Biochimica et Biophysica Acta 1828 (2013) 2230–2237

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

# New insights into membrane-active action in plasma membrane of fungal hyphae by the lipopeptide antibiotic bacillomycin L

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ARTICLE INFO

*Article history:*
Received 19 March 2013
Received in revised form 17 May 2013
Accepted 31 May 2013
Available online 10 June 2013

*Keywords:*
Bacillomycin L
Iturin
*Rhizoctonia solani* Kühn
Membrane permeabilization
Mechanism of action

ABSTRACT

Bacillomycin L, a natural iturinic lipopeptide produced by *Bacillus amyloliquefaciens*, is characterized by strong antifungal activities against a variety of agronomically important filamentous fungi including *Rhizoctonia solani* Kühn. Prior to this study, the role of membrane permeabilization in the antimicrobial activity of bacillomycin L against plant pathogenic fungi had not been investigated. To shed light on the mechanism of this antifungal activity, the permeabilization of *R. solani* hyphae by bacillomycin L was investigated and compared with that by amphotericin B, a polyene antibiotic which is thought to act primarily through membrane disruption. Our results derived from electron microscopy, various fluorescent techniques and gel retardation experiments revealed that the antifungal activity of bacillomycin L may be not solely a consequence of fungal membrane permeabilization, but related to the interaction of it with intracellular targets. Our findings provide more insights into the mode of action of bacillomycin L and other iturins, which could in turn help to develop new or improved antifungal formulations or result in novel strategies to prevent fungal spoilage.



## 1. Introduction

Lipopeptides from *Bacillus* species, synthesized in a non-ribosomal manner, encompass surfactins, iturins, and fengycins. Iturins are a crucial family of these cyclic lipopeptides and they derived only from *B. subtilis* and *B. amyloliquefaciens* [1]. This lipopeptide family consists of iturin A and C, bacillomycin D, F, L, and LC, and mycosubtilin, which share several structural and functional similarities. Indeed, these amphiphilic compounds are featured by a heptapeptide, with the LDDLLDL α-amino acid configuration including invariable D-Tyr$^2$ and D-Asn$^3$, cyclized in a ring with a β-amino fatty acid chain with a length of 14 to 17 carbons [2,3]. These properties may play a critical role in their mechanism of action and antibiotic activity towards microbial cells. They also exhibit strong antifungal activities against a wide variety of yeasts and plant pathogenic fungi but limited antibacterial activities.

In light of the potent antimicrobial activity of these iturins, a knowledge of the mode of action of them is important for attempts to increase their potency and specificity. A large number of studies aimed at understanding the mechanism of iturinic antibiotics targeted model membrane or some biological membrane by using various experimental approaches have been reported for the last 30 years [2,4–7]. It has been proposed that antimicrobial activity of iturins depends predominantly on its capacity to increase membrane permeabilization, which is being attributed to aggregates formed by iturin molecules, iturin–phospholipid complex or iturin–phospholipid–sterol complex [2]. Nevertheless, the question is still open whether membrane permeabilization is the only mechanism by which these iturinic lipopeptides kill bacteria, or peptides have various intracellular targets. In addition, most of the works regarding the membrane-permeabilization mode of action stem from experiments conducted on model membrane systems [4–6,8,9], while there are only few limited analyses of the iturin interaction with real cell membrane of yeast cells [10]. Hence, additional studies on biological membrane, particularly the plasma membrane of filamentous fungi are needed.

Bacillomycin L belonging to the iturin family was originally isolated from the culture broth of *B. amyloliquefaciens* K103 in our previous work. It displays a strong in vitro antifungal action against a wide variety of plant fungal pathogen, including the *Rhizoctonia solani* Kühn which is a pathogen of considerable economic importance. Due to the common structural and functional characteristics, the mechanism of action of bacillomycin L is similar to that of all these antibiotics of the iturinic group [11]. At present, little is known about the interaction between the bacillomycin L and the plasma membranes of filamentous fungi. To address this, we investigated the inhibitory effects of the antibiotic in relation to its ability to permeabilize the membrane of *R. solani* Kühn in a comparison study with amphotericin B, a polyene antibiotic

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0005-2736 
http://dx.doi.org/10.1016/j.bbamem.2013.05.033

that kills the fungal cells via membrane permeabilization. The interaction and morphological changes that bacillomycin L induces on fungal hyphae cells were also assayed by using electron microscopy, diverse fluorescent dyes, and gel retardation experiments.

## 2. Methods

### 2.1. Peptide synthesis

Bacillomycin L, which shows a strong antagonism against *R. solani* Kühn, was isolated in pure from the culture broth of *B. amyloliquefaciens* K103 by a combination of Sephadex LH-20 gel permeation chromatography and reversed-phase high-performance liquid chromatography using a Shim-pack PREP-ODS C18 column (20 mm × 250 mm, 10 μm, Shimadzu) with gradient elution of 20% to 80% acetonitrile with 0.1% trifluoroacetic acid (TFA) at a flow rate of 1 ml/min. The HPLC-purified peptide was identified based on its amino acid composition and quadrupole-time of flight mass spectrometry (Q-TOF-MS) analysis (unpublished data). Bacillomycin L was dissolved in PBS before use in the assay.

