Orenia metallireducens sp. nov. Strain Z6, a Novel Metal-Reducing Member of the Phylum Firmicutes 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 the genus Orenia, strain Z6 reduces various metal oxides [Fe(III), Mn(IV), Co(III), and Cr(VI)], using H2 as the electron donor. Strain Z6 actively reduced ferrihydrite over broad ranges of pH (6 to 9.6), salinity (0.4 to 3.5 M NaCl), and temperature (20 to 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-Fe2O3). 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 [Fe3(PO4)2] and siderite (FeCO3). 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 scopes 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.

IMPORTANCE
A novel iron-reducing species, Orenia metallireducens sp. nov., strain Z6, was isolated from groundwater collected from a geological formation located 2.02 km below land surface in the Illinois Basin, USA. Phylogenetic, physiologic, and genomic analyses of strain Z6 found it to have unique properties for iron reducers, including (i) active microbial iron-reducing capacity under broad ranges of temperatures (20 to 60°C), pHs (6 to 9.6), and salinities (0.4 to 3.5 M NaCl), (ii) lack of c-type cytochromes typically affiliated with iron reduction in Geobacter and Shewanella species, and (iii) being the only member of the Halanaerobiales capable of reducing crystalline goethite and hematite. This study expands the scope of phylogenetic affiliations, metabolic capacities, and catalytic mechanisms for iron-reducing microbes.

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. A phylogenetic variety of organisms has been reported for the capacity to reduce iron in a 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 (10–20). These subsurface environments contain a significant amount of the Earth’s total microbial biomass (21) and exhibit unique physical and geochemical features (e.g., anoxia, high salinity, and 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 ferreic iron, manganese, or other oxidized metals (e.g., *Geobacter* spp.) (10, 20, 22) or by utilizing these metals as an electron sink for fermentative pathways (e.g., *Fervidicella* and *Caloramator* spp.) (23, 24). The DMRB derived from the 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, 20) or amorphous ferrihydrite (22). More crystalline ferri oxides, like goethite and hematite, are more typical of the iron oxide minerals found in subsurface sediments (25) and can also act as an electron sink under both fermentative and respiratory conditions for DMRB (26), yet the extent to which deep-subsurface DMRB can reduce these minerals is largely unknown.

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 (25). The presence of these iron minerals along with millimolar concentrations of dissolved ferrous iron in the groundwater suggests that microbially mediated iron reduction is active in this environment (27). This groundwater is thermal (50°C), briny (19.1% total dissolved solids [TDS]), anoxic, and under considerable pressure (21 × 10⁶ Pa) (27). Our previous work identified indigenous microbial communities within different stratigraphic horizons in the Mt. Simon formation, and using this water, we successfully developed enrichment cultures capable of reducing ferric iron compounds (27, 28). 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.

**MATERIALS AND METHODS**

**Media.** If not mentioned otherwise, strain Z6 was grown in modified groundwater medium, which consisted of anoxic basal medium (18) and filter-sterilized formation water (0.2-μm pore size; Thermo Fisher Scientific, MA) (see Table S1 in the supplemental material) at a ratio of 9:1 (vol/vol). The basal medium was buffered with 3.3 mM piperazine-N₂-tetracarbonitrile (Pipes) and 30 mM NaHCO₃ sparged with N₂-CO₂ (80:20 [vol/vol]) and had a final pH of 7.0 to 7.2 (18). Trace metals (18) and vitamins (ATCC MD-VS, ATCC, VA) were supplemented from sterile stock solutions (1 ml/liter). Anoxic conditions were maintained by amending the medium with Na₂S (100 μM) and cysteine (250 μM). The cultures were prepared in 10 ml of medium in 27-ml serum tubes or 80 ml of medium in 160-ml serum bottles (Wheaton Industries, Inc., NJ). Under ferrihydrite-reducing conditions, the groundwater medium was amended with 10 mmol/liter Fe(III) (100 μmol/tube or 800 μmol/bottle) as the electron acceptor. H₂ (202 μmol/tube or 807 μmol/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 in the supplemental material. A synthetic *Orenia* medium was also developed to culture strain Z6 without amendment of formation groundwater. The synthetic *Orenia* medium contained NaCl (342 mM), MgCl₂·6H₂O (7.4 mM), KCl (13.4 mM), NH₄Cl (9.3 mM), KH₂PO₄ (2.5 mM), CaCl₂ (10 mM), NaHCO₃ (23.8 mM), yeast extract (0.2 g/liter), cysteine (200 μM), resazurin (1 mg/liter), trace metals (1 ml/liter) (29), and vitamins (ATCC MD-VS, 1 ml/liter) at pH 7.0 to 7.2. To confirm the extent and rates of iron reduction by strain Z6 in this synthetic *Orenia* medium, cultures were grown in the presence of 10 mmol/liter ferrihydrite, 202 μmol/tube H₂, and 5 mM acetate. The iron-reducing activity in this medium was then compared to that in the FeR medium.

