Characterization of Fe(III)-reducing enrichment cultures and isolation of Fe(III)-reducing bacteria from the Savannah River site, South Carolina

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Abstract

The Savannah River site, South Carolina (SRS), has been subjected to heavy metal and radionuclide contamination. Dissimilatory Fe(III)-reducing bacteria, by reducing insoluble Fe(III) to soluble Fe(II), may enhance contaminant mobility through subsurface environments. In order to investigate populations of the indigenous iron-reducing microbes from the SRS, duplicate enrichment cultures were initiated using a 10% inoculum of 7 sediment/soil samples, and serial dilutions were made into Wolfe’s minimal salts media amended with 50 mM Fe(III) floc, 10 mM acetate and 0.01% yeast extract. Terminal restriction fragment length polymorphism (T-RFLP) analysis was used to generate fingerprints of the cultures and track changes in the microbial communities through the dilutions. Cluster analysis determined the relatedness of individual fingerprints. Initial enrichment cultures exhibited complex fingerprints consisting of many individual T-RF peaks, and demonstrated low similarity between sites. After four serial dilutions the fingerprints were less complex and clustered at higher similarities. Several individual T-RF peaks became dominant in a majority of the fingerprints. Cloning and sequence analysis revealed the presence of microbes closely related to Clostridium and Bacillus species and to known iron reducers such as Geobacter species and Pantoea agglomerans. Several Fe(III)-reducing isolates related to Aeromonas, Bacillus and Clostridium species were obtained.

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1. Introduction

Dissimilatory Fe(III) reduction is an anaerobic respiratory pathway in which the reduction of Fe(III) is coupled to the oxidation of organic matter. Dissimilatory iron reduction occurs in natural soil and sediment systems. While many electron acceptors are available for anaerobic organic matter oxidation in these systems, Fe(III) is often the most abundant [30,51,63]. Bacterial species that mediate Fe(III) reduction are phylogenetically diverse, include both facultative and obligate anaerobes, and can be classified as fermentative, sulfur-oxidizing, hydrogen-oxidizing or organic-acid-oxidizing.

During dissimilatory Fe(III) reduction, extra electrons from energy production are transferred to solid Fe(III)-bearing minerals outside of the bacterial cell. The Fe(III) minerals are converted either to soluble Fe(II) which can be leached from a soil or sediment system or to Fe(II)-bearing minerals which remain. This increase in iron solubility may cause the release of inorganic compounds, such as many toxic metals and radionuclides, which are frequently bound to Fe(III) oxides. Field studies give indications that Fe(III) reduction is responsible for trace metal release to aquatic sediments [13,20,23,36] and groundwater [7]. In addition, laboratory studies demonstrate the release of nickel, cobalt, copper and manganese [30]; chromium [15]; cadmium, nickel and zinc [16]; and radium from uranium mill tailings [30].

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The U.S. Department of Energy has a legacy of heavy metal and radionuclide pollution from the production of enriched uranium and high grade plutonium used in atomic weapons. In particular, the Savannah River site, South Carolina (SRS), has been subjected to both heavy metal and radionuclide contamination. These inorganic compounds are known to bind to amorphous Fe(III) oxides. Given the effects of microbial Fe(III) reduction on iron mineral phases it will likely have an effect on heavy metal and radionuclide mobility. Enrichment cultures from various sites within the SRS were used to investigate and identify indigenous iron reducers. Each stage of the enrichment was characterized by terminal restriction length polymorphism (T-RFLP) analysis. T-RFLP analysis is a powerful tool that has been used to compare microbial community structure and diversity in a variety of laboratory and natural settings [5,6,9,19,40,58]. After four serial dilutions of the enrichment cultures, several Fe(III)-reducing pure cultures, including three similar to Aeromonas sp., were isolated.

2. Materials and methods

2.1. Study site and sample collection

The U.S. DOE SRS, located in Aiken, SC was established to produce basic materials for nuclear weapons, as well as for storing and processing spent nuclear fuel. During the fulfillment of this mission, the SRS has been subjected to both heavy metal and radionuclide contamination. Sediment samples were collected from floodplains at SRS that are subjected to alternating periods of oxidizing and reducing conditions. Lake-bottom sediment samples and floodplain soil samples were collected from Par Pond and Lower Three Runs (LTR), respectively. The LTR soil samples (~top 20 cm) were collected with hand-held instruments and stored in plastic bags. The bags containing samples were purged with argon in the laboratory. The Par Pond samples (~top 15 cm) were collected with a grab sampler, split into 2 fractions, placed in screw top polypropylene bottles which were then filled completely with site water.

2.2. Enrichment conditions

All enrichment cultures were set up as a 10% (vol/vol) sediment slurry in Wolfe’s minimal media (WMM) or in water collected at the site. The defined medium contained the following salts (g l⁻¹): NaHCO₃, 2.5; KCl, 0.1; Na₂HPO₄·H₂O, 0.6; NH₄Cl, 1.5. In addition, the following components were added (in ml l⁻¹): Wolfe’s vitamins, 10.0; Wolfe’s trace mineral solution, 10.0 [64]; 1% yeast extract, 10.0. Amorphous FeOOH (formed by the reaction of FeCl₃ with NaOH) and acetate were provided at ~50 and 10 mM, respectively. 10 ml of sediment slurry was added to 90 ml of FeOOH suspended in site water in 160 ml serum bottles under an atmosphere of 70% N₂/30% CO₂. Bottles were sealed with Teflon-coated, butyl rubber stoppers (Emsco, Philadelphia, PA) and crimp-sealed. Duplicate enrichments were started with 4 sediments from Par Pond (labeled P1–P4) and 3 soils from LTR. (D1, S1, T1). The cultures were incubated at room temperature in the dark without shaking. Strict anaerobic procedures were followed at all times. Subsequent passages were performed by transferring 10 ml of the enrichment into 90 ml of FeOOH suspended in WMM.