### 2.2. Microorganisms

The plant fungal pathogen used in this study was *R. solani* Kühn (No. 3.2871), purchased from the China General Microbiological Culture Collection Center. It was cultured on potato dextrose broth (PDB) for 3 days at 28 °C to obtain cells in the middle of rapid growth period.

### 2.3. In vitro antimicrobial activity assays

Antimicrobial activities against *R. solani* Kühn were assessed as described previously with some modifications [12,13]. Hyphae were grown in half-strength PDB from 3-day-old fungal culture in liquid medium. Hyphal suspension (180 μl) was then transferred to 96-well microtiter plates along with 20 μl of filter-sterilized bacillomycin L or amphotericin B with different concentrations to give a final volume of 200 μl. The plates were incubated in the dark at 28 °C for 24 h was determined by measuring absorbance at 595 nm using a microtiter plate reader (SpectraMax Pro M5e, Molecular Devices). The $IC_{50}$ of a peptide was defined as the concentration required to obtain 50% inhibition, and the minimum fungicidal concentration (MFC) was defined as the lowest peptide concentration that showed no growth at the end of the experiment (after 24 h of incubation) in all the experiments carried out with that peptide. Each test was conducted in triplicate.

### 2.4. Scanning electronic microscopy (SEM) and transmission electronic microscopy (TEM) analysis

Hyphae grown for 3 days in half-strength PDB were treated with bacillomycin L at the concentration of 100 μg/ml for 24 h, while hyphae treated with PBS without lipopeptide were used as a control. Specimens were resuspended in a 2.5% (v:v) glutaraldehyde solution in 0.1 M phosphate buffer (PBS, pH 7.2) and fixed for 24 h. After the primary fixation, hyphal cells were pelleted and the glutaraldehyde was removed, and a 1% osmium tetroxide solution (pH 7.2) was added. After 1.5 h, the cells were rinsed with PBS for 3 times. The cells were dehydrated in a series of cold ethanol solutions for 10 min each, starting with a 10% ethanol solution continuing through 30%, 50%, 70% and 90% solutions. Then the cells were rinsed in 100% ethanol at 10 min intervals for 2 times. For SEM assay, the cells were rinsed with 50%, 70%, 90% and 100% isoamyl acetate for 3 min each, critical point dried, and coated with gold–palladium. Observation and photomicrographs were then carried out with a Hitachi S-3400N SEM (Hitachi Instruments Inc., Japan). For TEM assay, the cells were infiltrated with the solution of acetone and epon-araldite over a period of 24 h, and polymerized at 60 °C for 48 h. Fifty nanometer sections were sectioned, then the sections were placed on copper sieves and contrasted with uranyl acetate and lead citrate for 30 min each. The sections were viewed on a JEM-1230 TEM (JEOL Japan Electronics Co., Ltd., Japan).

### 2.5. Fluorescence microscopy

Hyphae grown for 3 days in half-strength PDB were incubated in the presence of bacillomycin L with a final concentration of 20–300 μg/ml or PBS alone. After 24 h of incubation, SYTOX Green (SG) (Molecular Probes, Invitrogen, USA) was added to a final concentration of 0.8 μM, and samples were incubated for 15 min in the dark.

Subsequently, hyphal cells were washed, resuspended in 20% glycerol solution, and visualized under the fluorescence microscopy. Fluorescence was examined and photographed with a Leica CTR 5000 fluorescence microscope (Leica, Germany) with the filters set at an excitation wavelength of 488 nm and an emission wavelength at 538 nm for SG detection.

### 2.6. SYTOX Green uptake quantification assay

*R. solani* hyphae were treated with bacillomycin L or amphotericin B for 24 h prior to incubation with SYTOX Green, as described for the fluorescence microscopy assay. Samples (200 μl) were then transferred to 96-well microtiter plates and SYTOX Green quantification was measured using a microtiter plate reader (SpectraMax Pro M5e, Molecular Devices) with excitation and emission wavelengths of 488 and 538 nm, respectively. Three replicates were prepared for each treatment.

### 2.7. Time course analysis of SYTOX Green uptake

*R. solani* hyphae were grown in half-strength PDB grown for 3 days as described above. Hyphal suspension (180 μl) was then transferred to 96-well microtiter plates incubated with SYTOX Green (0.5 μM) for 10 min prior to the addition of different drug solutions with different concentrations to give a final volume of 200 μl. SYTOX Green quantification was measured using a microtiter plate reader (SpectraMax Pro M5e, Molecular Devices) with excitation and emission wavelengths of 488 and 538 nm, respectively. Each test was performed in triplicate.

### 2.8. Fluorescence polarization measurements with DPH

Membrane fluidity was determined by measuring fluorescence polarization using 1,6-diphenyl-1,3,5-hexatriene (DPH) (Molecular Probes, Invitrogen, USA) as a fluorescent probe. The method was described previously with some modifications. *R. solani* hyphae were treated with bacillomycin L or amphotericin B for 24 h prior to incubation with DPH (final concentration of 1 μM), as described for the SYTOX Green uptake quantification assay. Samples (200 μl) were then transferred to 96-well microtiter plates and fluorescence polarization was measured using a microtiter plate reader (SpectraMax Pro M5e) with excitation and emission wavelengths of 350 and 452 nm, respectively. Three replicates were prepared for each treatment.