**Isolation of strain Z6.** Strain Z6 was isolated from an iron-reducing enrichment culture created using the groundwater taken at a depth of 2.02 km in the Illinois Basin as an inoculum (27). Unless otherwise specified, this isolate was cultivated anaerobically in 25-ml anaerobic culture tubes or 120-ml serum bottles. All the amendments were added from autoclaved and anoxic stock solutions using sterile N₂-flushed syringes. The culture tubes/bottles were sealed with blue butyl rubber stoppers (Chемglass Life Sciences, NJ) and aluminum crimp seals. The cultures were grown in the dark at 42°C in a static incubator and manually shaken once per day.

Isolates were obtained from the enrichment culture using the agar shake method (30). Anoxic culture tubes were loaded with 6 ml of basal medium (18) and amended with low-melting-point agarose (Thermo Fisher Scientific, Inc., MA). After autoclaving, 4 ml of N₂-bubbled and degassed filter-sterilized formation water (see Table S1 in the supplemental material) (27) was added to each tube, giving a final concentration of 1.5% agarose. Each anoxic culture tube was then amended with 5 mM ferri citrate, H₂ (202 μmol/tube), and 5 mM (each) formate, acetate, pyruvate, and lactate that had been used in the enrichment culture (27). The agarose-containing medium was kept in a 45°C water bath before inoculation to prevent solidification. The parent enrichment was serially diluted 1:10 using sterilized basal medium in anoxic culture tubes. One milliliter of each dilution (1:10 to 1:10⁵) was inoculated into individual agar shake tubes, mixed well, and promptly solidified on ice. Five milliliters of filter-sterilized H₂ was injected into the headspace. After 5 to 10 days of incubation at 42°C, clearing zones formed around individual colonies, putatively indicating active Fe(III) reduction. To isolate single colonies from the agar shake, a sterile syringe containing ~0.1 ml of liquid anoxic medium was fitted with a 23-gauge 2-in. 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 N₂ 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 that 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 (31). PCR was conducted using Ex Taq polymerase (TaKaRa Bio USA, Madison, WI) and a Mastercycler (Eppendorf, Germany). PCR products were verified by using agarose gel electrophoresis and then purified with the QiAqick 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 (version 1.4.0), and classified using RDP10 (32). A phylogenetic tree based on the...
The observed iron reduction activity was due to the abiotic reduction of ferric products, determined to develop stoichiometric equations for 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.

**Physiological and biochemical characteristics.** A Gram stain of strain Z6 cells was performed using standard methods (34). The catalase activity of this organism was determined using 15% H2O2 on centrifuged cell pellets (35). Oxidase activity was determined using K520 oxidase test strips (Key Scientific Products Co., TX), according to 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 at 600 nm (OD600) of cell suspensions using a SpectroScan 200D+ UV-Vis spectrophotometer (Thermo Scientific, MA). The morphology of strain Z6 was examined using both scanning electron microscopy (SEM) and transmission electron microscopy (TEM) (27).

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, gluconate, glutamate, glucose, glycine, glycerol, lactate, malate, mannitol, mannose, peptone [1 g/liter], starch [1 g/liter], sucrose, trehalose, and yeast extract [1 g/liter]) were tested for their ability to support growth, as indicated by an increase in OD600, 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 otherwise specified): acetate, benzoate, butyrate, citrate, ethanol, formate, fumarate, glycine, glycerol, glutamic acid (202 μmol/tube), lactate, melitionate, propionate, phenol (2.5 mM), succinate, yeast extract (1 g/liter), starch (1 g/liter), tryptone (1 g/liter), peptone (1 g/liter), 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 [S0], thiocyanate, and sulfate) was evaluated by monitoring reduction activity in cultures amended with H2 (202 μmol/tube) and acetate (5 mM). The ability to reduce other metals [i.e., 5 mM Co(III)-EDTA, CrO42−, or MnO2] was also evaluated under 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/liter 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 HEPES. The fermentation and iron reduction products were determined to develop stoichiometric equations and calculate thermodynamics (36) under these two growth conditions.

Several control experiments were also performed to evaluate whether the observed iron reduction activity was due to the abiotic reduction of Fe(III) by medium components or fermentation by-products. Specifically, the medium components (e.g., Na2S, cysteine, and organic buffers), fermentation products, and proteinaceous products were evaluated for their ability to reduce ferrihydrite at pH 7.2, 0.4 M NaCl, and 42°C. The controls included (i) abiotic controls, without cell inoculation; (ii) inoculation-only controls, no electron donors added; (iii) autoclaved cultures with both cells and an electron donor; and (iv) autoclaved cultures pre-grown with 5 mM glucose and spent fermentation medium.

