Ann Microbiol (2013) 63:1075–1081
DOI 10.1007/s13213-012-0564-y

ORIGINAL ARTICLE

Purification and characterization of antifungal compounds from *Bacillus coagulans* TQ33 isolated from skimmed milk powder

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Received: 13 January 2012 / Accepted: 8 October 2012 / Published online: 1 November 2012

Abstract *Bacillus coagulans* TQ33, isolated from skimmed milk powder, displays strong antifungal activity against plant pathogenic fungi. The antifungal compound of the *B. coagulans* TQ33 culture was extracted by thin-layer chromatography and column chromatography, and its structure was elucidated based on HPLC, LC-MS, and NMR analysie. The antifungal compound was identified as phenyllactic acid (PLA), and it was found to have a minimum inhibitory concentration on *Phytophthora drechsleri* Tucker of 18 mg/mL. Bio-control activity tests indicated that PLA has a wide spectrum of antagonistic effects against *Fusarium oxysporum*, *Botrytis cinerea*, *Glomerella cingulata*, *Penicillium citrinum*, *Penicillium digitatum*, particularly against *F. oxysporum*. PLA is the most notable antimicrobial compound with broad and effective antimicrobial activity against both bacteria and fungi that has been isolated and identified to date. These results indicate that *B. coagulans* TQ33 has the potential for application in biological pesticides.

Keywords Plant pathogenic fungi · Antifungal activity · *Bacillus coagulans* TQ33 · Phenyllactic acid

Introduction

Plant pathogenic fungi cause many diseases that result in devastating biomass and crop losses to a wide variety of plants (Kuhajek et al. 2003) and in some cases the production of mycotoxins (Strange and Scott 2005). Many composite chemical fungicides have been used to inhibit fungal growth in various environments, but while some chemical fungicides are generally effective in controlling the growth of harmful fungi, the use of chemicals is considered undesirable because of concerns over potentially harmful residues (Matsumo 1990). The use of microorganisms to prevent fungal pollution has been gaining increasing interest due to the public's demand to reduce the potential damaging effects of chemical fungicides on the environment (Prema et al. 2008). Consequently, biocontrol has become a significant alternative to conventional methods (Raspor et al. 2010).

Bacillus coagulans is a lactic acid-forming and facultative anaerobic bacterial species within the genus *Bacillus* that is resistant to acid and heat. It is easy to culture and can be stored and preserved with stability over a long period. Several studies have examined the antimicrobial properties of the substances produced by *B. coagulans*. Hyronimus and Urdaci (1998) reported a bacteriocin-like inhibitory substance produced by the *B. coagulans* I-4 strain that is a protease-sensitive antibacterial substance. Another strain of *B. coagulans* was isolated from an industrial wastewater drainage site and selected for its antimicrobial activities against bacteria and fungi. Antimicrobial activity was subsequently found against Gram-positive and Gram-negative bacteria and a yeast strain (Abada 2008). An efficient conversion of phenylpyruvic acid to phenyllactic acid (PLA) by whole cells of *B. coagulans* SDM was reported by Zheng et al. (2011). Lactosporin is a novel antimicrobial compound produced by a strain of *B. coagulans* ATCC 7050 with inhibitory activity against Gram-positive microorganisms (*Micrococcus luteus* and *Listeria monocytogenes*), but not against Gram-negative bacteria. Its antimicrobial activity against pathogenic microorganisms indicates that it may have a potential for application in foods and personal care products (Riazi et al. 2009).

Anti-plant pathogenic fungi strain *B. coagulans* TQ33, which was isolated from skimmed milk powder, has anti-

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plant pathogenic fungal properties; specifically, it produces antifungal compounds that can inhibit the growth of *Botrytis cinerea*, *Phytophthora drechsleri* Tucker, *Alternaria solani*, *Fusarium oxysporum*, and *Glomerella cingulata*. The focus of the study reported here was to purify and identify the structure of these antifungal compounds (Wang et al., Antifungal activity of *Bacillus coagulans* TQ33, isolated from skimmed milk powder, against *Botrytis cinerea*, unpublished).

Materials and methods

Strains and culture conditions

Bacillus coagulans TQ33 was isolated from skimmed milk powder and transferred to slants (in g/l of distilled water: peptone 20, yeast extract 3, glucose 2, agar 2; pH adjusted to approx. 7.0–7.2 using 2 mol/L NaOH solution; sterilization at 121 °C for 20 min). The slants were then incubated at 37 °C for 48 h.

