Journal of Applied Microbiology 2003, **94**, 159–166

An antifungal compound produced by *Bacillus subtilis* YM 10–20 inhibits germination of *Penicillium roqueforti* conidiospores

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2002/114: received 18 March 2002, revised 11 September 2002 and accepted 17 September 2002

ABSTRACT

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Aims: To identify and characterize an antifungal compound produced by *Bacillus subtilis* YM 10-20 which prevents spore germination of *Penicillium roqueforti*.

Methods and Results: The antifungal compound was isolated by acid precipitation with HCl. This compound inhibited fungal germination and growth. Identification by HPLC and mass spectrometry analysis showed high similarity to iturin A. Permeabilization and morphological changes in *P. roqueforti* conidia in the presence of the inhibitor were revealed by fluorescence staining and SEM, respectively.

Conclusions: The iturin-like compound produced by *B. subtilis* YM 10-20 permeabilizes fungal spores and blocks germination.

Significance and Impact of the Study: Fluorescence staining in combination with flow cytometry and scanning electron microscopy are efficient tools for assessing the action of antifungal compounds against spores. Iturin-like compounds may permeabilize fungal spores and inhibit their germination.

Keywords: antifungal, *Bacillus subtilis*, germination, inhibition, lipopeptides, spores.

INTRODUCTION

Penicillium roqueforti is a common fungal contaminant of bakery and silage products. It may produce several mycotoxins, such as roquefortine C, isofumigaclavine A and B, PR toxin and mycophenolic acid, which can cause economic losses and may affect human health (Samson *et al.* 1995).

The approaches that have been used to control fungal contamination in food are usually based on heat treatment or chemical treatment (Samson *et al.* 1995; Pitt and Hoching 1997). The antifungal food additives commonly used as preservatives are inorganic compounds, e.g. sulphite and nitrite, or weak organic acids, such as acetic, propionic, sorbic and benzoic acid (Kabara and Eklund 1991; Holyoak *et al.* 1996; Stratford and Anslow 1998). Alternatively, antifungal agents produced by micro-organisms may be used as biocontrol agents. In particular, *Bacillus subtilis* is known to produce a number of antifungal compounds including Alboleutin, Bacitracin, Botrycidin, Clorotetain, Fengycin, Iturins and Rhizocticins (Zuber *et al.* 1993). These antifungal peptides inhibit the growth of a large number of fungi, such as *Aspergillus*, *Penicillium* and *Fusarium* species (Munimbazi and Bullerman 1998), as well as yeasts, i.e. *Candida albicans* and *Saccharomyces cerevisiae* (Besson *et al.* 1979; Quentin *et al.* 1982; Latoud *et al.* 1990; Thimon *et al.* 1995). Whereas most of these antifungals have been tested against mycelial growth, very little information is available about their effect on fungal spore survival and germination.

Penicillium produces conidia as agents of reproduction, dispersal and/or survival. Conidia can survive for a long

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time under environmental stress, and outgrowth in food can result in food spoilage and, potentially, in the production of mycotoxins. Since germination is the starting event of the asexual life cycle of this fungus, the antifungal compounds should preferably prevent germination.

Microscopic assessment of germination has long been the standard method for determining the viability of fungal conidia. However, such germination assays are time-consuming. Fluorescence methods using probes such as carboxyfluorescein diacetate (cFDA) and propidium iodide (PI) have been used to evaluate conidial viability (Schading *et al*. 1995). cFDA is an enzyme activity probe that can pass through the membrane of the spores. Once inside the spores, it is cleaved by non-specific esterases to release the fluorescent carboxyfluorescein, which is retained inside the spores. Thus, viability can be correlated with the ability of the spores to accumulate carboxyfluorescein (Gahan 1984). PI is a nucleic acid probe that is supposed to cross only through damaged membranes in cells (Brul *et al*. 1997). Fluorescent labelling in combination with flow cytometry (FCM) allows fast measurement of individual cells for viability assessment (Brul 1997; Bunthof *et al*. 2001). Several methods have been developed using FCM to assess the effects of antibacterial agents on different cellular characteristics or parameters, including membrane permeability, membrane potential and respiration (Novo *et al*. 2000).

