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Inhibition of NO_3^- and NO_2^- Reduction by Microbial Fe(III) Reduction: Evidence of a Reaction between NO_2^- and Cell Surface-Bound Fe^{2+}

Aaron J. Coby and Flynn W. Picardal*

Environmental Science Research Center, School of Public and Environmental Affairs, Indiana University, Bloomington, Indiana 47405

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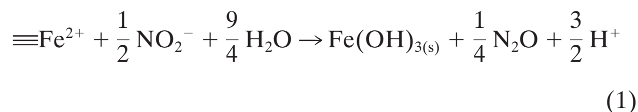
A recent study (D. C. Cooper, F. W. Picardal, A. Schimmelmann, and A. J. Coby, Appl. Environ. Microbiol. 69:3517–3525, 2003) has shown that NO_3^- and NO_2^- (NO_x^-) reduction by *Shewanella putrefaciens* 200 is inhibited in the presence of goethite. The hypothetical mechanism offered to explain this finding involved the formation of a Fe(III) (hydr)oxide coating on the cell via the surface-catalyzed, abiotic reaction between Fe^{2+} and NO_2^-. This coating could then inhibit reduction of NO_x^- by physically blocking transport into the cell. Although the data in the previous study were consistent with such an explanation, the hypothesis was largely speculative. In the current work, this hypothesis was tested and its environmental significance explored through a number of experiments. The inhibition of ~3 mM NO_3^- reduction was observed during reduction of a variety of Fe(III) (hydr)oxides, including goethite, hematite, and an iron-bearing, natural sediment. Inhibition of oxygen and fumarate reduction was observed following treatment of cells with Fe^{2+} and NO_2^-, demonstrating that utilization of other soluble electron acceptors could also be inhibited. Previous adsorption of Fe^{2+} onto *Paracoccus denitrificans* inhibited NO_x^- reduction, showing that Fe(II) can reduce rates of soluble electron acceptor utilization by non-iron-reducing bacteria. NO_2^- was chemically reduced to N_2O by goethite or cell-sorbed Fe^{2+}, but not at appreciable rates by aqueous Fe^{2+}. Transmission and scanning electron microscopy showed an electron-dense, Fe-enriched coating on cells treated with Fe^{2+} and NO_2^-. The formation and effects of such coatings underscore the complexity of the biogeochemical reactions that occur in the subsurface.

Dissimilatory reduction of ferric (hydr)oxide minerals has been documented for a large number of microorganisms in a wide range of environments (18, 19, 22, 26) and has become recognized as an important constituent of the global carbon and iron cycles (21, 23, 32, 44). Microbial reduction of Fe(III) and other metals has a profound effect on groundwater geochemistry and plays a key role in the fate of contaminant metals (5, 8, 13, 14, 20, 33) and organic compounds (16, 23, 24) in anoxic groundwater.

Nitrate has both natural and anthropogenic sources, but the extensive use of NO_3^- as an agricultural fertilizer threatens the quality of many groundwater systems (41). The use of denitrifying microorganisms to remediate NO_x^--contaminated soils may be a solution for its removal in some environments (27). Since contaminant metals and NO_3^- may coexist in some iron-bearing sediments (12, 31), it is important to understand the complex, biogeochemical interactions that can occur during the microbial reduction of NO_x^- and Fe(III) (hydr)oxides. *Shewanella putrefaciens* 200 is a facultative anaerobe capable of utilizing NO_3^-, NO_2^-, and Fe(III), as well as O_2, Mn(IV), trimethylamine *N*-oxide, thiosulfate, fumarate, and a number of other compounds as terminal electron acceptors for carbon metabolism (9, 29, 30). This makes *S. putrefaciens* an ideal organism for studying biogeochemical interactions during redox transformations of NO_3^-/NO_2^- and Fe(III)/Fe(II).

NO_x^- reduction by microorganisms typically precedes ferric (hydr)oxide reduction in experimental or subsurface systems, in part because NO_x^- tends to be more available to cells than the highly insoluble (at neutral pH) forms of Fe(III) (1, 9, 29, 30, 39). Recently, though, Cooper et al. reported that the reduction of NO_3^- and NO_2^- by *S. putrefaciens* 200 was inhibited by the presence of the solid-phase ferric (hydr)oxide goethite (α-FeOOH) (6). In addition, they observed that the presence of goethite in NO_x^--reducing incubations resulted in greatly enhanced production of N_2O. Cooper et al. speculated that the inhibition and enhanced N_2O production resulted from reaction of small amounts of biogenic Fe(II) with NO_2^- to form an Fe (hydr)oxide coating on the cell surface that inhibited transport of soluble electron acceptors into the cell. Although the stoichiometry and products are subject to speculation, reaction 1 shows such a reaction between surface-sorbed Fe^{2+} ($\equiv Fe^{2+}$) and NO_2^- that could lead to production of a solid Fe (hydr)oxide.



$$\equiv Fe^{2+} + \frac{1}{2} NO_2^- + \frac{9}{4} H_2O \rightarrow Fe(OH)_{3(s)} + \frac{1}{4} N_2O + \frac{3}{2} H^+ \quad (1)$$

Sørensen et al. described a similar surface-catalyzed reaction between Fe^{2+} and NO_2^- for which reduction of NO_2^- to N_2O was very slow in the absence of a mineral surface but proceeded rapidly in the presence of lepidocrocite (γ-FeOOH)

* Corresponding author. Mailing address: Environmental Science Research Center, School of Public and Environmental Affairs, Indiana University, Bloomington, IN 47405. Phone: (812) 855-0733. Fax: (812) 855-7802. E-mail: picardal@indiana.edu.

