African Journal of Microbiology Research Vol. 5(4), pp. 340-345, 18 February, 2011
Available online http://www.academicjournals.org/ajmr
DOI: 10.5897/AJMR10.679
ISSN 1996-0808 ©2011 Academic Journals

Full Length Research Paper

Evaluation of the efficacy of endophytic *Bacillus amyloliquefaciens* against *Botryosphaeria dothidea* and other phytopathogenic microorganisms

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Accepted 13 January, 2011

Endophytic bacteria were isolated from poplar trees in Shandong province, China, where canker disease caused by *Botryosphaeria dothidea* has reached epidemic proportions. On the basis of the results of classical bacteriological tests and sequence analysis of the *16S rRNA* gene, the PEBA20 strain isolated from poplar was identified as a strain of *Bacillus amyloliquefaciens*. *In vitro* PEBA20 inhibited the mycelium growth of *B. dothidea* and canker disease incidence on the cut shoots of poplar 60% than that in the control 100%. The optimum culture conditions for the propagation of the antagonistic strain against *B. dothidea* were determined using an orthogonal experiment design. The strain showed antifungal and antibacterial activity against a number of microorganisms, including mycelial fungi, yeast, gram-positive and gram-negative bacteria. *TasA* and *aiiA*—2 genes associated with antagonistic activity were detected in PEBA20. The results of our study indicate that *B. amyloliquefaciens* PEBA20 has the potential to serve as a biological control agent for the poplar canker disease caused by *B. dothidea* and for diseases caused by other phytopathogens.

Key words: *Bacillus amyloliquefaciens*, endophytic bacteria, isolation, biological control agent.

INTRODUCTION

Poplar (*Poplus* spp.) is a widely cultivated plant and is used as a source of wood and as an energy crop for the production of renewable sources of energy. Poplar canker disease is caused by *Botryosphaeria dothidea* (Moug.:Fr.) Ces. and De Not. and is an important fungal disease of the stem in China and other countries throughout the world (Morgan-Jones and White, 1987). Disease management strategies for poplar canker typically include the use of genetically resistant varieties of the plant, integration of select culture practices, application of chemical pesticides, and combinations thereof. However, the use of some of these practices is not always viable, and the continuous use of pesticides has caused severe ecological problems. Owing to their environment-friendly nature, biological control methods have emerged as an alternative to chemical treatments for plant disease control (Daughtrey and Benson, 2005; Strange and Scott, 2005). Microorganisms can colonize the tissues of healthy plants. Such endophytic bacteria have been reported to prevent disease development by controlling the spread of plant pathogens or by enhancing plant resistance (Stein, 2005; Ryan et al., 2008). Therefore, there is a considerable potential for finding new and beneficial endophytic bacteria that can serve as biocontrol agents.

Few studies have reported the biological control of poplar canker disease. Previous studies have revealed some strains, including *Bacillus subtilis*, *Bacillus pumilus*, *Pseudomonas fluorescens*, *Trichoderma* spp., *Eudarluca quinqueseptata*, and *Eudarluca biconia,* that are potential biocontrol agents against *B. dothidea* (Katumoto, 1986; Jin and Shen, 1989). However, these strains were isolated from either soil or the rhizosphere and

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Table 1. Antagonistic activity of Bacillus amyloliquefaciens PEBA20 against mycelial fungi.

Indicator fungi	Inhibitory rate (%) [a]	
	Treated with bacterial suspension of PEBA20	Treated with fermentation filtrate of PEBA20
B. dothidea	69.95	73.2
Fusarium oxysporum	85.94	63.55
Fusicladium tremulae	45.91	40.99
Gloeosporium fructigenum	75.75	64.87
Glomerella cingulata	60.35	46.05
Myrothecium roridum	38.44	26.26
Phytophthora capsici	81.06	71.3
Rhizoctonia solani	39.05	19.2
Sphaceloma euonymi-japonici	55.46	52.05
Trichothecium roseum	64.02	53.7
Valsa sordida	89.37	82.62

[a] Values are the mean of 4 replicates; the formula for inhibitory rate is as follows: Inhibitory rate (%) = [(colony radius in controls - colony radius in treatments)/colony radius in controls] x 100.

Table 2. Antagonistic activity of Bacillus amyloliquefaciens PEBA20 against yeast and bacteria.

