APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Oct. 1992, p. 3211–3216 Vol. 58, No. 10
0099-2240/92/103211-06$02.00/0

A Hydrogen-Oxidizing, Fe(III)-Reducing Microorganism from the Great Bay Estuary, New Hampshire

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Received 30 March 1992/Accepted 15 July 1992

A dissimilatory Fe(III)- and Mn(IV)-reducing bacterium was isolated from bottom sediments of the Great Bay estuary, New Hampshire. The isolate was a facultatively anaerobic gram-negative rod which did not appear to fit into any previously described genus. It was temporarily designated strain BrY. BrY grew anaerobically in a defined medium with hydrogen or lactate as the electron donor and Fe(III) as the electron acceptor. BrY required citrate, fumarate, or malate as a carbon source for growth on H_2 and Fe(III). With Fe(III) as the sole electron acceptor, BrY metabolized hydrogen to a minimum threshold at least 60-fold lower than the threshold reported for pure cultures of sulfate reducers. This finding supports the hypothesis that when Fe(III) is available, Fe(III) reducers can outcompete sulfate reducers for electron donors. Lactate was incompletely oxidized to acetate and carbon dioxide with Fe(III) as the electron acceptor. Lactate oxidation was also coupled to the reduction of Mn(IV), U(VI), fumarate, thiosulfate, or trimethylamine *n*-oxide under anaerobic conditions. BrY provides a model for how enzymatic metal reduction by respiratory metal-reducing microorganisms has the potential to contribute to the mobilization of iron and trace metals and to the immobilization of uranium in sediments of Great Bay Estuary.

Microbial reduction of Fe(III) plays an important role in the cycling of carbon, trace metals, and nutrients in a variety of anaerobic sedimentary environments (14). Food chains of Fe(III)-reducing microorganisms have the potential to oxidize complex sedimentary organic matter to carbon dioxide with Fe(III) as the sole electron acceptor (14). Fe(III) reducers which directly oxidize aromatic compounds to carbon dioxide (15, 19) or incompletely oxidize lactate or pyruvate to acetate and carbon dioxide (26) contribute to this organic-matter oxidation. However, much of the Fe(III) reduction is thought to be carried out by Fe(III) reducers which oxidize important fermentation products such as short-chain fatty acids and hydrogen (14). Two Fe(III)-reducing organisms, "*Geobacter metallireducens*" (formerly strain GS-15) (22, 28) and strain 172 (17), are known to completely oxidize multicarbon fermentation acids such as acetate to carbon dioxide. H_2 and/or formate is likely to be the other important fermentation product in Fe(III)-reducing sediments (14). Only two Fe(III) reducers, a *Pseudomonas* sp. (2) and *Shewanella putrefaciens* (26), have been reported to oxidize H_2, and only one, *S. putrefaciens*, is known to oxidize formate (26).

Metal remobilization in the sediments of the Great Bay estuary, New Hampshire, is directly influenced by microbial activity and seasonal effects (9, 10, 32). In the summer, sulfate reduction rates are rapid, promoting the removal of dissolved Fe(II) and oxygen from pore waters (10). During the spring, the level of dissolved O_2 decreases, dissolved-Fe(II) concentrations increase rapidly, and sulfate reduction rates remain low (9). Microbial Fe(III) reduction was proposed to have a significant role in creating the spring dissolved-Fe(II) maximum (9, 10), and enrichment cultures which enzymatically reduced Fe(III) could be recovered from the sediments (32).

The purpose of this study was to attempt to isolate in pure culture some of the Fe(III)-reducing microorganisms responsible for Fe(III) reduction in Great Bay estuarine sediments. Here we report the isolation of a hydrogen-oxidizing Fe(III)-reducer which may provide a model for microbial Fe(III) reduction in this environment. The isolate was temporarily designated BrY.

MATERIALS AND METHODS

Source of organism. Inocula for enrichments were collected by taking cores of near-shore sediments in Great Bay, New Hampshire (9). The top 3 cm of sediment was extruded into glass bottles and immediately transported back to the laboratory, where it was flushed with N_2 to maintain anaerobic conditions.

