Journal of Applied Microbiology 2003, **95,** 1143–1151 doi:10.1046/j.1365-2672.2003.02097.x

Antimicrobial activity of *Paenibacillus peoriae* strain NRRL BD-62 against a broad spectrum of phytopathogenic bacteria and fungi

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2003/0119: received 12 February 2003, revised 18 June 2003 and accepted 6 August 2003

ABSTRACT

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Aims: To investigate the potential antagonistic activity of *Paenibacillus peoriae* strain NRRL BD-62 against phytopathogenic micro-organisms and to determine the physiological and biochemical characteristics of the antimicrobial compound produced by this strain.

Methods and Results: Strain NRRL BD-62 showed a broad inhibition spectrum with activity against various phytopathogenic bacteria and fungi. Physico-chemical characterization of the antimicrobial activity showed that it was stable during heat treatment and was retained even after autoclave at 121°C for 10 min. The compound was also stable after the treatment with organic solvents, hydrolytic enzymes and its activity was preserved at a wide range of pH. The partial purification carried out by Sephadex G25 gel filtration showed two profiles of inhibition against the indicator strains tested, suggesting at least two different substances with distinct molecular weight.

Conclusions, Significance and Impact of the Study: This is the first report on the production of antimicrobial substances in *P. peoriae*. Besides the antimicrobial inhibition capability, the strain NRRL BD-62 is also able to effectively fix molecular nitrogen, and produce chitinases and proteases as well, suggesting that further studies should be addressed to use *P. peoriae* strain NRRL BD-62 as a plant growth promoter and/or as a biocontrol agent in field experiments.

Keywords: antimicrobial substance, biocontrol agent, chitinase, *Paenibacillus peoriae*, protease.

INTRODUCTION

Biological control, the use of one living organism to eliminate or control another, has received considerable attention over the last decade as an alternative to the use of chemical bactericides and/or fungicides. It has stemmed from increasing public concern over the use of chemicals in the environment in general, as well as a reduction in the availability of previously widely used, effective substances. The potential of biocontrol of soil-borne plant pathogens by antagonistic micro-organisms offers a nonpolluting complement, or alternative, to existing disease management strategies that depend basically in chemical pesticides.

Various mechanisms have been reported to explain or contribute to biocontrol activity and consequently, the pathogen suppression. Competition in the rhizosphere for nutrients and preferred colonization sites have been proposed as mechanisms of disease control with biological agents (Paulitz 1990). The production of iron-chelating siderophores by pseudomonads has also been shown to play a role in disease abolition for certain pathogens by intensifying competition for iron (O'Sullivan and O'Gara 1992). It has been shown that the production of antibiotic

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metabolites, such as phenazine carboxylic acid (Thomashow and Weller 1988) and 2,4-diacetylphloroglucinol (Vincent *et al.* 1991) can be involved in the elimination of fungal pathogens. In the same way, some works demonstrated that cell wall degrading enzymes such as β-1,3-glucanases, cellulases, proteases and chitinases could be involved in antagonism towards phytopathogenic fungi (Chernin *et al.* 1995; Dunn *et al.* 1997; Jijakli and Lepoivre 1998; Budi *et al.* 2000).

Paenibacillus peoriae (previously *Bacillus peoriae*) was first isolated from soil (Montefusco *et al.* 1993). However, no data concerning the role of this species in the rhizosphere have been reported. Previous studies indicated a close relationship between *P. peoriae*, *P. polymyxa* and *P. brasilensis*, also a soil-inhabiting bacterium (von der Weid *et al.* 2002). Several *Bacillus* and related genera have been tested as potential biological control agents as they produce a range of antibiotics and form resistant spores (Edwards *et al.* 1994). In the same context, several works have shown that strains of *P. polymyxa* can produce different antimicrobial substances (Rosado and Seldin 1993; Piuri *et al.* 1998; Dijksterhuis *et al.* 1999; Seldin *et al.* 1999). Also in this genus, lytic enzymes such as proteases and chitinases could have a role in the interaction between antagonistic micro-organisms and soil-borne plant pathogens, as shown by Budi *et al.* (2000).

Considering that *in vitro* studies of antagonism are generally the preliminary studies in most biocontrol work and also the high phylogenetic similarity among *P. peoriae* and *P. polymyxa*, the aims of the present study were to investigate the potential antagonistic activity of *P. peoriae* against phytopathogenic micro-organisms, and to describe the inhibitory spectrum, the biochemical characteristics and the production conditions for the antimicrobial compound produced by *P. peoriae* strain NRRL BD-62. This is the first report on the production of antimicrobial substances in *P. peoriae*.

