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Isolation and Characterization of Metal-Reducing *Thermoanaerobacter* Strains from Deep Subsurface Environments of the Piceance Basin, Colorado

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Five bacterial strains were isolated from anaerobic enrichment cultures that had originated from inoculations with samples collected from the deep subsurface environments of the millions-of-years-old, geologically and hydrologically isolated Piceance Basin in Colorado. Small-subunit rRNA gene-based analyses indicated that all of these bacteria were closely related to *Thermoanaerobacter ethanolicus*, with similarities of 99.4 to 99.5%. Three isolates (X513, X514, and X561) from the five bacterial strains were used to examine physiological characteristics. These thermophilic bacteria were able to use acetate, glucose, hydrogen, lactate, pyruvate, succinate, and xylose as electron donors while reducing Fe(III), cobalt(III), chromium(VI), manganese(IV), and uranium(VI) at 60°C. One of the isolates (X514) was also able to utilize hydrogen as an electron donor for Fe(III) reduction. These bacteria exhibited diverse mineral precipitation capabilities, including the formation of magnetite (Fe_3O_4), siderite ($FeCO_3$), rhodochrosite ($MnCO_3$), and uraninite (UO_2). The gas composition of the incubation headspace and the ionic composition of the incubation medium exerted profound influences on the types of minerals formed. The susceptibility of the thermophilic Fe(III)-reducing cultures to metabolic inhibitors specific for ferric reductase, hydrogenase, and electron transport indicated that iron reduction by these bacteria is an enzymatic process.

Microbial Fe(III) reduction is an important geochemical process in weathering, the formation of minerals, and the oxidation of organic matter in a variety of sedimentary environments, including freshwater aquatic sediments, submerged soils, marine sediments, deep pristine aquifers, and shallow aquifers contaminated with organic compounds (7, 11, 12, 13, 14, 25, 26, 27, 28, 39, 58, 59, 60). Many Fe(III)-reducing microorganisms also reduce other forms of oxidized metals, including radionuclides such as uranium(VI) (13, 18, 23, 30, 31) and technetium(VII) (13, 18, 22, 23) and trace metals including arsenic(V) (20), chromium(VI) (13, 18, 55, 57), cobalt(III) (16, 18, 57), manganese(IV) (18, 26), and selenium(VI) (41).

Information about microbial Fe(III) reduction under thermophilic conditions is very limited. Only recently, several phylogenetically different thermophilic iron-reducing bacteria have been isolated and characterized (4, 17, 19, 48, 51). Our previous studies showed that microbial Fe(III) reduction and the formation of iron minerals likely occurred in the deep subsurface environments several kilometers below land surface at a high temperature (65°C) (21, 40, 58, 59, 60). Small-subunit (SSU) rRNA gene-based molecular analyses showed that the microbial communities of the thermophilic iron-reducing enrichment cultures were diverse (21, 61). Molecular analyses also revealed that some bacteria in Fe(III)-reducing enrichment cultures from Piceance Basin samples were capable of utilizing H_2 as the electron donor for growth (21, 61). In this paper, we describe the isolation and characterization of pure bacterial strains derived from the hydrogen enrichment cultures obtained from the Piceance Basin samples. To our knowledge, these bacteria, recovered from some of the most geologically and hydrologically isolated microbial habitats examined so far, represent a unique group of microorganisms capable of reducing Fe(III) and several other metals.

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MATERIALS AND METHODS

Isolation of pure bacterial strains. Thermophilic Fe(III)-reducing enrichment cultures were established by inoculation of core sediment samples obtained from the various depths of a drilling in the Piceance Basin in Colorado and drilling fluids collected after they circulated through the various depths of the geological formations. The low-porosity deeply buried sandstone environment had likely been geologically and hydrologically isolated for several million years (8). The original enrichment cultures were incubated under a H_2-CO_2 (80:20) headspace (2 atm of pressure) with 70 mM Fe(III) oxyhydroxide in a basal medium supplemented with 0.05% yeast extract. The basal medium contained the following ingredients: 2.5 g of $NaHCO_3$/liter, 0.08 g of $CaCl_2 \cdot 2H_2O$/liter, 1.0 g of NH_4Cl/liter, 0.2 g of $MgCl_2 \cdot 6H_2O$/liter, 10.0 g of NaCl/liter, 7.2 g of HEPES/liter, 1.0 g of resazurin (0.01%)/liter, 0.5 g of yeast extract/liter, trace minerals (10 ml), and vitamin solutions (1 ml) (43). No reducing agents were added to the medium. The trace mineral solution contained (per liter) 1,500 g of nitrilotriacetic acid, 200 g of $FeCl_2 \cdot 4H_2O$, 100 g of $MgCl_2 \cdot 6H_2O$, 20 g of sodium tungstate, 100 g of $MnCl_2 \cdot 4H_2O$, 100 g of $CoCl_2 \cdot 6H_2O$, 1,000 g of $CaCl_2 \cdot 2H_2O$, 50 g of $ZnCl_2$, 2 g of $CuCl_2 \cdot 2H_2O$, 5 g of H_3BO_3, 10 g of sodium molybdate, 1,000 g of NaCl, 17 g of Na_2SeO_3, and 24 g of $NiCl_2 \cdot 6H_2O$. The vitamin solution contained (per liter) 0.02 g of biotin, 0.02 g of folic acid, 0.1 g of vitamin B_6 (pyridoxine) HCl, 0.05 g of vitamin B_1 (thiamine) HCl, 0.05 g of vitamin B_2 (riboflavin), 0.05 g of nicotinic acid (niacin), 0.05 g of pantothenic acid, 0.001 g of crystalline vitamin B_{12} (cyanocobalamin), 0.05 g of *p*-aminobenzoic acid, and 0.05 g of lipoic acid (thioctic). Subcultures were established in a phosphate- and carbonate-buffered basal medium amended with 0.005 to 0.05% yeast extract (21). The subcultures contained 70 mM Fe(III) oxyhydroxide as an electron acceptor and different electron donors, with either pyruvate (10 mM), acetate (10 mM), or hydrogen (80% in a headspace with 2 atm of pressure) present in the media. SSU rRNA gene-based molecular analyses indicated that the enrichment with hydrogen as the electron donor was more diverse than those with pyruvate and acetate as

electron donors (21, 61). Thus, the enrichment culture with hydrogen as an electron donor was used for bacterial isolation.

