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# Induction of Chlamydospores in *Trichoderma harzianum* and *Gliocladium roseum* by Antifungal Compounds Produced by *Bacillus subtilis* C2

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## Abstract

One bacterial strain was isolated from a *Panax notoginseng* field and identified as *Bacillus subtilis* C2 by its physiological and biochemical characteristics. The culture filtrate of the bacterium showed strong antifungal activity against *Trichoderma harzianum* and *Gliocladium roseum*. The antifungal compound termed 10M was purified from the culture filtrates of *B. subtilis* C2 through organic solvent extraction, silica gel H and Sephadex LH-20 column chromatography. Chlamydospores were formed either inside the conidia or from hyphae, when the conidia of *T. harzianum* and *G. roseum* were cultured on the surface of the potato dextrose agar (PDA) containing relatively low concentrations of 10M for 48 h. While they were cultured on the surface of PDA containing relatively high concentrations of 10M for 48 h the cells were swollen and then lysed. The 10M had high similarity to iturin A as showed by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry. This is the first report that antifungal compounds produced by *B. subtilis* can induce chlamydospore formation in biocontrol fungi at relatively low concentrations.

## Introduction

*Trichoderma* spp. and *Gliocladium* spp. have received considerable attention as potential biological control agents against a wide range of soil-borne plant pathogenic fungi (Papavizas, 1985) in greenhouse (Papavizas et al., 1982; Kuter et al., 1983; Morandi et al., 2003) and field (Hadar et al., 1984; Knudsen et al., 1991). Chlamydospore formation by *Trichoderma* spp. and *Gliocladium* spp. were routinely mentioned in taxonomic papers (Domsch et al., 1980). Caldwell (1958) was among the first to observe that chlamydospores survived in soil better than conidia. Many researches have demonstrated the formation of chlamydospores by *T. hamatum*, *T. harzianum*, *T. viride* and *G. virens* under natural conditions after they were introduced into soil (Lewis and Papavizas, 1983, 1984; Bae and Knudsen, 2000). These chlamydospores may play an important role as survival structures of introduced *Trichoderma* spp. and *Gliocladium* spp. in natural ecosystems (Papavizas, 1985). But little is known about the factors that enhance their formation.

Substances in soil were reported to induce chlamydospore formation in *Fusarium* spp. (Ford et al., 1969a). Many researches (Venkat Ram, 1952; Ford et al., 1969b; Camyon and Gerhardson, 1997) supported the hypothesis that specific bacteria were responsible for the chlamydospore-inducing substances in soil and that changes in numbers and types of bacteria might affect the ability to produce chlamydospores in soil. These studies suggest that soil bacteria may be an important factor-inducing chlamydospores in fungi. However, there is no report that soil bacteria and their metabolites can induce chlamydospores in *Trichoderma* spp. and *Gliocladium* spp.

In our previous study on soil bacteria that could influence the growth and efficacy of the introduced fungi *T. harzianum* and *G. roseum*, we isolated a bacterial antagonist C2 of *T. harzianum* and *G. roseum* from the rhizosphere of *Panax notoginseng*. The bacterial strain C2, the culture filtrate of which showed significant antifungal activity, was identified to be *B. subtilis*. The culture filtrate of C2 could also induce chlamydospore formation in the fungi. The aim of our present study was to isolate and partially identify antifungal compounds produced by *B. subtilis* that can induce chlamydospore formation in biocontrol fungi.

## Materials and Methods

### Fungal isolates and bioassay for measuring antifungal activity

*Trichoderma harzianum* (CGMCC No. 0975) and *G. roseum* (reclassified as *Clonostachys rosea*; Schroers

et al., 1999; CGMCC No. 0807) originally obtained from the China general microbiological culture collection centre, were grown on potato dextrose agar (PDA) for 6 days at 28 ± 2°C. The conidia of the fungi were washed out with sterile water. The resulting suspensions were adjusted with sterile water to a concentration of $1.0 \times 10^7$ conidia/ml.

The bioassay followed was used to guide the purification of antibiotics produced by bacterial strain C2. A 0.10 ml fungal inoculum prepared above was spread on PDA Petri plate (9 cm diameter) by sterilized glass scraper. A filter paper (8 mm diameter) containing 20 μl sample was dried by electro-blower for complete removal of solvent and then placed on the surface of the Petri plate prepared above. Clear inhibition zone appeared after the plate was incubated for 3 days at 28 ± 2°C indicating activity of this sample.

