

Identification and Characterization of a *Pseudomonas mosselii* strain and its Antibacterial Function against *Agrobacterium tumefaciens*

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Research

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ABSTRACT

Agrobacterium tumefaciens is a gram negative bacterial that can infect a range of plants and result in root crown gall. A total number of 10328 bacterial strains were isolated from rhizosphere of cherry tree. One strain of LWB10 showed clear inhibition zone around the bacterial colony in YEB media inoculated with *A. tumefaciens* C58. Morphological, physiological, and biochemical characterization indicated that LWB10 belongs to member of the genus *Pseudomonas*. Results from the high-throughput matrix-assisted laser desorption/ionization biotypersmart system indicated that this strain had a score value of 2.247 relative to *Pseudomonas mosselii*. Also, phylogenetic analysis based on 16S rRNA gene sequence showed that strain LWB10 shared the highest similarity with *Pseudomonas mosselii* CIP 105259T. The antagonist strains also exhibit well in growth inhibition of other five *A. tumefaciens* strains. Coinoculation of LWB10 and plant pathogenic strain of *A. tumefaciens* CFCC1369 showed strongly inhibition of tumor formation in tomato stems. All the results demonstrated that the isolated strain is *P. mosselii* LWB10 and its antibacterial ability to *A. tumefaciens* may offer new way for management of crown gall disease in the future.

Keywords: *Agrobacterium tumefaciens*, *Pseudomonas mosselii*, antagonist, biological control

1. INTRODUCTION

Agrobacterium tumefaciens is a kind of soil-born pathogen that causes crown gall in roots [1, 2]. Crown gall had found to occur on nearly 40 economically important plants, such as *Amygdalus persica* L., *Cerasus* spp., *Malus domestica*, *Pyrus* spp. and *Vitis vinifera* L. [3, 4]. *Cerasus* sp. is widely cultivated in China for its beautiful blooming flowers. But recently, crown gall disease spread fast in cherry yard and people found that it is hard to control this disease.

As one of the destructive soil-born pathogen, *A. tumefaciens* will produce tumors in root and rhizome upon infection and thus affect water and nutrient absorption from roots. The disease developed slowly and disease symptoms will not be recognized until plants get weak or die [5]. Chemical control is the most common strategy to control the disease. But no symptoms appear in the early stage of infection and it is too late for the chemical control when we notice the disease. It will cause significant economically losses, when the pathogen once established *in the field*.

Therefore, researchers have been trying to find ways on dealing with the pathogen before they infect the roots. Among all the disease control methods, biological control is a kind of strategy that can protect plants away from pathogenic microorganisms. The most common way is to control of soil-born plant pathogens in the rhizosphere with bacteria. Bacterial genera studied for the control of crown gall disease include *Pseudomonas*, *Bacillus*, *Serratia* and *Agrobacterium*. The most successfully used antagonist strain is *A. radiobacter* K84 [6-10]. This strain was isolated in Australia, and it has an inhibitory action on most pathogenic *A. tumefaciens* containing the nopaline Ti plasmid (biotype I and II), although some strains of *A. tumefaciens* biotype I and II became resistant to K84 agrocin [11]. Dipping seeds or roots into a K84 suspension prior to planting can efficiently prevent crown gall formation in the field on the roots of roses [12] and cherry [13]. Moreover, a novel *B. methylotrophicus* strain 39b found to stop the growth of *A. tumefaciens* C58 and B6. Mass spectrometry analysis revealed surfactins as the active principle that acting against *Agrobacterium* strains [14].

The genus *Pseudomonas* is a group of Gram-negative bacteria that are rod shaped, aerobic, non-spore-forming and motile [15]. *Pseudomonas* strains are capable to survive in diverse niches, ranging from terrestrial and aquatic environments to tissues of eukaryotic hosts. Many members of this genus displayed remarkable physiological and metabolic activity against different pathogens [16-19]. The highly precision metabolic system and several secondary metabolites, including phenazines, pyrrolnitrin, pyoluteorin and lipopeptides help *Pseudomonas* against other bacteria and fungi [20].

