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Pathogenicity and repulsion for toxin-producing bacteria of dominant bacteria on the surface of American pine wood nematodes

Kan-Cheng Liu[1] | Fei-Li Zeng[2] | Ai-Ling Ben[3] | Zheng-Min Han[1]

[1]College of Forestry, Nanjing Forestry University, Nanjing, Jiangsu, China

[2]Chunan County Forestry Bureau, Hangzhou, Zhejiang, China

[3]School of Food Science, Nanjing Xiaozhuang University, Nanjing, Jiangsu, China

Correspondence
Z.-M. Han, College of Forestry, Nanjing Forestry University, Nanjing, Jiangsu, China.
Email: zmhan@njfu.edu.cn

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Abstract

Bacteria were isolated from the surface of two samples of American pine wood nematodes to identify methods of controlling pine wilt disease. The dominant bacterial strains were identified, and their toxicity and pathogenicity, in addition to their competitiveness with other pathogenic bacteria, were measured to ascertain how bacteria on the surface of American pine wood nematodes might be used to prevent and control pine wilt disease. The bacterial isolates show that the dominant bacteria carried by the two samples of pine wood nematodes are US4, US5, Smal-007 and Rrad-006. Based on routine staining, morphological observation and 16S rDNA sequence analysis, the four strains were identified as *Delftia lacustris*, *Pseudomonas putida*, *Stenotrophomonas maltophilia* and *Rhizobium nepotum*. The incubation of four dominant bacterial strains and Chinese dominant bacterial strains on the surface of aseptic nematodes and in nutrient broth showed that Smal-007 and Rrad-006 have strong competitiveness on the surface of pine wood nematodes. Using a bacterial culture medium to measure the propensity of pine seedlings to wilt, all the American dominant bacterial strains were shown to be less toxic than the Chinese dominant strains. If pine seedlings are inoculated with both bacterial and aseptic pine wood nematodes, American dominant bacterial strains present less pathogenicity than the Chinese dominant bacterial strains. In particular, Smal-007 and Rrad-006 show the lowest pathogenicity. If pine seedlings are inoculated with both bacterial and wild pine wood nematodes, American dominant bacterial strains significantly reduce the pathogenicity of wild pine wood nematodes isolated from Zhejiang Province, China. The effects of Smal-007 and Rrad-006 are confirmed as the most prominent. The American dominant strains Smal-007 and Rrad-006 satisfy two main requirements: excellent repulsion performance and low pathogenicity. Therefore, they can be used as candidate strains for biocontrol bacteria.

KEYWORDS

bacterial identification, *Bursaphelenchus xylophilus*, competition experiment, dominant bacteria, pathogenicity, toxicity

1 | INTRODUCTION

Pine wilt disease is one of the most significant threats to forestry, with the potential to cause widespread loss of pine trees. Although in-depth studies have been conducted on the disease, its pathogenesis remains unclear. The pine wood nematode (*Bursaphelenchus xylophilus*) has long been considered the sole causal agent of pine wilt disease (Kiyohara & Tokushige, 1971; Mamiya, 1983; Nobuchi, Tominaga,

Futai, & Harada, 1984; Tokushige & Kiyohara, 1969). In recent years, however, the pathogenicity of bacteria on the surface of pine wood nematodes has drawn increasing attention. Zhao, Guo, Gao, and Guo (2000) and Han, Hong, and Zhao (2003) discovered that only pine trees with both bacterial and pine wood nematodes were affected, and they proposed the hypothesis that pine wilt disease is caused by a mixed infection of nematodes and bacteria. The hypothesis states that pine wilt disease occurs because pine wood nematodes and the pathogenic bacteria they carry invade the host simultaneously, and the pathogenic bacteria secrete toxins under the protection and nutrition of pine wood nematodes, leading to the wilting and death of the host. Many scholars have subsequently demonstrated that the toxins produced in infected pine trees are related to the bacteria on the surface of pine wood nematodes (Chi, 2003; Guo, 2001; Hong, Zhao, Cao, & Han, 2003). In fact, many types of bacteria are present on the surface of pine wood nematodes. Kawazu, Yamishita, and Kanzaki (1998) isolated three types of *Bacillus* from black pine (*Pinus thunbergii* Parl.). Guo (2001) isolated *Palleronia cepacia* from Masson's pine (*Pinus massoniana* Lamb.) in Nanjing. Hong, Cao, Zhao, and Han (2002) isolated two bacterial strains of *Pseudomonas fluorescens* from Masson's pine in Nanjing. Wang, Han, and Zhao (2004) collected nematode isolates in different areas of China and isolated and identified more than 30 types of bacteria from different nematode isolates, of which *Pseudomonas* spp. were the most toxigenic. Nematodes in different areas carry different bacteria that produce different toxins, resulting in significant differences in the pathogenicity of different isolates occurring with pine wood nematodes (Hu, Wang, & Yang, 1994; Zhang, Zhang, Lin, Luo, & Xu, 2002).