**Identification of bacterium C2**

The bacterial strain C2, the culture filtrate of which showed strong antifungal activity against *T. harzianum* and *G. roseum*, was isolated from the rhizosphere of *Panax notoginseng*-growing areas in Wenshan country, Yunnan Province, located in the west of China. The strain C2 was cultured in Luria Bertani (LB) agar at 37 ± 2°C. Bacterial phenotypic characterization by physiological and biochemical tests was performed according to the standard methods (Krieg and Holt, 1984).

**Production and purification of antibiotics**

A loop of C2 cells from a slant culture of fresh nutrient agar was inoculated to a 300 ml flask containing 150 ml LB broth (pH 7.0). The flask was incubated on a rotary shaker at 180 rpm for 24 h at 36°C. This fresh culture was inoculated to other flasks containing the same medium, each 3 ml. These flasks were incubated under the same conditions as described above for 36 h. A total of 9 l of the cultures of *B. subtilis* strain C2 was obtained for further purification of antibiotics. The cells of *B. subtilis* strain C2 were removed from the culture broth (9 l) by centrifugation at 5159 ***g***. The culture filtrates were reduced to 300 ml in vacuo and extracted three times with an equal volume of acetic ether (EtOAc). The concentrated EtOAc layers were pooled and subjected to column chromatography over Silica gel H (200 g, 200–300 mesh; Qingdao Marine Chemical Ltd, Qingdao, China) eluted with chloroform containing increasing amount of methanol to yield 61 fractions. The elution fractions were combined based on antifungal bioassay. The active fractions were combined and subjected to column chromatography over Sephadex LH-20 resin (Amersham Bioscience AB, SE-75184 Uppsala, Sweden 2.5 × 105 mm) eluted with methanol to yield 20 fractions. Each fraction was subjected to the antifungal bioassay. The active fractions were combined to obtain the active fraction, tentatively termed 10M.

**Effect of temperature on antifungal activity**

The 10M was stored for 1 month at 25°C, or treated at 100°C for 10, 30, 50 or 70 min, or subjected to autoclaving for 15 min at 121°C. After allowing the heated samples to cool (25°C), all samples were subjected to the antifungal bioassay. Control samples were kept at −4°C in refrigerator. The experiment was carried out twice.

**Thin layer chromatographic analysis**

The 10M was examined by thin layer chromatography (TLC) on silica plates obtained from Qingdao (0.2 mm thick; G, Qingdao Marine Chemical Ltd, Qingdao, China). A chloroform : methanol : water mixture (65 : 25 : 4, v/v/v) was used as the mobile phase (Yu et al., 2002). Spots were visualized by charring with $H_2SO_4$ and heating the plates at 200°C for 30 min. Spots were also detected by spraying with water for detection of hydrophilic compounds, with ninhydrin for detection of peptide bonds or 4,4′-bis(dimethylamino)diphenyl-methane (TDM) reagent (Sigma Chemical Co., St Louis, MO, USA) for peptide bonds (Yu et al., 2002).

**Chlamydospore formation in the presence of 10M**

The 10M (20 mg) was dissolved in 20 ml dimethyl sulphoxide (DMSO) to obtain a homogenous active solution (HAS). Petri plates (5 cm diameter) filled with 100, 150, 200, 250, 300, 400 or 500 μl HAS, respectively, were mixed with PDA approximately 10 ml precooled to 45°C and each treatment had two replications. A 50 μl conidial suspension of *T. harzianum* or *G. roseum* (approximately $1 \times 10^7$ conidia/ml), respectively, was spread evenly on the surface of the Petri plate prepared above with a sterilized glass scraper. They were then incubated at 28 ± 2°C. As the control, the conidia were spread on the surface of the Petri plates filled with PDA containing 0, 100, 150, 200, 250, 300, 400 or 500 μl DMSO, respectively, in the absence of antibiotics. The chlamydospore formation was observed microscopically (Olympus BX51TF, Tokyo, Japan) from the uniform cork-borer discs of the media cut randomly every 4 h in one of the two replications. After the remnant replication was incubated for 48 h chlamydospore number induced by each concentration of 10M in unit area was determined microscopically with three uniform cork-borer discs of the media cut randomly in this replication. All experiments were carried out three times.