To determine the range of the iron reduction activity, strain Z6 was evaluated with different iron oxide minerals, including more crystalline types found in subsurface environments. Four ferric iron minerals (ferrihydrite, lepidocrocite, hematite, and goethite) were synthesized as described by Schwertmann and Cornell (37). Strain Z6 was cultured in modified groundwater medium amended with one of the above-mentioned iron oxide minerals (~10 mmol/liter) from the parental inoculant grown in FeR medium. Activity was monitored by quantifying Fe(II) over time. At selected time points, 0.4 ml of well-mixed culture was withdrawn into an anaerobic chamber using a sterile syringe and split into two 0.2-ml aliquots. One 0.2-ml aliquot was filtered through a 0.45-μm filter (GE Healthcare Life Sciences, NJ), and each aliquot was added with 0.2 ml of 1 M HCl. The filtered sample was used for the determination of aqueous ferrous iron; the unfiltered sample was used for the determination of acid-extractable ferrous iron and total iron.

To determine how iron reduction was impacted by environmental factors, the ability of strain Z6 to reduce ferrihydrite was evaluated at different temperatures (4 to 80°C), salinities (0 to 5.98 M NaCl), and pHs (4 to 11) in FeR medium (see Table S2 in the supplemental material). Susceptibility to antibiotics was tested by amending the iron-reducing cultures with one of the following antibiotics: 40 μg/ml ampicillin, anisomycin, tetracycline, erythromycin, or kanamycin plus 20 μg/ml chloramphenicol (38).

The 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) (39) 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 MARCAST/EnviroCAT 10-BM beamline (40) at the Advanced Photon Source, Argonne National Laboratory. Anoxic conditions were maintained during data collection by purging the sample chamber with N2. These procedures have been previously shown to maintain the anoxic integrity of the sample for the duration of the measurement (41). The energy of the incident X rays was scanned using an 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 to 5 consecutive scans.

The extended XAFS (EXAFS) region of the spectrum was extracted using the program Autobk (42). The contribution of spectroscopically distinct Fe species in the k3-weighted EXAFS data was quantified using linear combination (LC) analysis implemented in the program ATHENA (43).

In addition to ferrihydrite, goethite, hematite, and lepidocrocite, spectra from the following Fe(II)-bearing phases were considered possible components in the LC analysis: vivianite, magentite, Fe(II) adsorbed to carboxyl-functionalized beads at pH 7 (44), 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 (28). The genome was sequenced using the Roche 454 and Illumina HiSeq technologies, with average sequencing coverages of 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 (45), and internal gap closure was achieved using the GapCloser package of SOAPdenovo (version 1.12) (46). CheckM (47) was used to evaluate the completeness, quality, and contamination of the assembled genome for strain Z6. Open reading frames (ORFs) were predicted and annotated using the IMG/ER pipeline (48).

**Accession number(s).** The nucleotide sequences of the 16S rRNA genes of strain Z6 determined in this study have been deposited in GenBank under accession no. KP988732. The whole-genome sequence of strain Z6 was deposited in NCBI GenBank under the accession no. LWDV00000000. The version described in this paper is
H₂ (202 μmol/tube) was used as the electron donor, and acetate as the carbon source. The organism contains peritrichous pili as indicated by the enlargement of the framed area shown in the inset in panel b.

**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 acids and ferric citrate and was designated strain Z6. Electron micrographs showed that this organism was rod shaped, with dimensions of 0.5 by 2 to 20 μm. Depending on the growth phase, filamentous cells were observed during exponential growth (Fig. 1 and Table 1). In addition, numerous pili were observed on the surface of strain Z6 cells (Fig. 1). Analysis of the 16S rRNA genes of strain Z6 indicated that it is a member of the phylum Firmicutes with 96% sequence similarity to *Orenia marismortui*, a fermenter originally isolated from Dead Sea sediment (49) (Fig. 2). Strain Z6 stained Gram negative and produced negative results for oxidase and catalase tests (see Table S1). Genomic DNA showed a G+C content for this organism of 32.1%. The major membrane fatty acids for strain Z6 consisted of 16:0, 16:1, and 18:0 (Table 1). Depending on the growth phase, filamentous cells (Fig. 1). Analysis of the 16S rRNA genes of strain Z6 indicated that this organism was rod shaped, with dimensions of 0.5 by 2 to 20 μm. Depending on the growth phase, filamentous cells were observed during exponential growth (Fig. 1 and Table 1). In addition, numerous pili were observed on the surface of strain Z6 cells (Fig. 1). Analysis of the 16S rRNA genes of strain Z6 indicated that it is a member of the phylum Firmicutes with 96% sequence similarity to *Orenia marismortui*, a fermenter originally isolated from Dead Sea sediment (49) (Fig. 2). Strain Z6 stained Gram negative and produced negative results for oxidase and catalase tests (see Table S1). Genomic DNA showed a G+C content for this organism of 32.1%. The major membrane fatty acids for strain Z6 consisted of 16:0, 16:1, and 18:0 (Table 1).

