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Bacteriology

Isolation, identification and characterization of novel *Bacillus subtilis*

Zhenxiang LU[1)#*], Weina GUO[1)#] and Chang LIU[1)]

[1)]College of Animal Science, Anhui Sciences and Technology University, Fengyang, Anhui 233100, China

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ABSTRACT. In this study, we have identified a bacterium that can inhibit the growth of *Staphylococcus aureus*, and further analyzed its antibacterial activity and other biological characteristics and laid the foundation for its future application. Through isolation and culture of the unknown bacteria, the culture characteristics, morphology observation, biochemical test, preliminary antibacterial test, 16S rRNA PCR amplification, sequence analysis, and homology analysis were performed. It was found that the bacteria are Gram positive spore chain *Bacillus*. The bacteria could only ferment glucose for acid production, but could not utilize lactose and maltose. The VP test for this bacteria was positive, while indole and methyl red tests were negative. Further analysis showed that these bacteria shared a homology up to 99.4% with *Bacillus subtilis* DQ198162.1. Thus, this newly identified bacterium was classified as *Bacillus subtilis*. Importantly, the crude bacteriocin of this *Bacillus subtilis* could inhibit the growth of *Staphylococcus aureus*, *Escherichia coli*, *Enterococcus* and *Salmonella*, which implies its potential usage in the future.

KEY WORDS: *Bacillus subtilis*, bacteriocins, identification

The prevention and treatment of bacterial diseases in humans and animals rely heavily on antibacterial drugs and perpetuates the widespread phenomenon of irrational use of antibiotics. This eventually leads to the increasing resistance of pathogens to various antibacterial drugs, and even the appearance of super bacteria [4, 8, 9]. Presently, neither the non-resistant bacteria nor the bacteria that sensitive to antimicrobial drugs exists, and the increase of resistant pathogenic strains is a huge obstacle to the prevention and treatment of the disease, rendering the treatment more difficult [3]. Therefore, researchers are investigating that antibacterial active substance that hardly instigates drug resistance, in order to replace antibacterial drugs to treat bacterial diseases.

Bacteriocins are a group of proteins or peptides with antimicrobial activity that are secreted into the extracellular matrix during metabolic process of some bacteria. These proteins can impede the growth of certain microorganisms. Furthermore, these proteins can be degraded by enzymes and will not affect the human body pathogenically; thus, these are excellent potential alternatives for antibiotics. Bacteriocins, unlike antibiotics, belong to the protein substances [6, 10]. However, due to the threat of the bacteria producing its own bacteriocin, currently there are only a few bacterial bacteriocins such as Nisin produced nisin used in food as a biological preservative. Moreover, its antibacterial activity requires specific conditions and range which is a weakness when compared with the antibiotics [5, 6]. *Bacillus subtilis* are recognized as safe and reliable probiotics strains that are non-pathogenic to humans and animals. Bacteriocin protein or polypeptide is generated during the process of growth and reproduction of this bacterium. This substance has a high antibacterial activity, broad antibacterial spectrum and excellent thermal stability. Moreover, pH value has little effect on stability or activity. Therefore, it has a great potential to be used as an alternative to antimicrobial drugs. *Bacillus subtilis* is a typical germ, which is rod-shaped and Gram-positive. When cultured on ordinary nutrient agar, the morphology circular colony of this bacteria is rough, opaque, fuzzy white or slightly yellow with jagged edges [1, 7].

Currently, the identification of bacteria is conducted mainly through conventional methods, such as observing bacterial colony characteristics and morphology and biochemical tests for comprehensive judgments. Although these methods can identify the most common clinical bacteria, the current type and nature of the clinical pathogens causing infection is more complex and thus the identification results are often not satisfactory. With the improvement of the nucleic acid sequence analysis techniques, the conserved bacterial genomic regions are sequenced compared with that from the GenBank sequences. Therefore, homology analysis has become the preferred approach for rapid identification of bacteria due to its accuracy in identifying the bacteria to genus [12].

