistic activity by *Pseudomonas fluorescens* strain 96.578 on the plant pathogenic fungus *Rhizoctonia solani*.

Methods and Results: Strain 96.578 produced a new cyclic lipopeptide, tensin. High tensin production per cell was detected in liquid media with glucose, mannitol or glutamate as growth substrate while fructose, sucrose and asparagine supported low production. Tensin production was nearly constant in media with different initial C levels, while low initial N contents reduced production. When applied to sugar beet seeds, strain 96.578 produced tensin during seed germination. When challenged with strain 96.578 or purified tensin, *Rhizoctonia solani* reduced radial mycelium extension but increased branching and rosette formation.

Conclusion: The antagonistic activity of strain 96.578 towards *Rhizoctonia solani* was caused by tensin.

Significance and Impact of the Study: When coated onto sugar beet seeds, tensin production by strain 96.578 could be of significant importance for inhibition of mycelial growth and seed infection by *Rhizoctonia solani*.

INTRODUCTION

The use of antagonistic micro-organisms to control plant-pathogenic microfungi is receiving increasing attention as current legislation restricts the use of synthetic chemical pesticides. Several years of searching for bacterial strains capable of exhibiting biocontrol have resulted in numerous antagonistic isolates, in which the antagonistic activity has been attributed to release of antibiotics (Keel and Défago 1997). Among these, the peptide antibiotics are important in both Gram-positive (Katz and Demain 1977) and Gram-negative bacteria (Dowling and O'Gara 1994). Many peptide antibiotics are amphiphilic molecules with both hydrophilic and hydrophobic properties, due to cyclic peptide and fatty acid moieties (Katz and Demain 1977). Small changes in the chemical structure of the peptide moiety cause drastic changes in antagonistic activity (Trischman *et al*. 1994; Danders *et al*. 1982). Knowledge of the chemical structure of the antibiotic is therefore of importance for understanding the mechanism of antagonistic activity by these antibiotics.

Various bacterial genera produce peptide antibiotics showing antagonistic activity against a wide range of different plant-pathogenic fungi (Katz and Demain 1977; Miller *et al*. 1998) Hence, the inhibition of mycelial growth by viscosinamide (Nielsen *et al*. 1999), fengycin (Vanittanakom and Loeffler 1986) and tolaasin (Hutchison and Johnstone 1993) has been attributed to perturbations of cell membrane structure (Vanittanakom and Loeffler 1986), or to formation of ion channels in the cell wall (Endo *et al*. 1997; Thrane *et al*. 1999). Furthermore, the synthetic peptide antibiotic, pneumocandine, was shown to inhibit 1,3-β-D-glucan synthesis in the ascomycete *Aspergillus fumigatus* (Kurtz *et al*. 1998).

In this paper, a new antifungal cyclic lipopeptide is described isolated from the *Pseudomonas fluorescens* strain 96.578, previously selected for biocontrol against the plant pathogenic fungus *Rhizoctonia solani*. The work describes the production characteristics of the new compound when strain 96.578 was cultured in media with different C sources, and with different initial C or N levels in the

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media. The stress responses of *R. solani* when challenged with strain 96.578 or pure antibiotic are also reported.

MATERIALS AND METHODS

Micro-organisms

Strain 96.578 was isolated from sugar beet rhizosphere soil at Danisco Seed, Holeby, Denmark and tentatively identified as *Pseudomonas fluorescens* biovar VI by a combination of phenotypic tests and phylogenetic 16S rRNA analysis (Nielsen *et al*. 1998a).

The test fungus was strain 92009 of *Rhizoctonia solani* (Anastomosis group 4) isolated from sugar beets exhibiting symptoms of damping-off disease at Danisco Seed, Holeby, Denmark. The strain was maintained at 4 °C and cultured as described by Nielsen *et al*. (1998a).

Antibiotic purification and analysis

For isolation of the antagonistic compound responsible for fungal inhibition, strain 96.578 was cultured on Potato Dextrose Agar (PDA; Difco) plates for 7 days at 20 °C. The agar of 200 plates was homogenized and extracted twice with 600 ml ethyl acetate containing 6 ml 88% formic acid. The solvent was subsequently evaporated by vacuum centrifugation and the residues dissolved in methanol (HPLC grade). The active compound was isolated by fractionation of the eluent after semi-preparative HPLC using a Hewlett Packard Model 1100 with a LiChroCART 250-10 HPLC-Cartridge containing a LiChrosphere 100 RP-18 (10 μm) preparative column (Struers KEBO Lab., Denmark) maintained at 20 °C. Samples of 200 μl were injected and eluted in a gradient increasing from 80% methanol in Milli-Q water to 100% methanol over a 20 min period. The flow rate was 3 ml min^{-1}. Fractions were collected every minute, evaporated by vacuum centrifugation, and each fraction was dissolved in 200 μl pure methanol.

Active fractions, which were identified by a filter inhibition test against *R. solani* (Nielsen *et al*. 1999), were further analysed by HPLC for preliminary characterization. The analytical HPLC was performed using the Hewlett-Packard Model 1100 with a diode array detector and a LiChroCART 250-4 HPLC-Cartridge containing a LiChrosphere 100 RP-18 (5 μm) column (Struers KEBO Lab., Denmark) at 40 °C. Acetonitrile (HPLC grade) and 0·1% phosphoric acid (85%) were mixed in a multistep gradient with 50% acetonitrile increasing to 65% acetonitrile (0–13 min), 65–99% acetonitrile (13–24 min) and 99% acetonitrile (24–26 min). The flow rate was 1 ml min^{-1}. Absorbance was determined at 210 ± 8 nm and chromatograms were analysed using the Hewlett-Packard HPLC 3D Chemstation software (Hewlett-Packard GmbH, Waldbronn, Germany).

