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Identification and Characterization of *Bacillus cereus* SW7-1 in *Bombyx mori* (Lepidoptera: Bombycidae)

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ABSTRACT. The bacterial diseases of silkworms cause significant reductions in sericulture and result in huge economic loss. This study aimed to identify and characterize a pathogen from diseased silkworm. SW7-1, a pathogenic bacterial strain, was isolated from the diseased silkworm. The strain was identified on the basis of its bacteriological properties and 16S rRNA gene sequence. The colony was round, slightly convex, opaque, dry, and milky on a nutrient agar medium, the colony also exhibited jagged edges. SW7-1 was Gram-positive, without parasporal crystal, and 0.8–1.2 by 2.6–3.4 μm in length, resembling long rods with rounded ends. The strain was positive to most of the physiological biochemical tests used in this study. The strain could utilize glucose, sucrose, and maltose. The results of its 16S rRNA gene sequence analysis revealed that SW7-1 shared the highest sequence identity (>99%) with *Bacillus cereus* strain 14. The bacterial strain was highly susceptible to gentamycin, streptomycin, erythromycin, norfloxacin, and ofloxacin and moderately susceptible to tetracycline and rifampicin. It exhibited resistance to other antibiotics. SW7-1 had hemolytic activity and could produce extracellular casease, lipase, and amylase. SW7-1 could reproduce septicemia-like symptoms with high mortality rate when re-fed to healthy silkworm. .The median lethal concentration (LC_{50}) was 5.45×10^4 cfu/ml. Thus, SW7-1 was identified as *B. cereus*, which is a pathogen for silkworm and human infections are possible.

Key Words: silkworm (*Bombyx mori*), identification, characterization, *Bacillus cereus*, pathogen

The silkworm, *Bombyx mori* L. (Lepidoptera: Bombycidae), is an important economic insect that not only can produce silk but has high nutritive value. Over 30 million silkworm farmers are presently involved in the sericultural production in China across 10 provinces (Li et al. 2011, Liang et al. 2014). The silkworm is susceptible to silkworm diseases, but massive outbreaks are generally rare. Studies have investigated the pathogenicity of *Nosema bombycis* (Li et al. 2015b) and *Beauveria bassiana* (Xu et al. 2015). However, a few studies have evaluated the bacterial diseases of silkworms. Bacteria can attack physiologically weak silkworms; as a result, great losses in sericulture occur (Tao et al. 2011). Hence, unknown pathogenic bacteria should be identified and effectively controlled.

Bacterial disease commonly affect silkworm, but the etiology is not fully understood because multiple bacterial types are involved in bacterial infections (Choudhury et al. 2002, Kaito et al. 2002). Many silkworm pathogenic bacteria, including *Bacillus*, *Enterobacter*, *Serratia*, *Aeromonas*, *Streptococcus*, *Pseudomonas,* and *Staphylococcus*, have been identified (Tao et al. 2011). Although previous studies have characterized the pathogenicity of *Bacillus bombysepticus* and *Bacillus thuringiensis* to silkworm (Cheng et al. 2014, Li et al. 2014b), few studies have identified or characterized other pathogenic bacteria in the *Bacillus* genus.

In our study, a pathogenic bacterial strain, named SW7-1, was isolated from the diseased silkworm and then cultured on nutrient agar medium. The isolated strain proved to be pathogenic to healthy silkworm, and its LC_{50} was investigated. Simultaneously, SW7-1 was examined in detail to confirm its taxonomic status, and its hemolytic activity, antibiotic susceptibility, and extracellular enzyme activity were recorded. This study aimed to identify and characterize a pathogen of silkworm, as well as provide a basis for its pathogenesis and control.

Materials and Methods

Experimental Silkworm. Diseased silkworm specimens were collected from the Laboratory of Genetics and Breeding of Silkworm, College of Biotechnology of Southwest University (Chongqing, China), and they were used as a source of inocula to isolate the pathogenic microorganisms. Healthy newly molted fifth-instar larvae were used for bioassays. Silkworm variety 734 was used for the test.