The present study has isolated and identified a unknown bacteria, which was occasionally found in the *S. aureus* culture medium and has a significant inhibition effect on its growth. 16S rRNA sequence tests and homology analysis were first performed and then its activated culture was processed on and transferred to LB (Luria-Bertani) liquid medium to obtain fermentation broth by culture

*Correspondence to: Lu, Z.: luzhenxiang2002@126.com #These authors contributed equally to this work.

propagation. Subsequently, centrifugation, acid precipitation, and other methods were used to obtain the crude extract of fermented supernatant and bacteriocins. An antimicrobial round paper containing the fermentation broth and crude extract bacteriocins was further prepared inhibition test carried out.

MATERIALS AND METHODS

Materials

Strain: Isolation and identification of a unknown bacterium, which is occasionally found in the *S. aureus* culture medium exert a significant inhibitory effect on its growth.

The antibiotic test consists of a cohort of 19 bacteria that were obtained from the Laboratory of Animal Medicine at Anhui Science and Technology University (Anhui, China).

Reagents and culture medium: For preparation of culture medium, peptone, beef extract powder, sodium chloride, potassium dihydrogen phosphate, agar strip, dipotassium hydrogen phosphate, tryptone, yeast extract, pH 6.5 phosphate buffer, and concentrated hydrochloric acid were used. Nutrient agar, MacConkey agar, rabbit blood agar, TSA agar containing 5% fetal calf serum, Chapman agar, LB agar, LB broth, peptone water, aqueous glucose peptone, glucose fermentation tube semi-solid, semi-solid maltose fermentation tube and semi-solid lactose fermentation tube were utilized in this research. Reagents (analytical grade) were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

Equipment: PCR instrument (Germany Biometra Co.), gel imaging system, electrophoresis apparatus (Bio-Rad, San Francisco, CA, U.S.A.), weighing balance (Shanghai Medical Laser Instrument Factory, Shanghai, China), Centrifuge 5424 (Eppendorf, Germany), Mastercycle Limited Nexus PCR Cycler, incubator (Shanghai Boxun Industrial Co., Ltd., Shanghai, China), LDZX-SOFBS vertical pressure steam sterilizer (Shanghai Shen An Instrumentarija, Shanghai, China), clean benches (Shanghai three rounds of Scientific Instruments Co., Shanghai, China), and optical microscope (SA3000, the Beijing Tektronix Instrument Co., Beijing, China) were used.

Methods

Isolation and culture of the unknown bacteria: The unknown bacteria were streaked on nutrient agar, MacConkey agar, Chapman agar, rabbit blood agar and TSA agar containing 5% fetal calf serum, and LB agar. The culture was inoculated into liquid LB culture medium and cultured at 37°C for 18–24 hr with vigorous shaking.

Bacteria smearing, staining, and morphology: The unknown bacteria were stained by Gram method and observed by optical microscope under the oil-immersion lens.

Biochemical tests of the bacteria: The unknown bacteria were subjected to carbohydrate fermentation test, indole formation, methyl red (MR) and VP tests, respectively.

Preparation of the fermentation broth by unknown bacteria: A single colony inoculation of the unknown bacterium was inoculated into the LB liquid medium, shaking at 37°C for 48 hr. Then the culture was centrifuged at 8,000 rpm at 4°C for 10 min and the supernatant was collected. The fermentation broth was equally divided into two parts, one for preparing a fermentation broth containing antibacterial round paper, another part for the preparation of a crude extract of bacteriocins.

Crude extract of bacteriocins from unknown bacteria and preparation of antimicrobial round paper: According to Wellinghausen *et al.* [15], acid precipitation methods was used to obtain the bacteriocins crude extracts from unknown bacteria. With concentrated hydrochloric acid adjusted to pH 2.0 at 4°C, the fermentation supernatants precipitated overnight, followed by centrifugation at 10,000 rpm at 4°C for 20 min. The supernatant was discarded, and the precipitate was solubilized in phosphate buffer with pH 6.5. The crude extract of bacteriocin, was stored at −20°C until further use.

