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Acetobacter bacteria are found in Zhenjiang vinegar grains

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ABSTRACT. Zhenjiang vinegar, the grains of which contain a unique microbial flora, is one of the four famous traditional Chinese vinegars. We investigated the components of Zhenjiang vinegar grains. Unique acetic acid bacteria were randomly isolated from Zhenjiang vinegar grains, and the obtained strains were qualitatively analyzed to compare their capacities for acetate decomposition and acid production. Acetic acid bacteria with a high acid-producing rate were identified by 16S rDNA sequencing, and further confirmation was performed using the Basic Local Alignment Search Tool comparison method. Six significant strains of acetic acid bacteria were isolated. Qualitative analysis showed that these strains produced no brown precipitate and had a capacity for acetate decomposition. Based on physiological and biochemical evaluation, the two strains with the highest acid yield were sequenced, and the results identified strain W1 as *Acetobacter aceti* and strain W6 as *A. pasteurianus*.

Key words: Vinegar; Acetic acid bacteria; High-acid producer; 16S rDNA sequencing; *Acetobacter* bacteria

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INTRODUCTION

Vinegar is a highly nutritious acidic seasoning that originated in China over 3,000 years ago. The fermentation techniques for vinegar can be roughly divided into solid-state fermentation and liquid-state fermentation. In China, most manufacturers use the traditional solid-state fermentation, whereas in Europe and the USA, liquid-state fermentation is more popular. The different fermentation techniques result in the diverse characteristics of the different types of vinegar.

Studies on vinegar have primarily focused on the isolation and identification of acetic acid bacteria, the taxonomic study of acetic acid bacteria, the selection and culturing of efficient acetic acid bacteria, improvements in brewing techniques, and the development of new types of fruit vinegar (González et al., 2006; Gullo et al., 2006; Cleenwerck and De Vos, 2008). Because the conversion rate of liquid-state fermentation, the speed of acid production, and the flavor of vinegar are all dependent on the quality of the acetic acid bacterial strains, the selection and culture of excellent acetic acid bacteria have attracted much attention from scholars. Currently, studies on the selection, culture, and improvement of acetic acid bacteria are focused in two directions: the selection and culturing of heat-resistant bacteria (Lu et al., 1999; Ndoye et al., 2006, 2007a,b; Matsutani et al., 2013) and the selection and culturing of high-acid-producing acetic acid bacterial strains (Beppu, 1993-1994; Steiner and Sauer, 2003; Nakano et al., 2004, 2006; Wu et al., 2007; Nakano and Fukaya, 2008). Heat-resistant acetic acid bacteria are selected and cultured because optimum ethanol oxidation by these bacteria requires a stable culture temperature (usually 30°C), and strains that produce high levels of acid play an essential part in the production of highly acidic vinegar, which is one way to satisfy the increasing global demand for vinegar produced by liquid-state fermentation.

Zhenjiang aromatic vinegar is traditional in China. It is popular across the country owing to its rich fragrance and mild acidity, and because of its principal raw materials, especially high-quality sticky rice. Another reason for its popularity is that it is produced by a unique solid-state layered fermentation brewing technique that uses self-provided vinegar grains. The high total acidity (an average of 6.82%) of Zhenjiang aromatic vinegar indicates that it contains abundant acetic acid- and organic acid-producing microbes. However, studies on Zhenjiang aromatic vinegar are scarce. Zhu et al. (2008) performed a randomized screen for acid-producing bacteria in Zhenjiang aromatic vinegar and successfully isolated five bacterial strains, which were identified as *Staphylococcus* sp, *Paenibacillus* sp, *Lactobacillus paracasei*, *Acetobacter pasteurianus*, and *Bacillus ruris*, with only one strain belonging to the acetic acid bacteria. Xu et al. (2007) analyzed the bacterial populations of the Zhenjiang Hengshun aromatic vinegar grains during acetic acid fermentation using a culture-independent method. According to sequence analysis, the bacteria were divided into 16 genera: five belonging to *Lactobacillus* sp and two belonging to *Acetobacter* sp. However, both of these studies used qualitative methods, and quantitative studies on Zhenjiang vinegar have not been published.

