Orenia metallireducens sp. nov. strain Z6, a Novel Metal-reducing *Firmicute* from the Deep Subsurface

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ABSTRACT

A novel halophilic and metal-reducing bacterium, *Orenia metallireducens* strain Z6, was isolated from briny groundwater extracted from a 2.02 km-deep borehole in the Illinois Basin, IL. This organism shared 96% 16S rRNA gene similarity with *Orenia marismortui*, but demonstrated physiological properties previously unknown for this genus. In addition to exhibiting a fermentative metabolism typical of genus *Orenia*, strain Z6 reduces various metal oxides [Fe(III), Mn(IV), Co(III), and Cr(VI)] using H_2 as the electron donor. Strain Z6 actively reduced ferrihydrite over broad ranges of pH (6-9.6), salinity (0.4-3.5 M NaCl) and temperature (20-60 °C). At pH 6.5, strain Z6 also reduced more crystalline iron oxides such as lepidocrocite (γ-FeOOH), goethite (α-FeOOH) and hematite (α-Fe_2O_3). Analysis of X-ray absorption fine structure (XAFS) following Fe(III) reduction by strain Z6 revealed spectra from ferrous secondary mineral phases consistent with the precipitation of vivianite [$Fe_3(PO_4)_2$] and siderite ($FeCO_3$). The draft genome assembled for strain Z6 is 3.47 Mb in size and contains 3,269 protein-coding genes. Unlike the well understood iron-reducing *Shewanella* and *Geobacter* species, this organism lacks the *c*-type cytochromes for typical Fe(III) reduction. Strain Z6 represents the first bacterial species in the genus *Orenia* (order *Halanaerobiales*) reported to reduce ferric iron minerals and other metal oxides. This microbe expands both the phylogenetic and physiological scope of iron-reducing microorganisms known to inhabit the deep subsurface and suggests new mechanisms for microbial iron reduction. These distinctions from other *Orenia* spp. support the designation of strain Z6 as a new species, *Orenia metallireducens* sp. nov.

47 **IMPORTANCE**

48 A novel iron-reducing species, *Orenia metallireducens* sp. nov. strain Z6, was isolated from
49 groundwater collected from a geological formation located 2.02 kilometers below land
50 surface in the Illinois Basin, USA. Phylogenetic, physiologic and genomic analyses of strain
51 Z6 found it to have unique properties for iron reducers including: 1) active microbial iron-
52 reducing capacity under a broad range of temperature (20-60 °C), pH (6-9.6), and salinity
53 (0.4-3.5 M NaCl); 2) lack of *c*-type cytochromes typically affiliated with iron reduction in
54 *Geobacter* and *Shewanella* species; and 3) the only member of the *Halanaerobiales* capable
55 of reducing crystalline goethite and hematite. This study expands the scope of phylogenetic
56 affiliations, metabolic capacities, and catalytic mechanisms for iron-reducing microbes.

INTRODUCTION

The reduction of ferric (Fe(III)-bearing) minerals by microorganisms is widespread in both terrestrial and marine environments (1, 2) and is potentially one of the earliest forms of metabolism (3, 4). Due to the abundance of ferric minerals in the Earth's crust and the ubiquity of dissimilatory metal-reducing bacteria (DMRB), Fe(III) reduction is of global environmental significance, particularly in the subsurface. Phylogenetic variety of organisms has been reported for the capacity to reduce iron with dissimilatory manner (1, 5). Dissimilatory iron reduction can be classified into two major groups. Many of the iron-reducing organisms (e.g., fermentative iron reducers) use Fe(III) as a minor side reaction in their metabolism but do not appear to conserve energy to support growth from this electron transfer. In comparison, the respiratory iron reducers conserve energy to support growth from Fe(III) via an electron transfer chain (1, 5). DMRB strongly influence the biogeochemical cycling of metals and mineralization of organic matter in aquatic and sedimentary environments (1, 6-8). In addition, DMRB play a key role in the bioremediation of hazardous contaminants such as heavy metals, radionuclides, and hydrocarbons (9).

DMRB capable of reducing ferric minerals have previously been isolated from gold mines, petroleum reservoirs, and other deep geological formations (10-21). These subsurface environments contain a significant amount of the Earth's total microbial biomass (22) and exhibit unique physical and geochemical features (e.g., anoxia, high salinity, elevated temperature and pressure) (12). Similar to those from many other natural environments, the DMRB isolated from the deep subsurface have been found to conserve energy through two primary ways: by directly respiring ferric iron, manganese, or other oxidized metals (e.g. *Geobacter* spp.) (10, 21, 23) or by utilizing these metals as an electron sink for fermentative pathways (e.g. *Fervidicella* and *Caloramator* spp.) (24, 25). The DMRB derived from deep subsurface typically show optimal activity at the elevated temperature and salinity of the

environment from which they were isolated, suggesting that they are native to the deep subsurface environments (12). In most previous studies, however, the ability of these DMRB to reduce Fe(III) was primarily assayed using Fe(III)-citrate (10, 19, 21) or amorphous ferrihydrite (23). More crystalline ferric oxides like goethite and hematite are more typical of the iron oxide minerals found in subsurface sediments (26) and can also act as an electron sink under both fermentative and respiratory conditions for DMRB (27), yet the extent to which deep subsurface DMRB can reduce these minerals is largely unkonwn.

The Mt. Simon formation within the Illinois Basin, IL, USA, is composed primarily of highly porous and permeable quartz sandstones coated with crystalline ferric minerals, including hematite and goethite (26). The presence of these iron minerals along with millimolar concentrations of dissolved ferrous iron in the groundwater suggests microbially-mediated iron reduction is active in this environment (28). This groundwater is thermal (50° C), briny [19.1 % total dissolved salts (TDS)], anoxic, and under considerable pressure (210 bar) (28). Our previous work identified indigenous microbial communities within different stratigraphic horizons in the Mt. Simon, and using this water we successfully developed enrichment cultures capable of reducing ferric iron compounds (28, 29). In this paper we describe a bacterium isolated from these enrichments that is capable of reducing a broad suite of iron minerals. Phylogenetic, physiologic and genomic analyses showed that this isolate is a member of the genus *Orenia* in the phylum *Firmicutes* and exhibits distinct physiological properties not previously reported for this genus or other known DMRB.

METHODS AND MATERIALS

Media. If not mentioned otherwise, strain Z6 was grown in modified groundwater medium, which consisted of anoxic basal medium (18) and filter-sterilized (0.2 µm pore size, Thermo Fisher Scientific, MA) formation water (see Table S1 in the supplemental material (SI)) at a ratio of 9:1 (v:v). The basal medium was buffered with 3.3 mM piperazine-N,N′-bis(2-ethanesulfonic acid (PIPES) and 30 mM $NaHCO_3$, sparged with N_2:CO_2 (80:20, v:v), and had a final pH of 7.0-7.2 (18). Trace metals (18) and vitamins (ATCC® MD-VS™, ATCC, VA) were supplemented from sterile stock solutions (1 mL/L). Anoxic conditions were maintained by amending the media with Na_2S (100 µM) and cysteine (250 µM). The cultures were prepared in 10 mL medium in 27 mL serum tubes or 80 mL medium in 160 mL serum bottles (Wheaton Industries Inc., NJ). Under ferrihydrite-reducing conditions, the groundwater medium was amended with 10 mmol/L Fe(III) of that mineral (100 µmoles/tube or 800 µmoles/bottle) as the electron acceptor. H_2 (202 µmoles/tube or 807 µmoles/bottle) and acetate (5 mM) were used as the electron donor and carbon source, respectively (FeR medium). FeR medium was used to test strain Z6 for temperature tolerance. Modifications to the FeR medium were made to characterize specific physiological traits of strain Z6 such as activity at different pH, salt concentrations and utilization of other electron donors and acceptors. These changes are described below as well as in Table S2 of the SI. A synthetic Orenia medium was also developed to culture strain Z6 without amendment of formation groundwater. The synthetic Orenia medium contained: NaCl (342 mM), $MgCl_2 \cdot 2H_2O$ (7.4 mM), KCl (13.4 mM), NH_4Cl (9.3 mM), KH_2PO_4 (2.5 mM), $CaCl_2$ (10 mM), $NaHCO_3$ (23.8 mM), yeast extract (0.2 g/L), cysteine (200 µM), resazurin (1 mg/L), trace metals (1 mL/L) (30), and vitamins (ATCC® MD-VS™, 1 mL/L) at pH 7.0-7.2. To confirm the extent and rates of iron reduction by strain Z6 in this synthetic Orenia medium, cultures were grown in

126 the presence of 10 mmol/L ferrihydrite, 202 µmoles/tube H_2 and 5 mM acetate. Iron-reducing
127 activity in this medium was then compared to that in the FeR medium.