Isolation and Culture. SW7-1 was isolated according to the method described by Zhang et al. (2013) with slight modifications. SW7-1 was grown on nutrient agar medium at 37°C for 48 h. The medium comprised the following (per liter): tryptone, 10 g; NaCl, 5 g; beef extract, 3 g; and agar, 20 g and neutral pH. The medium was autoclaved at 121°C for 15 min. To prepare the suspension culture, we cultivated strain SW7-1 at 37°C and 200 rpm (THZ-22 oscillator; Bing Lab Equipment Co., Ltd., Suzhou, China) for 24 h in nutrient broth medium (nutrient agar medium without agar) and stored at 4°C. Media for physiological and biochemical identification tests were prepared as described in relevant references (Barrow and Feltham 2004, Li et al. 2014a). The culture has been deposited to China General Microbiological Culture Collection Center (CGMCC), and its pathogenicity was established using Koch's laws (Liu et al. 1995).

Morphologic and Biochemical Characterization of SW7-1. Colony characteristics of SW7-1 were observed after growing on a nutrient agar plate at 37°C for 48 h, and cellular morphology was determined using light microscopy and Gram staining (Feng et al. 2011). Cell morphology was also observed under a JCM-5000 (Nikon, Japan) scanning electron microscope under ×5,400 magnification.

Physiological and biochemical analyses were performed by referring to Bergey's Manual (Holt 1994) and the Manual for the Microbiology Experiment (Shen et al. 1999).

DNA Extraction and Polymerase Chain Reaction Amplification of 16S rRNA Sequence. The genomic DNA of SW7-1 was extracted using a TIANamp Bacteria DNA Kit (Tiangen Biotech Co., Ltd., Beijing, China), quantified with a NanoDrop 2000 Spectrophotometer (Thermo, USA), and stored at −20°C until used. Polymerase chain reaction (PCR) amplification of SW7-1 was performed with a universal set of primers for the bacterial 16S rRNA gene. The forward primer was 27F: AGAGTTTGATCATGGCTCAG, and the reverse primer was 1492R: ACGGTTACCTTGTTACGACTT (Lane et al. 1985). PCR amplification was performed in a total volume of 50 µl containing 5 µl of DNA extract, 1 µl of each primer (10 mmol/liter), 4 µl of deoxyribonucleotide triphosphate mixture (each 2.5 mM), 5 µl of 10× PCR buffer (Mg^{2+} plus), and 0.3 µl of *Taq* DNA polymerase (TaKaRa, Japan). PCR was performed in a thermocycler (ABI, USA) with initial denaturation at 94°C for 4 min, followed by 35 cycles of denaturation for 40 s at 94°C, annealing at 55.0°C for 40 s, extension for 1 min at 72°C, and a final extension at 72°C for 10 min. The amplified products (∼1,500 bp) were subjected to 1.2% agarose gel electrophoresis, cloned into pMD19-T Vector (TaKaRa, Japan), and sequenced directly in Sangon Biotech Co., Ltd (Shanghai, China). The sequence was deposited in GenBank, and an accession number was obtained for it.

The sequence was analyzed online (http://rdp.come.msu.edu). Homology comparison of the almost complete 16S rRNA gene sequence of SW7-1 was performed by running it through the BLAST database on NCBI (http://www.ncbi.nlm.nih.gov/Blast.cgi). Sequence alignment was carried out using CLUSTAL X software. The phylogenetic tree was performed using the MEGA 5.05 software package (Hall 2005, Li et al. 2015a) and was constructed using the neighbor-joining method.

Hemolysis Test and Antibiotic Susceptibility Assay. The hemolysis test of SW7-1 was performed by placing the strain on a Columbia agar base (Cr-microbio Trade Co., Ltd., Jiangmen, China) at 37°C for 48 h, and hemolytic activity was observed. The antibiotic susceptibility test of SW7-1 employed the K-B disc diffusion method using 14 kinds of paper discs (Hangzhou Microbial Reagent Co., Ltd., Hangzhou, China). The zone of inhibition was measured (mm), and antibiotic sensitivity was recorded as different grades based on their activity (Thankappan et al. 2014).

Extracellular Enzyme Test. Casease medium, lipase medium, and soluble starch medium were prepared according to Li et al. (2015a) to survey casease, lipase, and amylase-producing activity of SW7-1. The bacterial isolate, SW7-1, grown overnight was inoculated on three selective media for the determination of extracellular enzyme activity. Plates were incubated at 37°C for 48 h, and the halos or clear zone around colonies were recorded.