2.3. DNA extraction

The presence of 50 mM FeOOH in the enrichments inhibited extraction of DNA following standard protocols. Therefore, total genomic DNA was extracted as follows: 3 ml of the enrichment cultures were sampled anaerobically using a 5 cc syringe and 21 gauge needle, dispensed into 1.5 ml centrifuge tubes and spun at 16 000 g in a microcentrifuge for 3 min. The supernatant was decanted and the iron floc was solubilized by resuspending the pellet in 1 ml of a filter-sterilized solution of ammonium oxalate (28 g l⁻¹) and oxalic acid (15 g l⁻¹) and incubated until complete solubilization of iron was achieved [35]. Total genomic DNA was then extracted from the remaining cells using phenol/chloroform/isoamyl alcohol extraction [4].

2.4. T-RFLP fingerprinting

The DNA was purified using CsCl density gradient ultracentrifugation. The 27F (5'-AGAGTTTGATCCTGGCTCAG) and 1525R (5'-AAGAGGTGTGCTGGTG) primers were used to amplify ~1500 bp of the 16S rRNA gene. The 27F primer was labeled with 5-hexachlorofluorescein (HEX; Perkin–Elmer). PCR conditions were 1 cycle at 94°C for 5 min, followed by 30 cycles of 95°C for 0.5 min, 55°C for 0.5 min, 72°C for 1.5 min and a final extension step at 72°C for 10 min. After amplification 15 μl of the PCR products were digested separately with HaeIII, HhaI, MnlI, Sau3AI or TaqI restriction enzymes at 37°C for 6 h. The digested DNA was precipitated with 0.1 volume 3 M sodium acetate and 2.0 volumes 95% ethanol followed by spinning at 16 000 g in an Eppendorf microcentrifuge for 15 min. The DNA pellet was washed with 70% ethanol, dried and resuspended in a mixture of 14.5 μl deionized formamide and 0.5 μl of DNA fragment length internal standard (TAMRA 500; Perkin–Elmer). The mixture was denatured for 2.5 min at 94°C and then immediately placed on ice. Electrophoresis was performed on an ABI 310 automated DNA sequencer using the GeneScan mode with POP4 gel matrix and a 47 cm by 50 µm capillary column. Lengths of T-RFs were determined relative to the internal TAMRA 500 standard (35–500 bp) using the ABI GeneScan software.

2.5. Cloning and identification of T-RF peaks

16S rRNA gene PCR products were cloned using the CLONEAMP pAMP1 system (Gibco BRL, Life Technologies, Gaithersburg, MD). The 27F and 1525R primers were modified with the 12 bp linkers required for use with this system. Recombinant plasmids were digested with HaeIII restriction endonuclease and unique clones were identified by agarose gel electrophoresis using 2% Metaphor agarose (FMC BioProducts, Rockland, ME). Plasmid DNA from unique transfor-
mants was purified using the FlexiPrep kit (Pharmacia, Piscataway, NJ) and sequenced on an ABI 373 automated sequencer (Perkin–Elmer/ABI). Double-stranded sequence for approximately 500 bp of the 16S rRNA gene was obtained by sequencing with 27F and 519R (5′-GWATTACCCGCGGCKGCTG-3′). Subsequently, predicted T-RF lengths were determined, via in silico restriction digestion, for each restriction enzyme used to generate T-RFLP fingerprints (HaeIII, HhaI, MnlI, Sau3AI, or TaqI). These predicted T-RF lengths were compared with the actual fingerprints in order to identify specific T-RF peaks. Sau3AI, which has a recognition sequence within the 27F primer, was used to verify sequence consistency among the clones and isolates, and to ensure complete digestion of labeled PCR products (i.e. absence of cryptic restriction sites).

2.6. Pairwise similarity calculations and cluster analysis

For each T-RFLP fingerprint generated, all peaks exceeding 50 units above background fluorescence were aligned relative to the internal size standard using the GeneScan software. T-RFs below 35 bp and above 500 bp were excluded from analysis in order to minimize sizing errors. In order to facilitate comparison of fingerprints between different enrichments, the peak area for individual T-RFs was normalized to percent of the total peak area for all T-RFs in sample A, and Nj/(Na + Nb), where Na is the total peak area for all T-RFs in sample A, Nj is the total peak area for all T-RFs in sample B, and Nb is the sum of the lower of the two areas recorded for T-RFs found in both samples. S_ab ranges from 0, when two fingerprints share no common T-RFs, to 1, when two fingerprints are identical [27, 37,38].