In this paper, the isolation, characterization and identification of an antifungal compound produced by *Bacillus subtilis* YM 10-20 is described. HPLC and mass spectrometry analysis revealed the compound to be similar to iturin A. Using fluorescence probes in combination with FCM, and using scanning electron microscopy (SEM), it is shown that this compound efficiently permeabilizes and disrupts fungal spores.

MATERIALS AND METHODS

Bacterial culture

Bacillus subtilis strain YM 10-20 (LU 1004 – NRRL B 23189), isolated from pre-harvest maize (Nout *et al*. 1997), was maintained on Plate Count Agar (PCA) slants and stored at 4°C. The inoculum for production of antifungal compounds was prepared by growing the cells in brain heart infusion (BHI) medium on a rotary shaker at 130 rev min^{-1} at 30°C for 48 h.

Fungal suspension

Penicillium roqueforti LU 510 was isolated from sugar beet press pulp (Nout *et al*. 1993) and was grown on malt extract agar medium (MEA; Oxoid) at 25°C for a maximum of 7 days. A spore suspension was prepared by adding 9 ml peptone physiological salt solution (8·5 g l^{-1} NaCl with 1 g l^{-1} bacteriological peptone (Oxoid) + 0·1% Tween 80) to the agar slant. Subsequently, the suspension was filtered through a 17 μm nylon filter, centrifuged twice at 4000 *g* for 3 min, and the pellet containing the spores resuspended in malt extract broth (MEB) medium (Oxoid), adjusted to pH 4·0 with lactic acid. The concentration of spores was determined using a haemocytometer and adjusted to $1{\cdot}0 \times 10^6$ conidia per ml.

Production and isolation of antifungal compounds from *B. subtilis* YM 10-20

The method of Arrendale *et al*. (1988) with some modifications was used to extract the antifungal compound produced by *B. subtilis* strain YM 10-20. *Bacillus subtilis* cells grown in BHI were harvested after 48 h of incubation, final $O.D._{600}$ approximately 3·5, and centrifuged at 4000 *g* for 15 min at 20°C. The supernatant fluid was filtered through a sterile 0·2 μm poreny size filter. Subsequently, the pH of the supernatant fluid was adjusted to 2 with concentrated HCl. After centrifugation at 16200 *g* for 10 min at 20°C, the precipitate was collected and dissolved in methanol:water (50:50 v/v) pH 8, filtered through 0·2 μm membrane filter, and then stored at − 20°C.

Antifungal activity of *B. subtilis* YM 10-20

The supernatant fluid of *B. subtilis* was tested for antagonistic activity against *P. roqueforti*, *Aspergillus niger*, *A. parasiticus*, *A. flavus*, *Mucor* sp., *Fusarium culmorum* and *Rhizopus* sp. Agar plates were prepared by mixing 50 ml MEA medium with spore suspensions to obtain final concentration of 10^4 spores per ml of each fungus. Two wells, 8 mm in diameter, were made in each plate; the bottom of the wells were sealed by filling with 80 μl 2% bacteriological agar. Subsequently, the wells were filled with 200 μl of *B. subtilis* strain YM 10-20 supernatant fluid or HCl precipitate suspended in methanol:water (50:50 v/v). The methanol:water (50:50 v/v) solution was used as a control. The plates were incubated for 3 days at 30°C and subsequently, the diameter of the inhibition zone was measured. All experiments were carried out with two replicates per fungus.

Germination test

A volume of 500 μl of the *P. roqueforti* LU 510 spore suspension of $1{\cdot}0 \times 10^6$ conidia per ml was centrifuged, and the pellet containing the spores was resuspended in 500 μl medium (MEB) pH 4 in the presence of 10, 25 and 50% of the supernatant fluid of *B. subtilis* YM 10-20. As a control, the pellet was resuspended in 500 μl MEB with

sterile distilled water (50:50 v/v) pH 4. After incubation at 25°C for 8 h, 100 conidia of *P. roqueforti* were analysed with an Olympus Optical CO microscope at 1000× magnification (Ltda BX40, Tokyo, Japan) and the percentage of germinated conidia of each suspension was calculated. A conidium was considered germinated if the germ tube was longer than one-half of the diameter of the conidium. The HCl precipitate of *B. subtilis* was also used for the germination test at identical conditions and concentrations as those presented above. The experiment was performed in triplicate, and the results of a typical experiment are presented.