Pathogenicity Test. A bacterial suspension of SW7-1 was prepared with 10.0 ml of sterile PBS (2 mol/liter, pH 7.4) and centrifuged at 2,314*g* (Allegra X-22R; BACKMAN, USA) for 3 min. The sediment was again suspended in 10.0 ml PBS. Its concentration was determined according to the method described by Tao et al. (2011). The feedstuff of silkworm was purchased from Shandong Academy of Agricultural Sciences (Yantai, China). The feedstuff was mixed well with ddH_2O, autoclaved at 105°C for 1 h, and stored at room temperature. Forty healthy newly molted fifth-instar larvae were included in each experimental group, and each treatment was performed in triplicate. Simultaneously, the control group was set with PBS only. The feedstuff of silkworm inoculated with an appropriate number of bacteria was fed to the larvae three times daily. The larvae were reared under hygienic conditions at 28.0 ± 1.0°C. The symptoms of the silkworms were observed and the mortality of the silkworms was recorded. Data were analyzed using SPSS 20, and the median lethal concentration (LC_{50}) was obtained (Mohanta et al. 2014).

Results

Description of SW7-1 Colonies and Microscopic Morphology. Bacteria were isolated after plating onto agar solidified nutrient medium. After routine culture on nutrient agar at 37°C for 48 h, milky, large, convex, opaque, dry, crude colonies of SW7-1 developed. The colonies had jagged edges, and a waxy substance appeared around the colony. Moreover, SW7-1 was Gram-positive and could produce spores. No crystals were found in the sporulated cultures of the SW7-1 strains examined through light and electron microscopies. Electron microscopy revealed that the cells were rods shaped with rounded ends, and the sizes of the cells were 0.8–1.2 µm in width by 2.6–3.4 µm in length (Fig. 1).

Physiological and Biochemical Characteristics of SW7-1. SW7-1 was aerobic and motile. It was positive for urease, catalase, oxidase, deoxidization of nitrate, and Voges−Proskauer reaction. However, it was negative for methyl red, indole, H_2S production, and phenylalanine deaminase reaction tests. SW7-1 produced oxidase and catalase. It could utilize sodium citrate, glucose, fructose, and sucrose, except lactose (Table 1).

On the basis of these observations, we propose that SW7-1 may be a strain of *Bacillus* sp.

16S rRNA Gene Sequence and Phylogenetic Analysis. The 16S rRNA gene sequence of strain SW7-1 was about 1,500 bp in length (GenBank accession no. KP698984). The sequence was analyzed using the RDP online software, and the taxonomic status of SW7-1 was determined directly as follows: Bacteria (domain), Firmicutes (phylum), Bacilli (class), Bacillales (order), Bacillaceae (family), and *Bacillus* (genus).

The 16S rRNA gene sequence was also compared using BLASTN, and SW7-1 showed high similarity to *Bacillus* spp. (>99%). A total of 29 sequences of different strains were selected and aligned, and a phylogenetic tree was constructed (Fig. 2). The phylogenetic tree revealed that *Bacillus cereus* strain 14 had high homology with SW7-1. Hence, SW7-1 could be a *B. cereus* (CGMCC No. 10652).

Hemolytic Activity and Antibiotic Susceptibility. The hemolysis test indicated that SW7-1 exhibited hemolytic activity and produced hemolysin. SW7-1 was highly susceptible to gentamycin, streptomycin, erythromycin, norfloxacin, and ofloxacin (zone of inhibition of >6 mm) and moderately susceptible to tetracycline and rifampicin. It was resistant to other antibiotics (Table 2).

Extracellular Enzyme Activity. SW7-1 exhibited strong amylase activity as observed by the clear inhibition zone (4–5 mm) around the colony. However, SW7-1 possessed only moderate casease activity (2–4 mm) and lipase activity.

Pathogenicity Analysis of Strain SW7-1. The SW7-1 suspension was fed to the fifth-instar larvae, and SW7-1 exerted an obvious lethal effect on the silkworms after 48 h. The peak mortality of silkworms occurred between 48 and 72 h. Simultaneously, the control groups all survived. The silkworms fed with SW7-1 exhibited symptoms similar

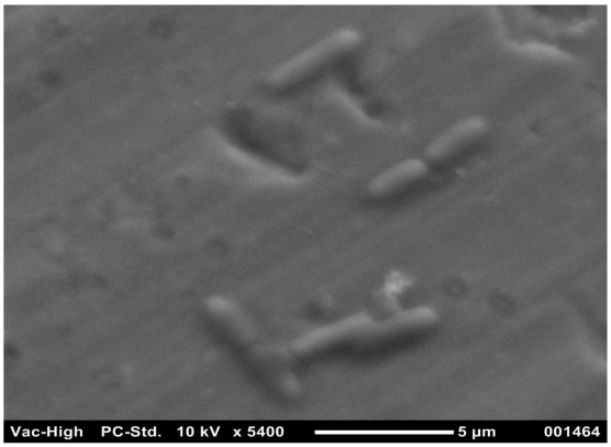

Fig. 1. Morphological features of SW7-1 under scanning electron microscope.