Six mm diameter filter papers with uniform thickness were high-pressure sterilized and dried. The paper was soaked in the crude extract of bacteriocin for 30 min and air dried for future use.

Antibacterial spectrum detection

Culture of indicative bacteria: The aerogenes of the indicated bacteria such as, *Bacillus subtilis*, *Staphylococcus aureus*, Staphylococcus white (a total of four from healthy chicken skin, colon, oral, and intranasal were isolated, respectively), chicken white grapes cocci (isolated from dead chickens' liver), *Escherichia coli* (four isolated from healthy cows, within cattle, goats, and chicken dung), *Escherichia coli* (isolated from dead chickens liver), chicken pathogenic *E. coli* (isolated from dead chickens liver), enterococci (from healthy rats, cows, cattle isolated total 3), chicken Salmonella (isolated from dead chickens liver) and a total of 19 strains of *Pseudomonas aeruginosa* were provided by Anhui Science and Technology University Animal Medicine Laboratory. The strains mentioned above were streaked on ordinary nutrient agar medium and cultured at 37°C for 18–24 hr.

Antibacterial tests: The antibacterial tests were performed using the spread plate method. With a right amount of scrape coating bar, it can develop into a good bacterial indicator when uniformly applied on the surface of the nutrient agar. Ophthalmic forceps were used and the fermentation broth containing the crude extract of bacteriocin antibacterial paper affixed to a uniform coating on the medium, placed at 37°C for 18–24 hr, and the results were observed and recorded.

Preparation of the bacterial DNA template: Single bacterial colonies were inoculated into LB liquid medium and agitated at 37°C overnight and then 1 m*l* medium was centrifuged at 8,000 rpm for 5 min. The supernatant was mixed with 50 μl water and heated for 10 min, and the ice-cooled for 5 min. The reaction was then centrifuged again at 12,000 rpm for 5 min, and the supernatant was collected as the bacterial DNA template.

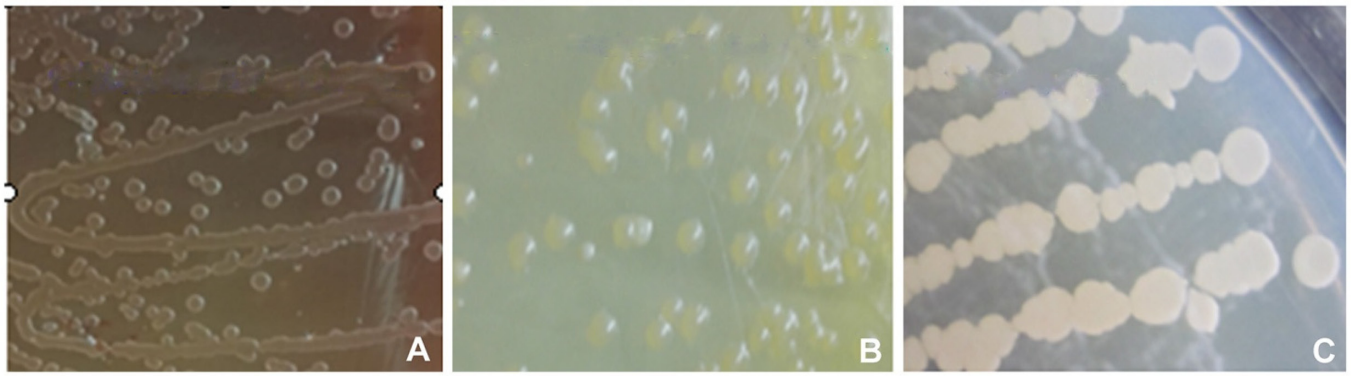

Fig. 1. Growth performance of the bacteria on a variety of media. A. Chicken blood agar. B. TAS agar containing 5% fetal calf serum. C. Nutrient agar.