Plant pathogenic fungi *B. cinerea*, *A. solani*, *P. drechsleri* Tucker, *F. oxysporum*, and *G. cingulata*, used as the indicator species, were obtained from the Institute of Plant Protection, Tianjin, PR China. All pathogenic fungal specimens were stored at −70 °C in 15 % glycerol and subsequently grown on potato dextrose agar (PDA) plates at 28 °C for 5–7 days.

Production of antifungal substance

A *Bacillus coagulans* TQ33 seed culture was prepared in a 100-mL flask with 20 mL of seed medium (in g/l of distilled water: peptone 20, yeast extract 3, glucose 2, $MgSO_4 \cdot 7H_2O$ 0.5; pH 7.2–7.4) and incubated at 38 °C and 140 rpm for 20 h. Fermentation was performed in a 500-mL flask containing 100 mL of fermentation medium (in g/l of distilled water: peptone 10, yeast extract 10, glucose 6, $MgSO_4 \cdot 7H_2O$ 1, K_2HPO_4 2; pH 7.0; sterilization at 121 °C, for 20 min; 5 % volume of seed culture was used as inoculum. The flasks were incubated at 38 °C and 140 rpm for 67 h.

Well-developed *B. coagulans* TQ33 cultures were centrifuged (8,000 rpm, 10 min) and filtered under sterile conditions (pore size 0.22 μm; Millipore, Billerica, MA). In subsequent analyses, antifungal compounds were isolated, purified, and identified from the cell-free supernatant.

Bio-control activity assays

Two types of bio-control activity assays were performed. The first was a Poison Food Technique in which the inhibitory effect of the test compounds on the mycelial growth of the plant pathogenic fungi was assayed (Wang et al. 2011). PDA was used as the medium for all test fungi. Media incorporating the test compounds at a concentration of 10 % (v/v) were inoculated with agar discs of the test fungi (5 mm) at the center of the assay plate. A sterile puncher was used to remove the 5-mm agar discs from fungi cultures incubated on PDA solid medium at 28 °C for 5 days. Three replicate plates for each fungus were incubated at 27±2 °C for all test fungi. Control plates containing media mixed with sterile water (10 %, v/v) were also included in the experiment. Following a 2- to 6-day incubation period, the mycelial growth of the fungi (in millimeters) in both the treatment (T) and control (C) petri dishes was measured diametrically in perpendicular directions until the fungi growth in the control dishes was almost complete. The mean of the two perpendicular colony diameters was used in the following calculation. The percentage of growth inhibition (I), which is considered to accurately reflect the antifungal activity of different substances, was calculated using the formula: $I(\%) = [(C - T)/C] \times 100$. The corrected inhibition (IC) was then calculated as: $IC(\%) = [(C - T)/(C - C0)] \times 100$. In the above formula C0 indicates the diameter of the test fungi agar discs (5 mm), C is the diameter of the mycelia in the control group, and T is the diameter of the mycelia in the test group.

The inhibition of the test compounds on the conidium growth of the pathogenic fungi was assayed in the second bio-control activity assay. Ten millilitres of 2 % agar was poured into a sterile Petri dish and allowed to harden, following which sterile Oxford cups (diameter7.64 mm) were punched into the plates and held in place by the solid agar. The plates were then overlaid with PDA (agar 0.8 %) containing 10^6 fungi conidia per milliliter; the overlaid PDA did not extend beyond the upper edge of the Oxford cups. The oxford cups were removed after the upper medium had solidified and the wells filled with 180 uLl PLA (concentration 12 mg/mL; test plates) or an equal volume of sterile water (control plates). Three replicate dishes for the test fungi were incubated at 27± 2 °C. The diameters of the inhibitory zones in the test dishes were measured in millimeters until fungal growth was almost complete in the control plates. The inhibition was graded according to the size of inhibited growth area: −, no visible inhibition; +, 0.1- to 7-mm zone of inhibition; ++, 7.1- to 14-mm zone of inhibition; +++, >14-mm zone of inhibition.