MATERIALS AND METHODS

Micro-organisms and culture conditions

The strain NRRL BD-62 of *P. peoriae* was originally described as belonging to the genus *Bacillus* (Montefusco *et al.* 1993) and then reclassified by Heyndrickx *et al.* (1996). The glucose broth (GB; Seldin *et al.* 1983) was used as growth medium, unless otherwise stated. Cells were incubated at 30°C without agitation for 18 h. Working cultures were kept on GB agar plates at 4°C, while long-term storage was followed in GB slants supplemented with 1% (wt/vol) $CaCO_3$ (Seldin *et al.* 1983). Other bacterial and fungal strains together with their sources and growth conditions are listed in Table 1. Bacterial strains were kept frozen with 20% (vol/vol) glycerol at −20°C, while fungal strains were stored at 4°C in 0·1× trypticase soy broth (TSB) plates containing 0·2% sucrose.

Antimicrobial activity assays and susceptibility range

Two assays, the overlay method and the agar-well diffusion method, were used to detect antimicrobial activity. The overlay method was performed as described by Rosado and Seldin (1993). *Paenibacillus peoriae* strain NRRL BD-62 was spot-inoculated (5 μl) on the surface of agar plates containing different media [GB, TBN (thiamine/biotin/nitrogen; Seldin and Penido 1986), TSB and 0·1× TSB with 0·2% sucrose] and, after incubation at 30°C for 48 h, it was killed by exposure to chloroform vapours for 15 min. These plates were then flooded with the indicator strains (*Micrococcus* sp. and *Agrobacterium tumefaciens*) and clear zones of inhibition around the spot inoculum indicated antimicrobial production. For the agar-well diffusion assay, plates were also prepared with both indicator strains spread on the surface of the agar. Wells, with a diameter of 5 mm, were then cut in the agar using a sterile loop. Then, 50 μl of NRRL BD-62 culture supernatant prepared as described below were added to the wells and allowed to diffuse into the agar, followed by incubation at 30°C for 48 h. The strains listed in Table 1 were tested by either the overlay method, containing spot-culture grown in 0·1× TSB with 0·2% sucrose, or the agar-well diffusion method, using 10-fold-concentrated supernatant of 96-h culture of NRRL BD-62 also grown in 0·1× TSB plus sucrose. Diameters of inhibition zones were scored for Gram-positive bacterium, Gram-negative bacteria and fungal strains as follows: (−) no inhibition, (+) weak inhibition of the tested strain around the spot or well (clear zones of inhibition <7 mm), (++) moderate inhibition (clear zones between 7 and 12 mm) and (+++) strong inhibition of the tested strain around the spot or well (large and clear zones bigger than 12 mm).

Influence of growth media on the production of secreted antimicrobial activity

The culture supernatant was prepared in five different media: GB, TBN, TSB, 0·1× TSB with 0·2% sucrose and a chemically defined medium [DM; glucose (10 g l^{-1}), $(NH_4)_2SO_4$ (1 g l^{-1}), NaCl (2 g l^{-1}), micronutrients solution (1 ml l^{-1}; Jurgensen and Davey 1971), $MgSO_4 \cdot 7H_2O$ (0·25 g l^{-1}), L-arginine (0·7 g l^{-1}), L-glutamine (0·1 g l^{-1}), biotin (1 mg l^{-1}), thiamine (1 mg l^{-1}) and 0·08% phosphate buffer (K_2HPO_4), pH 7·2]. After incubation of the culture of strain NRRL BD-62 in those different media for 96 h, the cultures were then centrifuged (15 000 *g*, 20 min) and the supernatants (crude preparations) were filtered in 0·22-μm pore filter (Millipore). Then, the supernatants were

Table 1 Antagonistic activity of *Paenibacillus peoriae* NRRL BD-62 against different phytopathogenic micro-organisms