128 **Isolation of strain Z6.** Strain Z6 was isolated from an iron-reducing enrichment
129 culture created using the groundwater taken at a depth of 2.02 km in the Illinois Basin as
130 inoculum (28). Unless otherwise specified, this isolate was cultivated anaerobically in 25 mL
131 anaerobic culture tubes or 120 mL serum bottles. All the amendments were added from
132 autoclaved and anoxic stock solutions using sterile N_2-flushed syringes. The culture
133 tubes/bottles were sealed with blue butyl rubber stoppers (Chemglass Life Sciences, NJ) and
134 aluminum crimp seals. The cultures were grown in the dark at 42 °C in a static incubator and
135 manually shaken once per day.

136 Isolates were obtained from the enrichment culture using the agar shake method (31).
137 Anoxic culture tubes were loaded with 6 mL of basal medium (18) and amended with low-
138 melting-point agarose (Thermo Fisher Scientific Inc., MA). After autoclaving, 4 mL of N_2-
139 bubbled and degassed filter-sterilized formation water (see Table S1 in the SI) (28) was
140 added to each tube, giving a final concentration of 1.5% agarose. Each anoxic culture tube
141 was then amended with 5 mM ferric citrate, H_2 (202 µmoles /tube) and 5 mM each of formate,
142 acetate, pyruvate, and lactate that had been used in the enrichment culture (28). The agarose-
143 containing medium was kept in a 45 °C water bath before inoculation to prevent
144 solidification. The parent enrichment was serially diluted 1:10 using sterilized basal medium
145 in anoxic culture tubes. One milliliter of each dilution (1:10 to $1:10^5$) was inoculated into
146 individual agar shake tubes, mixed well and promptly solidified on ice. Five milliliters of
147 filter sterilized H_2 was injected into the headspace. After 5-10 days of incubation at 42° C,
148 clearing zones formed around individual colonies, putatively indicating active Fe(III)
149 reduction. To isolate single colonies from the agar shake, a sterile syringe containing ~0.1
150 mL of liquid anoxic medium was fitted with a 23 gauge, 2 inch needle and used to remove a

single, well-separated colony from the culture tube. Anoxia was maintained during this process by passing a continuous stream of sterile N_2 gas into the culture tube. The individual colony was drawn into a syringe, and then promptly injected into sterile anoxic FeR medium. The agar-shake isolation protocol was repeated twice more for the picked colonies to ensure a pure culture had been obtained. Isolate purity was verified morphologically using an optical microscope and phylogenetically by sequencing the 16S rRNA gene of the isolated culture.

Phylogenetic characterization. Genomic DNA was extracted from a pellet centrifuged from 1 mL of liquid culture using the FastDNA® Spin Kit for Soil (MP Biomedicals, CA). Full-length 16S rRNA genes for cloning were amplified using the universal Bacterial primers 8F and 1492R (32). PCR was conducted using TaKaRa *Ex Taq* polymerase (TaKaRa Bio USA, Madison) and an Eppendorf MasterCycler (Eppendorf, Germany). PCR products were verified by using agarose gel electrophoresis and then purified with QIAquick PCR Purification Kit (QIAGEN Inc., CA), after which a clone library was generated using the pGEM®-T Easy kit (Promega U.S., Madison, WI). Cloned 16S rRNA gene fragments were sequenced using M13 primers (ACGT Inc., Wheeling, IL), assembled with Sequencher (v. 1.4.0) and classified using RDP10 (33). A phylogenetic tree based on the 16S rRNA genes for strain Z6, close phylogenetic relatives, and representative DMRB was created using RAxML (34).

Physiological and Biochemical Characteristics. A Gram stain of strain Z6 cells was performed using the standard methods (35). Catalase activity of this organism was determined using 15% H_2O_2 on centrifuged cell pellets (36). Oxidase activity was determined using K520 oxidase test strips (Key Scientific Products Co., TX) following the manufacturer's recommendation. To test the capacity to grow aerobically, strain Z6 was cultured in aerobic basal medium (18) amended with 10 mM glucose. After inoculation, growth was determined by measuring the optical density of cell suspensions at 600 nm

(OD$_{600}$) using a SPECTRONIC 20D+ UV-Vis spectrophotometer (Thermos Scientific, MA). The morphology of strain Z6 was examined using both scanning electron microscopy (SEM) and transmission electron microscopy (TEM) (28).

A suite of culture conditions was used to determine the metabolic capacity of strain Z6. Fermentable substrates (5 mM unless stated otherwise) (i.e., betaine, cellobiose, fructose, fumarate, galactose, glucosamine, glutamate, glucose, glycine, glycerol, lactose, maltose, mannitol, mannose, peptone (1 g/L), starch (1 g/L), sucrose, trehalose, yeast extract (1 g/L)) were tested for their ability to support growth as indicated by an increase in OD$_{600}$ over several days of incubation. To determine which electron donors would support iron-reducing activity, cultures were incubated at 42 °C for 21 days in the FeR medium with one of the following substrates (5 mM unless specified): acetate, benzoate, butyrate, citrate, ethanol, formate, fumarate, glycerol, glycine, H_2 (202 µmoles/tube), lactate, methanol, propionate, phenol (2.5 mM), succinate, yeast extract (1 g/L), starch (1 g/L), tryptone (1 g/L), peptone (1 g/L), glucose, fructose, cellobiose, lactose, galactose, sucrose and trimethylamine. An increase in Fe(II) concentration was used as an indicator of iron-reducing activity. The capacity to reduce other electron acceptors (5 mM unless mentioned otherwise) (i.e., nitrate, nitrite (2.5 mM), fumarate, elemental sulfur [S(0)], thiosulfate and sulfate] was evaluated by monitoring reduction activity in cultures amended with H_2 (202 µmoles /tube) and acetate (5 mM). The ability to reduce other metals (i.e., 5 mM one of Co(III)-EDTA, CrO_4^{2-} and MnO_2) was also evaluated using the same conditions.

To determine if ferrihydrite reduction benefited the isolate energetically, we compared cultures grown with glucose only (fermentation) to those grown with glucose and 10 mmol/L ferrihydrite (fermentative iron reduction). The medium for these cultures was the same as the modified groundwater medium except that it was buffered solely with 10 mM PIPES and no bicarbonate was amended. The fermentation and iron reduction products were

201 determined to develop stoichiometric equations and calculate thermodynamics (37) under
202 these two growth conditions.
203 Several control experiments were also performed to evaluate whether the observed
204 iron reduction activity was due to the abiotic reduction of Fe(III) by medium components or
205 fermentation byproducts. Specifically, the medium components (e.g., Na_2S, cysteine, organic
206 buffers), fermentation products, and proteinaceous products were evaluated for their ability to
207 reduce ferrihydrite at pH 7.2, 0.4 M NaCl and 42 °C. The controls included: 1) abiotic—
208 without cell inoculation; 2) inoculation only—no electron donors added; 3) autoclaved
209 cultures with both cells and electron donor; and 4) autoclaved cultures pre-grown with 5 mM
210 glucose—spent fermentation media.
211 To determine the range of the iron reduction activity, strain Z6 was evaluated with
212 different iron-oxide minerals, including more crystalline types found in subsurface
213 environments. Four ferric iron minerals (ferrihydrite, lepidocrocite, hematite, and goethite)
214 were synthesized as described by Schwertmann and Cornell (38). Strain Z6 was cultured in
215 modified groundwater medium amended with one of the above iron-oxide minerals
216 (~10 mmol/L) from the parental inoculant grown in FeR medium. Activity was monitored by
217 quantifying Fe(II) over time. At selected time points, 0.4 mL of well-mixed culture was
218 withdrawn inside an anaerobic chamber using a sterile syringe and split into two 0.2 mL
219 aliquots. One 0.2 mL aliquot was filtered through a 0.45 μm filter (GE Healthcare Life
220 Sciences, NJ) and each aliquot was combined with 0.2 mL of 1 M HCl. The filtered sample
221 was used for determination of aqueous ferrous iron; the unfiltered sample was used for
222 determination of acid-extractable ferrous iron and total iron.
223 To determine how iron reduction was impacted by environmental factors, the ability
224 of strain Z6 to reduce ferrihydrite was evaluated at different temperatures (4-80 °C), salinities
225 (0-5.98 M NaCl), and pH (4-11) in FeR medium (Table S2). Susceptibility to antibiotics was

tested by amending the iron-reducing cultures with one of the following antibiotics: 40 µg/ml ampicillin, anisomycin, tetracycline, erythromycin and kanamycin plus 20 µg/mL chloramphenicol (39).

Details about culture setup, chemical analyses of different ferric and ferrous iron species (e.g., total iron and 0.5 M HCl extractable Fe(II)), fermentation substrate/products, and calculation of kinetic constants for iron reduction by strain Z6 are described in detail in the supplemental material.

