Wide-range antifungal antagonism of *Paenibacillus ehimensis* IB-X-b and its dependence on chitinase and β-1,3-glucanase production

G. Aktuganov, A. Melentjev, N. Galimzianova, E. Khalikova, T. Korpela, and P. Susi

Abstract: Previously, we isolated a strain of *Bacillus* that had antifungal activity and produced lytic enzymes with fungicidal potential. In the present study, we identified the bacterium as *Paenibacillus ehimensis* and further explored its antifungal properties. In liquid co-cultivation assays, *P. ehimensis* IB-X-b decreased biomass production of several pathogenic fungi by 45%–75%. The inhibition was accompanied by degradation of fungal cell walls and alterations in hyphal morphology. Residual medium from cultures of *P. ehimensis* IB-X-b inhibited fungal growth, indicating the inhibitors were secreted into the medium. Of the 2 major lytic enzymes, chitinases were only induced by chitin-containing substrates, whereas β-1,3-glucanase showed steady levels in all carbon sources. Both purified chitinase and β-1,3-glucanase degraded cell walls of macerated fungal mycelia, whereas only the latter also degraded cell walls of intact mycelia. The results indicate synergism between the antifungal action mechanisms of these enzymes in which β-1,3-glucanase is the initiator of the cell wall hydrolysis, whereas the degradation process is reinforced by chitinases. *Paenibacillus ehimensis* IB-X-b has pronounced antifungal activity with a wide range of fungi and has potential as a biological control agent against plant pathogenic fungi.

Key words: antifungal activity, antagonism, chitinase, β-1,3-glucanase, *Paenibacillus ehimensis*.

Résumé : Nous avons précédemment isolé une souche de *Bacillus* qui possédait une activité antifongique et qui produisait des enzymes lytiques à potentiel fongicide. Dans la présente étude, nous avons identifié la bactérie comme *Paenibacillus ehimensis* et nous avons exploré davantage ses propriétés antifongiques. Lors d'essais de co-culture en milieu liquide, la présence de *P. ehmensis* IB-X-b diminuait la production de biomasse de champignons pathogènes de 45 % à 75 %. L'inhibition était accompagnée par la dégradation de la paroi cellulaire des champignons et modifiait la morphologie des hyphes. Le milieu de culture résiduel de *P. ehmensis* IB-X-b inhibait la croissance des champignons, ce qui signifie que des inhibiteurs étaient secrétés dans le milieu. Parmi les deux enzymes lytiques majeures, les chitinases n'étaient induites que par des substrats contenant de la chitine, alors que la β-1,3-glucanase était exprimée à des niveaux constants sur toutes les sources de carbone. La chitinase et la β-1,3-glucanase purifiées dégradaient toutes deux la paroi cellulaire des mycéliums macérés alors que seule la dernière dégradait aussi la paroi cellulaire de mycéliums intacts. Les résultats indiquent qu'il existe une synergie entre les mécanismes d'action antifongiques de ces enzymes dans lesquels la β-1,3-glucanase est l'initiatrice de l'hydrolyse de la paroi cellulaire alors que le processus de dégradation est renforcé par les chitinases. *Paenibacillus ehimensis* IB-X-b possède une activité antifongique importante sur une vaste gamme de champignons et démontre un potentiel en tant qu'agent de contrôle biologique des champignons pathogènes.

Mots-clés : activité antifongique, antagonisme, chitinase, β-1,3-glucanase, *Paenibacillus ehimensis*.

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Introduction

Strains in the genus *Bacillus* are highly prevalent in soil and have an important role in physiology and ecology of soil microbial balance. Growth properties, heat tolerance, and the ability to form endospores, thus, make them attractive candidates for biocontrol agents over genetically modified strains (Chet and Inbar 1994; Mari et al. 1996; Podile and Prakash 1996; Nielsen and Sorensen 1997; Pleban et al. 1997). Furthermore, the debate about the effects of genetically modified organisms (GMOs) on biodiversity and increases in antibiotic resistance make them less favorable

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G. Aktuganov. Institute of Biology, Ufa Research Centre of Russian Academy of Sciences, Prospect Oktyabrya 69, Ufa 450054, Russia; Joint Biotechnology Laboratory (JBL), University of Turku, BioCity 6A, 20520 Turku, Finland.
A. Melentjev and N. Galimzianova. Institute of Biology, Ufa Research Centre of Russian Academy of Sciences, Prospect Oktyabrya 69, Ufa 450054, Russia.
E. Khalikova and T. Korpela. Joint Biotechnology Laboratory (JBL), University of Turku, BioCity 6A, 20520 Turku, Finland.
P. Susi.[1] Joint Biotechnology Laboratory (JBL), University of Turku, BioCity 6A, 20520 Turku, Finland; Department of Virology, Institute of Microbiology and Pathology, University of Turku, Kiinamyllynkatu 13, 20520 Turku, Finland.

[1]Corresponding author (e-mail: pesusi@utu.fi).

agents for biocontrol. As natural soil microbes, *Bacillus* strains are less likely to pose a threat to environmental balance even when introduced to habitats where they are not naturally found.

Bacterial antifungal potential is often determined by analysis of extracellular chitinases (Ordentlich et al. 1988; Lim et al. 1991; Pleban et al. 1997; Nielsen et al. 1998; Gohel et al. 2006), since chitinases hydrolyze chitin, which is the major structural component of fungal cell walls (Peberdy 1990). The ability to produce not only chitinase (EC 3.2.1.14) but also other fungal cell wall-degrading enzymes and a broad spectrum of antibiotics is common to aerobic spore-forming bacteria (Katz and Demain 1977; Tanaka and Watanabe 1995; Bertagnolli et al. 1996; Nielsen and Sorensen 1997; Budi et al. 2000; San-Lang et al. 2002; Hong and Meng 2003; Kim and Chung 2004). Chitinolytic bacteria found in poor soil habitats (limited in carbon sources) are facultative mycoparasites, which degrade fungal hyphae (De Boer et al. 1998). This property is widely distributed among *Bacillus* species (Mitchell and Alexander 1962; Tominaga and Tsujisaka 1975; Podile and Prakash 1996), allowing them to compete successfully with saprophytic fungi for chitin as a carbon source (De Boer et al. 1998).

There are several possible molecular and cellular mechanisms used by bacteria that are antagonistic to fungi. The involvement of chitinases and other lytic enzymes, as well as certain small molecular compounds, has been shown in previous studies (Ordentlich et al. 1988; Lim et al. 1991; Pleban et al. 1997; Nielsen et al. 1998; Nagarajkumar et al. 2004). Chitinases from *Bacillus amyloliquefaciens* 5PVB and V656 strains, which do not produce antibiotics, were effective against *Botrytis cinerea* (grey mould) and *Fusarium oxysporum* (Mari et al. 1996; San-Lang et al. 2002). In contrast, some reports indicate that antibiotics also play a role in the antifungal processes (Roberts and Selitrennikoff 1988; Frändberg and Schnürer 1998).