Isolation and assay of the antifungal compounds

The cell-free supernatant of *B. coagulans* TQ33 was initially extracted (1:3, v/v) with an eluotropic series of organic solvents, namely *n*-hexane, cyclohexane, dichloromethane, chloroform, ethyl acetate, and *n*-butanol; the entire process of extraction, concentration under vacuum, and evaporation (by rotary evaporator) at a temperature of <40 °C was repeated three times. The residue (partially isolated active fraction) was detected to have antifungal activity and subjected to further purification. The active fraction (concentrated organic phase) was separated by thin-layer chromatography (TLC) on silica gel plates (Merck, Kieselgel, Germany; 60 F254, 0.25 mm).

The TLC analysis was carried out with different ratios and kinds of organic solvents as solvent systems according to the method of Wilson (2005). The spots were visualized by exposing the plates to UV radiation and spraying with 10 % H_2SO_4 in CH_3OH, followed by heated at 110 °C for 10 min (Lavermicocca et al. 2000). The active fraction (concentrated organic phase) was then separated by column chromatography (CC) on silica gel (mesh size 200–400; Merck, Darmstadt, Germany), the concentrated active residue (10 g) was subjected to column chromatography (CC) on silica gel (mesh size 200–400; Merck, Darmstadt, Germany), and carried out at different ratios and kinds of organic solvent as the mobile phase. The isolated antifungal compounds fractions were collected and evaporated to dryness. The inhibitory activity against *P. drechsleri* Tucker was then determined using the Poison Food Technique as described.

High-performance liquid chromatography

The purified, active portion was further purified with preparative high-performance liquid chromatograph (HPLC) performed on an Agilent 1200 HPLC system (Agilent Technologies, Palo Alto, CA) using an Agilent PrepHT C18 column (21.2×150 mm, 5 μm; model 300SB). The elution was monitored using a UV detector at 210 nm and methyl cyanide–water (from 3:97 to 100:0) as a gradient elution for 30 min, with a flow rate of 10 mL/min and a column temperature of 35 °C. All peak fractions were collected and concentrated in vacuo to detect the antifungal activity.

Identification of the antifungal compounds

The active fraction obtained from preparative HPLC was further purified and determined by liquid chromatography-mass spectrometry (LC-MS) and nuclear magnetic resonance (NMR). LC-MS was performed on an UltraPerformance liquid chromatography system and an ESQUIRE-LC quadrupole ion trap mass spectrometer (Bruker Daltonics, Billerica, MA) with electrospray ionization. For chromatographic separation, a BEH C18 column (50×2.1 mm, 1.7 μm; Thermo Fisher Scientific, Waltham, MA) was kept at 25 °C. The mobile phases were: (1) acetonitrile with 0.1 % formic acid and (2) water with 0.1 % formic acid.

NMR spectra were recorded on a spectrometer equipped with a 2.5-mm microprobe using DMSO-d6 as a solvent. All spectra were recorded at 30 °C.

Minimum inhibitory concentration of PLA on *P. Drechsleri* Tucker

Phenyllactic acid was diluted to different concentrations (18, 14, 12, 10 5 mg/mL), and the inhibition of PLA on *P. drechsleri* Tucker was determined by the Poison Food Technique.

Results

Purification of the antifungal compounds

Liquid–liquid extraction was performed, and both organic and aqueous phases were obtained. The antifungal compounds were primarily found in the ethyl acetate fraction (Table 1), and the combined ethyl acetate of the culture filtrate was subsequently concentrated in vacuo to obtain 12.0 g of residue. TLC analysis of the residue was carried out in one of two solvent systems (methanol and ethyl acetate). The results showed that the residue was partially purified by preparative TLC to give three fractions, i.e., 1, 2, and 3, respectively. The ethyl acetate residue (10 g) was subjected to CC on silica gel using ethyl acetate–methanol (from 50:1 to 20:3) as the eluent. The results indicated (three partial) were recovered after fractional collection using silica gel (200–400 mesh). The rude extract (5.5 mg/disk) and three partially purified extracts were assayed by the Poison Food Technique using *P. drechsleri* Tucker as the indicator. Only the crude extract and the three partial extracts showed predominant antifungal activity; fractions 1 and 2 (partial extracts) displayed weaker effects (Table 2). The three partial extracts was further purified by preparative reverse-phase HPLC with a C18 column based on hydrophobicity to obtain retention times of 1.565, 5.590, and 16.154, respectively (Fig. 1). However, only the second partial extract (retention time 5.590) had strong antifungal activity.

