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RESEARCH ARTICLE OPEN ACCESS

Screening of Antagonistic Bacteria from Endophytes against Walnut Blight Pathogen *Xanthomonas arboricola* pv. *juglandis*

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Abstract

Walnut blight caused by *Xanthomonas arboricola* pv. *juglandis* (Xaj) is the most important bacterial disease in walnut production worldwide. To seek biocontrol agents against *Xaj*, we screened 152 endophytic bacteria isolated from 87 plants. Through dual-culture method screening, we obtained four antagonistic bacteria, ATE17, BME17, CIE17, and OFE17 which were isolated from *Amaranthus tricolor, Bambusa multiplex, Canna indica,* and *Osmanthus fragrans* plants respectively. The inhibition ratios of ATE18, BME17, CIE18, and OFE17 against *Xaj* on plates were 1.5, 1.6, 1.3, and 1.6, respectively. These indicated they have good biocontrol potential for walnut bacterial blight. Subsequently, the four endophytic bacteria were identified by morphology, Gram staining, Microbial Identification System (fatty acid methyl ester analysis), as well as 16S rDNA and *gyrB* sequencing. It turns out that all four strains were identified as *Bacillus* sp. Furthermore, the two strains BME17 and OFE17 can suppress multiple plant fungal pathogens and bacterial pathogens on plates.

Keywords: Antagonism, *Bacillus* sp., *Bambusa multiplex,* Biocontrol agent, Endophytic bacteria, *Osmanthus fragrans*

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INTRODUCTION

English walnut or Persian walnut (*Juglans regia* L) belongs to the Juglandaceae family Juglans genus. It is an important edible nut, wood oil crop, and valuable timber tree species[1]. Walnut kernels are composed largely of polyunsaturated fatty acid, alpha-linolenic acid, which gives them anti-atherogenic properties and brain health benefits. Walnut oil may even serve as a helpful natural remedy for hyperlipidemic patients with type 2 diabetes[2]. So walnuts have both nutritional and medicinal benefits[3]. Walnuts are widely spread both natively and commercially in Europe, Asia, and part of the United States[4].

Walnut planting areas have increased sharply over the past several decades, particularly in Asia. In 2018, the global walnut harvest area was 1159484 ha with production 3 662 507 tonnes. China alone has harvested an area of 390 224 ha with production 1 586 367 tonnes[5]. The harvest area and production of China accounting for 33.65% and 43.31%, respectively of global totals.

With the walnut planting area increasing substantially, walnut bacterial blight caused by *Xanthomonas arboricola* pv. *juglandis* (*Xaj*) is also spreading around the world[6,7]. Currently, the disease is the most important bacterial disease affecting walnut production. The disease has sparked international concern and has been added to CABI (Centre for Agriculture and Bioscience International) invasive species list[8]. Walnut bacterial blight negatively affects almost all walnut crown tissues. It causes early defoliation, fruit blackening and decay, immature dropping, kernel drying, oil and yield reducing, then serious walnut productivity losing and quality decreasing[9].

The management of walnut bacterial blight has to date mainly relied on chemical pesticides such as copper-based compounds and streptomycin-dominated antibiotics[10,11]. However, these conventional control agents are now recognized to produce adverse residue and environmental problems, as well as having decreased effectiveness due to emerging pesticide resistance[10,12,13].

Using biological control methods based on microbes or their derivatives has become an important direction in plant disease control. Among them, the use of plant endophytes has more advantages compared to other microorganisms in plant disease control, therefore it is one of the current hot spots in biological control research [14,15,16,17]. Endophytes are facultative or obligate symbiotic microorganisms that live in apparently healthy internal plant tissues, without causing disease[17]. Endophytes have a capacity for disease prevention, as well as plant growth promotion, aid nitrogen fixation, and are beneficial to host plant development[18,19,20].

There are many research reports on the screening, identification, and utilization of plant endophytes in plant disease control. For instance, there are fungal endophytes for suppression of *Rhizoctonia solani*[21]. Wheat endophytes inhibit the growth of Fusarium head blight. *Fusarium graminearum* inhibition of 30–51% and *Fusarium culmorum* inhibition of 15–53% have been established by dual culture assays *in vitro*[22]. Soybean endophytic bacteria *Bacillus* sp. and *Burkholderia* sp. have been proved that they can control bacterial and fungal pathogens of the host effectively *in vitro*, such as *Sclerotinia sclerotiorum, Phomopsis sojae,* and *Rhizoctonia solani*[23]. Dogwood (*Cornus florida*) endophyte A22F1 has high potential as a biological control agent for *Phytophthora capsici* in pepper[24]. Some endophytes have been translated into commercial products[14].

