Hydrolytic enzyme activity of *Paenibacillus* sp. strain B2 and effects of the antagonistic bacterium on cell integrity of two soil-borne pathogenic fungi

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Received 31 May 1999;
revised 1 December 1999;
accepted 23 March 2000.
Available online 4 August 2000.

Abstract

Paenibacillus sp. strain B2, isolated from the mycorrhizosphere of *Sorghum bicolor* and having an antagonistic activity towards soil-borne fungal pathogens, possessed extracellular cellulolytic, proteolytic, chitinolytic and pectinolytic enzyme activities. The eventual role of these lytic enzymes in cellular interactions between *Paenibacillus* sp. strain B2 and *Phytophthora parasitica* and *Fusarium oxysporum* was investigated by electron microscopy and molecular cytology. Electron microscopic observations showed that the presence of *Paenibacillus* sp. strain B2 resulted in disorganisation of cell walls and/or cell contents of *P. parasitica* and *F. oxysporum*. However, when *P. parasitica* was treated with commercial purified cellulase, protease, chitinase and pectinase, only protease had an inhibitory effect on mycelial growth. It is proposed that the inhibitory effect of *Paenibacillus* sp. strain B2 on the growth of soil-borne fungal pathogens is probably derived from more than one mechanism.

Author Keywords: Hydrolytic enzymes; *Paenibacillus* sp. strain B2; Cell integrity; Wall metabolism; *Phytophthora parasitica*; *Fusarium oxysporum*

Article Outline

1. Introduction

Pesticides applied to circumvent damage caused by pathogens and insects in order to increase plant production are a major chemical input in modern agriculture. However, their excessive use has led to problems of environmental degradation and pollution. Such chemicals can, for example, be lethal to useful soil insects and to key micro-organisms in the rhizosphere (e.g. mycorrhizal fungi), and they may also enter the food chain. Moreover, there are increasing examples where their efficiency is decreased due to the development of resistant pathogens (Rosenberger, 1991). The potential of biological control of soil-borne plant pathogens by antagonistic micro-organisms (Dunn et al., 1997) offers a non-polluting complement, or alternative, to existing disease management strategies that depend heavily on chemical pesticides. However, the use of antagonistic micro-organisms in agricultural practices should be compatible with the persistence and function of symbiotic mycorrhizal fungi which play a central role in plant health and survival (Paulitz and Linderman, 1989; Barea et al., 1998).

We have isolated previously a gram positive bacterium *Paenibacillus* sp. strain B2 from the mycorrhizosphere of *Sorghum bicolor* inoculated with surface sterilised sporocarps of *Glomus mosseae*, and demonstrated that although it possesses a broad spectrum of antagonistic activity against many important pathogenic fungi, it has no detrimental effect on AM fungi in vitro and in vivo. In particular, it inhibits in vitro sporangia production, germination and hyphal growth of zoosporangia of *Phytophthora parasitica* and reduces root necrosis caused by *P. parasitica* in tomato seedlings (Budi et al., 1999).

A variety of mechanisms have been reported to contribute to the biocontrol activity of microbes and it is, for example, known that cell wall degrading enzymes, such as β-1,3-glucanases, cellulases, proteases and chitinases are involved in the antagonistic activity of some biological control agents against phytopathogenic fungi (Ordentlich et al., 1988; Shapira et al., 1989; Harman et al., 1993; Chernin et al., 1995; Dunn et al., 1997). Understanding the mode of action of biocontrol agents is a prerequisite for: (i) developing rational procedures in order to select more effective antagonistic microbial strains, (ii) developing appropriate production and formulation methods that enhance biocontrol activity, and (iii) fulfilling some requirements of the toxicological and registration packages needed for commercial development (Jijakli and Lepoivre, 1998).