Table 1. Growth performance of the bacteria on a variety of media

Media types	Colony characteristics
Chapman agar and MacConkey agar	No growth
TSA agar containing 5% fetal calf serum	Gray-white, round, opaque, thick ridges, smooth, moist, medium-sized colony
LB agar	Gray-white, round, opaque, flat, drying, medium-size colony
Rabbit blood agar	Gray white, round, completely hemolytic, opaque, flat, drying, medium-size colony
Nutrient agar	Gray-white round, opaque, flat, drying, medium-size colony

Bacterium's 16s rRNA PCR amplification: The universal bacterial primers [11] were synthesized by the General Biological Systems (Anhui) Co., Ltd. as described previously. The following primers were used in this study:

Forward primer: 5′-AGAGTTGATCCTGGCTAAG-3′

Reverse primer: 5′-GGTTACCTTGTTACGACTT-3′

The constitution and condition for PCR reaction was described as follows:

10 × PCR Buffer 5 μl, 4 × dNTPs (10 mmol/l) 4 μl, TaqDNA polymerase (5 U/μl) 0.5 μl, upstream and downstream primer (concentration, 50 μmol/l) each 1 μl, template 1 μl, and ultra-pure water to a final volume of 50 μl.

The reaction conditions were: 94°C denaturation for 5 min, 35 cycles of 94°C denaturation 1 min, 55°C annealing 1 min, 72°C extension 1 min and a final extension at 72°C for 15 min.

Electrophoresis of bacterial PCR amplification products: After PCR amplification, 5 μl products was analyzed by 1% agarose gel electrophoresis at 110 V for 30 min along with a molecular weight standard DL 2000 Marker. The products were visualized on the gel imaging system, and images captured.

Sequencing of bacterial PCR products for homology analysis: The PCR products were sequenced by the General Biosystems (Anhui, China) Co., Ltd. BLAST program was used to align the sequence with that from the GenBank database for homology analysis.

RESULTS

Growth performance of the unknown bacteria on different media

The growth performance of the unknown bacteria on rabbit blood agar was firstly shown in Fig. 1A, from which we can see that the colony formed was gray white, round, opaque, flat, drying, medium-sized and completely hemolytic (Table 1). Figure 1B showed the growth performance of this strain on TSA agar with 5% fetal calf serum, it can be seen that the colony was also gray-white, round, opaque, medium-sized, but also has thick ridges, smooth and moist colony (Table 1). The growth performance of this strain on nutrient agar was shown in Fig. 1C, it can be seen that the colony formed was characterized by gray-white, round, opaque, flat and dry with medium-size, which was similar to the colony on LB agar. However, this unknown bacterium did not growth on Chapman or Mac Conkey agar (Table 1).

After shaking culture or stationary culture in LB liquid medium at 37°C for 24hr, the growth performance of this bacterium was shown in the left and right tube of Fig. 2, respectively. The results showed that the bacteria in left tube and right tube both formed biofilm, but the turbidity degree was different. The left tube was turbid while the right tube was clarity, and the turbidity was decreased slightly from top to bottom in the left tube. In addition, there was flocculent precipitate in the bottom by shaking culture (Fig. 2).

Interestingly, an unknown bacterium was accidentally isolated from *Staphylococcus* aureus culture plate which significantly

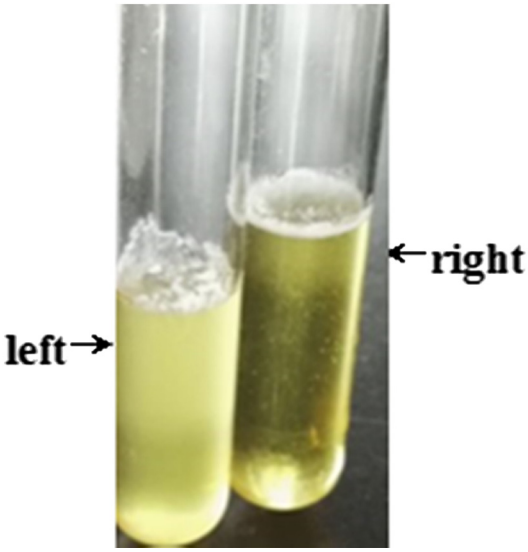

Fig. 2. The growth performance of unknown bacterium was shown in LB liquid medium. Bacteria were cultured for 24 hr in LB liquid medium at 37°C, left tube is denoted shaking culture, and the right tube was by stationary culture.

