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Thermophily in the *Geobacteraceae*: *Geothermobacter ehrlichii* gen. nov., sp. nov., a Novel Thermophilic Member of the *Geobacteraceae* from the "Bag City" Hydrothermal Vent

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Little is known about the microbiology of the "Bag City" hydrothermal vent, which is part of a new eruption site on the Juan de Fuca Ridge and which is notable for its accumulation of polysaccharide on the sediment surface. A pure culture, designated strain SS015, was recovered from a vent fluid sample from the Bag City site through serial dilution in liquid medium with malate as the electron donor and Fe(III) oxide as the electron acceptor and then isolation of single colonies on solid Fe(III) oxide medium. The cells were gram-negative rods, about 0.5 μm by 1.2 to 1.5 μm, and motile and contained *c*-type cytochromes. Analysis of the 16S ribosomal DNA (rDNA) sequence of strain SS015 placed it in the family *Geobacteraceae* in the delta subclass of the *Proteobacteria*. Unlike previously described members of the *Geobacteraceae*, which are mesophiles, strain SS015 was a thermophile and grew at temperatures of between 35 and 65°C, with an optimum temperature of 55°C. Like many previously described members of the *Geobacteraceae*, strain SS015 grew with organic acids as the electron donors and Fe(III) or nitrate as the electron acceptor, with nitrate being reduced to ammonia. Strain SS015 was unique among the *Geobacteraceae* in its ability to use sugars, starch, or amino acids as electron donors for Fe(III) reduction. Under stress conditions, strain SS015 produced copious quantities of extracellular polysaccharide, providing a model for the microbial production of the polysaccharide accumulation at the Bag City site. The 16S rDNA sequence of strain SS015 was less than 94% similar to the sequences of previously described members of the *Geobacteraceae*; this fact, coupled with its unique physiological properties, suggests that strain SS015 represents a new genus in the family *Geobacteraceae*. The name *Geothermobacter ehrlichii* gen. nov., sp. nov., is proposed (ATCC BAA-635 and DSM 15274). Although strains of *Geobacteraceae* are known to be the predominant Fe(III)-reducing microorganisms in a variety of Fe(III)-reducing environments at moderate temperatures, strain SS015 represents the first described thermophilic member of the *Geobacteraceae* and thus extends the known environmental range of this family to hydrothermal environments.

Fe(III)-reducing microorganisms play an important role in the decomposition of natural and contaminant organic compounds in marine and freshwater sediments and in the production of geologically significant minerals (24, 25, 54). Molecular studies have demonstrated that strains of *Geobacteraceae* are the predominant Fe(III)-reducing microorganisms in a variety of sedimentary environments in which Fe(III) reduction is the terminal electron-accepting process (16, 47, 48, 51, 52). The competitive advantage of strains of *Geobacteraceae* over other well-known Fe(III) reducers can be attributed, in part, to their ability to completely oxidize organic electron donors, such as acetate, the key intermediate in anaerobic organic matter degradation (24). Another key feature may be the ability of strains of *Geobacteraceae* to find Fe(III) oxides through chemotaxis to Fe(II) and directly attach to Fe(III) oxides in order to reduce them (9, 24). This strategy may be a more energetically favorable method for Fe(III) reduction in sedimentary environments than the strategy of other Fe(III) reducers, which release extracellular electron shuttles or chelators in order to alleviate the need for cell-Fe(III) oxide contact (9, 40, 41, 42).

Although the microbial reduction of Fe(III) to Fe(II) has been studied in detail in aquatic sediments and aquifers at moderate temperatures (26, 27), there has been little investigation of Fe(III) reduction in hotter environments. Fe(III) is considered to be an important electron acceptor in modern hot environments, such as deep subsurface and hydrothermal systems, and may also have been an important electron acceptor in hot environments during Earth's early history (36, 57). Recent studies demonstrated that the potential for dissimilatory Fe(III) reduction is a common characteristic of hyperthermophilic members of the *Archaea* and *Bacteria* (21, 55, 57). Novel hyperthermophiles with Fe(III) serving as the electron acceptor have been isolated (19, 21), and it has been shown that the metabolic potential of some previously described hyperthermophiles is significantly enhanced when Fe(III) is provided as an electron acceptor (56, 57). Studies with Fe(III)-reducing hyperthermophiles have documented for the first time the potential for the oxidation of important organic compounds, such as acetate, long-chain fatty acids, and aromatic compounds, in environments hotter than 80°C (21, 55, 56).

Previous studies on moderately thermophilic Fe(III)-reducing microorganisms focused on subsurface organisms. For ex-

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ample, the first thermophilic microorganism shown to conserve energy to support growth through Fe(III) reduction was *Bacillus infernos*, an obligate anaerobe isolated from deep terrestrial subsurface environments (6). This organism can incompletely oxidize lactate to acetate or oxidize formate with the reduction of Fe(III) at temperatures of 40 to 65°C, with an optimum temperature of 60°C (6). *Deferribacter thermophilus*, which was isolated from production waters of a petroleum reservoir in the North Sea (15), is capable of coupling the oxidation of a wide variety of electron donors to the reduction of Fe(III), Mn(IV), and NO_3^- at an optimum growth temperature of 60°C. *Thermoterrabacterium ferrireducens*, which was isolated from hot springs in Yellowstone National Park and New Zealand (50), incompletely oxidizes lactate, pyruvate, and glycerol with the reduction of Fe(III) at an optimum temperature of 65°C. A *Thermus* sp. isolated from the waters of a deep gold mine incompletely oxidizes lactate with the reduction of Fe(III) at an optimum temperature of 65°C (23).

As part of a study of the role of microbial Fe(III) reduction at elevated temperatures, microorganisms associated with a marine hydrothermal vent named "Bag City" were investigated. Here we report on the isolation and characterization of a novel thermophilic bacterium which was isolated from diffuse-flow vent fluid from Bag City and which can couple the oxidation of acetate to the reduction of Fe(III) or nitrate. The isolate, designated strain SS015, has an optimal temperature of 55°C and can grow at temperatures as high as 65°C; this isolate represents a novel genus in the family *Geobacteraceae*. These findings expand the known environmental range of the family *Geobacteraceae* and suggest that members of this family, which are known to predominate in many cooler Fe(III)-reducing environments, may also be important Fe(III) reducers in hotter environments.

MATERIALS AND METHODS

Source of organism and sampling. Strain SS015 was isolated from diffuse-flow vent fluid samples collected from the hydrothermal chimney named Bag City in July 1999 by using a hydrothermal fluid and particle sampler. This vent is located at the southern end of the new seafloor lava flow of the Axial Seamount, a very seismically active submarine volcano at 46°N, 130°W. Axial Seamount rises 1,100 m above the surrounding seafloor to a minimum depth of 1,400 m below sea level at the intersection of the Juan de Fuca Ridge and the Cobb Seamount chain in the northeastern Pacific Ocean, approximately 300 mi west of Cannon Beach, Oreg. (The New Millennium Observatory Vents Program Network [http://newport.pmel.noaa.gov/nemo/realtime/nemosite.html and http://newport.pmel.noaa.gov/nemo/logbook/teacher071300.html]).

