Gene sdaB Is Involved in the Nematocidal Activity of Enterobacter ludwigii AA4 Against the Pine Wood Nematode Bursaphelenchus xylophilus

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Bursaphelenchus xylophilus, a plant parasitic nematode, is the causal agent of pine wilt, a devastating forest tree disease. Essentially, no efficient methods for controlling B. xylophilus and pine wilt disease have yet been developed. Enterobacter ludwigii AA4, isolated from the root of maize, has powerful nematocidal activity against B. xylophilus in a new in vitro dye exclusion test. The corrected mortality of the B. xylophilus treated by E. ludwigii AA4 or its cell extract reached 98.3 and 98.6%, respectively. Morphological changes in B. xylophilus treated with a cell extract from strain AA4 suggested that the death of B. xylophilus might be caused by an increased number of vacuoles in non-apoptotic cell death and the damage to tissues of the nematodes. In a greenhouse test, the disease index of the seedlings of Scots pine (Pinus sylvestris) treated with the cells of strain AA4 plus B. xylophilus or those treated by AA4 cell extract plus B. xylophilus was 38.2 and 30.3, respectively, was significantly lower than 92.5 in the control plants treated with distilled water and B. xylophilus. We created a sdaB gene knockout in strain AA4 by deleting the gene that was putatively encoding the beta-subunit of L-serine dehydratase through Red homologous recombination. The nematocidal and disease-suppressing activities of the knockout strain were remarkably impaired. Finally, we revealed a robust colonization of P. sylvestris seedling needles by E. ludwigii AA4, which is supposed to contribute to the disease-controlling efficacy of strain AA4. Therefore, E. ludwigii AA4 has significant potential to serve as an agent for the biological control of pine wilt disease caused by B. xylophilus.

Keywords: sdaB, L-serine dehydratase, Enterobacter ludwigii, nematocidal activity, Bursaphelenchus xylophilus, pine wilt disease, methuosis
INTRODUCTION

The pine wood nematode (PWN) Bursaphelenchus xylophilus causes serious damage to forest ecosystems and massive economic losses by inducing pine wilt disease (PWD) (Zhao et al., 2014; Lee et al., 2019; Guo et al., 2020). PWD may result in destruction of conifer forests and has long been a huge threat to Asian and European forestry for a long time (Kikuchi et al., 2011; Faria et al., 2015). As a pathogenic nematode native to North America (Li et al., 2015; Proenca et al., 2017), B. xylophilus, feeding on live trees and fungi colonizing dead or dying trees, is a migratory endoparasite transmitted by the insect vector Monochamus alternatus (Japanese pine sawyer beetle) (Kim et al., 2019, 2020). The annual economic cost of the PWN (Soliman et al., 2012) in the European Union (EU) alone is estimated at a billion euros for each of the past 22 years. The rapid death of pine trees infected by the PWN could be attributed to the dysfunction of the water-conducting system caused by the death of parenchyma cells. The secreted enzymes and surface coat proteins of B. xylophilus are involved in its pathogenicity (Putai, 2013; Nunes da Silva et al., 2015; Wen et al., 2021). The molecular mechanisms of PWN pathogenesis are still largely unknown, hindering the prospects for control of this pathogen and PWD. Chemical insecticides and nematicides used to control B. xylophilus by jet-sprays or trunk injections for decades (Qiu et al., 2019; Guo et al., 2020; Faria et al., 2021) have become a major social concern. More environmental-friendly strategies, such as beneficial microorganisms that suppress PWN, have recently gained more attention (Tian et al., 2007; Wu et al., 2013; Cai et al., 2022).

Biological control of plant parasitic nematodes (PPNs) using nematicidal bacteria or their metabolites, which are toxic to nematodes, is a potentially sustainable alternative to chemical nematicides (Haegeman et al., 2009; Kumar and Dara, 2021). Nematicidal prokaryotes mainly belonging to bacterial genera, such as Bacillus (Crickmore, 2005; Ponpadian et al., 2019; Park et al., 2020), Streptomyces (Kang et al., 2021), Serratia (Paiva et al., 2013; Nascimento et al., 2016; Abd El-Aal et al., 2021), Stenotrophomonas (Huang et al., 2009; Ponpadian et al., 2019), Pseudomonas (Fang et al., 2019; Abd El-Aal et al., 2021), Novosphingobium (Topalović et al., 2020), Pasteuria (Tian et al., 2007), Pseudomonas (Chan et al., 2020), Enterobacter (Munif et al., 2000; Oh et al., 2018), and Curtobacterium (Kumar and Dara, 2021), are capable of suppressing PPNs by diverse modes of action, including parasitism (Wu et al., 2013; Proenca et al., 2017; Ponpadian et al., 2019), production of toxins (Abd El-Aal et al., 2021; Kahn et al., 2021), antibiotics plus enzymes (Yang et al., 2007; Huang et al., 2009), competition for nutrients (Proenca et al., 2019), and induction of systemic resistance of plants (Liang et al., 2019; Han et al., 2021). Within these nematicidal groups, some bacterial strains have exhibited efficient killing activity against the PWNs. Bacillus thuringiensis z8c85 caused 90% mortality of B. xylophilus by producing a Cry protein named Cry5Ba3 (Kahn et al., 2021). Two Streptomyces strains did kill the hatched PWNs and affected egg hatching via biosynthesis of the toxic compounds, teleocidin B4, and spectinabilin, which effectively suppressed the development of PWD under field conditions (Kang et al., 2021). A 70 kD serine protease produced by Serratia sp. A88copa13 was majorly responsible for the toxicity of this PWN-killing strain (Paiva et al., 2013). Thus, the application of nematicidal bacteria is a promising strategy in suppressing PWD.

Enterobacter is an exceptionally diverse genus of bacteria found in various habitats in association with soil (El-Sayed et al., 2014; Habibi et al., 2019; Danish et al., 2020), plants (Park et al., 2015; Andres-Barrao et al., 2017; Sarkar et al., 2018), and animals, including humans (Mokracka et al., 2004; Peng et al., 2009). Many Enterobacter strains are characterized as plant beneficial bacteria. Such procaryotic microbes promote the growth of their plant hosts under favored or adverse abiotic conditions by producing indole-acetic acid (IAA) (Park et al., 2015; Srisuk et al., 2018; Habibi et al., 2019; Li et al., 2022), siderophores (Mokracka et al., 2004; Nurjadi et al., 2021; Li et al., 2022), hydrocyanic acid (Mpongwana et al., 2016; Mahdi et al., 2020; Javaheri Safa et al., 2021), salicylic acid (Kang et al., 2015) plus exopolysaccharides (Sayed et al., 2015; Niu et al., 2018; Dhanya et al., 2021), solubilizing phosphate (Adhikari et al., 2020; Roslan et al., 2020; Aeroen et al., 2021), by fixing nitrogen (Kämpfer et al., 2005; Peng et al., 2009; El-Sayed et al., 2014), reducing Na+ uptake (Um et al., 2017; Sarkar et al., 2018), and inducing the activity of enzymes with action as antioxidants (Andres-Barrao et al., 2017; Danish et al., 2020). At present, it has been demonstrated that some Enterobacter species exhibit inhibitory effects against the oomycete pathogen Pythium ultimum (Lohrke et al., 2002; Kayegyama and Nelson, 2003; Windstam and Nelson, 2008), the fungal pathogens Fusarium moniliforme (Hinton and Bacon, 1995; Demirci et al., 2000; Rodrigues et al., 2018), F. oxysporum (El-Sayed et al., 2014; Del Barrio-Duque et al., 2019; Abdelshafy Mohamad et al., 2020), Aspergillus niger (Yadav et al., 2016; Kaushik et al., 2017; Batyrova et al., 2020), and Aspergillus flavus (Etcheverry et al., 2009; Mahmood et al., 2020), and the bacterial pathogen Ralstonia solanacearum (Sarkar and Chaudhuri, 2015; Yin et al., 2020; Zaki et al., 2021), indicating their protective efficacy on plants. A few Enterobacter strains exhibited significant nematicidal activity against the root-knot nematodes (Munif et al., 2000; El-Sayed et al., 2014; Oh et al., 2018). Enterobacter asburiae HK169 was able to reduce root gall formation rate by 66% and killed all juveniles of Meloidogyne incognita within 48 h (Oh et al., 2018). Similarly, an endophytic E. intermedius strain isolated from tomato roots remarkably decreased the number of root galls (Munif et al., 2000). Our knowledge of the molecular mechanisms underlying the inhibitory effects of Enterobacter strains against PPNs is still very limited.

In the present study, we identified a powerful PWN-killing bacterial strain, Enterobacter ludwigi AA4, which was previously isolated from maize roots (Niu et al., 2017) by screening the nematicidal activities of 374 bacterial strains against B. xylophilus using a new fluorescent staining-based PWN-killing activity test. E. ludwigi AA4 had a remarkable inhibitory effect against PWD under greenhouse conditions. After deleting the sdaB gene, presumed to encode the beta subunit of L-serine dehydratase, the nematicidal and disease-suppressing effects of the mutant strain were significantly reduced. The robust colonization of P. sylvestris seedling needles by E. ludwigi AA4, presumed to contribute to
the disease-controlling efficacy of strain AA4, was quantified and compared to the strain carrying a sdaB gene deletion.