Identification by HPLC and mass spectrometry analysis

HPLC analysis was performed by injecting 50 μl of the extracted material of *B. subtilis* on a Spherisorp ODS-2 column, 4·6 mm × 250 mm (Chrompack, Bergen op Zoom, the Netherlands) and monitoring at 214 and 280 nm. Elution (0·9 ml min^{-1}) was performed in a linear gradient in methanol:water (50:50 v/v) during 0–20 min, methanol:water (80:20 v/v) during 20–60 min, 100% methanol during 60–65 min and methanol:water (50:50 v/v) during 65–75 min. Individual fractions of the HCl precipitate of *B. subtilis* were collected manually and were subsequently tested for antagonistic activity against *P. roqueforti* as described previously. The mass spectrometry analysis was performed on a Perseptive Biosystems Voyager DE^{TM}-RP matrix assisted laser desorption/ionization (MALDI) – Time of flight (TOF) (Basingstoke, UK) to determine the molecular weight of the compounds. Three matrices were applied in this study: sinapinic acid (proteins and peptides mol. wt > 10 000 Da), α-cyano-4 hydroxy cinnamic acid (proteins and peptides mol. wt < 10 000 Da) and 2,4,6-trihydroxiacetophenone (THAP) (peptides). Purified iturin A was kindly provided by Prof. Dr Françoise Peypoux (Laboratoire de Biochimie Analytique et Synthese Bioorganique, Lyon, France).

Monitoring germination capacity

The spore suspension of *P. roqueforti* at a concentration of 10^6 spores per ml was incubated in the presence of *B. subtilis* supernatant fluid for 1, 2 or 3 h, and also in the presence of *B. subtilis* HCl precipitate for 2 h. Subsequently, the spores were harvested by centrifugation at 2500 *g* for 3 min. The pellet was washed in MEB and incubated for 10 h. Each sample was observed every 2 h under the microscope to determine the percentage of spore germination. The control used in the HCl precipitate solution consisted of spores suspended in MEB medium with methanol and water (50:25:25 v/v/v).

SEM analysis

Samples of fungal mycelium were removed from the border of the inhibition zone and transferred to a Nuclepore polycarbonate filter with 1 μm pores (Costar, Cambridge, MA, USA). These filters are glued with Tissue Freezing Medium (Electron Microscopy Sciences, Washington D.C., USA) on a brass specimen holder. The specimens were frozen in liquid nitrogen and subsequently transferred to the cryo-preparation chamber (Oxford CT 1500 HF, Oxford Instruments, High Wycombe, UK), which was dedicated to FESEM. Inside the cryo-preparation chamber, the sample was kept for 3 min at −95°C to sublime water contamination. The samples were coated at −95°C with 5 nm platinum by magnetron sputtering and observed in an FESEM (JSM 6300F, Tokyo, Japan) at −180°C and 5 kV. Digital images were recorded.

Fluorescence of conidiospores

The fluorescent probes, cFDA and PI (Molecular Probes, Eugene, OR, USA) were applied to assess the viability of *P. roqueforti* conidiospores. Spores were labelled with cFDA and PI. A stock solution of cFDA was prepared in acetone (4·6 mg ml^{-1}) and stored at −20°C in the dark. A stock solution of PI (1·0 mg ml^{-1}) was prepared in distilled water and stored in the refrigerator. Conidiospores of *P. roqueforti* LU 510 at a concentration of 10^6 spores per ml were incubated for 2 h in the absence and presence of 1·0 ml of HCl precipitate of *B. subtilis* YM 10-20. Samples containing 1·0 ml of spore suspension of *P. roqueforti* were incubated with cFDA and PI at a final concentration of 10 μmol l^{-1}. The samples were incubated for 15 min at 25°C and subsequently, the staining of the conidiospores was determined microscopically. The Axioskop epifluorescence microscope (Carl Zeiss, Oberkochen, Germany) was equipped with a 50 W mercury arc lamp, a fluorescein isothiocyanate filter set (excitation wavelength of 450–490 nm, emission wavelength >520 nm) and a Plan-Neofluar objective lens.