(40). The hypothetical mechanism suggested by Cooper et al. requires the sorption of Fe^{2+} to the cell surface before reacting with aqueous NO_2^-. Dissimilatory iron-reducing bacteria are known to sorb Fe^{2+} to their cell surfaces, and such sorption is believed to passivate the surface and impede further Fe(III) reduction (34, 46, 47). Fe^{2+} is relatively soluble at neutral pH and, in anoxic, iron-reducing environments, there may also be significant sorption of Fe^{2+} to cell surfaces of bacteria incapable of iron reduction. NO_2^-, whether from anthropogenic sources or produced via nitrification or denitrification, will have the opportunity to react with that sorbed Fe^{2+}. The oxidation of surface-bound Fe^{2+} by NO_2^- and resulting formation of a Fe (hydr)oxide mineral on the surface of the cell could have a rapid, inhibitory affect on the transport of soluble electron acceptors into the cell. Although recent work has established the ability of some bacteria to catalyze Fe(II)-dependent reduction of NO_3^- (45, 49), little is known about how abiotic NO_x^- reduction by cell-sorbed Fe^{2+} will affect microbial metabolism.

Processes that limit the rate or extent of NO_x^- reduction may have notable environmental effects. Attempts to bioremediate a NO_x^--contaminated site by addition of a suitable substrate, for example, would take longer and be more costly. Since NO_3^- must often be consumed prior to bioremediation of metals and radionuclides (5, 11), remediation of these compounds may also be negatively affected by processes that limit NO_x^- removal. The same inhibition mechanism may also affect the reduction of other environmentally relevant electron acceptors, which has broad implications for the understanding of subsurface geochemical cycles. Since N_2O, a product of the proposed reaction, is also a potent greenhouse gas also believed to be implicated in stratospheric ozone depletion (7, 10, 48), increased knowledge about possible N_2O sources may improve our modeling of global climate change and other environmental stresses.

The inhibitory mechanism proposed by Cooper et al. was supported primarily by nitrogen stable isotope experiments that showed that the increased N_2O production was primarily of chemical origin, i.e., produced by the abiotic reduction of NO_2^- by surface-bound Fe^{2+} produced during microbial reduction of synthetic goethite. Their proposed mechanism, however, was largely speculative, direct evidence of such Fe-rich cell coatings was not provided, and the potential environmental significance of the reaction was not apparent. In the current work, we demonstrate that NO_x^- reduction is inhibited during reduction of other Fe(III) minerals, that the utilization of soluble electron acceptors other than NO_x^- are similarly inhibited, and that NO_x^- reduction by microorganisms incapable of dissimilatory iron reduction can also be inhibited by sorption of aqueous Fe^{2+}. In addition, we provide direct evidence of an Fe-rich, electron-dense coating formed on bacteria in the presence of NO_2^- and Fe^{2+}.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *S. putrefaciens* 200 is a gram-negative motile rod with an obligate respiratory metabolism (37), originally isolated from a Canadian oil pipeline by Obuekwe (28). *Paracoccus denitrificans* (ATCC 13543) is a facultative, denitrifying bacterium incapable of reducing Fe(III) that was used in this study for comparison to *S. putrefaciens*. The cultures were maintained at −80°C in a 10% glycerol–90% nutrient broth solution. Liquid cultures were grown aerobically to late log phase in a 1-liter flask on a shaker table in Westlake medium (30). Cells were subsequently harvested by centrifugation and resuspended to a target optical density (λ, 600 nm) of approximately 1.2 in low-ionic-strength, artificial groundwater (AGW) medium. The full composition of AGW medium is described elsewhere (5). This medium contains 15 mM lactate as an electron donor, and primary buffering capacity is provided by 10 mM HEPES buffer. Phosphate (0.044 mM) and bicarbonate (0.50 mM) concentrations are relatively low to minimize the potential for precipitation of vivianite or siderite.

Fe(III) and NO_x^- reduction experiments. Although the general methodology employed in the Fe(III) and NO_3^- reduction experiments has been previously described (6), a summary of the procedure is presented here. All experiments were conducted in 150-ml serum bottles, crimp sealed with butyl rubber stoppers under a 95% N_2–5% H_2 headspace. Except where noted, slurries were established that contained 75 ml AGW medium and the appropriate solid phase. Three solid phases were used in separate experiments as sources of ferric iron: goethite (α-FeOOH,) hematite (Fe_2O_3), and an iron-containing natural sediment. Goethite was prepared as described by Schwertmann and Cornell (36), hematite was purchased from J.T. Baker/Mallinckrodt Baker Inc., and the natural sediment (MNC-71) was collected from iron-bearing, clayey sediments at Marshall, N.C. (J. Zachara, PNNL, personal communication). The amount of each sediment required for the 50 mM Fe^{3+} (by citrate dithionate extraction) used in these experiments was approximately 0.34 g goethite, 0.6 g hematite, and 4.9 g MNC-71 in 75 ml of medium. When required, NO_3^- or NO_2^- was added to serum bottles from sterile stock solutions of $NaNO_3$ and $NaNO_2$. In experiments requiring a chelator to reduce Fe^{2+} adsorption to cell surfaces, 1.0 ml sterile anaerobic nitrilotriacetic acid (NTA) stock solution was added to the AGW medium for a final concentration of 5.0 mM.

Experiments were initiated by inoculating slurries with 1 ml of washed *S. putrefaciens* suspension under anoxic conditions (ca. 2×10^6 cells ml^{-1}). Serum bottle reactors were wrapped in foil and incubated horizontally on a shaker table at room temperature. Initial samples were taken prior to inoculation, and subsequent samples were taken at regular intervals thereafter. During sampling, bottles were transferred to the anaerobic chamber, shaken vigorously, and immediately sampled with a 3-ml syringe (23-gauge needle). A 1.5-ml aliquot of slurry was transferred to a microcentrifuge tube, and solids were separated by centrifugation. The supernatant was immediately sampled for pH, NO_3^-, and NO_2^-. To determine if the observed inhibition of NO_x^- reduction was due to unknown goethite toxicity, a different bacterium (*Paracoccus denitrificans*) was substituted for *S. putrefaciens*. *P. denitrificans* is able to utilize NO_3^- as a terminal electron acceptor but is unable to reduce Fe(III). The same growth and experimental procedures described above for goethite and NO_3^- reduction were followed, using *P. denitrificans* in place of *S. putrefaciens*.

