Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Isolation, purification and characterization of an antifungal molecule produced by *Bacillus licheniformis* BC98, and its effect on phytopathogen *Magnaporthe grisea*

S.R. Tendulkar[1], Y.K. Saikumari[3], V. Patel[1], S. Raghotama[2], T.K. Munshi[1], P. Balaram[3] and B.B. Chattoo[1]

1 Department of Microbiology and Biotechnology Centre, The M.S. University of Baroda, Vadodara, India
2 Sophisticated Instrumentation Facility, Indian Institute of Science, Bangalore, India
3 Molecular Biophysics Unit, Indian Institute of Science, Bangalore, India

Keywords
antagonist, *Bacillus licheniformis* BC98, lipopeptide, *Magnaporthe grisea,* surfactin.

Correspondence
Bharat B. Chattoo, Department of Microbiology and Biotechnology Centre, The M.S. University of Baroda, Vadodara 390002, India. E-mail: chattoo@bcmsu.ac.in

2006/1192: received 21 August 2006, revised 14 April 2007 and accepted 12 May 2007

doi:10.1111/j.1365-2672.2007.03501.x

Abstract

Aims: Isolation of bacterial antagonist for use in the biological control of phytopathogenic fungi like rice blast fungus, *Magnaporthe grisea*, and to further purify and characterize the antifungal molecule produced by the antagonist.
Methods and Results: Bacterial antagonist exhibiting highest antifungal activity against the rice blast fungus *M. grisea* was isolated from soil and identified as *Bacillus licheniformis* BC98. Besides *M. grisea*, the isolate also inhibited the growth of other phytopathogens such as *Curvularia lunata* and *Rhizoctonia bataticola*. Biologically active fractions were isolated from the culture filtrate and further fractionated by reverse-phase high-performance liquid chromatography (HPLC) enabling detailed structural characterization of a component of molecular mass 1035 Da. The active peptide was identified as surfactin after 500 MHz ^{1}H NMR analysis. Microscopic analysis of the effect of the antagonist on *M. grisea* revealed bulbous hyphae showing patchy and vacuolated cytoplasm when observed under the electron microscope.
Conclusions: The antagonistic lipopeptide secreted by *B. licheniformis* BC98 and identified as surfactin, induced morphological changes in *M. grisea*, inhibiting its further growth, and thus exhibiting fungicidal activity.
Significance and Impact of the Study: The antagonist inhibits germination of *M. grisea*, a potent rice phytopathogen, and therefore appears to be a potential candidate for control of rice blast disease.

Introduction

Rice is the staple diet for more than half the population of the world (Khush *et al.* 1984), and globally nearly 10–15% of rice yield is lost because of diseases caused by phytopathogens (Ou 1985). Among these diseases, rice blast caused by *Magnaporthe grisea* (Herbert) Barr (anamorph. *Pyricularia grisea*, Sacc. formerly *P. grisea*), is considered to be the most serious and widely distributed disease (Ou 1972; Mew 1991). Although efforts are being made to control the disease, alternative approaches and new bioactive compounds need to be used to control plant diseases to reduce the negative environmental impact because of the use of chemicals.

Microbial compounds exhibiting antagonistic activities include a wide variety of surface and interfacially active compounds, such as glycolipids, lipopeptides, polysaccharide–protein complexes, phospholipids, fatty acids and neutral lipids; however, among the many classes of biosurfactants, lipopeptides are particularly interesting because of their high surface activities and antagonistic potential (Georgiou *et al.* 1992; Neu 1996; Ron and Rosenberg 2001; Kim *et al.* 2004). The consequences of the interaction of these compounds with eukaryotic cells are well known and include pyrogenicity, toxicity, immunogenicity and other

© 2007 The Authors

molecular effects (Wicken and Knox 1980). Moreover, biosurfactants are easily biodegradable and less toxic than chemical surfactants, and thus are particularly suited for environmental and agricultural applications (Ron and Rosenberg 2001; Kim *et al.* 2004).

Biosurfactants are produced by a wide variety of diverse micro-organisms. Various bacterial species like *Enterobacter* (Chernin *et al.* 1995), *Pseudomonas* (Levy *et al.* 1992; Ganeshan and Kumar 2005), *Bacillus* (Javaheri *et al.* 1985; Cooper and Goldenberg 1987; Rosales *et al.* 1993; Peypoux *et al.* 1999), *Streptomyces* (Gomes *et al.* 2001) and *Erwinia* (Bryk *et al.* 1998) have been described as producers of effective biological control agents antagonistic to many fungal phytopathogens. However, data pertaining to the antagonistic activity of bacteria against *M. grisea*, a potent rice pathogen, are still restricted to only a few strains of *Pseudomonas* (Lee *et al.* 2003), *Streptomyces* (Gomes *et al.* 2001) and *Bacillus* (Cottyn *et al.* 2001). In view of this, the aim of the study was to investigate bacterial isolates from soil for their antagonistic activity for use in the biological control of phytopathogenic fungi like *M. grisea*. The bacterial antagonist was identified, and the biocontrol agent produced by the antagonist was purified, characterized and examined for fungicidal activity against *M. grisea* and other phytopathogens.

Materials and methods

Fungal strains

Sclerotium sclerotinii, Phomopsis phyllanthi, Rhizoctonia bataticola, Aspergillus niger N 573, *Curvularia lunata, Helminthosporium* sp., *Chaetomium* sp., *Fusarium verticillioides, Pestaliopsis magnifera, Gleosporium magnifera* were kindly provided by the strain collection centre of Hans-Knöll-Institut für Naturstoff-Forschung, Jena. *Magnaporthe grisea* B157 was isolated in our laboratory from infected rice leaves (Kachroo *et al.* 1994). Fungal cultures were grown and maintained on YEG medium (glucose 10 g l^{-1}, yeast extract 2 g l^{-1}) or oat meal agar (Hi Media, Mumbai, India). Fungal conidia were harvested by scraping the biomass grown on oat meal agar plates with a sterile surgical blade, resuspended in sterile water and purified by passing through glass wool column.

