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Evidence for Microbial Fe(III) Reduction in Anoxic, Mining-Impacted Lake Sediments (Lake Coeur d'Alene, Idaho)

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Mining-impacted sediments of Lake Coeur d'Alene, Idaho, contain more than 10% metals on a dry weight basis, approximately 80% of which is iron. Since iron (hydr)oxides adsorb toxic, ore-associated elements, such as arsenic, iron (hydr)oxide reduction may in part control the mobility and bioavailability of these elements. Geochemical and microbiological data were collected to examine the ecological role of dissimilatory Fe(III)-reducing bacteria in this habitat. The concentration of mild-acid-extractable Fe(II) increased with sediment depth up to 50 g kg^{-1}, suggesting that iron reduction has occurred recently. The maximum concentrations of dissolved Fe(II) in interstitial water (41 mg $liter^{-1}$) occurred 10 to 15 cm beneath the sediment-water interface, suggesting that sulfidogenesis may not be the predominant terminal electron-accepting process in this environment and that dissolved Fe(II) arises from biological reductive dissolution of iron (hydr)oxides. The concentration of sedimentary magnetite (Fe_3O_4), a common product of bacterial Fe(III) hydroxide reduction, was as much as 15.5 g kg^{-1}. Most-probable-number enrichment cultures revealed that the mean density of Fe(III)-reducing bacteria was 8.3×10^5 cells g (dry weight) of $sediment^{-1}$. Two new strains of dissimilatory Fe(III)-reducing bacteria were isolated from surface sediments. Collectively, the results of this study support the hypothesis that dissimilatory reduction of iron has been and continues to be an important biogeochemical process in the environment examined.

Nearly a century of sulfidic ore mining in the Coeur d'Alene River (CDAR) watershed in northern Idaho (Fig. 1) has resulted in enrichment of Lake Coeur d'Alene sediments with iron and trace elements, such as lead, zinc, and arsenic (19, 27). The mean concentrations of these elements in sediments of the CDAR delta and the region of the lake immediately surrounding the delta are 82,486 mg of Fe kg^{-1} 3,820 mg of Pb kg^{-1} 2,995 mg of Zn kg^{-1}, and 201 mg of As kg^{-1} (24). Previous research has revealed that there is a pattern of arsenic and iron distribution in the CDAR delta sediments, suggesting that some elements have undergone postdepositional mobilization (24). The sediments also support bacterial communities whose concentrations range from 10^4 to 10^8 cells g (wet weight) of $sediment^{-1}$ (mean, ca. 10^7 cells g [weight weight] of $sediment^{-1}$) (15, 24). Such observations underscore the need to understand interactions between the resident microflora and the metal contaminants in this unique environment.

In the absence of molecular oxygen, many bacteria can respire alternative electron acceptors, such as nitrate, manganese and iron oxides, and sulfate. It has been shown that microbial Fe(III) reduction [i.e., respiration of Fe(III) oxides] alters the geochemistry of submerged soils and sediments, as well as the geochemistry of both surface water and subsurface water (10, 22, 29, 32, 33, 44). Typically, fermentation end products, such as acetate and molecular hydrogen, are oxidized as dissimilatory iron-reducing bacteria (DIRB) concomitantly reduce Fe(III) to Fe(II) (32). Theoretical thermodynamic considerations indicate that oxidation of organic compounds with soluble Fe(III) as the terminal electron acceptor should yield more energy than oxidation of compounds using either SO_4^{2-} or CO_2 as terminal electron acceptor. Accordingly, microcosm studies have shown that DIRB can outcompete both sulfate-reducing bacteria and methanogens for limiting electron donors when bioavailable Fe(III) is provided (10, 35). Because most oxidized iron in freshwater lake sediments is present as insoluble hydrous ferric oxides (HFO) (13), reduction of Fe(III) may or may not be a more competitive respiratory strategy than reduction of less oxidized molecules, such as SO_4^{2-} or CO_2, depending on the surface area and degree of crystallinity of the natural HFO.

Trace elements, such as arsenic and phosphorus, readily adsorb onto the surfaces of HFO (5, 39, 40, 46, 48, 53). Reducing conditions can promote the subsequent release of such trace elements stored in soils and sediments (41, 52). Ribet et al. (47) suggested that reductive dissolution of HFO in weathered mine tailings may promote the release of adsorbed trace elements. Recent findings have demonstrated that this release may be due in part to the reduction of HFO and crystalline iron oxide minerals by DIRB (15). Specifically, dissolution of iron-trace element complexes results in solubilization of the trace element, which is then free to migrate along its aqueous phase concentration gradient. The Fe(III)-reducing activity of DIRB may, therefore, be indirectly responsible for mobilizing trace elements in iron-rich soils and sediments.

Iron is by far the most abundant metal in CDAR delta sediments, accounting for 8 to 10% of the mass on a dry weight basis (24). Iron is redox active and readily transformed abiotically and biotically. These transformations of iron could profoundly influence the biogeochemistry of micronutrients and contaminating trace elements, such as phosphorus and arsenic.

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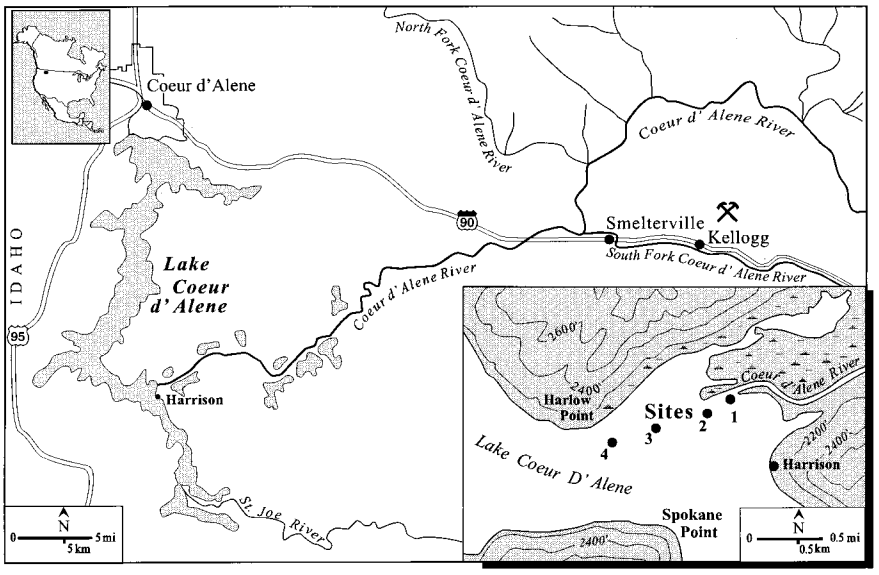

FIG. 1. Map of Lake Coeur d'Alene, Idaho, and the CDAR delta (inset), showing the sampling sites used in this study. The CDAR is the source of metal contamination in the sediments.

