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Dissimilatory Iron-Reducing Bacteria Can Influence the Reduction of Carbon Tetrachloride by Iron Metal

ROBIN GERLACH,*,† AL B. CUNNINGHAM,† AND FRANK CACCAVO, JR.‡

Center for Biofilm Engineering, Montana State University, Bozeman, Montana 59717, and Department of Microbiology, University of New Hampshire, Durham, New Hampshire 03824

Little is known about the long-term performance of zerovalent iron (Fe^0) subsurface barriers. Groundwater exposure induces corrosion processes that can passivate the Fe^0 surface and decrease barrier reaction rates. We present evidence that dissimilatory iron-reducing bacteria (DIRB) can stimulate the rate of carbon tetrachloride (CT) transformation in the presence of corroded iron. The DIRB, *Shewanella alga* BrY, adhered to the corroded Fe surfaces that showed little or no capacity to transform CT. The addition of BrY to these systems with decreased CT transformation rates resulted in increased ferrous iron concentrations and increased CT transformation to chloroform (CF). The results suggest that DIRB can have an influence on the long-term performance of Fe^0 barriers.

Introduction

Permeable, reactive, subsurface barriers of zerovalent iron (Fe^0) have been successfully employed for the remediation of contaminated groundwater. Fe^0 is effective in degrading or transforming a wide range of contaminants including chlorinated organics (*1*−*9*), heavy metal oxyanions (*10*, *11*) and oxycations (*12*), nitroaromatics (*13*, *14*), and nitrate (*9*, *15*, *16*). The corrosion of Fe^0 provides the electrons necessary for the reduction of these contaminants.

Laboratory studies simulating long-term performance of Fe^0 have indicated that contaminant degradation rates can either decrease (*2*, *17*) or more rarely increase (*4*) as a function of time. This temporal effect on contaminant degradation rates is usually attributed to the development of corrosion products that develop on the metal surface (*4*, *18*, *19*). Johnson and Tratnyek (*18*), Scherer et al. (*19*), and Odziemkowski et al. (*20*) have proposed that the accumulation of corrosion products on Fe^0 surfaces either inhibits contaminant access to the metal surface or forms new sites for contaminant adsorption, reaction, and catalysis. A decrease in reaction rates can be due to the accumulation of thick layers of amorphous corrosion products on Fe^0 surfaces, which may function as a physical barrier between dissolved contaminants and the underlying reactive sites. The accumulation of highly redox-reactive corrosion products such as green rust, however, can also significantly contribute to the reduction of contaminants in Fe^0 systems (*21*).

* Corresponding author phone: (406)994-4770; fax (406)994-6098; e-mail: robin_g@erc.montana.edu.

† Montana State University.

‡ University of New Hampshire.

A wide range of Fe^0 corrosion products has been found in laboratory and field studies, and corrosion product distribution appears to be dependent on the type of Fe^0 used, the contaminant present, geochemical conditions, and the time of exposure to contaminated groundwater. The corrosion products commonly found in the presence of contaminated groundwater include goethite (α-FeOOH (*22*)), magnetite (Fe_3O_4 (*20*, *23*−*25*)), maghemite (γ-Fe_2O_3 (*23*)), haematite (α-Fe_2O_3 (*22*)), mixed chromium−iron-hydroxides (*26*, *27*), green rust (mixed Fe(II)-Fe(III)-hydroxy salts (*25*)), and siderite ($FeCO_3$ (*28*)).

Many of these iron minerals are used by dissimilatory iron-reducing bacteria (DIRB) as electron acceptors (*29*−*34*). DIRB are widely distributed in both pristine and contaminated terrestrial, aquatic, and subsurface environments (*35*) and are thus likely to contact Fe^0 barriers in situ. DIRB gain energy for growth by coupling the oxidation of organic matter to the dissimilatory reduction of Fe(III) minerals.

Gorby et al. (*36*) demonstrated that DIRB can reduce structural Fe(III) oxides, which can in turn reductively dechlorinate carbon tetrachloride (CT) to chloroform (CF). The bacterial reduction of Fe(III) to Fe(II) allowed for the following reaction to occur

$$2Fe^{2+} + CCl_4 + H^+ \rightarrow 2Fe^{3+} + CHCl_3 + Cl^-$$

DIRB have also been shown to influence the corrosion of mild steel by solubilizing the protective ferric iron film on the passivated metal surface (*37*, *38*). A decrease in corrosion layer thickness due to solubilization of corrosion products in Fe^0 systems could also lead to increased reaction rates of iron metal with chlorinated aliphatics (*2*, *17*, *19*).

We hypothesized that DIRB can influence the performance of Fe^0 subsurface barriers by adhering to corroded iron and reducing Fe(III) corrosion products to Fe(II)-compounds. The reduction of Fe(III) to Fe(II) can lead to the formation of surface reactive Fe(II) species or result in the removal of passivating ferric precipitates on the Fe^0 surface. Either one of these processes will potentially increase the reductive transformation of carbon tetrachloride (CT).