Enrichment of strain SS015. Strict anaerobic culturing techniques were used throughout (4, 39). Gases were passed through a column of hot copper filings to remove all traces of oxygen. Basal marine enrichment medium for Fe(III) reducers was prepared as previously described (21) and was supplemented with the following (per liter): Difco yeast extract, 0.10 g; Na_2SeO_4, 0.02 g; vitamin mixture (34), 10 ml; trace mineral solution (45), 10 ml; and poorly crystalline Fe(III) oxide (33), 100 mmol/liter. For initial enrichments and isolations, DL-malate was used as the electron donor (final concentration, 10 mM). The culture medium (10 ml) was dispensed into 26-ml anaerobic pressure tubes (Bellco Glass, Inc., Vineland, N.J.) and sparged with oxygen-free N_2-CO_2 (80:20 [vol/vol]) gas for 12 to 15 min to remove dissolved oxygen. The tubes were then sealed with thick butyl rubber stoppers and autoclaved. After autoclaving, the medium in each tube was supplemented with 0.25 mM L-cysteine HCl and 1.3 mM $FeCl_2 \cdot 2H_2O$ from concentrated anaerobic stock solutions. The final pH of the medium was ca. 6.8 to 7.0 (at room temperature). This medium (10 ml) was inoculated with anoxic diffuse-flow vent fluid samples (1 ml) collected from the Bag City hydrothermal vent and incubated at various temperatures (50 to 100°C) in the dark. The production of Fe(II) was monitored over time as described below.

Isolation of strain SS015 on solid medium. A modification of the roll tube method (17) for the cultivation of strict anaerobes with serial dilution of an enrichment culture on solid medium was used to isolate single colonies (20, 22), with the following modifications: pyruvate was replaced by DL-malate (10 mM) as the electron donor, and the medium was supplemented with Difco yeast extract (0.10 g/liter). The final pH of the medium was ca. 6.8 to 7.0 (at room temperature). Once prepared, all roll tubes were incubated vertically at 55°C in the dark.

Evaluation of electron donors and electron acceptors utilized. To determine the ability of strain SS015 to utilize substrates other than DL-malate, alternate electron donors from concentrated anoxic and sterile stock solutions were added to freshly prepared media. The alternate electron donors used were as follows: pyruvate (10 mM), acetate (10 mM), malate (10 mM), formate (10 mM), starch (0.1%), peptone (0.1%), tryptone (0.1%), Casamino Acids (0.1%), sodium glutamic acid (MSG; 8.4 mM), isoleucine (1.0 mM), arginine (1.0 mM), asparagine (1.5 mM), glutamine (1.0 mM), histidine (6.4 mM), serine (2.0 mM), butyrate (1 to 5 mM), propionate (1 to 5 mM), maltose (10 mM), fructose (10 mM), ethanol (10 mM), methanol (10 mM), isopropanol (5 mM), hydrogen (as H_2-CO_2, 80:20 [vol/vol]; 101 kPa), lactate (10 mM), citrate (10 mM), succinate (10 mM), fumarate (10 mM), yeast extract (0.1%), glycerol (20 mM), glycine (13.3 mM), L-cysteine (8.3 mM), alanine (11.2 mM), aspartic acid (7.5 mM), proline (8.7 mM), stearate (1 mM), palmitate (1 mM), valerate (1 to 10 mM), catechol (1 mM), phenol (0.25 to 0.50 mM), benzoic acid (0.5 to 1 mM), 4-hydroxybenzoic acid (0.5 to 1 mM), Fe^{2+} (amorphous FeS; 20 mM), and ribose (10 mM). Substrate utilization was monitored by measuring growth and Fe(II) production during incubation at 55°C and under N_2-CO_2 (80:20 [vol/vol]).

To evaluate the utilization of alternate electron acceptors, poorly crystalline Fe(III) was omitted from the media. Alternate electron acceptors from concentrated anaerobic and sterile stock solutions were added individually to freshly prepared media with DL-malate as the electron donor at 55°C and under N_2-CO_2 (80:20 [vol/vol]). The alternate electron acceptors used were as follows: poorly crystalline Fe(III) oxide (100 mM), NO_3^- (10 mM), NO_2^- (1.0 mM), dimethyl sulfoxide (1 to 2mM), oxygen (0.3 to 1% and 20%), Fe(III) citrate (20 and 50 mM), Fe(III) pyrophosphate (3 mM), MnO_2 [poorly crystalline Mn(IV); 20 mM], anthraquinone-2,6-disulfonate (5 mM), goethite (50 mM), hematite (50 mM), citrate (25 to 50 mM), fumarate (25 to 50 mM), malate (10 to 20 mM), $S_2O_3^{2-}$ (10 mM), SO_4^{2-} (10 to 20 mM), SO_3^{2-} (2 to 4 mM), and S^0 (20%).

Temperature, pH, and salt optima. The rates of growth of strain SS015 at various temperatures, pHs, and salt concentrations were determined spectrophotometrically by measuring changes in the absorbances (at 600 nm) of cultures growing in enrichment medium amended with DL-malate as the electron donor and nitrate as the electron acceptor. Growth rates were also determined by direct cell counting with DL-malate as the electron donor and poorly crystalline Fe(III) oxide or nitrate as the electron acceptor. The effect of temperature on the rate of growth of strain SS015 was determined by incubating inoculated pressure tubes at temperatures ranging from 20 to 90°C. The cultures were incubated in either temperature-controlled hot air incubators or water baths containing a calibrated thermometer. The pH range investigated for the growth of strain SS015 was 2.0 to 9.0. For pHs ranging from 2.0 to 5.5, the pH was adjusted with a solution of HCl (1 M sterile anoxic stock). For pHs ranging from 5.5 to 8.0, a bicarbonate buffer solution (1 M sterile anoxic stock) was added to the medium, which was kept under N_2-CO_2 (80:20 [vol/vol]). For pHs ranging from 8.0 to 9.0, different amounts of a solution of NaOH (1 M sterile anoxic stock) were added to the medium, which was kept under N_2. All media were then inoculated with 1 ml of a freshly grown culture of strain SS015 and incubated in the dark at 55°C. The effect of NaCl on growth was determined by varying its concentration from 0 to 72 g liter^{-1} in freshly prepared medium inoculated with strain SS015 (10% [vol/vol]) at 55°C.