The purpose of this study was to evaluate geochemical and microbiological evidence that microbial Fe(III) reduction occurs in these mining-impacted sediments. Specifically, we set out to test the following hypotheses: if microbial iron reduction is an important process in this environment, then (i) recently reduced iron should be abundant, (ii) dissolved Fe(II) concentrations in the pore water should be elevated, (iii) common solid-phase reduction products of DIRB should be present, (iv) DIRB should be abundant, and (v) bacteria isolated from iron-reducing enrichment cultures should be capable of growth coupled to Fe(III) reduction.

MATERIALS AND METHODS

Sample collection and preparation. In June 1997, June 1998, and September 1998, intact sediment cores were retrieved either by hand or with a gravity coring device (43) fitted with 2.5-in polyvinyl chloride pipe. Cores were retrieved from several locations around the CDAR delta (Fig. 1). Sediments in the vicinity of sites 1 and 2 have previously been shown to contain the highest levels of metal contaminants in the CDAR delta (24). Sites 3 and 4 are located in the transition between the river delta and the lake proper, and sediments in this area are less contaminated than sediments at sites 1 and 2 (24). Cores were capped under a column of lake water and returned to the laboratory on ice, where they were extruded and segmented by depth in an anoxic glove box (Labconco) containing an N_2-CO_2-H_2 (75:15:10) atmosphere. Some of the segments were made into slurries by using equal volumes of sterile anoxic lake water. Triplicate 1-ml slurry samples were placed in preweighed glass vials, dried at 65°C for 24 h, and then reweighed to determine sediment dry weight per unit volume of slurry. Measurements were obtained and enrichment cultures were prepared by using a second set of slurry subsamples, and the values were normalized to the dry weight of the sediment. Whole sediments (nonslurries) were used for pore water Fe(II) analysis and isolation enrichment cultures.

Estimation of sediment E_h and pH. In June 1995, 13 sediment cores were retrieved from random locations around the CDAR delta, extruded immediately, and analyzed to determine the reduction potentials (E_h) and pH values. E_h was measured with a platinum electrode that was adjusted to the standard hydrogen electrode and was checked by using a standard between measurements. pH values were determined for the same cores with a portable pH probe and meter.

Iron analyses. All analyses of iron in sediment and pore water samples were performed by using three cores taken from a single square meter in shallow water (depth, 1.25 m) at the mouth of the CDAR in September 1998 (Fig. 1, site 1). The surface of the sediment at this location was clearly oxidized (as revealed by a bright orange hue) and contained sparse vegetation. Each core was split into two halves lengthwise; one half was used to make slurries, and the other was used for pore water extraction. The sediment was grainy at the surface and became more claylike with depth. The color ranged from bright orange at the surface (depth, 0 to 1 cm) to black below the surface (1 to 15 cm) to gray-black (gleyed) deeper in the sediment column (15 to 30 cm). We consistently observed this pattern in all cores obtained from the CDAR delta. Analyses were performed by using 5-cm segments extending from the sediment-water interface to a sediment depth of 30 cm. The total Fe content was determined by concentrated hydrochloric acid-nitric acid (3:1) digestion of whole dried sediments, followed by inductively coupled plasma spectrophotometric analysis (ACME Analytical Labs, Vancouver, Canada). Bulk pore water samples were extracted with N_2 (50 kPa) by using a pressurized ultrafiltration cell (Amicon, Danvers, Mass.) and were immediately refrigerated at 4°C under N_2. Within 3 days pore water samples were filtered (Nalgene nylon syringe filters; pore size, 0.2 μm) to remove suspended solids and were analyzed to determine dissolved Fe(II) contents by using ferrozine (see below).

Water- and weak-acid-soluble Fe(II) was quantified with the ferrozine reagent of Stookey (50), as described by Lovley and Phillips (34). To determine the concentration of dissolved Fe(II) in pore water, a 100-μl sample was acidified with 0.5 N HCl, and a 100-μl subsample of the acidified pore water was reacted with the ferrozine reagent [1 g of 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. The absorbance at 586 nm of the ferrozine-Fe(II) complex was determined with a Perkin-Elmer Lambda Bio II spectrophotometer and was compared to values obtained for standards prepared with ferrous ethylenediammonium sulfate (GFS Chemicals, Columbus, Ohio). To determine the concentrations of weak-acid-soluble Fe(II) in whole sediments, 1 ml of slurry was added to 9 ml of 0.5 N HCl and incubated overnight. One hundred microliters of the acidified slurry was reacted with the ferrozine reagent, and the ferrozine-FeII complex was quantified as described above.

Magnetic minerals were recovered from each slurry with a hand-held magnet. These minerals were rinsed twice with double-distilled water, retained on a preweighed Whatman no. 42 filter, dried at 65°C for 24 h, and weighed.

X-ray diffraction was performed with a Siemans Kristalloflex model D5000 diffractometer by using Cu K_α radiation.

Raman spectroscopy. After the mass was determined, magnetic separates were pooled, and Raman spectroscopy was used to identify the principal components of the solid phase. Raman spectra were obtained with a Kaiser Hololab Raman microscope equipped with a 785-nm diode laser and a charge-coupled device detector with a resolution of 4 cm^{-1}. The laser was operated at an average power of 0.5 mW to reduce sample degradation. To identify minerals, the laser was focused through a ×100 objective in order to maximize the signal intensity; for quantitative measurements we used either ×10 or ×50 objectives. In order to obtain satisfactory spectra, a minimum collection time of 30 s per spectrum was required, and at least 30 spectra obtained over a Raman shift range of 100 to 3,500 cm^{-1} were averaged.