Strains	Code	Origin and relevant characteristics*	Growth conditions†	Inhibition profile‡
Bacteria				
Micrococcus sp.	LMG2	(I)	a	+++
Agrobacterium tumefaciens	IPO 1508	(II)	b	++
Escherichia coli	DH5α	(I)	a	++
Ralstonia solacenarum	1609	(II)	b	++
Erwinea carotovora subsp. *carotovora*	IPO161	(II)	a	++
Burkholderia cepacia	P2	(II)	b	+
Fungi				
Fusarium moliniforme		(III); Isolated from maize seeds	b	+++
Fusarium oxysporum		(I)	b	++
Diplodia macrospora	1	(III); Isolated from maize grains	b	+++
Diplodia macrospora	2	(III); Isolated from maize	b	++
Diplodia maydis		(III); Isolated from maize	b	++
Cephalosporium acremonium		(III)	b	+++
Penicillium corylophilum	4211	(IV)	b	+++
Verticillium dahliae	PD79/116	(III)	b	++
Rhizoctonia solani	IPO4R22	(II)	b	–
Aspergillus parasiticus		(I)	b	–
Colletotrichum gloeosporioides	2	(III); Isolated from papaya	b	++
Colletotrichum graminicola		(III); Isolated from maize	b	+++
Colletotrichum graminicola	13E	(III); Isolated from sorghum	b	++
Colletotrichum graminicola	31A	(III); Isolated from sorghum	b	++

*(I) strains provided by members of Instituto de Microbiologia, UFRJ, Brazil; (II) strains provided by members of Plant Research Institute, Wageningen, The Netherlands; (III) strains provided by members of EMBRAPA/CNPMS; (IV) strain from the Culture Collection of FIOCRUZ, Rio de Janeiro, Brazil.

†a, strains were grown in LB medium at 32°C; b, grown in 0·1× TSB supplemented with 0·2% sucrose at 32°C.

‡Activity was scored after three independent experiments using the agar-well diffusion assay and the overlay method containing spot-culture grown in 0·1× TSB with 0·2% sucrose, as follows: (–) no suppression, (+) weak suppression (with narrow clear zones around bacterial spots), (++) medium suppression or (+++) strong suppression (with large, clear zones around bacterial spots).

lyophilized and resuspended (to a 10-fold-concentration) in PBS (150 mmol l^{-1} NaCl, 20 mmol l^{-1} phosphate buffer, pH 7·2).

Influence of temperature, pH and aeration on the production of antimicrobial compound

In these set of experiments, we used the defined medium developed in this study to grow NRRL BD-62 for 96 h. Different temperatures (30 and 37°C), pH (6–8) and aeration conditions (still or shaking cultures, 100 rev min^{-1}) were tested for the determination of the best conditions for the production of the antimicrobial compound. *Micrococcus* sp. and *Ag. tumefaciens* were used as indicator strains.

Effects of proteolytic enzymes, organic solvents, heat and pH on the secreted antimicrobial activity

Crude preparations (100 μl of 10-fold-concentrated supernatant obtained after growth of strain NRRL BD-62 in DM) were subjected to treatments with proteolytic enzymes (0·5 mg ml^{-1} of pronase E, proteinase K and trypsin) and organic solvents (acetone, methanol, ethanol and chloroform – all at 10% v/v) for 4 h at 37°C. The supernatant fluids were also subjected to treatments with 2·5 mmol l^{-1} dithiothreitol (DTT), 3 mol l^{-1} urea, 0·05% Tween-80 and RNase (5 mg ml^{-1}) for 4 h at 37°C. For heat treatment, the preparation was heated to either 42, 65, 100°C for 1 h or autoclaved at 121°C for 10 min. The pH effect was investigated by adjusting the pH values from 3·2 to 9·6. Antimicrobial activity was determined before and after different treatments using the agar-well diffusion assay.

Antimicrobial activity and sporulation

Antimicrobial activity was monitored during the growth cycle of *P. peoriae* NRRL BD-62 in DM. Viable counts and spores (heat-treated counts after 80°C for 10 min) were recorded and supernatant fluids were lyophilized, 10-fold concentrated and tested for activity on indicator bacterial

lawns. Activity was determined by the highest dilution giving a halo. Arbitrary units (AU) per millilitre were defined as the result of: (reciprocal of the greatest dilution of the supernatant that showed a zone of inhibition)/(volume of supernatant applied on the spot) × 1000.

Mode of action

An aliquot of 10 μl of the indicator bacterial suspensions (about 3×10^8 CFU ml^{-1}) was co-inoculated with 40, 80 or 400 AU ml^{-1} of crude preparations obtained after the growth of NRRL BD-62 in defined medium. At various times, these cultures were streaked onto an agar plate without the antimicrobial compound to observe a bacteriostatic or a bacteriocidal effect on the indicator strains tested.