Antibiotic sensitivities. Sensitivities to ampicillin (200 μg ml^{-1}), chloramphenicol (100 μg ml^{-1}), erythromycin (150 μg ml^{-1}), kanamycin (100 μg ml^{-1}), novobiocin (100 μg ml^{-1}), penicillin G (200 μg ml^{-1}), phosphomycin (200 μg ml^{-1}), rifampin (100 μg ml^{-1}), streptomycin (200 μg ml^{-1}), vancomycin HCl (100 μg ml^{-1}), trimethoprim (300 μg ml^{-1}), and tetracycline (75 μg ml^{-1}) were tested. A 1-ml aliquot of an exponentially growing culture was transferred to fresh marine enrichment medium containing potassium nitrate (10 mM), DL-malate (10 mM), and filter-sterilized antibiotic. The cultures were incubated at 50°C to minimize thermal inactivation of the antibiotics.

Analytical techniques. Fe(III) reduction was monitored by measuring the production of Fe(II) over time. The amount of Fe(II) solubilized after 2 h of incubation in anaerobic oxalate solution was determined with ferrozine as previously described (44). Cell numbers were counted by using epifluorescence microscopy as previously described (34). Nitrate and nitrite concentrations were measured by ion chromatography (DX100 ion chromatograph; Dionex Corp., Sunnyvale, Calif.) with 2.65 mM $Na_2CO_3 \cdot H_2O$– 1.15 mM $NaHCO_3$ as the eluent

and a Dionex IonPac AS4A column with conductivity detection. Ammonium concentrations were determined by using a previously described (46, 49) spectrophotometric assay. Briefly, culture supernatants sampled at different time points were added to an alkaline solution of sodium nitroprusside, and the resulting color was quantified by measuring the absorbance at 650 nm.

Cytochromes. For the detection of cytochromes, strain SS015 was grown under optimal conditions in marine enrichment medium with nitrate (10 mM) as the electron acceptor and DL-malate as the electron donor. For the detection of cell-associated cytochromes, whole-cell suspensions were prepared by harvesting the cells under anaerobic conditions, washing the cells twice with 0.1 M Tris buffer (pH 6), and resuspending the cells in the same buffer. For the detection of soluble (periplasmic and cytoplasmic) cytochromes, cell extracts were prepared by harvesting the cells as described above. Harvested cells were then lysed by rapid freezing and thawing (dry ice-ethanol and a hot water bath at 60°C, respectively) in the presence of lysozyme (50 μg ml^{-1}) and under an N_2 atmosphere. Cell debris was removed by centrifugation (12,000 × *g* for 10 min at room temperature). Dithionite-reduced and air-oxidized spectra of whole-cell suspensions and cell extracts were analyzed on a Varian 50 Bio spectrophotometer.

Epifluorescence and electron microscopy. Cells were routinely examined with a Zeiss Axioskop 20 phase-contrast microscope equipped with a UV lamp, an excitation filter (LP 420), and a red attenuation filter (BG 38). Electron microscopy was carried out as previously described (20). The presence of an exopolysaccharide (EPS) matrix associated with cells of strain SS015 was assessed by transmission electron microscopic analysis of ruthenium red-stained samples (58).

DNA G+C content. DNA isolation (8) and G+C content analysis (38) were performed with nitrate-grown cells by the Identification Service of the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH.

Isolation of DNA and amplification of 16S ribosomal DNA (rDNA) and *fusA*. Cultures (10 ml) grown with poorly crystalline Fe(III) oxide as the electron acceptor were treated with 30 ml of filter-sterilized oxalate solution (44) to remove solid Fe(III) oxide, which inhibits PCR. Cells were collected by centrifugation, and genomic DNA was extracted with a FastDNA SPIN kit (Bio 101 Systems, Carlsbad, Calif.). Primers 8F (14) and 1492R (1, 3) were used to amplify a 1,484-bp fragment of the 16S rRNA gene of strain SS015.

A 590-bp fragment of the *fusA* gene, which encodes elongation factor G (Ef-G), was amplified by using degenerate primers that were designed from conserved amino acids within the Ef-G protein (5). PCR mixtures, in a total volume of 100 μl, contained 200 ng of DNA template, 10 μl of 10× buffer (15 mM $MgCl_2$) (Qiagen Inc.), 20 μl of buffer Q (Qiagen Inc.), 12 μl of 25 mM $MgCl_2$ solution, 8 μl of 0.25 μM deoxynucleoside triphosphate solution, and 60 pmol of forward and reverse primers. The following parameters were used to amplify this gene fragment: initial denaturation at 92°C for 5 min; 20 cycles of denaturation at 92°C (60 s), annealing starting at 50°C (90 s) and decreasing 0.5°C with each cycle, and elongation at 71°C (60 s); 20 cycles of 92°C for 60 s, 43°C for 60 s, and 71°C for 60 s; and a final extension at 71°C for 10 min. Cloning of PCR-amplified fragments and sequencing were performed by standard procedures.

Phylogenetic analyses of 16S rDNA sequences and Ef-G protein. Complete bidirectional sequences of the 16S rRNA and *fusA* genes were compared to the GenBank nucleotide and protein databases by using BLASTN and BLASTX algorithms (2). 16S rDNA nucleotide and Ef-G amino acid sequences were manually aligned, and hypervariable regions were masked by using the Genetics Computer Group sequence editor (Wisconsin Package, version 10; Genetics Computer Group, Madison, Wis.). Aligned sequences were imported into PAUP 4.0b 4a (53), and phylogenetic trees were inferred. Branching order was determined and compared by using character-based (maximum-parsimony and maximum-likelihood) algorithms and distance-based algorithms (HKY85 and among-site rate variations). Comparisons of 1,100 nucleotide positions from the 16S rRNA gene and 200 amino acid positions from the Ef-G protein were used for the generation of phylogenetic trees. Bootstrap analysis was performed by using the parsimony-based character state optimization model with 100 replicates.

The similarity matrix program (37) available at the Ribosomal Database Project II website and LFASTA, version 3.2 (43), were used to generate similarity matrices with 1,400 bp considered for the 16S rRNA gene and 200 amino acids considered for the translation product of *fusA* (Ef-G protein).

Nucleotide sequence accession numbers. The sequences were deposited in GenBank under the following accession numbers: SS015 16S rDNA gene fragment, AY155599; SS015 *fusA* gene fragment, AF547801; *Desulfuromusa bakii fusA* gene fragment, AY155597; *Desulfuromusa kysingii fusA* gene fragment, AY155598; *Geobacter sulfurreducens fusA* gene fragment, AF546867; and *Pelobacter acetylenicus fusA* gene fragment, AY155600. The GenBank accession numbers for other known 16S rDNA nucleotide sequences used in the phylogenetic analyses are as follows: *Aeromonas hydrophila*, X60404; *Ferrimonas balearica*, X93021; *Ferribacterium limneticum*, Y17060; *Thiobacillus ferrooxidans*, AB039820; *Desulfobacter hydrogenophilus*, M34412; *Malonomonas rubra*, Y17712; *D. bakii*, X79412; *D. kysingii*, X79414; *Desulfuromonas acetoxidans*, M26634; *Desulfuromonas thiophila*, Y11560; *Geobacter pelophilus*, U96918; *Geobacter metallireducens*, L07834; *D. thermophilus*, U75602; *Geovibrio ferrireducens*, X95744; and *Thermus* sp. strain SA-01, AF020205. The accession numbers for Ef-G proteins from known organisms used in the phylogenetic analyses are as follows: *Haemophilus influenzae*, F64078; *Xylella fastidiosa*, NP_299906; *Chlamydophila pneumoniae*, NP_300605; and *Borrelia burgdorferi*, NP_212825.