Enrichment and isolation. Standard anaerobic techniques (3, 12, 30) were used throughout as previously described (22). All anaerobic incubations were in anaerobic pressure tubes or serum bottles capped with thick butyl rubber stoppers. All anaerobic media were extensively bubbled with N_2-CO_2 (80:20) to remove dissolved O_2. The gas phase overlying the medium was N_2-CO_2 (80:20) unless specified otherwise. Medium constituents were sterilized by autoclaving (121°C, 20 min). All incubations were at 30°C in the dark.

The Fe(III) enrichment medium, described previously (22), contained major and minor trace metals, vitamins, and ca. 250 mmol of a synthetic, poorly crystalline Fe(III) oxide per liter (20). Acetate (20 mM) and yeast extract (0.05 g/liter) were provided as potential electron donors and carbon sources. The enrichment was initiated by adding anaerobic sediment (1 ml) to sterile enrichment medium (9 ml) under a stream of N_2-CO_2 (80:20).

The basal culture medium was the same as that described previously for the culture of "*G. metallireducens*" (22). Fe(III) or Mn(IV) was added to the basal medium prior to

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bubbling with N_2-CO_2. Fe(III) forms were typically either poorly crystalline Fe(III) oxide (ca. 100 mmol/liter) or Fe(III) citrate (20 mM). However, in one study, in which sediment Fe(III) oxides served as the Fe(III) source, surficial freshwater sediments from the Potomac River (20) were diluted 2:1 (vol/vol) with water and oxidized by stirring them under air for 3 days. The oxidized sediment slurry (5 ml/10 ml of medium) was added to the medium prior to bubbling with N_2-CO_2. In order to evaluate the potential for Mn(IV) reduction, a synthetic MnO_2 (22) was added to provide 10 to 30 mmol of Mn(IV) per liter. When noted, sodium nitrate, sodium thiosulfate, sodium sulfite, sodium sulfate, fumaric acid, or trimethylamine *n*-oxide was added from anaerobic stock solutions to provide 20 mM. Elemental sulfur was added as sublimed flowers of sulfur (10 g/liter). Agar slants of nitrate medium were made by adding a final concentration of 1.5% (wt/vol) purified agar.

For aerobic growth, cells were cultured on nutrient agar (Difco Laboratories, Detroit, Mich.). In order to determine the optimal salt concentrations for aerobic growth, cells were grown aerobically in media that contained (grams per liter of water) KH_2PO_4 (2.5), KCl (0.1), NH_4Cl (1.5), and sodium lactate (30 mM) with various concentrations of NaCl. Anaerobic growth at various salt concentrations was determined in the basal culture medium described above with sodium lactate (20 mM) as the electron donor and Fe(III) citrate (10 mM) as the electron acceptor.

Time course and stoichiometry studies. For growth on H_2, sodium citrate (10 mM) was added as a carbon source. H_2 was added to an initial partial pressure of 60 kPa. For hydrogen stoichiometry and threshold studies, the H_2 pressure was initially 2 to 5 kPa.

The stoichiometry of lactate utilization was determined by inoculating BrY into Fe(III) oxide medium (10 ml) containing ca. 5 mM lactate and [U-^{14}C]lactate (1 μCi; 154 mCi/mmol). $^{14}CO_2$ concentrations were determined by acidifying replicate culture tubes and trapping the $^{14}CO_2$ in NaOH prior to liquid scintillation counting (23). Acidified culture fluid (2 ml) was filtered (0.2-μm-pore-diameter filter), and the filtrate was neutralized with NaOH and then filtered again to remove any precipitated Fe(III) oxide. As previously described (23), [^{14}C]lactate and [^{14}C]acetate in the filtrate were separated by high-pressure liquid chromatography, collected, and quantified with liquid scintillation counting.

Uranium reduction. BrY was examined for its potential to reduce U(VI) in a manner similar to that previously described for *Desulfovibrio desulfuricans* (24). BrY grown in Fe(III)-citrate medium with lactate as the electron donor were collected by centrifugation and washed three times in sodium bicarbonate buffer (2.5 g/liter) under N_2-CO_2. Washed cells were suspended in bicarbonate buffer that was amended with uranyl acetate (0.5 mM) and lactate (5 mM). Subsamples (0.5 ml) were withdrawn over time and analyzed for U(VI).