In this study, a total number of 10328 bacterial strains were isolated from rhizosphere of cherry tree to test their antibiotic ability for the control of *A. tumefaciens*. One strain named LWB10 showed strong antibiotic activity to *A. tumefaciens* C58 *in vitro*. Zone of inhibition and co-culture assay demonstrated that *P. mosselii* LWB10 significantly inhibited the growth of several pathogenic *A. tumefaciens* strains. When coinoculated LWB10 with two pathogenic *A. tumefaciens* strains in tomato, a significant decrease of tumor observed. Then we used a series of methods to identify this strain. Results from morphological, physiological, biochemical characterization, molecular identification and MALDI-TOF analysis indicated that this strain belonged to *P. mosselii*. Moreover, the antibiotic components could secrete outside the cell. Collectively, our results revealed that *P. mosselii* LWB10 is a promising biocontrol agent for inhibiting the growth of *A. tumefaciens*.

2. MATERIALS AND METHODS

2.1. Sample collection

We collected soil samples from the root area of a cherry tree in Ningbo, China. The entire collected samples were isolated immediately for the microbiological experiments in the laboratory.

2.2. Isolation of antibacterial strains

The soil samples were serially diluted and inoculated on LB agar media (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 1% agar-agar per 1 liter) at 28 °C for 48 h. After that we isolated and ordered all the clear monoclonal colonies in individual tubes with LB liquid media

in a shaker under the same temperature. All the bacteria were potential antibiotic agent for *A. tumefaciens* C58. Then *A. tumefaciens* was grown at 28 °C in YEB medium (0.5% tryptone, 0.1% yeast extract, 0.5% beef extract, 0.5% sucrose and 0.05% MgSO₄ per 1 liter) until the optical density at 600 nm (OD₆₀₀) reached about 0.8. Inhibition zone assays conducted to reveal the antibiotic ability of isolated strains against C58. Top agar medium was prepared according to the following steps. There are three layers in the top agar medium. In the first layer, we poured 15 mL YEB solid medium in sterilized petrol dish. The second layer is the mixture of 1 ml *A. tumefaciens* C58 (density at OD₆₀₀ about 1.0) and 10 mL YEB solid medium (cold till 60 °C). Nine sterilized paper discs (0.6 cm in diameter) placed on the surface of the plate to form the third layer. Then 10 µL of each candidate antibacterial strains at OD₆₀₀ of 1.0 were loaded on the paper discs [21, 22]. The zone of inhibition of bacterial growth appeared after 24 h incubation at 28 °C. Other strains of *A. tumefaciens* used in this study including GV3101, LBA4404, EHA105, ACCC19197 (ordered from Agricultural Culture Collection of China) and CFCC1369 (ordered from China Forest Culture Collection Center). The antibiotic ability of candidate strains against to other *A. tumefaciens* strains were tested with the same procedure.

2.3. Morphological, physiological and biochemical characterization

Transmission electron microscopes (TEM) used for examining the cell morphology of the candidate strain after cells grown for 12 h on YEB medium. Gram staining conducted according to the procedure described by Murray [23]. Bacteria was cultured on YEB medium at different temperature of 4, 16, 22, 28, 42 °C for 2-4 d to detect cell growth. Biochemical and physiological characterization examined using non-fermenting microorganism identification tube according to the manufactures' instructions (HuanKai Microbial, China).

2.4. Identification bacteria by MALDI Biotyper platform

First, candidate bacteria grew on the YEB agar plate for 24 h at 28 °C. Then cells collected and washed

twice with sterilized water for protein extraction according to the ethanol/formic acid extraction method. Four technical replicates were spotted onto a MALDI target plate and analyzed by MALDI-TOF MS (Matrix-assisted laser desorption/ionization time of flight mass spectrometry) according to the manufacturer's instructions [24]. Later, the spectra were loaded into Biotyper software. The Biotyper-derived scores obtained according to the comparison against the MSP database library.

2.5. Phylogenetic analysis

Genomic DNA extracted using the TIANamp Bacteria DNA Kit (Cat. No DP302, TIANGEN BIOTECH (BEIJING) CO. LTD) according to the manufacturer's instructions. The 16S rRNA gene amplified by PCR using the universal primer pair: F8 (5'-AGAGTTTGATCCTGGCTCAG-3')/ R1492 (5'-ACGGCTACCTTGTACGACTT-3'). Fragment sequenced and sequence similarity was determined using BLAST server of NCBI. The available sequences from NCBI database which showing >99% similarity retrieved by BLAST N program available at NCBI server (www.ncbi.nlm.nih.gov). Phylogenetic tree based on 16S rRNA genes reconstructed using MEGA of version 7.0 [25]. Bootstrap analysis based on 1000 replications used to estimate the confidence level of tree topologies.

