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Diversity of Geobacteraceae Species Inhabiting Metal-Polluted Freshwater Lake Sediments Ascertained by 16S rDNA Analyses

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ABSTRACT

The abundance, distribution, and phylogenetic diversity of members of the Fe(III)-reducing family Geobacteraceae were studied along a gradient of metal contaminants in Lake Coeur d'Alene, Idaho. Partial 16S rRNA gene fragments were amplified by PCR using primers directed toward conserved regions of the gene within the family Geobacteraceae. Analysis of amplicons separated by denaturing gradient gel electrophoresis (DGGE) suggested within-site variation was as great as between-site variation. Amplicons were cloned and grouped by RFLP type and DGGE migration distance and representatives were sequenced. Grouping clones with 3% or less sequence dissimilarity, 15 distinct phylotypes were identified compared to 16 distinct DGGE bands. Only 1 phylotype was recovered from all sites. This clone, B14, is most closely related to *Geobacter metallireducens* and constituted a greater portion of the pristine community than of the contaminated communities. A second phylotype, Q2, predominated in the contaminated communities and was notably absent from the pristine libraries. Clone Q2 presents a high degree of sequence similarity to two *Geobacter* spp. previously isolated from this region of Lake Coeur d'Alene. Six phylotypes were unique to the contaminated sediments, whereas two were found only in the pristine sediments. Indices of diversity (Shannon and Simpson) were consistently

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higher when calculated with DGGE data than when clone library data were used. Most-probable-number PCR and real-time PCR suggested that the Geobacteraceae phylotypes were spread relatively evenly across all three sites along the gradient. Our data indicate that the Geobacteraceae are diverse and abundant in Lake Coeur d'Alene sediments, regardless of metals content. These results provide insight into the ability of dissimilatory Fe(III)-reducing bacteria to colonize habitats with elevated metal concentrations, and they have important implications for the management and remediation of metal-contaminated sites.

Introduction

Dissimilatory iron-reducing bacteria (FeRB) are a phylogenetically and metabolically diverse group of microorganisms unified by their ability to couple the oxidation of organic matter and hydrogen to the reduction of oxidized metals (metals, metalloids, and radionuclides). Under appropriate conditions they can catalyze the precipitation of metals [20, 34], the dehalogenation of haloorganics [29, 47], and the mineralization of recalcitrant aromatic compounds [3]. These transformations can be mediated by specific enzymes [22, 24, 39] or may occur nonspecifically following the generation of reactive ferrous ions [5, 18, 31]. FeRB appear to be involved in both the sequestration and remobilization of Fe(III) oxide-associated trace elements such as arsenic, zinc, cobalt, and nickel [12, 13, 60]. Because of their diverse capabilities, the FeRB have been proposed as bioremedial agents for some anoxic contaminated sites [19, 36, 38].

Because of its phylogenetic conservation and apparent ubiquity in anoxic environments, we chose to focus the present investigation on the Fe(III)-reducing family Geobacteraceae in the δ subdivision of the Proteobacteria. The proposed family [35] includes the four genera *Geobacter, Pelobacter, Desulfuromonas,* and *Desulfuromusa.* In addition, 16S rDNA sequence analysis places a recently described trichloroacetic acid–dechlorinating isolate, *Trichlorobacter thiogenes,* within the family, although its ability to reduce Fe(III) is unclear [16, 51]. Data increasingly suggest that the Geobacteraceae represent an ecologically important group of FeRB. Its members have been isolated from or detected in a wide range of natural habitats including freshwater sediments [21, 37, 53], marine or estuarine sediments [6, 11, 40], and subsurface environments [4, 8, 26, 50, 62]. In addition to naturally pristine environments, members of the Geobacteraceae have also been identified in a variety of organically contaminated media [1, 3, 10, 49]. Relatively little is known, however, of their ability to colonize sediments containing elevated levels of inorganic contaminants. Information regarding their likely success in metal-contaminated environments is important if we are to make use of their proven capacity to reductively precipitate metals and radionuclides from the groundwater of contaminated sites.

The purpose of this study was to compare the diversity of Geobacteraceae spp. in metal-contaminated sediments with that of nearby pristine sediments in mining-impacted Lake Coeur d'Alene, Idaho. Our results indicate that a diverse community of Geobacteraceae spp. has successfully colonized the metal-contaminated sediments with densities similar to those observed in pristine sediments.

Methods

Sample Collection and Preparation

A steep gradient in the total concentration of metal contaminants exists between the Coeur d'Alene River delta at Harrison, ID, and the pristine St. Joe River delta to the south (Fig. 1) [25]. Both microbiological and geochemical evidence suggest that microbial Fe(III) reduction has been an important process in these sediments [14]. In August 1999, sediments were sampled in duplicate at each of three sites along a known gradient of metals (Fig. 1). Previous studies reported that these sediments are anoxic and circumneutral [14, 25]. Intact sediment cores were collected by use of a gravity coring device [43] fitted with sleeves of PVC pipe (6.35 cm × 50 cm), capped under approx. 500 mL lake water, and transported on ice to the laboratory under flowing N_2. In the laboratory, cores were extruded into an anoxic glove box having an atmosphere of N_2–CO_2–H_2 (75:15:10), where they were sectioned into 5-cm depth intervals and homogenized manually with a sterile spatula. Cores varied from 20 to 30 cm in length. All analyses described herein were performed upon subsamples of hand-homogenized sediments.

Analytical Procedures

To extract sediment pore waters, subsamples were centrifuged at 5000 *g*, 10 min, at room temperature. The supernatant was drawn off the top of the solid fraction using a syringe and filtered to remove suspended solids (pore size 0.22 μm, Fisher Scientific).