**Physiological characteristics.** In addition to reducing dissolved ferric citrate, strain Z6 also reduced solid-phase ferrihydrite. Strain Z6 was able to survive under a broad range of pH, temperature, and salinity conditions when using ferrihydrite as the electron acceptor, H₂ as the electron donor, and acetate as the carbon source, respectively. Iron reduction occurred at pH values of 6 to 9.6, with an optimal initial iron reduction rate at pH 7. However, more iron was reduced at lower pH (i.e., pH 6) (Fig. 3a), even though the corresponding initial rate of iron reduction was lower. Iron reduction activity occurred at salinity levels ranging from 0.4 to 3.5 M NaCl (Fig. 3b). The highest initial iron reduction rate occurred with 1.2 M NaCl and decreased correspondingly with increasing salt concentrations, while the amount of ferric iron reduced was similar from 1.2 to 3.5 M NaCl (Fig. 3b). At pH 7 and with 0.4 M NaCl, robust iron reduction occurred over a broad range of temperatures (20 to 60°C), with similar amounts of iron reduced between 20 and 50°C (Fig. 3c). Initial rates of reduction dropped sharply at 60°C, but significant iron reduction was still observed.

Substrates that support iron reduction were evaluated in the media with a ferrihydrite suspension and one of several inorganic or organic compounds. Positive results were indicated by ferrous iron generation described above that was observed in the abiotic and inoculation-only controls (Fig. 4; see also Fig. S1a in the supplemental material). Among the nonfermentable substrates, only H₂ and trimethylamine supported the 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 HCO₃⁻ (including HCO₃⁻ and CO₃²⁻), and 88.6 μmol/bottle H₂ (see Table S4 in the supplemental material). The stoichiometry of fermentative iron reduction with glucose and ferrihydrite determined was

\[
\begin{align*}
C₆H₁₂O₆ + (6/5)Fe(OH)₃ &\rightarrow (4/3)C₂H₅OH \\
&+ (2/3)CH₃COO⁻ + (2/3)HCOO⁻ + (12/5)HCO₃⁻ \\
&+ (6/5)Fe^{2+} + (1/15)H₂ + (4/15)H⁺ + (2/5)H₂O
\end{align*}
\]

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 (see Fig. S1b in the supplemental material). 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 with the genus *Orenia*, strain Z6 rapidly grew on many mono- and polysaccharides, including cellobiose, fructose, glucose, glycine, glyceral, mannose, maltose, sucrose, and trehalose, based on an increase in OD₆₀₀ (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 primary products (i.e., acetate, ethanol, formate, CO₂, and H₂) similar to...
TABLE 1 Physiological characterization of strain Z6 and its closest phylogenetic relativesa

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Strain Z6</th>
<th>Orenia chitinitrophaT (60)</th>
<th>Orenia marismortuiT (38, 49)</th>
<th>Orenia salinariaT (38)</th>
<th>Orenia sivashensisT (59)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample characteristics</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Habitat</td>
<td>2.1-km depth, Illinois Basin, IL, USA</td>
<td>Saline lake, Russia</td>
<td>Dead Sea</td>
<td>Solar salterns, France</td>
<td>Saline lagoons, Crimean Peninsula</td>
</tr>
<tr>
<td>Morphology (depth by length) (μm)</td>
<td>Rod (0.5 by 2–20)</td>
<td>Rod (0.25–0.3 by 5–15)</td>
<td>Rod (0.6 by 3–13)</td>
<td>Rod (1 by 6–10)</td>
<td>Rod (0.5–2.5 by 2.5–10)</td>
</tr>
<tr>
<td>Spore formation</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Gram stain</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>G+C content of DNA (%)</td>
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<td>32</td>
<td>29.6</td>
<td>33.7</td>
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<td>NA</td>
<td>–</td>
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<td>NA</td>
<td>NA</td>
<td>–</td>
<td>NA</td>
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<td>NaCl range (optimal) (%)</td>
<td>2–20 (2.8)</td>
<td>5.8–16 (8.8)</td>
<td>3–18 (3–12)</td>
<td>2–25 (5–10)</td>
<td>5–25 (7–10)</td>
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<td>Temp range (optimal) (°C)</td>
<td>20–60 (30–50)</td>
<td>45 (40)c</td>
<td>25–50 (36–45)</td>
<td>10–50 (40–45)</td>
<td>25–50 (40–45)</td>
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<td>pH range (optimal)</td>
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<td>6.8–8.2 (7.2–7.4)</td>
<td>NA</td>
<td>5.5–8.5 (7.2–7.4)</td>
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<td>Major membrane fatty acids</td>
<td>16:0, 16:1, 18:0</td>
<td>140, 16:1, 16:0</td>
<td>14:0, 16:0, 18:0</td>
<td>14:0, 16:0, 16:1, 18:0</td>
<td>NA NA</td>
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Fermentationb