Reaction of NO_2^- with sorbed Fe^{2+}. Experiments were done to measure N_2O production from the abiotic reduction of NO_2^- by aqueous Fe^{2+} or Fe^{2+} sorbed to a goethite surface. Previously deoxygenated AGW medium lacking vitamins and lactate was dispensed into replicate (n = 3) sets of serum bottle batch reactors in an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI). The first set contained 3 mM Fe^{2+} added from a stock solution of $FeCl_2$ that had been prepared anoxically, adjusted to pH 7, and filtered (0.45 μm) to remove fine particulates. This filtration step was required to ensure that only aqueous Fe^{2+} was utilized and Fe(III) oxides present as contaminants on the $FeCl_2$ crystals or formed in trace amounts during stock solution preparation were not inadvertently added. The second and third sets of replicates contained 50 mM goethite and 50 mM goethite plus 3 mM Fe^{2+}, respectively. After 24 h, all systems received an amendment of NO_2^- as $NaNO_2$ to a final concentration of 3 mM. N_2O production was measured in all three systems over time as described below.

Fe^{2+} was sorbed to the surface of *S. putrefaciens* and *P. denitrificans* (4, 38) to compare the inhibition of NO_x^- reduction by bacteria able and unable to reduce Fe(III). *S. putrefaciens* and *P. denitrificans* were grown and harvested as described above and then resuspended in anaerobic AGW medium containing 50 mM $FeCl_2$ (pH, ~7) in the anaerobic chamber. The resuspension was gently mixed for 15 min to allow adsorption of Fe^{2+} onto the cell surfaces. The cells were then centrifuged again in tightly sealed centrifuge tubes to prevent Fe^{2+} oxidation and washed with anaerobic, Fe-free AGW before being used to inoculate replicate reactors containing 3 mM NO_3^- or NO_2^-. Control reactors were set up identically except they were initially resuspended in Fe-free AGW medium.

Additional experiments were similarly done (only with *S. putrefaciens*) to determine if sorption of Fe^{2+} and subsequent exposure to NO_2^- would impede the utilization of soluble electron acceptors other than NO_3^- or NO_2^-. In these experiments, Fe^{2+} was sorbed to cells and suspensions were harvested and washed as described above. The washed cells were then incubated in anaerobic AGW medium containing 5 mM NO_2^- which was allowed to react for 1 h with the Fe^{2+} sorbed to cells. Cells were washed and subsequently incubated in AGW

amended either with 5 mM fumarate or ~8.6 mg liter^{-1} O_2. Consumption of the electron acceptor over time was compared with identical suspensions that had not undergone the Fe^{2+} sorption step. Where reported, initial and final cell numbers were determined by acridine orange direct counting (15).

Electron microscopy and electron dispersion spectroscopy. Cells of *S. putrefaciens* for examination using transmission and scanning electron microscopy (TEM and SEM) were prepared by sequential incubation with Fe^{2+} and NO_2^- as described above to abiotically simulate the formation of the Fe(III) (hydr)oxide coating believed to form in NO_x^-- reducing incubations containing goethite. Control cells were incubated solely with either Fe^{2+} or NO_2^- for comparison. All preparation of samples for TEM and SEM analyses was performed in an anaerobic chamber to prevent oxidation of Fe^{2+}. For TEM analysis, prepared cells were placed on parlodian support films on 300 mesh copper grids and allowed to dry in the anaerobic chamber. Dried grids with cells were placed in an anaerobic container (BBL GasPak system) for transport to the TEM facility and were exposed to the air for less than 1 minute while being transferred to the TEM vacuum chamber. Treated and control cells were observed at 100 kV on a JEOL JEM-1010 transmission electron microscope, and images were taken at a magnification of ×12,000. For SEM analysis, prepared cells were filtered onto a 0.45-μm membrane filter, dehydrated using graded alcohol-water washes, and critical point dried in hexamethyldisilazane. Following gold coating, treated and control cells were observed at 20.0 kV on a FEI Quanta 400F scanning electron microscope at a magnifications between ×23,000 and ×78,000. Surface point elemental analysis was performed while the samples were being viewed in the SEM using a Princeton Gamma-Tech energy-dispersive spectrometer.

Analytical methods. Samples for NO_3^- and NO_2^- analyses were diluted twofold in Milli-Q water, frozen, and stored for later analysis by ion chromatography as previously described (6). NH_4^+ was quantified in some experiments using the colorimetric phenate method (15). N_2O production was quantified by removing 25.0 μl of headspace gas from the batch reactors using a gas-tight syringe and analyzed via gas chromatography as previously described (6).

When applicable, aqueous and cell-bound Fe^{2+} was analyzed via a modification of the ferrozine method (35, 42). Surface areas of the various iron oxides used were determined by multipoint Brunauer-Emmett-Teller (BET) N_2 adsorption on a NOVA 1000 surface area analyzer. Oxygen utilization rate was determined using a YSI biological oxygen monitor. Fumarate concentrations were quantified on a Waters high-performance liquid chromatography apparatus using an absorbance detector at a wavelength of 238 nm.