MATERIALS AND METHODS

Microbial Strains and Growth Conditions

Bacterial strains kept frozen with 15% (v/v) glycerol at −80°C were streaked on Luria-Bertani (LB) agar plates and incubated at 30°C for 16 h. Then, a single colony of each strain was inoculated into 5 ml LB liquid medium and shaken at 30°C, 200 rpm for another 16 h. Antibiotics were supplemented where necessary at the following concentrations: kanamycin 50 µg/ml, tetracycline 50 µg/ml, gentamicin 25 µg/ml, streptomycin 50 µg/ml, and gentamicin 25 µg/ml (Table 1).

The spores of Botrytis cinerea were deposited at −80°C in 15% (v/v) glycerol. Ten microliters of fungal spores were dropped on a potato dextrose agar (PDA) plate and incubated at 25°C in the dark. When B. cinerea mycelia covered PDA plates, the fungal culture was used for inoculating B. xylophilus.

The Source and Culture of Pine Wood Nematodes

Cultures of the PWN, Bursaphelenchus xylophilus, were obtained from Dr. Hongtao Li at the Hebei Academy of Agriculture and Forestry Sciences and Dr. Kai Guo at Zhejiang A&F University. The fungus B. cinerea was purchased from the Shanghai Bioresource Collection Center (SHBBC).

Bursaphelenchus xylophilus was inoculated on a PDA culture of B. cinerea and incubated at 25°C in the dark until the fungal mycelia were completely consumed by B. xylophilus. Nematodes were collected using the modified Baermann funnel technique (Kitazume et al., 2018; Cesarz et al., 2019; Maehara et al., 2020) and washed with a mixture of 0.1% streptomycin sulfate and 0.002% actinomycin three times to remove surface microbial contaminants (Liu et al., 2016). Then, these nematodes were used for PWN-killing activity test and in planta biocontrol assays.

For microscopic image analysis of PWN morphology, about 10,000 nematodes were decanted into a burette containing 25 ml of 0.3% carboxymethyl cellulose (CMC) solution. After 12 h, the second-stage juveniles were collected from the top of the burette (Qiu et al., 2016, 2019). These worms were fed with B. cinerea. After 48 h, the L4 juveniles were collected in sterile water and washed with a mixture of 0.1% streptomycin sulfate and 0.002% actinomycin three times. These L4 juveniles were used for the image analysis.

Preparation of Cell Extracts of E. ludwigi AA4

Wild-type AA4, AA4∆sdaB (derivative of wild-type AA4 that lacks sdaB), and C0∆sdaB (AA4∆sdaB complemented with wild-type sdaB gene) kept frozen with 15% (v/v) glycerol at −80°C were streaked on LB agar plates with corresponding antibiotics (Table 1) and incubated at 30°C for 16 h. Then, a single colony of each strain was inoculated into 5 ml LB liquid medium and shaken at 30°C, 200 rpm for another 16 h. One milliliter of the culture of each strain was transferred into 50 ml LB liquid medium and shaken at 30°C, 200 rpm for 2–3 h until Optical Density at 600 nm (OD$_{600}$) value reached 0.6–0.8 (early stationary phase). Cells were harvested by centrifuging at 9,100 rpm (8,000 × g) at 4°C for 20 min (Thermo, Multifuge X1R, Germany) and washed by phosphate-buffered saline (PBS). The cell precipitations were resuspended in 5 ml PBS before sonication. An ultrasound processor (Sonics, VCX130PB, United States) was used to sonicate the resuspended cell suspensions. The probe was immersed into the suspensions and sonicated the cells on ice for 60 min at a power of 130 W (pulse duration: 2 s on, 1 s off). The resulting cell lysates were centrifuged at 15,300 rpm (14,000 × g) for 30 min. The supernatant was collected and used as cell extracts. The concentration of total protein in cell extract was measured by using a biuret protein assay reagents kit (Solarbio, PC0010, China).

Fluorescent Staining-Based Pine Wood Nematode-Killing Activity Test

The nematocidal activity of the 43 efficient PWN-killing bacterial strains of AA4∆sdaB and C0∆sdaB was evaluated by the fluorescent staining-based PWN-killing activity test, while Bacillus pumilus YLT40 and Paenibacillus polymyxa M-1 were utilized for confirming the reliability of the assay. The cell density of bacterial culture suspension was adjusted to OD$_{600}$ = 1.0 for each strain used in the test. Each well of the 96-well microplates was added with 40 µl of bacterial culture suspension and 20 µl of worm suspension containing 40–50 nematodes, while 40 µl of LB liquid medium or PBS and 20 µl of worm suspension was employed as negative control. Five wells were used for each treatment designated with the code of the strain listed in Supplementary Tables 1, 2. The inoculated microplates were incubated at 25°C in the dark for 24 h. Then, a plate washer (Biobase, BK-9622, China) was employed to change the liquid cultures with sterile water. 4′,6-diamidino-2-phenylindole (DAPI, Solarbio, PC0010, China) was used to stain the PWNs, and the final concentration of DAPI in each well was 5 µg/ml. After being stained for 1 h, the nematodes were observed by a confocal laser scanning microscope (Zeiss, LSM 800, Germany) using an excitation laser of 353 nm and collecting emission of 465 nm, where the dead worms displayed bright fluorescence while the live ones did.

To test the nematocidal activity of the cell extract of wild-type AA4, AA4∆sdaB, and C0∆sdaB, the extract containing total proteins of 5 mg/ml was mixed with 20 µl of worm suspension containing 40–50 nematodes in one well of a 96-well microplate. The remaining steps are the same as those for the assays with bacterial cell suspensions.

The mortality and corrected mortality of B. xylophilus were calculated as follows (Faria et al., 2013; Guo et al., 2016; Liu et al., 2019):

\[
\text{The mortality (\%) } = \frac{\text{number of dead nematodes}}{\text{number of all nematodes}} \times 100
\]
TABLE 1 | Strains and plasmids used for genetic manipulation of Enterobacter ludwigii AA4.

| Strain or plasmid         | Description                                                      |
|---------------------------|------------------------------------------------------------------|
| Strains                   |                                                                  |
| T1                        | Tn10 transposon inserting into genome of wild-type AA4; KanR.   |
| AA4 sdaB                  | Derivative of wild-type AA4 that lacks sdaB; KanR.              |
| CΔsdaB                    | AA4 ΔsdaB complemented with wild-type sdaB gene; KanR, TetR.    |
| AA4-GFP                   | Green fluorescent protein (GFP)-labeled wild-type AA4; TetR, GenR. |
| AA4ΔsdaB-GFP              | GFP-labeled AA4 ΔsdaB; KanR, TetR, GenR.                        |
| CΔsdaB-GFP                | GFP-labeled CΔsdaB; KanR, TetR, GenR.                           |
| DH5a                      | Competent cells for cloning.                                     |
| Plasmids                  |                                                                  |
| pKD4                      | Kanamycin cassette; KanR.                                        |
| pKD46                     | Template plasmid containing Red recombinase system under arabinose-inducible promoter; KanR. |
| pUC19                     | Vector for construction of recombinant plasmid; TetR.           |
| pBBR1                     | Vector for construction of complement plasmid; TetR.            |
| pGPF78                    | Vector with green fluorescent protein (GFP); TetR, GenR.        |

KanR, kanamycin resistance; TetR, tetracycline resistance; GenR, gentamicin resistance.

TABLE 2 | Primers used in this study.

| Primer             | 5′–3′ sequence       |
|--------------------|----------------------|
| For 16S rRNA gene sequencing analysis |                     |
| 27F                | AGAGTTGATCATGCTGCAG  |
| 1492R              | TACGTTACCTGTATACGCTT |
| For gene deletions |                      |
| sdaB-H1up          | TCTGATTCCGCTGATCATCATGCTATGCTATGCTCTCC |
| sdaB-H1down        | GAAAAAGCCTCGATAAACAGAGGTTCCTGGAAGGCAATCTCCTTTGTTCTTTCC |
| sdaB-H2up          | GTGCGATATGTTGAAAGCCACAGGAAAGGGAG |
| sdaB-H2down        | GCGTCCCTGAAAGGCGATATCCCTCTTATTGCC |
| Kup                | AGCAATGAAACGCGATCGATGAGGAAAGGGCCAG |
| Kdown              | CATATGACGAGCGATCGAGGAAAGGACAG |
| pUC19up            | CATGTACGCAGAGCGATCGAGGAAAGGACAG |
| pUC19down          | CTCCTTCGAGCTGATCGACGAGCGAAAGGACAG |
| For complementing the mutation |                   |
| sdaB-DF            | ATGGAACACCCAATTAAACCAAGCCGCTCGTTGCTGATTG |
| sdaB-UR            | CGAAGCGCGCTGTTGAGGCGGATACGCAAGCAGAAAG |
| sdaB-PBBR1-DF      | AAGACAGAATACGAAATCCAATCGGCTGCTATAAGTGAGGCTGATTATA |
| sdaB-PBBR1-UR      | TTGTAGACCTCGAGATTTGTTGCTGCTATTAG |

Corrected mortality (%) = \[
\frac{\text{mortality in treatment} - \text{mortality in control}}{100 - \text{mortality in control}} \times 100
\]

Two-tailed t-test (GrapPad Prism 8) was used for the statistical analysis. All these experiments above were repeated five times.