RESULTS

Enrichment and isolation. Several of the hydrothermal fluid vent samples from the Axial Seamount eruption site yielded active Fe(III)-reducing enrichment cultures. However, only the results for strain SS015, enriched and isolated from the samples collected from the Bag City hydrothermal vent, will be summarized here. Within 10 days of inoculating DL-malate–Fe(III) oxide medium at 55°C with vent fluid samples, the reddish brown, nonmagnetic Fe(III) oxide changed to a black, magnetic mineral which was probably magnetite (35). Inocula (10%) from the enrichment were transferred to the same medium and incubated at 50, 55, 60, 65, 70, 75, 80, 85, 90, or 100°C. The enrichment at 55°C was successfully transferred five times, and the fifth transfer was serially diluted 10-fold. The 10^{-8} dilution, which was the highest dilution exhibiting Fe(III) reduction, was serially diluted, and the serial dilution procedure was repeated a third time. The highest positive dilution from the third serial transfer was used to inoculate roll tubes of DL-malate– Fe(III) oxide medium solidified with GELRITE (Sigma-Aldrich Chemical Co.). After 2 weeks of incubation at 55°C, colonies (0.5 to 1.0 mm in diameter) appeared in the more dilute solutions. Individual colonies were picked from the highest positive dilution and transferred to 2 ml of liquid medium amended with poorly crystalline Fe(III) oxide (100 mM) and DL-malate (10 mM). The liquid culture with the highest rate of Fe(III) reduction was passed through the serial dilution procedure three times to further ensure that it was a pure culture. This isolate was designated SS015.

Morphology. Cells were rod shaped, ca. 1.2 to 1.5 μm in length, and 0.5 μm in diameter and appeared as single cells or in chains (Fig. 1a). The cells were highly motile in wet mounts, even at room temperature. Spores were not observed. Electron micrographs of negatively stained cells revealed a single subpolar flagellum (8 to 10 nm thick, up to 6 μm long) as well as pili that densely covered the cell surface (Fig. 1b). The cells were Gram stain negative, and ultrathin sections revealed a membrane structure typical of that of gram-negative organisms (Fig. 1c). Cells from exponentially growing cultures in the presence of antibiotics such as trimethoprim and tetracycline appeared very sticky. Electron microscopic examination of ruthenium red-stained cells treated with antibiotics revealed a thick layer of electron-dense material around the cells that was suggestive of an EPS matrix (Fig. 1d). Similar results were obtained with cells from cultures grown under stress conditions, such as temperatures below and pHs above the optima. No layer of ruthenium red-stained EPS material was observed in cultures grown in the absence of antibiotics (mentioned above) and/or under optimal conditions (data not shown).