**Identification by mass spectrometry analysis**

The mass spectrometry analysis of 10M was performed on a matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) instrument (API, Qstar, AB Ltd, USA, Q-TOF) with a 337 nm nitrogen laser for desorption and ionization. Approximately 1–2 μl of 10M was mixed with an equal volume of matrix medium (a saturated solution of β-cyano-4-hydroxycinnamic acid in 70% aqueous acetonitrile containing 0.1%, v/v, TFA (trifluoroacetic acid)). Ions were accelerated with a voltage of 5.5 kV. Positive ion detection and the reflector mode were used.

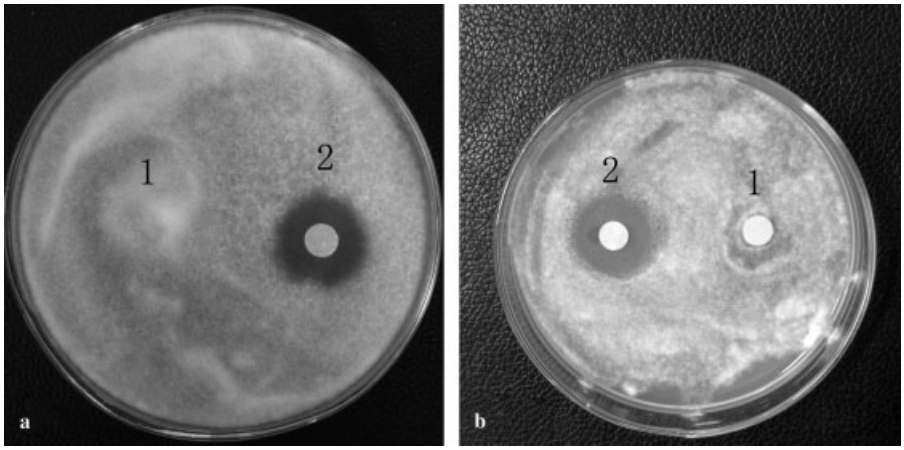


Fig. 1 *In vitro* growth inhibition of the biocontrol fungi caused by 10M produced by *Bacillus subtilis* strain C2. (a) An inhibition zone for growth inhibition of *Trichoderma harzianum* on potato dextrose agar (PDA) containing 10ML: (1) a control disc obtained by drying a filter paper with 20 $\mu$l methanol; (2) a disc obtained by drying a filter paper with 20 $\mu$l 1.5 mg/ml 10M in methanol. (b) An inhibition zone for growth inhibition of *Gliocladium roseum* on PDA containing 10M: (1) a control disc obtained by drying a filter paper with 20 $\mu$l methanol; (2) a disc obtained by drying a filter paper with 20 $\mu$l 1.5 mg/ml 10M in methanol

## Results

### Characterization and identification of bacterial strain C2

The cell size of strain C2 was found to be 0.9–1.5 $\mu$m by 1.7–2.0 $\mu$m. Strain C2 was identified as an aerobic, motile, endospore forming, catalase-positive, Gram-positive and lecithinase-negative bacterium. Nitrate reduction to nitrite was found. Indole or $SO_2$ was not produced. The strain C2 utilizes citrate. Casein was hydrolysed. No hydrolysis of tyrosine was observed. It metabolizes mannitol, glycerol, arabinose, xylose and starch. It also utilizes glucose, but gas production was not found. Strain C2 grew at nutrient broth (pH 5.7). It could tolerate well up to 10% NaCl in the basal medium. These tests indicated that the most probable identity of the isolate was *B. subtilis* according to Bergey's Manual of Systematic Bacteriology (Krieg and Holt, 1984).

### Purification

The EtOAc extract (6.0 g) was subjected to column chromatography over Silica gel to produce 61 fractions (EE-1 to EE-61). Eight consecutive fractions (EE-46 to EE-50) had antifungal activity and were combined. The combined fraction (0.5 g) was subjected to column chromatography over Sephadex LH-20 to yield 20 factions (EES-1 to EES-20). Four consecutive fractions (EES-6 to EES-8) had antifungal activity and were combined to give the active fraction 10M (approximately 200 mg).