Z′ factor, a combination of signal interval and variation, is a parameter used to evaluate the reliability of results obtained from an experiment for guiding a larger-scale experimentation. It is calculated as:

\[
Z' \text{factor} = 1 - \frac{3(\sigma_p + \sigma_n)}{|\mu_p - \mu_n|}
\]

where σp and σn represent the standard deviations of positive control and negative control, respectively, and μp and μn represent the means of positive control and negative control, respectively. Z′ factor was calculated by using the software Excel (Microsoft). The theoretical value of Z′ factor is 1, and a Z′ factor value between 0.5 and 1 indicates the outcomes of a given experiment is highly reliable, while a test of low reliability usually possesses a Z′ factor value of less than 0.5 (Zhang X. D. et al., 2020).

Identification of the Efficient Bacterial Pine Wood Nematode-Killing Strains

Identification of the efficient bacterial PWN-killing strains were carried out by using 16S rRNA gene sequencing analysis. Genomic DNA was extracted from an overnight LB culture of...
each strain by utilizing a bacterial genomic DNA extraction kit (Tiangen, DP302-02, China) following the manufacturer’s protocol. The 16S rRNA gene was amplified in 25-μl PCR reactions using the universal primers 27F and 1492R (Table 2) (Monciardi et al., 2002; Niu et al., 2013; López and Alippi, 2019). The reaction mixture contained 1 μl of template DNA, 12.5 μl of master mix (Takara, RR350Q, Japan), 1 μl of each of the forward and reverse primers, and 9.5 μl of sterile deionized water. Amplifications were performed using a T100 Thermal Cycler (Bio-Rad, 621BR47532, United States) with the following cycle conditions: initial denaturation at 95°C for 5 min; 30 cycles at 94°C for 30 s, 58°C for 30 s, and 72°C for 1.5 min; and a final extension at 72°C for 10 min. Amplicons were purified using a PCR purification kit (Omega, D6492-02, China) and sequenced using 27F primer at Tsingke Biotechnology Co., Ltd. DNA sequences were inspected for ambiguous trailing or leading bases using the software GAP4 in the STADEN Package1. The sequences were compared with those of the reference organisms by Basic Local Alignment Search Tool (BLAST) at the National Center for Biotechnology Information (NCBI) website2 and by Sequence Match at the Ribosomal Database Project (RDP) website3. The 16S rRNA gene sequences of efficient nematocidal strains were deposited in the GenBank database. The accession numbers were listed in Supplementary Table 1.

**In planta Assays for Biocontrol Effect Against Pine Wilt Disease**

The biological control effects of wild-type AA4, AA4ΔsdaB, and CΔsdaB were examined by using both 3-year-old and 1-month-old Scots pine (Pinus sylvestris) tree seedlings grown under greenhouse conditions. The 3-year-old seedlings were purchased from Longsheng nursery at Harbin. Ten milliliters of bacterial suspension (OD600 = 1.0) were inoculated on the 3-year-old seedlings by spraying. Then, a 2–4 cm slit was made on the surface of bark located at approximately 10–15 cm above the soil. One hundred microliters of PWN suspension containing about 5,000 worms were injected into the needles. A small piece of sterile cotton was fixed on the needles to cover the pinhole. The plants were incubated in greenhouse under the same condition on which the 3-year-old seedlings grew. The number of dead seedlings and symptoms were recorded on the ninth day after inoculation. Ten plants were used for each of the 11 treatments designated as PWN (inoculation of seedlings with PWN alone), PWN + ΔsdaB (inoculation of seedlings with PWN jointly with AA4ΔsdaB cell suspension), PWN + CΔsdaB (inoculation of seedlings with PWN jointly with AA4CΔsdaB cell suspension), PWN + WT (inoculation of seedlings with PWN jointly with wild-type AA4 cell suspension), PWN + ΔsdaBCE (inoculation of seedlings with PWN jointly with AA4ΔsdaB cell extract), PWN + WTCE (inoculation of seedlings with PWN jointly with wild-type AA4 cell extract), PWN + ΔsdaBS (inoculation of seedlings with PWN jointly with AA4ΔsdaB culture supernatant), PWN + CΔsdaBS (inoculation of seedlings with PWN jointly with AA4CΔsdaB culture supernatant), PWN + WTS (inoculation of seedlings with PWN jointly with wild-type AA4 culture supernatant), and DW (treatment with distilled water), respectively.

The severities of PWD were evaluated based on the disease ranks shown in Supplementary Figure 3. The disease severity indices were calculated as follows:

\[
\text{Disease Index (DI)} = \frac{\sum (X_i \times A_i)}{(X \times A_{max})}
\]

where Xi is the number of pine trees in each disease rank, Ai is the disease rank, X is the total number of pine trees, and Amax is the maximum rank (Qiu et al., 2019; Xue et al., 2019). Two-tailed t-test (GrapPad Prism 8) was used for the statistical analysis. These experiments were repeated three times.

**Image Analysis of Pine Wood Nematode Morphology**

The cell extract of E. ludwigii AA4 was prepared as above. Forty microliters of cell extract containing total proteins of 5 mg/ml and 20 μl of worm suspension containing 40–50 L4 juveniles of PWNs were added in a single well of the 96-well microplates. At the meantime, the combination of 40 μl of PBS and 20 μl of worm
After being washed three times, the worms were transferred to a suspension and put into 10 μl of PBS by injector pinhead. After being washed three times, the worms were transferred to a slide. Ten microliters of PBS were then dropped on the samples. The morphological changes of *B. xylophilus* were observed and recorded by a light microscope (Olympus, BX43, Japan).

**Construction of Mutants of *E. ludwigii* AA4**

The *sdaB* gene knock-out mutant of *E. ludwigii* AA4 was created by using the Red recombinase system (Datsenko and Wanner, 2000; Dahyot et al., 2020; Guérin et al., 2020). Primers were designed according to the information obtained from the whole genome sequence of strain AA4 (Niu et al., 2017). The upstream border sequence of the AA4 *sdaB* gene was amplified from AA4 chromosomal DNA using primers *sdaB*-H1up and *sdaB*-H1down (Table 2), while the downstream border sequence of *sdaB* was amplified with primers *sdaB*-H2up and *sdaB*-H2down (Table 2). A kanamycin resistance cassette, flanked by 23-bp-long sequences homologous with *sdaB* gene, was amplified from plasmid pKD4 DNA with primers Kup and Kdown (Table 2). Another fragment flanked by 22-bp-long sequences homologous with *sdaB* gene was amplified from plasmid pUC19 by using primers pUC19up and pUC19down (Table 2). The purified PCR products were assembled by Gibson DNA assembly technology, using a SoSoo Cloning Kit (Tisingke, T-TSV-S1, China) following the manufacturer's instructions and then cloned into *Escherichia coli* DH5α. Clones containing the resulting recombinant pU19ΔsdaB vector were selected on LB agar supplemented with kanamycin. The recombinant plasmid pUC19ΔsdaB was isolated with a plasmid extraction kit (Omega, D6943-02, China) following the manufacturer's instructions and used as template DNA for PCR amplification with the primers *sdaB*-H1up and *sdaB*-H2down (Table 2). After purification, the PCR products were introduced into the competent cells of wild-type AA4, AA4 *sdaB*, or CAΔsdaB by electroporation at 2.5 KV. The resulting transformants were selected on LB agar supplemented with tetracycline, or kanamycin and tetracycline, or a combination of kanamycin, tetracycline, and gentamicin where necessary after incubation at 30°C for 24 h. The correctness of the transformants were then confirmed by PCR, sequencing, and fluorescence microscopy.

**Assay for L-Serine Dehydratase Activity**

The cell extract of *E. ludwigii* AA4 was prepared as above, except washing and resuspending in the extraction buffer (50 mM K₂HPO₄, 2.5 mM serine protease inhibitors) (Velayudhan et al., 2004). Three aliquots of 1 ml of cell extract containing total proteins of 10 mg/ml was incubated with 500 μl of 300 mM L-serine at 37°C for 6 min. Then, the concentration of pyruvate was determined by using a pyruvate assay kit (Solarbio, BC2205, China). The L-serine hydrolase activity was calculated as:

\[
l\text{serine hydrolase activity} \text{ nmol/min-mg of protein} = \frac{c \times v}{M \times T \times m} \times 10^3
\]

where \(c\) is the concentration of pyruvate in μg/ml, \(v\) is the total reaction volume in ml, \(M\) is the relative molecular mass of pyruvate, \(T\) is the reaction time in minutes, and \(m\) is the weight of cell extract in mg.

