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Effects of arsenic on the biofilm formations of arsenite-oxidizing bacteria

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ABSTRACT

Arsenite-oxidizing bacteria (AOB) play a key role in the biogeochemical cycle of arsenic in the environment, and are used for the bioremediation of As contaminated groundwater; however, it is not yet known about how arsenic affects biofilm formations of AOB, and how biofilm formations affect bacterial arsenite-oxidizing activities. To address these issues, we isolated seven novel AOB strains from the arsenic-contaminated soils. They can completely oxidize 1.0 mM As(III) in 22–60 h. Their arsenite oxidase sequences show 43–99% identities to those of other known AOB. Strains Cug1, Cug2, Cug3, Cug4, and Cug6 are able to form biofilms with thickness of 15–95 μm, whereas Cug8 and Cug9 cannot form biofilms. It is interesting to see that arsenite inhibited the biofilm formations of heterotrophic AOB strains, but promoted the biofilm formations of autotrophic strains in a concentration-dependent manner. The arsenite-oxidizing rates of Cug1 and Cug4 biofilms are 31.6% and 27.6% lower than those of their suspension cultures, whereas the biofilm activities of other strains are similar to those of their suspension cultures. The biofilm formation significantly promoted the bacterial resistance to arsenic. This work is the first report on the complex correlations among environmental arsenic, bacterial biofilm formations and bacterial arsenite-oxidizing activities. The data highlight the diverse lifestyle of different AOB under arsenic stress, and provide essential knowledge for the screening of efficient AOB strains used for constructions of bioreactors.

1. Introduction

Biological oxidation of arsenite [As(III)] by As(III)-oxidizing bacteria (AOB) play key roles in the arsenic (As) biogeochemical cycles (Xie et al., 2014; van Lis et al., 2013; Zhang et al., 2015a). Under aerobic conditions, some AOB are able to convert As(III) into arsenate [As(V)] by using As(III) as the terminal electron donor and oxygen as the terminal electron acceptor (Santini et al., 2000; Bagade et al., 2016), whereas under anaerobic conditions, other AOB can oxidize As (III) into As(V) by using nitrate, chlorate, ferric iron or selenate as the terminal electron acceptor (Zhang et al., 2015a; Sun et al., 2011; Rhine et al., 2008; Fisher and Hollibaugh, 2008). AOB are either chemoautotrophic, using carbon dioxide as the sole carbon source (Oremland et al., 2002; Garcia-Dominguez et al., 2008), or heterotrophic, using organic carbon as the sole carbon source (Bahar et al., 2013; Zhang et al., 2016). For heterotrophic AOB, As(III) oxidation is generally considered to be a detoxification mechanism rather than one that can support growth. In the absence of organic carbon source, however, As (III) oxidation is a thermodynamically exergonic reaction that provides sufficient energy to support the growth of chemoautotrophic microbes (Oremland et al., 2002; Donahoe-Christiansen et al., 2004; Kulp et al., 2008). Anaerobic oxidation of As(III) can also support the growth of AOB cells (Fisher and Hollibaugh, 2008; Sun et al., 2009; Zhang et al., 2017).

As(III) oxidase is responsible for microbial As(III) oxidation. It is generally an $\alpha_1\beta_1$ heterodimer, consisting of a large catalytic subunit AioA and a small subunit AioB (Ellis et al., 2001; Inskeep et al., 2007). The gene *aioA*, which codes for AioA subunit, is used as a genetic marker to detect the existence, distribution and diversity of AOB in the environment (Quéméneur et al., 2008; Hamamura et al., 2009; Heinrich-Salmeron et al., 2011). AOB widely distribute in As-contaminated paddy soils, waste soils, surface water, groundwater, wastewater, sediments/aquifers, realgar mine tailing, gold mine, geothermal environments, watershed, plants, and alkaline saline lake (Das et al., 2016; Bahar et al., 2013; Chang, 2015; Ghosh et al., 2014; Zeng et al., 2016; Andres et al., 2013; Yang et al., 2017). They play critical roles in the transformation of As and the coupling of As, Fe and N biogeochemical cycles in the environment (Hassan et al., 2015; Zhang

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et al., 2015a). It was also found that nitrate-dependent anaerobic oxidation of As(III) can significantly limit As motility in As-contaminated paddy soils (Zhang et al., 2017).

Approximately 30 cultivable AOB strains have been isolated from diverse environments so far. Most of these bacteria are affiliated to the families *Alphaproteobacteria*, *Betaproteobacteria* and *Gammaproteobacteria* (Quéméneur et al., 2010; Das et al., 2014; Yamamura et al., 2014). In addition to As(III), AOB may also oxidize sulfur (Garcia-Dominguez et al., 2008; Rhine et al., 2008). A few of AOB strains were found to mobilize As from pyrite in acid-mine drainage or under circumneutral pH (Rhine et al., 2008).

Some AOB were used for the bioremediation of As-contaminated groundwater (Michon et al., 2010; Battaglia-Brunet et al., 2012; Wan et al., 2010; Dastidar and Wang, 2012; Ito et al., 2012). The most commonly used method to remove As from contaminated groundwater involves oxidation of As(III) into As(V), and subsequent treatment by filtration, ion exchange, coagulation, absorption, and inverse osmosis (Mondal et al., 2006, 2013). Biological oxidation of As(III) by AOB is obviously better than use of chemical oxidants because the latter needs more energy and usually causes environmental pollution by the by-products and residual oxidants (Mondal et al., 2013). To achieve high oxidation efficiency, AOB consortia or single strains were used to construct biofilm reactors (Michon et al., 2010; Battaglia-Brunet et al., 2012; Wan et al., 2010; Dastidar and Wang, 2012; Ito et al., 2012; Katsoyiannis et al., 2002; Li et al., 2016). Although these biological As (III) oxidation systems are efficient in the laboratory, their stability, scalability and suitability need to be further improved prior to putting into industrial applications.