Chitinolytic *Bacillus* strain IB-X-b was originally isolated because of its ability to degrade crystalline and colloidal chitin in soil, and the secreted enzymes were characterized and partially purified (Helistö et al. 2001). Ribosomal RNA (rRNA) sequencing identified this species as *Paenibacillus ehimensis*. Antifungal activities of *P. ehimensis* IB-X-b against plant pathogenic and saprophytic fungi with high economical importance (e.g., *Fusarium* spp., *Drechlera sorokiniana*, and *Alternaria alternata*) were found. The present study describes in detail the qualitative and quantitative effects of the antifungal factors secreted by *P. ehimensis* IB-X-b on various fungi. The results reinforce the previous view that *P. ehimensis* IB-X-b could serve as a potential wide-range biocontrol agent against plant pathogenic fungi and deserves further studies in field conditions.

Materials and methods

Isolation and identification of chitinolytic bacteria

Soil was enriched with powder of crab shell chitin 16 weeks prior to sampling. Enrichment cultures were suspended in sterile water (5% *m*/*v*) in glass tubes and heated in a water bath at 80 °C for 15 min. The heat-treated suspension was inoculated onto solid agar medium containing 0.5% (*m*/*v*) colloidal chitin, which was used for isolation of chitinolytic bacteria. The agar plates were incubated at 37 °C for 7 days. *Paenibacillus ehimensis* IB-X-b was isolated from soil based on its strong chitinolytic activity (Helistö et al. 2001). The morphological and physiological properties of the bacterial species were described according to Gordon et al. (1973) and Garrity (1986). The strain was identified as a *P. ehimensis* IB-X-b by the 16S rRNA sequencing in the bacterial diagnostic unit (UTULab, Turku, Finland) using commercial laboratory procedures. The 16S rRNA gene was deposited in the GenBank database under accession No. EU623973. The strain was deposited to the Russian National Collection of Industrial Microorganisms (Moscow, Russia) under accession No. VKPM B-9517.

Microbial cultures

Fungal species used in the study were mainly plant-pathogenic fungi that cause root rot in wheat and other cereals. Strains obtained from the All-Russian Collection of Microorganisms, Institute of Physiology of Microorganisms, Pushino, Russia, included *Alternaria alternata* (Fr.) Keissl. VKM F-3047, *Fusarium culmorum* (W.G. Smith) Sacc. VKM F-844, *Fusarium graminearum* Schwabe VKM F-1668, *Fusarium moniliforme* Sheild. VKM F-670, *Fusarium nivale* (Fr.) Ces. VKM F-3106, *Fusarium oxysporum* (Schlecht.) Snyd. et Hans. VKM F-137, *Fusarium semitectum* Berkeley et Ravenel VKM F-1938, *Fusarium solani* Mart. App. et Wr. VKM F-142, *Fusarium sporotrichiella* Bilai. var. *sporotrichioides* (Sherb.) Bilai VKM F-815, and *Rhizoctonia solani* Kuhn VKM F-935. Other fungal species including *Aspergillus glaucus* Lk. IB F-6, *Aspergillus niger* van Tieghem IB F-7, *Drechlera sorokiniana* (Sacc.) Subram. et gain. (= Bipolaris sorokiniana) IB F-12, *Paecilomyces variotii* Bainier IB F-27, *Penicillium variabile* Sopp. IB F-36, and *Trichoderma viride* Pers. IB F-40 were isolated from soil or wood samples and characterized in the Laboratory of Applied Microbiology of the Institute of Biology, Ufa Research Centre, Russian Academy of Sciences, Ufa, Russia (Ainsworth et al. 1973). These fungal strains are deposited in The Microbial Collection of the Institute of Biology, Ufa Research Centre, Russian Academy of Sciences, Ufa, Russia. All fungal species were tested as a single passage during a period of 3 months. The fungal species were maintained on slants of potato dextrose agar (PDA) or Czapek agar.

Media for cell cultivation

Paenibacillus ehimensis IB-X-b was screened and purified on 1.6% agar plates prepared in PE medium (0.5% colloidal chitin from crab shells, 0.2% polypeptone, 0.1% corn steep liquor, 0.1% KH_2PO_4, 0.1% K_2HPO_4, 0.05% $MgSO_4$, and 0.03% $CaCl_2$, with final pH of 6.5). For evaluation of the induction of lytic enzymes, bacteria were cultivated in liquid PE medium and its modified variants supplemented, instead of colloidal chitin, with 0.5% potato starch, 0.5% laminarin, 0.5% β-glucan from barley, 0.5% (*m*/*v*) mycelia of *F. culmorum* VKM F-844, or fruit bodies of basidiomycete *Macrolepiota procera* (Fr.) Sing as the main carbon source. In the last case, fungal fruit bodies were finely cut, dried, and powdered prior to use. The substrate from the mycelia of *F. culmorum* was prepared in the same way for the determi-

nation of cell wall-degrading activity (see the following). The colloidal chitin was prepared using a modified method of Rodriguez-Kabana et al. (1983). For preparation of the crude enzyme extract, bacterial cells were grown in liquid medium containing 0.4% (*m*/*v*) crushed fruit bodies of *M. procera* and 0.15% colloidal chitin. Bacteria were precultivated for 16–18 h in 50 mL of PE medium supplemented with 0.5% starch, instead chitin, on a rotary shaker at 160 r/min at 36 °C ± 0.5 °C. One millilitre of initial cultivation medium (>10^6 colony-forming units (CFU)) was inoculated into the chitin-containing medium and cultivated under the same conditions for 60 h. Intact fungal mycelia were grown in liquid Czapek medium for 6–10 days at 28 °C then washed twice with sterile sodium phosphate buffer (25 mmol/L, pH 6.0) containing 0.9% (*m*/*v*) NaCl, and dried. For preparation of powdered mycelia, fungal material was autoclaved in the culture medium at 121 °C for 20 min. The mycelium was washed twice in distilled water, air-dried at 37 °C, and ground in a mortar using a pestle.

