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Isolation and characterization of *Pseudomonas stutzeri* QZ1 from an anoxic sulfide-oxidizing bioreactor

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ABSTRACT

Bacterial strain QZ1 was isolated from sludge of anoxic sulfide-oxidizing (ASO) reactor. Based on 16S rDNA sequence analysis and morphological characteristics, the isolate was identified as *Pseudomonas stutzeri*. The isolate was found to be a facultative chemolithotroph, using sulfide as electron donor and nitrite as electron acceptor. The strain QZ1 produced sulfate as the major product of sulfide oxidation, depending on the initial sulfide and nitrite concentrations. The isolate was capable of growth under strictly autotrophic conditions. The growth and substrate removal of *Pseudomonas stutzeri* QZ1 were optimal at an initial pH of 7.5–8.0 at 30 °C. The specific growth rate (μ) was found as 0.035 h^{-1} with a doubling time of 21.5 h. For isolate QZ1, the EC_{50} values both for sulfide and nitrite were found to be 335.95 mg S L^{-1} and 512.38 mg N L^{-1}, respectively, showing that the sulfide oxidation into sulfate by *Pseudomonas stutzeri* QZ1 was badly affected beyond these substrate concentrations.

1. Introduction

Anoxic sulfide oxidation using nitrite as electron acceptor has been demonstrated in our laboratory to be a very successful biotechnological process capable of bioremediation of nitrite and sulfide rich wastewaters simultaneously employing mixed culture [1]. It was gratifying to identify and characterize the bacterial species involved in sulfide biooxidation in mixed culture of an anoxic sulfide-oxidizing (ASO) reactor.

Many microorganisms can exploit H_2S oxidation to gain energy [2]. A variety of bacterial genera can oxidize reduced sulfur compounds under aerobic or anaerobic conditions, including:

- green sulfur bacteria (anaerobic, phototrophic, e.g. *Chlorobium*, etc.);
- purple sulfur bacteria (anaerobic, phototrophic, e.g. *Chromatium*, *Thiocapsa*, etc.);
- colorless sulfur bacteria (aerobic, e.g. *Thiobacillus*, *Beggiatoa*, *Thiothrix*, etc.).

Thiobacillus species are thought to account for the bulk of sulfide oxidation, via the sulfite-oxidase pathway [3].

1.1. Objectives of the study

In this paper, we report the isolation, identification and characterization of a chemolithoautotrophic sulfide-oxidizing, nitrite-reducing bacterial strain present in an ASO reactor.

2. Materials and methods

2.1. Media and culture conditions

The minimal medium used for isolation contained (grams per liter each): $NaHCO_3$, 1; trace element solution, 1 mL; $NaHCO_3$, 1; $MgCl_2$, 1; KH_2PO_4, 1; $(NH_4)_2SO_4$, 0.24; $Na_2S.9H_2O$, 512 (mg L^{-1}); $NaNO_2$, 528.75 (mg L^{-1}); $(NH_4)_2SO_4$, 0.5; KH_2PO_4, 1; $MgCl_2$, 1; and

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$NaHCO_3$, 1. The trace element solution contained EDTA, 5; NaOH, 11; $CaCl_2.2H_2O$, 7.34; $FeCl_2.4H_2O$, 3.58; $MnCl_2.2H_2O$, 2.5; $ZnCl_2$, 1.06; CoCl2.6H2O, 0.5; $(NH_4)_6Mo_7O_{24}.4H_2O$, 0.5; $CuCl_2.2H_2O$, 0.14; in 1 L of distilled water. All experimental cultures were incubated at the fixed temperature designated by every test. The agar (2%) was added as solidifying agent.

The pH of the medium was adjusted between 7.0 and 7.5 following autoclaving and cooling. The pH was adjusted using a PHS-9V pH meter (China). Then sulfide (1.0 mM) was added aseptically from a sterile stock solution of 1.0 M $Na_2S\cdot 9H_2O$. The culture medium was poured into Petri plates in the presence of sterilized air and was allowed to cool down to 45 °C. The culture medium was flushed with oxygen-free argon gas for 5 min to create anaerobic conditions. Between 0.2 and 0.3 mL of sludge dilutions (10^{-2}, 10^{-3}, and 10^{-4}) were sprinkled over the sterilized medium and incubated at 30 °C in anaerobic growth chamber.

2.2. Isolation of sulfide-oxidizing nitrite-reducing bacteria

The sludge sample used to isolate the sulfide-oxidizing nitrite-reducing bacterium was taken from the anoxic sulfide-oxidizing (ASO) bioreactor operated for more than 2 years in the department of Environmental Engineering, Zhejiang University Hangzhou, China. The sludge sample (5 mL) was diluted to 10^{-2}, 10^{-3} and 10^{-4} times. After dilution, the liquid culture was spread onto the solidified agar plates containing sulfide and nitrite using the dilution plate method, and these were incubated at 30 °C for 48–72 h. Endpoint dilution was carried out several times to further purify strains until pure isolates were obtained. The purified isolate with a higher capacity for sulfide oxidation and nitrite reduction was named strain QZ1. The purified isolate obtained was transferred to liquid culture medium for enrichment and performing the sulfide oxidation–nitrite reduction ability test. The composition of the minimal medium was as described in Section 2.1.