Analytical techniques. Fe(III) reduction was monitored by measuring the accumulation of Fe(II) over time. The amount of Fe(II) solubilized after 15 min in 0.5 N HCl was determined with ferrozine as previously described (22). Cell numbers in cultures growing on Fe(III) were determined by a modification of the epifluorescent microscopy technique (11) as previously described (22). Cell densities in cultures without Fe(III) were monitored by directly inserting the culture tubes into a Spectronic 20 spectrophotometer and measuring the A_{540}.

H_2 was quantified by gas chromatography using either a thermal conductivity detector (stoichiometry studies) or a reduction gas analyzer (threshold studies). U(VI) was measured under anaerobic conditions, as previously described (8, 25), with a Kinetic Phosphorescence Analyzer (KPA-10; Chemchek Instruments).

Moles percent guanosine plus cytosine (G+C) was determined by the thermal denaturation procedure described by Marmur and Doty (29).

The presence of poly-β-hydroxybutyrate was detected by fluorescence staining with Nile Blue-A (31). Cells stained with uranyl acetate were examined with a JEOL 100 transmission electron microscope at 60 kV.

RESULTS AND DISCUSSION

Enrichment and isolation. Three successive transfers (10% inoculum) of a positive enrichment continued to actively reduce Fe(III) in the enrichment medium. The third transfer was streaked on agar slants of medium with acetate as the sole electron donor and nitrate as the sole electron acceptor. An isolated colony was used to inoculate liquid acetate-nitrate medium, which was subsequently diluted to extinction. The highest dilution (10^{-7}) which had cell growth was subcultured aerobically on nutrient agar. An isolated colony was selected for further study. With continued aerobic subculture on nutrient agar, an isolate of uniform cell and colony morphology was obtained.

Well-isolated colonies on nutrient agar were pink, round, and glossy. Cells were straight rods 0.75 to 1.5 μm in length by 0.5 μm in width (Fig. 1). They were motile and possessed one or two polar or bipolar flagella. Cells were gram negative, nonfermentative, and oxidase positive (1% dimethyl-*p*-phenylenediamine hydrochloride) and contained granules of poly-β-hydroxybutyrate. These phenotypic characteristics are consistent with the genus *Pseudomonas* (4). However, the G+C content was 53 mol%, which does not fall in the range of 58 to 70 mol% typically associated with *Pseudomonas* spp. (4). Furthermore, analysis of the cellular fatty acids by MICRO, Inc. (Northfield, Vt.) found no match between the fatty acid profiles of BrY and those of a wide variety of known microorganisms, including *S. putrefaciens* and more than 40 species of *Pseudomonas*. Given the uncertain taxonomic status of the organism, it was temporarily designated strain BrY.

H_2 oxidation. BrY grew in medium with H_2 as the electron donor and poorly crystalline Fe(III) oxide (Fig. 2) or Fe(III) citrate (data not shown) as the electron acceptor. Growth on H_2 required the presence of a carbon source. Citrate, fumarate, and malate at 10 mM supported reduction of poorly crystalline Fe(III) oxide with H_2, but acetate and succinate did not. None of the carbon sources which supported growth on H_2 could serve as the sole electron donor for Fe(III) reduction. When grown with H_2 and Fe(III) oxide, duplicate cultures consumed an average of 13 mmol of H_2 per liter and produced an average of 31 mmol of Fe(II) per liter. These results suggest that BrY oxidizes H_2 according to the following reaction: $H_2 + 2Fe(III) \rightarrow 2Fe(II) + 2H^+$.

Two other microorganisms, a *Pseudomonas* sp. (2) and *S. putrefaciens* (26), have previously been shown to obtain energy to support growth from this reaction. These organisms also need organic carbon sources for growth on H_2 and Fe(III). The *Pseudomonas* sp. requires additions of yeast extract (2), and *S. putrefaciens* requires the presence of an amino acid mixture (26).