Determination of enzyme activity

Secreted enzymes with hydrolyzing activity were separated and partially purified by ammonium sulphate fractionation, chitin–chitosan adsorption, anion-exchange chromatography (DEAE-Sepharose Fast Flow; Pharmacia, Uppsala, Sweden) and gel chromatography (Bio-Gel P-100; Bio-Rad Laboratories, Hercules, California) as described previously (Helistö et al. 2001). The activities of chitinase, chitosanase, and β-1,3-glucanase were determined by hydrolysis of colloidal chitin, colloidal chitosan, and laminarin, respectively, and the enzyme activities in units (U) were defined as formation of 1 μmol/min of *N*-acetyl-D-glucosamine from colloidal chitin by chitinase, D-glucosamine from colloidal chitosan by chitosanase, and D-glucose from laminarin by β-1,3-glucanase (Helistö et al. 2001). Protease activity was assayed as described by Haab et al. (1990). Lipase activity was defined as release of 1 μmol/min of *p*-nitrophenol from *p*-nitrophenol butyrate (*p*-Np-butyrate) at 37 °C (Helistö et al. 2001). All reagents were from Sigma-Aldrich (St. Louis, Missouri).

The fungal cell wall-degrading activity of crude enzyme complex and purified chitinase and other enzymes were examined with intact and powdered mycelia. Enzyme activity was estimated as the rate of liberation of soluble reducing sugars. The crude and purified enzymes were added into tubes containing intact (2% *m*/*v*) or powdered (1% *m*/*v*) fungal biomass suspended in 50 mmol/L sodium phosphate buffer, pH 6.0, to the final volume of 10 mL. The mixtures were incubated for 6 h at 37 and 50 °C for intact and macerated mycelia, respectively. Concentration of specific reaction products was calculated using *N*-acetyl-D-glucosamine, D-glucosamine, and D-glucose as standards for purified chitinase, chitosanase, and β-1,3-glucanase, respectively. The control included crude enzyme complex, which was heat inactivated by autoclaving at 121 °C for 15 min.

Determination of fungal growth inhibition by *P. ehimensis* IB-X-b and secreted enzymes

The effect of *P. ehimensis* IB-X-b on pathogenic fungi was assayed in liquid cocultures at optimal fungal growth temperature (28 °C). Fungi from Czapek agar plates 7–10 days postcultivation were washed with a sterile solution of NaCl (0.9% *m*/*v*) and gently collected by sterile spreaders. The suspension was passed through sterile Whatman No. 1 filter paper (Maidstone, UK). One milliliter of the filtrate (the titer ranged from 10^3 to 10^5 CFU for several fungal species of different spore sizes) was inoculated into potato dextrose broth (PDB). Bacterial cells grown in starch-containing medium at 37 °C for 24 h were centrifuged and resuspended in sterile NaCl (0.9% *m*/*v*). One millilitre of the bacterial suspension (~10^6 CFU/mL) was added to PDB either at the same time as the fungal culture or 24 h after fungal inoculation. The densities of the cells or spores in the inoculated bacterial and fungal suspensions were calculated by standard methods after serial dilutions and plating on meat peptone agar (MPA) or Czapek agar, respectively. The fungi and bacteria were cocultivated to stationary phase for 6 days in flasks containing 50 mL of PDB. After co-cultivation the mycelia were collected on paper filters (Whatman No. 1) and washed with distilled water. The dry mass of washed mycelia was determined after drying at 105 °C for 90 min. Fungal growth inhibition was calculated as the decrease in dry mass (%) of fungal biomass against control fungi grown in the absence of bacteria.

Fungal growth inhibition by the secreted enzyme complex of *P. ehimensis* IB-X-b was also evaluated in PDB and Czapek agar diffusion plates. Enzymes were precipitated from the supernatant of bacterial culture grown in starch- and chitin-containing medium (60 h, 37 °C, 160 r/min) by 65% (*m*/*v*) ammonium sulphate. The precipitate was collected, dialyzed against MQ water, and filter sterilized (0.2 μm; Schleicher & Schuell, BioScience, Germany). The sterilized preparation was mixed with PDB (1 mL in 50 mL of medium) and pipetted (50 μL) into wells punctured onto Czapek agar plates over-layered with fungal spore suspensions (2×10^2 to 2×10^5 CFU). The fungal growth inhibition was evaluated after 6 days (for the PDB assay) of incubation using the procedures mentioned above. For the Czapek agar assay, the degree of inhibition was described as the diameter of the halo around the wells after 3 days of incubation at 28 °C.

For determination of small molecular antifungal compounds secreted by the bacteria during cocultivation with 5 fungal species, the supernatants were filtered and fractionated by ultra filtration (MicroSep FILTRON 30K; Amicon, Danvers, Massachusetts). Both the flow-through fraction (<30 kDa), which was concentrated by vacuum evaporation at 45–50 °C, and the retained fraction (>30 kDa), were collected and used for the analysis. Spore suspension of *Paecilomyces variotii* (0.1 mL) was seeded evenly onto the surface of a Czapek agar plate and then wells (diameter = ~4 mm) were punctured into the agar. Samples (50 μL) from the fractions were pipetted into the wells. The degree of inhibition was estimated as the diameter of the halo on the Czapek agar assay.

The effects of bacteria and secreted enzymes on the hyphal growth and morphology were examined by light and phase-contrast microscopy. Washed living mycelia from the fungal culture grown for 6–10 days were suspended in sterile sodium phosphate buffer (25 mmol/L, pH 6.0) containing 0.9% NaCl (*m*/*v*). The prepared suspension (1 mL) was transferred into sterile plastic tubes containing different con-

centrations of the enzymes. The control included 1 mL of the mycelial suspension without enzymes. The mixtures were incubated at 28 °C for 12–24 h.

Statistical analyses

All experiments were carried out in triplicate. The cell wall-degrading activity means were calculated with Origin 6.0 software as the average of 3 measures with standard deviations (SDs). The rate of hydrolysis of fungal cell walls was calculated as the average of means measured at different time intervals. The values reflecting the fungal growth inhibition determined by the dry mycelial mass and Czapek agar diffusion methods are presented as means with standard errors (SEs). For values of enzymatic activities, statistical analysis was performed using the Student's *t* test at a confidence limit of 95%.

Results

Genetic identification of chitinolytic *Bacillus* sp. strain IB-X-b

When soil samples enriched with crab shell powder were screened for bacteria with chitinolytic activity, a bacterial strain was isolated, characterized, and named using standard microbiological classification schemes as *Bacillus* sp. strain IB-X-b (Gordon et al. 1973; Garrity 1986; Helistö et al. 2001). The strain was further subjected to genetic typing using standard rRNA sequencing procedures at the UTULab Bacterial Diagnostic Unit, Turku, Finland. The first 20 hits were for *Paenibacillus* or *Bacillus* species with the closest match to *Paenibacillus ehimensis* IB5 (GenBank accession No. EF025575; (X.L. Rao, D.L. Shen, J. Li, unpublished results)) at a 100% identity level in 2 consecutive runs with 2 samples within the length of an ~400 bp PCR fragment (UTULab Bacterial Diagnostic Unit, Turku, Finland; Olsen et al. 1986; Kuroshima et al. 1996; Lee et al. 2004). These fragments possessed 99% identity in pairwise comparison with 3 nucleotide gaps. Therefore, the strain was designated as *P. ehimensis* IB-X-b. The 16S rRNA gene was deposited in the GenBank database under accession No. EU623973.