Analysis of active compounds

Approximately 7 mg of the second partial extract (retention time 5.590) was obtained for further analyzation by LC-MS. The compounds were identified by comparing their electron impact (EI)-MS spectra with those recorded in the mass

Table 1 Antifungal activity of the raffinate phase and extraction phase against *Phytophthora drechsleri* Tucker

Extraction phase	Extraction phase (%)	Raffinate phase (%)
Chloroform	20.4±2.1	79.9±1.5
Cyclohexane	15.3±1.7	85.5±1.2
Dichloromethane	10.4±1.4	87.3±1.7
Ethyl acetate	81.0±2.0	20.3±1.4
n-Hexane	16.5±1.9	77.5±1.1
n-Butanol	9.8±2.0	89±2.1

Data are presented as percentage of growth inhibition (I) and are the means of triplicate determinations ± the standard deviation (SD)

Table 2 Antifungal activity of the crude extract obtained by column chromatography against *P. drechsleri* Tucker

Sample	Inhibition (%)
Ethyl acetate–methanol	10±1.2
1	14.5±1.6
2	12.4±1.4
3	88.3±1.8

Data are presented as percentage of growth inhibition and are the means of triplicate determinations ± the SD

spectrum library; identification was considered to be reliable only if there was 90 % similarity with the reference spectrum. Based on the [ES+] and [ES−] spectra, the molecular mass of the second partial extract (retention time 5.590) was determined to be ESI-MS (*m/z*): 165 (Fig. 2). According to the NMR spectra (Figs. 2, 3, 4, 5), the properties of the compound were: ^{13}C-NMR (400 MHz, DMSO-d6): δ175.57 (C-1), 71.53 (C-2), 40.21 (C-3), 138.60 (C-4), 128.42 (C-5 and C-9), 129.85 (C-6 and C-8), 126.56 (C-7); ^{1}H-NMR (400 MHz, DMSO-d6): δ7.23 (H-5 and H-9), 7.21 (H-6 to H-8), 4.17 (H-2), 2.99 (H-3a), 2.78 (H-3b). The ligands formed for the compound produced were similar to those of commercial PLA. Thus, according to the HPLC, LC-MS, and NMR analyses, one of the inhibiting compounds from *B. coagulans* TQ33 was PLA.

Minimum inhibitory concentration of PLA on *P. Drechsleri* Tucker

The minimum inhibitory concentration (MIC) of PLA on the *P. drechsleri* Tucker was examined. The results (Fig. 5) indicated that the MIC of PLA was 18 mg/mL (Fig. 5a). When the concentration of PLA was below 5 mg/mL, there was almost no inhibition (Fig. 5e). The inhibition activity was gradually higher with the increase in PLA concentration (Fig. 5b–e). When the concentration of PLA was 14 mg/mL,

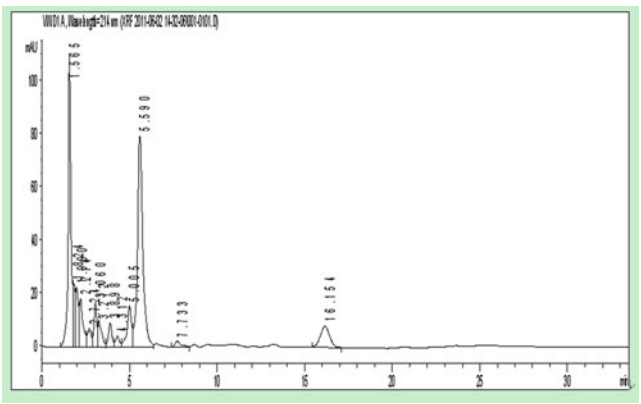

Fig. 1 Preliminary high-performance liquid chromatography profile of antifungal compounds produced by *Bacillus coagulans* TQ33

