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Isolation and Characterization of Novel Iron-Oxidizing Bacteria That Grow at Circumneutral pH

DAVID EMERSON[1]* AND CRAIG MOYER[2]†

American Type Culture Collection, Rockville, Maryland 20852 and Biology Department, George Mason University, Fairfax, Virginia 22030,[1] and Center for Microbial Ecology, Michigan State University, East Lansing, Michigan[2]

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A gel-stabilized gradient method that employed opposing gradients of Fe^{2+} and O_2 was used to isolate and characterize two new Fe-oxidizing bacteria from a neutral pH, Fe^{2+}-containing groundwater in Michigan. Two separate enrichment cultures were obtained, and in each the cells grew in a distinct, rust-colored band in the gel at the oxic-anoxic interface. The cells were tightly associated with the ferric hydroxides. Repeated serial dilutions of both enrichments resulted in the isolation of two axenic strains, ES-1 and ES-2. The cultures were judged pure based on (i) growth from single colonies in tubes at dilutions of 10^{-7} (ES-2) and 10^{-8} (ES-1); (ii) uniform cell morphologies, i.e., ES-1 was a motile long thin, bent, or S-shaped rod and ES-2 was a shorter curved rod; and (iii) no growth on a heterotrophic medium. Strain ES-1 grew to a density of 10^8 cells/ml on FeS with a doubling time of 8 h. Strain ES-2 grew to a density of 5×10^7 cells/ml with a doubling time of 12.5 h. Both strains also grew on $FeCO_3$. Neither strain grew without Fe^{2+}, nor did they grow with glucose, pyruvate, acetate, Mn, or H_2S as an electron donor. Studies with an oxygen microelectrode revealed that both strains grew at the oxic-anoxic interface of the gradients and tracked the O_2 minima when subjected to higher O_2 concentrations, suggesting they are microaerobes. Phylogenetically the two strains formed a novel lineage within the γ *Proteobacteria*. They were very closely related to each other and were equally closely related to PV*B* OTU 1, a phylotype obtained from an iron-rich hydrothermal vent system at the Loihi Seamount in the Pacific Ocean, and SP*B* OTU 1, a phylotype obtained from permafrost soil in Siberia. Their closest cultivated relative was *Stenotrophomonas maltophilia*. In total, this evidence suggests ES-1 and ES-2 are members of a previously untapped group of putatively lithotrophic, unicellular iron-oxidizing bacteria.

The role that prokaryotes play in the oxidation of ferrous iron at near-neutral pH has been enigmatic for both geochemical and microbiological reasons. From a geochemical perspective it is often noted that at a pH of ≥5, Fe^{2+} will rapidly and spontaneously oxidize to Fe^{3+} (4). In fully aerated freshwater at pH 7, the $t_{1/2}$ of Fe^{2+} oxidation is <15 min (30). Under these conditions Fe-oxidizing prokaryotes may find it difficult to compete with the chemical oxidation. From a microbiological perspective, the most pervasive problem has been the inability to isolate or study prokaryotes in the laboratory that are capable of conserving energy from iron oxidation at circumneutral pH under oxic conditions. This problem is reinforced by the fact that environments with high iron concentrations are characterized by the remains of a few morphologically distinct "iron bacteria," such as the sheaths of *Leptothrix ochracea* and the stalks of *Gallionella ferruginea*, but otherwise appear largely devoid of microbial cells when viewed by light microscopy (6, 11).

To begin to understand the relevancy of microbial iron oxidation, it is most important to consider the niche where high concentrations of Fe^{2+} undergo oxidation. Generally these environments occur where water is moving from an anoxic into an oxic zone. As a result of Fe reduction in the anoxic region the water can become highly charged with Fe^{2+}, and when the Fe^{2+} is exposed to air ocherous mats, or loose aggregations of filaments coated with iron hydroxides, may form. Since these occur at anoxic-oxic transition zones, the oxygen concentrations can be very low, i.e., <10% of ambient, aerated water. At these concentrations the chemical oxidation of Fe(II) is significantly slower than it is in fully aerated water (21). It is in just this kind of environment that *G. ferruginea* thrives. To date, this stalk-forming organism is the only aerobic, neutralophilic iron-oxidizer that has been obtained in either purified enrichments or pure culture in the laboratory, where it grows at very low oxygen tensions in opposing gradients of oxygen and Fe^{2+} (15). There is both strong circumstantial evidence and accumulating biochemical evidence that this microbe can grow lithotrophically on iron and fix CO_2, although it appears capable of limited mixotrophic growth as well (13, 14). One of the most visible tenants of iron seeps, *L. ochracea* leaves behind copious amounts of Fe hydroxide-encrusted, refractile tubular sheath material, most of which is vacant of cells. Despite its striking visual appearance at these sites, *L. ochracea* has never been isolated from them, although it has been speculated that this organism may be a lithotroph. Recent discoveries now make it important to distinguish between anoxic and oxic Fe oxidation. It has been demonstrated that anoxygenic phototrophic bacteria can catalyze the oxidation of Fe^{2+} to Fe^{3+} (3, 31), and still more recently, a thermophilic archaeum, *Ferroglobus placidus*, was isolated that can couple Fe^{2+} oxidation to NO_3 reduction for growth at neutral pH (12).

A recent study of a microbial iron mat in Denmark revealed that there were up to 10^9 cells/ml of mat material (6). Most of these were nonappendaged, unicellular microbes intimately associated with the oxides. They were only visible when stained with a DNA-binding fluorescent dye, acridine orange, and viewed by epifluorescence microscopy. This explained why cursory examination of these samples by phase-contrast micros-

* Corresponding author. Mailing address: American Type Culture Collection, 12301 Parklawn Dr., Rockville, MD 20852. Phone: (301) 231-5541. Fax: (301) 816-4365. E-mail: demerson@gmu.edu.

† Present address: Biology Dept., Western Washington University, Bellingham, WA 98225.

copy revealed a paucity of cells. Laboratory microcosm studies using material collected at this site revealed that the microbes catalyzed up to 80% of the Fe oxidation (7). Ferrous iron stimulated growth of the microorganisms, including *L. ochracea* and *Gallionella*, in the microcosms; however, as in the mat itself, the numerically dominant microbes were unicellular, nonappendaged organisms. These results suggested that while classic iron bacteria such as *Leptothrix* and *Gallionella* were important in laying down the matrix of the mat, there was an even larger population of unicellular prokaryotes that might be playing a key role in iron oxidation.