In this study, unique acetic acid bacteria were isolated from Zhenjiang vinegar grains. The acetate-decomposing and acid-producing capacities of the screened bacterial strains were compared quantitatively, and those with a high acid-producing rate were identified by 16S rDNA sequencing and compared using the Basic Local Alignment Search Tool (BLAST). The results of this study may provide a theoretical basis for the discovery of the distribution and function of the unique brewing microbes that are used in Zhenjiang vinegar, and may provide important fundamentals for the further improvement of bacterial strains for industrial application.

MATERIAL AND METHODS

Materials

Vinegar grains were purchased from the Zhenjiang Hengshun Vinegar Factory. The liquid culture media consisted of distilled water (100 mL), yeast cream (1 g), glucose (1 g), calcium carbonate (2 g), and absolute ethyl alcohol (3.5 mL), and was modulated to a final pH of 6.5-6.8 (Zheng et al., 2010). The solid culture media consisted of distilled water (100 mL), yeast cream (1 g), glucose (10 g), calcium carbonate (2 g), and agar (1.5 g); and the liquid culture media contained distilled water (100 mL), yeast cream (1 g), glucose (1 g), and absolute ethyl alcohol (3.5 mL), and was modulated to a final pH of 5.5. The culture media for the acid production test consisted of distilled water (100 mL), yeast cream (1 g), and glucose (1 g), and was modulated to a final pH of 4.5; 7 mL ethyl alcohol was added after sterilization. The major devices included a MyGene polymerase chain reaction (PCR) amplifier (Shimadzu, Japan), a WV-BP330/CH gel-imaging system (Jiangsu Jieda, China), and a LY-HPCCD image device (Chengdu Liyang, China).

Isolation and purification of acetic acid bacteria

After sampling 3% of the vinegar grains, the bacteria were inoculated into liquid culture media and cultured at 30°C and 100 revolutions per min (rpm) for 48 h. The solid culture medium was coated with an aliquot of the sample liquid, and after culturing at 30°C for 72 h, isolated colonies with obvious crystal rings were selected for purification. The strains were then seeded onto an agar slant, cultured at 30°C for 72 h, and preserved at 4°C in a refrigerator. The isolation procedure consisted of the following: sampling, selection of single colonies, isolation and culture, morphology observations, liquid fermentation and screening culture, determination of the acid-producing rate, selection of high acid-yielding strains, and molecular biology identification.

Preliminary screening and re-screening of acetic acid bacteria

The re-purification of strains was performed as in the preliminary screening. The selected strains were marked as W1, W2, W3, W4, W5, or W6, inoculated into conical flasks (three flasks for each strain) containing liquid culture media, and cultured at 30°C and 100 rpm for 72 h. The total acidity of the fermentation liquid was determined, and the acid-producing efficiency of each strain was calculated.

Identification of strains

Based on "The handbook of systemic identification of common bacteria", the isolated strains were qualitatively analyzed and identified (Dong and Cai, 2001). For the qualitative analysis, 3 mL fermentation liquid was transferred to a tube, and the liquid was alkalized by the addition of 1 M NaOH. Three drops of 10% $FeCl_3$ were then added, and the liquid was boiled. A bronze-colored sediment indicated acetic acid bacteria. Calcium acetate was used as the carbon source when evaluating the decomposition of acetate. Yeast cream was added to a plate, and colonies were observed after cultivation. A milk-white ring around the colonies

