Microbial Reduction of Fe(III) in Acidic Sediments: Isolation of *Acidiphilium cryptum* JF-5 Capable of Coupling the Reduction of Fe(III) to the Oxidation of Glucose

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To evaluate the microbial populations involved in the reduction of Fe(III) in an acidic, iron-rich sediment, the anaerobic flow of supplemental carbon and reductant was evaluated in sediment microcosms at the in situ temperature of 12°C. Supplemental glucose and cellobiose stimulated the formation of Fe(II); 42 and 21% of the reducing equivalents that were theoretically obtained from glucose and cellobiose, respectively, were recovered in Fe(II). Likewise, supplemental H_2 was consumed by acidic sediments and yielded additional amounts of Fe(II) in a ratio of approximately 1:2. In contrast, supplemental lactate did not stimulate the formation of Fe(II). Supplemental acetate was not consumed and inhibited the formation of Fe(II). Most-probable-number estimates demonstrated that glucose-utilizing acidophilic Fe(III)-reducing bacteria approximated to 1% of the total direct counts of 4′,6-diamidino-2-phenylindole-stained bacteria. From the highest growth-positive dilution of the most-probable-number series at pH 2.3 supplemented with glucose, an isolate, JF-5, that could dissimilate Fe(III) was obtained. JF-5 was an acidophilic, gram-negative, facultative anaerobe that completely oxidized the following substrates via the dissimilation of Fe(III): glucose, fructose, xylose, ethanol, glycerol, malate, glutamate, fumarate, citrate, succinate, and H_2. Growth and the reduction of Fe(III) did not occur in the presence of acetate. Cells of JF-5 grown under Fe(III)-reducing conditions formed blebs, i.e., protrusions that were still in contact with the cytoplasmic membrane. Analysis of the 16S rRNA gene sequence of JF-5 demonstrated that it was closely related to an Australian isolate of *Acidiphilium cryptum* (99.6% sequence similarity), an organism not previously shown to couple the complete oxidation of sugars to the reduction of Fe(III). These collective results indicate that the in situ reduction of Fe(III) in acidic sediments can be mediated by heterotrophic *Acidiphilium* species that are capable of coupling the reduction of Fe(III) to the complete oxidation of a large variety of substrates including glucose and H_2.

Reclamation of surface mining of lignite in wide areas of Germany, Poland, and the Czech Republic has led to the formation of numerous acidic mine lakes (45). These lakes have high concentrations of sulfate and iron due to the oxidation of sulfide-containing minerals of the mine tailings (35). The oxidation of elemental sulfur and Fe(II) by chemolithotrophic acidophilic bacteria, such as species of *Thiobacillus* and *Leptospirillum*, has been extensively investigated (21, 40). However, reductive processes [e.g., the dissimilatory reduction of sulfate and Fe(III)] under acidic conditions have received relatively little attention (15, 20, 24, 49).

In pH-neutral aquifers and aquatic and marine sediments, the reduction of Fe(III) is regarded as an important process for the degradation of naturally occurring organic compounds and anthropogenic matter (10, 30). Although the capacity to reduce Fe(III) is widespread among neutrophilic bacteria, fermentative microorganisms do not effectively couple the reduction of Fe(III) to the oxidation of organic compounds (28). In contrast, bacteria that conserve energy via the reduction of Fe(III) utilize short-chain organic acids, H_2, or aromatic compounds; such organisms include species of *Shewanella*, *Pelobacter*, *Geobacter*, or *Desulfuromonas* (27, 28, 37, 40). Acetate is a key intermediate in anaerobic habitats and has been used

as a substrate for the isolation of Fe(III)-reducing organisms from sediments and aquifers (9, 12, 31).

High rates of Fe(III) reduction in iron-rich sediments of acidic coal mine lakes were recently reported (6, 16, 38). Under these low-pH conditions, the reduction of Fe(III) might be mediated by *Thiobacillus ferrooxidans* and *Thiobacillus thiooxidans*, which can use elemental sulfur as an electron donor (8, 21, 40, 41). Some heterotrophic acidophiles of the genus *Acidiphilium* also have the capacity to reduce Fe(III) under aerobic or anaerobic conditions (23, 24). To better understand the microbial populations potentially involved in the reduction of Fe(III) in these acidic sediments, (i) the effects of various electron donors on the reduction of Fe(III) were evaluated, (ii) the Fe(III)-reducing microflora was enumerated, and (iii) Fe(III)-reducing microorganisms of these sediments were isolated and characterized.

MATERIALS AND METHODS

Field site and sampling. Sediments were obtained from an acidic coal mine lake situated in the Lusatian mining area in east central Germany. The pH of the lake water and the maximum summer temperature of the upper sediment approximated to 3 and 12°C, respectively. No oxygen was detected at the water-sediment interface. The upper sediment zone (0 to 4 cm) was enriched with amorphous iron and contained no reduced sulfur components (FeS, FeS_2, and S^0) (38). Replicate sediment cores were collected in August 1996, February 1997, and August 1998 with a gravity corer in Plexiglas tubes (inner diameter, 5.9 cm), transported to the laboratory, and sectioned under an N_2 atmosphere within 24 h. Detailed descriptions of the site and the solid and the pore water phases of the sediment are given elsewhere (38).