The work in the present study describes a new gradient method for cultivating Fe-oxidizing prokaryotes and describes two new bacteria isolated from a groundwater-fed iron seep that appear to grow lithotropically on ferrous iron. These new isolates are unicellular and do not produce stalks or sheaths and thus they may represent a portion of the numerically dominant population of microbes that have been observed in other iron-oxidizing communities.

(A portion of this work has been presented previously [8].)

MATERIALS AND METHODS

Source. The source material for enrichments was groundwater from a basement tile drain in East Lansing, Mich. The drain was 70 cm deep by 40 cm wide and was filled to a water depth of approximately 50 cm. Nearly the entire water column was filled with a loose amorphous, rust-colored floculant material. The pH of the water was 7.1, the temperature was approximately 10°C, and the water contained 3 to 12 μM Fe^{2+}. The flow rate was quite slow, barely visible to the eye. Samples of the iron oxides were collected into sterile glass vials on two separate occasions and used as the source for enrichments.

Gradient method for isolation and enrichment. Enrichments were carried out in opposing gradients of oxygen and Fe^{2+} that were established in 60- by 15-mm screw-cap glass tubes by using a technique modified from that originally described by Kucera and Wolfe (20) for the enrichment of *Gallionella*. The tubes had a plug of either FeS or $FeCO_3$ overlaid with a semisolid mineral salts–bicarbonate-buffered medium and a headspace of air. The FeS was prepared according to Hanert (15); $FeCO_3$ was prepared according to Hallbeck et al. (14). The FeS or $FeCO_3$ precipitate was mixed 1:1 with modified Wolfe's mineral medium (MWMM) (15) in a flask and 1% (wt/vol) agarose (Pharmacia) was added. In a separate flask the overlayer was prepared by adding 0.15% (wt/vol) agarose to the MWMM medium. Both mixtures were autoclaved at 121°C for 20 min. A 0.75-ml aliquot of the molten FeS was added to a presterilized tube, and the agarose solidified to form a solid plug. Sodium bicarbonate (5 mM final concentration; 1 M sterile, stock solution) and 1 ml of vitamins (filter sterilized [32])/liter was added to the overlayer while it was still molten. Then 3.75 ml of this mixture was placed on the surface of the FeS plug. While still molten, the overlayer was bubbled with filter-sterilized CO_2 dispensed through a cannula at a flow rate of 1.8 ml/s for 4 s, and the final pH was between 6.2 and 6.4. The tubes were capped with butyl rubber septa or stoppers and allowed to sit for 6 to 24 h prior to inoculation. The tubes were inoculated by removing the top, inserting a pipette tip (Rainin pipetman; 1 to 20 μl) containing 10 to 15 μl of inoculant into the gel almost to the FeS plug, and expelling the contents of the pipette as the tip was withdrawn. The tubes were incubated in the dark in a constant temperature incubator at 21°C (± 1°C).

Other potential growth substrates were also tested by using gradient tubes. For Mn, the FeS was replaced with 0.5, 5, or 10 mM $MnCl_2$; for acetate (1, 5, or 10 mM), pyruvate (5 or 10 mM), and glucose (5 or 10 mM), the respective substrates were added to the agarose plug in the bottom of the tube or in some cases to the overlayer. Thiosulfate (2.5 mM) was added to the agarose plug, and sulfide gradients were established as described by Nelson and Jannasch (26). For those substrates that did not spontaneously consume O_2, in some cases the headspace was gassed briefly (2 to 3 s) with N_2 to decrease the $[O_2]$ in the headspace.

When larger volumes of cell material were required this whole procedure could be scaled up by using 5- by 9-cm glass vials (custom-made; Department of Chemistry glass shop, Michigan State University) that were sealed with no. 11 rubber stoppers. In this case, the volume of the FeS plug was 16 ml, the MWMM overlayer was 70 ml, sodium bicarbonate and vitamins were added as described above, and the molten overlayer was bubbled for 10 s with CO_2. Each of the large vials was inoculated at five evenly spaced points within the gel.

Growth studies. Cell growth rates on different substrates were determined by acridine orange direct counts. A series of gradient tubes was inoculated at the same time with identical amounts of cell material. The tubes were incubated together, and at daily intervals (or as specified), the entire semisolid overlayers from a duplicate pair of tubes were removed into separate glass test tubes and vortexed for 30 s to homogenize the growth band. For cell counts, two 10-μl aliquots of this material were smeared in defined circles on an agar-coated microscope slide and allowed to air dry; two slides were prepared for each growth tube. Cell counts were performed by adding a solution of 0.0005% acridine orange and counting all the cells in 15 microscope fields at ×1,000 magnification with an epifluorescence microscope (Optiphot; Nikon) (6). For the determination of the total amount of Fe that accumulated during growth, a 1-ml aliquot was removed from the original mixed sample and placed in an Eppendorf tube and frozen at −20°C for later analysis.

To determine whether the isolates could carry out the reduction of the Fe oxides that they formed, the growth bands from several growth tubes per isolate were harvested and washed once by centrifugation in PO_4 buffer. The material was placed in vials containing anaerobic medium and incubated as described previously (6).

Fe determination. Total Fe concentration was determined as described previously (6). A sample of the Fe oxides was diluted into a known amount of a solution of 0.25 M hydroxylamine–0.25 M HCl. After 1.5 to 2 h of incubation at 30°C with gentle agitation, a subsample of the reduced oxides was diluted into ferrozine and the absorbance of the resulting colored product was measured at 562 nm (29).

Microelectrode measurements. Clark-type oxygen microelectrodes (28) were constructed in our laboratory (2). The microelectrodes were mounted in a motorized micromanipulator (World Precision Instruments, Sarasota, Fla.), and the tip was lowered into the gel in the gradient tube at increments ranging from 50 to 250 μm. The progress of the electrode tip was monitored with a stereomicroscope to determine precisely when it contacted and pierced the bacterial bands that formed in the gradient tubes. The oxygen microelectrode was connected to a picoammeter (Diamond-General, Ann Arbor, Mich.) and the current output was recorded on a strip chart recorder.