The United States is considered the origin of pine wilt disease. Although the pine wood nematode is distributed in 36 states, the area of dead pine trees is small, and the harm is manageable (Bergdahl, 1988). The American pine forest is not unique, and the pine species, vector insects and climatic conditions are in line with the typical epidemic characteristics of pine wilt disease (Bergdahl, 1988; Dropkin & Foudin, 1979; Robbins, 1982). Therefore, pine wilt disease is not serious in America, likely because of the nature of the bacteria on the surface of the nematodes. After long-term evolution and continuous succession, the bacteria on the surface of American nematodes have developed to an advanced stage, and these bacteria have a strong ability to survive and multiply, coupled with weak pathogenicity and other characteristics (Zeng, 2010). Accordingly, the bacteria with strong pathogenicity on the surface of Chinese or Japanese pine wood nematodes could be replaced with the bacteria with weak pathogenicity carried by the American pine wood nematode, which would contribute to reducing the pathogenicity of the pine wood nematode and achieve biological control of pine wilt disease. To verify this possibility, American pine wood nematode isolates were collected, and bacteria were isolated from their surfaces. Then, the bacteria on the surface of the American nematodes were compared to the bacteria on the surface of the Chinese pine wood nematode isolates in terms of their ability to produce toxins and competitiveness on the nematode surface to identify a bacterial strain that could be used to control pine wilt disease.

2 | MATERIALS AND METHODS

2.1 | Experimental material

The American pine wood nematode isolates US-Bx1 and US-Bx2 were isolated from an inbound packing container of American pine trees seized by the Jiangsu Entry-Exit Inspection and Quarantine Bureau (provided by An Yulin and Su Han) and kept at the Jiangsu Inbound Inspection and Quarantine Center.

The Zhejiang nematode isolate ZJ-Bx was isolated from dead Masson's pine in Ningbo, Zhejiang Province, China, and kept by the Forest Pathology Laboratory of Nanjing Forestry University.

The isolate ZJ-Bx was used as an aseptic nematode prepared in accordance with Ben Ailing's method (Ben, Han, Han, & Sun, 2008).

The virulent bacterial strains of *P. fluorescens*, CH1, CH2, CH3 and CH4 isolated by Hong et al. (2002) and Wang et al. (2004) from wild nematode isolates in Jiangsu Province, Zhejiang Province, Anhui Province and Fujian Province, respectively, represent the dominant bacterial strains on the surface of nematodes and display strong pathogenicity or toxicity for pine seedlings. All bacterial strains were kept by the Forest Pathology Laboratory of Nanjing Forestry University.

The two reference strains used during strain identification were *Staphylococcus aureus* and *Escherichia coli*, which were isolated and kept by the Forest Pathology Laboratory of Nanjing Forestry University.

2.2 | Procedure for the isolation of bacteria on the nematode surface

Under aseptic conditions, three to five adult nematodes were selected and placed in an aseptic mortar with 1 ml of sterile water and a small amount of quartz sand and thoroughly ground. Then, 10-fold serial dilutions with sterile water were performed to achieve a dilution of 1×10^{-2}. Three replicates were performed. Then, 200 μl of the diluent was spread on a plate with King's B medium (King, Campbell, & Eagles, 1948) and cultured for 48 hr at a constant temperature of 28°C. Later, the colony size, morphological features, fluorescence and other characteristics of the bacterial colonies on the medium were observed under a dissecting microscope, identified, isolated and purified. In addition, the bacterial colonies and the number and occurrence probability of each colony were calculated. After continuous streaking and purification of the strains onto the plate, a single bacterial colony was selected and implanted into nutrient agar (NA) slant culture medium (Lapage, Shelton, & Mitchell, 1970), cultured for 1–2 days at a constant temperature of 28°C and stored at 4°C.

2.3 | Identification of bacterial strains

The morphological features of the colonies of each strain on NA culture medium were observed, and general biochemical tests were conducted, with reference to Bergey's Manual of Systematic Bacteriology (Second Edition).