Indicator yeast and bacteria	Diameter of the inhibitory zone (mm) [a]	
	Treated with bacterial suspension of PEBA20[b]	Treated with fermentation filtrate of PEBA20[c]
Saccharomyces cerevisiae	28.51	9.04
Xanthomonas campestris	19.14	15.76
Pectobacterium carotovorum subsp. *carotovorum*	9.97	9.21
Ralstonia solanacearum	13.30	13.31
Clavibacter michiganense subsp. *sepedonicum*	16.61	16.68
Staphylococcus aureus	10.01	5.1
B. subtilis	13.57	6.67
B. cereus	18.95	9.45
B. megaterium	18.52	19.05

[a] Values are the mean of 4 replicates; the diameter of the inhibitory zone includes the diameter of the well (5 mm), [b] Treatment by adding 20 µl of bacterial suspension in a well with diameter 5 mm, [c] Treatment by adding 20 µl of fermentation filtrate in a well with diameter 5 mm.

phyllosphere; to date, no study has reported the isolation of endophytic antagonistic bacteria from poplar. Although endophytic bacteria from poplar plants have been reported, these studies did not assess the biological control mediated by these organisms (Doty et al., 2005; Frey et al., 2008; Ulrich et al., 2008). In this study, we isolated a bacterial strain from poplar as a potential biocontrol agent and assessed its inhibitory activity against *B. dothidea*.

MATERIALS AND METHODS

Bacterial and fungal strains

The fungi and bacteria used as indicators for evaluating antagonistic activity are listed in Tables 1 and 2. The fungi were stored at 4℃ and the bacteria, at –20℃. Before being used for evaluating antagonistic activity, the fungal cultures were routinely incubated on potato dextrose agar (PDA) at 25℃, while the bacterial cultures were incubated on nutrient agar (NA) at 28℃.

Isolation of endophytic bacteria and screening for antagonistic activity against *B. dothidea*

Endophytic bacteria were isolated from five 6-year-old poplar trees (*Populus tomentosa* Carr.). A tissue sample (10 mm in depth and 40 mm in length and breadth) comprising the epidermis, phloem, and xylem was cut from the outer surface of the tree trunk at a height of 1.5 m from the ground. The samples were surface sterilized with 70% alcohol and the epidermis and phloem were discarded. Then, the remaining tissue was surface sterilized with 70% alcohol for 1 min and 2% sodium hypochlorite for 10 min and rinsed 5 times in sterile distilled water. Hundred microliters of the last wash of each sample was spread onto NA plates to check the sterility of the sample. Then, the tissue samples were aseptically ground in sterile distilled water. The supernatant was diluted up to 0.01 $g \cdot ml^{-1}$, pour-

plated on NA plates, and incubated at 28℃. After incubation, individual bacterial colonies were selected and subcultured on NA plates. An *in vitro* dual culture technique was used to screen the isolates for antagonistic activity against *B. dothidea* (Katumoto, 1986; Ryan et al., 2008). A mycelial plug of *B. dothidea* (diameter, 5 mm) and a paper disc (diameter, 5 mm) dipped in overnight cultures of the isolates were placed 25 mm apart on a Petri dish (90 mm) containing PDA. Inhibition of fungal growth was assessed on the basis of the presence or absence of an inhibition zone. Isolate 080120 showed strong inhibitory activities and was selected for further studies.

Bacterial characterization and *16S rRNA* gene sequence analysis

Cultural, morphological, and physiological characteristics of isolate 080120 were assessed using conventional methods (Colwell and Grigorova, 1987; Holt, 1994). Genomic DNA was extracted by the cetyltrimethylammonium bromide (CTAB) method (Ausubel et al., 1992). For polymerase chain reaction (PCR) amplification of the *16S rRNA* gene, primers P3—5′-AGAGTTTGATCMTGGCTCAG-3′—and P5—5′-TACGGYTACCTTGTTACGACTT-3′—were designed using DNAman 6.0 on the basis of the previously described universal primers and the *16S rRNA* gene sequences of *Bacillus* species in GenBank (Moreno et al., 2002). PCR amplification consisted of initial denaturation at 94℃ for 5 min followed by 30 cycles of denaturation at 94℃ for 60 s, annealing at 58℃ for 60 s, elongation at 72℃ for 90 s, and a final extension at 72℃ for 10 min. Subsequently, the PCR product was subcloned and sequenced. Two clones from each independent PCR reaction were sequenced twice on both strands. Two independent PCR reactions were performed to obtain the consensus sequence. Nucleotide sequence alignment and analyses were performed using DNAman 6.0 and Molecular Evolutionary Genetic Analysis (MEGA) version 4.1.