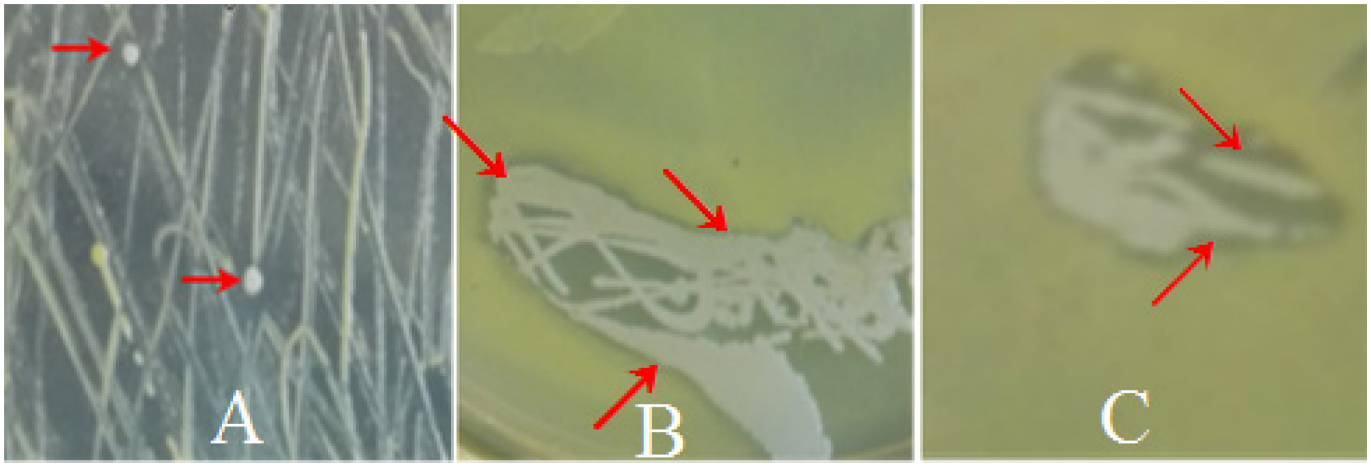

Fig. 3. Unknown bacterium significantly inhibited the growth of S. aureus. The gray-white colony is the unknown bacterium, and the surrounding *Staphylococcus aureus* is not growing. The arrows in A, B and C of Fig. 3 were all denoted the inhibitory effect of this unknown bacterium.

inhibited the growth of S. aureus (Fig. 3A–C). The arrows in Fig. 3 indicated that the bacteria growth around the colony or lawn of *Staphylococcus aureus* were all inhibited.

Gram-stained bacterial smears

We then carried out gram staining to analyze the characteristic of this unknown bacterium. The results was presented in Fig. 4, from which we can see that this bacterium belong to the Gram-positive class by morphological observations and was identified as Gram-positive *bacillus*, which can form spores (Fig. 4).

Biochemical test results of the bacteria

Biochemical test was further employed to analyze the characteristic of this bacterium. The results clearly showed that the bacteria could only ferment glucose acid production, but not lactose or maltose. The VP test was positive, whereas the indole formation and methyl red tests were negative.

Preliminary bacteriostatic test of the supernatants and crude bacteriocin of the bacteria

Table 2 shows that the bacteria fermentation broth is inferior to crude bacteriocins inhibitory effect, indicating that the content of the bacterial factors influences the size of the inhibition zone. The bacteriocins content is positively correlated with the inhibitory effect. In addition, the fermentation of the other metabolites is seen in the serum fatty acid and ammonia without a significant inhibitory effect.