Identification of new compounds took place from NMR spectra recorded on Bruker 750 MHz and Varian 400 FT-NMR spectrometers (Varian NMR Instruments, CA, USA) operated at 400·0 and 100·6 MHz to obtain ^{1}H- and ^{13}C-NMR data, respectively. For NMR, samples of the compound were dissolved in DMSO-d_6. ROESY (Rotating frame nuclear Overhauser Enhancement SpectroscopY) data were determined using a mixing time of 200 ms. Further, FAB-MS data were obtained on a JEOL JMS-HX/HX110A tandem mass spectrometer (JEOL Ltd, Tokyo, Japan) and circular dichroism (CD) spectra were measured on a JASCO J-710 spectropolarimeter (JASCO International Co. Ltd, Tokyo, Japan). The structure was confirmed by a single crystal X-ray structural analysis (Henriksen *et al*. 1999).

Antibiotic production in different media

Antibiotic production by *Ps. fluorescens* strain 96.578 at different C and N levels was studied in Davis Minimal Broth (DMB) which contained 30 mmol l^{-1} K_2HPO_4, 14 mmol l^{-1} KH_2PO_4, 0·4 mmol l^{-1} $MgSO_4$, 7·5 mmol l^{-1} $(NH_4)_2SO_4$, 60 mmol l^{-1} C (glucose) and 1 ml of trace elements solution l^{-1} Milli-Q water; 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 l Milli-Q water. In these experiments, pre-cultures of strain 96.578 were grown in 20 ml medium in 100 ml Capsenberg glass flasks placed on a rotary shaker (200 rev min^{-1}) for 2 days at 20 °C. Cells were harvested by centrifugation (7000 *g*, 10 min, 4 °C), washed twice, and resuspended in 0·9% NaCl solution. Inoculation in the subsequent experiments was made at an optical density (O.D.) of 0·1 at 600 nm.

In some experiments, antibiotic production was studied in DMB medium containing 10 mmol l^{-1} glucose, fructose, sucrose, mannitol, glutamate or asparagine. In others, the effect of C limitation was studied in DMB medium containing 15, 30, 60, 120, or 150 mmol l^{-1} C (glucose) and a standard concentration of 7·5 mmol l^{-1} $(NH_4)_2SO_4$ corresponding to 15 mmol l^{-1} N. Finally, the effect of N limitation was studied in DMB medium containing 0·1, 0·5, 1, 2·5, 5, 7·5 or 15 mmol l^{-1} $(NH_4)_2SO_4$ and the standard concentration of 10 mmol l^{-1} glucose corresponding to 60 mmol l^{-1} C. All media were incubated with shaking (150 rev min^{-1}) for 2 d at 20 °C. The cultures were then subsampled for O.D. determinations and analysis of antibiotic accumulation in both cellular and supernatant fractions, as described by Nielsen *et al*. (1999). Concentrations of antibiotic in the two fractions were expressed in μmol l^{-1} of

culture. All experiments were performed twice, taking triplicates at each sampling time.

Supplementing control experiments were performed to determine whether altered pH or oxygen levels during incubation could affect antibiotic production in the cultures. Both pH and oxygen were thus followed in cultures grown to stationary phase in DMB medium with 150 mmol l^{-1} C as glucose. Small subsamples were retrieved in late exponential and early stationary phase from the cultures, and the oxygen measurements were conducted by quickly inserting an oxygen microelectrode into the sample.

Inhibition of the plant-pathogenic fungus *Rhizoctonia solani*

Growth of *R. solani* in the presence of purified tensin was studied on Potato Dextrose Agar (PDA; Difco) or glucose–asparagine–mineral agar (GAsnM; Olsson 1995) with 0·1 g KH_2PO_4, 0·05 g KCl, 0·05 g $MgSO_4.7H_2O$, 0·0024 g Fe(EDTA), 0·18 g asparagine, 3 g glucose and 15 g agar l^{-1} Milli-Q water, pH 6·5. Antagonistic activity of tensin was initially registered by measuring radial growth using *in vitro* plate tests (Nielsen *et al.* 1999). In one set of plates, *Ps. fluorescens* 96.578 was inoculated 10 mm from the edge of an agar plug with fungal mycelium. In another set, a 5 mm Whatman GF/C filter with 21 nmol tensin was placed 10 mm from the edge of such an agar plug. The plates were incubated at 25 °C and inhibition zones were measured after 1 and 2 days. A Nikon Eclipse TE 300 inverted microscope with a high pressure Hg 100 W lamp was used to observe details of hyphal growth patterns.

To determine whether tensin affected total mycelium growth (dry weight production), different amounts of pure antibiotic dissolved in methanol were added to GAsnM liquid media inoculated with *R. solani*. Fungal inoculum was initially grown on a disc of cellophane sheet placed on a PDA plate for 3 days at 25 °C. The fungal mycelium was subsequently scraped off the cellophane sheet and ground in 10 ml 0·33 mol l^{-1} mannitol in a Sorvall Omnimixer (Ivan Sorwall Inc., Norwalk, USA) (Crowe and Olsson 1999). Aliquots of 400 μl inoculum suspension were pipetted into Petri dishes containing 20 ml liquid GAsnM. Pure tensin dissolved in methanol was added to a final concentration of 0, 1·4, 2·8, 4·3, 5·7 and 7·1 μmol l^{-1} (0, 2, 4, 6, 8 and 10 μg ml^{-1}) in the medium. Samples with added methanol were used as controls. The plates were incubated for 3 days before fungal biomass production (dry weight) was determined after drying overnight at 55 °C. Finally, tensin concentration in the GAsnM medium was verified by HPLC analysis of spent medium, using the HPLC protocol described above.

Search for other antibiotics in strain 96.578

In order to determine whether *Ps. fluorescens* 96.578 produced more than one active antibiotic against *R. solani*, cell and supernatant fractions from spent DMB media with either glucose, fructose, sucrose, mannitol, glutamate or asparagine were tested to see if they inhibited the fungus. The cell and supernatant fractions were obtained from 15 ml medium separated by centrifugation (7000 *g*, 10 min, 4 °C) using 2-day-old stationary-phase cultures.