Isolation of bacterial antagonist

One gram of each soil sample, collected from different locations in Gujarat, India, was suspended in 10 ml of sterile water and vortexed for 45 s. The sample was serially diluted and 100 μl of each dilution was added to molten YEG agar maintained at 55°C along with 10^5 spores of *M. grisea* and poured in sterile petri dishes. After incubation at 28°C for 2–3 days, bacterial colonies showing zones of inhibition were selected and transferred to fresh YEG agar medium. Further, the bacterial antagonists were grown in Luria-Bertani broth (LB) (Hi Media) at 28°C, 200 rev min^{-1} for 48 h. The supernatant was separated by centrifugation at 13 000 ***g*** for 10 min followed by filtration through 0·45-μm filter (Millipore, Schwalbach, Germany) to remove bacteria (crude supernatant). Further, the supernatant was concentrated 10-fold by lyophilization and used for confirming antifungal activity on a test plate. Nystatin (Sigma, St Louis, MO, USA) was used as a positive control at a concentration of 5·7 U ml^{-1}. Long-term storage of bacterial isolates was performed either on LB slants or in glycerol (stored at –80°C).

Test plate for checking the activity of the bacterial antagonists was prepared by mixing *M. grisea* conidia (10^5 spores ml^{-1}) with molten YEG agar maintained at 55°C and then poured in sterile petri plates. Concentrated culture supernatant (10-fold) was added in the wells, made in YEG agar using sterile metal cylinders, and the plates were incubated at 28°C for 48 h.

For further analysis, fungal strains were inoculated (10^6 conidia) and grown in 50 ml of YEG medium for 2 days at 28°C, 200 rev min^{-1} in the presence or absence of the culture supernatant (1% v/v) of antagonist bacterial, grown in LB for 48 h at 28°C. The biomass was separated, dried at 105°C for 14 h and weighed to obtain the dry weight. The dry weight of treated biomass (fungus grown in the presence of the antagonist) was subtracted from the control biomass (fungal biomass grown in the absence of the antagonist) and the values were expressed in percentage in order to obtain reduction in biomass of the aforementioned fungal strains.

Identification of bacterial antagonist

The bacterial antagonist showing highest antifungal activity was identified by the Biolog system (Mauchline and Keevil 1991), and by 16S rRNA gene sequence analysis. Polymerase chain reaction (PCR) amplification of 16S rRNA gene was carried out using the *Eubacteria*-specific primers 19 F (5′-AGAGTTTGATCCTGGCTCA-3′) and 20R (5′-GCTCGTTGCGGGACTTATCC-3′). The thermal cycle used was as follows: initial denaturation at 94°C for 3 min followed by 30 cycles of 94°C, 30 s; 58°C, 30 s and 72°C, 1 min; in a Minicycler (MJ Research, Waltham, MA, USA). The final step included final elongation at 72°C for 5 min. Amplified PCR product was purified and sequenced with CEQ 8000 series Genetic Analysis System (Beckman Coulter, Fullerton, CA).

© 2007 The Authors

Sensitivity of the culture supernatant of *Bacillus licheniformis* BC98 to proteolytic enzymes, surfactants, heat and pH

The concentrated crude supernatant (15 μl) was subjected to treatments for 2 h at 37°C (for enzymes) or room temperature (for surfactants). The proteolytic enzymes (Sigma) were used at a final concentration of 1 mg ml^{-1} in 10 mmol^{-1} potassium phosphate buffer, pH 7·0. The concentrate crude supernatant in buffer without enzymes as well as the enzyme solutions was exposed to the same conditions. The surfactants used were sodium dodecyl sulfate (SDS), Tween 80, Triton X-100 and urea, which were used at a final concentration of 1% (v/v) and incubated at 37°C for 5 h. All surfactants (Sigma) were prepared at 10% in water and filter-sterilized before use. For the heat treatment, the preparations were incubated at 25°C or heated at 37°C, and 60°C and autoclaved (121°C, 15 min). For pH stability, 10 μl of concentrated supernatants were mixed to the same volume of citric acid–Na_2HPO_4 buffer to achieve different pH values lower than 6·0 and with Tris-HCl buffer for pH 8·0 and 10·0. Antifungal activities were checked before and after all treatments on a test plate made with *M. grisea* conidia as mentioned earlier.

Purification and characterization of bulb-inducing compound

Media and process parameters for better production and purification of antifungal molecule from *B. licheniformis* were performed as reported (Patel *et al.* 2004). For large-scale production of the antifungal molecule for purification and characterization, *B. licheniformis* BC98 was grown in CYD medium (glucose 1% w/v, casein enzyme hydrolysate 1% w/v, KH_2PO_4 0·5% w/v, yeast nitrogen base without amino acids and ammonium sulfate 0·65% w/v) for 16 h at 28°C at 200 rev min^{-1}. The bacteria were separated from the culture broth by centrifugation at 10 000 ***g*** for 10 min at 4°C. The antifungal peptides were extracted from the supernatant with chloroform : methanol (9 : 1). The combined organic extract was concentrated *in vacuo* yielding a brownish gummy residue, which was further purified by reverse-phase high-performance liquid chromatography (HPLC) (HP 1100; Hewlett-Packard, Palo Alto, CA), on a Vydac C_{18} column, 250 mm × 10 mm, 4 μm. Purification was carried out using methanol–water gradient containing 0·1% v/v TFA (triflouroacetic acid), monitored at A_{226}. A gradient of 65–75% in 20 min and 75–95% in 35 min was used with a flow rate of 1·5 ml min^{-1}. This protocol yielded 52 mg of purified molecule from 20 l of culture supernatant.

MALDI-TOF-MS (matrix assisted laser desorption/ionization time-of-flight mass spectrometry) was carried out on a Kompact SEQ spectrometer (Kratos Analytical, Manchester, UK) operating with a N_2 laser of 337 nm. All of the analyses were carried out in the positive ion mode. Spectra were averaged over 25 laser shots.

