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Metal Reduction and Iron Biomineralization by a Psychrotolerant Fe(III)-Reducing Bacterium, *Shewanella* sp. Strain PV-4

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A marine psychrotolerant, dissimilatory Fe(III)-reducing bacterium, *Shewanella* sp. strain PV-4, from the microbial mat at a hydrothermal vent of Loihi Seamount in the Pacific Ocean has been further characterized, with emphases on metal reduction and iron biomineralization. The strain is able to reduce metals such as Fe(III), Co(III), Cr(VI), Mn(IV), and U(VI) as electron acceptors while using lactate, formate, pyruvate, or hydrogen as an electron donor. Growth during iron reduction occurred over the pH range of 7.0 to 8.9, a sodium chloride range of 0.05 to 5%, and a temperature range of 0 to 37°C, with an optimum growth temperature of 18°C. Unlike mesophilic dissimilatory Fe(III)-reducing bacteria, which produce mostly superparamagnetic magnetite (<35 nm), this psychrotolerant bacterium produces well-formed single-domain magnetite (>35 nm) at temperatures from 18 to 37°C. The genome size of this strain is about 4.5 Mb. Strain PV-4 is sensitive to a variety of commonly used antibiotics except ampicillin and can acquire exogenous DNA (plasmid pCM157) through conjugation.

Microbial metal reduction and mineral formation play an important role in the iron and carbon geochemistry and organic matter mineralization in natural subsurface environments. Dissimilatory metal reduction is proposed to be an early form of microbial respiration (16). Reduction of metals by bacteria is generally coupled with the oxidation of organic matter (16, 28, 29). Therefore, the ability to reduce metals can be exploited not only for the bioreduction or immobilization of many toxic metals, including cobalt, chromium, uranium, and technetium, but also for the biotransformation of organic contaminants to benign products such as carbon dioxide (7).

A number of metal-reducing bacteria have been isolated and characterized from a variety of habitats, and much work has focused on *Shewanella oneidensis* and *Geobacter* spp. (17). The genus *Shewanella* was first described two decades ago (20). All members of this genus reported so far are facultatively anaerobic, gram-negative, motile by polar flagella, rod-like, and generally associated with aquatic or marine habitats (4, 5, 11, 12, 13, 25, 26, 31, 38). Although most *Shewanella* species are mesophilic, psychrotolerant and psychrophilic bacteria in the *Shewanella* genus have been isolated recently (4, 43). Since a vast majority of the world's surface is covered by oceans, cold deep-sea environments (≤4°C) represent a significant portion of potential microbial habitants, and thus, psychrophilic and psychrotolerant *Shewanella* species could be important in the overall biogeochemical processes of metals and carbon.

We have previously isolated more than 10 *Shewanella* strains from a variety of marine environments (33). Strain PV-4, isolated from iron-rich microbial mats at an active, deep-sea, hydrothermal Naha vent of Loihi Seamount, Hawaii, is the first *Shewanella* strain capable of reducing metals in a wide range of temperatures, from 0 to 37°C. Although strain PV-4 was chosen for genome sequencing by the Shewanella Federation at the Department of Energy Joint Genome Institute and the genome draft was released recently (http://www.jgi.doe.gov), phenotypic and physiological characteristics of this isolate remain undefined. In this study, we have examined the characteristics of metal reduction and iron mineralization of strain PV-4 under various conditions. In addition, we have determined the genetic traits that allow the genetic manipulation of this strain. Our results demonstrated that strain PV-4 has unique physiological characteristics and is transformable by conjugation.

MATERIALS AND METHODS

Site characteristics, bacterial isolation, and phylogenetic characterization. Strain PV-4 was isolated from samples obtained from iron-rich microbial mats at an active, deep-sea, hydrothermal Naha vent (1,325 m below sea level) located on the South Rift of Loihi Seamount, Hawaii (http://www.soest.hawaii.edu/GG/HCV/loihivents.html). Loihi Seamount is an active submarine hotspot volcano rising more than 3,000 m above the floor of the Pacific Ocean. Since August 1996, the Loihi volcano has been intermittently active. The Naha vent field (ca. 20 by 30 m), first discovered in 1996, is heavily covered with nontronite deposits and tan bacterial mats. This spot consists of many small vents as well as diffuse flow through fractured pillows and large fissures. The vent water on the Loihi Seamount vents was characterized as being relatively high in dissolved Fe (ca. 1 mM) and CO_2 (ca. 30 mM) concentrations. The plumes from the Naha vent at Loihi

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were acidic (as low as pH 5.6 [October 1996]). Temperatures measured at the Naha vent were 23°C (October 1996) and 11°C (September 1997) (39). When the microbial mat samples were collected, the in situ water temperature at the vent was 11°C, and the ambient seawater temperature was 4°C. Sample collection, handling, bacterial enrichments, and isolation were described previously (33). The small-subunit rRNA gene of strain PV-4 was sequenced to determine phylogeny (33). Strain PV-4 belongs to the *Shewanella* genus, as confirmed by the analysis of the complete 16S rRNA gene.