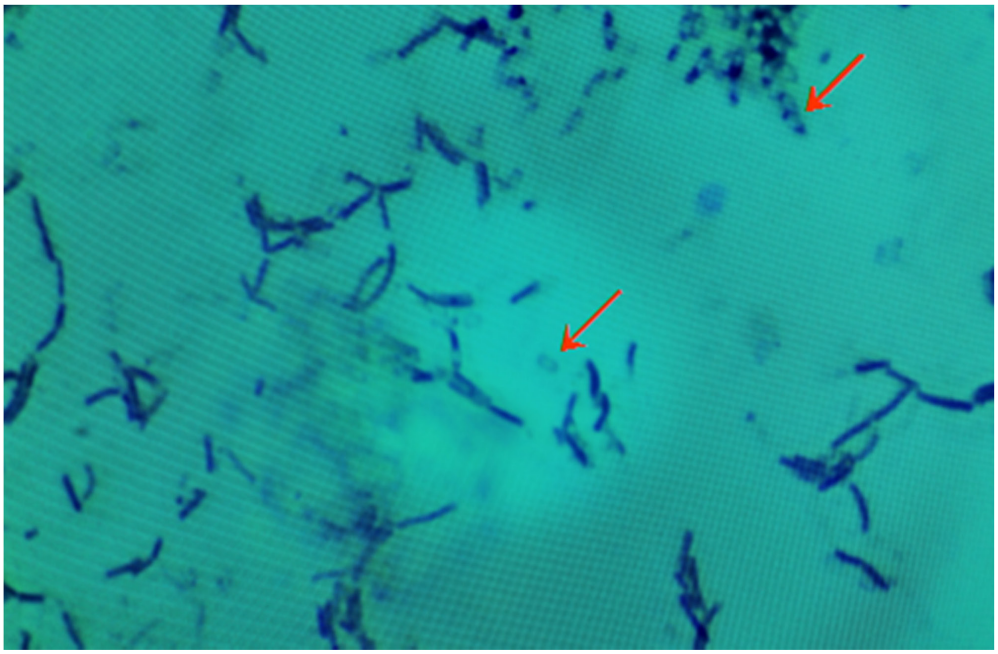

Fig. 4. Gram staining to analyze the characteristic of unknown bacterium. The arrows showed the forming spores of this unknown bacterium.

Table 2. Antibacterial test results of bacteria fermentation broth and crude bacteriocins

Indicator bacteria	Inhibition zone diameter (mm)	
	The crude extract bacteriocin	Fermentation broth
The unknown bacteria	0	0
Bacillus subtilis	11.0	0.0
Staphylococcus aureus	9.0	7.0
Staphylococcus albus isolated from the large intestine	8.5	7.0
Staphylococcus albus isolated from skin	9.0	8.0
Staphylococcus albus isolated from mouth	10.0	7.5
Staphylococcus albus isolated from nasal cavity	8.0	8.0
Chicken *Staphylococcus albus*	8.0	6.5
E. coli isolated from healthy dairy cows	10.0	7.0
E. coli isolated from healthy cattle	7.0	7.5
E. coli isolated from healthy chickens	12.0	7.5
E. coli isolated from healthy goats	9.0	0
E. coli isolated from the liver of dead chickens	7.5	8.0
Enterococci isolated from healthy rat	8.5	7.5
Enterococci isolated from healthy dairy cows	7.0	0
Enterococci isolated from healthy cattle	11.0	0
Salmonella gallinarum	7.5	6.5
Aerobacter aerogenes	0	0
Pseudomonas aeruginosa	0	0

Bacteria PCR amplification results

Furthermore, PCR method was used to amplify the target fragment from the genomic DNA of the bacteria. After electrophoresis analysis, we can see that the size of the PCR product is consistent with the expectation (Fig. 5).

