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ORIGINAL PAPER

Influence of carbon sources and electron shuttles on ferric iron reduction by *Cellulomonas* sp. strain ES6

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Abstract Microbially reduced iron minerals can reductively transform a variety of contaminants including heavy metals, radionuclides, chlorinated aliphatics, and nitroaromatics. A number of *Cellulomonas* spp. strains, including strain ES6, isolated from aquifer samples obtained at the U.S. Department of Energy's Hanford site in Washington, have been shown to be capable of reducing Cr(VI), TNT, natural organic matter, and soluble ferric iron [Fe(III)]. This research investigated the ability of *Cellulomonas* sp. strain ES6 to reduce solid phase and dissolved Fe(III) utilizing different carbon sources and various electron shuttling compounds. Results suggest that Fe(III) reduction by and growth of strain ES6 was dependent upon the type of electron donor, the form of iron present, and the presence of synthetic or natural organic matter, such as anthraquinone-2,6-disulfonate (AQDS) or humic substances. This research suggests that *Cellulomonas* sp. strain ES6 could play a significant role in metal reduction in the Hanford subsurface and that the choice of carbon source and organic matter addition can allow for independent control of growth and iron reduction activity.

Keywords Fermenters · Anthraquinone-2,6-disulfonate (AQDS) · Humics · HFO · Ferrihydrite · Goethite · Magnetite · Maghemite · Hematite

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Introduction

The reductive transformation of oxidized contaminants by ferrous iron, Fe(II), has received increasing interest in the recent years. Electron transfer from Fe(II) usually occurs fast and is non-specific so that a broad spectrum of compounds can react with surface-associated and soluble Fe(II). This makes Fe(II)-based remediation technologies an attractive alternative or addition to traditional cleanup strategies. Potentially treatable contaminants include heavy metals such as

Cr(VI), radionuclides such as U(VI), chlorinated aliphatics such as carbon tetrachloride (CT) and trichloroethylene (TCE) as well as nitroaromatics such as 2,4,6-trinitrotoluene (TNT) (Eary and Rai 1988; Erbs et al. 1999; Amonette et al. 2000; Fredrickson et al. 2000b; Lee and Batchelor 2002a, b; Hofstetter et al. 2003; Borch et al. 2005).

In subsurface environments, Fe(II) can be produced chemically or biologically and engineered in situ remediation schemes utilizing the reactivity of subsurface Fe(II) have been demonstrated (Heijman et al. 1995; Yin and Allen 1999; Chilakapati et al. 2000). Remediation based on the biological reduction of ferric [Fe(III)] minerals has been suggested as a low-cost technology for heavy metal and radionuclide immobilization at US Department of Energy (DOE) and other sites (Istok et al. 1999). It has been shown that a wide variety of bacteria, including but not limited to iron-respiring, fermenting, sulfate-reducing, halorespiring, and methanogenic bacteria, are able to reduce dissolved and solid phase ferric iron, either directly or indirectly, in the presence of electron shuttling compounds (Lovley et al. 1991; Benz et al. 1998; Cervantes et al. 2002).

Past research regarding biological Fe(III) reduction has focused mainly on Gram-negative metal-reducers such as *Geobacter*, *Shewanella* and *Desulfovibrio* spp. (Nealson and Saffarini 1994; Roden and Zachara 1996; Zachara et al. 1998; Lovley and Blunt-Harris 1999; Dong et al. 2000; Newman 2001; Liu et al. 2002; Royer et al. 2002b; Saffarini et al. 2002). In general, Gram-positive fermenters have received much less attention for their potential role in Fe(III) reduction and contaminant remediation. Previously, isolates were obtained from contaminated and uncontaminated subsurface locations at the DOE site in Hanford, Washington that were capable of Cr(VI) reduction (Smith et al. 2002; Viamajala et al. 2007). Of the nine isolates obtained, eight were Gram-positive of which four were from the *Cellulomonas* genus. Additional studies with three of the *Cellulomonas* isolates—strain ES5, strain WS01 and strain WS18, demonstrated their ability to reduce U(VI) as well as soluble Fe(III) using lactate as the electron donor (Sani et al. 2002). Finally, bench scale column experiments using *Cellulomonas* sp. strain ES6 indicated prolonged periods of Cr(VI) reduction in columns containing microbially reduced hydrous ferric oxide (HFO) via abiotic reduction of Cr(VI) by surface associated Fe(II) and regeneration of Fe(II) through microbial reduction by strain ES6 (Viamajala et al. 2008). This study continued to focus on strain ES6 in order to further investigate relevant microbial reduction mechanisms. In specific, the direct and indirect reduction of Fe(III) minerals by strain ES6 was assessed in order to better understand the potential for Fe(II)-based remediation through stimulation of indigenous bacteria at the Hanford site.

The choice of electron donor for Fe(III) reduction will likely determine the growth of a particular group of organisms and is important in order to achieve effective Fe(II) production to maintain electron flow necessary for biologically mediated contaminant reduction. The electron donor that is ultimately selected for field use will likely depend on regulatory approval, cost, availability, and ability to sustain growth of the microorganisms of interest.

While microorganisms can directly reduce Fe(III) through the addition of an electron donor, Fe(III) can also be reduced indirectly through the use of electron shuttles such as naturally occurring organic matter or the addition of quinone moieties. Previous studies have already shown that the presence of an electron shuttle can increase Fe(III) reduction rates and extent (Fredrickson et al. 1998, 2000a; Lovley et al. 1998; Lovley and Blunt-Harris 1999; Nevin and Lovley 2000; Royer et al. 2002b; Jiang and Kappler 2008; Bauer and Kappler 2009; Wolf et al. 2009). To design and implement effective Fe-based bioremediation strategies, it is important to clearly understand interactions of site-specific bacteria, such as strain ES6, with the numerous types of iron minerals that might be present (Lovley and Phillips 1986a; Lovley 1997).