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Preparation of sediment microcosms. Sediment from five replicate cores was pooled under anoxic conditions, and 40 g (fresh weight) of the upper iron-rich sediment was transferred to sterile 150-ml infusion bottles (Merck ABS, Dietikon, Switzerland) inside a Mecaplex anaerobic chamber (100% N_2 gas phase). Bottles were closed with rubber stoppers and screw-cap seals, flushed with sterile argon for 15 min, and incubated in the dark at 12°C with an initial overpressure of 20 to 25 kPa of argon at room temperature. Substrates were added as sterile stock solutions or as sterile gas.

Enumeration of the sediment microflora. Numbers of cultured cells were determined by the most-probable-number (MPN) technique with three replicates incubated at 15°C in various media as indicated; MPN values were calculated from standard MPN tables and were within 95% certainty (1). Total bacteria in sediments were enumerated by the 4′,6-diamidino-2-phenylindole (DAPI) procedure (39) as modified for sediment samples (7). The DAPI-stained bacteria were counted by using a Nikon Optiphot microscope equipped with an EF-D episcopic fluorescence attachment (Nippon Kogaku K.K., Tokyo, Japan).

Media and isolation. For culturing acidophilic Fe(III)-reducing bacteria, an acidic Fe-tryptone soya broth (Fe-TSB) medium (23) was used. The medium contained 0.025% TSB-basal salts at a pH of 2.5 and was supplemented with 5 mM glucose. The medium was boiled, cooled, and dispensed under N_2. Ferric sulfate was added from an anoxic 500 mM stock solution (pH 1.7, sterilized by membrane filtration [0.2-μm pore size]) to a concentration of 35 mM, leading to the partial formation of an orange precipitate that might be jarosite (4). The final pH approximated to 2.3. Reduction of Fe(III) was determined visually by the disappearance of the orange precipitate, the complete decolorization of the orange medium, and the formation of a white precipitate and also analytically by measuring the accumulation of Fe(II). For culturing pH-neutral Fe(III)-reducing bacteria, an FePP$_i$ medium (9) was used; the medium contained 2.5 g of NaHCO$_3$, 1.5 g of NH$_4$Cl, 0.6 g of KH$_2$PO$_4$, 0.1 g of KCl, and 0.5 g of yeast extract per liter; 10 ml of vitamins (13); and 10 ml of trace metals (13). Soluble ferric pyrophosphate [Fe$_4$(P$_2$O$_7$)$_3$, 3 g liter^{-1}] was added as the electron acceptor, and glucose (5 mM) was added as the electron donor. Alternative supplemental electron donors were cellobiose (3 mM), acetate (5 mM), and H$_2$ (15 mM). The gas phase was N_2-CO_2 (80:20); the final pH of the medium approximated to 6.7. Reduction of Fe(III) was determined visually by a change of the medium from yellow to colorless and the formation of a white precipitate and also analytically by measuring the accumulation of Fe(II). Enrichment cultures were streaked on solidified media supplemented with either 1% agarose for the acidic Fe-TSB medium or 1.5% agar for the pH-neutral FePP$_i$ medium. Isolated colonies were transferred to liquid medium and then sequentially restreaked three times on solidified medium. Cultures were considered to be pure based on uniform colony and cell morphologies. Unless otherwise indicated, isolates were cultivated at 30°C. Growth in media lacking Fe(III) was monitored as optical density at 660 nm with a Spectronic 501 spectrophotometer (Bausch and Lomb, Rochester, N.Y.). Growth of JF-5 in Fe-TSB medium was monitored by direct counting of cells in a Thoma chamber.

Analytical techniques. The rate of Fe(III) reduction by sediments was estimated by determining the amount of Fe(II) formed (44). Aliquots (0.2 ml) of the sediment were taken with sterile syringes, transferred to 9.8 ml of 0.5 N HCl, and incubated for 1 h at room temperature (29). Fe(II) was measured after the addition of acetate by the phenanthroline method (47). Total iron was determined with an inductively coupled plasma optical emission spectrometer (GBC Scientific Equipment, Melbourne, Victoria, Australia); Fe(III) was calculated as the difference between Fe(II) and total iron.

Headspace gases (H$_2$, O$_2$, CO$_2$, and CH$_4$) were measured with Hewlett-Packard Co. (Palo Alto, Calif.) 5980 series II gas chromatographs (26). Gas values were estimated by Henry's law and included the total amounts in both the liquid and the gas phases. To compare the amount of a specific gas (millimoles per bottle or tube filled with variable amounts of water depending on the experiment) with the concentration of a substrate consumed or of Fe(II) formed, the total amount of the gas for 1,000 ml of water to achieve a millimolar concentration was calculated. In this study, no distinction was made between CO$_2$ and its carbonate forms. Concentrations were corrected for the changing liquid-to-gas-phase volume ratio due to liquid samplings (0.7 ml). Aliphatic acids, aromatic compounds, alcohols, and sugars were determined with Hewlett-Packard 1090 series II high-performance liquid chromatographs (26). Nitrate and sulfate were analyzed by ion chromatography (26). Sediment pH was measured with an Ingold U457-S7/110 combination pH electrode.