Detection of *TasA* and *aiiA* genes associated with antagonistic activity

Detection of the *TasA* and *aiiA* genes was performed using PCR amplification (Lee et al., 2002; Branda et al., 2006). The PCR primers to detect these genes were designed using DNAman 6.0 on the basis of homologous sequences obtained from GenBank. The primers *aii*A1—5′-GGAATTCATGACAGTAAARAARCT-3′—and *aii*A2—5′-CCCTCGAGTATATAYTCHGGGAAC-3′—were used for *aii*A amplification and TasA1—5′-GAATTCATGGGTATGAAAAAGAA-3′—and TasA2—5′-CTGCAGTTAATTTTTATCCTCGCT-3′—were used for *TasA* amplification (*EcoR* I site for *aiiA*1 and TasA1, *Xho* I site for *aiiA*2 and *Pst* I site for TasA2 have been underlined). The *aiiA* gene was amplified under the following conditions: initial denaturation at 94℃ for 5 min, 30 cycles of denaturation at 94℃ for 60 s, annealing at 50℃ for 45 s, elongation at 72℃ for 60 s, and a final extension at 72℃ for 7 min. For *TasA* amplification, touchdown PCR was performed with annealing at 56℃ for 45 s after which the temperature was decreased to 52℃ at the rate of 2℃ per 2 cycles; this step was followed by 28 cycles at 50℃ for 45 s. The amplified products were subcloned and sequenced. The sequence was compared with homologous sequences in GenBank.

Assessment of antifungal and antibacterial activity

To determine antifungal activity, a paper disc (diameter, 5 mm) dipped in overnight suspension of isolate 080120 was placed on a PDA plate at a distance of 50 mm from the mycelial plug (diameter, 5 mm) of the indicator fungi. For the fermentation filtrate test, the bacteria were inoculated in 250 ml Erlenmeyer flasks containing nutrient broth (NB; 150 ml) and grown at 28℃ and 180 rpm for 120 h. The cell-free fermentation filtrate thus obtained was previously added to the PDA plate at a concentration of 200 µl of fermentation filtrate per 10 ml PDA in a Petri dish (90 x 15 mm). The mycelial plug of the indicator fungi was placed at the center of the PDA plate. Then, the PDA plates were incubated at 25℃ for 3~5 days, depending on the growth rate of the fungi. The antagonistic activity against *Saccharomyces cerevisiae* and bacteria was assessed using well-diffusion inhibition assays (Cintas et al., 1995). The overnight cultures of indicator yeast and bacteria (100 µl) were added to the base plates containing PDA and NA, respectively and incubated to obtain lawns of the organisms on the plates. A well (diameter, 5 mm) was bored into the base plate and overnight culture of isolate 080120 (20 µl) or the cell-free fermentation filtrate was added to the well. The plates were then incubated at 28℃ for 2 days. To establish the controls, the fungi and bacteria were incubated under the same conditions without the bacterial suspensions and cell-free fermentation filtrate. Four replications were performed for each treatment.

Optimization of culture conditions for the propagation of an antagonist strain

To determine the optimum culture medium for the propagation of an antagonist strain against *B. dothidea*, NB, Luria-Bertani (LB), BPY (beef, peptone, and yeast), and PD media were used. The inoculum of isolate 080120 was incubated in 250 ml Erlenmeyer flasks containing 150 ml of different culture media at 28℃ and 180 rpm for 120 h. To determine the antifungal activity of the cell-free fermentation filtrate, the optimum medium was chosen for optimization of the culture conditions. The optimization of culture conditions for the propagation of the antagonist strain was performed using an orthogonal experimental design. Five factors at 5 levels, viz., volume of the medium (50, 100, 125, 150 and 175 ml), initial pH (5, 6, 7, 8 and 8.67), temperature (20.0, 24.2, 28.5, 32.8 and 37℃), shaking speed (100, 120, 140, 160, and 180 rpm) and cultivation time (24, 48, 72, 96 and 120 h) were selected and an orthogonal array layout of 5^5 was performed. The antifungal activity of the cell-free fermentation filtrate was determined by the methods described above.

Cut shoots assay for suppression of poplar canker

The efficacy of isolate 080120 in suppressing canker caused by *B. dothidea* was assessed using stem cuttings of poplar. The basal end of healthy shoots of *P. tomentosa* (length, about 40 cm) were dipped in a bacterial suspension (10 CFU ml^{-1}) for 30 min. Then, the shoots were hydroponically cultured at room temperature (about 28℃) for a week. A mycelial plug of *B. dothidea* was inoculated on the stem and incubated at 28℃ in a moisture chamber. Dark brown to black lesions were observed on the stem. This test was performed in June; 30 cuttings were used for the treatments and 10 were used as controls.