Mineralogical characterization. The solid phases in the cultures were analyzed by X-ray absorption fine structure spectroscopy (XAFS) (40) to determine the chemical speciation of Fe. Solids were collected by filtering 2 mL of suspension through a 0.22 µm nylon filter membrane. The hydrated solids retained on the membrane were sealed between two layers of Kapton film in an anaerobic chamber. XAFS measurements at the Fe K-edge (7,112 eV) were performed at the MRCAT/EnviroCAT 10-BM beamline (41) at the Advanced Photon Source, Argonne National Laboratory. Anoxic conditions were maintained during data collection by purging the sample chamber with N_2. These procedures have been previously shown to maintain the anoxic integrity of the sample for the duration of the measurement (42). The energy of the incident X-rays was scanned using a Si(111) water-cooled double-crystal monochromator. Harmonic content was removed by detuning the second crystal to 50% of the maximum intensity. Data were collected in transmission mode using gas-filled ionization detectors. Monochromator energy calibration was maintained by the simultaneous collection of spectra from a metallic Fe standard using X-rays transmitted through the samples. The final spectrum for each sample was produced by averaging 3-5 consecutive scans. The extended XAFS (EXAFS) region of the spectrum was extracted using the program Autobk (43). The contribution of spectroscopically-distinct Fe species in the k^3-weighted EXAFS data was quantified using linear combination (LC) analysis implemented in

the program ATHENA (44). In addition to ferrihydrite, goethite, hematite, and lepidocrocite, spectra from the following Fe(II)-bearing phases were considered as possible components in the LC analysis: vivianite, magnetite, Fe(II) adsorbed to carboxyl-functionalized beads at pH 7 (45), siderite, green rust, and amorphous mackinawite. The best-fit combination was chosen based on the quality of the fit as determined by the lowest reduced-chi-square value. Linear combination analyses were also performed on the derivative of the X-ray absorption near edge structure (XANES) data to corroborate the results of the EXAFS analysis.

Genomic reconstruction. Genomic DNA for strain Z6 was extracted using the phenol:chloroform extraction method as described previously (29). The genome was sequenced using the Roche 454 and Illumina HiSeq technologies with the average sequencing coverage approximately 26× and 140×, respectively. The sequencing reads were assembled using Newbler 2.7 (Roche Holding AG, Swiss). The draft assembly was then error-corrected using iCORN 0.97 (46) and internal gaps closure was achieved using the GapCloser package of SOAPdenovo (v.1.12) (47). CheckM (48) was used to evaluate the completeness, quality and contamination of the assembled genome for strain Z6. The open reading frames (ORFs) were predicted and annotated using the IMG/ER pipeline (49).

Nucleotide sequence accession number. The nucleotide sequences of the 16S rRNA genes of strain Z6 determined in this study have been deposited in GenBank under accession number KP898734. The whole-genome sequence of strain Z6 was deposited in NCBI GeneBank under the accession no. LWDV00000000. The version described in this paper is LWDV00000000. The IMG Genome ID for strain Z6 is 2687453649.

RESULTS

Isolation of strain Z6. A pure culture of an iron-reducing bacterium was obtained after sequential colony transfers from agar-shake medium containing a mixture of volatile fatty

276 acids and ferric citrate and is designated as strain Z6. Electron micrographs showed that this
277 organism was rod-shaped with dimensions of 0.5×(2-20) µm. Depending on the growth
278 phase, filamentous cells were observed during exponential growth (Fig. 1 and Table 1 in SI).
279 In addition, numerous pili were observed on the surface of strain Z6 cells (Fig. 1). Analysis
280 of the 16S rRNA genes of strain Z6 indicated that it is a member of phylum *Firmicutes* with
281 96% sequence similarity to *Orenia marismortui*, a fermenter originally isolated from Dead
282 Sea sediment (50) (Fig. 2). Strain Z6 also stained Gram-negative and produced negative
283 results for oxidase and catalase tests (Table 1 in SI). Genomic DNA showed a G+C content
284 for this organism of 32.1%. The major membrane fatty acids for strain Z6 consisted of 16:0,
285 16:1 and 18:0 (Table 1).

286 **Physiological characteristics.** In addition to reducing dissolved ferric citrate, strain
287 Z6 also reduced solid phase ferrihydrite. Strain Z6 was able to survive a broad range of pH,
288 temperature and salinity conditions when using ferrihydrite as the electron acceptor, H_2 as the
289 electron donor and acetate as carbon source, respectively. Iron reduction occurred at pH
290 values from 6-9.6, with an optimal initial iron reduction rate at pH = 7. However, more iron
291 was reduced at lower pH (i.e. pH 6) (Fig. 3a), even though the corresponding initial rate of
292 iron reduction was slower. Iron reduction activity occurred at salinity ranging from 0.4 to 3.5
293 M NaCl (Fig. 3b). The highest initial iron reduction rate occurred with 1.2 M NaCl and
294 decreased correspondingly with increasing salt concentrations, while the amount of ferric
295 iron reduced was similar from 1.2-3.5 M NaCl (Fig. 3b). At pH 7 and with 0.4 M NaCl,
296 robust iron reduction occurred over a broad range of temperatures (20-60 °C), with similar
297 amounts of iron reduced between 20 and 50 °C (Fig. 3c). Initial rates of reduction dropped
298 sharply at 60° C, but significant iron reduction was still observed.

299 Substrates that support iron reduction were evaluated in media with a ferrihydrite
300 suspension and one of several inorganic or organic compounds. Positive results were

indicated by ferrous iron generation above that observed in the abiotic and inoculation-only controls (Fig. 4 and Fig. S1a in SI). Among the non-fermentable substrates, only H_2 and trimethylamine supported reduction of ferrihydrite (Fig. 4), while short chain fatty acids, alcohols, and aromatic compounds did not. In contrast, the selected fermentable substrates (i.e., cellobiose, fructose, galactose, glucose, starch, sucrose and yeast extract) supported fermentative ferrihydrite reduction (Fig. 4). In the presence of ferrihydrite, fermentation of 4.55 mM glucose produced 2.61 mM acetate, 5.53 mM ethanol, 2.65 mM formate, 6.07 mM HCO_3^- (including HCO_3^- and CO_2), and 88.6 µmoles/bottle H_2 (see Table S4 in the supplemental material). The stoichiometry of fermentative iron reduction with glucose and ferrihydrite determined was:

$$C_6H_{12}O_6 + (6/5)Fe(OH)_3 \rightarrow (4/3)C_2H_5OH+(2/3)CH_3COO^-+(2/3)HCOO^-+(12/5)HCO_3^- +(6/5)Fe^{2+}+(1/15)H_2+(4/15)H^++(2/5)H_2O \quad \text{(Eq. 1)}$$

Although no significant iron reduction was observed in the two controls, one with substrate only and the other with cells only, it was not clear whether the fermentation products generated were directly involved in the abiotic reduction of the ferrihydrite. In order to exclude the possibility of these abiotic reactions, a series of strain Z6 cultures were grown by fermenting glucose without iron. After growth leveled off, the cultures were autoclaved before different iron oxides were amended. No reduction of ferrihydrite, lepidocrocite, goethite or hematite occurred in any of these cultures (Fig. S1b in SI). This indicated that the observed iron-reducing activity was due to biologically mediated enzymatic reactions.

The capacity to grow on different substrates in the absence of iron was also evaluated. Similar to other species affiliated to genus *Orenia*, strain Z6 rapidly grew on many mono- and poly-saccharides, including cellobiose, fructose, glucose, glycine, glycerol, mannose, maltose, sucrose, and trehalose based on an increase in OD_{600} (Table 1). Yeast extract and

starch alone also supported fermentative growth. However, strain Z6 did not grow on fumarate, galactose, glutamate, glucosamine, lactose, mannitol and peptone. Fermentation of glucose alone yielded similar primary products (i.e., acetate, ethanol, formate, CO_2, H_2) as those observed in the fermentative iron-reducing cultures, except for observation of a trace amount of lactate (0.15 mM) (see Table S4 in SI). In the absence of ferrihydrite, however, about 30% of the initial glucose remained unfermented (Fig. S2), while H_2 produced under this condition was about 1.6 times higher than in the fermentative iron-reducing cultures (see Table S4 in SI). The stoichiometry of glucose fermentation alone was:

$$C_6H_{12}O_6+(5/3)H_2O \rightarrow (4/3)C_2H_5OH+(2/3)CH_3COO^- +HCOO^-+HCO_3^- +(1/2)H_2 + (8/3)H^+ \quad \text{(Eq. 2)}$$

In addition to iron reduction and fermentation, strain Z6 also showed capacity to reduce a number of other transition metal compounds, such as Co(III) [Co(III)-EDTA], Cr(VI) [chromate] and Mn(IV) [MnO_2] based on changes in the media colors. The medium changed from purple to clear, yellow to clear and brown to pink for Co^{III}, Cr^{VI} and Mn^{IV}, respectively. No assessment was made whether these reactions supported growth of strain Z6. In contrast to ferric iron reduction, no significant reduction of substrate was observed when strain Z6 was provided with nitrate, nitrite, S(0), thiosulfate, sulfate, or fumarate (Table 1).

