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Research article

Coupling heavy metal resistance and oxygen flexibility for bioremoval of copper ions by newly isolated *Citrobacter freundii* JPG1

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ABSTRACT

The potential for bioremoval of copper ions was investigated by a novel strain of bacterium *Citrobacter freundii* JPG1, which was newly isolated from gold mining tailing in China and grew either aerobically or anaerobically. The strain cross-tolerated heavy metals of Ag^{+}, Cd^{2+}, Co^{2+}, Cr^{6+}, Cu^{2+} and Ni^{2+} and removed copper under both aerobic and anaerobic conditions with the stress of copper. Under aerobic conditions, the cells grew rapidly and exhibited higher biomass at low copper concentrations ($< 1\ mmol\ L^{-1}$), while the growth of cells was almost completely inhibited at high copper concentrations ($2\ mmol\ L^{-1}$). However, the cell growths were less affected by copper under anaerobic conditions. Similarly, the copper-removal efficiency was affected by oxygen and the capability of copper removal by anaerobic cells was significantly higher than that of aerobic cells ($P < 0.05$). The quantitative measurement of extracellular biosorption and intercellular bioaccumulation of copper indicated that biosorption efficiencies for aerobic cells (37%) and anaerobic cells (38%) were similar but the bioaccumulation by anaerobic cells was almost ten-fold higher than that by aerobic cells, indicating bioaccumulation contributed most in copper reduction under anaerobic conditions. Overall, the results suggested the facultative strain *C. freundii* JPG1 had great potential in the treatment of copper-laden industrial wastewater under both aerobic and anaerobic conditions.

1. Introduction

Heavy metal pollution is a widespread environmental concern due to its toxic, persistent, and non-biodegradable properties (Fashola et al., 2016). Because municipal sewage treatment plants are not designed or equipped to deal with toxic wastes, heavy metals should be removed from industrial effluent before being discharged into the environment. The use of microbes, especially bacteria, for the removal of metal ions from wastewater is an economical alternative to physicochemical methods. Under their thresholds, metal-resistant growing cells exhibit better removal capacities of heavy metals than inactive cells because both biosorption and bioaccumulation participate in heavy metal reduction (Malik, 2004).

Due to bacterial metabolism can be adversely affected by an elevated concentration of heavy metals, great efforts have been made to isolate metal-resistant bacterial strains to remove heavy metal efficiently, the work is still largely limited in the lab-scale so far (Fu and Wang, 2011). One of the challenges is that a large-scale bioprocess is greatly influenced by variable environmental parameters, such as temperature, pH, and dissolved oxygen (Gadd, 2009; Timoumi et al., 2017). In most of cases, the removal of heavy metals is carried out by aerobic bacteria and dissolved oxygen is often a limited factor in packed-bed or slurry bioreactors (Chan et al., 2009).

Facultative bacteria are able to grow aerobically and anaerobically because they have both sets of metabolic mechanisms. These bacteria can benefit from their metabolic flexibility in a microenvironment with limited oxygen (Unden et al., 1994). Previous studies have investigated the effects of oxygen on the growth of some facultative bacterial species, for instance *Bacillus subtilis*, *Shewanella alga*, and *Shewanella oneidensis* MR-1(Clements et al., 2002; Guha et al., 2001). The overall effect of oxygen on the growth of these facultative bacteria is to increase both the lag phase and the generation time. Nevertheless these studies were carried out in absence of

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heavy metals. Moreover, studies on the capability of facultative bacteria removal of metal ions under anaerobic conditions are rarely reported. To date only copper bioaccumulation by the bacterium *Escherichia coli* under anaerobic conditions is reported (Outten et al., 2001). Additionally, cadmium (VI) reduction by the yeast *Saccharomyces cerevisiae* under anaerobic conditions is available in literature (Guha et al., 2001). Interestingly, anaerobic cells of *E. coli and S. cerevisiae* all accumulated larger amount of Cu and Cr than did the aerobic cells. These results motivate us to think whether other microbial species show the same effect. Hence, it is deserved to study on heavy metal removal by a certain metal-resistant and oxygen-flexible bacterium under both aerobic and anaerobic conditions for a thorough understanding of the microbial mechanisms in heavy metal bioremediation.

In this study, the novel facultative strain of C. *freundii* JPG1, which was able to grow under the pressure of multiple heavy metals (Ag, Cd, Co, Cr, Cu and Ni), was investigated for its growth with the copper stress and its capacity of removal of copper under aerobic and anaerobic conditions.

2. Materials and methods

2.1. Medium, growth and conditions

The medium used for culturing, enriching and isolating bacteria was Luria Bertani (LB), containing 10 g tryptone, 5 g yeast extract and 10 g NaCl per liter. LB agar plates were made as LB medium with addition of 15 g L^{-1} agar. The media involved in the experiment of copper removal, biosorption and bioaccumulation were LB culture amended different concentration CuSO4 solution (0–4 mmol L^{-1}).