The identification of novel biocontrol agents is a critical step in the development of commercial biocontrol products[25]. Some attempts have been made in walnut bacterial blight biocontrol agent exploration. These include the examination of medicinal plant essential oils and aqueous extracts[26] and bacteriophages isolated from walnut orchard soils in New Zealand and Chile[27,28,29]. Screening, identification, and evaluation for antagonistic bacteria led to four *Pseudomonas fluorescens* strains being identified as putative antagonists of *Xaj* and being shown to significantly reduce symptoms on walnut leaves (41% to 82%)[30].

To search for more potential biocontrol agent candidates from plant endophytes resources, we investigated 50 families and 86 species of plants to isolate and screen their antagonistic effects on *Xaj in vitro*. Through conventional and modern techniques, we identified four antagonistic

Table 1. Two endophytes against different plant pathogens on plates

Diseases	Pathogens	OFE17	BME17
Wheat Fusarium Head Blight	*Fusarium graminearum*	0.63	0.55
Orchid black spot	*Alternaria alternata*	0.72	0.65
Walnut anthracnose	*Gloeosporium fructigenum.*	0.63	0.67
Maca root rot	*F. avenaceum.*	0.51	0.53
Camellia black spot	*A. alternate*	0.61	0.48
Citrus scab	*Sphaceloma fawcettii*	0.30	0.33
Corn ear rot	*F. graminearum*	0.76	0.78
Gray leaf spot of corn	*Cercospora sorghi*	0.66	0.68
Corn northern leaf blight	Setosphaeria turcica	0.52	0.67
Rice blast	*Magnaporthe grisea*	0.73	0.74
Rice bacterial leaf streak	*Xanthomonas oryzae* pv. *oryzae*	2.45	1.21
Rice bacterial blight	*X. oryzae pv. oryzicola*	1.86	2.18

Note: The values for fungal pathogens mean suppression ratio; for bacterial pathogens means D/d ratio. Each value represents the mean of three replicates.

endophytes with great potential biocontrol capacity against Xaj from four different plant species.

MATERIALS AND METHODS

Plants and target pathogen strains

Ninety-four samples from various parts and tissues of healthy plants were collected from Xiaogan city, Hubei province from May to September in 2017. They are distributed across 50 families, 84 genera, and 86 species (Supplement Table 1). Screening target pathogen strain Xaj BW3F3[31] was isolated in 2015 from Baokang county, Hubei province, and maintained on YPGA (Yeast powder 5 g, peptone 5 g, D-glucose 10 g, deionized water 1000 mL, agar 15 g, pH 7.2)[32] and storage at -80 °C in 30% glycerol. More plant fungal and bacterial pathogens for antagonistic spectrum tests are listed in Table 1.

Isolation of plant endophytes

Plant samples were washed with tap water and sterilized water three times. They were then cut into around 1 cm^2 patch, surface disinfected in 75% ethanol for 3 min then in 0.1% $HgCl_2$ for 1 min. These were mashed up and suspended in ddH_2O. The tissue debris suspension was spread onto a selective medium plate based on YPGA supplement with Cephalexin (30 µg/L) and Cycloheximide (100 µg/L)[33], incubated at 28 °C for 2 days. Endophytes were picked for purification and cultured on a YPGA plate at 28 °C.

Screening of antagonistic bacteria

The antagonistic effect was tested via the dual culture method[34]. In short, fresh endophyte suspension 5 µl (OD_{600}=0.5) inoculate to the center of the plate spread with 200 µl Xaj DW3F3 (OD_{600}=0.5). After incubating for 2-3 d at 28 °C, the inhibitory zone and endophyte colony size are measured. Inoculation of ddH_2O (2 µl) and Kanamycin (50 µg/mL) (2 µl) as the negative and positive control, respectively. The inhibitory rate (D/d) is calculated by dividing the inhibitory zone diameter (D, mm) by the endophyte colony diameter (d, mm). Each treatment was conducted