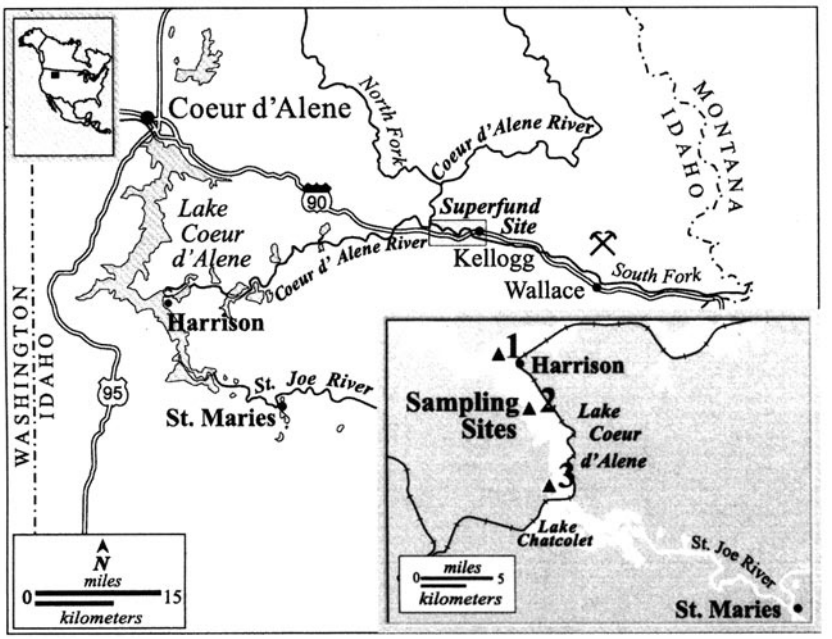

Fig. 1. Lake Coeur d'Alene and the St. Joe and Coeur d'Alene Rivers. Disposal of mine wastes into the South Fork Coeur d'Alene River has contaminated the lake with metals and other trace elements. The sediment load carried by the St. Joe River is pristine. *Inset*: Transect sampled in this study.

Filtered pore waters were diluted (1:1 or 1:9) in ultrapure water and acidified with 1 drop of concentrated nitric acid. The concentrations of dissolved Fe, Mn, As, Cd, Pb, and Zn were determined by inductively coupled plasma (ICP) spectrophotometric analysis. Very little porewater could be extracted from core 1B, making analysis of dissolved metals impossible.

To determine the concentration of dissolved Fe^{2+}, a 100-µL porewater sample was acidified in 1 mL HCl (0.5 N), and a 100-µL subsample of the acidified porewater was reacted with ferrozine reagent (1 g 3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine per liter of 50 mM HEPES buffer; pH 6.5) for 15 s [37, 54]. Absorbance of the ferrozine–Fe(II) complex was determined spectrophotometrically at $\lambda = 562$ nm and compared to standards prepared with ferrous ethylenediammonium sulfate (GFS Chemicals, Columbus, OH). To determine the concentration of weak acid-soluble Fe(II) in the sediments, 1 mL of sediment was added to 9 mL HCl (0.5 N) and incubated 1 h. One hundred µL of the acidified sediment was reacted with ferrozine reagent and quantified as described above.

The solids remaining after centrifugation were placed in a preweighed glass dish and baked for 3 days at 100°C. The glass dishes were reweighed after baking to determine the dry mass of the solids extracted. Concentrations of solid-phase metals (precipitated, coprecipitated, and adsorbed) were determined by digestion of dried sediments in concentrated hydrochloric and nitric acids, and demineralized water (in equal proportions) followed by ICP analysis (Acme Analytical Labs, Vancouver, Canada). Total sulfur concentrations were determined by Leco (Acme Analytical Labs, Vancouver, Canada).

DNA Extraction and PCR

Microbial community DNA was extracted from duplicate 0.5-g samples of homogenized sediments using the FastDNA SPIN Kit for Soil (Bio 101, Vista, CA). Extracts from all depths were pooled for each core, providing a composite DNA extract that represented the entire core length. Optimal polymerase chain reaction (PCR) conditions were determined empirically. Each 50-µL reaction contained the following (stock concentrations are in parentheses): 5 µL 10× PCR buffer (Invitrogen), 1 µL dNTPs (10 mM each) (Invitrogen), 1 µL bovine serum albumin (20 mg mL^{-1}) (Roche Diagnostics Corp.), 2 µL $MgCl_2$ (50 mM) (Invitrogen), 2 µL each forward and reverse primer (12.5 µM) (Invitrogen), 0.25 µL *Taq* DNA polymerase (5 U μL^{-1}) (Invitrogen), and 35.75 µL HPLC-grade water (Aldrich). Primers Geo564F (5′-AAG CGT TGT TCG GAW TTA T-3′) [9] and Geo840R (5′-GGC ACT GCA GGG GTC AAT A-3′) (employed for the first time in this study), corresponding to approximate positions 564 and 840 of the 16S rRNA gene (*E. coli* numbering), respectively, were used to target the 16S rRNA genes of Geobacteraceae spp. A GC-rich 40-mer (5′-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC G-3′) was added to the 5′ end of Geo840R for DGGE (see below). One µL of extracted DNA (approx. 50 ng) was used as template. PCR reactions were performed with the Mastercycler Gradient thermal cycler (Eppendorf). Reactions were initially incubated at 94°C (4 min) to denature all of the DNA, followed by 35 cycles of 94°C (30 s) (template denaturation), touchdown from 65 to 55°C in 0.5°C increments over 20 steps (30 s) (primer annealing), and 72°C (30 s) (extension). Reactions were finished with an extra 72°C extension (3 min).

DGGE

Denaturing gradient gel electrophoresis (DGGE) was performed with the D Code System (Bio-Rad) at a temperature of 60°C, a constant voltage of 65 V, for 15 h in TAE (40 mM Tris-acetate, 2 mM disodium EDTA). Acrylamide (38:2 acrylamide:*N,N′*-methylenebisacrylamide, 7.5% wt/vol) was amended with the DNA

denaturants urea and formamide (100% denaturants defined as 7 M urea and 40% formamide). Optimal gradients and running time were determined empirically. A gradient of denaturants from 40 to 60% was chosen for all DGGE analyses reported herein. After electrophoresis, gels were stained for 15 min in 1 L deionized water with 100 μg ethidium bromide, and destained in deionized water another 15 min. Stained gels were transilluminated, photographed, and analyzed using an AlphaImager System and AlphaEase software (Alpha Innotech, San Leandro, CA). Those bands that could be identified by eye were scored according to migration position and intensity.