The goal of these studies was to determine the influence of various carbon sources (electron donors) and electron shuttling compounds, such as synthetic and natural organic matter, on the growth and Fe(III) reduction capabilities of *Cellulomonas* sp. strain ES6 (henceforth referred to as strain ES6) of dissolved and solid phase Fe(III).

Materials and methods

Experimental

Organism and culture conditions

Strain ES6 was maintained in frozen stock cultures containing tryptic soy broth (TSB, 30 g/l, Difco

Laboratories) with 20% glycerol at –70°C. Cells were pre-cultured in TSB for 24 h on a horizontal shaker at 150 rpm and 30°C, transferred into fresh TSB, and grown again for 18 h in TSB (each 1% initial inoculum). Cultures were then harvested via centrifugation (5,860 × *g*, 20 min, 4°C), washed in oxygen-free synthetic groundwater (SGW), and re-suspended in SGW to the desired cell concentration.

Media composition

Synthetic groundwater (SGW) modified from Petersen et al. (1994) was used for all experiments. The final concentrations of the constituents are listed in Table 1. Sodium metasilicate, sodium carbonate, sodium sulfate, yeast extract, and casamino acids were dissolved in deionized (DI) water and autoclaved at 121°C. The autoclaved solution was boiled under an oxygen-free atmosphere of N_2/CO_2 (80:20) for 10 min and cooled to room temperature under the same oxygen-free atmosphere to avoid re-dissolution of atmospheric oxygen. Sterile, oxygen-free stock solutions of sodium bicarbonate, potassium chloride, calcium chloride, magnesium hydroxide, and the trace minerals were added. The medium was dispensed into Balch tubes (used as experimental reactors as described below) under the N_2/CO_2 atmosphere to maintain oxygen-free conditions.

Table 1 Composition of synthetic groundwater medium (SGW)

Name	Formula	Concentration in SGW (mg/l)
Aluminium sulfate	$Al_2(SO_4)_3 \cdot 18H_2O$	0.123
Boric acid	H_3BO_4	0.6
Calcium chloride dihydrate	$CaCl_2 \cdot 2H_2O$	8
Casamino acids		100
Cobalt sulfate	$CoSO_4 \cdot 7H_2O$	0.109
Cupric sulfate	$CuSO_4 \cdot 5H_2O$	0.08
Ferrous sulfate	$FeSO_4 \cdot 7H_2O$	0.3
Lithium chloride	LiCl	0.021
Magnesium hydroxide	$Mg(OH)_2$	33.5
Manganese chloride	$MnCl_2 \cdot 4H_2O$	0.629
Nickel chloride	$NiCl_2 \cdot 6H_2O$	0.11
Potassium bromide	KBr	0.03
Potassium chloride	KCl	26.5
Potassium iodide	KI	0.03
Sodium bicarbonate	$NaHCO_3$	2519.7
Sodium carbonate	Na_2CO_3	160
Sodium metasilicate	$Na_2SiO_3 \cdot 9H_2O$	455
Sodium molybdate	Na_2MoO_4	0.01
Sodium selenate	Na_2SeO_4	0.05
Sodium sulfate	Na_2SO_4	6
Yeast extract		50
Zinc sulfate	$ZnSO_4 \cdot 7H_2O$	0.106

Iron minerals

Goethite (α-FeOOH, BET specific surface area (SSA) 16.7 m^2/g), maghemite (γ-Fe_2O_3, SSA 43.9 m^2/g), hematite (α-Fe_2O_3, SSA 7.8 m^2/g), and magnetite (Fe_3O_4, SSA 6.6 m^2/g) were obtained from Alfa Aesar (Ward Hill, MA) with purities of 97% or greater. Hydrous ferric oxide (HFO, SSA 34.5 m^2/g) was synthesized using the procedure of Lovley and Phillips (1986b). In brief, 13.8 g $FeCl_3$ (Fisher Scientific) was dissolved in 400 ml of deionized water and the solution pH was slowly adjusted to 7.0 using NaOH. The resulting HFO suspension was diluted in SGW and used for the experiments within 1 month. The concentrations of total iron in the experiments were between 1.74 and 3.58 mM depending on iron mineral and experimental goal. Stock solutions of Fe(III)-citrate (50 mM) and Fe(III)-NTA (100 mM) were prepared by dissolving 1.225 g of Fe(III)-citrate (Sigma, St. Louis, MO) and 1.62 g $FeCl_3$ with 1.91 g NTA in 70 ml of deionized water, respectively. The Fe(III)-citrate solution was heated until dissolved and the pH of both solutions was slowly adjusted to 6.5 using $NaHCO_3$. Both solutions were sparged with oxygen-free nitrogen gas, transferred into an oxygen-free glovebag, stirred overnight, filled to a total volume of 100 ml each, and finally filter sterilized. It was determined that less than 1% of the Fe present in these solutions and suspensions was Fe(II).