RESULTS AND DISCUSSION

Fe(III) reduction and inhibition of NO_x^- reduction. Our hypothetical mechanism of inhibition relies on a surface-catalyzed reaction between Fe^{2+} and NO_2^- and the subsequent formation of an Fe (hydr)oxide coating on the surface of cells. Fe^{2+} produced from the microbial reduction of Fe(III) sorbs to the surface of the cell and then is reoxidized by NO_2^-. Goethite was the only Fe (hydr)oxide used in previous experiments which resulted in the inhibition of NO_x^- (6). When hematite was used as the Fe(III) oxide, NO_3^- reduction and NO_2^- production were severely inhibited after 4 h (Fig. 1). In controls without hematite, NO_3^- reduction was complete after 7 h, and NO_2^- reduction was complete in less than 25 h. In incubations with hematite and NO_3^-, inhibition of NO_3^- reduction was not apparent for about 4 h, which may be related to the reduced Fe(III) reduction rates (not shown) and lower surface area of the hematite used in our experiments. The surface area of this hematite (~9 m^2 g^{-1}) is much less than that of the goethite (~72 m^2 g^{-1}) used in previous experiments where inhibition of NO_3^- reduction occurred almost immediately (6). The microbially accessible surface area of an iron oxide has a significant effect on the rate and extent of iron oxide reduction (34, 35). Less Fe(III) reduced to Fe^{2+} would in turn affect the rate of the hypothesized surface reaction between Fe^{2+} and NO_2^-.

Nitrate reduction in the presence of sediment MNC-71 also progressed more slowly than in sediment-free systems. After approximately 12 h, more than 10 times as much NO_3^- remained in systems containing MNC-71 (Fig. 2). NO_3^- took at least twice as long (>24 h) to be completely reduced in the systems containing MNC-71. The inhibition of NO_x^- reduction in the MNC-71 systems was less pronounced than in pure iron oxide systems. This may be due to the presence of clay and sand particles which make up a significant portion of the natural sediment (A. Coby, unpublished results). The negatively charged surfaces of clays in particular could compete with the bacterial surfaces for sorption of Fe^{2+}. Indeed, clays have been used as solid-phase complexants to adsorb microbially produced Fe^{2+} and thereby increase the rate and extent of Fe(III) reduction that would otherwise be limited by Fe^{2+} adsorption to cells and cause cell surface passivation (47). Still, a significant lag in NO_x^- reduction resulted, which is in agreement

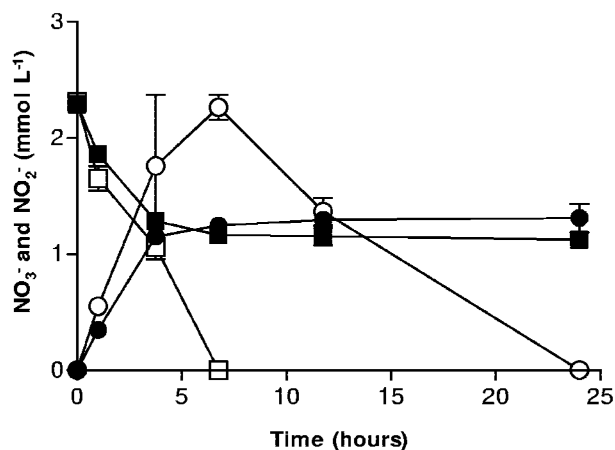


FIG. 1. NO_3^- reduction by *S. putrefaciens* in the presence of 50 mmol liter^{-1} hematite (■) and without hematite (□) and NO_2^- production/reduction in the presence of 50 mmol liter^{-1} hematite (●) and without hematite (○). Error bars indicate the standard deviations of the means (n = 3).

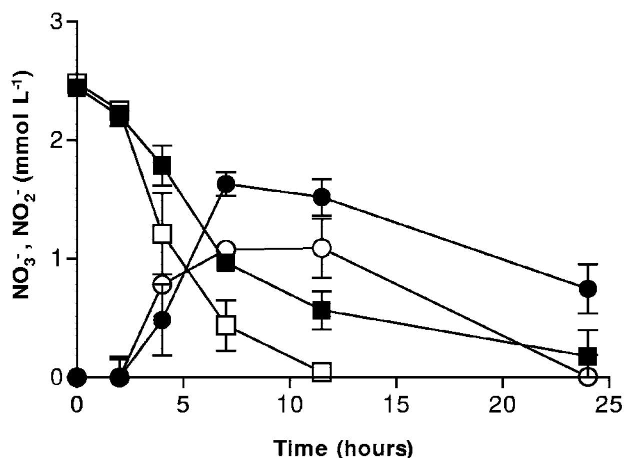


FIG. 2. NO_3^- reduction by *S. putrefaciens* in the presence of the natural sediment MNC-71 (■) and without MNC-71 (□) and NO_2^- production/reduction in the presence of MNC-71 (●) and without MNC-71 (○). Error bars indicate the standard deviations of the means (n = 3).

with results using goethite or hematite (6). This suggests that the hypothetical surface reaction between Fe^{2+} and NO_2^- and the putative cell coating are not restricted to pure iron oxides and may be significant in a variety of iron-containing natural sediments and soils.

In the studies by Cooper et al. using goethite (6), the inhibition of NO_x^- was incomplete, i.e., both NO_3^- and NO_2^- were eventually exhausted after approximately 50 and 800 h, respectively. With the hematite incubations shown in Fig. 1, although NO_3^- and NO_2^- were both still present when the experiment was stopped at 24 h, it is possible that they would have been slowly utilized and eventually exhausted. In incubations with sediment MNC-71 (Fig. 2), NO_3^- was almost exhausted at the conclusion of the experiment and NO_2^- was clearly being consumed. This lack of complete inhibition may be due to incomplete formation of cell coatings on some cells or the ability of cells to eventually "clear" themselves of such coatings. During studies of microbial Fe(III) reduction by *S. putrefaciens* CN32, Liu et al. used TEM and energy-dispersive spectroscopy to show that cells released small (100-nm) membrane vesicles that apparently helped clear them of Fe precipitates that formed on the cells and limited Fe(III) reduction rates (17). Although the nature of the precipitates formed in our NO_x^--containing systems is likely different from those observed by Liu et al., it is possible that release of Fe (hydr)oxide-coated membrane blebs could eventually relieve inhibition caused by mineral coatings.