Cloning and Screening 16S rDNA Amplicons

16S rRNA gene fragments amplified from each of the six cores were cloned into the pGem-T Easy Vector (Promega) according to the manufacturer's protocol. *E. coli* JM109 cells were transformed with the ligated vector and spread onto Luria-Bertani agar plates with ampicillin (sodium salt, 100 μg mL^{-1}) and IPTG/X-gal on the surface for standard blue/white screening.

Fifty white colonies from each of the six libraries were used directly in whole-cell PCR. Whenever possible, if no PCR product was obtained, additional colonies were picked in order to have approximately 50 colonies from each core. In the end, between 46 and 52 clones were examined from each core. The whole-cell PCR mix was the same as that described above with two exceptions: cells from a single transformed *E. coli* colony were used as the template in place of extracted DNA, and primers M13F (5′-GTA AAA CGA CGG CCA G-3′) and M13R (5′-CAG GAA ACA GCT ATG AC-3′), flanking the insertion site on the vector, were used to reamplify the insert. Whole-cell PCR reactions were cycled through the following protocol: 99°C (15 min) in buffer and water alone to lyse the cells, 80°C (10 min) during which time the remaining reaction components were added, followed by 25 cycles of 94°C (1 min), 50°C (1 min), and 72°C (1 min), and a final extension step at 72°C (1 min). Reamplified inserts were digested with the restriction endonucleases *Msp*I and *Hin*P1I (New England BioLabs) in NEB2 buffer (37°C, 5 h) for restriction fragment length polymorphism (RFLP) analysis, and resolved in a 3% agarose gel (NuSieve agarose). Clones with like RFLP patterns were grouped and subjected to DGGE analysis. GC-clamped PCR products for DGGE were generated by reamplifying the M13 PCR products used in the RFLP analysis (25 cycles), and separated by DGGE as described above. M13 PCR products of clones with unique DGGE migration positions were purified using the Wizard PCR Preps DNA Purification System (Promega) and sequenced.

Sequencing and Phylogenetic Analysis of Unique 16S rDNA Amplicons

PCR products (approx. 100 ng) were sequenced using primers M13F and Geo840R (no GC clamp) for 2× coverage. Sequencing reactions employed the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) and Model 3700 Automated DNA Sequencer (Applied Biosystems). Sequences were initially aligned against known sequences (GenBank database) using the BLAST tool [2] provided by the National Center for Biotechnology Information prior to phylogenetic analyses. The Ribosomal Database Project (RDP) [41] Chimera Check program and secondary structure determination were used to check the partial 16S rRNA gene sequences for potential chimeric artifacts [28, 32]. Using our alignments as a guide, sequences were mapped onto the secondary structure of a related organism (in this case, the secondary structure model of *Desulfovibrio desulfuricans* found at http://www.rna.icmb.utexas.edu/) [7]. Sequences were analyzed using BLAST and Similarity Matrix (RDP) in order to find the most similar available database sequences. Sequences were then manually aligned with closely related 16S rRNA gene sequences from RDP and GenBank using the graphical user interface SeqLab (Wisconsin Package version 10; Genetics Computer Group [GCG], Madison, WI). Only those sequence regions that could be aligned with confidence were included in the analyses, and gaps were treated as missing nucleotides. Phylogenetic trees were inferred from unambiguously aligned sequence data using the distance, maximum-likelihood, and maximum-parsimony tools of PAUP* [56].

Distance and maximum likelihood analyses were performed using heuristic tree searching via simple stepwise addition with tree bisection reconnection rearrangement. Unweighted parsimony analysis used the branch-and-bound algorithm. The distance tools (neighbor-joining of Kimura distances) of TREECON for Windows 1.3b [58] were also used. Three different methods of phylogenetic tree construction were used with the same data set in order to test the robustness of the generated tree topology. One thousand bootstrap replicates were performed on the data set. Phylogenetic trees inferred using the two different software packages described above showed the same topologies.

Distinct sequences have been deposited in the GenBank, EMBL, and DDBJ databases; accession numbers are reported in Table 5.

Diversity Indices

As a quantitative means of comparing the resulting communities described by DGGE and clone data, both Shannon and Simpson indices of diversity were calculated using the paleoecology statistical software PAST v. 0.98 [23]. For both calculations, band intensity (DGGE) and clone frequency (clone libraries) were used to estimate abundance. Indices calculated for duplicate DGGE profiles were averaged. Richness was calculated as the number of unique bands (DGGE) or clones (clone libraries) identified.

Quantitative PCR Methods

Geobacteraceae cell densities were estimated by 3-tube most probable number (MPN)-PCR [46]. Genomic DNA was serially diluted in 10-fold steps, and a 3-μL subsample was used as template in triplicate PCR reactions. PCR conditions were as described above.

Table 1. Total concentrations of select elements in the Lake Coeur d'Alene sediments examined in this study

	Site 1		Site 2		Site 3	
Element (mg kg^{-1})[a]	Core 1A	Core 1B	Core 2A	Core 2B	Core 3A	Core 3B
Pb	18,108 ± 3,420	1,905 ± 851	5,956 ± 3,149	3,718 ± 1,915	36 ± 19	31 ± 12
Zn	14,470 ± 4,157	4,239 ± 1,225	4,212 ± 1,257	3,947 ± 497	140 ± 106	153 ± 85
Cd	101 ± 32	26.8 ± 8.3	34.2 ± 12.4	46.8 ± 9.2	1.3 ± 0.8	1.2 ± 0.8
As	70 ± 16	69 ± 21	62 ± 31	97 ± 21	7 ± 3	8 ± 3
Mn	10,860 ± 1,434	8,000 ± 1,425	5,061 ± 1,932	5,353 ± 927	257 ± 112	281 ± 81
Fe	104,000 ± 10,100	81,000 ± 15,800	64,600 ± 11,000	61,200 ± 9,240	23,000 ± 6,500	25,100 ± 3,980
Fe(II)[b]	9,710 ± 1,270	2,220 ± 125	8,290 ± 3,120	8,310 ± 2,370	1,850 ± 420	1,900 ± 546
S	13,300 ± 3,500	4,700 ± 1,000	2,500 ± 800	2,300 ± 600	960 ± 450	790 ± 210

[a] Mean ± 1 standard deviation; data from 5-cm depth intervals of each core were pooled, $n = 3$ to 6.
[b] 0.5 N HCl-soluble Fe(II).