The centrifugation pellet represented the cell fraction and was redissolved in 0·5 ml methanol. Tensin concentration in this extract was determined by HPLC analysis as described above. An aliquot of 100 μl extract was subsequently evaporated to dryness on a 5 mm wide glass fibre filter (GF/C, Whatman). Each filter was then placed on a GAsnM agar plate at a distance of 10 mm from the edge of an agar plug with *R. solani*. The latter was from a 2-day-old fungal mycelium growing on PDA plates. The antagonistic activity was registered by measuring radial growth of the fungus relative to growth towards filters, without added cell fractions, and to filters with known concentrations of purified antibiotic.

Aliquots of 250 μl filter-sterilized (0·2 μm) supernatant fractions were pipetted into 10 mm wide wells in the agar of GAsnM plates, approximately 15 mm from an agar plug with *R. solani* mycelium. Inhibition zones with no or limited radial growth were monitored after 24 h of incubation at 25 °C. For comparison with inhibition by purified tensin, 250 μl samples of DMB medium (without C source) with different amounts of tensin were pipetted into other wells on the GAsnM plates.

Antibiotic production by *Ps. fluorescens* 96.578 inoculated on sugar beet seeds

A simple agar model system was used to determine whether germinating sugar beet seeds inoculated by strain 96.578 were able to sustain antibiotic production. A comparison was made between antibiotic concentrations obtained from extracts of strain 96.578 in pure culture and by sugar beet seeds inoculated with strain 96.578. A minor background signal in the HPLC chromatogram from sugar beet seeds without bacteria was subtracted. To lower the density of indigenous soil bacteria, the sugar beet seeds were washed in hypochlorite solution (5 ml 15% NaOCl, 45 ml sterile water, 500 μl 10% Tween 20), as modified from Miller *et al.* (1989), for 30 min, followed by a wash in sterile water. The bacterial inoculum was grown and harvested as described above and the antibiotic concentration was then determined. Two sets of 18 mineral salt agar plates (0·25 g KH_2PO_4, 0·25 g K_2HPO_4, 0·001 g $ZnSO_4$, 0·001 g $FeSO_4$, 0·001 g $MgCl_2$, 20 g agar l^{-1}) were inocu-

lated with 100 μl of cell suspension and left for 1 h on the bench. One set of the plates (and a control set without bacterial inoculum) received 25 sugar beet seeds per plate. Sterile tweezers were used to gently push the seeds into the agar.

After incubation at 20 °C for 5 days, the total contents of six plates were harvested and bulked as one of the triplicate samples for each treatment. The sample was subsequently extracted twice with 60 ml ethyl acetate solution containing 0·6 ml formic acid. After centrifugation, a known volume of the organic phase was removed. This phase was then evaporated by vacuum centrifugation and dissolved in 800 μl methanol before HPLC analysis as described above.

RESULTS

Structural analysis of a new antibiotic, tensin

A new antibiotic compound was isolated from *Ps. fluorescens* 96.578 and is here proposed to be named 'tensin'. Figure 1 shows the chemical structure, which was inferred from the FABMS pseudomolecular ions at m/z 1409·89 $(M+H)^+$ (1% relative intensity) and 1431·89 $(M+Na)^+$ (0·3% relative intensity) in glycerol matrix, 1431·72 $(M+Na)^+$, 1453·75 $(M\text{-}H+2Na)^+$ in *m*-nitrobenzylic alcohol (NBA) matrix and 1415·93 $(M+li+H)^+$ (17% relative intensity) in a LiCl-containing matrix. These data are compatible with the composition $C_{67}H_{116}N_{12}O_{20}$, representing an exact molecular weight of 1408·84. The data further predict a cyclic lipopeptide composed of a 3-hydroxydecanoyl residue in combination with 11 amino acid residues: five leucine (Leu), one iso-leucine (Ile), one aspartic acid (Asp), one glutamine (Gln), one glutamic acid (Glu), one threonine (Thr) and one serine (Ser). The three-dimensional structure was depicted from an X-ray structure determination of a single crystal of tensin (Henriksen *et al.* 1999).

Tensin production in liquid cultures with different sources and levels of C and N

Figure 2a shows that growth took place on all C sources when *Ps. fluorescens* 96.578 was inoculated into DMB medium with glucose, fructose, sucrose, mannitol, glutamate or asparagine. Strain 96.578 showed high cell yields when cultured with glucose, mannitol and glutamate, while fructose, sucrose and asparagine gave lower yields. Total tensin production varied in the media; C sources providing high cell yields also gave high tensin production. A large majority of the tensin production accumulated in the supernatant fraction, while the cellular fraction in most cases represented only 5% or less of the total. Figure 2b shows that when the tensin released to the supernatant fluid was calculated per unit of cell yield (O.D.), glucose, mannitol and glutamate media again gave the highest values, approximately 20–30 μmol l^{-1} O.D. $unit^{-1}$. This was clearly higher than the