Antifungal effect of *P. ehimensis* IB-X-b on fungi in cocultures

The antifungal effect of *P. ehimensis* IB-X-b was confirmed when it was cocultivated with the pathogenic fungi in PDB. The results showed almost total growth suppression of a majority of the tested fungi as indicated by the reduction in the biomass production (Table 1). When fungi were added to the growth medium 24 h prior to the bacteria, the growth of *Alternaria alternata*, *A. glaucus*, *D. sorokiniana*, *F. culmorum*, *F. graminearum*, *F. semitectum*, and *R. solani* were affected, whereas other fungi, such as *A. niger* and *T. viride*, were more resistant to bacterial growth inhibition. Microscopic examination showed that cocultivation resulted in alterations in mycelial morphology compared with the fungi grown in the absence of bacteria. This was species-dependent and included either extensive degradation or swelling of hyphae with subsequent formation of spheroplast-like structures. The most severe damages were detected in *F. culmorum* and *D. sorokiniana* (Fig. 1), whereas in some fungi (*F. solani* and *F. nivale*) only minor changes in mycelial morphology were seen even though the growth rate and biomass production were decreased (data not shown). These results indicate that *P. ehimensis* IB-X-b possesses antifungal activities that strongly inhibit growth of several plant pathogenic fungi.

Table 1. Fungal growth inhibition by *Paenibacillus ehimensis* IB-X-b.

	Growth inhibition (%)	
Fungal species	Co-inoculation[a]	Postinoculation[b]
Alternaria alternata	>95	80±6
Aspergillus glaucus	>95	62±7
Aspergillus niger	25±5	<5
Drechlera sorokiniana	>95	78±8
Fusarium culmorum	>95	62±5
Fusarium graminearum	79±8	60±6
Fusarium moniliforme	>95	8±2
Fusarium nivale	>95	46±5
Fusarium oxysporum	>95	16±4
Fusarium semitectum	>95	61±6
Fusarium solani	>95	41±5
Fusarium sporotrichiella	>95	16±4
Paecilomyces variotii	>95	40±6
Rhizoctonia solani	>95	>95
Trichoderma sp.	20±5	<5

Note: Values are means ± standard errors.

[a]Bacteria were inoculated into the potato dextrose broth at the same time with fungi. All incubations were at 28 °C.

[b]Bacteria were inoculated into the potato dextrose broth 24 h after fungal inoculation. All incubations were at 28 °C.

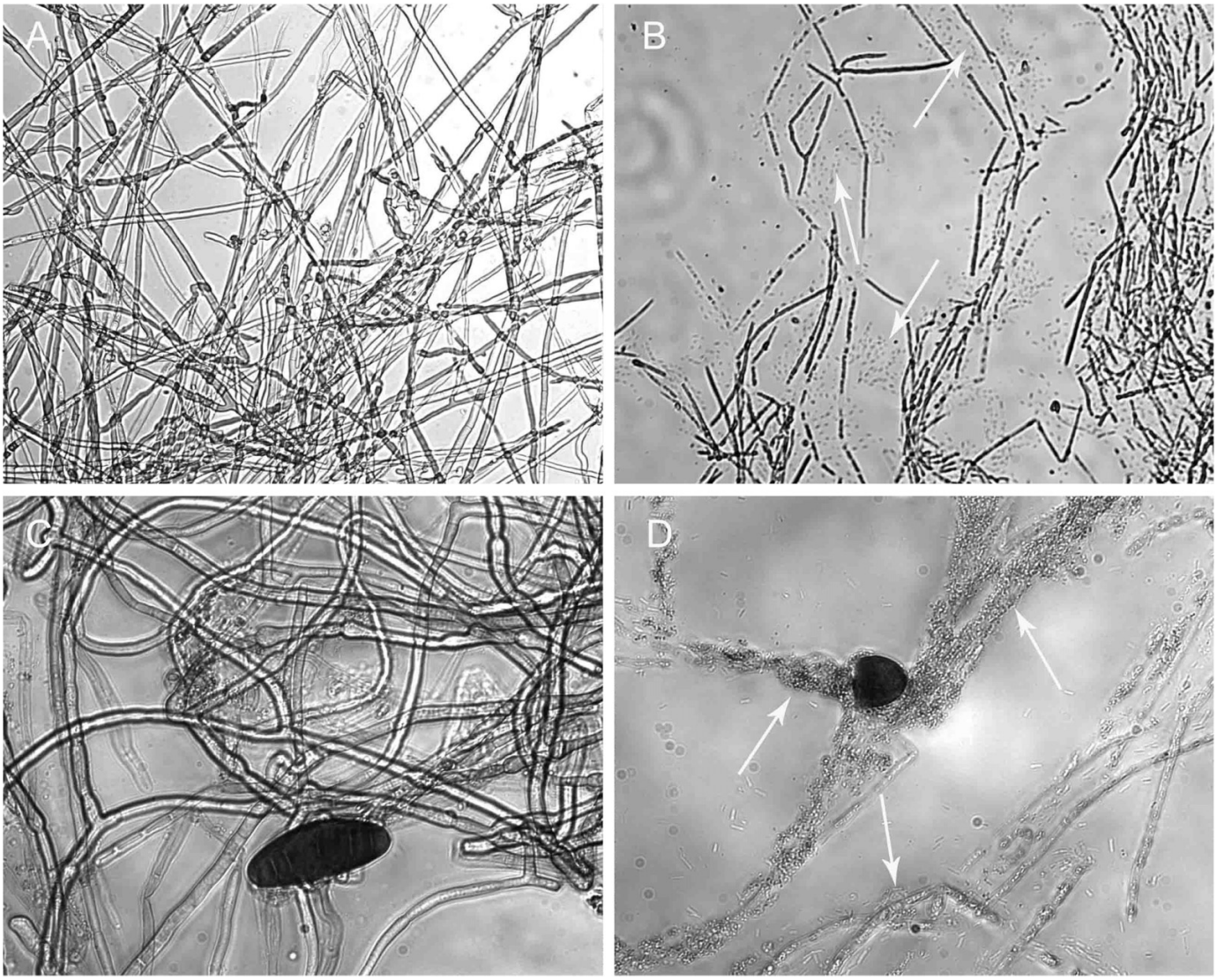
Analysis of lytic enzymes secreted by *P. ehimensis* IB-X-b