After calculating S_ab for all possible comparisons between enrichment fingerprints, clustering was performed by the UPGMA (unweighted pair group mean average) method. All indices and clusters were calculated using the COMBinatorial Polythetic Agglomerative Hierarchical clustering package (COMPAH96; Dr. Eugene Gallagher, University of Massachusetts, Boston, http://www.es.umass.edu/edgwebp.htm).

2.7. Phylogenetic analyses

Approximately 500 bp of the 16S rRNA gene were aligned using ClustalX, version 1.83 [62]. For pairwise alignments, a gap opening value of 10.00 and a gap extension of 0.10 were used. For multiple alignments, gap opening and gap extension values were set to 10.00 and 0.20, respectively. The generated alignment was visually inspected and adjusted using the delay divergent sequences criterion to ensure gaps were not introduced erroneously.

The sequence alignment was then used as input for generating the phylogenetic dendrogram via the Phylogenetic Inference Package 3.63 (PHYLIP) [14]. First, the DNADIST program was used to generate a genetic distance matrix assuming the Kimura 2-parameter model (transitions:transversions = 2.0). Then, this matrix was used to generate the dendrogram using the program NEIGHBOR, with E. coli K-12 as the out-group and randomization of the input order of species. Finally, the dendrogram was drawn using the drawgram program. Phylogenetic analyses were verified by performing 1000 bootstrap iterations using SEQBOOT.

2.8. Isolation of Fe(III)-reducing bacteria

Isolates were obtained by spread plating 100 µl from one Par Pond enrichment (P4b-5) and one LTR enrichment (S1b-5) onto agar WMM plates. The media for the plates is as described above, with the following exceptions: HEPES (4.8 g) replaced the carbonate buffer and Fe(III) citrate (12.0 g) replaced the FeOOH floc. Plates were incubated anaerobically at room temperature in a Coy chamber, under an atmosphere of 3% H2/97% N2. Individual colonies were transferred anaerobically into 10 ml serum bottles containing WMM and 25 mM Fe(III)-floc. Fe(II) production was measured spectrophotometrically using the ferrozine method [34].

2.9. Nucleotide accession numbers

Nucleotide sequences generated in this study were filed in GenBank under accession numbers AF400089–AF400106 (P1a4, Dsa3, Tcb3 clonal libraries, respectively); DQ138302–DQ138310 (P1b3 clonal library); and AF427149–AF427155 (Fe(III)-reducing isolates).

3. Results

3.1. Enrichment cultures

Duplicate Fe(III)-reducing enrichment cultures were initiated for 4 Par Pond sediment samples, and 3 Lower Three Run (LTR) soil samples. All 14 enrichments demonstrated Fe(III) reduction, as evidenced by Fe(II) production determined by the ferrozine method [34]. The enrichments consistently reduced about half of the 50 mM Fe(III) floc available and maintained Fe(III) reduction throughout all (1:10) serial passages (data not shown).

3.2. T-RFLP fingerprinting and cluster analysis of similarity values

Five restriction enzymes were used to create different fingerprints for each of the initial enrichment culture samples. HhaI and MnlI consistently yielded the highest number of resolvable peaks and were used in subsequent analyses. HaeIII and TaqI produced fingerprints with significantly lower numbers of resolvable peaks most likely due to conservation of enzyme recognition site sequences. Sau3AI, which was predicted to yield T-RFs of 7 bp, produced fingerprints with no resolvable peaks.

Over the course of the dilutions, the enrichment communities became less complex (i.e. fewer resolvable peaks) as well as
Fig. 1. Comparison of the contribution of individual T-RFs toward total fingerprint fluorescence for the 1st through 3rd serial passages of the Lower Three Runs, Donora Station (DSa) enrichment. Fluorescence of individual peak areas was normalized to total fingerprint fluorescence. For example, T-RF 168 contributed over 60% of the total fingerprint fluorescence in enrichment DSa-3 and less than 10% in enrichment DSa-1. Note that the x axis, T-RF size in base pairs, is not linear. T-RF fingerprints were generated using the MnlI restriction enzyme.

3.3. Cloning and identification of T-RF peaks

Clonal libraries were generated from four different enrichment cultures in order to identify the dominant peaks in the various fingerprints. Approximately 100 clones were screened from the libraries generated from DSa-3, Tcb-3, P1b-3, and P1a-4 enrichments. Table 1 lists the percent similarity of the clones obtained from each library to sequences deposited in the GenBank database. The T-RF size for each clone as generated by the restriction enzymes HhaI and MnlI is also listed.

Known iron-reducing bacteria such as the Geobacter species and Pantoea agglomerans were common in the Tcb-3, P1a-4, P1b-3 libraries. However, none of these species was found in the 100 clones screened for the DSa-3 library. Several clones similar to subsurface organisms and those isolated from anoxic soils, as well as Clostridium species, were identified for this library.

3.4. Isolation of Fe(III)-reducing bacteria from SRS

Seven Fe(III)-reducing isolates were obtained from the SRS enrichment cultures, 6 from the Par Pond enrichment P4b-5 and 1 from the LTR enrichment SBb-5. Table 2 lists tentative identification of these isolates based on percent similarity of 16S rRNA sequence data (∼1500 bp) to sequences deposited in GenBank. With the exception of PAR4, all isolates were able to reduce approximately 50% of the 50 mM Fe(III)-citrate within 30 days (Table 2).

