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Isolation and identification of cellulose-degrading endophytic bacteria from Tomoceridae (springtails)

Wei Deli[1,2], Miao Xiulian[1], Tian Yue[1], Du Jing[2] and Wang Meng[1*]

[1]School of Life Science, Liaocheng University, Liaocheng 252059, People's Republic of China.
[2]Department of Reproductive Medicine, Liaocheng People's Hospital, Liaocheng 252000, People's Republic of China.

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Springtails are considered as an important candidate bioindicator to assess soil quality spiked with trace metals, but little is known of their endophytic bacteria. In this study, a kind of Tomoceridae springtail was used, and a total of 45 effective sequences were obtained through the process of endophytic bacteria isolation, culture, polymerase chain reaction (PCR) amplification and sequencing. After NCBI-BLAST, the results showed that there were 20 bacterial colonies belonging to the genus *Staphylococcus*, 12 belonging to the genus *Bacillus*, 7 belonging to the genus *Paenibacillus*, 1 belonging to the genus *Exiguobacterium* and 5 belonging to *Acinetobacter lwoffii*. Furthermore, five bacterial strains from these five genera (named TomoRZH14, TomoRZH26, TomoRZH30, TomoRZH37, TomoRZH40) were selected for cellulose degradation analysis. The results showed that TomoRZH26 (*Bacillus* sp.) seemed to have a stronger cellulose degradation ability than those of the other four strains, while the three main components cellulase endo-β-glucanase, exo-β-glucanase and β-glucosidase in TomoRZH26 showed significantly higher enzymatic activity than in the other strains. Viscosity analysis also showed that the TomoRZH26 bacterium degraded relatively quickly in cellulase fermentation medium. In general, in this study, we preliminarily revealed several endophytic bacteria of Tomoceridae springtails and found that they had potentially strong cellulose degradation activity, which may be one of the important reasons behind springtail adaptation to this kind of soil ecological environment.

Key words: springtail, endophytic bacteria, cellulose.

INTRODUCTION

It is well known that endophytic bacteria play very important roles in host animal behavior and many flies with hyperactive locomotor behavior return to normal levels (Schretter et al., 2018). In bees, endophytic bacteria may also be associated with their social regulation (Kwong and Moran, 2016). In addition to host behaviors, endophytic bacteria may also affect insect development, lifespan, fecundity and so on (Gould et al., physiological processes. In *Drosophila melanogaster*, the commensal bacterium *Lactobacillus brevis* can help the 2018). In mammals, studies have demonstrated that specific endophytic bacteria can modulate the nutritional status, health, and disease susceptibility of the host (Huda et al., 2020). In general, endophytic bacteria play an important role in animal organisms, and to a certain extent, the microbiota has great similarity between some

*Corresponding author. E-mail: mengw1986@yeah.net.

kinds of insects and mammals. According to the impact on the host, there are mainly beneficial bacteria, harmful bacteria, and opportunistic pathogens. These endophytic bacteria have different effects on animal bodies.

Springtail is the general name of Collembola order animal species and plays very important roles in the process of soil ecological environment regulation. Springtails mainly feed on living plants, as well as fungi, animal debris, humus, bacteria and others, with a relatively miscellaneous feeding habit (Fujii et al., 2021). At the same time, they also have a certain resistance to heavy metals in the environment, such as nickel (Lin et al., 2019), mercury (Buch et al., 2016), and lead (Dai et al., 2020), or involve in certain related metabolic activities. A recent study showed that the gut bacteria of springtail had complex interactions within the gut community (Agamennone et al., 2018). In view of the complex feeding habits and diverse ecological functions of springtails, we believe that these characteristics are inseparable owing to the role of endophytic bacteria, although there is still a lack of in-depth research, especially in terms of cellulose degradation. These endophytic bacteria may play important roles in assisting the degradation of cellulose in soil and maintaining the normal energy flow of the ecological environment.