Consistent with observations that *P. ehimensis* IB-X-b caused visible damage to fungal hyphae, it was evident that several lytic enzymes were found in the growth medium (Table 2). Particularly, β-1,3-glucanase was expressed at high levels in media containing several defined carbon sources. The highest activities were observed in media with either colloidal chitin or starch. Inclusion of either laminarin or β-glucan into the growth medium yielded significantly lower β-1,3-glucanase activity, but nevertheless, resulted in increased growth rates by *P. ehimensis* IB-X-b (data not shown). In contrast to β-1,3-glucanase, significant increases in chitinase activities were observed only in the presence of chitin from various sources. Maximal activity of chitinolytic enzymes by *P. ehimensis* IB-X-b was detected in the medium supplemented with fungal biomass from *M. procera* and colloidal chitin (0.20 and 0.35 U/mL for chitinase and chitosanase, respectively; Table 2).

Antifungal activities of crude enzyme mixture produced by *P. ehimensis* IB-X-b

The effects of lytic enzymes secreted during the cultivation of *P. ehimensis* IB-X-b on chitin- and starch-containing media or in cocultivation assay with fungi were tested on several pathogenic fungi or *Paecilomyces variotii*, respectively (Tables 3–5). While chitinolytic activities in supernatants induced by chitin were markedly higher compared with starch-containing medium (see Table 2), there were only minor differences in the antifungal activities between

Fig. 1. Effects of *Paenibacillus ehimensis* IB-X-b on mycelia in a cocultivation assay. *Fusarium culmorum* (B) and *Drechlera sorokiniana* (D) were cocultivated with *P. ehimensis* IB-X-b, whereas control incubations were performed in the absence of *P. ehimensis* IB-X-b (A and C). Cocultivations were performed in potato dextrose broth at 28 °C, and mycelia were examined under a light microscope after 48 h. The arrows indicate some of the sites where damage to the fungal spores and hyphae is visible.



chitin- and starch-induced lytic enzymes (Table 3). Among the saprophytic fungi, the growth of *Paecilomyces variotii* and *T. viride* were most strongly inhibited, whereas *A. glaucus* and *A. niger* were inhibited only in the initial stage of growth. Significantly, *Alternaria alternata* and *D. sorokiniana*, which cause root rots of cereals, were very sensitive to the action of lytic enzymes (Table 3).

Lytic enzymes with a molecular mass below and above 30 kDa were fractioned and tested against *Paecilomyces variotii* on plate assays (Table 4). Both fractions had specific inhibitory effects on specific pathogenic fungi. When the >30 kDa fraction was analyzed in more detail, increased activities of chitinase and β-1,3-glucanase were detected as expected because of their high molecular masses (Table 5). Notably, chitinolytic activity was detected only by using *p*-NP-*N*,*N*′-diacetyl-β-D-chitobioside substrate. Colloidal chitin was hydrolyzed only very weakly (data not shown). As the results in Tables 2 and 3 indicate, *P. ehimensis* IB-X-b secretes lytic enzymes and exhibits antifungal properties particularly in carbon sources, which contain the major fungal cell wall component, chitin. When the secreted fraction was fractioned, it was evident that there were many antifungal compounds that possessed specific activities against fungi. Particularly, the major enzyme activities detected were chitinase and β-1,3-glucanase.

Degradation of fungal mycelia by enzymes secreted by *P. ehimensis* IB-X-b

When intact fungal mycelia were treated with crude enzyme mixtures from *P. ehimensis* IB-X-b, hyphal damage was visible under the microscope (Fig. 2). Specifically, damaged hyphae and cytoplasmic leakage were evident in saprophytic *Paecilomyces variotii* and *T. viride* (Fig. 2). Vacuole formation was substantial in other saprophytic fungal species. Among the plant pathogenic fungi, the lysis was most evident in *F. culmorum* and *D. sorokiniana* and almost sim-

Table 2. Lytic enzymes secreted by *Paenibacillus ehimensis* IB-X-b in various carbon sources.

	Enzyme activity (U/mL) in culture supernatant				
Main carbon source[a]	Chitinase	Chitosanase	β-1,3-Glucanase	Lipase	Protease
D-Glucose	0	0	0.15±0.02	ND	ND
Laminarin	<0.01	0	2.95±0.35	ND	0.10±0.02
β-Glucan	<0.01	0	2.80±0.30	0.50±0.10	0.05±0.01
Starch	0.03±0.01	0.02±0.005	5.25±0.50	2.60±0.30	0.85±0.10
Colloidal chitin[b]	0.17±0.03	0.09±0.02	5.25±0.55	ND	0.10±0.02
Colloidal chitosan[c]	0.03±0.01	0.05±0.01	2.30±0.25	ND	ND
Fungal biomass[d]	0.15±0.03	0.06±0.01	3.50±0.40	ND	0.35±0.04
Fungal biomass	0.11±0.02	0.24±0.03	6.10±0.67	6.00±0.55	1.20±0.15
Colloidal chitin + fungal biomass[e]	0.20±0.03	0.35±0.04	8.15±0.90	ND	1.05±0.11

Note: ND, activity not determined.

[a]Concentration was 0.5% (*m*/*v*) except for the mixture of colloidal chitin and fungal biomass, which was 0.25%.

[b]Prepared from coarse crab shell chitin (Sigma-Aldrich, St. Louis, Missouri).

[c]Degree of acetylation of colloidal chitosan was not more than ~5%.

[d]Macerated mycelia from *Fusarium culmorum*.

[e]Macerated mycelia from crushed fruit bodies of *Macrolepiota procera*.

Table 3. Inhibition of fungal growth by enzyme mixture secreted by *Paenibacillus ehimensis* IB-X-b in solid and liquid media containing starch and chitin.

	Diameter of inhibition zone (mm)[a]		Growth inhibition (%)[b]	
Fungal species	Starch	Chitin	Starch	Chitin
Alternaria alternata	19±1	18±1	70±6	60±7
Aspergillus glaucus	10±0.5	9±1	50±5	35±5
Aspergillus niger	8±0.5	7±0.5	5±1	10±1
Drechlera sorokiniana	22±2	20±1	80±10	70±8
Fusarium culmorum	10±0.5	11±0.05	45±5	35±5
Fusarium moniliforme	16±1	15±1	25±4	20±4
Fusarium oxysporum	11±0.5	10±0.05	45±5	50±6
Fusarium solani	9±0.5	8±0.05	50±5	40±5
Paecilomyces variotii	24±2	22±1	65±10	35±5
Penicillium variabile	14±1	12±1	ND	ND
Rhizoctonia solani	8±0.5	9±0.5	>95	>95
Trichoderma viride	19±1	21±1.5	ND	ND

Note: Values are means ± standard deviations. ND, activity not determined.