Supernatant partition

The 10-fold-concentrated supernatant was submitted to ultrafiltration in a 10 kDa molecular weight cut-off Centricon microconcentrator (Amicon, Bedford, MA, USA). Two distinct fractions (one bigger than 10 kDa and other smaller or equal to 10 kDa) were obtained after this process. Both fractions were treated with an equal volume of SDS–PAGE sample buffer (125 mmol l^{-1} Tris–HCl, pH 6·8; 4% SDS; 20% glycerol; 0·002% bromophenol blue) as described by Santos *et al.* (1999). These fractions were then tested for protein content, chitinolytic and proteolytic activities, as described below.

Protein analysis

Samples containing 20 μl of each fraction (50 μg of proteins) were mixed with 10% 2-β-mercaptoethanol, followed by heating at 100°C for 5 min. Proteins were analysed in 10 or 20% SDS–PAGE by the method described by Laemmli (1970). Electrophoresis was carried out at 4°C at 100 V, and the gels were silver stained. Molecular masses of sample polypeptides were calculated from mobility of Gibco BRL (Grand Island, NY, USA) molecular weight standards.

Chitinase activity

For the detection of chitinase activity, electrophoresis was performed according to Trudel and Asselin (1989) in a 10 or 20% SDS–PAGE containing 0·01% glycol chitin. Supernatant samples (50 μg) were incubated for 24 h in 50 mmol l^{-1} sodium acetate buffer, pH 5·5 at 37°C to look for chitinase activity. Lytic zones were revealed by incubation of the gel in a freshly prepared 0·001% calcofluor white solution in 50 mmol l^{-1} Tris–HCl, pH 8·9. The calcofluor solution was removed and the gel was incubated for about 1 h at room temperature in distilled water. The chitinolytic activity was observed under u.v. light.

Protease activity

Protease was assayed by electrophoresis on 10 or 20% SDS–PAGE with 0·1% gelatin as substrate incorporated into the gel (Santos *et al.* 1999). Supernatant samples (50 μg) were incubated for 48 h at 37°C in 50 mmol l^{-1} sodium phosphate buffer, pH 5·5, supplemented with 2 mmol l^{-1} DTT to detect the proteolytic activity. The gel was stained for 2 h with 0·2% Coomassie brilliant blue R in methanol–acetic acid–water (50 : 10 : 40) and destained in the same solvent.

Preliminary purification

The cell culture supernatant (cells grown in DM), filtered with a 0·22-μm membrane (Millipore), was also submitted to Amicon Diaflo membrane with a cut-off molecular weight of 10 kDa. The sample obtained (≤10 kDa) was collected and lyophilized. This fraction was applied in 5-ml (20 mg of protein) aliquots to a Sephadex G25 (Pharmacia Biotech, Cambridge, UK) column (2 × 55 cm) equilibrated and eluted with ammonium bicarbonate buffer (pH 7·4) at a flow rate of 0·5 ml min^{-1}. Two molecular weight standards were used to calibrate the column: aprotinin (6·5 kDa) and cytochrome C (12·5 kDa). Fractions of 2 ml were collected in 140 tubes, lyophilized and suspended in 500 μl of distilled water. The antimicrobial activity, protein (Lowry *et al.* 1951) and carbohydrate (Dubois *et al.* 1956) concentrations were determined with 10 μl of each fraction. The antimicrobial activity was based on the halos obtained when the indicator strain used was the Gram-positive bacterium *Micrococcus* sp., the Gram-negative bacterium *Ag. tumefaciens* and the phytopathogenic fungus *Cephalosporium acremonium*.

RESULTS

Antimicrobial activity by *P. peoriae* NRRL BD-62

During a screening procedure for antimicrobial activity-producing strains of *Paenibacillus* against phytopathogenic organisms, the strain NRRL BD-62 of *P. peoriae* showed a broad inhibition spectrum with activity against most of the strains tested. Several taxonomic groups of bacteria and fungi had their growth inhibited by this strain, as shown in Table 1.

The antimicrobial activity could be observed either in solid medium (as a clear zone around the producing strain) or in liquid medium, in which filter-sterilized supernatant was used in agar-well diffusion assays producing clear zones around the well. Four media (GB, TBN, TSB and 0·1×

TSB) were first used to screen the antimicrobial activity and the best results corresponding to the highest suppression effect on the indicator strains (*Micrococcus* sp. and *Ag. tumefaciens*) could be observed in 0·1× TSB supplemented with 0·2% sucrose. Therefore, the 0·1× TSB was selected as the medium for determination of the inhibition spectrum presented in Table 1.