| Betaine                     | +/−       | NA                        | NA                            | −                     | −                          |
| Cellobose                   | +         | +                         | NA                            | +                     | +                          |
| Chitin                      | NA        | +                         | −                             | NA                    | −                          |
| Fructose                    | +         | +                         | NA                            | +                     | +                          |
| Fumarate                    | –         | NA                        | −                             | −                     | −                          |
| Galactose                   | –         | −                         | −                             | −                     | −                          |
| Glucosamine                 | −         | +                         | −                             | −                     | NA                         |
| Glutamate                   | −         | NA                        | −                             | −                     | +                          |
| Glucose                     | +         | +                         | +                             | +                     | +                          |
| Glycine                     | +         | NA                        | −                             | −                     | −                          |
| Glycerol                    | +         | NA                        | −                             | −                     | −                          |
| Lactose                     | −         | −                         | −                             | −                     | −                          |
| Mannose                     | −         | +                         | +                             | −                     | +                          |
| Maltose                     | +         | +                         | +                             | +                     | +                          |
| Mannitol                    | −         | NA                        | NA                            | +                     | +                          |
| Peptone                     | −         | NA                        | NA                            | NA                    | NA                         |
| Starch                      | +         | −                         | −                             | −                     | −                          |
| Sucrose                     | +         | +                         | +                             | +                     | +                          |
| Trehalose                   | −         | +                         | −                             | +                     | +                          |

a, +, −, and +/− indicate positive, negative, and marginal responses for the physiological properties or activity; NA, not studied or not available.
b, Growth of the fermenting cultures was identified by measuring OD600.
c, Only the maximum growth temperature with chitin and optimum temperature are available (60).

Those observed in the fermentative iron-reducing cultures, except for observation of a trace amount of lactate (0.15 mM) (see Table S4 in the supplemental material). In the absence of ferrihydrite, however, about 30% of the initial glucose remained unfermented (see Fig. S2 in the supplemental material), while H2 produced under this condition was about 1.6 times higher than that in the fermentative iron-reducing cultures (see Table S4). The stoichiometry of glucose fermentation alone was

\[
\text{C}_6\text{H}_{12}\text{O}_6 + (5/3)\text{H}_2\text{O} \rightarrow (4/3)\text{C}_3\text{H}_4\text{OH} + (2/3)\text{CH}_2\text{COO}^- + \text{HCOO}^- + \text{HCO}_3^- + (1/2)\text{H}_2 + (8/3)\text{H}^+ \quad (2)
\]

In addition to iron reduction and fermentation, strain Z6 also showed a capacity to reduce a number of other transition metal compounds, such as Co(III) [Co(III)-EDTA], Cr(VI) (chromate), and Mn(IV) (MnO₂) based on changes in the medium 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 as to 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 H2 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 the supplemental material).

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

Fe K-edge XANES analyses of the solids confirmed the presence of reduced iron species in the active cultures, as indicated by the shift in the edge position to lower energy than that of the corresponding parent oxide and the control sample spectrum (see Fig. S5 in the supplemental material). Linear combination (LC) analyses of the XANES data indicated that the ferrous iron content in the solid phase \([\text{Fe(II)}_{\text{solid}}/\text{Fe(III)}_{\text{solid}}]\) at the end of the experiment ranged from 7.5 to 38.8% (Table 2). The EXAFS data also showed differences between the uninoculated controls and the active culture incubations, with larger differences corresponding to greater extents of iron reduction, as determined by XANES and the Fe(II) measurements of the HCl extracts (Fig. 6; see also Fig. S5). LC analysis of the EXAFS data determined that ferrous iron was present as O-coordinated Fe(II) species [inclusive of disordered Fe(II) precipitates or adsorbed Fe(II)], vivianite \([\text{Fe}_3(\text{PO}_4)_2]\], and/or siderite (FeCO\(_3\)) (Table 2 and Fig. 6). The distribution of the Fe(II) products in the best fit 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\(_3\)O\(_4\)) and green rust, two common Fe(II)-bearing minerals frequently observed as biomineralization products by DMRB (50–54), were not detected under the culture conditions here.