Table 1. Physiological and biochemical characteristics of SW7-1

Tested items	SW7-1	Tested items	SW7-1	Tested items	SW7-1
Gram strain	+	Parasporal crystal	–	H_2S production	–
Spore	+	Deoxidization of nitrate	+	Glucose fermentation	+
Motility	+	Utilization of sodium citrate	+	Lactose fermentation	–
Catalase	+	Gelatin liquefaction test	+	Sucrose utilization	+
Oxidase	+	Phenylalanine deaminase	–	Maltose utilization	+
Urease test	+	M.R. reaction	–		
V–P test	+	Indole test	–		

+, Positive; –, negative.

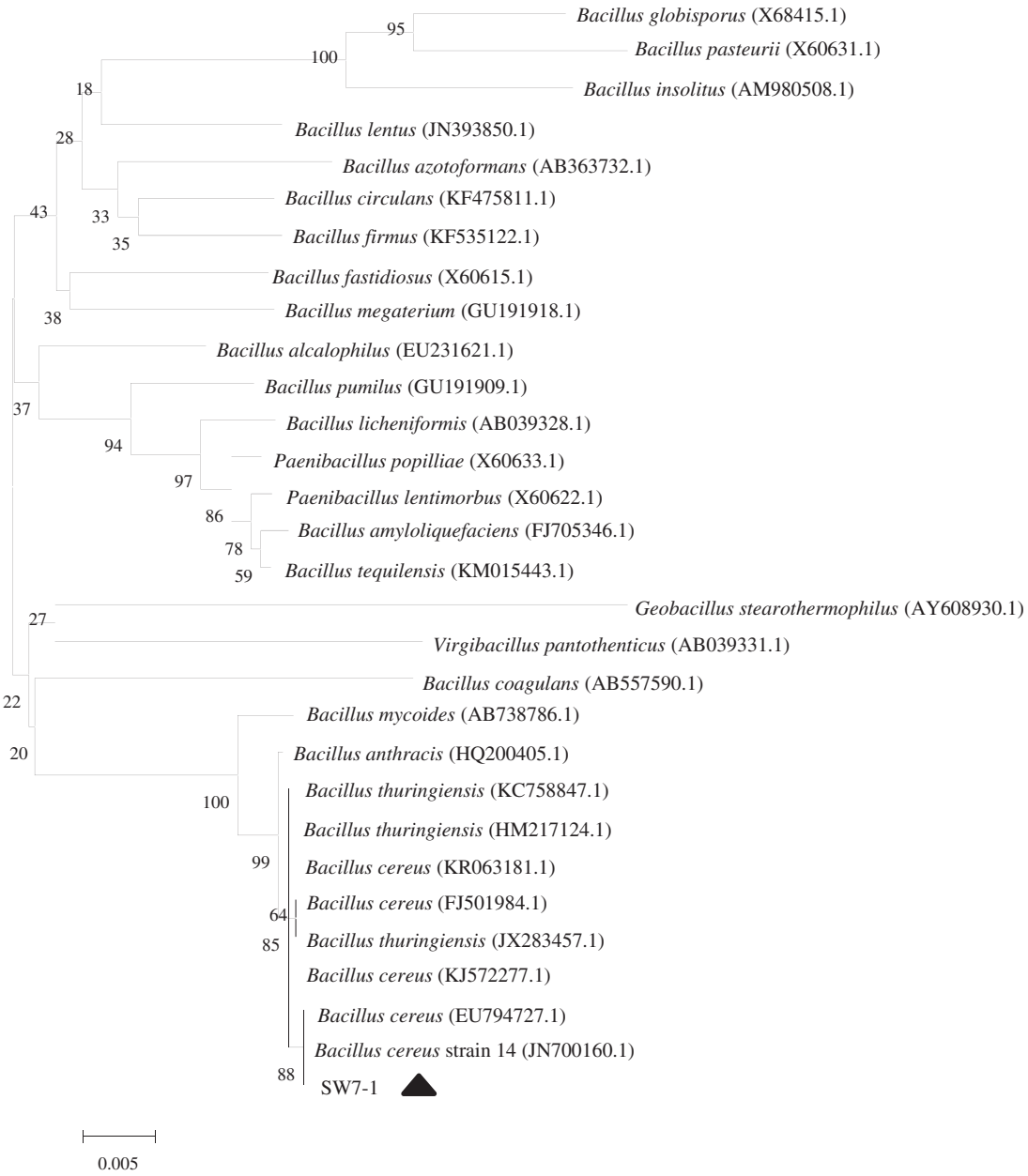

Fig. 2. Phylogenetic tree of the 16S rRNA sequences of the SW7-1 strain. The tree was constructed by the neighbor-joining method. Numbers in parentheses represent the sequences accession numbers in GenBank. The neighbor-joining consensus tree used 1,000 bootstrap replicates. The number at each branch point represents the percentage of bootstrap values.

to diseased silkworms. The mortality rate of the larvae increased with increasing bacterial concentration. Pathogenicity could be depicted as a linear regression of the logarithm (y) of SW7-1 concentration against probability (x) ($y = 1.85 + 0.6651x$). LC_{50} was found to be 5.45×10^4 cfu/ml (Table 3). The silkworm infected with SW7-1 exhibited symptoms such as loss of appetite, vomiting, diarrhea, softening of larvae, crimping of cadavers, and rotting, which were similar to those of bacterial septicemia (Fig. 3).

Discussion

The silkworm is an insect with high economic value. It occupies an important position in human economic life and cultural history. In China, sericulture is an important part of agriculture that has been in existence for more than 5,000 years. However, bacterial diseases often cause severe economic losses in sericulture. Although several bacterial diseases have been researched and described, the pathogenic mechanism of many bacteria remains unknown. Therefore, the identification and characterization of new pathogenic bacteria for silkworm is very important. We found masses of dead silkworms with the same symptom when we worked on the genetics and breeding of silkworm. Therefore, we collected diseased silkworm specimens, which we used as source of inocula to isolate pathogenic microorganisms.