Physiological characterization. This study focused mainly on Fe(III) reduction and biomineralization by strain PV-4 in a wide range of temperatures, from 0 to 37°C, which was examined quantitatively. The reduction of other metals was tested in a qualitative way. For experiments under anaerobic conditions, a defined medium (DM) was used in pressure tubes unless otherwise noted. The basal part of DM contained the following ingredients (per liter): ($NaHCO_3$, 2.5 g; $CaCl_2 \cdot 2H_2O$, 0.08 g; NH_4Cl, 1.0 g; $MgCl_2 \cdot 6H_2O$, 0.2 g; NaCl, 10.0 g; HEPES, 7.2 g; resazurin (0.01%), 0.1 g; yeast extract, 0.5 g; trace minerals, 10 ml; vitamin solution, 1 ml (16). No exogenous electron carrier substance (i.e., anthraquinone disulfonate) or reducing agent (i.e., cysteine) was added to the medium. The trace mineral solution contained (per liter) 1.5 g of nitrilotriacetic acid, 0.2 g of $FeCl_2 \cdot 4H_2O$, 0.1 g of $MgCl_2 \cdot 6H_2O$, 0.02 g of sodium tungstate, 0.1 g of $MnCl_2 \cdot 4H_2O$, 0.1 g of $CoCl_2 \cdot 6H_2O$, 1 g of $CaCl_2 \cdot 2H_2O$, 0.05 g of $ZnCl_2$, 0.002 g of $CuCl_2 \cdot 2H_2O$, 0.005 g of H_3BO_3, 0.01 g of sodium molybdate, 1 g of NaCl, 0.017 g of Na_2SeO_3, and 0.024 g of $NiCl_2 \cdot 6H_2O$. The vitamin solution contained (per liter) 0.02 g of biotin, 0.02 g of folic acid, 0.1 g of B_6 (pyridoxine) HCl, 0.05 g of B_1 (thiamine) HCl, 0.05 g of B_2 (riboflavin), 0.05 g of nicotinic acid (niacin), 0.05 g of pantothenic acid, 0.001 g of B_{12} (cyanobalamine) crystalline, 0.05 g of *p*-aminobenzoic acid, and 0.05 g of lipoic acid (thioctic).

To assess the bacterial capability of metal reduction and mineral formation, various electron acceptors, including ferric citrate (10 to 20 mM), ferric EDTA (10 mM), akaganeite (~70 mM), Co(III)-EDTA (1.5 mM), potassium chromate (0.5 mM), and uranyl carbonate (5 mM), were examined at 8°C with lactate (10 mM) as an electron donor. A high concentration of U(VI) was used so that the resulting color change (yellow to colorless) could be confidently recorded. The effects of temperature (0 to 45°C), pH (6.5 to 9.6), and salinity (0.05% to 7%) on microbial metal reduction and mineral formation were also examined using akaganeite (70 mM) as an electron acceptor and lactate (10 mM) as an electron donor. The medium at 0°C was maintained on ice and was not frozen because of its high ionic strength. The pH was adjusted using NaOH or HCl and monitored following the incubation period. Growth of strain PV-4 on Fe(III)-citrate (20 mM) with lactate (10 mM) as an electron donor was quantitatively assessed under anaerobic conditions. The optimal growth temperature was determined based on the combination of growth rate and Fe(III) reduction rate. In all experiments, uninoculated controls were used to rule out contamination or abiotic metal reductions.

A variety of factors that influenced metal reduction and mineral formation, including medium pH, incubation temperature, electron donors, electron acceptors, metals, and the headspace gas composition, were examined. To determine the range of growth substrates, PV-4 cells were inoculated in anaerobic basal medium with lactate (10 mM), acetate (10 mM), pyruvate (10 mM), succinate (10 mM), xylan (1%), cellulose (1%), or H_2 (80% H_2–20% CO_2, vol/vol) as an electron donor and akaganeite (β-FeOOH, ~70 mM) as an electron acceptor and incubated at 8°C in the dark for several days. The akaganeite was prepared by neutralizing a solution of 0.4 M $FeCl_3$ with 10 M NaOH as described previously (27, 42). X-ray diffraction (XRD) analysis confirmed the structure of akaganeite (29).

To understand the effect of headspace atmosphere on iron mineralogy, the anaerobic media were prepared and incubated under three different headspace atmospheres: N_2, N_2-CO_2 (80:20, vol/vol), and H_2-CO_2 (80:20, vol/vol) with akaganeite (~70 mM) as an electron acceptor (28). M1, which is used extensively for *Shewanella* strains (http://www.shewanella.org), was tested to examine the effect of CO_2 on iron biomineralization in addition to the bicarbonate-buffered DM. In the case of using lactate (10 mM) as an electron donor, N_2 and N_2-CO_2 headspaces were used, while H_2-CO_2 headspace atmosphere was used when H_2 served as the electron donor. Incubation time ranged from 2 weeks to 6 months. Abiotic controls were established for each treatment.

Quantitative assessment of HFO reduction. Quantitative assessment of hydrous ferric oxide (HFO) (~50 mM) reduction by strain PV-4 was conducted as described previously (6, 8). To determine the capability of strain PV-4 to reduce HFO, *Shewanella putrefaciens* strain CN-32 was included for comparison. Briefly, exponentially growing cells in DM with 30 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] and 20 mM lactate were diluted in Balsch tubes containing 50 mM HFO to a final optical density at 600 nm of 0.075. HFO treatments were sampled at 4 h, 8 h, 24 h, 48 h, 7 days, 14 days, and 21 days by adding 0.5 ml of cultures to 0.5 ml 1 M HCl in an anaerobic glove bag and allowing the cultures to sit overnight. Fe(II) concentrations in the samples and in the abiotic controls were determined by the ferrozine method (41).

Bacterial cell counting and chemical analysis. Bacterial growth with Fe(III)-citrate was quantified by direct cell counting using epifluorescence microscopy of acridine orange-stained samples as described previously (10, 14, 40). Light microscopy was used to visualize wet mounts and acridine orange-stained filters with a Nikon Phase Contrast Optiphot microscope (Nikon, Japan).

To examine the chemical conditions of metal reduction and mineral formation by PV-4, subsamples (0.1 ml) of bacterial cultures and abiotic controls were taken from the culture bottles at different times, and E_h and pH were measured at room temperature in an anaerobic chamber (41). Fe(II) concentrations in the cultures and in abiotic controls were determined by the ferrozine method (34, 41). Using this method, a 0.1-ml sample was added to 2 ml of anaerobic 0.5 M HCl solution. After 15 min, 0.1 ml of the mixture was added to 3 ml of ferrozine (1 g/liter) in 50 mM HEPES buffer at pH 7. The sample was mixed, filtered through a Whatman syringe filter (13-mm filter diameter, 0.2-μm pore diameter), and measured for maximum absorbance at 562 nm. A standard for the ferrozine assay was prepared with ferrous ethylene diammonium sulfate dissolved in 0.5 M HCl. Reduction of Co(III) to Co(II) was measured as the decrease in the Co(III) concentration. Subsamples (0.5 ml to 1 ml) were diluted with 2 ml of distilled water and filtered as described above. The concentration of Co(III) was measured for maximum absorbance at 548 nm (40).