Batch experiments, Fe(III) reduction

Oxygen-free SGW in Balch tubes containing iron minerals was amended with a carbon source, anthraquinone-2,6-disulfonate (AQDS, Sigma, St. Louis, MO), Elliott soil humic acid standard (International Humic Substances Society, MN, USA), and finally inoculated with strain ES6 before the tubes were crimp sealed using thick butyl rubber stoppers

(Bellco Glass, Vineland, NJ). All manipulations were performed under an oxygen-free N_2/CO_2 atmosphere, and all additions were made using sterile, purged syringes and needles. The carbon sources investigated were added from oxygen-free stock solutions and included acetate, lactate, glycerol, xylose, sucrose, and molasses ("Brer Rabbit Molasses—Unsulphured", B&G Foods Inc., Roseland, NJ). All carbon sources were added from 1 M oxygen-free stock solutions to a final concentration of 10 mM. Molasses, which has a high content of sucrose (Prescott and Dunn 1983) was added from a concentrated stock solution to a final molasses concentration of 3.42 g/l. The initial bacterial concentrations were between $2.23 \times 10^8 \pm 2.08 \times 10^7$ and $6.73 \times 10^9 \pm 2.48 \times 10^8$ colony forming units (CFU) per ml (equivalent to between 25 and 770 mg per litre of protein) depending on the experiment. Experiments were performed in triplicate with appropriate controls lacking bacteria, carbon source, AQDS, or humic substances. The vials were incubated statically at ambient temperature in the dark, sampled periodically using purged, sterile syringes and needles, and analyzed to determine the concentration of ferrous and total iron, protein, CFU, substrate, and metabolites as described below.

Analytical

Iron quantification

Total iron (Fe_{tot}) and Fe(II) were determined using the ferrozine method. For Fe(II) analysis, sample aliquots were extracted with 0.5 or 2.5 N HCl. Extractions for Fe_{tot} were performed in the additional presence of 0.25 M NH_2OH as a reductant. Commonly, the extraction time was 2 h but in some cases longer extraction times were used to verify complete extraction (Cooper et al. 2000; Royer et al. 2002b). Aliquots of acid extracts were added to a solution of 1 g ferrozine (Sigma, St. Louis, MO) in 1 l HEPES buffer at pH 7 and the absorbance was determined spectrophotometrically at 540 nm (EL 808 Microplate Reader, BIO-TEX Instruments, Winooski, VT).

Colony forming units (CFU) and protein

The concentration of CFU was determined after serial dilution of 0.1 ml aliquots in phosphate buffered saline solution (pH 7). Aliquots were spread onto tryptic soy agar (TSA, 40 g/l, Difco Laboratories), incubated for 48 h at 30°C, and colonies were counted. No statistically significantly different counts were observed if plates were incubated for 72 h or longer. Total protein was measured by a modification of the Coomassie method (Bradford 1976). Cells were disrupted by mixing 0.5 ml of sample with 0.5 ml of 2 N NaOH. The mixture was heated for 30 min at 95°C. After cooling the sample to ambient temperature, the pH was lowered to approximately 2 by adding 0.167 ml of 6 N HCl. To this solution, 1 ml of Coomassie Plus reagent (Pierce, Rockford, IL) was added and the absorbance was measured at 595 nm on a UV–vis spectrophotometer (Milton Roy Company Spectronic® GENESYS 5™, Rochester, NY). The assay was calibrated using 2 g/l bovine serum albumin fraction V protein standard (Pierce, Rockford, IL) diluted to a concentration range of 0–30 mg/l protein.

Carbohydrate and fatty acid analysis

Sucrose, glucose, fructose, glycerol, and lactate were quantified using a Dionex DX-300 ion chromatograph equipped with a Dionex PA10 column and a pulsed amperometric detector. Samples (10 µl) were injected into a mobile phase consisting of 52 mM NaOH pumped at a flowrate of 1.2 ml/min. Lactate, acetate, formate, propionate, iso-butyrate, and *n*-butyrate were analyzed using the same ion chromatograph equipped with a AS10 column, 25 µl sample loop, and conductivity detector. The eluent consisted of a 3.5 mM potassium tetraborate solution pumped at a flowrate of 1 ml/min.

Results

Carbon source and electron acceptor influence on growth

Figure 1 shows the extent of bacterial growth [measured as change in protein (Δ protein)] for lactate-, xylose-, and sucrose-amended ES6 cultures in the presence of several potential electron acceptors including Fe(III)-NTA, Fe(III)-citrate, HFO, fumarate, and oxygen. Protein data suggest that after 21 days xylose supported more bacterial growth than

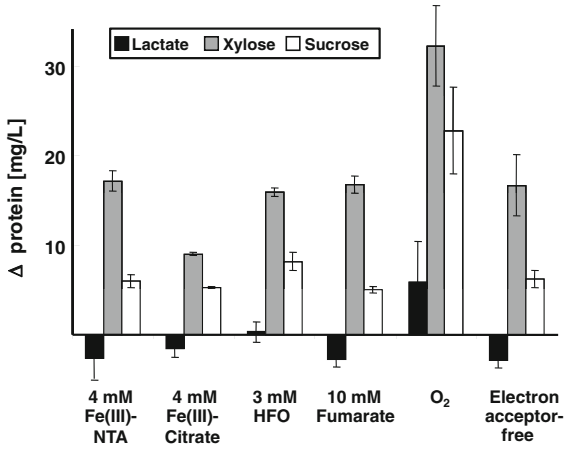

Fig. 1 Change in protein after 21 days of incubation of strain ES6 in the presence of lactate, xylose, sucrose, and several different potential electron acceptors. The electron acceptors tested included Fe(III)-NTA, Fe(III)-citrate, hydrous ferric oxide (HFO), fumarate, and oxygen. The initial protein concentration was 0.19 ± 0.01 mg/l. *Error bars* represent ±one standard deviation ($n = 3$)

sucrose regardless of the terminal electron acceptor used, while lactate supported bacterial growth only if oxygen was the terminal electron acceptor. For all three carbon sources tested, the greatest increase in protein was observed when oxygen was the terminal electron acceptor. When any of the other electron acceptors were used, no significant differences in growth were observed between the electron acceptor-free control and the other potential electron acceptors. These results suggest that both the electron donor and acceptor influence bacterial growth.