In order to test whether the reaction between NO_2^- and Fe^{2+} was indeed surface catalyzed, the chelator NTA was used to complex with Fe^{2+} produced during the reduction of goethite. The formation of an NTA-Fe^{2+} complex would reduce Fe^{2+} sorption and, if the reaction with NO_2^- was indeed surface catalyzed, we expected that this would minimize the formation of the hypothetical Fe (hydr)oxide coating on the cell and allow NO_3^- reduction to proceed normally. Previous studies under similar conditions have reported no detectible $Fe(III)_{aq}$ in NTA-amended (5 mM) systems containing 50 mM goethite and inoculated with *Shewanella alga* (47). The NTA amendments were therefore not expected to significantly contribute to the dissolution of Fe(III) from goethite. Figure 3 summarizes the results, which support the surface reaction hypothesis. Without NTA, NO_3^- reduction was inhibited by up to 200 h in systems containing both goethite and NO_3^-. When NTA was present at 5 mM in goethite-containing systems, NO_3^- reduction was complete within 50 h. In goethite-free controls (with and without NTA), NO_3^- reduction was complete by 30 h. The temporary inhibition seen in the system containing goethite and NTA may be due to Fe^{2+} sorbing to the cell surface before having a chance to complex with NTA. The rate and extent of Fe(III) mineral reduction is increased in the presence of NTA (2, 47), and it is reasonable to postulate that a portion of the Fe^{2+} formed at the mineral-microbe interface may be temporarily sorbed to the cell before removal by the chelators.

The possibility exists that the observed inhibition of NO_x^- reduction in the presence of goethite (6) was a result of an unknown toxic effect of goethite on the cells in the sediment slurries. *Paracoccus denitrificans*, a gram-negative, denitrifying organism unable to reduce Fe(III), was chosen as a surrogate for *S. putrefaciens* in additional experiments. The rate of NO_3^- reduction, in systems with or without goethite, was nearly identical when using *P. denitrificans* (data not shown). This suggests that there is no toxic effect of goethite in the absence of Fe(III) reduction and supports our hypothesis that a small amount of Fe(III) needs to be reduced to Fe^{2+} for the inhibition of NO_x^- reduction to occur.

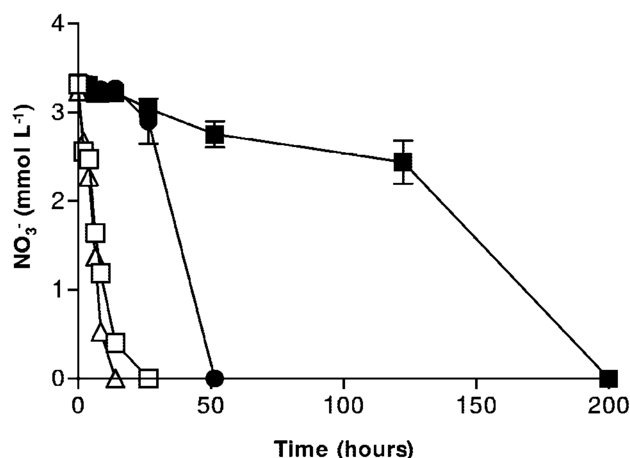


FIG. 3. NO_3^- reduction by *S. putrefaciens* with 5 mmol liter^{-1} NTA (Δ), without NTA (□), with 50 mmol liter^{-1} goethite (■), and with 5 mmol liter^{-1} NTA plus 50 mmol liter^{-1} goethite (●). Error bars are not shown for cultures lacking goethite, for the sake of clarity.

Sorption of Fe^{2+} and reaction with NO_2. In the absence of an iron (hydr)oxide, *S. putrefaciens* 200 primarily reduces NO_3^- and NO_2^- to ammonia and typically produces only trace amounts of N_2O (6), presumably as an enzymatic side reaction during dissimilatory reduction of nitrate to ammonia (3, 43). In systems containing small amounts of biogenic Fe^{2+}, goethite, and *S. putrefaciens*, though, Cooper et al. found N_2O production in amounts 10-fold greater than when goethite was absent (6). Additional experiments were done to clearly determine if the enhanced N_2O production observed in microbial systems containing NO_x^- and goethite could be explained solely by the abiotic reduction by surface-bound Fe^{2+}. The first set of abiotic, replicate batch reactors contained 3 mM aqueous Fe^{2+} and 3 mM NO_2^-; the second set contained 50 mM of goethite and 3 mM NO_2^-; the third contained all three components, Fe^{2+}, NO_2^-, and goethite. As can be seen in Fig. 4, bottles containing only aqueous Fe^{2+} and NO_2^- generated only 30 μM N_2O after 125 h. In contrast, bottles containing Fe^{2+}, NO_2^-, and goethite had generated 225 μM N_2O after 30 h and, eventually, 260 μM after 125 h. Controls containing only NO_2^- and goethite amendments did not produce any N_2O over the course of the experiment. These results are in agreement with studies by Sørensen et al., in which very little N_2O was produced by reaction of aqueous Fe^{2+} and NO_x^- in systems lacking a surface catalyst (40). The results also confirm that, under our experimental conditions, Fe^{2+} will sorb to a surface such as goethite and abiotically reduce NO_2^- to N_2O. Although the mechanistic details of the surface reaction remain unknown, it is possible that surface sorption of Fe^{2+} increases the local electron density over that in aqueous solution, thereby increasing the possibility of reduction reactions