Experimental Section

Organism and Culture Conditions. *Shewanella alga* BrY (*34*) was used as a model DIRB in these studies. *S. alga* BrY was maintained on tryptic soy agar (40 g/L, Difco Laboratories) at ambient temperature. *S. alga* BrY cultures were grown in tryptic soy broth (30 g/L, Difco Laboratories) at 28 °C and 150 rpm for 15 h. Cultures were centrifuged at 5520 × g for 20 min at 4 °C, washed once in anaerobic 10 mM HEPES (*N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]) buffer (pH 7.0) amended with 2.5 g L^{-1} NaCl, and suspended in the same buffer. All buffers were made anaerobic by boiling and cooling under a constant stream of O_2-free N_2 (*39*). All manipulations were performed under an O_2-free N_2 purge.

CT Reduction. The ability of *S. alga* BrY to influence CT reduction by passivated Fe^0 was examined. Fe^0 powder (20 mg, 100 mesh, Fisher Scientific) was added to 10 mL of aerobic HEPES buffer (pH 7.0), sealed in culture tubes under an aerobic headspace, and vigorously shaken on a wrist action shaker for ca. 40 h. This method was found to be efficient for pre-exhausting the CT-reducing capacity of the Fe^0 powder. Following pretreatment, the tubes were purged with O_2-free N_2 for 15 min by lowering a stainless steel cannula into the bottom of each tube and letting the anaerobic gas percolate through the aqueous phase. Anaerobic sodium

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lactate (final concentration 10 mM) and either heat-killed or active cells of *S. alga* BrY (final concentration of 10^{10} cells mL^{-1}) were added to the tubes using degassed syringes and needles. The tubes were then filled to their maximum capacity (27.5 mL) with anaerobic HEPES buffer to minimize loss of CT into the headspace. The gas-sparging cannula was slowly removed from the tube as a PTFE-lined silicon septum was inserted. Control tubes containing either heat-killed or active *S. alga* BrY cells without iron powder were also established. The experiments were initiated by injecting 0.5 mL of a CT (Sigma) stock solution into the tubes. The stock solution was made by adding 1 mL of pure phase CT to 40 mL of deionized water. Excess aqueous phase was allowed to escape from the tube by allowing it to equilibrate with a N_2 filled syringe. Aqueous phase samples (0.1 mL) were taken with a gastight syringe through the PTFE lined septum immediately after CT addition and periodically over the duration of the experiment. At each time-point the sampling-syringe was flushed with N_2, and approximately the same volume of N_2 was injected into each tube. The sample was injected into 1 mL of hexane and allowed to equilibrate for at least 10 min on a horizontal shaker (150 rpm). The hexane phase was then used for gas chromatographic analysis. The total headspace volume at the end of the experiment was less than 5% of the vessel volume, ensuring that less than 4% of CT remaining and less than 0.5% of CF produced in the tubes were in the headspace (using the Henry constants given by Gossett in ref *40*). All CT reduction treatments were conducted in triplicate.

Iron Reduction. The ability of *S. alga* BrY to use Fe^0 corrosion products as a terminal electron acceptor was examined in an experiment analogous to that described above, omitting the CT and replacing the PTFE-lined septa with blue butyl rubber stoppers. The initial cell concentration was 1.60×10^{10} cells mL^{-1}. Sampling was performed by removing aqueous phase-aliquots with a disposable syringe and 22-gauge needle. Samples were injected directly into 0.5 N HCl for ferrous iron analysis. All iron reduction treatments were conducted in triplicate.

Adhesion of BrY to Iron. Adhesion studies were performed as described previously (*41*) with the exception that the Fe(III) oxide-bicarbonate buffer was replaced with Fe^0-HEPES buffer. Anaerobic tubes of HEPES-pretreated iron were prepared as described above. A final concentration of 4.2×10^8 cells mL^{-1} was used, and adhesion assays were performed in triplicate.

Analytical Methods. CT, CF, and dichloromethane (DCM) were analyzed using a HP 6890 gas chromatograph (GC) equipped with an electron capture detector. An aliquot (10 μL) of hexane phase of the liquid−liquid extracts was injected onto a 60/80 Carbopack B/1% SP-1000 column (8 ft × 1/8 in.). The GC was operated under the following conditions: injector temperature of 275 °C, initial temperature of 100 °C, increased at 15 °C/min up to 200 °C, detector temperature of 250 °C, and N_2 flow of 40 mL/min. The GC was controlled by HP Chem Software, Vers. A.04.01. This method permitted for the detection of concentrations of less than 10 $\mu g\ L^{-1}$ of CT, CF, or DCM. Ferrous iron analysis was performed with Ferrozine as described by Lovley and Phillips (*42*).