Carbon source influence on Fe(II) production

Both, xylose and sucrose, as electron donors supported some, though very little, reduction of hydrous ferric oxide (HFO, 3.22 mmol/l) by strain ES6 as compared to uninoculated controls after 14 days (Fig. 2). The presence of 100 μM AQDS significantly increased both the rate and extent of HFO reduction regardless of which carbon source was present. After 14 days, approximately 90% of the Fe(III) present had been reduced to Fe(II) by strain ES6 when AQDS was present and xylose was the carbon source utilized; only 7% had been reduced to Fe(II) in the absence of AQDS. When sucrose was the carbon source 61% of the available Fe was reduced to Fe(II) in the presence of AQDS compared

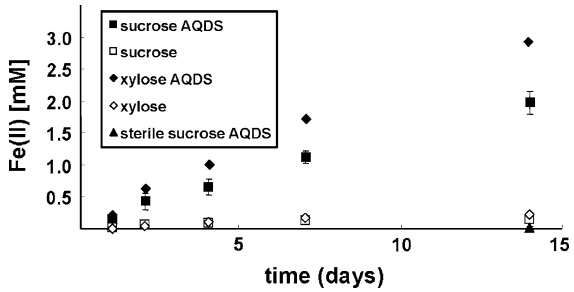

Fig. 2 Fe(II) production from hydrous ferric oxide (HFO) by strain ES6 over time in the presence and absence of 10 mM xylose or sucrose and 100 μM AQDS. *Error bars* represent ±one standard deviation ($n = 3$). *Error bars* are smaller than markers if not visible

to only 4% when AQDS was absent. The slight reduction of HFO after longer incubation times might be due to the production and release of compounds by strain ES6, which allowed for some reduction of solid phase Fe(III) even in the absence of AQDS.

While the most Fe(II) was produced in the treatment containing xylose and AQDS after 14 days, significant amounts of Fe(II) were also produced when molasses, glycerol or sucrose were present as well as AQDS (Fig. 3). When acetate was utilized as a carbon source, minimal Fe(II) was produced regardless of whether or not AQDS was present. Significantly lower Fe(II) production was observed in AQDS-free treatments. Interestingly, if AQDS was present, xylose-amended treatments showed the greatest Fe(II) production [2.9 mM Fe(II)] but if AQDS was absent, molasses-amended treatments

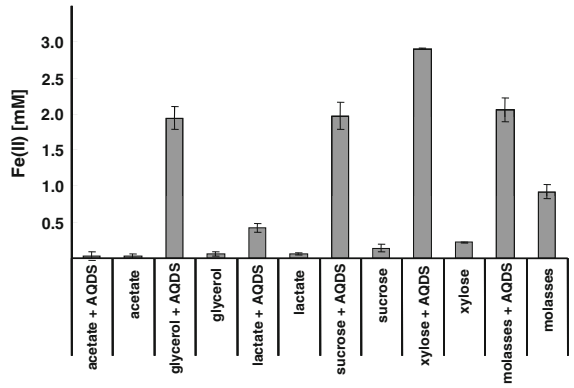

Fig. 3 Fe(II) production from hydrous ferric oxide (HFO) by strain ES6 after 14 days of incubation in the presence and absence of different carbon sources (10 mM, 3.42 g/l for molasses) and AQDS (100 μM). *Error bars* represent ±one standard deviation ($n = 3$)

showed the greatest Fe(II) production [0.92 mM Fe(II)] compared to the other carbon sources. When AQDS was absent, no significant amounts of Fe(II) were produced except when molasses was the carbon source utilized.

Neither lactate, glycerol, nor acetate appeared to support bacterial growth (protein, CFU, and optical density, data for lactate in the presence of 3 mM HFO can be seen in Fig. 1, other data not shown), but HFO reduction was observed in the presence of glycerol and lactate if 100 μM of AQDS were present. Sterile controls with each carbon source ± AQDS did not show significant Fe(III) reduction suggesting the reduction observed was not abiotic. Results suggest that bacterial growth was not necessary for Fe(III) reduction to occur. For each of the electron donors tested, Fe(II) production was lower in the absence of AQDS than in its presence. No statistically significant differences (*t*-test, $P > 0.05$) in biomass production were observed between AQDS amended and not AQDS amended treatments (data not shown). Hence, the increased production of Fe(II) in AQDS amended treatments was not the result of increased biomass production but was likely due to enhanced electron transfer from the bacterial cells to HFO.

These observations were reconfirmed in studies in which sucrose metabolism by strain ES6 was monitored in the presence and absence of HFO and AQDS. Substrate and metabolite measurements using ion chromatography indicated that lactate concentrations increased by approximately 25% in the presence of HFO and 40% in the presence of HFO with 100 μM AQDS (data not shown). Formate concentrations also increased approximately 10% in these treatments indicating a more complete oxidation of sucrose in the presence of HFO and HFO plus AQDS. The presence of 100 μM AQDS in the absence of HFO did not lead to any significant differences in metabolite patterns.