Two-tailed *t*-test (GrapPad Prism 8) was used for the statistical analysis. This experiment was repeated five times.

**Assays for Colonization of Scots Pine Needles**

In order to investigate the ability of *E. ludwigii* AA4 to colonize Scots pine needles, 50 ml of LB cultures (OD₆₀₀ = 1.0) of GFP-labeled wild-type AA4, AA4ΔsdaB, and CAΔsdaB were sprayed on 21 1-month-old seedlings, respectively. The plants were placed in a greenhouse under the following conditions: 16 h of light (day) and 8 h of dark (night), 25°C, and a relative humidity of 70%. Three aliquots of 50 mg of needles were sampled at the 4th, 48th, 72th, 96th, 120th, and 144th, and 168th hour post inoculation, respectively, for bacterial quantification. The needles were crushed with 1 ml of PBS by using a sterile mortar. The resulting suspensions were diluted and spread on LB agar plates supplemented with tetracycline and incubated at 30°C for 16 h. The colony-forming unit (CFU) numbers were recorded. Two-tailed *t*-test (GraphPad Prism 8) was used for the statistical analysis. This experiment was repeated five times.
FIGURE 1 | Screening and taxonomy of the nematocidal bacterial strains against pine wood nematodes (PWNs). (A) Screening of strong PWN-killing bacterial strains against *Bursaphelenchus xylophilus*. The green dots represent the 43 strong nematocidal strains causing the death of more than 85% of *B. xylophilus* treated with them, while the blue and red dots are bacterial cultures of *Bacillus pumilus* YLT40 and *Paenibacillus polymyxa* M-1 (Continued).

For image analysis, 48 h after inoculation, the needle slices were washed with PBS and transferred to a slide. Ten microliters of PBS were dropped on the samples. The colonization of needles by bacteria was visualized by a Confocal Laser Scanning Microscopy (CLSM; Zeiss, LSM 800, Germany) using an excitation laser of 480 nm.

RESULTS

Screening of Bacterial Strains for Nematocidal Activity Against *Bursaphelenchus xylophilus*

To find microorganisms possessing strong nematocidal activity against *B. xylophilus* for potential biological control of the PWD, we performed a PWN-killing test with 374 bacterial strains from our lab collection. First, we used an efficient PWN-killer *Bacillus pumilus* YLT40 and a moderate PWN-killing strain *Paenibacillus polymyxa* M-1 (Niu et al., 2013), identified previously (data not shown), as controls (Figure 1A) to verify if the PWN-Bacterium interaction system employed in this study was reliable for a large-scale screening. The corrected mortality rates of the nematode *B. xylophilus* treated by *B. pumilus* YLT40 and *P. polymyxa* M-1 were more than 85% and less than 25% (Figure 1A), respectively. We calculated a Z' factor score of 0.682 (Figure 1A) which indicated that the screening procedure utilized is sufficiently reliable for selecting powerful PWN-killing strains.

We detected 43 efficient nematocidal bacterial strains exhibiting *B. xylophilus* mortality rates above 85% (Figure 1A, Supplementary Figure 1, and Supplementary Table 1). Based on 16S rRNA gene sequencing, we found that these bacterial strains belong to 21 species from seven genera, including *Bacillus*, *Pseudomonas*, *Enterobacter*, *Chryseobacterium*, *Stenotrophomonas*, *Pantoea*, and *Paenibacillus* (Figure 1B), all genera with known nematocidal activity. Among the seven genera, *Bacillus* harbored 19 nematocidal strains, while *Pantoea* and *Paenibacillus* possessed only one PWN-killing strain (Figure 1B). Interestingly, seven *Enterobacter* strains, including the plant beneficial bacterium *E. ludwigii* AA4 isolated from maize roots (Niu et al., 2017), exhibited high nematocidal activity against the PWN (Figure 1B). In addition, AA4 did kill almost all of the *B. xylophilus* clones (96%) in our assay (Figure 1C). In our collection, we found 43 strong PWN-killing strains involving...
E. ludwigii AA4. Next, we validated the PWN-killing function of strain AA4 in both in vitro and in planta experiments.

**Biocontrol Effect of E. ludwigii AA4 Against B. xylophilus**

To confirm the nematocidal effect of E. ludwigii AA4 against B. xylophilus, we developed a fluorescent dye-exclusion viability test to visualize PWN-killing activity. We used DAPI which differentially stains live and dead PWNs. Dead worms take up DAPI rapidly and show bright fluorescence, while the live ones are relatively non-fluorescent and look dim (Figure 2A).

In our assay, the majority (68%) of PWNs co-cultured with wild-type E. ludwigii AA4 cells in very low density (OD$_{600} = 0.063$) for 24 h were killed (Figure 2B). The corrected mortality rate of the nematodes treated with wild-type AA4 cells tends to increase with enhanced cell density. The percentage of dead worms dramatically increased from less than 70% to more than 92% when the OD$_{600}$ value of the bacterial culture was raised from 0.063 to 0.5. The corrected mortality rate rose slowly to a maximum of 98.3% when the OD$_{600}$ value of the cell suspension reached 2 (Figure 2B). Our visual test documented that nearly all the nematodes incubated with wild-type AA4 emitted intense fluorescent signals, while almost no fluorescence was detected without bacteria (Figure 2A). These results well corroborated the high mortality rate of B. xylophilus when exposed to wild-type AA4. E. ludwigii AA4 exhibited a remarkable in vitro PWN-killing effect.

We then investigated the disease-suppressing efficacy of E. ludwigii AA4 against PWD on 3-year-old Pinus sylvestris greenhouse-grown seedlings. Thirty days after inoculation, we found that the needles of the seedlings treated with PWNs together with wild-type AA4 were much greener and less wilted than those inoculated with B. xylophilus only (Figure 3A). The disease index of PWD on the P. sylvestris plants treated with PWNs together with wild-type AA4 (19.5) was significantly lower than that treated with B. xylophilus alone (86.9) (Figure 3B), indicating that the PWD severity was substantially reduced by treatment with wild-type AA4. E. ludwigii AA4 was capable of efficiently suppressing PWNs not only under in vitro conditions but also in in planta and was a strong bacterial biocontrol strain against PWD caused by B. xylophilus.

**E. ludwigii AA4 Causes Morphological Defects in B. xylophilus**

To further understand the PWN-killing effect of E. ludwigii AA4, culture supernatants and cell extracts were prepared for the DAPI-staining based nematocidal activity test. Treatment of B. xylophilus with the culture supernatant of wild-type AA4 did not affect the mortality rate of the nematode (Figure 2A). Wild-type AA4 cells did dramatically enhance fluorescence of the B. xylophilus nematodes (Figure 2A). This result was corroborated by the mortality rate of the PWNs, suggesting that the death rate of worms exposed to wild-type AA4 culture supernatant (less than 20%) was significantly less than that of B. xylophilus incubated with cells of wild-type AA4 (more than 60%) (Figure 2B). By contrast, we detected intense fluorescent signals from the worms treated with wild-type AA4 cell extract (Figure 4A) and found that more than 80% of PWNs were dead after being exposed to the extract (Figure 4A), indicating its strong nematocidal effect. Thus, our data indicates that the cell extract of wild-type AA4 rather than the culture supernatant contains the nematocidal activity.

To elucidate the mode of action of the nematocidal effect of E. ludwigii AA4 against PWN, we choose its cell extract for investigating morphological variations of AA4-treated B. xylophilus. The L4 stage nematodes were incubated with wild-type AA4 cell extract. Afterward, the morphological changes of the worms were observed and recorded using an optical light microscope. Exposure of PWN to cell extract-induced formation of vast amounts of vacuoles filled the internal tissues of nematodes completely (Figures 4Bi,iii). Newly formed small vacuoles accumulate and progressively fuse into giant ones, leading to membrane rupture and death (Maltese and Overmeyer, 2014; Armenta and Dixon, 2020). This process is defined as methuosis (Cai et al., 2013; Chen et al., 2014; Nirmala and Lopus, 2020), a type of non-apoptotic cell death widely described in animals (Jang et al., 2016; Rajasekharan et al., 2017; Song et al., 2021). The death of B. xylophilus caused by AA4 might be attributed to methuosis induced by the damage to cell membranes.

In these vacuolated worms, some vital organs, e.g., intestine, pharynx, and genital glands, disappeared in the mass of bubbles. We also failed to capture a clear structure of stylet at the head of PWN treated with wild-type AA4 cell extract (Figures 4Bi,iii). The reproductive organ of male worms, the copulatory spicule, dispersed after being incubated with cell extract (Figure 4Bv). In contrast, we observed fewer vacuoles in the control nematodes exposed only to PBS. All the critical internal organs were intact and clearly visible in the control worm bodies (Figures 4Bii,iv,v). Therefore, E. ludwigii AA4 appeared to destroy crucial internal organs of B. xylophilus through causing a series of morphological defects.