In this paper, we describe the ability of the isolated *Paenibacillus* sp. strain B2 to produce hydrolytic enzymes that may contribute to its antagonistic activity, and the effect of the bacterium on cell integrity of two pathogenic fungi *P. parasitica* and *Fusarium oxysporum.*

2. Material and methods

2.1. Micro-organisms and culture conditions

Eight isolates of bacteria were isolated originally from an in vitro pot culture of *Sorghum bicolor* L. inoculated with surface disinfected sporocarps of *Glomus mosseae* BEG12 (Budi et al., 1999). The isolates were kept on Luria Broth (LB) media supplemented with 10% glycerol at −80°C. When needed, they were transferred to LB agar media plates and grown overnight at 37°C.

2.2. Plate assays for hydrolytic (cellulolytic, pectinolytic, proteolytic and chitinolytic) activities

Bacterial isolates were grown in Erlenmeyer flasks for 24 h on a culture shaker at 30°C in LB buffered medium. Ten microlitres of each bacterial suspension 10^{-3}–18^{-8} CFU/μ/l was spotted onto plates containing the substrate of the enzyme to be tested and grown at 25°C for 48 h.

Cellulolytic activity was assessed as described by Teather and Wood (1982) using a solid medium, containing $MgSO_4 \cdot 7H_2O$ (0.1 g/l), $CaCl_2 \cdot 2H_2O$ (0.2 g/l), $FeSO_4 \cdot 7H_2O$ (0.04 g/l), NaCl (0.2 g/l), KH_2PO_4 (0.3 g/l), K_2HPO_4 (0.5 g/l), CMC (carboxymethylcellulose) (Sigma) (5 g/l), yeast extract (0.1 g/l) and Bacto agar (15 g/l). For visualisation of β-D -glucan hydrolysis, the agar medium containing CMC was flooded with an aqueous solution of congo red (1 mg/ml) for 15 min.

Pectinolytic activity was studied as described by Hankin et al. (1971). The reaction medium contained $(NH_4)_2SO_4$ (2.0 g/l), KH_2PO_4 (4 g/l), Na_2HPO_4 (6 g/l), $FeSO_4 \cdot 7H_2O$ (1 mg/l), $MgSO_4$ (0.2 g/l), $CaCl_2$ (1 mg/l), H_3BO_3 (10 μg/l), $MnSO_4$ (10 μg/l), $ZnSO_4$ (70 μg/l), $CuSO_4$ (50 μg/l), MoO_3 (10 μg/l), apple pectin (Sigma) (5 g/l), yeast extract (1 g/l) and Bacto agar (15 g/l). Pectinolytic activity was revealed by flooding plates for 10 min with a 1% solution of hexadecyl trimethyl ammonium bromide in water. Enzyme activities were identified by the development of a zone of clearing (halo) around the bacterial colonies.

Proteolytic activity was performed in an agar medium containing skimmed milk (Difco) (100 g/l), yeast extract (1.5 g/l) and Bacto agar (15 g/l) according to Dunn et al. (1997) and visualised directly on the plates after 48 h. Chitinolytic activity was revealed according to the method of O'Brien and Colwell (1987). Bacteria were grown on LB agar medium for 24–48 h. One to five colonies from culture plates were blotted vigorously onto Whatman No. 1 filter paper and 20 μl of the 4-methylumbelliferyl-*N*-acetyl-β-D -glucosaminide-buffered substrate solution was added. Controls included the substrate only and the bacteria plus the solvent. After incubation at 37°C for 10 min, each test spot was covered with a drop of a saturated sodium bicarbonate solution and exposed to UV light. The reactions were graded as positive, weakly positive or negative. In positive reactions, substrate–bacteria mixtures produced a strong light-blue fluorescence.