### 2.9. DNA-binding assay

Assays of peptide DNA binding activity were performed by gel retardation experiments essentially as described previously [14,15]. Briefly, 250 ng of *R. solani* hyphal cells DNA was mixed with increasing molar concentrations of antibiotics in 20 μl of binding buffer (10 mM Tris–HCl, 1 mM EDTA buffer, pH 8.0). Reaction mixtures were incubated at room temperature for 30 min. Samples were subjected to 1.0% agarose gel electrophoresis and stained with ethidium bromide.

## 3. Results

### 3.1. Antimicrobial activity

The $IC_{50}$ and MFC of bacillomycin L or amphotericin B against the fungal pathogen *R. solani* Kühn are provided in Table 1. At 50 μg/ml, bacillomycin L retarded the growth of *R. solani* by 50%, whereas killed hyphal cells at 150 μg/ml. As shown in Table 1, the MFC of bacillomycin L against *R. solani* is 3-fold higher than its MIC, whereas the MFC of amphotericin B is 13-fold higher than its MIC. It indicated that bacillomycin L killed hyphal cells more effectively than amphotericin B with increasing concentrations of antibiotic.

### 3.2. Effect of bacillomycin L on morphology and ultrastructure transformation of R. solani

The morphology and ultrastructure changes of hyphal cells treated with bacillomycin L were analyzed by SEM and TEM. As shown in Fig. 1, untreated hyphal cells were intact, smooth and had a fine structure. After treating with bacillomycin L, the surface of the hyphal cells was rough with a lumpy appearance and abnormal configuration (Fig. 1).

The ultrastructure of fungal hyphae in ultrathin sections was examined by TEM (Fig. 2). Untreated *R. solani* hyphae featured cells with regular submicroscopic structure, even distribution, good integrity in their cytoplasms, and clearly identifiable organelles, such as vacuole, mitochondria, and lipid body (Fig. 2A to C). Hyphal segments typically contained the nuclei surrounded by the nuclear membrane and nucleoli were visible in many cases (Fig. 2A and C). The numerous mitochondria were obviously visible with membranous cristae. Compared to the untreated cells, intracellular organization of treated hyphae cells were noticeably disrupted, showing uneven distribution, condensation of the cytoplasms and abnormal architecture of the nuclei (Fig. 2D to F). The nuclei were less well defined and often displayed discontinuous or missing nuclear membrane. The mitochondria became disorganized and decreased in the hyphal cytoplasm. These features appeared frequently, but not all of them occurred in every cell.

### 3.3. Membrane permeabilization by bacillomycin L

To feature the mode of action and interaction of bacillomycin L with *R. solani* hyphae, we used fluorescence microscopy and an assay based on the uptake of the fluorescent dye SYTOX Green, a high-affinity nucleic acid stain. Because the dye easily penetrates cells with compromised plasma membranes and yet will not cross the membranes of noncompromised living cells, it has been used to assess the integrity of biological membranes [16,17].

As shown in Fig. 3, no appreciable fluorescent signal was discerned in untreated hyphal cells as the controls. After treatment with 50 μg/ml ($IC_{50}$) bacillomycin L, only very weak fluorescent signal was detected by using fluorescence microscopy. With increasing concentrations of bacillomycin L, the fluorescent signal emitted by hyphal cells was more and more intense. Increasing the bacillomycin L concentration to 300 μg/ml, the fluorescent signal was distinct, clear and strong, indicating that the membrane of these cells was absolutely permeabilized.

SYTOX Green uptake quantification assay was used to quantify permeabilization and assess the correlation between the permeabilization and growth inhibition of bacillomycin L-treated hyphae. As shown in Fig. 4, bacillomycin L-induced permeabilization increased in a concentration-dependent manner. As the concentration of bacillomycin L increased, the fluorescence intensity and inhibition ratio values increased accordingly. Growth inhibition and permeabilization curves of amphotericin B added to hyphal cells, run in parallel to bacillomycin L experiments, were similar to those of bacillomycin L in a concentration-dependent manner. However, at the concentrations of $IC_{50}$ (50 μg/ml) bacillomycin L had an effect on fungal growth while only limitedly inducing permeabilization. This result is in agreement with the poor SG uptake observed at $IC_{50}$ concentrations by fluorescence microscopy. In contrast, the fluorescence intensity values for amphotericin B at the concentrations of $IC_{50}$ (1 μg/ml) were much more higher than those of bacillomycin L at the concentrations of $IC_{50}$ (50 μg/ml), even though they had a similar antifungal effect at this point.

The rate at which bacillomycin L permeabilized fungal hyphal membranes was monitored over time for 2 h by measuring the uptake of SYTOX Green, which was performed immediately after the addition of peptides and SG (Fig. 5). The rate of permeabilization was partially concentration-dependent and increased with increasing concentrations of bacillomycin L. At a concentration of 300 μg/ml, the permeabilization increased over time within 2 h or more time. Surprisingly, in the case of amphotericin B, this increase in permeabilization was clearly low even increasing its concentrations up to 300 μg/ml (2–3 times MFC), indicating that 2 h was too short for amphotericin B to permeabilize *R. solani* hyphae substantially.