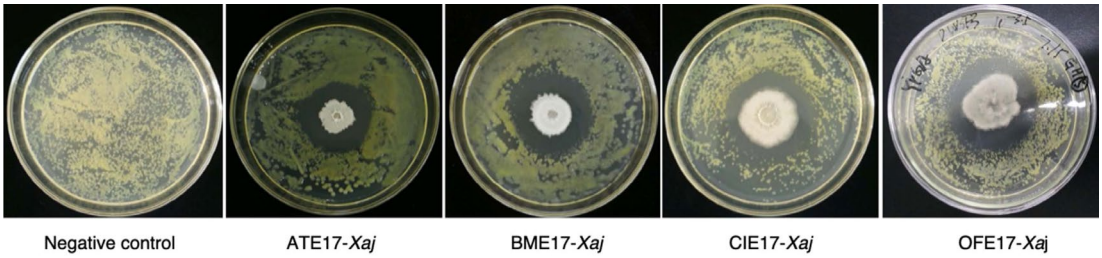


Fig. 1. Antagonistic effects of four endophytes against Xaj DW3F3 on YPGA plates.

Table 2. Endophytic bacteria identification by microbial identification system based on Fatty Acid Analysis (MIDI)

Antagonistic bacteria	Entry Name	Percent Named	Sim Index	Library
ATE17	*Bacillus subtilis*	91.70%	0.583	RTSBA6 6.21
BME17	*Bacillus subtilis*	94.14%	0.777	RTSBA6 6.21
CIE17	*Bacillus subtilis*	93.52%	0.77	RTSBA6 6.21
OFE17	*Bacillus subtilis*	93.31%	0.756	RTSBA6 6.21

in triplicate with three plates. Those endophytes with obvious inhibitory zones on plates were stocked in 30% glycerol at -80 °C.

Identification of antagonistic bacteria

Four strains with strong antagonistic effects against Xaj DW3F3 on YPGA plates were chosen to identify. They were denoted as ATE17 (*Amaranthus tricolor* endophyte isolated in 2017), BME17 (*Bambusa multiplex* endophyte isolated in 2017), CIE17 (*Canna indica* endophyte isolated in 2017), and OFE17 (*Osmanthus fragrans* endophyte isolated in 2017).

The cellular morphology of these strains was observed by Gram staining. The strains were then subjected to identification by the Microbial Identification System (MIDI Inc) based on Fatty Acid Methyl Ester (FAME) profiles. Bacterial genomic DNA was then extracted using EZ-10 Spin Column Bacterial Genomic DNA Mini-Preps Kit (Sangon Biotech Shanghai Co., Ltd.) for subsequent molecular identification. The PCR procedure and two sets of primers were used, 16S rDNA 27 F□5'-AGAGTTTGATCCTGGCTCAG-3' and 1492R□5'-GGTTACCTTGTTACGACTT-3', and gyrB UP-1: 5’-GAAGTCATCATGACCGTTCTGCAYGCNGGNGGN AARTTYGA-3’ and UP-2:5’-AGCAGGGTACGG ATGTGCGAGCCRTCNACRTCNGCRTCNGTCAT-3’ sequences came from Bavykin et al[35]. The 16S rDNA and gyrB amplicons were sequenced by Sangon Biotech (Shanghai) Co., Ltd. Then the sequences were subjected to phylogenetic tree construction using the MEGAX software[36].

Test of BME17 and OFE17 against multiple plant pathogens

To assay the potential ability of the endophytes against other plant pathogens, we selected two endophytes BME17 and OFE17 which showed a high inhibition ability isolated from two different plants. We tested the antagonistic effects of the two endophytes on 10 different plant fungal pathogens and two bacterial pathogens collected in our laboratory. The method used was the same as described above except the fungal confrontation culture was carried out on PDA plates. The suppression effect on fungal pathogens was calculated by (negative control radius-treat radius)/negative control radius (radius in mm).

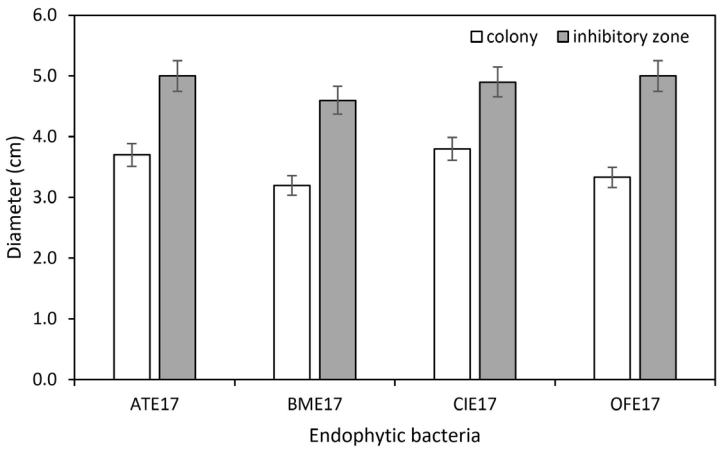


Fig. 2. Inhibitory zone size of four endophytes against Xaj DW3F3 on YPGA plates.