TEM. When cells were grown in agarose and concentrated by centrifugation, the agarose concentrated with the cells and effectively diluted them to the point where it was very difficult to observe any cells by transmission electron microscopy (TEM). For this reason the high-molecular-weight polymer polyvinylpyrolidone (PVP) (5% [wt/vol]) was substituted for agarose in the gradient tubes. Medium made with PVP was viscous enough that the cells could maintain themselves in a band a few millimeters above the FeS plug. When these cells were harvested by centrifugation and washed in deionized H_2O, they formed a tight pellet of concentrated Fe oxides and cells that was more suitable for thin sectioning and the observation of cells by TEM. The washed, concentrated cell material was fixed for 24 h in 2.5% glutaraldehyde and then treated with osmium for an additional 24 h. The long fixation times were required to get adequate penetration of the fixatives into the iron oxide matrix to preserve cell structure. The fixed material was placed in resin and sectioned. The sections were poststained with uranyl acetate and then viewed under a Philips TEM operating at 80 kV.

Phylogenetic analysis. Each strain was grown in four large vials. After 10 to 14 days of growth, a sterile pipette was used to remove as much of the agarose that overlaid the growth band as possible without disturbing the band itself. The gel which included the growth band was placed into sterile, plastic 15-ml screwcap test tubes. These were heated at 70°C in a water bath for 12 min to melt the agarose and then quickly transferred into a clinical centrifuge at a room temperature of 37°C and spun at the maximum setting for 5 min. The supernatant was discarded, the tight pellet of iron oxides and cells in each tube was resuspended in 100 to 200 μl of deionized water, and the samples were pooled. The pooled sample was placed in a presterilized, screwcap Corex glass centrifuge tube, and 0.5 M oxalic acid (filter sterilized) was added in a ratio of 2:1 (final oxalic acid concentration, 0.33 M). This mixture was incubated at 30°C on a table shaker set at 150 rpm for 20 min, during which time the sample turned from deep reddish brown to light yellow as the oxides were reduced. The cell material was concentrated by centrifugation (10 min, 7,700 × *g*; Sorvall RC-5 centrifuge). Often after this first reduction the cell pellet was still dark brown due to the presence of residual Fe oxide, and the oxalic acid treatment was repeated. Based on visual inspection this removed additional oxides but there was always a small amount of oxide that remained with the pellet. Following the second reduction, the cell pellet was washed twice in 15 ml of deionized water, transferred to an Eppendorf tube, and washed two more times in 1.5 ml of deionized water. After the final spin, the supernatant was removed and the pellet was stored at −20°C until it was used for DNA extraction.

The extraction of genomic DNA and PCR amplification of small-subunit (SSU) rRNA gene products from each strain were as described elsewhere (24). The complete sequences, in both directions, of representative bacterial SSU ribosomal DNAs (rDNAs) were determined with an automated DNA sequencer (Applied Biosystems, Foster City, Calif.) at the sequencing facility at Michigan State University. Novel sequences were manually aligned to a database of SSU rDNA sequences obtained from the Ribosomal Database Project (23). Sequence alignments were based on primary and secondary structures and were submitted to the CHECK_CHIMERA program to detect for the presence of chimeric artifacts (19, 23). Phylogenetic analyses were restricted to the comparison of highly to moderately conserved nucleotide positions that were unambiguously alignable in all sequences, corresponding to residues 101 to 183, 220 to 451, 846 to 1005, 1037 to 1133, and 1141 to 1445 (*Escherichia coli* numbering system). Phylogenetic analyses were conducted by maximum likelihood analysis using the fastDNAml program distributed by the Ribosomal Database Project (23). Phylogenetic trees were constructed by using jumbled orders for the addition of taxa

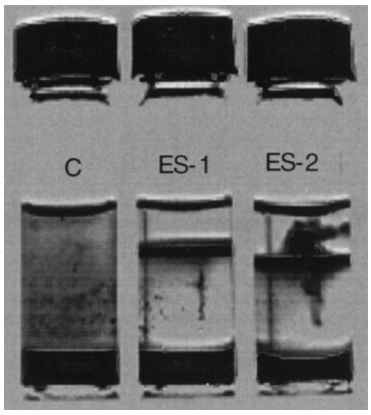

FIG. 1. Gradient growth tubes. The black layer on the bottom is the FeS-agarose plug. The tube on the left (C) is a control tube that was not inoculated with cells, Fe oxidation occurred throughout the semisolid overlayer in this tube. The tubes labeled ES-1 and ES-2 were inoculated with pure cultures of the respective organisms, and distinct, horizontal bands of iron oxidation have formed. The more faint vertical lines of oxidation are vestiges of the initial inoculation point in the gel.

and allowed for the global swapping of branches option. Using these parameters, the search for an optimal tree was repeated until the best log likelihood score was reached in at least three independent searches. Bootstrapping methods were conducted so that node reproducibility for the overall tree topology could be estimated (10). Bootstrapping occurred 100 times with the jumbled addition of taxa, and the search for an optimal tree was repeated until the best log likelihood score was reached in at least two independent searches each time.

Nucleotide sequence accession number. The SSU rRNA sequences representing strains ES-1 and ES-2 have been submitted to GenBank and have been assigned accession no. AF012541.

RESULTS

Enrichment and isolation. The most striking feature of the groundwater from the tile drain source was the high density of floculant iron hydroxides. When these flocs were stained with acridine orange and viewed by epifluorescence microscopy it was evident that they harbored a large population of prokaryotic cells of diverse morphology (results not shown). While evidence for both the sheaths characteristic of *L. ochracea*, and the stalks characteristic of *Gallionella* were visible, it appeared that most of the oxides were amorphous and that these harbored the largest populations of unicellular bacteria. This was quite similar to what has been seen in other high iron environments (11).

On two occasions, 6 weeks apart, enrichments were done by inoculating Fe gradient tubes with the Fe floculant-containing water. In both cases, 24 to 48 h after inoculation, a zone of cell growth visible by the appearance of reddish-brown iron oxides began forming. During this initial period the oxides were often distributed in a flare-like pattern spreading out from the point of inoculation in the center of the tube. Over the next 48 to 72 h these patterns typically coalesced into a discrete, rust-colored band at an intermediate distance between the FeS plug in the bottom of the tube and the air interface at the surface of the gel (Fig. 1). Once a stable band had formed, usually within 4 to 6 days, it was subsampled and diluted into a new gradient tube. At dilutions of 10^{-7} or 10^{-8}, cell growth began from individual colonies within the tubes; those tubes that initiated growth from between one to three colonies were chosen for further passage. After two or three passages of cells from the tube with the highest dilution, the cultures were assessed for purity by repeated passage in Fe gradient tubes and visual inspection by light microscopy. In addition, material was streaked onto plates of R2A medium to check for the presence of heterotrophs. The cultures were deemed pure if there was no growth on the R2A plates and if the cells associated with the iron oxides were of uniform morphology. In one case a heterotrophic, rod-shaped bacterium was still present after several high-dilution transfers; however, by subsampling and diluting the iron-oxidizing bacterium in the early stages of growth it was possible to rid the culture of this heterotroph. Ultimately, each of the initial enrichments yielded a pure culture of a morphologically unique iron-oxidizing microorganism (Fig. 2). These were designated isolates ES-1 and ES-2. Both isolates have been deposited with the American Type Culture Collection (ATCC) (ES-1, ATCC 700298; ES-2, ATCC 700299).