Electron shuttle influence on Fe(II) production

As AQDS was shown to increase HFO reduction rates, increasing AQDS concentrations were tested to determine if an increased concentration further increased HFO reduction rates. Results suggest increasing AQDS concentrations increased the rate of HFO reduction by strain ES6 in the presence of sucrose (Fig. 4). Initial Fe(III) reduction rates (inset to Fig. 4), normalized by the cell concentration (CFU), increased approximately tenfold from $2.27 \times 10^{-12} \pm 2.23 \times 10^{-13}$ μmol Fe(III)/CFU/h in the absence of AQDS to $3.31 \times 10^{-11} \pm 3.34 \times 10^{-12}$ μmol Fe(III)/CFU/h in the presence of 500 μM AQDS. Cell-free control treatments containing the highest AQDS concentration tested in this research (500 μM) did not lead to significant HFO reduction again suggesting this was not an abiotic reaction.

Fe(III) reduction of different iron mineral phases

In the presence of 100 μM AQDS and sucrose as the electron donor, HFO was the most readily reducible iron mineral among the ones tested as at least 10 times more Fe(II) was produced when HFO was the iron mineral present compared to any others (Fig. 5). No significant differences in Fe(II) produced were observed between the other iron minerals until day

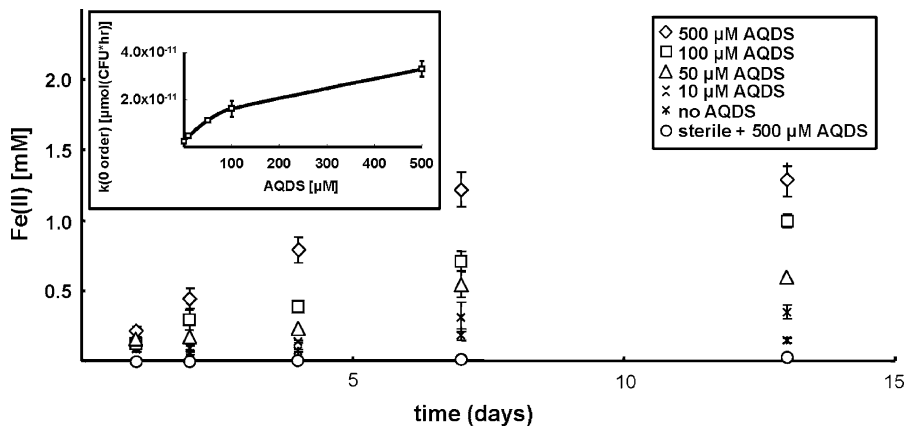

Fig. 4 Influence of AQDS concentration on Fe(II) production from hydrous ferric oxide (HFO) by strain ES6 over time in the presence of sucrose. *Inset* Zero order Fe(II) production rates normalized to cell number. *Error bars* represent ±one standard deviation ($n = 3$). *Error bars* are smaller than markers if not visible. Total amount of Fe(III) available in these experiments was 1.74 mmol/l

14. Results indicate 1.56 mM Fe(II) was produced from HFO after 14 days while only 0.23 and 0.15 mM Fe(II) was produced by maghemite and magnetite, respectively. Goethite and hematite were not significantly reduced over the 14 days period although slight reduction of these minerals was observed in long term experiments (>50 days) in the presence of AQDS (data not shown). It is possible that some of the Fe(II) produced in these experiments is originating from less crystalline Fe(III) phases present as impurities (up to 3%) in the purchased iron mineral phases.

Since magnetite (Fe_3O_4) already contains Fe(II) as part of its mineral structure, an increase in mild acid-extractable Fe(II) could have been due to an increase in extractability over time. However, controls containing magnetite but lacking strain ES6 or AQDS did not show an increase in acid-extractable Fe(II) over the duration of the experiment. Thus, results indicate the increase in 0.5 N HCl-extractable Fe(II) observed for magnetite is due to microbial reduction of ferric iron.

Dissolved Fe(III) reduction in the presence of an electron shuttle

While the enhancement of Fe(III) mineral reduction in the presence of AQDS has been described and attributed to enhanced electron transport between the bacterial cell and solid phase Fe(III) (Lovley et al. 1998; Newman 2001; Royer et al. 2002b), reports of electron shuttling compound-enhanced dissolved phase Fe(III) reduction appear to be rare. The results of these studies indicate that the presence of the electron shuttle AQDS drastically enhanced the reduction of Fe(III)-citrate by strain ES6 in the presence of sucrose as electron donor (Fig. 6). Reduction rates of Fe(III)-citrate were generally higher than for HFO; ranging from $9.85 \times 10^{-12} \pm 3.10 \times 10^{-13}$ μmol Fe(III)/CFU/h without AQDS to $7.34 \times 10^{-11} \pm 6.19 \times 10^{-12}$ μmol Fe(III)/CFU/h for systems containing 500 μM AQDS (inset to Fig. 6). These results indicate that soluble quinones, such as AQDS, can enhance the electron transport to solid phases and also to dissolved phase electron acceptors by strain ES6.