### Antifungal activity and stability of the 10M

The purified 10M could clearly inhibit growth of *T. harzianum* and *G. roseum* (Fig. 1). The antifungal activity did not reduce after the 10M was stored for 1 month at room temperature. No activity loss was observed even after it was heated at 100°C for 30 min indicating the 10M was thermostable. However, longer heating (70 min) would decrease the activity by 40%. Autoclaving for 15 min at 121°C reduced activity by 60%. Furthermore, the 10M was found to be resistant to proteolytic enzymes, including pronase E, proteinase K and trypsin (data not shown).

Table 1
Effect of different concentrations of 10M on conidial cells of *Trichoderma harzianum*

| Series | Concentration of 10M (mg/ml) in medium | Number of chlamydospores formed per unit area |
|---|---|---|
| A | $1 \times 10^{-h}$ | 12 |
| B | $1.5 \times 10^{-2}$ | 83 |
| C | $2.0 \times 10^{-2}$ | 146 |
| D | $2.5 \times 10^{-2}$ | 214 |
| E | $3.0 \times 10^{-2}$ | 71 |
| F | $4.0 \times 10^{-2}$ | 25 |
| G | $5.0 \times 10^{-2}$ | 0 |
| Water control | 0 | 0 |
| DMSO control | 0 | 0 |

The numbers above were recorded after incubation of the conidia for 48 h. Chlamydospore numbers were determined by counting chlamydospores in microscope visual fields (×100 magnification) from each uniform cork-borer disc of the media. For each disc of the media the chlamydospores of six microscope visual fields (×100 magnification) were counted. Thus, the number of chlamydospores induced by each concentration of 10M is the mean values of 18 counts. Mean values within the chlamydospore number column are significantly different according to an ANOVA test followed by Duncan's multiple range test ($P \leq 0.05$).

### TLC analysis

The 10M appeared as a single spot on the TLC plate at $R_F$-value of 0.49. It was ninhydrin-negative, and positive to TDM reagent. These results indicated the absence of free amino groups and the presence of peptide bonds in the compounds. A white spot formed when the plate was sprayed with water indicating that the compounds were lipophilic (Yu et al., 2002).

### Chlamydospore formation by 10M

The effect of 10M on the morphology of *T. harzianum* conidia varied depending upon the concentrations of the reagent (Table 1). Relatively lower concentrations of 10M could induce chlamydospores formation in

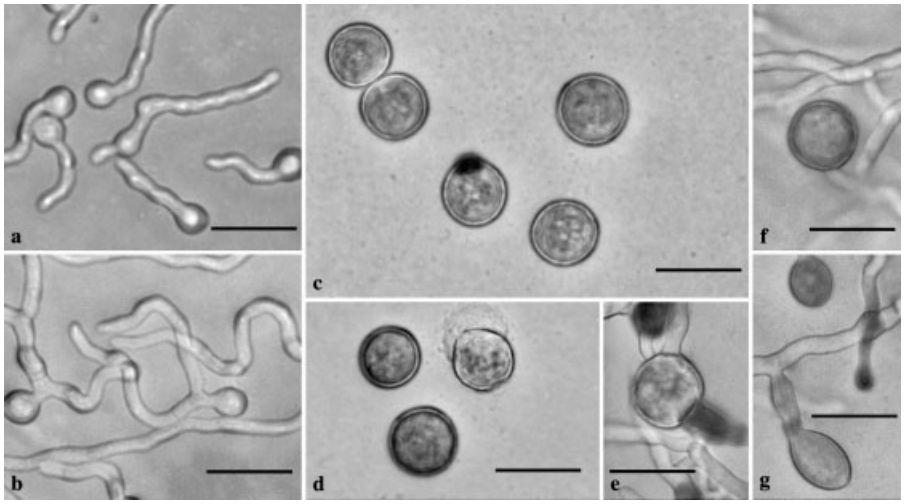


Fig. 2 Effect of different concentrations of 10M on chlamydospore formation in *Trichoderma harzianum*. (a) Control (the conidia of *T. harzianum* germinated normally when they were cultured on potato dextrose agar (PDA) in the absence of 10M for 12 h); (b) control (normal mycelia of *T. harzianum* cultured on PDA mixed with dimethyl sulphoxide (DMSO) in the absence of 10M for 16 h); (c and d) conidial chlamydospores formed on PDA containing $2.0 \times 10^{-2}$ mg/ml 10M; (e) chlamydospore formed intercalarily in hyphae on PDA containing $1 \times 10^{-2}$ mg/ml 10M; (f and g) chlamydospores formed terminally in hyphae tip on PDA containing $1.5 \times 10^{-2}$ mg/ml 10M (the scale bars are 20 $\mu$m)

*T. harzianum*, while at higher 10M levels, the cells were swollen and then lysed.