Standard spectra of several minerals that we hypothesized could be present in the magnetic samples were collected for comparison. These minerals included magnetite, lepidocrosite, hematite, goethite, siderite, iron sulfide, and other iron (hydr)oxides. The spectra which we obtained were compared to the previously published Raman spectra of other iron oxides (54, 57). The laser power was reduced for quantitative measurements to ensure sample uniformity. Curve fitting was accomplished by using Grams/32 Spectral Notebase (Galactic Industries Corp., Salem, N.H.) in order to determine peak areas. The area of the

strongest Raman band for each standard was used for quantification of iron oxides. Quantification of iron sulfide, whose spectrum contained no discernible Raman bands, was based on random sampling of the sediment. An approximate representative sample composition could be obtained by examining a large number of mineral grains. Spectra which indicated that iron sulfide was present were obtained for 3 of 44 samples or about 7% of the pooled magnetic separates.

Microbiological growth conditions. Standard anaerobic techniques were used throughout this study (2, 8). All media were boiled and cooled under flowing N_2-CO_2 (80:20) and dispensed into culture tubes under the same gas phase, and the tubes were capped with butyl rubber stoppers, sealed with aluminum crimps, and sterilized by autoclaving (121°C, 15 min). All incubations were carried out at 25°C in the dark.

The bicarbonate-buffered basal medium used contained (per liter) 2.5 g of $NaHCO_3$, 1.5 g of NH_4Cl, 0.6 g of KH_2PO_4, 0.1 g of KCl, vitamins, and trace minerals (1). The final pH was approximately 6.8. Portions of sterile anoxic stock preparations were added to anaerobic tubes containing the basal medium with a needle and syringe (2).

Amorphous Fe(III) hydroxide was prepared as described by Lovley and Phillips (34).

MPN enrichment cultures. The most probable number (MPN) (12) of Fe(III)-reducing bacteria was determined by using three cores retrieved from each of two locations in the CDAR delta in June 1998 (Fig. 1, sites 2 and 4). The first location was directly in the mouth of the river in relatively shallow water (depth, 2 m), and the second location was approximately 1 km from the mouth toward Harlow Point at a depth of 10 m. Slurries were prepared by using surficial core segments (depth, 0 to 15 cm) and deep core segments (depth, 15 to 30 cm). Enrichment cultures contained basal medium amended with 5 mM sodium acetate as the sole electron donor and approximately 100 mM amorphous Fe(III) hydroxide as the sole electron acceptor. A 1-ml subsample of each slurry was incubated for 1 h in 4 ml of anoxic basal medium amended with disodium pyrophosphate (final concentration, 1.0 g $liter^{-1}$) to dislodge the cells and then diluted in 10-fold steps to a dilution of 10^{-7} in anoxic basal medium. A 0.5-ml portion of each dilution was inoculated into 4.5 ml of the MPN enrichment medium and incubated for 6 months at room temperature in the dark. Enrichment cultures were analyzed to determine whether Fe(II) was produced by using ferrozine, and cell densities were estimated by using a modification of the simple formula of Thomas (55).

Isolation of pure DIRB strains. The sediments used in isolation enrichment cultures were obtained in June 1997 from nine random locations in the CDAR delta. Sediment cores obtained at each location were divided into 5-cm segments in an anaerobic chamber. Dissimilatory Fe(III)-reducing enrichment cultures were started by adding 1.0 g (wet weight) of sediment to 10 ml of sterile basal medium containing sodium acetate (10 mM) as the sole electron donor and amorphous Fe(III) hydroxide (approximately 100 mM) as the sole electron acceptor in 20-ml pressure tubes. The headspace atmospheres of the enrichment cultures were immediately replaced with N_2-CO_2 (80:20) after each enrichment bottle was removed from the chamber. Enrichment cultures which had reduced the amorphous Fe(III) hydroxide after 3 months were transferred to the same medium containing Fe(III) pyrophosphate (3.0 g $liter^{-1}$; Sigma Chemical Co.) in place of the amorphous Fe(III) hydroxide. Two enrichment cultures prepared by using the top 5 cm of sediment from locations near Harlow Point (Fig. 1, sites 3 and 4) reduced the Fe(III) to Fe(II) (as shown by the production of a white precipitate that was presumably siderite) in less than 48 h. Each culture was serially transferred in the same medium until a uniform cell morphology was obtained and then streaked onto slants of anoxic basal medium amended with Fe(III) pyrophosphate (3.0 g $liter^{-1}$) and Bacto Agar (15 g $liter^{-1}$; Difco). Pinpoint white colonies were restreaked until uniform colony morphology was obtained. The two strains obtained were designated CdA-2 and CdA-3.

Pure-culture studies. The bacterial isolates were grown in the presence of acetate (10 mM) and Fe(III) pyrophosphate (3.0 g $liter^{-1}$) to determine whether growth was coupled to reduction of Fe(III), as described previously (16). Growth in the presence of various electron donors and acceptors was examined by using basal medium amended with compounds from anoxic stock solutions. Elemental sulfur was baked overnight at 65°C and added anoxically as sublimed sulfur flower. Cytochromes were examined by using dithionite-reduced-minus-air-oxidized difference spectra as described previously (16). The cell densities of Fe(III)-grown cultures were determined by direct cell counting performed with 4′,6-diamidino-2-phenylindole (DAPI) stain and a Zeiss Axioskop epifluorescent microscope (25). The amounts of growth resulting from other electron-accepting processes were determined by measuring the optical density at 600 nm. The stoichiometry of acetate oxidation was determined by using cultures grown in basal medium supplemented with 500 μM sodium acetate and excess Fe(III) pyrophosphate; Fe(II) contents were determined after 4 days and again after 6 days to confirm that Fe(III) reduction had ceased. The ability to ferment 2,3-butanediol was examined in both basal medium and in a medium specifically formulated for *Pelobacter propionicus* (DSMZ medium 298). The optimal pH and pH tolerance ranges were estimated by using cells grown with acetate (5 mM) as the sole electron donor and Fe(III) pyrophosphate (3 g $liter^{-1}$) as the sole electron acceptor in the medium described above; the bicarbonate buffer was replaced with 10 mM acetate buffer (pH 4.1, 4.5, 4.9, or 5.5), phosphate buffer (pH 6.5, 6.9, or 7.2), or Tris-HCl buffer (pH 7.6 or 8.1). Cultures were analyzed to determine the amount of Fe(II) produced after 11 days of incubation by using the ferrozine assay described above.

