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Characterization of Fe (III)-reducing enrichment culture and isolation of Fe (III)-reducing bacterium *Enterobacter* sp. L6 from marine sediment

Hongyan Liu* and Hongyu Wang

Tianjin Key Laboratory of Marine Resources and Chemistry, College of Marine and Environmental Sciences, Tianjin University of Science & Technology, Tianjin 300457, PR China

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To enrich the Fe (III)-reducing bacteria, sludge from marine sediment was inoculated into the medium using Fe $(OH)_3$ as the sole electron acceptor. Efficiency of Fe (III) reduction and composition of Fe (III)-reducing enrichment culture were analyzed. The results indicated that the Fe (III)-reducing enrichment culture with the dominant bacteria relating to *Clostridium* and *Enterobacter* sp. had high Fe (III) reduction of (2.73 ± 0.13) mmol/L-Fe (II). A new Fe (III)-reducing bacterium was isolated from the Fe (III)-reducing enrichment culture and identified as *Enterobacter* sp. L6 by 16S rRNA gene sequence analysis. The Fe (III)-reducing ability of strain L6 under different culture conditions was investigated. The results indicated that strain L6 had high Fe (III)-reducing activity using glucose and pyruvate as carbon sources. Strain L6 could reduce Fe (III) at the range of NaCl concentrations tested and had the highest Fe (III) reduction of (4.63 ± 0.27) mmol/L Fe (II) at the NaCl concentration of 4 g/L. This strain L6 could reduce Fe (III) with unique properties in adaptability to salt variation, which indicated that it can be used as a model organism to study Fe (III)-reducing activity isolated from marine environment.

[**Key words:** Marine sediment; Denaturing gradient gel electrophoresis; *Enterobacter* sp. L6; Fe (III) reduction]

Microbial dissimilatory Fe (III) reduction is a process that can cause the release of soluble Fe (II) by coupling the oxidation of organic matter to the reduction of Fe (III) (1). The process plays an important role in the geochemical cycling of iron and organic matter in anoxic ecology systems (2,3). The organisms capable of coupling the oxidation of organic matter to the dissimilatory reduction of Fe (III) have been described as Fe (III)-reducing bacteria. Dissimilatory Fe (III) reduction may occur during the respiratory, fermentative or photosynthetic metabolism of various Fe (III)-reducing bacteria (4–6). These bacteria could transfer electrons derived from the oxidation of organic matter to carbon dioxide with Fe (III) as the sole electron acceptor (7).

Microbial Fe (III) reduction can contribute to purification of pollutes and has the application of environmental protection (8,9). *Geobacter metalli-reducens* GS-15 was the first reported Fe (III)-reducing bacterium, which could simultaneously decompose benzene, toluene and other aromatic compounds (10). Iwahori et al. (11) demonstrated that Fe (III)-reducing microbial enrichment cultures had the ability of removal of toxic metal cations from water. Zhou et al. (12) reported that *Klebsiella* sp. strain FD-3 could reduce Fe (III) EDTA and remove NOx efficiently. The Fe (III)-reducing bacteria are being increasingly recognized as an ecological and environmental important group of microorganisms (13). Many Fe (III)-reducing bacteria were isolated and had been reported for significant contribution to iron and organic matter cycling in fresh condition (14–16). However, few studies have been reported on Fe (III) reduction by Fe (III)-reducing bacteria from the marine sediments.

The marine sediments with increasing depth below the seafloor have been the important habitats for the Fe (III)-reducing bacteria due to the special anaerobic environment. Dissimilatory Fe (III) reduction is the most reaction among a series of microbial-mediated redox reactions occurs in the marine sediments (17,18). Bohai is one of the inland seas in China. Due to the rapid development in coastal regions, Bohai has been subjected to both heavy metal and organic contamination. These pollutants will be eventually accumulated in submarine sediments. Fe (III)-reducing bacteria can change the formation of insoluble Fe (III) to the soluble Fe (II), which influence the distribution of toxic trace metals and strengthen the liquidity of pollutants in the sediments. Therefore, Fe (III)-reducing bacteria using Fe (III) as the most potentially electron acceptors in marine sediments will provide an alternative approach for the purification of the contaminants.

In this work, by using sediment from Bohai Sea, China, we determined the efficiency of Fe (III) reduction and composition of microbial community of the Fe (III)-reducing enrichment culture. In the enrichment culture, a new Fe (III)-reducing bacterium was isolated. The level of microbial Fe (III) reduction by the isolated strain was optimized under different carbon sources and salt concentration conditions.

MATERIALS AND METHODS

Source of the organism The marine sediment was collected from Bohai Sea, in Tianjin, China (longitude of 116 and latitude of 38). The sediment sample was taken from approximately 15 m below the sea surface and placed in sterile plastic

* Corresponding author: Tel./fax: +86 22-60601305.
E-mail address: hongyanliu1214@163.com (H. Liu).