Considering further physico-chemical characterization and purification of the antimicrobial compound secreted by the strain NRRL BD-62, a chemically defined medium (presented in the Materials and methods) was developed and the influence of temperature, aeration and pH were considered on the production of the antimicrobial activity. Supernatants showing inhibitory activity were obtained when NRRL BD-62 was grown in a range of pH from 6 to 8, temperatures of 30 and 37°C and in either still or shaking cultures. However, the maximum antimicrobial activity was observed against both *Micrococcus* sp. and *Ag. tumefaciens* in DM when the strain NRRL BD-62 was cultivated as a still culture, pH 7·0, at 30°C for 96 h (data not shown).

Effects of temperature, pH, organic solvents and proteolytic enzymes on the secreted antimicrobial activity

The antimicrobial activity of the 10-fold-concentrated supernatant was found to be stable during at least 4 months at −20°C, while the lyophilized supernatant could be stored either at 4°C or at room temperature for the same time without any loss of activity. However, the activity rapidly decreased after 1 week at 4°C in the nonconcentrated supernatant. The crude preparation also showed a high stability after treatments with proteolytic enzymes (0·5 mg ml^{-1} proteinase K, pronase E and trypsin), organic solvents at 10% (acetone, ethanol, methanol and chloroform), 2·5 mmol l^{-1} DTT, 3 mol l^{-1} urea, 0·05% Tween-80 and 5 mg ml^{-1} RNase. The pH of culture supernatant was 4·9 ± 0·2 after 96 h of culture incubation; however, the antimicrobial activity remained stable over a broad pH range (3·2–9·6). Also, the activity of the 10-fold-concentrated supernatant was not lost after 1 h at 100°C or after autoclaved at 121°C for 10 min. However, when the crude preparation was treated with 0·5 N NaOH for 4 h, no inhibitory activity was detected against *Micrococcus* sp., but no decrease of its activity was observed against *Ag. tumefaciens*.

Antimicrobial activity after Centricon partition

The concentrated supernatant of NRRL BD-62 was partitioned in two fractions (bigger and lower than 10 kDa) after ultrafiltration in Centricon system. These two fractions were tested for antimicrobial activity and only the fraction lower than 10 kDa showed inhibitory activity against *Micrococcus* sp. and *Ag. tumefaciens*.

Protein, protease and chitinase profiles

Strain NRRL BD-62 secretes a complex profile of polypeptides to the extracellular environment during its growth in DM for 96 h, with molecular weight ranging from 3 to 100 kDa (Fig. 1a, lanes A, B). In order to correlate the inhibitory activity shown by this strain with the secretion of proteolytic and chitinolytic enzymes to the extracellular medium, the presence of both classes of enzymes was investigated in the concentrated culture supernatant. The released protease profile showed at least three major distinct acidic proteolytic activities with apparent molecular masses of 25, 45 and 120 kDa (Fig. 1b, lane A). Additionally, at this pH we also detected two different chitinolytic activities with

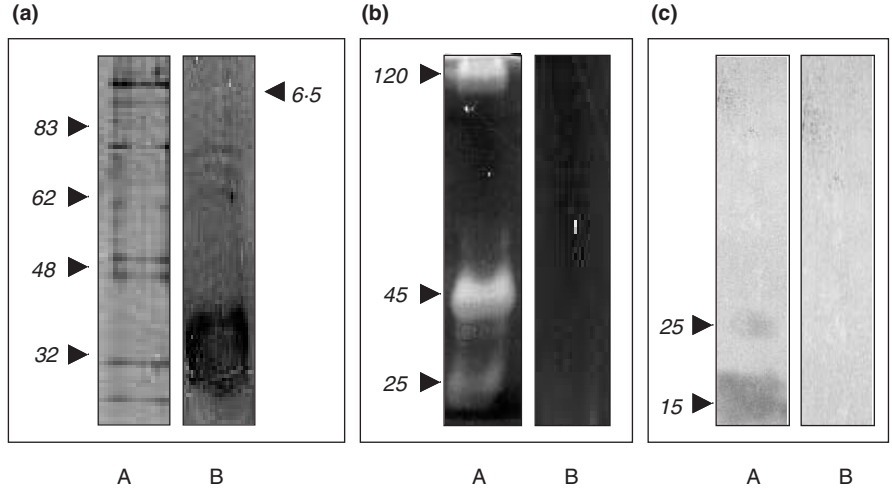

Fig. 1 Expression of released protein (a), protease (b) and chitinase (c) to the culture supernatant of *P. peoriae* NRRL BD-62. After ultrafiltration in Centricon system, the crude supernatant was partitioned in two fractions (>10 and ≤10 kDa). The polypeptidic and enzymatic profiles were analysed in 10% (A) and 20% (B) SDS–PAGE without (a) or with gelatin (b) and glycol-chitin (c) as substrates incorporated to the gels. The numbers indicate relative molecular masses expressed in kilodaltons