indicated the ability to decompose acetate. The acid-producing capability of the selected strains was evaluated using an acid-producing test with shaking cultivation. The total volume of the culture ferment was 150 mL, giving culture ferment a loading capacity of 50 mL of fermentation broth. The culture was incubated at 30°C and 100 rpm for 5 days, and the final acidity was determined using NaOH acid-base titration with phenolphthalein as the indicator. A 1-mL sample was transferred to a 125-mL conical flask to which 30 mL distilled water was added followed by two drops of phenolphthalein. NaOH (0.1 M) was added until the liquid became red. The volume needed for titration was recorded, and the acetic acid content was calculated using the formula:

$$\text{Acetic acid (g/100 mL)} = 0.0937 \times V_{NaOH} \times 0.6 \times 100 \qquad \text{(Equation 1)}$$

where V_{NaOH} is the volume of NaOH.

Molecular biology identification (Qiao et al., 2011)

DNA extraction

Eugonic colonies were selected for inoculation and cultivation in liquid culture media until the logarithmic phase of growth was reached. A small amount of bacteria was removed by centrifugation, and DNA was extracted using cetyltrimethylammonium bromide. After purification with phenol chloroform, the DNA samples were stored at -20°C.

PCR

The PCR amplification of 16S rDNA was performed using the total DNA of the TY.GF1 strain as a template and universal primers for the 5'- and 3'-termini of bacterial 16S rDNA [F (8F): AGAGTTTGATCATGGCTCAG; R (1492R): GGTTACCTTGTTACGACTT] (20). The total volume was 25 µL, and the mixture contained 2.0 µL template DNA, 2.5 µL 10X Ex Taq Buffer (Takara, Dalian, China), 1 µL dNTPs (10 mM), 0.5 µL forward primer (20 µM), 0.5 µL reverse primer (20 µM), 0.5 µL Ex Taq polymerase, and 18 µL double-distilled H_2O. The PCR regimen comprised: denaturation at 94°C for 4 min; 30 cycles of 94°C for 30 s; 52°C for 30 s; 72°C for 1 min; extension at 72°C for 10 min; and storage at 4°C. The PCR products were separated by agarose gel (1%) electrophoresis and detected.

Recycling of PCR products and target fragment ligation

After agarose gel electrophoresis of the PCR products, the DNA was recovered and purified using a quick gel extraction kit (TaKaRa, Japan). The target DNA fragment and the pMD18-T vector were then ligated overnight in 15 µL containing 9 µL of the extracted products, 0.5 µL T vector, 4.5 µL solution I and 1 µL double-distilled H_2O. The ratio of the PCR products to vector DNA was optimized based on the concentration of the PCR products. In this study, the molar ratio of vector DNA:PCR products was 1:5.

Plasmid DNA transformation

After recovery (37°C, 24 h) of *Escherichia coli* DH5α from storage conditions at

-70°C, a single colony was transferred to 3 mL Luria-Bertani (LB) culture medium and cultured at 37°C for 12 h. The bacterial suspension was transferred to 100 mL LB culture media at a ratio of 1:100 and cultured at 37°C for 3 h until the optical density measured at 600 nm approached 0.5, and competent cells were prepared using a kit. The procedures included transferring 1 mL bacterial media to a 1.5-mL Eppendorf tube, centrifugation at 4000 rpm for 5 min, transferring the supernatant, adding 0.1 mL pre-cooled 1X Single Step Competent Cell Preps Kit (SSCS) solution (Sangon Biotech, Shanghai, China), gently suspending the pellet and then storing the bacteria at -70°C for further application. The products of the overnight ligation were added to the prepared competent bacteria (100 μL), mixed gently, incubated in an ice-bath for 30 min, heat-shocked at 42°C for 90 s, and then quickly transferred to ice and incubated for 10 min. LB medium (900 μL) at 37°C was then added. After cultivation at 37°C and 180 rpm for 1.5 h and centrifugation at 8000 rpm for 5 min, the supernatant was discarded. Two hundred microliters of supernatant remained, to which 5 μL ampicillin (Amp) was added. LB plates with Amp/IPTG/X-Gal were inoculated with the supernatant, and the plates were placed upright for 30 min and then in an inert position for overnight cultivation.