The conidia of *T. harzianum* germinated and grew normally when they were cultured on PDA or PDA mixed with DMSO, respectively (Fig. 2a,b). Chlamydospores were produced in the conidial cells (Fig. 2c,d), hyphae (Fig. 2e), or terminally in the hyphae tips (Fig. 2f,g) when conidia of *T. harzianum* were cultured on PDA mixed with 100, 150 or 200 $\mu$l HAS for 48 h; when the conidia of *T. harzianum* were cultured on PDA mixed with 250 $\mu$l HAS even for 16 h most of them were converted directly into chlamydospores. The development of conidial chlamydospores is illustrated in Fig. 3. As illustrated

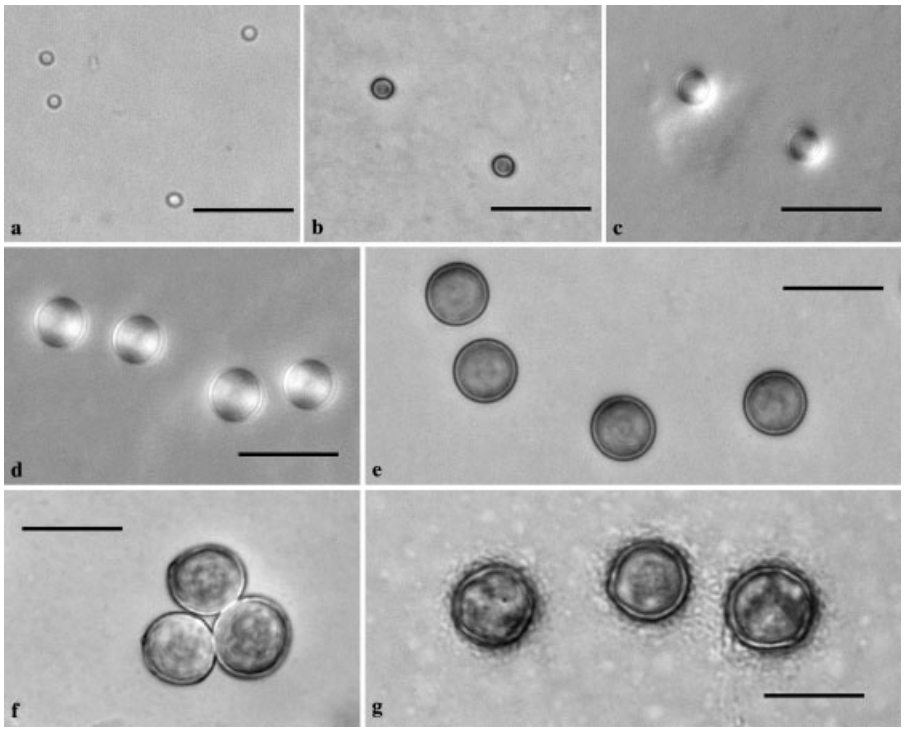


Fig. 3 Development of conidial chlamydospores of *Trichoderma harzianum* cultured on potato dextrose agar (PDA) containing $2.5 \times 10^{-2}$ mg/ml 10M. (a) Conidia of *T. harzianum* used as starting inoculum for chlamydospore development on PDA containing 10M; (b) swollen conidia cultured for 4 h; (c and d) 'changed' conidia cultured for 8 h or 12 h respectively; the wall of them is a little thicker than swollen conidia; (e–g) conidial chlamydospores with thick wall cultured for 16 h, 24 h or 48 h respectively (the scale bars are 20 $\mu$m)