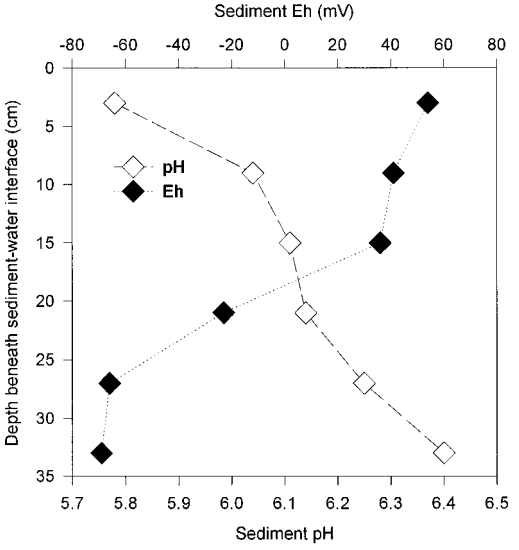

FIG. 2. E_h and pH as a function of depth in the CDAR delta sediments. The E_h decreases beneath the surface, indicating that there is a lack of oxygen and reducing conditions.

Phylogenetic analysis. Cells of Fe(III)-reducing isolates were used directly for PCR amplification of almost full-length bacterial 16S rRNA gene fragments (58). The resulting PCR products were purified with a Prep-A-Gene DNA purification kit (Bio-Rad, Munich, Germany) and were sequenced by using a LICOR automated sequencer (MWG Biotech, Ebersberg, Germany). Cycle sequencing protocols based on the chain termination technique were used with a Thermo Sequenase fluorescently labeled primer cycle sequencing kit (Amersham, Braunschweig, Germany). The new sequences were added to an alignment containing about 10,000 previously published and unpublished homologous primary structures for bacteria by using the alignment tool of the ARB program package (37). Phylogenetic analyses were performed by using the maximum-parsimony (ARB, PHYLIP), distance matrix (ARB, PHYLIP) (20), and maximum-likelihood (fastDNAml) (38) methods with different data sets.

Nucleotide sequence accession numbers and strain numbers. The 16S ribosomal DNA sequences of strains CdA-2 and CdA-3 have been deposited in the EMBL, GenBank, and DDBJ nucleotide sequence databases under accession no. Y19190 and Y19191, respectively. Cultures of strains CdA-2 and CdA-3 have been deposited in the American Type Culture Collection as strains ATCC 700775 and ATCC 700776, respectively.

RESULTS

Sediment E_h and pH. The E_h of the sediment was generally between 100 and 0 mV at the surface and decreased to values between 0 and −100 mV at depths below 20 cm (Fig. 2). The sediment pH values ranged from 5.2 to 6 at the surface, and the sediment became less acidic with depth. Of 13 cores, 3 were found to be circumneutral throughout; all of the other cores exhibited the general pattern shown in Fig. 2.

Iron in the sediments. The geochemical signature of iron reduction is summarized in Table 1. The concentrations of total iron in the sediments were ca. 80 g kg (dry weight)$^{-1}$ at the sediment-water interface and increased with depth to 110 g kg (dry weight)$^{-1}$ at a depth of 30 cm (Fig. 3A). These data are broadly consistent with the pattern of iron distribution observed previously with multiple cores taken along four transects in the CDAR delta (24). The concentration of Fe(II) soluble in 0.5 N HCl increased with depth from ca. 20 g kg (dry weight)$^{-1}$ at the sediment-water interface to 50 g kg (dry weight)$^{-1}$ at a depth of 30 cm (Fig. 3B). Although both the weak-acid-soluble Fe(II) content and the total Fe content increased with depth, the ratio of weak-acid-soluble Fe(II) to total Fe generally increased with depth from 0.21 at the sediment-water interface to 0.46 at a depth of 30 cm (Fig. 3C). The

TABLE 1. Summary of the various forms of iron found in the sediments of the CDAR delta

Form of Fe	Concn		
	Range	Mean	SD
Total Fe[a]	76–112	100.2	10.7
Weak-acid-soluble Fe(II)[a]	20–49	32.28	9.4
Magnetic minerals[a,b]	8.4–15.6	11.34	1.8
Dissolved pore water Fe(II)[c]	<0.5–41	17.34	9.9

[a] Grams per kilogram (dry weight) ($n = 18$).
[b] The magnetic minerals were determined to be approximately 77% magnetite. See text.
[c] Milligrams per liter ($n = 18$).

dissolved Fe(II) concentration in the pore water ranged from <0.5 to 41 mg liter^{-1} (Fig. 3D). Although there was variability in the pore water Fe(II) concentration among the cores, all three cores exhibited a peak at a depth of 10 to 15 cm. The concentrations of magnetic minerals ranged from 8.5 to 15.5 g kg (dry weight)$^{-1}$ (0.85 to 1.55% of the total sediment) (Fig. 3E).

Analysis of magnetic materials. Figure 4 shows the Raman spectra of individual mineral grains obtained after magnetic separation of the CDAR delta sediments. Magnetite was identified by its broad background and the presence of one broad band centered at approximately 665 cm^{-1}. Crystalline magnetite and an amorphous magnetitelike phase were found throughout the pooled sample. Magnetically isolated materials were insoluble in 0.5 N HCl (<2% soluble) and were not sensitive to reduction by hydroxylamine HCl (0.25 N hydroxylamine HCl in 0.25 N HCl) or to atmospheric oxidation.

Isolated regions of ferrihydrite, siderite, and pyrrhotite ($Fe_{1-x}S$) were also found in the sample. Amorphous iron oxides, including ferrihydrite, are weak Raman scatterers that have partially resolved peaks centered around 350 cm^{-1}. Ferrihydrite was present as unconsolidated masses in association with magnetite that cemented larger mineral grains together. Siderite, which produces strong bands at 1,088 and 295 cm^{-1}, was found in isolated regions without any clear mineral association.

On the basis of Raman spectroscopy, magnetite comprised approximately 77% of the sample, while siderite and pyrrhotite each comprised approximately 7% of the sample. The balance of the magnetic separates was a mixture of ferrihydrite and an array of other minerals and organic matter that could not be conclusively identified. Some constituents exhibited intense fluorescence, which is commonly observed with humic materials.