RESULTS

Isolation and identification of antagonistic bacteria

A total of 126 bacterial isolates were collected from the surface-sterilized internal tissues of poplar trees. The results of screening for antagonistic activity against

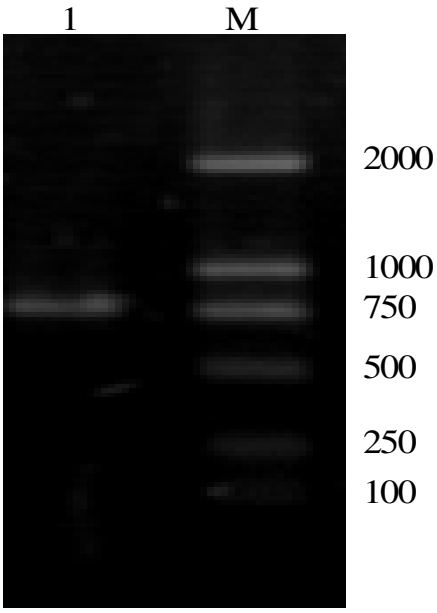

Figure 1. PCR amplification of aiiA gene from *B. amyloliquefaciens* PEBA20. Lane 1: products of PCR amplification, lane M: molecular weight marker.

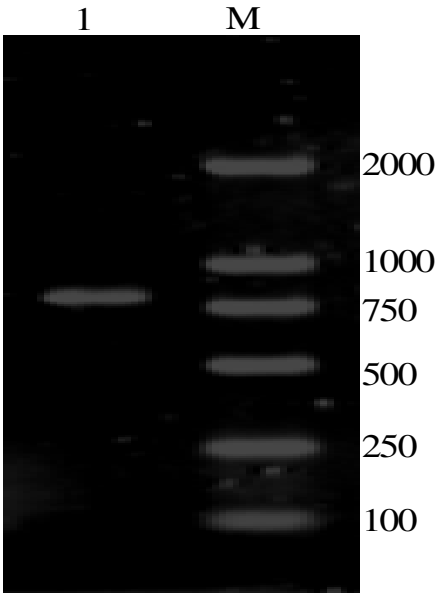

Figure 2. PCR amplification of TasA gene from *B. amyloliquefaciens* PEBA20. Lane 1: products of PCR amplification, lane M: molecular weight marker.

B. dothidea showed inhibition zones with different areas for different isolates. Isolate 080120 showed strong inhibitory activity against *B. dothidea*. After incubation for 96 h, solitary bacterial colonies of isolate 080120 were obtained on NA medium; the colonies were circular, undulated, raised, rough, opaque, and greyish white in color with no diffusible pigment. Classical bacteriological tests showed that the isolate consisted of motile, aerobic Gram-positive long rods that showed endospore formation; were positive for catalase activity, oxidase activity, Voges-Proskauer test, gelatin hydrolysis, and starch degradation; and could utilize D-glucose, D-xylose, L-arabinose, and D-mannitol.

The partial *16S rRNA* gene sequence was amplified by using the genomic DNA of isolate 080120; this sequence has been deposited in GenBank with accession number FJ685773. Basic Local Alignment Search Tool (BLAST) analysis and a neighbor-joining dendrogram constructed using MEGA revealed that the bacterium belonged to the genus *Bacillus* and was closely clustered together with *Bacillus amyloliquefaciens.* The amplified *16S rRNA* gene sequence of isolate 080120 was most closely related to that of *B. amyloliquefaciens* FZB42 (GenBank accession number, CP000560) and showed 100% identity with the sequence from *B. amyloliquefaciens* FZB42. The sequence showed 99.87 to 99.93% identity with the sequences obtained from most of the *B. amyloliquefaciens* strains, whereas it showed 99.73 to 99.80% identity with the sequences obtained from most of the *B. subtilis* strains. On the basis of the results of the classical bacteriological tests and the analysis of the *16S rRNA* gene, we concluded that the isolate was a strain of *B. amyloliquefaciens* and was named PEBA20.