In addition to MPN-PCR, *Geobacter* spp. 16S rRNA genes were enumerated in each core using real-time quantitative PCR (TaqMan). Primers specific to the genus *Geobacter* 561F (5′-GCG TGT AGG CGG TTT CTT AA-3′) and 825R (5′-TAC CCG CRA CAC CTA GTT CT-3′), along with TaqMan probe Gbc1 (5′-FAM-CAC TTC CTG GGT TGA GCC CAG-TAMRA-3′), specific for *Geobacter* spp. and close relatives [55], were used in a Ruggedized Advanced Pathogen Identification Device (R.A.P.I.D., Idaho Technology) for amplification and real-time quantification. The LightCycler-FastStart DNA Master Hybridization Probes kit (Roche Diagnostics Corp.), optimized for use with glass capillaries, was used as the master mix base for the reactions. Capillaries were heated at 95°C for 10 min (hot start) followed by an amplification cycling regime consisting of 50 cycles at 95°C for 15 s and 55°C for 60 s. Reagent concentrations were as follows: 5.0 mM $MgCl_2$; 300 nM each primer; and 100 nM probe. Fluorescence was measured in Ch 1 with a gain of 32. Genomic DNA from strain CdA-2, a *Geobacter* sp. previously isolated from the contaminated area of Lake Coeur d'Alene [14], was quantified spectrophotometrically and used to generate the standard curve. The lower detection limit for the standard control was 7.2 fg of genomic DNA. The number of 16S rDNA copies in the strain CdA-2 genome is not known, nor could we estimate the average number of copies per genome across the genus *Geobacter*. Therefore, TaqMan results are reported as the mass of *Geobacter*-derived genomic DNA per g sediment rather than target gene copy numbers. Representative TaqMan reaction mixtures were removed from the capillaries and products separated by agarose gel electrophoresis. In each case, a discrete PCR product of the expected molecular weight was observed with minimal nonspecific products found in select samples (data not shown).

Results

Gradient of Metal Contamination between the Coeur d'Alene and St. Joe Rivers

A large fraction of the metals load that is carried by the river system is deposited in the Coeur d'Alene River delta at Harrison, which is now the most contaminated region of the lake [27, 59]. To confirm the presence of a gradient of metal contaminants along the transect sampled in this study, total and dissolved metal concentrations were measured. Core 1A consisted of fine-grained, densely packed clay (determined visually), much like most sediments in the Coeur d'Alene River delta that are out of the main channel (unpublished observations). Core 1B, however, consisted primarily of sandy clay and contained lower concentrations of total metals than core 1A (Table 1). Since previous observations by our laboratory [14, 25] and others [27] have shown this region to be most heavily contaminated, with metals concentrations generally being similar to those measured in core 1A, we considered core 1B to be an atypical representative from this section of the Coeur d'Alene River delta.

Generally, mean total metals concentrations decreased with distance from the Coeur d'Alene River delta (Table 1). Mean total Pb concentrations, for example, decreased from approximately 18,000 mg kg^{-1} at site 1 (core 1A) to approximately 30 mg kg^{-1} at site 3, a 600-fold decrease over the 15-km transect (Table 1). Likewise, dissolved Pb decreased from approximately 300 μg L^{-1} at site 1 (core 1A) to as low as 57 μg L^{-1} at site 3, a >5-fold decrease (Table 2). Total S also decreased approximately 17-fold along the transect (Table 1), indicative of a decrease in pyritic ore materials, the ultimate source of metals in the lake. It should be noted, however, that sediment texture may play a role in metals distribution, as suggested by the low metals concentrations in the unusually sandy core 1B. A systematic, quantitative examination of sediment texture, metals content, and microbial diversity would shed further light on this subject, but was beyond the scope of the present study.

Total concentrations of Pb, Zn, Cd, Mn, and Fe all decreased in the order site 1 > site 2 > site 3, whereas total As

Table 2. Concentrations of select elements in porewaters of the Lake Coeur d'Alene sediments examined in this study

Element (mg kg^{-1})[a]	Site 1		Site 2		Site 3	
	Core 1A	Core 1B	Core 2A	Core 2B	Core 3A	Core 3B
Pb	0.295 ± 0.145	NA	0.102 ± 0.067	0.060 ± 0.035	0.057 ± 0.061	0.125 ± 0.130
Zn	1.48 ± 0.780	NA	0.422 ± 0.274	0.246 ± 0.241	0.066 ± 0.067	0.110 ± 0.133
Cd	0.003 ± 0.003	NA	BDL	BDL	BDL	BDL
As	0.465 ± 0.237	NA	0.635 ± 0.099	0.647 ± 0.344	0.093 ± 0.050	0.040 ± 0.040
Mn	1.06 ± 0.37	NA	11.2 ± 2.86	13.5 ± 0.8	1.04 ± 0.36	0.994 ± 0.344
Fe[b]	34.7 ± 12.53	NA	36.4 ± 6.44	24.7 ± 7.1	23.9 ± 3.4	18.4 ± 7.1
Fe^{2+}	29.5 ± 10.1	NA	32.9 ± 8.6	21.1 ± 7.6	18.0 ± 3.6	15.3 ± 6.1

NA, not available; BDL, below detection limit (i.e., < 1 μg L^{-1}).
[a] Mean ± 1 standard deviation; data from 5-cm depth intervals of each core were pooled, n = 3 to 6.
[b] Unspeciated soluble Fe.

concentrations were roughly equivalent at sites 1 and 2 but lowest at site 3 (Table 1). Aqueous-phase Pb, Zn, and Cd, elements that are relatively insensitive to redox changes, followed this same trend (Table 2). By contrast, aqueous-phase concentrations of the redox-active elements As, Fe, and Mn were either equivalent at sites 1 and 2 (Fe), or maximal at site 2 (As, Mn). The observation that trends in aqueous-phase redox-active metal concentrations are not perfectly concordant with solid-phase values may reflect microbial transformations of differentially soluble species.