molecular masses of 15 and 25 kDa (Fig. 1c, lane A). However, no proteolytic or chitinolytic enzymes with molecular mass lower than 10 kDa could be detected being produced by this strain in these growth conditions (Fig. 1b,c, lane B).

Antimicrobial activity during growth

For the determination of the growth stage in which NRRL BD-62 produces the antimicrobial compound, supernatant samples were obtained at various stages during its growth in DM, and were tested for their activity using *Micrococcus* sp. as indicator strain. Changes in cell numbers (viable counts) and spores were recorded, as well as the antimicrobial activity (AU ml^{-1}) released, as shown in Fig. 2. High activity could be recovered even after 24 h of growth, when the cells were still in the logarithmic growth phase. However, the maximum antimicrobial activity was observed after 96 h of growth, late in the stationary phase, and no decline was observed after 168 h. Similar results were obtained when *Ag. tumefaciens* was used as indicator strain (data not shown).

Mode of action

When exponential phase cultures of *Micrococcus* sp. were mixed together with different concentrations of the antimicrobial compound produced by *P. peoriae* NRRL BD-62 (40, 80 or 400 AU ml^{-1} of crude preparation), it was observed that 40 AU ml^{-1} had a bacteriostatic effect in this strain, while 80 AU ml^{-1} showed a bacteriocidal effect. However, a higher concentration of this compound (400 AU ml^{-1}) was needed for the observation of this effect when the Gram-negative strain of *Ag. tumefaciens* was tested in the same conditions.

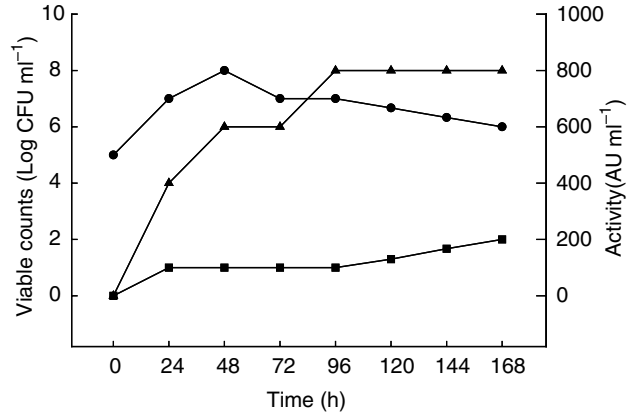

Fig. 2 Production of antimicrobial substance during growth in a chemically defined medium and sporulation. (●) Viable counts (log CFU ml^{-1}), (■) spores (log CFU ml^{-1}) and (▲) antimicrobial activity (AU ml^{-1}). *Micrococcus* sp. was used as indicator strain in this assay

Preliminary purification

After gel filtration on Sephadex G25 column of the crude aqueous extract (fraction of ≤10 kDa) of the antimicrobial compound from *P. peoriae* NRRL BD-62, antimicrobial activity was determined for each of the 140 fractions collected (Fig. 3). It could be observed that this crude extract from *P. peoriae* NRRL BD-62 supernatant showed more than one profile of activity, one with activity against the Gram-positive indicator strain (column fraction tubes from number 92 to 136; Fig. 3, dark line) and another one against the Gram-negative indicator strain (column fraction tubes from number 55 to 77; Fig. 3, hachured line). The range of activity against *C. acremonium* showed the same profile observed for *Micrococcus* sp. (data not shown). However, column fractions obtained from tube numbers 78 to 91 were still active against all the three micro-