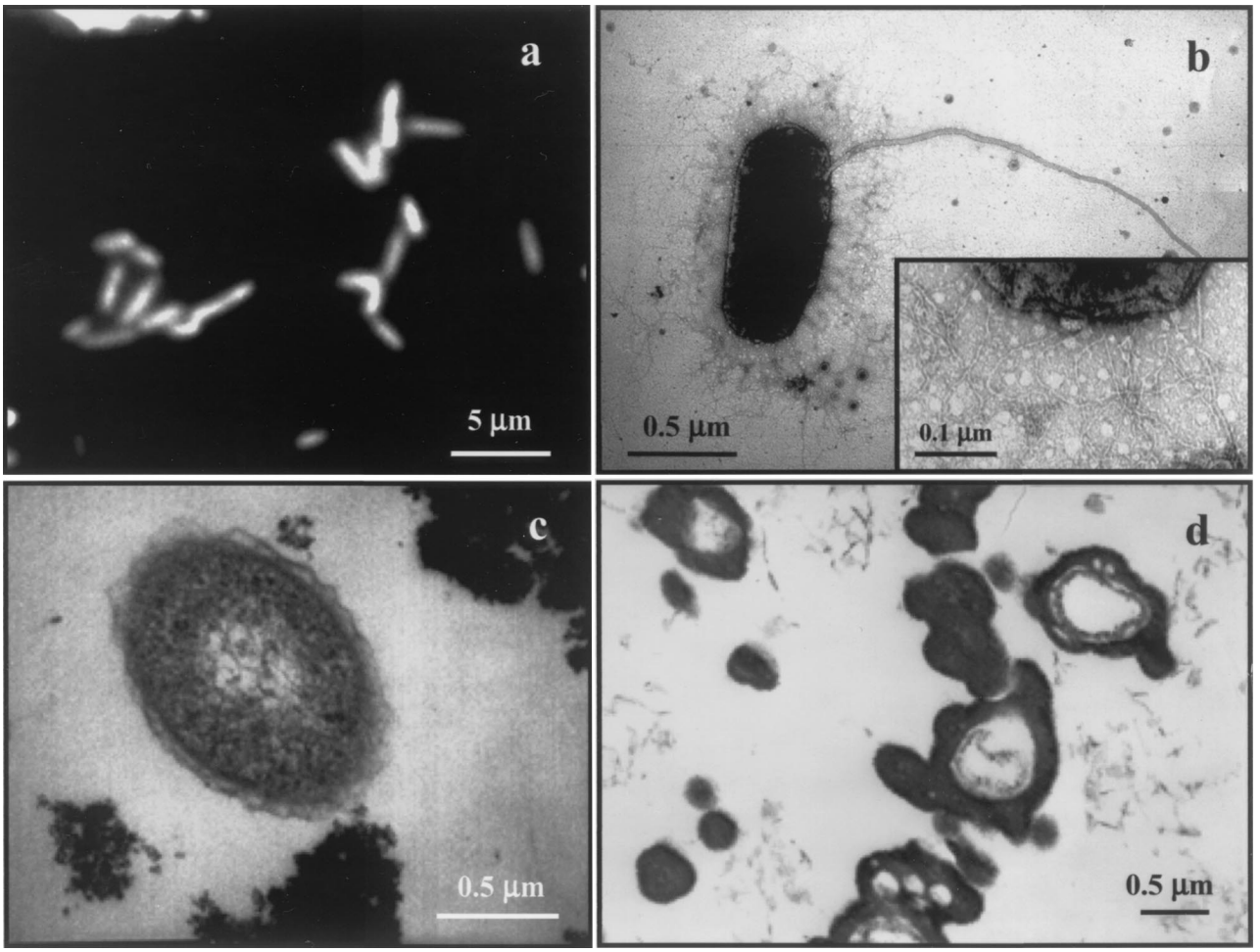

FIG. 1. Microscopic characterization of strain SS015. (a) Phase-contrast micrograph of strain SS015 in late log phase. (b) Electron micrograph of negatively stained cells of strain SS015 showing a single subpolar flagellum and pili densely covering the cell surface. The inset shows a higher magnification of the pili. (c) Electron micrograph of a thin section of strain SS015 grown in marine enrichment medium with poorly crystalline Fe(III) oxide as the electron acceptor and DL-malate as the electron donor; the micrograph shows a typical gram-negative bacterium. (d) Electron micrograph of a thin section of strain SS015 stained with ruthenium red; the micrograph shows the production of an EPS material around the cells in the presence of antibiotics.

Electron donors and electron acceptors utilized. Strain SS015 readily grew in liquid medium at 55°C with DL-malate as the sole electron donor and poorly crystalline Fe(III) oxide as the sole electron acceptor (Fig. 2). Strain SS015 also grew with malate as the electron donor and nitrate as the electron acceptor (Fig. 3). Nitrate was reduced to ammonia (Fig. 3).

In addition to using DL-malate as an electron donor, the new isolate also grew by oxidizing acetate to carbon dioxide with the reduction of Fe(III). Strain SS015 could also reduce Fe(III) with a number of other organic electron donors (pyruvate, acetate, malate, formate, starch, peptone, tryptone, Casamino Acids, MSG, isoleucine, arginine, asparagine, glutamine, histidine, serine, butyrate, propionate, maltose, fructose, ethanol, methanol, and isopropanol). Hydrogen, lactate, citrate, succinate, fumarate, yeast extract, glycerol, glycine, L-cysteine, alanine, aspartic acid, proline, stearate, palmitate, valerate, catechol, phenol, benzoic acid, 4-hydroxybenzoic acid, Fe^{2+}, and ribose were not utilized as electron donors. Growth on the reducing sugars with poorly crystalline Fe(III) oxide could not be investigated because these sugars reacted abiotically with

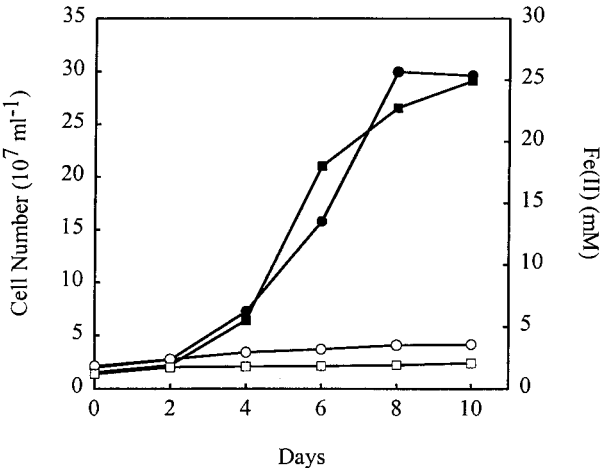

FIG. 2. Growth of strain SS015 at 55°C in medium with DL-malate (10 mM) as an electron donor and poorly crystalline Fe(III) (100 mM) as an electron acceptor. Closed circles, cells with donor; closed squares, Fe(II) with donor; open circles, cells without donor; open squares, Fe(II) without donor. The results are the means for duplicate cultures.

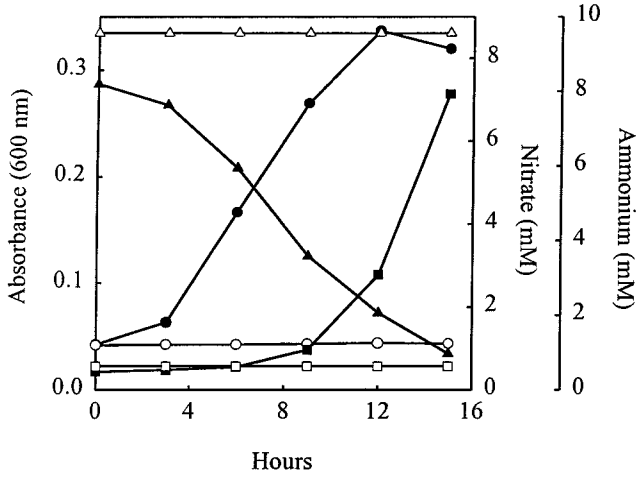

FIG. 3. Growth of strain SS015 at 55°C in medium with DL-malate (10 mM) as an electron donor and nitrate (10 mM) as an electron acceptor. Closed circles, cells with donor; open circles, cells without donor; closed triangles, nitrate with donor; open triangles, nitrate without donor; closed squares, ammonium with donor; open squares, ammonium without donor. The results are the means for duplicate cultures.

the Fe(III) oxide at 55°C (20, 22). However, the growth of strain SS015 with sugars such as maltose and fructose as the sole electron donor could be demonstrated by using nitrate as the sole electron acceptor. Several amino acids and short-chain fatty acids, such as glutamine, histidine, propionate, and butyrate, could serve as the sole electron donor for both Fe(III) and nitrate reduction. Furthermore, strain SS015 utilized starch as an electron donor for Fe(III) reduction. Attempts to grow strain SS015 with starch as the electron donor and nitrate as the electron acceptor were unsuccessful. The long-chain fatty acids palmitate and stearate did not support growth with either Fe(III) or nitrate added to the medium. Strain SS015 did not grow with hydrogen as the sole electron donor for Fe(III) reduction, even when organic carbon sources such as yeast extract were provided.

Attempts to grow strain SS015 on a wide variety of commonly used electron acceptors were unsuccessful [oxygen, Fe(III) citrate, Fe(III) pyrophosphate, MnO_2, anthraquinone-2,6-disulfonate, geothite, hematite, citrate, fumarate, malate, $S_2O_3^{2-}$, SO_4^{2-}, SO_3^{2-}, and S^0]. No growth was observed with sulfate (10 to 20 mM), thiosulfate (10 mM), or sulfite (2 to 4 mM) as an electron acceptor and (i) H_2 (as H_2-CO_2, 80:20 [vol/vol]; 101 kPa), lactate (10 mM), pyruvate (10 mM), acetate (10 and 20 mM), yeast extract (0.05% [wt/vol]), or peptone (0.1% [wt/vol]) or (ii) a combination of H_2-lactate (2 and 10 mM), H_2-pyruvate (2 and 10 mM), H_2-acetate (2 and 10 mM), or H_2-yeast extract (0.02%) and peptone (0.05%) as electron donors. In addition, multiple attempts to grow strain SS015 with S^0 (20% [wt/vol]) as the sole electron acceptor and malate, lactate, pyruvate, acetate, succinate, ethanol, or ethanol-succinate as the electron donor were also unsuccessful. No growth was observed when an inorganic donor such as Fe(II) (as amorphous FeS, 20 mM) or H_2 (as H_2-CO_2, 80:20 [vol/vol]; 101 kPa) was added to the medium with nitrate (10 mM) as the electron acceptor. Poorly crystalline Fe(III) oxide, NO_3^-, NO_2^-, and dimethyl sulfoxide were utilized as electron acceptors.