To infer microbial Fe(III)-reducing activity [30], we examined geochemical profiles of total Fe, weak and strong acid-soluble Fe(II), dissolved Fe^{2+}, and total dissolved Fe (unspeciated). Concordant with a decrease in total Fe from approximately 100,000 mg kg^{-1} at site 1 to 23,000 mg kg^{-1} at site 3 (Table 1), dissolved Fe^{2+} decreased from approximately 30 mg L^{-1} to 15 mg L^{-1} (Table 2) and accounted for most of the dissolved Fe (unspeciated). The differences between dissolved Fe (unspeciated) and dissolved Fe^{2+} may be due to local differences in the abundance of organically bound Fe^{3+} [57], or possibly to differences in the two methods used to measure the dissolved Fe. Whereas the absolute mass of weak acid-soluble Fe(II) decreased from site 1 to site 3, the proportion of total Fe consisting of weak acid-soluble Fe(II) was similar at sites 1 and 3 (between 7.6 and 9.3%) and maximal at site 2 (approx. 13%).

Community PCR-DGGE

Migration of PCR amplicons in a denaturing gradient gel is a function of both fragment length and nucleic acid sequence [33] and enables separation of equal-length fragments that differ in sequence. When used in combination with 16S rDNA-targeted PCR, the number of DGGE bands resolved reflects the number of distinct 16S rDNA genes amplified from the DNA extract. To the extent that each ribotype is phylogenetically distinct, application of these methods enables researchers to estimate the number of individual species present in the original sample [44]. Sequence data indicated that our PCR primers amplified not only the Geobacteraceae, but some other closely related δ-Proteobacteria as well (see below). Therefore, the DGGE profiles presented here represent a community defined not by the Geobacteraceae alone, but rather by the scope of the targets of primers Geo564F and Geo840R, which includes the Geobacteraceae.

Amplification of sediment DNA with primers Geo564F and Geo840R resulted in PCR products from each core along the metals gradient (Fig. 2). A total of 16 different bands were identified. Jaccard similarity coefficients (r) were calculated with unweighted data (binary data representing migration position only) to compare community fingerprints (Table 3). DGGE profiles from duplicate extractions and amplifications were identical in almost every case, with the exception of the duplicates from core 2B (r = 0.933). Duplicate cores from each site showed only moderate similarities (0.400 < r < 0.533), indicating high within-site variance. The most similar cores were 1A and 2A (r = 0.769), and the least similar cores were 2B and 3B (r = 0.167). When the weighted data (continuous data representing both migration position and band intensities) were considered, the same general observations could be made (data not shown).

Distribution and Diversity of Geobacteraceae Phylotypes

Despite relatively stringent touchdown thermal cycling conditions, 16S rRNA genes from Geobacteraceae spp. were not the only templates amplified (Table 4). Also

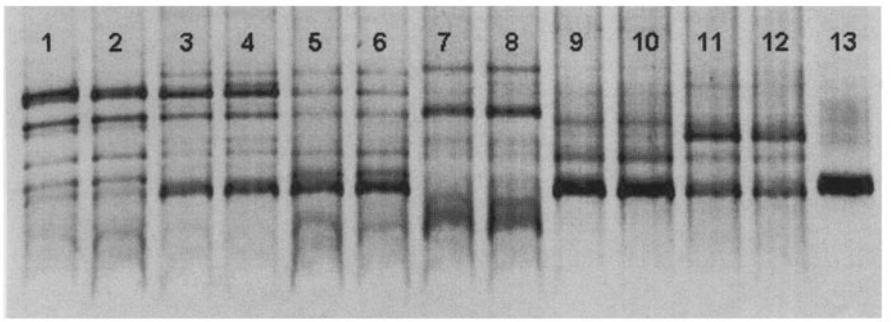

Fig. 2. DGGE profiles of 16S rDNA fragments amplified from duplicate DNA extracts from Lake Coeur d'Alene sediments. Lanes 1 and 2: core 1A. Lanes 3 and 4: core 1B. Lanes 5 and 6: core 2A. Lanes 7 and 8: core 2B. Lanes 9 and 10: core 3A. Lanes 11 and 12: core 3B. Lane 13: *Geobacter* sp. strain CdA-2. Electrophoresis was performed at 60°C, 65 V, for 15 h, 40–60% gradient of denaturants.

amplified were the genera *Syntrophus, Desulfomonile,* and *Anaeromyxobacter,* all members of the δ-Proteobacteria. When compared against the 16S rRNA gene sequences in GenBank from 4 *Syntrophus* strains, 3 *Desulfomonile* strains, and 5 *Anaeromyxobacter* strains, the primers used in this study had very few mismatches (0–2 of 19 nucleotides per primer). Compared to the Geobacteraceae, primer Geo564F has only one mismatch with *G. pelophilus* at position 1 of the primer, and no other mismatches with the 16S rRNA gene sequences of the other reference strains shown in Fig. 3. Geo840R has four mismatches with the 16S rRNA genes of members of the genus *Desulfuromusa,* one mismatch with members of the genus *Pelobacter,* and no mismatches with any of the other reference strains.

Of 295 clones examined across all three sites, 148 were from Geobacteraceae spp., 116 were from closely related δ-Proteobacteria, and 31 clones did not produce high quality sequence data and so were disregarded. Between 50% and 75% of the clones from a given core were most closely related to Geobacteraceae spp. with the exception of core 2B, which produced the lowest frequency of Geobacteraceae clones (15.5%); core 1B produced the greatest (75%). Curiously, Core 1A, the most contaminated core, produced the greatest number of clones that we were unable to sequence.