Strain Z6 also exhibited different degrees of susceptibility to antibiotics. When supplied with H_2 and ferrihydrite, the cultures amended with chloramphenicol, tetracycline, kanamycin or erythromycin showed significant inhibition in iron-reducing capacity, while no reduction in iron-reducing activity was seen with ampicillin or anisomycin compared to the antibiotic-free controls (see Fig. S3 in SI).

Reduction of iron oxides with different crystallinity. Because hematite and goethite are common iron minerals in the environment, we evaluated the capacity of strain Z6 to

351 reduce these naturally occurring ferric iron oxides. In most previous studies of iron-reducing
352 microorganisms, the more crystalline goethite and hematite resulted in significantly less
353 Fe(II) production than chelated Fe(III) compounds or ferrihydrite (2). Strain Z6, however,
354 was able to substantially reduce crystalline minerals such as lepidocrocite, goethite and
355 hematite (Fig. 5). At pH 6.5, strain Z6 reduced 23-43% of the total Fe(III) present in the
356 parent iron oxides within 17 days. Despite a significantly faster initial iron reduction rate for
357 ferrihydrite than the other minerals, similar extents of total iron reduction (40.1-43.2 %) were
358 determined for ferrihydrite and lepidocrocite. In comparison, 23.5 and 29.5 % of the Fe(III)
359 was reduced for goethite and hematite, respectively (Fig. 5 and Table 2). In addition to the
360 FeR medium containing 10% filter-sterilized groundwater, active ferrihydrite reduction by
361 strain Z6 can also be observed in the synthetic Orenia medium in the absence of groundwater
362 amendment when the same electron donor and carbon source were supplied (see Fig. S4 in
363 SI).

364 Fe K-edge XANES analyses of the solids confirmed the presence of reduced iron
365 species in the active cultures, as indicated by the shift in the edge position to lower energy
366 compared to the corresponding parent oxide and the control sample spectrum (see Fig. S5 in
367 the supplemental material). Linear combination (LC) analyses of the XANES data indicated
368 that the ferrous iron content in the solid phase ($Fe(II)_{solid}/Fe(III)_{solid}$) at the end of the
369 experiment ranged from 7.5 to 38.8% (Table 2). The EXAFS data also showed differences
370 between the un-inoculated controls and the active culture incubations, with larger differences
371 corresponding to greater extents of iron reduction as determined by XANES and the Fe(II)
372 measurements of the HCl extracts (Figs. 6 and S5 in the supplemental material). LC analysis
373 of the EXAFS data determined that ferrous iron was present as O-coordinated Fe(II) species
374 (inclusive of disordered Fe(II) precipitates or adsorbed Fe(II)), vivianite [$Fe_3(PO_4)_2$], and/or
375 siderite ($FeCO_3$) (Table 2 and Fig. 6). The distribution of the Fe(II) products in the best fits of

the data varied depending on the starting ferric iron mineral and the extent of iron reduction, but all showed the presence of siderite (Table 2). In contrast, magnetite (Fe_3O_4) and green rust, two common Fe(II)-bearing minerals frequently observed as biomineralization products by DMRB (51-55), were not detected under the culture conditions here.

Genomic reconstruction. The draft genome sequence of strain Z6 is 3,456,989 bp (3.47 Mb). Evaluation of this assembled genome using CheckM (48) indicates that the genome for strain Z6 is 99.1% in completeness, 0% in heterogeneity and <2.5% in contamination. The assembled genome comprises 12 scaffolds, ranging from 2.2 to 903.3 kb in size. N50 for this genome is 177.7 kb. Comparison of the genomes of strain Z6 and other bacterial genomes showed that it shared no more than 90 % pairwise average nucleotide identity (ANI) with any other organism and was 78.1% in ANI with *Orenia metuimatui* DSM5156. A total of 3,269 protein-coding genes were predicted from the genome. The reconstructed pathways from the ORFs (Fig. 7) are consistent with the phenotypic features of this strain as described above. Strain Z6 harbors fermentation pathways, including glycolysis and pentose phosphate pathway (Fig. 7). As expected for a fermentative organism, it does not contain a complete TCA cycle and lacks the genetic capability to use short-chain fatty acids and alcohols (e.g., acetate, formate, lactate and ethanol). This is consistent to the observation that none of these compounds supported iron reduction by strain Z6 (Fig. 4). The genome contains coded regions for 15 hydrogenases. Some of these appear to be homologous to bifurcating hydrogenases that generate proton motive force for energy coupling (56) (Fig. 7). Although not confirmed physiologically, a gene cluster containing nitrogenase (i.e., *nif*D, *nif*H, *nif*K, *nif*E, *nif*N, *nif*B, *nif*V) and associated transporters and regulators are identified in the genome. Despite the capacity of using trimethylamine as an electron donor for iron reduction, no ORFs associated with the pathway(s) for trimethylamine metabolism were identified in the genome of strain Z6. Sulfate adenylyltransferase, adenylylsulfate kinase,

adenylylsulfate reductase and sulfite reductase were predicted in the genome of strain Z6, however, no active sulfate reduction was observed in culture test (Table S3). No annotated sulfur reductase or observed sulfur-reducing activity by strain Z6 suggest cryptic sulfur cycle that mediates electron shuttling during iron reduction by some iron reducers (e.g., *Shewanella oneidensis* MR-1) (57) is not feasible for strain Z6. The genes for biosynthesis and assembly of flagella and Type IV pili were also identified (Fig. 7). Based on the gene prediction and annotation by IMG pipeline (49), in contrast to many other dissimilatory iron-reducing bacteria (e.g., *Geobacter* and *Shewanella* spp.), strain Z6 lacks *c*-type cytochromes thought to be critical for ferric iron reduction in these other organisms (2, 58, 59).

DISCUSSION

Strain Z6 is a thermophilic and halophilic metal-reducing bacterium of the genus *Orenia* that was enriched and isolated from formation water taken from a depth of at 2.02 km in the Illinois Basin, USA (28). This organism possesses 92-96% 16S rRNA gene sequence identity related to the previously isolated *Orenia* species (*O. marismortui*, *O. salinaria*, *O. sivashensis* and *O. chitinitropha*) (Fig. 2) (39, 50, 60, 61). A screen of the NCBI and RDP reference databases (33) also shows that closely related sequences or phylotypes are not abundant. The only phylotypes with a similarity >96 % are from the parental enrichment culture (28) and four sequences from a high-temperature North Sea oil field (62).

Strain Z6 represents the first *Orenia* species isolated from terrestrial deep subsurface. All previous *Orenia* species (*O. marismortui*, *O. salinaria*, *O. sivashensis* and *O. chitinitropha*) (Fig. 2) were isolated from saline or hypersaline salterns or lakes (39, 50, 60, 61). The natural habitats for these known *Orenia* populations reflect their capacity to tolerate a broad range of temperature and salt concentrations. Previous physiological characterization of these *Orenia* species, however, has focused on the fermentation of saccharides or

426 polysaccharides (e.g., chitin) (39, 50, 60, 61). As with other *Orenia* populations, strain Z6
427 exhibits rod-shaped morphology, low G+C content, tolerance of comparable ranges of pH,
428 temperature, salinity, as well as active fermentation of sugars (Table 1). However, strain Z6
429 shows distinct physiological characteristics compared to other *Orenia* isolates. For example,
430 in contrast to *O. salinaria* and *O. chitinitropha* but similar to *O. marismortui*, strain Z6
431 ferments starch. In contrast, glycerol supports growth of strain Z6, but not for other *Orenia*
432 species (Table 1) (39, 50, 60, 61). The findings of dissimilatory reduction of iron (Figs. 3-5)
433 and other metal compounds (e.g., Co(III)-EDTA, MnO_2 and CrO_4^{2-}) (Table S3) by strain Z6
434 have not been previously tested for genus *Orenia*. Overall, the phylogenetic and
435 physiological data suggest that this strain represents a new species of the genus *Orenia*.
436 In contrast to previously studied deep subsurface iron-reducing bacteria, strain Z6 is
437 distinct in both phylogenetic and physiological features. In comparison, other deep
438 subsurface iron reducers belong to many phylogenetic lineages, such as other classes of
439 *Firmicutes*, and populations associated with phyla *Thermotogae*, *Thermodesulfobacteria*,
440 *Deferribacteres*, *Deinococcus-Thermus*, and *Proteobacteria*) (Fig. 2). All of these organisms
441 are only distantly related to strain Z6, sharing ≤ 81% 16S rRNA gene sequence identity (10-
442 21). Physiologically, strain Z6 tolerates a broader range of temperature than those reported
443 for other iron reducers derived from deep subsurface environments, which are typically
444 characterized as either mesophilic or thermophilic organisms (10-21). In terms of salt
445 tolerance, these previously characterized deep subsurface iron reducers are also capable of
446 surviving or growing at elevated salt concentrations, but typically no higher than 1.7 M or
447 10 % NaCl. Their ability to reduce iron over this broad range of salinities and temperatures,
448 especially for the crystalline iron oxides, however, has not been confirmed for all of these
449 bacteria as we have shown for strain Z6 (10-21).