Matrix solution was prepared by dissolving α-cyano-4-hydroxycinnamic acid powder (Sigma Aldrich Chemicals Pvt. Ltd., Bangalore, India) in acetonitrile : water (1 : 1 v/v) with 0·1% (v/v) trifluoroacetic acid to obtain a saturated solution. MALDI sample plates were first spotted with 1 μl of matrix solution followed by 1 μl of sample (0·1 mg ml^{-1}). After air drying of the sample, one-dimensional (1D) and 2D nuclear magnetic resonance (NMR) experiments were carried out in CD_3OH solvent (Sigma Aldrich Chemicals Pvt. Ltd.) on a BRUKER-DRX-500 MH_Z spectrometer. All experiments were performed at 323 K as it provided a better dispersion. Peptide concentration was *c.* 4 mmol l^{-1}. Resonance assignments were performed using the experiments. The TOCSY and ROESY 2D data were collected in phase-sensitive mode. The spectral widths were set to 6000 Hz. 16 K data points were used for 1D and for 2D, sets of 1024 and 450 data points were used in t_2 and t_1 dimensions, respectively. For TOCSY and ROESY, 32 and 64 transients were collected, respectively for each increment of 2D data set. Final data matrix size after zero filling was 2 K × 1 K. A shifted square sine-bell window was used before Fourier transformation.

Detection of *in vitro* antifungal activity

In order to determine inhibitory activity of the antagonistic molecule against *M. grisea*, 100 μl of spore suspension (10^4 spores ml^{-1}) of *M. grisea* was allowed to germinate on a glass slide under high humidity in presence of 0·1, 1·0 and 10 μg ml^{-1} concentrations of purified antifungal compound. The germination of spores was allowed at 28°C for 24 h and change in morphology was monitored microscopically. The experiment was repeated twice and each time it was performed in triplicate.

Microscopy

Light microscopy images were taken with the help of Nikon eclipse E1000 equipped with DXM1200 CCD camera (Nikon, Japan). Effect of antifungal molecule on *M. grisea* spores was observed by germinating 10^3 spores in the presence or absence of 0·1 μg ml^{-1} of antifungal compound on glass slides.

For electron microscopic studies, 10^5 spores of *M. grisea* were inoculated in 50 ml of YEG medium and grown at 28°C and 200 rev min^{-1}. After 48 h, the biomass obtained was further incubated with 0·1 μg ml^{-1} of

© 2007 The Authors

bulb-inducing compound (BIC) under the aforementioned conditions for 2 h and embedded in 0·2 % w/v agarose and cut into 2-mm^2 blocks. Samples were fixed in 4% glutaraldehyde in 50 mmol l^{-1} of cacodylate buffer, pH 7·2; for 2 h, followed by three cacodylate buffer washes of 10 min each. Further fixing was performed in osmium tetroxide (1% w/v) overnight at 28°C. The samples were washed four times in buffer followed by dehydration in ethanol of increasing concentration (10%, 30%, 50%, 70%, 95%, 100%; v/v), ethanol : acetone (1 : 1) and 100% acetone. Infiltration of spur was performed in acetone, and finally sample, was embedded in fresh 100% spur at 70°C overnight. One-micron sections were observed under the microscope. Ultra-thin sections were prepared by ultramicrotome (Jeol JEM, Tokyo, Japan). The sections were mounted in 200 mesh Formvar coated copper grids (Sigma Aldrich Chemicals Pvt. Ltd.) and were stained with 1% w/v lead citrate and 2% w/v uranyl acetate and the grids were preserved under desiccation before observation under the electron microscope (JEM-2500SE).

Nucleotide sequence accession number

The nucleotide sequence of 16S rDNA of *B. licheniformis* strain BC98 has been deposited in the GenBank database under the accession number EF545137.

Results

Screening and identification of bacterial antagonist

To screen for a potential bacterial antagonist, over 150 bacterial isolates from soil were screened for their ability to inhibit the mycelial growth of rice blast fungus *M. grisea*. Eight bacterial isolates showed significant antifungal activity against this phytopathogen. Interestingly, bacterial isolate I-6 not only showed the strongest inhibitory activity against the fungus, but also induced morphological changes in *M. grisea*. The isolate was a facultative anaerobe, gram-positive, motile, endospore-former, growing at 65°C and tolerated upto 10% NaCl. Using the Biolog system and 16S rRNA gene analysis, the isolate was identified as belonging to the genus *Bacillus.* 16S rDNA sequence analysis showed that this *Bacillus* strain had 100% similarity with *B. licheniformis* (Fig. 1), and was designated as *B. licheniformis* BC98. Preliminary screening to study the spectrum of fungal growth inhibition by crude extracts from *B. licheniformis* BC98 showed that it produced a potential antagonistic compound, which had significant activity against different phytopathogenic fungi including *M. grisea* (Table 1). Furthermore, the antifungal activity was found to be highly stable at extreme pH and temperatures, and also after treatments with pepsin, trypsin and different detergents (Table 2).

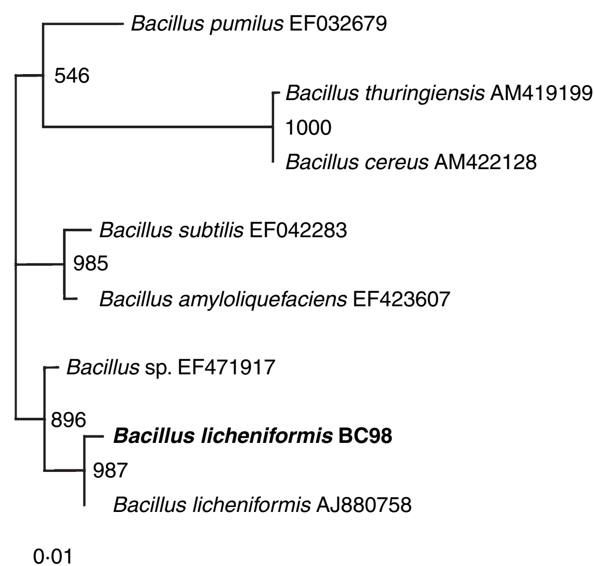

Figure 1 Neighbour-joining phylogenetic tree showing relationships between strain BC98 and several other strains of *Bacillus*, based on their 16S rDNA sequences. Numbers at nodes represent the percentages of occurrence of nodes in 1000 bootstrap trials. The scale bar represents the expected number of substitutions per nucleotide position.