Marchesi and co-workers [42] have suggested reporting what they refer to as "coverage" of a clone library, the percent of clones that are at least duplicated in the library, as an indicator of the adequacy of the number of clones examined. Applying this concept, our six libraries ranged in coverage from 82% to 93%, with total library coverage of 96% for the entire 295 clones.

Speksnijder and co-workers [52] discovered that as much as 3% sequence error can be introduced during the PCR, cloning, and sequencing of closely related phylotypes. The authors found that the frequency of aberrant clones increased with an increasing number of targets, and much of the error was attributable to chimera and heteroduplex formation during amplification. To allow for the occurrence of procedural artifacts, then, we clustered those clones with less than 3% sequence dissimilarity,

Table 3. Jaccard similarity matrix comparing each of the 12 DGGE profiles in Fig. 2 to one another[a]

Core	1A (1)	1A (2)	1B (3)	1B (4)	2A (5)	2A (6)	2B (7)	2B (8)	3A (9)	3A (10)	3B (11)	3B (12)
1A (1)	1.00											
1A (2)	1.00	1.00										
1B (3)	0.533	0.533	1.00									
1B (4)	0.533	0.533	1.00	1.00								
2A (5)	0.769	0.769	0.750	0.750	1.00							
2A (6)	0.769	0.769	0.750	0.750	1.00	1.00						
2B (7)	0.308	0.308	0.375	0.375	0.429	0.429	1.00					
2B (8)	0.286	0.286	0.471	0.471	0.400	0.400	0.933	1.00				
3A (9)	0.667	0.667	0.333	0.333	0.400	0.400	0.200	0.182	1.00			
3A (10)	0.667	0.667	0.333	0.333	0.400	0.400	0.200	0.182	1.00	1.00		
3B (11)	0.364	0.364	0.571	0.571	0.500	0.500	0.167	0.308	0.500	0.500	1.00	
3B (12)	0.364	0.364	0.571	0.571	0.500	0.500	0.167	0.308	0.500	0.500	1.00	1.00

[a] The number in parentheses represents the lane in Fig. 2 that is being compared.

Table 4. Disposition of 295 clones generated with primers Geo564F and Geo840R-GC

Clone identity	Frequency by core						Total
	1A	1B	2A	2B	3A	3B	
Geobacteraceae spp.	24	33	30	7	30	24	148
Other δ-Proteobacteria	14	11	12	38	17	24	116
Unable to sequence	8	7	7	3	2	4	31
Totals	46	51	49	48	49	52	295

considering them indistinguishable from one another. Applying this minimum threshold for sequence dissimilarity, we recovered 15 unique Geobacteraceae clones (Table 5, Fig. 3). Table 5 reports the distribution of these clones within the 6 cores examined. All of the clones reported in Table 5, with the exception of clone B14, clustered unambiguously into groups with 3% or less sequence dissimilarity; clone B14 required the stringency of the threshold to be relaxed to 4% in order to account for all of the closely related clones. Clone B14 was the only clone identified in all 6 cores, and is most similar to *Geobacter metallireducens* (Fig. 3). In addition, it was the most abundant clone found in the sediments, accounting for 45% of the total Geobacteraceae clones, and 70% and 67% of those from cores 3A and 3B, respectively. By contrast, clone B14 only comprised 17% of the library from core 1A, the most contaminated core in the study.

Clone Q2 was the next most frequently encountered clone, accounting for 27% of the total Geobacteraceae clones, and was found only in cores from sites 1 and 2. Unlike clone B14, Q2 dominated the library from core 1A (75%) and was undetectable in cores from site 3. Remarkably, a search of the GenBank database revealed that clone Q2 was most closely related to two strains previously isolated from this region of the lake, strains CdA-2 and CdA-3 [14]. This suggests that these two isolates may represent predominant metal reducers in the contaminated region of the lake, warranting further study of their physiology.

Two clones, A276 and C75, were only encountered in core 1A. Core 1B, the low-metals, unusually sandy core from the contaminated area, produced four clones that were unique to that core, clones B13, C101, C102, and O175. Two abundant clones, C109 and C130, were restricted in their distribution to sites 2 and 3, or just to site 3, respectively; neither was encountered in the site 1 cores. Clone B54 was also restricted to site 3.

Even though none of the six communities was completely distinct from the others in composition, core 1A and cores from site 3 shared only one phylotype (clone B14). This distinction was not evident in the comparison of DGGE profiles from the two sites (Table 3). Core 1B, the sandy core with metals concentrations similar to those at site 2, also shared a single clone with site 3; in fact, clone S246 was found in at least one core from each of the three sites. Perhaps reflecting similarity in metals chemistry, core 1B shared four of its nine phylotypes with site 2. Six clones were identified that were unique to site 1, and two were unique to site 3. Clone C109 was distributed across the four cores from sites 2 and 3, but was not found at site 1.

Figure 3 illustrates the phylogenetic diversity of the 15 clones with respect to previously described Geobacteraceae spp. All clones but one belonged to the freshwater *Geobacter* cluster [35]. Clone C75, found only in core 1A, was the only phylotype identified that appears to belong to the marine *Desulfuromonas* cluster [35]. Clones C102 and C119 form a relatively deeply branching cluster with previously described clones BVB33 and BVB66 [48] and no cultured representatives. Clones C109, C130, and C112, most closely related to *Geobacter hydrogenophilus*, also formed a distinct cluster with no immediate cultured relatives. Three clones, B24, B54, and S247, clustered with the recently described *Trichlorobacter thiogenes*. The appropriate name for this organism is disputed and its role in metal reduction is unclear [16, 51].