In this study, we used a kind of Tomoceridae springtail, which is a common species that lives in temperate forests, for endophytic bacterial analysis. To isolate these bacteria, we sterilized the surface of the springtails. After further culture and identification of these bacteria, we continued to analyze their ability to degrade cellulose. In summary, in this study, we wanted to know the general endophytic bacterial composition and their potential role in this kind of springtail. This study lays a foundation for further study of the physiology and ecology of bacteria and their hosts.

MATERIALS AND METHODS

Springtail acquisition and culturation

The springtails used in this study were of the family Tomoceridae and were collected from Wulian Mountain, Rizhao city of Shandong Province, China. The springtails were fed in a culture bottle containing gypsum/activated carbon matrix that was covered with a permeable transparent lid, cultured in a constant temperature incubator at 25°C, and fed with dry yeast every day.

Isolation and identification of endophytic bacteria

Ten springtail adults were placed in a 1.5 ml centrifuge tube, washed with sterile water, and then placed in 75% alcohol for body surface sterilization for 30 min. Then, springtails were washed twice with sterile water and ground with an electric pestle. After grinding, 150 µl sterile water was added to the grinding solution, which was then serially diluted to dilution factors of 10-1, 10-2, 10-3, 10-4 and 10-5. These dilutions were then spread evenly on Luria-Bertani (LB) plates and incubated at 30°C for 36 h. Then, single colonies were isolated and cultured at a speed of 200 r/min in LB liquid medium overnight. To eliminate the interference of epiphytic bacteria, the last time for springtails washed water was flatted on LB plate and cultured as the negetive control.

The bacteria were identified through Polymerase chain reaction (PCR) amplification and Sanger sequencing. PCR was carried out using 16S universal primer pairs F: AGAGTTTGATCCTGGCTCAG; R: GGTTACCTTGTTACGACTT(Gao et al., 2017). Each 50 µl amplification system included: Taq enzyme 0.25 µl, 10 × PCR buffer 5 µl, deoxynucleotide triphosphates (dNTP) 4 µl, bacteria solution 2 µl, upstream and downstream primers 1 µl. The reaction conditions were as follows: First step: 94°C 10min; second step: 35 cycles: 94°C 30 s, 58°C 30 s, 72°C 30 s; third step: 72°C 5 min. The amplified product was tested with 1% agarose gel, and the amplified single sample was selected for Sanger sequencing. The sequencing results were retrieved by the NCBI-BLAST online database, and the result with the highest consistency was selected as the candidate species.

CMC-Na plate experiment

The candidate cellulose-degrading bacteria were inoculated on the CMC-Na plate, which was used as the sole carbon source for culture. After 72 h of culture at a constant temperature of 30°C, 1 mg/ml Congo red was used for staining for 20 min, and 1 mol/L NaCl solution was used for decolorization for 30 min. A transparent hydrolytic ring was observed.

Determination of endophytic cellulase activity

Cellulase is a kind of complex enzyme with synergistic effects, mainly composed of endo-β-1,4-glucanase, exo-β-1,4-glucanase and β-glucosidase (Mihajlovski et al., 2016). Since the main product catalyzed by cellulase is glucose, in this study, an enzyme unit was defined as the enzyme activity that catalyzes the production of 1 g of glucose in one minute, and the content of glucose was determined by the 3,5-dinitrosalicylic acid (DNS) method.

Glucose standard curve

First, 0, 0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 ml were taken from a 1 g/L standard glucose solution and placed into 7 scale test tubes, and then distilled water was added to brighten the solutions to 2.0 ml. Then, 1.5 ml of DNS solution was added to the solution, mixed well and boiled for 5 min. After cooling to room temperature, distilled water was added to a final volume of 20 ml and mixed well. Next, 200 µl of solution was taken from each tube and placed into a 96-well plate. The absorbance at 540 nm was measured in a multifunctional EPOCH2 microplate reader (BioTek, USA). The standard curve was made according to the absorbance value of different glucose contents.

