Isolation and identification of a pathogenic strain of *Serratia marcescens* against the red palm weevil *Rhynchophorus ferrugineus* Olivier

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Background

The red palm weevill, *Rhynchophorus ferrugineus* Olivier is an important quarantine pest, which has caused serious economic losses.Finding effective biocontrol resources to prevent and control the insect is important.

Methods

To screen for effective biocontrol resources against the red palm weevil, a pathogenic strain named HJ-01 was isolated from infected and dead pupa of *Tenebrio molitor*. The HJ-01 strain was streak cultured and purified, and its morphological, physiological, biochemical characteristics, and 16S rDNA homology were identified after conducting a pathogenicity test on red palm weevil larvae.

Results

The results of the test revealed that larvae inoculated with HJ-01 exhibited reduced movement, decreased appetite, and eventual death. Over time, the larvae's bodies turned red, became soft, and started to rot, resulting in the discharge of red liquid. HJ-01 demonstrated the ability to produce scarlet pigment after 24 hours of culture on a basic medium. Colonies of HJ-01 appeared convex, bright red, moist, and viscous. They displayed opacity in the center, irregular edges, and emitted an unpleasant odor. Under microscopic observation, the cells of HJ-01 appeared as short rod-shaped and flagellate, with a size ranging from (1.2~1.8) μm × (1.0~1.2) μm. Genomic DNA extraction was performed on the strain, and the 16S rDNA sequence was amplified, yielding a sequence length of 1445 bp. This sequence displayed a 99.72% similarity to the sequence of *Serratia marcescens*. Phylogenetic tree analysis further confirmed that strain HJ-01 belonged to *S. marcescens*.

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Abstract

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Keywords *Serratia marcescens*;16S rDNA;Proxypene;*Rhynchophorus ferrugineus*; Biological control

Introduction

The red palm weevil (*Rhynchophorus ferrugineus* Olivier), belonging to the family Curculionidae in the order Coleoptera, is a significant global quarantine pest native to Southern Asia and Melanesia (Faleiro 2006; EPPO 2008; Roda et al. 2011;Huang 2013;Wakil et al. 2015). It poses a major threat to various palm plants, including *Cocos nucifera*, *Elaeis guinensis*, *Phoenix dactylifera*, *Areca catechu*, and other ornamental palms (Dembilio et al. 2010a; Wang et al. 2013; Dembilio and Jaques 2015; Lü et al. 2020). The red palm weevil primarily causes damage through larval burrowing, characterized by its destructive nature, high lethality, and difficulty in early detection. In the Middle East, the annual economic losses due to the red palm weevil are estimated to range from $5-25 million, with Saudi Arabia alone accounting for $1.74-8.69 million (Massoud 2012). In China, the red palm weevil was first reported in Zhongshan, Guangdong Province (Wan et al. 2005), and has since spread to 15 provinces and cities, causing severe damage to palm plants in Guangdong, Hainan, Yunnan, and other regions (Han et al.2013). Infestations by the red palm weevil significantly weaken the palm trunks, reducing their productivity and compromising their ability to withstand environmental conditions, such as strong winds (Saleh 2018). Detecting and controlling this pest is challenging due to the difficulty in early detection. Currently, the primary methods of control involve the use of chemical insecticides (Llácer et al. 2010; Llácer and Jacas 2010; Liu et al.2011; Meng et al. 2013; Alhewairini 2019) and pheromone trapping (Vacas et al. 2013; Sewify et al.2014; Chen 2016; El-Shafie and Faleiro 2017; Al Ansi 2022). Research on biological control mainly focuses on entomopathogenic nematodes (Dembilio et al. 2010b), entomopathogenic bacteria (Salama et al. 2004; Manachini et al.2009), and entomopathogenic fungi (Gindin et al. 2006; Dembilio et al. 2010c; Cito et al. 2014; Yasin et al. 2019).

