Project 1012275

Formation and Reactivity of Biogenic Iron Microminerals

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RESULTS TO DATE: Fe(II)-induced inhibition of dissimilatory bacterial reduction of metals and radionuclides: the role and reactivity of cell-surface precipitates Interim Report

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August 10, 2002

Overall objective

Radionuclide and heavy metal contaminants at DOE sites pose immediate and long-term environmental problems. Under the NABIR program, bacteria are being considered for their role in the cycling of these contaminants because they influence many redox reactions in the subsurface. Dissimilatory metal reducing bacteria (DMRB) are particularly important to controlling the biogeochemistry of subsurface environments through enzymatic reduction of iron and manganese minerals. During reduction of FeIII, biogenic FeII phases form at the cell-mineral interface which may profoundly influence metal reduction. The purpose of the project is to characterize these minerals and the reactions that form them, investigate the effects of FeII on the cell surface and to identify conditions that minimize inhibitory effects of FeII. Towards achieving these goals, we have directed our efforts in the last year to: 1) understanding the formation and nature of precipitates formed during Fe reduction by Shewanella putrefaciens CN32, 2) investigating intracellular Fe oxide particles formed during Fe reduction by CN32, 3) conducting hydrophobicity/hydrophilicity studies on Shewanella and 4) investigating how these characteristics affect Fe oxide adhesion.

Task #1: Formation of extracellular mineral precipitates

Approach

Understanding overall mineral dynamics during Fe reduction will help elucidate pathways of Fe2+ in the bulk solution and partitioning of Fe2+ to the cell surfaces. Fe reduction experiments were carried out using batch cultures of Shewanella putrefaciens CN32 and hydrous ferric oxide (HFO). Cultures were grown in anaerobic defined mineral medium consisting of mineral salts, 20 mM lactate, and 4 mM NaHPO4H2O, and were held in an aerobic chamber with a H2/Ar atmosphere for the duration of the experiment. Cell viability was monitored by agar plate counts, Fe2+ by the ferrozine method, and P by the phosphomolybdate method. Samples were taken for TEM and prepared as whole mounts and embedded samples for sectioning. XRD scans were made in an N2 atmosphere on culture precipitates that were washed and dried in the anaerobic chamber.

Progress

The rate of FeIII reduction appears to be key to the formation of post-reduction minerals. Rapid reduction favors the formation of large vivianite crystals away from the cells in the bulk solution. It appears that Fe2+ produced by the actively respiring bacteria diffuses away from the cells and into aggregates of HFO, catalyzing the further reduction of HFO. Slower reduction of FeIII results in higher proportions of

magnetite, which crystallizes within flocs of cell-associated HFO at the cell surface. The formation of magnetite does not, however, appear to be limiting cell growth. Evidence for this comes from cultures where FeIII is reduced more slowly, which exhibit the highest survivability. Green rust is a precursor to both vivianite and magnetite, and it was observed both within HFO aggregates and associated with the cell surface. Goethite was also observed at the cell surface and was temporally and spatially associated with the magnetite. It appears that under our culture conditions phosphate has the greatest effect on the viability of the bacteria; once soluble P was consumed by vivianite precipitation, the cultures began to die off. We aim to eliminate P limitation by implementing continuous culture studies and by using mineral forms of P, such as brushite, rather than soluble P salts, to achieve constant low levels of P.

Task #2: Intracellular granules

Approach

We previously observed that CN32 forms intracellular granules of Fe oxide during reduction of HFO and Fe-citrate. The granules form at the cell poles, with up to 60 particles visible in active cells. This phenomenon represents a potential pathway for removal of Fe^{2+} from the surface and uptake to the cytoplasm. The mechanism for formation of the granules and their function is still unknown although we speculate that they play a role in respiration. To determine if the granule phenomenon was specific to Fe metabolism or extended to other redox-active metals, we carried out reduction studies identical to the ones described under Task #1, but replacing HFO with birnessite and pyrolusite. Both minerals are MnIV oxides; birnessite exhibits poorly crystalline layer structures composed of MnIV octahedra, whereas pyrolusite consists of MnIV octahedra in well-crystalline tunnel structures. MnIV reduction was monitored by measurement of soluble Mn^{2+} using atomic absorption spectroscopy. Granule formation was followed by EM on samples taken at regular intervals. At the same time, we have continued to investigate the intracellular Fe particles via batch culture studies. Cells were grown anaerobically in Fe-citrate until most cells (> 90 %) contained more than 10 distinct granules. The cultures were then exposed to air (with stirring) and the bacteria were monitored over 24 h by electron diffraction. In addition, electron nanodiffraction techniques have been applied to characterize the mineral structure of the granules.