X-ray diffraction (XRD) was used to verify the presence of magnetite and to determine whether iron sulfides were isolated with the magnetic separates (data not shown). Iron sulfides formed long crystals and amorphous regions too small to distinguish by optical microscopy. Using XRD, we identified magnetite, small quantities of quartz, and small broad peaks corresponding to iron sulfides. Iron sulfide has a highly variable composition, and when XRD was used, we could not easily distinguish among the different stoichiometries because of the similar crystalline dimensions. Generally, the iron sulfide diffraction lines most closely matched the characteristics of troilite (Fe_7S_8), although other iron-deficient sulfides may have been present.

MPN enrichment cultures. An MPN analysis of the CDAR delta sediments (Fig. 1, sites 2 and 4) revealed that between 5.4×10^3 and 4.8×10^6 Fe(III)-reducing cells g (dry weight)$^{-1}$ (mean, 8.3×10^5 cells g [dry weight]$^{-1}$; $n = 12$) were present. When the depth from the sediment-water interface was considered the top 0 to 15 cm of the sediments contained on average 1.6×10^6 Fe(III)-reducing cells g (dry weight)$^{-1}$ ($n = 6$), while the bottom 15 to 30 cm of the sediments contained 2.9×10^4 cells g (dry weight)$^{-1}$ ($n = 6$). The difference was analyzed by using Student's t test ($P = 0.107$).

Characterization of strains CdA-2 and CdA-3. Two unique DIRB, strains CdA-2 and CdA-3, were isolated from surface sediments (depth, 0 to 5 cm) located at the outer edge of the CDAR delta near Harlow Point, approximately 50 m from one another (Fig. 1, sites 3 and 4). The growth of each strain was tightly coupled to Fe(III) reduction and was dependent on the presence of an electron donor (Fig. 5). Strains CdA-2 and CdA-3 were cultured in anoxic basal media containing no terminal electron acceptor and acetate as the sole electron donor. After 11 days, the counts of DAPI-stained cells decreased to values below the initial cell concentration, presumably due to cell death and lysis (data not shown). The measured stoichiometry of Fe(III) reduction coupled to acetate oxidation for each strain was close to the theoretical stoichiometry when acetate is oxidized completely to CO_2 (34). The values obtained were 7.0 and 7.8 mol of Fe(III) per mol of acetate for CdA-2 and CdA-3, respectively. Both strains were strictly anaerobic, motile, nonfermentative, gram-negative rods. Dithionite-reduced-minus-air-oxidized difference spectra for both strains had peaks at 423, 523, and 551 nm, which are indicative of type *c* cytochromes. When acetate (10 mM) was provided as the sole electron donor, both strains produced more than 5 mM Fe(II) after 2 weeks of incubation with Fe(III) citrate, Fe(III) pyrophosphate, amorphous Fe(III) hydroxide, and ferrihydrite. Cultures of each strain incubated for 2 weeks with goethite, hematite, or magnetite as the electron acceptor and acetate as the electron donor produced less than 0.5 mM Fe(II). In addition to the forms of iron described here, both strain CdA-2 and strain CdA-3 reduced 9,10-anthraquinone-2,6-disulfonate (10 mM), manganese(IV) (as synthetic MnO_2; approximately 100 mM), nitrate (10 mM), and elemental sulfur (1 g liter^{-1}). Neither strain was able to reduce fumarate, malate, trimethylamine *N*-oxide, nitrite, sulfate, sulfite, thiosulfate, or arsenate (each at a final concentration of 10 mM) when acetate (10 mM) was the sole electron donor.

Strain CdA-2 produced more than 4 mM Fe(II) from Fe(III) pyrophosphate when acetate, formate, benzoate, or succinate was the electron donor (each at a final concentration of 10 mM). Less than 0.5 mM Fe(II) was detected in cultures of strain CdA-2 incubated for 2 weeks with hydrogen (101 kPa), lactate, propionate, glucose, citrate, methanol, ethanol, isopropanol, or phenol (1 mM) as the electron donor (each at a final concentration of 10 mM unless noted otherwise). In contrast, under identical conditions strain CdA-3 produced more than 4 mM Fe(II) when acetate, formate, or hydrogen was the electron donor. Less than 0.5 mM Fe(II) was detected when CdA-3 cells were incubated with lactate, propionate, succinate, benzoate, phenol, citrate, glucose, methanol, ethanol, or isopropanol as the electron donor.

Both strains exhibited maximal Fe(II) production at pH 6.5, 6.9, and 7.2 (data not shown). However, at pH values below 6.5 or above 7.2, CdA-2 did not produce detectable amounts of Fe(II). Strain CdA-3 was able to reduce Fe(III) at a broader range of pH values. Limited iron reduction occurred at pH 5.5, as well as at pH 7.6 and 8.1. Iron reduction did not occur at pH values lower than 5.5; Fe(III) reduction at pH values above 8.1 was not tested.

Comparative 16S ribosomal DNA sequence analyses placed both strains in the family *Geobacteraceae* as proposed by Lon-

