APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 1988, p. 1472–1480
0099-2240/88/061472-09$02.00/0

Vol. 54, No. 6

Novel Mode of Microbial Energy Metabolism: Organic Carbon Oxidation Coupled to Dissimilatory Reduction of Iron or Manganese

DEREK R. LOVLEY* AND ELIZABETH J. P. PHILLIPS

U.S. Geological Survey, Water Resources Division, 432 National Center, Reston, Virginia 22092

Received 1 February 1988/Accepted 17 March 1988

A dissimilatory Fe(III)- and Mn(IV)-reducing microorganism was isolated from freshwater sediments of the Potomac River, Maryland. The isolate, designated GS-15, grew in defined anaerobic medium with acetate as the sole electron donor and Fe(III), Mn(IV), or nitrate as the sole electron acceptor. GS-15 oxidized acetate to carbon dioxide with the concomitant reduction of amorphic Fe(III) oxide to magnetite (Fe_3O_4). When Fe(III) citrate replaced amorphic Fe(III) oxide as the electron acceptor, GS-15 grew faster and reduced all of the added Fe(III) to Fe(II). GS-15 reduced a natural amorphic Fe(III) oxide but did not significantly reduce highly crystalline Fe(III) forms. Fe(III) was reduced optimally at pH 6.7 to 7 and at 30 to 35°C. Ethanol, butyrate, and propionate could also serve as electron donors for Fe(III) reduction. A variety of other organic compounds and hydrogen could not. MnO_2 was completely reduced to Mn(II), which precipitated as rhodochrosite ($MnCO_3$). Nitrate was reduced to ammonia. Oxygen could not serve as an electron acceptor, and it inhibited growth with the other electron acceptors. This is the first demonstration that microorganisms can completely oxidize organic compounds with Fe(III) or Mn(IV) as the sole electron acceptor and that oxidation of organic matter coupled to dissimilatory Fe(III) or Mn(IV) reduction can yield energy for microbial growth. GS-15 provides a model for how enzymatically catalyzed reactions can be quantitatively significant mechanisms for the reduction of iron and manganese in anaerobic environments.

Geochemical evidence suggests that the primary terminal electron acceptors for organic matter decomposition in anaerobic sediments are nitrate, Mn(IV), Fe(III), sulfate, and carbon dioxide (40). It is frequently reported that there are distinct zones in sediments in which the metabolism of organic matter is coupled to the reduction of only one of these electron acceptors at any one time (13, 17, 39, 40). Microorganisms or microbial consortia which can completely metabolize organic matter to carbon dioxide with nitrate (36, 48) or sulfate (8, 37, 45) as the sole electron acceptor have been isolated, as have consortia which can completely convert organic matter to carbon dioxide and methane (54). Microorganisms which can completely oxidize organic matter with Fe(III) or Mn(IV) as the sole electron acceptor have not been previously reported.

Numerous bacteria which reduce Fe(III) during growth on organic substrates have been described (23). However, quantitative studies on electron donor metabolism and Fe(III) reduction (18, 19, 31, 32, 41) have suggested that previously described Fe(III)-reducing organisms use Fe(III) reduction as a minor pathway for the disposal of electron equivalents (23). Fermentation is their major mode of metabolism. In a similar manner, previously described Mn(IV)-reducing isolates (11, 31, 32, 50) also appear to have a primarily fermentative metabolism. It can be calculated from the data of those studies that less than 5% of the reducing equivalents in the glucose metabolized were transferred to Mn(IV) reduction. It has recently been reported that *Alteromonas putrefaciens* can reduce Fe(III) (42) or Mn(IV) (C. R. Myers and K. H. Nealson, Science, in press) during anaerobic growth. However, lactate is the only organic electron donor known to support iron reduction in this organism, and lactate is only incompletely oxidized to acetate (42; unpublished data). Thus, previously described Fe(III)- and Mn(IV)-reducing organisms cannot account for the complete oxidation of organic matter coupled to Fe(III) or Mn(IV) reduction because their metabolism results in the accumulation of fermentation products.

Environments in which microorganisms oxidize organic matter with Fe(III) or Mn(IV) as the sole electron acceptor can only be established if the microorganisms gain energy for maintenance and growth from these reactions. Fe(III) or Mn(IV) reduction may be linked to electron transport chains in some organisms (2, 9, 10, 14, 18, 22, 34, 35, 43, 50), but none of these organisms has been shown to derive energy for growth from Fe(III) or Mn(IV) reduction. The addition of Fe(III) did increase the growth yield of a malate-fermenting *Vibrio* sp. (19). However, most of the increased cell yield had to be attributed to some factor other than Fe(III) reduction because the amount of Fe(III) reduced could have accounted for, at most, a minor increase in cell yield (19). The finding that a *Pseudomonas* sp. could grow by coupling the oxidation of hydrogen to the reduction of Fe(III) (3) does demonstrate that energy for microbial growth can be obtained from dissimilatory Fe(III) reduction.

We are studying the microbially catalyzed reduction of Fe(III) and Mn(IV) in near surface and deep subsurface sedimentary environments to more fully understand the factors influencing the geochemistry of iron and manganese in aquatic systems. Previous studies suggested that sediments of the Potomac River contain microorganisms which completely oxidize organic matter to carbon dioxide with the reduction of Fe(III) or Mn(IV) (25, 26, 28; D. R. Lovley and E. J. P. Phillips, submitted for publication). Here we describe an isolate from these sediments which grows under anaerobic conditions by oxidizing organic compounds to carbon dioxide with Fe(III), Mn(IV), or nitrate as the sole electron acceptor.

MATERIALS AND METHODS

Source of organism. As previously described (25), freshwater sediments from the Potomac River, Maryland, were

* Corresponding author.

used to start an enrichment culture with acetate and yeast extract as potential electron donors and amorphic Fe(III) oxide as the electron acceptor. The location of this site and various aspects of Fe(III) reduction in these sediments have been discussed previously (25–28).