Restriction enzyme digestion

After blue-white selection, single, white colonies were transferred to 5 mL LB medium containing 5 μL Amp and cultured overnight. Plasmid DNA was then extracted using a UNIQ-10 column-type plasmid extraction mini kit. Restriction enzyme digestion was performed to identify recombinant plasmids using a reaction system that contained 1 μg plasmid, 1 μL *Bam*HI, 1 μL *Eco*RI, 2 μL Buffer K, 2 μL bovine serum albumin, and double-distilled H_2O in a total volume of 20 μL. The procedures included thermostatic water bath digestion at 37°C, slight centrifugation, collection of the reaction solution, and agarose gel (1%) electrophoresis identification.

Preparation and sequencing of bacteria

Bacterial culture (100 μL) that was identified as containing the recombinant plasmid was transferred to 5 mL LB medium and cultured overnight. PCR amplification was then performed using DNA from these bacteria as the template and M13 primers (F: 5'-TGTAAAA CGACGGCCAGT-3'; R: 5'-CAGGAAACAGCTATGAC-3') to identify the strains. Strains with the expected results were stored in 50% glycerol and sent to Shanghai Sangon, China, for identification through sequencing.

The sequencing results were edited and analyzed using EditSeq (DNASTAR, USA), and sequence joining and assembly were performed using SeqMan (DNASTAR). Clustal X 1.83 was used for sequence alignment analysis and heritable variation analysis, and phylogenetic tree constructions were accomplished with Mega 5.05. Phylogenetic trees were established using the neighbor-joining method (Kumar et al., 2004). Genetic distances were calculated using the Kimura 2-parameter method, and the stability of the relationships was assessed using 2000 bootstrap analyses (Wu et al., 2012).

Statistical analysis

The values are reported as the mean of individual, triplicate experiments. The statisti-

cal significance was analyzed using the Student *t*-test and regression analysis, and the data were fitted using the SPSS 19.0 software.

RESULTS AND DISCUSSION

Qualitative analysis

The qualitative analysis of the six strains mentioned above was performed, and the results of the bronze-colored sediment test indicated that they were all acetic acid bacteria. Based on further evaluation, these strains were shown to be capable of decomposing acetate (Table 1).

Table 1. *Acetobacter* identification results.

	W1	W2	W3	W4	W5	W6
Brown-colored sediment[a]	+	+	+	+	+	+
Milk-white ring[b]	+	+	+	+	+	+
Acidity (g/100 mL)	5.58 ± 0.15*	3.62 ± 0.13*	4.97 ± 0.18*	4.03 ± 0.25*	5.49 ± 0.14*	5.66 ± 0.12*

[a]Qualitative analysis: (+) indicates a positive result. [b]Decomposition of acetate: (+) indicates a positive result. *P < 0.05 *vs* acetic acid.

Acid-producing rate

The acid-producing rate test demonstrated that W6 and W1, followed by W5 and W3, were high-acid producers (the final acidity was higher). However, W2 and W4 were low-acid producers. The results are summarized in Table 1.

Acetic acid was used as the reference, and the acidities of the screened strains were compared. As shown in Table 1, the acidities of all screened strains were significantly different from that of acetic acid. Among these strains, W6, followed by W1, had the highest acid-producing rate, and W4 had the lowest rate. The differences in the acid-producing rates of these strains are presumably associated with the different organic acids that are produced during fermentation. As well as acetic acid, the acid-producing bacteria in Zhenjiang aromatic vinegar have the ability to produce lactic acid, fumaric acid, succinic acid, pyruvic acid, tartaric acid, and oxalic acid (Zhu, 2011). All of these acids greatly reduce the harshness of the vinegar and improve its flavor, making the vinegar sour, smooth, soft, and palatable, thereby endowing Zhenjiang aromatic vinegar with its unique aroma and texture.