2.3. Susceptibility of P. parasitica to commercial cellulase, protease, chitinase and pectinase

The effect of commercial hydrolytic enzymes on *P. parasitica* growth was studied in vitro. Agar plugs (5 mm in diameter) of *P. parasitica* were taken from the periphery of growing mycelia maintained on malt agar medium and incubated in sterile water (control), cellulase from *Aspergillus niger* (Sigma EC 3.2.1.4, 1.0 U/ml), proteinase K from *Tritirachium album* (Boehringer, 1.0 U/ml), chitinase from *Serratia marcescens* (Sigma EC 3.2.1.14, 1.0 U/ml) and pectinase from *A. niger* (Merck EC 3.2.1.15, 1.0 U/ml) for 24 or 48 h at 24°C. The plugs were then rinsed with sterile water and transferred to malt agar medium. Fungal growth was measured as colony diameter after incubating plates for 5 days at 24°C.

2.4. Ultrastructural observations

Phytophthora parasitica (syn. *P. parasitica* B. de Haan var. parasitica (Dastur) Waterh) isolate 201 (kindly provided by P. Bonnet, INRA, Antibes, France) and *F. oxysporum* isolate Foeu 1 (kindly provided by G. Lori, La Plata University, Argentina) were used. Agar plugs of *P. parasitica* and *F. oxysporum* cultures were placed on nutrient agar either containing or not containing CMC (5 g/l) or apple pectin (5 g/l), and in the presence or not in the presence of drops of a suspension of *Paenibacillus* sp. strain B2. The cultures were incubated in the dark at 27°C for 3 days. Mycelia were then sampled at the fungal colony margin adjacent to the bacterium, fixed overnight at 4°C in 2% glutaraldehyde buffered in 0.1 M Pipes (pH 7.2), and washed four times in Pipes buffer. Half of the samples of each treatment was post-fixed 1 h in 1% osmium tetroxide. All samples were subsequently dehydrated through a graded ethanol series, and embedded in Epon or LR White medium grade resin as described by Gianinazzi and Gianinazzi-Pearson (1992). Ultrathin sections of 85–95 nm were cut onto carbon-coated gold grids using a Reicher Ultracut microtome. For ultrastructural observations, sections of osmium tetroxide-fixed, epon-embedded samples were subjected to the periodic acid-thiocarbohydrazide-silver proteinate (PATAg) reaction to detect β(1-4) and β(1-6) glucans (Thiéry, 1967). An indirect immunogold labelling technique was carried out to localize β(1-3) glucans. For this, LR White embedded sections were incubated overnight at 4°C with a mouse monoclonal antibody raised against β(1-3) glucopyranose polymers (Biosupplies Australia, Australia) and diluted 1:50 in saline Tris-buffer containing 1% bovine serum albumin. After washing, sections were incubated 1 h in a gold-labelled (15 nm) polyclonal goat antimouse antibody. Immunological controls were performed by omitting the primary antibodies. *N*-acetyl glucosamine residues (chitin) were localised in *F. oxysporum* mycelium by incubating LR White embedded sections 1 h at room temperature in 10 nm gold-labelled wheat germ agglutinin lectin (WGA, Sigma) diluted 1:50. All treated sections were counterstained for 10 min with 2% aqueous uranyl acetate before being observed using a Hitachi 600 electron transmission microscope at 75 kV.

2.5. Statistical analysis

In vitro experiments were performed with four replicates and repeated twice. Data were analysed by one-way ANOVA and Newman–Keuls test at a probability of $p<0.05$.