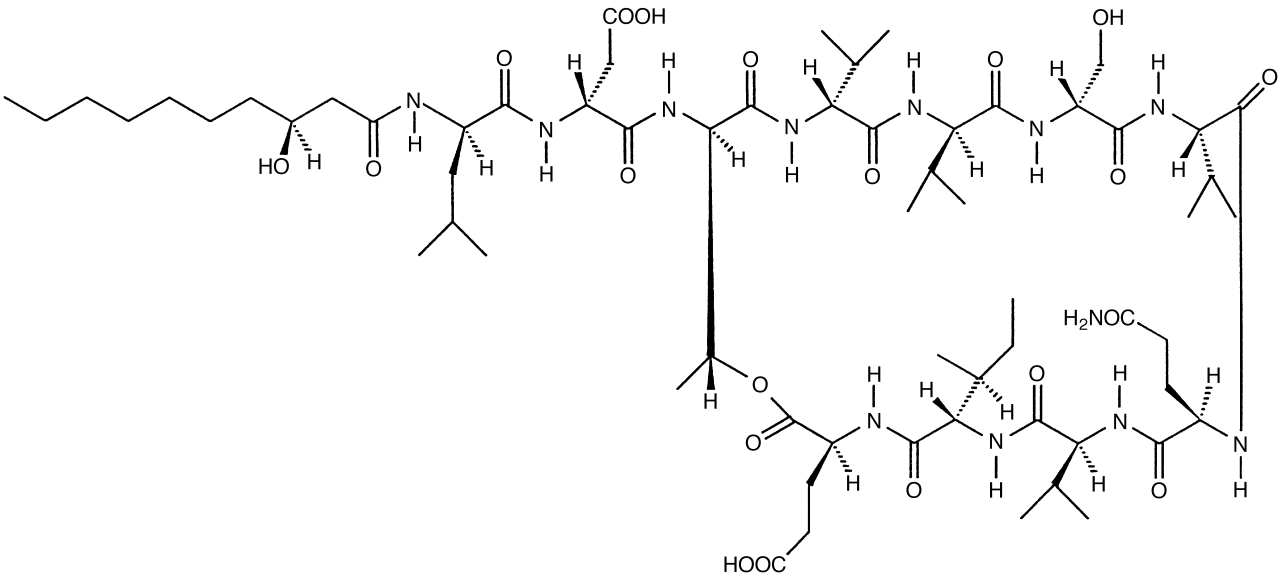

Fig. 1 Chemical structure of tensin

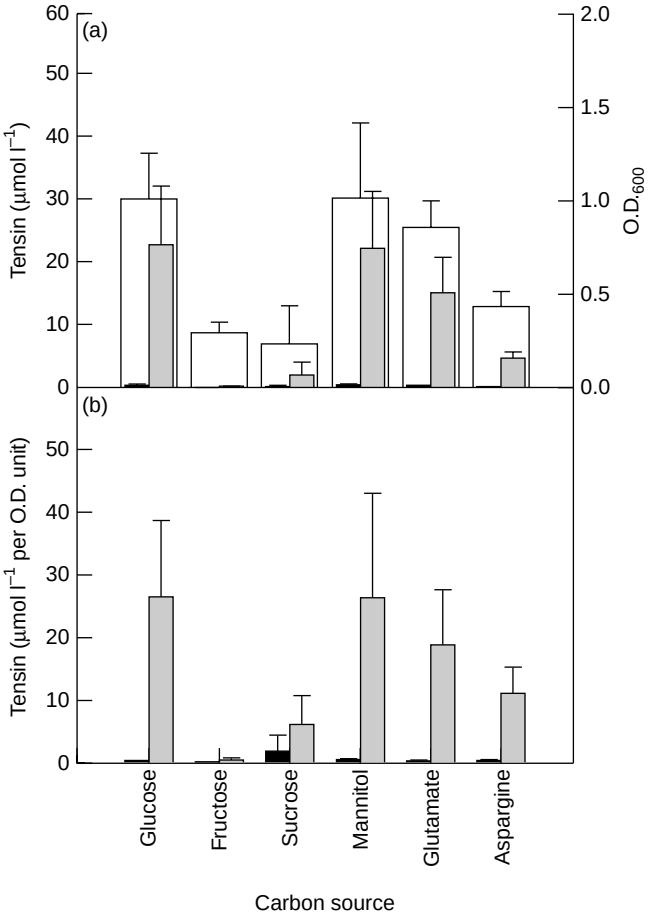

Fig. 2 Tensin production and O.D. (optical density) units of cell mass in batch cultures of *Pseudomonas fluorescens* 96.578 grown in DMB medium with different initial carbon additions (a). Production of tensin per O.D. unit is shown for each initial carbon addition (b). (■), Cells; (▨), supernatant fluid; (□), O.D.

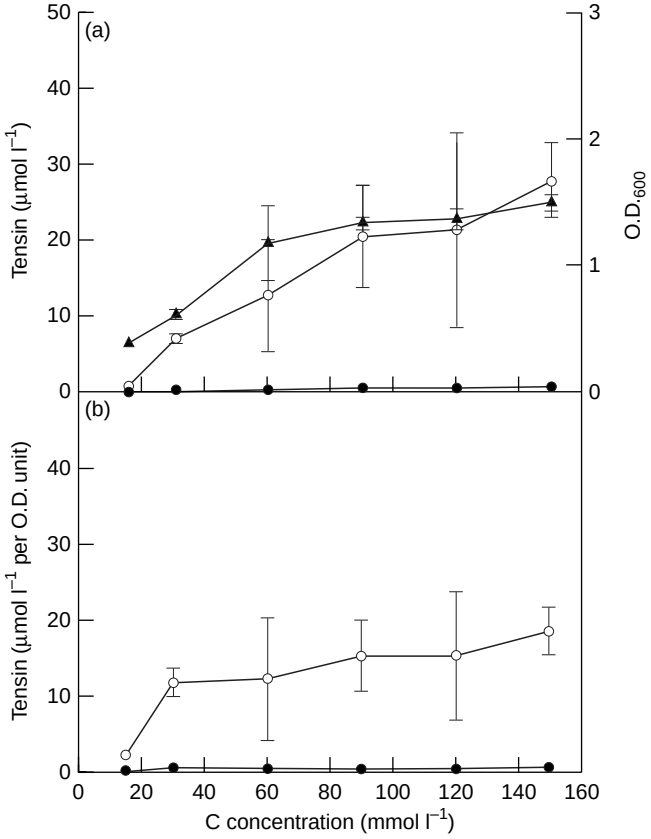

Fig. 3 Tensin production and O.D. (optical density) units of cell mass in batch cultures of *Pseudomonas fluorescens* 96.578 grown in DMB medium with different initial carbon levels (a). Production of tensin per O.D. unit is shown for each initial carbon level (b) ($n = 3 \pm$ S.D.). (●), Cells; (○), supernatant fluid; (▲), O.D.$_{600}$

values of 5–10 μmol l^{-1} O.D. unit^{-1} or less for the other C sources.

Figure 3a shows the tensin release at different initial C concentrations in the medium, where the N concentration was held at 15 mmol l^{-1} N. Generally, the accumulation found in both the supernatant fluid and the cell yield increased as the initial C concentrations increased to approximately 60 mmol l^{-1}; only small increases were observed above this initial C level. On the other hand, as shown in Fig. 3b, the lowest initial C concentrations resulted in relatively lower tensin production when expressed per unit of cell mass (O.D.).