Analysis of sequence evolution of bacterium AH1005

The 16S rRNA sequence of isolate AH1005 bacteriocin-producing strain has been registered in the GenBank Database with the accession number (KT804652) and The sequence was aligned to those available in the NCBI database. The alignment results showed that the isolated strain AH1005 was closely related to *Bacillus subtilis* DQ198162.1, which with an identity of 99.4% (Fig. 6). The eight Bacillus strains, including *Bacillus licheniformis* AY601721.1, amyloliquefaciens AY651023.2, *Bacillus subtilis* DQ198162.1, *Bacillus circulans* AB215100.1, *Bacillus cereus* DQ207729.2, *Bacillus pumilus* AB098578.1, vegetables *Bacillus*

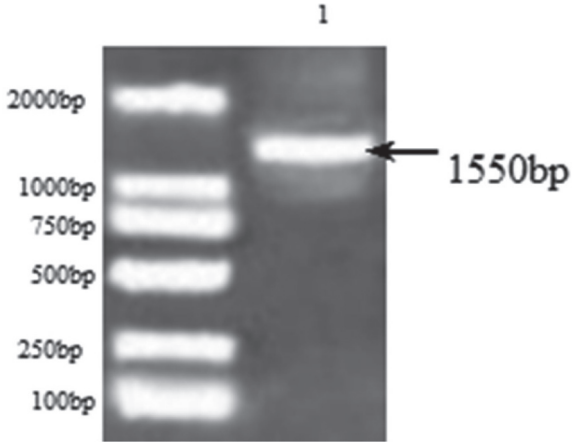

Fig. 5. Results of PCR amplification and electrophoresis analysis of 16SrRNA from of AH1005. Lane M: DL2000 DNA ladder; Lane 1: PCR amplified 16S rRNA gene product from the AH1005.

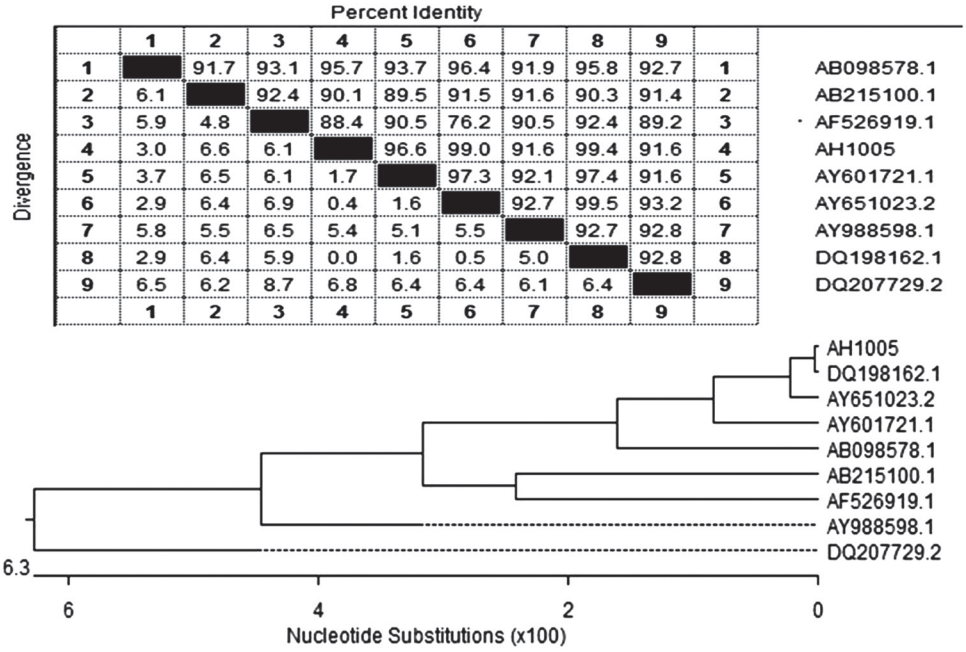

Fig. 6. Analysis of sequence evolution of unknown bacterium. The AH1005 bacteria and eight strains of Bacillus bacteria 16S rRNA gene homology and the phylogenetic tree map.