Bacterial 16S rDNA sequence analysis for bacterial identification: a test strain was implanted into a 150-ml Erlenmeyer flask

with 30 ml of NB culture medium (Ohta & Hattori, 1980) for the extraction of bacterial DNA after 20 hr of shaking the culture in an incubator shaker (120 rpm, 30°C). A GK1071 bacterial genomic DNA extraction Kit (GENERAY BIOTECH) was used for the bacterial DNA extraction, separation and purification. With bacterial genomic DNA as a template, the universal primer of the bacterial 16S rRNA gene was used for PCR amplification (Hilyard, Jones-Meehan, Spargo, & Hill, 2008). The universal primer sequences were as follows: Primer 1, 8f: 5′-AGAGTTTGATCCTGGCTCAG-3′; Primer 2, 1541r: 5′-AAGGAGGTGATCCAGCCGCA-3′ (synthesized by Sangon Biotech (Shanghai) Co., Ltd.). Hilyard's method (Hilyard et al., 2008) was adopted for the amplification reaction and conditions of the 16S rRNA gene. Five microlitres of the PCR product was taken with bromophenol blue, electrophoresed with agarose gel and stained with ethidium bromide. It was then observed in a gel imaging system to obtain pictures and was recovered and sequenced after the amplified band was detected (DNA sequencing was performed by Nanjing GenScript Biotechnology Co., Ltd.). After the sequencing was completed, the sequences of the obtained bacterial 16S rDNA were searched (at www.ncbi.nlm.nih.gov), and the highly homologous type strains were selected as references to determine the taxonomic status of the strains (Kawazu et al., 1998).

2.4 | Preparation of the bacterial cell-free culture medium

The bacteria were first implanted into NB culture medium and cultured for 48 hr in an incubator shaker (135 rpm, 28°C) before the bacteria culture solution was placed in a 10-ml centrifuge tube and centrifuged for 10 min (8,000 *g*, 4°C). The supernatant fluid was then filtered with a 0.22-μm microporous filtering film to obtain the bacterial filtrate, which was tested on NA culture medium to determine whether it carried bacteria. Finally, the supernatant was stored at 4°C.

2.5 | Preparation of the bacterial and nematode suspensions

Bacterial colonies were inoculated onto NB medium, and the bacterial fluid was diluted with NB culture medium after 36 hr of shaking the culture at 28°C at a speed of 135 rpm and adjusted to a bacterial suspension with a concentration of 1×10^8 cfu/ml (Hanks & James, 1940). The nematodes were inoculated into *Botrytis cinerea* (Han et al., 2003). After the nematodes consumed the *B. cinerea*, the developed nematodes were washed with sterile water, and the nematode suspension was adjusted to 10,000 nematodes/ml suspension. The aseptic nematodes were tested to determine whether the nematode grinding fluid carried bacteria with an NB culture medium. Then, the nematode suspension was placed into sterilized 5-ml centrifuge tubes with 3 ml per tube, and 50 μl of prepared bacterial suspension was added to each tube for 1 hr of mixed processing, during which the tubes were constantly inverted to fully integrate the nematodes and bacteria. After 5 min of centrifugation at 200 *g* and 4°C, the supernatant fluid was removed, and 3 ml of sterile water was added to prepare a suspension, which was then preserved.

2.6 | Toxicity test of bacterial cell-free culture medium

Han's method (Han et al., 2003) was applied to culture aseptic black pine (*P. thunbergii*) seedlings. Two vials were placed in a jar, and a small amount of water was placed around it to keep it moist. It was then sealed with sealing film and sterilized. Under sterile conditions, 3 ml of bacterial cell-free culture medium was added to each vial, and 10 repetitions were conducted for each processing. Sterile water and NB culture medium were taken as a reference. Then, aseptic root-cut black pine seedlings were inserted with one seedling per vial, and the vials were sealed with sealing film and placed flat in an illuminated incubator. The seedlings were cultured under the conditions of 25°C and 16 hr of illumination per day to observe and record the signs of wilting. In this experiment, the wilting pine seedlings were divided into five levels: "4" indicated seriously withered and allochroic; "3" indicated wilting due to complete water loss; "2" indicated wilting due to significant water loss; "1" indicated slight water loss; and "0" indicated fresh and no water loss.

2.7 | Procedure to determine the pathogenicity of mixed suspensions of bacteria and aseptic nematodes for pine seedlings

Two vials were placed in a jar, and 3 ml of water was added to each vial. The jar was sealed with sealing film and sterilized. Under sterile conditions, one aseptic root-cut seedling was placed in each vial, the terminal bud of the aseptic seedling was cut-off with a sterile blade to produce a wound, and a small absorbent cotton ball (able to absorb 200–300 μl of suspension) was attached to the wound. Two hundred microlitres of a mixed suspension of bacteria and aseptic nematodes was dropped onto the absorbent ball, which was kept moist for 24 hr. Ten repetitions were performed for each processing. The jar was sealed after inoculation. The seedlings were cultured under the conditions of 25°C and 16 hr of illumination per day. The evidence of wilting was regularly observed and recorded using the method for classifying the damage given in Section 2.6.