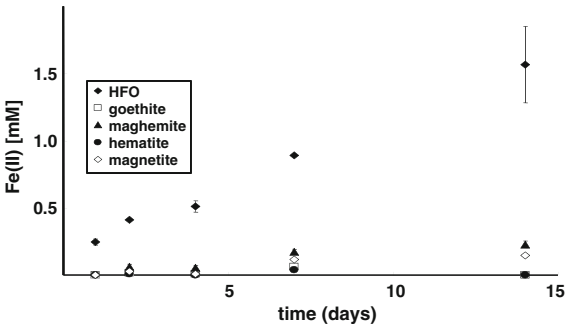

Fig. 5 Fe(II) production over time from different Fe-minerals by strain ES6 in the presence of 100 μM AQDS and sucrose. *Error bars* represent ±one standard deviation ($n = 3$). *Error bars* are smaller than markers if not visible. Total amounts of Fe(III) available in these experiments were 3.58 mmol/l in the HFO containing treatments and 6 mmol/l Fe(III) in the goethite, maghemite, hematite, and magnetite containing treatments

Fe(III) reduction in the presence of humic substances

While the addition of synthetic electron shuttling compounds such as AQDS to the environment might lack regulatory approval, the addition of natural organic matter such as humic and fulvic acids or humic substances should not encounter significant regulatory resistance. Hence, the influence of humic substances obtained from the International Humic Substance Society on the ability of strain ES6 to reduce HFO was also tested. Similar to the studies with AQDS, results demonstrate that the addition of the humic substances lead to an increase in Fe(II) production compared to controls lacking humic substances or lacking strain ES6 after 21 days (Fig. 7).

Discussion

Reductive transformation reactions for enhanced in situ bioremediation of oxidized environmental contaminants typically require an external electron donor. In most subsurface remediation scenarios, carbon sources such as those used in this study are considered the most convenient electron donor. The injection of readily available carbon can lead to biofouling of the injection wells or the surrounding formation (Roberts et al. 1991; Semprini et al. 1991; Shouche et al. 1993). Hence, it is important to be able to control the permeability of injection wells and the surrounding formation while maximizing microbial

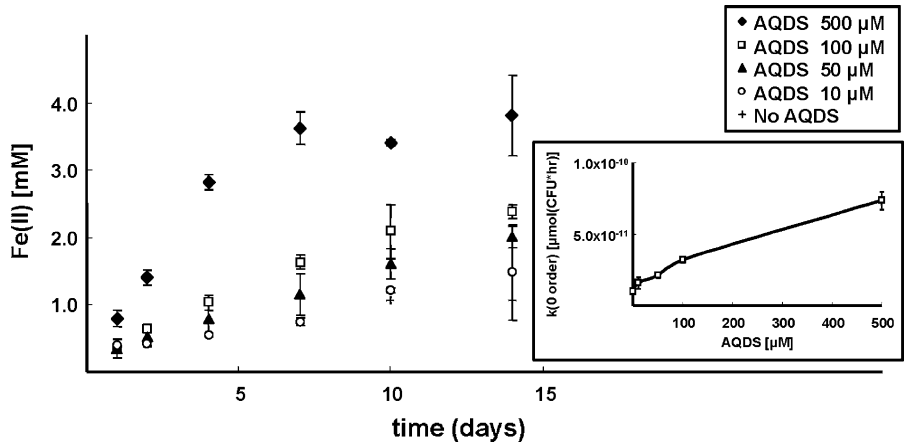

Fig. 6 Fe(II) production from Fe(III)-citrate by strain ES6 in the presence of sucrose and different AQDS concentrations over time. *Inset* Zero order reduction rates of Fe(II) production normalized to cell number. *Error bars* represent ±one standard deviation ($n = 3$). *Error bars* are smaller than markers if not visible. Total amount of Fe(III) available: 4.26 mmol/l

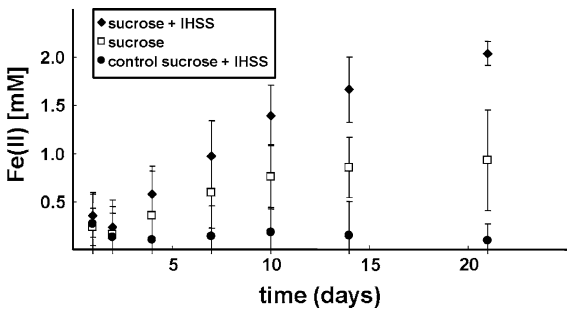

Fig. 7 Fe(II) production over time from hydrous ferric oxide by ES6 in the presence and absence of humic substances with sucrose as electron donor. *Error bars* represent ±one standard deviation ($n = 3$)

activity. In this case, maximizing Fe(III) reduction while limiting microbial growth. The choice of electron donor will depend on which indigenous organisms are present and the type of electron acceptor(s) present. Facultative anaerobes, such as the strain ES6 investigated in this study, capable of respiration in the presence of oxygen and fermentation under anaerobic conditions can be selectively controlled by adding substrates that either do or do not support growth under the existing conditions.

Control of growth and Fe(III) reduction

Under anaerobic conditions, xylose appeared to support more growth than sucrose, while lactate and other short chain organic compounds supported little if any growth. *Cellulomonas* species are known to be capable of electron transport chain phosphorylation in the presence of oxygen (aerobic respiration) and substrate level phosphorylation (fermentation) in its absence (Stackebrandt et al. 2006). The energy yield is several times greater during aerobic respiration than during fermentation, thus explaining the lower amount of biomass produced in the absence of oxygen (Fig. 1). Lactate and other short chain organic compounds are not easily fermented explaining the lack of biomass production in their presence. In regards to lactate, these findings are consistent with Sani et al. (2002) who reported that lactate supported the reduction of NTA-chelated Fe(III) by *Cellulomonas* spp. under non-growth conditions, but anaerobic growth on Fe(III) as the terminal electron acceptor was not observed.