Electron microscopy. Cells of JF-5 were cultivated at 30°C either anaerobically in Fe-TSB medium supplemented with glucose and various concentrations of Fe(III) (10, 35, and 65 mM) or aerobically in TSB medium lacking Fe(III) and supplemented with glucose. Cells were fixed by adding glutaraldehyde to a final concentration of 2% (vol/vol) and harvested by centrifugation. For negative staining (50), aqueous solutions of neutralized phosphotungstic acid (1% [wt/vol]) were used for visualizing the blebs on the cell periphery, whereas uranyl acetate (2% [wt/vol], pH 4.5) was adequate for visualizing the detached blebs. For thin-section preparations, pelleted cells were embedded in agar (2% [wt/vol]) and fixed in glutaraldehyde-OsO$_4$ (48). After being stained for 7 min with 2% uranyl acetate and, subsequently, for 5 min with lead citrate (43), specimens were examined in a model CEM 902A microscope (Zeiss, Oberkochen, Germany).

DNA isolation and determination of G+C content of DNA. The DNA was isolated by standard methods (11). The G+C content of the DNA was determined by high-performance liquid chromatography (34).

16S rRNA gene sequencing and phylogenetic analysis. Genomic DNA was extracted, amplified by PCR, and purified (42). Purified PCR products were sequenced by using an ABI PRISM Ready Reaction dye terminator kit (Applied Biosystems, Foster City, Calif.). Sequence reaction mixtures were electrophoresed with an Applied Biosystems model 373A DNA sequencer. Alignments of the sequence were done manually, and determinations of similarity values were done by using the ae2 editor (33). Accession numbers for sequences used for comparison of JF-5 with the closest relatives are as follows: *Acidiphilium cryptum* ATCC 33463^T, D30773 (25); *A. cryptum* B-Het4, X75265 (17); and *Acidiphilium organovorum* ATCC 443141^T, D30775 (25). The accession number of the sequence of the closest relative of CH-1 (*Clostridium butyricum* MMP3 DSM 2478) is X68177.

Nucleotide sequence accession number. The 16S rRNA gene sequence of strain JF-5 has been deposited in the EMBL database under accession no. Y18446. The accession numbers of *A. cryptum* DSM 2389^T and DSM 9467 are Y18445 and Y18447, respectively.

RESULTS

Effects of supplemental electron donors on the formation of Fe(II) in acidic sediments. In unsupplemented sediment microcosms, the concentration of Fe(II) increased with time at a rate of 232 μmol liter^{-1} day^{-1}. Since nitrate was negligible, and since sulfate (20 mM) was not consumed and methane was not produced, the reduction of Fe(III) appeared to be the main terminal electron-accepting process that was coupled to the oxidation of naturally occurring electron donors. In glucose-supplemented sediment microcosms, glucose was consumed without apparent delay and stimulated the formation of Fe(II) and CO$_2$ (Fig. 1A and data not shown). No fermentation products (e.g., short-chain organic acids, alcohols, or H$_2$) were detected during the consumption of glucose. Compared to controls lacking glucose, the production of CO$_2$ was enhanced from 5.3 to 12.8 mM. Forty-two percent of the reducing equivalents that were theoretically obtained from glucose were recovered in Fe(II). The pH of the sediment increased from 3.2 to 5.8 during the time in which glucose was consumed; in contrast, the pH of the control increased only slightly, from 3.2 to 3.6. In microcosms supplemented with cellobiose (2.8 mM), 21% of the reducing equivalents theoretically obtained from cellobiose were recovered in Fe(II). Cellobiose was initially converted to glucose. During the subsequent consumption of glucose, lactate, acetate, and propionate were detected in low concentrations (0.5, 1.9, and 1.2 mM, respectively) and the pH increased to 5.7.

When H$_2$ was added to sediment microcosms, H$_2$ was consumed concomitantly with the formation of Fe(II) (Fig. 1B). Sulfate was not consumed, and neither acetate nor methane was detected (data not shown). Approximately 93% of the reducing equivalents theoretically obtained from H$_2$ were recovered in Fe(II). The pH increased from 3.2 to 3.8. In microcosms supplemented with lactate (2 mM), small amounts of lactate were consumed (0.7 mM) and yielded acetate (0.4 mM) (data not shown). The formation of Fe(II) was not stimulated by lactate. In the presence of supplemental acetate, the formation of Fe(II) increased with a lower rate during the first 12 days of incubation than that of unsupplemented controls and then ceased completely (Fig. 1C). Acetate was not consumed over an incubation period of 140 days, and the final pH remained relatively stable at 3.3.