Figure 4a shows comparable results for tensin release at different N concentrations in the glucose medium held at an initial level of 60 mmol l^{-1} C. In these experiments, it was again demonstrated that tensin was mainly released to the supernatant fraction, and that both total tensin production and cell yield generally increased as the initial N concentrations increased to 5–7·5 mmol l^{-1} N; no further increases were observed above this N level. As shown in Fig. 4b, tensin production expressed per unit of cell yield (O.D.) decreased and eventually became very low at the lowest initial N concentrations.

Control experiments conducted using the glucose medium with 150 mmol l^{-1} C verified that altered pH or oxygen levels had no effect on the tensin production in the culture flasks. An initial pH of 7·3 decreased to 6·5 as these cultures entered stationary phase, but control experiments conducted with a wider range of pH values (5·8–7·8) actually demonstrated that tensin production was not affected (data not shown). Oxygen measurements in the cultures

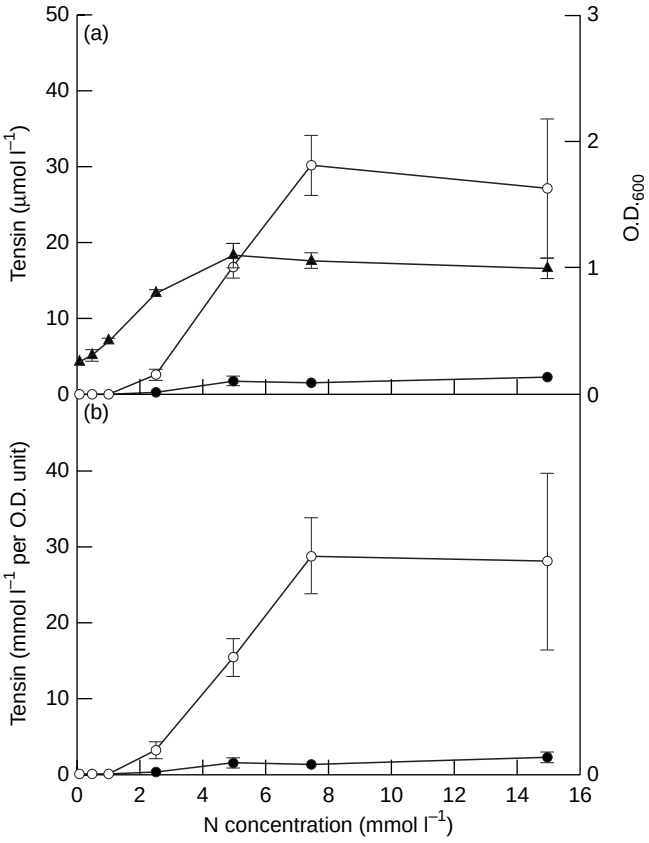

Fig. 4 Tensin production and O.D. (optical density) units of cell mass in batch cultures of *Pseudomonas fluorescens* 96.578 grown in DMB medium with different initial nitrogen levels (a). Production of tensin per O.D. unit is shown for each initial nitrogen level (b) ($n = 3 \pm$ S.D.). (●), Cells; (○), supernatant fluid; (▲), O.D.$_{600}$

further showed that the oxygen tension never decreased to values below 50–65% of air saturation (data not shown), indicating that aerobic conditions were maintained throughout growth and hence, that tensin production was not influenced by low oxygen tensions in the cultures.

Assays of fungal inhibition

Tensin reduced radial growth of *R. solani* towards the antibiotic source and increased branching of hyphae as shown in Fig. 5, using *in vitro* tests of fungal inhibition on PDA plates. The mycelial growth towards a tensin source on a small filter (Fig. 5a, left) and towards a colony of strain 96.578 (Fig. 5a, right) was compared after 2 days of incubation at 25 °C. Similar results were obtained on GAsnM plates (data not shown). After 2 days of incubation or more, inhibition zones remained more distinct (showing sharp edges of inhibited fungal mycelium) around the bacterial colonies than around filters with antibiotics, probably because of the continued production of antibiotic in the colonies.

The early events of hyphal growth after 1 day of incubation were studied in detail by microscopy, as shown in Fig. 5b. Whereas the unchallenged hyphae showed normal tips at the edge of the mycelium (Fig. 5b, left), the tensin-challenged hyphae typically became hyaline and swollen (Fig. 5b, right). A surprising observation was that a few hyphae of the tensin-challenged mycelium were able to grow across the inhibition zone and directly into the tensin source in filters (Fig. 5c, left) or in bacterial colonies (Fig. 5c, right). As also shown in Fig. 5c, these hyphae formed small rosettes of highly branched mycelium.

To determine whether tensin affected total mycelium production (biomass production), dry weight determinations were made on mycelia growing in liquid media at different tensin concentrations. In such cultures, the control mycelium grew and branched normally over the whole surface of the medium, while the tensin-challenged mycelium showed a reduced outgrowth on the medium surface, but formed a dense mycelium with extensive branching and dark coloration. Surprisingly, the biomass production was significantly higher ($P \leq 0{\cdot}03$) in the tensin-containing medium than in the control samples (data not shown). HPLC analysis of the growth media further showed that the tensin concentrations did not change during the incubation period (data not shown).

Absence of other active metabolites in *Ps. fluorescens* 96.578

When screening for antagonistic activity against *R. solani* in the preparative HPLC fractions, it was evident that only fractions containing tensin were active (data not shown). This did not exclude, however, the production of other antagonistic compounds (non-extractable by ethyl acetate) by strain 96.578. To test this possibility, *in vitro* inhibition studies were conducted with cell fractions dissolved in methanol or filter-sterilized supernatant fluid. Cell fractions deposited in filters on GAsnM agar plates showed detectable, but low inhibition of *R. solani* as measured by radial growth towards the filters. However, filter-sterilized supernatant fluids gave inhibition zones corresponding to those expected from the tensin concentrations in the supernatant fluids and from comparisons with standards of pure tensin (data not shown). This suggested that filter-sterilized supernatant fluids did not contain additional active metabolites which could interfere with fungal growth in the inhibition tests for tensin.