Table 1 Broad-spectrum antifungal activity of culture supernatant of *Bacillus licheniformis* BC98 towards phytopathogenic fungi

Fungal phytopathogen	Reduction in growth (%)*	Diameter of inhibition zone (cm)**
Sclerotium sclerotinii	95·90 ± 2·3	2·5 ± 0·35
Phomopsis phyllanthi	83·90 ± 4·1	1·8 ± 0·23
Rhizoctonia bataticola	94·23 ± 2·6	2·3 ± 0·21
Aspergillus niger N 573	14·20 ± 1·9	1·1 ± 0·26
Curvularia lunata	98·11 ± 1·3	2·9 ± 0·41
Magnaporthe grisea	96·90 ± 2·7	2·7 ± 0·31
Helminthosporium sp.	97·64 ± 1·2	2·8 ± 0·25
Chaetomium sp.	91·10 ± 3·3	2·2 ± 0·31
Fusarium verticillioides	75·70 ± 4·2	1·7 ± 0·21
Pestaliopsis magnifera	95·90 ± 1·8	2·6 ± 0·32
Gleosporium magnefera	97·68 ± 1·3	2·6 ± 0·37

*The dry weight of treated biomass (fungus grown in the presence of antagonist) was subtracted from the control biomass (fungal biomass grown in the absence of antagonist) and expressed in percentage in order to obtain reduction in biomass. The data represents the average of three replicates.

**Data represents the average of three replications.

Purification of the antifungal peptide

Assays for isolation of antifungal antagonists was devised based on morphological changes induced in the germinating spores of *M. grisea*, leading to a number of bacterial

© 2007 The Authors

Table 2 Sensitivity of culture supernatant of *Bacillus licheniformis* BC98 to heat, pH, protease and surfactants

Treatment*	Residual activity (%)
Control	98·20 ± 1·35
Heat	
25°C for 30 min	98·2 ± 1·05
37°C for 30 min	97·3 ± 2·15
60°C for 30 min	98·2 ± 1·12
Autoclaving (121°C, 20 min)	96·5 ± 3·2
pH	
02.0	98·6 ± 1·12
04.0	98·2 ± 1·45
06.0	97·6 ± 1·16
08.0	98·2 ± 1·32
10.0	97·9 ± 2·05
Enzymes**	
Pepsin	98·6 ± 1·12
Trypsin	97·6 ± 2·14
Surfactant	
SDS	96·9 ± 2·74
Triton X-100	97·2 ± 1·89
Tween 80	95·3 ± 3·12

*Tests were performed on the 10-fold concentrated supernatant of *B. licheniformis* BC98.

**All enzymes were used at a final concentration of 1 mg ml^{-1}.

antagonists, and one of these highly effective against development of appressoria in this fungus, was selected for further studies. The conditions for maximum yield of antifungal peptide had been optimized earlier (Patel *et al.* 2004). The specific growth rate (μ) was calculated to be 0·52 and the doubling time (t_D) was found to be 80 min. To purify the antifungal compound from the culture supernatant of *B. licheniformis* was precipitated by adjusting the pH with 3 N HCl and extracted using chloroform/methanol (2 : 1, v/v). Antifungal peptides soluble in chloroform-methanol were readily extracted yielding a group of microheterogeneous molecules with masses ranging from 992 to 1077 Da. Antagonistic activity was checked for each fraction during chloroform : methanol purification. Two major peaks were fractionated, and antifungal activity was examined (Fig. 2). Peak of 1035-Da peptide showing antifungal activity against phytopathogenic fungi, including *M. grisea*, and was used for further study.

Effect of antifungal peptide on *Magnaporthe grisea*

When the induction of morphological changes was assayed using culture supernatant, bulb formation in the mycelia of *M. grisea* was observed after 8 h of growth, indicating production of antifungal compound. To validate that among the various lipopeptides produced by *B. licheniformis*, the 1035-Da peptide was the one inducing morphological changes, and inhibiting the growth of

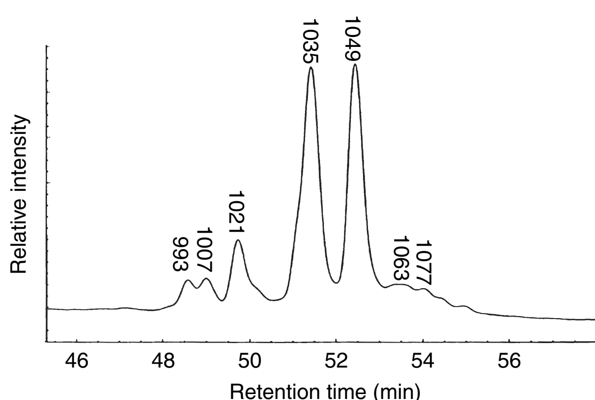

Figure 2 Fractionation of the crude chloroform methanol (9 : 1) extract of the culture supernatant of *Bacillus licheniformis* BC98. High-performance liquid chromatographic profile is RP C_{18}, 65–95% methanol water gradient. Masses of the individual component are indicated above the peaks.

fungus, we further studied the effect of the purified antifungal compound on the germination of *M. grisea.* The spores were germinated on glass slides in the presence or absence of the BIC. *Magnaporthe grisea* formed appressoria on the glass surface (Fig. 3a). BIC inhibited germination of *M. grisea* spores at concentration as low as 1 μg ml^{-1} (Table 3). Although fungus germinated at lower concentrations of BIC, the germ tube formed was abnormal (Fig. 3b). Light microscopic observations of the bulbous and swollen germinating spores and hyphal tips revealed shrunken, granulated and vesicular cytoplasm as compared with the hyaline, healthy cytoplasm of control untreated hyphae. The swollen bulbous structure subsequent to treatment with BIC measured from 18 to 25 μm in diameter, whereas normal fungal hyphae had a diameter of 2–2·5 μm. The maximum bulb-inducing activity observed was 68 bulbs per mm^2.