Flares or bands of oxides did not appear in gradient tubes to which either no inoculum or 1 mM sodium azide was added or in which the inoculum was pasteurized at 70°C for 20 min prior to inoculation. Instead, over several days the gel turned uniformly reddish orange as the Fe^{2+} released from the FeS plug was chemically oxidized by oxygen (Fig. 1).

Microscopy. Light microscopy revealed that the highest densities of cells for both isolates were associated with the Fe oxide matrix (Fig. 2). Both strains were motile, and in young cultures it was common to see free swimming cells that appeared unencumbered by Fe oxides. The number of motile, free-swimming cells appeared to decrease with the age of the culture. Isolate ES-1 (Fig. 2A) was a bent or helical-shaped rod. Isolate ES-2 (Fig. 2B) was a shorter bent rod that was often U-shaped. Light microscopy revealed no visibly obvious differences in the morphologies of the Fe oxides precipitated by the two strains.

Electron microscopy revealed that Fe oxides were tightly associated with the cell walls of both isolates (Fig. 3). It also appeared that the Fe oxides that formed around the cells were very fine-grained and amorphous in the sense that well-defined capsules of Fe oxides were not observed. The presence of the iron did appear to impede the diffusion of the TEM fixatives to the cells; even with extra-long fixative times it was difficult to get good enough fixation to assess cell wall structure, and this was especially true for strain ES-1. The diameter of strain ES-1 was 0.32 μm; the diameter of strain ES-2 was 0.73 μm.

Growth. The growth curves of strains ES-1 and ES-2 are shown in Fig. 4 and 5, respectively. ES-1 grew on FeS with a doubling time of approximately 8 h. The addition of acetate to the growth medium did not appear to stimulate the growth of ES-1 above that of Fe^{2+} alone. When ES-1 was grown in the presence of agarose alone it did not show any sign of growth (Fig. 4). When it was grown in the presence of acetate, pyruvate, or glucose without added iron, there was no visible sign of growth, nor was there any sign of oxygen consumption in tubes containing these carbon sources when oxygen profiles were measured with an oxygen microelectrode (results not shown). This was also true for tubes which had a reduced $[O_2]$ in the headspace. ES-1 also appeared to grow well on $FeCO_3$, although actual growth rates and doubling times were not calculated. ES-1 did not grow on Mn^{2+} or sulfide. On one occasion it did show some growth on thiosulfate, but we were unsuccessful in repeating this.

Strain ES-2 grew on FeS with a doubling time of approximately 12.5 h and had a comparable doubling time when grown on $FeCO_3$ (Fig. 5). It too did not grow with acetate, pyruvate, or glucose as a C source, nor did it show any indication of oxygen consumption in the presence of these substrates. ES-2 also did not grow on Mn^{2+}, sulfide, or thiosulfate. The cell yield of ES-2 was not as high, 5×10^7 cells/ml, as that of ES-1, which reached a cell density of 10^8 cells/ml. Both strains required vitamins for growth; when the vitamins were omitted