Purity of the isolates was determined by microscopic investigation as well as by plating onto minimal and rich media plates. Uniform colony morphology was obtained for all plates. Microscopic investigation initially showed that the PAR2 sample contained two distinct microbes. Dilution spread-plating and isolation of individual colonies resulted in obtaining 2 closely
related *Aeromonas* species, PAR2A and PAR2B, which displayed different rates of iron reduction (Table 2).

PAR2B demonstrated the highest rate of Fe(III)-citrate reduction of all the isolates (2.2 mM Fe(II) produced per day) when yeast extract was added to the media. It did not grow with acetate as the sole carbon source. In addition, it grew poorly on Fe(III)-floc with yeast extract or yeast extract/acetate as the carbon source. Growth on Fe(III)-floc and acetate as the sole carbon source was not above background controls. (Fig. 3). In a similar experiment, PAR2B was grown with ferric citrate and acetate as the sole carbon source; there was no Fe(II) production over background controls (data not shown).

### 3.5. Phylogenetic analysis of clones and isolates

A neighbor-joining dendrogram was generated for the clones and isolates presented in this study, in order to elucidate their relationship to known Fe(III)-reducing and non-Fe(III)-reducing microorganisms (Fig. 4). The Fe(III)-reducing isolates PAR2A, PAR2B and PAR3 were most closely related to *Aeromonas* species and were grouped with the *Aeromonas/Shewanella* cluster (990 out of 1000 bootstraps). Isolates PAR1, PAR4, PAR5, and LTR1, as well as multiple clones from all four clonal libraries screened formed a large cluster of Gram-positive organisms (961/1000 bootstraps). This group included *Bacillus*, *Clostridium* and *Rhodococcus* species. Eight clones from the P1b-3, P1a-4, and TCb-3 clonal libraries formed a very tight cluster with several identified *Geobacter* species (1000/1000 bootstraps). And finally, 3 clones from the P1a-4 and TCb-3 clonal libraries were clustered with two different isolates of *Pantoea agglomerans*.

### 4. Discussion

Given the history of metal and radionuclide contamination at DOE sites throughout the United States, including the SRS, it is
The percent similarity of the clonal sequences to the most closely related sequences deposited in GenBank, as determined using the BLAST algorithm [1], as well as relevant accession numbers are indicated. The predicted T-RF length for each clone, as generated by the BLAST algorithm [1], is also listed.

extremely important to understand the factors affecting mobility of these contaminants in subsurface environments. In addition to geological parameters such as pore structure, microbial processes can greatly affect the fate of inorganic contaminants in subsurface environments. Therefore, the specific goal of this research was to characterize Fe(III) reducing enrichment cultures from the SRS as well as to obtain pure Fe(III)-reducing isolates directly relevant to the SRS.

The 14 Fe(III)-reducing enrichment cultures obtained in this study all showed iron reduction capability through several serial passages. T-RFLP analysis demonstrated that despite large variability in the total microbial population in the initial soil/sediment samples, the fourth dilution enrichment communities were very similar. This convergence of the communities indicates that the enrichment cultures obtained in this study may be more representative of the actual iron-reducing microorganisms at the SRS compared to laboratory estimates of pure isolates. Of course, given that all enrichment cultures were subjected to the same selective pressures (e.g., carbon substrate, Fe(III) source, temperature), it is not surprising that they demonstrated higher similarity over time.