Temperature, pH, and salt optima. Strain SS015 grew at 35 to 65°C, with an optimum temperature of about 55°C (Fig. 4a), in malate-amended medium with Fe(III) oxide as the electron acceptor. There was no growth at 32°C or below and at 68°C or above after 2 weeks of incubation. The pH range for growth was 5.0 to 8.0, with an optimum at 6.0 (Fig. 4b). Strain SS015 grew at NaCl concentrations ranging from 5.0 to 50 g liter^{-1}, with an optimum at 19 g liter^{-1} (Fig. 4c).

Antibiotic sensitivity. The growth of strain SS015 was inhibited by ampicillin, chloramphenicol, erythromycin, kanamycin, novobiocin, penicillin G, phosphomycin, rifampin, streptomycin, and vancomycin HCl. Trimethoprim and tetracycline did not inhibit growth.

Cytochromes. Dithionite-reduced verses air-oxidized spectral analyses of whole-cell suspensions of strain SS015 grown on nitrate– DL-malate revealed α, β, and γ peaks at 552, 523, and 420 nm, respectively, suggesting the presence of a c_3-

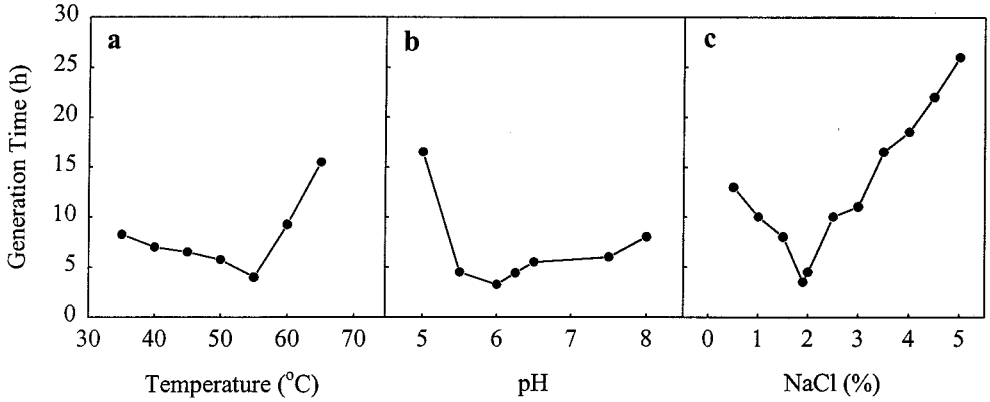

FIG. 4. Effect of temperature, pH, and salt on growth of strain SS015. (a) Optimal growth temperature for strain SS015. Doubling times were calculated from the slopes of the growth curves (not shown) at pH 6.0. (b) Influence of pH on the growth of strain SS015. Doubling times were calculated from the slopes of the growth curves (not shown) at 55°C. (c) Effect of NaCl concentrations on the growth of strain SS015 at 55°C. Doubling times were calculated from the slopes of the growth curves (not shown) at pH 6.0. All growth experiments were done with DL-malate as an electron donor and nitrate as an electron acceptor.

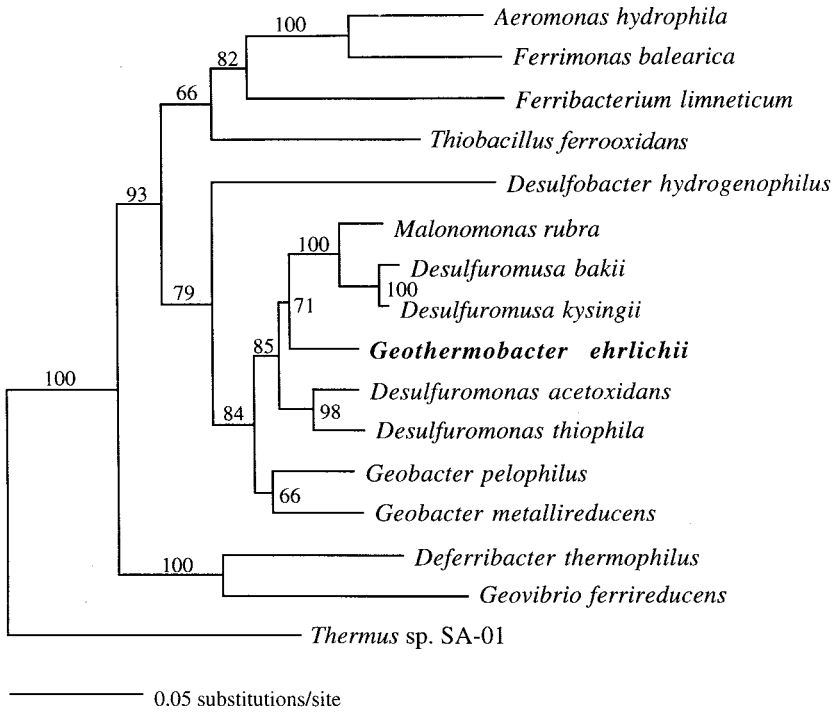

FIG. 5. Phylogenetic tree generated by maximum-parsimony analysis of 16S rDNA nucleotide sequences. The tree shows the relationship of strain SS015 to previously described bacterial sequences. *Thermus* sp. strain SA-01 was used as the outgroup, and bootstrap values were determined from 100 replicates by the character state optimization model.

cytochrome (data not shown). Similar results were obtained for cell extracts (data not shown).

DNA base composition and phylogenetic analyses of 16S rDNA and Ef-G proteins. The moles percent G+C content of strain SS015 was 62.6%. Phylogenetic analysis of the 16S rDNA sequence of strain SS015 indicated that this strain is most similar to members of the family *Geobacteraceae* (Fig. 5). The closest relatives of strain SS015 are *D. kysingii* (93.9% similar), *D. bakii* (93.6% similar), and *M. rubra* (93.7% similar). When phylogenetic comparisons of 16S rDNAs from other members of the family *Geobacteraceae* were made, strain SS015 was found to be 91.7% similar to *D. acetoxidans*, 92.8% similar to *D. thiophila*, 89.6% similar to *G. metallireducens*, and 83.4% similar to *G. pelophilus* (Fig. 5).