Indices of Diversity

Three diversity indices were calculated using weighted DGGE data or clone data (Table 6). The two approaches were compared using Wilcoxon's paired-sample rank sum test [61]. Richness varied from 3 to 9 phylotypes per core based on DGGE, and 4 to 9 based on clone data; there was no statistically significant difference between the richness data obtained by the two methods. DGGE produced a total of 16 identifiable bands, while 15 unique clones were identified. The two methods did, however, produce significantly different Shannon and Simpson indices. Both indices were consistently higher using weighted DGGE

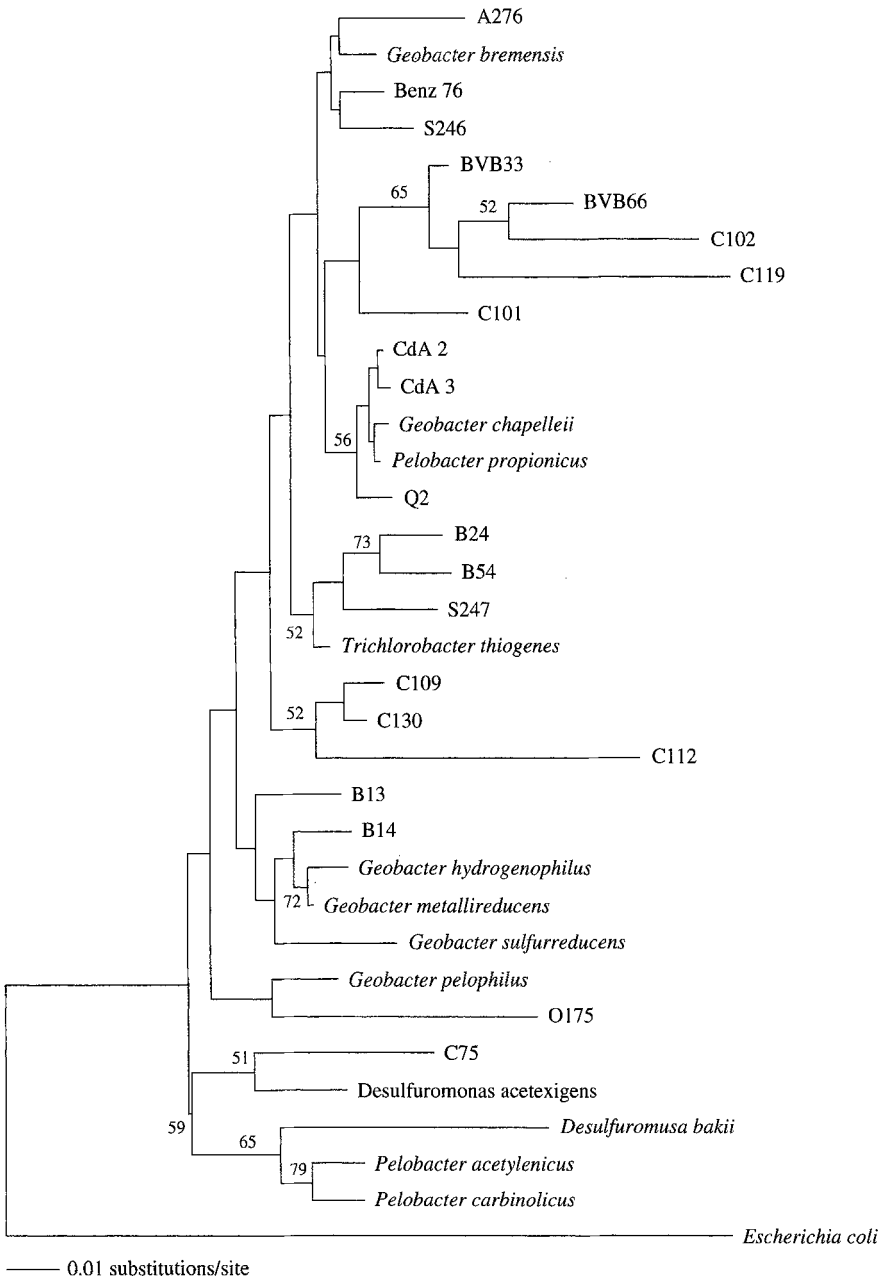

Fig. 3. The most parsimonious tree inferred from an heuristic search using maximum parsimony showing the relationships of Coeur d'Alene clones to other members of the family Geobacteraceae inferred from partial 16S rDNA sequences; 275 base positions were considered.

data than when they were calculated using clone data. When calculated from DGGE data, the Shannon index varied from 1.079 to 2.110 and the Simpson index varied from 0.6533 to 0.8679. Clone data produced Shannon indices ranging from 0.7792 to 1.536 and Simpson indices from 0.4063 to 0.7347.

Quantitative Estimates of Geobacteraceae Densities

A 3-tube MPN-PCR was performed on DNA extracts. The estimated number of genomes targeted by the primers Geo564F and Geo840R, which includes the Geobacteraceae and a few closely related genera (see previous section), varied from 1.42×10^5 g^{-1} (wet weight) at site 1 (core 1B) to 3.08×10^6 g^{-1} at site 3 (core 3B) (Fig. 4). According to real-time PCR, the concentration of genomic DNA from *Geobacter* spp. ranged from $2.96 \times 10^6 \pm 6.05 \times 10^5$ fg (core 1A) to $9.33 \times 10^7 \pm 1.69 \times 10^7$ fg (core 3A) g^{-1} sediment (Fig. 4). Considerable within-site variance resulted in no significant difference in real-time PCR-determined *Geobacter* DNA concentration among the three sites (ANOVA $P = 0.51$).