Anaerobic technique. Standard anaerobic techniques (4, 15, 30) were used throughout. Gases were passed through a column of hot reduced copper filings to remove traces of oxygen. All transfers and samplings of the cultures were performed with syringes and needles that had been flushed with oxygen-free gas or were performed with a transfer loop or needle under a stream of oxygen-free gas.

Media. As previously specified (25), enrichments were started in freshwater enrichment medium with the following constituents (in grams per liter of deionized water): $NaHCO_3$, 2.5; $CaCl_2 \cdot 2H_2O$, 0.1; KCl, 0.1; NH_4Cl, 1.5; $NaH_2PO_4 \cdot H_2O$, 0.6; NaCl, 0.1; $MgCl_2 \cdot 6H_2O$, 0.1; $MgSO_4 \cdot 7H_2O$, 0.1; $MnCl_2 \cdot 4H_2O$, 0.005; $NaMoO_4 \cdot 2H_2O$, 0.001; $NaCH_3COO$, 2.7; and yeast extract (BBL Microbiology Systems, Cockeysville, Md.), 0.05. Fe(III) was provided in the form of amorphic Fe(III) oxide at ca. 250 mmol of Fe(III) per liter of medium. The amorphic Fe(III) oxide was synthesized by neutralizing a solution of $FeCl_3$ as previously described (25). The gas phase was N_2-CO_2 (80:20).

FWA-Fe(III) medium contained the following constituents (in grams per liter of deionized water): $NaHCO_3$, 2.5; $CaCl_2 \cdot 2H_2O$, 0.1; KCl, 0.1; NH_4Cl, 1.5; $NaH_2PO_4 \cdot H_2O$, 0.6; and $NaCH_3COO$, 6.8. Vitamins and trace minerals were added from stock solutions (24). The medium contained ca. 200 mmol of Fe(III) per liter in the form of amorphic Fe(III) oxide. The gas phase was N_2-CO_2 (80:20). The pH of the autoclaved medium was ca. 6.7.

For studies on the stoichiometry of acetate metabolism coupled to Fe(III) reduction, the organism was grown in a modified FWA-Fe(III) medium in which the $NaHCO_3$ was omitted and the gas phase was N_2. The pH of the autoclaved medium was ca. 7.

FWA-Fe(III)–citrate medium had the same constituents as the FWA-Fe(III) medium with the exception that the amorphic Fe(III) oxide was replaced with 20 mM Fe(III)-citrate and the $CaCl_2 \cdot 2H_2O$ was deleted.

FWA-Mn(IV) medium had the same constituents as FWA-Fe(III) medium with the exception that the amorphic Fe(III) oxide was omitted and ca. 15 mmol of Mn(IV) per liter of medium were provided as MnO_2. The MnO_2 was synthesized by slowly adding a solution of $MnCl_2$ (30 mM) to a basic solution of $KMnO_4$ (20 mM) which was stirred with a magnetic stir bar. This procedure is similar to a previously described technique (33) which yields a poorly crystalline MnO_2 similar to the naturally occurring MnO_2 mineral, birnessite. The MnO_2 that was formed was allowed to settle to the bottom of the beaker and then washed with deionized water by centrifugation until the chloride in the associated water was less than 1 mM.

FWA-NO_3 medium had the same constituents as FWA-Fe(III) medium with 20 mM $NaNO_3$ replacing amorphic Fe(III) oxide as the electron acceptor. For liquid FWA-NO_3, the 0.1 g of $CaCl_2 \cdot 2H_2O$ per liter was omitted. In studies on the stoichiometry of ammonia production, the NH_4Cl was omitted from the medium. Studies to determine whether the organism produced N_2 during nitrate reduction were conducted with He-CO_2 (80:20) replacing the N_2-CO_2 gas phase. The medium to develop the inoculum for the stoichiometry studies with MnO_2 as the electron acceptor contained 10 mM acetate and 7 mM nitrate.

For most studies, 10 ml of medium was dispensed in anaerobic pressure tubes (Bellco Glass, Inc., Vineland, N.J.) and bubbled for at least 6 min with the appropriate gas phase to remove dissolved oxygen. For the studies on the stoichiometry of acetate metabolism with the reduction of amorphic Fe(III) oxide, 100 ml of medium was dispensed into 120-ml serum bottles and bubbled for at least 15 min with N_2. Although no reducing agent was added to the medium, the Fe(II) that was transferred with the inoculum would have consumed any traces of oxygen that were not removed by flushing with oxygen-free gas (29). The tubes or bottles were capped with butyl rubber stoppers (Bellco Glass) and an aluminum crimp. Agar slants of the media were made in agar streak tubes (Bellco Glass) by adding a final concentration of 1.5% (wt/vol) purified agar (Difco Laboratories, Detroit, Mich.) to the appropriate media.

The incubation temperature for the enrichment culture was 30°C. The organism was isolated with incubations at 35°C. All subsequent studies were done at 33°C unless otherwise specified.

Fe(III) forms. Synthetic Fe(III) forms were synthesized or purchased as previously described (26). The naturally formed amorphic Fe(III) oxide was generated when an anaerobic Fe(II)-rich groundwater was exposed to oxygen upon reaching the surface. The material was kindly provided by David Parkhurst of the U.S. Geological Survey. The Fe(III) forms were added to FWA-Fe(III) medium with the amorphic Fe(III) oxide replaced with the Fe(III) forms at ca. 200 mmol of Fe(III) per liter of medium.

Time course measurements of acetate metabolism. For studies of acetate metabolism coupled to Fe(III) or nitrate reduction, the cultures were anaerobically sampled with a syringe and needle and analyzed as outlined below for acetate and either Fe(III) and Fe(II) or nitrate and ammonia as appropriate. With cultures reducing MnO_2, the formation of a precipitate of $MnCO_3$ made it difficult to reproducibly subsample the culture for Mn(II) determinations. Therefore, initial concentrations of Mn(IV), Mn(II), and acetate in 12 replicate tubes of FWA-Mn(IV) medium were determined as outlined below. At each time point, samples from three tubes were taken for acetate and cell counts, and then these tubes were used for estimates of Mn(II) concentrations.

Cell growth was monitored in nitrate-reducing cultures by directly inserting the culture tubes into a Spectronic 20 spectrophotometer and measuring the A_{540}.