AY988598.1 and *Bacillus firmus* AF526919.1 were selected for further comparison. Finally, an phylogenetic tree was constructed using DNASTAR software and the genetic distance of each strain was analyzed. The results are illustrated in Fig. 6,

DISCUSSION

Occasionally, on nutrient agar medium, we found *S. aureus* culture contaminated with another bacteria with gray, flat, opaque, rough surface, approximately circular colonies, around the medium-sized, golden-yellow colonies of *S. aureus*. Thus, the *S. aureus* colonies did not grow out long, and the contaminant bacterial metabolites can inhibit its growth and reproduction. After analysis through various methods, the strain was identified as Gram-positive bacteria of *Bacillus subtilis* DQ198162.1 with a homology of 99.4%. Bosshard *et al*. [2] demonstrated that 95–99% of the similarity of 16S rRNA gene sequence between two bacteria hints towards a similar species while >99% indicates the same bacteria. Therefore, the strain identified in this study belongs to the *Bacillus subtilis*. The crude extract of bacteriocin in addition to the other bacterial aerogenes and *Pseudomonas aeruginosa* have a different inhibition capability on the growth of the bacteria. The crude extract of bacterial bacteriocins of Gram-positive bacteria has an excellent antibacterial effect, probably due to a film produced by the ion channel that can change the cell permeability,

amino acid infiltration and lead to cell death finally. The susceptible membrane receptor activates its role upon the binding of bacteriocins, and therefore, their close correlation with the general microorganisms have a strong antibacterial activity [13, 14]. Consecutively, previous studies have shown that the bacteriocins from Gram-positive bacteria can also lead to bacterial cell autolysis by subtilisin. Herein, the bacteriocins from Gram-negative bacteria *E. coli* also has antibacterial effects that might work through bacteriocins, and thus, *E. coli* cells also lead to autolysis. In the present study, the crude extract of bacteriocin was prepared by the acid precipitation method. Compared to the conventional ammonium sulfate precipitation method, the precipitation from this study contained more bacteriocins that resulted in increased bacteriocin activity potency. Therefore, deeming this extraction process as a satisfactory choice [15]. In order to exclude the interference of other metabolites of fatty acids and ammonia produced during fermentation of the test, the fermentation supernatant in this test served as a control group. Moreover, the bacteriocins we get in this study was only a crude extract using the conventional culture method. Therefore, future studies of bacterial fermentation conditions are required to optimize the extraction method to increase the yield of bacteria used in actual production.

The strain of *Bacillus subtilis* found on MacConkey agar and Chapman agar medium did not grow; however, it grow better on TSA agar medium containing 5% fetal calf serum with circular ridges, smooth, moist, sticky and medium-sized colonies. LB agar and nutrient agar showed nearly circular, flat, dried colonies. The rabbit blood agar displayed completely smooth, moist, uplifted, hemolytic colonies. In LB broth medium, surface biofilm generation, fluid and transparent culture, bottom floc after the shock, indicate that the bacteria are aerobic, consistent with the characteristics of *Bacillus subtilis*. Further, during the preparation of the broth, the strain in the liquid medium surface readily forms a biofilm, culture broth remains transparent for 48 hr. Thus, it was suspected that the biofilm bacteria interfering with the growth and reproduction of bacteria are aerobic, and upon agitation, the liquid culture turns muddy (Fig. 2).

Based on the results above, we concluded that the unknown bacterium is *Bacillus subtilis*. The strain of *Bacillus subtilis* generated crude bacteriocin has a broad antibacterial spectrum: it can inhibit the the growth and reproduction of *Bacillus subtilis, Staphylococcus aureus*, *Enterococcus*, *Salmonella* and chicken *E. coli* and other bacteria, such as Gram-positive bacteria. All these results support that metabolic products of *E. coli* and other Gram-negative bacteria have a strong antibacterial effect, which will be wiht great potential for further development and utilization.

COMPETING INTERESTS. The authors have declared no competing interests.

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