Enumeration of Fe(III) reducers. The above findings indicated that these acidic sediments contained microorganisms capable of coupling the reduction of Fe(III) to the oxidation of glucose and H$_2$. Since H$_2$ is a known substrate for neutrophilic Fe(III)-reducing microorganisms (27), Fe(III) reducers were enumerated both under acidic and under pH-neutral conditions. The cultured number of microorganisms capable of
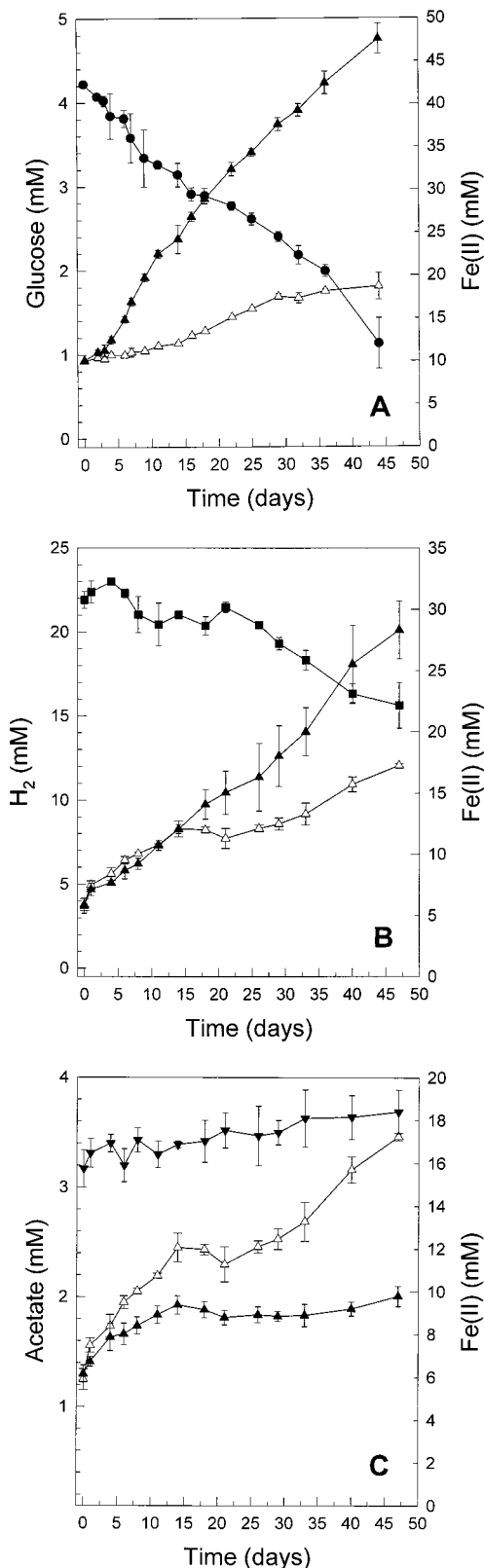
FIG. 1. Effect of supplemental electron donors on the formation of Fe(II). Sediment was supplemented with glucose (A), H_2 (B), or acetate (C) and incubated under an argon gas phase at 12°C. Presented are the averages (± standard deviations) of triplicate microcosms. Symbols: ●, glucose; ■, H_2; ▼, acetate; ▲, Fe(II); △, Fe(II) control.

TABLE 1. MPN values of Fe(III)-reducing microbes and total counts of DAPI-stained microbes obtained from acidic sediments[a]

Metabolic type	Medium	pH	MPN (g [wet wt] of sediment^{-1})[b]
Glucose-utilizing Fe(III) reducer	Fe-TSB	2.3	4×10^3 (8.6×10^2–1.9×10^4)
Glucose-utilizing Fe(III) reducer	FePP$_i$	6.7	2.3×10^3 (4.9×10^2–1.1×10^4)
H$_2$-utilizing Fe(III) reducer	FePP$_i$	6.7	<3
Acetate-utilizing Fe(III) reducer	FePP$_i$	6.7	<3
Total DAPI-stained microbes	NA[c]	NA	7.2×10^5 (5.8×10^5–8.6×10^5)

[a] MPN dilutions were incubated in three replicates at 15°C for 9 months.
[b] Values in parentheses represent the ranges of the MPN values within 95% certainty.
[c] NA, not applicable.

Fe(III) reduction with glucose as the electron donor approximated 4×10^3 (g [wet weight] of sediment^{-1}) in acidic Fe-TSB medium compared to 2.3×10^3 (g [wet weight] of sediment^{-1}) in pH-neutral FePP$_i$ medium (Table 1). In acidic Fe-TSB medium, supplemental glucose was partially consumed (up to 2 mM) and no fermentation products were detected. Fe(III) was totally reduced to Fe(II). In pH-neutral FePP$_i$ medium, the complete consumption of supplemental glucose (5 mM) yielded formate, acetate, propionate, and butyrate (2.0, 6.8, 1.6, and 1.3 mM, respectively), and approximately 3 mM Fe(II) was formed. The numbers of cultured acetate- or H$_2$-utilizing Fe(III) reducers in pH-neutral FePP$_i$ medium were negligible (Table 1). In FePP$_i$ medium, supplemental acetate was not consumed. Supplemental H$_2$ was consumed in FePP$_i$ medium; however, its consumption was coincident with the production of acetate. The average H$_2$-to-acetate ratio in all positive dilutions approximated to 4.3:1, a value indicative of H$_2$-dependent acetogenesis (13). The total number of DAPI-stained microbes in the sediment approximated to 7.2×10^5 (g [wet weight] of sediment^{-1}).

Isolation of JF-5. Repeated transfers from the highest growth-positive dilution of the MPN series in acidic Fe-TSB medium supplemented with glucose yielded a stable, Fe(III)-reducing, glucose-utilizing enrichment culture. No fermentation products were detected. On solidified Fe-TSB medium incubated under an N$_2$ gas phase, colonies appeared after 8 weeks simultaneously with a complete decolorization of the orange plates. Colonies were shiny, convex, and white with a slightly beige center and had a maximum diameter of 2 mm. One representative strain, JF-5, was selected for further characterization.

