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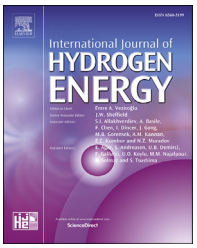

Microbial oxidation of antimonite and arsenite by bacteria isolated from antimony-contaminated soils

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ABSTRACT

Two Sb(III)-oxidizing bacteria were isolated from Sb(III)-contaminated soil. By analyzing the *16S rRNA* gene, two isolated strains were designated *Shinella* sp. NLS1 and *Ensifer* sp. NLS4. Both strains could oxidize Sb(III) to produce Sb(V) under nutrient-limited conditions. Kinetic studies indicated the NLS1 Sb(III) oxidation rate (V_{max} = 0.28 μM min^{-1}, K_m = 279.13 μM) was comparable to that of NLS4 (V_{max} = 0.32 μM min^{-1}, K_m = 248.43 μM). NLS1 could also perform aerobic As(III) oxidation and simultaneous oxidation of Sb(III) and As(III). NLS4 was able to perform Sb(III) oxidation under anaerobic conditions with nitrate as an electron acceptor. NLS1 possesses the *aioA* gene, which might function as both arsenite and antimonite oxidase, whereas strain NLS4 does not. This implies that Sb(III) oxidation in strains NLS1 and NLS4 occurred through two different pathways. This study demonstrates the potential of both isolates for bioremediation of Sb-contaminated sites.

Introduction

Antimony (Sb) and arsenic (As) are two toxic metalloids in group 15 of the periodic table of elements. The occurrence of Sb and As in water systems originates from natural weathering of rocks, mining activities, and industry [1,2]. Both Sb and As are listed as priority pollutants by the United States Environmental Protection Agency [3]. Sb is considered to be more toxic than As; therefore, the maximum acceptable concentration of Sb in drinking water is set at 5 μg L^{-1} [4], whereas that of As is set at 10 μg L^{-1} [5] by the World Health Organization. The production and application of Sb as a flame-retarding agent and decolorizing agent for the electronics industry continuously increases. Therefore, Sb pollution in the environment and its remediation is a focus area for the scientific community.

Because they are both in group 15, Sb shares some chemical properties with As such as existing in the +5 redox state in the oxygenated environment and in the +3 redox state in anoxic conditions. Although both forms of Sb are toxic, Sb(III) compounds are 10 times more toxic than Sb(V) compounds [6,7]. Aqueous Sb(III) is more mobile than Sb(V) [8]. In addition, the recently established methods for Sb removal from water sources by adsorption on Fe–Mn binary oxides demonstrates that the oxidation of Sb(III) to Sb(V) has a positive effect on Sb(III) removal [9,10]. The oxidation of Sb(III) to Sb(V) not only reduces the toxicity of Sb, but also changes its mobilization in the environment and benefits some treatment technologies.

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Biological oxidation of Sb(III) is preferable to chemical oxidation because the known chemical methods are not environmentally friendly. Microbial oxidation of Sb(III) to Sb(V) is based on the enrichment and activation of microorganisms that can transform Sb(III) into Sb(V) through enzymatic pathways. Since the first Sb(III)-oxidizing bacterium, *Stibiobacter sernamontii*, was reported by Lialikova's group [11,12], microbial Sb(III) oxidation has not been explored to the extent of As(III) oxidation. More than 60 Proteobacteria have been isolated from environmental samples contaminated with Sb [13–17]. The bacteria capable of oxidizing Sb(III) and As(III) simultaneously were also explored [16]. The *aioA* gene, which encodes the arsenite oxidase large subunit, is partially involved and functions as an antimonite oxidase, but other unknown pathways are also implicated [17,18]. Recently, an oxidoreductase designated *anoA* was found to be capable of Sb(III) oxidation [19]. The Sb(III) oxidation kinetics by purified *aioA* and *anoA* were well studied *in vitro*, but the kinetics of microbial Sb(III) oxidation by microorganisms are unknown.

In this study, the Sb(III)-oxidizing bacteria were isolated from Sb-contaminated soil collected near a Sb trioxide-producing factory. The isolated bacteria were tested for growth with and without yeast extract and under aerobic and anaerobic conditions. Kinetics of microbial Sb(III) oxidation to Sb(V) by the isolates was studied under aerobic conditions. In addition, the isolated bacteria were also tested for aerobic As(III) oxidation ability and simultaneous As(III) and Sb(III) oxidation when grown in a mixture of various Sb(III) and As(III) concentration ratios. The arsenite oxidase (*aioA*) gene, which might be involved in Sb(III) and As(III) oxidation, was identified and characterized.