RESULTS

Obtained four potential biocontrol agent endophytes

One hundred fifty-two endophytic bacterial strains were isolated and purified from 86 species of plants. Through dual culture screening of the target pathogen *Xaj* DW3F3 one by one, four strains with significant antagonistic effects on *Xaj* DW3F3 were obtained and verified more than five times (Fig.1). The inhibition ratio (D/d) of four endophytes ATE17, BME17, CIE17, and OFE17 were 1.5, 1.6, 1.3, and 1.6, respectively (Fig.2).

Morphology and fatty acid identification

After being cultured on YPGA plates for 24 to 48h, four antagonist strain colonies were nearly round, light white, or slightly yellow. Taking a fresh colony to Gram staining and observing it under a microscope (Nikon ECLIPSE Ni-U), the cells are rod-shaped and the Gram stain is positive.

The microbial identification system based on fatty acid is fully automated for bacterial identification. It showed that the strains ATE17, BME17, CIE17, and OFE17 were most similar to *Bacillus subtilis* in the software's library database (Table 2).

16S rRNA and gyrB molecular identification

The 16S rDNA partial sequences of four antagonist strains ATE17, BME17, CIE17, and OFE17 were run through the Blastn against the NCBI Standard databases (nr, etc.) Nucleotide collection (nr/nt). These four strains are judged to be similar to dozens of different *Bacillus* sp., with query coverage 100%, E value 0.0, and percentage identity 100%. A phylogenetic tree was constructed based on 17 *Bacillus* species and one out group species *Virgibacillus halodenitrificans* through the Neighbor-Join algorithm (bootstrap =500) (Fig. 3). Their classification position cannot be inferred yet on this basis.

The *gyr*B partial sequences of four antagonist strains ATE17, BME17, CIE17, and OFE17 were sequenced. These sequences were subjected to an NCBI Blastn search as above. They are judged to be highly similar to many different *Bacillus* sp. with query coverage 100%, E value 0.0, and percentage identity 100% as well. A