Cell growth in Fe(III)- and Mn(IV)-reducing cultures was monitored by direct cell counts. In Fe(III)-reducing cultures with either amorphic Fe(III) oxide or Fe(III)-citrate, samples (1 ml) of the cultures were removed over time and fixed with glutaraldehyde (2.5% final concentration). A particle-free oxalate solution (8.9 ml of 28 g of ammonium oxalate and 15 g of oxalic acid per liter) was added to dissolve the iron forms. The iron was extracted for at least 15 min with occasional mixing on a Vortex mixer. A subsample (1 ml) was passed through a Nuclepore filter (0.2-μm pore diameter). The cells on the filter were progressively dehydrated with solutions containing 20, 50, 75, 90, and 100% ethanol in water; 50% ethanol in amyl acetate; and then amyl acetate alone. The filter was mounted on an aluminum stub, critical point dried, and coated with gold. The filters were examined at ×3,000 with a JEOL JSM 840 scanning electron microscope.

Cell counts in Mn(IV)-reducing cultures were made by epifluorescence microscopy. Samples (0.3 ml) were fixed with glutaraldehyde (2.5% final concentration), and 5 ml of 0.25 N hydroxylamine hydrochloride in 0.25 N HCl was added to dissolve solid manganese. A subsample (0.1 to 0.5

ml) was diluted with particle-free medium to give a final volume of 2 ml. A particle-free acridine orange solution (0.2 ml) was added to give a final acridine orange concentration of 0.01%. After 2 min, the sample was filtered onto a black Nuclepore filter (0.2-μm pore diameter). The filters were observed under oil immersion (×1,000) with a Zeiss microscope.

To measure production of carbon dioxide, we quantified the amount of carbon dioxide in the headspace of the culture and the total dissolved inorganic carbon at an initial and final time point in five cultures. Dissolved inorganic carbon was measured by removing a subsample (1 ml) and injecting it into an N_2-filled anaerobic pressure tube containing 0.25 ml of 85% phosphoric acid. The carbon dioxide released was allowed to equilibrate with the headspace and then quantified. Carbon dioxide was measured by gas chromatography as outlined below.

Analytical techniques. With the exception of the stoichiometric studies on acetate metabolism with amorphic Fe(III) oxide, Fe(II) production was monitored by measuring the accumulation of HCl-soluble Fe(II) over time. As previously described in detail (26), the amount of Fe(II) that was soluble after a 15-min extraction in 0.5 N HCl was determined with ferrozine.

For the stoichiometric studies on acetate metabolism with amorphic Fe(III) oxide, Fe(III) and Fe(II) were determined by a modification of the anaerobic oxalate extraction method (38). Subsamples (ca. 0.1 ml) of the culture were injected into preweighed serum bottles (10-ml capacity) that were covered with aluminum foil to exclude light and had an N_2 headspace. The weight of the added culture was determined. The oxalate extractant (5 ml) was added, and after 30 min, the oxalate-extractable Fe(III) and Fe(II) were determined as previously described (38).

Mn(II) production was estimated by measuring the accumulation of manganese soluble in 0.5 N HCl over time. For the initial time point, subsamples (ca. 0.1 ml) were removed from the tubes and placed into preweighed vials containing 5 ml of 0.5 N HCl. The weight of the added sample was determined. After 10 min, the sample was filtered (Nuclepore filter, 0.2-μm pore diameter) and the manganese concentration was determined by atomic absorption spectrophotometry with an acetylene flame. For subsequent time points, concentrated HCl (0.4 ml) was added to replicate culture tubes to give a final HCl concentration of 0.5 N. After 10 min, the acidified culture medium was filtered (Nuclepore filter, 0.2-μm pore diameter) and the manganese concentration in the filtrate was determined. $MnCO_3$, which was the primary form of Mn(II) in the cultures, was completely dissolved by the HCl, and less than 10% of the MnO_2 was extracted by the treatment. The initial concentration of Mn(IV) in the culture tubes was determined by taking subsamples (ca. 0.1 ml), dissolving the Mn(IV) in a solution (5 ml) of 0.25 N hydroxylamine hydrochloride in 0.25 N HCl, and measuring the manganese content.

Acetate, nitrate, nitrite, and ammonia analyses were conducted on filtrates (Gelman filter, 0.45-μm pore diameter) of the cultures. Acetate concentrations were determined by gas chromatography as previously described (25). Nitrate and nitrite concentrations were determined by high-performance liquid chromatography. The ions were separated on a Partisil PXS 10/25 SAX column (Whatman Inc., Clifton, N.J.) with 50 mM phosphate buffer (pH 3.0) as the eluant at a flow rate of 2 ml/min. Nitrate and nitrite were detected by A_{190}. Samples for ammonia determinations were diluted 200-fold and analyzed by the phenol-hypochlorite method (46) with the modification that color was allowed to develop overnight, rather than for 1 h.

Carbon dioxide and N_2 were quantified with a thermal conductivity detector. The gases were separated with a 3-m stainless-steel column of Porapak N with helium (20 ml/min) as the carrier gas at 110°C (carbon dioxide analyses) or 30°C (N_2 analyses).

RESULTS

Enrichment and isolation. The previously described anaerobic enrichment culture (25), in which acetate and yeast extract were potential electron donors and amorphic Fe(III) oxide was provided as an electron acceptor, actively reduced Fe(III) when transferred into FWA-Fe(III) medium. The enrichment was purified by twice diluting it in FWA-Fe(III) medium and selecting the highest dilution that reduced Fe(III) (10^{-6} or 10^{-7}) for further transfers. Only rod-shaped organisms (2 by 0.5 μm) were observed by phase-contrast microscopy of this purified culture. Attempts to grow the organism on agar slants of FWA-Fe(III) medium were unsuccessful. However, when the culture was streaked on agar slants of FWA-NO_3 medium, small red colonies were visible within 14 days of incubation. An isolated colony was subcultured on agar slants of FWA-NO_3 medium. Based on the uniformity of cell and colony morphology in repeated subcultures and on tests (outlined below) for growth in various media, the isolate was judged to be a pure culture of an organism with an obligately anaerobic respiratory metabolism. The isolate was designated Geological Survey-15, abbreviated GS-15. GS-15 is a gram-negative rod that is 2 to 4 by 0.5 μm (Fig. 1). Motility or spores have not been observed.