Determination of cellulase activity

The isolated bacteria were inoculated into the enzyme-producing fermentation medium containing 20 g/L CMC-Na, 2 g/L KH_2PO_4, and 14 g/L$(NH_4)_2SO_4$ and cultured for 7 days at 30°C and 150 r/min in a constant temperature oscillating incubator. The enzyme activity was measured every 24 h. The substrates used for endo-β-1,4-glucanase, exo-β-1,4-glucanase and β-glucosidase enzyme activity measurements were 1% CMC-Na solution, 1% microcrystalline cellulose solution and 1% salicin solution, respectively. The supernatant obtained from 1 ml of fermentation broth, which was centrifuged at 4°C and 6000 r/min for 10 min, was used as the crude enzyme solution. Then, 0.5 ml of citric acid buffer (pH 4.5)

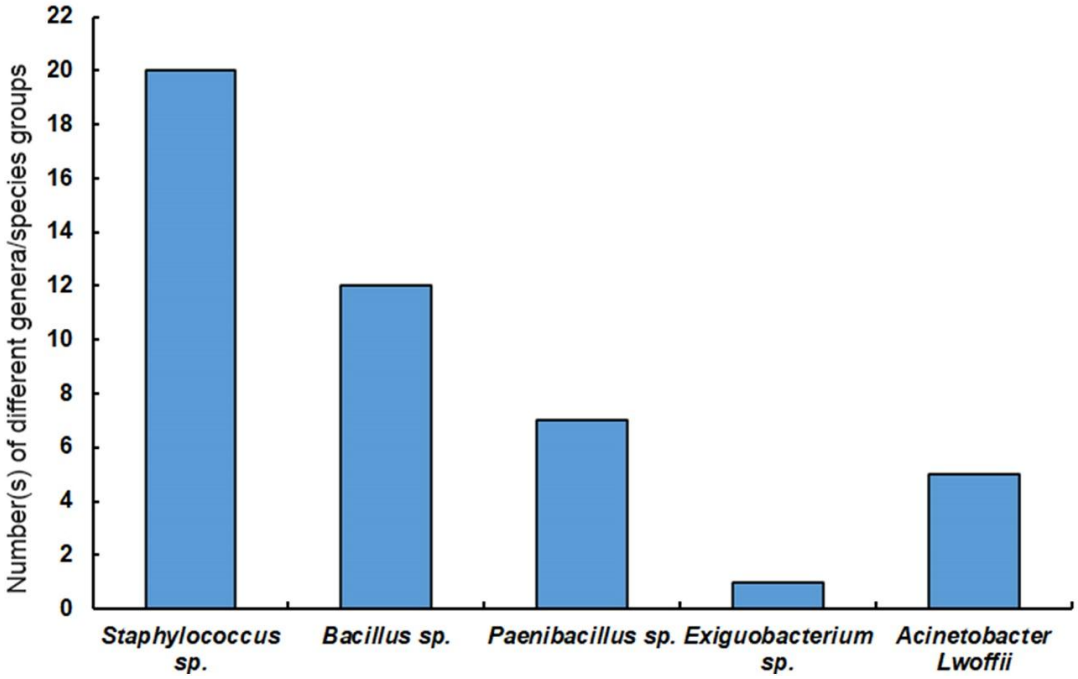

Figure 1. Number(s) of different genera/species groups of 45 endophytic bacteria isolated and selected in Tomoceridae springtails.

and 0.5 ml of crude enzyme solution were added into each 1 ml substrate solution. Next, the mixture was incubated in a metal block bath at 50°C for 30 min, and then 1.5 ml of DNS reagent was added to each tube. The reaction tube was then incubated in the metal bead bath at 100°C for 5 min. After quickly cooling to room temperature, the solutions were replenished with distilled water to 20 ml. After blending, the absorbance value at 540 nm was measured in a multifunctional micrometer. In the control group, no enzyme solution was added, and the amount of buffer solution was increased to 1.0 ml without reacting at 50°C.

