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Isolation of *Ochrobactrum* sp.QZ2 from sulfide and nitrite treatment system

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ABSTRACT

A bacterial strain QZ2 was isolated from sludge of anoxic sulfide-oxidizing (ASO) reactor. Based on 16S rDNA sequence analysis and morphology, the isolate was identified as *Ochrobactrum* sp. QZ2. The strain was facultative chemolithotroph, able of using sulfide to reduce nitrite anaerobically. It produced either elemental sulfur or sulfate as the product of sulfide oxidation, depending on the initial sulfide and nitrite concentrations. The optimum growth pH and temperature for *Ochrobactrum* sp. QZ2 were found as 6.5–7.0 and 30 °C, respectively. The specific growth rate (μ) was found as 0.06 h^{-1} with a doubling time of 19.75 h; the growth seemed more sensitive to highly alkaline pH. *Ochrobactrum* sp. QZ2 catalyzed sulfide oxidation to sulfate was more sensitive to sulfide compared with nitrite as indicated by IC_{50} values for sulfide and nitrite utilization implying that isolate was relatively more tolerant to nitrite. The comparison of physiology of *Ochrobactrum* sp. QZ2 with those of other known sulfide-oxidizing bacteria suggested that the present isolate resembled to *Ochrobactrum anthropi* in its denitrification ability.

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 desirable to identify and characterize the bacterial species involved in the sulfide biooxidation found in mixed culture of anoxic sulfide-oxidizing (ASO) reactor.

Many microorganisms can use H_2S oxidation to gain energy [2]. Various groups of organisms can oxidize reduced sulfur compounds under aerobic or anaerobic conditions, including:

- Green sulfur bacteria (anaerobic, phototrophic, i.e., *Chlorobium*, etc.)
- Purple sulfur bacteria (anaerobic, phototrophic, i.e., *Chromatium*, *Thiocapsa*, etc.)
- Colorless sulfur bacteria (aerobic, i.e., *Thiobacillus*, *Beggiatoa*, *Thiothrix*, etc.)

Colorless sulfur-oxidizing bacteria are most widely used to oxidize H_2S using oxygen as an electron acceptor. This process is preferred because growth rates are significantly higher and there are no light intensity requirements. *Thiobacillus* species are thought to account for a majority of sulfide oxidation, via the sulfite-oxidase pathway [3].

Numerous other bacterial types are involved in the cycling of sulfur. For example, sulfur compounds of intermediate oxidation state (elemental sulfur, thiosulfate, and sulfite) can be actively disproportionated [4–6], and some organisms use disproportionation reactions as their sole means of energy conservation [7]. At present, all of the bacteria known to disproportionate sulfur compounds of intermediate oxidation state are found within the δ-subdivision of the purple bacteria.

The ability of organisms to oxidize sulfur compounds chemolithoautotrophically is widespread between the two prokaryotic domains (Bacteria and Archaea). Among the hyperthermophiles, deep-branching members of the Aquifex/Hydrogenobacter group in the Bacterial Domain can oxidize hydrogen sulfide using O_2 [8], as can do several known members of the Archaeal Domain [8]. Aquifex/Hydrogenobacter is a rather strange group of organisms, as its microaerophilic habitat is rather inconsistent with its deep-branching placement. In general, deeper-branching lineages house anaerobic organisms while aerobic organisms tend to occupy

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higher positions on the tree [9], reinforcing well-accepted notions [10] that life originated in an anaerobic environment. It has been suggested that the aerobic lifestyle of Aquifex/Hydrogenobacter developed over the course of evolution [11] and does not represent the ancestral metabolism of this group.

Of considerable significance for the process of sulfide oxidation is the large radiation of sulfide-oxidizing bacteria emerging from the base of the α- and β-subdivisions of the purple bacteria [12]. Most known non-photosynthetic, sulfide-oxidizing bacteria are found within these two subdivisions, and this radiation is, therefore, of some evolutionary significance. It has been proposed that evolutionary radiations of organisms are promoted by profound environmental changes [9].