Further, 2 genes—*aiiA* and *TasA*—from the genomic DNA of PEBA20 were amplified using PCR to facilitate gene sequencing to confirm the identity of the strain (Figures 1 and 2). The intact open reading frame (ORF) of the *aiiA* gene (GenBank accession number, FJ713589) consists of 753 base pairs, which encode a protein of 250 amino acid residues. The gene showed 87.0 to 99.3% identity with the homologues of the *aiiA* genes cloned from strains of *Bacillus thuringiensis, Bacillus cereus, Bacillus anthracis, Bacillus weihenstephanensis*, and *B. subtilis*. The ORF of the *TasA* gene (GenBank accession number, FJ713580) consists of 786 base pairs, which encode a protein of 261 amino acid residues. The gene showed 98.60% identity with the *TasA* gene of *B. amyloliquefaciens* FZB42 (GenBank accession number, CP000560) and 76.02% identity with 3 homologues from *B. subtilis* strains (GenBank accession numbers, BACJH642; AJ871386; and AL009126). The results of the gene sequence analysis indicated that PEBA20 has the *aiiA* and *TasA* genes and that it is a strain of *B. amyloliquefaciens*.

Antifungal and antibacterial spectrum

B. amyloliquefaciens PEBA20 showed antimicrobial activity against a wide range of fungi and bacteria. The mycelial fungi, yeast, and gram-positive and negative

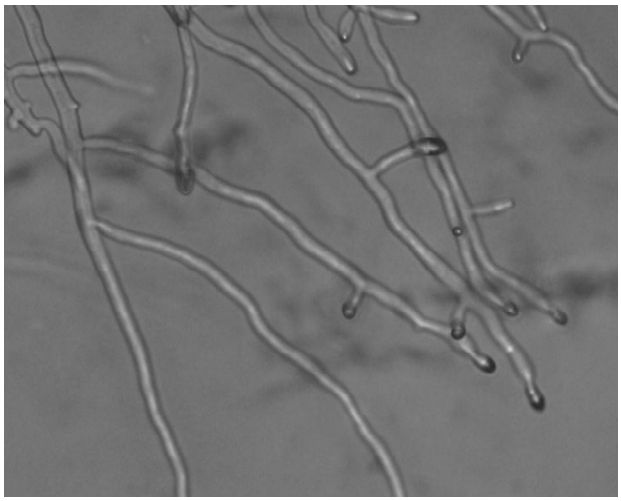

Figure 3. Normal hyphae of *B. dothidea* at the edge of a colony cultured on a potato dextrose agar (PDA) plate at 25℃ for 120 h (magnification, 400 x).

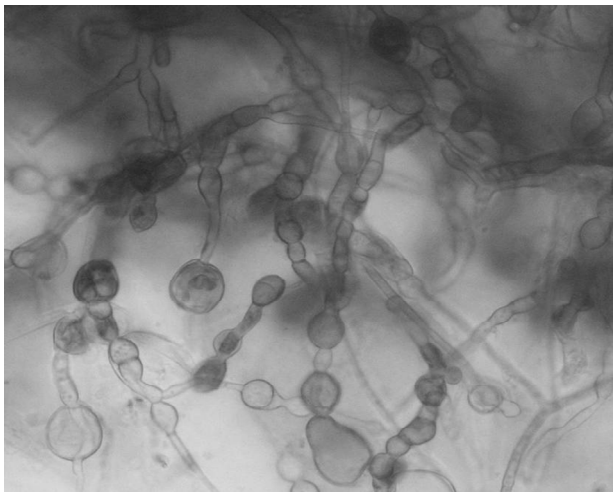

Figure 4. Aberrant hyphae of *B. dothidea* treated with Bacillus amyloliquefaciens PEBA20 (magnification, 400×) * distorted hyphae at the edge of a colony cultured on potato dextrose agar (PDA) plate at 25℃ for 120 h after treatment with bacterial suspensions of PEBA20 (a paper disc immersed in the bacterial suspension was placed on the PDA plate at a distance of 50 mm from the mycelial plug).

bacteria tested in the study showed various degrees of sensitivity to bacterial suspensions or fermentation filtrates of PEBA20 (Tables 1 and 2). The efficacy of the bacterial suspension in inhibiting fungal growth *in vitro* ranged from 38.44 to 89.37%, while that of the fermentation filtrate ranged from 19.2 to 82.62%. The diameter of the inhibition zone after treatment with bacterial suspension and fermentation filtrate of PEBA20 ranged from 9.97 to 19.14 mm and from 5.1 mm (very weak effect) to 19.05 mm. However, no relationship was seen either among the different indicators or between the treatments using bacterial suspensions and fermentation filtrate.