2.6. The antibiotic activity *in vivo*

Tomato plants were used as model to evaluate the antibiotic ability of isolated strains. Seeds were sown in individual pot for 1-2 month in growth chambers (at 25 ± 2 °C, 65% relative humidity, and 12/12 h light/darkness photoperiod). Plants main stems with 6-8 true leaves were pin-prick inoculated with pathogenic *A. tumefaciens* strains of CFCC1369 or the antibiotic strain alone or the mixture of antibiotic strain with *A. tumefaciens*. The procedures were conducted as following: first, dipped the sterilized absorbent cotton in the bacterial solution (1×10⁸ cfu mL⁻¹). Then attached the main stems with absorbent cotton after pin-prick and covered with plastic wrap for 2 d in growth chamber. After that the absorbent cotton were detached from main stems and disease symptoms were evaluated at 30-60 days post inoculation

(dpi) by calculating the size of tumor in inoculated sites. Each treatment conducted with 15 individual replications.

3. RESULTS

3.1. LWB10 shows strong inhibitory activity against C58

A total number of 10328 culturable bacterial colonies obtained from cherry trees root area. Among them, nine isolates demonstrated various degrees of inhibitory activities against C58 growth (zone of inhibition area >10 mm) after two days of cocultivation. Notably, a bacterial isolate (termed LWB10) showed strong inhibition zone to *A. tumefaciens* C58 than other bacterial strain (LWB11) (Fig. 1a). The diameter of inhibition zone was > 20 mm after 2 d. Further study revealed that LWB10 could produce diffusible yellowish pigments on the YEB agar medium after incubation for 1-5 days (Fig. 1b). Moreover, we co-culture LWB10 with C58 for several days and found that LWB10 showed strong inhibition zone to *A. tumefaciens* C58. The inhibition zone had a diameter of 26, 40 and 62 mm after coculture with *A. tumefaciens* C58 after 2, 4 and 6 days (Fig. 1c, in 90 mm petri dish). These results indicate that LWB10 effectively inhibited the growth of *A. tumefaciens* C58.

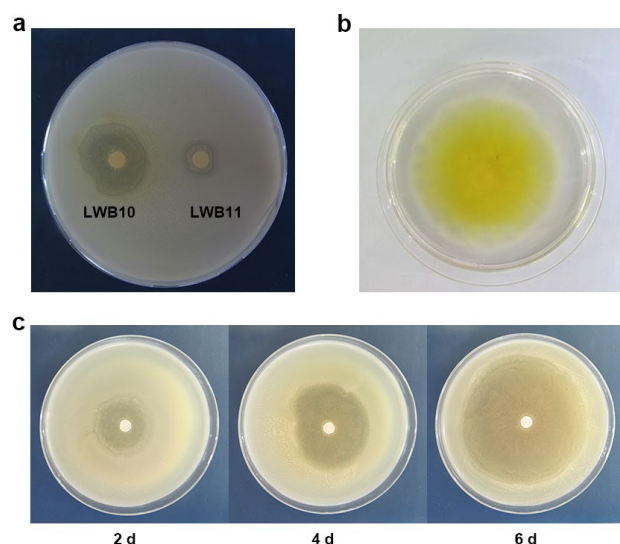


Fig. 1 Strain LWB10 shows strong inhibitory activity against C58. (a), LWB10 showed bigger inhibition zone than LWB11; (b) LWB10 produces diffusible yellowish pigments on YEB agar plate after 1 day of incubation, picture was taken at 5 dpi; (c) Zone of

inhibition test showed that after incubation for 2, 4 and 6 days, the inhibition zone of LWB10 to C58 was 26, 40 and 62 mm, respectively.

3.2. Molecular identification of LWB10

Blast analysis demonstrated that LWB10 closely related to the genus *Pseudomonas*. The phylogenies of LWB10 and other 32 members of the genus *Pseudomonas* were determined by using the neighbor-joining method in the program MEGA7.0 [25]. As showed in Fig. 2, the tree has two big branches. One branch contains *Pseudomonas pachastrellae* CCUG 46540T, *Pseudomonas azotoformans* DSM 18862T, *Pseudomonas composti* CCUG 59231T and *Pseudomonas punonensis* CECT 8089T. Strain LWB10 clustered in another branch with *Pseudomonas mosselii* CIP 105259T. The sequence similarity of LWB10 to *Pseudomonas mosselii* CIP 105259T is as high as 98%. All these results indicated that LWB10 belongs to *Pseudomonas mosselii*.