Morphological and physiological characteristics of JF-5. Cells of JF-5 were facultative anaerobic, motile, gram-negative (Fig. 2D), short rods (Fig. 2C). Electron microscopy revealed peritrichous inserted flagella. JF-5 did not grow aerobically on solidified glucose-supplemented TSB medium lacking Fe(III). In liquid glucose-supplemented aerobic TSB medium lacking Fe(III), growth appeared after a lag phase of 3 to 7 days. In general, the cultivation conditions in TSB medium appeared to favor growth under Fe(III)-reducing conditions. However, growth occurred rapidly and without a lag phase in an aerobic basal mineral salt solution supplemented with yeast extract (0.3 g liter^{-1}) (19). Neither growth nor the utilization of glucose was observed in anaerobic glucose-supplemented TSB medium lacking Fe(III). JF-5 grew at temperatures ranging from 12 to
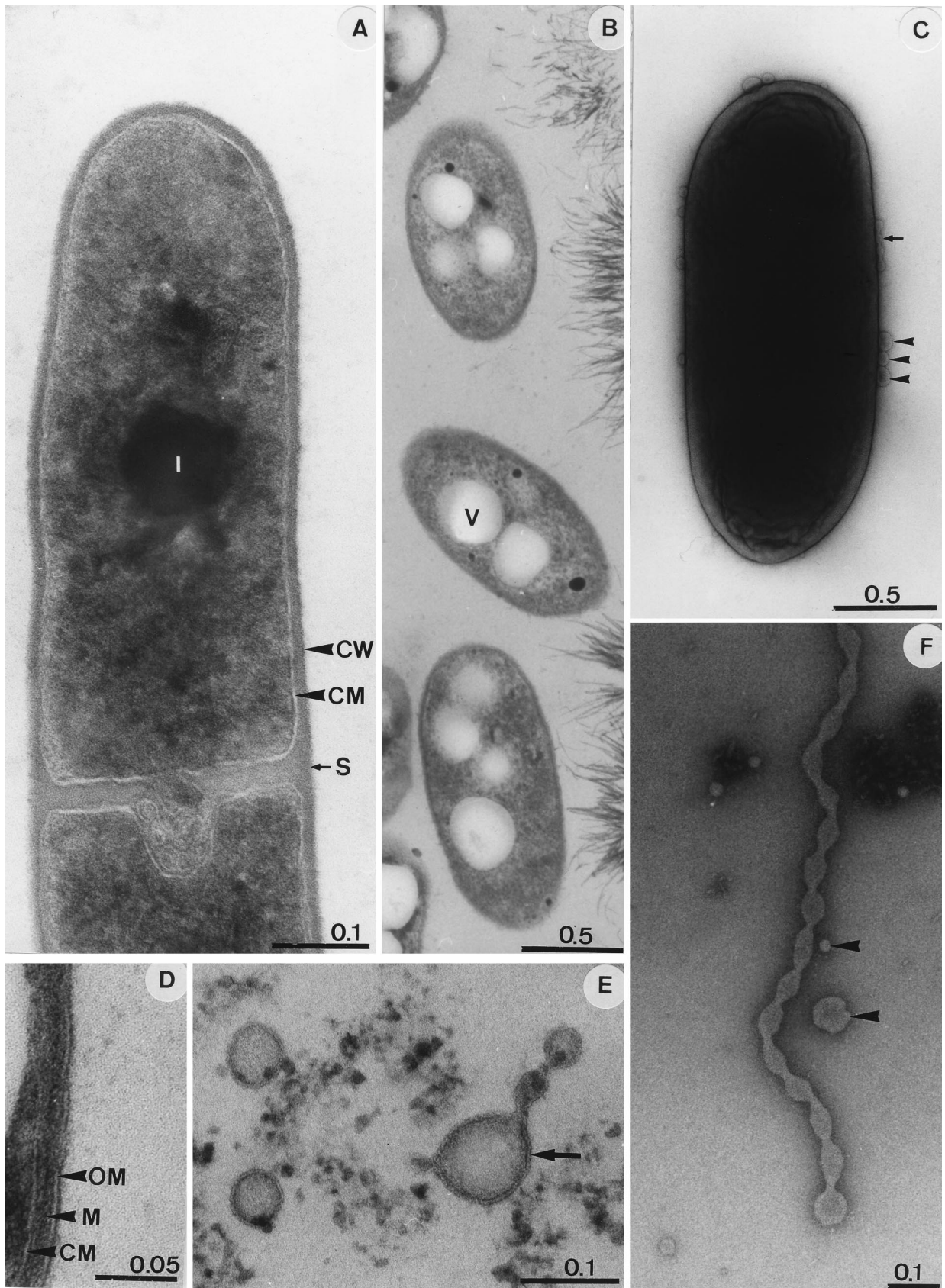
FIG. 2. Electron micrographs of strain JF-5 grown in aerobic TSB medium lacking Fe(III) (A) or in Fe-TSB medium (B to F). (A, B, and D to F) Thin-section micrographs. (C) Whole cell, negatively stained with 2% phosphotungstate. Single blebs (arrowheads) and a short "chain" consisting of four blebs (small arrow) are visible on the cell periphery. (D) Cell envelope of JF-5 with features of a gram-negative cell. (E) Micrograph showing that blebs and extrusions are membrane surrounded (arrow). (F) The long extrusion which originates from the cytoplasmic membrane reveals constrictions. Arrowheads indicate single free blebs of different sizes. Bar lengths are shown in micrometers. Abbreviations: CM, cytoplasmic membrane; CW, cell wall; I, inclusion body; S, septum (division point); M, murein layer; OM, outer membrane; V, vesicle.
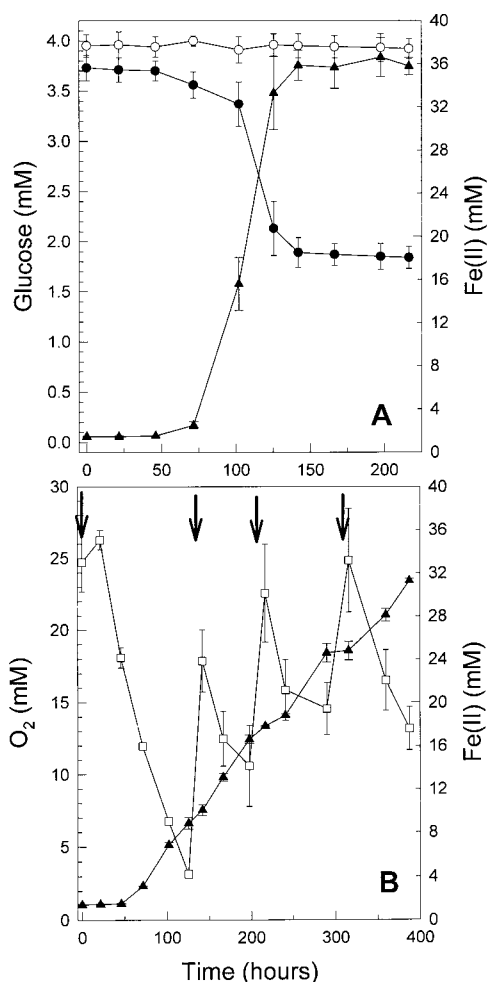
FIG. 3. Formation of Fe(II) and consumption of glucose by strain JF-5 incubated in Fe-TSB medium (A) and formation of Fe(II) and consumption of O_2 by strain JF-5 incubated in Fe-TSB medium supplemented with O_2 (B). O_2 and glucose were added at the indicated time intervals (arrows). Presented are the averages (± standard deviations) of triplicate experiments. Symbols: ●, glucose; ○, glucose-supplemented TSB medium lacking Fe(III); ▲, Fe(II); □, O_2.