To isolate pure cultures, a series of 10-fold dilutions of the hydrogen-enriched Fe(III)-reducing cultures were made with cell numbers ranging from 10^1 to 10^8 by vortexing them in an anaerobic chamber. Bacterial numbers were determined by directly counting cells, which were stained with 0.1% acridine orange, under an epifluorescence microscope (57). A 0.1-ml sample of each dilution was inoculated into 28-ml pressure tubes containing 10 ml of basal medium and 0.05% yeast extract, with ferric citrate (10 mM) as the electron acceptor and hydrogen as the primary electron donor under a H_2-CO_2 gas headspace. The tubes were incubated at 65°C for 12 to 16 h, and Fe(III) reduction was assessed visually according to color changes. The highest dilution showing iron reduction was serially diluted again and used for the second round of purification as described above. This process was repeated at least 20 times prior to initiating the isolation of pure cultures.

Pure cultures of microorganisms were obtained by plating the enrichment cultures on 1.8% agar medium prepared with the basal medium buffer plus HEPES and phosphate. Ferric citrate (10 mM) was used as an electron acceptor, and lactate was used as an electron donor (10 mM). The plates were incubated under a N_2 gas headspace in an anaerobic transparent plastic jar at 65°C for 48 h. A preliminary assessment of the diversity of the iron-reducing bacteria was based on the differences in colony morphologies. Approximately 20 isolates were selected for further screening. Individual colonies were transferred 10 times as described above. Enterobacterial repetitive intergenic consensus (ERIC)-, repetitive extragenic palindromic (REP)-, and BOX-PCR were used to differentiate identical or closely related strains, and these assays were accomplished as described elsewhere (9, 44, 52). The isolates with different genetic fingerprinting patterns were tested again for iron reduction to ensure that they were capable of reducing ferric iron.

Physiological characterization. A variety of factors were examined for their influence on metal reduction and mineral formation, including medium pH, incubation temperature, electron donors, electron acceptors, different metal species, and headspace gas composition. To determine the range of growth substrates, the purified isolates (X513, X514, and X561) were inoculated into anaerobic basal medium with lactate (10 mM), acetate (10 mM), pyruvate (10 mM), succinate (10 mM), xylose (1%), or H_2 (100% H_2 or H_2-CO_2 at an 80:20 ratio) as an electron donor and a Fe(III) oxyhydroxide (~70 mM) as an electron acceptor and incubated at 60°C in the dark for several days. The Fe(III) oxyhydroxide was prepared by neutralizing a solution of 0.4 M $FeCl_3$ with 10 M NaOH as described previously (46). Analysis of X-ray diffraction (XRD) showed that most of the Fe(III) oxyhydroxide was poorly crystalline akaganeite (FeOOH) and a small portion was amorphous. In this paper, we refer to this material as Fe(III) oxyhydroxide.

To assess the capabilities for metal reduction and mineral formation of the pure cultures (X513, X514, and X561), various electron acceptors, such as ferric citrate (10 to 20 mM), Fe(III) oxyhydroxides (~70 mM), cobalt-EDTA [Co(III)-EDTA, 1.5 mM], potassium chromate (0.5 mM), Mn(IV) oxide (10 mM), gold chloride ($AuHCl_3$, 0.1 mM), and uranyl carbonate (1 mM), were examined at 60°C with lactate (10 mM) as an electron donor. The Mn(IV) oxide was prepared as described by Lovley and Phillips (29), and uranyl carbonate [$UO_2(CO_3)_2^{2-}$] was prepared by mixing uranyl nitrate [$UO_2(NO_3)_2^{2-}$] solution with sodium bicarbonate (45). The effects of temperatures (25 to 75°C), pH (6.5 to 9.6), and salinity (0.05 to 7% NaCl) on microbial Fe(III) reduction and mineral formation were also examined using an Fe(III) oxyhydroxide (70 mM) as an electron acceptor and lactate (10 mM) as an electron donor. In addition, the optimal growth temperature for the isolate X514 was determined under anaerobic iron-reducing conditions with Fe(III) citrate (15 mM) as an electron acceptor and lactate (10 mM) as an electron donor.

To examine the ability to produce ethanol concomitantly with Fe(III) reduction, the three isolates (X513, X514, and X561) were incubated at 60°C with xylose (10 mM) and Fe(III) oxyhydroxide (70 mM). The isolates were also used to examine their fermentative capabilities on glucose (10 mM) while simultaneously reducing Fe(III) in non-energy-generating side reactions. In addition, the ethanol-producing thermophilic anaerobe *Thermoanaerobacter ethanolicus* (ATCC 31938) was obtained from the American Type Culture Collection (Manassas, Va.) and grown anaerobically with Fe(III) oxyhydroxide as an electron acceptor.