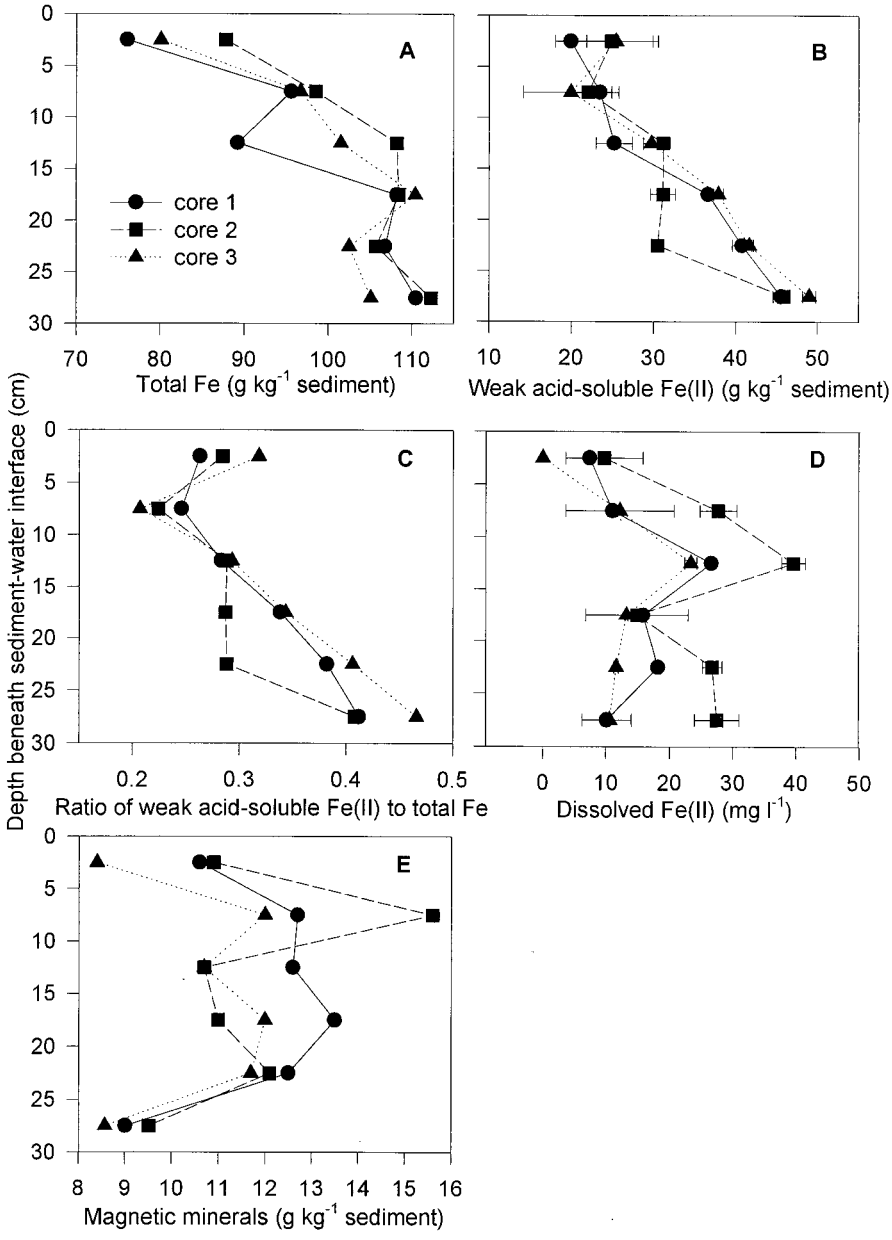

FIG. 3. Vertical distribution of iron phases in CDAR delta sediments. (A) Total iron. (B) Weak-acid-soluble Fe(II). (C) Ratio of weak-acid-soluble Fe(II) to total iron. (D) Fe(II) dissolved in pore water. (E) Mass of magnetic minerals. The error bars represent standard deviations from the means ($n = 3$) for weak-acid-soluble Fe(II) data. Symbols without error bars show data from single determinations.

ergan et al. (31) in the δ subclass of the class *Proteobacteria* (Fig. 6). Both isolates are members of the phylogenetically defined *Geobacter* cluster in the *Geobacteraceae*. The most closely related validly described species is *Pelobacter propionicus*. It is noteworthy that neither strain could grow fermentatively on 2,3-butanediol, a diagnostic trait of the genus *Pelobacter*. The partially characterized species "*Geobacter chapelleii*" is even more closely related, exhibiting levels of sequence similarity of 97.6 and 97.9% with CdA-2 and CdA-3, respectively. The level of sequence similarity for the two new isolates is 97.2%; this value is in the same range as the similarity values obtained with sequences of *P. propionicus* or "*G. chapelleii*", suggesting that CdA-2 and CdA-3 belong to different species. We do not propose new taxa for these microorganisms here because of the difficulties in distinguishing the members of the *Geobacter* cluster. The iron-reducing isolates retrieved by Straub et al. (51) (Dfr1 and Dfr2) and by Coates et al. (11) (Ala-5, JW-3, and TC-4), which also belong to this cluster, may not have been described as members of species for similar reasons.

DISCUSSION

In this report we show that the anticipated geochemical and microbiological signatures of bacterial Fe(III) reduction occur in mining-impacted sediments of the CDAR delta.

Geochemical evidence. The vertical distribution of total Fe is consistent with the history of local mining activities. Strict mine

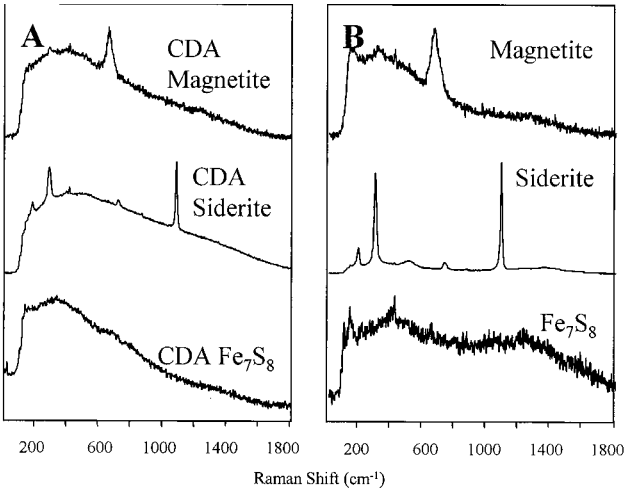

FIG. 4. Raman spectra of mineral grains from the magnetic fraction of CDAR delta sediments (A) and natural mineral standards (B).