Progress

MnIV reduction began several hours (birnessite) to 1 day (pyrolusite) after inoculation. Mn-rich granules were observed forming within 3 days, and 10-15 particles per cell were counted for apparently active cells. The granules were similar in size and shape to those observed during Fe reduction. In contrast, however, the granules did not preferentially cluster to the poles and were dispersed throughout the cell, although there was a tendency for particles to accumulate at the poles over time. Over four weeks the number of particles per cell did not increase. Bacteria appeared to become closely associated with the birnessite and pyrolusite minerals during reduction. In additions, thin sections of embedded culture samples revealed periplasmic precipitates that have not been seen for similar studies with Fe. We are currently working to characterize these precipitates. With respect to the intracellular Fe granules, exposing anaerobic cultures CN32 to air had no apparent effect. The bacteria grew vigorously and soluble Fe^{2+} produced during FeIII reduction rapidly oxidized and formed reddish precipitates that coated the cells. The fact that cells with granules were still observed after 24 h indicates that the granules are conserved during cell division. This agrees with TEM observations of dividing cells, during which granules can be distributed between the cells. The stability of the granules against oxidation supports that they consist of FeIII, since FeII phases are unstable in air. However, extensive nano-diffraction studies on intracellular particles formed during respiration on FeIII-citrate and HFO indicate no regular crystalline structure. The interpretation of this result is complicated by the fact that hydroxide mineral phases are often highly unstable in the electron beam. Diffraction patterns recorded at a rate of 30 frames per second indicate crystal structures similar to magnetite, but these were not consistent for all particles examined. We are continuing characterization of the granules using synchrotron-based spectroscopies at the Advanced Light Source in Berkeley, and Argonne in Illinois.

Task #3: Cell surface physico-chemical properties of Shewanella species

Approach The rate of bacterially-mediated reduction of FeIII-minerals may depend on the strength and closeness of cell adhesion to the mineral surface. Structural components of the Shewanella outer membrane (OM; such as proteins [OMPs], lipopolysaccharide [LPS], and capsular polysaccharide [CPS]) determine the surface physico-chemical properties of the cell, and are responsible for establishing and maintaining contact with mineral surfaces. Adhesion mechanisms of Shewanella to iron minerals, however, are not completely understood. Previously we characterized different Shewanella species with respect to surface composition (LPS profiles, PS structure) and ultrastructure. Now we haveexamined hydrophobicity and surface charge of nine Shewanella strains/species. Cultures were grown aerobically on trypticase soy broth and/or anaerobically on defined mineral lactate/fumarate medium, and the cells were harvested at the mid-exponential phase. Relative surface hydrophobicity of these strains was determined using hydrophobic interaction chromatography. Relative cell surface charge was assayed using electrostatic interaction chromatography.

Progress Hydrophobic interaction chromatography revealed that LPS rough strains possessing no LPS side chains (e.g., CN32, 63) were very hydrophobic over a full range of ionic strength (0.1-4.0 M of NaCl) while other strains (OK-1, BrYDL) and encsapsulated strains (MR-4, DLM-7, BrYFC) were hydrophilic. At the same time, all rough strains were highly electronegative. Strains that possessed capsules displayed much less electronegativity than rough strains. However, the negative charge of encapsulated strains was still quite high; about 100% of the cells were bound to the anion-exchange resin at a phosphate concentration up to 0.2M. This work was presented at the International Union of Microbiological Societies (IUMS) Congress in Paris this summer.

Task #4: Adhesion of Shewanella to FeIII minerals.

Approach To relate the cell surface properties (see task #3) to FeIII mineral adhesion we conducted adsorption experiments. Shewanella spp. grown aerobically or anaerobically were washed twice in 0.2 M HEPES buffer pH 7.4 and resuspended in the buffer. Then, cell suspensions were mixed with hematite and placed on a rotary shaker at 125 rpm for 30 min. In the case of anaerobic cultures, all steps were carried out under anaerobic conditions. Initial cell concentrations were 0.3-0.4 mg of dry cell wt./ml and hematite concentrations varied between 0.05-4.0% on a wt to wt basis. In these experiments we used commercially available hematite with a grain size 10-20 micrometer. Samples were taken for transmission electron microscopy (TEM) and were prepared as unstained whole mounts to minimise stain-mineral alterations.

Progress

Cell adhesion to hematite occurred within minutes of contact. To characterize cell adhesion to hematite we determined the hematite sorption capacity for Shewanella at different cellular equilibrium concentrations. The highest sorption affinity and capacity to hematite was observed for rough LPS strains. Encapsulated strains displayed the lowest adhesion. Correlation analysis (relative cell surface hydrophobicity or relative cell surface charge versus adhesion to hematite) suggested that cell electronegativity played an important role in cell adhesion and that electrostatic interactions between cells and FeIII minerals prevail over hydrophobic interactions. TEM observations of whole-mount preparations revealed that cells adhered to the surface of hematite particles via their poles as well as their sides. At low cell concentrations, bacteria formed monolayers over the mineral surface. However, at high equilibrium concentrations of cells multiple bacterial layers formed around mineral particles so that many bacteria bound not only to the mineral but also to their neighbouring cells. In this case, co-operative cell-to-cell binding took place due to hydrophobic interaction. We are currently trying to establish relationship between cell adhesion properties and the rate of mineral reduction. Furthermore, we hope to establish if adsorption of different anions and cations (e.g., Fe2+) influences Shewanella adhesion to FeIII-minerals and, therefore, the rate of mineral reduction.

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