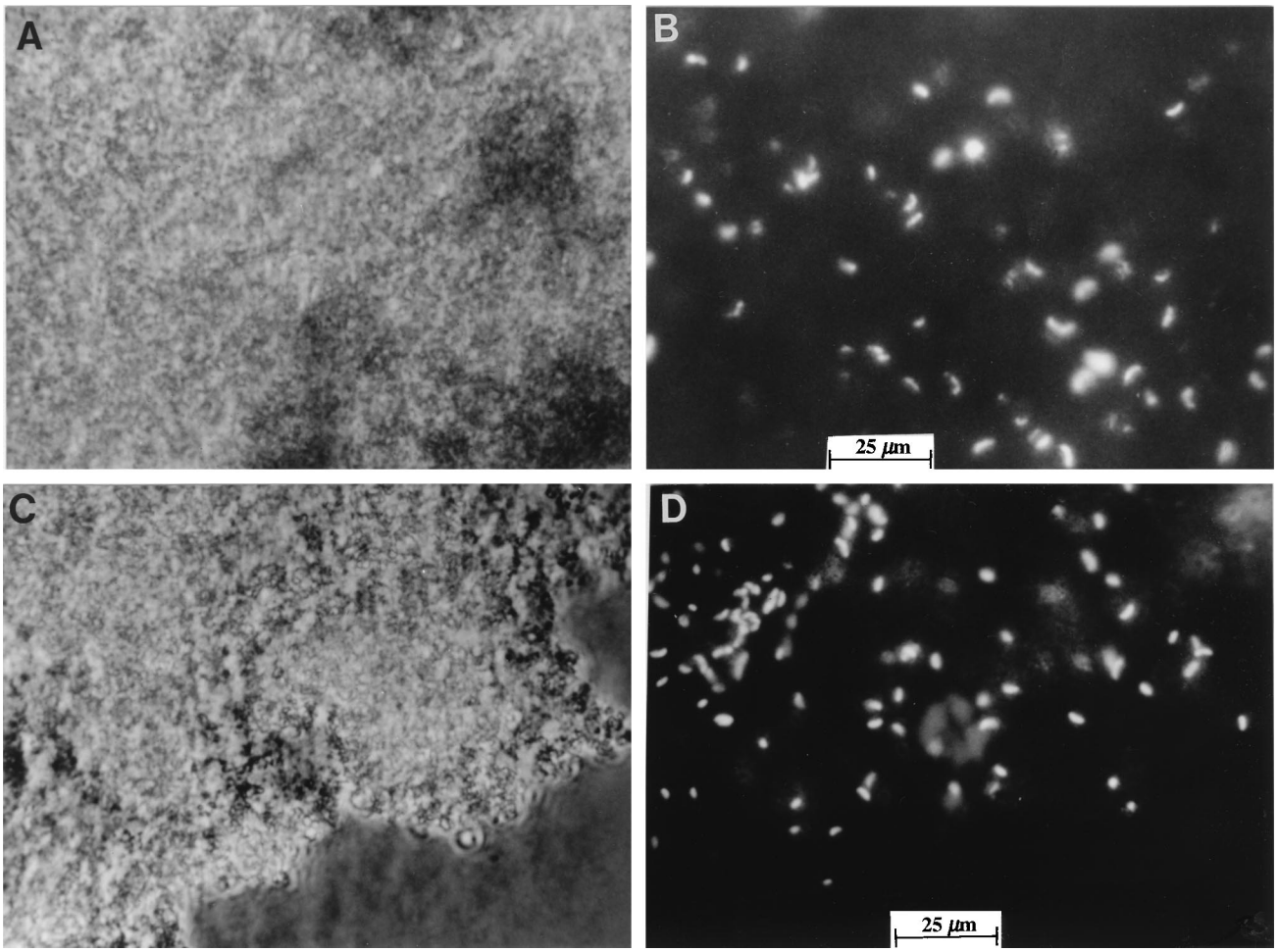

FIG. 2. Photomicrographs of strains ES-1 and ES-2 stained with acridine orange. Phase-contrast images of the Fe oxides and cells of ES-1 (A) and ES-2 (B) are shown. (B and D) The same fields shown in panels A and C (B and D, respectively) were viewed by epifluorescence to reveal the presence of cells associated with the oxides.

their growth diminished markedly after two or three transfers. If bicarbonate was omitted from the medium and 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer was substituted as a buffer at pH 6.5, it also appeared that the growth of the strains was diminished, as judged by the slower than normal formation of a growth band. Strain ES-2 appeared to be more adversely affected by the absence of bicarbonate than strain ES-1; however, these effects were not quantitated for either organism.

The total amount of Fe oxidized was similar in inoculated versus uninoculated tubes, suggesting that the amount of Fe^{2+} released into the medium was similar in both cases. Measured at a time scale of days, there did not appear to be major differences between the rates of abiotic and biotic Fe oxidation (results not shown). The difference in the oxidation rates, as stated above, was that in inoculated tubes all the iron oxidation occurred in a very localized zone where the cells were growing. Neither of these strains showed any evidence for Fe reduction when grown anaerobically in the presence of acetate or succinate.

Oxygen microelectrode measurements. Tubes inoculated with either strain ES-1 or ES-2 had steeper oxygen gradients than did the corresponding uninoculated gradient tubes (Fig. 6). The growth bands for both ES-1 and ES-2 defined the oxic-anoxic interface in their respective gradient tubes in that when the tip of the microelectrode penetrated the bottom of the growth zone, no O_2 was detected. In uninoculated control tubes, the O_2 profile extended all the way down to the FeS layer (Fig. 6). These results suggested that both these strains are microaerophiles. To demonstrate this further, some tubes were resealed after the first O_2 profile was made and were incubated for another 24 h. During this time a new band of iron oxidation appeared below the first band, and a second oxygen profile showed that a new oxic-anoxic interface had formed right at this lower band (Fig. 7). Apparently, the O_2 in the headspace was partially depleted due to growth of the bacteria. Exposure to the air replenished the O_2 in the headspace, which caused it to diffuse further into the gel, and the microbes tracked the O_2 to a new minimum, where they established a new oxic-anoxic interface.

Phylogeny. The two strains, ES-1 and ES-2, had identical SSU rRNA gene sequences, placing them into the same phylotype. Phylogenetically, they fell within a novel lineage of the γ subclass of the *Proteobacteria* (Fig. 8). What was most striking was their close relationship with the environmental phylotypes PV*B* OTU 1 (24, 25) and SP*B* OTU 1 (27), which were detected at deep-sea hydrothermal vents and ancient Siberian permafrost sediments, respectively. The former site, Loihi Sea-