Serratia marcescens, also known as Bacillus spiritus, belongs to *Serratia* of enterobacteriaceae and is a gram-negative bacterium, which is a kind of entomogenic bacteria widely existing in nature (Montaner and Pérez-Tomás 2003; Grimont and Grimont 2006; Petersen et al. 2013). In the growth process, this bacterium can produce a secondary metabolite, linomycin, which is highly pathogenic to a variety of agricultural and forestry pests include Lepidoptera, Coleoptera, Orthoptera, Diptera and Hemiptera (Sikorowski et al. 2001; Feng et al. 2002; Ke et al. 2006; Mohan et al. 2011; Babashpour et al. 2012; Wang et al. 2013; Fu et al. 2019), has attracted increasing attention worldwide. In this work, we isolated a bacterium from the dead mealworm pupae. After purification and back splicing tests on larvae and pupae of *Tenebrio molitor*, it was identified as an insect pathogenic bacterium, named HJ-01. The morphological characteristics, physiological and biochemical properties of the bacterium were observed, and the 16S rDNA of the strain was extracted for homology analysis. The strain was ultimately identified as *Serratia*

marcescens. Its pathogenicity against red palm weevil larvae was tested to explore its potential for biological control. The findings aim to provide valuable insights for the selection of biological control resources and the development of biological control technologies for the red palm weevil.

Materials and methods

Isolation and culture of the bacterial strain

Naturally infected and deceased pupae of *T. molitor* were collected from Wenchang, Hainan Island, China. The samples underwent a series of steps for preparation. Firstly, they were immersed in 70% alcohol for 1 minute and then rinsed with sterile distilled water. Next, the samples were surface-sterilized using 0.1% mercury chloride and washed three times with sterile distilled water. Subsequently, sections of the tissues were cut and inoculated onto Luria-Bertani solid medium (LB), which consisted of 10 g/L peptone, 5 g/L yeast, 5 g/L sodium chloride, and 15 g/L agar. The inoculated tissues were placed on separate sterile petri dishes, sealed with Parafilm, and incubated at 28±1°C with a relative humidity of 75±5% for 24 hours. A single colony exhibiting red pigment production was selected and cultured on LB solid medium for purification. To confirm the strain's ability to produce red pigment, a backgrafting test was conducted by introducing the bacterial solution to a healthy *T. molitor* specimen. This process aimed to restore the strain capable of producing red pigment, which was designated as HJ-01.

Pathogenicity determination of strain HJ-01 against red palm weevil

Red palm weevil larvae of the same age and the same size were selected for the test. The purified strain was prepared in sterile water containing aqueous 0.05% Tween-80, and the mixture was vortexed to attain homogenization. A dilution series of bacterial suspension (1.0×10^8, 1.0×10^7, 1.0×10^6, 1.0×10^5, 1.0×10^4 cfu/mL) was prepared thorough mixing, then sprayed on larvae. Then the larvae were transferred to the artificial feed cups for further incubation, 1 larva per cup, 20 larvae per treatment, replicated 3 times. The larvae were kept in controlled conditions (28±1℃, 75±5%RH) and checked daily for mortality. The dead larvae were reisolated use moisturizing the culture and verifying the pathogenicity of their isolates according to Koch's rule.

Morphological, physiological and biochemical identification of strain HJ-01

The morphology was observed using an optical microscope, and the physiological and biochemical reaction tests were identified by reference to methods such as bacterial classification and systematic identification (Buchanan et al. 1984; Dong et al. 2001).

16S rDNA amplification and sequence analysis of strain HJ-01

Genomic DNA extraction

The purified strain was inoculated in a triangular flask with sterilized LB liquid medium and incubated on a shaker at 28℃ and shaken at 180 rpm for 24 h. The genomic DNA of strain HJ-01

was extracted according to the procedure of the bacterial genome extraction kit (TIANGEN kit DP302).