Table 1

<table>
<thead>
<tr>
<th>Clone</th>
<th>Accession No.</th>
<th>Similarity (%) to published sequences</th>
<th>T-RF size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tb3-J</td>
<td>AF400100</td>
<td>98%, uncultured clone ZZ12C10 (AY214184)</td>
<td>Hha1 216</td>
</tr>
<tr>
<td>Tb3-R</td>
<td>AF400101</td>
<td>99%, uncultured clone 189up (AY212641)</td>
<td>208</td>
</tr>
<tr>
<td>Tb3-T</td>
<td>AF400102</td>
<td>99%, uncultured clone Gly0346 (AY527737)</td>
<td>150</td>
</tr>
<tr>
<td>Tb3-AH</td>
<td>AF400103</td>
<td>99%, uncultured Enterobacteriaceae Ko' (AY635978)</td>
<td>371</td>
</tr>
<tr>
<td>Tb3-AK</td>
<td>AF400104</td>
<td>94%, uncultured DGGE type E (AY521561)</td>
<td>94</td>
</tr>
<tr>
<td>Tb3-AM</td>
<td>AF400105</td>
<td>98%, Geobacter psychrophilus str. P11 (AY653551)</td>
<td>91</td>
</tr>
<tr>
<td>Tb3-BU</td>
<td>AF400106</td>
<td>98%, uncultured clone ZZ12C10 (AY214184)</td>
<td>94</td>
</tr>
<tr>
<td>Pa4-5</td>
<td>AF400089</td>
<td>99%, uncultured Enterobacteriaceae Ko' (AY635978)</td>
<td>371</td>
</tr>
<tr>
<td>Pa4-9</td>
<td>AF400090</td>
<td>100%, uncultured clone PL-4B6 (AY570632)</td>
<td>208</td>
</tr>
<tr>
<td>Pa4-20</td>
<td>AF400091</td>
<td>98%, Geobacter psychrophilus str. P11 (AY653551)</td>
<td>92</td>
</tr>
<tr>
<td>Pa4-25</td>
<td>AF400092</td>
<td>97%, uncultured clone TSAL18 (AB186866)</td>
<td>229</td>
</tr>
<tr>
<td>Pa4-32</td>
<td>AF400093</td>
<td>98%, uncultured Enterobacteriaceae Ko' (AY635978)</td>
<td>371</td>
</tr>
<tr>
<td>Pa4-38</td>
<td>AF400094</td>
<td>98%, uncultured clone TSAC03</td>
<td>94</td>
</tr>
<tr>
<td>Da3-33</td>
<td>AF400095</td>
<td>99%, uncultured clone 189up (AY212641)</td>
<td>208</td>
</tr>
<tr>
<td>Da3-38</td>
<td>AF400096</td>
<td>95%, unidentified eubacterium from anoxic soil (AJ229194)</td>
<td>–</td>
</tr>
<tr>
<td>Da3-39</td>
<td>AF400097</td>
<td>99%, uncultured clone ICBTG1 (AF390941)</td>
<td>–</td>
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<tr>
<td>Da3-41</td>
<td>AF400098</td>
<td>95%, Clostridia clone X3Ba26 (AY071233)</td>
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<tr>
<td>Da3-45</td>
<td>AF400099</td>
<td>98%, uncultured Rhodococcus sp. clone (AY694585)</td>
<td>–</td>
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<tr>
<td>Pb3-2</td>
<td>DQ138302</td>
<td>99%, uncultured clone SIA-65 (AJ009473)</td>
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</tr>
<tr>
<td>Pb3-6</td>
<td>DQ138303</td>
<td>96%, uncultured Actinobacterium S15A-MN100 (AJ534681)</td>
<td>360</td>
</tr>
<tr>
<td>Pb3-7</td>
<td>DQ138304</td>
<td>97%, uncultured clone TSAL18 (AB186866)</td>
<td>229</td>
</tr>
<tr>
<td>Pb3-20</td>
<td>DQ138305</td>
<td>98%, uncultured Clostridium (AY261814)</td>
<td>–</td>
</tr>
<tr>
<td>Pb3-22</td>
<td>DQ138306</td>
<td>97%, uncultured clone TSAL18 (AB186866)</td>
<td>229</td>
</tr>
<tr>
<td>Pb3-25</td>
<td>DQ138307</td>
<td>98%, anaerobic bacterium U3P-1 (AY422344)</td>
<td>–</td>
</tr>
<tr>
<td>Pb3-30</td>
<td>DQ138308</td>
<td>95%, thermophilic bacterium (AJ242834)</td>
<td>370</td>
</tr>
<tr>
<td>Pb3-31</td>
<td>DQ138309</td>
<td>94%, uncultured DGGE type E (AY521561)</td>
<td>94</td>
</tr>
<tr>
<td>Pb3-33</td>
<td>DQ138310</td>
<td>95%, uncultured delta-proteobacterium AKYH1470 (AY922116)</td>
<td>94</td>
</tr>
</tbody>
</table>

The percent similarity of the clonal sequences to the most closely related sequences deposited in GenBank, as determined using the BLAST algorithm [1], as well as the relevant accession numbers are indicated. The predicted T-RF length for each clone, as generated by HhaI or MnlI, is also listed.

Table 2

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Accession No.</th>
<th>Similarity (%) to published sequences</th>
<th>Fe(II) prod. (mM per day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAR1</td>
<td>AF427149</td>
<td>96%, Clostridium aff. estertheticum</td>
<td>0.78</td>
</tr>
<tr>
<td>PAR2A</td>
<td>AF427150</td>
<td>96%, Aeromonas salmonicida</td>
<td>1.11</td>
</tr>
<tr>
<td>PAR2B</td>
<td>AF427151</td>
<td>99%, Aeromonas caldicaica str. 03037TRG</td>
<td>2.20</td>
</tr>
<tr>
<td>PAR3</td>
<td>AF427152</td>
<td>98%, Aeromonas salmonicida</td>
<td>1.28</td>
</tr>
<tr>
<td>PAR4</td>
<td>AF427153</td>
<td>98%, Bacillus megaterium isolate AC46b1</td>
<td>0.48</td>
</tr>
<tr>
<td>PAR5</td>
<td>AF427154</td>
<td>99%, Bacillus sp. TUT1008</td>
<td>1.02</td>
</tr>
<tr>
<td>LTR1</td>
<td>AF427155</td>
<td>94%, Clostridium hydroxybenzoicium</td>
<td>1.08</td>
</tr>
</tbody>
</table>

Fe(II) production was measured spectrophotometrically using the ferrozine method [34]. Approximately 1500 bp of the 16S rRNA gene was sequenced. The percent similarity of the isolate sequences to the most closely related sequences deposited in GenBank, as determined using the BLAST algorithm [1], as well as the relevant accession numbers are indicated.
Fig. 3. Fe(II) production by isolate PAR2A. The isolate was grown in Wolfe’s Mineral Media (WMM) with either (A) 25 mM of Fe(III)-floc or (B) 50 mM of Fe(III)-citrate as the terminal electron acceptor. Fe(II) production was measured spectrophotometrically using the ferrozine method [34]. All Fe(II) measurements were performed in duplicate and standard deviation is shown for each data point. Acetate was provided at 10 mM and yeast extract at 0.01% (vol/vol).