3. Results

3.1. Plate assays for hydrolytic activities

The pectinolytic, cellulolytic, chitinolytic and proteolytic activities of the different strains of bacteria isolated from the mycorrhizosphere of sorghum are summarised in Table 1. Hydrolytic activities of *Paenibacillus* sp. strain B2, as indicated by the formation of clear zones surrounding the bacterial colony, were detected in medium supplemented with apple pectin, CMC and skimmed milk with diameters of lytic zones of 18, 22.5 and 22 mm, respectively (after 48 h incubation at 25°C). The other isolates B6 and B8 which did not display any antagonistic activity towards soil-borne fungal pathogens (Budi et al., 1999) also produced cellulolytic and pectinolytic activities revealed by the formation of clear zones of 17 and 20 mm diameter for cellulolytic activity and 12 and 22 mm diameter for pectinolytic activity, respectively. No proteolytic activity was observed for these two isolates. The two other isolates B3 and B7, which have a very weak antagonistic activity (Budi et al., 1999), also produced proteolytic activity with the formation of clear zones of 14 and 15 mm diameter, respectively. No cellulolytic and pectinolytic activities were observed for these two isolates. The chitinolytic activity was detected under UV light as a strong light-blue fluorescence for *Paenibacillus* sp. strain B2 and a weak light-blue fluorescence for isolates B6 and B8. No fluorescence was observed for isolates B3 and B7.

Table 1. Presence (+) or absence (−) of cellulolytic, proteolytic, chitinolytic and pectinolytic activity in different strains of bacteria isolated from the mycorrhizosphere of *Sorghum bicolor*

Bacteria isolates	Hydrolytic activity			
	Cellulase	Protease	Chitinase	Pectinase
B1	−	−	−	−
B2	+	+	+	+
B3	−	+	−	−
B4	−	−	−	−
B5	−	−	+	−
B6	+	−	+	+
B7	−	+	−	−
B8	+	−	+	+

3.2. Effect of commercial hydrolytic enzymes on P. parasitica

The effect of commercial hydrolytic enzymes on *P. parasitica* mycelial growth are shown in Table 2. No effects were observed on the growth of *P. parasitica* up to 48 h of incubation in cellulase, chitinase or pectinase, but protease inhibited mycelial extension by approximately 25%.

Table 2. Effect of commercial hydrolytic enzymes on *P. parasitica* mycelial growth assessed 5 days after immersion for 24 or 48 h in the solutions

Treatment	Mycelial growth (cm)[a]	
	24 h	48 h
Control	4.85a	4.75a
Cellulase	4.75a	4.65a
Protease	3.65b	3.60b
Chitinase	4.75a	4.75a
Pectinase	4.55a	4.50a

3.3. Ultrastructural observations

In the absence of *Paenibacillus* sp. strain B2, hyphae of *P. parasitica* taken at colony margins had active cell contents characterised by large individual vacuoles, a polysome-rich cytoplasm and numerous organelles (Fig. 1 and Fig. 3). Hyphal cells were delimited by a thin, electron-translucent wall (Fig. 1), the cellulosic component of which was strongly stained by the PATAg reaction (Fig. 3). Immunolabelling of β(1-3) glucans was associated with the cell wall (Fig. 4). Hyphal extension was inhibited about 70% by the presence of *Paenibacillus* sp. strain B2 (see also Budi et al., 1999). When mycelium of *P. parasitica* was sampled in apical regions, where contact between the micro-organisms had not occurred, hyphal contents showed different degrees of alteration. In some hyphae, the cytoplasm was extremely electron-dense, vacuoles were hardly visible and large lipid droplets accumulated (Fig. 2), whilst in others, cell contents were completely degraded (Fig. 5). The wall of most hyphae was distorted and paramural deformations developed intensely into the fungal cell (Fig. 2 and Fig. 5). Like the normal cell wall, these wall proliferations were PATAg-positive and contained β(1-3) glucans (Fig. 5 and Fig. 6).


Full-size image (17K)

Fig. 1. Transmission electron micrographs of hyphae of *P. parasitica* grown in the absence of *Paenibacillus* sp. strain B2. Sections were contrasted with uranyl acetate. The fungal cell has a thin electron-translucent wall (arrowhead) and an electron-opaque cytoplasm containing numerous vacuoles (v). Bar=2 μm.

 Full-size image (15K)

Fig. 3. Transmission electron micrographs of hyphae of *P. parasitica* grown in the absence of *Paenibacillus* sp. strain B2. Sections were stained by the PATAg reaction. The fungal wall reacts strongly to the PATAg reaction (arrowhead) and the cytoplasm is rich in mitochondria (m) and vacuoles (v). Bar=2 μm.