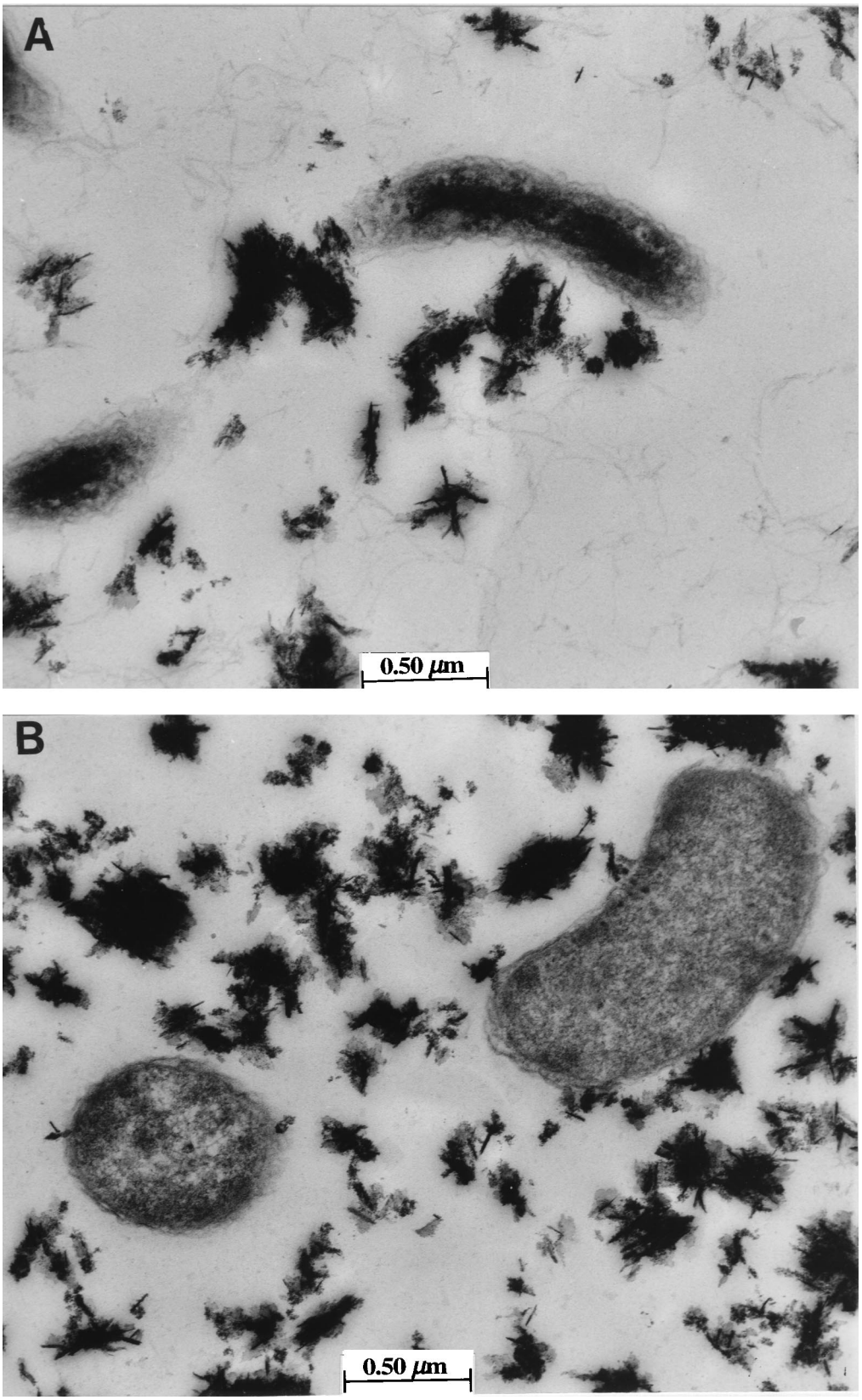

FIG. 3. Transmission electron micrographs of thin sections of ES-1 (A) and ES-2 (B). Note the differences in cell shape of the two isolates but also the similarities in the morphologies of the Fe oxides and the close association of the oxides with the cells.

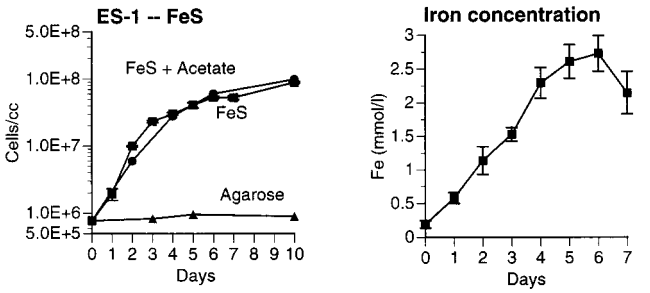

FIG. 4. Growth curves for strain ES-1 on FeS (■), FeS plus 5 mM acetate in the overlayer (●), and agarose alone (▲). The panel on the left shows cell growth, and the panel on the right shows accumulation of total Fe in the overlayer during growth on FeS alone. The error bars represent standard deviations from the means.

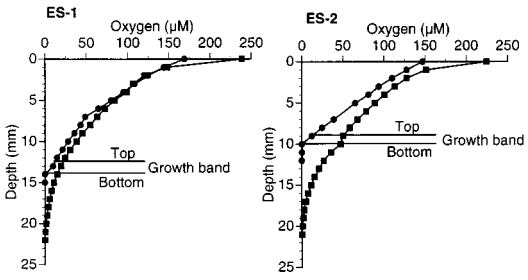

FIG. 6. Oxygen microelectrode profiles in gradient tubes of pure cultures of ES-1 and ES-2. Tubes inoculated with cells in which a growth plate had formed are represented by circles, and uninoculated tubes that were incubated for the same amount of time as the inoculated tubes are represented by squares. The horizontal lines are included to indicate the position of each growth plate relative to the oxygen concentration.

mount, is a low-temperature hydrothermal vent system that is characterized by a high output of Fe^{2+}. Large accumulations of amorphous iron hydroxides precipitate in the vicinity of the vents, including the morphotypes of sheathed and stalked iron oxidizers (18). The closest-known relative to this lineage is *Stenotrophomonas maltophilia*, a ubiquitous free-living bacterium isolated from aquatic habitats but more often associated with soils and especially the plant rhizosphere (17).

DISCUSSION

Growth on iron. The iron-oxidizing isolates ES-1 and ES-2 represent a new phenotypic group of unicellular, microaerobic bacteria that appear to harness energy from the oxidation of Fe^{2+} to Fe^{3+} for growth at circumneutral pH. Both of these strains grew in a gel-stabilized, bicarbonate-buffered, mineral salts medium and oxidized iron at the oxic-anoxic interface in opposing gradients of Fe^{2+} and O_2. Neither strain grew by utilizing organic substrates in the absence of iron, and their growth on iron was not stimulated by the presence of acetate. The results suggest that these organisms are true or obligate lithotrophs, unable to utilize reduced organic carbon compounds as an energy source. In growth tubes, all the iron oxidation occurred within a narrow band at the oxic-anoxic transition zone, which was a niche created by the bacteria consuming the O_2 diffusing down from the headspace and the Fe^{2+} diffusing up from the FeS or $FeCO_3$ layer. By comparison, in uninoculated tubes or tubes containing metabolic poisons, abiotic Fe oxidation occurred throughout most of the agarose layer. It does remain to be shown that these microbes are autotrophs. While this would seem the most likely explanation given that the only organics present in the growth medium were vitamins and trace contaminants in the electrophoresis-grade agarose, definitive proof of chemolithoautotrophic growth awaits demonstration of uptake of radiolabeled $^{14}CO_2$ and/or demonstration of enzymatic activities of a CO_2 fixation pathway.

The energetics of growth on iron are complicated by the effects that both pH and the different chemical states of Fe have on the reaction. At circa pH 2, where *Thiobacillus ferrooxidans* grows optimally, the complete reaction for Fe oxidation is generally stated as follows: $2Fe^{2+} + 0.5O_2 + 5H_2O \rightarrow 2Fe(OH)_3 + 4H^+$, which is exergonic with a $\Delta G° = -8.6$ kcal · mol^{-1} (5), barely enough energy to produce an ATP. However, as Widdel et al. (31) have pointed out, at a pH of 6 or 7, the Fe^{2+} may be in the form of either dissociated Fe^{2+} or $FeCO_3$ (siderite), and upon oxidation, the Fe^{3+} will all almost instant-

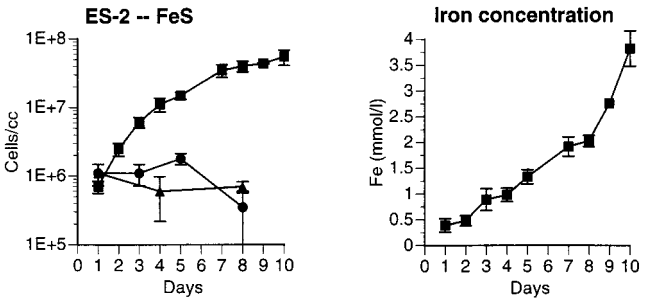

FIG. 5. Growth curves for strain ES-2 on FeS (■), 10 mM acetate without FeS (●), and agarose alone (▲). The panel on the left shows cell growth, and the panel on the right shows accumulation of Fe in the overlayer during growth on FeS. The error bars represent standard deviations from the means.