37°C; no growth was observed at 5, 10, or 40°C. The optimal temperature was 35°C. Although no spores could be detected, growth and Fe(III) reduction were observed after heating cultures for 15 min at 80°C and for 5 min at 95°C. Under aerobic conditions, growth was observed over a pH range of 2.1 to 5.8; the pH optimum was 3.2.

Cells grown in Fe-TSB medium contained large intracellular vesicles (Fig. 2B); in comparison, cells grown in aerobic TSB medium lacking Fe(III) had a dense cytoplasm (Fig. 2A). Cells grown in Fe-TSB medium had blebs (i.e., protrusions still in

contact with the cytoplasmic membrane [46]) on the cell surface (Fig. 2C). Over 80% of the cells examined formed blebs. In contrast, cells grown in aerobic TSB medium lacking Fe(III) rarely formed blebs. The number of bleb-forming cells in Fe-TSB medium was independent of the amount of Fe(III) added (data not shown). In thin sections, projections and detached vesicles were also detected (Fig. 2F). Both the projections and detached vesicles were enclosed by a membrane (Fig. 2E). The morphological continuity between the cytoplasmic membrane, the blebs, and projections indicated that the membranes of the blebs and projections originated from the cytoplasmic membrane.

Oxidation of organic compounds coupled to the reduction of Fe(III) by JF-5. Substrate utilization and the reduction of Fe(III) by JF-5 were observed with the following substrates (in Fe-TSB medium): glucose, fructose, xylose, ethanol, glycerol, malate, glutamate, fumarate, citrate, and H_2. No fermentation products were detected. No Fe(III) reduction or substrate utilization was observed with lactose, cellobiose, lactate, formate, pyruvate, acetate, vanillate, or benzoate. JF-5 could not respire even low amounts of acetate (300 μM). In the presence of acetate (5 mM; 300 μM), growth and the reduction of Fe(III) were inhibited in glucose-, ethanol-, and H_2-supplemented cultures. In aerobic TSB medium lacking Fe(III), growth and substrate utilization were observed with glucose, glycerol, citrate, succinate, and malate but not with lactose, glutamate, fumarate, ethanol, and H_2. In aerobic TSB medium supplemented with nitrate (5 mM) and glucose, substrate utilization or growth was not observed.

In Fe-TSB medium supplemented with glucose, the consumption of 1.8 mM glucose was concomitant with the reduction of approximately 35 mM Fe(III) (Fig. 3A) and yielded an increase in cell numbers from 1.2×10^7 to 2.6×10^8 cells ml^{-1}, indicating that growth-supportive energy was conserved. Glucose oxidation, CO_2 formation, and Fe(III) reduction approximated the following stoichiometry: $C_6H_{12}O_6 + 24Fe(III) + 24OH^- \rightarrow 6CO_2 + 24Fe(II) + 18H_2O$ (Table 2). In aerobic Fe-TSB medium supplemented with glucose, the formation of Fe(II) was concomitant with the consumption of oxygen (Fig. 3B), indicating that oxygen and Fe(III) were corespired.