When FWA-Fe(III) medium was inoculated with GS-15, the Fe(III) was reduced to Fe(II) (Fig. 2 and 3). There was no Fe(III) reduction if the FWA-Fe(III) medium was not inoculated with GS-15 or if the inoculated medium was heat killed by pasteurization (80°C, 15 min) before incubation. No Fe(III) was reduced if physical contact between GS-15 and the amorphic Fe(III) oxide was prevented by placing the amorphic Fe(III) oxide within dialysis tubing. Cell-free filtrates of actively growing cultures did not reduce Fe(III). The temperature optimum for Fe(III) reduction was 30 to 35°C, with no detectable Fe(III) reduction at 50°C or at temperatures of 10°C or lower (Fig. 2). In FWA-Fe(III) medium with the bicarbonate omitted and only N_2 in the gas phase, Fe(III) reduction was observed in medium with an initial pH of 7 but not in medium at pH 5, 6, 8, or 9. The results summarized in this paragraph indicate that GS-15 enzymatically catalyzed Fe(III) reduction, but only when there was direct contact between GS-15 and the Fe(III).

Acetate metabolism and growth with Fe(III) reduction. GS-15 was transferred more than 50 times (inoculum of 10% or less) through FWA-Fe(III) medium with continued active Fe(III) reduction. This demonstrated that GS-15 was reproducing in the FWA-Fe(III) medium.

The growth of GS-15 corresponded with the oxidation of acetate to carbon dioxide and the concomitant reduction of Fe(III) to Fe(II) (Fig. 3; Table 1). The reported cell numbers in the later stages of growth may be underestimates. The cells aggregated extensively as the culture grew, and this made counting the individual cells difficult, even by scanning electron microscopy. Cell numbers decreased dramatically after Fe(III) reduction stopped.

As previously described (29), the Fe(II) resulting from the reduction of amorphic Fe(III) oxide was primarily in the

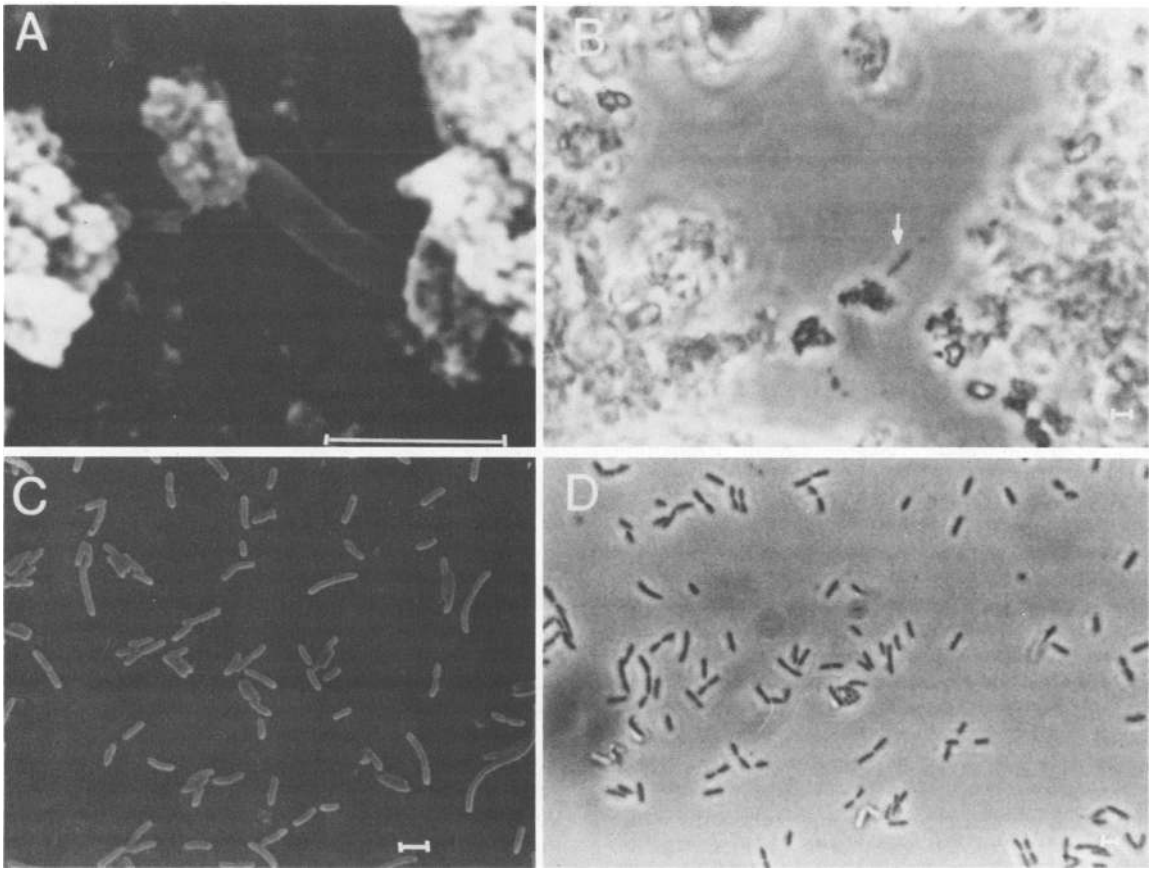

FIG. 1. (A and C) Scanning electron micrographs of GS-15 collected on Nuclepore filters (0.2-μm pore diameter) after growth on FWA-Fe(III) (A) or FWA-Fe(III)–citrate (C) medium. (B and D) Phase-contrast micrographs of GS-15 in FWA-Fe(III) (B) or FWA-Fe(III)–citrate (D) medium. Bars, 1 μm.