Phylogenetic comparison of the Ef-G proteins from several members of the family *Geobacteraceae* showed a similar trend

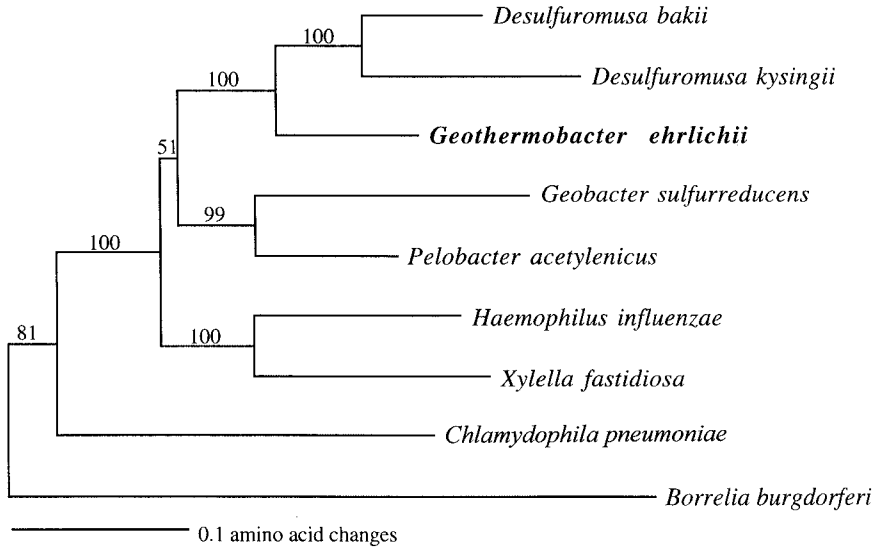

FIG. 6. Phylogenetic tree generated by maximum-parsimony analysis of a translated *fusA* gene fragment from strain SS015. The tree shows the relationship of Ef-G protein from SS015 to Ef-G proteins from other bacteria. *B. burgdorferi* was used as the outgroup, and bootstrap values were determined from 100 replicates by the character state optimization model.

(Fig. 6). The Ef-G protein from strain SS015 was most similar to that from *D. kysingii* (80.6% similar; 200 amino acids considered), 76.4% similar to that from *D. bakii*, 74.3% similar to that from *P. acetylenicus*, and 63.1% similar to that from *G. sulfurreducens*.

DISCUSSION

Although molecular (16, 47, 48, 51, 52) and culturing (7, 10, 11, 12, 13, 34, 35) studies have suggested that members of the family *Geobacteraceae* are important components of Fe(III)-reducing sedimentary environments at cooler temperatures, this study is the first report of a thermophilic member of the family *Geobacteraceae*. Strain SS015 also represents the first description of a thermophilic Fe(III)-reducing microorganism recovered from a marine hydrothermal vent area. As detailed below, these results emphasize the continued need for isolation and characterization studies in order to describe the microbiology of hydrothermal vent areas, as none of the unique physiological capabilities of strain SS015 (such as growth at elevated temperatures; the utilization of sugars, starch, and amino acids; or the production of copious quantities of EPS) could have been predicted from typical molecular analyses and/or the physiology of previously described members of the family *Geobacteraceae*.

Novel physiological characteristics and potential environmental significance. Although molecular analyses are valuable for describing the phylogenetic diversity of microorganisms found in unusual environments, the understanding of physiological diversity is so limited that such molecular analyses can fail to provide a meaningful description of the physiological potential of microorganisms in those environments (20). For example, strain SS015 has a number of physiological characteristics not found in previously described members of the family *Geobacteraceae* which may provide insights into the Bag City hydrothermal vent environment. Most notable is the ability of strain SS015 to grow at temperatures significantly higher than those previously reported for other members of the *Geobacteraceae*. Strain SS015 grew at temperatures of 35 to 65°C, with an optimum temperature of ca. 55°C, compared with optima of 25 to 40°C for previously described members of the *Geobacteraceae*. The temperature growth range of strain SS015, which is comparable to the temperatures at many of the diffuse-flow areas at the site, suggests that members of the *Geobacteraceae*, like strain SS015, can function in hot waters and/or sediments associated with the Bag City vent.

Another unique physiological characteristic of strain SS015 is its ability to use sugars, starch, and some amino acids as electron donors. It was previously considered that members of the *Geobacteraceae* do not use carbohydrates or amino acids as electron donors but rely primarily on acetate and other short-chain fatty acids or alcohols as electron donors. Thus, the presence of strain SS015 suggests that members of the family *Geobacteraceae* in hot environments may have a metabolic niche which is broader than that of mesophilic members of the *Geobacteraceae*.

A physiological property that is commonly found in members of the family *Geobacteraceae* but that is missing from strain SS015 is the ability to use S^0 as an electron acceptor. Although S^0 may be produced in hydrothermal vent areas from the oxidation of sulfide in vent fluids, strain SS015 was not capable of utilizing this electron acceptor. Furthermore, strain SS015 did not reduce the humic acid analogue anthraquinone-2,6-disulfonate, an electron acceptor utilized by the vast majority of members of the *Geobacteraceae* and other Fe(III)-reducing microorganisms (30, 29, 32, 36).

Yet another unexpected physiological property of strain SS015 was the production of large quantities of EPS when the strain was grown under stress conditions, a feature not previously described for members of the family *Geobacteraceae*. The production of polysaccharide is environmentally relevant because of the accumulation of polysaccharide (the 1- to 3-cm translucent "bag creatures" that were found and later identified as polysaccharide coagulants) found on the surface of the sediments at the Bag City site, for which the site is named (The New Millennium Observatory Vents Program Network). Although there is not enough information to determine whether microorganisms like strain SS015 are responsible for the polysaccharide production at the site, these results do demonstrate that there are microorganisms living there that are capable of producing polysaccharide in abundance.

Fe(II) emitted in vent fluids is oxidized to Fe(III) by oxygenated surface waters, and the Fe(III) oxides precipitate into the hot sediments, where they may serve as electron acceptors for microbial metabolism (18, 21, 26, 27). Thus, Fe(III)-reducing microorganisms may be important components of the sediments surrounding vent areas. Given the predominance of members of the family *Geobacteraceae* in Fe(III)-reducing sediments at cooler temperatures, it is interesting that strain SS015 is also a member of this family. However, an analysis of microbial community structure in Fe(III)-reducing hot sediments with unbiased molecular techniques will be required before any conclusions about the importance of *Geobacteraceae* in hot marine sediments can be made.

Comparison with other thermophiles and proposal for a new genus in the family *Geobacteraceae*. Strain SS015 represents the first moderately thermophilic Fe(III)-reducing microorganism isolated from a marine hydrothermal vent area. The isolation of microorganisms utilizing poorly crystalline Fe(III) oxide as an electron acceptor previously proved instrumental for isolating novel hyperthermophilic microorganisms (19–21).