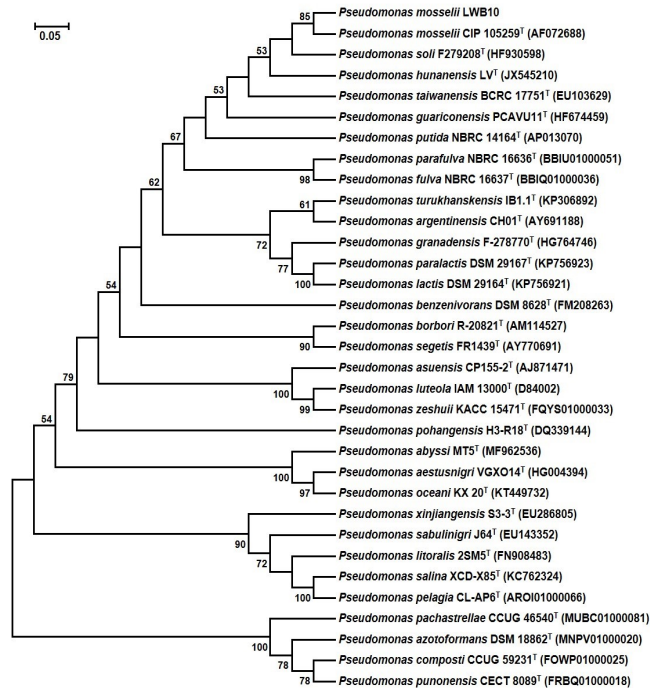


Fig. 2 Phylogenetic tree analysis of LWB10 based on the nucleotide sequence of 16S rRNA gene. The tree was generated by the neighbor joining (NJ) method. Bootstrap probability values of above 50% are indicated at branch-points.

3.3. Morphological, physiological and biochemical characterization

Results from morphological, physiological and biochemical tests showed that strain LWB10 was determined to be G-, non-spore-forming rods (0.5-0.8 μm in wide and 1.5-1.8 μm in long) with polar flagella, motile as shown in Fig. 3a and 3b. Cell colonies on blood agar medium are circular and smooth. Colonies have a 2 mm diameter length after growth for 24 h (Fig. 3c). Bacteria could not growth under 4 and 42 $^{\circ}\text{C}$. The biochemical characteristics of strain LWB10 listed in table 1. According to the biochemical characteristics, we concluded the bacteria was *P. mosselii*.

Table 1. The biochemical characteristics of strain LWB10

Biochemical tests	
Gram reaction	-
Pigment production	+
MacConkey Agar Medium growth	+
Growth at 4 $^{\circ}\text{C}$	-
Growth at 42 $^{\circ}\text{C}$	-
Catalase test	+
Oxidase test	+
Nitrite reduction	-
Arginine dihydrolase	+
Assimilation of Glucose	+
Mannitol	+
Maltose	+
Xylose	+
Simon's citrate	++
Acetamide	-
DNA	-

To provide convincing evidence, we applied other two approaches to identify strain LWB10. Results from MALDI biotypersmart system revealed that LWB10 had a high score value of 2.247 relative to *P. mosselii* (Fig. 3d). Moreover, we also performed the gas chromatography cellular fatty acid analysis (GC-FA) to identify this strain. Unfortunately, the version of the database is too old that lack of *P. mosselii*. GC-FA analysis indicated LWB10 was *P. putida* while *P. putida* was in the second place of MALDI results and showed a low score value of

1.742 (data not showed). These two procedures together indicated that strain LWB10 is *P. mosselii*.