The T-RFLP technique, like all RFLP-based methods, is inherently biased toward underestimating the diversity of sequences in a given sample due to conservation of restriction enzyme site recognition sequences in a given gene across different organisms. And since the diversity of sequences in an environmental sample is impossible to predict accurately a priori, it is important to test a variety of restriction enzymes when attempting to assess sequence diversity. In this study, five restriction enzymes were tested.

The same five restriction enzymes were also used to predict T-RF lengths for the clonal library sequences in order to identify a specific clone with a particular T-RF peak in the fingerprints. Again, HaeIII, Sau3AI, and TaqI were found to generate similar T-RF lengths for the sequenced clones. Sau3AI has a conserved recognition site in the 16S rDNA PCR primer (27F) and therefore the predicted T-RF length for all the clones (7 bp) is identical. With the exception of one clonal sequence, TaqI was predicted to produce either a 55 bp fragment or to not cut at all. HaeIII was able to differentiate between several of the clones, but in many cases yielded T-RF lengths so similar that they would not be resolvable under the T-RFLP procedure.

The BC_sim value reached close to 0.80 for later dilutions of different origin, also indicating a trend toward increasing similarity of the enrichments (Fig. 2). Finally, Table 3 shows that 5 particular T-RFs were common to many of the final dilutions, in some cases accounting for over 85% of the total fingerprint area.

There are many diversity measurements in use today and the choice of an appropriate index can be daunting. In a previous study [58], the Sorenson index was used to examine the relatedness of fingerprints between different sites in marine sediments. While this presence–absence index is easy to calculate and interpret, it neglects the relative abundance of species. Thus, a peak which dominates a given fingerprint would have the same weight as one of considerably less area. Therefore, the Bray–Curtis similarity index was chosen. This meets several suitability criteria outlined in Magurran [38]: the value of the index is 1 for identical samples; 0 for samples which share no common T-RFs; the value is unchanged by including/excluding T-RFs which occur in neither sample; and the index reflects differences in total, rather than just relative, abundance.

The T-RFLP technique, like all RFLP-based methods, is inherently biased toward underestimating the diversity of sequences in a given sample due to conservation of restriction enzyme site recognition sequences in a given gene across different organisms. And since the diversity of sequences in an environmental sample is impossible to predict accurately a priori, it is important to test a variety of restriction enzymes when attempting to assess sequence diversity. In this study, five restriction enzymes were tested.

The same five restriction enzymes were also used to predict T-RF lengths for the clonal library sequences in order to identify a specific clone with a particular T-RF peak in the fingerprints. Again, HaeIII, Sau3AI, and TaqI were found to generate similar T-RF lengths for the sequenced clones. Sau3AI has a conserved recognition site in the 16S rDNA PCR primer (27F) and therefore the predicted T-RF length for all the clones (7 bp) is identical. With the exception of one clonal sequence, TaqI was predicted to produce either a 55 bp fragment or to not cut at all. HaeIII was able to differentiate between several of the clones, but in many cases yielded T-RF lengths so similar that they would not be resolvable under the T-RFLP procedure.

Table 3
Dominant TR-F distribution data, as generated by digestion with MnlI

<table>
<thead>
<tr>
<th>Putative identification</th>
<th>T-RF</th>
<th>P1a-3</th>
<th>P1b-3</th>
<th>P2a-4</th>
<th>P2b-4</th>
<th>P3a-3</th>
<th>P3b-3</th>
<th>P4a-4</th>
<th>P4b-4</th>
<th>DSa-3</th>
<th>DSb-3</th>
<th>SBa-4</th>
<th>SBB-4</th>
<th>TCa-3</th>
<th>TCB-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>–</td>
<td>72</td>
<td>29.7</td>
<td>14.3</td>
<td>12.9</td>
<td>2.4</td>
<td>22.9</td>
<td>–</td>
<td>5.3</td>
<td>6.6</td>
<td>–</td>
<td>1.6</td>
<td>4.2</td>
<td>–</td>
<td>3.6</td>
<td>–</td>
</tr>
<tr>
<td>Tb3-R, P1a4-9 Dsa3-33</td>
<td>168</td>
<td>2.7</td>
<td>6.1</td>
<td>26.9</td>
<td>52.1</td>
<td>8.2</td>
<td>10.7</td>
<td>19.0</td>
<td>62.7</td>
<td>14.5</td>
<td>5.5</td>
<td>11.5</td>
<td>11.7</td>
<td>10.9</td>
<td>–</td>
</tr>
<tr>
<td>Tb3-J, Tb3-BU P1a4-38</td>
<td>216</td>
<td>–</td>
<td>–</td>
<td>14.0</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>4.6</td>
<td>–</td>
<td>4.9</td>
<td>17.9</td>
<td>21.3</td>
<td>9.2</td>
<td>15.7</td>
<td>42.6</td>
</tr>
<tr>
<td>PAR4, PAR5</td>
<td>247</td>
<td>–</td>
<td>–</td>
<td>9.2</td>
<td>4.5</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>26.0</td>
<td>13.2</td>
<td>21.3</td>
<td>13.1</td>
<td>–</td>
<td>34.0</td>
<td>31.7</td>
</tr>
<tr>
<td>Tb3-AM, P1a4-20</td>
<td>343</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>26.0</td>
<td>53.5</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

Five individual TR-F’s dominated the fingerprints for the final serial dilution of each enrichment. Shown are the percentages of each TR-F for the individual enrichments. Percentages were calculated for each TR-F as (TR-F peak area/total peak area of the individual fingerprint) × 100. Also indicated is the corresponding clone or isolate for each T-RF, as determined by in silico restriction digestion analysis.
used in this study. Since MnlI produced the greatest number of resolvable T-RFs for the individual fingerprints, it was chosen as the enzyme to generate the T-RFLP data for the community analysis.