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bags at 4°C before enrichment. Data on temperature and salinity of the sediment slurry were 23°C and 28%, respectively.

Enrichment conditions The sludge was inoculated into Fe (III)-reducing medium with different Fe (III) concentration, 0, 4, 8, 11 and 15 mmol/L, respectively. The Fe (III)-reducing medium was consisted of (per liter): 10.0 g of glucose, 2.0 g of yeast extract, 1.0 g of tryptone, 30.0 g of NaCl, 1.5 g of K_2HPO_4 and 0.5 g of L-cysteine. Fe (III) was provided in the form of Fe $(OH)_3$. Fe $(OH)_3$ suspension was prepared by adding 0.5 mol/L NaOH to a solution of 0.4 mol/L $FeCl_3$ with continuous stirring and the pH value was adjusted to 7.0–7.5. Then, the formation of reddish brown precipitate was washed three times and amorphous Fe $(OH)_3$ was provided at 50 mmol/L. The pH of the medium was adjusted initially to 7.0. The enrichment culture was set up as 10% (w/v) sediment slurry in the Fe (III)-reducing medium using 150 mL serum bottles. The serum bottles were purged with nitrogen gas for 1 min and sealed with a 12-mm-thick butyl rubber septum for the required anaerobic condition. After cultivation at 30°C for 2 days, 1.0 mL culture broth was inoculated into 100 mL of fresh Fe (III)-reducing medium to enrich the mixed microbial culture. The above procedure was repeated three times.

Composition analysis of microbial community Composition of Fe (III)-reducing enrichment culture was analyzed by the denaturing gradient gel electrophoresis (DGGE). The V3 region of 16S rDNA was amplified by performing a PCR (GC-357F: 5′-CCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGGCAGCAG-3′, 517R: 5′-ATTACCGCGGCTGCTGG-3′). A PCR was performed under conditions of initial denaturation of DNA for 5 min at 94°C; 30 cycles of 30 s at 94°C, 30 s at 58°C, 30 s at 72°C and extension of incomplete products for 10 min at 72°C. The PCR samples were applied directly to 8% (w/v) polyacrylamide gels with a denaturing gradient ranging from 30% to 60%. DGGE analysis was performed with a DCode universal mutation detection system (Bio-Rad, USA). Electrophoresis was run at a constant voltage of 130 V at 60°C. After 4.5 h of electrophoresis, the gel was stained by ethidium bromide (0.5 mg/mL). The bands from DGGE gel were excised. DNA was purified by gel recovery purification kit and re-amplified. The PCR products were sequenced by AuGCT DNA-SYN Biotechnology Co. (Beijing, China). The corresponding results were submitted to GenBank.

Isolation and identification of the strain The enrichment culture with high Fe (III)-reducing activity was used for isolation of Fe (III)-reducing bacterium. Fe (III)-reducing bacteria were screened by the Hungate roll-tube technique (19) and many modifications. The mixed culture was spread onto Fe (III)-reducing medium agar plates. After cultivation 2 days at 30°C, single colonies were picked and inoculated to the same liquid media for 24 h. The strains with the highest Fe (III)-reducing activity among the isolates were re-streaked for further purification. This procedure was repeated five times to ensure the purity and high Fe (III)-reducing activity of the isolated strain.

The identification of the isolated strain was carried out by 16S rRNA gene sequence analysis. The 16S rRNA genes were amplified using a pair of eubacteria universal primers: 27f: 5′GAGTTTGATCCATGGCTCAG-3′; 1541r: 5′-AAGGAGGTGATCCAGCC-3′. The PCR conditions were as follows: an initial denaturation step for 4 min at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 58°C, 60 s at 72°C and then final extension for 10 min at 72°C. The sequence of PCR products was performed by AuGCT DNA-SYN Biotechnology Co. (Beijing, China). 16S rRNA gene sequence was compared to the closely related strains from GenBank database using the Basic Local Alignment Search Tool (BLAST) search. These sequences were aligned with the closest matches found in the GenBank. Phylogenetic analysis was performed using the neighbor-joining methods. Alignment and neighbor joining phylogenetic trees was analyzed using the Mega program.

Culture condition To describe the effects of culture condition on biomass accumulation and Fe (III) reduction of the isolated strain, a serial of batch fermentation was performed. The isolated strain was inoculated into the Fe (III)-reducing medium in a 150 mL serum bottle (working volume 100 mL) with a shaking speed of 120 rpm at 30°C. The batch operations were conducted at different NaCl concentration (0–60 g/L) using various carbon sources such as glucose, pyruvate, acetate, formate, lactate and propionate fixed at 20 mmol/L. All tests were performed in triplicate to assure data reproducibility.