2.8 | Determination of pathogenicity of mixed suspensions of bacteria and wild nematodes for pine seedlings

First, a wound with a depth of one-fourth of the seedling diameter at the trunk of greenhouse-grown seedlings was made with a sterile blade, and sterile absorbent cotton balls (able to absorb 500–600 μl of suspension) were placed on the wounds of the seedlings and fixed with funnel-shaped adhesive tape. Five hundred microlitres (5,000 nematodes) of a mixed suspension of bacteria and nematodes was added, and the cotton balls were kept moist for 24 hr. Twenty repetitions were performed for each processing. The seedlings were cultivated in a greenhouse to observe and record the evidence of wilting. Seedlings with signs of yellowing or wilting were considered diseased.

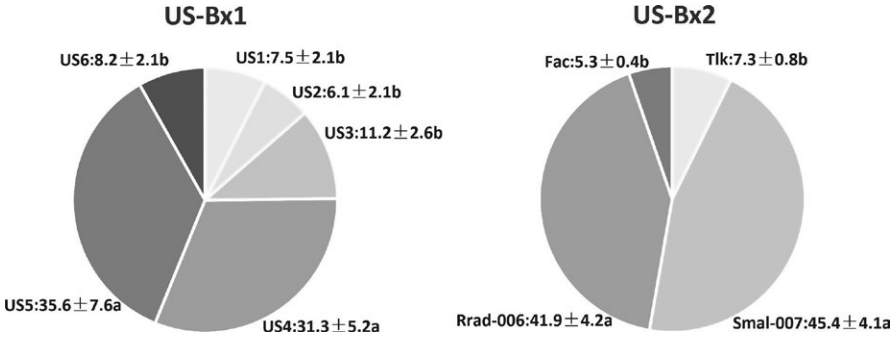

FIGURE 1 Proportion of each bacterial strain on the surface of corresponding nematodes (%). US-Bx1 and US-Bx2 are American pine wood nematode isolates. Data in the figure are the means ± *SD*; the different normal letters indicate significant differences amongst the treatments at the 0.05 level (n = 3) by Duncan's multiple range test

2.9 | Competition between bacteria on the surface of aseptic pine wood nematodes

The precultured aseptic pine wood nematodes were washed with sterile water and adjusted to a suspension of 1,000 nematodes/ml; the suspension was placed in aseptic 5-ml centrifuge tubes. Fifty microlitres of two types of competitive bacterial suspensions (1×10^8 cfu/ml) were added to centrifuge tubes with 5 ml of aseptic nematode suspension and processed for 1 hr, during which the tubes were constantly inverted for uniform mixing and to prevent the nematodes from sinking to the bottom of the tubes. After the nematodes and bacteria were fully integrated, they were centrifuged at 4°C (200 *g*, 5 min), and the supernatant liquid was discarded and washed with sterile water three times. Then, the nematodes were drawn and cultured on a PDA culture medium of *B. cinerea*, three repetitions were performed, and the medium was placed in an incubator at a constant temperature of 28°C. After the nematodes consumed the *B. cinerea* in the culture medium (7 days), the nematodes were washed with sterile water, centrifuged at 4°C (200 *g*, 5 min), and the supernatant liquid was discarded and washed with sterile water three times. Three to five nematodes were then selected and placed on a sterilized mortar, and 1 ml of sterile water was added. They were fully ground with a small amount of aseptic quartz sand to create 10-fold serial dilutions of 10^{-1}, 10^{-2} and 10^{-3} with sterile water. Two hundred microlitres of dilutions of different concentrations were taken and spread onto a plate. After culturing for 48 hr in an incubator at 28°C, two types of competing bacterial colonies in the culture medium were distinguished and quantified according to their morphological features and fluorescence.

2.10 | Competition between bacteria in the liquid culture medium

Fifty microlitres of two types of competing bacterial suspensions (1×10^8 cfu/ml) were taken and added to an NB culture medium. This was repeated three times for each processing. The medium was then placed in an incubator shaker (135 rpm) and cultured for 48 hr at 28°C. To conduct 10-fold serial dilutions with sterile water, 200 μl of dilutions diluted 10^5, 10^6 and 10^7 times were spread onto a plate. They were cultured for 48 hr in an incubator at 28°C to quantify the two types of competing bacterial colonies, and the frequency of occurrence of the colonies was calculated.

3 | RESULTS

3.1 | Bacterial isolation results

Six bacterial strains were isolated from US-Bx1 and labelled US1, US2, US3, US4, US5 and US6. Four bacterial strains were isolated from US-Bx2 and labelled Tlk, Smal-007, Rrad-006 and Fac (see Figure 1 for the proportion of each bacterial strain on the surface of the corresponding nematodes). Four strains, namely, US4, US5, Smal-007 and Rrad-006, were identified as dominant strains.