**Table 1**
Growth inhibition effects of bacillomycin L or amphotericin B towards *Rhizoctonia solani* Kühn hyphae.

| | $IC_{50}$ (μg/ml) | MFC (μg/ml) |
|---|---|---|
| Bacillomycin L | 50.0 | 150.0 |
| Amphotericin B | 1.0 | 13.0 |

### 3.4. Fluorescence polarization measurements with DPH

In order to assess the effect of bacillomycin L on membrane destabilization, changes in membrane fluidity were further examined with a fluorescent membrane probe DPH. DPH is a hydrophobic molecule, and this property makes it possible to associate with the hydrocarbon tail region of phospholipids within the cytoplasmic membrane, without disturbing the structure of the lipid bilayer [18–20]. As the cytoplasmic membrane fluidity decreases, the fluorescent polarization values increase, and vice versa [21]. As shown in Fig. 6, the fluorescence polarization values for bacillomycin L decreased with increasing concentrations from 50 to 300 μg/ml. Similarly, the fluorescence polarization of amphotericin B-treated hyphal cells decreased significantly. This suggests that the plasma membrane was structurally perturbed when exposed to bacillomycin L or amphotericin B.

### 3.5. DNA-binding activity

The DNA binding abilities of bacillomycin L or amphotericin B were examined by analyzing the electrophoretic mobility of *R. solani* hyphal cells' DNA bands at the various weight ratios of peptides to DNA on 1.0% agarose gel. As shown in Fig. 7, with increasing concentrations of bacillomycin L up to 100 μg/ml or higher concentration (such as 300 μg/ml), the migration of DNA through the gel decreased and the disappearance of free DNA was observed. It seemed that bound DNA appeared as a low-mobility smear, indicative of nonspecific binding of the peptide to the nucleic acid. Compared with bacillomycin L, amphotericin B could not inhibit the migration of DNA or smear the DNA bands at concentrations even above 100 μg/ml, suggesting that amphotericin B could not bind to *R. solani* hyphal cells' DNA.

## 4. Discussion

Most studies performed on artificial membrane demonstrated that the antifungal activity of iturin lipopeptides was the consequence of membrane permeabilization, which was due to aggregates (lipopeptide aggregates or lipopeptide–phospholipid complex aggregates). However, the interaction of iturinic antibiotics with actual fungal membranes, different significantly from the model membrane that may not be representative of in vivo activities, has been far from being fully understood. To address this, the antifungal activity of bacillomycin L which involves

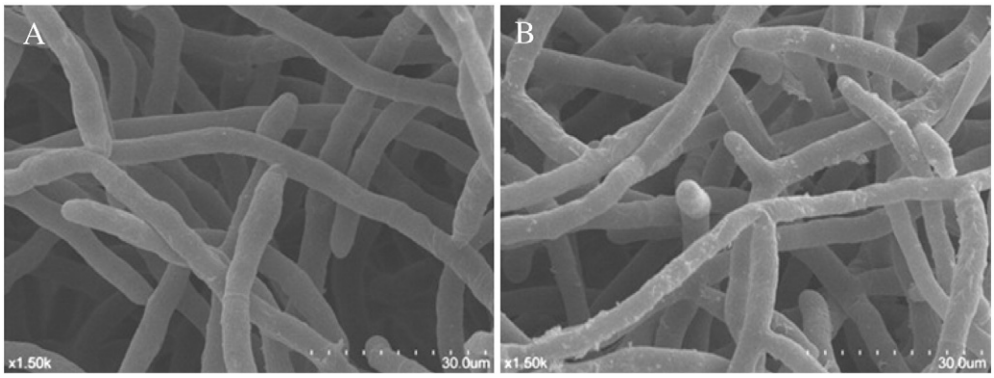


**Fig. 1.** Scanning electron micrographs of untreated hyphae (A) or bacillomycin L-treated hyphae (B).

specific interaction with the intact fungal hyphae of *R. solani* Kühn was investigated in detail by means of various fluorescent techniques, electron microscopy and gel retardation experiment assay.

SEM and TEM observations revealed that growth inhibition of *R. solani* as a response to bacillomycin L was accompanied by marked morphological and cytological changes, including rough cell surface,