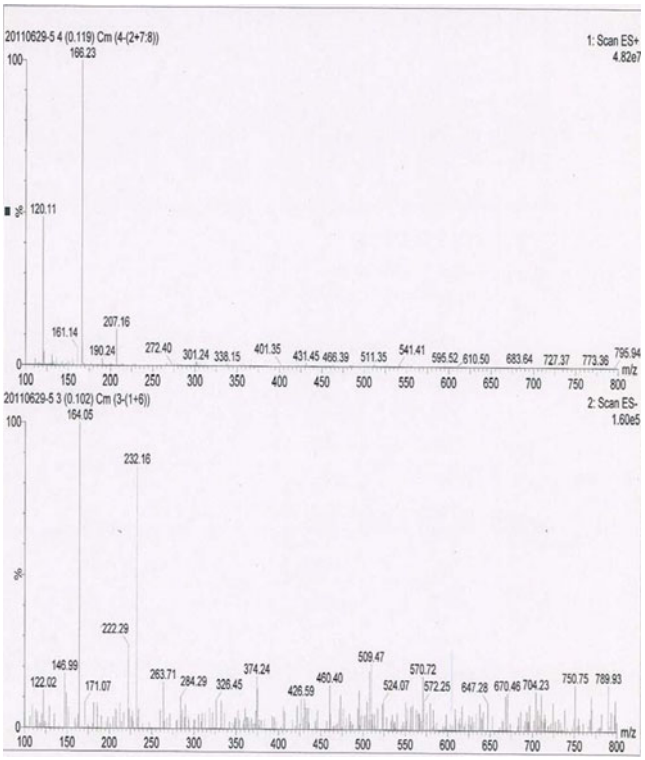

Fig. 2 Liquid chromatography-mass spectrometry profile of antifungal compounds produced by *Bacillus coagulans* TQ33

the mycelia still have a small amount of growth (Fig. 5b). However, when *P. drechsleri* Tucker was treated with 18 mg/mL PLA, the mycelium displayed a nearly complete inhibition of growth (Fig. 5a). These results indicated that the MIC of PLA on *P. drechsleri* Tucker was 18 mg/mL.

Bio-control activity of PLA on fungi

The effects of the antifungal spectra of PLA against *Fusarium oxysporum*, *Botrytis cinerea*, *Glomerella*

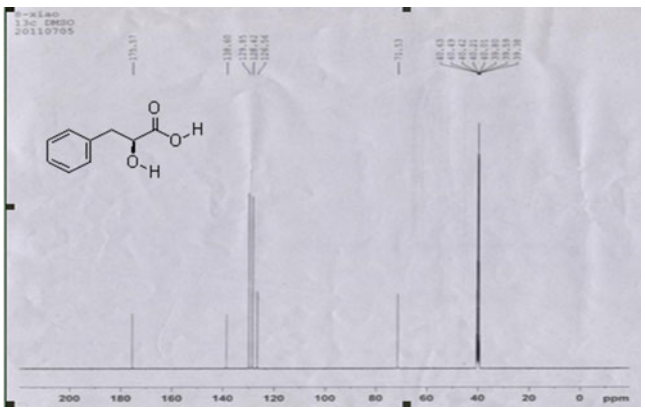

Fig. 3 Nuclear magnetic resonance (NMR) ^{13}C-NMR profile of antifungal compounds produced by *Bacillus coagulans* TQ33. ^{13}C-NMR (400 MHz, DMSO-d6): δ175.57 (C-1), 71.53 (C-2), 40.21 (C-3), 138.60 (C-4), 128.42 (C-5 and C-9), 129.85 (C-6 and C-8), 126.56 (C-7)

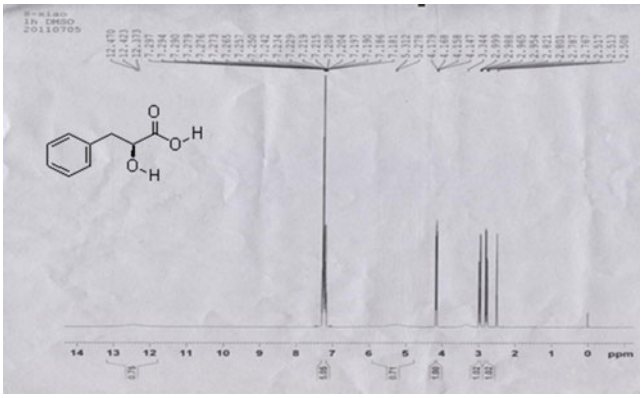

Fig. 4 NMR [1]H-NMR profile of antifungal compounds produced by *Bacillus coagulans* TQ33. [1]H-NMR (400 MHz, DMSO-d6): δ7.23 (H-5 and H-9), 7.21 (H-6 to H-8), 4.17 (H-2), 2.99 (H-3a), 2.78(H-3b)

cingulata, *Penicillium citrinum*, and *Penicillium digitatum* are shown in (Tables 3 and 4). The results indicate that PLA has a wide spectrum of antagonistic activities against *F. oxysporum*, *B. cinerea*, *P. citrinum*, *P. digitatum*, and *G. cingulata*, particularly against *F. oxysporum*.