Antagonistic activity against *B. dothidea*

Bacterial suspensions and the fermentation filtrate of PEBA20 inhibited the growth of *B. dothidea in vitro*, thereby showing the marked antagonistic activity of PEBA20 (Table 1). The inhibitory effect of the fermentation filtrate varied in different culture media; the maximum rate of inhibition (84.33%) was obtained when PEBA20 was incubated in NB, and the rate of inhibition decreased to 77.81, 75.2 and 60.31% when PEBA20 was incubated in LB, BPY, and PD, respectively. Therefore, NB medium was chosen as the optimum culture medium. The optimum culture conditions for the propagation of the strain with antagonistic activity against *B. dothidea* were as follows: 100 ml of NB in a 250 ml flask; initial pH, 5; temperature, 28.5℃; shaking speed, 140 rpm; and cultivation time, 120 h.

Aberrant hyphae were detected when the fungus were treated with either the bacterial suspensions or fermentation filtrate. Compared with the normal hyphae (Figure 3), the hyphae at the edge of the colony showed obvious hyphal aberration, such as increasing branches, swelling, and bulb formation in the middle and/or at the top (Figure 4). Our results indicated that the hyphal aberration increased with treatment duration; some hyphae had a bead-like appearance, while some formed cluster-like structures.

We used cut shoots of poplar to determine the efficacy of PEBA20 against *B. dothidea* infection. Our results showed that the incidence of poplar canker in the cut shoots treated with the bacterial suspension of PEBA20 (60%) was lower than that in the control (100%). In addition, the canker symptoms in the treated cut shoots were different from those in the controls. The diseased cut shoots treated with the PEBA20 bacterial suspension showed delayed incidence of typical canker symptoms and decrease in the average radius of the lesion.

DISCUSSION

Endophytic microorganisms have attracted the attention of researchers because of their potential to serve as biocontrol agents (Stein, 2005; Ryan et al., 2008). In this study, *B. amyloliquefaciens* strain PEBA20 was isolated from poplar. The efficacy of this strain as a biocontrol agent against poplar canker caused by *B. dothidea* was evaluated by *in vitro* bioassay and cut shoots assay. Our work provides evidence for the antagonistic activity of endophytic bacteria against *B. dothidea* infection in poplar (Katumoto, 1986; Doty et al., 2005; Frey et al., 2008; Ulrich, 2008). Since the PEBA20 strain was obtained from poplar, it has the advantage of being a “native” antagonist isolated from the same habitat in

which it is expected to be used for biological control.

Species of the genus *Bacillus*, particularly *B. amyloliquefaciens* and *B. subtilis*, have been shown to produce a range of antimicrobial dipeptides or cyclic lipopeptides. Some of the metabolites are strain-specific and may be associated with certain species and subspecies of *B. amyloliquefaciens* and *B. subtilis* (Stein, 2005; Romero et al., 2007). Although we did not detect the antibiotics produced by PEBA20 in the present study, the detection of antagonistic activity and aberrant hyphae indicated the production of antimicrobial compounds.

In this study, 11 mycelial fungi, 1 yeast, 5 Gram positive bacteria, and 3 Gram negative bacteria were found to be sensitive to PEBA20, thereby implying that PEBA20 has a wide spectrum of antimicrobial activity; however, since a large antimicrobial spectrum is an important attribute for a successful biocontrol agent, more fungi and bacteria should be tested in further studies to establish the antimicrobial spectrum of this strain. We also detected the *aiiA* and *TasA* genes in PEBA20. AiiA, encoded by the *aiiA* gene, is a quorum-quenching agent and can be a promising agent for biological control of bacterial diseases (Dong et al., 2001; Dong and Zhang, 2005). *TasA* is a spore-associated protein encoded by *TasA* in *B. subtilis* and is a protein with the property of antibacterial activity (Stover and Wong, 1999). In addition, *TasA* is a predominant protein component of the extracellular matrix of biofilms, which contribute to important biological function of bacteria (Branda et al., 2006). PEBA20 was found to possess *TasA* and *aiiA*, which suggested its potential antagonistic activity and indicated that it serves as a quorum-quenching agent in biological control of bacterial diseases. The results indicate that PEBA20 has immense potential to serve as a biocontrol agent.

ACKNOWLEDGEMENTS

The authors thank the anonymous reviewers for their valuable suggestions. This work was supported by grants from the National Natural Science Foundation of China (30972367) and the Advanced Postdoctoral Science Foundation of Shandong Province (200603059).

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