Strain SS015, unlike most previously described thermophilic Fe(III)-reducing microorganisms, can use acetate as an electron donor for Fe(III) reduction. Only one other thermophilic Fe(III) reducer, *D. thermophilus*, has this type of metabolism. The ability to use acetate is an important physiological factor for competing in anaerobic environments, since acetate is a key intermediate in the anaerobic degradation of organic matter in sedimentary environments (28, 55, 56). Most previously described thermophilic Fe(III) reducers do not use acetate and only incompletely oxidize other multicarbon organic acid substrates to acetate (27). These incomplete oxidizers are unlikely to be abundant components of the microbial communities in hot sediments because of the much smaller quantities of electrons available to them for Fe(III) reduction.

The 16S rDNA sequence of strain SS015 places it within the family *Geobacteraceae*. Strain SS015 shares many of the hallmark physiological characteristics of the most well-studied members of the family *Geobacteraceae*, most notably the ability to oxidize acetate with the reduction of Fe(III) and an abun-

dance of *c*-type cytochromes. To date, *Pelobacter* species are the only members of the *Geobacteraceae* which lack the ability to utilize acetate and which do not contain *c*-type cytochromes (27). Like *G. metallireducens* (31, 34), strain SS015 can also use nitrate as an electron acceptor by reducing it to ammonia. However, as noted above, strain SS015 also has important physiological differences from previously described strains of *Geobacteraceae*.

Strain SS015 is most closely related to members of the genus *Desulfuromusa*, but its 16S rDNA sequence is no more than 93% similar to those of previously described members of this genus. Under current taxonomic practice, an evolutionary distance of this range is considered sufficient justification for establishing a new genus. The significant differences in the *fusA* sequences of strain SS015 and previously described members of the *Geobacteraceae* also suggest that strain SS015 represents a new genus. Furthermore, the ability to reduce S^0 is a defining physiological characteristic of the genus *Desulfuromusa*, but strain SS015 cannot reduce S^0. The thermophilic nature of strain SS015 further emphasizes its uniqueness relative to microorganisms in the genus *Desulfuromusa*. Thus, both phylogenetic and physiological factors suggest that strain SS015 represents a new genus in the family *Geobacteraceae*. The name *Geothermobacter ehrlichii* (named after Henry Lutz Ehrlich, a pioneer in the field of geomicrobiology) is proposed.

Description of *Geothermobacter* gen. nov. *Geothermobacter* (Ge.o.ther.mo.bac′ter. Gr. n. *ge*, the earth; Gr. n. *thermos*, heat; Gr. hyp. masc. n. *bakter*, a rod; N.L. masc. n. *Geothermobacter*, a rod from hot earth). Cells are rod shaped, about 0.5 μm by 1.2 to 1.5 μm, occur singly or in chains, and are highly motile (even at room temperature) by means of monotrichous flagellation. Cells are densely piliated. Cell wall structure is typical of that of a gram-negative bacterium. It has a temperature optimum of approximately 55°C and an optimum salt concentration of 19 g $liter^{-1}$. It is a strictly anaerobic chemoorganotroph which conserves energy to support growth by coupling the oxidation of acetate, pyruvate, DL-malate, glutamate, propionate, butyrate, ethanol, and methanol to the reduction of Fe(III) and nitrate. The reduction of poorly crystalline Fe(III) oxide results in the accumulation of what appears to be extracellular, ultrafine-grained magnetite. Nitrate is reduced to ammonia. Accumulations of cells are pink. Dithionite-reduced verses air-oxidized spectral analyses of whole-cell suspensions and cell extracts of strain SS015 exhibit absorption maxima at 420, 552, and 523 nm, reminiscent of a *c*-type cytochrome(s) (data not shown). Cells also secrete a thick layer of an EPS matrix when grown under unfavorable conditions. The G+C content of the DNA is 62.6 mol%. The genus *Geothermobacter* is in the family *Geobacteraceae* in the delta subclass of the *Proteobacteria*.

Habitat. The habitat of strain SS015 is the Bag City hydrothermal vent at the Axial Seamount, an eruption site in the center of the Juan de Fuca Ridge (approximately 300 mi west of Cannon Beach, Oreg.).

Description of *Geothermobacter ehrlichii* sp. nov. *Geothermobacter ehrlichii* (ehrlichi.i. N.L. gen. n. *ehrlichii*, in honor of Henry Lutz Ehrlich, in recognition of his fundamental contributions to the field of geomicrobiology and the area of microbial interactions with metals in particular). Gram-negative rods, 0.5 μm by 1.2 to 1.5 μm, occur singly or in chains and are motile (even at room temperature). Cells have a single flagellum in the subpolar position. *G. ehrlichii* grows by oxidizing malate, pyruvate, acetate, formate, starch, peptone, tryptone, Casamino Acids, MSG, alanine, arginine, asparagine, glutamine, histidine, proline, butyrate, propionate, maltose, fructose, ethanol, methanol, and isopropanol, coupled to the reduction of Fe(III) and nitrate. Only poorly crystalline Fe(III) oxide, nitrate, and nitrite are utilized as electron acceptors. No growth occurs with electron acceptors such as $S_2O_3^{2-}$, SO_4^{2-}, SO_3^{2-}, S^0, O_2, Mn(IV), anthraquinone-2,6-disulfonate, malate, and fumarate (see Results). No growth occurs with hydrogen (H_2-CO_2, 80:20 [vol/vol]; 101 kPa), lactate, citrate, succinate, fumarate, ribose, yeast extract, glycerol, isoleucine, glycine, L-cysteine, aspartic acid, serine, stearate, palmitate, valerate, catechol, phenol, benzoic acid, 4-hydroxybenzoic acid, and Fe(II) (in the form of amorphous FeS) as electron donors.

Growth occurs at temperatures of 35 to 65°C (optimum temperature, approximately 55°C), in the presence of NaCl concentrations ranging from 5.0 to 50.0 g $liter^{-1}$ (optimum NaCl concentration, 19 g $liter^{-1}$), and pHs ranging from 5.0 to 7.75 (optimum pH, 6.0). *G. ehrlichii* is sensitive to ampicillin, chloramphenicol, erythromycin, kanamycin, novobiocin, penicillin G, phosphomycin, rifampin, streptomycin, and vancomycin HCl but resistant to trimethoprim and tetracycline. The organism was isolated from diffuse-flow vent samples taken from the Bag City hydrothermal vent system of the newly erupted Axial Seamount, a submerged volcano on the Juan de Fuca Ridge axis (approximately 300 mi west of Cannon Beach, Oreg., at a depth of 2,500 m).

The strain has been deposited in the American Type Culture Collection (ATCC BAA-635) and in the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSM 15274). The 16S rDNA sequence has been deposited in GenBank under accession number AY155599.

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