RESULTS

Endophytic bacterial identification

In this study, we obtained 45 effective sequences by Sanger sequencing after endophytic bacterial isolation, cultivation and PCR amplification. These 45 sequences were input into the NCBI online database, and nucleic acid BLAST analysis was performed. The results are shown in Figure 1. Among the 45 sequences, 20 belonged to the genus *Staphylococcus*, 12 belonged to *Bacillus*, 7 belonged to *Paenibacillus*, 5 were identified as *Acinetobacter lwoffii*, and 1 belonged to *Exiguobacterium*. *Staphylococcus* was the most common springtail endophytic bacteria in this study.

Determination of cellulose degradation capacity and cellulase activity

We chose one strain of each genus to further study whether these bacteria had cellulose-degrading activity among which the sample TomoRZH14 belonged to Paenibacillus, TomoRZH26 belonged to *Bacillus*, TomoRZH37 belonged to *Exiguobacterium*, TomoRZH30 was *A. lwoffii*, and TomoRZH40 belonged to *Staphylococcus*. The five selected bacterial strains were inoculated on Sodium carboxymethyl cellulose (CMC-Na) plates and cultured at 30°C for 72 h. After Congo red staining and NaCl decolorization, the resulting transparent hydrolytic rings are shown in Figure 2. The results showed that TomoRZH26 had an obvious hydrolytic ring, followed by TomoRZH37 and TomoRZH30, which had smaller rings, while TomoRZH14 and TomoRZH40 had the smallest hydrolytic rings (Table 1).

The standard curve of glucose was generated with glucose content (mg/ml) as the abscissa and 540 nm absorption value as the ordinate. The equation of the generated curve is y = 0.2803 x - 0.0039, and the correlation coefficient R2 is 0.9994. The enzyme activity of endo-β-1,4-glucanase in the fermentation broth of these five bacteria was detected by the DNS method every 24 h for 7 days. The results are shown in Figure 3A. It is easy to see that the TomoRZH26 strain fermentation broth presented consistently high enzyme activity in the 7 days, and the enzyme activity of this strain was also the highest among the five strains. The highest enzyme activity was observed on the 4th day, reaching 8.3 U/ml. The other four strains, TomoRZH14, TomoRZH37, TomoRZH30 and TomoRZH40, had relatively low enzyme activities in the 7 days.