Electron microscopic studies

For further analysis by electron microscopy, the treated fungal samples were embedded in agarose; sections were taken and observed under the microscope. The cell wall of *M. grisea* hyphae treated with antifungal molecule was thick and showed distinct cytoplasmic membrane. The mitochondria were elongated by three- to fourfold, compared with the control. In contrast to the cytoplasm of untreated biomass, which was dense, homogenous and uniformly distributed (Fig. 4a) cytoplasm of treated samples was distinctly granular, patchy, fibrous and vacuolated (Fig. 4b). Moreover, the cytoplasmic membrane was not very distinct. This indicated that treatment of *M. grisea* with BIC led to unpolarized growth of the tip, thereby inhibiting normal development of *M. grisea*.

© 2007 The Authors

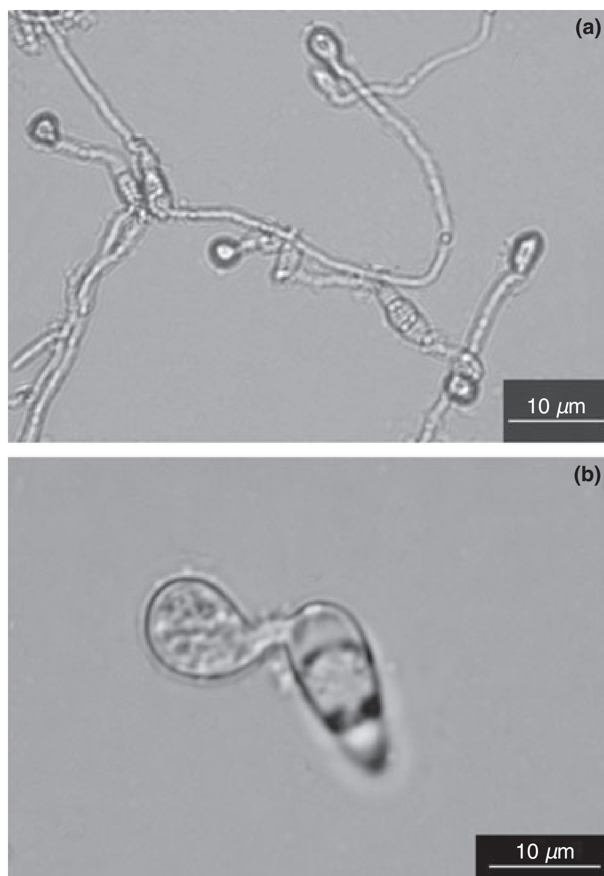

Figure 3 Light microscopic studies on *Magnaporthe grisea* biomass treated with the antifungal molecule. (a) Conidia germinated and formed appressorial structure on glass surface. (b) Conidia germinated in the presence of 0·1 μg ml^{-1} of purified antifungal compound. The inhibition of further development of germ tube by antifungal molecule inhibited appressoria formation as well as branched hyphae.

Table 3 Effect of purified bulb-inducing compound on germination of phytopathogen, *Magnaporthe grisea*. The experiment was performed in triplicate and repeated twice

Concentration (μg ml^{-1})	Germination (%)*	Bulb formation (%)**
0·0	98·2 ± 1·5	–**
0·1	95·3 ± 2·3	93·2 ± 1·3
1·0	–	–
10·0	–	–

*The conidia were germinated at 28°C for 24 h under high humidity.
**No bulb formation observed.

Structural analysis

Mass spectral analysis

MALDI TOF/MS was chosen initially to determine the molecular mass. Inspection of the MALDI mass spectrum of the crude peptide mixture revealed two major groups of substances with masses centered around 1000 and 1400 Da (Fig. 5a). Within each group, the observed microheterogeneity corresponded to peaks related by mass difference of 14 Da, indicating possible replacements of $–CH_2–$ groups. This polypeptide fraction was further separated by reverse-phase HPLC, and one pure peptide with a mass of 1035 Da was subjected to further characterization by NMR analysis.

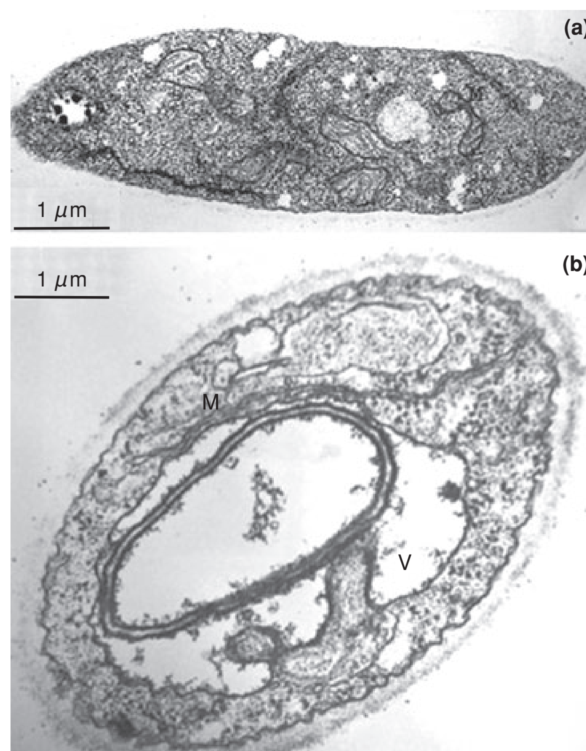

Figure 4 Electron microscopic studies of *Magnaporthe grisea* treated with the antifungal molecule. Control and biomass treated with antifungal molecule was embedded in 0·2% agarose and stained as mentioned in Materials and methods. (a) Untreated biomass showing normal distribution of cytoplasm, and subcellular organelle-like mitochondria. (b) *M. grisea* treated with 0·1 μg ml^{-1} of purified antifungal compound. The treated hyphae were vacuolated and showed patchy, granulated cytoplasm. The mitochondria were elongated and morphology was lost. M, mitochondria; V, vacuolated cytoplasm.