Phylogenetic analysis of the 16S rRNA gene and G+C content of JF-5. Phylogenetic analysis of the almost complete 16S rRNA gene sequence (93.3% of the *Escherichia coli* sequence) with the database of 16S rRNA gene sequences (33) indicated that JF-5 is a member of the alpha subclass of the *Proteobacteria*. The highest sequence similarity value (99.6%) was to an Australian isolate of *A. cryptum* (strain B-Het4 [17]). A sequence similarity value of 99.2% was obtained with the sequence of the type strain of *A. organovorum*, ATCC 43141[T]. The presence of almost identical 16S rRNA gene sequences in the type strains of *A. cryptum* and *A. organovorum* has been reported previously (25). The DNA base composition of strain JF-5 was 68.3 mol% G+C. The G+C content of the type strain of *A. cryptum*, ATCC 33463[T], is 69.8 mol% (19).

TABLE 2. Carbon and reductant balances obtained from glucose cultures of isolate JF-5[a]

Culture no.	Fe(III) added (mM)	Fe(II) formed[b] (mM)	Glucose consumed (mM)	CO_2 produced (mM)	Reductant obtained (mM)	Carbon recovered (%)	Reductant recovered (%)
1	65.2 ± 2.8	66.0 ± 1.3	2.7 ± 0.1	16.9 ± 0.8	64.8	104.3	101.9
2	35.0 ± 1.5	34.0 ± 0.8	1.6 ± 0.3	10.3 ± 1.3	38.4	107.3	88.5
3	10.0 ± 1.7	12.0 ± 1.1	0.6 ± 0.2	4.2 ± 0.9	14.4	116.7	83.3

[a] JF-5 was cultivated in triplicate in Fe-TSB medium supplemented with 4.0 mM glucose and various concentrations of Fe(III) (as indicated).
[b] Concentrations of Fe(II) were corrected for the concentration of Fe(II) present at the beginning of the experiment and for the concentration of Fe(II) formed in controls lacking glucose.

Enrichment and isolation of CH-1. Since sugar-fermenting microorganisms were present and possibly involved in the reduction of Fe(III) in acidic sediments, a stable cellobiose-fermenting, Fe(III)-reducing culture was enriched in pH-neutral FePP$_i$ medium. Isolate CH-1 was obtained on solidified FePP$_i$ medium. Colonies were surrounded by a decolorized zone and were white, smooth, irregular, and convex with a diameter of 3 to 4 mm. CH-1 was a strictly anaerobic, gram-labile, spore-forming rod (3 to 6 by 0.6 μm). Cellobiose (4.0 mM) was fermented to formate, acetate, butyrate, and H$_2$ (7.3, 3.3, 6.0, and 4.1 mM, respectively). Concomitant with the consumption of cellobiose, 4.4 mM Fe(II) was formed, indicating that approximately 2.3% of the cellobiose-derived reducing equivalents were utilized in the reduction of Fe(III). In the absence of cellobiose, no Fe(II) was formed. The reduction of Fe(III) was also coupled to the fermentation of glucose. CH-1 was able to grow at pH 4.4 but not at pH 3.9. Phylogenetic analysis of the 16S rRNA gene sequence indicated that CH-1 was 100.0% identical to *C. butyricum* MMP3 DSM 2478.

DISCUSSION

The geochemistry of this acidic sediment differs from that of other sedimentary environments in which Fe(III) reduction is a main terminal electron-accepting process (29, 30, 44). In these acidic coal mine lakes, Fe(III) is sedimented permanently at high rates, yielding an orange, fluffy sediment zone of reactive amorphous Fe(III)-(hydr)oxide which consists mainly of schwertmannite (38). Schwertmannite is a poorly crystallized Fe(III)-oxyhydroxysulfate (5) that can be formed in iron- and sulfate-rich waters (3, 4). Since the reactivity of Fe(III)-(hydr)oxides as electron acceptors for microbial processes is linked to their degree of crystallinity (29, 36, 40), schwertmannite might be easily degraded due to its amorphous structure. Sediments that contain high amounts of crystalline Fe(III)-(hydr)oxides are readily depleted of microbially reducible Fe(III) (29, 44). The linear rate of Fe(II) formation by sediments during the 44-day incubation period (Fig. 1A) implies that the availability of reducible Fe(III) was not a limiting factor. However, under in situ conditions, electron donors might be limited, because only very low quantities of autochthonous organic matter reach the water-sediment interface (38).

In most pH-neutral sedimentary Fe(III)-reducing environments, H$_2$ and acetate are expected to be major intermediates in the oxidation of fermentable organic substrates (12). *Geobacter* and *Shewanella* species which can metabolize these substrates and can be easily isolated from those sediments are thought to be important catalysts of Fe(III) reduction in sedimentary environments (12, 37). In sediment microcosms, supplemental H$_2$ stimulated the formation of Fe(II) in a 1:1.9 ratio (Fig. 1B). This ratio approximates the following stoichiometry: H$_2$ + 2Fe(III) → 2H$^+$ + 2Fe(II), indicating that the oxidation of H$_2$ was completely coupled to the reduction of Fe(III). Isolate JF-5 was obtained from the highest growth-positive dilution of the MPN series on the sediment and was capable of H$_2$ utilization at in situ pH. The number of cultured neutrophilic H$_2$-utilizing Fe(III) reducers was negligible. The capacity of mesophilic acidophiles to oxidize H$_2$ under aerobic conditions is also known for *T. ferrooxidans* (14).