Results and Discussion

A number of studies have suggested that contact between DIRB cells and insoluble Fe(III) minerals is a necessary prerequisite for Fe(III) mineral reduction (*34*, *43*−*46*). The ability of *S. alga* BrY to catalyze reactions on the surface of Fe^0 particles would thus be predicated on the ability of *S. alga* BrY cells to adhere to those particles. The results in Table 1 demonstrate that 82% of the *S. alga* BrY cells adhered to 2 mg mL^{-1} of HEPES-pretreated iron, while cells were unable to adhere to untreated Fe^0. When 20 mg mL^{-1} of HEPES-pretreated iron were provided, more than 99% of the cells adhered while only 10% adhered to 20 mg mL^{-1} of untreated Fe^0. The difference in the ability of *S. alga* BrY to adhere to each of these forms of Fe^0 is most likely due to the presence of Fe(III) oxides on the surface of the corroded iron. Previous studies have demonstrated that DIRB preferentially adhere to Fe(III) coated surfaces (*41*, *47*). The results of the adhesion experiments suggest that DIRB would be more likely to catalyze reactions on the surface of corroded iron than on the surface of untreated Fe^0.

TABLE 1. Adhesion of *S. alga* BrY Cells to HEPES-Pretreated Iron and Fe^0

treatment	% adhered cells[a]
2 mg of HEPES-pretreated iron	82.2 ± 6.2
2 mg of Fe^0	−5.4 ± 8.2
20 mg of HEPES-pretreated iron	99.3 ± 5.8
20 mg of Fe^0	10.6 ± 6.3

[a] Numbers represent the means of three replicate assays ± the standard errors of those means.

Following adhesion, active *S. alga* BrY cells were capable of restoring the capacity of HEPES-pretreated iron to transform CT to CF. After 20 h of incubation CT concentrations were statistically significantly lower (*t*-test, *P*-value ≤ 0.025) in treatments with active *S. alga* BrY than in controls containing HEPES-pretreated iron without cells or cells without iron (Figure 1A). In fact, neither *S. alga* BrY nor HEPES-pretreated iron caused significant transformation of CT. No direct CT transformation by BrY was observed, which agrees with previous work by Gorby et al. (*36*) and Workman et al. (*48*). Workman et al. (*48*) were able to stimulate CT transformation in the presence of BrY through the addition of Vitamin B12. However, the levels of Vitamin B12 used by Workman et al. (*48*) were much higher than normally observed in natural systems and are unlikely to have existed in these experiments.

Treatments containing both HEPES-pretreated iron and active cells also accumulated more CF than the controls (Figure 1B) ($P \leq 0.0003$). Approximately 60% of the added CT were converted to CF in tubes containing both HEPES-pretreated iron and *S. alga* BrY. No dichloromethane (DCM) or other transformation products were detected in any of the setups.

Active cells of *S. alga* BrY were capable of generating ferrous iron with lactate as an electron donor and HEPES-pretreated iron as a sole terminal electron acceptor (Figure 2). These experiments were conducted in the absence of CT since the presence of CT could have resulted in instantaneous reoxidation of microbially produced Fe(II) to Fe(III), thus masking the production of Fe(II) by DIRB. The sum of dissolved and colloidal Fe(II) was measured in these systems. Dissolved Fe(II) is reported to be a weak reductant for CT transformation to CF (*49*), while surface associated Fe(II) is thought to be a stronger reductant (*50*−*52*). Using conventional extraction methods, however, it is difficult to measure the quantity of adsorbed Fe(II) selectively, because the grains of Fe^0 provide a large reservoir of extractable metal ions (*6*). Figure 2 clearly shows that, over a period of ca. 6 days, the treatments with active *S. alga* BrY cells resulted in significantly higher Fe(II) concentrations than either control ($P \leq 0.027$).

The influence of microorganisms on the performance of Fe^0 subsurface barriers has only recently been recognized. Methanogenic and denitrifying bacteria have been shown to have a positive effect on the degradation of CT and CF by Fe^0 (*16*, *53*, *54*).