Morphological and mineralogical characterization. The morphology, mineralogy, and chemistry of the precipitated or transformed mineral phases were examined under a JEOL JSM-35CF (JEOL Ltd., Tokyo, Japan) scanning electron microscope with energy-dispersive X-ray analysis. The mineralogical composition of the precipitated or transformed phases was determined using XRD. All XRDs were performed using a Scintag (Scintag, Inc., Sunnyvale, CA) XDS 2000 diffractometer (40 kV, 35 mV) equipped with Co-Kα radiation with a scan rate of 2° 2θ/min. Transmission electron microscopy (TEM) was used to study the morphology of the isolate and precipitated crystalline iron minerals (42). Cells of strain PV-4 were grown either aerobically in tryptic soy broth (Difco, Detroit, Mich.) at 30°C or anaerobically in DM using Fe(III)-citrate (15 mM) as an electron acceptor and lactate (10 mM) as an electron donor at 8°C. Culture medium containing microorganisms, organic matter, and iron minerals was fixed with 2.5% glutaraldehyde in 0.1 M cacodylate. After being washed with buffer and an alcohol-water solution, samples were dehydrated with propylene oxide and embedded in a low-viscosity, thermally curving epoxy resin. Ultrathin sections (70 to 80 nm) were cut from resin blocks with a diamond knife and transferred to 300-mesh, formvar-coated Cu TEM grids for image analysis with a JEOL FX 2000 apparatus equipped with an energy-dispersive X-ray detector.

Genetic characterization. Luria-Bertani (LB) medium (Difco, Detroit, Mich.) was generally used for characterization under aerobic conditions unless otherwise noted. To determine the genome size of strain PV-4, agarose-embedded chromosomal DNA was prepared using a modification of protocols described in the instruction manual and application guide that accompanied the CHEF-DRII pulsed-field electrophoresis system (Bio-Rad). Endonuclease cleavage of DNA in agarose blocks was done as described previously (15, 35). The genome size of PV-4 was calculated based on the estimated sizes of resultant fragments derived from enzyme digestion.

The antibiotic susceptibility of strain PV-4 was assessed at room temperature using a method described previously (1). Experiments were conducted in triplicate. The ability of strain PV-4 to acquire exogenous DNA was examined with either conjugation or electroporation. Conjugation was performed with *Escherichia coli* WM3064 (30) hosting plasmid pCM157 (21) as described previously (36). Electroporation was conducted using a BTX Electro Cell Manipulator ECM 600 (BTX Electronic Genetics, San Diego, CA) according to either the standard protocol for *E. coli* provided by the manufacturer or the protocol for *S. oneidensis* MR-1 as described previously (24).

Nucelotide sequence accession number. The nucleotide sequence of the 16S rRNA gene of strain PV-4 determined in this study has been deposited in GenBank under accession number DQ286387.

RESULTS

Cell morphology. Cells of strain PV-4 grown in tryptic soy broth aerobically at 30°C or anaerobically in DM using Fe(III)-citrate (15 mM) as an electron acceptor and lactate (10 mM) as an electron donor at 8°C were examined. Electron micrographs showed that strain PV-4 is a rod-shaped bacterium with a polar

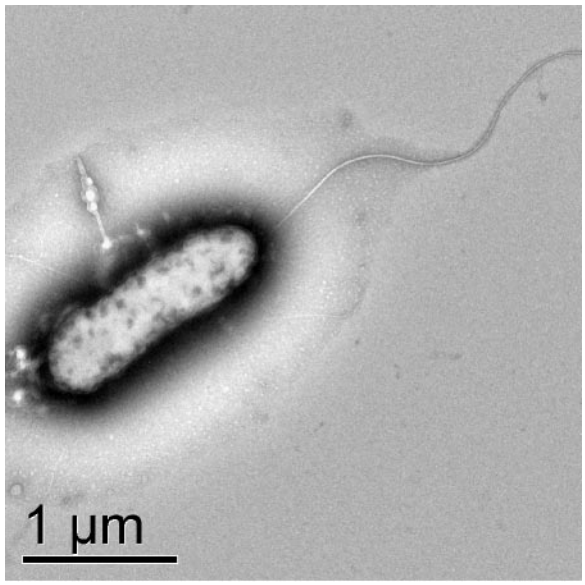

FIG. 1. Transmission electron micrograph of *Shewanella* sp. strain PV-4 grown in tryptic soy broth aerobically at 30°C.

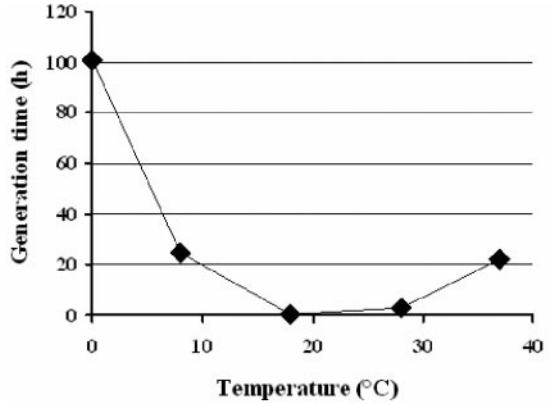

FIG. 2. Generation time of PV-4 cells. Cells were grown using Fe(III)-citrate (15 mM) as an electron acceptor and lactate (10 mM) as an electron donor at each temperature tested.

flagellum (Fig. 1). Under aerobic conditions, cells were ~1.6 μm in length and ~1.0 μm in diameter. However, the cell morphologies varied under different growth conditions. Under anaerobic conditions at 8°C, cells were narrower (~1.6 μm in length and ~0.4 μm in diameter) and exhibited less condensed cytoplasmic materials (data not shown). Similar morphologies were observed in cells grown at room temperature under anaerobic conditions (data not shown).