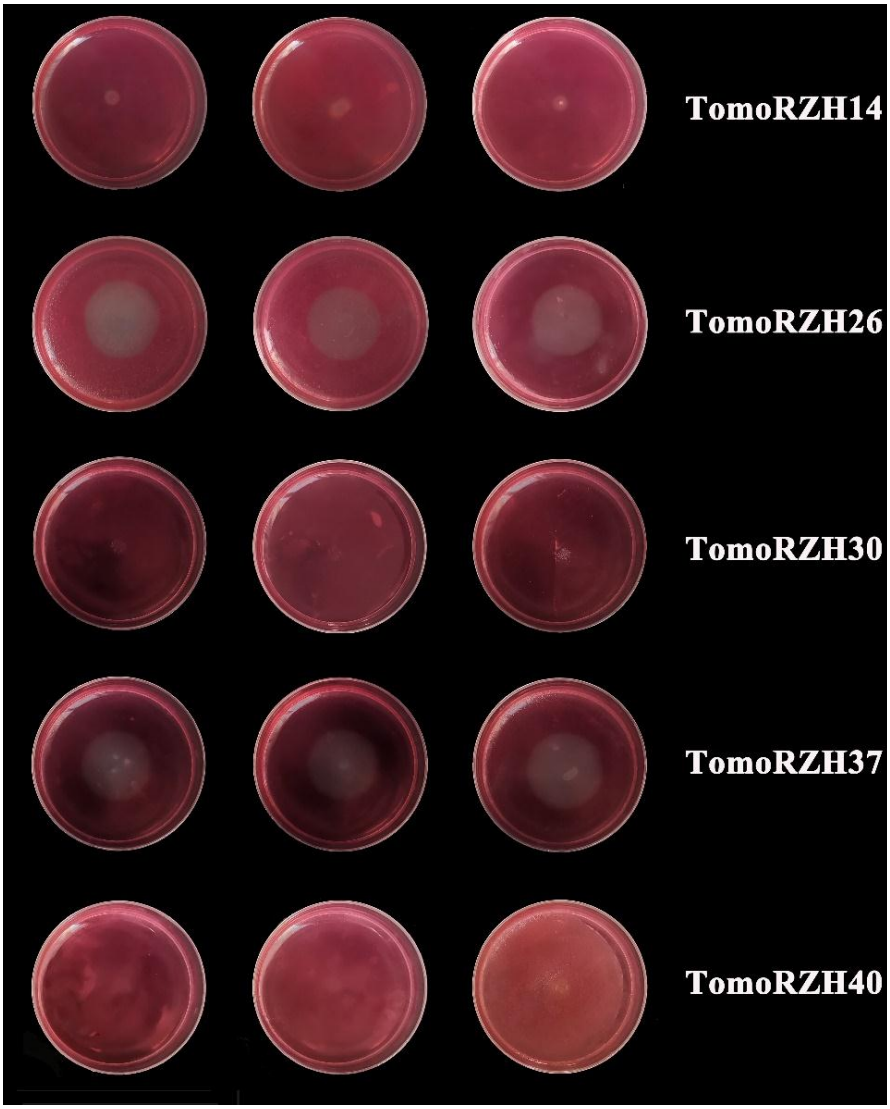

Figure 2. Hydrolytic zones of five springtails endophytic bacteria on CMC-Na plates.

Table 1. Hydrolytic zones of five springtails endophytic bacteria on CMC-Na plates.

Bacteria strains		TomoRZH14	TomoRZH26	TomoRZH30	TomoRZH37	TomoRZH40
Diameters of hydrolytic zones (D, mm)	Rep.1	6.07±0.12	21.47±0.55	6.13±0.12	19.00±0.50	7.03±0.26
	Rep.2	7.20±0.89	20.63±0.35	6.53±0.12	21.43±0.25	5.10±0.10
	Rep.3	5.43±0.16	18.7±0.23	6.46±0.06	18.70±0.26	6.87±0.12
Diameters of the colonyies (d, mm)	Rep.1	5.60±0.53	25.43±0.15	4.90±0.10	23.37±0.15	6.73±0.55
	Rep.2	7.38±1.14	24.50±0.50	5.33±0.28	25.27±0.25	5.67±0.58
	Rep.3	8.87±0.32	23.80±0.26	5.06±0.15	23.13±0.21	6.80±0.26
D/d	Total	1.01±0.08	1.21±0.05	0.80±0.02	1.22±0.03	1.022±0.08

Similar to endo-β-1,4-glucanase, as shown in Figure 3B, the TomoRZH26 strain fermentation broth presents a relatively higher exo-β-1,4-glucanase enzyme activity than those of the other four strains. However, the trends of enzyme activity for all five bacteria were roughly the same, with a lower level in the first two days than in the