To understand the effect of headspace atmosphere on iron mineralogy, the anaerobic media were prepared and incubated under three different headspace atmospheres, N_2, N_2-CO_2 (80:20), and H_2-CO_2 (80:20), with Fe(III) oxyhydroxide (~70 mM) as an electron acceptor. In the case of the N_2 and N_2-CO_2 headspaces, lactate (10 mM) was used as an electron donor, while H_2 was used as an electron donor when a H_2-CO_2 headspace atmosphere was used. Incubation time ranged from 2 to 3 weeks. Abiotic controls were established for each treatment. To determine whether iron reduction and mineral formation with three strains (X513, X514, and X561) were enzyme-linked biological processes, we used various inhibitors (final concentrations are indicated within parentheses), including the ferric reductase inhibitor *p*-chloromercuriphenyl sulfonate (*p*CMPS) (0.1 mM), the hydrogenase inhibitor quinacrine dihydrochloride (1 mM), the protonophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (0.02 mM), and the electron transport inhibitors 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide (HOQNO) (0.05 mM) and dicumarol (0.05 mM) (1, 15, 21, 35, 36, 37)

Phylogenetic analyses of bacterial isolates. Genomic DNA was recovered from the isolates as described previously (62). SSU rRNA genes were amplified from genomic DNA by using conserved primers 27f and 1592r (56, 62). Amplified products were purified with the WizardPrep kit (Promega, Madison, Wis.). Nucleic acid sequences were determined by the automated *Taq*-cycle sequencing method with an ABI 373A sequencer (Applied Biosystems, Foster, Calif.) by using 12 primers as described previously (62).

Analyses of SSU rRNA sequences were initially conducted by searching current databases with the FASTA program from the Genetics Computer Group software package (10). The genetic data environment was used to manually align the sequences from the isolates with representative sequences from the Ribosomal Database Project (RDP) (33). The phylogenetic relationships of these isolates with previously described bacteria were established with the neighbor-joining DNA distance program in the ARB package (32) and the maximum-likelihood method with the program fastDNAml from the RDP. The maximum-likelihood tree was constructed with a transition/transversion ratio of 2.0 by using jumbled orders of 10 for the addition of taxa.

Chemical and mineralogical characterization. To examine the chemical conditions of metal reduction and mineral formation by the thermophilic Fe(III)-reducing bacteria, subsamples (1 ml) of bacterial cultures and abiotic controls were taken from the culture bottles at different times and measured for redox potential (E_h) and pH at room temperature in an anaerobic chamber (58). Fe(II) concentrations (0.5 N HCl, soluble) were determined by measuring the absorbance at 562 nm on a Hewlett-Packard model 8453 spectrophotometer by the ferrozine method with anaerobic water for sample dilution (50, 57).

A JSM-35CF (JEOL, Ltd., Tokyo, Japan) scanning electron microscope (SEM) with an energy-dispersive X-ray (EDX) detector was used for the analysis of the morphology, mineralogy, and chemistry of the mineral phases precipitated or transformed by the thermophilic Fe(III)-reducing bacteria (46). The mineralogical compositions of the precipitated or transformed phases were determined by using XRD. All XRDs were performed on an XDS 2000 diffractometer (40 kV, 35 mV; Scintag, Inc., Sunnyvale, Calif.) equipped with Co-Kα radiation. To understand the morphology of the transformed crystalline iron minerals, mineralogical characteristics of the precipitates were also examined by transmission electron microscopy (TEM). Culture media containing bacterial cells, organic matter, and inorganic solids [magnetite and Fe(III) oxyhydroxides] were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate. After being washed with a HEPES buffer and an alcohol-water solution (1:1 ratio), samples were dehydrated with propylene oxide and embedded in a low-viscosity, thermally curing epoxy resin. Ultrathin sections (70 to 80 nm) were cut from resin blocks with a diamond knife and transferred to a 30-mesh Formvar-coated Cu TEM grid for image analysis on an FZ 2000 TEM (JEOL, Ltd.) equipped with an EDX detector (59).

Nucleotide sequence accession numbers. The sequences obtained by this study have been submitted to GenBank under accession numbers AF542517 (X514), AF542518 (X561), AF542519 (X521), AF542520 (X513), and AF542521 (X524).

RESULTS

Molecular analyses of bacterial isolates. After a series of dilution and plating, five different pure cultures were obtained and designated X513, X514, X521, X524, and X561. These strains were closely related, as indicated by the fingerprinting patterns revealed by BOX-, ERIC-, and REP-PCR. Strains X513, X514, X524, and X561 showed the same fingerprinting patterns by BOX- and REP-PCR, but they showed different fingerprinting patterns by ERIC-PCR (data not shown). This suggests that the ERIC-PCR method may have a higher level of resolution in differentiating these anaerobic thermophilic Fe(III)-reducing bacteria.

Preliminary sequence analysis based on the FASTA search agreed with results of microscopic examinations indicating that