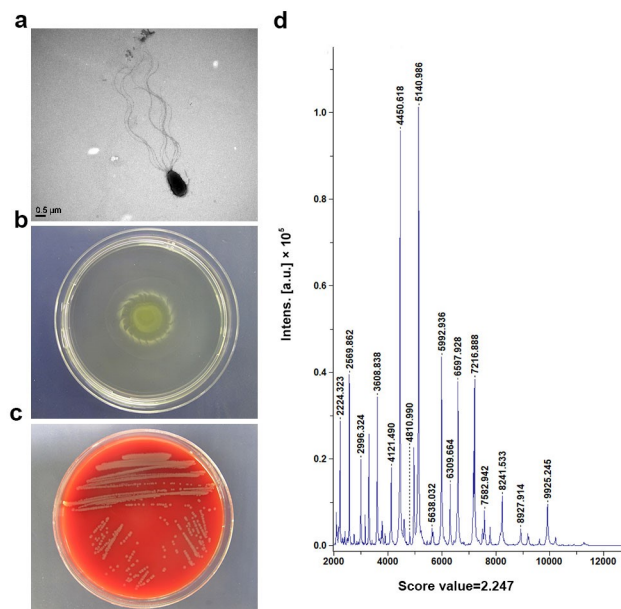


Fig. 3 Morphological, physiological and biochemical characterization of LWB10. (a) Transmission electron microscopy of LWB10, showing polar flagella and rod-shaped cell; (b) Motility activity test of LWB10. (c) Growth on blood agar medium showed a circular and smooth colony; (d) LWB10 profile generated by the high-throughput MALDI Biotypersmart system.

3.4. In vitro antagonism of LWB10 to other *A. tumefaciens* strains

In order to test the antagonism ability of LWB10 to other *A. tumefaciens* strains (ACCC19197, CFCC1369, GV3101, LBA4404 and EHA105), we conducted *in vitro* assays by the inhibition zone test (in 60 mm petri dish). Results showed that LWB10 had diverse inhibition ability to the test strains of *A. tumefaciens* after 2 days. The diameter of inhibition zones to each strains were 23, 35, 31, 18 and 16 mm (Fig. 4). Strain LWB10 displayed strong antibiotic activity to ACCC19197, CFCC1369 and GV3101. Our results showed that LWB10 has strongly antagonism to other *A. tumefaciens* strains.

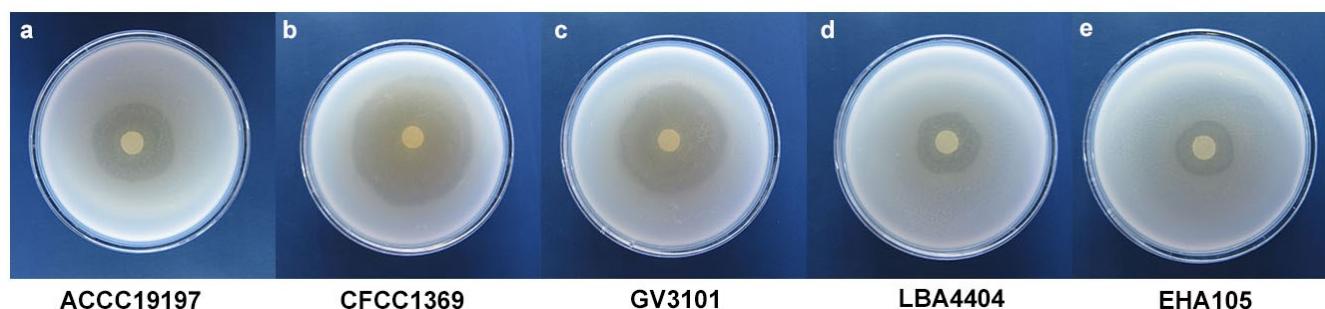


Fig. 4 *P. mosselii* LWB10 suppresses *A. tumefaciens* growth *in vitro*. LWB10 showed significantly growth inhibition of five strains, including (a) ACCC19197, (b) CFCC1369, (c) GV3101, (d) LBA4404 and (e) EHA105. The diameter of inhibition zone was 23, 35, 31, 18 and 16 mm, respectively. Pictures were taken at 2 days after inoculation. Each treatment conducted at least 5 replications.

3.5. *In vitro* antagonism of LWB10 to other *A. tumefaciens* strains

In order to test the antagonism ability of LWB10 to other *A. tumefaciens* strains (ACCC19197, CFCC1369, GV3101, LBA4404 and EHA105), we conducted *in vitro* assays by the inhibition zone test (in 60 mm petri dish). Results showed that LWB10 had diverse inhibition ability to the test strains of *A. tumefaciens* after 2 days. The diameter of inhibition zones to each strains were 23, 35, 31, 18 and 16 mm (Fig. 4). Strain LWB10 displayed strong antibiotic activity to ACCC19197, CFCC1369 and GV3101. Our results showed that LWB10 has strongly antagonism to other *A. tumefaciens* strains.