Growth characteristics. Strain PV-4 was able to use O_2 and fumarate as electron acceptors but not nitrate, nitrite, sulfate, sulfite, thiosulfate, or dimethyl sulfoxide for growth with lactate or formate as the electron donor (data not shown). Strain PV-4 was able to reduce Fe(III)-citrate, as indicated by the color change of the culture solution from brownish gray [Fe(III)-citrate] to colorless at 8°C. Strain PV-4 was also able to reduce Co(III) [Co(III)-EDTA] to Co(II), Cr(VI) (potassium chromate) to Cr(III), and U(VI) (uranyl carbonate) to U(IV) with lactate (10 mM) as an electron donor, as indicated by the color change of the culture solution from purple [Co(III)-EDTA] and yellow (potassium chromate and uranyl carbonate) to colorless at 8°C. Furthermore, strain PV-4 was able to reduce manganese oxide using lactate (10 mM) as an electron donor and formed rhodochrosite ($MnCO_3$) at 8°C under an H_2-CO_2 atmosphere. However, the cells did not reduce Fe(III)-EDTA as an electron acceptor using lactate (10 mM) as an electron donor at 8°C under an N_2 atmosphere.

Among the organic compounds tested, PV-4 cells were able to use lactate, formate, and pyruvate as well as hydrogen (H_2) as the electron donors for the reduction of akaganeite and formed magnetite at 8°C. However, growth was not observed when cells were provided with succinate, xylan, cellulose, or acetate as an electron donor to reduce Fe(III).

The temperature range for growth using Fe(III)-citrate (15 mM) as an electron acceptor and lactate (10 mM) as an electron donor was between 0°C and 37°C (Fig. 2). While cells grew at virtually the same rate at temperatures ranging from 17 to 24°C under the conditions tested, the fastest rate of Fe(III)-citrate reduction was observed at 18°C. We therefore concluded that the optimum temperature was approximately 18°C. The generation time was approximately 101 h at 0°C, 24.5 h at 8°C, 0.7 h at 18°C, 2.8 h at 28°C, and 22 h at 37°C. At −4°C and over 45°C, the cells remained viable but did not reproduce. Although PV-4 was isolated from a microbial mat in seawater, a high NaCl concentration was not essential for growth, as cells were able to grow in a salinity range of 0.05 to 5% (wt/vol) NaCl at 8°C using lactate (10 mM) as an electron donor. When the isolate was grown in the presence of Fe(III)-citrate or akaganeite at 8°C, the pH optimum was approximately 8.0, with a pH range of 6.5 to 9.5.

Changes in the pH and E_h of the medium were monitored. The initial pH and E_h of the medium using akaganeite (β-FeOOH) as an electron acceptor and lactate (10 mM) as an electron donor were 8.0 and 40 to 50 mV, respectively. The E_h of the medium was reduced to −200 mV (under N_2 headspace gas) and −450 mV (under H_2-CO_2 headspace gases), and the pH was reduced to 7.4 after 30 days of incubation at 8°C.

Quantitative reduction of ferrihydrites. Since the iron-rich microbial mats at Loihi are dominated by noncrystalline ferrihydrites, HFO reduction by strain PV-4 was quantitatively determined. In this experiment, *S. putrefaciens* CN-32 was used as a positive control. Both PV-4 and CN-32 were grown under anaerobiosis in DM with 30 mM PIPES and 20 mM lactate. The samples were collected and processed as described in Materials and Methods. The result is presented in Fig. 3, which clearly shows that HFO reduction occurred in strain PV-4. No significant difference in the reduction rates between these two strains was observed in the first 50-h period. However, after 50 h, PV-4 exhibited a slightly weaker capability of reducing HFO than that of *S. putrefaciens* CN-32.

Mineralogy. Strain PV-4 was able to reduce nonmagnetic reddish-brown akaganeite (β-FeOOH) to black magnetite using lactate (10 mM) as an electron donor at a wide range of temperatures, from 0°C to 37°C. The result was verified by XRD (Fig. 4). The magnetic minerals were not found in the absence of bacterial cells or at temperatures higher than 42°C. Although strain PV-4 formed magnetite using akaganeite (β-FeOOH) as a magnetite precursor over a wide temperature

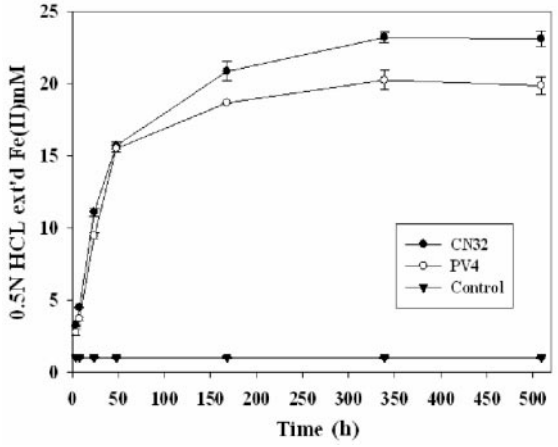

FIG. 3. Reduction of 50 mM HFO by strain PV-4 and *S. putrefaciens* CN-42 with 20 mM lactate in DM at room temperature.

range (0 to 37°C), both crystal size and shape varied with temperature. TEM images of the magnetite formed by PV-4 cells at temperatures below 8°C revealed aggregates of small crystals ranging in size from 10 to 40 nm (Fig. 5A and B). In contrast, the magnetite formed at temperatures between 18 and 37°C consisted of aggregates of magnetite crystals ranging in size from 30 to 200 nm (Fig. 5C, D, and E). No fine intracellular magnetite was observed, and the crystals were not aligned in chains. These results suggested that temperature has a significant effect on the sizes of magnetite crystals.