form of extracellular, ultrafine-grained magnetite. Magnetite (Fe_3O_4) is a mixed ferric-ferrous mineral ($Fe^{+3}Fe^{+3}Fe^{+2}O_4$). GS-15 could not reduce the Fe(III) contained in magnetite. Thus, only a third of the Fe(III) initially added as amorphic Fe(III) oxide could be reduced to Fe(II) even if the

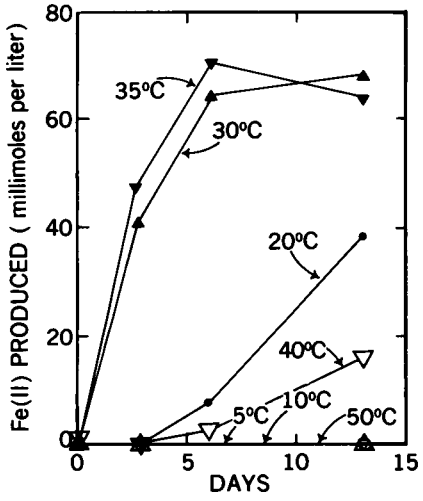

FIG. 2. Fe(III) reduction by GS-15 in FWA-Fe(III) medium incubated at various temperatures.

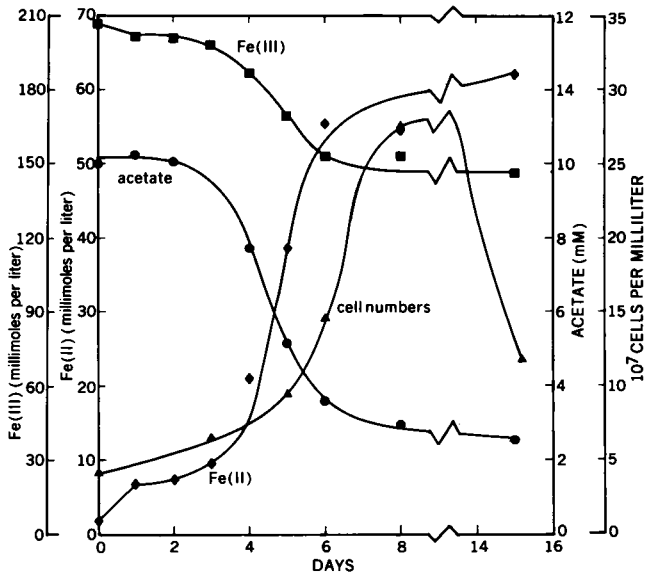

FIG. 3. Concentrations of acetate, cell numbers, and oxalate-extractable Fe(III) and Fe(II) over time in FWA-Fe(III) medium inoculated with GS-15 that had been grown in FWA-Fe(III) medium.

TABLE 1. Stoichiometry of Fe(II) and carbon dioxide production during acetate metabolism by GS-15 with amorphic Fe(III) oxide as the electron acceptor

Culture	Acetate consumed (μmol)	Carbon dioxide produced (μmol)	Fe(II) produced (μmol)	% of expected Fe(II) produced[a]
1	728	1,395	5,520	99
2	729	1,515	6,018	99
3	798	1,312	5,950	113
4	665	1,130	4,385	97
5	497	924	4,085	111

[a] Computed as: [moles of Fe(II) produced/(moles of carbon dioxide produced × 4)] × 100.

initial acetate concentration was increased to 50 mM (Fig. 3; data not shown).

The stoichiometry of acetate consumption and production of Fe(II) and carbon dioxide (Fig. 3; Table 1) indicate that for each mole of acetate oxidized to carbon dioxide, 8 mol of Fe(III) were reduced to Fe(II), with the subsequent incorporation of the Fe(II) along with 16 mol of Fe(III) to form 8 mol of magnetite (Table 2, reaction 1). A small portion of the acetate consumed was probably incorporated into cellular constituents.

When Fe(III) was provided in a soluble form as Fe(III)-citrate (Fig. 4), GS-15 grew more rapidly than with amorphic Fe(III) oxide (Fig. 3). The faster growth rate could not be attributed to citrate serving as an electron donor for Fe(III) reduction because GS-15 did not grow or reduce Fe(III) in FWA-Fe(III)–citrate medium which did not contain acetate. Furthermore, the stoichiometry of acetate consumption and Fe(II) production (see below) indicated that acetate was the sole electron donor for Fe(III) reduction. With Fe(III)-citrate, no magnetite was formed and all the Fe(III) was reduced to Fe(II). During the course of Fe(III) reduction, the FWA-Fe(III)–citrate medium changed color from amber to green. Approximately 1 day after the completion of Fe(III) reduction, a white precipitate formed and the medium became clear. X-ray diffraction analysis indicated that the white precipitate was vivianite, $Fe_3(PO_4)_2 \cdot 8H_2O$.

The stoichiometry of acetate consumption and Fe(II) accumulation was consistent with reaction 2 (Table 2) in which 1 mol of acetate is oxidized to 2 mol of carbon dioxide with the reduction of 8 mol of Fe^{3+} to 8 mol of Fe^{2+}. Of course, the actual reaction was more complex than written, as some of the acetate metabolized must have been used for cell synthesis, Fe(III) was complexed with citrate, and Fe(II) was in various forms.

Reduction of various Fe(III)-containing minerals. In addition to synthetic amorphic Fe(III) oxide and Fe(III)-citrate, GS-15 readily reduced a natural amorphic Fe(III) oxide form. However, more crystalline ferric oxides were poorly reduced even with extended incubation (Table 3).