450 The ability of strain Z6 to reduce both amorphous and crystalline ferric iron oxides is
451 found in only a few other iron-reducing organisms (e.g., *Geobacter* and *Shewanella* species)
452 (27, 63-65). This feature is also distinct from most previously studied deep-subsurface iron
453 reducers, which have typically been shown to reduce chelated Fe(III) or amorphous
454 ferrihydrite but not more crystalline forms of Fe(III) (10-21). For example, one *Thermus*
455 species isolated from the 3.2-km depth of a South African gold mine actively reduces
456 dissolved Fe(III)-NTA but performs poorly when reducing ferrihydrite in the absence of a
457 soluble electron shuttle (21). Although ferrihydrite is comparatively bioavailable and is
458 found in a wide range of environments, it is metastable and often undergoes phase transitions
459 to more crystalline ferric iron oxides (e.g., hematite and goethite). Studies have shown
460 accelerated transformation of ferrihydrite to hematite and/or goethite occurs at the conditions
461 of elevated temperature and salt concentration, typical of a kilometer deep subsurface
462 environment (66, 67). Therefore, these more crystalline ferric iron minerals may be more
463 representative of the Fe(III) oxide minerals found in deep subsurface environments and are
464 likely to be available to sustain survival and growth of the DMRB.
465 Intensive studies of iron-reducing *Geobacter* and *Shewanella* spp. have revealed that
466 transmembrane electron-transfer complexes are involved in iron reduction, either directly by
467 assemblies of *c*-type cytochromes and nanowires or indirectly by means of electron shuttles
468 or reduced sulfide complexes (2, 57, 59). Moreover, the availability of specific Fe(III)
469 phases may have important implications for the energetics of microbial iron reduction. A
470 recent study shows that *G. sulfurreducens* operates multiple iron-related respiratory
471 pathways depending on the redox potential of substrate being reduced (68). In this case, as
472 the crystallinity of the ferric substrates increases, the amount of energy generated by their
473 reduction decreases. In contrast to *Geobacter* or *Shewanella* species, strain Z6 does not
474 contain any multi-heme cytochromes. It is therefore unlikely to share similar respiratory

475 pathways with these well-understood iron-reducing organisms for this metabolism. Due to
476 its capacity to reduce multiple ferric iron compounds with differing crystallinity, electrical
477 potential, solubility, and reducibility, it is possible that strain Z6 uses multiple iron reduction
478 pathways.

479 Although microbial iron oxide reduction becomes thermodynamically less favorable
480 as pH increases (69), alkaliphilic DMRB have likely evolved distinct metabolic strategies to
481 adapt to these conditions (57). The capacity of strain Z6 to reduce ferric iron at up to pH 9.6
482 (Fig. 3a) is similar to other Gram-positive alkaliphilic iron reducers (e.g., *Bacillus*
483 *pseudofirmus* and *Alkaliphilus metalliredigens* (70-72)). However, the capacity of Z6 to
484 reduce iron under weakly acidic condition has not been observed for these other alkaliphilic
485 organisms. *B. pseudofirmus* MC02 has been hypothesized to have functional groups on the
486 outer cell wall that strongly sorb Fe(III) or secrete Fe(III) chelating agents to enhance Fe(III)
487 reduction (70). A community of Gram-positive alkaliphilic bacteria was also reported to
488 synthesize and use flavins as electron shuttles for ferric iron reduction at pH up to 9.2 (57,
489 72). The ability of strain Z6 to reduce ferric iron under alkaline conditions suggests that it
490 may also synthesize or utilize chelating compounds or electron shuttles to facilitate iron
491 reduction. In the present study, some medium components (e.g., riboflavin from yeast extract
492 and vitamins, resazurin, or cysteine) may act as electron shuttles and facilitate iron reduction
493 by strain Z6. Comparison shows that the redox potential for these electron-shuttling
494 compounds (e.g., resazurin (-0.05~-0.11V) (73), cysteine (-0.34V) (74), riboflavin (-0.18~-
495 0.25V) (72)) is between that for H_2 (-0.42V) and the investigated ferric iron minerals (i.e.,
496 ferrihydrite (0.014V), lepidocrocite (-0.088V), goethite (-0.274V) and hematite (-0.287V))
497 (2, 75). These redox potential values also suggest that the contribution of the electron
498 shuttles on reduction of crystalline hematite and goethite may not be as significant as that for
499 lepidocrocite and amorphous ferrihydrite. Active reduction of crystalline hematite and

500 goethite by strain Z6 also suggests that other mechanisms than electron shuttles may also be
501 responsible for iron reduction by this organism.

502 The subsurface biosphere that inhabits sedimentary basins is sustained by organic
503 carbon buried during deposition or transported there by flowing groundwater as well as
504 reduced gases such as H_2 that are generated in situ by geochemical processes (15, 17, 76).
505 Previous studies have revealed that iron-reducing organisms derived from deep terrestrial
506 environments are able to utilize a diverse array of substrates. For example, *Fervidicella*
507 *metallireducens* and *Caloramator australicus* only use fermentable sugars as the energy and
508 carbon source for iron reduction (24, 25), while others (e.g., *Bacillus infernus*,
509 *Thermoanaerobacter ethanolicus* and some *Thermus* species) utilize a broader range of
510 fermentable substrates, organic acids, alcohols and/or H_2 (10, 21, 23). The broad nutrient
511 source for some iron reducers is in contrast to strain Z6 in that only H_2, trimethylamine, and a
512 few fermentable sugars support its iron reduction. Indeed, acetate, an important electron
513 donor for iron reducers in many sedimentary environments (1) does not support iron
514 reduction by this organism (Fig. 4). This is consistent to the lack of complete TCA cycle and
515 other metabolic pathways involved in further metabolizing these organic compounds in the
516 genome of strain Z6 (Fig. 7).

517 Physiological characterization suggests that H_2 provides electron equivalents to
518 support iron reduction by strain Z6. This is based on the observations: 1) strain Z6 reduces
519 iron when H_2 is added but does not when H_2 is replaced by one of the fermentation products
520 (formate, ethanol, lactate, acetate and CO_2) (Fig. 4); 2) a much lower amount of H_2
521 accumulate during glucose fermentation in the presence of ferric iron oxide compared to
522 glucose fermentation alone (see Table S4 in the supplemental material); and 3) hydrogenases
523 were annotated in the genome of strain Z6 (Fig. 7). Alignment shows that one gene cluster in
524 the genome of strain Z6 contains 4 protein-coding genes that share 46-65% identity in amino

acids with four subunits of the electron bifurcating dehydrogenases of *Acetobacterium woodii*, which catalyze NAD^+ dependent reduction of ferredoxin with H_2 ($2H_2+NAD^++Fd_{ox} \rightleftharpoons 3H^++NADH+Fd_{red}^{2-}$) and build-up an electrochemical proton ion potential for this exergonic reaction (77). Hydrogenase has been reported to be directly associated with dissimilatory reduction of metalloids (e.g., Tc (VII)) (78). Therefore, without the electron transfer conduit comprising cytochromes typically used by many iron-reducing organisms (e.g., *Shewanella* and *Geobacter*) (2), ferric compounds may couple with H_2 for the hydrogenase-facilitated redox reaction by strain Z6. These observations also suggest that during glucose fermentation, H_2 is the only source of electron equivalents that sustain iron reduction by strain Z6. Among the predicted hydrogenases, there exist both [Ni-Fe] hydrogenase enzymes and Fe-only hydrogenase enzymes including the bifurcating hydrogenases homologous to those for *Alkaliphilus metalliredigens* (70-72), which suggest catalysis of the reactions of production and consumption of molecular H_2 (79, 80). This type of electron transfer process has been identified in phylogenetically diverse fermentative iron reducers (e.g., *Bacillus*, *Clostridium*, *Thermoterrabacterium*, *Thermotoga*, *Thermoanae*, *Thermoanaerobacter*, and *Thermococcus* spp.) (1, 10, 81-83). In all these cases, iron reduction itself does not significantly contribute to growth because only a small fraction (typically less than 5 %) of the electron equivalents from fermentation is transferred to ferric iron (10, 83). This is consistent to the calculated 2.4-4.7 % of the electron equivalents transferred to ferrihydrite during fermentative iron reduction by strain Z6 in the presence of different mono- and di-saccharides (Eq. 1 and Fig. 4). However, consumption of fermentation products such as H_2, by means of their use as electron donor(s) for iron reduction, creates more thermodynamically favorable conditions that result in better carbohydrate decomposition and elevated biomass production (5, 83-85). This may be important in natural habitats where available organic matter and other electron donors are limited.