XRD was also used to determine the end product of HFO reduction by strain PV-4. Based on the defined peaks of the XRD pattern, magnetite was present in the samples (data not shown). To verify the result, the end products of HFO reduction by strains CN-32 and PV-4 at room temperature were examined in parallel by XRD. Diffractograms from these two strains matched, confirming the presence of magnetite (data not shown).

The gas composition of the headspace atmosphere appeared to affect mineral formation. Under N_2 headspace, the akaganeite was predominantly reduced to magnetite, indicated by color changes from reddish brown to black and further confirmed by XRD analysis in either bicarbonate-buffered DM or M1. In contrast, under N_2-CO_2 and H_2-CO_2, a mixture of magnetite and siderite was formed, indicated by reddish-to-brownish-black color changes and confirmed by XRD analysis (Fig. 6A to E). Energy-dispersive X-ray spectroscopy analysis of bacterial cells showed that the cells were significantly enriched with Fe (Fig. 6F). Interestingly, siderite formed by PV-4 at various temperatures appeared globular, suggesting that temperatures may not have a role in the process. However, the globule was different from the cube-shaped siderite formed by a mesophilic Fe(III)-reducing bacterium, *Geobacter metallireducens* GS-15 (19). Further analysis revealed that under an H_2-CO_2 atmosphere, siderite ($FeCO_3$) was the principal reduced iron mineral. It appeared that an H_2-CO_2 atmosphere provides a favorable environment for PV-4 to complete the reduction of a poorly crystalline iron oxide, akaganeite (β-FeOOH).

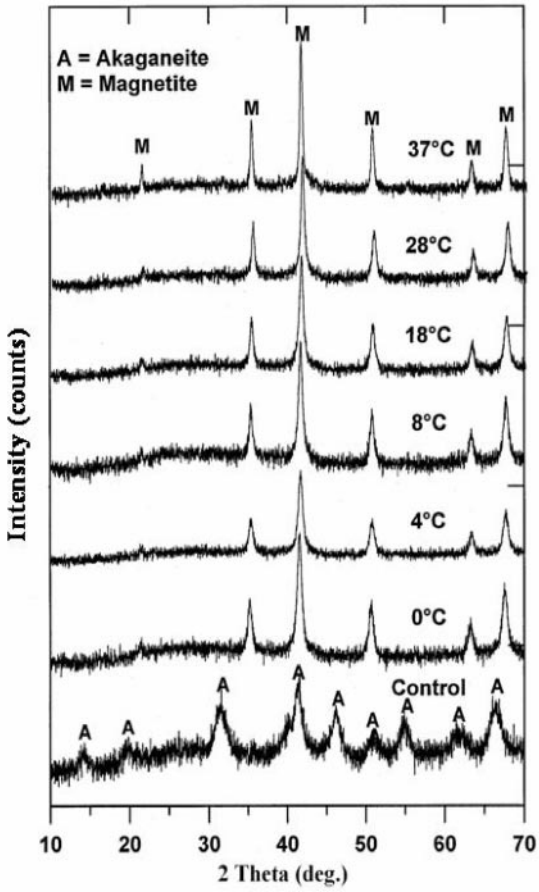

FIG. 4. XRD analysis of the black magnetic minerals formed at various temperatures. Cells were grown using akaganeite (β-FeOOH; 70 mM) as an electron acceptor and lactate (10 mM) as an electron donor, harvested, and prepared for transmission electron microscopy as described in Materials and Methods. Reduction of akaganeite was observed at all tested temperatures ranging from 0 to 37°C.

Genome size, antibiotic susceptibility, and uptake of foreign DNA. The genome size of strain PV-4 was required for the genome sequencing project and was determined using a standard pulsed-field gel electrophoresis (PFGE) method. Several rarely cutting endonucleases, including AscI (GGCGCGCC), AsiSI (GCGATCGC), NotI (GCGGCCGC), PacI (TTAATTAA), PmeI (GTTTAAAC), and SwaI (ATTTAAAT), were used for determining genome size. Only AscI and SwaI gave a small number of distinguishable fragments that could be separated efficiently by PFGE (Fig. 7). The sum of four fragments (1,900, 1,120, 820, and 700 kb) from AscI digestion was around 4,540 kb, while five fragments (820, 800, 790, 750, and 680 kb) from SwaI added up to 3,840 kb. The different estimates in genome size from two digests may be due to two fragments of similar size (680 kb) in the SwaI digest, as supported by a much stronger band (680 kb) in the SwaI digest than the 700-kb band in the AscI digest. With the addition of a 680-kb fragment, the estimated genome size from SwaI digestion was around 4,520 kb. Thus, the genome size of PV-4 was determined to be ~4.5 Mb, consistent with the result based on the genome draft released by the Joint Genome Institute. The result suggests