NMR analysis

500 MHz ^1H NMR spectra in CD_3OH showed seven distinct amide resonances establishing the presence of seven amino acid residues (Fig. 5b). TOCSY and ROSEY spectra enabled assignment of seven spin systems, Glu/Gln (1), Leu (4), Val (1), Asp/Asn (Fig. 5c). The absence of primary amide –NH– resonance suggested that the residues present were Glu and Asp. At this stage, the comparison with the known sequences pointed to a similarity with surfactin characterized earlier (Lin *et al.* 1994). Based on

© 2007 The Authors

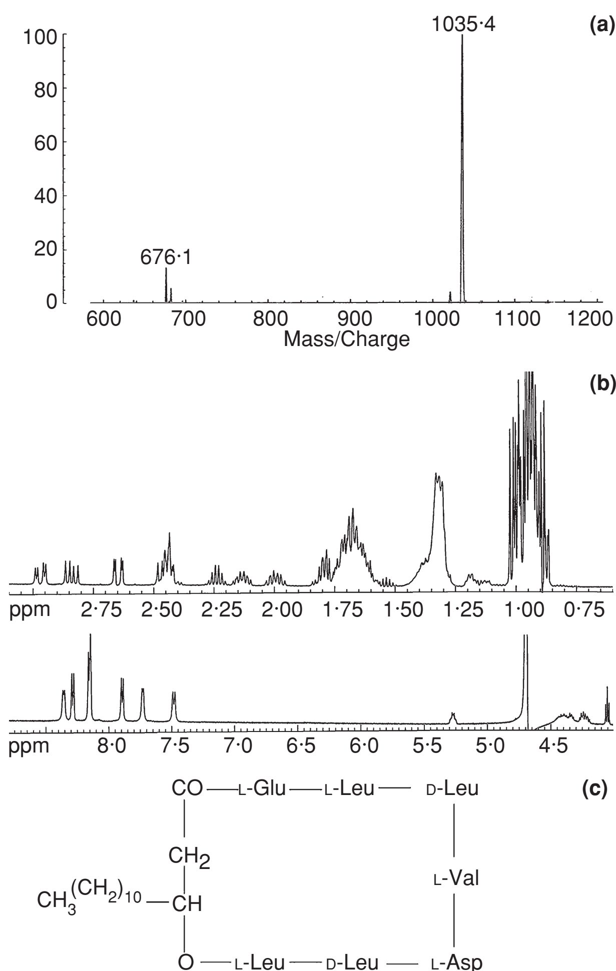

Figure 5 Structural analysis of antagonistic peptide. (a) MALDI-TOF-MS of the component corresponding to the high-performance liquid chromatographic (HPLC) peak with a retention time of 51 min. (b) ^{1}H nuclear magnetic resonance (NMR) spectrum of HPLC fraction (1035 Da) in CD_3OH at 323 K. (c) TOCSY and ROSEY spectral assignment of seven amino acid residues to the antagonistic lipopeptide identified as surfactin.

the observed mass, the fatty acid moiety was assigned as 3-hydroxy tetradecanoic acid. Thus, the inhibitory peptide against *M. grisea* produced by *B. licheniformis* BC98 was identified to be surfactin. This class of lipopeptides is closely related to the lichenysin which has been characterized from *B. licheniformis* (Peypoux *et al.* 1999).

Discussion

Biological control using antagonists provides an alternative to the use of pesticides for controlling plant diseases. Among 150 putative bacterial antagonists obtained, detailed studies were carried out on a gram-positive, motile, endospore-forming culture identified to be *B. licheniformis*. In presence of the supernatant from *B. licheniformis* BC98, *M. grisea* showed bulbous hyphae which inhibited further development of the fungus. Furthermore, the activity of antagonistic lipopeptide was found to be highly stable at extreme pH and temperature and it was also resistant to protease treatment. *Bacillus* species are known to produce various antibiotics and antifungal compounds that inhibit phytopathogenic fungi (Katz and Demain 1977; Kang *et al.* 1998; Hiradate *et al.* 2002; Yu *et al.* 2002; Cho *et al.* 2003; Thaniyavarn *et al.* 2003; Souto *et al.* 2004; Korenblum *et al.* 2005). *Bacillus licheniformis*, e.g. has been reported to produce different antifungal molecules such as fungimycin M4 (3·4 kDa hydrophilic peptide), which inhibits the growth of fungi such as *Sporothrix schenckii*, and *Microsporum canis* (Scott *et al.* 1987; Lebbadi *et al.* 1994).

In order to further characterize the effect of the antagonistic compound on *M. grisea*, microscopic studies were performed. Scanning electron and optical microscopic studies showed that the lipopeptide was capable of affecting the development of the phytopathogenic fungus. Light microscopic studies showed that hyphae of *M. grisea* treated with the purified antagonistic compound were 8–10-fold larger in diameter. Thus, the treatment caused abnormal germ tube formation and as a result the fungus could not grow further to form appressoria, necessary for penetration of the host tissue. Similar swelling at the hyphal tips has been observed in case of *Aspergillus nidulans* when treated with cytochalsin A, which is a known inhibitor of actin polymerization (Torralba *et al.* 1998). The cytoskeleton of *M. grisea* when treated with the antifungal peptide had collapsed leading to unpolarized growth at the tip of the hyphae. The results indicate that the peptide affected the distribution of cytoskeletal elements, actin and tubulin, and as a consequence, directional growth of the fungus is lost, leading to a bulbous morphology.