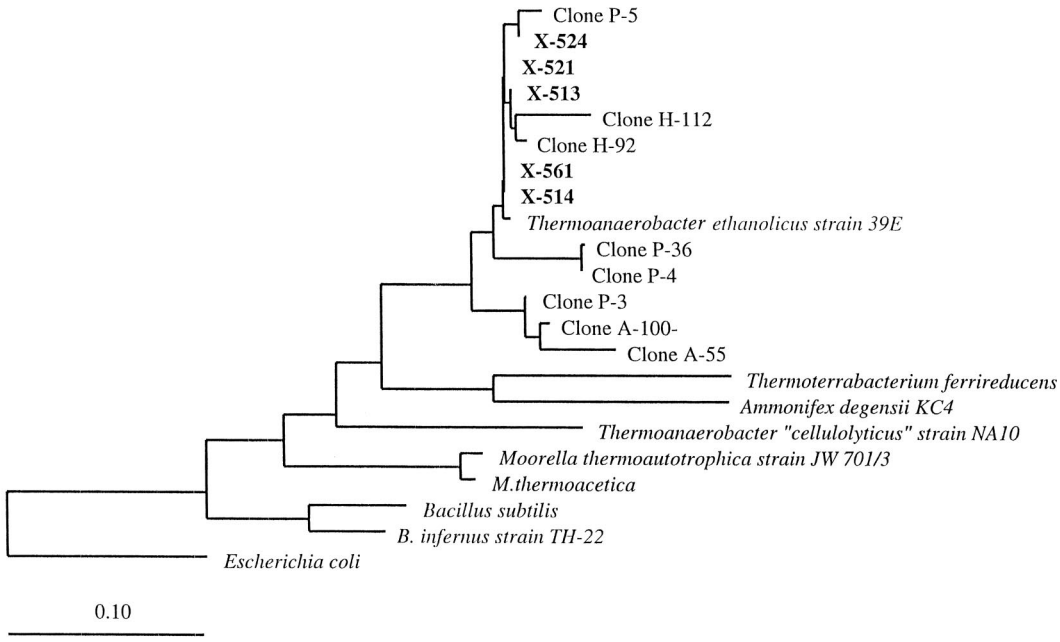

FIG. 1. Phylogenetic relationships of the thermophilic iron-reducing bacteria from the deep subsurface environments. The tree was constructed by the maximum-likelihood method. *Escherichia coli* was used as the out-group.

these isolates were gram-positive bacteria. To further determine their phylogenetic relationships, their SSU rRNA gene sequences were compared with those of gram-positive bacteria listed in the RDP. Maximum-likelihood analysis indicated that these isolates were closely related to *Thermoanaerobacter ethanolicus* 39E, with 99.4 to 99.5% similarity (Fig. 1), and to the clones P-5, H-92, and H-112 from pyruvate- and hydrogen-enriched cultures (61). These isolates were 81.4 to 83.2% similar to the thermophilic metal-reducing bacterium *Bacillus infernus* and 81.0 to 83.5% similar to *Thermoterrabacterium ferrireducens*. A similar topology for the SSU rRNA phylogenetic tree was obtained with the neighbor-joining distance method (data not shown).

Physiological characteristics. Since these isolates were very closely related, only three isolates (X513, X514, and X561) were selected for further physiological and biogeochemical characterization. All three isolates examined were able to use lactate, acetate, succinate, xylose, and glucose as electron donors in reducing Fe(III) oxyhydroxide and producing magnetite under a N_2 atmosphere. Although these isolates are closely related phylogenetically, they showed some physiological differences. For example, X514 was able to use both pyruvate and hydrogen in reducing Fe(III) oxyhydroxide and producing magnetite, whereas X561 was not. X513 was able to utilize pyruvate but not hydrogen for iron reduction and magnetite production. The non-hydrogen utilizers, including X513 and X561, may use yeast extract (0.05%) as an energy source. No growth, Fe(III) reduction, or magnetite formation was observed in controls without microbial inocula. These strains were capable of producing ethanol from glucose (<0.1%), and their growth was unaffected in media containing 1% ethanol. However, a much slower growth was observed in incubated cultures containing 2% ethanol, and no growth was evident in incubated cultures containing 3% or more ethanol.

The effects of incubation temperature on microbial growth rates and Fe(III) reduction rates were examined with the isolate X514 (Fig. 2). The maximum Fe(III) reduction rates and bacterial growth were observed at 60°C (Fig. 2A), and generation times of 6 to 10 h were typically observed (data not shown). The initial concentration of Fe(III) citrate (15 mM) was reduced biotically by 36% after 24 h of incubation and by 50% after 48 h of incubation at 60°C. The cell density increased from 5×10^4 cells/ml at time zero to 7.5×10^5 cells/ml at 24 h after incubation at 60°C (Fig. 2A). Although the abiotic reduction of Fe(III) increased as the temperature increased, the Fe(III) reduction occurring without cells was significantly less than that occurring with cells (Fig. 2B). The abiotic reduction of Fe(III) at 55 and 75°C after 48 h of incubation accounted for 1.5 and 38% of the total reduced Fe(II), respectively. No significant increase in abiotic Fe(III) reduction [<3% of the initial Fe(III) concentration] was detected at 45°C after 24 or 48 h of incubation. These results indicate that the optimal temperature for the reduction of Fe(III) citrate and for bacterial growth was about 60°C, and the minimum bacterial population doubling time of the cultures was approximately 6 h.

Three isolates (X513, X514, and X561) fermented glucose (10 mM) and reduced Fe(III) oxyhydroxide (70 mM) and produced magnetite simultaneously. An ethanol-producing anaerobe, *Thermoanaerobacter ethanolicus* (ATCC 31938), also fermented glucose while simultaneously reducing Fe(III) citrate (15 mM). However, *Thermoanaerobacter ethanolicus* (ATCC 31938) did not grow with Fe(III) citrate as an electron acceptor and lactate (10 mM) as an electron donor. Three isolates