**The sdaB Gene Is Involved in the Nematocidal Effect of E. ludwigii AA4 Against B. xylophilus**

To resolve the molecular mechanisms underlying the strong nematocidal effect of E. ludwigii AA4, we characterized genes involved in its PWN-killing efficacy using transposon and PCR-targeted mutagenesis. First, by screening a miniTn10 transposon mutant library of strain AA4, we identified a mutant with greatly reduced nematocidal activity against B. xylophilus. We further analyzed the genomic site where the transposon was located and found a miniTn10 element that was inserted into an open reading frame (ORF) located between kilobase positions 3,956,912 and 3,955,545 (Supplementary Figure 4). This ORF was identified as the sdaB gene encoding the l-serine dehydratase beta subunit, indicating that the sdaB gene might be involved in the PWN-killing effect of AA4.
We next created a knock-out mutant of the sdaB gene by employing the Red ($\lambda$, $\beta$, exo)-mediated DNA homologous recombination system. The L-serine hydrolase activity of the sdaB gene knock-out deletion mutant AA4$\Delta$sdaB was significantly lower than that of the wild-type AA4 or the AA4$\Delta$sdaB mutant strain complemented with the sdaB wild-type gene (Figure 5B), which indicated that the interruption of the sdaB gene affected the biosynthesis of L-serine dehydratase. Then, we compared the PWN-killing activities of the wild-type AA4 and AA4$\Delta$sdaB by using the fluorescent staining-based nematocidal activity assay (Figure 2A). Nearly no fluorescence could be detected in the worms treated with AA4$\Delta$sdaB cells or their extracts, which confirmed the loss of nematocidal activity of the miniTn10 insertionional mutant. Complementation of the mutant with a recombinant plasmid harboring sdaB partially restored the PWN-killing activity (Figure 2A). These results were corroborated by the nematode mortality curves, which documented that the corrected death rate of the PWNs treated by AA4$\Delta$sdaB was significantly lower than those incubated with wild-type AA4, while the mortality of nematodes exposed to the mutant strain complemented with the sdaB wild-type gene increased remarkably (Figure 2B). Therefore, sdaB gene product has a major impact on the nematocidal activity of E. ludwigii AA4.

Then, we investigated the role of the sdaB gene product on the protective effect of E. ludwigii AA4 on P. sylvestris against PWD. Using a Pine-Nematode-Bacterium tripartite interaction system with 1-month-old P. sylvestris seedlings, a lower disease index was estimated when wild-type AA4 cells or their extracts were added to the system. Under these conditions, much less wilted and fewer chlorotic pine seedlings were observed compared
FIGURE 4 | Nematocidal effect of cell extracts of *E. ludwigii* AA4 and its sdaB gene knock-out mutant against *B. xylophilus*. (A) Mortality of the *B. xylophilus* nematode treated with bacterial cell extracts. The worms were incubated with the cell extracts of wild-type AA4 and AA4ΔsdaB for 24 h or 48 h, respectively. WT24, PWNs treated with a cell extract of wild-type AA4 for 24 h; WT48, PWNs treated with a cell extract of wild-type AA4 for 48 h; ΔsdaB24, PWNs treated with a cell extract of AA4ΔsdaB for 24 h; ΔsdaB48, PWNs treated with a cell extract of AA4ΔsdaB for 48 h; CΔsdaB24, PWNs treated with a cell extract of AA4ΔsdaB complemented with a wild-type sdaB gene for 24 h; CΔsdaB48, PWNs treated with a cell extract of AA4ΔsdaB complemented with a wild-type sdaB gene for 48 h. (B) Morphological changes of *B. xylophilus* incubated with the cell extract of wild-type *E. ludwigii* AA4. The L4 stage worms exposed to a wild-type AA4 cell extract containing total proteins of 5 mg/mL for 24 h, are shown in the left column, where the vacuoles in front (i) and back (iii) sections of worm bodies as well as dispersed copulatory spicule (v) are indicated by arrows. Non-treated L4 stage nematodes (ii, iv, vi) are presented in the right row, where the arrows indicate the intestine, pharynx, and stylet (ii), genital gland (iv), copulatory spicule and vacuoles (vi), respectively (scale bars: 1 mm).

FIGURE 5 | L-serine hydrolase activity of *E. ludwigii* AA4 and its sdaB gene knock-out mutant. (A) Colorimetrical quantification of pyruvate generated in a L-serine hydrolase activity assay. The color shown in each well of the microplate corresponds to the pyruvate concentration measured by a spectrophotometer at 520 nm. The values of optical density (OD) range from 0 (white) to 0.8 (orange). (B) Comparison of L-serine hydrolase activities of wild-type *E. ludwigii* AA4, its sdaB gene knock-out mutant, and AA4ΔsdaB complemented with a wild-type sdaB gene. DW, distilled water; WT, *E. ludwigii* AA4 wild-type strain; ΔsdaB, AA4 sdaB gene knock-out mutant; CΔsdaB, AA4ΔsdaB complemented with a wild-type sdaB gene. The asterisks indicate that differences among the means represented by the columns are statistically significant (*P* < 0.0001). Two-tailed *t*-test (GrapPad Prism 8) was used for the analysis.
to the control containing B. xylophilus alone (Figure 6B). We found more wilted and chlorotic seedlings when AA4ΔsdaB cells or their extracts were added instead of wild-type AA4 cells or their extracts. The number of diseased plants was reduced when the seedlings were treated with PWNs together with cells or cell extracts of the AA4ΔsdaB mutant strain complemented with the sdaB wild-type gene (Figure 6A). These findings were supported by disease severity analysis, where the disease index of plants treated with PWNs and AA4ΔsdaB mutant cells or cell extracts was significantly higher than that with PWNs treated with the cells or cell extracts of wild-type AA4 or the sdaB mutant complemented with the sdaB wild-type gene (Figure 6B). The indices of the PWN infected seedlings treated with cells or cell extracts of the AA4ΔsdaB mutant complemented with the wild-type sdaB gene and seedlings treated with PWNs plus the wild-type AA4 strain cells or cell extracts showed no significant differences. The culture supernatants of the wild-type AA4, the AA4ΔsdaB mutant strain, or AA4ΔsdaB mutant complemented with the sdaB wild-type gene not only exhibited nearly no nematocidal activity against B. xylophilus in vitro, but also failed to display biocontrol efficacy against PWD (Figure 6). The sdaB gene encoding the beta subunit of L-serine dehydratase, is highly related to the PWN-killing activity of E. ludwigii AA4 and may be a key molecular element controlling the interplay between strain AA4 and PWNs.

Colonization of E. ludwigii AA4 on Needles of P. sylvestris

Efficient colonization of plant surfaces and tissues is fundamental for successful inhibition of pathogens by biological control agents. To investigate the ability of E. ludwigii AA4 to colonize P. sylvestris, we sprayed the needles with a GFP-labeled transgenic strain of AA4. We observed by CLSM that strain AA4 was capable of adhering to the surface of P. sylvestris needles by forming robust biofilms (Figure 7A). Although there was a dramatical reduction of the abundance of AA4 cells sticking to the needles, from around 1.9 × 10^7 CFU (colony-forming unit)/g (of pine needle fresh weight) 4 h after inoculation to 3.8 × 10^4 CFU/g 144 h after inoculation, the colonization rates of AA4 at days 6 and 7 (2.8 × 10^4 CFU/g) showed no significant difference (Figure 7B). This indicates that E. ludwigii AA4 can colonize the needles of P. sylvestris, presumably a precondition for its disease-controlling efficacy against PWD. AA4ΔsdaB cells were equally able to colonize pine needles compared to the wild-type AA4 (Figure 7A), demonstrating that the sdaB gene function is not needed for colonization. The biomass accumulation rate of both strains was at the same level along our 7-day-long experiment (Figure 7B). Therefore, E. ludwigii AA4 may be an efficient colonizer of pine needles, and that the eradication of sdaB gene did not affect its adherence to needles.

DISCUSSION

Compared to the control of phytopathogens in agroecosystems, forest disease suppression puts more emphasis on the sustainable management of pests due to the vital functionality of forests in maintaining the balance and health of their ecosystems (Nascimento et al., 2015; Proença et al., 2017; Khan et al., 2020; Ozair et al., 2020). To this end, biological control of forest pathogens, including the PWNs, by beneficial microbes have been receiving increasing attention (Nunes da Silva et al., 2015; Ponpandian et al., 2019; Proença et al., 2019). A considerable number of nematocidal microbial strains suppressing B. xylophilus have been identified and characterized. These strains are, mainly, species of Bacillus (Crickmore, 2005; Niu et al., 2011; Park et al., 2020), Streptomyces (Kang et al., 2021), Serratia (Paiva et al., 2013; Nascimento et al., 2016; Abd El-Aal et al., 2021), and Stenotrophomonas (Huang et al., 2009; Ponpandian et al., 2019).