The inhibitory peptide from *B. licheniformis* was purified by solvent extraction and reverse-phase HPLC and characterized by MALDI-TOF-MS and NMR analysis. This antifungal peptide was identified to be a surfactin. Although surfactin was discovered about 30 years ago, there has been a revival of interest in this compound recently, triggered by an increasing demand for effective biosurfactants for difficult contemporary ecological problems. Earlier, lipopeptide bioemulsifier such as surfactin had been reported from *Bacillus* species and the structure was elucidated by 2D NMR analysis combined with molecular modelling (Bonmatin *et al.* 1994). Surfactin has also found wide applications in industries (Razafindralambo *et al.* 1998) and medicine (Arima *et al.* 1968; Kameda *et al.* 1972; Vollenbroich *et al.* 1997) and has been shown to be active against prokaryotes, yeasts and

© 2007 The Authors

fungi (Jenny *et al.* 1991; Peypoux *et al.* 1999); however, its activity against rice blast fungi *M. grisea* has not been investigated earlier. Preliminary studies at the green house level have also indicated protection of rice by purified antifungal compound from *B. licheniformis* BC98 against rice blast disease (data not shown).

Thus, in the present study we identified the antifungal agent produced by *B. licheniformis* BC98 as an antagonist to *M. grisea* as well as other phytopathogens. This lipopeptide was highly potent in its antagonistic activity as it completely inhibited the growth of *M. grisea* at a concentration as low as 1 μg ml^{-1}. Therefore, we suggest that it may be a potential bioactive agent that should be further explored for field application.

Acknowledgements

This project was funded by the grants from Department of Biotechnology and Council of Scientific and Industrial Research, Ministry of Science and Technology, Government of India.

References

Arima, K.A., Kakinuma, A. and Tamura, G. (1968) Surfactin a crystalline peptidelipid produced by *Bacillus subtilis*: isolation, characterisation and its inhibition of fibrin clot formation. *Biochem Biophys Res Commun* **31**, 488.

Bonmatin, J.M, Genest, M., Babbe, H. and Ptak, M. (1994) Solution three dimensional structure of surfactin: a cyclic lipopeptide studied by 1H-NMR. Distance geometry and molecular dynamics. *Biopolymers* **34**, 975–986.

Bryk, H., Sobiczewski, P. and Dyki, B. (1998) Antagonistic effect of *Erwinia herbicola* on *in vitro* spore germination and germ tube elongation of *Botrytis cinerea* and *Penicillium expansum*. *BioControl* **43**, 97–106.

Chernin, L., Ismailov, Z., Haran, S. and Chet, I. (1995) Chitinolytic *Enterobacter agglomerans* antagonistic to fungal plant pathogens. *Appl Environ Microbiol* **61**, 1720–1726.

Cho, S.-J., Lee, S.K., Cha, B.J., Kim, Y.H. and Shin, K.-S. (2003) Detection and characterisation of the *Gloeosporium gloeosporioides* growth inhibitory compound iturin A from *Bacillus subtilis* strain KS03. *FEMS Microbiol Lett* **223**, 47–51.

Cooper, D.G. and Goldenberg, B.G. (1987) Surface-active agents from two *Bacillus* species. *Appl Environ Microbiol* **53**, 224–229.

Cottyn, B., Regalado, E., Lanoot, B., De Cleene, M., Mew, T.W. and Swings, J. (2001) Bacterial populations associated with rice seed in the tropical environment. *Phytopathology* **91**, 282–292.

Ganeshan, G. and Kumar, M.A. (2005) *Pseudomonas fluorescens*, a potential bacterial antagonist to control plant diseases. *J Plant Interact* **1**, 123–134.

Georgiou, G., Lin, S.C. and Sharma, M.M. (1992) Surface-active compounds from microorganisms. *Bio/Technology* **10**, 60–65.

Gomes, R.C., Sêmedo, L.T.A.S., Soares, R.M.A., Linhares, L.F., Ulhoa, C.J., Alviano, C.S. and Coelho, R.R.R. (2001) Purification of a thermostable endochitinase from *Streptomyces* RC1071 isolated from a cerrado soil and its antagonism against phytopathogenic fungi. *J Appl Microbiol* **90**, 653–661.

Hiradate, S., Yoshida, S., Sugie, H., Yada, H. and Fujii, Y. (2002) Mulberry anthracnose antagonists (iturins) produced by *Bacillus amyloliquefaciens* RC-2. *Phytochemistry* **61**, 693–698.

Javaheri, M., Jenneman, G.E., McInerney, M.J. and Knapp, R.M. (1985) Anaerobic production of a biosurfactant by *Bacillus licheniformis* JF-2. *Appl Environ Microbiol* **50**, 698–700.

Jenny, K., Kappeli, O. and Fiechter, A. (1991) Biosurfactants from *Bacillus licheniformis*: structural analysis and characterisation. *Appl Microbiol Biotechnol* **36**, 5–13.

Kachroo, P., Leong, S.A. and Chattoo, B.B. (1994) Pot2, an inverted repeat transposon from the rice blast fungus *Magnaporthe grisea*. *Mol Gen Genet* **245**, 339–348.

Kameda, Y., Matsui, K., Hisato, K., Yamada, T. and Sagai, H. (1972) Antitumor activity of *Bacillus* natto III. Isolation and characterisation of a cytolytic substance on Ehrlich ascites carcinoma cells in the culture medium of Bacillus natto KMD 1126. *Chem Pharmacol Bullet (Tokyo)* **20**, 1551–1533.

Kang, Y., Carlson, R., Tharpe, W. and Schell, M.A. (1998) Characterisation of genes involved in biosynthesis of a novel antibiotic from *Burkholderia cepacia* BC11 and their role in biological control of *Rhizoctonia solani*. *Appl Environ Microbiol* **64**, 3939–3947.

Katz, E. and Demain, A.C. (1977) The peptide antibiotics of *Bacillus*: chemistry, biogenesis, and possible functions. *Bacteriol Rev* **41**, 449–474.