Supplemental acetate was not consumed in sediment microcosms; indeed, acetate appeared to inhibit the formation of Fe(II) (Fig. 1C). Even low concentrations of acetate also inhibited growth and the reduction of Fe(III) by JF-5. The inhibitory effect of acetic acid on growth of acidophilic bacteria has been previously described (19). Under pH-neutral conditions in FePP$_i$ medium, supplemental acetate also did not

stimulate the reduction of Fe(III) in sediment MPN dilutions (Table 1). Thus, acetate does not appear to be an important electron donor for the reduction of Fe(III) in these acidic sediments. Lactate, a substrate for many Fe(III)-reducing organisms (27, 37), also did not stimulate the formation of Fe(II) in sediment microcosms and was not utilized by JF-5. These collective results indicate that the Fe(III)-reducing microorganisms present in this acidic sediment are distinct from most well-described neutrophilic nonfermentative Fe(III) reducers.

Fermentable substrates like glucose or cellobiose were consumed in sediment microcosms and stimulated the formation of Fe(II) (Fig. 1A and data not shown). Many sugar-fermenting microorganisms are capable of the reduction of Fe(III) (28). However, the amounts of reducing equivalents usually recovered in Fe(II) are in a range of 0.03 to 3%, demonstrating that the reduction of Fe(III) is only a minor pathway for these microorganisms (28). The fermentative isolate CH-1 transferred approximately 2.3% of the reducing equivalents obtained from cellobiose or glucose to Fe(III). The 16S rRNA gene sequence of CH-1 was identical to that of *C. butyricum*, which is known to have the capacity to reduce Fe(III) (18). In sediment microcosms, however, high amounts of reducing equivalents obtained from glucose were recovered in Fe(II), suggesting that nonfermentative processes were also involved in the reduction of Fe(III). This observation is contradictory to other studies on glucose metabolism in Fe(III)-reducing sediments that demonstrate that sugars are utilized in a microbial food chain by the combined activity of fermentative bacteria and fatty acid-oxidizing Fe(III)-reducing bacteria (28, 32). To date, the complete oxidation of sugars to CO$_2$ with Fe(III) as the sole electron acceptor by a pure culture has not been clearly demonstrated (23, 28). The nonfermentative isolate JF-5 readily reduced Fe(III) via the oxidation of glucose, with 83 to 102% of the glucose-derived reducing equivalents being recovered in Fe(II) (Table 2).

On the basis of the 16S rRNA gene sequence similarity, JF-5 was closely related to an Australian isolate of *A. cryptum* that was isolated from an acidic leaching environment (17). In general, species of *Acidiphilium* are described as being obligate aerobes; however, some isolates of *Acidiphilium*, including *A. cryptum*, have the capacity to reduce Fe(III) under aerobic or microaerophilic conditions (21–23, 40). In the absence of oxygen, the growth of *Acidiphilium* strain SJH is coupled to the reduction of Fe(III) in glucose-TSB liquid medium; however, stoichiometries for glucose consumption and Fe(III) reduction for this strain have not been determined (23). Indeed, the general capacity of *Acidiphilium* species to completely oxidize organic substrates to CO$_2$ via the reduction of Fe(III) has not been adequately resolved.

In contrast to *A. cryptum* and other species of acidophilic heterotrophs that are heat sensitive (19, 23), JF-5 grew after pasteurization without delay and could survive boiling. The formation of blebs by JF-5 (Fig. 2C) has not been described for *A. cryptum* (19). In general, blebs that originate from the cytoplasmic membrane have not been observed in other gram-negative bacteria (46). The formation of numerous blebs and the appearance of intracellular vesicles under Fe(III)-reducing conditions appear to be characteristic features of JF-5. The function of these blebs is not resolved. It can be speculated that an enlargement of a cytoplasmic membrane might enhance cellular contact in order for the cell to get into contact with nonsoluble Fe(III) hydroxides that are utilized as electron acceptors. The capacity of JF-5 and other *Acidiphilium* species to corespire O$_2$ and Fe(III) (23) is dissimilar to that of other facultative Fe(III)-reducing organisms (e.g., *Shewanella* species) that do not significantly reduce Fe(III) until O$_2$ is completely

removed (2). Under acidic conditions, substantial amounts of dissolved Fe(III) are available in solution, and the redox potential for the reduction of soluble Fe(III) to soluble Fe(II) ($+0.77$ V) is close to the redox potential for the reduction of O_2 to H_2O ($+0.82$ V) (28, 37). *Shewanella* does not grow at low pH values, and the redox potential for the reduction of solid Fe(III)-(hydr)oxides is much lower than that of soluble Fe(III). Thus, the small difference in these redox potentials might facilitate the use of soluble Fe(III) as an alternative electron acceptor even in the presence of the energetically more favorable O_2. The capacity of *Acidiphilium* species to utilize a variety of substrates and to reduce Fe(III) both in the presence and in the absence of oxygen indicates that they might be of ecological significance in the turnover of iron at oxic-anoxic interfaces in acidic sediments.

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