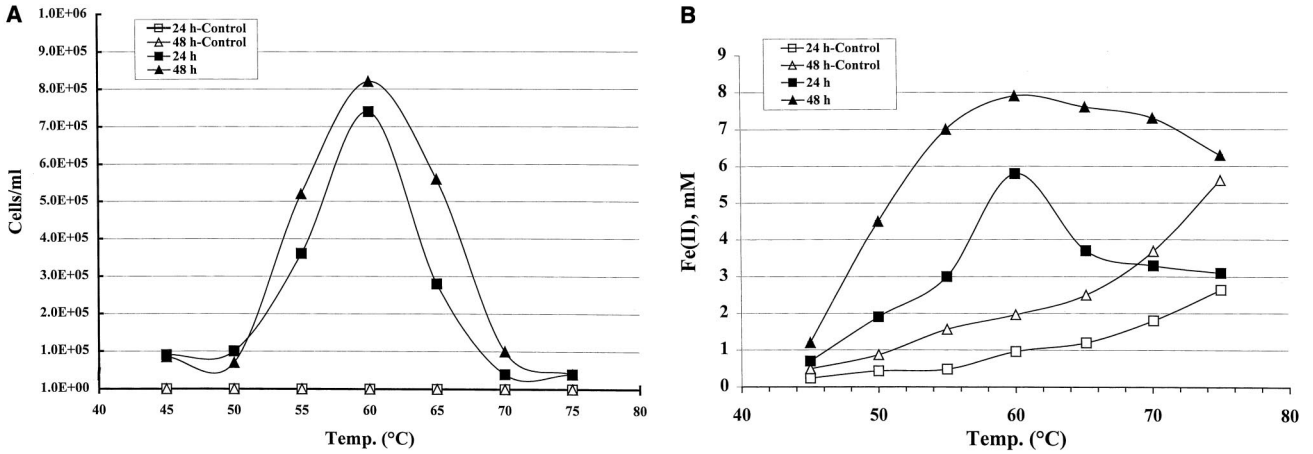

FIG. 2. Temperature profiles of growth (a) and iron reduction (b) rates by the thermophilic iron-reducing bacteria (X514) at 24 and 48 h. The results shown are the means of results from triplicate cultures.

(X513, X514, and X561) fermented xylose and produced ethanol (<0.1%) concomitantly with the reduction of Fe(III) oxyhydroxide and magnetite formation. The ability of three strains (X513, X514, and X561) to reduce metals besides iron was also examined. These isolates were able to reduce Co(III) [Co(III)-EDTA, 1 mM] to Co(II) and Cr(VI) (potassium chromate, 0.5 mM) to Cr(III) with lactate (10 mM) as an electron donor, as indicated by the color change of the culture solution from purple [Co(III)-EDTA] and yellow (potassium chromate) to colorless at 60°C. Three strains were also able to reduce U(VI) ($UO_2CO_3^{2-}$, 1 mM) and produced black precipitate with lactate as an electron donor. XRD analysis showed that the precipitated phase was uraninite (UO_2) (Fig. 3C), thereby demonstrating the reduction of U(VI) to U(IV). SEM and EDX analyses showed that the crystalline phase contained significant quantities of U (Fig. 3B) and was ball shaped (Fig. 3A). Three strains were also able to reduce Mn(IV) oxide (10 mM), producing a reddish-brown precipitate with lactate (10 mM) as an electron donor. XRD analysis showed that the precipitated phase was rhodochrosite ($MnCO_3$) (Fig. 3F); therefore, Mn(IV) was reduced to Mn(II). SEM with EDX analysis showed that this crystalline phase contained significant quan-

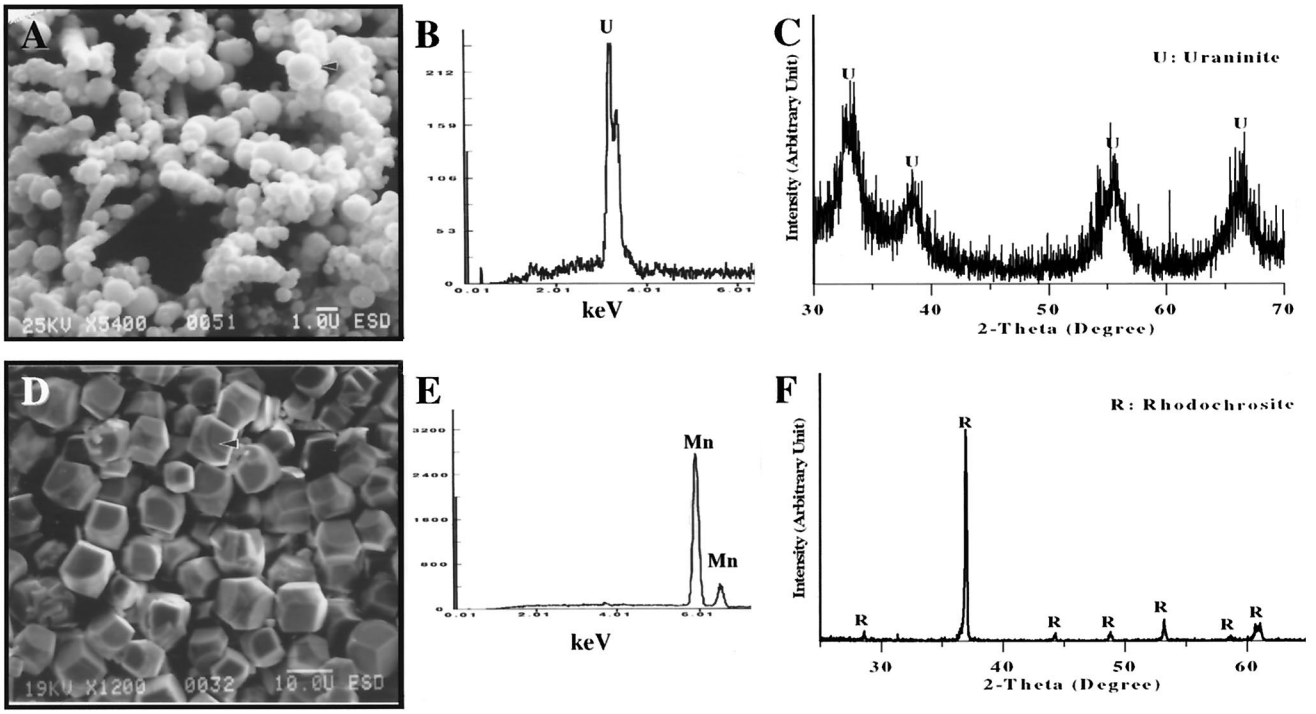

FIG. 3. SEM (A), EDX analysis (B), and XRD analysis (C) of uranium precipitated by X514. SEM (D), EDX analysis (E), and XRD analysis (F) of rhodochrosite formed by the reduction of Mn(IV) oxide by X514.

tities of Mn (Fig. 3E) and was shaped like a rhombohedron with curved faces (Fig. 3D). These results indicated that these thermophilic *Thermoanaerobacter ethanolicus* strains can reduce several other transition metals besides iron as long as their concentrations are held below the toxic levels. Gold (0.1 mM) was reduced from Au(I) to Au(0), as evidenced by a color change from yellow to brownish black, but it likely proved lethal to the cells, as transfers from gold-containing cultures did not grow.