Add 2 mL of fresh bacterial solution into a centrifuge tube, centrifuge at 10,000 rpm for 1 min, discard the supernatant, add 200 μL of buffer GA, shake until the bacteria are thoroughly suspended, add 20 μL of Proteinase K, mix well, then add 220 μL of buffer GB, shake for 15 sec, leave at 70°C for 10 min, centrifuge briefly to remove the cap. Add 220 μL of anhydrous ethanol, shake for 15 sec, centrifuge briefly, then add the resulting solution to an adsorbent column CB3, centrifuge at 12,000 rpm for 30 sec, pour to waste, add 500 μL of buffer GD, centrifuge at 12,000 rpm for 30 sec, pour to waste, then add 600 μL of rinse solution, centrifuge at 12,000 rpm for 30 sec, pour to waste. Centrifuge the column CB3 back into the collection tube, centrifuge at 12,000 rpm for 2 min, pour off the waste solution and leave it at room temperature for a few minutes to dry the residual rinse solution, then transfer the column CB3 into a clean centrifuge tube, add 100 μL of Elution Buffer TE dropwise, leave it at room temperature for 5 min, centrifuge at 12000 rpm for 2 min and finally collect the solution The solution was then collected in a centrifuge tube.

Amplification and Determination of 16S rDNA Sequence

The 16S rDNA sequences of strain HJ-01 were amplified using universal primer sets 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′), with an expected amplification fragment length of about 1400 kb. The PCR reactions (50 μL) contained: 25 μL of 2× Taq PCR premix reagent, 1 μL each of primers 27F and 1492R at 20 μmol/L; 2 μL of template DNA; 21 μL of double-distilled water. The PCR protocol for amplification of 16S rDNA regions included initial denaturation at 94°C for 5 min, 35 cycles at 94°C for 30 s, 58°C for 30 s, and 72°C for 90 s, followed by a final elongation at 72°C for 10 min. PCR products were kept at 4°C.The size and quality of PCR products were determined by gel electrophoresis using 1% agarose gel, which was stained with ethidium bromide (0.5 mg/mL) and visualized under UV light. Then sequenced in an automated system (Sangon Bioengineering Co., Ltd, Shanghai, China).

Construction of phylogenetic tree for strains

The sequences obtained were analyzed against nucleic acid data in GenBank using NCBI's BLAST tool, 16S rDNA sequences of related strains were downloaded, homology analysis was performed using multiple sequence alignment with MEGA6.0 and phylogenetic tree was constructed. The conformation and stability of the phylogenetic tree was determined by sampling and analysis 1000 times with MEGA6.0 software.

Results

Isolation of strains and pathogenicity of red palm weevil

A red pigment-producing strain was isolated from infected *T. molitor* pupae (Figure 1a), which was purified on LB medium and then tested against mealworm pupae, allowing the strain to be isolated again and named HJ-01 (Figure 1b).

HJ-01 was incubated in a constant temperature shaker for 24 hours and then inoculated with red palm weevil larvae to observe their infection status. After 8 hours, the larvae displayed reduced mobility and loss of appetite. By the 24-hour mark, the larvae started to show signs of mortality, including a gradual reddening and softening of their bodies (Figure 1c). As time progressed, the larvae further deteriorated, with their bodies decaying and releasing red liquid.

Strain HJ-01 of *S. marcescens* exhibits remarkable pathogenicity towards red palm weevil larvae. Regardless of the concentration tested, the strain effectively eliminates the larvae, and the mortality rate increases with longer treatment duration (Figure 2). The highest larval mortality rates were observed at suspension concentrations of 1.0×10^8 and 1.0×10^7 cfu/mL, reaching cumulative mortality rates of 82.22% and 77.78%, respectively, which were significantly higher compared to other treatments. For a concentration of 1.0×10^8 cfu/mL, the half lethal time (LT50) of red palm weevil larvae caused by strain HJ-01 was 4.72 days (Table 1, $p<0.05$).

Morphological observation, physiological and biochemical characteristics of strain HJ-01

The strain was cultured on LB solid medium for 24 hours and began to produce red pigment, the colony was raised, bright red, moist and sticky, opaque in the center, irregular at the edges, and smelly. Under the microscope, the bacterium was short rod-shaped, flagellated, and the size was (1.2~1.8) μm×(1.0~1.2) μm.