Amplification of 16S rDNA

Based on the identification results, the two high-acid-producing strains, W1 and W6, were further characterized. Using universal 16S rDNA PCR primers and the genomes of the W1 and W6 strains as templates, PCR amplification was performed, and the gel electrophoresis results presented a clear band at around 1500 bp (Figure 1). The subsequent results of the gel extraction, ligation, transformation, plasmid extraction, and double-restriction enzyme digestion are shown in Figure 2 and are consistent with those reported in the literature (Xu et al., 2007; Zhu et al., 2008; Song, 2013).

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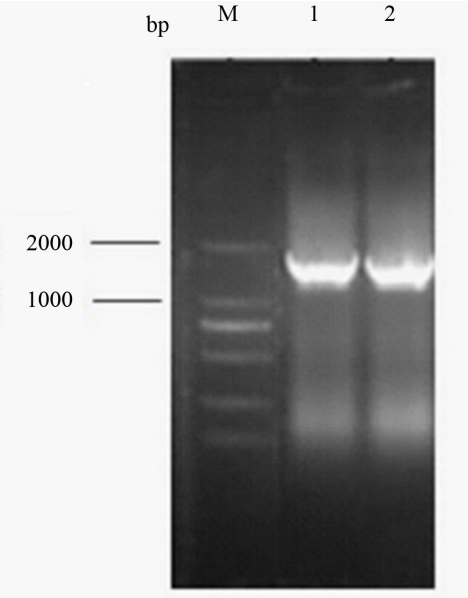

Figure 1. Photograph of the polymerase chain reaction products of 16S rDNA analysis of the W6 and W1 strains. *Lane M*, DNA marker DL2000; *lane 1*, strain W6; *lane 2*, strain W1.

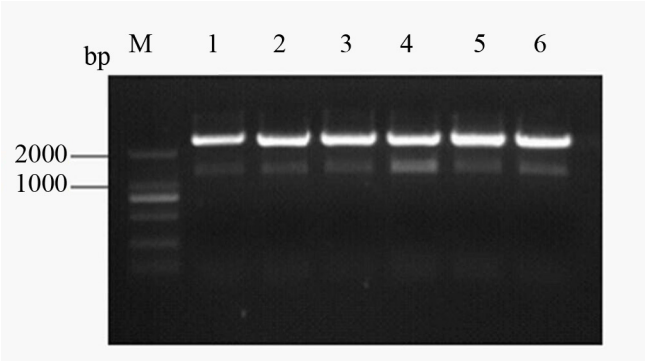

Figure 2. Double-restriction enzyme digestion products for the identification of the pMD18-T-W1 and pMD18-T-W6 recombinant plasmids. *Lane M*, DNA marker DL2000; *lanes 1-3*, pMD18-T-W1; *lanes 4-6*, pMD18-T-W6.

Sequencing determined that the PCR-amplified 16S rDNA of W1 was 1449 bp and that of W6 was 1513 bp. The 16S rDNA sequences of W1 and W6 are given in **Table S1**.

The sequences were submitted to GenBank (accession numbers KC662508 and KC662507), and sequence alignment was performed using BLAST (National Center for Biotechnology Information). The results showed that the 16S rDNA of W1 shared the same sequence (100% homology) with *Acetobacter aceti* (NR_026121) and was 99% homologous to *Acetobacter nitrogenifigens* (AB682235). Additionally, 21 other strains with high homology to W1 rDNA were selected, and *Bacillus subtilis* was set as an outgroup to construct a phylogenetic tree (Figure 3).