We report here for the first time that DIRB can influence the transformation of a chlorinated aliphatic in the presence of passivated iron. The results indicate that DIRB can have

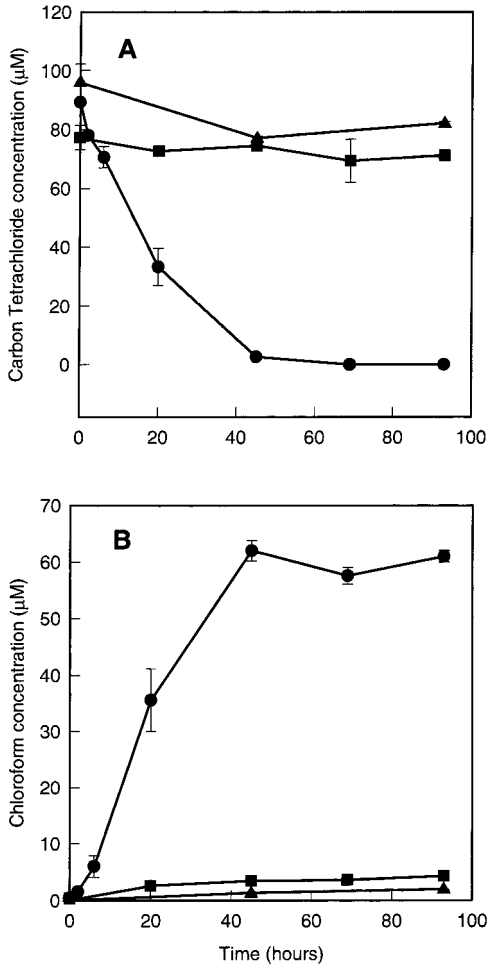

FIGURE 1. (A) Carbon tetrachloride degradation and (B) chloroform production by (■) passivated Fe^0, (●) passivated Fe^0 with active *S. alga* BrY cells, and (▲) active *S. alga* BrY cells alone. Errors bars represent the standard error of the means ($n = 3$).

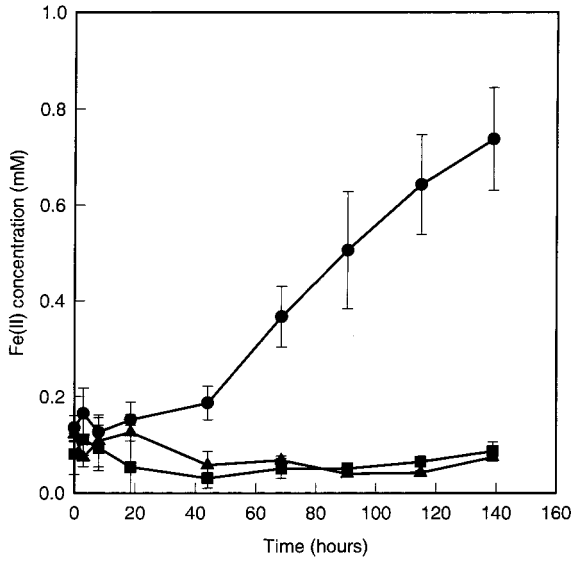

FIGURE 2. Production of Fe(II) by (■) passivated Fe^0, (●) passivated Fe^0 with active *S. alga* BrY cells, and (▲) passivated Fe^0 with heat-killed *S. alga* BrY cells. Errors bars represent the standard error of the means ($n = 3$).

a positive influence on the performance of zerovalent iron by restoring the reactivity of passivated iron to transform CT to CF.

The work described herein did not allow for the identification of the exact reaction mechanism. It is possible that DIRB restore the reactivity of corroded iron by producing surface-bound reactive Fe(II) sites through microbial reduction of Fe(III) precipitates or by reductive dissolution of Fe(III) corrosion products. Reductive dissolution of ferric iron precipitates would lead to a decrease in corrosion layer thickness, which could increase the mass transport of contaminants to surface reactive sites or facilitate the electron transport from the Fe^0 to reaction sites on the corrosion product-covered surface of the iron grains (*19*). Thin passive films composed of magnetite, maghemite, and other mixed valent (hydr)oxides have been modeled as semiconducting Fe_2O_3 doped with Fe(II) (*55*). Thicker corrosion layers tend to be amorphous with fewer semiconductor characteristics (*19*). The reductive dissolution of amorphous iron (oxy)-hydroxides from the grain surface would lead to a decrease in corrosion layer thickness and could result in enhanced electron transport from the underlying iron metal to surface reactive sites.

More research is needed to identify the mechanisms by which DIRB influence the performance of Fe^0. Specifically designed experiments involving surface analytical equipment should allow for the identification of the reaction mechanisms. Future research should also address the role of iron-reducing bacteria in pilot and field scale barriers. DIRB could be isolated from core samples, and their effects could be evaluated under more field realistic conditions (alkaline pH, field-weathered iron material).

A thriving DIRB population in Fe^0 barriers could also have a negative effect on the reactivity of Fe^0. The development of thick biofilms could inhibit mass transport of dissolved contaminant to the iron surface (*56*, *57*), thus lowering the overall reactivity of Fe^0 barriers. Although currently not observed in field applications, excessive microbial growth within subsurface barriers could potentially lead to a decrease in porosity, which could result in hydraulic failure of the barrier. DIRB activity in Fe^0 barriers could also result in enhanced dissolution of reactive corrosion products thus decreasing the overall reactivity of Fe^0 subsurface barriers.

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