We selected a PWN-killing Enterobacter ludwigii strain, designated as AA4 from a large-scale screening of nematocidal activity and inhibition of PWD (Figures 1C, 3). Representatives of the genus Enterobacter are able to inhibit a wide range of phytopathogens (Demirci et al., 2000; Kageyama and Nelson, 2003; Windstam and Nelson, 2008; Del Barrio-Duque et al., 2019; Batyrova et al., 2020), including plant pathogenic nematodes, such as root-knot nematodes (Munif et al., 2000; El-Sayed et al., 2014; Oh et al., 2018). To our knowledge, E. ludwigii AA4 is the first Enterobacter strain described as a nematocidal agent suppressing B. xylophilus. Our findings expand the knowledge of the biocontrol potential of the genus Enterobacter, one of the most expanding bacterial taxa in recent decades (Taghavi et al., 2010; Ibort et al., 2018; François et al., 2021).

Accurate and rapid determination of the mortality of nematodes is the key to efficient screening and selection of microbial strains with nematocidal activity. In earlier studies on PPN-bacterium interactions, the killing rate of nematodes was estimated mainly by the morphology of the worms, where rigid nematodes were considered dead, while the curved ones were considered alive (Liu et al., 2016, 2019; Ponpandian et al., 2019; Qiu et al., 2019). Such criteria do not always work well. We found that a significant number of apparently rigid nematodes could still move after being touched with a needle, suggesting that they were still alive. In addition, counting dead nematodes under a microscope is time consuming and tedious.

We developed a visual PWN-killing activity DAPI dye exclusion which is faster and more reliable (Figure 2). DAPI passes slowly through intact cell membranes of living nematodes, and thus preferentially stains the dead nematodes. Once entering the cells and binding to the minor groove of A-T-rich regions of DNA, DAPI fluorescence is greatly enhanced (Atale et al., 2014). As a result, dead worms display intense fluorescent signals. A similar method was employed in a previous study, where the dead Caenorhabditis elegans nematodes were stained with the fluorophore Sytox Orange (Natalia et al., 2013; Rajamuthiah et al., 2015; Xie et al., 2020). Applying fluorescence staining in the identification of PWN-killing bacterial strains may be amenable to high throughput or automated screening for biocontrol agents against other plant pathogenic nematodes.

In present study, we observed that the PWNs treated with cell extracts of wild-type E. ludwigii AA4 displayed dramatic morphological defects associated with the accumulation of numerous vacuoles, recognized as a sign of reversible cell
FIGURE 6 | Disease-suppressing effect of *E. ludwigii* AA4 against PWD caused by *B. xylophilus*. (A) Biocontrol effect of *E. ludwigii* AA4 and its *sdaB* gene knock-out mutant against PWD on 1-month-old *P. sylvestris* seedlings. (B) Severity of PWD on 1-month-old *P. sylvestris* seedlings treated with PWN jointly with wild-type AA4 or AA4ΔsdaB. The horizontal bars within boxes represent the median. The tops and bottoms of boxes represent the 75th and 25th quartiles, respectively. The upper and lower whiskers extend from 75th quartiles to the maxima and from the 25th quartiles to the minima, respectively. The asterisks indicate that differences among the means represented by the boxes are statistically significant (*p* ≤ 0.0345). Two-tailed *t*-test (GraphPad Prism 8) was used for the analysis. PWN, treatment with PWN alone; PWN + ΔsdaB, joint treatment with PWN and AA4ΔsdaB; PWN + CΔsdaB, joint treatment with PWN and AA4CΔsdaB; PWN + WT, joint treatment with PWN and wild-type AA4; PWN + CΔsdaBCE, joint treatment with PWN and AA4ΔsdaB cell extract; PWN + CΔsdaBCE, joint treatment with PWN and AA4CΔsdaB cell extract; PWN + WTCE, joint treatment with PWN and wild-type AA4 cell extract; PWN + ΔsdaBS, joint treatment with PWN and AA4ΔsdaB culture supernatant; PWN + CΔsdaBS, joint treatment with PWN and AA4CΔsdaB culture supernatant; PWN + WTS, joint treatment with PWN and wild-type AA4 culture supernatant; DW, treatment with distilled water (scale bars: 2 cm).
injury (RCI) and methuosis, resulting in the destruction of internal organs of B. xylophilus needed for absorbing nutrients, pathogenicity, and reproduction (Figure 4). Similar changes in morphology were also reported in B. xylophilus incubated with indoles and abamectin (Rajasekharan et al., 2017). Another plant pathogenic nematode, Meloidogyne incognita, the causal agent of root knot in several crops, showed bubbling in its body when meeting toxic compounds as well (Jang et al., 2016). A large number of vacuoles were present in the transgenic C. elegans with an ectopically expressed and activated mek-1 gene (Koga et al., 2000). Intracytoplasmic vacuolation may commonly occur when nematodes live under stressful conditions, which can be triggered by fluid accumulation within the cytoplasm due to Ca$^{2+}$ and water influx caused by RCI (Jurkowitz-Alexander et al., 1992; Basavappa et al., 1998; Rajasekharan et al., 2017). The death of B. xylophilus caused by AA4 might be attributed to methuosis induced by damage to the cell membrane caused by this nematocidal bacterial strain. The mechanisms underlying the PWN-killing function of E. ludwigii AA4 remains to be determined.

Some mechanisms of nematocidal activity against B. xylophilus have been recently documented, such as parasitization (Proenca et al., 2017; Ponpandian et al., 2019), production of toxins (Abd El-Aal et al., 2021; Kahn et al., 2021), antibiotics and destructive enzymes (Yang et al., 2007; Huang et al., 2009) or competing for nutrients (Proenca et al., 2019), and inducing systemic resistance of plants (Liang et al., 2019; Han et al., 2021). The molecular mechanisms underlying these PWN-killing effects are still largely unknown. In this work, we demonstrate that the sdaB gene encoding the beta subunit of L-serine dehydratase is involved in the nematocidal activity of E. ludwigii AA4 against PWNs. The deletion of this gene caused significant impairment in both L-serine hydrolase activity (Figure 5) and ability to inhibit PWN (Figures 2, 6), but not the growth of E. ludwigii AA4 in vitro or in planta (Supplementary Figure 2). L-serine dehydratase activity is widely found in diverse organisms. It specifically deaminates L-serine to produce pyruvate and ammonia (Xu et al., 2011; Grant, 2012; Thoden et al., 2014), and plays a critical role in maintaining amino acid homeostasis during shifts in nutrient availability (Velayudhan et al., 2004; Zhang et al., 2010;
Chen et al., 2012). Until now, little or no data about the contribution of L-serine dehydratase to microbial nematocidal activities has been reported. L-serine is an important biomolecule, and excessive amounts of intracellular serine inhibit the biosynthesis of other amino acids (Zhang et al., 2018; Soeriyadi et al., 2021).

Here, we assume that the sdaB gene product might control B. xylophilus by regulation of potential anti-nematode compounds in E. ludwigii AA4. Considering the tight relationship between L-serine and methylation reactions (Kalhan and Hanson, 2012; Datta et al., 2016; Bignell et al., 2018; Kriner and Subramaniam, 2020), such modulation may be linked to bacterial epigenetics. We noticed that the supernatant of strain AA4 cell culture has almost no effect (Figures 2, 6), which may be due to the low concentration of nematocidal compounds in the broth or suggests that the PWN-killing efficacy of AA4 might be contact-dependent. In future work, we will more deeply analyze the function of the sdaB gene in the nematocidal activity of E. ludwigii AA4.

Colonization of plant hosts is a necessary precondition for efficient pathogen inhibition by microbial biocontrol agents (Vurukonda et al., 2018; Niu et al., 2020; Zhang et al., 2020; Chen et al., 2012). Until now, little or no data about the great adaptability of the genus Enterobacter, which appears to be highly adaptable to diverse environments (Shoebitz et al., 2009; Singh et al., 2017; Shastrty et al., 2020; Ranawat et al., 2021). These features may facilitate the development of an AA4-based biopesticide against B. xylophilus. Identification of genes and proteins involved in resistance to pests and pathogens is of long-term interest because such molecules represent the next generation of targets for creation of nematode resistant plants through genetic engineering or targeted mutagenesis. Our findings also pave the way for introducing agriculture biocontrol agents in forest disease management.

DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in the Genbank repository, accession numbers CP018785, OM883853, OM899759-OM899796, OM899810, OM900025, and OM900026.

AUTHOR CONTRIBUTIONS

BN and RB designed the project. YZ and BN wrote the manuscript. BN, RB, ZY, RS, and HS revised the manuscript. YZ and ZY performed the experiments. YZ, SW, DW, and BN analyzed the data. YC created the random transposon library. HW, HY, JP, and MP performed the in planta biocontrol assays. All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.870519/full#supplementary-material

Supplementary Figure 1 | The corrected mortality of 43 efficient pine wood nematode (PWN)-killing bacterial strains. Bacillus pumilus YLT40 and Paenibacillus polymyxa M1 were used as positive and negative controls, respectively. Asterisk indicates that differences among the means represented by the columns are statistically significant (*p < 0.0001). Two-tailed t-test (GrapPad Prism 8) was used for the analysis.

Supplementary Figure 2 | Growth curves of wild-type E. ludwigii AA4, its sdaB gene knock-out mutant, and AA4ΔsdaB complemented with a wild-type sdaB gene. WT, E. ludwigii AA4 wild-type strain; ΔsdaB, AA4 sdaB gene knock-out mutant; CΔsdaB, AA4 ΔsdaB complemented with a wild-type sdaB gene; LB, sterile Luria-Bertani liquid medium.