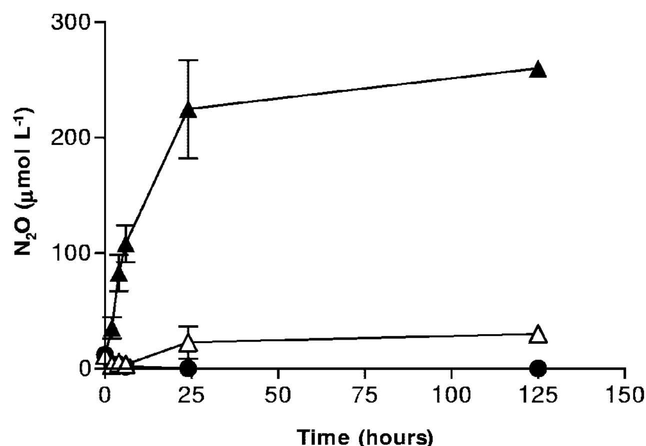


FIG. 4. N_2O production in systems containing aqueous Fe^{2+} (2 mmol $liter^{-1}$) and NO_2^- (3 mmol $liter^{-1}$) (Δ), aqueous Fe^{2+}, NO_2^-, and 50 mmol $liter^{-1}$ goethite (▲), and NO_2^- and 50 mmol $liter^{-1}$ goethite (●). Error bars indicate the standard deviations of the means (n = 3).

involving multiple electrons. It is possible that a similar reaction could occur on cell surfaces which have adsorbed Fe^{2+}.

To test the hypothesis that Fe^{2+} sorbed to cell surfaces will react with NO_2^-, producing an Fe (hydr)oxide coating on the cell and an inhibition of NO_x^- reduction, Fe^{2+} was sorbed to cells by equilibration of aqueous Fe^{2+} with a concentrated bacterial culture. Those cells were then incubated with a medium containing NO_3^- or NO_2^- as the sole electron acceptor. After 20 h, all the NO_3^- had been reduced in systems not treated with Fe^{2+}, while approximately 1.5 mM NO_3^- remained in systems containing treated cells (Fig. 5a). Inhibition of NO_2^- reduction by prior sorption of Fe^{2+} was even more dramatic. Reduction of 3.25 mM of NO_2^- by untreated cells was complete within 50 h, whereas treated cells only reduced 1/10 of the original NO_2^- after 50 h (Fig. 5b). After 5 days of incubation in NO_2^--reducing systems, we observed only 5.3 ± 2.9 μM N_2O in systems containing untreated cells, whereas 14.1 ± 2.6 μM N_2O was produced in systems containing cells treated with Fe^{2+}. These results all support the hypothesis that Fe^{2+} sorbs to the cell surface and reacts with NO_2^- to form an Fe (hydr)oxide coating that may be responsible for the observed inhibition of NO_x^- reduction.

The above experiment was repeated using *P. denitrificans* to see if NO_3^- and NO_2^- reduction by a non-Fe(III)-reducing organism could be inhibited after treatment with Fe^{2+} and NO_2^-. In these experiments the inhibition of NO_x^- reduction was less pronounced, but still significant. In Fig. 6a, after 6 h, 1.7 times more NO_3^- had been reduced in untreated systems versus those treated with Fe^{2+}. This is in close agreement with the analogous experiment inoculated with *S. putrefaciens* after 7 h (Fig. 5a). In Fig. 6b, almost 2 mM of NO_2^- still remained after 24 h in the systems treated with Fe^{2+}, while untreated cells had reduced nearly all the NO_2^- at the same time point. These results are particularly significant because they suggest that, in environments where Fe(II) is being produced, the reduction of NO_x^- or other soluble electron acceptors could be inhibited across a wide range of microorganisms. These results show that the inhibition of NO_x^- reduction is not due to the putative iron (hydr)oxide coating serving as an alternate electron acceptor and competitively inhibiting NO_x^- reduction, since *P. denitrificans* is unable to utilize Fe(III) as an electron acceptor. The less-pronounced inhibition of NO_3^- reduction with *P. denitrificans* compared to *S. putrefaciens* may be due to interspecies differences in the nature of the cell surface that might affect the extent of Fe^{2+} sorption. Alternately, *P. denitrificans* may be able to recover more quickly by clearing coatings from its surface.

If reduction of NO_x^- is inhibited as a result of formation of mineral coatings on the cell, the utilization of other soluble, terminal electron acceptors should also be inhibited. A greater environmental significance of the Fe (hydr)oxide coating could be argued if it were seen to have an effect on utilization of other respiratory substrates. Following incubation of Fe^{2+}-treated cells with 5 mM NO_2^-, we measured O_2 utilization rates and fumarate reduction rates. The O_2 utilization rate for cells that were incubated only with 5 mM NO_2^- (0.41 mg $liter^{-1}$ min^{-1}) was about 2.2 times faster than for cells that had

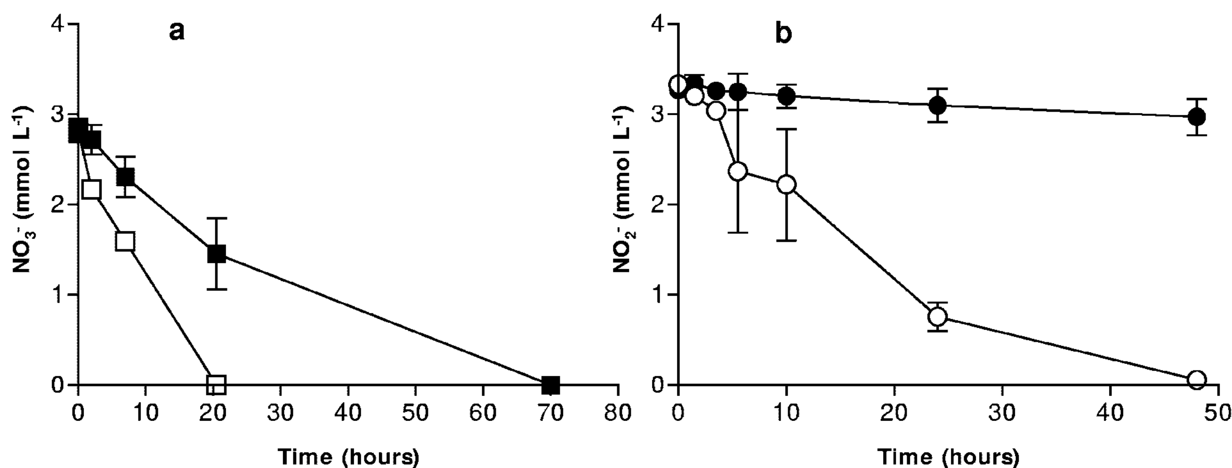


FIG. 5. (a) NO_3^- reduction by *S. putrefaciens* treated with Fe^{2+} (■) and without treatment (□). (b) NO_2^- reduction by *S. putrefaciens* treated with Fe^{2+} and NO_2^- (●) and without treatment (○). Error bars indicate the standard deviations of the means (n = 3).