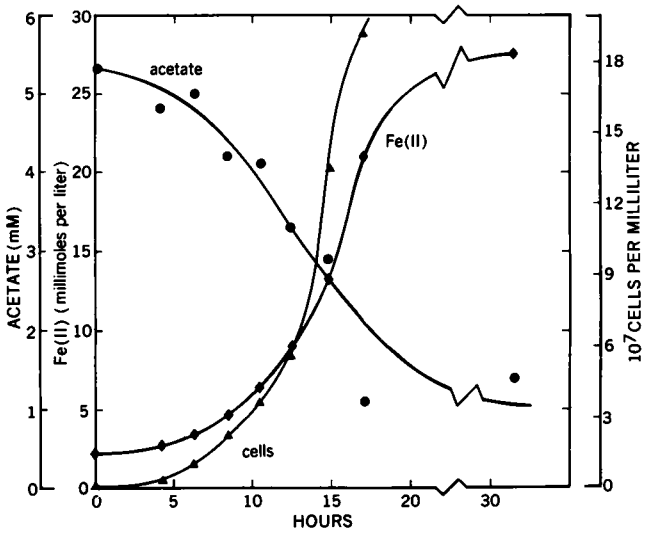

FIG. 4. Concentrations of acetate, cell numbers, and Fe(II) over time in FWA-Fe(III)–citrate medium inoculated with GS-15 that had been grown in FWA-Fe(III)–citrate medium.

Acetate metabolism and growth with Mn(IV) reduction. When Fe(III)- or nitrate-grown cells were inoculated into FWA-Mn(IV) medium, the brown precipitate of MnO_2 at the bottom of the culture tube was converted to a white precipitate within 1 week. X-ray diffraction analysis indicated that the white precipitate was the Mn(II)-containing mineral rhodochrosite, $MnCO_3$. MnO_2 was not reduced to Mn(II) in uninoculated media. GS-15 was subcultured through more than 10 transfers (inoculum of 10% or less) through FWA-MnO_2 medium with continued active reduction of MnO_2.

The reduction of Mn(IV) was associated with acetate metabolism and cell growth (Fig. 5). After Mn(IV) reduction was completed, there was a significant decline in cell numbers as was seen in cultures with Fe(III) as the electron acceptor. The Mn(IV) in the culture medium was completely reduced to Mn(II) (Fig. 5). The metabolism of acetate coupled to the reduction of Mn(IV) was consistent with reaction 3 (Table 2) in which the oxidation of 1 mol of acetate results in the reduction of 4 mol of Mn(IV) to Mn(II). The Mn(II) produced combines with the carbon dioxide produced and carbon dioxide in the culture medium to form $MnCO_3$.

Acetate metabolism and growth with nitrate reduction.

TABLE 2. Stoichiometry and standard free energy of reactions related to the metabolism of acetate, Fe(III) reduction, and Mn(IV) reduction

Reaction no.	Reactants	Products	$\Delta G^{0'}$ (kJ/reaction)[a]
1	$CH_3COO^- + 24Fe(OH)_3$	$8Fe_3O_4 + HCO_3^- + CO_2 + 37H_2O$	−712
2	$CH_3COO^- + 8Fe^{3+} + 3H_2O$	$8Fe^{2+} + HCO_3^- + CO_2 + 8H^+$	−814
3	$CH_3COO^- + 4MnO_2 + 2HCO_3^- + 3H^+$	$4MnCO_3 + 4H_2O$	−737
4	$CH_3COO^- + NO_3^- + 2H^+$	$NH_4^+ + HCO_3^- + CO_2$	−500
5	$CH_3COO^- + 2O_2$	$HCO_3^- + CO_2 + H_2O$	−849
6	$CH_3COO^- + SO_4^{2-} + H^+$	$HS^- + HCO_3^- + CO_2 + H_2O$	−52
7	$CH_3COO^- + H_2O$	$CH_4 + HCO_3^-$	−31
8	$Fe^{2+} + 2Fe(OH)_3$	$Fe_3O_4 + 2H_2O + 2H^+$	−90

[a] Free energy calculated from the standard free energies of formation of the products and reactants (47) and by assuming standard conditions except for pH 7.

TABLE 3. GS-15 reduction of various Fe(III) forms with acetate as the electron donor

Fe(III) form	Fe(II) produced after incubation for[a]:		
	4 days	14 days	53 days
Synthetic amorphic Fe(III) oxide	47 ± 2	66 ± 1	59 ± 4
Natural amorphic Fe(III) oxide	57 ± 6	92 ± 3	97 ± 5
Goethite (α-FeOOH)	0.7 ± 0.3	2 ± 0.2	2 ± 0.5
Akaganeite (β-FeOOH)	0.9 ± 0.8	3 ± 0.1	6 ± 1
Hematite (Fe_2O_3)	0.6 ± 0.4	4 ± 3	0.5 ± 0.7
Magnetite (Fe_3O_4)	0.7 ± 0.7	2.3 ± 0.3	3 ± 2

[a] Millimoles per liter; mean and standard deviation for triplicate cultures.

GS-15 grew well in liquid medium with acetate as the sole electron donor and nitrate as the sole electron acceptor (Fig. 6). The metabolism of acetate was accompanied by the reduction of nitrate to ammonia. Nitrite was not detected as an intermediate. No N_2 was produced during growth with nitrate. The results suggest that acetate metabolism coupled to nitrate reduction follows reaction 4 (Table 2) in which the oxidation of 1 mol of acetate results in the reduction of 1 mol of nitrate to ammonia.

Other electron acceptors and donors. GS-15 did not grow with acetate as the electron donor and oxygen (atmospheric), fumarate (20 mM), sulfate (20 mM), or elemental sulfur (ca. 1 g/liter) as the electron acceptor. GS-15 would not grow fermentatively in anaerobic nutrient broth or glucose-yeast extract broth. There was no growth if air was added to the headspace of anaerobic media with acetate as the electron donor and amorphic Fe(III) oxide, Fe(III) citrate, MnO_2, or nitrate as the electron acceptor. These results indicate that GS-15 is a strict anaerobe. GS-15 grew readily (as evidenced by active FE(III) reduction) in a highly reducing medium which had been prepared by boiling the FWA-Fe(III) medium under N_2-CO_2 and then reducing it with the addition of L-cysteine hydrochloride (0.25 g/liter) and sodium sulfide (0.25 g/liter).