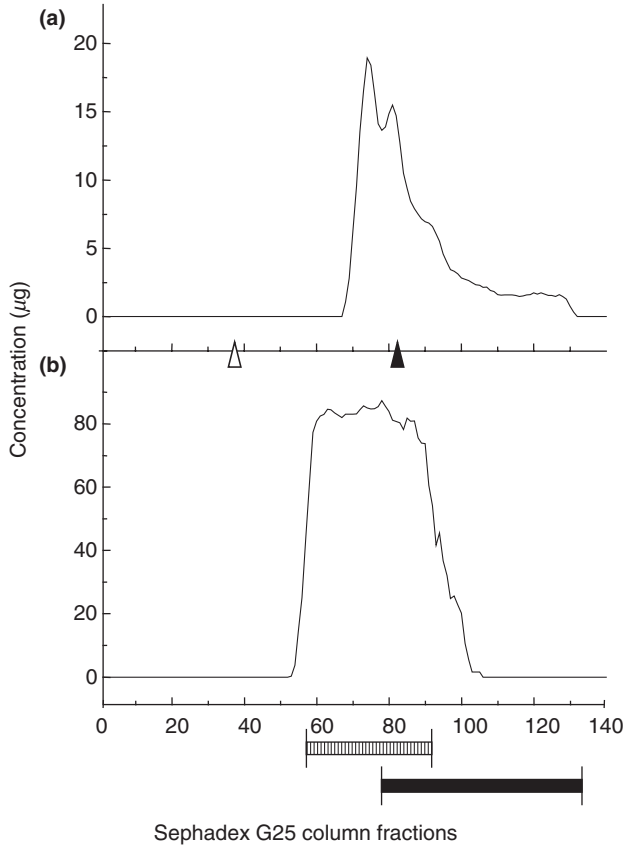

Fig. 3 Gel filtration of crude aqueous extract (fraction ≤10 kDa) on Sephadex G-25. (a) Protein, (b) carbohydrate contents, ■ activity against *Micrococcus* sp., ▥ activity against *Agrobacterium tumefaciens*. The molecular weight patterns: (△) cytochrome C (12.5 kDa) and (▲) aprotinin (6.5 kDa) are marked on the protein curve in (a)

organisms tested. Different amounts of protein (Fig. 3a) and carbohydrate (Fig. 3b) were detected in all fractions, which showed antimicrobial activity.

Estimation of the molecular weight of the antimicrobial substances was determined with aprotinin and cytochrome C as patterns, resulting in the detection of active substances around 6·5 kDa or lower against *Micrococcus* sp. and *C. acremonium*, and between 6·5 and 12·5 kDa against *Ag. tumefaciens*.

DISCUSSION

In addition to the need to provide effective control of plant diseases, a successful biocontrol agent must be robust enough to survive the processing and storage conditions involved in its formulation and use. In this context, endospore-forming bacteria have especially desirable properties. The endospore is resistant to desiccation, heat, u.v. irradiation and organic solvents; characteristics that allow for further formulation and commercialization procedures. Moreover, it is well documented that several members of the genus *Bacillus*, including the reclassified *Brevibacillus* and *Paenibacillus* produce antimicrobial substances, many of them already characterized (Katz and Demain 1977; Edwards *et al.* 1994; Piuri *et al.* 1998; Seldin *et al.* 1999; Beatty and Jensen 2002). Secondary metabolites produced by these species demonstrated antibacterial and/or antifungal activity against phytopathogenic and food-borne pathogenic micro-organisms, suggesting that some of these strains or their metabolites could be used as an alternative or supplementary method to chemical plant protection (Katz and Demain 1977; Leifert *et al.* 1995; Beatty and Jensen 2002). These properties combined with their natural ability to flourish in the soil environment make them well suited for biocontrol applications.

The close relationship between *P. peoriae* and *P. polymyxa* and *P. brasilensis*, both species able to produce antimicrobial substances (Kajimura and Kaneda 1997; von der Weid *et al.* 2000, 2002) was demonstrated genetically and phenotypically. For this reason, we decided to investigate whether strains of *P. peoriae* also have antimicrobial properties. After a screening procedure using different strains of *P. peoriae* against a wide range of phytopathogenic micro-organisms, the strain NRRL BD-62 was selected for further investigation. The antagonistic activity against a variety of bacteria and fungi, including some responsible for costly agricultural crop diseases, was demonstrated in NRRL BD-62 using both liquid and solid media. Furthermore, various antibiotics (Ito and Koyama 1972; Shoji *et al.* 1977; Umezawa *et al.* 1978; Pichard *et al.* 1995; Piuri *et al.* 1998) and antifungal compounds (Kurusu *et al.* 1987; Kajimura and Kaneda 1997; Beatty and Jensen 2002) produced by strains of *P. polymyxa* demonstrated a spectrum of activity against fungi and bacteria, similar to the antimicrobial compound described herein for *P. peoriae* NRRL BD-62.