Table 2 presents the results indicating that strain HJ-01 is Gram-negative and facultative aerobic. It exhibited positive reactions for the Voges-Proskauer (V-P) test, motility test, glucose acid production, and gas production. However, it showed negative results for the methyl red test and phenylpropyl amino acid decarboxylase reaction. In terms of carbohydrate utilization, the strain produced acid when grown on media containing sucrose, maltose, sorbitol, and mannitol, while it did not produce acid when grown on media containing lactose, raffinose, fibrinous disaccharide, xylose and arabinose. These physiological and biochemical characteristics, as determined through standard methods outlined in the Manual of Systematic Identification of Common Bacteria and Bergey's Manual of Determinative Bacteriology, confirm that this strain belongs to *S. marcescens.*

Amplification and analysis of 16S rDNA sequence of strain HJ-01

The PCR amplification product of strain HJ-01 was analyzed using 1% agarose gel electrophoresis, revealing a distinctive band of approximately 1400 bp in size (Figure 3a). The amplification product was subsequently sent to Sangon Bioengineering Co., Ltd (Shanghai) for sequencing, resulting in a full-length sequence of 1445 bp (Figure 3b). The obtained sequence was uploaded to GenBank, and its accession number is OP317557. By performing a BLAST

search in the NCBI nucleic acid database, it was found that the 16S rDNA nucleotide sequence of strain HJ-01 exhibited a high similarity to that of Serratia marcescens strain whpu-5 (accession number: MK157269.1), with a sequence similarity of 99.72%. These findings suggest that strain HJ-01 is likely to be *Serratia marcescens.*

Phylogenetic tree of Strain HJ-01

A total of 12 closely related strains belonging to the Serratia genus were selected from the nucleic acid database for multiple sequence alignment with the ITS sequences of HJ-01. The aligned sequences were then used to construct a phylogenetic tree using MEGA 6.0 software, employing the Neighbor-Joining (NJ) method with a bootstrap value of 1000. The phylogenetic analysis revealed that strain HJ-01 shared the highest similarity with Serratia marcescens strains, specifically with accession numbers MK157269.1 and AB680122.1, exhibiting a self-extension value of 90% (Figure 4).

In conclusion, based on the morphological characteristics, physiological and biochemical traits, as well as the identification results of 16S rDNA, it has been established that strain HJ-01 belongs to the species Serratia marcescens within the genus Serratia.

Discussions

Biological control refers to the utilization of organisms, microorganisms, and their byproducts to manage pests. It is an essential component of integrated pest management, offering a safe and environmentally friendly approach. Therefore, the discovery of safe and effective biological control resources is of utmost importance (de Queiroz and de Melo 2006; Roberts et al. 2007). Among the bacteria suitable for pest control, Serratia is widely distributed in nature and can be isolated from healthy, infected, or deceased insects. Among the Serratia genus, *S. plymuthica* and *S. entomophila* have been extensively studied. *S. plymuthica* HRO-C48, registered and commercialized in Germany under the trade name Rhizostar is primarily used to combat root rot and wilt in strawberry plants (Berg 2009). On the other hand, *S. entomophila* is predominantly employed for the biological control of scarab beetles (Nuñez-Valdez et al. 2008).

Studies have found that *S. marcens* also has pathogenicity against a variety of Coleoptera insects. Yang et al. (2014) isolated a strain of *S. marcescens* PS-1 from diseased *Phyllotreta striolata* larvae, which was highly pathogenic to *P. striolata* adults after feeding treatment. Deng et al. (2008a,b) isolated a strain of *S. marcescens* from the carved grooves of *Anoplophora glabripennis* (Motschulsky) and applied the bacterial solution to the larvae using a microinjector. The fatality rate reached 80.6% when the treatment concentration was 7.8×10^{10} cfu/mL. Zhang et al. (2011) isolated *S. marcescens* subspecies HN-1 from eggs and dead larvae of the red palm weevil, and using this bacterium to infect larvae resulted in a 60% mortality rate and an 80% reduction in egg hatching rate. In this study, a red pigment-producing strain HJ-01 was isolated

from the dead mealworm pupae, and the morphological characteristics, physiological and biochemical characteristics were identified to be consistent with those of *S. marcescens*. By extracting 16S rDNA sequence of the strain, the similarity of 16S rDNA sequence between HJ-01 and S. marcescens was 99.72%. Therefore, the strain HJ-01 could be identified as *S. marcescens.*