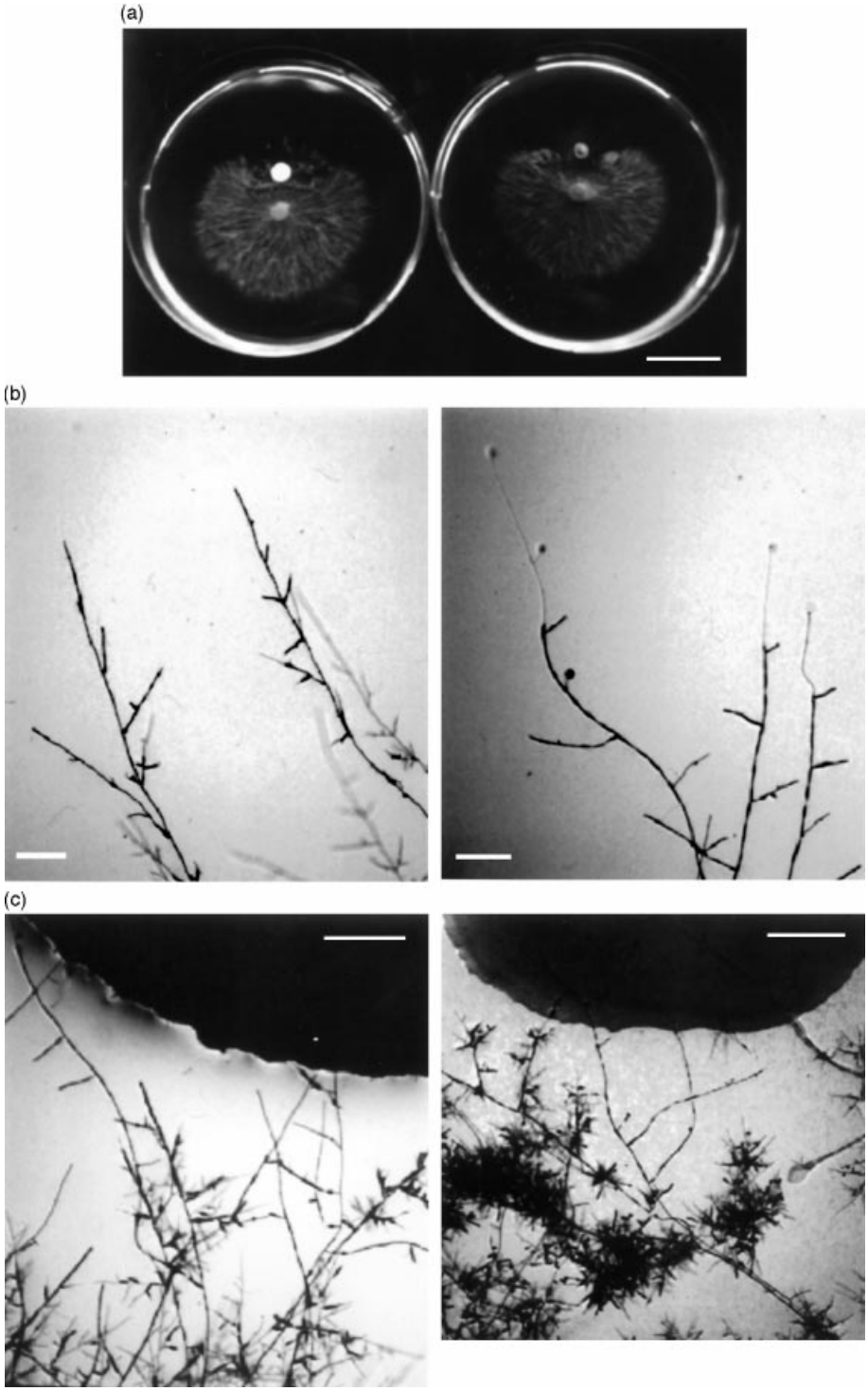

Fig. 5 Photographs of *Rhizoctonia solani* grown on PDA plates at 25 °C for 2 days, as challenged with purified tensin added to a sterile glass fibre filter 30 μg per filter (5a left) or cells of *Pseudomonas fluorescens* 96.578 (5a, right). Unchallenged hyphal tips were relatively straight after 1 day of incubation (5b, left) while challenged hyphal tips were swollen and apparently dead (5b, right). After 2 days of incubation, increased branching and rosette formation were observed for most hyphae challenged with purified tensin (5c, left) or *Pseudomonas fluorescens* 96.578 cells (5c, right)

Tensin production by strain 96.578 when incubated on sugar beet seeds

The ability of *Ps. fluorescens* 96.578 to produce tensin when the strain was cultured on mineral salt agar with and without sugar beet seeds was tested. The tensin production in agar medium with and without seeds was 2·0 and 0·1 nmol l^{-1} tensin per agar plate, respectively, indicating that unknown compounds associated with the sugar beet seeds stimulated tensin production strongly. However, the nature of these compounds was not further investigated in this study.

DISCUSSION

Tensin structure

Several cyclic lipodepsipeptides, all containing a 3-hydroxy fatty acyl residue attached to an *N*-terminal group in a ring structure of eight to nine amino acids, have been isolated from *Pseudomonas* spp. The list of compounds now includes a number of nonapeptides such as the eight different massetolides from an unidentified *Pseudomonas* sp. (Gerard *et al*. 1997), the WLIP (white line inducing principle) compound from *Ps. reactans* (Mortishire-Smith *et al*. 1991), viscosin from *Ps. viscosa* (Laycock *et al*. 1991) and viscosinamide from *Ps. fluorescens* (Nielsen *et al*. 1999). The latter was isolated in this laboratory in a recent search for new potential biocontrol agents among *Pseudomonas* spp. from sugar beet rhizospheres (Nielsen *et al*. 1998a). It was found that the compound had both antibiotic and biosurfactant properties, and several studies have already been undertaken to test the efficacy of the producing strain, *Ps. fluorescens* DR54, in biological control (Nielsen *et al*. 1998a; Thrane *et al*. 2000).