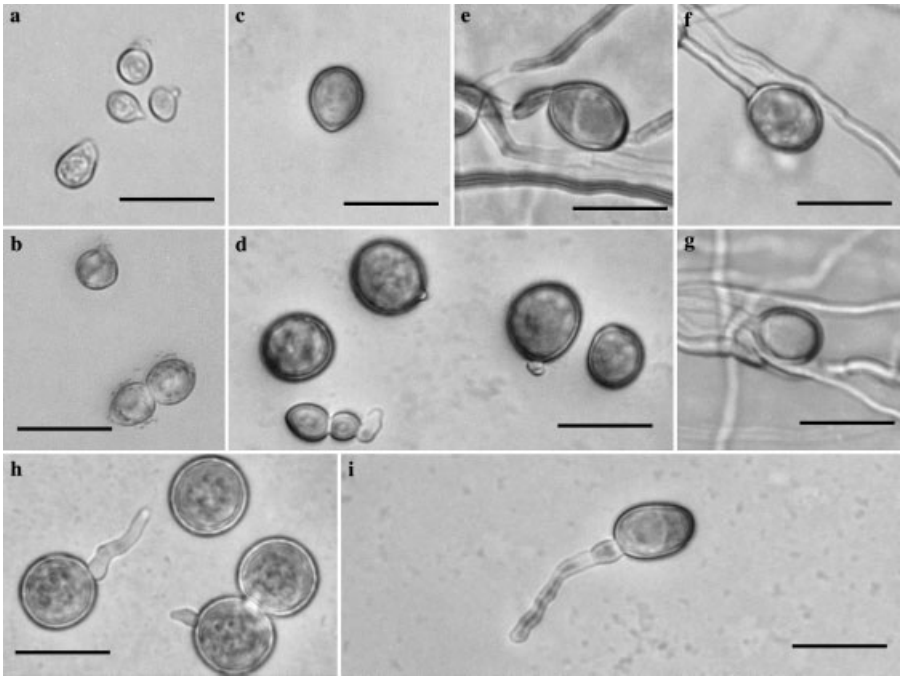


Fig. 4 Lysis of conidia and germination of chlamydospores in *Trichoderma harzianum* and *Gliocladium roseum*. (a) Lysis of swollen *T. harzianum* conidia cultured on potato dextrose agar (PDA) containing $5.0 \times 10^{-2}$ mg/ml 10M; (b) lysis of swollen *G. roseum* conidia cultured on PDA containing $5.0 \times 10^{-2}$ mg/ml 10M; (c and d) conidial chlamydospores of *G. roseum* formed on PDA containing $3.0 \times 10^{-2}$ mg/ml 10M; (e) a chlamydospore of *G. roseum* formed intercalarily in hyphae on PDA containing $2.0 \times 10^{-2}$ mg/ml 10M; (f and g) chlamydospores of *G. roseum* formed terminally in hyphae tip on PDA containing $2.5 \times 10^{-2}$ mg/ml 10M; (h) germinated chlamydospores of *T. harzianum* cultured on PDA in the absence of 10M; (i) a germinated chlamydospore of *G. roseum* cultured on PDA in the absence of 10M (the scale bars are 20 $\mu$m)

in Fig. 3b, the conidia were swollen after being cultured for 4 h. Then the swollen conidia continued to enlarge and the wall became thick at 8 and 12 h (Fig. 3c,d). Conidia of *F. sulphureum* that showed similar changes in distilled water were described by Schneider and Seaman (1974) as 'changed' conidia. After incubation for 16 h many conidia were observed to differentiate into chlamydospores (Fig. 3e) and the conidia chlamydospores became larger in diameter for longer incubation (Fig. 3f,g). On PDA mixed with 300, 400 or 500 $\mu$l HAS after 48 h most conidia of *T. harzianum* were swollen, and then lysed (Fig. 4a).

Most chlamydospores induced by 10M were globular or subglobular, with smooth and thick walls, ranging in size from 9 to 16 $\mu$m. The biggest diameter of chlamydospores of *T. harzianum* reached 20 $\mu$m (Fig. 3g). These chlamydospores could germinate after they were moved to PDA in the absence of 10M, indicating that these chlamydospores were alive (Fig. 4h).

Chlamydospores were produced in the conidia (Fig. 4c,d), intercalarily in the hyphae (Fig. 4e), or terminally in the hyphae tips (Fig. 4f,g), when conidia of *G. roseum* were cultured on PDA mixed with 200, 250 or 300 $\mu$l HAS for 48 h (Fig. 4). The conidia of *G. roseum* were swollen and then lysed (Fig. 4b) when being cultured on PDA mixed with high concentrations of 10M. Chlamydospores of *G. roseum* germinated after being transferred to PDA in the absence of 10M, indicating that the chlamydospores were alive (Fig. 4i).