The pathogenicity of *S. marcescens* strains from different sources to different insect species is very different. *S. marcescens* isolated from *Helicoverpa armigera* (Hubner) by Bulla et al. (1975) has pathogenicity against not only *H. armigera*, but also *Pieris rapae* L., a member of the family *Pieridae*. However, it was less pathogenic to the larvae of *Spodoptera exigua* Hiibner, which belongings to the same family. *S. marcescens* PS-1 obtained by Yang et al. (2014) not only had high pathogenicity against the larvae and adults of *P. striolat*a, but also had a significant inhibitory effect on the population growth of the beet armyworm *S. exigua.* Mónica L et al. (2015) showed that oral and injection bioassays using healthy *Phyllophaga blanchardi* larvae fed with the S. marcescens isolates showed different degrees of antifeeding effect and mortality. But no insecticidal activity was observed for *Spodoptera frugiperda* larvae by oral inoculation. In this study, strain HJ-01 of *S. marcescens* isolated from mealworm pupae had a fatality rate of 82.22% against red palm weevil larvae. It can be seen that the insecticidal effect of *S. marcescens* varies according to its source, application method and pest species.

Conclusions

A strain named HJ-01, exhibiting insecticidal properties against the red palm weevil (RPW), was isolated from infected mealworms. Through a comprehensive analysis of its physiological, biochemical, and molecular characteristics, it was identified as *Serratia marcescens* HJ-01. Upon infection with this strain, RPW larvae displayed reduced activity, a softer texture, and eventually succumbed to the treatment. Notably, the deceased insects emitted red pus upon gentle contact. The concentration of the HJ-01 suspension used in the experiments was 1.0×10^8 cfu/mL, resulting in an impressive mortality rate of 82.22% among the red palm weevils. Furthermore, the half-lethal time (LT50) for RPW larvae was determined to be 4.72 days.

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Figure 1

Figure1 Strain HJ-01 and infected insects

a Diseased *T. molitor* pupae. **b** Strain HJ-01 of *Serratia marcescens*. **c** Red palm weevil larvae infected by strain HJ-01

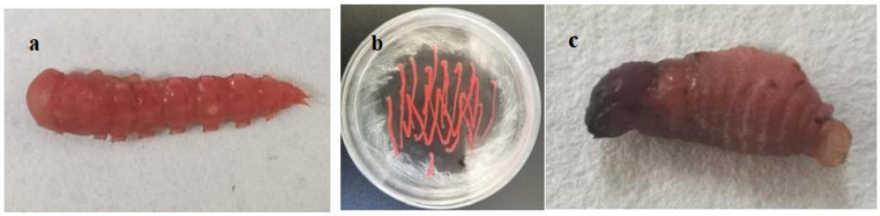

Figure 1 Strain HJ-01 and infected insects. **a** Diseased *T. molitor* pupae. **b** Strain HJ-01 of *Serratia marcescens*. **c** Red palm weevil larvae infected by strain HJ-01

Figure 2

Figure 2 Mortality rates of *R. ferrugineus* larvae treated with different doses of *S. marcescens*

Mortality of red and brown weevils at different times after spraying using different concentrations of bacterial suspensions. The error bars in the figure indicate the standard error of three repetitions.