Based upon the results obtained from screening clonal libraries generated from the DSa-3, TCh-3, P1b3, and P1a-4 libraries (Donora Station, Tabernacle Church, or Par Pond enrichments, respectively), and from the isolate data, the domi-
nant peaks listed in Table 3 can potentially be identified. Clones Tb3-J, Tb3-BU, and P1a4-38 all produce a 216 bp T-RF upon in silico digestion with MnlI. Based on 16S rDNA phylogenetic analysis, Tb3-J, Tb3-BU, and P1a4-38 cluster closely with Geobacter sp. JW3 (Fig. 4). The 343 bp T-RF is identified as either Tb3-AM or P1a4-20, which both correspond to Geobacter psychrophilus str. P11.

The PAR4 and PAR5 isolates, both identified as Bacillus species, are predicted to produce a 251 bp T-RF and therefore are most likely responsible for the 247 bp T-RF. Small differences between observed and predicted T-RF lengths are fairly common, and therefore observed T-RFs within ±4 bp of the predicted T-RF length are considered acceptable [24,28]. Th3-R, P1a4-9, and Da3-33 all produce a TR-F of 169, and most likely contribute to the prevalence of T-RF 168 in the enrichment fingerprints.

No clones or isolates were found to produce in silico T-RFs corresponding to 72 bp. This is in part due to the fact this peak was not present in either the Da3 or Tb3 fingerprints. However, the T-RF of 72 bp constituted 29.7 and 14.3% of the total fingerprint area for P1a-3 and P1b-3, respectively, and therefore it is surprising that no clones from these libraries are predicted to produce T-RFs of this length. This could be due to insufficient clonal library screening or to biases introduced during the cloning process.

Clostridia and Bacillus species were identified in the SRS enrichment cultures and isolates (Tables 1 and 2). These microbes anaerobically reduce iron primarily through fermentative processes, although this reduction represents only a minor pathway for electron flow [30]. In this study, acetate was present as the main electron donor both in the enrichments and during the isolation of pure cultures, however the additional presence of 0.01% yeast extract in the medium or residual organic matter from the sediment inocula may have provided sufficient organic substrates to support fermentation. In fact, the first Fe(III)-reducing organisms to be characterized were fermentative bacteria growing under anaerobic conditions [8,44,45]. Organisms involved in this process include Escherichia coli, Clostridium pasteurianum, Lactobacillus lactis, C. sporogenes, and Bacillus polymyxa [30,31]. Many other fermentative bacteria that can reduce ferric iron have also been isolated [21,22,36].

The PAR1 and LTR1 isolates were identified, by 16S rDNA analysis, as belonging to the Clostridia, while PAR4 and PAR5 represent Bacillus species (Table 2). PAR4 was closely related to the thermophile Bacillus infernus. Curiously, a sequence closely related to B. infernus was detected in Fe(III)-reducing enrichments from sandy aquifer sediments that had been amended with humics to stimulate iron reduction [60].

While these microbes themselves may not be especially important iron reducers, their fermentation products (e.g., acetate and H₂) can provide excellent substrates for iron reduction by other organisms [30–32,42]. Indeed, several Clostridium-like DNA sequences were detected in Fe(III)-reducing enrichments from sandy aquifer sediments that had been amended with glucose [60]. Recently, ferricyanide reduction by various Geobacter species was found to be stimulated by the presence of secondary bacteria, including the fermenter Lactococcus lactis [61]. Furthermore, even if metal reduction in these organisms is not growth-related they are commonly found in enrichment cultures and their role in total Fe(III) reduction warrants further investigation [18,42].

In a study of a co-culture of Aeromonas veronii and Shewanella alga BrY, the synergism between a fermentative and an iron-reducing microbe demonstrated the potential for wider substrate utilization as well as for increasing the rate and extent of iron reduction [26]. In this study the initial iron reduction rates of PAR5 and LTR1 were comparable to those of the PAR2A, PAR2B and PAR3 isolates, indicating that interaction between different bacteria may be relevant in overall iron-reduction extent and rates for a particular site.

PAR2A, PAR2B, and PAR3 are all closely related species of the Aeromonas group and demonstrated the highest Fe(III) reduction rates of all the isolates generated in this study (Table 2). In particular, PAR2B demonstrated the highest initial reduction rates and upon subsequent transfers the Fe(III) reduction rate doubled. PAR2B consistently reduced iron floc as well as Fe(III)-citrate (Fig. 3) both in liquid media (WMM) and agar plates. In both cases, however, yeast extract was added (0.01%) to aid growth and Fe(II) production was identical between cultures provided only yeast extract and those provided with yeast extract/acetate; therefore iron reduction was most likely not coupled to acetate oxidation.