Analytical methods Morphological examination was carried out by scanning electron microscopy (Hitachi, Japan). The biomass accumulation was determined by measuring the optical absorbance at 600 nm using a spectrophotometer (UV757CRT, China). Fe (III) reduction was monitored by measuring the accumulation of Fe (II) in the medium. The produced Fe (II) was determined spectrophotometrically by ferrozine assay of HCL-extractable Fe (II) (20). Briefly, 0.5 mL cell suspension of the isolated strain were taken using sterile syringes and immediately transferred to a screw top plastic tube containing 4.5 mL HCL (0.5 mol/L). After 24 h of extraction at 30°C, samples were centrifuged at 4500 × *g*. 100 μL aliquots of supernatant were mixed with 10% hydroxylammonium chloride, 1 mol/L sodium acetate buffer, pH 5.0, 0.1% *o*-phenanthroline in a test volume of 5 mL and the A510 values measured.

RESULTS AND DISCUSSION

Fe (III) reduction by enrichment culture After three times of enrichment procedure, cell growth and Fe (III)-reducing activity of the enrichment culture with different Fe (III) concentration, 0, 4, 8, 11 and 15 mmol/L, respectively were investigated (Fig. 1). Bacterial growth of the mixed culture was significantly stimulated by the addition of Fe (III) as an electron acceptor and sensitive to the supplement of different Fe (III) concentrations in the medium. The maximum optical absorbance (1.4335 ± 0.12) and the highest Fe (III) conversion (2.73 ± 0.13) mmol/L-Fe (II) were obtained by the Fe (III)-reducing enrichment culture inoculated in the medium with Fe (III) concentration of 8 mmol/L. The result was similar with the previous study that the bacteria could produce greater accumulations of Fe (II) with respect to the increased initial supply of Fe (III) during cell growth (21,22). There was a decrease on Fe (III) reduction with the increase of Fe (III) concentration above 8 mmol/L. At the Fe (III) concentration of 15 mmol/L, the significant decrease on cell growth and Fe (III) reduction was found. The addition of excess amount of Fe (III) may negatively impact the enrichment of Fe (III)-reducing bacteria.

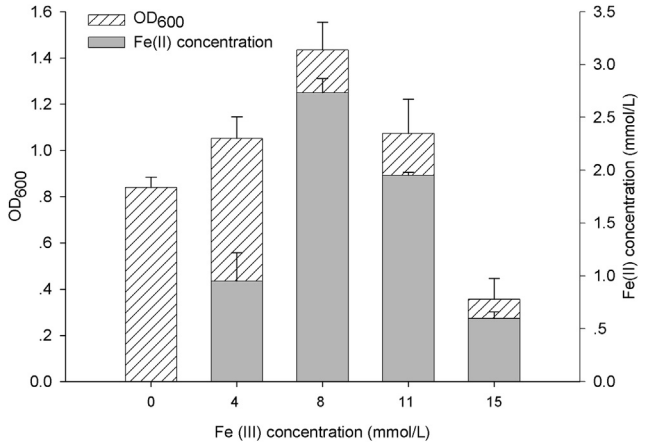

FIG. 1. Cell growth and Fe (III) reduction of the mixed culture with Fe $(OH)_3$ of different concentrations ($n = 3$, error bars = s.e.m.).

Composition analysis of microbial community We used PCR-DGGE to determine composition of Fe (III)-reducing enrichment cultures. Fig. S1 shows the profile of the DGGE bands for the microbial community in the medium with Fe $(OH)_3$ of different concentrations (4, 8, 11 and 15 mmol/L). The major bands (B1, B2 and B3) in the DGGE gels were excised and purified to determine the sequence. The nucleotide sequences of the 16S rRNA gene of major bands have been deposited in GenBank, with accession number of KP278236, KP278237 and KP278238. The result indicated that the dominant bacteria inoculated with these three different Fe (III) concentrations (4, 8 and 11 mmol/L) were basically identical and closely related to *Clostridium* and *Enterobacter* species. It meant that the effect of Fe (III) concentration on Fe (III) reduction was microbial activity rather than composition of the microbial community. The major bands in the DGGE gels of the mixed culture enriched at Fe (III) concentration of 15 mmol/L were not formed. It meant that the number of bacteria enriched at this Fe (III) concentration was decreased significantly with the increasing of Fe (III) concentration. The cell growth of Fe (III)-reducing bacteria was inhibited by high Fe (III) concentration. The result was similar as the report that the excess amount of Fe (III) may lead to the formation of iron mineral coatings on cell surface of the Fe (III) reducing organisms, which may be unfavorable for cell growth and microbial activity (23).

Isolation and identification of the strain After five times of solid–liquid separation, a strain named as L6 was selected for the highest Fe (III)-reducing activity. The bacterial colonies by