3.6. Antibiotic activity in the host plant

At 45 dpi, the plants inoculated with CFCC1369 alone showed severe tumor formation in inoculated sites, while in LWB10 added treatments displayed fewer tumor formation in inoculation sites and *P. mosselii* LWB10 alone showed no symptoms of tumor formation (Fig. 5). The average size of the tumor is 4×10 mm in CFCC1369 inoculated stems, and there is few tumors in co-inoculated sets. Symptoms first observed after 30 dpi in tomato main stems of CFCC1369 treated sets, while tumors not format until 40 dpi. Each treatments has at least 15 replications. These results indicated that LWB10 had strong

inhibition ability of tumor formation by *A. tumefaciens* CFCC1369.



Fig.5 *In vivo* test of antibiotic activity of LWB10. Main stems of tomato plants were inoculated with CFCC1369 alone, LWB10/CFCC1369 mixture solutions and LWB10 alone. The existence of LWB10 strongly suppressed the tumor formation in tomato stems. Symptoms were taken at 45 dpi. This test was repeated for three times and each time at least 15 replications.

4. DISCUSSION

Crown gall disease caused by *A. tumefaciens* has long been a big threat to agricultural economic. *A. tumefaciens* was a soil born, gram negative, plant pathogen that exist in the rhizosphere of host plant. After infection, it will produce tumors on infection sites, such as roots and stems. The disease symptoms developed slowly and when the pathogen once established, it will be hard to cure. Increasing evidence has shown that

plants recruit protective bacteria to their rhizosphere to enhance microbial activity to suppress pathogens [26-28]. Many researchers have been trying to use biological control method for this disease. Moreover, some other antibiotic strains also found to stop the growth and tumor formation in host plants. As the disease become more and more severe all around the world, we need more new biological control agents to cure this disease.

Among all the 10328 bacterial strains isolated from rhizosphere of cherry tree, only LWB10 strain exhibited strong antibiotic activity to *A. tumefaciens*. A series of methods, such as biochemical characterization, molecular identification and MALDI-TOF analysis, indicated that LWB10 closely related to *P. mosselii*. *P. mosselii* LWB10 was deposited to the China Center for Type Culture Collection (CCTCCas M2019081.

Pseudomonas mosselii regarded as a specie of the *P. putida* group [29, 30]. It is an environmental species detected in rhizospheric soil and is an overall unusual human opportunistic pathogen [31, 32]. Reports had showed that *P. mosselii* also has antibacterial ability to other pathogens [33, 34]. Moreover, a novel insecticidal protein, PIP-47Aa, isolated from *P. mosselii* is toxic to three corn rootworm species. This protein is a novel insecticidal protein for controlling of the corn rootworm pests [35]. *P. mosselii* has been reported to been characterized in biocontrol against plant disease. Recently, Zhou and colleges generated an engineered *P. mosselii* strain to express *Ralstonia solanacearum* *ripAA* gene, which determines incompatible interactions with tobacco plants to control tobacco bacterial wilt [36]. A gene cluster named *c-xtl* from *P. mosselii* BS011 documented to be required for inhibitory activity against the fungus *Magnaporthe oryzae* [37]. While there are no such reports for the biocontrol of *P. mosselii* to crown gall formation bacteria *Agrobacterium tumefaciens*.

Gene deletion experiments demonstrated that the gene cluster named *c-xtl* from *P. mosselii* BS011 documented to be required for the inhibitory activity [35]. Engineering of *Ralstonia solanacearum* *ripAA* gene to *P. mosselii* strain is efficient to control tobacco

bacterial wilt [36]. A drafted genome sequence of *P. mosselii* Gil3 predicted for the synthesis of antimicrobial compound xantholysin and a *ppyS* homologous gene for synthesis of antibiotic pseudopyronines [38]. In this study, we isolated a *P. mosselii* strain LWB10 that show strong antibiotic activity to *A. tumefaciens*. And further study is needed to prove out the exact antibiotic agent of *P. mosselii* LWB10.

5. CONCLUSION

The results of the present study provide evidence of the potential antibiotic ability exhibited by *P. mosselii* LWB10 to control crown gall disease in tomato. It is also the first report on the antibiotic ability of *P. mosselii* LWB10 to *A. tumefaciens*. Future research should focus on extraction assays to prove out the exact antibiotic agent of *P. mosselii* LWB10. *P. mosselii* LWB10 provided a new strain for the biocontrol of plant crown gall disease.

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