Oxidation of organic compounds. Acetate did not serve as an electron donor for Fe(III) reduction. This was unexpected, because the enrichment that yielded BrY contained

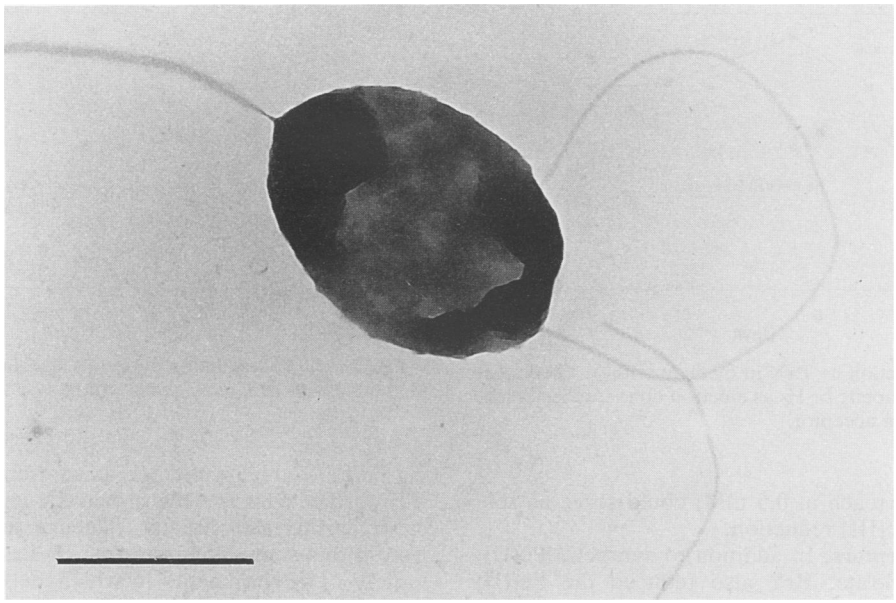

FIG. 1. Transmission electron micrograph of negatively stained BrY. Bar, 0.5 μm.

acetate as the primary electron donor. This finding and the lack of good growth on nitrate (see below) suggest that BrY was not the principal component(s) of the original enrichment or the dilutions on acetate-nitrate medium. Other organisms that were active in the enrichment phase were apparently unable to grow aerobically on nutrient agar. This is consistent with the fact that the acetate-oxidizing Fe(III)-reducing microorganism "*G. metallireducens*" is a strict anaerobe (22).

BrY grew in medium with lactate as the sole electron donor and poorly crystalline Fe(III) oxide (Fig. 3) or Fe(III) citrate (data not shown) as the electron acceptor. In order to determine the stoichiometry of lactate metabolism coupled to Fe(III) reduction, BrY was grown in the presence of [U-^{14}C]-lactate. The accumulation of radioactivity in acetate was twice that in carbon dioxide (Table 1). These results indicate that lactate oxidation proceeded via the following reaction: lactate$^-$ + $2H_2O$ + 4Fe(III) → acetate$^-$ + HCO_3^- + $5H^+$ + 4Fe(II). This partial oxidation of lactate to acetate and carbon dioxide is similar to the lactate metabolism during Fe(III) reduction in *S. putrefaciens* (16, 26).

Reduction of Fe(III) oxide in the presence of lactate could be attributed to an enzymatic reaction, as would be expected for a reaction which yields energy to support growth. In lactate-Fe(III) oxide medium, there was no Fe(III) reduction if the culture was treated with heat (80°C, 15 min) prior to incubation. When contact between BrY and poorly crystalline Fe(III) oxide was prevented by placing the poorly crystalline Fe(III) oxide in dialysis tubing, there was no Fe(III) reduction. The cell-free filtrate of an actively growing Fe(III)-reducing culture of BrY did not reduce poorly crystalline Fe(III) oxide. Fe(III) reduction was not the result of a decrease in pH, as growth and Fe(III) reduction by BrY did not lower the pH of Fe(III) oxide medium.

BrY was similar to the previously described H_2-oxidizing Fe(III)-reducing *Pseudomonas* sp. (2) in that formate did not serve as an electron donor to support Fe(III) reduction. This is in contrast to *S. putrefaciens*, which can use formate as sole electron acceptor to support growth on Fe(III) (26).