Supplementary Figure 3 | Disease ranks of the pine wilt disease (PVD) caused by B. xylophilus under greenhouse conditions on day 9 after inoculation (scale bars: 20 μm). In rank 0, stems and needles are green and plump. In rank 1, yellowish speckles are presented on stems, and stems and needles appear slightly shriveled. In rank 2, stems turn yellow and shriveled, and less than 25% needles are partially wilted. In rank 3, stems turn yellow and shriveled, among which 25–50% of needles are partially wilted. In rank 4, stems turn brownish and completely shriveled, and more than 50% of needles are completely wilted and turn yellowish. W, the whole views of P. sylvestris seedlings; T, the top views of P. sylvestris seedlings; S, the stems of P. sylvestris seedlings; N, the needles of P. sylvestris seedlings.

Supplementary Figure 4 | The location of miniTn10 transposon on the genome sequence of E. ludwigii AA4. The site of miniTn10 transposon insertion is indicated by a black triangle.

Supplementary Table 1 | Corrected mortality rates for the 43 efficient pine wood nematode (PWN)-killing strains.
Guo, Q., Du, G., He, H., Xu, H., Guo, D., and Li, R. (2016). Two nematicidal furcocoumarins from Ficus carica L. leaves and their physiological effects on pine wood nematode (Bursaphelenchus xylophilus). Nat. Prod. Res. 30, 1969–1973. doi: 10.1080/14786419.2015.1094804

Habibi, S., Djjeddi, S., Olskama-Otso, N., Sarhadi, W. A., Kojima, K., Ralloos, R. V., et al. (2019). Isolation and screening of indigenous plant growth-promoting rhizobacteria from different rice cultivars in Afghanistan soils. Microbes Environ. 34, 347–355. doi: 10.1264/jme.ME18166

Haegeman, A., Vanholme, B., Jacob, J., Vandezande, H., Claes, M., Borgonie, G., et al. (2009). An endosymbiotic bacterium in a plant-parasitic nematode: member of a new Wolbachia supergroup. Int. J. Parasitol. 39, 1045–1054. doi: 10.1016/j.ijpara.2009.01.006

Han, G., Mannaa, M., Kim, N., Jeon, H. W., Jung, H., Lee, H. H., et al. (2021). Response of pine rhizosphere microbiota to foliar treatment with resistance-inducing bacteria against pine wilt disease. Microorganisms 9:688. doi: 10.3390/microorganisms9040888

Hinton, D. M., and Bacon, C. W. (1995). Enterobacter cloacae is an endophytic symbiont of corn. Mycopathologia 129, 117–125. doi:10.1007/BF01034371

Huang, X., Liu, J., Ding, J., He, Q., Xiong, R., and Zhang, K. (2009). The investigation of nematocidal activity in Stenotrophomonas maltophilia G2 and characterization of a novel virulence serine protease. Can. J. Microbiol. 55, 934–942. doi: 10.1139/w09-045

Ibort, P., Molina, S., Ruiz-Lozano, J. M., and Aroca, R. (2018). Molecular insights of the plant-parasitic nematode Meloidogyne incognita on the surface of pine tree logs: a promising biocontrol strategy for the Japanese pine sawyer beetle (Monochamus alternatus). ACS Omega 5, 25312–25318. doi:10.1021/acsomega.0c03585

Kang, M. K., Kim, M. H., Liu, M. J., Jin, C. Z., Park, S. H., Lee, J., et al. (2021). Modelling of nematicidal constituents of Chenopodium quinoa willd. Microorganisms 9:688. doi:10.3390/microorganisms9040888

Kämpfer, P., Ruppel, S., and Remus, R. (2005).

Khan, M. A., Ahmed, L., Mandal, P. K., Smith, R., and Haque, M. (2020). Modelling the dynamics of pine wilt disease with asymptomatic carriers and optional control. Sci. Rep. 10:11412. doi:10.1038/s41598-020-67090-7

Kikuchi, T., Cotton, J. A., Dalzell, J. I., Hasagawa, K., Kanzaki, N., McVeigh, P., et al. (2011). Genomic insights into the origin of parasitism in the emerging plant pathogen Bursaphelenchus xylophilus. PLoS Pathog. 7:e1002219. doi: 10.1371/journal.ppat.1002219