FCM analysis

Analysis of individual cells was performed with a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) equipped with an air-cooled argon ion laser at 15 mW (excitation wavelenth 488 nm). Conidiospores of *P. roqueforti* LU 510 at a concentration of 10^7 spores per ml were incubated for 3 h in the absence and presence of 1·0 ml of HCl precipitate of *B. subtilis* YM 10-20. Subsequently, samples were diluted to 10^6 spores per ml in 50 mmol l^{-1} KP_i buffer (pH 7·0) and delivered at a low flow. The instrument was set up to collect

five parameters: forward scatter (FSC), side scatter (SSC) and three fluorescent signals. A band pass filter of 530 nm (515–545 nm) was used to collect the green fluorescence (FL-1), a band pass filter of 585 nm (564–606 nm) was used to collect the yellow–orange fluorescence (FL-2) and a long pass filter of 670 nm was used to collect the red fluorescence (FL-3). FSC was collected with a diode detector. SSC and the three fluorescence signals were collected with photomultiplier tubes. All signals were collected using logarithmic amplifications. Data were analysed with the CELLQuest program (version 3·1f; Becton Dickinson) and the WinMDI program (version 2·8; Joseph Trotter, John Curtin School of Medical Research, Canberra, Australia). The sample analysis time was 1 min and approximately 5000 particles were analysed.

RESULTS

Inhibition of germination and fungal growth

The supernatant fluid of *B. subtilis* strain YM 10-20 inhibited growth of *P. roqueforti*, *Aspergillus niger*, *A. parasiticus*, *A. flavus*, *Mucor* sp. and *F. culmorum*, whereas growth of *Rhizopus* sp. was not affected. Analysis of the inhibition zone diameters revealed *F. culmorum* to be the most sensitive fungus, followed by *P. roqueforti* and *A. niger* (data not shown).

The germination efficiency of *P. roqueforti* spores after 8 h of incubation in the absence of *B. subtilis* strain YM 10-20 supernatant fluid was 84% (Fig. 1). In the presence of 10, 25 and 50% of supernatant fluid, the percentage of germination decreased to 7, 1 and 0%, respectively.

Penicillium roqueforti was chosen as the model organism to test the inhibitory activity of the HCl precipitate of the *B. subitilis* YM 10-20 supernatant fluid. The diameter of the inhibition zone of the HCl precipitate and that of the supernatant fluid was 25 and 17 mm, respectively. The inhibitory zones remained the same for several days (data not shown).

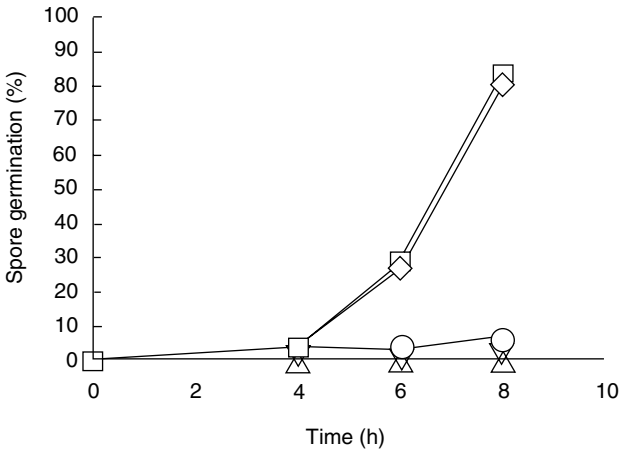

Fig. 1 Germination of *Penicillium roqueforti* in (□) malt extract broth (MEB), (◇) MEB/distilled water (50:50 v/v) and MEB containing 10% (○), 25% (▽) and 50% (△) of supernatant fluid of *Bacillus subtilis* YM 10-20

Effect of temperature and cholesterol on antifungal activity

The antifungal compound produced by *B. subtilis* YM 10-20 was shown to be heat stable. After heat treatment at 70°C and 100°C for 1 h, growth of *P. roqueforti* was still inhibited and the inhibitory zones were 18 and 8 mm, respectively (data not shown). Furthermore, the antifungal compound was found to be resistant to proteolytic enzymes, including Pronase E, proteinase K and α chymotrypsin (data not shown).

The antifungal activity was also tested in the presence of cholesterol (Sigma). After 9 h of incubation, the percentage of spore germination of *P. roqueforti* in the control (MEB:water [50:50 v/v]) was 90%, whereas it was completely inhibited in the presence of the antifungal compound (MEB:supernatant fluid [50:50 v/v]). However, addition of cholesterol (15 μg ml^{-1}) resulted in the latter case in 61% of spore germination, which points to a significant reduction of the antifungal activity under these conditions. Germination of *P. roqueforti* in the control was not affected by cholesterol (data not shown).