3.2 | Bacterial identification results

After the bacteria were cultured for 24–36 hr on NA culture medium, the forms of the tested bacterial colonies were observed (see Table 1 for the Gram-staining results of the strains and the detailed characteristics of the bacteria).

With the genomes of US4, US5, Smal-007 and Rrad-006 as templates, the universal primers of 16S rDNA were amplified to obtain approximately 1,400 bp PCR products. The amplification products were sequenced to obtain 16S rDNA sequence fragments of US4, US5, Smal-007 and Rrad-006, which were 1,307, 1,307, 1,428 and 1,359 bp long, respectively. Then, the 16S rDNA sequences of the four strains were compared to those at NCBI. The 16S rDNA sequence of the bacterial strain US4 (ID: KX261514) was most similar to *Delftia lacustris*, with a similarity of 100%. The 16S rDNA sequence of the bacterial strain US5 (ID: KX260958) was most similar to *Pseudomonas putida*, with a similarity of 100%. The 16S rDNA sequence of the bacterial strain Smal-007 (ID: KX260960) was most similar to *Stenotrophomonas maltophilia*, with a similarity of 100%. The 16S rDNA sequence of the bacterial strain Rrad-006 (ID: KX260959) was most similar to *Rhizobium nepotum*, with a similarity of 99.8%.

3.3 | Competition results of American dominant bacteria and toxigenic bacteria

The competition of four dominant bacteria carried by American nematodes (US4, US5, Smal-007 and Rrad-006) with the four Chinese strains (CH1, CH2, CH3 and CH4) of *P. fluorescens*, a bacterium with strong pathogenicity, on the surface of aseptic nematodes was assessed. After the nematodes consumed *B. cinerea* in a

TABLE 1 Gram stain and morphological features of the bacteria

Bacterial strain	Features of bacterial colonies on the plate of NB culture medium	Gram stain	Bacteria shape	Bacteria size/μm
US4[a]	The colonies are white, round, translucent, bulge in the middle and are neat-edged with low viscosity. Diameter will increase by 2–3 mm within 36 hr	G	Short rod-shaped	0.5–0.8 × 1.7–2.2
US5[a]	The colonies are pale yellow, round, translucent, bulge in the middle, neat-edged, have low viscosity and are fluorescent. Diameter will increase by 1–2 mm within 36 hr	G	Rod-shaped	0.7–0.8 × 2.3–3.3
Smal-007[a]	The colonies are pale yellow, round, translucent, bulge in the middle, neat-edged and have low viscosity. Diameter will increase by 1–2 mm within 36 hr	G	Short rod-shaped	0.6–0.9 × 1.4–2.0
Rrad-006[a]	The colonies are pale yellow, round, translucent, bulge in the middle, neat-edged and have low viscosity. Diameter will increase by 0.5–1.0 mm within 36 hr	G	Short rod-shaped	0.5–0.6 × 1.4–1.9
Staphylococcus aureus[b]	The colonies are thick, glossy, round and slightly bulging. Diameter will increase by 1–2 mm within 36 hr	G+	Ball-shaped	0.7–0.8 × 0.7–0.8
Escherichia coli[b]	The colonies are round, translucent, slightly bulging, and neat-edged with a smooth surface. Diameter will increase by 1–2 mm within 36 hr	G	Rod-shaped	0.4–0.5 × 1.8–2.8

[a]US4, US5, Smal-007 and Rrad-006 are dominant strains of American pine wood nematode isolates US-Bx1 and US-Bx2.
[b]The reference strains.

culture medium, the bacteria were isolated from the nematodes. Two types of competing colonies were distinguished by the morphological features and fluorescent characteristics of *P. fluorescens* on King's B medium (King et al., 1948) to quantify the two types of competing bacterial colonies that appeared (see Table 2 for results). US4, US5, Smal-007 and Rrad-006 were able to grow and reproduce on the nematodes. Smal-007 and Rrad-006 were isolated after competition with *P. fluorescens* on aseptic nematodes and had more bacterial colonies than US4 and US5.

Consequently, a competition culture of US4, US5, Smal-007 and Rrad-006 was set-up separately with *P. fluorescens* in NB medium to observe the reproductive capacity of these two types of bacteria in the culture medium. After 48 hr in a competition culture of the bacterial strains in pairs, 10-fold serial dilutions were made with sterile water and spread onto King's B medium. The two types of competing bacteria could be distinguished according to their colony characteristics and the fluorescent characteristics of *P. fluorescens* on King's B medium. The two types of competing bacterial colonies that appeared were quantified (the results are shown in Table 2). We showed that US4, US5 and Rrad-006 were weaker when competing with *P. fluorescens* strains CH2, CH3 and CH4 in liquid medium, and their colonies were dominant only when competing with CH1. However, the competitive capacity of Smal-007 was weaker than that of all four *P. fluorescens* strains when competing in liquid medium.