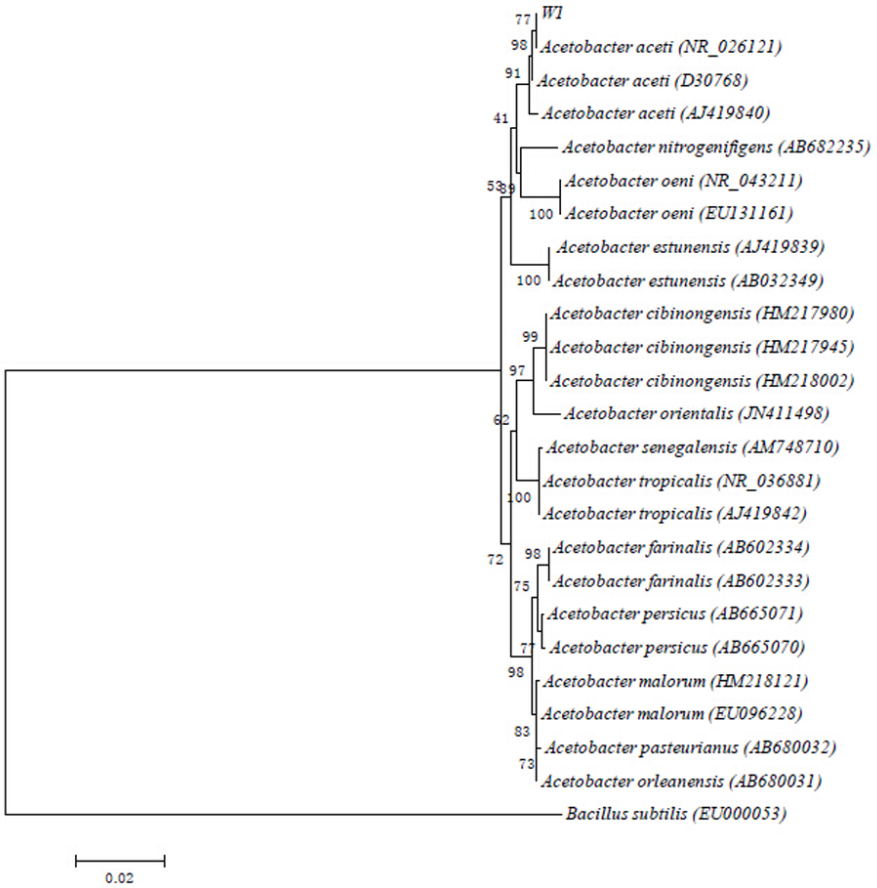

Figure 3. Phylogenetic tree of strain W1 and other bacteria based on their 16S rDNA sequences.

The phylogenetic tree of strain W1, based on the 16S rDNA sequence, showed that strain W1 and *A. aceti* clustered on the same branch, with a bootstrap support rate of 77%, followed by 88% for *A. aceti* (D30768) and 91% for *A. aceti* (AJ419840). Considering its physiological and biochemical features, strain W1 was identified as *A. aceti*, which is an important microorganism for the vinegar industry and has the capacity to produce gluconic acid and vitamin C. Wu et al. (2013) reported two *A. aceti* strains during the isolation of acetic bacteria from apricot dreg vinegar. However, to date, *A. aceti* has not been reported in Zhenjiang aromatic vinegar.

The sequence alignment of the 16S rDNA of W6 was performed similarly, and the results showed that the 16S rDNA of W6 was 100% homologous to *Acetobacter pasteurianus* (NR_044607), and it shared 99% homology with *A. pasteurianus* (GU385849), *Acetobacter estunensis* (FM178867), and *Lysinibacillus parviboronicapiens* (AB681953). Moreover, 14 other strains whose sequences were highly homologous to the rDNA sequence of W6 were selected, and *B. subtilis* was set as an outgroup to construct the phylogenetic tree (Figure 4).