Identification of the HCl precipitate

Analysis of the HCl precipitate by HPLC showed one fraction that produced a clear inhibition zone against growth of *P. roqueforti*. Comparison of the HPLC profile of the HCl precipitate of strain YM 10-20 with that of purified iturin A revealed similar retention times and peak heights (data not shown). The mass spectrum of the compound of *B. subtilis* YM 10-20 showed homologous molecular ion peaks differing by 14 mass units at m/z 1029 and 1043. Other homologous peaks were observed at 1068 and 1082. The difference of 22 mass units in the molecular ion peaks at m/z 1046 and 1068, and peaks at 1060 and 1082, indicates the presence of salt, i.e. sodium ions. A difference of 17 mass units in the molecular ion peaks at m/z 1029 and 1046, and peaks at 1082 and 1098, was also found. Comparison of the mass spectrum of the HCl precipitate with that of purified iturin A showed identical molecular ion peaks (1029, 1043, 1046, 1060, 1068, 1074 and 1082) (Fig. 2), from which it is concluded that the antifungal compound of *B. subtilis* strain (HCl precipitate) is highly similar to iturin A.

SEM and fluorescent probes

SEM analysis showed major damage of the hyphae and the spores of *P. roqueforti* in the presence of the HCl precipitate

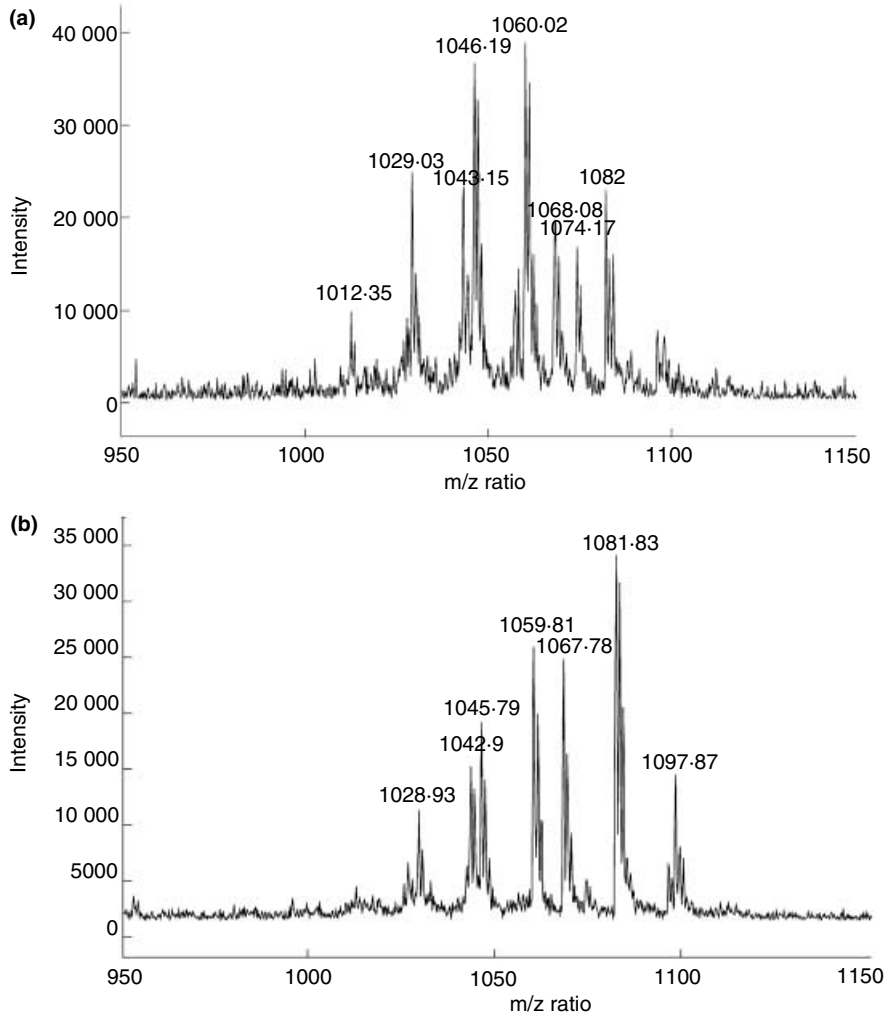

Fig. 2 Mass spectrometry analysis (MALDI-TOF) of iturin A (a) and the HCl precipitate of *Bacillus subtilis* YM 10-20 (b)