In the present study, a new cyclic lipopeptide, tensin, was isolated from *Ps. fluorescens* 96.758. The X-ray crystallographic analysis reveals that the structure (Henriksen *et al*. 1999) is similar to the cyclic peptides isolated from both Gram-positive and Gram-negative bacteria, e.g. fengycin from *Bacillus cereus* (Vanittanakom and Loeffler 1986), surfactin from *B. subtilis* (Arima *et al*. 1968) and WLIP from *Ps. reactans* (Mortishire-Smith *et al*. 1991).

Tensin production on different media

Tensin production was observed on all media when *Ps. fluorescens* 96.578 was cultured with a number of different carbohydrates and amino acids as C sources. However, the tensin production per O.D. unit was quite different on the different media, showing the highest yields of tensin production (tensin produced per biomass unit) on media supporting high growth rates and high maximum yields of cells (glucose, mannitol and glutamic acid). Similar observations were made for viscosinamide production by *Ps. fluorescens* DR54 using the same media (Nielsen *et al*. 1998b).

Further attempts to characterize tensin production by *Ps. fluorescens* 96.578 included the comparison of tensin yields under C- or N-limiting conditions in the growth medium. By varying the initial C and N concentrations in the medium, it was evident that both total growth yields (maximum O.D. values) and specific yields of tensin (tensin produced per O.D. unit) of strain 96.578 decreased at C levels below approximately 30 mmol l^{-1} C and at N levels below approximately 7 mmol l^{-1} N. Using the same range of initial C and N levels in the media (Nielsen *et al*. 1999), viscosinamide production by *Ps. fluorescens* DR54 showed similar trends, although viscosinamide was mainly associated with the cell fraction and not the supernatant fluid. In the present experiments, there were no indications that nutrient limitations by C and N resulted in elevated tensin production; similar results were observed for viscosinamide production (Nielsen *et al*. 1999). Comparison of the previous work on viscosinamide (Nielsen *et al*. 1999) and the present study on tensin production (Figs 3 and 4) thus suggested that production of the cyclic lipopeptides may be completely suppressed during growth at very low C levels (15 mmol l^{-1} C or less) and at very low N levels (1 mmol l^{-1} N or less). This demonstrated that lipopeptide production in these strains is under metabolic control during cell proliferation.

Antifungal effect of tensin

When screening for antagonistic activity in the culture extracts separated by preparative HPLC fractionation, and in spent media, it was evident that the activity against *R. solani* was most likely only due to the tensin compound. It should be noted, however, that *Ps. fluorescens* strain 96.578 also releases chitinolytic enzymes (Nielsen and Sørensen 1999), which may contribute to growth inhibition of *R. solani*. As the basidiomycete fungus has a high chitin content cell wall, a possible interaction between tensin and chitinolytic enzymes in the antagonism should be further investigated. The possibility that tensin acted in synergism with cell wall-degrading enzymes could not be excluded from this study.

The changes in mycelial growth patterns observed in the tensin-challenged *R. solani* were comparable to observations made with other cyclic lipopeptide compounds. Notably, the increased branching and darkening of the mycelium resembled the response of *Aspergillus fumigatus* when challenged with synthetic pneumocandine lipopeptide (Kurtz *et al*. 1998) inhibiting 1,3-β-D-glucan synthase and fungal wall synthesis. Hyaline and balloon-shaped tips of affected hyphae further demonstrated the growth-inhibiting effect

of tensin. However, further studies are needed to clarify whether inhibition of cell wall synthesis at the hyphal tips is responsible for the observed anti-fungal effect of tensin.

The recently described viscosinamide also had biosurfactant properties (Nielsen *et al.* 1999) and showed a dramatic effect on the mycelial growth pattern in *R. solani* (Thrane *et al.* 1999). Increased branching was also observed, but without dark coloration. Rather than hyaline and balloon-shaped tips, the hyphal ends were more swollen in the case of viscosinamide. It was suggested that viscosinamide inhibited *R. solani* by forming ion channels in the cell membrane, thus disturbing the intracellular Ca^{2+} level (Thrane *et al.* 1999). The three-dimensional structure of tensin revealed both hydrophobic and hydrophilic properties (Henriksen *et al.* 1999), suggesting that tensin may interact with the cell membrane of *R. solani*. However, the different morphological changes observed in *R. solani* by treatments with viscosinamide and tensin suggested that the two cyclic lipopeptides affected the fungus by different inhibitory mechanisms.

Ps. fluorescens 96.578 as a seed inoculant in biological control

In the present study, a new cyclic lipopeptide, tensin, has been described from *Ps. fluorescens* strain 96.578 showing promising features for biological control of plant pathogenic microfungi. Further, the compound shows little binding to the producing cells and is thus easily released to the environment. Tensin production appears to be tightly coupled to metabolism and cell proliferation in strain 96.578. Several laboratory media thus supported tensin production, and a strongly stimulated production of the compound was also observed when strain 96.578 was grown in the proximity of sugar beet seeds. When *R. solani* was challenged with tensin, mycelia and hyphal growth showed stress symptoms, which may be important for the antagonistic activity of strain 96.578. The production of tensin on several media and on germinating seeds is promising for production of the compound *in situ* when strain 96.578 is coated on sugar beet seeds and established in the rhizosphere.

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REFERENCES

Arima, K., Kakinuma, A. and Tamura, G. (1968) Surfactin, a crystaline peptidolipid surfactant produced by *Bacillus subtilis*: isolation, characterization and its inhibition of fibrin clot formation. *Biochemical and Biophysical Research Communications* **31**, 488–494.

Crowe, J. and Olsson, S. (1999) Induction of laccase activity in *Rhizoctonia solani* by antagonistic *Pseudomonas fluorescens* strains and a range of chemical treatments. *Applied and Environmental Microbiology* Submitted.

Danders, W., Marahiel, A., Krause, M. et al. (1982) Antibacterial action of gramicidin S and tyrocidines in relation to active transport, *in vitro* transcription, and spore outgrowth. *Antimicrobial Agents and Chemotherapy* **22**, 785–790.