In the presence of oxygen, relatively inexpensive substrates such as lactate or glycerol as well as more complex substrates such as glucose, sucrose, or molasses can be used to build up biomass and consume oxygen. Once oxygen is consumed and no more biomass growth is desired, substrates can be added, which support reductive transformation reactions but no bacterial growth (e.g., lactate or glycerol). If additional growth of biomass is desired after oxygen has been consumed, sugars such as xylose or sucrose, or complex fermentable carbon sources such as molasses can be injected again.

Purified carbon sources, such as sucrose and xylose, are likely to be significantly more expensive than molasses, which often has sucrose as its main constituent (Prescott and Dunn 1983). Hence, molasses was tested in this study in order to include a fermentable carbon source with a high potential to be used in the field. In the absence of the electron shuttle

AQDS, molasses supported the greatest amount of Fe(III) reduction from HFO after 14 days (Fig. 3), which is important as the addition of AQDS may not be approved for in situ applications or be far too expensive. Molasses also supported significantly more growth, based on viable cell counts, than any of the other carbon sources tested with no significant difference between AQDS and non-AQDS supplemented treatments (data not shown). The greater Fe(II) production in the molasses amended treatments could thus potentially be explained by the observed increased biomass production. Based on our research, it is however more likely that some of the molasses constituents other than sucrose either more readily provided electrons for HFO reduction or enhanced the electron transfer from strain ES6 to HFO, i.e., functioned as an electron shuttle. Separate experiments conducted in our laboratories investigated the reductive transformation of Cr(VI) (manuscript in preparation) by strain ES6. These experiments provided evidence that the increased reduction rates in the presence of molasses were due to molasses constituents that increase electron transfer rates from the bacterial cells and not due to increased biomass production since the addition of molasses in these treatments significantly increased reduction rates without increasing biomass production rates over the duration of these experiments.

AQDS-enhanced Fe(III) reduction

AQDS and other synthetic quinones are often used as model compounds for the quinone moieties of humic substances and have been shown to enhance the reduction of amorphous and crystalline solid phase Fe(III) by a wide range of bacteria (Benz et al. 1998; Lovley et al. 1998; Cervantes et al. 2002; Dong et al. 2003; Kappler et al. 2004; Luijten et al. 2004; Wolf et al. 2009). Our results demonstrate that the addition of AQDS and humic substances can significantly enhance the ability of strain ES6 to reduce solid phase Fe(III). The same is true for other *Cellulomonas* strains isolated from the Hanford site (data not shown).

The ability of strain ES6 to reduce HFO and Fe(III)-citrate increased with increasing AQDS concentrations, which indicates that the addition of compounds rich in quinone moieties could be used to enhance electron transfer processes in subsurface environments containing microbial communities capable of fermentation. The influence of iron chelating or electron shuttling compounds on the reduction of solid phase Fe(III) has been investigated by other researchers (Zachara et al. 1998; Lovley and Blunt-Harris 1999; Royer et al. 2002a, b; Saffarini et al. 2002; Turick et al. 2002) and the results of this study are similar to their findings.

The results of this study suggest that strain ES6 was capable of reducing solid phase Fe(III) to some extent and the amount of Fe(II) produced increased with the addition of an electron shuttle. While overall these findings are similar to those studies performed with other microorganisms, the rate and extent of Fe(II) produced from these solid phase iron minerals is lower than some well-studied iron reducing microorganisms including *Shewanella* spp. (Kostka and Nealson 1995; Roden and Zachara 1996; Zachara et al. 1998; Dong et al. 2000; Royer et al. 2002b). However, it was observed that all of these solid phase iron minerals were reduced to some extent by strain ES6 even in the absence of an electron shuttle such as AQDS which was not observed in the case of the iron-reducing bacterium *Deinococcus radiodurans* (Fredrickson et al. 2000a). Additionally, Benz et al. (1998) demonstrated that *Propionibacterium freudenreichii,* a fermenting bacterium, was only capable of reducing HFO, maghemite and hematite in the presence of AQDS. Together, these results suggest that strain ES6 may not be able to reduce Fe(III) to the same extent or at the same rate as most iron respiring bacteria, but it may possess a greater ability to reduce iron than other fermenting bacteria. Therefore, *Cellulomonas* species may play a valuable role in Fe(III) reduction and subsequent contaminant transformations in subsurface environments.

The exact mechanism of how different compounds enhance solid phase Fe(III) reduction is not always obvious since some compounds are capable of both chelation of Fe(III) and electron shuttling. Humic substances for instance are commonly believed to both, chelate iron and shuttle electrons (Lovley et al. 1996; Lovley and Blunt-Harris 1999; Royer et al. 2002b). AQDS, a frequently used model compound in laboratory experiments, has been widely recognized as an electron shuttle only (Lovley et al. 1998; Newman 2001; Royer et al. 2002b). While Shyu et al. (2002) reported that AQDS can diffuse into the membrane of *Shewanella oneidensis* MR1 cells, the

mechanism of electron transfer from bacterial cells to AQDS is not completely understood.

Several groups have reported that fermenting bacteria are able to shift their fermentation patterns to slightly more oxidized compounds in the presence of humic substances, electron shuttling compounds, or other potential electron acceptors (Emde and Schink 1990; Benz et al. 1998). With sucrose as the substrate, strain ES6 produced approximately 25% more lactate in the presence of HFO and approximately 40% more if 100 μM AQDS were present in addition to HFO. The increased oxidation of sucrose in the presence of AQDS and HFO and the increased Fe(II) concentrations in these treatments indicate that more electrons can be transferred from sucrose to HFO in the presence of an electron shuttling compound. However, despite increased electron transfer to HFO and more oxidized fermentation products, no statistically significant difference in bacterial growth was observed. Assuming that the more extensive oxidation of sucrose in the presence of HFO or HFO and AQDS allowed strain ES6 to gain additional energy via substrate level phosphorylation, this additional energy was apparently not used to produce additional biomass. It is however possible that this additional energy was used to produce carbon storage compounds which would agree with observations made by Sani et al. (2002) and Viamajala et al. (2008).