**Genomic reconstruction.** The draft genome sequence of strain Z6, phylogenetically related organisms, and previously studied representative iron-reducing organisms. Strain Z6, isolated in this study, is shown in red bold type. Previously published iron-reducing bacteria type strains are underlined, and the ones isolated from deep terrestrial subsurface are shown in red type. The NCBI accession numbers of the type strains are listed in parentheses. *Sulfolobus acidocaldarius* DSM 639 was used as the outgroup and is not shown. Statistical confidence for the evolutionary tree was assessed by bootstrap analysis (500 replicates) and is shown as bootstrap values in percentages. Values lower than 60 are not shown. The scale bar indicates 0.2 change per nucleotide position.

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**FIG 2** 16S rRNA gene-based phylogenetic tree of strain Z6, phylogenetically related organisms, and previously studied representative iron-reducing organisms. Strain Z6, isolated in this study, is shown in red bold type. Previously published iron-reducing bacteria type strains are underlined, and the ones isolated from deep terrestrial subsurface are shown in red type. The NCBI accession numbers of the type strains are listed in parentheses. *Sulfolobus acidocaldarius* DSM 639 was used as the outgroup and is not shown. Statistical confidence for the evolutionary tree was assessed by bootstrap analysis (500 replicates) and is shown as bootstrap values in percentages. Values lower than 60 are not shown. The scale bar indicates 0.2 change per nucleotide position.
Z6 is 3,456,989 bp (3.47 Mb). Evaluation of this assembled genome using CheckM (47) indicates that the genome of 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. The \( N_{50} \) 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 had

FIG 3 Effects of different geochemical factors on initial iron reduction rates and fractional amounts of ferrous iron produced. All the samples were prepared in modified groundwater medium. Ferrihydrite (10 mmol/liter) was used as the electron acceptor. \( \text{H}_2 \) (202 \( \text{mol/tube} \)) was used as the electron donor, and acetate (5 mM) was used as the carbon source. (b and c) The culture pH was 7.0 to 7.2. Duplicate samples were prepared, and the error bars indicate standard deviation of replicate samples. The initial Fe(II) concentrations (Conc.), due to the introduction of Fe(II) from parental cultures and/or reduction by the chemical reducers (Na2S and cysteine) present as medium components, are indicated by the dashed lines.

FIG 4 Use of different organic and inorganic substrates to support reduction of ferrihydrite by Z6. All the samples were prepared in modified groundwater medium (pH 7.0 to 7.2). Ferrihydrite (10 mmol/liter) was used as the electron acceptor. The uninoculated control samples are labeled as “Blank,” and those inoculated with strain Z6 but without electron donor are labeled as “Control.” \( \text{H}_2 \) was added as the electron donor for blank samples. Under all the other conditions, the corresponding blanks were prepared in parallel with the active cultures, and no significant changes in Fe(II) were observed. Error bars indicate average standard deviations of duplicate samples. The dashed lines show the final Fe(II) in the control to illustrate the iron reduction due to nutrient carryover from the parental cultures.
Fe(II) is oxidized to Fe(III) by iron-reducing organisms. The oxidation rate depends on the initial concentration of ferrous iron and the type of mineral substrate. The observed rates range from 0.046 to 13.6 mol Fe(II)/h/m². The most common minerals observed by EXAFS fit are siderite, vivianite, goethite, hematite, and ferrihydrite. The relative content of Fe(II)/Fe(III) as a percentage of the total Fe in the wet filtered solids based on the linear combination fits of the XANES data is shown in Table 2. The uncertainties are estimated as +5% and are not shown. Sorbed Fe(II) stands for the standard of Fe(II) adsorbed to carboxyl functionalized beads, which is used as the spectral representative of disordered Fe(II) associated with the mineral or bacterial surfaces in the system.

### Table 2: Iron dynamics and secondary biomineralization products as a function of initial Fe oxide substrate and iron-reducing organisms

<table>
<thead>
<tr>
<th>Dynamic</th>
<th>Ferrihydrite [Fe(OH)3]</th>
<th>Lepidocrocite [γ-FeO(OH)]</th>
<th>Goethite (α-FeOOH)</th>
<th>Hematite (Fe2O3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe(III)int (mmol/liter)</td>
<td>9.66 ± 0.66</td>
<td>10.19 ± 0.99</td>
<td>10.02 ± 0.15</td>
<td>15.27 ± 0.22</td>
</tr>
<tr>
<td>Fe(II) speciation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fe(II) produced (mM)</td>
<td>3.87 ± 0.24</td>
<td>4.40 ± 1.35</td>
<td>2.35 ± 0.18</td>
<td>4.51 ± 0.23</td>
</tr>
<tr>
<td>Fe(II)int/Fe(III)int (%)</td>
<td>40.1 ± 3.9</td>
<td>43 ± 13</td>
<td>23.5 ± 1.8</td>
<td>29.5 ± 1.6</td>
</tr>
<tr>
<td>Fe(II)/Fe(II)tot (%)</td>
<td>7.5</td>
<td>25</td>
<td>38.8</td>
<td>13.6</td>
</tr>
<tr>
<td>Fe(III) reduction rates</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial [μmol Fe(II)/h]</td>
<td>2.67 ± 0.16</td>
<td>1.51 ± 0.61</td>
<td>1.02 ± 0.33</td>
<td>1.252 ± 0.021</td>
</tr>
<tr>
<td>Initial [μmol Fe(II)/m²/h]</td>
<td>0.742 ± 0.046</td>
<td>1.79 ± 0.80</td>
<td>0.88 ± 0.28</td>
<td>0.572 ± 0.036</td>
</tr>
</tbody>
</table>

