Isolation and Identification of Nitrite-oxidizing Bacteria

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Abstract In order to select the strain that can degrade nitrite, we use the screening plate with nitrite as the sole nitrogen source to select the strain with ability to degrade nitrite, and get a strain with nitrite degrading capacity from the silt of shrimp farming pond in Hepu City, Guangxi Zhuang Autonomous Region. By identifying the strain from colony morphology, physiological and biochemical characteristics and 16S rRNA sequence, we finally get a bacteria strain that can degrade nitrite, and this strain can grow well on the culture medium with nitrite concentration of 2 g/L. Based on morphology, nitrogen source requirements and evolutionary tree analysis of the above 16S rRNA sequence, it is found that this strain belongs to *Pannonibacter phragmitetus*. According to the screening location, it is named HPPP007 strain.

Key words Nitrite degradation, *Pannonibacter phragmitetus*, Strain screening

1 Introduction

With the rapid development of China's high-density aquaculture, the nitrite accumulation in the water is becoming very prominent. Excessive nitrite content can not only cause a variety of metabolism disorders but also directly lead to aquaculture animal diseases or death, so nitrite poses a serious threat to farming and intensive aquaculture[10, 24]. Therefore, the prevention of nitrite accumulation in water has become one of the key measures to promote sustainable development of intensive aquaculture. Currently we can use physical, chemical and biological methods to degrade nitrite. Noticeably, using chemical method for nitrite degradation generates little success, and it easily leads to a series of environmental and social issues. The use of microbial agents for nitrite degradation will cause low costs and infinitesimal environmental pollution, so it is a good way to restore the environment, and reduce nitrite concentrations in aquaculture waters[20]. For example, nitrobacteria and *Bacillus subtilis*, *etc.* have achieved remarkable results in many countries. Based on this, we take strains with nitrite degradation capacity from the shrimp farming pond silt in Hepu City, and after screening and physiological, chemical and 16S rRNA identification, it is identified as *Pannonibacter phragmitetus*, which lays the foundation for further study.

2 Materials and methods

2.1 Experimental materials Aquaculture silt is collected from shrimp farming pond in Hepu City. Nitrite-oxidizing bacteria selective medium: $Na_2HPO_412H_2O$; 5 g/L, KH_2PO_4; 1 g/L, $MgSO_47H_2O$; 0.1g/L, sodium succinate 6 g/L, $NaNO_2$ 2 g/L, agar 2 g/L; trace element solution 2 mL; pH = 8. Trace element solution (g/L): EDTA 50 g/L; $ZnSO_4$ 2.2 g/L; $CaCl_2$ 5.5 g/L; $MnCl_2$ $4H_2O$ 5.6 g/L; $FeSO_47H_2O$ 5 g/L; $(NH_4)_6Mo_7O_24H_2O$ 1.1 g/L; $CuSO_45H_2O$ 1.6 g/L; $CoCl_2$ $6H_2O$ 1.6 g/L; pH = 7. Nacl solution: 0.9%. The above culture medium and solutions are sterilized at 121 ℃ for 20 min, and stored in the refrigerator. PCR primers are synthesized by Shanghai Sangon Company; Taq polymerase, PCR buffer, Mg^{2+} and dNTPs are provided by Takara Company; the gel extraction kit is provided by Shanghai Sangon Company; other reagents are analytically pure reagents produced at home.

2.2 Isolation, purification, screening and biochemical identification of nitrite-oxidizing bacteria 10g of the soil sample is dissolved in 100ml of sterile 0.9% Nacl solution and kept stationary at 28 ℃ after overnight incubation. 0.9% Nacl solution is used to dilute the culture solutions $10 - 10^8$ fold, and the culture solutions of different dilution rates are evenly coated on the denitrifying bacteria culture plate for the separation and purification of nNitrite-oxidizing bacteria.

2.3 Molecular identification of nitrite-oxidizing bacteria The 16S rRNA sequence identification method for strain HP007 is as follows: the activated HP007 is inoculated on denitrifying bacteria medium plate, and the single colonies are picked directly for PCR amplification. The bacterial universal primers are used for PCR amplification[9], and the sequence is as follows: Primer 1 (F27):5′-AGAGTTTGATCATGGCTCAG-3′; Primer 2 (R1492) 5′-TACGGTTACCTTGTTACGACTT-3′. PCR reaction system: 10 × Buffer 2.5 μl, $MgCl_2$ 2 μl (25 mmol/L), dNTPs Mixture (10 mmol/L) 0.5 μl each, Primer 1 and Primer 2 (10 μmol/L) 1 μl each, DNA Polymerase (5u. μ/L) 0.2 μl, Template 1 μl, dd H2O 16.8 μl, total volume of 25 μl. PCR reaction conditions: 94℃ 5 min; 94 ℃ 30 s; 50℃ 1 min; 72 ℃ 2 min, 30 cycles; 72℃ 10min. After completion of the reaction, 5 μl of the reaction solution is taken for electrophoresis using 1% agarose gel. The PCR products are purified by DNA gel extraction kit, and it is commissioned by Shanghai Sangon Company. After using DNAMAN software for editing the identification results, the BLAST software and the known 16S rRNA sequence in GenBank database are used for homology comparison to select the sequences with high homology. After multiple comparisons using BioEdit 7.0 and

Received: January 26, 2015 Accepted: March 31, 2015
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MEGA 6.0, the neighbor-joining method is used to build the phylogenetic tree. The strain type is determined based on colonial morphology and physiological and biochemical characteristics.