TABLE 2 Proportion of American strains after competition between American dominant bacteria and toxigenic bacteria (%)[a]

American strain	Competition on the surface of aseptic nematodes				Competition in NB medium			
	CH1[c]	CH2[c]	CH3[c]	CH4[c]	CH1[c]	CH2[c]	CH3[c]	CH4[c]
US4[b]	73.7 ± 2.2e[d]	78.4 ± 3.8de	75.1 ± 5.4e	90.0 ± 2.8abc	88.1 ± 2.8bcd	2.5 ± 0.7k	24.6 ± 2.9hi	23.3 ± 2.7hi
US5[b]	15.3 ± 3.4ij	58.9 ± 2.7f	94.0 ± 5.5ab	31.0 ± 14.3h	73.8 ± 7.2e	10.3 ± 3.6jk	5.8 ± 2.0jk	48.6 ± 2.7g
Smal-007[b]	91.7 ± 5.4ab	90.8 ± 3.5ab	100.0 ± 0.0a	89.3 ± 1.4abc	43.8 ± 3.0g	30.6 ± 12.5h	0.0 ± 0.0k	21.1 ± 3.4hi
Rrad-006[b]	97.6 ± 1.6ab	99.2 ± 1.2ab	98.4 ± 2.3ab	91.7 ± 2.1ab	79.9 ± 6.5cde	0.0 ± 0.0k	0.0 ± 0.0k	3.6 ± 5.2k

[a]Proportion = $\frac{A}{A+B} \times 100\%$; A = the number of colonies of American strains; B = the number of colonies of Chinese strains.
[b]US4, US5, Smal-007 and Rrad-006 are dominant strains of the American pine wood nematode isolates US-Bx1 and US-Bx2.
[c]The virulent strains of *Pseudomonas fluorescens*, CH1, CH2, CH3 and CH4 isolated from wild nematode isolates in Jiangsu Province, Zhejiang Province, Anhui Province and Fujian Province, respectively, represent the dominant bacterial strains on the surface of nematodes that display strong pathogenicity or toxicity for pine seedlings.
[d]Data in the table are the means ± *SD*; the different normal letters indicate significant differences amongst treatments at the 0.05 level (n = 3) by Duncan's multiple range test.

TABLE 3 Performances of *Pinus thunbergii* Parl. seedlings processed with different bacterial cell-free culture media

Processed strain	Harm on the seventh day (0/1/2/3/4)[c]	Days for complete wilting/d
US4[a]	0/5/4/1/0	9~11
US5[a]	0/2/5/2/1	7~10
Smal-007[a]	0/5/3/2/0	9~12
Rrad-006[a]	0/6/4/0/0	12~13
CH1[b]	0/0/0/0/10	5~7
CH2[b]	0/0/0/0/10	6~7
CH3[b]	0/0/0/0/10	3~5
CH4[b]	0/0/0/0/10	4~5
Sterile water	10/0/0/0/0	>15
Culture medium	10/0/0/0/0	>15

[a]US4, US5, Smal-007 and Rrad-006 are dominant strains of the American pine wood nematode isolates US-Bx1 and US-Bx2.
[b]The virulent strains of *Pseudomonas fluorescens*, CH1, CH2, CH3 and CH4 isolated from wild nematode isolates in Jiangsu Province, Zhejiang Province, Anhui Province and Fujian Province, respectively, represent the dominant bacterial strains on the surface of nematodes that display strong pathogenicity or toxicity for pine seedlings.
[c]"4" indicates seriously withered and allochroic; "3" indicates wilting due to complete water loss; "2" indicates wilting due to significant water loss; "1" indicates slight water loss; "0" indicates fresh and no loss of water.

3.4 | Toxigenic measurement results of the bacterial cell-free culture medium

To understand the toxigenicity of the bacteria, a bacterial cell-free culture medium was used to measure the biological toxicity of one-month-old aseptic root-cut black pine (*P. thunbergii*) seedlings. The results are shown in Table 3. The cell-free culture medium of the dominant bacteria CH1, CH2, CH3 and CH4 carried by the Chinese nematode exhibited strong toxicity for pine seedlings, which caused the seedlings to wilt on the third to seventh day. However, the culture medium of the dominant bacteria US4, US5, Smal-007 and Rrad-006 carried by the American nematode showed weaker toxicity, which caused wilting of the pine seedlings after the seventh day and complete death on the thirteenth day at the latest, whereas the reference seedlings processed with sterile water and non-inoculated blank medium did not wilt within 2 weeks.