BrY reduced Fe(III) in the presence of pyruvate (20 mM). Neither butyrate, citrate, succinate, fumarate, ethanol, glycerol, glucose, nor fructose (each at 20 mM) and neither

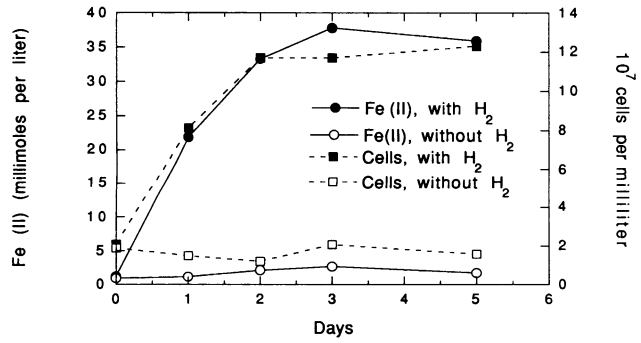

FIG. 2. Growth of BrY in medium with H_2 as electron donor and poorly crystalline Fe(III) oxide as electron acceptor. Citrate was provided as carbon source.

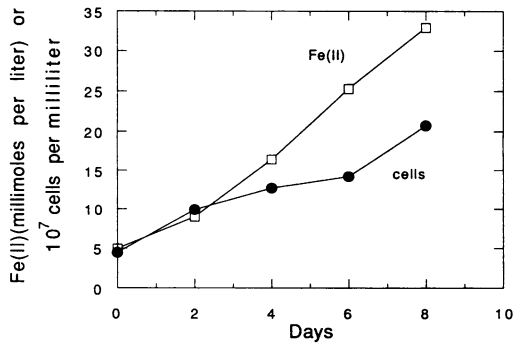

FIG. 3. Growth of BrY in medium with lactate as electron donor and poorly crystalline Fe(III) oxide as electron acceptor.

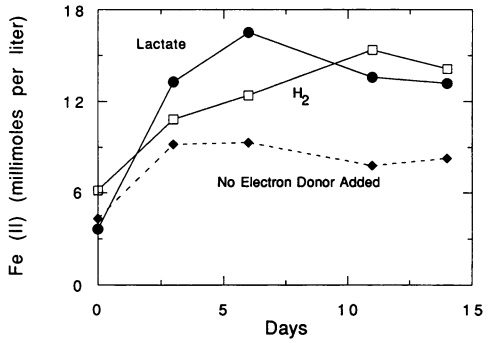

FIG. 4. Fe(III) reduction by BrY in medium with just sediment organic matter or with lactate or H_2 as electron donor and sediment Fe(III) oxide as electron acceptor.

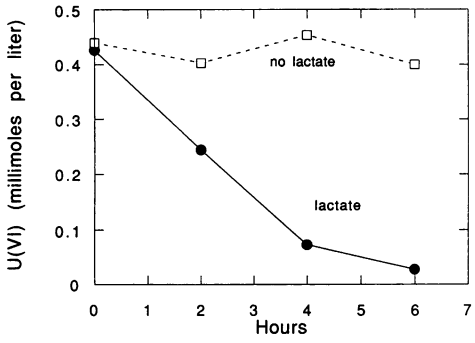

FIG. 6. U(VI) reduction by cell suspensions of BrY in bicarbonate buffer with lactate as electron donor.

benzoate nor phenol (each at 0.5 mM) could serve as sole electron donor for Fe(III) reduction.

Other electron acceptors. In addition to synthetic Fe(III) oxide and Fe(III) citrate, BrY also reduced the Fe(III) oxides in sediments from the Potomac River (Fig. 4). BrY grew in medium with lactate (30 mM) or H_2 as an electron donor and Mn(IV) as an electron acceptor (Fig. 5A). BrY did not grow in Mn(IV) medium without lactate or H_2. Production of Mn(II) was not quantified. However, it was routinely observed that during growth in the presence of Mn(IV), the brown MnO_2 precipitate was converted to a white precipitate. Previous studies have demonstrated that under similar culture conditions, microbial Mn(IV) reduction converts MnO_2 to the white, Mn(II)-containing mineral rhodochrosite, $MnCO_3$ (22, 26).