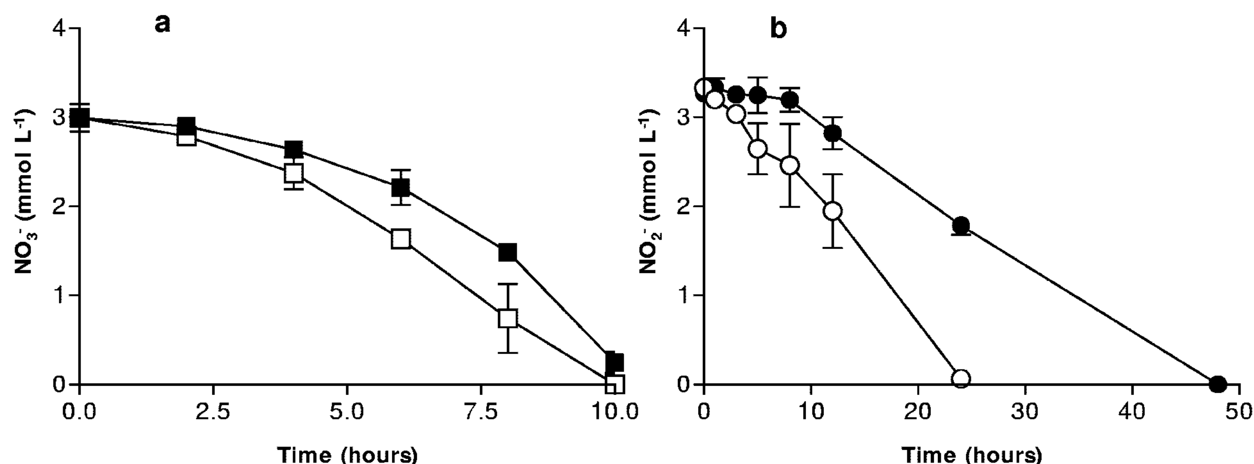


FIG. 6. (a) NO_3^- reduction by *P. denitrificans* treated with Fe^{2+} and NO_2^- (■) and without treatment (□). (b) NO_2^- reduction by *P. denitrificans* treated with Fe^{2+} and NO_2^- (●) and without treatment (○). Error bars indicate the standard deviations of the means (n = 3).

been incubated with Fe^{2+} prior to NO_2^- (0.19 mg liter^{-1} min^{-1}). This experiment was repeated using twice the cell number (2 $\times 10^8$ cells ml^{-1}) with comparable results.

Fumarate reduction by cells treated with Fe^{2+} and NO_2^- was also severely inhibited compared to cells exposed to just NO_2^- (Fig. 7). After 12 h, about 3.5 times more fumarate remained in systems containing cells exposed to both Fe^{2+} and NO_2^- than in those systems containing cells previously exposed to NO_2^- alone. Fumarate reduction in treated systems was still not complete after 48 h. Both the O_2 and fumarate reduction results suggest that the inhibitory effect of the Fe (hydr)oxide coating on the reduction of soluble electron acceptors is not restricted to NO_3^- and NO_2 but may have broad environmental implications concerning the utilization of environmentally significant electron acceptors.

Electron microscopy and energy-dispersive spectroscopy. The six panels shown in Fig. 8 show the effect of NO_2^- reacting with Fe^{2+} which had been previously sorbed to the surface of the cells. Panels A and D are TEM images of *S. putrefaciens* incubated in AGW medium containing 5 mM NO_2^- without previous Fe^{2+} sorption to the cell surface. Panels B and E are of cells which were treated with 50 mM aqueous Fe^{2+} for 30 min but without subsequent NO_2^- incubation. The cells and surrounding medium in the TEM image (Fig. 8B) are slightly darker, indicating perhaps the presence of Fe^{2+} both sorbed to the cell surface and in solution. Panels C and F are of cells that have been treated with 50 mM aqueous Fe^{2+} and then allowed to react with NO_2^- (5 mM) for 3 hours. This is the same treatment used on cells in the previous experiments examining the reduction of soluble electron acceptors. The much darker quality and thickened appearance of the cells in panel C suggest an electron-dense coating on the cells, likely a Fe (hydr)oxide precipitate resulting from the surface-catalyzed reaction between sorbed Fe^{2+} and aqueous NO_2^-. This is supported by the presence of a rough coating on the surface in the SEM images shown in panel F. The EDS data for each of the SEM cell treatments indicate a clear enrichment of Fe on the surface of the cells treated with both Fe^2 and NO_2^- (Fig. 8F). Taken together, these data clearly show that electron-dense, Fe-enriched coatings on cells exposed to NO_2^- are formed only following sorption of Fe^{2+}. Sorption of Fe^{2+} alone is also insufficient to form such coatings.

