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NOTE

Inhibitory Effect of Nitrate on Fe(III) and Humic Acid Reduction in *Shewanella putrefaciens* DK-1

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The inhibitory effects of nitrate on Fe(III) and humic acid reduction were examined in *Shewanella putrefaciens* DK-1. There is no difference in Fe(III) reduction until 25 hours between cultures using Fe(III) alone as an electron acceptor and using Fe(III) and nitrate as electron acceptors, but after 25 hours Fe(II) production was decreased drastically when Fe(II) and nitrate were used as electron acceptors. The production of AHQDS (2,6-anthrahydroquinon disulfonate) showed similar patterns when AQDS alone and both AQDS and Fe(III) were used as electron acceptors. When AQDS (2,6-anthraquinon disulfonate) and nitrate were used as electron accepors, the production of AHQDS was completely inhibited.

Key words: Humic acid reduction, electron acceptor

Shewanella putrefaciens is capable of dissimilatory reduction of many different electron acceptors including O_2, iron, manganese, nitrite, fumarate, thiosulfate, dimethyl sulfoxide, trimethylamine N-oxide, and elemental sulfur as terminal electron acceptors (9). Dissimilatory metal reducing bacteria gain energy to support anaerobic growth by coupling the oxidation of H_2 or organic matter to the reduction of various multivalent metals (3).

Many environmental microbiologists have noted that *S. putrefaciens* makes use of Fe(III) and Mn(IV) as electron acceptors. Many researchers have focused on microbiological-geochemical investigations of Fe(III) reduction as a model for microbial transformations of harzadous metals such as U(VI) and Cr(V). It is also known that humic substances are chemically oxidized by ferric iron or manganese oxides (8). These results are considered encouraging signs in bioremediating metal contaminated areas. *In situ* bioremediation strategies using dissimilatory metal reducing bacteria would rely on either stimulating naturally occurring dissimilatory metal reducing bacterial population, or inoculating preadapted or genetically engineered dissimilatory metal reducing bacteria into the contaminated environment.

DiChristina (1) showed that nitrogen oxide reduction was not affected by the presence of Fe(III) and that Fe(III) reduction was partially inhibited by the presence of either NO_3^- or NO_2^-. This study was carried out to show the inhibitory effect of nitrate on Fe(III) and humic acid reduction.

S. putrefaciens DK-1, which could use nitrate, Fe(III), and humic acid as electron acceptors, is a facultative anaerobic bacterium that was isolated from oil contaminated sediment in Korea and was identified by API-20NE and MIDI systems. It was cultivated in mineral media. One liter of mineral media contained the following : $NaHCO_3$ 2.5 g, $(NH_4)_2SO_4$ 30 mg, NaCl 2 g, K_2HPO_4 0.5 g, $MgSO_4$-$7H_2O$ 50mg, $CaCl_2$-H_2O 20 mg, and trace metal solution 2ml, whose composition was described previously (4). Also, sodium lactate (10mM) as an electron donor, Fe(III)-citrate (10 mM), AQDS (2.6-anthraquinone disulfonate) (10 mM) for a model compound of humic acid and KNO_3 (10 mM) as an electron acceptor were added. Anaerobic condition was constructed by the substitution of headspace with mixed gas (N_2: CO_2= 85:15). Cultures were grown in triplicates, each in a 250 ml serum vial containing 100 ml of mineral medum capped with silicon rubber and an aluminium seal. Inoculations of *S. putrefaciens* DK-1 strain were performed with washed cell suspensions suspended in saline, to an OD_{440} of 0.1 in 100 ml of mineral media and the controls for measurement of abiotic reduction rates were not inoculated. All cultures were incubated at 28°C without agi-

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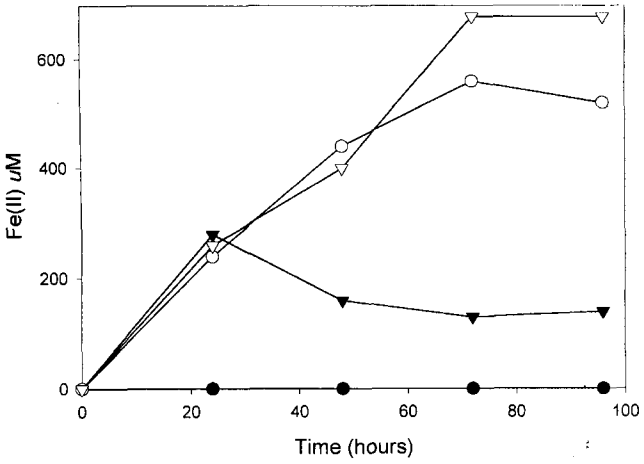

Fig. 1. Fe(III) reduction with lactate (10 mM) as the electron donor and NO_3 and AQDS as electron acceptors (● : control ; ○ : Fe(III) only ; ▽ : Fe(III)+AQDS; ▼ : Fe(III)+NO_3)