With 30 mM lactate as the sole electron donor, BrY grew with fumarate, thiosulfate, or trimethylamine *n*-oxide (each at 20 mM) as the sole electron acceptor (Fig. 5B). Although part of the enrichment procedure leading to the isolation of BrY involved growth in an acetate-nitrate medium, the pure culture of BrY grew poorly on nitrate (Fig. 5B). Cells did not utilize sulfate, sulfite, or elemental sulfur as terminal electron acceptors. Cell suspensions of BrY reduced U(VI) (Fig. 6). The ability of BrY to grow with U(VI) as the sole electron acceptor was not evaluated.

Biogeochemical implications. The isolation of an H_2-oxidizing Fe(III)-reducing microorganism from sediments of Great Bay further confirms the previous suggestion (32) that microorganisms can directly influence the remobilization of iron in this saline environment. H_2 has been detected in a variety of environments in which Fe(III) reduction is the predominant terminal-electron-accepting process (6, 18, 21) and is expected to be an important intermediate in the decomposition of fermentable compounds in such sediments (14). Therefore, H_2 consumption by organisms with a metabolism like that of BrY could play an important role in organic-matter oxidation coupled to Fe(III) reduction in Great Bay. In contrast, the metabolism of lactate and pyruvate by BrY is not expected to be an important component of carbon and electron flow, because lactate and pyruvate are expected to be minor extracellular intermediates in Fe(III)-reducing environments (14, 23). Furthermore, the partial oxidation of lactate or pyruvate to acetate still leaves the majority of the electron equivalents in acetate, which must be metabolized by organisms with a metabolism like that of "*G. metallireducens*" (22, 28) or strain 172 (17).

With Fe(III) citrate as the electron acceptor, BrY metabolized H_2 down to concentrations of less than 0.015 Pa. Under similar conditions, *S. putrefaciens* has a minimum threshold of 0.04 Pa (26). In comparison, pure cultures of sulfate reducers typically have minimum thresholds for hydrogen uptake of ca. 1 Pa (7). These findings provide further pure-culture evidence for the hypothesis that when

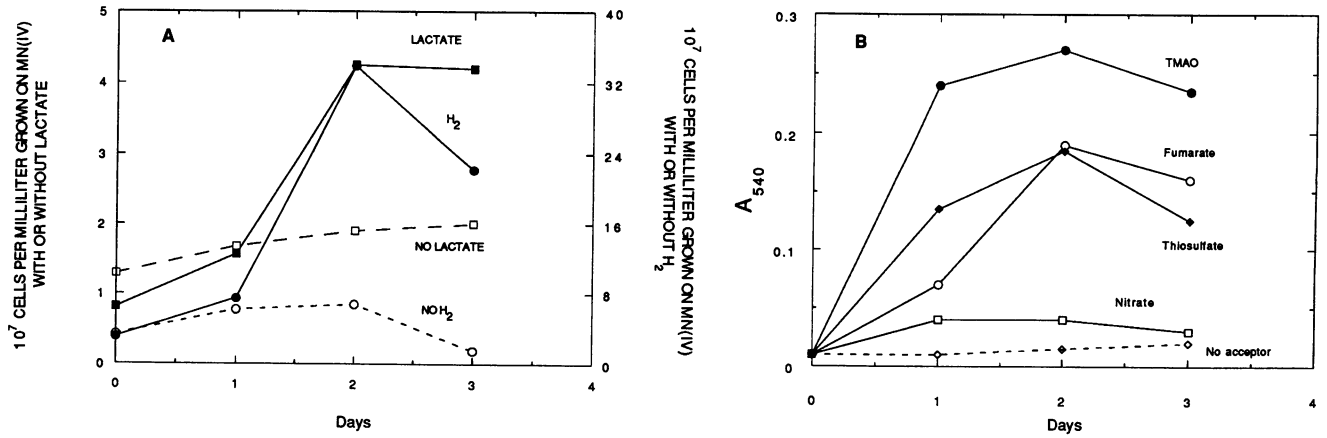

FIG. 5. Growth of BrY with various electron acceptors. (A) Growth in medium with lactate or H_2 as electron donor and MnO_2 as electron acceptor. For growth on H_2, citrate (10 mM) was provided as carbon source. (B) Growth in medium with lactate as electron donor and trimethylamine *n*-oxide, fumarate, thiosulfate, or nitrate as electron acceptor.