Three strains (X513, X514, and X561) were examined for Fe(III) reduction with Fe(III) oxyhydroxide as an electron acceptor and lactate (10 mM) as an electron donor under different salinities (0.05 to 7% NaCl) and pHs (6.5 to 9.6). Thermophilic iron reduction and magnetite formation occurred at salinities ranging from 0.05 to 5% NaCl (wt/vol) with lactate as an electron donor and Fe(III) oxyhydroxide as an electron acceptor. Magnetite formation was not observed at 6% or higher NaCl. The thermophilic bacteria exhibited optimum growth at 3% NaCl with Fe(III) citrate (15 mM) as an electron acceptor and lactate (10 mM) as an electron donor. These results are consistent with the effect of salinity on the growth of the thermophilic metal-reducing bacteria isolated at the subsurface environments (4, 21). Microbial transformation of Fe(III) oxyhydroxides (FeOOH) to magnetite (Fe_3O_4) was observed at a pH range of 6.9 to 8.9. The E_h of the initial medium (−40 to −50 mV) was decreased to −200 mV (under N_2 headspace gas) and −450 mV (under H_2-CO_2 headspace gases), and pH was also decreased from 8.0 to 7.4 upon incubation. Measured E_h and pH were consistent with the thermodynamic stabilities of magnetite and siderite (3, 46, 58). In other experiments at pH values slightly below neutral (6.6 to 6.9), minor amounts of magnetite were observed upon incubation with the thermophilic microorganisms.

The Fe(III) reduction process was sensitive to oxygen and metabolic inhibitors as well. Three isolates (X513, X514, and X561) were viable and fermented glucose on exposure to O_2. However, exposure of the thermophilic iron-reducing bacteria to oxygen decreased the Fe(III) reduction rate, indicating the anaerobic nature of the Fe(III)-reducing microorganisms. The formation of magnetic iron oxides by three thermophilic strains was also prevented by the addition of the ferric reductase inhibitor *p*CMPS, the hydrogenase inhibitor quinacrine dihydrochloride, the protonophore CCCP, and the electron transport inhibitors HOQNO and dicumarol. The susceptibility of the thermophilic Fe(III)-reducing cultures to these metabolic inhibitors indicated that the iron reduction by these bacteria appears to be an enzymatic process.

Mineralogical characteristics. All three strains were able to reduce Fe(III) oxyhydroxide and produce magnetite (Fe_3O_4) under temperatures ranging from ~45°C to 70°C (Fig. 4). TEM analysis revealed elongated prismatic magnetite particles in X514 samples after 21 days of incubation at 60°C (Fig. 5B). The crystal morphology of the elongated prismatic magnetite particles formed by the X514 strain differed from that of the magnetite crystals formed by the *Thermoanaerobacter ethanolicus* strain (TOR-39) isolated from Taylorsville Triassic Basin, because TOR-39 produced sharp and well-formed octahedral crystals (59). The magnetite particles were associated with acicular goethite (Fe_2O_3) and thin, needle-like Fe(III) oxyhydroxides. Also, degradation of the texture of magnetite, such as

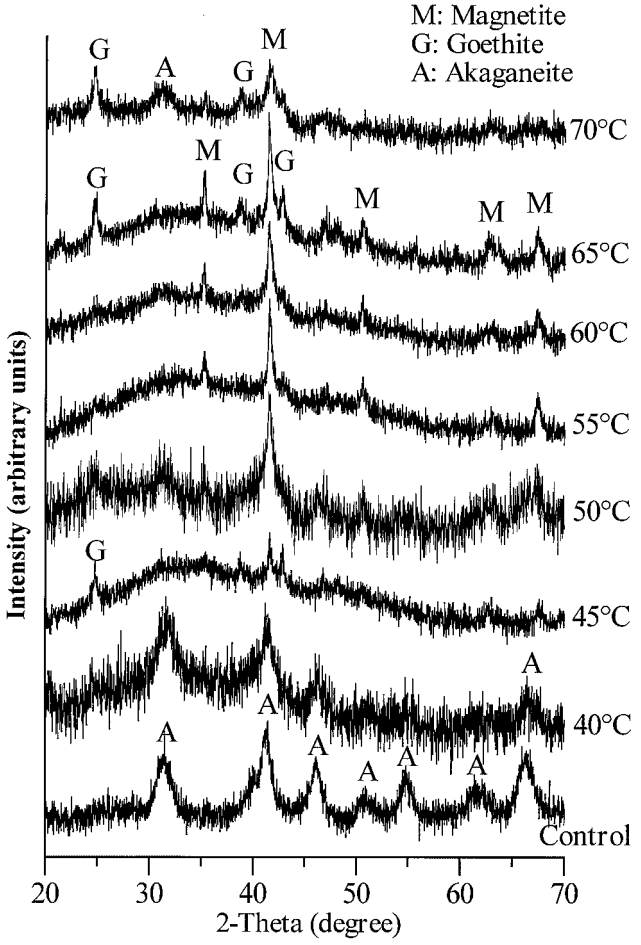

FIG. 4. XRD analysis of iron minerals formed by the thermophilic iron-reducing bacteria (X514) at different temperatures.

rounded margins, was observed at temperatures of 70°C after 21 days of incubation (Fig. 5C). In addition, poorly crystalline magnetite crystals associated with fine-grained Fe(III) oxyhydroxides were observed at 50°C (Fig. 5A). Goethite (FeOOH) was also formed at 45, 65, and 70°C during the microbial transformation of the Fe(III) oxyhydroxides. The presence of goethite in some samples suggests that Fe(III) oxyhydroxides may be abiotically transformed into goethite through a dehydration process during incubation (47). This likely occurred due to the experimental temperature (>45°C) used in the incubations (47). Neither Fe(III) reduction nor magnetic iron mineral formation was observed below 40°C or above 75°C. No magnetic mineral formation was observed in the abiotic control experiments.