3.5 | Pathogenicity test results of mixed inoculations with bacteria and aseptic nematodes

Two-month-old aseptic black pine (*P. thunbergii*) seedlings were inoculated with the mixed suspension of bacterial and aseptic nematodes to measure the pathogenicity of the dominant bacteria. Seedlings were taken and inoculated separately with aseptic nematodes, Zhejiang nematodes, American nematodes and sterile water as the reference. See Table 4 for the results. Amongst them, the dominant bacteria *P. fluorescens* carried by Chinese nematodes displayed strong pathogenicity, and pine seedlings displayed different degrees of wilting within one to 2 weeks after inoculation. The dominant bacteria carried by American nematodes presented low pathogenicity, with pine seedlings showing almost no changes within the first week after inoculation, followed by partial withering after 2 weeks. Pine seedlings inoculated with Zhejiang nematodes began to wither within 2 weeks and died on the 17th day, whereas the pine seedlings inoculated with American nematodes and aseptic nematodes did not wilt within a month.

TABLE 4 Disease status of *Pinus thunbergii* Parl. seedlings after mixed inoculation with bacterial and aseptic nematodes

Processing	Harm on the 21st day (0/1/2/3/4)[e]	Days for complete wilting/d
US4[a] + aseptic nematodes	0/7/3/0/0	28–30
US5[a] + aseptic nematodes	0/6/4/0/0	27–30
Smal-007[a] + aseptic nematodes	2/6/2/0/0	>30
Rrad-006[a] + aseptic nematodes	1/6/3/0/0	>30
CH1[b] + aseptic nematodes	0/0/3/7/0	22–23
CH2[b] + aseptic nematodes	0/0/4/6/0	23–24
CH3[b] + aseptic nematodes	0/0/0/0/10	19–21
CH4[b] + aseptic nematodes	0/0/0/0/10	19–21
ZJ-Bx[c]	0/0/0/0/10	17–18
US-Bx1[d]	3/6/1/0/0	>30
US-Bx2[d]	4/6/0/0/0	>30
Sterile water	10/0/0/0/0	>30
Sterile water + aseptic nematodes	7/3/0/0/0	>30

[a]US4, US5, Smal-007 and Rrad-006 are dominant strains of American pine wood nematode isolates US-Bx1 and US-Bx2.
[b]The virulent strains of *Pseudomonas fluorescens*, CH1, CH2, CH3 and CH4 isolated from wild nematode isolates in Jiangsu Province, Zhejiang Province, Anhui Province and Fujian Province, respectively, represent the dominant bacterial strains on the surface of nematodes that display strong pathogenicity or toxicity for pine seedlings.
[c]ZJ-Bx is the Zhejiang pine wood nematode.
[d]US-Bx1 and US-Bx2 are American pine wood nematodes.
[e]"4" indicates seriously withered and allochroic; "3" indicates wilting due to complete water loss; "2" indicates wilting due to significant water loss; "1" indicates slight water loss; "0" indicates fresh and no loss of water.

3.6 | Pathogenicity test results of mixed inoculations with bacteria and wild nematodes

Two-year-old Masson's pine (*P. massoniana*), black pine (*P. thunbergii*), Chinese red pine (*Pinus tabulaeformis*) and slash pine (*Pinus elliottii*)

TABLE 5 Disease status in pine seedlings after mixed inoculation with bacteria and wild nematodes

Processing	Disease incidence of *Pinus massoniana* (%)	Disease incidence of *Pinus thunbergii* (%)	Disease incidence of *P. tabulaeformis* (%)	Disease incidence of *P. elliottii* (%)
US4[a] + ZJ-Bx[b]	10	10	10	5
US5[a] + ZJ-Bx[b]	5	15	20	20
Smal-007[a] + ZJ-Bx[b]	0	0	0	0
Rrad-006[a] + ZJ-Bx[b]	0	0	0	10
US-Bx1[c]	0	0	0	0
US-Bx2[c]	0	0	0	5
ZJ-Bx[b]	100	100	100	100
Sterile water	0	0	0	0

[a]US4, US5, Smal-007 and Rrad-006 are dominant strains of American pine wood nematode isolates US-Bx1 and US-Bx2.
[b]ZJ-Bx is the Zhejiang pine wood nematode.
[c]US-Bx1 and US-Bx2 are American pine wood nematodes.

seedlings were inoculated with a mixed suspension of American dominant bacteria and Zhejiang nematode isolates. The seedlings were separately inoculated with aseptic nematodes, Zhejiang nematodes, American nematodes and sterile water as the reference. Ninety days later, the disease status in the pine seedlings was calculated and analysed (the results are shown in Table 5). All four types of pine seedlings inoculated with Zhejiang nematode isolates became diseased. Compared to the disease incidence in pine seedlings inoculated with Zhejiang nematode isolates, the disease incidence of pine seedlings inoculated with both American dominant bacteria and Zhejiang nematode isolates significantly decreased, and the pine seedlings inoculated with Smal-007 and Rrad-006 and Zhejiang nematode isolates almost lost their pathogenicity. Amongst the four types of pine seedlings inoculated with two American nematode isolates, only one slash pine seedling inoculated with US-Bx2 had disease symptoms within 90 days.