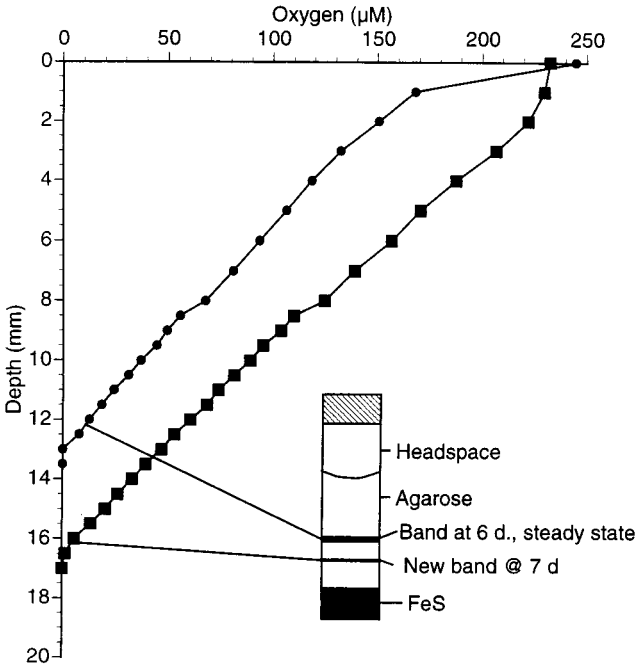

FIG. 7. Migration of cell band in response to oxygen. An initial oxygen profile was determined on day 6 (●), and the tube was resealed and incubated for another 24 h. On day 7 a new band appeared below the first band. The day 7 oxygen profile (■) indicated that the oxygen minimum now corresponded exactly to the position of this new band.

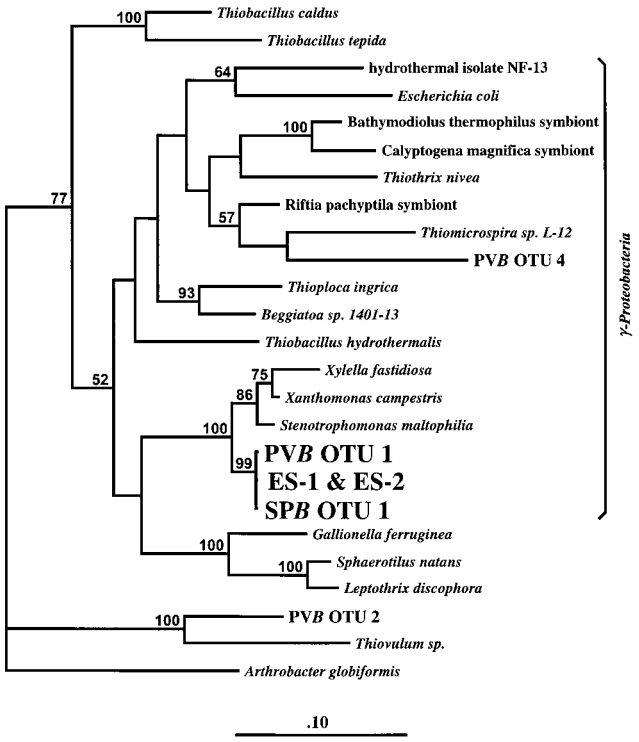

FIG. 8. Phylogenetic tree demonstrating the relationships of the ES-1 and ES-2 phylotype, which is included in the Siberian permafrost bacteria (SP*B* OTU 1) and Pele's Vents bacteria (PV*B* OTU 1) lineage, with other gamma *Proteobacteria* and additional representative iron and sulfur oxidizers as determined by maximum likelihood analysis of SSU rDNA sequences. Numbers at nodes represent bootstrap values (percent) for that node (based on 200 bootstrap resamplings). An outgroup is represented by *Arthrobacter globiformes*. The scale bar represents 0.10 fixed mutations per nucleotide position. Bootstrap values are shown for frequencies at or above a threshold of 50%.

ly form an insoluble amorphous hydroxide, e.g., $Fe(OH)_3$ or FeOOH. This results in the product of the oxidation being rapidly and continuously removed from solution. At pH 7 the redox potential of the couples for ferric hydroxide formation from either the siderite, $Fe(OH)_3 + HCO_3^-/FeCO_3$ ($E'_o = +0.200$ V), or the completely dissociated form of Fe^{2+}, $Fe(OH)_3/Fe^{2+}$ ($E'_o = -0.236$ V), are substantial compared to the redox couple of O_2/H_2O ($E'_o = +0.810$ V) (31). Thus, Fe^{2+} oxidation coupled to O_2 respiration can generate a significant redox potential (30). Indeed, it is this interpretation of the energetics of growth on Fe that explains why anoxic phototrophic bacteria are able to couple the oxidation of Fe^{2+} to Fe^{3+} as an electron donor for anoxygenic photosynthesis (3, 31). In addition, the complete oxidation of 1 mol of Fe^{2+} to FeOOH or $Fe(OH)_3$ yields 2 mol of H^+. If this reaction occurs in close proximity to the cell wall, as TEM evidence suggests, then these H^+ may be available to contribute to the ΔpH of the proton motive force.

Taxonomy and comparison with other Fe oxidizers. The taxonomic characteristics of strains ES-1 and ES-2 are summarized in Table 1. Besides having a different morphology, strain ES-1 tolerated more mesophilic temperatures than ES-2 and it had a more rapid doubling time on FeS.

Both the morphological and phylogenetic evidence indicate that these isolates are not closely related to the more commonly recognized iron bacteria *G. ferruginea* and *L. ochracea*. Isolates ES-1 and ES-2 are unicellular and produce neither stalks nor sheaths; instead these microbes appear to encapsulate themselves in a coating of amorphous iron hydroxides. In this regard, they are similar to members of the *Siderocapsaceae* family. This ill-defined group contains four genera, *Siderocapsa*, *Naumanniella*, *Siderococcus*, and *Ochrobium*, consisting of 18 species (16). The species descriptions are based primarily on the morphology of the oxide coatings that form around the cells. Based on enrichment studies, this group is thought to be predominantly heterotrophic; however, none of these organisms have been maintained in pure culture so both their physiological and taxonomic status are virtually unknown. Morphologically the strain ES-2 does bear a resemblance to *G. ferruginea*, although it tends to be more U-shaped compared to the classic description of *G. ferruginea* as a bean-shaped cell. It has been reported that when grown at a pH of >6, *G. ferriginea* does not form a stalk. When ES-2 was grown at a pH as low as 5.4, stalk formation was not observed.