The gas composition of the headspace atmosphere appeared to affect the mineral formation. All reddish-brown Fe(III) oxyhydroxides in culture bottles under a N_2 headspace changed to black, with the black precipitate being magnetite, as determined by XRD analysis. All reddish-brown solids under N_2-CO_2 and H_2-CO_2 changed to brownish black. Tubes incubated under a N_2-CO_2 atmosphere contained a mixture of magnetite and siderite, as determined by XRD analysis, whereas under a H_2-CO_2 atmosphere, siderite ($FeCO_3$) was the principal re-

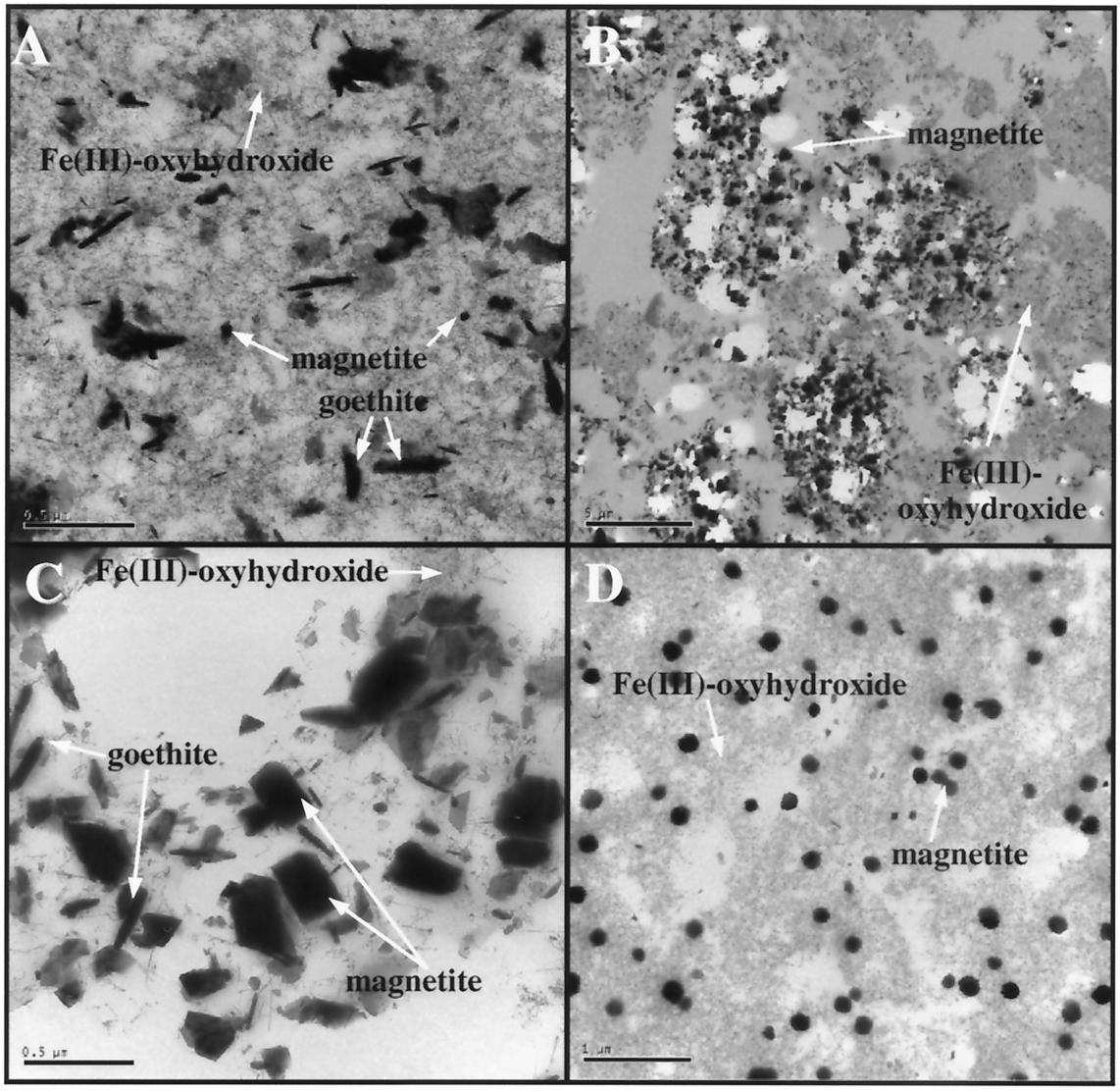

FIG. 5. TEMs of magnetite formation in X514 cultures at 50°C (A), 60°C (B and C), and 70°C (D).

duced iron mineral. It appeared that a H_2-CO_2 atmosphere provides a favorable energetic environment, allowing the complete reduction of Fe(III) oxyhydroxides plus considerable carbon dioxide and facilitating the formation of siderite rather than less-reduced magnetite (3, 46, 58). This study indicated that geochemical and environmental factors of iron biomineralization include atmospheric composition, pH, incubation temperature, and salinity. The presence of CO_2 gas was an important factor allowing the complete reduction of Fe(III) oxyhydroxide and the formation of siderite in subsurface environments.