**Identification of the antibiotics**

The MALDI-TOF mass spectrum of the purified compound 10M is illustrated in Fig. 5. Three $[M + H]^+$ peaks occurred at $m/z$ 1043.1, 1057.3, 1071.4. These peaks were shifted to $m/z$ 14 lower indicating isoforms

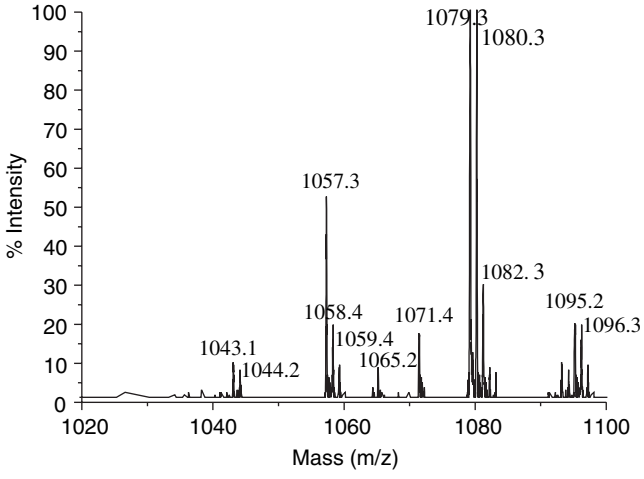


Fig. 5 Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrum of the lipopeptide iturin fraction of *Bacillus subtilis* C2. The cyclic lipopeptide appeared as a complex mixture of isoform that show variations in the length of their $\beta$-amino fatty acid moiety ($n = 10$–$12$ $CH_2$ groups). The $[M + H]^+$ peaks at $m/z$ 1043.1, 1057.3, 1071.4 were accompanied by the corresponding $[M + Na]^+$ peaks ($m/z$ 1065.2 and 1079.3)

of iturin A. One series of ion was found at 1065.2 and 1079.3 indicating sodium adducts (Fig. 5).

## Discussion

*Bacillus subtilis* strain C2 isolated in our laboratory-excreted metabolites with antifungal activity against growth of *T. harzianum* and *G. roseum*. The antifungal activity of the purified 10M produced by *B. subtilis* strain C2 was resistant to high temperature and the action of many hydrolytic enzymes. It was negative to ninhydrin and positive to TDM reagent indicating the presence of cyclic peptide. These characteristics indicated that the antifungal compound might belong to the iturin group of antibiotics (Chitarra et al., 2003). The iturin group consists of iturin A–E, bacillomycins D, F and L, and mycosubtilin (Bland, 1996). MALDI-TOF mass spectrometry was successfully used to detect and characterize lipopeptides directly in crude culture filtrates of *B. subtilis* with complex compositions (Vater et al., 2002). Compared with the mass numbers reported for the lipopeptide complexes from other *B. subtilis* strains (Leenders et al., 1999; Vater et al., 2002), this group of peaks of 10M might be attributed to iturin A isomers.

When the conidia of *T. harzianum* and *G. roseum* were cultured on the surface of PDA mixed with relatively high concentrations of 10M for 48 h, they were swollen and then died (Fig. 4a,b). The mechanism of action for iturin is that penetration into the cytoplasmic membrane by the hydrophobic tail is followed by auto-aggregation to form a 'pore' that causes cellular leakage (Maget-Dana et al., 1985). It is conceivable that auto-aggregation in lipid membrane needs enough quantity of antifungal compounds.

In the present study, the antifungal compound 10M isolated from the culture filtrate of *B. subtilis* C2 could induce chlamydospore formation in *T. harzianum* and *G. roseum* at relatively low concentrations, as PDA and PDA mixed with DMSO themselves did not promote appreciable chlamydospore formation. Moreover, these chlamydospores could be produced in appreciable amounts from conidia of *T. harzianum* and *G. roseum*. Previously, chlamydospores of *T. harzianum* were shown to form from conidia in natural soil and organic debris (Lewis and Papavizas, 1983). A similar differential reaction of various fungal isolates to chlamydospore formation in mixed cultures with bacteria was reported by many researchers (Venkat Ram, 1952; Ford et al., 1969a; Camyon and Gerhardson, 1997) in *F. solani* f. sp. *phaseoli* and *Phoma foveata*, who suggested that these bacteria produce antifungal compounds *in situ* that could induce chlamydospore formation by *F. solani*. However, insufficient amounts of metabolic products from the bacteria were isolated and detected to account completely for the induction of chlamydospore formation. This is one of the few reports about induction of chlamydospore formation in *T. harzianum* and *G. roseum* by purified antifungal compounds produced by *B. subtilis*.