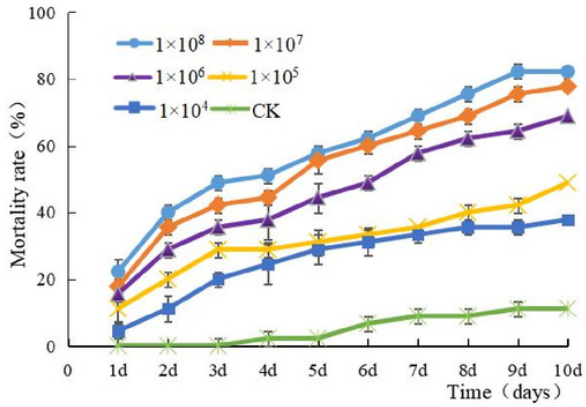

Figure 2 Mortality rates of *R. ferrugineus* larvae treated with different doses of *S. marcescens*

Note: The error bars on figure 2 are corresponding to SE.

Figure 3

Figure 3 Electrophoresis and Nucleotide sequence of 16S rDNA PCR products of strain HJ-01

a Electrophoresis of 16S rDNA PCR products of strain HJ-01, M□DL 2 000 marker. 1 and 2□product of 16S rDNA. **b** Nucleotide sequence of 16S rDNA of strain HJ-01.

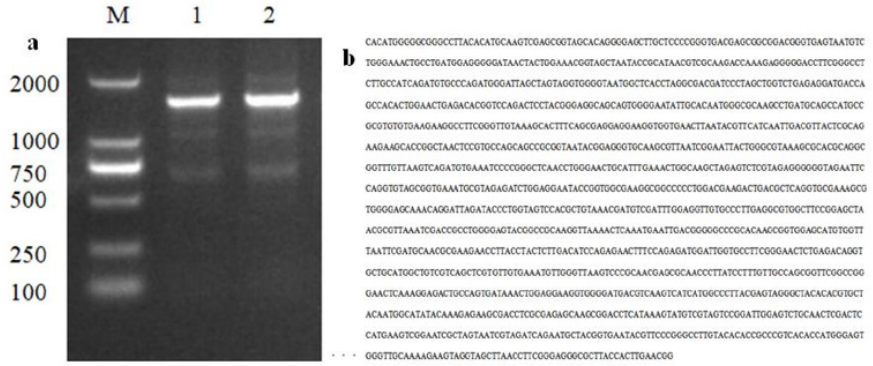

Figure 3 Electrophoresis and Nucleotide sequence of 16S rDNA PCR products of strain HJ-01. **a** Electrophoresis of 16S rDNA PCR products of strain HJ-01, M：DL 2 000 marker. 1 and 2：product of 16S rDNA. **b** Nucleotide sequence of 16S rDNA of strain HJ-01.

Figure 4

Figure 4. Phylogenetic placement of strain HJ-01 based on 16S rDNA

The aligned sequences of 12 closely related strains belonging to the Serratia genus were selected from the nucleic acid database and then were used to construct a phylogenetic tree using MEGA 6.0 software, employing the Neighbor-Joining (NJ) method with a bootstrap value of 1000.The scale bar represents a genetic variability of 0.002 for the genome.