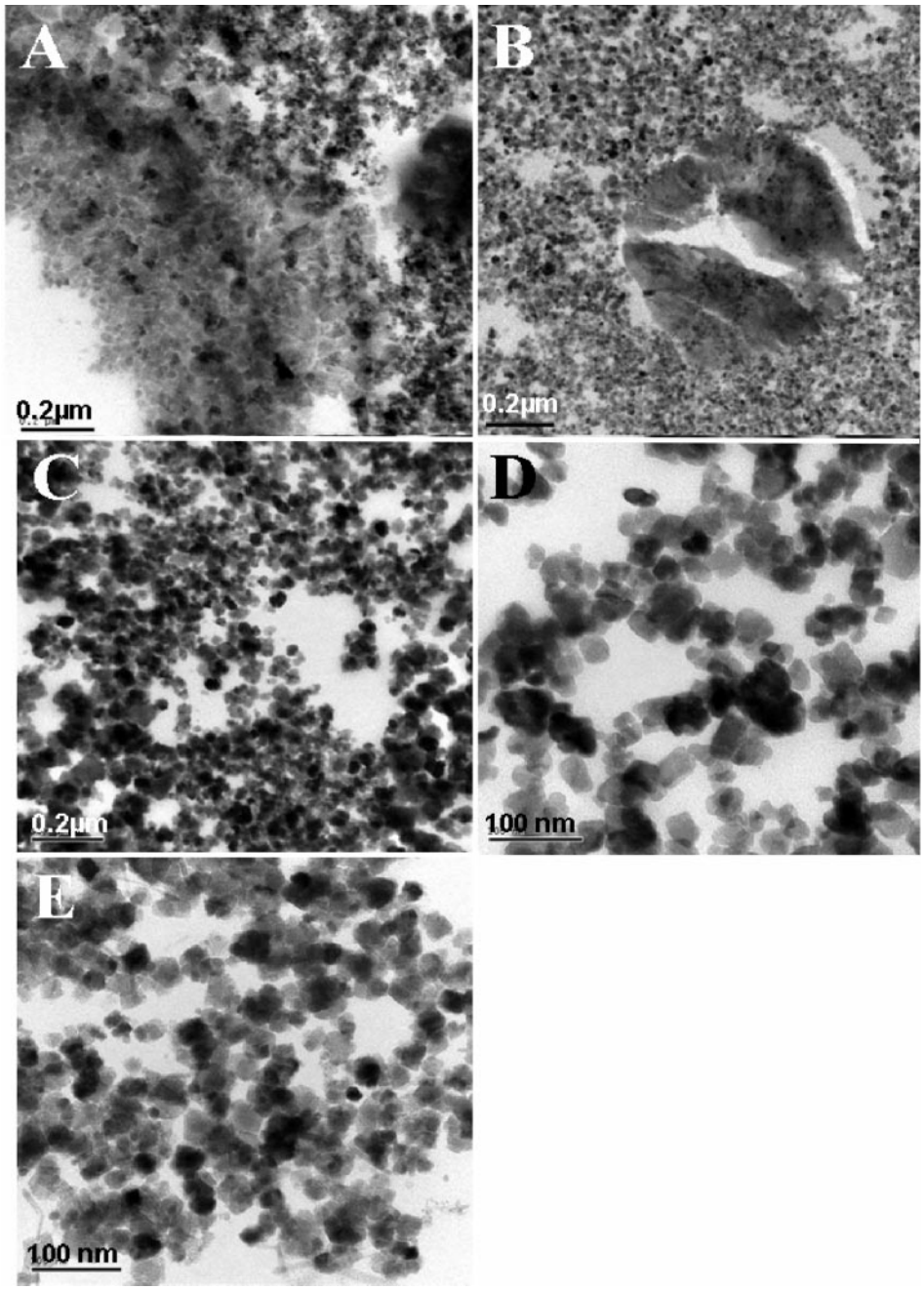

FIG. 5. TEM images of magnetite formed by PV-4 at various temperatures. A, 4°C; B, 8°C; C, 18°C; D, 28°C; E, 37°C. Small crystals were formed at temperatures below 8°C (A and B), while large crystals were produced at higher temperatures (C, D, and E).

that PFGE is a useful and reliable method for determining genome size.

Strain PV-4 was also tested for naturally occurring resistance to nine antibiotics at room temperature (Table 1). Strain PV-4 showed strong resistance to ampicillin and intermediate resistance to spectinomycin and streptomycin. For all other antibiotics tested, strain PV-4 exhibited virtually no resistance.

Foreign DNA can be taken up by PV-4 through conjugation, as indicted by tetracycline-resistant PV-4 colonies. For confirmation, plasmid pCM157, extracted from PV-4 tetracycline-resistant cells, was introduced into *E. coli* WM3064 (plasmid free and tetracycline sensitive), resulting in tetracycline-resistant *E. coli* colonies. In addition, plasmids extracted from both donor cells and PV-4 tetracycline-resistant cells exhibited the same restriction map with three restriction enzymes. Strain PV-4, however, was not able to acquire exogenous DNA through electroporation.

DISCUSSION

Strain PV-4 was recovered from the microbial mat at hydrothermal vents, and it is able to grow at temperatures ranging