550 The kinetics of microbial iron oxide reduction are influenced by many factors,
551 including mineral surface area, the extent of particle aggregation, crystal structure, solubility,
552 and the amount of usable energy available for microbial metabolism (27, 63, 69, 86). In the
553 present study, strain Z6 follows the general trend that less crystalline ferric iron minerals are
554 more rapidly reduced and to a greater extent compared to the more crystalline iron oxides
555 (Fig. 5). Further analyses indicate a generally linear relationship between total initial surface
556 area for the different ferric oxides under investigation and iron reduction rates for strain Z6
557 (Fig. 5b). This observation is similar to that reported for *Shewanella alga* strain BrY (64). It
558 was suggested that surface area positively correlates with concentration of surface sites
559 present for enzymatic contact and/or solubility of iron oxides (64). The relationship between
560 surface area and solubility has been shown for different ferric iron minerals whereby smaller
561 size minerals exhibited higher solubility due to greater structural disorder and surface tension
562 effects (87). Thus, the available active sites associated with ferric iron oxide surface area
563 might be the most significant factor for iron reduction by strain Z6.

564 **Conclusions.** In this study, strain Z6 was isolated from groundwater sampled from
565 the Cambrian-aged Mt. Simon sandstone, 2.02 km beneath the Earth's surface in the Illinois
566 Basin, USA (28). The capacity of this species to reduce a diverse array of ferric iron
567 minerals expands our understanding of the metabolic capabilities of members of the genus
568 *Orenia*, which have typically been characterized solely as fermenters (39, 60). It underscores
569 the significance of understanding environmental backgrounds of the habitats and
570 comprehensive physiological characterizations of novel organisms beyond the characteristics
571 for their known phylogenetic relatives (e.g., iron reduction by strain Z6). The capacity of
572 strain Z6 to tolerate a broad range of physical and geochemical conditions in terms of
573 temperature, pH, salinity and its capacity to reduce both amorphous and crystalline ferric
574 iron oxides suggest that this organism is indeed indigenous to the deep subsurface of Illinois

575 Basin. Moreover, strain Z6 also broadens the phylogenetic affiliation and iron reduction
576 mechanisms that have been demonstrated for the previously known iron reducers (2, 57).
577 The observations of phylogenetically and physiologically diversified iron-reducing
578 organisms from the geographically distant deep subsurface environments support the claim
579 that metal reduction may be a widespread characteristic in the domain *Bacteria* (1). In
580 addition, it also suggests adaptive evolution of these iron-reducing organisms in response to
581 a broad range of physical and geochemical conditions that enable them to survive and carry
582 out biogeochemical processes in extreme environments that control the states, forms and
583 conversion of iron minerals.
584
585 **Description of *Orenia metallireducens* sp. nov**.
586 *Orenia metallireducens* (me.tal'li.re.du'cens. L. n. *metallum* metal; L. part. Adj. *reducens*, in
587 chemistry, converting to a different oxidation state; N. L. part. Adj. *metallireducens* reducing
588 metal).
589 Cells are rod shaped, ca. 0.5 µm wide by 2-20 µm long. They contain pili and are
590 motile in liquid media. Colonies are formed in agar shake amended with Fe(III)-citrate.
591 Growth is obligately anaerobic. Cellobiose, fructose, glucose, glycine, glycerol, maltose,
592 mannose, starch, sucrose, trehalose and yeast extract support fermentation by strain Z6, but it
593 is unable to ferment fumarate, galactose, glutamate, glucosamine, mannitol, lactose, or
594 peptone. Only H_2, trimethylamine and some fermentable substrates (e.g., yeast extract,
595 glucose, fructose, cellobiose, galactose, glycerol, starch and sucrose) act as the electron donor
596 or supply electron equivalents (e.g., H_2) for iron reduction. Ferric iron oxides with different
597 crystallinity, including ferrihydrite, lepidocrocite, goethite, and hematite can be actively
598 reduced by this organism. Other electron acceptors include Co(III) [Co(III)-EDTA], Cr(VI)
599 [chromate] and Mn(IV) [MnO_2]. The isolate tolerates a broad range of pH 6-9.6, salinity of

0.4-3.5 M NaCl, and temperature 20-60 °C. 16S rRNA gene analysis indicates that this isolate is affiliated with the *Orenia* cluster in the *Halobacteroidaceae* of *Firmicutes.* Its habitat is anoxic deep subsurface environments. Phenotypic characteristics and the 16S rRNA gene sequence distinguish this new isolate from previously described members of the genus *Orenia*. Strain Z6 is the type strain of the new species *Orenia metallireducens.* Strain Z6 has been deposited at the American Type Culture Collection (ATCC, BAA-2645) and Japan Collection of Microorganisms (JCM, JCM 31419).

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887 minerals used in this study. We especially appreciate Bhoopesh Mishra, Drew Latta, and the
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889 Table 1. Physiological characterization of strain Z6 and its closest phylogenetic relative

Character	Strain Z6	*Orenia chitinitropha*^{T} (61)	*Orenia marismortui*^{T} (39, 50)	*Orenia salinaria*^{T} (39)	*Orenia sivashensi*^{T} (60)
Sample characteristics					
Habitat	2.1 km depth, Illinois Basin, IL, USA	Saline lake, Russia	Dead sea	Solar salterns, France	Saline lagoons, Crimea Peninsula
Morphology (D×L) (μm)	Rod (0.5×(2-20))	Rod ((0.25-0.3)×(5-15))	Rod (0.6×(3-13))	Rod (1×(6-10))	Rod ((0.5-2.5)×(2.5-10))
Spore formation	+[a]	+	+	-	+
Gram Stain	-	-	-	-	-
G+C content of DNA (%)	32.1	32	29.6	33.7	28.6
Oxidase	-	NA	-	-	NA
Catalase	-	NA	-	-	NA
NaCl range (optimal) (%)	2-20 (2.8)	5.8-16(8.8)	3-18(3-12)	2-25(5-10)	5-25 (7-10)
Temp. range (optimal) (°C)	20-60 (30-50)	-45(40)	25-50 (36-45)	10-50(40-45)	25-50 (40-45)
pH range (optimal)	6-9 (6-7)	6.8-8.2(7.2-7.4)	NA[b]	5.5-8.5 (7.2-7.4)	5.5-7.8 (6.3-6.6)
Major membrane fatty acids	16:0, 16:1 18:0	14:1, 14:0, 16:0	14:0, 16:0, 16:1, 18:0	NA	NA
Fermentation[c]					
Betaine	+/-	NA	NA	-	-
Cellobiose	+	+	NA	+	+
Chitin	NA	+	-	NA	-
Fructose	+	NA	+	+	-
Fumarate	-	NA	-	NA	-
Galactose	-	-	-	-	-
Glucosamine	-	+	-	-	NA
Glutamate	-	NA	-	-	+
Glucose	+	+	+	+	+
Glycine	+	NA	-	-	-

Glycerol	+	NA	-	-	-
Lactose	-	-	NA	-	-
Mannose	+	-	+	-	+
Maltose	+	+	+	+	+
Mannitol	-	NA	NA	+	+
Peptone	-	NA	NA	NA	NA
Starch	+	-	+	-	+
Sucrose	+	+	+	+	+
Trehalose	+	-	NA	+	+

[a]+, - and +/- indicate positive, negative and marginal response for the physiological properties or activity; [b] Not studied or not available; [c] Growth for the fermenting cultures was identified by OD_{600}.

903 Table 2. Iron dynamics and secondary biomineralization products as a function of initial Fe
904 oxide substrate and iron-reducing organism

	Ferrihydrite $Fe(OH)_3$	Lepidocrocite γ-FeO(OH)	Goethite (α-FeOOH)	Hematite (Fe_2O_3)
$Fe(III)_{int}$ (mmol/L)[a]	9.66±0.66	10.19±0.99	10.02±0.15	15.27±0.22
Fe(II) speciation[b]				
Fe(II) produced (mM)	3.87±0.24	4.40±1.35	2.35±0.18	4.51±0.23
$Fe(II)_{tot}/Fe(III)_{initial}$ (%)[c]	40.1±3.9	43±13	23.5±1.8	29.5±1.6
$Fe(II)_{solid}/Fe(III)_{solid}$ (%)[d]	7.5	25	38.8	13.6
Fe(III) reduction rates				
Initial (μmole Fe(II)/h)	2.67±0.16	1.51±0.61	1.02±0.33	1.252±0.021
Initial (μmole Fe(II)/m^2/h)	0.742±0.046	1.79±0.80	0.88±0.28	0.572±0.036
Observed minerals by EXAFS fit[e]				
Siderite	9	20	11	12
Vivianite	-[f]	5	10	-
Sorbed Fe(II)[g]	-	-	-	2
Ferrihydrite	23	-	-	-
Lepidocrocite	-	75	-	-
Goethite	-	-	79	-
Hematite	68	-	-	86
Magnetite	-	-	-	-
FeS	-	-	-	-

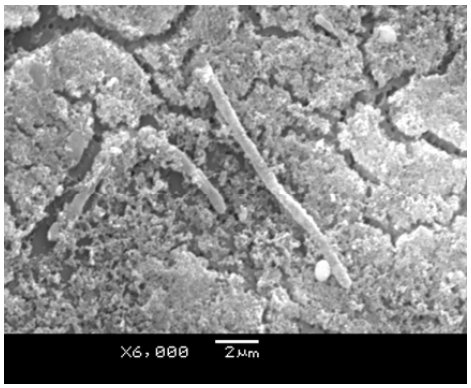
905 [a]$Fe(III)_{int}$ means the initial Fe(III) concentrations. [b]The initial concentrations of ferrous iron in the samples were
906 lower than 0.29 mM. [c]$Fe(II)_{tot}/Fe(III)_{int}$ indicate the fraction of ferric minerals reduced. [d]$Fe(II)_s/Fe(II)_{tot}$ is
907 defined as the ratio of Fe(II) in the form of solid phase versus total Fe(II). [e] The values show the relative content
908 of Fe(II)/Fe(III) as percentage of the total Fe in the wet, filtered solids based on the linear combination fits of
909 the XANES data. Results were obtained by a two-component XANES LC fit and the uncertainties are estimated
910 as ±5%. [f] - denotes the values or minerals below detection limit or no statistical production occurred. Results
911 were obtained by considering up to three of the components in a LC fit of the EXAFS data. [g]Sorbed Fe(II)
912 stands for the standard of Fe(II) adsorbed to carboxyl functionalized beads, which is used as the spectral
913 representative of disordered Fe(II) associated with the mineral or bacterial surfaces in the system.