4 | DISCUSSION

The bacteria carried by different pine wood nematodes are not the same. That is, a successional phenomenon exists in the bacteria on the surface of pine wood nematodes that can be demonstrated by the variations in the pathogenicity of the different nematode isolates. The pathogenicity of the pine wood nematode has changed significantly since it was introduced into China in 1982. The most immediate change is that the pathogenicity of pine wood nematodes for Masson's pine (*P. massoniana*) was originally weak, but Masson's pine later became the most susceptible species (Hu et al., 1994; Tan, Yang, & Wu, 2000). Another major piece of evidence is the pathogenicity of cedar (*Cedrus deodara*). Cedars are susceptible in Japan and the United States, but they are completely immune in China (Ju, Zhao, & Pu, 2007; Zheng, 2003). The explanation for such changes is that the bacteria attached to the surface of pine wood nematodes have changed. The reported information demonstrates that the main bacteria on the surface of Japanese nematodes include several types of *Bacillus* (Kawazu et al., 1998), whereas China's main bacteria are *Pseudomonas* spp. (Han et al., 2003; Wang, 2003). All these phenomena demonstrate that a successional phenomenon exists in the bacteria on the surface of pine wood nematodes.

The United States is considered the origin of pine wilt disease. Although the disease is distributed in 36 states, there are few large areas of dead pine trees, showing that the development of the disease in the United States has stabilized. An analysis of the reasons for the stabilization of pine wilt disease in the United States should be limited to the pathogen, media, host and environment. Differences in environmental conditions and host resistance to disease should be excluded. The United States and China lie at nearly the same latitude, and their temperature and humidity conditions present no significant differences; thus, environmental conditions cannot be the main factor. With respect to the host, pine wood nematodes can infect all *Pinus* plants. Many susceptible pine species exist in the United States, including *Pinus strobus*, *Pinus clanusa*, *Pinus sylvestris*, *P. elliottii* and *Pinus banksiana*, and these pine trees have been shown to be susceptible (Yang et al., 1993). Therefore, the disease resistance of the pine trees can also be eliminated. There is also the view that *Monochamus alternatus* is not present in the United States, whereas it is the major agent of the disease in China and Japan. However, *Monochamus carolinensis* is found in the United States, and it can also effectively be spread by pine wood nematodes (Ning, Fang, Tang, & Sun, 2004). In this way, the focus of the problem should be on pathogenic nematodes. In other words, the pathogenicity of the American pine wood nematode is weak, which has also been shown in this study. The changes in the pathogenicity of the pine wood nematode derive from changes in the bacteria on its surface. That is, the bacteria on the surface of American nematodes have experienced a long-term and complete succession, resulting in bacteria with low pathogenicity becoming dominant. In addition to low pathogenicity or weak toxicity, the bacteria on the surface of American nematodes also show another characteristic, namely, an absolute advantage. In our study, the dominant bacteria Smal-007 and Rrad-006 on the surface of American nematode isolates revealed an absolute advantage when competing with Chinese dominant

strains on the surface of nematodes, and they could also significantly reduce the pathogenicity of wild nematode isolates in Zhejiang colonized by these bacteria. However, we also found that Smal-007 and Rrad-006 strains grow well when cultured on the surface of pine wood nematodes; furthermore, they are more competitive compared to the Chinese strains. Opposite results were obtained when they were cultured in NB medium. Such completely opposite results may be caused by differences between the medium components and substances on the surface of pine wood nematodes. Bacteria that can grow well on the surface of pine wood nematodes do not necessarily grow well in an NB medium. The substances on the surface of pine wood nematodes are mainly polysaccharides and some unknown substances (Cao, 1997; Kawazu, 1998), which are completely different from the components in NB medium.

Although Smal-007 and Rrad-006 strains have advantages such as low pathogenicity, strong exclusiveness and the potential for preventing pine wilt disease, the natural situation is complex. Biocontrol strains will be subjected to ultraviolet radiation and rainwater washing and must survive and reproduce for a long time on the surface of pine wood nematodes to successfully replace the pathogenic strains. Pine wood nematodes not only live in pine trees but can also be attached to the surface of long-horned beetles or exposed to the outside of trees; therefore, the bacteria on the surface of pine wood nematodes must withstand such complex environmental conditions. Only laboratory results were obtained in our study; thus, extensive and detailed research should be conducted to examine whether American bacterial strains can replace Chinese bacterial strains in nature.

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