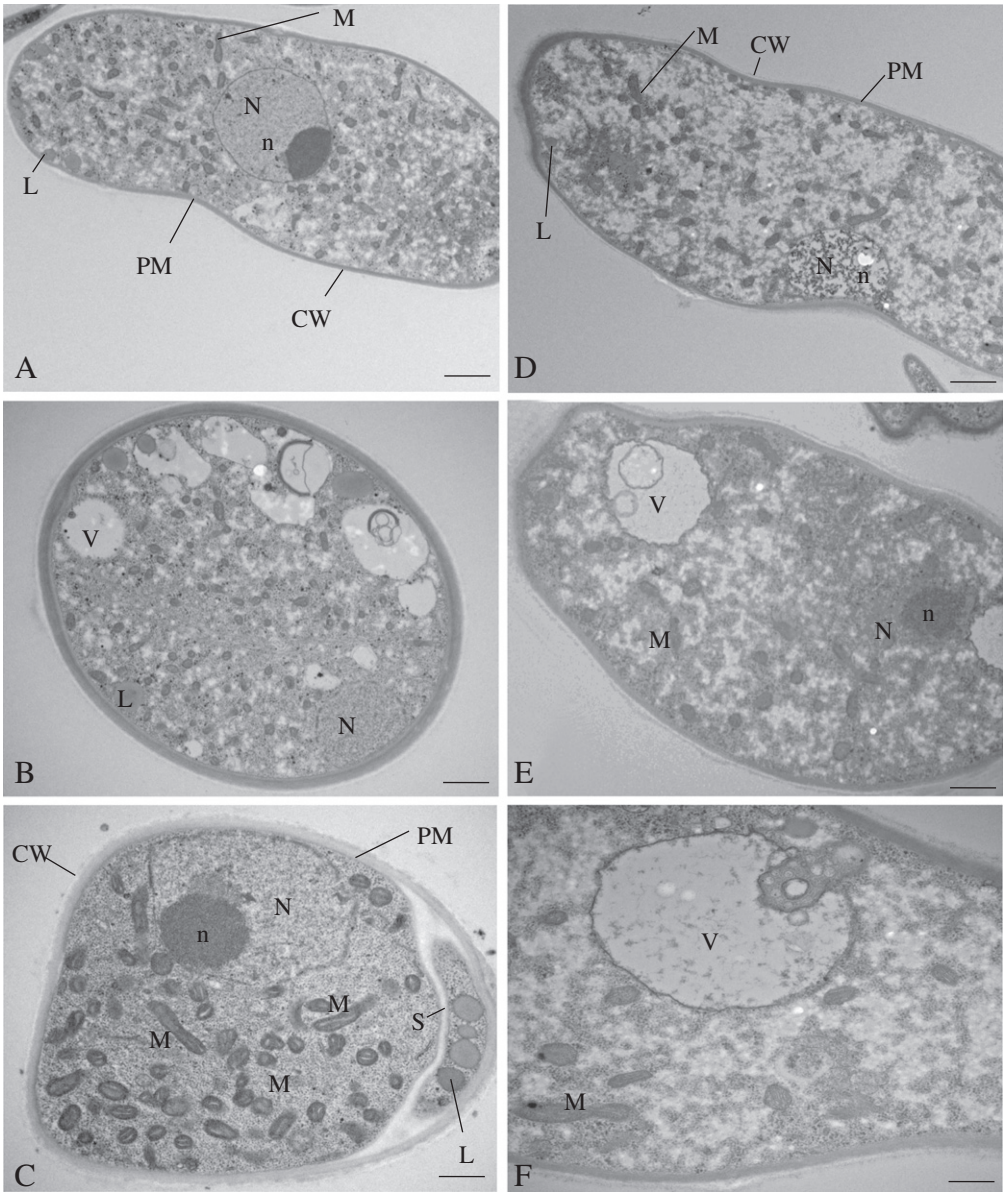


**Fig. 2.** TEM images of the cellular ultrastucture of hyphal cells. (A to C) Untreated control; (D to F) specimens were treated with bacillomycin L. CW, cell wall; PM, plasma membrane; M, mitochondrion; N, nucleus; n, nucleolus; V, vacuole; L, lipid body; S, septum. Scale bars: 1.5 μm (A, B, D, and E) and 0.5 μm (C and F).