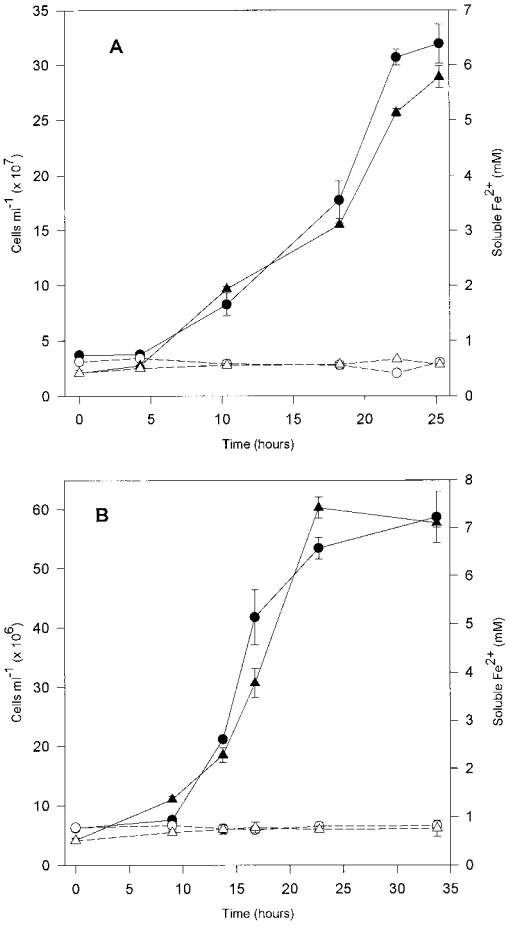

FIG. 5. Growth coupled to Fe(II) generation by strains CdA-2 (A) and CdA-3 (B). The error bars represent standard deviations from the means ($n = 3$). Symbols: ●, cell density in the presence of acetate; ○, cell density in the absence of acetate; ▲, amount of Fe(II) produced in the presence of acetate; △, amount of Fe(II) produced in the absence of acetate.

waste treatment regulations, including the construction of tailings ponds to restrict mine waste discharge, were implemented in 1968 (27). The implementation of these measures probably accounts for the decrease in total Fe content in surficial sediments. Recent iron deposition is due to resuspension and secondary transport of materials previously deposited in riverbanks and flood plains (60). However, we observed that compared to total iron abundance, the abundance of reduced iron species increased disproportionately with depth, which indicates that reducing conditions prevail beneath the sediment-water interface. This is consistent with observations that the Fe(II) content increases (15) and the E_h decreases (24; this study) with depth in these sediments. The fraction of Fe(II) that is soluble in weak acid is often interpreted as the fraction which has been reduced most recently (28). The high levels of acid-soluble Fe(II) in CDAR delta sediments suggest that iron reduction is active in this environment.

Dissolved Fe(II) concentrations greater than 41 mg $liter^{-1}$ at a depth of 10 to 15 cm indicate that molecular oxygen is absent and iron is being reduced in this region. This observation also suggests that the rate of sulfidogenesis in this zone is less than the rate of dissimilatory Fe(III) reduction. Elevated dissolved iron concentrations are commonly observed in acid-generating tailings (21, 59) due to the increased solubility of iron at low pH values. However, the dissolved Fe(II) concentration does not appear to be controlled by pH (Fig. 2 and 3D). Because of the nature of the transport process (i.e., suspension in oxic river water), few Fe(II) minerals are likely to have been deposited in the CDAR delta, and those that were deposited were probably highly insoluble. Thus, the majority of the Fe(II) in the sediment pore water probably resulted from reductive dissolution of ferric hydroxides, a microbially mediated process. Moore et al. (42) also found high concentrations (up to 44 mg $liter^{-1}$) of dissolved Fe(II) in the circumneutral pore water of mining-impacted reservoir sediments in northwestern Montana. Although the role of bacteria in generating the reduced soluble iron was not determined, DIRB may reasonably be implicated in this process.

It has been demonstrated that mixed populations and pure cultures of Fe(III)-reducing microorganisms reduce hydrous ferric oxides to magnetite (4, 36, 61). Recently, evidence that bacterial magnetite is present was reported for new lake sediments enriched in ferric chloride (23). Magnetite was the principal mineral which we observed in magnetically isolated CDAR sediments. Other magnetic minerals, including maghemite, were not detected by our analytical methods.

The U.S. Geological Survey has reported that magnetite is an accessory mineral which is found in the parent bedrock of the general region that includes northern Idaho, northeastern Washington, northwestern Montana, southeastern British Columbia, and southwestern Alberta (26). If the magnetite observed is ore-derived, it should have been deposited in a manner similar to the manner in which other mine wastes, including bulk iron, were deposited. However, the vertical profile of magnetite is very different from the vertical profile of total iron or other metals (24) and suggests that postdepositional diagenesis occurred (Fig. 3A and E). Although entrainment of magnetite which originated as an accessory mineral in the ore seems to be an unlikely explanation for the large quantities which we observed in the CDAR delta sediments, this explanation cannot be ruled out at this point.

Magnetotactic bacteria (6) are also an unlikely source of the CDAR delta magnetite. These bacteria typically produce only 10 to 20 crystals of magnetite per cell in a cell's lifetime. Even if the population density was relatively high, the magnetite contributed by magnetotactic bacteria could not by itself explain the dominant magnetic character of the sediments stud-

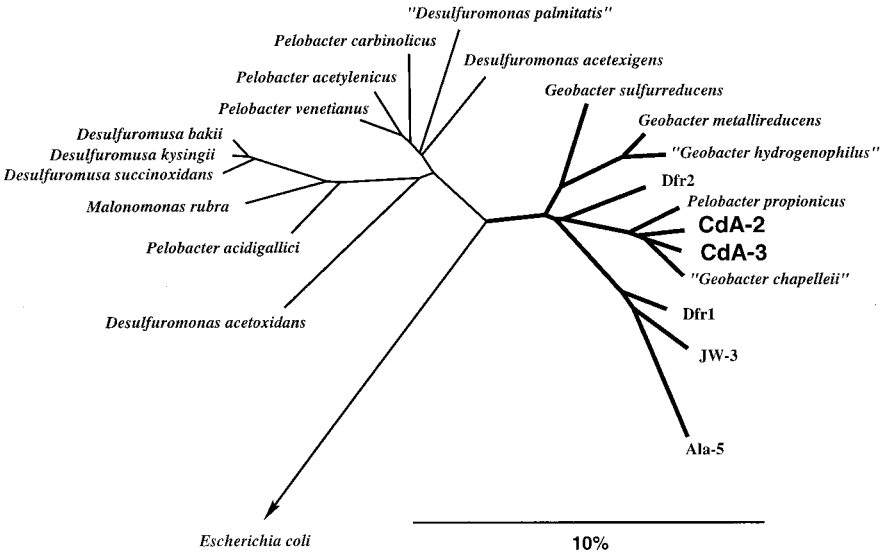

FIG. 6. Phylogenetic tree based on 16S rRNA gene sequences, showing the positions of newly isolated strains CdA-2 and CdA-3 in the family *Geobacteraceae* (29). The *Geobacter* cluster in this group is indicated by boldface lines. Phylogenetic distances were calculated as described by Felsenstein (20). The sequence of *Escherichia coli* was used as an outgroup. Bar = 10% estimated sequence divergence.