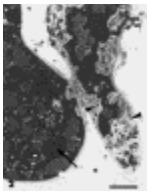 Full-size image (20K)

Fig. 4. Transmission electron micrographs of hyphae of *P. parasitica* grown in the absence of *Paenibacillus* sp. strain B2. Sections were stained by the immunogold labelled with β(1-3) antibodies: Gold particles (arrowheads) indicate the presence of a β(1-3) glucan wall component. Bar=2 μm.

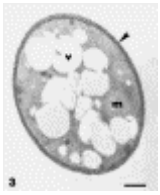 Full-size image (14K)

Fig. 2. Transmission electron micrographs of hyphae of *P. parasitica* grown in the presence of *Paenibacillus* sp. strain B2. Sections were contrasted with uranyl acetate. Fungal cell contents are extremely electron dense (arrow) in the presence of the bacterium and paramural deformations (arrowheads) develop into the cytoplasm. Bar=2 μm.

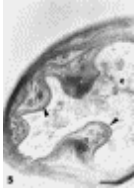 Full-size image (21K)

Fig. 5. Transmission electron micrographs of hyphae of *P. parasitica* grown in the presence of *Paenibacillus* sp. strain B2. Sections were stained by the PATAg reaction. PATAg reactive wall proliferations (arrowheads) protrude into the degraded cell lumen (c). Bar=0.5 μm.

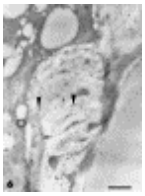 Full-size image (20K)

Fig. 6. Transmission electron micrographs of hyphae of *P. parasitica* grown in the presence of *Paenibacillus* sp. strain B2. Sections were immunogold labelled with β(1-3) antibodies. β(1-3) glucans (arrowheads) are located in the paramural wall deposits. Bar=0.5 μm.

F. oxysporum did not react to the same extent as *P. parasitica* to the presence of *Paenibacillus* sp. strain B2. Hyphal growth was less affected (about 30% inhibition, see also Budi et al., 1999), and fungal cells appeared metabolically active in the absence (Fig. 7) and in the presence (Fig. 9) of the bacterium. In both cases, cytoplasmic contents were dense, and rich in organelles and membrane systems, with vacuolisation occurring only in older parts (not shown). The outer part of the fungal wall stained with the PATAg reaction (Fig. 7) and WGA labelling indicated the presence of chitin (Fig. 8). Glycogen granules were present in most cells of *F. oxysporum* growing in bacteria-free conditions (Fig. 7) but they were less abundant or absent in hyphae growing in the presence of *Paenibacillus* sp. strain B2 (Fig. 9). A consistent modification induced by the bacterium was the deposition of polymorphic vesicle inclusions in an amorphous matrix between the cell wall and the fungal protoplast (Fig. 9 and Fig. 10). In these cells, the wall was distorted and the plasma membrane was retracted and convoluted around the inclusions. This material, easily distinguished from the cell wall by its lack of reaction to PATAg (Fig. 9), was labelled intensely with WGA indicating its cell wall origin (Fig. 10). These different alterations in the cell integrity and wall organisation of *P. parasitica* and *F. oxysporum* were induced similarly by *Paenibacillus* sp. strain B2 in the presence and in the absence of cellulose or pectin in the nutrient medium.

 Full-size image (24K)

Fig. 7. Transmission electron micrographs of hyphae of *F. oxysporum* grown in the absence of *Paenibacillus* sp. strain B2. Sections were stained by the PATAg reaction. The outer fungal wall (arrowhead) reacts strongly to the PATAg reaction. The cytoplasm is rich in glycogen (g), organelles and membrane systems. Bar=0.5 μm.