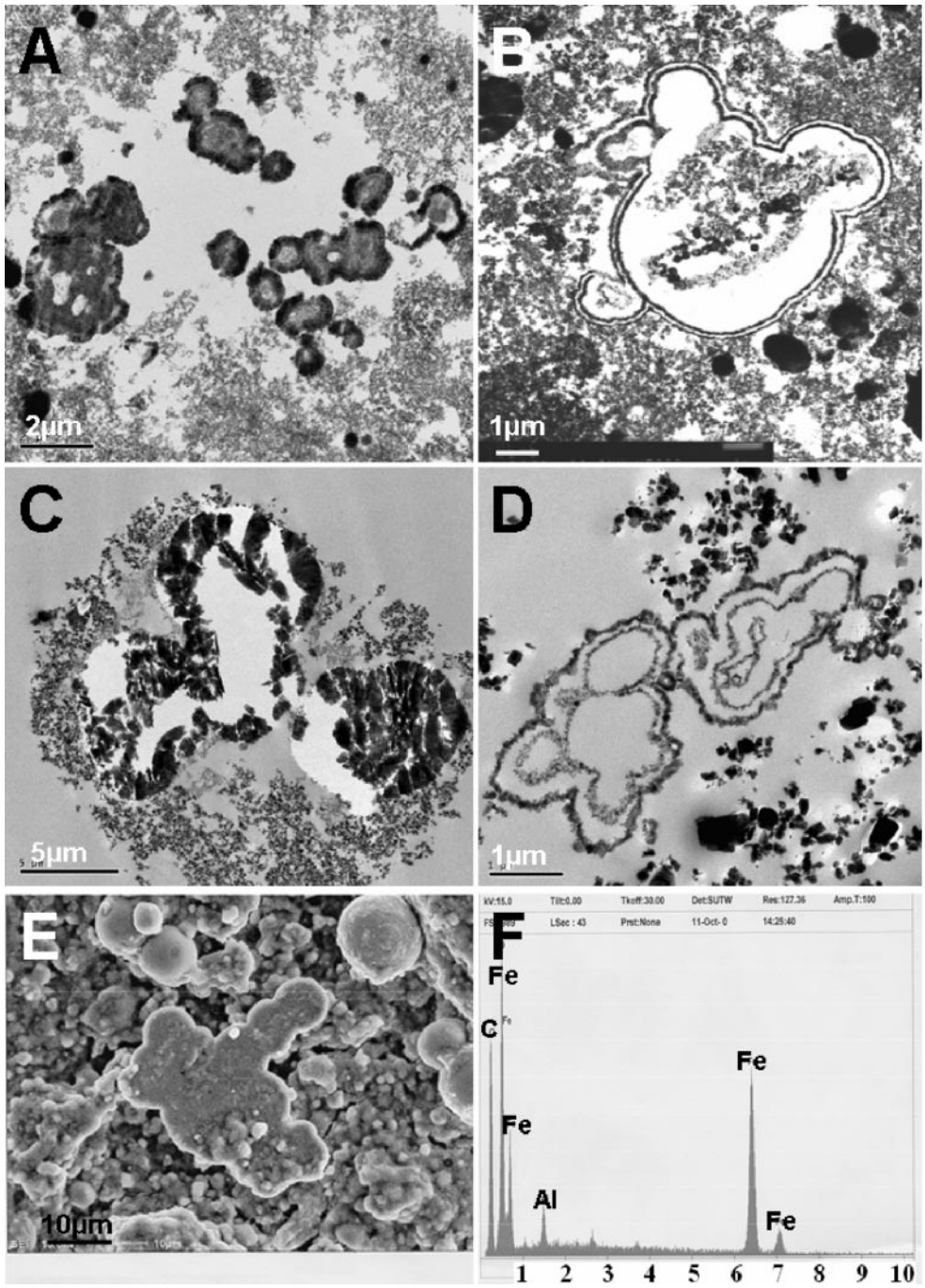

FIG. 6. Transmission electron microscope images (A to D) and scanning electron microscopic image (E) with energy-dispersive X-ray analysis (F) of siderite crystals formed by PV-4 with N_2-CO_2 as headspace gas at various temperatures. A, 4°C; B, 8°C; C, 18°C; D, 37°C; E, 28°C. Similar crystal globules were formed at all temperatures tested. Energy-dispersive X-ray analysis (F) of crystal globules (E) was used to confirm the composition of the siderite crystals.

from 0 to 42°C aerobically. In anaerobic environments, strain PV-4 is able to grow by reducing akaganeite at temperatures ranging from 0 to 37°C, with the optimum rate at approximately 18°C. Such a wide range of growth temperatures could be reflective of its natural habitat, where hot, anoxic fluids from deep-sea hydrothermal vents mix suddenly with cold seawater. Strain PV-4 can be described as a psychrotolerant bacterium because of its capability of growing at 5°C or lower according to the revised definition of Morita and Moyer (22). More importantly, strain PV-4 exhibits remarkable rates of growth at temperatures as low as 8 °C and as high as 37°C. Although strain PV-4 is closely related to recently described marine psychrotolerant *Shewanella* species, including *S. frigidimarina*, *S. gelidimarina*, *S. waksmanii*, *S. japonica*, and *S.*

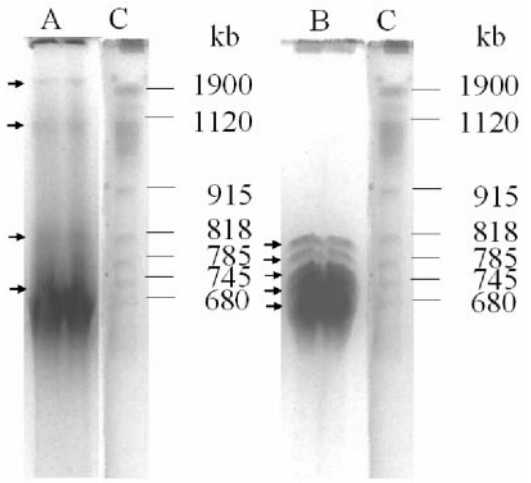

FIG. 7. PFGE of PV-4 DNA digested with 8-base restriction enzymes. Lane A, AscI; lane B, SwaI; lane C, yeast chromosome marker.

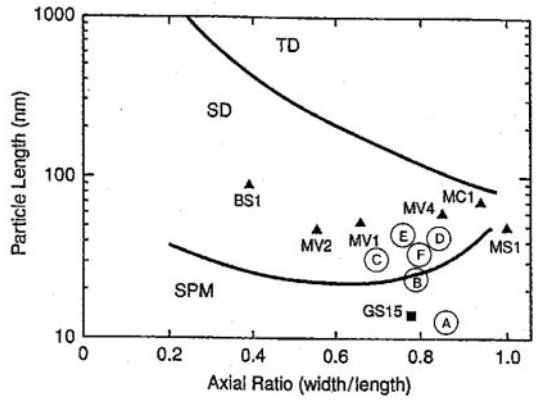

FIG. 8. Modified diagram (2) showing theoretical domain states for magnetite. SPM, superparamagnetic; SD, single domain; TD, two domains. Solid triangles represent particles formed by magnetotactic bacterial strains BS-1, MV-1, MV-4, MC-1, and MS-1 (1), and the solid square represents particles formed by the metal-reducing bacterium *Geobacter metallireducens* GS-15 (30). Open circles represent magnetite particles formed by PV-4 in this study. A, 4°C; B, 8°C; C, 14°C; D, 18°C; E, 28°C; F, 37°C.

fidelis (4, 11, 12, 13), it has a much higher temperature tolerance. *S. frigidimarina* grows from below 0 to 28°C, and *S. gelidimarina* cannot grow at 23°C or higher. *S. waksmanii*, *S. japonica*, and *S. fidelis* grow at temperatures ranging from 4 to 30°C (4, 11, 12, 13). Therefore, strain PV-4 can potentially function well in environments with great temperature variations. It will be a good model organism for studying cold adaptation and the associated metal reduction characteristics.