Materials and methods

Bacterial enrichment and isolation

The enrichment experiment was carried out using Sb-contaminated soil and minimal medium containing 0.1 g L^{-1} of KH_2PO_4, 0.1 g L^{-1} of NH_4Cl, 0.01 g L^{-1} of $CaCl_2$, 0.05 g L^{-1} of KCl, 0.5 g L^{-1} of $NaHCO_3$, 1 g L^{-1} of yeast extract, and 1 mL L^{-1} of vitamin solution (Gamborg's vitamin solution 10 ×, Sigma-Aldrich Co., LLC, South Korea). The concentration of Sb(III) in the enrichment medium was fixed at 500 μmol L^{-1} by adding potassium antimony(III) tartrate ($KSbC_4H_4O_7 \cdot 0.5H_2O$).

The Sb-contaminated soil was collected near a Sb-producing factory in Chungcheongnam-do, Republic of Korea. The total Sb concentration in the soil sample was 29.1 ± 0.2 g kg^{-1}, which was determined by inductively coupled plasma optical emission spectrometry (ICP-OES; Agilent Technologies 7700 series, Wilmington, DE, USA) after digesting the soil sample by aqua regia. This soil sample was classified as very toxic because of its heavy Sb contamination.

The enrichment experiment started with 10 g of the soil sample added into 100 mL minimal medium stored in a 250-mL flask capped with a silicon stopper. The enrichment culture was carried out in duplicate and incubated at 120 rpm and 25 °C in a shaking incubator. The enrichment was then sub-cultured every 5 d by transferring 10 mL of the culture supernatant into fresh medium. After 3 sub-cultivations, the enrichment culture was serially diluted and spread on agar medium containing the same ingredients as the enrichment medium. The agar culture was then incubated under aerobic conditions in an incubator at 25 °C. Four single colonies corresponding to 4 bacterial strains (named NLS1, NLS2, NLS3, and NLS4) were isolated and purified. The purified cultures on agar plate were then recultured in the liquid medium to test the Sb(III)-oxidizing ability. When cultured in the medium containing the same ingredients as the enrichment medium, only strains NLS1 and NLS4 aerobically oxidized Sb(III) to Sb(V), whereas the other two strains did not show Sb(III) oxidation ability (data not shown).

Aerobic Sb(III) oxidation with and without yeast extract

Experiments on aerobic Sb(III) oxidation with and without yeast extract were performed with strains NLS1 and NLS4, which showed Sb(III) oxidation ability. Yeast extract was the common carbon sources for microorganisms and was added at 0.1% (1 g L^{-1}). Sb(III) was added to the minimal medium at the concentration of 500 μM. The inoculum (1%, v/v) from both bacterial strains were obtained from the pre-cultures in the exponential growth phase (after 24 h incubation). The pre-cultures were cultivated under the same conditions. These experiments were carried out in triplicate using Erlenmeyer flasks incubated in a shaking incubator at 120 rpm and 25 °C. The mean values with standard deviation error bars are presented in each figure. The abiotic controls in which no inoculum was added were implemented to validate the results obtained by microbial performance.

Kinetic study of Sb(III) oxidation by isolated strains

Microbial Sb(III) oxidation kinetics were investigated under aerobic conditions for both strains NLS1 and NLS4. The kinetic experiments were carried out under the same conditions as the above growth experiment using minimal medium without yeast extract. The initial Sb(III) concentration investigated was in the range of 50–2000 μM. The kinetic rates of microbial Sb(III) oxidation were calculated following the Michaelis-Menten equation:

$$v = \frac{V_{max}[S]}{K_m + [S]} \quad (1)$$

where, v (μM·min^{-1}) is the reaction rate of microbial Sb(III) oxidation, [S] (μM) is the initial molar concentration of Sb(III), V_{max} (μM·min^{-1}) is the maximum velocity of the Sb(III) oxidation reaction, and K_m is the Michaelis-Menten constant. The K_m value indicates the initial Sb(III) concentration at which the Sb(III) oxidation rate achieved is half of V_{max}. The V_{max} and K_m were obtained from the Lineweaver-Burk plot derived by linearization with the selected data from culture experiments. The V_{max} and K_m values were optimized using Solver in Microsoft Excel 2010 to build the kinetic model.

Anaerobic Sb(III) oxidation with nitrate as the electron acceptor

Both isolated strains were tested for anaerobic growth with nitrate as an electron acceptor using 150-mL serum bottles. Sb(III) (200 μM) was added to the minimal medium as the

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