[a]Solid assay was performed by inoculating 50 μL of concentrated crude enzyme mixture onto Czapek agar plates with 3-day-old fungal cultures.

[b]Liquid assay was performed by cocultivating 6-day-old fungal cultures with 1 mL of filter-sterilized (0.20 μm) enzyme solution. The following enzyme activities (U/mL) were used in solid and liquid assays, respectively: chitinase, 0.2 and 2.2; chitosanase, 0 and 1.2; β-1,3-glucanase, 8.2 and 10.4; protease, 14.0 and 24.0; lipase, 3.8 and 10.4; and total protein, 7.1 and 12.3 mg/mL.

Table 4. Growth inhibition of *Paecilomyces variotii* by secreted compounds isolated from cocultures of *Paenibacillus ehimensis* IB-X-b with fungi.

	Diameter of inhibition zone (mm)[b]	
Fungal species used in cocultivation[a]	M_r < 30 kDa	M_r ≥ 30 kDa
Aspergillus niger	8±0.5	0
Drechlera sorokiniana	8±0.5	0
Fusarium culmorum	0	5±0.5
Fusarium moniliforme	0	16±2
Paecilomyces variotii	11±1	6±0.5

Note: Values are means ± standard deviations. M_r, molecular mass.

[a]Fungi were grown on plates for 2 days at 28 °C before inhibition assay.

[b]Two fractions (<30 and >30 kDa) were isolated from cocultures by ultrafiltration and precipitated with ammonium sulfate. Precipitates were dialyzed against MQ-water and used for inhibition analysis.

ilar to the action of cocultivation with the same fungal species (Fig. 1). Therefore, the results suggest that the fungal growth inhibition was, at least partially, due to the disrupting effects of the lytic enzymes.

Fungal cell wall-degrading activity of crude enzyme mixture and purified chitinase and β-1,3-glucanase

Enzymatic digestion of the cell walls of intact and disrupted fungal mycelia was compared by measuring soluble products released from the cell walls. The crude enzyme mixture was effective in releasing reducing sugars from many fungi; the most susceptible species were *R. solani*, *Paecilomyces variotii*, and *T. viride* (Fig. 3). Among purified enzymes, chitinase and β-1,3-glucanase alone displayed high cell wall-degrading activities, and their activities accounted for 50%–60% of the activity of the crude enzyme preparation containing similar activities of these enzymes (Fig. 4). The most likely explanation for that result is a synergistic (cumulative) effect of several enzymes in the culture supernatant. The activity of the 2 purified enzymes together against intact mycelia was higher than the sum of separate enzymes, implying that they had differential targets (Fig. 5). In addition, the lytic activity seemed to be independent of fungal species, since similar effects were observed with 3 fungal species (Figs. 3 and 5). The results suggest that chiti-

Table 5. Chitinase and β-1,3-glucanase activities in supernatant (fraction ≥30 kDa) isolated from 6-day-old cocultivation of *Paenibacillus ehimensis* IB-X-b with fungi.

	Control[a]		Mixed culture	
Fungal species	Chitinase[b]	β-1,3-Glucanase[c]	Chitinase[b]	β-1,3-Glucanase[c]
Aspergillus niger	0.02±0.005	0.01±0.004	0.13±0.02	0.03±0.005
Drechlera sorokiniana	0.03±0.005	0	0.12±0.01	0.14±0.02
Fusarium culmorum	0.06±0.01	0.01±0.005	0.13±0.01	0.08±0.01
Fusarium moniliforme	0.05±0.01	0.01±0.005	0.13±0.02	0.17±0.02
Paecilomyces variotii	0.05±0.01	0.01±0.003	0.13±0.01	0.15±0.02

Note: Values are means ± standard deviations.

[a]Fungal culture cultivated in potato dextrose broth served as a control.

[b]Chitinase was measured using *p*-NP-$(GlcNAc)_2$ as the substrate (μmol·(L of liberated *p*-nitrophenyl)$^{-1}$·mL^{-1}·min^{-1}).

[c]β-1,3-Glucanase (U·mL^{-1}) was measured using 0.2% laminarin as the substrate (Helistö et al. 2001).

Fig. 2. Mycolytic action of crude enzyme mixture secreted by *Paenibacillus ehimensis* IB-X-b on mycelia of *Paecilomyces variotii* (B) and *Trichoderma viride* (D). As controls (for *P. variotii* (A) and *T. viride* (C), respectively), fungi were incubated in the absence of enzyme mixture. Mycelia were examined with a phase-contrast microscope after incubation for 24 h at 28 °C. The arrows indicate the sites of lysis in hyphae (B and D). The bar represents ~50 μm.