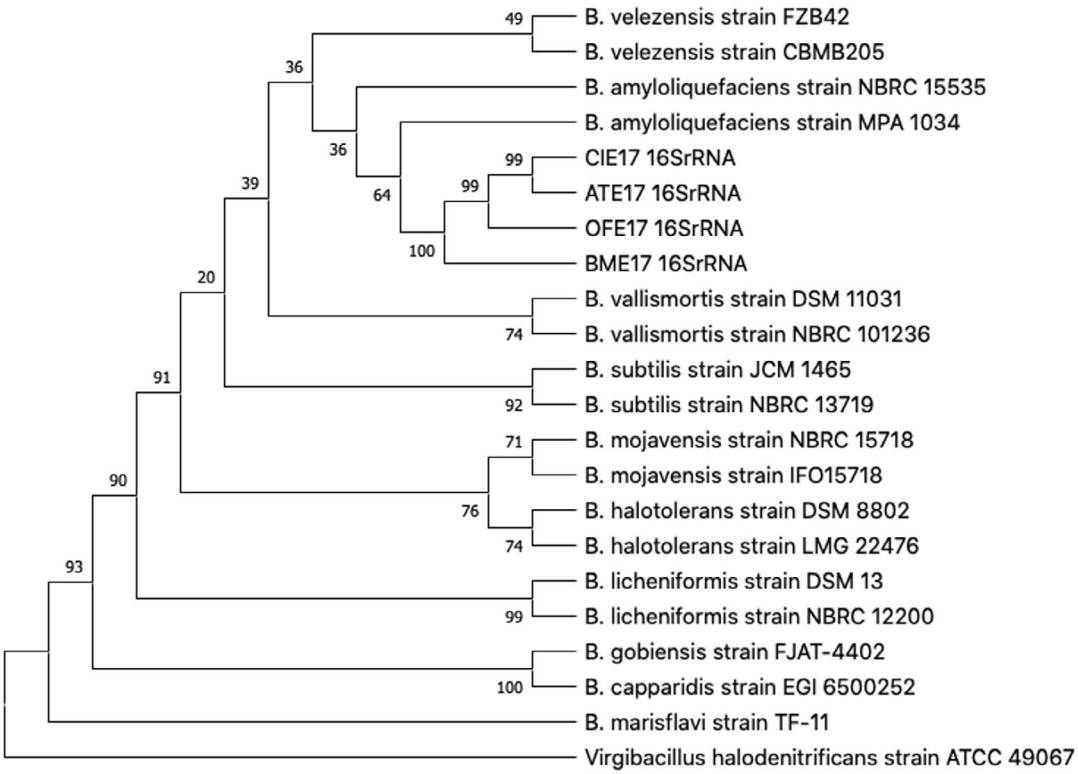


Fig. 3. Phylogenetic tree based on Neighbor-joining tree of 22 16S-rRNA gene partial sequences.

phylogenetic tree was constructed based on highly similar sequences with more than 98% identity and 98% query coverage (bootstrap =1000) (Fig. 4). Based on the tree, we cannot infer species positions for these strains.

Hereby, we tentatively identified the four antagonist bacteria as *Bacillus* sp. based on combined morphology, microbe identification system, and housekeeping genes.

Inhibition of different pathogens by BME17 and OFE17

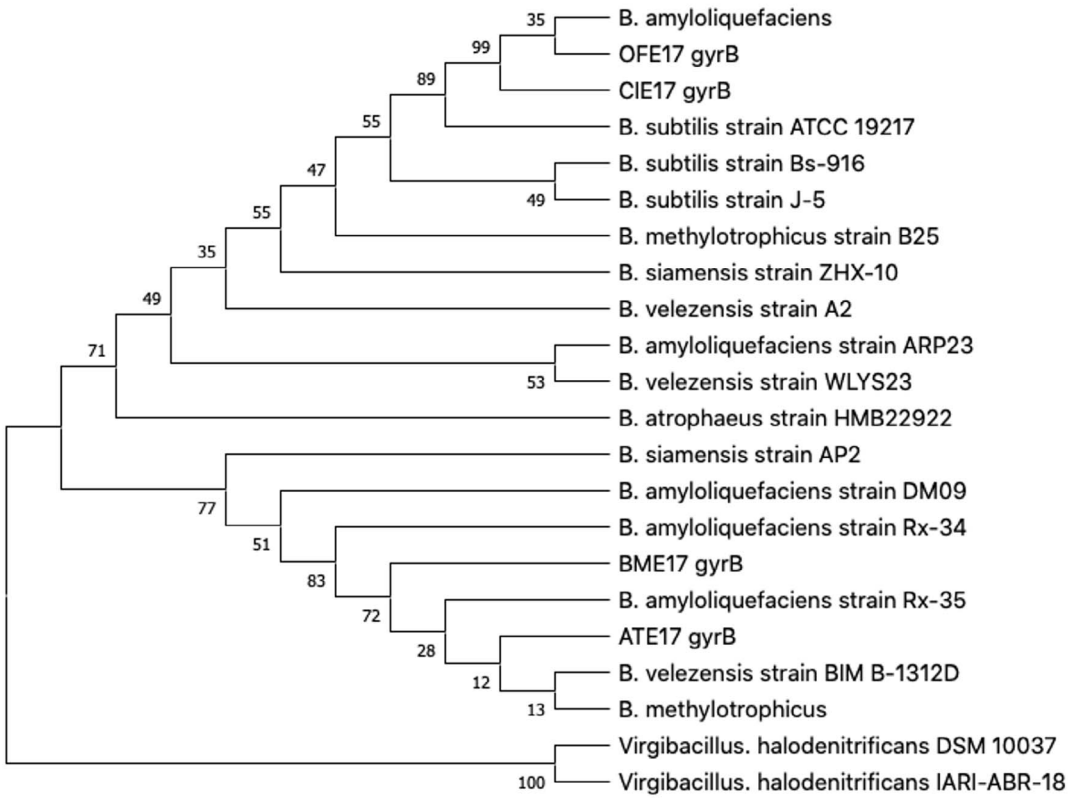
We selected the two endophytes BME17 and OFE17 for assessing their antagonistic properties against various plant pathogens. The two endophytes presented antagonistic effects on all 10 crop fungal pathogens and two rice bacterial pathogens (Table 1). The result suggests the strains have broad-spectrum antagonistic roles instead of specific functions and have potential biocontrol roles against both fungal and bacterial diseases.