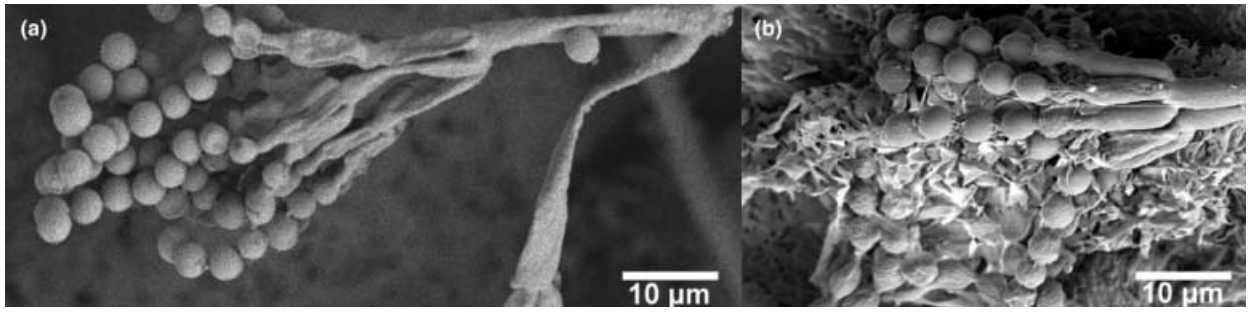

Fig. 3 Scanning electron microscopy of *Penicillium roqueforti* grown on malt extract agar in the absence (a) and presence (b) of *Bacillus subtilis* YM 10-20 supernatant fluid

(Fig. 3). Furthermore, germination of *P. roqueforti* was not observed after pre-incubation of the conidiospores with the HCl precipitate for 2 h followed by transfer to fresh MEB:methanol:water (50:25:25 v/v/v), suggesting irreversible damage of the conidiospores (data not shown).

To study the effect of the antifungal compound in more detail, fluorescent probes were applied to assess the membrane permeabilization and viability of the conidiospores of *P. roqueforti*. Untreated, control conidiospores could be stained with cF, but not with PI. In contrast, conidiospores

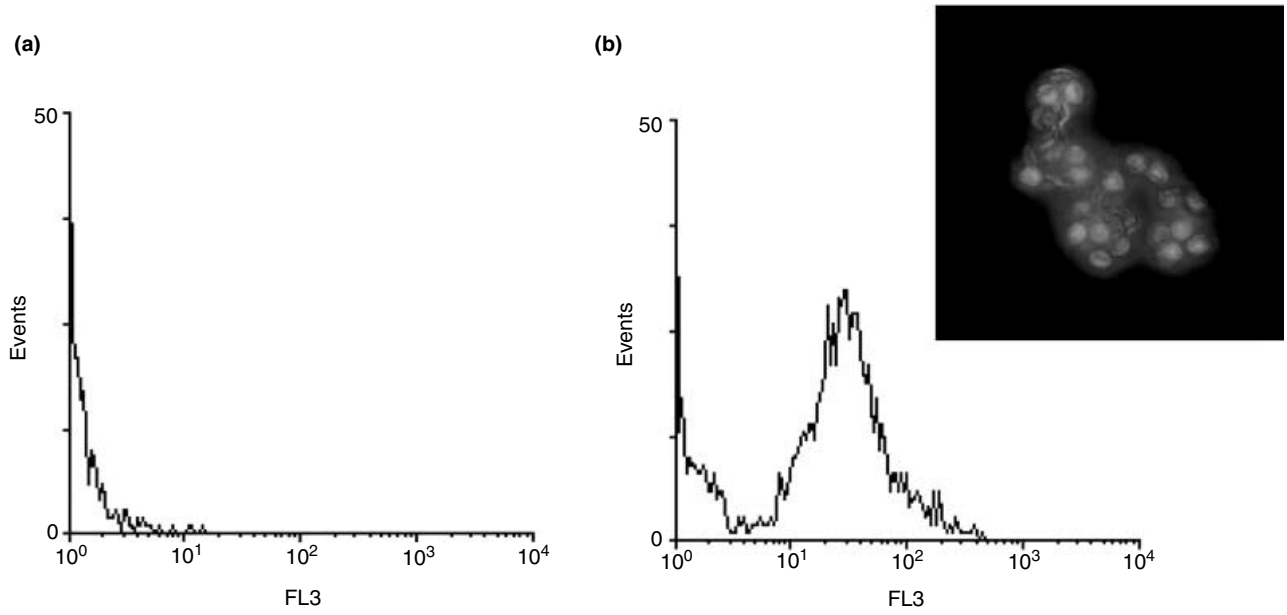

Fig. 4 Histograms of propidium iodide-stained control spores (a) and HCl precipitate-treated spores (b). The insert in (b) shows a fluorescence microscopy picture of PI-labelled spores.