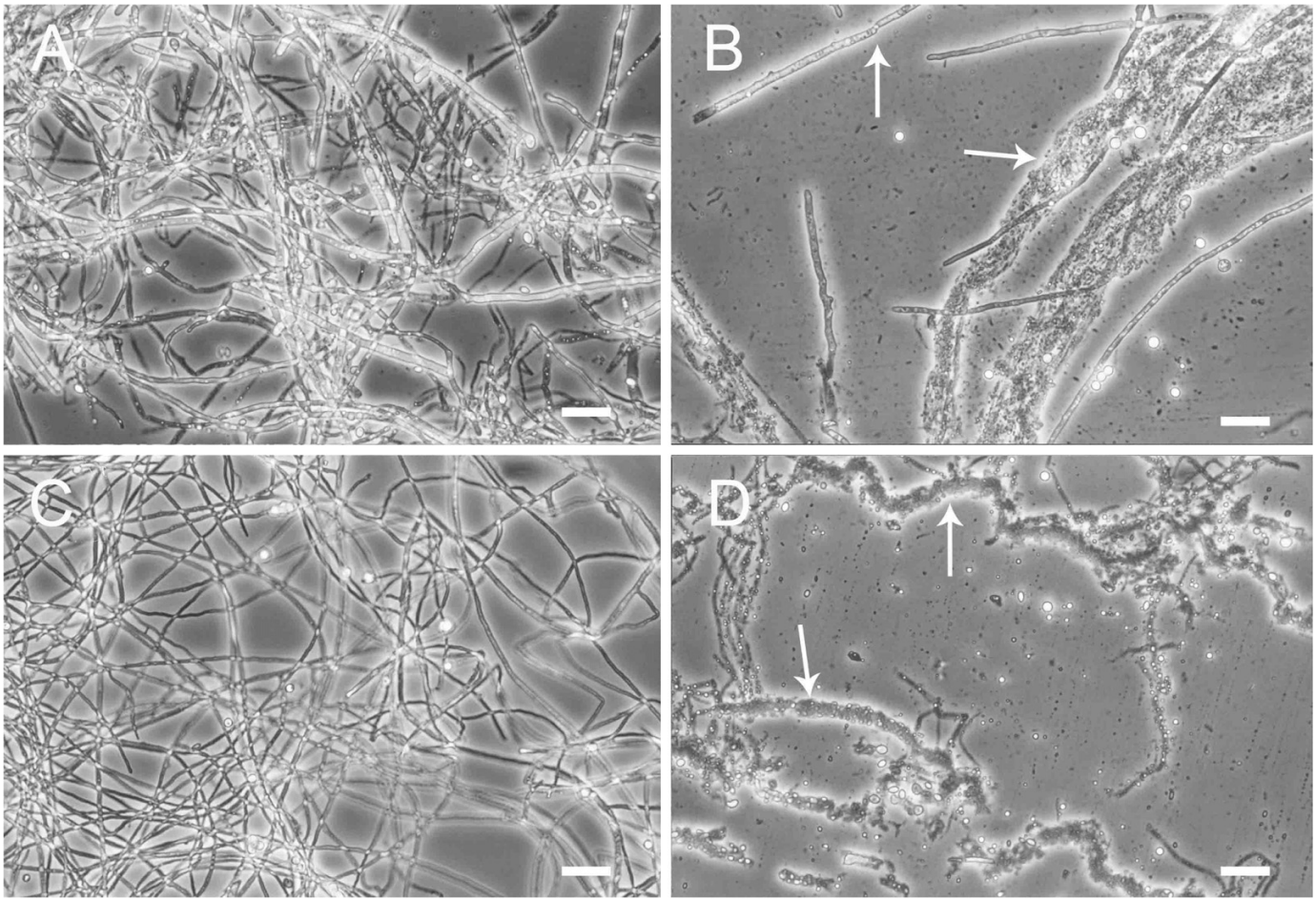


Fig. 3. Average rates of hydrolysis of fungal cell walls by crude enzyme mixture secreted by *Paenibacillus ehimensis* IB-X-b. The reducing sugars released during reaction between lytic enzymes and fungi were determined by a modified Schale's method (Imoto and Yagishita 1971). Native mycelia were incubated for 6 h at 37 °C in sodium phosphate buffer (50 mmol/L, pH 6.0) with an enzyme solution containing partially purified chitinase (0.44 U/mL), chitosanase (0.22 U/mL), β-1,3-glucanase (2.70 U/mL), protease (0.24 U/mL), and lipase (2.80 U/mL) isolated from a crude enzyme mixture of *P. ehimensis* IB-X-b (Helistö et al. 2001).

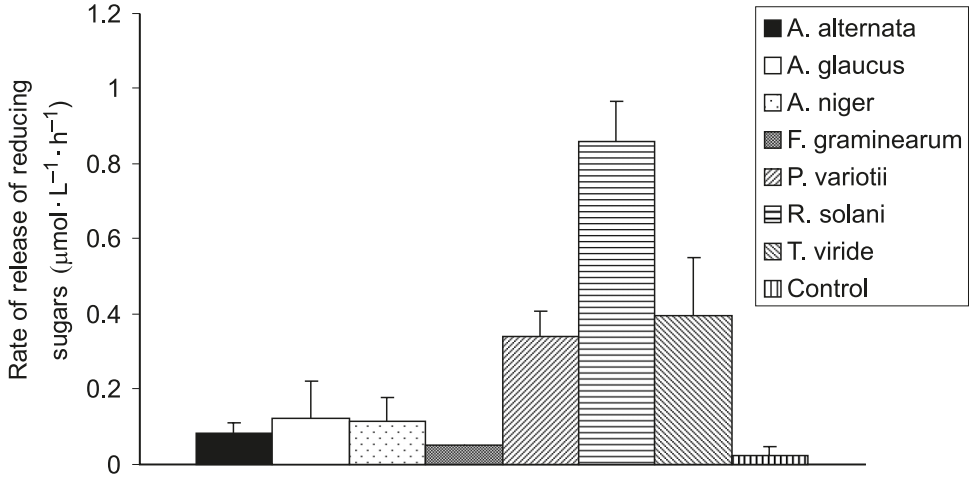
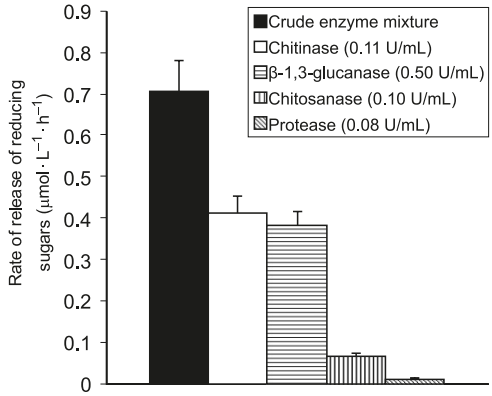


Fig. 4. Fungal cell wall-hydrolyzing activities of a crude enzyme mixture and purified chitinase, β-1,3-glucanase, chitosanase, and protease isolated from the culture supernatant of *Paenibacillus ehimensis* IB-X-b. Degraded mycelia from *Paecilomyces variotii* were used as substrates. The crude enzyme mixture was the same as in Fig. 3 and was used as a 5-fold dilution.

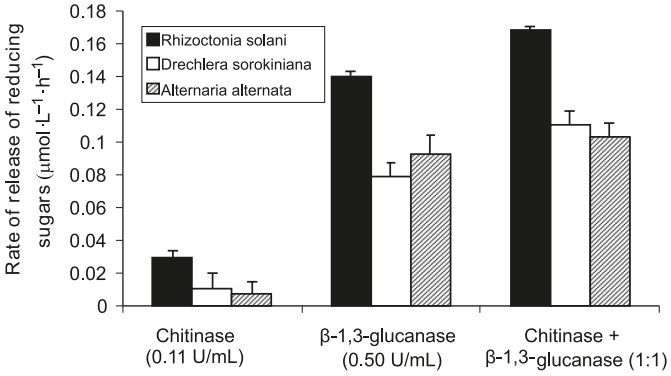


nase and β-1,3-glucanase function in asynergistic manner when bacteria attack fungi; β-1,3-glucanase degrades outer cell walls, whereas chitinase is more effective after the disruption of the mycelial surface.