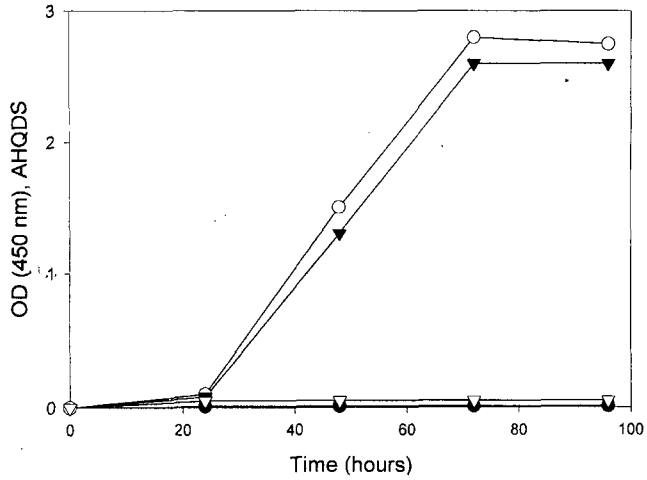

Fig. 2. AQDS reduction with lactate (10 mM) as the electron donor and NO_3 and AQDS as electron acceptors (● : control ; ○ : AQDS only ; ▽ : AQDS+NO_3; ▼ : AQDS+Fe(III))

tation.

Analytical samples were removed periodically with 10 ml syringes. Fe(III) reduction was monitored by measuring the accumulation of Fe(II) over time. Fe(II) was solubilized in 0.5N HCl for 15 min and the amount of Fe(II) was determined with ferrozine as previously described (4). The humic acid reduction rate was measured by the rate of AQDS reduction to AHQDS (2,6-anthrahydroquinon disulfonate) of which concentrations were measured by the increase of absorbance at 450 nm (8).

In the experiment of Fe(III) reduction (Fig. 1), 550 μM of Fe(II) were produced at 75 hours of cultivation when Fe(III) was used as an electron acceptor. However 630 μM of Fe(II) were produced at the same time in the presence of Fe(III) and AQDS as electron acceptors. Microbial humic reduction also enhances the capacity of microorganisms to reduce other less accessible electron acceptors such as insoluble Fe(III) oxides because humic substances can shuttle electrons between the humic reducing microorganisms and Fe(III) oxide (8). AQDS could accelerate the reduction of Fe(III). It seemed that humic acid could act as an electron shuttle to Fe(III), which elevated the Fe(III) reduction rate. Although there is no difference in Fe(III) reduction until 25 hours between cultures using Fe(III) alone as an electron acceptor and using Fe(III) and nitrate as electron acceptors, after 25 hours Fe(II) production decreased drastically when Fe(III) and nitrate were used as electron acceptors. In the presence of Fe(III) and either nitrate or nitrite, both Fe(III) and nitrogen oxide reduction occurred simultaneously (1). Nitrogen oxide was not affected by the presence of Fe(III), suggesting that *S. putrefaciens* 200 expressed a set of at least three physiologically distinct terminal reductases that served as electron donors to nitrate, nitrite and Fe(III). However Fe(III) reduction was partially inhibited by the presence of either nitrate or nitrite. Nitrate inhibition of electron transfer to Fe(III) is caused by a preferential shuttling of respiratory electrons after nitrite reductase activity is fully saturated (1).

In the experiment of AQDS reduction (Fig. 2), the production of AHQDS showed similar patterns when AQDS alone and both AQDS and Fe(III) were used as electron acceptors. When AQDS and nitrate were used as electron acceptors, the production of AHQDS was completely inhibited. The finding that microorganisms can donate electrons to humic acid has important implications in mechanisms by which microorganisms oxidize both natural and contaminated organics in anaerobic soils and sediments, and suggests a biological source of electrons for humic mediated reduction of contaminated metals and organics (8). The mineralization of aromatic hydrocarbons in a contaminated aquifer by indigenous populations of dissimilatory metal reducing bacteria could be stimulated by the addition of synthetic iron chelators (2,6,7,8,) or natural humic acids (7). Fe(III) reducers also have the potential to aid in the remediation of metal and metalloid contaminants in the subsurface. Fe(III) reducers have the ability to substitute several metal and metalloid contaminants for Fe(III) as electron acceptors. The most intensively studied substance of these contaminants has been uranium (3,5). Microbial U(VI) reduction is environmentally significant because U(VI) is highly soluble whereas U(IV) is generally insoluble. Reductive precipitation of uranium is likely to be an important mechanism sequestering uranium in anaerobic aquatic sediments and may account for the formation of some forms of uranium deposits.

Fe(III)-reducing microorganisms could help the remediation of metal and metalloid contaminants in subsurface environments by their ability to use some of these contaminants as electron acceptors. Unfortunately, nitrate which is prevalent in the environments can act as an inter-

fering substance in metal bioremediation using dissimilatory metal reducing bacteria such as *S. putrefaciens*. Our results indicate that studies concerning on these inhibitory materials must continue in order to pursue successful metal bioremediation methods.

Acknowledgments

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