Observed minerals by EXAFS fit:
- Siderite
- Vivianite
- Sorbed Fe(II)
- Ferrihydrite
- Lepidocrocite
- Goethite
- Hematite
- Magnetite
- FeS

---

*a* Fe(III)int, initial Fe(III) concentrations. The values following the ± symbol indicate standard deviations for replicate samples.

*b* The initial concentrations of ferrous iron in the samples were lower than 0.29 mM.

*c* Fe(II)/Fe(III)int, the ratio of Fe(II) in the form of solid phase versus total Fe(II).

*d* The values show the relative content of Fe(II)/Fe(III) as a percentage of the total Fe in the wet filtered solids based on the linear combination fits of the XANES data. Results were obtained by a two-component XANES LC fit. The uncertainties are estimated as ±5% and are not shown.

*e* Sorbed Fe(II) stands for the standard of Fe(II) adsorbed to carboxyl functionalized beads, which is used as the spectral representative of disordered Fe(II) associated with the mineral or bacterial surfaces in the system.
otic iron reduction by some iron reducers (e.g., Shewanella oneidensis MR-1) (56) 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 (48), and 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, 57, 58).

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 2.02 km in the Illinois Basin, USA (27). This organism possesses 92 to 96% 16S rRNA gene sequence identity to the previously isolated Orenia species (O. marismortui, O. salinaria, O. sivashensis, and O. chitinitropha) (Fig. 2) (38, 49, 59, 60). A screen of the NCBI and RDP reference databases (32) also shows that closely related sequences or phylotypes are not abundant. The only phylotypes with a similarity of >96% are from the parental enrichment culture (27) and four sequences from a high-temperature North Sea oil field (61).

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 (38, 49, 59, 60). 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 polysaccharides (e.g., chitin) (38, 49, 59, 60). As with other Orenia populations, strain Z6 exhibits rod-shaped morphology, low G+C content, tolerance of comparable ranges of pH, temperature, and salinity, as well as active fermentation of sugars (Table 1). However, strain Z6 shows distinct physiological
Orenia ports the growth of strain Z6 but not other marismortui, strain Z6 ferments starch. In contrast, glycerol suppling iron reduces growth. In comparison, other deep-subsurface iron-reducing bacteria, strain Z6 is distinct in both phylogenetic and physiological features. Due to its capacity to reduce multiple ferric iron compounds with differing crystallinity, electrical potential, solubility, and reducibility, it is possible that strain Z6 uses multiple iron reduction pathways. Physiologically, strain Z6 tolerates a broader range of temperature than that reported for other iron reducers derived from deep-subsurface environments, which are typically characterized as either mesophilic or thermophilic organisms (10–20). In terms of salt tolerance, these previously characterized deep-subsurface iron reducers are also capable of surviving or growing at elevated salt concentrations, but typically no higher than 1.7 M or 10% NaCl. Their ability to reduce iron over broad ranges of salinities and temperatures, especially for the crystalline iron oxides, however, has not been confirmed for all of these bacteria, as we have shown for strain Z6 (10–20).

The ability of strain Z6 to reduce both amorphous and crystalline ferric iron oxides is found in only a few other iron-reducing organisms (e.g., Geobacter and Shewanella species) (26, 62–64). This feature is also distinct from most previously studied deep-subsurface iron reducers, which have typically been shown to reduce chelated Fe(III) or amorphous ferrihydrite but not more crystalline forms of Fe(III) (10–20). For example, one Thermus species isolated from 3.2-km depth of a South African gold mine actively reduces dissolved Fe(III)-nitrilotriacetic acid (NTA) but performs poorly when reducing ferrihydrite in the absence of a soluble electron shuttle (20). Although ferrihydrite is comparatively bioavailable and is found in a wide range of environments, it is metastable and often undergoes phase transitions to more crystalline ferric iron oxides (e.g., hematite and goethite). Studies have shown that accelerated transformation of ferrihydrite to hematite and/or goethite occurs under conditions of elevated temperature and salt concentration, typical of a kilometer-deep subsurface environment (65, 66). Therefore, these more crystalline ferric iron minerals may be more representative of the Fe(III) oxide minerals found in deep-subsurface environments and are likely to be available to sustain survival and growth of the DMRB.