ied. Furthermore, direct microscopy revealed that the average diameter of the CDAR delta magnetite particles was between 3 and 40 μm, which is far too large for multiple crystals to be accommodated in a single magnetotactic bacterial cell, which has an average length of 1 μm (6). Mining-impacted sediments in the CDAR delta contain sufficient iron to support large populations of DIRB (up to 4.8×10^6 cultivable cells g [dry weight]$^{-1}$). Such organisms could produce magnetite continuously throughout every cell's lifetime. Thus, the magnetite found in CDAR delta sediments may represent a signature of active DIRB populations. Unfortunately, there is currently no sure method to determine whether the environmental magnetite is biogenic or lithogenic. However, recent advances in the area of Fe isotope fractionation (3) may prove to be useful in the future.

Microbiological evidence. The activity of DIRB has been largely overlooked when the geochemistry of disposed mine tailings has been considered. Acidophilic lithotrophic bacteria typically are the predominant organisms in aerobic tailings heaps (21, 49). However, some of these organisms are capable of dissimilatory Fe(III) reduction (7, 17, 18, 45). Schippers et al. (49) detected (by enrichment culturing) abundant nitrate-reducing bacteria in a uranium mine waste heap, but DIRB were detected in only a small percentage of their samples. Wielinga et al. (59) recently found more than 10^6 DIRB cells g (dry weight)$^{-1}$ in a mine tailings slicken in northwestern Montana. The pH values in this habitat ranged from 2 to 7. The tailings at the bottom of Lake Coeur d'Alene differ from many other disposed tailings in that they are completely submerged, which may account for the lack of substantial acid generation. The anoxic nature of these sediments was confirmed by the high concentrations of both dissolved and insoluble Fe(II), as well as E_h values that typically are well below 0 mV.

While it has been shown that reduced organic compounds reduce small amounts of Fe(III) under some conditions (30, 47), we hypothesized that DIRB were principally responsible for generating the Fe(II) in the environment which we studied. The presence of DIRB in the sediments supports this hypothesis. Virtually all CDAR delta sediments enriched with acetate and amorphous Fe(III) hydroxide have produced Fe(II) (14). *Ferribacterium limneticum* CdA-1 was previously enriched and isolated from deeper sediments (depth, 35 to 40 cm) near site 1 (16). The discovery of *F. limneticum* established that DIRB reside in the most contaminated regions of Lake Coeur d'Alene. Strains CdA-2 and CdA-3, which were isolated in this study, were also both capable of respiratory Fe(III) reduction. These microorganisms were enriched and isolated from surficial sediments (depth, 0 to 5 cm) near the edge of the delta. The discovery of these bacteria is significant because it establishes that (i) bacteria capable of Fe(III) reduction reside in other regions of the lake besides the heart of the CDAR delta, (ii) DIRB reside at the sediment-water interface, where exchange with the overlying water is possible, and (iii) members of the *Geobacteraceae*, a family in which the capacity for dissimilatory Fe(III) reduction is widespread, are a part of the DIRB community in Lake Coeur d'Alene sediments.

Lake Coeur d'Alene sediments contain between 10^4 and 10^8 total microbial cells per g (wet weight) of sediment (15, 24). Our MPN analyses indicated that at least 10^6 acetate-oxidizing, Fe(III)-reducing cells g (dry weight)$^{-1}$ can be found in some regions of the CDAR delta. This estimate does not include non-acetate-degrading, Fe(III)-reducing bacteria. Thus, DIRB may comprise 0.01 to 100% of the total microbial community in a given subhabitat in the benthic environment. Our MPN enrichment cultures almost certainly underestimated the abundance of Fe(III)-reducing bacteria. The actual densities are probably higher inasmuch as we have imperfect knowledge of the specific nutritional requirements and optimal culture conditions for every DIRB species. The MPN estimates of DIRB densities in this study, therefore, represent the minimum number of cells capable of respiring Fe(III). Because of the variety of alternative terminal electron acceptors that many Fe(III)-reducing bacteria are able to use (32, 33), we acknowledge that our data may not accurately reflect the numbers of bacteria that actually respire Fe(III) in situ. Furthermore, it is our experience that the sizes of many cells at sediment depths below 10 to 15 cm appear to be dramatically reduced (14). These cells resemble ultramicrobacteria (56), organisms which may be metabolically inactive for indefinite periods (9) and

which may be less easily recovered by traditional enrichment methods than the corresponding vegetative forms. It is interesting that far fewer Fe(III)-reducing bacteria were detected in MPN enrichment cultures prepared with the deeper sediments, in which ultramicrobacteria predominated, than in MPN enrichment cultures prepared with the upper sediments, in which the maximum dissolved Fe(II) concentration was observed.

While recent sulfidogenesis may be used to explain the increasing proportion of weak-acid-soluble Fe(II) with depth, it does not explain the elevated levels of magnetite or dissolved Fe(II). The only explanation for these profiles is enzymatic Fe(III) reduction.

Summary. Our results suggest that long-term aging of anoxic sediments enriched with mine tailings includes bacterial reduction of the iron oxides present to magnetite and other Fe(II) minerals, as well as generation of increased levels of dissolved iron. The ability of sediments or mine tailings to retain trace elements may be highly dependent on oxidized iron species, the reduction of which can lead to trace element solubilization. In addition, high concentrations of dissolved Fe(II) may complex hydrogen sulfide that is produced by sulfate-reducing bacteria and leave other more dangerous metals in solution.

The contribution of DIRB to the geochemical cycling of iron in sediments of the CDAR delta was evaluated by identifying the geochemical signatures and the microbiological potential for iron reduction. Our results suggest that microbial Fe(III) reduction is an important process in this environment, which has markedly altered the distribution and speciation of iron. The goals of future research include developing methods to accurately determine in situ biotic iron reduction rates and establishing vertical and horizontal patterns of the distribution of this key biogeochemical process.

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