In this study, the isolated bacterium was characterized by various morphological, biochemical, and molecular biological techniques and named as SW7-1. Based on morphological, physiological, and biochemical characteristics, SW7-1 was preliminarily identified as *Bacillus* sp. The phylogenetic tree and 16S rRNA gene sequence analysis showed that SW7-1 had high sequence homology with *B. cereus* strain 14 (>99%). Bacterial taxonomists believe that bacteria can be identified as the same species when their 16S rRNA gene sequence homology is higher than 99% (Fry et al. 1991, Laurentiu et al. 2014). Most microbiologists believe that *B. thuringiensis* and *B. cereus* can generally be classified on the basis of parasporal crystals, despite the difficulty in distinguishing these strains in terms of bacteriological properties (Gillis and Mahillon 2014). The presence of crystals can be determined through light or scanning electron microscopy (Priest et al. 2004). Thus, our experiments support the SW7-1 is a strain of *B. cereus*. *B. cereus* is a highly threatening hemolytic bacterium that causes diarrhea to the host organism (Thankappan et al. 2014). This study confirmed that SW7-1 was pathogenic and hemolytic against the silkworm and red blood cell, respectively, which verified its pathogenicity for sericulture and other livestock production purposes. Using an antibiotic susceptibility assay, we found that SW7-1 was highly susceptible to gentamycin, streptomycin, erythromycin, norfloxacin, and ofloxacin. However, SW7-1 is highly resistant to β-lactam antibiotics (Yim et al. 2015), such as penicillin, ampicillin, carbenicillin, amoxicillin, oxacillin, cefazolin, and ceftriaxone. This resistance may be associated with β-lactamase production (Park et al. 2009). The result of antibiotic susceptibility assay indicated that the SW7-1 bacterium was a multidrug-resistant pathogen that might lead to sudden infectious disease outbreaks.