DISCUSSION

Developing novel, economical, and environment-friendly walnut bacterial blight biocontrol agents is challenging. Amongst, exploiting plant endophytes is an important methodology in this direction.

Screening biocontrol agents from many plant resources is the first step in this important work. However, to date, only a few plant endophytes have been screened for the treatment of disease. Ozaktan et al[30] assayed 29 epiphytes from healthy walnut leaves against Xaj, 18 strains have antagonistic effects with inhibitory zone 3.0 to 13.0mm. Bacteria measured as around 60% antagonistic, have decreased walnut bacterial blight 41-77% on walnut seedlings.

In this study, we screened 152 endophytes from 86 plant species, and obtained four strains with strong antagonistic effects on the target pathogen Xaj. Through multiple identification



methods, we determined that they are all *Bacillus* sp. (this identification is tentative, due to *Bacillus genera* classification complexity). These antagonistic bacteria do not belong to the same genera as those identified as *P. fluorescens* against *Xaj*[30]. The *in vitro* inhibition provides the first evidence that these endophytes are potential biocontrol agents of walnut bacterial blight. Our research has added to the potential arsenal of a biological agent in combating walnut bacterial blight.

Although we screened 152 endophytes, only four potential biocontrol agent strains were discovered (their effects verified through experimental replication). The positive selection frequency is 2.6%. This low frequency is similar to that found in research in coffee berry disease antagonistic bacteria screening, in which from a total of 4 323 microorganisms isolates around 3% exhibited detectable inhibition[37].

We obtained antagonistic bacteria isolated from four plants common in our location, *A. tricolor, B. multiplex, C. indica,* and *O. fragrans*. In each of these plants, only some endophytes were studied for their biocontrol ability. For example, there were 46 endophytic bacteria and 17 fungi isolated from amaranth plant, among them six bacterial endophyte isolates and one fungal endophyte were found to be efficient in inhibiting the growth of its leaf blight pathogen *Rhizoctonia solani in vitro*[38]. Endophytic fungi isolates from bamboo (*Phyllostachys edulis*) branches were found to have high bioactivity against *Botryotinia fuckeliana* and *Thanatephorus cucumeris*[39]. Endophytic bacteria were isolated from *C. indica* proved to be *P.fluorescens, Enterobacter* sp., *Erwinia* sp. and all of them have plant growth promoting ability[40]. There were two endophytes isolated from O. fragrance that were identified as Bacillus sp. too. One endophyte *Bacillus* sp. isolated from *O. fragrance* can be used in honey wine[41]. Another endophyte strain B. safensis B21, isolated from *O. fragrans* fruits, showed antifungal activity against *Magnaporthe oryzae*[42]. These are thus broad antimicrobe spectrum bacteria we tested in this study. These *Bacillus* spp. are not only antagonistic against bacteria but also fungi.

In our search process, we only isolated 152 endophytic bacteria from 86 plant species. On one hand, we added antibiotics into the isolation medium and on the other hand, the bacterial colony was hard to distinguish. We simply picked up those distinct single colonies obvious to the naked eye. Possibly we ignored some isolates on the plates with similar colony morphology.

Bacillus bacteria is one of the most important biocontrol resources. Many active substances have been discovered, and even already used in the field. In our projects, the evaluation of biocontrol effectiveness on plants, and active substances are under investigation. Although we obtained four potential antagonistic bacteria, there is much additional work to be done on the way to developing powerful biocontrol agents for disease control. The present results will lay a foundation for further exploring the function and application of such agents in the future.

SUPPLEMENTARY INFORMATION

Supplementary information accompanies this article at https://doi.org/10.22207/JPAM.15.3.30

Additional file: Additional Table S1.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

BF designed the experiments. LY and BW performed the experiments. BF, L Y and BW analyzed the data. BF wrote the manuscript. CZ reviewed and edited the manuscript. All authors read and approved the manuscript.

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DATA AVAILABILITY

The sequences obtained during this study are included in the manuscript with a public database deposit number.

ETHICS STATEMENT

This article does not contain any studies with human participants or animals performed by any of the authors.

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