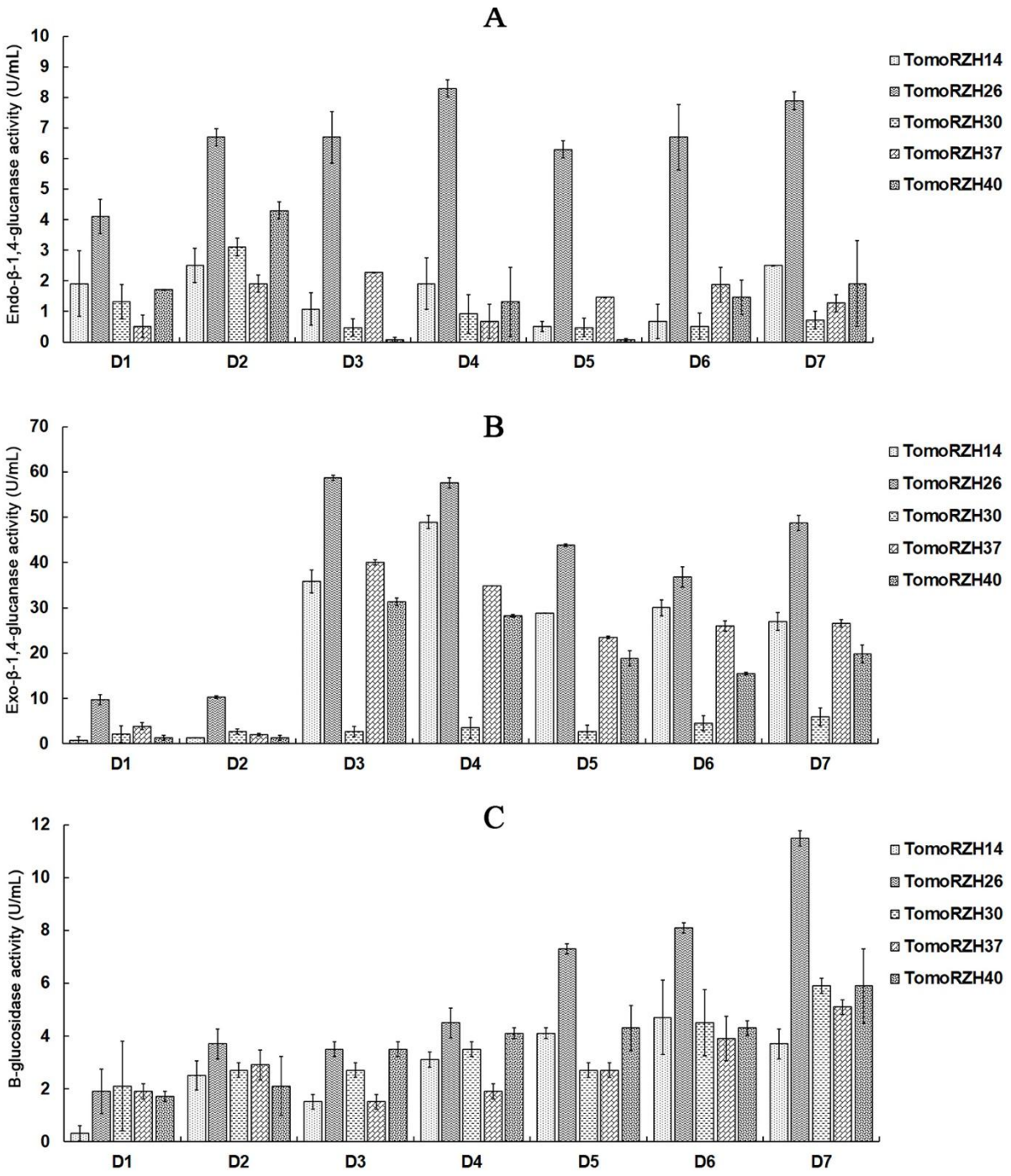

Figure 3. Cellulase activity identification of five springtails endophytic bacteria by DNS method. A) The enzyme activity of endo-β-1,4-glucanase; B) The enzyme activity of exo-β-1,4-glucanase; C) The enzyme activity of β-glucosidase.

proceeding five says. The enzyme activity of strains TomoRZH26, TomoRZH37, and TomoRZH40 reached the highest level on the 3rd day, which was 58.7, 40.0 and 31.4 U/ml, respectively, and strains TomoRZH14 and TomoRZH30 reached their highest levels of β-1,4-glucanase activity on the 4th day, which were 49.0 and 37.2 U/ml, respectively. In the following days, their enzyme activity decreased slightly. Therefore, these five bacteria all had certain enzyme activities of exo-β-1,4-glucanase and might be involved in some processes of cellulose degradation.

Unlike endo- and exo-β-1,4-glucanases, the enzyme activity of β-glucosidase continuously increased during the seven days of cultivation (Figure 3C). However, strain

TomoRZH26 also had higher enzyme activity than the other four strains after the 4th day. On the 7th day, the highest enzyme activity reached 11.5 U/ml. There was no significant difference in enzyme activity among the other four strains. On the 6th day, the enzyme activity of TomoRZH14 reached a maximum of 4.7 U/ml but decreased slightly on the 7th day.