914

915

916

(a)



(b)

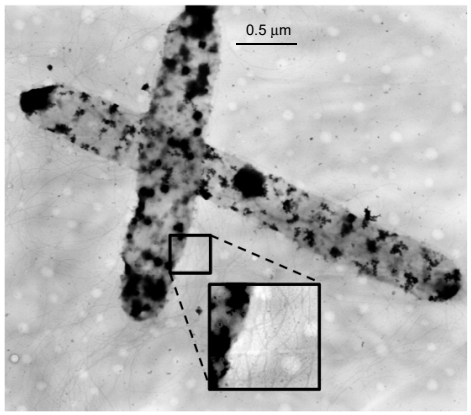
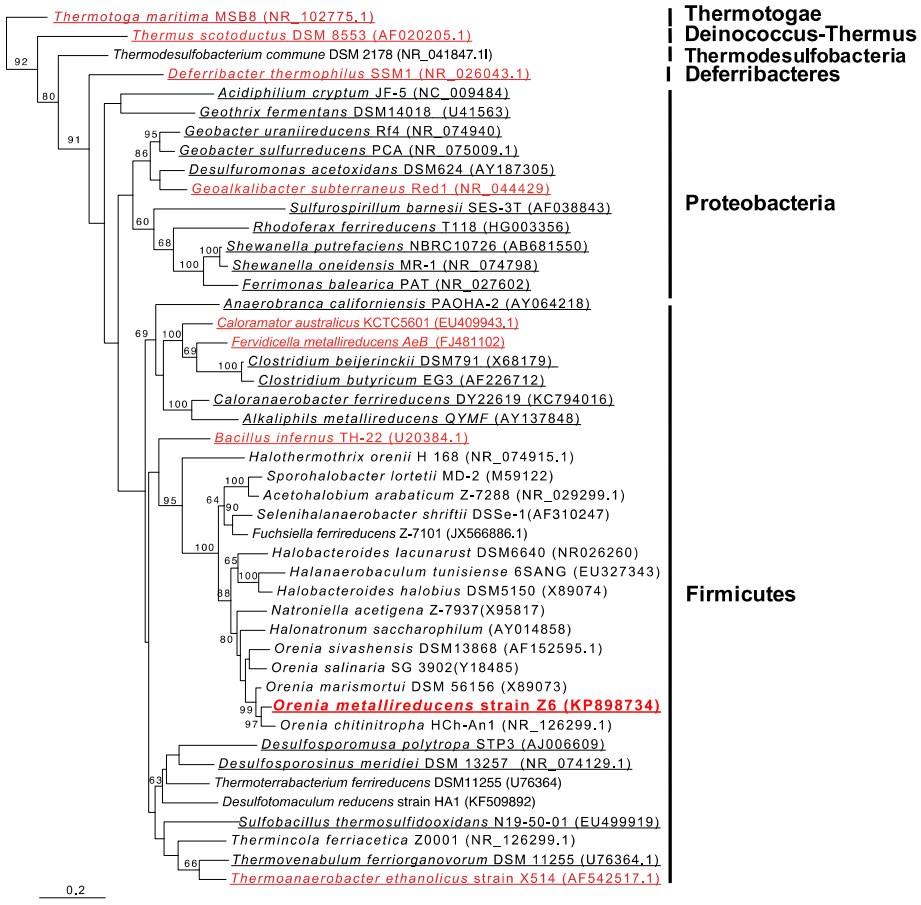


Figure 1. SEM (a) and TEM (b) photo micrographs of strain Z6. The isolates used for cell morphology identification were grown in the presence of ferric citrate at 42 °C in modified groundwater medium. The culture was prepared in modified groundwater medium (pH 7.0-7.2). Ferric citrate (10 mM) was used as the electron acceptor. H_2 (202 µmol/tube) was used as the electron donor and acetate (5 mM) was used as the carbon source. The organism contains peritrichous pili as indicated by the enlargement of the framed area shown in subfigure in (b).



939

940 Figure 2. 16S rRNA gene-based phylogenetic tree of strain Z6, phylogenetically related
941 organisms, and previously studied representative iron-reducing organisms. Strain Z6, isolated
942 in this study, is shown in red boldface type. Previously published iron-reducing bacteria type
943 strains are underlined and the ones isolated from deep terrestrial subsurface are shown in red
944 type. The NCBI accession numbers of the type strains are listed in parentheses. *Sulfolobus*
945 *acidocaldarius* DSM 639 was used as the out-group and is not shown. Statistical confidence
946 for the evolutionary tree was assessed by bootstrap analysis (500 replicates) and shown as
947 bootstrap values in percentage. The values lower than 60 are not shown. The scale bar
948 indicates 0.2 change per nucleotide position.

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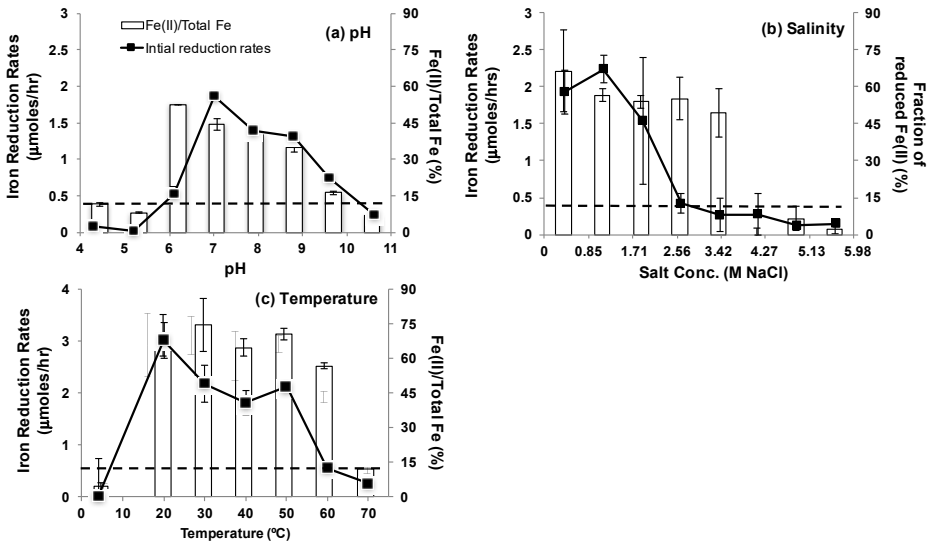
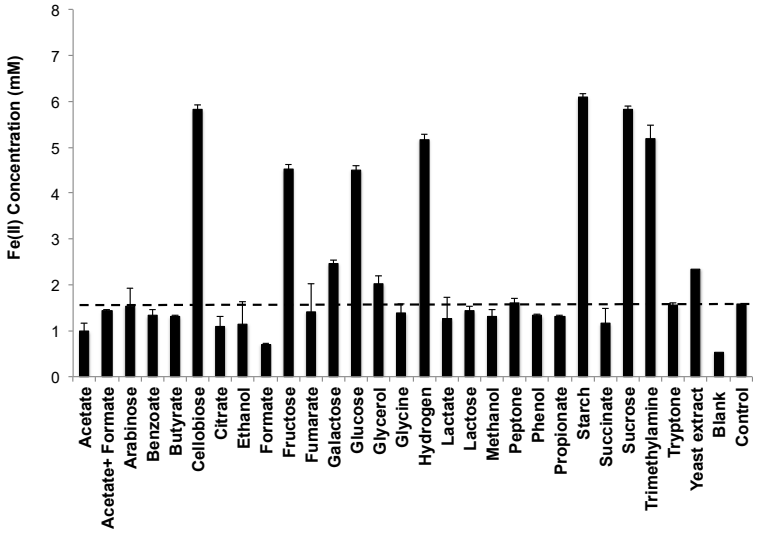


952

953 Figure 3. Effects of different geochemical factors on initial iron reduction rates and fractional
954 amounts of ferrous iron produced. All the samples were prepared in modified groundwater
955 medium. Ferrihydrite (10 mmol/L) was used as the electron acceptor. H_2 (202 μmol/tube)
956 was used as the electron donor and acetate (5 mM) was used as the carbon source. In (b) and
957 (c), the culture pH was 7.0-7.2. Duplicate samples were prepared and the error bars indicate
958 standard deviation of replicate samples. The initial Fe(II) concentrations—due to the
959 introduction of Fe(II) from parental cultures and/or reduction by the chemical reducers (Na_2S
960 and cysteine) present as medium components—are indicated by the dashed lines.