 Full-size image (17K)

Fig. 8. Transmission electron micrographs of hyphae of *F. oxysporum* grown in the absence of *Paenibacillus* sp. strain B2. Sections were lectin-gold labelled with wheat germ agglutinin. Scattered gold particles (arrowheads) indicate the presence of chitin in the fungal wall. Bar=0.2 μm.

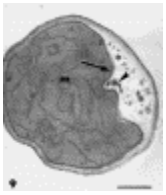 Full-size image (17K)

Fig. 9. Transmission electron micrographs of hyphae of *F. oxysporum* grown in the presence of *Paenibacillus*sp. strain B2. Sections were stained by the PATAg reaction. A PATAg non-reactive paramural matrix with vesicle inclusions (arrowhead) is bordered by a retracted plasma membrane (arrow). The cytoplasm is rich in mitochondria (m) and membrane systems. Bar=0.5 μm.

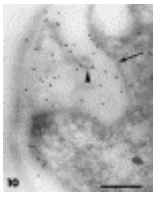 Full-size image (16K)

Fig. 10. Transmission electron micrographs of hyphae of *F. oxysporum* grown in the presence of *Paenibacillus* sp. strain B2. Sections were lectin-gold labelled with wheat germ agglutinin. The matrix between the fungal wall and the retracted plasma membrane (arrow) is labelled for chitin (arrowhead). Bar=0.2 μm.

4. Discussion

Although the exact mechanisms by which *Paenibacillus* sp. strain B2 operates to reduce disease incidence is not elucidated, one possibility is that this biocontrol agent exerts a direct inhibitory effect on hyphal growth of fungal pathogens in the rhizosphere (Budi et al., 1999). Of the eight bacteria isolated from the mycorrhizosphere of *Sorghum bicolor*, only *Paenibacillus* sp. strain B2 secreted all four hydrolytic enzyme activities tested. However, the role of these enzymes in controlling the growth of soil-borne pathogens is not clear since only protease inhibited significantly the in vitro growth of *P. parasitica*.

The electron microscope investigations from this study revealed that growth inhibition of *P. parasitica* and of *F. oxysporum* induced in the presence of the bacterium is accompanied by marked cellular changes in the two fungi. Effects on *P. parasitica* were much more pronounced with rapid disorganisation of hyphal contents and cell death. However, bacterial-induced alterations in both fungi were associated with the deposition of material on the inner side of the hyphal wall. The occurrence of cell wall components in these abnormal deposits raises the question of why and how the fungi react in reorganising wall material in such an unusual way. Disturbances in the regulation of enzymes involved in the biosynthesis of wall compounds may explain deposition of wall polymers at sites where this does not normally occur. The cellulolytic and chitinolytic activity of *Paenibacillus* sp. strain B2 could affect the structural integrity of the walls of *P. parasitica* and of *F. oxysporum*, respectively, leading to the release of molecules which may be responsible for deregulation of membrane-bound enzymes. Such a mechanism has been proposed to explain cell wall abnormalities induced by chitosan in *F. oxysporum* and *Pythium aphanidermatum* (Benhamou, 1992; El Gaouth et al., 1994) or by the fungicide cyproconazole in *Sclerotium rolfsii* (Fuller et al., 1990). Changes in cell vitality and modifications in cell walls are likely to have a deleterious effect on the growth of *P. parasitica* and of *F. oxysporum*. However, it seems unlikely that hydrolytic enzymes are the primary antifungal factors involved since none of the commercial wall-degrading enzymes except protease have such pronounced effects on fungal growth. The inhibitory effect of *Paenibacillus* sp. strain B2 on the growth of soil-borne fungal pathogens and disease development is probably derived from more than one mechanism. It would be interesting to isolate the active antifungal compound and to investigate the possibility that the bacterium elicits resistance mechanisms in planta.

Acknowledgements

We are grateful to the Center Grand Program, Directorate General of Education, Republic of Indonesia and the INCO-DC EU Project No. ERBIC18 CT 970180 for supporting this work. The authors thank Denise Dubois for technical assistance.

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