Studies have shown that the extracellular enzymes of *B. cereus* might be related to pathogenicity (Orhan et al. 2005). The result of the extracellular enzyme test showed that SW7-1 could produce extracellular casease, lipase, and amylase, and these enzymes might contribute to pathogenicity. *B. cereus* is a pathogenic strain that does not only infect insects but also causes human diseases, such as septicaemia (Lysenko 1972, Casella and Monzani 1984, Fagerlund et al. 2007). Thus, SW7-1 was a pathogen for silkworm, and human infections are possible. The LC_{50} of SW7-1 was 5.45×10^4 cfu/ml. Previous studies have reported the LC_{50} values of several pathogens of silkworm: *Pseudmonas aurantiaca*, LC_{50} of 2.12×10^4 cfu/ml; *Serratia marcescens*, 4.32×10^4 cfu/ml; *Bacillus thuringensis*, 2.80×10^4 cfu/ml; *Providencia rettgeri*, 1.44×10^7 cfu/ml; and *Klebsiella granulomatis*, 2.54×10^7 cfu/ml (Tao et al. 2011, Zhang et al. 2013, Mohanta et al. 2014).

Table 2. Antimicrobial susceptibility and resistance pattern of SW7-1

Antibiotics (μg)	SW7-1	Antibiotics (μg)	SW7-1	Antibiotics (μg)	SW7-1
Penicillin-G (10)	R	Gentamycin (10)	S	Amoxicillin (10)	R
Oxacillin (1)	R	Streptomycin (10)	S	Rifampicin (5)	M
Ampicillin (10)	R	Tetracycline (10)	M	Ofloxacin (5)	S
Cefazolin (30)	R	Erythromycin (15)	S	Carbenicillin (100)	R
Ceftriaxone (30)	R	Norfloxacin (10)	S		

S, zone of inhibition above 6 mm; M, zone of inhibition between 4 and 6 mm; R, resistant.

Table 3. Pathogenicity of *B. cereus* SW7-1 against silkworm

Concentration of bacteria (cfu/ml)	Corrected mortality (%)	Linear regression equation	LC_{50}	95% confidence limits	Pearson correlation
1.91×10^3	23.84	$y = 1.85 + 0.661x$	5.45×10^4	28433.59-104432.18	0.961
1.91×10^4	36.47				
1.91×10^5	52.16				
1.91×10^6	78.42				
1.91×10^7	97.53				

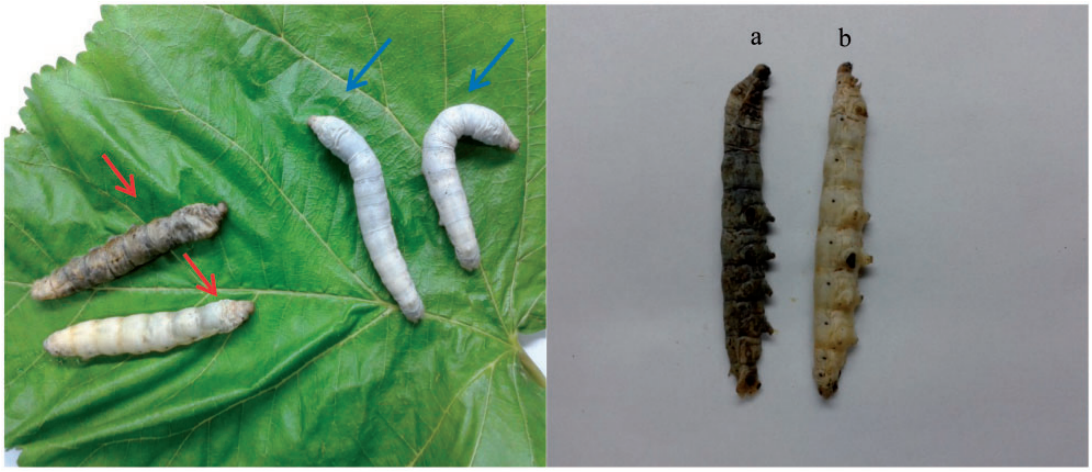

Fig. 3. Symptoms of silkworm infected by SW7-1. Blue arrow: healthy silkworm; red arrow, silkworm infect with SW7-1; a: after 48 h infection; b: after 24 h infection.

In conclusion, the silkworm pathogen SW7-1 was identified and characterized. It was hemolytic and highly pathogenic to silkworm ($LC_{50} = 5.45 \times 10^4$ cfu/ml), and this pathogen could produce extracellular enzyme and resistance to β-lactam antibiotics. Detailed studies are needed to confirm the pathogenic mechanism of the silkworm disease caused by this bacterium. These results contribute to prevent and control of disease caused by this bacterium. Hence, investigations on this bacterium have important implications in sericulture.

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