Physiological and biochemical characteristics of the antimicrobial compound produced by NRRL BD-62 were obtained using a chemically defined medium developed in this study. The antimicrobial activity remained stable during heat treatment and also after treatment with a wide range of enzymes, chemicals and different pH. However, when the crude preparation was treated with 0·5 N NaOH, no inhibitory activity was detected against *Micrococcus* sp., but no decrease in its activity was observed against *Ag. tumefaciens*. This observation was the first indication that we were probably leading with at least two antimicrobial substances with different spectrum of action.

Peptides antibiotics, an abundant class of special metabolites, are produced by many microbial species including Gram-positive bacteria. Many of these special metabolites are produced under conditions of nutritional stress, and an accumulation of these substances can be often observed in stationary-phase cultures (Zuber *et al.* 1993). When the inhibitory activity was measured during the growth curve of the strain NRRL BD-62 in a chemically defined medium, it was shown that the sporulation was greatly reduced in this medium when compared with the TBN medium (data not shown), and that the activity seems to be induced under non-sporulate conditions as a secondary metabolite. The activity could be detected early in the logarithmic growing-phase but with an increasing activity during late stationary-phase. The same kind of expression has been reported for antimicrobial substances in *P. polymyxa* (Piuri *et al.* 1998), *Bacillus cereus* (Naclerio *et al.* 1993) and *Bacillus licheniformis* (Galvez *et al.* 1993). Although sporulation seems not to be required for activity detection, a common early regulatory pathway could be involved in this process, as described for other antimicrobial compounds (Leifert *et al.* 1995).

Considering the fact that nutritional stress increases the expression of the antimicrobial substance(s), different media and growth conditions were tested in order to produce large amounts of the antimicrobial substance(s), for further purification. During the partial purification procedure, it was demonstrated that the activities were found in the Amicon/Centricon fraction lower than 10 kDa. This fraction was then applied to a Sephadex G25 column and 140 fractions were analysed for antimicrobial activity. The results obtained after this procedure showed a splitting of the activity in which the activity against the Gram-positive bacterium and fungal strain, and the activity against Gram-negative bacterium could be detected in different fractions, suggesting that more than one active substance with distinct molecular weight was released to the culture supernatant. Furthermore, the antimicrobial activity secreted by NRRL BD-62 was eluted in fractions presenting high quantities of carbohydrate and protein, which suggest a proteinaceous

and/or glycopeptide nature. Notwithstanding, any reduction in antimicrobial activity was observed after treatment with proteolytic enzymes. If the antimicrobial compound is a peptide, it must be cyclic, N- and C-terminally blocked or composed of unnatural amino acids. Cyclic peptides can be resistant to hydrolysis by proteases because their cyclic structure renders them relatively inflexible, which may make cleavage sites inaccessible because of steric hindrance (Eckart 1994).

It has been described that proteases and chitinases produced by certain micro-organisms could also take part in the antagonistic process (Mavingui and Heulin 1994; Budi *et al.* 2000; de Marco and Felix 2002). To make sure that the antagonistic activity observed against the indicator strains was not due to these enzymes, protease and chitinase profiles were generated with the fractions lower and bigger than 10 kDa. The results obtained demonstrated that neither proteases nor chitinases with molecular mass lower than 10 kDa were released by this strain in these growth conditions. Although the antimicrobial activity studied here was not related to these classes of enzymes, we cannot exclude the possibility that the enzymes detected in the fraction bigger than 10 kDa might have an important role or synergistic effect in the potential antagonism *in vivo*. The studies in this sense are in progress in our laboratory.

The present study documents the production of antimicrobial substance(s) by *P. peoriae* NRRL BD-62 with bacteriocidal activity against a broad spectrum of micro-organisms. Besides the antagonistic activity, the strain NRRL BD-62 is also able to fix molecular nitrogen (von der Weid *et al.* 2002) and to produce enzymes as proteases and chitinases, suggesting that further studies should be addressed to use *P. peoriae* strain NRRL BD-62 as a plant growth promoter and/or as a biocontrol agent in field experiments.

ACKNOWLEDGEMENTS

The authors would like to specially thank Dr L. Nakamura (National Center for Agricultural Utilization Research, USDA, Peoria) for providing the strain of *P. peoriae* and all other researchers who kindly provided strains used in this study. This work was supported by grants from the National Research Council of Brazil (CNPq) and FAPERJ.

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