DISCUSSION

Springtails are hexapod arthropods that live in soil. These have complex feeding habits and play important roles in the circulation of soil materials, soil development microstructure formation, soil physical and chemical property improvement and soil biome maintenance (Oliveira Filho et al., 2016). The ecological roles of springtails are likely to have a strong relationship with their endophytic bacteria. In this study, we isolated five different bacteria belonging to the genera *Staphylococcus*, *Bacillus*, *Paenibacillus*, and *Exiguobacterium* and the species *A. lwoffii*. The first four genera belong to the same order Bacillales and the phylum Firmicutes, while *A. lwoffii* belongs to the phylum Proteobacteria. The bacteria were not as abundant as expected because more endophytic bacteria were isolated from the springtail *Proisotoma ananevae*, and the isolated bacteria belonged to 6 genera and 3 phyla (Wang et al., 2018). The different living habits of these two different springtail species might be a reason for the difference in endophytic bacterial abundance; however, in this study, springtails were fed and cultured for a relatively long period of time, which might have caused the loss of some endophytic bacteria. As reported earlier, the phyla Firmicutes and Proteobacteria were also the most isolated bacteria from insect guts, which might be related to their coevolution process (Chen et al., 2016).

As reported earlier, many *Staphylococcus* species cannot cause disease and normally reside on the skin and mucous membranes of humans and other animals and are also a small component of the soil microbiome (Jacquemyn et al., 2013). Moreover, staphylococci were not commonly found to have effective cellulase activity, but cellulolytic compounds were occasionally isolated from soil-living small arthropods, such as termites (Pourramezan et al., 2012) and springtails in this study. However, the cellulase activity of staphylococci was found to be relatively low.

Bacillus species are well known in the food industry as troublesome spoilage organisms, two of which are parasitic pathogenic species, *B. anthracis* and *B. cereus*. Many *Bacillus* species can produce copious amounts of enzymes, such as α-amylase, subtilisin, surfactins, and mycosubtilins, which are used in various industries (Favaro et al., 2016). Additionally, some *Bacillus* species have been proven to have a significantly high cellulose degradation ability (Thomas et al., 2018; Cubas-Cano et al., 2020), which also varied in our study.

The other two genera, *Paenibacillus* and *Exiguobacterium*, also belong to the same order Caryophanales. Bacteria belonging to *Paenibacillus* have been detected in a variety of environments and vary with many effects in agriculture and horticulture and industrial and medical applications. Some *Paenibacillus* bacteria, such as *P. lautus* and *P. lactis*, with cellulolytic potential have been studied (Yadav and Dubey, 2018). Therefore, *Paenibacillus* bacteria also have a large potential application for cellulose degradation. *Exiguobacterium* bacteria have also been found in a variety of environments worldwide, and some species can grow within a wide range of pH values, tolerate high levels of UV radiation, and undergo heavy metal stress (Ordonez et al., 2013). A study showed that *Exiguobacterium* may have high β-glucosidase activity and would help with the fermentation of cellulose (Gao et al., 2015). In this study, the TomoRZH37 strain of *Exiguobacterium* exhibited relatively low β-glucosidase activity but with relatively high exo-β-1,4-glucanase enzyme activity, which need for further study.

The fifth type of bacterium found in this study was *A. lwoffii*, which is often considered a normal member of the human skin flora. However, most *Acinetobacter* bacteria are important soil organisms and contribute to aromatic compound mineralization. Some studies also found that the genus *Acinetobacter* bacteria could hydrolyze cellulose efficiently (Pourramezan et al., 2012; Karthika et al., 2020).

Overall, we isolated and identified five different genera of bacteria from Tomoceridae springtails. Although they exhibited different enzyme activities in the degradation of cellulose, these bacteria have potential application value in the degradation of cellulose. These bacteria may be a specific manifestation of springtails with extensive adaptation to the ecological environment. Therefore, more in-depth studies of the interaction between these bacteria and their springtail hosts will be needed in the future. Given the cellulose degradation activity of these bacteria, they also provide the possibility for large-scale biodegradation application for cellulose in industry.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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