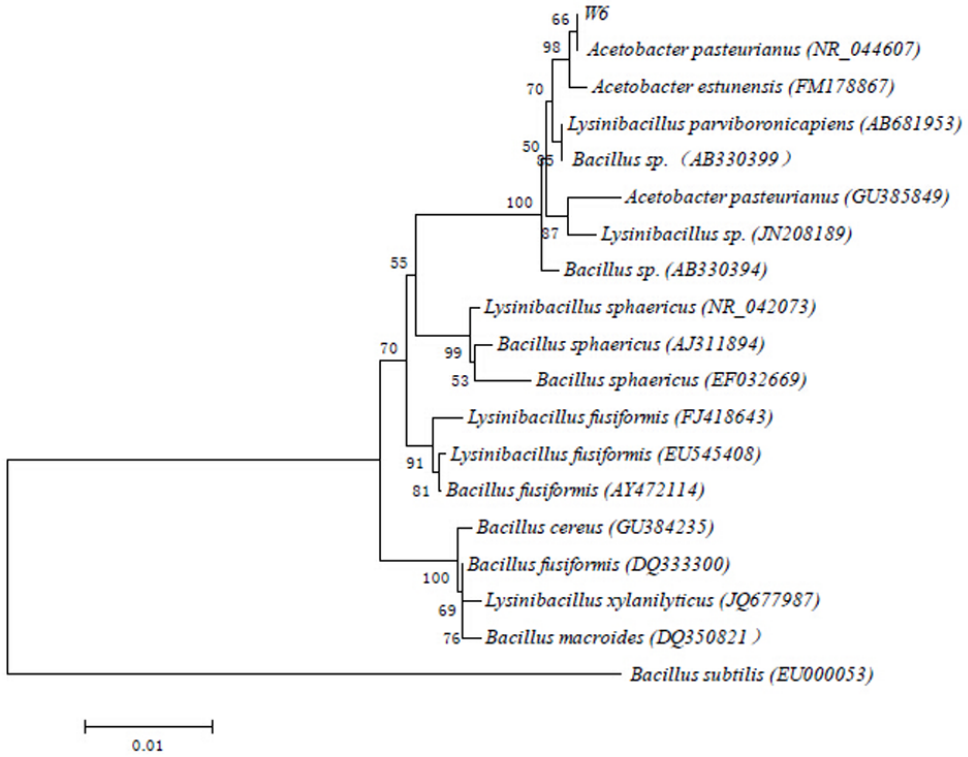

Figure 4. Phylogenetic tree of strain W6 and other bacteria based on their 16S rDNA sequences.

The phylogenetic tree based on the 16S rDNA of strain W6 showed that strain W6 clustered on the same branch as *A. pasteurianus*, followed by *A. estunensis* (FM178867). Although the bootstrap support rate was as high as 98%, strain W6 clustered earlier with *A. pasteurianus*. Therefore, when considering its physiological and biochemical features, strain W6 was identified as *A. pasteurianus*, which was consistent with the literature (Xu et al., 2007; Zhu et al., 2008).

Unlike with other vinegars, the brewing process for Zhenjiang vinegar is a hybrid-type, solid-state fermentation that uses multiple bacterial strains. The complexity and uncontrollability of solid-state fermentation creates the unique flavor and microbiological diversity of Zhenjiang vinegar. Generally, lactobacilli, yeast, and acetic acid bacteria constitute the major flora in vinegar brewing, of which acetic acid bacteria play an essential role. The isolation of microbes from vinegar grains demonstrates the great complexity of the vinegar flora, which includes unexpected microbes with acid-producing ability, such as bacilli. Presumably, long-term acclimation to the acidic environment has endowed some varieties of bacilli with this ability.

Six major strains of acetic acid bacteria were isolated and identified, and comparisons of their acid-producing rates showed that strains W1 and W6 were both preferred acetic acid bacteria. In further studies, both the associated features of the two strains and their usefulness in liquid-state fermentation for vinegar production will be further explored. In addition, high numbers of microbes in Zhenjiang vinegar grains, including additional acetic acid bacteria, remain to be discovered. This research may provide more perspectives on this field of study.

Conflicts of interest

The authors declare no conflict of interest.

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Supplementary material

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