Although strain PV-4 was isolated from microbial mats in seawater, it is able to reduce akaganeite and form magnetite in the near absence of NaCl at 8°C using lactate (10 mM) as an electron donor. Strain PV-4 has a preference for slightly alkaline conditions. Strain PV-4 grows well aerobically in the pH range of 5.5 to 10. However, the cells showed a much lower pH tolerance under anaerobic conditions with metals. Metal reduction was observed in the pH range of 7.0 to 8.9, and little growth was observed at a pH below 6.5 or above 9.5. This is in agreement with the fact that microbial transformation of akaganeite to magnetite by Fe(III)-reducing bacteria generally occurs at a pH between 7.0 and 8.9, suggesting that such a process is favored by slightly alkaline conditions (3, 19, 27, 29). As observed in other metal-reducing bacteria, the decreased E_h and pH were recorded from cultures of strain PV-4 (24, 27, 41). This observation may be due to the decomposition of organic matter used as electron donors, which produces organic acids and CO_2 (41).

TABLE 1. Susceptibility of strain PV-4 to nine antibiotics

Antibiotic	Concn (μg/ml) of antibiotic at which strain PV-4 is[a]:		
	Resistant	Intermediate resistant	Susceptible
Ampicillin	1,000	ND	ND
Chloramphenicol	1	2	4
Erythromycin	1	2	4
Gentamycin	2	5	10
Kanamycin	2	5	10
Rifampin	ND	1	2
Spectinomycin	10	20	40
Streptomycin	10	20	40
Tetracycline	ND	1	2

[a] ND, no data.

In addition to akaganeite, iron species such as Fe(III)-citrate, iron oxyhydroxide, and HFO can be reduced by strain PV-4. Among them, HFO is the principal form of ferrihydrites, which are dominant iron species in the iron-rich microbial mats at Loihi. Furthermore, HFO has great potential in environmental science because it is an efficient sorbent for inorganic and organic pollutants. Much work on HFO bioreduction has focused on *S. putrefaciens* strain CN32, a well-studied dissimilatory Fe-reducing model bacterium (6, 8). Similar to strain CN32, PV-4 is able to effectively reduce HFO. This could be significant in iron biogeochemistry, because strain PV-4 has a much wider temperature and pH tolerance.

Strain PV-4 is able to reduce a number of metals other than iron species, Co(III), Cr(VI), Mn(IV), and U(VI) as electron acceptors while using lactate, formate, pyruvate, and hydrogen as electron donors. It is natural to speculate that the bacterial capability of transforming these heavy metals is reflective of its living environments, where hydrothermal fluids are often enriched in heavy metals. However, many other *Shewanella* strains isolated from different environments can also reduce heavy metals, suggesting that the ability may come mainly from their common ancestor (38).

One of the most striking characteristics of strain PV-4 was that it produced single-domain magnetite crystals using akaganeite at temperatures between 18°C and 37°C. Magnetite formed by closely related mesophilic dissimilatory Fe(III)-reducing bacteria (i.e., *Geobacter* and most *Shewanella* spp.) under mesophilic environments was usually poorly crystalline, impure, and mostly in the superparamagnetic size range (diameter, <30 nm), with a few known examples of single-domain grains (Fig. 8) (2, 18, 32). Furthermore, magnetite crystals produced by *Shewanella* marine isolate W3-6-1 at temperatures ranging from 0 to 30°C were also in this size range (33). Poorly crystallized and superparamagnetic magnetite particles

are not stable, arguing against their importance in contributing to the permanent remanent magnetism in sediments (9). The well-crystallized particles in strain PV-4 are more similar to the particles produced by magnetotactic bacteria than those formed by the above-mentioned bacteria (23, 32). This finding is significant, because single-domain magnetite is more likely attributed to magnetization of the marine sediments in subsurface environments (9). Strain PV-4 forms magnetite upon HFO reduction at room temperature as well. However, the magnetite is poorly crystallized, similar to those formed upon akaganeite reduction at low temperatures. The phenomenon cannot be readily explained, and more studies are needed to understand the mechanism of magnetite formation by microbes.

A mixture of magnetite and siderite was formed by strain PV-4 in the presence of N_2-CO_2, and the more complete reduction of iron oxide was observed with H_2-CO_2. In contrast, magnetite was formed predominantly in cultures with N_2 headspace, although the medium was bicarbonate buffered. This is in agreement with previous findings reported for some other *Shewanella* strains (e.g., *Shewanella alga* BrY) and *Thermoanaerobacter ethanolicus* TOR-39 (28, 29). Temperature does not have an effect on the formation of siderite. It has been previously reported that carbon dioxide facilitates the formation of siderite rather than less reduced magnetite (3, 29, 41). Microbial Fe(III) reduction is believed to be critical for forming a "biological carbon pump" through which CO_2 is consumed in surface waters and transported to the deep sea as sinking particulate organic carbon (37). Given the abundance of Fe in anaerobic sedimentary systems, the sequestration of CO_2 by iron biomineralization in oceans could play important roles in iron and carbon biogeochemistry in cold natural environments over geologic times. In addition to CO_2 sequestration, microbial reduction of iron oxide to siderite may be potentially important for bioremediation because it allows more thorough oxidation of organic matter or contaminants than when magnetite is the end product.

While all results presented thus far suggest that strain PV-4 is a good model system for studying metal reduction at low temperatures, whether this organism can be genetically manipulated remains unknown. Uptake of foreign DNA is a prerequisite for all molecular operations. In this study, after determining the antibiotic susceptibility of strain PV-4, we successfully introduced a plasmid into the organism by a natural method (conjugation) but failed to do so by an artificial method (electroporation). This is not surprising, because *Shewanella* strains can hardly be transformed by electroporation (24). Our following work will focus on developing a site-specific mutagenesis system, which will enable us to pursue functional analysis of this organism.

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