961

962 Figure 4. Use of different organic and inorganic substrates to support reduction of ferrihydrite
963 by Z6. All the samples were prepared in modified groundwater medium (pH 7.0-7.2).
964 Ferrihydrite (10 mmol/L) was used as the electron acceptor. The uninoculated control
965 samples are labeled as "Blank" and those inoculated with strain Z6 but without electron
966 donor are labeled as "Control". H_2 was amended as the electron donor for "Blank". Under all
967 the other conditions, the corresponding blanks were prepared in parallel with the active
968 cultures and no significant changes in Fe(II) was observed. Error bars indicate average
969 deviation of duplicate samples. The dashed lines show the final Fe(II) in "Control" to
970 illustrate the iron reduction due to nutrient carryover from the parental cultures.
971

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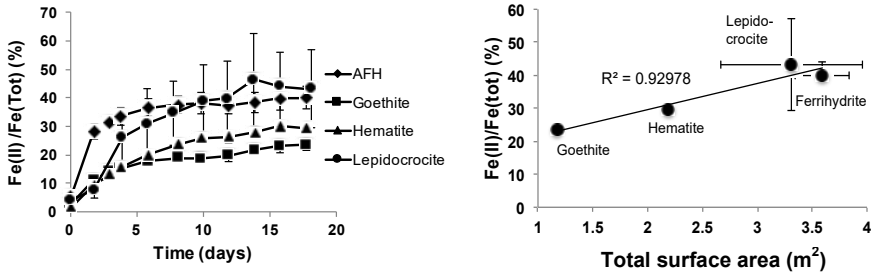
973

974 (a) (b)



975

976 Figure 5. Reduction of different ferric iron minerals by strain Z6 (a) and relation between
977 total mineral surface area of these minerals and fractional Fe(II) production (b). The samples
978 were prepared in modified groundwater medium (pH 6.5). H_2 (202 µmol/tube) was used as
979 the electron donor and acetate (5 mM) was used as the carbon source. The total surface area
980 was calculated by the specific surface area multiplied by the mass of the ferric iron oxide
981 mineral amended into each sample. All the samples were prepared in duplicate and the error
982 bars indicate standard deviation of the replicates. AFH: amorphous ferrihydrite.
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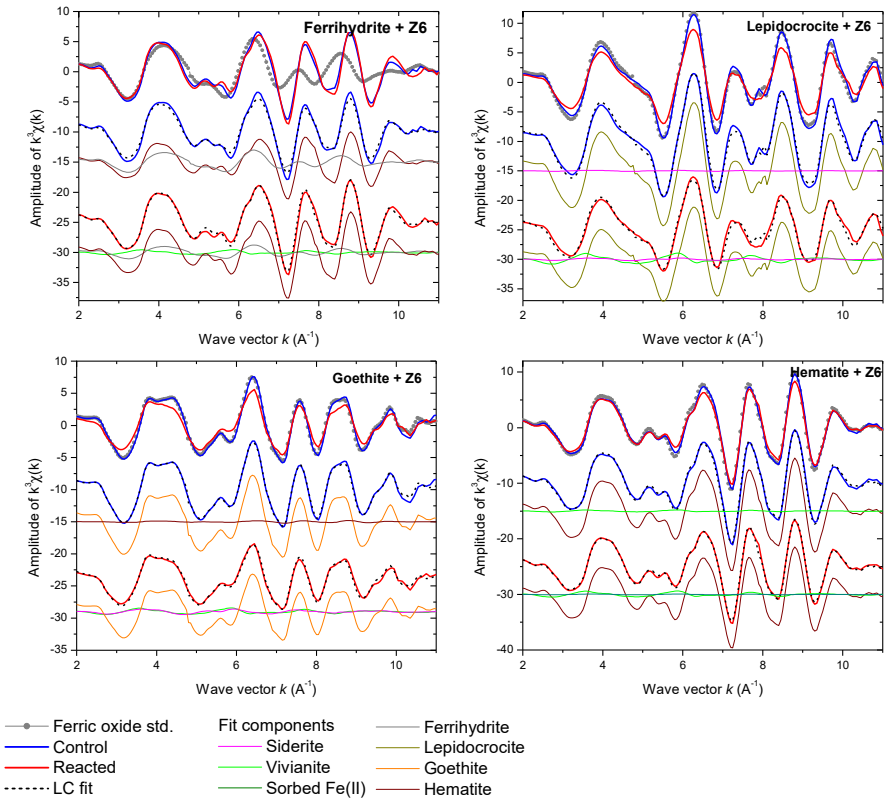
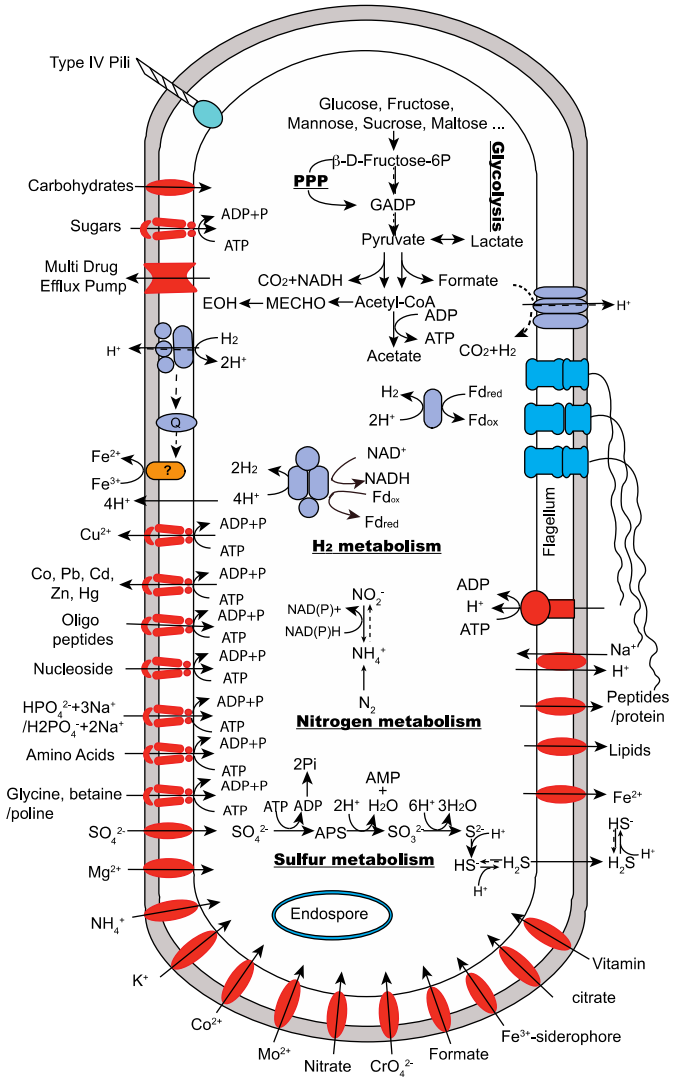


987

988 Figure 6. Fe K-edge EXAFS spectra and linear combination fits. Spectra from the reacted
989 solids (red) are compared to the control reactor (blue) and the corresponding parent iron
990 oxides at the top of each graph. The linear combination fit (black dashed line) of the control
991 sample (blue) is shown below the comparison plot, together with the scaled components in
992 the fit. The linear combination fit (black dashed line) of the bio-reduced solids (red) is shown
993 below the LC of the control, together with the scaled components in the fit. The numerical
994 results of the fits are listed in Table 1.
995



996

997 Figure 7. Genomic reconstruction for strain Z6 based on the predicted ORFs from its
998 genome. The solid lines indicate the metabolic functions with identified ORFs, while the
999 dashed lines mean those without ORFs annotated in the genome of strain Z6. ADP: adenosine
1000 diphosphate; ATP: adenosine triphosphate; AMP: adenosine monophosphate; APS:
1001 adenosine 5'-phosphosulfate; D-fructose-6P: D-fructose 6-phosphate; GADP: D-
1002 glyceraldehyde-3-phosphate; MECHO: acetylaldehyde; EOH: ethanol; NADH/NAD+:
1003 nicotinamide adenine dinucleotide; PPP: pentose phosphate pathway.
1004