DISCUSSION

Five pure cultures of thermophilic metal-reducing bacteria closely related to *Thermoanaerobacter ethanolicus* were isolated from the hydrogen-fed enrichment cultures derived from drilling fluid samples that had circulated to depths of approximately 2,000 m below land surface in a core hole at the Piceance Basin in Colorado (21). Thermophilic microorganisms were not cultured from the 2,000-m-deep sandstone samples, though thermophilic bacterial growth was observed in samples from depths of 860 m and thermophilic microorganisms were detected in drilling fluids that had circulated at depths greater than 860 m (S. V. Liu, unpublished data). We cannot ascertain the exact depths from which these thermophilic metal-reducing microorganisms were obtained, but their growth temperatures in laboratory media range from 45 to 75°C. *Thermoanaerobacter ethanolicus* strains were not detected in core samples from the 860-m depth with an in situ temperature of 42°C (8). Thus, these thermophiles were likely derived from fractured habitats below the 860-m depth but above the 1,996-m depth, which exhibited an in situ temperature of 81°C (21).

The reduction of Fe(III) appears to be coupled with growth, as indicated by the following evidence: (i) no iron reduction or

growth was observed without the addition of living cells; (ii) the production of Fe(II) was concomitant with cell growth; (iii) no growth or Fe(III) reduction was observed when various metabolic inhibitors were added; and (iv) Fe(III) reduction and magnetite production were observed when nonfermentative substrates, such as lactate, pyruvate, acetate, and/or hydrogen, were used as electron donors. Interestingly, as CO_2 partial pressures increased, a greater percentage of the iron was reduced, with siderite being the predominant product of iron reduction whenever bicarbonate concentrations exceeded the ferric iron concentration. Siderite formation under a CO_2 atmosphere indicates that the microbial siderite formation via reduction of Fe(III) oxides may occur naturally when such a ligand (HCO_3^-) and appropriate electron donors are in sufficient concentration. Given the abundance of Fe in anaerobic sedimentary systems, the capacity of Fe(III)-reducing bacteria to precipitate siderite by using iron oxides and dissolved Fe ion species could have a significant impact on carbon sequestration in subsurface environments.

Phylogenetic analysis indicated that these isolates are very closely related to *Thermoanaerobacter ethanolicus* and are distinctly different from many other known thermophilic iron-reducing bacteria isolated from different environments. The SSU rRNA gene sequences from these isolates were about 81 to 84% similar to that from *Thermoterrabacterium ferrireducens* isolated from a hot spring (48), 81.1% similar to that from *Deferribacter thermophilus* isolated from a petroleum reservoir (17), 84% similar to that from *B. infernus* from the deep subsurface (4), and ~80% similar to that from the *Thermus* isolate (19). Although these bacteria are similar to cultured bacteria from the Taylorsville Triassic Basin in Virginia (21), these bacteria are distinctly different from all of the known mesophilic iron-reducing bacteria, such as *Geobacter* and *Shewanella* species (24). Thus, these bacteria appear to represent a novel group of thermophilic bacteria capable of reducing Fe(III). The results of this study support the claim that metal reduction may be a characteristic that is widespread in the domain Bacteria (24).

Although the isolated thermophilic Fe(III)-reducing bacteria are phylogenetically closely related, some metabolic differences indeed exist among these isolates. Even though all cultures were obtained by enrichment in a medium with H_2 as a presumed chief energy source, only the isolate X514 was able to utilize hydrogen as an electron donor. Other isolates, including X513 and X561, were not able to use hydrogen as an electron donor. These isolates (X513 and X561) may be presumed to use citrate or yeast extract as an energy source. Also, some of these isolates appear to be more metabolically diverse than the isolates previously isolated from subsurface environments, such as *B. infernus* (4) and TOR-39 (58, 59, 60). All of these isolates are able to utilize acetate and succinate as electron donors. *B. infernus* could not use these substrates. Neither *B. infernus* nor TOR-39 could utilize hydrogen as an electron donor for growth or for the reduction of Fe(III) oxyhydroxides.

It has been proposed (6, 38) that prior to the development of life on Earth, there was production of Fe(III) and hydrogen by hydrothermal systems. The oxidation of hydrogen and organic matter coupled to the reduction of Fe(III) species carried out by *Thermoanaerobacter ethanolicus* provides a biological model for geochemical reactions on early Earth. Hydrogen gas was generated by geochemical processes in subsurface environments and probably constitutes a sustainable source of energy for a subsurface biosphere ecosystem today (42, 49). Studies in the past 2 decades have clearly shown that Fe(III)-reducing microorganisms can oxidize organic compounds to carbon dioxide with Fe(III) as an electron acceptor (25, 26, 27, 39). Carbon isotopic signatures suggest that the reduction of Fe(III) to magnetite in the banded iron formations may be linked to the oxidation of organic matter to carbon dioxide during the early Precambrian period (2, 38, 53, 54). Our findings of different thermophilic Fe(III)-reducing bacteria, collectively capable of using organic carbons as well as H_2 as electron donors, from geologically isolated, millions-of-years-old deep subsurface environments thousands of kilometers apart suggest that Fe(III) reduction and the production of magnetite might indeed be ancient biological processes. The existence of phylogenetically distinct thermophilic iron-reducing bacteria in diverse environments (5, 19, 48, 51) supports the hypothesis that iron-reducing bacteria exhibited an early form of microbial respiration (18, 25). The iron reduction and magnetite formation by thermophilic iron-reducing bacteria (X514) with hydrogen as an electron donor indicate that hydrogen can serve as an electron donor for Fe(III) reduction in deep subsurface environments. This knowledge may have implications for understanding the potential of life processes on extraterrestrial bodies, where subsurface environments might be capable of sustaining life under otherwise adverse contemporary conditions (34).

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