The growth and iron-reducing capabilities of these Aeromonas sp. isolates is very interesting because until recently, neither anaerobic respiration nor Fe(III) reduction had been reported for members of the Aeromonas group [25]. In a study by Knight and Blakemore (1998), the growth and Fe(III) reduction of Aeromonas hydrophila were concomitant, and no growth was observed in the absence of Fe(III). A. hydrophila showed poor growth and Fe(III) reduction on amorphous Fe(III) oxyhydroxides and no reduction with acetate as the electron donor. Another isolate, PAR3, phylogenetically related to A. hydrophila, was found to actively reduce Fe(III), but could not oxidize acetate with ferric citrate as the electron acceptor [48].

Several organic acid-oxidizing organisms have been shown to completely mineralize organic acids to carbon dioxide while reducing ferric iron. The first such organism isolated was Geobacter metallireducens GS-15 [33,35,36]. Other organic acid-oxidizing organisms include other species of the Geobacter genus [10], Desulfuromonas acetoxidans [52] which is a close relative of the Geobacter genus, Shewanella (formerly Pseudomonas) putrefaciens strain 200 [2,3,49,50], S. putrefaciens MR-1 [41,47] and S. alga BrY [11,55].

Recently, a facultatively anaerobic bacterium, Pantoea agglomerans SP1, was described that can grow by coupling the oxidation of acetate or H₂ to Fe(III) reduction [17]. This is in contrast to other facultative organisms within the genera Shewanella [31], Aeromonas [25], and Ferrimonas [56], which are unable to use acetate as an electron donor. P. agglomerans sequences were also found to be abundant in an examination of contaminated, acidic subsurface sediments [46]. Pantoea agglomerans SP1-like 16S rDNA clones were present in both the Tabernacle Church and Par Pond (Tb-3 and P1a-4) clonal libraries.
In addition, several clones were closely affiliated with various strains of *Geobacter* sp. These sequences were not found in the Donora Station (Da-3) library, which contained clones similar to common Gram-positive bacteria (e.g. *Clostridia* sp.). Since bacterial populations have been shown to exhibit a wide range of temporal and spatial variability [39,40,58,59], these differing results may reflect the inherent heterogeneity of bacterial communities. The sampling regime itself as well as site-to-site variations in sediment properties are also important factors that can influence our understanding of bacterial community composition.

As molecular studies often highlight, bacterial strains that serve as excellent models for laboratory experiments are often not abundant in situ [39]. *Geobacter* and *Shewanella* species are excellent model organisms for studying Fe(III) reduction in the laboratory, and several studies have shown that members of the Geobacteraceae are quite dominant in varied environments, including a landfill leachate-polluted aquifer [53], a petroleum-contaminated aquifer [54], sandy aquifer sediments [60], and other sedimentary environments [12]. Still, it is likely that the actual assemblage of iron-reducing organisms in the environment is more complex [29]. For instance, *Shewanella* species were not detected in the initial enrichment inocula containing 10% site sediment. Nor were they detected during the screening of 100 colonies of a clonal library based on DNA extracted from a mixture of the Lower Three Run samples (data not shown). Also, in a recent study of contaminated acidic sediments, the most abundant sequences were found to belong to *Anaeromyxobacter* sp., a recently characterized Fe(III) reducer. *Geobacter* species sequences were more abundant in the pristine site used for comparison, and *Shewanella* species were not detected at either site. This study offers convincing evidence that geochemical parameters, such as pH, can greatly influence the Fe(III) reducing community structure [46].

*Geobacter* sp. and other closely related sequences were frequently detected in our clonal libraries. However, Fig. 4 indicates that clones from a variety of other bacterial groups were important as well. The importance of challenging assumptions about the contribution of specific groups of microbes to environmental processes is also highlighted by *Pantoea agglomerans* SPI [17]. In this instance, a group of organisms which were known to grow anaerobically by fermentation were revealed to possess alternative modes of anaerobic growth, including dissimilatory Fe(III)-reduction.

The *Aeromonas* species that were isolated in this study consistently reduced Fe(III), both as Fe(III)-citrate or amorphous Fe(III) floc. Other Fe(III)-reducing *Aeromonas* isolates have been reported which indicate the importance of this group as iron reducers [25,26]. Therefore, other microbes besides *Geobacter* or *Shewanella* species may be important in Fe(III)-reduction at the SRS. Given the iron reduction potential of PAR2B, this organism will be further characterized in order to compare its iron reduction rates with those of known iron reducers.

Molecular tools such as T-RFLP analysis can reveal important information about microbes in the environment. The molecular characterization of our enrichments coupled with information obtained on our SRS isolates have aided the specific goal of obtaining information on environmentally relevant iron-reducing microorganisms from the SRS. Since both cultivation and molecular techniques are subject to biases, the use of model organisms and enrichment techniques are equally necessary in order to obtain a more realistic view of microbial processes [39,57]. Identification of the Fe(III) reducers at a given site has become even more critical given the recent revelation that different genera of Fe(III) reducers may utilize different mechanisms for Fe(III) reduction [43]. Therefore, for a model of Fe(III) reduction to be mechanistically accurate, knowledge of the site-specific Fe(III)-reducing population is necessary.

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