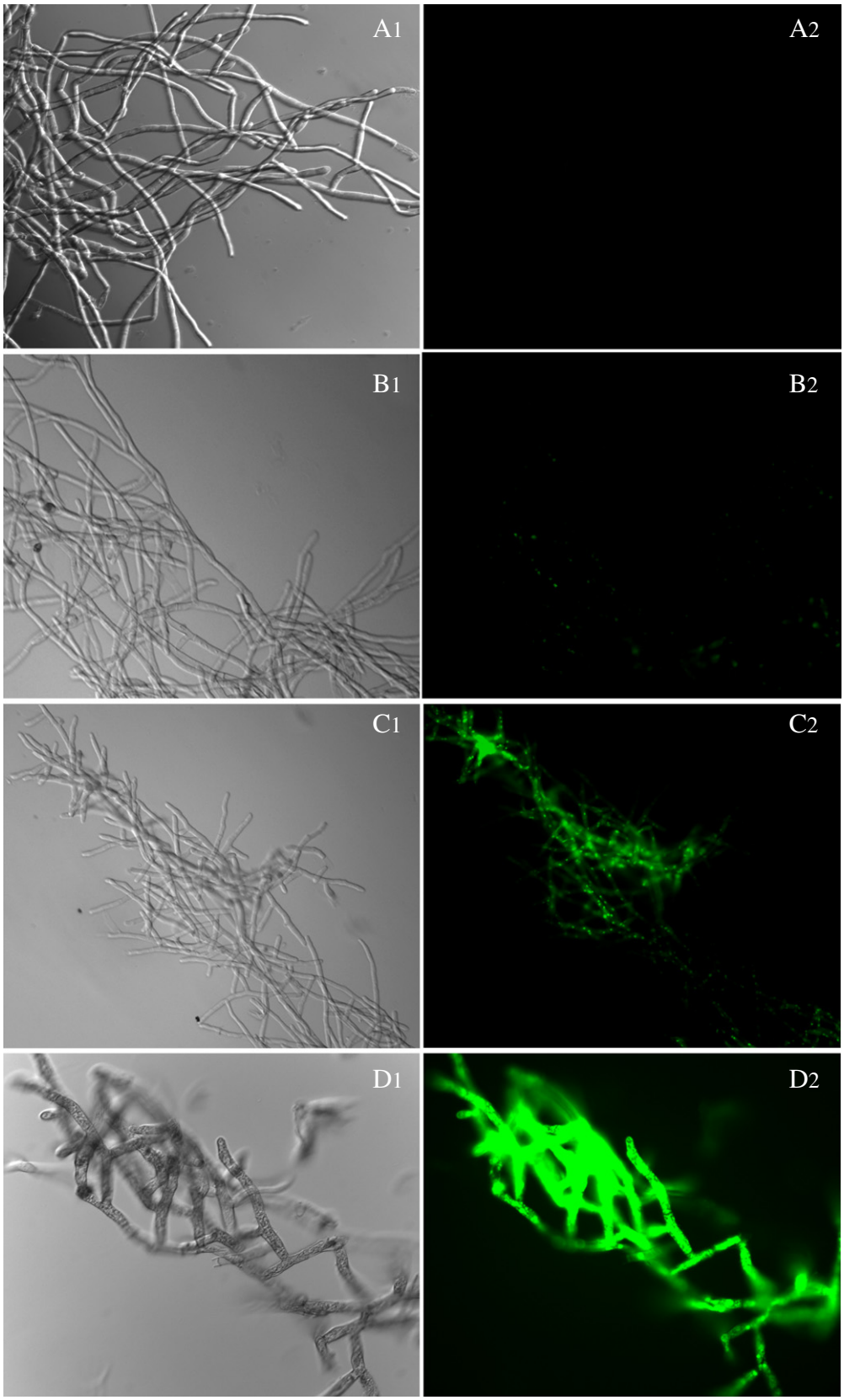


**Fig. 3.** Fluorescence microscopy of *Rhizoctonia solani* hyphae treated with bacillomycin L. Hyphal cells were incubated in half-strength PDB at 28 °C without lipopeptide (panels A1 and A2) or in the presence of bacillomycin L at final concentrations of 50 μg/ml (panels B1 and B2), 100 μg/ml (panels C1 and C2), or 300 μg/ml (panels D1 and D2). Panels represent bright-field images (A1 to D1 on the left) and green fluorescence indicative of SG uptake (A2 to D2 on the right) for the same fields. Magnification is 200× (panels A1 and A2, B1 and B2, and C1 and C2) or 400× (panels D1 and D2).

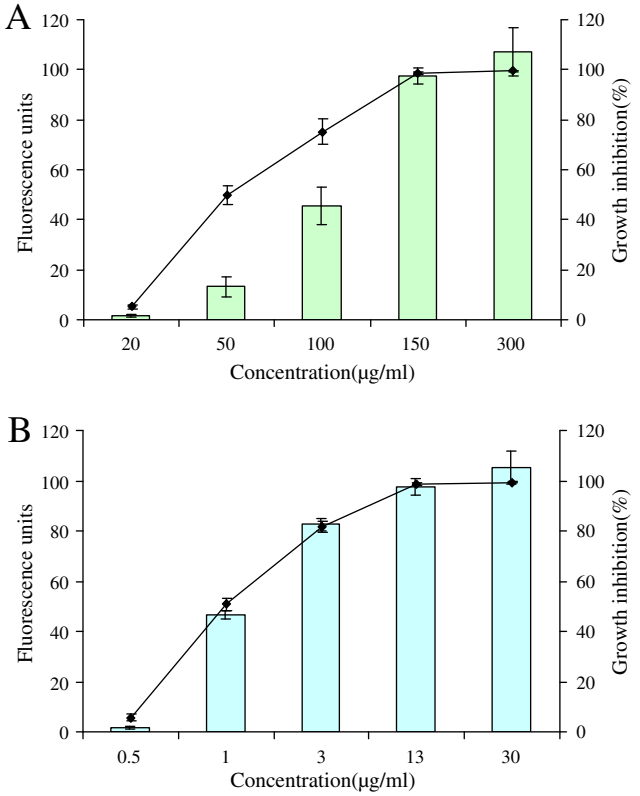


**Fig. 4.** Effects on growth and SG uptake by *Rhizoctonia solani* Kühn hyphae of bacillomycin L (A) and amphotericin B (B). Hyphae were treated with increasing concentrations of bacillomycin L or amphotericin B for 24 h prior to incubation with SG. Growth inhibition was measured relative to a non-antibiotic control (line, %, right axis), and permeabilization was measured by SG uptake quantification (column, fluorescence units, left axis). The figure is representative of three separate experiments, and error bars represent S.E.