Aerobic cultures were incubated in a shaker incubator at 160 rpm. The anaerobic cultures were continuously flushed with nitrogen for at least 20 min and subsequently incubated in Hungate tubes. All the growth and copper removal experiments were performed in triplicate at 30 °C. Bacterial growth was estimated by measuring the optical density at 600 nm (OD_{600}).

2.2. Study area, soil sampling and preparation

The Jiapigou gold mine (42°41′-43°00′ N and 127°15′-127°30′ E) is located in the valley area upstream of the Songhua River, Jilin Province, Northeast of China. Gold mining activity at Jiapigou was initiated in the 1820, and is still active today. A total of eight soil samples were collected in this study (Fig. 1). Three samples (0–15 cm in depth) were collected in each sampling site, and thoroughly mixed to be a representative sample.

All the samples were air-dried at room temperature and crushed to pass through 2 mm polyethylene sieve. 0.2000 g of such prepared soil sample was wet-digested by 4 ml of concentrated HNO_3 and 0.5 ml of concentrated HF in a closed polytetrafluoroethylene vessel with microwave heating for 10 min at 180 °C. Hg content in the soils was digested in a mixture of $HClO_4$—HNO_3—HF (2:3:5) with the addition of $KMnO_4$.

2.3. Isolation and identification of C. freundii JPG1

Samples for bacterial isolation were collected from a tailing pile of gold mining, located in the Jiapigou area in northeast China. Isolation was performed using the standard 10-fold dilution plate method. To do this, 10 g of fresh tailing samples was firstly added to 90 ml of sterile water and fully mixed for 30 min. Next, 1 ml of the suspension was transferred to 9 ml of sterile water and fully mixed. The dilution was carried out until the desired number of colonies was shown on agar plates. In each dilution, 0.1 m of the suspension was spread on LB agar plates containing different amounts of copper (0.78, 1.56, or 3.12 mmol L^{-1}). Plates were incubated aerobically and anaerobically at 30 °C for 3–7 days. Anaerobic Incubator (Shanghai Longyue Instrument Co., Ltd., China) was used to anaerobically grow bacteria, in which the anaerobic condition was maintained by nitrogen gas. Colonies with different morphologies were selected and isolated by further subculturing in the same media. One of the isolated bacterial strains, designated JPG1, was selected on the basis of its copper resistance under aerobic and anaerobic conditions.

JPG1 was characterized based on physiological characteristics and phylogenetic analyses. The physiological characteristics of the isolate were carried out according to Bergey's Manual of Systematic Bacteriology (second edition, 2004). The bacterial genomic DNA of JPG1 was extracted using a DNA extraction kit (Takara Biotechnology Co., Ltd., Dalian, China). The 16S rRNA genes were PCR-amplified from the genomic DNA using the bacterial universal primer set 27f (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1492r (5′-GGC TAC CTT GTT ACG ACT T-3′). The 16S rRNA gene sequence was compared to reference sequences in the GenBank database using BLAST to identify closely related bacteria. The sequence data of JPG1 was submitted to the NCBI GenBank as accession KU513787.

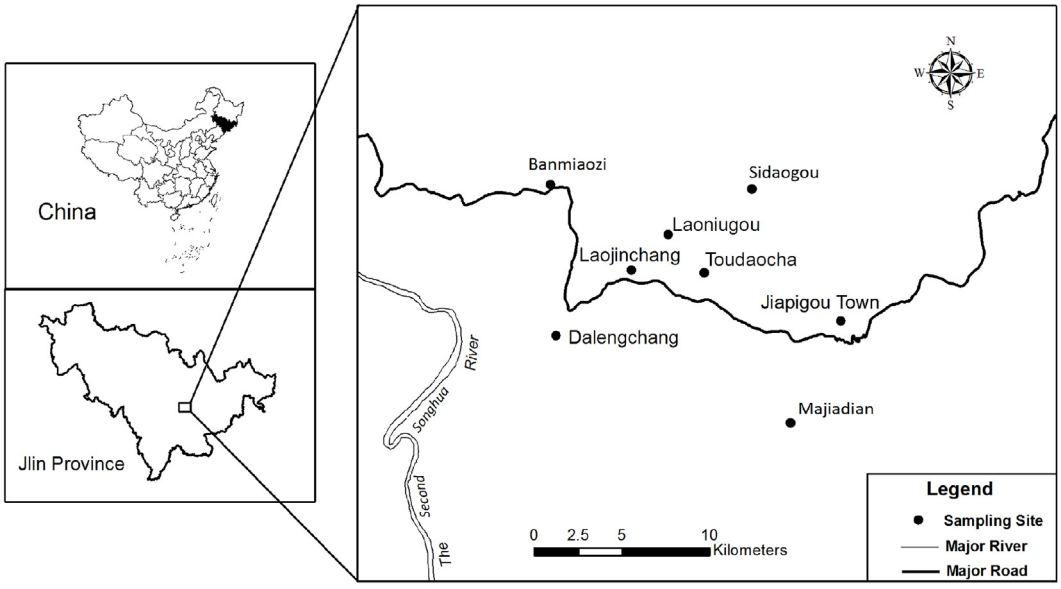

Fig. 1. Location of sampling points in Jiapigou gold mining area, northeast of China.