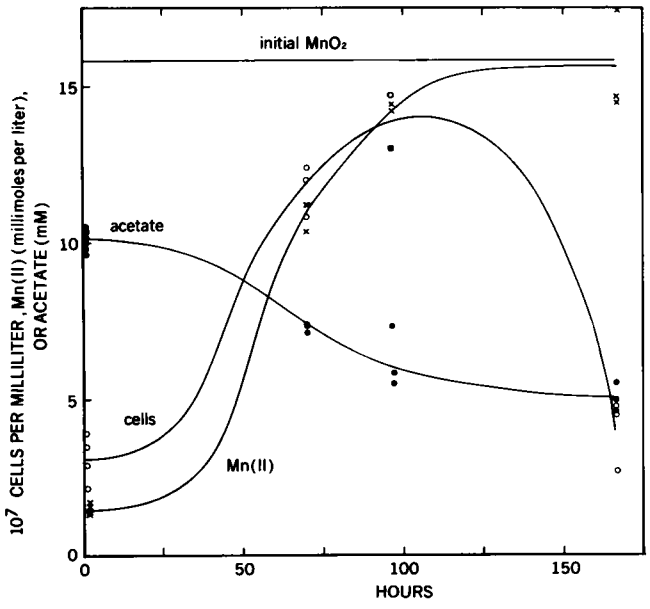

FIG. 5. Concentrations of acetate, cell numbers, and Mn(II) over time in FWA-Mn(IV) medium inoculated with GS-15 that had been grown in FWA-NO_3 medium until the nitrate was depleted. Concentrations at the time of inoculation were measured on subsamples from the cultures. At subsequent time points, three cultures were sacrificed for analysis. The results from all the analyses are shown, and the curves were drawn through the mean of the values. The initial concentration of MnO_2 is designated by the line near the top of the figure.

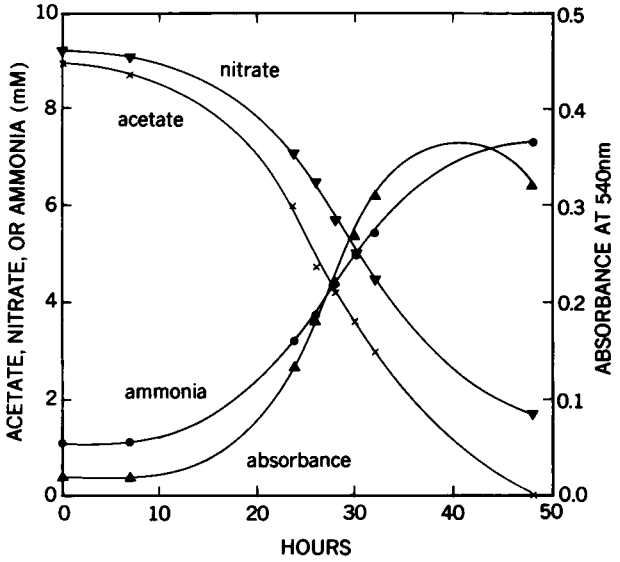

FIG. 6. Culture absorbance and concentrations of acetate, ammonia, and nitrate over time in FWA-NO_3 medium inoculated with GS-15 that had been grown in FWA-Fe(III)–citrate medium until the Fe(III) was depleted.

GS-15 reduced amorphic Fe(III) oxide with ethanol (20 mM) as the electron donor at a rate comparable to that with acetate. Slower rates of reduction of amorphic Fe(III) oxide were observed with butyrate (20 mM) or propionate (20 mM) as the electron donor. Glucose (20 mM), malate (20 mM), fumarate (20 mM), elemental sulfur (ca. 1 g/liter), methanol (20 mM), glycerol (20 mM), trimethylamine (20 mM), formate (20 mM), lactate (20 mM), and hydrogen (130 kPa) did not serve as sole electron donors for Fe(III) reduction. When hydrogen (2 kPa) was added to the headspace of FWA-Fe(III) medium at the time of inoculation, there was no net consumption of hydrogen during growth.

DISCUSSION

The results demonstrate that microorganisms can obtain energy for growth by coupling the oxidation of organic matter to the dissimilatory reduction of Fe(III) or Mn(IV). These results further indicate that there are microorganisms (or consortia of microorganisms) in sediments which are capable of completely oxidizing organic compounds with Fe(III) or Mn(IV) as the sole electron acceptor.

Energy for growth from Fe(III) and Mn(IV) reduction. The finding that microorganisms can gain energy for growth by catalyzing the reduction of Fe(III) or Mn(IV) by organic electron donors is not surprising when the energy available from these reactions is considered (Table 2). The standard free energy at pH 7 that was calculated to be available from acetate oxidation coupled to Fe(III) or Mn(IV) reduction (reactions 1 to 3) is nearly as great as that calculated to be available with oxygen as the electron acceptor (reaction 5). It is much greater than that calculated to be available from acetate oxidation with sulfate as the electron acceptor (re-

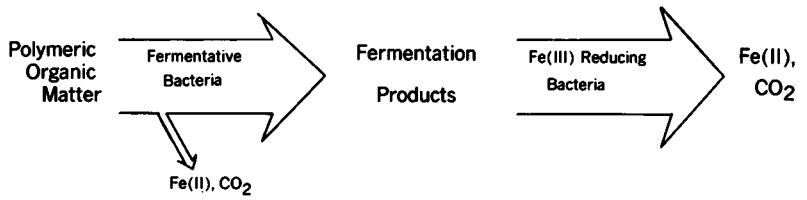

FIG. 7. Model for carbon and electron flow in sediments with Fe(III) reduction as the predominant terminal electron-accepting reaction. The width of the arrows is indicative of the relative quantity of carbon and electron flow proceeding by that pathway.

action 6) or from the conversion of acetate to methane (reaction 7), two reactions known to support growth of microorganisms (37, 54).

The actual amount of energy that is potentially available from acetate metabolism coupled to Fe(III) or Mn(IV) reduction in the culture tubes is as yet unknown. The speciation of the iron and manganese forms in the cultures was undoubtedly much more complex than as written in the reactions in Table 2. The concentrations of the reactants and products were far from standard conditions. The steps of the iron and manganese transformations from which GS-15 can obtain energy are also unknown. For example, the formation of magnetite from Fe^{2+} and amorphic Fe(III) oxide is exergonic at pH 7 (reaction 8). Magnetite does not form from Fe(II) and amorphic Fe(III) oxide in FWA-Fe(III) medium in the absence of GS-15 (29). However, the catalytic role of GS-15 in magnetite formation and whether it can gain energy from this reaction is not known.