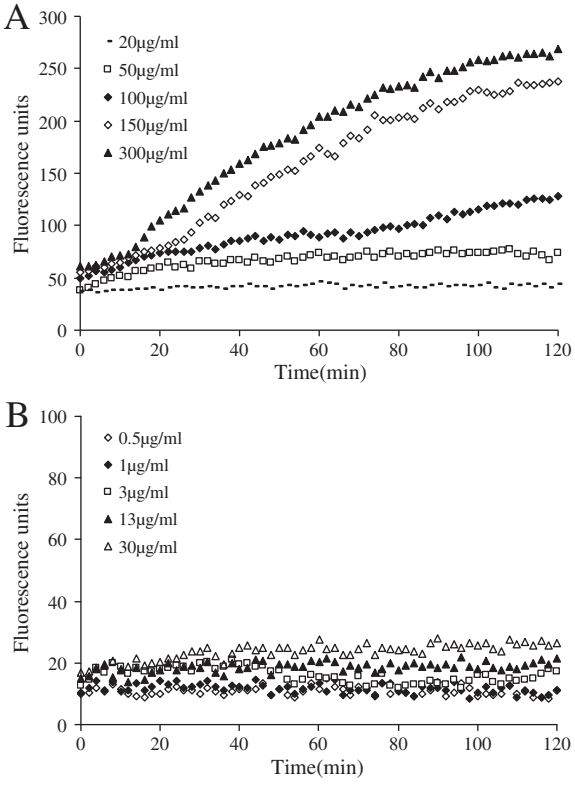


**Fig. 5.** Time course of SG uptake by *Rhizoctonia solani* Kühn hyphae of bacillomycin L (A) or amphotericin B (B). Hyphal cells were simultaneously exposed to antibiotics at varying concentrations in combination with 0.5 μM SG in half-strength PDB. Immediately after drugs and SG addition, fluorescence at 538 nm was recorded at 2-min intervals for 120 min. Data are from a single experiment representative of three individual replicates.

condensation of the cytoplasms and irregular architecture of organelles. In addition, no damage was detected on the cell wall. These effects were very similar to those occurring in targeted fungi treated with iturin A [10] and other antibiotics [22,23]. However, the differences are noteworthy. The formation of small vesicles, which is closely related to the antifungal activity, between the cell wall and yeast cell membrane or in artificial membrane treated with iturin A was observed in previous works [10]. In our case, this phenomenon has not occurred. This may be attributed to the differences of the plasma membrane or the insufficient concentration of bacillomycin L. Also of note, iturin A was reported to interact with intracellular membranes due to the marked alteration of the nuclear membrane in TEM images [10]. Consistent with this, in this study, the nuclear membrane of *R. solani* hyphae was badly damaged, indicating that bacillomycin L was probably able to interact with intracellular targets.

Unlike a few investigations involved in the interaction of iturinic antibiotics with intact fungal cells in terms of release of electrolytes and other cytoplasmic components [10,24], the permeabilization of the fungal hyphae by bacillomycin L using a membrane-impermeable fluorescent dye SG was studied and compared with that by amphotericin B for the first time in our study. This cyclic amphipathic molecule whose molecular weight is 1000, amphotericin B, shared many similarities of physicochemical property with bacillomycin L [2]. It is believed to exert its antibacterial activity results from its ability to increase the membrane permeability of target cells owing to the formation of conducting pores, which consist of an annulus of eight amphotericin B molecules linked hydrophobically to the membrane sterols forming the staves in a barrel-like structure [18,25].

Fluorescence microscopy observations in combination with SYTOX Green uptake quantification assay were utilized to determine permeabilization and evaluate the relevance between membrane permeabilization and antibiotic effects on intact fungal hyphae. Data presented in the study demonstrated that the permeabilization of the plasma membrane was dependent on antibiotic concentration, and the response curve correlated roughly with that found for growth inhibition. However, both fluorescence microscopy images and dose–response curves observed that bacillomycin L at the concentrations of $IC_{50}$ (50 μg/ml) had an effect on fungal growth while it permeabilized the fungal hyphae slightly. In contrast, amphotericin B at the concentrations of $IC_{50}$ (1 μg/ml) had much more intense permeabilization than that of bacillomycin L at $IC_{50}$, even though they had a similar antifungal effect at this point. The growth inhibition and membrane permeabilization by bacillomycin L were not completely relevant, as has been previously suggested for a number of linear antimicrobial peptides on filamentous fungi [12,17].

Time course analysis of SYTOX Green uptake showed that the permeabilization of fungal hyphae increased with increasing concentrations of bacillomycin L for 2 h.

Nevertheless, with all the given concentrations even up to 300 μg/ml (2–3 times MFC), amphotericin B had only a negligible effect in increasing permeabilization. This may be attributed to the diversity of the mechanism of action between these antibiotics. The interaction of amphotericin B with fungal membrane required the presence of sterol as the binding site, which was limited in plasma membrane, indicating that the time of 2 h was too short to permeabilize *R. solani* hyphae substantially. Compared with amphotericin B, bacillomycin L was not restricted to interaction with sterol and also with phospholipid molecules or other targeted sites. Nevertheless, it should be noted that no significant changes in fungal biomass would be observed within so short a time of 2 h, suggesting that a direct correlation between permeation and growth inhibition may be plausible in this experiment. Taken together, these results showed that the growth-inhibitory mechanisms of bacillomycin L and amphotericin