The presence of quinone-like compounds capable of shuttling electrons, such as AQDS or humic substances, drastically increased the ability of strain ES6 to transfer electrons to solid phase Fe(III). While it has been shown that electron shuttling compounds such as AQDS can enhance the ability of several bacterial strains to reduce solid phase ferric iron, enhanced electron transfer from the bacterial cell to a dissolved electron acceptor has rarely been described. Figure 6 shows a significant increase in Fe(II) production rates from Fe(III)-citrate in the presence of AQDS. Borch et al. (2005) showed that the addition of AQDS can considerably enhance the rate of TNT transformation by strain ES6 and we have shown in separate experiments that AQDS also drastically increases Cr(VI) reduction rates by strain ES6 (manuscript in preparation). The electron transfer from the reduced form of AQDS to dissolved Fe(III), TNT, or Cr(VI) is basically instantaneous. The rate of electron transfer to solid phase Fe(III) is likely limited by the number of available reducible surface sites.

The addition of synthetic compounds, such as AQDS, capable of increasing electron transfer from bacterial cells to oxidized contaminants or terminal electron acceptors, such as Fe(III)-minerals, could have economic potential. At this point in time however, the addition of synthetic electron shuttling compounds such as AQDS might lack regulatory approval because the effects of AQDS on the environment have not been studied extensively. AQDS has been shown to be toxic to a tolC mutant of *S. oneidensis* MR1 (Shyu et al. 2002) and methanogenic bacteria (Cervantes et al. 2000) in millimolar concentrations but no other toxicological data appear to be available. Concentrations used in this study were all below 500 μM, and other authors have reported that AQDS concentrations in the 50–100 μM range are sufficient for maximum Fe(III) reduction stimulation (Lovley et al. 1998; Royer et al. 2002b).

Humic substance-enhanced iron mineral reduction

The addition of natural organic matter such as humic and fulvic acids or humic substances should not encounter significant regulatory resistance. Thus, the addition of humic substances or fractions thereof have the potential to be successfully used in the field to enhance electron transfer from bacterial cells to oxidized contaminants or oxidized minerals. Humic substances obtained from the IHSS stimulated Fe(III) production from HFO although to a lesser extent than AQDS. A slight production of Fe(II) was observed in uninoculated controls containing these humic substances and is believed to be based on the abiotic reduction of Fe(III) by reduced humic substance moieties, such as reduced quinone structures (Kappler et al. 2004). The extent of stimulation by natural or synthetic organic matter is likely to depend on the number and reactivity of quinone-like structures, although compounds without quinone groups have also been reported to increase electron transfer from bacterial cells to Fe(III)-minerals (Hernandez et al. 2004). Additionally, the size and solubility of electron shuttling compounds are likely to play a role, such that insoluble fractions of natural organic matter for instance might not be as efficient in shuttling electrons as soluble fractions.

Conversely, in a flowing environment, such as a contaminated aquifer, soluble fractions will likely be transported away from the treatment area with the flowing groundwater faster than fractions with low solubility and higher sorption tendency.

Conclusions

Important reactions involving dissolved and solid phase iron can be significantly influenced by fermenting, sulfate-reducing, halorespiring, and methanogenic bacteria (Benz et al. 1998; Cervantes et al. 2002; Kappler et al. 2004). This study demonstrates that the type of organic electron donor and other organic matter can significantly change the ability of *Cellulomonas* sp. strain ES6, a fermenting bacterium isolated from the Hanford site, to reduce dissolved and solid phase Fe(III).

It appears from our results that, while strain ES6 possesses a limited ability for Fe(III) reduction compared to iron respiring bacteria, it might possess a greater ability to reduce iron than other fermenting bacteria. This makes strain ES6 a potentially important organism for in situ contaminant remediation. Results demonstrate that carbon sources can be selected to control growth at least partly independently from Fe(III) reduction. In the potential application of in situ biobarriers in the field, this could be an important method for controlling the bacterial density and activity in subsurface environments and hence provide a mechanism to avoid the plugging of injection wells by gaining some control over subsurface bacterial growth.

While the reduction of Fe(III) minerals by respiratory metal-reducing bacteria such as *Shewanella* and *Geobacter* has been described intensively over the past decade, the importance of other genera on the iron cycle and contaminant transformations have received much less attention. Although Lovley (1987) reported that a large number of bacteria can reduce, but not grow on Fe(III) as their sole terminal electron acceptor, it was only recently accepted that these bacteria can have a significant influence on oxidation–reduction reactions in the environment. Based on the results presented here and recent results by Benz et al. (1998), Cervantes et al. (2002), Kappler et al. (2004), and Luijten et al. (2004) we suggest that the presence of bacteria other than respiratory iron reducers, can be sufficient to achieve significant reduction of subsurface iron minerals especially in the presence of even very low concentrations of natural or synthetic organic matter. Reduced iron minerals could then serve to reductively transform oxidized contaminants such as Cr(VI), U(VI), trinitrotoluene, or chlorinated aliphatic compounds.

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