The nature of the process by which the conidia and the thin-walled vegetative portions of the fungi convert to the thickened, double walled and resting spores is still not clear. Our studies indicate that the process is initiated in the fungi as a response to the antifungal compounds produced by *B. subtilis*. Purification and identification of such substances could make it possible to perform a detailed experimental study of the mechanism of chlamydospore formation.

Previous studies have established the presence of substances in soils that induced chlamydospore formation by *Fusarium* and suggested that these substances may be antibiotics produced by bacteria (Venkat Ram, 1952; Ford et al., 1969a,b). Our studies showed that the antifungal compounds produced by *B. subtilis* C2 could induce chlamydospore in *T. harzianum* and *G. roseum* at relatively low concentrations. The production of antifungal compounds has been described for a wide range of soil micro-organisms including bacteria (Burgess et al., 1999) and many bacteria were demonstrated to produce antibiotics in the spermosphere and rhizosphere (Raaijmakers et al., 2002). Many lipopeptides produced by soil micro-organisms have the same antifungal mechanism as iturin A, e.g. syringopeptin and syringomycin produced by *Pseudomonas syringae* pv. *syringae* (Serra et al., 1999), viscosinamide produced by *P. fluorescens* DR54 (Thrane et al., 1999), surfactin and the iturinic group (Maget-Dana and Ptak, 1995). So it was reasonable that these antibiotics can induce chlamydospores in *Trichoderma* spp. and *Gliocladium* spp. at relatively low concentrations and are present in soil. Furthermore, evidence is emerging that the vegetative form of *B. subtilis* is prevalent in nutrient-rich environments such as the rhizosphere. There are numerous reports of isolation of *B. subtilis* from the rhizosphere of a range of plant species (Vullo et al., 1991; Mahaffee and Backman, 1993; Pandey and Palni, 1997) at concentrations as high as $10^7$/g of rhizosphere soil (Pandey and Palni, 1997). *Bacillus subtilis* RB14 was suggested to produce surfactin and iturin in a sterilized vermiculite-soil system (Asaka and Shoda, 1996). Pandey et al. (2001) reported that the dominant *B. subtilis* had antagonistic activity against *Trichoderma* species under *in situ* condition in the rhizosphere of established tea bushes, but it was not known whether the antagonism was induced by iturin. The diversity of bacteria that can produce antibiotics and prevalence of *B. subtilis* in soil would indicate that this fungus–antibiotics relationship may prevail in many soil types, and is potentially of great importance in the production of chlamydospores by *Trichoderma* spp. and *Gliocladium* spp. Further study on this fungus–antibiotics relationship in natural soils is necessary and now under investigation.

*Bacillus subtilis* was reported to be effective for the biocontrol of multiple plant diseases caused by soil-borne pathogens (Turner and Backman, 1991; Asaka and Shoda, 1996). Fravel (1988) discussed the possibility of deleterious effects of antibiotic compounds, produced by biocontrol agents, on beneficial

micro-organisms. In the present study, iturin produced by *B. subtilis* showed antibiosis against *T. harzianum* and *G. roseum in vitro*. But there was a report showing that the combination of *T. harzianum* and *B. subtilis* is compatible and effective in suppressing Fusarium wilt (Hervás et al., 1998). As for the compatibility of antagonists applied as strain mixtures, it is usually not only dependent on compatible utilization of nutrients, minerals and spaces but can also be influenced by molecular signalling between the individual antagonists (Duffy et al., 1996; Lutz et al., 2004). However, the result that antibiotic produced by *B. subtilis* can induce chlamydospores in *Trichoderma* at low concentrations gives a new perspective to explain this compatibility. Environmental factors influence the production of antibiotic compounds including iturin produced by *B. subtilis* (Raaijmakers et al., 2002). It is possible that an antibiotic may not reach the threshold concentration for antagonistic activity within certain microsites where *Trichoderma* spp. could form chlamydospores. Induction of chlamydospores may compensate for slight growth inhibitory effects and improve the control efficacy of *Trichoderma* spp. So further study to investigate the interaction between these biocontrol agents more thoroughly is required.

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