Dowling, D.N. and O'gara, F. (1994) Metabolites of *Pseudomonas* involved in the biocontrol of plant disease. *TIBTECH* **12**, 133–141.

Endo, M., Takesako, K., Kato, I. and Yamaguchi, H. (1997) Fungicidal action of aureobasidin A, a cyclic depsipeptide antifungal antibiotic, against *Saccharomyces cerevisiae*. *Antimicrobial Agents and Chemotherapy* **41**, 672–676.

Gerard, J., Lloyd, R., Barsby, T., Haden, P., Kelly, M.T. and Andersen, R.J. (1997) Massetolides A-H, antimycobacterial cyclic depsipeptides produced by two pseudomonads isolated from marine habitats. *Journal of Natural Products* **60**, 223–229.

Henriksen, A., Anthoni, U., Nielsen, T.H., Sørensen, J., Christophersen, C. and Gajhede, M. (1999) Cyclic lipoundecapeptide Tensin from *Pseudomonas fluorescens* strain 96.578. *Acta Crystallographic Section C.* 55.

Hutchison, M.L. and Johnstone, K. (1993) Evidence for the involvement of the surface active properties of the extracellular toxin tolaasin in the manifestation of brown blotch disease symptoms by *Pseudomonas tolaasii* on *Agaricus bisporus*. *Physiological and Molecular Plant Pathology* **42**, 373–384.

Katz, E. and Demain, A.L. (1977) The peptide antibiotics of *Bacillus*: Chemistry, Biogenesis and possible functions. *Bacteriological Reviews* **41**, 449–474.

Keel, C. and Défago, G. (1997) Interactions between beneficial soil bacteria and root pathogens: mechanisms and ecological impact. In *Multitropic Interactions in Terrestrial Systems* ed. Gange, A.C. and Brown, V.K. pp. 27–46. London: Blackwell Scientific.

Kurtz, M.B., Heat, I.B., Marrinan, J., Dreikorn, S., Onishi, J. and Douglas, C. (1998) Morphological effects of lipopeptides against *Aspergillus fumigatus* correlate with activities against (1,3)-β-D-glucan synthase. *Antimicrobial Agents and Chemotherapy* **38**, 1480–1489.

Laycock, M.V., Hildebrand, P.D., Thibault, P., Walter, J.A. and Wright, J.-L.C. (1991) Viscosin, a potent peptidolipid biosurfactant and phytopathogenic mediator produced by a pectolytic strain of *Pseudomonas fluorescens*. *Journal of Agricultural and Food Chemistry* **39**, 483–489.

Miller, C.M., Miller, R.V., Garton-Kenny, D. et al. (1998) Ecomycins, unique antimycotics from *Pseudomonas viridiflava*. *Journal of Applied Microbiology* **84**, 937–944.

Miller, H.J., Henken, G. and Van Veen, J.A. (1989) Variation and composition of bacterial populations in the rhizospheres of

maize, wheat and grass cultivars. *Canadian Journal of Microbiology* **35**, 656–660.

Mortishire-Smith, R.J., Nutkins, J.C., Packman, L.C. et al. (1991) Determination of the structure of an extracellular peptide produced by the mushroom saprotroph *Pseudomonas reactans*. *Tetrahedron* **47**, 3645–3654.

Nielsen, M.N. and Sørensen, J. (1999) Chitinolytic activity of *Pseudomonas fluorescens* isolates from barley and sugar beet rhizosphere. *FEMS Microbiology Ecology* **30**, 217–227.

Nielsen, M.N., Sørensen, J., Fels, J. and Pedersen, H.C. (1998a) Secondary metabolite- and endochitinase-dependent antagonism toward plant-pathogenic microfungi of *Pseudomonas fluorescens* isolates from sugar beet rhizosphere. *Applied and Environmental Microbiology* **64**, 3563–3569.

Nielsen, T.H., Christophersen, C., Anthoni, U. and Sørensen, J. (1998b) A promising new metabolite from *Pseudomonas fluorescens* for biocontrol of *Pythium ultimum* and *Rhizoctonia solani*. In *Molecular Approaches in Biological Control* ed. Duffy, B.K., Rosenberger, U. and Douglas, C. Bulletin OILB srop **21**, pp. 51–56. International Organization for Biological and Integrated Control of Noxious Animals and Plants, West Palaeartic Regional Section (IOBC/WPRS).

Nielsen, T.H., Christophersen, C., Anthoni, U. and Sørensen, J. (1999) Viscosinamide, a new cyclic depsipeptide with surfactant and antifungal properties produced by *Pseudomonas fluorescens* DR54. *Journal of Applied Microbiology* **86**, 80–90.

Olsson, S. (1995) Mycelial density profiles of fungi on heterogenous media and their interpretation in terms of nutrient reallocation patterns. *Mycological Research* **99**, 143–153.

Thrane, C., Nielsen, T.H., Nielsen, M.N., Olsson, S. and Sørensen, J. (2000) Viscosinamide-producing *Pseudomonas fluorescens* DR54 exerts a biocontrol effect on *Pythium ultimum* in sugar beet rhizosphere. *FEMS Microbiology Ecology* **1151**, 1–8.

Thrane, C., Olson, S., Nielsen, T.H. and Sørensen, J. (1999) The use of vital fluorescent probes for the detection of stress in the fungi *Pythium ultimum* and *Rhizoctonia solani* challenged with viscosinamide from *Pseudomonas fluorescens* DR54. *FEMS Microbiology Ecology* **30**, 11–23.

Trischman, J.A., Jensen, P.R. and Fenical, W. (1994) Halobacillin: a cytotoxic cyclic acylpeptide of the iturin class produced by a marine *Bacillus*. *Tetrahedron Letters* **35**, 5571–5574.

Vanittanakom, N. and Loeffler, W. (1986) Fengycin — A novel antifungal lipopeptide antibiotic produced by *Bacillus subtilis* F-29–3. *Journal of Antibiotics* **39**, 888–901.