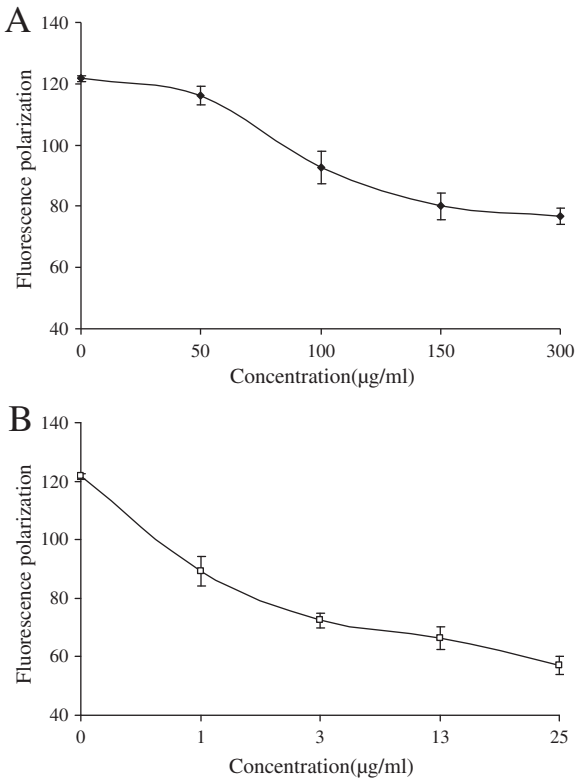


**Fig. 6.** Effect of bacillomycin L (A) or amphotericin B (B) on the polarization of *Rhizoctonia solani* hyphal membrane stained with DPH. Hyphae were treated with increasing concentrations of bacillomycin L or amphotericin B for 24 h prior to incubation with DPH. The error bars represent the S.E. values for three independent experiments performed in triplicate.

B on *R. solani* hyphae were different, and we can cautiously speculate that membrane permeabilization is primarily responsible for the activity of amphotericin B but not bacillomycin L.

Fluorescence polarization measurements with DPH were employed to evaluate the effect of bacillomycin L on membrane fluidity. From the data obtained, it was likely that both the bacillomycin L and amphotericin B altered fungal membrane fluidity in a dose-dependent manner. This may be due to the interaction of them with sterols, an important component of fungal plasma membrane which involves changes in membrane fluidity. This is consistent with earlier works on artificial membranes that iturins interact strongly with sterols [2,5,6]. This finding further supports the conclusion that the membrane disruption by bacillomycin L may play an essential role in the activity against *R. solani* hyphae.

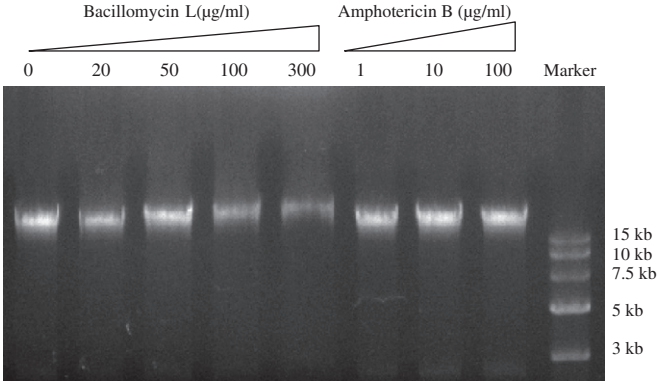


**Fig. 7.** Analysis of DNA-binding properties of bacillomycin L or amphotericin B by gel retarding assay. *R. solani* hyphal cells' DNA (250 ng) was incubated with increasing amounts of antibiotics (as given above the gel) for 30 min at room temperature before electrophoresis on a 1.0% agarose gel.

Iturin A had been proposed to interact with the plasma membrane and other cytoplasmic targets of yeast cells [10]. To confirm this similar effect in the presence of bacillomycin L, interaction of the lipopeptide with hyphal cells' DNA was examined firstly by gel retardation experiments. The results indicated that bacillomycin L had the ability to bind to *R. solani* hyphal cells' DNA and amphotericin B did not. This may be ascribed to the distinct diversity of structural characteristics. In contrast to amphotericin B, bacillomycin L is in possession of seven polar amino acid residues, several of which, such as Gln, Asn, or Ser residue, have been proposed to bind predominantly with DNA through hydrogen bonds [26–28]. Although a detailed experiment on the interaction between bacillomycin L and nucleic acids is needed, the present results lead us to believe that they interact with some intracellular targets via binding to DNA of fungal cells after penetrating the cell membranes, resulting in the cell death. Consistent with this concept, numerous antimicrobial peptides in addition to lipopeptides have been reported to have parallel effects on the nucleic acids of cells [12,15,29,30].

## 5. Conclusion

On the basis of the previous investigations about iturinic antibiotics on model membrane or yeast cells in combination with the aforementioned results in our study, we can cautiously conclude that the inhibitory activity of bacillomycin L against *R. solani* Kühn may not only solely involve membrane permeabilization, but also link to the interaction with the intracellular targets, such as cells' DNA. Our findings provide important new insights into the mechanism of action of iturins with the ability to suppress plant fungal pathogen, and pave the road for the design of new and promising antifungal peptides for a variety of biocontrol agents.

## Acknowledgements

This work was supported by grants from the National Natural Science Foundation of China (No. 31172001), and supported by the Special Fund for Agro-scientific Research in the Public Interest of China (201303014).

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