3 Results

3.1 The morphological character and physiological and biochemical identification of strain After 20 silt samples are diluted and screened, we get a strain with ability to degrade nitrite. This strain is cultured on solid nitrite culture medium for 15 d (28 ℃), and the colony diameter is 0.8 – 1.0 mm (Colonies on alkaline agar medium are whitish-beige, circular, convex, smooth and shiny with entire edges. Cells are non-spore-forming rods, motile with polar flagella, contain PHA and stain Gram-negative, and facultatively anaerobic, chemo-organotrophic.) (Fig. 1). The physiological and biochemical characteristics are shown in Table 1. Based on Bergey Bacterial Identification Manual 9[9, 11] and literature[2], it is found that the strain is in line with the Pannonibacter phragmitetus features.

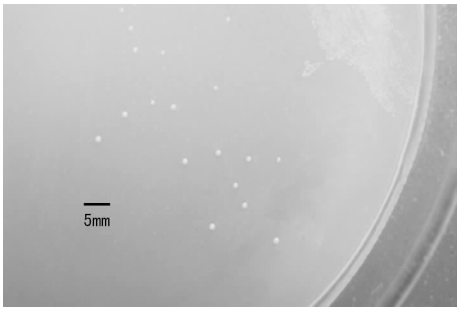
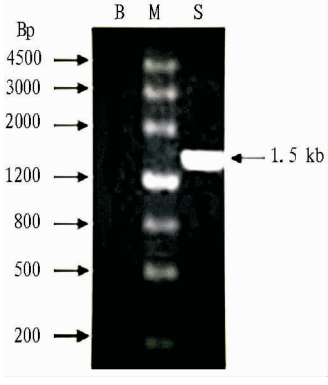


Fig. 1 Colonial morphology of HP 007 strain

3.2 Determination and analysis of 16S rDNA sequence of HP007 strain Using the bacterial 16S rDNA universal primers for single colony PCR amplification, we get 1.5 kb of DNA fragment from HP007 strain (Fig. 2), and directly send the PCR products to Shanghai Sangon Company for bi-directional DNA sequencing. By comparing the sequencing results with BLAST of GenBank, it is found that the sequence homology between 16S rDNA sequence of HP007 strain and *Pannonibacter phragmitetus* strain C6 – 19 reaches 99% (Fig. 3). Therefore, based on homology of 16S rDNA sequence, combined with comprehensive analysis of mycelial morphology and physiological and biochemical characteristics of HP007 strain, this strain is identified as *Pannonibacter phragmitetus*. According to the screening location, it is named HPPP007 strain.



S: PCR product; B: Negative control; M: Marker

Fig. 2 Amplification of the 16S rDNA fragment of HP007 strain

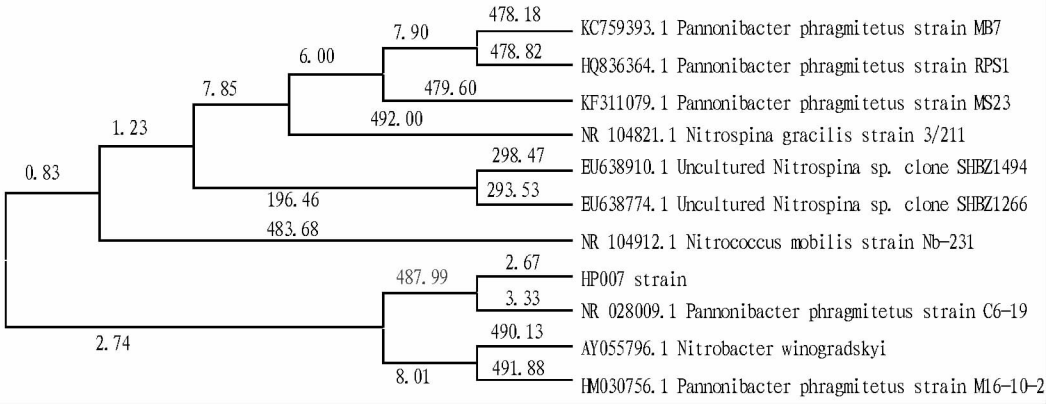


Fig. 3 Phylogenetic tree of HP007 strain based on 16S rRNA gene sequence homology

4 Conclusions and discussions

In order to select the strain that can degrade nitrite, we use the screening plate with nitrite as the sole nitrogen source to select the strain with ability to degrade nitrite, and get a strain with nitrite degrading capacity from the silt of shrimp farming pond in Hepu City, Guangxi Zhuang Autonomous Region. By identifying the strain from colony morphology, physiological and biochemical characteristics and 16S rRNA sequence, we finally get a bacteria strain that can degrade nitrite, and this strain can grow well on the culture medium with nitrite concentration of 2 g/L. Based on morphology, nitrogen source requirements and evolutionary tree analysis of the above 16S rRNA sequence, it is found that this strain belongs to *Pannonibacter phragmitetus*, which is completely different from the previously reported *Nitrobacter*, *Ni-trococcus*, *Nitrospina*, and *Nitrospira*[15-18]. This study is a complement to nitrite-oxidizing bacteria flora. *Pannonibacter phragmitetus* as a new spe-

cies was reported in 2003[2], and in recent years, its efficient Cr (Ⅵ) deoxidization function has been found[4]. There is a need to conduct further experiments on whether we can use Pannonibacter phragmitetus as the microbial agent to reduce nitrite damage in farming and repair environment.

Table 1 Physiological and biochemical characteristics of HP007 strain

Characteristics	Results
Colony pigmentation	Beige, whitish
D-Xylose	+
D-Fructose	+
D-Glucose	+
L-Arabinose	+
D-Galactose	–
Sucrose	–
Maltose	–
Lactose	–
Citrate utilization	+
Indole production	–
Urease activity	+
Phosphatase	+
Growth in the absence of NaCl	+
Temperature optimum (℃)	22 – 30
pH optimum	9 – 10

" + ": Positive; " – ": Negative.

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