Discussion

Biological control using bacterial antagonists of pathogenic fungi is an alternative to the use of chemical compounds in plant disease control. However, to exploit the full potential of biocontrol agents, antifungal mechanisms operating at cellular and molecular levels should be analyzed in more detail. Bacteria secreting chitinase and other fungal cell wall-degrading enzymes are commonly considered as potential biocontrol agents against plant-pathogenic fungi.

Fig. 5. Cell wall-degrading activities of purified chitinase and β-1,3-glucanase of *Paenibacillus ehimensis* IB-X-b and their combination against intact mycelia isolated from *Rhizoctonia solani*, *Drechlera sorokiniana*, and *Alternaria alternata* as substrates. The enzyme activities are the same as in Fig. 4.



However, there seems to be no common rule for the role of bacterial lytic enzymes in antagonistic microbial interactions. Many reports support the significant role of hydrolyzing enzymes in antagonism and directly demonstrate the occurrence of antifungal activity for purified chitinase and β-1,3-glucanase in vitro (Ordentlich et al. 1988; Lim et al. 1991; Nielsen and Sorensen 1997; Pleban et al. 1997; Budi et al. 2000; San-Lang et al. 2002; Hong and Meng 2003; Chen et al. 2004; Kim and Chung 2004; Nagarajkumar et al. 2004). Instead, other studies indicate an absence of antifungal activities in bacterial chitinases and their weak involvement in antagonism (Roberts and Selitrennikoff 1988; Frändberg and Schnürer 1998).

In the present study, a novel *P. ehimensis* IB-X-b with high chitinolytic activity almost completely suppressed the growth of several plant-pathogenic fungi in cocultivation assays. Many different enzymatic activities, which could be

involved in the fungal lysis, were detected in the bacterial growth medium. Even if the crude enzyme mixtures, induced by different compounds, may have possessed different enzyme compositions, they inhibited the growth of several plant-pathogenic and saprophytic fungi similarly. This suggests that the pattern of antifungal activity by *P. ehimensis* IB-X-b is independent of the nature of lytic enzymes. Involvement of chitinase and β-1,3-glucanase in the antifungal activity of *P. ehimensis* IB-X-b was further demonstrated by increased specific activities in cocultures and the degradation of fungal hyphae by purified enzyme preparations. The induction of hydrolyzing enzymes in cocultures was independent of fungal species (Table 5). In accordance to many previous studies, this indicates that chitinase and β-1,3-glucanase can be involved in a wide-range antagonism against various fungal species.

Nielsen and Sorensen (1997) reported 2 different strategies for enzymes in medium-independent antagonism by bacilli. The strategies assume either the constitutive production of cell wall-degrading enzymes or the induction of a repertoire of hydrolases substituting for each other in different media. As *P. ehimensis* IB-X-b was effective against a wide range of fungal pathogens, it is, therefore, tempting to speculate on the actual antifungal mechanism(s). One factor is the fast bacterial growth rate in the presence of various carbon sources and the ability to compete for nutritional resources, especially in poor soil habitats. This may be accompanied by the activity of hydrolyzing enzymes such as chitinase and β-1,3-glucanase, which not only degrade fungal cell walls liberating carbon source but also cause mechanical damage to the fungal structures. While β-1,3-glucanase was constitutively produced by the bacteria in the presence of various carbon sources, chitinase was induced only in the presence of chitin substrates. This suggests that β-1,3-glucanase is the primary lytic factor of *P. ehimensis* IB-X-b. Consequently, the results suggest that these enzymes have a dualistic function in the life cycle of *P. ehimensis* IB-X-b; supplementation of primary nutrients to the bacteria and antifungal activities, which provide the means to compete for limited carbon sources in soil (De Boer et al. 1998).

It is evident that chitinolytic and glucanolytic enzymes play a major role in antifungal activity of *P. ehimensis* IB-X-b against pathogenic fungi (Nielsen and Sorensen 1997; Budi et al. 2000). Chitinolytic enzymes are well-known for their ability to degrade fungal hyphae (Horikoshi and Iida 1958; Skujins et al. 1965; Tominaga and Tsujisaka 1975; Gupta et al. 1995). In agreement with this, the crude enzyme mixture as well as purified enzymes secreted by *P. ehimensis* IB-X-b were capable of degrading cell walls of various fungal species (Figs. 3–5). However, only purified β-1,3-glucanase effectively hydrolyzed the cell walls of intact mycelia (Fig. 5). This was coincidental with the fact that the antifungal effects of β-1,3-glucanase activity could be detected in the enzyme mixture secreted by *P. ehimensis* IB-X-b grown on any carbon source (Table 3). Evidently, β-1,3-glucanase plays the major role in the initial stage of enzymatic disruption of fungal cell walls because β-1,3-glucan is a common component of the outer layers of cell walls, whereas chitin fibrils form inner layers making them less accessible to chitinolytic enzymes (Peberdy 1990). Purified chitinase and β-1,3-glucanase showed a synergism in the degradation of intact fungal cell walls, which is also supported by previous data (Lim et al. 1991; Nagarajkumar et al. 2004). In support of the present results, San-Lang et al. (1999) also suggested that the combined action of enzymes and other antifungal compounds have an essential role during the degradation of hyphae by *Pseudomonas aeruginosa* K-187.

While it was demonstrated that there is a differential expression pattern for chitinase and β-1,3-glucanase on different carbon sources, whether or not antifungal action was solely caused by activities of lytic enzymes is not known. The highest production of the enzymes was observed in media containing complex polysaccharide carbon sources while they were produced by the bacteria only in small quantities in cocultures in PDB, (Tables 2 and 5, respectively). Since considerable antifungal effect was still detected in cocultures (Table 1), the possibility that unidentified antifungal compound(s) (Katz and Demain 1977; Stein 2005) are secreted into growth, cannot be excluded. Nevertheless, it is evident from these findings that *P. ehimensis* IB-X-b displays a wide-ranged and medium-independent antagonism against various soil fungi and produces significant amounts of chitinase and β-1,3-glucanase, which are involved in fungal cell wall lysis. Primarily, medium-independent antagonism is based on constitutive production of β-1,3-glucanase, which is the key lytic factor in degradation of fungal cell walls by *P. ehimensis* IB-X-b. Expression of chitinase and its activity on degraded mycelia contributes to the pattern of antifungal activity of *P. ehimensis* IB-X-b. The overall wide-range antifungal activity of *P. ehimensis* IB-X-b makes it an attractive candidate for further biocontrol studies against plant-pathogenic fungi.

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