2.3. Growth and maintenance

Routine growth and maintenance of the isolate was carried out in mineral medium. Stock cultures of QZ1 were stored as lyophiles at −20 °C for 15 days. Working cultures from the lyophiles were grown in mineral medium containing agar (2% agar) for 3–5 days. From the agar plates, the isolate was cultured into serum bottles containing mineral medium. Growth was detected from an increase in optical density (at 600 nm). In addition, growth on sulfide ($Na_2S\cdot 9H_2O$) and nitrite ($NaNO_2$), as an electron donor and acceptor, respectively, was detected as an increase in sulfate, sulfide and nitrite concentrations, respectively, relative to uninoculated controls.

2.4. Identification and characterization of bacterial isolates

2.4.1. Microscopic characterization

Colony structure and cell morphology of the strain QZ1 was observed under a light microscope (Olympus BH-2, Japan) and transmission electron microscope (TEM) (JEM-1200EX, Japan). All cells used in microscopic characterization were grown in mineral medium containing sulfide and nitrite. The morphology and dimensions of the isolate were determined from photomicrographs using scanning electron microscopy (SEM), and transmission electron microscopy (TEM). The width and length given represent the average measurements of several cells. Motility and the presence of spores were determined using phase microscopy of wet mounts. Gram staining was performed by the Hucker method [4].

For SEM and TEM, 1 mL of QZ1 cells grown in mineral medium (containing sulfide and nitrite) for about 24 h was centrifuged at 10,000 × *g* for 15 min. The pellet was resuspended in 1 mL of 0.1 M sodium phosphate (pH 7.0) containing 2.5% glutaraldehyde. After 30 min, the cells were washed thrice with 0.1 M phosphate buffer and then fixed in 0.1% (wt/vol) osmium tetroxide. Following three more washes in 0.1 M phosphate buffer, an aliquot of cell suspension was removed and washed thrice with distilled water. This suspension was pipetted onto a glass slide. Twenty minutes later, the slide was immersed in a liquid nitrogen-cooled isopentane bath. The frozen slides were dried overnight in a lyophilizer, sputter coated with Au–Pd, and observed with a scanning and transmission electron microscope.

2.4.2. 16S rDNA sequence determination and phylogenetic analysis

Chromosomal DNA of the isolate was extracted by a slightly modified procedure of Wilson [5]. The 16S rDNA was amplified using PCR [6] with *Taq* polymerase (Boya) and the universal primer pair of 27f (5-AGA GTT TGA TCC TGG CTC AG-3) and 1523R (5-GGT TAC CTT GTT ACG ACT T-3) described by Weisburg et al. [7], and determined by the Shanghai Boya Biological Technique Company.

PCR reactions were performed in 100 μL reaction volumes containing 1 μL of Taq DNA polymerase (2.5 U μL^{-1}) (Sangon), 10 μL of 10× PCR reaction buffer (Sangon), 2 μL of each of the primers (10 μM), 2 μL dNTPs (10 mM), (Sangon), 5 μL of the extracted DNA as the templates and 78 μL of sterile distilled water. The PCR amplification protocol was as follows: denaturation at 94 °C for 1 min, annealing at 52 °C for 1 min, and extension at 72 °C for 3 min, and all the three steps were repeated for 30 cycles.

Related sequences were obtained from the GenBank database (National Center for Biotechnology Information, NCBI) using the BLASTN search program. The 16S rDNA sequences determined and reference sequences obtained from Gene Bank databases were aligned using multiple sequence alignment software CLUSTAL W ver. 1.81. A phylogenetic tree was constructed with MegAlign software of DNASTAR based on the 16S rDNA sequences of 10 strains closer to QZ1.

2.5. Growth kinetics

Growth tests were carried out in 65-mL serum bottles sealed with butyl rubber stoppers. Cell suspension (5%) of QZ1 (OD = 0.31) was inoculated and incubated at 30 °C in 45 mL of denitrifying medium with initial pH 7.5 containing sulfide and nitrite. The initial pH in these vials was adjusted to 7.5 by adding Tris–HCl solutions. The bottles were flushed with oxygen-free argon to create anoxic conditions. Control experiments were carried out with cell-free medium. Three replicates were conducted for each experiment.

2.5.1. Aerobic substrate tests

Aerobic growth in mineral medium minus acetate and nitrate was tested for the sulfide in 250-mL flasks containing 50 mL of medium with shaking on an incubator–shaker at 250 rpm.

Microaerophilic (1% O_2) growth with sulfide (1.0 mM) as an electron donor was tested in mineral medium without nitrite. The oxygen was added as 0.5 mL of sterile air (21% O_2) to the headspace of each bottle containing 10 mL of N_2-CO_2 (90%:10%). A control for chemical oxidation of sulfide was run in sterile mineral medium without nitrite containing 1% oxygen in the headspace.

2.6. Physiological characteristics

The isolate was tested for its ability to grow on various carbon sources and other substrates using Biolog and Vitek systems. The Vitek system (GNI+) (bioMérieux Vitek, USA) and the Biolog microstation (GN) (Biolog Hayward, CA, USA) were used for carbon source utilization and to identify physiological characteristics of the isolate, respectively.