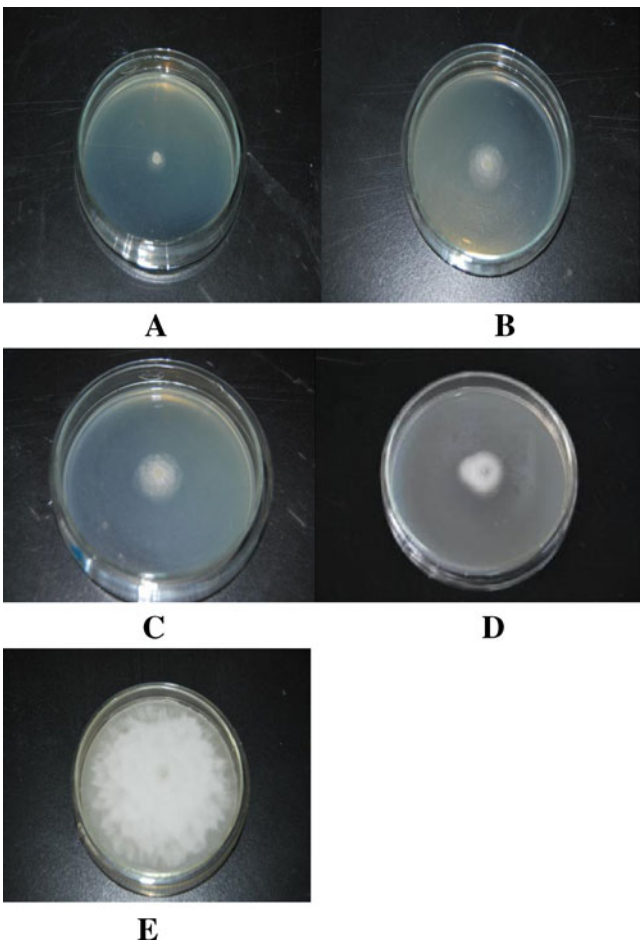

Fig. 5 Minimum inhibitory concentration of phenyllactic acid (PLA) on *Phytophthora drechsleri* Tucker. Treatment of *P. drechsleri* Tucker with 18 (**a**) 14 (**b**), 12 (**c**), 10 (**d**) and 5 mg/mL PLA (**e**)

Table 3 Antifungal activity of phenyllactic acid against mycelial growth of plant pathogenic fungi tested

Pathogenic fungi	Inhibitory antifungal activity (%)
Fusarium oxysporum	80.2±1.3
Botrytis cinerea	60.6±1.2
Glomerella cingulata	32.3±1.1
Penicillium citrinum	60.5±1.3
Penicillium digitatum	60.1±1.5

Data are presented as percentage of growth inhibition and are the means of triplicate determinations ± the SD

Discussion

Phenyllactic acid, a novel antimicrobial compound with broad and effective antimicrobial activity against both bacteria and fungi, is produced by many microorganisms, especially lactic acid bacteria (LAB). A number of studies on LAB found that some of the antifungal activity is caused by organic acids, proteinaceous compounds, and other end products (Park et al. 2009; Schnurer and Magnusson 2005). L-lactic acid was registered as a bio-pesticide in 2009, and it has become a widely used environmental protection pesticide in America and Japan. In their comparison of the inhibition rate of the organic acids, Gerez et al. (2010) found that PLA was about 66- and 600-fold more effective than acetic acid and lactic acid, respectively.

Jaroszewski et al. (2009) reported that the metabolites from LAB displayed inhibitory activity against certain kinds of important fungi. Sjogren et al. (2003) identified and chemically characterized four antifungal substances, i.e., 3-(*R*)-hydroxydecanoic acid, 3-hydroxy-5-*cis*-dodecenoic acid, 3-(*R*)-hydroxydodecanoic acid, and 3-(*R*)-hydroxytetradecanoic acid, from *Lactobacillus plantarum* strain MiLAB 14. Racemic mixtures of the saturated 3-hydroxy fatty acids showed antifungal activity against different molds and yeasts with MICs of between 10 and 100 μg/mL (Sjogren et al. 2003). Strom et al. (2002) isolated a *L. plantarum* strain (MiLAB 393) from grass silage that produces broad-

Table 4 Phenyllactic acid inhibition of the conidia of the plant pathogenic fungi tested