1.1. Objectives of the study

Some bacterial strains belonging to *Ochrobactrum* sp. have been isolated previously capable of nitrite reduction in aerobic or anaerobic conditions [13,14]. In this paper, we report the isolation, identification and characterization of a chemolithoautotrophic sulfide-oxidizing, nitrite-reducing bacterial strain present in ASO reactor.

2. Materials and methods

2.1. Media and culture conditions

The minimal medium used for isolation contained (: $NaHCO_3$ ($1\,g\,L^{-1}$), trace element solution (1 mL), $NaHCO_3$ ($1\,g\,L^{-1}$), $MgCl_2$ ($1\,g\,L^{-1}$), KH_2PO_4 ($1\,g\,L^{-1}$), $(NH_4)_2SO_4$ ($0.24\,g\,L^{-1}$), agar (2%), $Na_2S.9H_2O$ ($512\,mg\,L^{-1}$), $NaNO_2$ ($528.75\,mg\,L^{-1}$), $(NH_4)_2SO_4$ ($0.5\,g\,L^{-1}$), KH_2PO_4 ($1\,g\,L^{-1}$), $MgCl_2$ ($1\,g\,L^{-1}$), and $NaHCO_3$ ($1\,g\,L^{-1}$). The trace element solution contained EDTA ($50\,g\,L^{-1}$, NaOH ($11\,g\,L^{-1}$), $CaCl_2{\cdot}2H_2O$ ($7.34\,g\,L^{-1}$), $FeCl_2{\cdot}4H_2O$ ($3.58\,g\,L^{-1}$), $MnCl_2{\cdot}2H_2O$ ($2.5\,g\,L^{-1}$), $ZnCl_2$ ($1.06\,g\,L^{-1}$), $CoCl_2{\cdot}6H_2O$ ($0.5\,g\,L^{-1}$), $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ ($0.5\,g\,L^{-1}$), $CuCl_2{\cdot}2H_2O$ ($0.14\,g\,L^{-1}$), in distilled water. All experimental cultures are 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. 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 in 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. Then, 0.2–0.3 mL of sludge dilutions (10^{-2}, 10^{-3}, and 10^{-4}) were sprinkled over the cooled 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 100, 1000 and 10,000 times. After dilution, the liquid culture was spread onto the solidified agar plates containing sulfide and nitrite using the dilution plate method, and was incubated at 30 °C for 48–72 h. Several rounds of endpoint dilution were used to further purify strains until pure isolates were obtained. The purified isolates, with higher capacity for sulfide oxidation and nitrite reduction were named as strain QZ2. Purified isolate obtained was transferred to liquid culture medium for enrichment and performing the *sulfide oxidation–nitrite reduction* ability test of the isolate. The composition of the minimal medium was same as described above.

2.3. Growth and maintenance

Routine growth and maintenance of both isolates were in mineral medium (composition described above). Stock culture of QZ2 was stored as lyophiles at −20 °C. Working cultures from the lyophiles were grown in mineral medium containing agar (2% agar). From the agar plates, the isolates were 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 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 strain QZ2 was observed by 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 isolate were determined from photomicrographs using scanning electron microscopy (SEM), and transmission electron microscopy (TEM). The widths and lengths given represent the averages of measurements of several cells. Motility and the presence of spores were determined using phase microscopy of wet mounts.

For SEM and TEM, 1 mL of QZ2 cells grown in mineral medium (containing sulfide and nitrite) for about 24 h was centrifuged at $10{,}000 \times g$ 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 three times with 0.1 M phosphate buffer and then fixed in 0.1% (w/v) osmium tetroxide. Following three more washes in 0.1 M phosphate buffer, a small portion of the 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 [15]. The 16S rDNA was amplified using PCR [16] with *Taq* polymerase (Boya) and the universal primer pair of 27f (5-AGAGTTTGATCCTGGCTCAG-3) and 1523R (5-GGTTACCTTGTTACGACTT-3) described by Weisburg et al. [17], 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) (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 GenBank databases were aligned using multiple sequence alignment software CLUSTAL W