Khush, G.S., Singh, R.J., Sur, S.C. and Librojo, A.L. (1984) Primary trisomics of rice: origin, morphology, cytology and use in linkage mapping. *Genetics* **107**, 141–163.

Kim, P.I., Bai, H., Bai, D., Chae, H., Chung, S., Kim, Y., Park, R. and Chi, Y.-T. (2004) Purification and characterisation of a lipopeptide produced by *Bacillus thuringiensis* CMB26. *J Appl Microbiol* **97**, 942–949.

Korenblum, E., von der Weid, I., Santos, A.L.S., Rosado1, A.S., Sebastián, G.V., Coutinho, C.M.L.M., Magalhães, F.C.M., de Paiva, M.M. *et al.* (2005) Production of antimicrobial substances by *Bacillus subtilis* LFE-1, *B. firmus* H2O-1 and *B. licheniformis* T6-5 isolated from an oil reservoir in Brazil. *J Appl Microbiol* **98**, 667–675.

Lebbadi, M., Gálvez, A., Maqueda, M., Martinez-Bueno, M. and Valdivia, E. (1994) Fungimycin M4: a narrow spectrum peptide antibiotic from *Bacillus licheniformis* M-4. *J Appl Bacteriol* **77**, 49–53.

© 2007 The Authors

Lee, J.Y., Moon, S.S. and Hwang, B.K. (2003) Isolation and antifungal and Antioomycete activities of aerugine produced by *Pseudomonas fluorescens* strain MM-B16. *Appl Environ Microbiol* **69**, 2023–2031.

Levy, E., Gough, F.J., Berlin, K.D., Guiana, P.W. and Smith, J.T. (1992) Inhibition of *Septoria tritici* and other phytopathogenic fungi and bacteria by *Pseudomonas fluorescens* and its antibiotics. *Plant Pathol* **41**, 335–341.

Lin, S.-C., Minton, M.A., Sharma, M.M. and Georgiou, G. (1994) Structural and immunological characterisation of a biosurfactant produced by *Bacillus licheniformis* JF-2. *Appl Environ Microbiol* **60**, 31–38.

Mauchline, W.S. and Keevil, C.W. (1991) Development of the Biolog substrate utilization system for identification of *Legionella* spp. *Appl Environ Microbial* **57**, 3345–3349.

Mew, T.(1991) Disease management in rice. In *CRC Handbook of Pest Management in Agriculture*, **Vol. 3**. pp. 279–299. Boston: CRC press.

Neu, T.R. (1996) Significance of bacterial surface-active compounds in interaction of bacteria with interfaces. *Microbiol Rev* **60**, 151–166.

Ou, S.H. (1972) Studies on stable resistance to rice blast disease. In *Rice Breeding*. pp. 227–237. Manila: International Rice Research Institute.

Ou, S.H.(1985) *Rice Diseases*. pp. 109–200. Kew, United Kingdom: Commonwealth Mycological Institute.

Patel, V., Tendulkar, S. and Chattoo, B.B. (2004) Bioprocess development for the production of an antifungal molecule by *Bacillus licheniformis* BC98. *J Biosci Bioeng* **98**, 231–235.

Peypoux, F., Bonmatin, J. and Wallach, J. (1999) Recent trends in the biochemistry of surfactin. *Appl Microbiol Biotechnol* **51**, 553–563.

Razafindralambo, H., Popineau, Y., Deleu, M., Jacques, H.C., Thonart, P. and Paquot, M. (1998) Foaming properties of lipopeptides produced by *Bacillus subtilis*: effect of lipid and peptide structural attributes. *J Agric Food Chem* **46**, 911–916.

Ron, E.Z. and Rosenberg, E. (2001) Natural roles of biosurfactants. *Environ Microbiol* **3**, 229–236.

Rosales, A.M., Vantomme, R., Swings, J., De Ley, J. and Mew, T.W. (1993) Identification of some bacteria from paddy antagonistic to several rice fungal pathogens. *J Phytopathol* **138**, 189–208.

Scott, E.N., Kaufman, L., Brown, A.C. and Muchmore, H.G. (1987) Serological studies in the diagnosis and management of meningitis due to *Sporothrix schenckii*. *N Engl J Med* **317**, 935–940.

Souto, G.I., Correa, O.S., Montecchia, M.S., Kerber, N.L., Pucheu, N.L., Bachur, M. and Arcıa, A.F. (2004) Genetic and functional characterisation of a *Bacillus* sp. strain excreting surfactin and antifungal metabolites partially identified as iturin-like compounds. *J Appl Microbiol* **97**, 1247–1256.

Thaniyavarn, J., Roongsawang, N., Kameyama, T., Haruki, M., Imanaka, T., Morikawa, M. and Kanaya, S. (2003) Production and characterisation of biosurfactants from *Bacillus lichemiformis* F2.2. *Biosci Biotechnol Biochem* **67**, 1239–1244.

Torralba, S., Raudoski, M., Pedregosa, A.M. and Laborda, F. (1998) Effect of cytochalasin A on apical growth, actin cytoskeleton organization and enzyme secretion in *Aspergillus nidulans*. *Microbiology* **144**, 45–53.

Vollenbroich, D., Pauli, G., Ozel, M. and Vater, J. (1997) Antimycoplasma properties and application on cell cultures of surfactin, a lipopeptide antibiotic from *Bacillus subtilis*. *Appl Environ Microbiol* **63**, 43–49.

Wicken, A.J. and Knox, K.W. (1980) Bacterial cell surface amphiphiles. *Biochem Biophys Acta* **604**, 1–26.

Yu, G.Y., Sinclair, J.B., Hartman, G.L. and Bertagnolli, B.L. (2002) Production of iturin A by *Bacillus amyloliquefaciens* suppressing *Rhizoctonia solani*. *Soil Biol Biochem* **34**, 955–963.

© 2007 The Authors
Journal compilation © 2007 The Society for Applied Microbiology,