treated with the HCl precipitate for 2 h could not be stained with cF, whereas they were stained with PI. This indicates that the spores were permeabilized after treatment with the HCl precipitate. Indeed, FCM results show a distinct population of PI-labelled spores (Fig. 4).

DISCUSSION

In this study, the antifungal compound produced by *B. subtilis* YM 10-20 was shown to inhibit germination and growth of *P. roqueforti* conidiospores. The antifungal compound is very heat stable, its activity is reduced in the presence of cholesterol, and it is resistant to proteolytic degradation. These characteristics indicate that the antifungal compound may belong to the iturin group of antibiotics, which are known to interact with sterols of the cytoplasmic membrane of fungi (Maget-Dana *et al.* 1985, 1992; Latoud *et al.* 1990). Most of the known antifungal agents produced by *B. subtilis* are polypeptides (Babad *et al.* 1952; Besson *et al.* 1976; Pusey 1989; Munimbazi and Bullerman 1998), including iturins A–E, bacillomycins D, F and L, and mycosubtilin (Bland 1996). Recently, Moyne *et al.* (2001) described the antifungal action of two bacillomycin D variants. Iturins are cyclic lipopeptides characterized by the presence of seven α-amino acids (Peypoux *et al.* 1973, 1978; Isogai *et al.* 1982; Latoud *et al.* 1990). Iturin A has been shown to increase the permeability of lipid membranes of fungal cells by pore formation, resulting in the loss of essential macromolecular compounds (Thimon *et al.* 1995).

HPLC analysis of the HCl precipitate from YM 10-20 showed that this compound is similar to iturin A. In addition, mass spectra analysis of the *B. subtilis* compound and of purified iturin A revealed a high similarity between these compounds, i.e. identical molecular ion peaks were identified, suggesting that the compound produced by *B. subtilis* YM 10-20 is iturin A.

The HCl precipitate of strain *B. subtilis* YM 10-20 totally inhibited the germination of *P. roqueforti* conidiospores. SEM analysis of *P. roqueforti* conidiospores exposed to the *B. subtilis* supernatant fluid revealed destruction and morphology changes of spores. Germination of *P. roqueforti* was also inhibited in the presence of purified iturin A (data not shown). It appears that the HCl precipitate also affects the permeability of the membrane of conidiospores, preventing germination. Under these conditions, the spores will lose the ability to initiate biochemical activities, increase metabolism and initiate morphological changes. Notably, these studies reveal that the compounds may also act on spores.

Spore inactivation was also assessed by fluorescence techniques. Fluorescence microscopy and FCM revealed PI labelling of damaged cells, indicating permeabilization of the membrane of *P. roqueforti* conidiospores after exposure to the HCl precipitate. FCM analysis is a rapid, reliable and sensitive method for assessing viability (Roth *et al.* 1997; Novo 2000; Bunthof *et al.* 2001).

According to the present studies, the antifungal compound may be effective in the control of fungal growth. However, *Rhizopus* sp. was not sensitive (Pusey 1989). This may be explained by the low ergosterol content of the

Rhizopus sp. membrane (Besson *et al*. 1979; Schnurer 1993).

In conclusion, fluorescence staining in combination with FCM and SEM are efficient tools for assessing the action of antifungal compounds against spores. Iturin-like compounds may permeabilize fungal spores and inhibit their germination. This is the first study to show that iturin-like compounds may permeabilize fungal spores and inhibit their germination.

ACKNOWLEDGEMENTS

The authors thank Edwin Bakx and Gert-Jan van Alebeek for technical assistance, and F. Peypoux for providing iturin A. They also thank Luiz G. Chitarra for critically reading the manuscript. This work was conducted with support of the Brazilian CAPES Coordenação de Aperfeiçoamento de Pessoal de Nível Superior.

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