It is noteworthy that the main difference in the enrichments for *Gallionella* and for isolates ES-1 and ES-2 is the addition of a gel-stabilizing agent to the growth medium for the latter. To our knowledge, the prolific growth of nonappendaged, unicellular iron oxidizers has not been observed in *Gallionella* enrichments, and our attempts to culture ES-1 or ES-2 under the liquid-only conditions used for *Gallionella* were unsuccessful. In a nonstabilized or low-viscosity liquid environment the dense, Fe-encrusted cells would descend out of the anoxic-oxic transition zone to the anoxic FeS or $FeCO_3$ layer and be physically separated from their electron acceptor, O_2. However, the presence of an agarose gel or a high viscosity polymer (PVP) reduces or entirely eliminates the gravitational effect, and thus the cells can maintain their position and establish an optimum niche at the oxic-anoxic boundary. It is also important to note that cell numbers obtained in the gel-stabilized growth tubes are nearly 2 orders of magnitude greater than those of *G. ferruginea* grown in liquid gradient tubes.

Phylogenetically, ES-1 and ES-2 are very closely related to

TABLE 1. Characteristics of Fe-oxidizing strains ES-1 and ES-2

Characteristic	ES-1	ES-2
Phylogeny	γ *Proteobacteria*	γ *Proteobacteria*
Morphology	Curved or helical rod	Curved rod
Cell diameter (μm)	0.32	0.73
Motile	Yes	Yes
Growth substrate		
FeS	Yes (8)[a]	Yes (12.5)[a]
Thiosulfate	?[b]	No
Sulfide	No	No
Mn^{2+}	No	No
Acetate	No	No
Pyruvate	No	No
Glucose	No	No
Microaerophilic	Yes	Yes
Growth temperature		
6°C	No	Yes
30°C	Yes	No
Iron reduction potential	ND[c]	ND[c]

[a] Doubling time in hours.
[b] Growth on thiosulfate was detected on one occasion but this result could not be repeated.
[c] ND, not detected.

one another, but again they do not share a close relationship with *Gallionella*, which is a member of the β subdivision of the *Proteobacteria*. What is remarkable is that they are closely related to the PV*B* OTU 1 SSU rDNA phylotype that was described by Moyer et al. (24) from a hydrothermal vent system at the Loihi Seamount near Hawaii. This site is characterized by large accumulations of precipitated Fe hydroxides that contain the morphological remains of Fe bacteria (9, 18).

Physiology and ecology. The isolation and initial characterization of these iron oxidizers raises many intriguing questions about both their metabolic capabilities and their distribution in Fe^{2+}-rich environments around the world. As mentioned above, a systematic study of an iron seep in Denmark revealed up to 10^9 cells/ml of mat material. Most of these cells were unicellular, and several different morphologies were evident. We have also recently isolated another strain of iron-oxidizing bacterium in a 10^{-7} dilution tube from an iron seep in a Michigan wetland. This strain is morphologically distinct from ES-1 and ES-2; however, based on sequence analysis of the 16S rRNA gene, it too shares a common phylogeny with ES-1 and ES-2 (9). Thus, three enrichments with exactly the same selection and from similar environments have yielded three microbes that have unique phenotypes yet which share a close phylogenetic relationship. Furthermore, we have recently been successful at enriching Fe oxidizers with similar growth characteristics to ES-1 and ES-2 from low-temperature hydrothermal vent systems in the Pacific Ocean that are enriched in Fe^{2+}. These strains obligately require a marine salts solution for growth. In total, these results suggest that the importance of unicellular, nonappendaged Fe-oxidizing microbes in high iron environments may be much greater than had previously been realized.

Another interesting question concerning these microbes is how they cope with encasing themselves in an insoluble iron oxide matrix. Once encrusted in the oxides are they entombed or do they have a mechanism of escape? Preliminary evidence suggests that both ES-1 and ES-2 produce an extracellular matrix, and it is in this matrix that precipitation of the iron oxides occurs. Perhaps this matrix also affords them a means to avoid entrapment in a cocoon of iron oxide. In addition, TEM observations indicate that the Fe oxides are very fine-grained, which may afford the cells some freedom of movement within oxide matrix.

Biogeochemical importance. Iron is the fourth most abundant element in the Earth's crust. It has been recognized for many years that microbial iron oxidation in acidic environments is an important process, and a polyphyletic group of lithoautotrophic microbes that can utilize Fe^{2+} as an energy source have been described. The best-studied member of this group is *T. ferrooxidans* (5). In environments at a more neutral pH, the role microbes play in the oxidation of ferrous iron has largely been overlooked for reasons stated in the introduction. This is also despite the fact that over the last decade it has become apparent that the reduction of ferric iron coupled to anaerobic respiration is a very important microbial process that can significantly affect the structure of microbial ecosystems in groundwaters and sediments (22). Wherever biologically generated ferrous iron encounters an aerobic environment a potential habitat for iron-oxidizing bacteria is created. In turn, by producing amorphous, poorly crystalline Fe hydroxides, these iron-oxidizing microbes create an excellent substrate for Fe-reducing prokaryotes to utilize under anoxic conditions (6). Thus, it is possible that Fe oxidation and reduction could form a relatively tightly coupled cycle.

Finally, the recent discovery of Fe-oxidizing photosynthetic bacteria in anaerobic sediments has led to an elegant argument suggesting that these microbes could provide a biological explanation for the banded iron formations (BIFs) laid down in the Archaeon and early Proterozoic eras (3, 31). The crux of this argument is whether the earth's atmosphere was completely reducing or whether it contained small amounts of oxygen at the time BIFs formed between 1.8 and 3.85 billion years ago. If the atmosphere was completely reducing, then anoxygenic iron-oxidizing phototrophs would have been favored as a means of biological deposition of iron oxide layers. However, if portions of the biosphere were partially oxygenated, the presence of microaerophilic iron-oxidizing bacteria could have contributed to the formation of BIFs. In fact, if some BIFs formed in the deep sea, well below the photic zone, as has been suggested (1), then microaerophilic iron oxidizers might provide a more tenable biological means of oxidation in these environments.

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