Kim, H. M., Jeong, S. G., Choi, I. S., Yang, J. E., Lee, K. H., Kim, J., et al. (2020). Mechanisms of insecticidal action of Metarhizium anisopliae on adult Japanese pine sawyer beetles (Monochamus alternatus). J. Invertebr. Pathol. 165, 7033–7039. doi: 10.1016/j.jipath.2020.04.033
benzimidazole derivatives. J. Biomat. Struct. Dyn. 38, 1670–1682. doi: 10.1080/07391102.2019.1617783
Maltese, W. A., and Overmeyer, J. H. (2014). Methuosis: nonapoptotic cell death associated with vacuolization of macrophosome and endosome compartments. Am. J. Pathol. 184, 1630–1642. doi: 10.1016/j.ajpath.2014.02.028
Mokracka, J., Koczura, R., and Kaznowski, A. (2004). Yersiniabactin and other siderophores produced by clinical isolates of Enterobacter sp. and Citrobacter sp. FEMS Immunol. Med. Microbiol. 40, 51–55. doi: 10.1016/S0928-824X(03)00276
Monciardini, P., Sosio, M., Cavaletti, L., Chiocchini, C., and Donadio, S. (2002). New PCR primers for the selective amplification of 16S rDNA from different groups of actinomyces. FEMS Microbiol. Ecol. 42, 419–429. doi: 10.1111/j.1574-6941.2002.tb01331.x
Mpongwa, N., Ntwame, S. K., Mekuto, L., Akinpelu, E. A., Dyantyi, S., Monciardini, P., Sosio, M., Cavaletti, L., Chiocchini, C., and Donadio, S. (2002). Yersiniabactin and other siderophores produced by clinical isolates of Enterobacter sp. and Citrobacter sp. FEMS Immunol. Med. Microbiol. 40, 51–55. doi: 10.1016/S0928-824X(03)00276
Niu, X., Song, L., Xiao, Y., and Ge, W. (2018). Drought-tolerant plant species and their potential in alleviating drought stress. Front. Plant Sci. 11:1023. doi: 10.3389/fpls.2020.01023
Nascimento, F. X., Espada, M., Barbosa, P. M., Vicente, C. S., and Mokracka, J., Koczura, R., and Kaznowski, A. (2004). Yersiniabactin and other siderophores produced by clinical isolates of Enterobacter sp. and Citrobacter sp. FEMS Immunol. Med. Microbiol. 40, 51–55. doi: 10.1016/S0928-824X(03)00276
Park, J. M., Radiakrishnan, R., Kang, S. M., and Lee, J. I. (2015). IAA producing Enterobacter sp. I-3 as a potent bio-herbicide candidate for weed control: a special reference with lettuce growth inhibition. Indian J. Microbiol. 55, 207–212. doi: 10.1007/s12088-015-0515-y
Peng, G., Zhang, W., Luo, H., Xie, H., Lai, W., and Tan, Z. (2009). Enterobacter oryzae sp. nov., a nitrogen-fixing bacterium isolated from the wild rice species Oryza sativa. Int. J. Evol. Microbiol. 59, 1650–1655. doi: 10.1099/ijs.0.009567-0
Ponpandan, L. N., Rim, S. O., Shanmugam, G., Jeon, J., Park, Y. H., Lee, S. K., et al. (2019). Phylogenetic characterization of bacterial endophytes from four Pinus species and their nematicidal activity against the pine wood nematode. Sci. Rep. 9:12457. doi: 10.1038/s41598-019-48745-6
Proenca, D. N., Grass, G., and Morais, P. V. (2017). Understanding pine wilt disease: roles of the pine endophytic bacteria and of the bacteria carried by the disease-causing pine wood nematode. Microbiol. Open 6:e00415. doi: 10.1002/mbo3.415
Proenca, D. N., Heine, T., Senges, C. H. R., Bandow, J. E., Morais, P. V., and Tischler, D. (2019). Bacterial metabolites produced under iron limitation kill pine wood nematode and attract Caenorhabditis elegans. Front. Microbiol. 10:2166. doi: 10.3389/fmicb.2019.02166
Qiu, X. W., Wu, X. Q., Huang, L., and Ye, J. R. (2016). Influence of Bxpsel gene silencing by dsRNA interference on the development and pathogenicity of the pine wood nematode, Bursaphelenchus xylophilus. Int. J. Mol. Sci. 17:125. doi: 10.3390/ijms17010125
Qiu, X., Yang, L., Ye, J., Wang, W., Zhao, T., Hu, H., et al. (2019). Silencing of cyp-33C9 gene affects the reproduction and pathogenicity of the pine wood nematode, Bursaphelenchus xylophilus. Int. J. Mol. Sci. 20:4520. doi: 10.3390/ijms20184520
Rajamuthiah, R., Jayamani, E., Majed, H., Conery, A. L., Kim, W., Kwon, B., et al. (2015). Antibacterial properties of 3-(phenylsulfonyl)-2-pyrazinecarbonitrile. Biorg. Med. Chem. Lett. 25, 5203–5207. doi: 10.1016/j.bmcl.2015.09.066
Rajasekharan, S. K., Lee, J. H., Ravichandran, V., and Lee, J. (2017). Assessments of iodoindoles and abamectin as inducers of methuosis in pinewood nematode, Bursaphelenchus xylophilus. Int. J. Mol. Sci. 17:125. doi: 10.3390/ijms1710125
Rosa, M., Zulkiifi, N. N., Sohri, Z. M., Zuan, A., Cheak, S. C., and Abdul Rahman, N. A. (2020). Seed biopriming with P- and K-solubilizing Enterobacter hormaechei sp. improves the early vegetative growth and the P and K uptake of okra (Abelmoschus esculentus) seedling. PLoS One 15:e0232860. doi: 10.1371/journal.pone.0232860
Rövers, V., Marchesini, M. I., and Comerci, D. I. (2020). Brucella abortus controls pine wilt disease by elicitation of moderate hypersensitive reaction. Front. Plant Sci. 11:1023. doi: 10.3389/fpls.2020.01023
Rojas, M., Zulvi, N. N., Sohri, Z. M., Zuan, A., Cheak, S. C., and Abdul Rahman, N. A. (2020). Seed biopriming with P- and K-solubilizing Enterobacter hormaechei sp. improves the early vegetative growth and the P and K uptake of okra (Abelmoschus esculentus) seedling. PLoS One 15:e0232860. doi: 10.1371/journal.pone.0232860
Sarkar, A., Ghosh, P. K., Pramanik, K., Mitra, S., Soren, T., Pandey, S., et al. (2018). A halotolerant Enterobacter sp. displaying ACC deaminase activity promotes rice seedling growth under salt stress. Res. Microbiol. 169, 20–32. doi: 10.1016/j.resmic.2017.08.005
Sarkar, S., and Chaudhuri, S. (2015). New report of additional Enterobacter species causing wilt in West Bengal, India. Can. J. Microbiol. 61, 477–486. doi: 10.1139/cjm-2015-0017
Sayed, R. Z., Patel, P. R., and Shaikh, S. S. (2015). Plant growth promotion and root colonization by EPS producing Enterobacter sp.
RZ55 under heavy metal contaminated soil. Indian J. Exp. Biol. 53, 116–123.
Shastry, R. P., Welch, M., Rai, V. R., Ghate, S. D., Sandeep, K., and Rekha, P. D. (2020). The whole-genome sequence analysis of Enterobacter cloacae strain Ghats1: insights into epidemiologic lifestyle-associated genomic adaptations. Arch. Microbiol. 202, 1571–1579. doi: 10.1007/s00203-020-02184-5
Shoebitz, M., Ribaudo, C. M., Pardo, M. A., Cantore, M. L., Ciampi, L., and Curá, J. A. (2009). Plant growth promoting properties of a strain of Enterobacter ludwigii isolated from Lolium perenne rhizosphere. Soil Biol. Biochem. 41, 1768–1774. doi: 10.1016/j.soilbio.2007.12.031
Singh, R. P., Runthala, A., Khan, S., and Jha, P. N. (2017). Quantitative proteomics analysis reveals the tolerance of wheat to salt stress in response to Enterobacter cloacae SBP-8. PLoS One 12:e0183513. doi: 10.1371/journal.pone.0183513
Sooijadi, A. H., Ongley, S. E., Kehr, J. C., Pickford, R., Dittmann, E., and Neilan, B. A. (2021). Tailoring enzyme stringency marks the multispecificity of a L-lysine oxidase (Indolactam Alkaloid) nonribosomal peptide synthetase. ChemBiochem 23:e202100574. doi: 10.1002/chem.202100574
Soliman, T., Mouriès, M. C., Werf, W., Hengeveld, G. M., Robinet, C., and Lansink, A. G. (2012). Framework for modelling economic impacts of invasive species, applied to pine wood nematode in Europe. PLoS One 7:e45505. doi: 10.1371/journal.pone.0045505
Song, S., Zhang, Y., Ding, T., Ji, N., and Zhao, H. (2021). The dual role of macrocinocytosis in cancers: promoting growth and inducing meuthosis to participate in anticancer therapies as targets. Front. Oncol. 10:570108. doi: 10.3389/fonc.2020.570108
Srisuk, N., Sakpuntoon, V., and Nutaratat, P. (2018). Production of indole-3-acetic acid by Enterobacter sp. DMKU-RP206 using sweet whey as a low-cost feed stock. J. Microbiol. Biotechnol. 28, 1511–1516. doi: 10.4161/mib.180403
Taghavi, S., van der Lele, D., Hoffman, A., Zhang, Y. B., Walla, M. D., Vangronsveld, J., et al. (2010). Genome sequence of the plant growth promoting enteric bacterium Escherichia coli SBP-8. J. Bacteriol. 192, 5116–5123. doi: 10.1128/JB.01253w
Tian, B., Yang, J., and Qin, K. (2007). Bacteria used in the biological control of plant-parasitic nematodes: populations, mechanisms of action, and future prospects. FEMS Microbiol. Ecol. 61, 197–213. doi: 10.1111/j.1574-6941.2007.00349.x
Topalovic, O., Hussain, M., and Heuer, H. (2020). Plants and associated soil microbiota cooperatively suppress plant-parasitic nematodes. Front. Microbiol. 11:313. doi: 10.3389/fmicb.2020.00313
Um, J., Kim, D. G., Jung, M. Y., Saralale, G. D., and Oh, M. K. (2017). Metabolic engineering of Enterobacter aerogenes for 2,3-butanediol production from sugarcane bagasse hydrolysate. Bioresour. Technol. 245, 1567–1574. doi: 10.1016/j.biortech.2017.05.166
Vandana, U. K., Rajkumari, J., Singha, L. P., Satish, L., Avadi, H., Sudheer, V., et al. (2021). The endophytic microbiome as a hotspot of synergistic interactions, with prospects of plant growth promotion. Biology 10:101. doi: 10.3390/biolog10020101
Velayudhan, J., Jones, M. A., Barrow, P. A., and Kelly, D. J. (2004). L-serine catabolism via an oxygen-labile L-serine dehydratase is essential for colonization of the avian gut by Campylobacter jejuni. Infect. Immunity 72, 260–268. doi: 10.1128/IAI.72.1.260-268.2004
Vurukonda, S., Giovanardi, D., and Stefani, E. (2018). Plant growth promoting and plant-parasitic nematode endophytic bacterium in vitro. Microbiol. Res. 2113–6. doi: 10.1016/j.micres.2018.02.001
Wang, C., Wu, J., Shi, B., Shi, J., and Zhao, Z. (2020). Improving L-serine formation by Escherichia coli by reduced uptake of produced L-serine. Microb. Cell Fact. 19:66. doi: 10.1186/s12934-020-01323-2
Wen, T. Y., Wu, X. Q., Hu, L. J., Qiu, Y. J., Rui, L. Y., Zhang, X., et al. (2021). A novel pine wood nematode effector, BagCD1, suppresses plant immunity and interacts with an ethylene-forming enzyme in pine. Mol. Plant Pathol. 22, 1399–1412. doi: 10.1111/mpp.13121
Windham, S., and Nelson, E. B. (2008). Differential interference with Pythium ultimum sporangial activation and germination by Enterobacter cloacae in the corn and cucumber spermospheres. Appl. Environ. Microbiol. 74, 4285–4291. doi: 10.1128/AEM.00263-08
Xu, K. L., Chen, S., and Grant, G. A. (2011). Kinetic, mutagenic, and structural homology analysis of L-serine dehydratase from Legionella pneumophila. Arch. Biochem. Biophys. 515, 28–36. doi: 10.1016/j.abb.2011.08.005
Xue, Q., Xiang, Y., Wu, X. Q., and Li, M. J. (2019). Bacterial communities and virulence associated with pine wood nematode Bursaphelenchus xylophilus from different Pinus spp. Int. J. Mol. Sci. 20:3342. doi: 10.3390/ijms20203342
Yadav, M., Shukla, A. K., Srivasta, N., Upadhyay, S. N., and Dubey, S. K. (2016). Utilization of microbial community potential for removal of chlorpyrifos: a review. Crit. Rev. Biotechnol. 36, 727–742. doi: 10.3109/07388551.2015.1019598
Zhang, J., Ju, B., Li, J., Shi, Z., and Zhao, Z. (2015). Extracellular enzymes and the pathogenesis of nematophagous fungi. Appl. Microbiol. Biotechnol. 75, 21–31. doi: 10.1007/s00253-015-7188-x
Zhao, L., Mota, M., Vieira, P., Butcher, R. A., and Sun, J. (2014). Interspecific communication between pinewood nematode and its insect vector, and associated microbes. Cell Press 30, 299–308. doi: 10.1016/j.ctlp.2014.04.007
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