TABLE 1. Metabolism of U-^{14}C in medium with lactate (ca. 5 mM) as electron donor and poorly crystalline Fe(III) oxide as electron acceptor

Time of incubation (days)[a]	Radioactivity (dpm) in:			Fe(II) (mmol/liter)
	Lactate	Acetate	CO_2	
0	2,509,350	0	972	4.5
	2,401,179	0	972	3.6
	2,300,046	0	995	3.4
14	468,185	1,272,585	547,382	21.5
	554,453	1,190,113	578,928	18.6
	569,477	1,165,679	562,124	18.6

[a] Results were from three replicate tubes sacrificed at time 0 and after 14 days of incubation.

Fe(III) in aquatic sediments is not limiting, Fe(III)-reducing microorganisms have a higher affinity for H_2 than do sulfate reducers and thus can inhibit sulfate reduction by maintaining H_2 concentrations too low for sulfate reducers to metabolize (18, 21). Fe(III) reducers outcompeting sulfate reducers for hydrogen and other electron donors could account for the spring maximum of dissolved Fe(II) that is observed in Great Bay sediments (9, 10). A similar phenomenon has been proposed to account for the high level of dissolved Fe(II) in deep aquifers of the Atlantic coastal plain (6).

BrY has several characteristics which suggest it would be well suited for life in Great Bay sediments. BrY was capable of anaerobic (Fig. 7) and aerobic (data not shown) growth over a salinity range that closely approximated the salinity fluctuations (<1 to 4‰, wt/vol) that were observed in Great Bay during the course of 1 year (5). The ability of BrY to use O_2 and a number of alternative electron acceptors should be adaptive in an environment such as Great Bay sediments, in which the availability of electron acceptors in the surficial sediments varies seasonally.

The reduction of soluble U(VI) to insoluble U(IV) is an important sink for the removal of dissolved uranium from aquatic environments (1, 13, 33). Recent studies have suggested that U(VI) reduction in aquatic sediments is the result of an enzymatic reaction catalyzed by Fe(III)- or sulfate-reducing organisms (8, 24, 25). The finding that BrY can also reduce U(VI) suggests that U(VI) reduction may be a common capability of dissimilatory Fe(III) reducers.

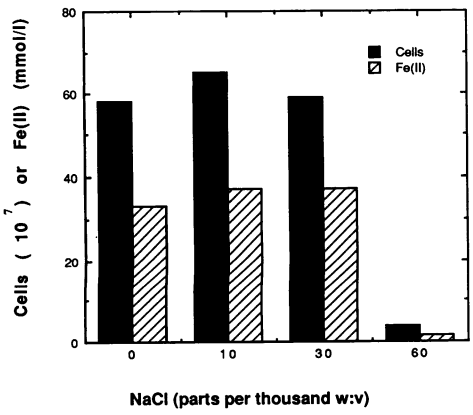

FIG. 7. Cell numbers and Fe(II) concentrations 36 h after BrY had been inoculated into lactate-Fe(III) citrate medium. Initial cell numbers and Fe(II) concentrations were 1.5×10^{-7} and 1.2 mM, respectively.

In summary, BrY represents a new addition to the relatively small group of organisms that are known to obtain energy to support growth by coupling the oxidation of organic compounds or H_2 to the reduction of Fe(III). It is becoming increasingly apparent that enzymatic mechanisms are responsible for much of the reduction of Fe(III) (27), U(VI) (25), and possibly other metals in aquatic sediments. Thus, continued isolation and characterization of respiratory metal-reducing microorganisms like BrY is likely to yield further insight into the factors controlling the rate and extent of metal reduction.

ACKNOWLEDGMENTS

We thank Elizabeth Phillips, Yuri Gorby, Joan Woodward, and Ray Van Hoven for technical assistance.

F.C. was supported in part by a grant from the Central University Research Fund of the University of New Hampshire and by the National Research Program of the Water Resources Division of the U.S. Geological Survey.

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