Discussion

Dissimilatory Fe(III)-reducing bacteria have been studied in a wide range of environments, yet information on their

Table 5. Frequency and distribution of Geobacteraceae clones from Lake Coeur d'Alene

Clone ID	GenBank Accession Number	Frequency by core						Total
		1A	1B	2A	2B	3A	3B	
B14	AY157585	4	11	13	2	21	16	67
Q2	AY157586	18	14	7	1			40
A276	AY157587	1						1
C75	AY157588	1						1
S246	AY157589		2	1	2		1	6
S247	AY157590		1	1				2
B24	AY157591		1				2	3
B13	AY157592		1					1
C101	AY157593		1					1
C102	AY157594		1					1
O175	AY157595		1					1
C109	AY157596			6	2	1	1	10
C112	AY157597			2				2
C130	AY157598					7	3	10
B54	AY157599					1	1	2
Totals		24	33	30	7	30	24	148

ecology in metal-polluted habitats is notably lacking. In this study we examined the abundance, distribution, and phylogenetic diversity of the Fe(III)-reducing family Geobacteraceae along a gradient of sedimentary metal contaminants in Lake Coeur d'Alene, Idaho. Our results indicate that Geobacteraceae spp. have successfully colonized the most polluted sediments in the lake, which contain as much as 2% lead and greater than 10% iron, in addition to elevated concentrations of dissolved metals in the porewaters. While some clones were shared among different sites along the gradient, others were unique to a given site. The number of distinct phylotypes identified at each site did not appear to vary in a predictable manner. Quantitative PCR methods suggested that nearly equal concentrations of genomic DNA from Geobacteraceae spp. were present at all three sites.

While clone S246 was indeed present at all three sites, only clone B14 was found in all six cores. Many clones were unique to a given core or site. This distribution of Geobacteraceae phylotypes along the gradient of metals suggests that some species may be able to colonize either medium (contaminated or pristine), while others may be adapted to the metals content of the sediments in which they reside. These observations must be interpreted with caution, however, as many uncontrolled variables may also influence their distribution, such as inoculum source (i.e., the Coeur d'Alene River vs the St. Joe River), organic content, local heterogeneities at each site, etc.

Two modes of quantitative analysis indicated that Geobacteraceae have been able to colonize both contaminated and pristine sediments. The MPN-PCR estimates of 1.42×10^6 and 1.42×10^5 target genomes g^{-1} in cores 1A and 1B, respectively (Fig. 4), were very similar to the enrichment-based MPN estimates of 5.4×10^3 to 4.8×10^6 acetate-oxidizing Fe(III)-reducing bacteria previously reported [14]. Although these analyses (MPN PCR and enrichment-based MPN) were not performed on exactly the same cores at exactly the same time, their comparison suggests that Geobacteraceae spp. may constitute a large proportion of the microorganisms in these sediments capable of coupling acetate oxidation to dissimilatory Fe(III) reduction.

Table 6. Diversity indices based on either weighted DGGE data or 16S rDNA clone libraries

Core	DGGE			Clones		
	Richness	Shannon	Simpson	Richness	Shannon	Simpson
1A	6	1.722	0.8083	4	0.7792	0.4063
1B	9	2.110	0.8679	9	1.536	0.6997
2A	7	1.889	0.8393	6	1.431	0.7111
2B	7.5	1.957	0.8503	4	1.352	0.7347
3A	3	1.079	0.6533	4	0.8160	0.4533
3B	5	1.536	0.7702	6	1.135	0.5278

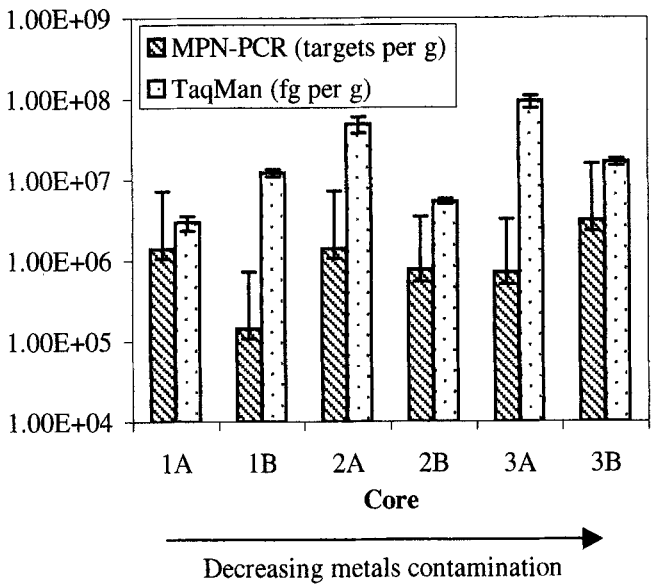

Fig. 4. Quantitative estimates of Geobacteraceae cell densities in Lake Coeur d'Alene sediments. Error bars represent 95% confidence limits for MPN-PCR data and ± 1 standard deviation (n = 3) for TaqMan data. Note: Units are different for the two methods.

Few studies have examined native metal-reducing populations found in metal-rich soils. In one of the few studies of its kind, Stein et al. [53] reported on bacterial and archaeal populations associated with Fe and Mn nodules in freshwater lake sediments. Although these communities were not compared to low-metal sediments, the results were instructive: the metal-rich sediments were populated by Mn-oxidizers and metal-reducing Geobacteraceae spp., among others. In fact, 16S rRNA genes from Geobacteraceae spp. comprised the largest group of bacterial clones from the nodules [53].

Lake Coeur d'Alene, Idaho provides a unique environment in which to study microbe-metal interactions. An increasing body of information on both the geochemistry [14, 17, 24, 25, 27, 59] and microbiology [14, 15, 24, 45] of the bottom sediments continues to amass. The high Fe, low redox character of the sediments [13, 25] provides a suitable habitat for FeRB [14], and previous work has established that microbial Fe(III) reduction is a prominent feature of their geochemistry [14]. Our group has described four novel Bacteria isolated from Lake Coeur d'Alene capable of respiratory Fe(III) reduction [14, 15, 45], two of which belong to the family Geobacteraceae. In combination with these two *Geobacter* isolates [14], the findings reported in this study represent the first evidence that FeRB, and the Geobacteraceae in particular, can successfully inhabit metal-polluted environments.

The implications of these findings extend beyond the Lake Coeur d'Alene ecosystem. The biological reduction of Fe(III) and other metals holds great promise for the remediation of metal- and radionuclide-contaminated environments [19, 36, 38]. Until now, however, the ability of metal-reducing bacteria to successfully inhabit sediments with elevated metals concentrations was largely unknown. These results suggest that the Geobacteraceae may have a role in the anaerobic activities of numerous metal-polluted sites across the nation and around the world.

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