Pathogenic fungi	Antifungal activity–zone of inhibition
Fusarium oxysporum	+++
Botrytis cinerea	++
Glomerella cingulata	+
Penicillium citrinum	++
Penicillium digitatum	++

The inhibition was graded according to the size of inhibited growth area: −, no visible inhibition; +, 0.1- to 7-mm zone of inhibition; ++, 7.1- to 14-mm zone of inhibition; +++, >14-mm zone of inhibition.

spectrum antifungal compounds: cyclo (L-Phe-L-Pro), cyclo (L-Phe-trans-4-OH-L-Pro), and 3-PLA. PLA is a novel antimicrobial compound derived from phenylalanine (Phe). *Lactobacillus* sp. SK007 has high PLA-producing ability; however, when 6.1 mM phenylpyruvic acid (PPA) was used to replace Phe as substrate at the same concentration, PLA production increased 14-fold and the fermentation time decreased from 72 h to 24 h with growing cells (Li et al. 2007). Prema et al. also isolated a *L. plantarum* strain from grass silage that produces a broad spectrum of antifungal compounds. Subsequent structural characterization of the antifungal compound by NMR spectroscopy, infrared spectroscopy, and gas chromatography revealed that the produced compound (PLA) acted as a fungistatic and delayed the growth of a variety of fungal contaminants. Compared to LAB, *B. coagulans* is resistant to acid and heat; it can also be easily cultured and preserves well over a long period of time. In our study, *B. coagulans* TQ33 also had the ability to produce PLA. *B. coagulans* TQ33 with antifungal activities therefore has the potential to be implemented as a natural fungicide, preventing the growth of pathogens in plant species. The preliminary characterization and biocontrol effect of the antifungal compounds produced by strain *B. coagulans* TQ33 showed a very broad spectrum of activity and inhibited *Botrytis cinerea*, *Alternaria solani*, *Phytophthora drechsleri* Tucker, *Fusarium oxysporum*, and *Glomerella cingulata*.

The safety for chronic human consumption of *B. coagulans* has recently been demonstrated, with various reports indicating that *B. coagulans* can prevent respiratory infections, ramp up the immune system, and be used to prevent cancer or the formation of cancer-causing agents. There is also some interest in using it as an addictive to vaccines to improve their effectiveness. *B. coagulans* is currently available in commercial probiotic products, including GanedenBC30 (*B. coagulans* GBI-30, 6086), a proprietary probiotic preparation that is considered safe for human consumption and has been associated with easing of gastrointestinal symptoms (such as bloating and abdominal pain) in irritable bowel syndrome (Dolin 2009; Hun 2009). A toxicological safety assessment was published in 2009 on the proprietary preparation of *B. coagulans*–GanedenBC (30)™—a novel probiotic. The conclusion of this assessment was that GanedenBC(30)™ is safe for chronic human consumption based upon scientific procedures which are supported by a history of safe usage (Endres et al. 2009). A 1-year chronic oral toxicity study combined with a one-generation reproduction study has also been conducted to investigate still further the safety of long-term consumption. During this 1-year study, GanedenBC(30)™ was administered to male and female HsdBrlHan:Wistar rats through their diet; the rats showed no signs of toxicity at the highest dose tested. The conclusion drawn by the authors of the reproduction toxicity study is that the administration of GanedenBC(30)™ in the diet caused no signs of toxicity in the parental generation (male or female) nor in the F1 offspring (Endres et al. 2011). Therefore, it would appear that *B. coagulans* TQ33 strain has a large potential for practical application for plant pathogenic fungi control. It also has many unprecedented advantages compared with the applied fungicides currently on the market, including environmentally safe implementation and cost-effectiveness.

In this study, one of the substances inhibiting fungal growth was isolated from *B. coagulans* strain TQ33 and subsequently identified as PLA. In terms of marketing environmentally safe fungicides, this finding is a really exciting result due to the three most notable features of *B. coagulans* TQ33: its resistance to acid and heat, its ability to be cultured easily, and its ability to be stably preserved. It is environmentally safe and has high thermal stability. The antifungal mechanism of *B. coagulans* TQ33 and the presence of these antifungal compounds and other unidentified substances should be evaluated in greenhouse tests to further their elucidate protective abilities for crops.

Acknowledgments Financial support provided by the National Natural Science Foundation of China (No. 30900961 and No. 20876116) and TianJin Social Science Planning Program (No. TJGLWT11-08) is gratefully acknowledged.

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