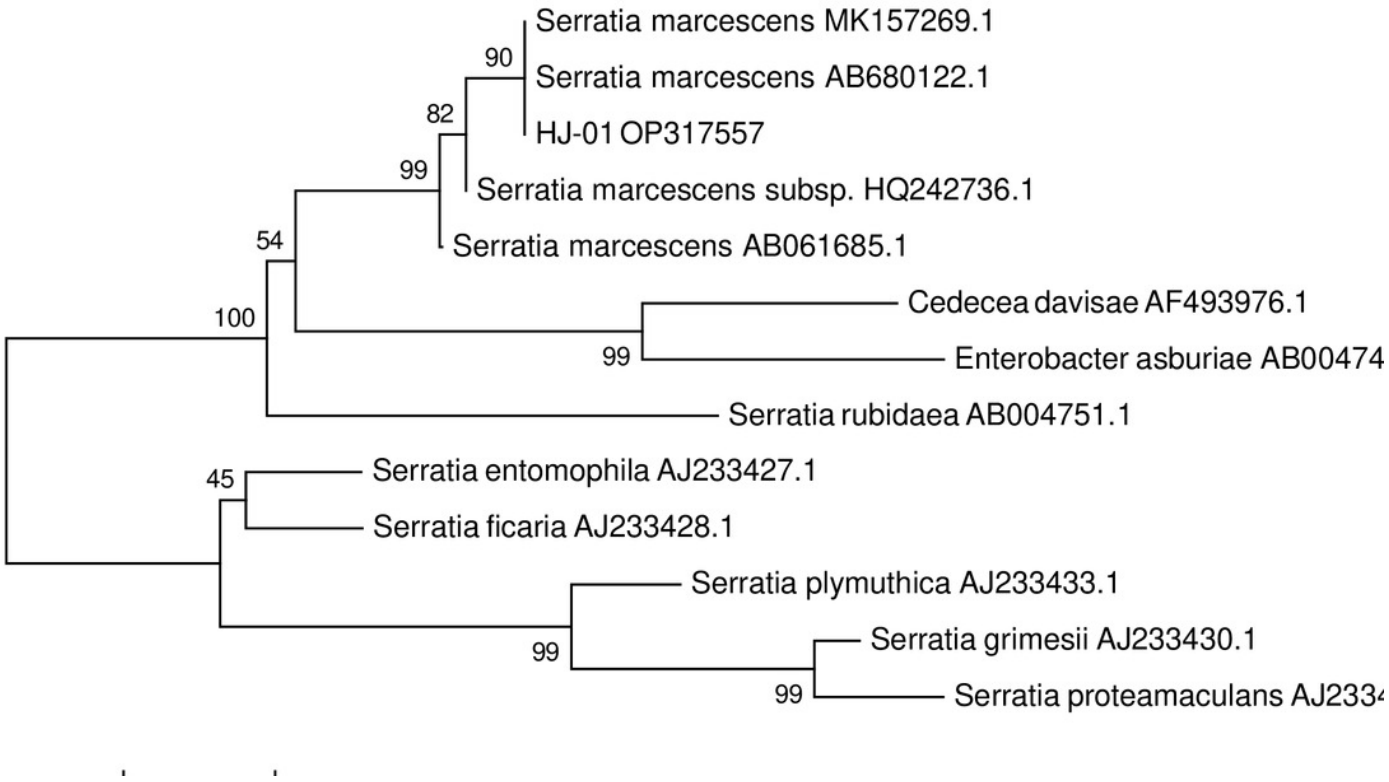

Table 1(on next page)

Table 1. LT_{50} values of *R. ferrugienus* infected by *S. marcescens* Strain HJ-01

LT_{50}, lethal time for 50 % mortality.

Table 1. LT_{50} values of *R. ferrugienus* infected by *S. marcescens* Strain HJ-01

Bacterial suspension （cfu/mL）	LT_{50} （days）	Correlation coefficient r	95% confidence interval	
			Lower	Upper
1.0×10^8	4.72	0.9845	4.24	5.26
1.0×10^7	5.30	0.9932	4.70	5.98
1.0×10^6	6.83	0.9950	5.90	7.92
1.0×10^5	14.81	0.9632	9.43	23.26
1.0×10^4	22.66	0.9867	9.08	56.58

Note: LT_{50}, lethal time for 50 % mortality.

Table 2(on next page)

Table 2. Physiological and biochemical characteristics of strain HJ-01

"+" is positive; "-" indicates negative.

Table 2. Physiological and biochemical characteristics of strain HJ-01

Characteristics	*Serratia marcescens*	HJ-01	Characteristics	*Serratia marcescens*	HJ-01
Gram staining reaction	—	—	Maltose	+	+
Methyl Red	—	—	Sucrose	+	+
V-P	+	+	Lactose	—	—
Movement test	+	+	Raffinose	—	—
Glucose acid production	+	+	Fibrinose	—	—
Glucose gas production	+	+	D-xylose	—	—
Phenylpropyl amino acid decarboxylase	—	—	Arabinose	—	—
D-Mannitol	+	+	D-sorbitol	+	+

Note: "+" is positive; "-" indicates negative.