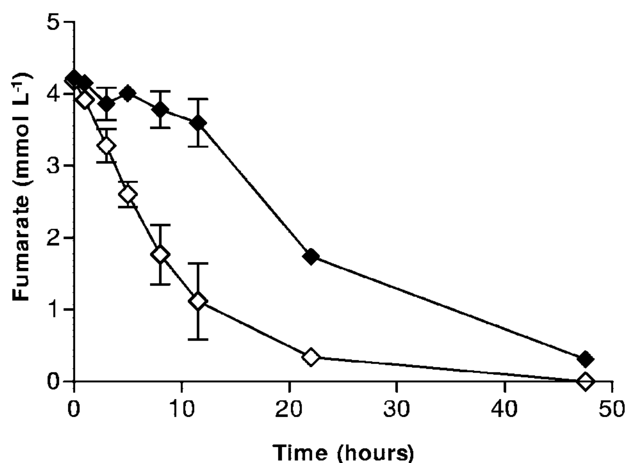


FIG. 7. Fumarate reduction by *S. putrefaciens* treated with Fe^{2+} and NO_2^- (◆) and without treatment (◇). Error bars indicate the standard deviations of the means (n = 3).

The identity of the Fe (hydr)oxide formed on the surface of *S. putrefaciens* in our systems is not known, but it does not appear that the cells are able to rapidly utilize this precipitate as an electron acceptor. Some forms of Fe minerals, such as magnetite [$Fe^{2+}(Fe^{3+})_2O_4$] or green rusts [$Fe^{2+}_{(1-x)}Fe^{3+}_{(x)}(OH)_2]^{x+}$ are resistant to microbial reduction (25). Experiments by Cooper et al. have demonstrated the formation of magnetite following the reduction of lepidocrocite by *S. putrefaciens* 200 (5), and Sørensen et al. reported the formation of magnetite as a product of the reaction of Fe^{2+} with NO_2^- on the surface of lepidocrocite (40). Although our studies did not utilize lepidocrocite, it is clear that the Fe(III) (hydr)oxide coatings formed in our experiments were not readily or completely reducible by *S. putrefaciens* over the duration of our experiments.

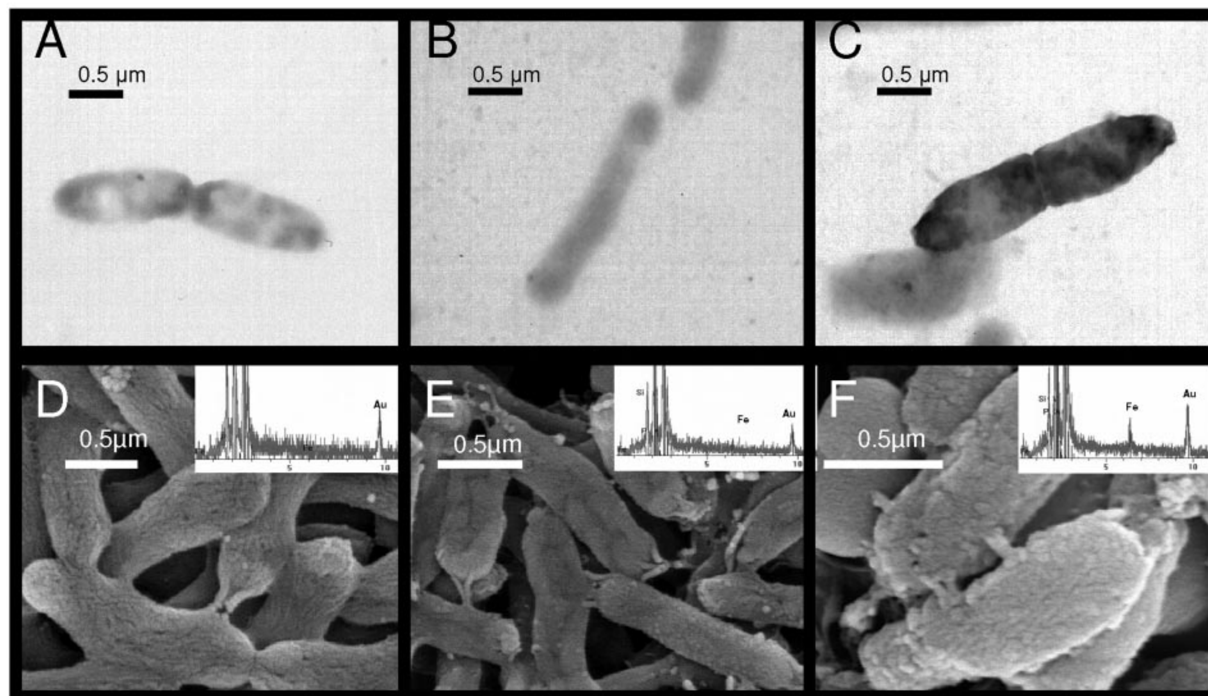


FIG. 8. Transmission and scanning electron micrographs of *S. putrefaciens* under three treatments. (A and D) Cells treated with NO_2^-; (B and E) cells treated with Fe^{2+}; (C and F) cells treated with both Fe^{2+} and NO_2^-. The electron-dense coating on the cells in panel C and the rough surface on the cells in panel F represent an Fe (oxy)hydroxide coating formed from the reaction between surface-bound Fe^{2+} and NO_2^-. The inset on each SEM image is an EDS point scan that corresponds to the cells in the image.

Conclusions. Support for the proposed inhibition mechanism involving the reaction of NO_2^- and surface-bound Fe^{2+} is evident in the results demonstrating that the presence of Fe^{2+} on the surface of cells leads to the production of N_2O and an inhibition of NO_x^- reduction even at small concentrations of Fe^{2+}. This reaction occurs using a variety of Fe oxides, including an iron-bearing natural sediment, can occur with bacterial species incapable of Fe(III) reduction if they are in environments containing aqueous Fe^{2+}, and affects several soluble terminal electron acceptors. The exact composition of the Fe (hydr)oxide coating is not yet known. The extent and significance of N_2O formation via this reaction in the environment is not known but could be significant in iron-bearing soils during microbial remediation of NO_x^- contamination. The potential increase of both Fe^{2+} and NO_2^- production during bioremediation efforts could significantly increase the formation of N_2O as a by-product, and the implications of this reaction for metal bioremediation efforts, geochemical cycling, and greenhouse gas production make it an important subject for further investigation.

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