Although there were many investigations on the distributions, activities, biogeochemical roles and bioreactor constructions of AOB, little is known about the biofilm properties of different AOB strains, especially about how As affects the bacterial biofilm formations, and how biofilm formation affects the bacterial As(III)-oxidizing activities. In this study, we isolated 7 AOB strains from As-contaminated soils by an enrichment technique. We found that the interactions between AOB and environmental As are more complicated than expected. This work highlights diverse effects of As on the biofilm formations of different AOB strains, and provides new insights into the strategies for AOB strains to adapt themselves to the As stress in the environment. The data of this study also provides essential knowledge on the screening of efficient AOB strains for the constructions of bioreactors.

2. Materials and methods

2.1. Chemical analysis

The concentrations of soluble As(V) and As(III) were determined using the high-performance liquid chromatography coupled with inductively coupled plasma spectrometry (HPLC-ICP-MS) (LC-20A, Shimadzu, Japan; ELAN, DRC-e, PerkinElmer, USA) as described elsewhere (Chen et al., 2017).

2.2. Isolation of As(III)-oxidizing bacteria from As-contaminated soils

Samples were collected from a depth layer of 0.9–1.0 m of the As-contaminated soils in the Shimen County of Changde City affiliated to the Hunan Province, China. Approximately 1.0 g of soils was inoculated into 9.0 mL of synthetic mineral salts (SMS) medium (Rhine et al., 2006) amended with 1.0 mM As(III) and 10.0 mM $NaHCO_3$ for three rounds of enrichment as described previously (Zeng et al., 2016). The enriched culture was serially diluted and plated onto SMS agar plates containing the same supplements. After three days of aerobic incubation at 30 °C, visible individual colonies were further purified by streaking method.

2.3. Cloning and analysis of bacterial 16S rRNA and As(III) oxidase genes

Bacterial 16S rRNA and As(III) oxidase genes were cloned, sequenced and analyzed as described previously (Xu et al., 2014; Wang et al., 2017; Zhang et al., 2015b). A neighbor-joining phylogenetic tree was constructed based on the multiple sequence alignment of the obtained sequences and their closely related homologues using MEGA (Luo et al., 2014; Zhong et al., 2017).

The 16S rRNA gene sequences of the strains Cug1, Cug2, Cug3, Cug4, Cug6, Cug8, and Cug9 have been deposited into the GenBank database under the accession numbers MF621569, KT992333, MF621570, MF621571, MF621573, KT992332, and KT992327, respectively; their *aioA* gene sequences have also been deposited with the accession numbers MF621575, KT992350, MF621576, MF621578, MF621579, KT992353, KT992342, respectively.

2.4. Examination of As(III) oxidation activities of the isolates

A bacterial colony was inoculated into 50 mL of Tryptic Soy Broth (TSB) (Mu et al., 2016a, 2016b) medium amended with 1.0 mM As(III). Controls were the same medium without inoculation of bacterial cells. All the flasks in triplicate were incubated at 30 °C with shaking at 150 RPM. At an interval of 2–5 h, approximately 1.0 mL of each culture was removed for measuring the concentrations of As(V) and As(III) using HPLC-ICP-MS.

2.5. Examination of bacterial biofilm formation capacity

A mid-log phase culture of a bacterial strain was diluted into an $O.D_{600}$ of 0.05 using TSB medium. The diluted culture was placed into a 48-well flat-bottom polystyrene microplate amended with 0, 0.5, 1.0, 1.5, 2.0, 2.5, or 3.0 mM As(III). The plates were incubated at 30 °C for 6 days. Biofilm formation capacity was quantified by standard crystal violet assay (Balasubramanian et al., 2017).

2.6. Confocal imaging of biofilms

A mid-log phase culture of a strain was diluted into an $O.D_{600}$ of 0.05 using TSB medium. The diluted culture was placed into a 12-well flat-bottom polystyrene microplate (with coverslips) amended with 0, 1.0, or 2.0 mM As(III). The microplates were incubated for 6 days at 30 °C. Biofilms attached to coverslips were stained with 0.2% acridine orange for 5 min, and observed under the Laser Scanning Confocal Microscope. Images were captured using the VK confocal software. Image stacks were collected from 5 randomly-selected points of the biofilms. The biofilm thickness was calculated using the Z-Stack software (Shukla and Rao, 2013; Graham et al., 2017).

2.7. Determination of As(III) oxidation activities of biofilms and their suspended cells

Biofilms of each strain were formed in a 12-well microplate in the presence or absence of 2.0 mM As(III). Duplicate wells were used for cell counting using a hemocytometer. Biofilms in triplicate wells were suspended by sonicating six times at 60 Hz for a total of 30 min. Both bacterial films and suspended cells in triplicate were rinsed twice with PBS (Li et al., 2016). Suspended cells were adjusted to the same cell numbers as biofilms. Bacterial As(III)-oxidizing activities of the biofilms and suspended cells were evaluated in 5.0 mL of PBS amended with 1.0 mM As(III) by incubating at 30 °C with shaking. At an interval of 0.5 or 5.0 h, approximately 0.5 mL of cultures was removed for measuring the concentrations of As(V) and As(III).

2.8. Detection of the As resistance of biofilms and planktonic cells

A mid-log phase culture of a strain was diluted with TSB medium to