It is hypothesized that the mechanism for the generation of cellular energy with Fe(III) and Mn(IV) is an electron transport chain coupled to an Fe(III) or Mn(IV) reductase(s). This is currently under investigation.

Complete oxidation of organic matter with Fe(III) or Mn(IV) reduction. Fe(III) reduction is an important process for organic matter decomposition in some recent sediments (1, 23, 26), and Fe(III) may have been the major electron acceptor for the oxidation of organic matter in some ancient sedimentary environments (52). The preponderance of evidence suggests that microorganisms are the catalysts for the oxidation of organic matter coupled to the reduction of Fe(III) in sedimentary environments (23). However, before the isolation of GS-15, there was no organism which could serve as a model for the mechanisms by which microorganisms could completely oxidize organic matter with Fe(III) or Mn(IV) as the sole electron acceptor. As discussed in the Introduction, previously described Fe(III)- and Mn(IV)-reducing bacteria oxidized little of their substrate to carbon dioxide. Fermentation acids, ethanol, and hydrogen were the major products of their metabolism.

The ability of organisms to couple the oxidation of acetate to Fe(III) or Mn(IV) reduction was predicted by earlier studies. The oxidation of [2-^{14}C]acetate to $^{14}CO_2$ during the period of Fe(II) accumulation in submerged paddy soils suggested that acetate could be oxidized with Fe(III) as the electron acceptor (20). Acetate added to Fe(III)-reducing sediments was shown to be rapidly consumed (28) with the stimulation of Fe(III) reduction (25). Enrichment cultures that reduced Fe(III) (25) or Mn(IV) (7, 55) with acetate as a potential electron donor were previously reported.

GS-15 uses only a restricted number of organic acids and ethanol as electron donors for Fe(III) reduction. The electron donors for its metabolism are typical fermentation products of other organisms, including the previously described Fe(III)-reducing organisms which metabolize fermentable substrates (19, 41). Because of our difficulty in enriching for an Fe(III)-reducing organism which can completely oxidize glucose to carbon dioxide without the accumulation of fermentation products (25; unpublished data), we propose that the complete oxidation of complex organic matter coupled to Fe(III) reduction may require the cooperation of a consortium of Fe(III)-reducing organisms (Fig. 7). In this model, organisms with a primarily fermentative metabolism initially metabolize the complex organic matter in the sediments. The fermentative organisms primarily produce fermentation products, but some of them may also reduce minor amounts of Fe(III). Most of the organic matter oxidation and Fe(III) reduction takes place in the second stage of metabolism when a separate group of bacteria oxidizes the fermentation products with the reduction of Fe(III). This model is similar to that for sediments in which sulfate reduction is the terminal electron-accepting process for organic matter oxidation (53).

Similar models may also apply to sediments in which Mn(IV) reduction or dissimilatory reduction of nitrate to ammonia is the terminal electron-accepting process. With the exception of the recently isolated *Desulfobacter catecholicum*, all the previously known dissimilatory nitrate-reducing organisms incompletely oxidized their substrates (48). Although organisms such as *D. catecholicum* and GS-15 can completely oxidize organic acids with dissimilatory nitrate reduction, in aquatic sediments where most readily degradable organic matter is in the form of complex carbohydrates and proteins, these organisms would be dependent on the metabolism of others to supply most of their substrate.

Geochemical significance. The finding that microorganisms can effectively couple the oxidation of organic matter to the reduction of Fe(III) and Mn(IV) is of geochemical significance to processes other than organic matter diagenesis. For example, the reduction of amorphic Fe(III) oxide to ultrafine-grained magnetite by GS-15 has important implications for paleomagnetic studies as well as for understanding the accumulation of magnetite in ancient iron formations and near hydrocarbon deposits (12, 29). The results presented here demonstrated that Fe(III) reduction by GS-15 does not always result in magnetite production, and further studies on the factors controlling magnetite production are required.

The reduction of Fe(III) and Mn(IV) greatly influences the quality and chemistry of surface water and groundwater by increasing the concentrations of dissolved iron and manganese and by releasing phosphate and trace metals bound to Fe(III) and Mn(IV) oxides (6, 16). Although the standard approach to modelling iron and manganese geochemistry has been to treat Fe(III) and Mn(IV) reduction as nonbiological redox reactions, the metabolism of GS-15 may provide a more appropriate model to help further elucidate the mechanisms controlling these geochemical phenomena.

Ecological niche. The ability of GS-15 to use nitrate, Mn(IV), and Fe(III) as electron acceptors suggests that it is well adapted for life in what is frequently termed the suboxic

(13) or postoxic zone (5) of sediments. This zone contains sediments which are depleted of oxygen but in which sulfate reduction or methane production has not commenced. Organisms which reduce nitrate to ammonia are expected to be better competitors for sediment organic matter than denitrifiers at the low concentrations of nitrate in many suboxic sediments (21, 44, 49). With the depletion of nitrate, organisms such as GS-15 should remain successful competitors for sediment organic matter by switching their metabolism to Mn(IV) and Fe(III) reduction.

In summary, although geochemical evidence had previously suggested that organic matter could be completely oxidized to carbon dioxide with Fe(III) or Mn(IV) as the sole electron acceptor, the isolation of GS-15 has provided the first example of a microbiological mechanism for these reactions. The characteristics of this organism do not appear to be consistent with any previously described genus of bacterium. Preliminary results indicate that organisms with a similar metabolism may be isolated from brackish water estuarine sediments (25) and deep subsurface environments (D. R. Lovley and F. Chapelle, unpublished data). Further studies on these organisms and GS-15 are required before it will be appropriate to attempt to classify them taxonomically.

ACKNOWLEDGMENTS

We thank Daniel Webster for the X-ray diffraction analyses; Steve Goodwin for helpful discussions during the initial attempts to isolate the organism; Ron Oremland, John Stolz, and Greg Ferry for helpful comments on the manuscript; and Carol Lee for preparing the text and tables.

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