Intensive studies of iron-reducing Geobacter and Shewanella spp. have revealed that transmembrane electron transfer complexes are involved in iron reduction, either directly by assembly of c-type cytochromes and nanowires or indirectly by means of electron shuttles or reduced sulfide complexes (2, 56, 58). Moreover, the availability of specific Fe(III) phases may have important implications for the energetics of microbial iron reduction. A recent study shows that G. sulfurreducens operates multiple iron-related respiratory pathways depending on the redox potential of the substrate being reduced (67). In this case, the crystallinity of the ferric substrates increases, the amount of energy generated by their reduction decreases. In contrast to Geobacter or Shewanella species, strain Z6 does not contain any multiheme cytochromes. It is therefore unlikely to share similar respiratory pathways with these well-understood iron-reducing organisms for this metabolism. Due to its capacity to reduce multiple ferric iron compounds with differing crystallinity, electrical potential, solubility, and reducibility, it is possible that strain Z6 uses multiple iron reduction pathways.

Although microbial iron oxide reduction becomes thermodynamically less favorable as pH increases (68), alkaliphilic DMRR have likely evolved distinct metabolic strategies to adapt to these conditions (56). The capacity of strain Z6 to reduce ferric iron at...
up to pH 9.6 (Fig. 3a) is similar to other Gram-positive alkalophilic iron reducers (e.g., Bacillus pseudofirmus and Alkaliphilus metallireducens [69–71]). However, the capacity of Z6 to reduce iron under weakly acidic conditions has not been observed for these other alkalophilic organisms. B. pseudofirmus MC02 has been hypothesized to have functional groups on the outer cell wall that strongly sorb Fe(III) or secrete Fe(III)-chelating agents to enhance Fe(III) reduction (69). A community of Gram-positive alkalophilic bacteria was also reported to synthesize and use flavins as electron shuttles for ferric iron reduction at pH up to 9.2 (56, 71). The ability of strain Z6 to reduce ferric iron under alkaline conditions suggests that it may also synthesize or utilize chelating compounds or electron shuttles to facilitate iron reduction. In the present study, some medium components (e.g., riboflavin from yeast extract and vitamins, resazurin, or cysteine) may act as electron shuttles and facilitate iron reduction by strain Z6. A comparison shows that the redox potential for these electron-shuttling compounds (e.g., resazurin \([-0.05 \text{ to } -0.11 \ V]\) [72], cysteine \([-0.34 \ V]\) [73], and riboflavin \([-0.18 \text{ to } -0.25 \ V]\) [71]) is between that for \(H_2\) \((-0.42 \ V)\) and the investigated ferric iron minerals (i.e., ferrihydrite \(0.014 \ V\), lepidocrocite \(-0.088 \ V\), goethite \(-0.274 \ V\), and hematite \(-0.287 \ V\)) (2, 74). These redox potential values also suggest that the contribution of the electron shuttles on the reduction of crystalline hematite and goethite may not be as significant as those for lepidocrocite and amorphous ferrihydrite. Active reduction of crystalline hematite and goethite by strain Z6 also suggests that other mechanisms than electron shuttles may also be responsible for iron reduction by this organism.

The subsurface biosphere that inhabits sedimentary basins is sustained by organic carbon buried during deposition or transported there by flowing groundwater as well as reduced gases, such as \(H_2\), that are generated in situ by geochemical processes (15, 17, 75). Previous studies have revealed that iron-reducing organisms derived from deep terrestrial environments are able to utilize a diverse array of substrates. For example, Fervidicella metallireducens and Caloramator australicus use only fermentable sugars as the source of energy and carbon for iron reduction (23, 24), while others (e.g., Bacillus infernus, Thermoanaerobacter ethanolicus, and some Thermus species) utilize a broader range of fermentable substrates, organic acids, alcohols, and/or \(H_2\) (10, 20, 22). The broad nutrient source for some iron reducers is in contrast to strain Z6 in that only \(H_2\), trimethylamine, and a few fermentable sugars support its iron reduction. Indeed, acetate, an important electron donor for iron reducers in many sedimentary environments (1), does not support iron reduction by this organism (Fig. 4). This is consistent with the lack of a complete TCA cycle and other metabolic pathways involved in further metabolizing these organic compounds in the genome of strain Z6 (Fig. 7).

Physiological characterization suggests that \(H_2\) provides electron equivalents to support iron reduction by strain Z6. This is based on the observations that (i) strain Z6 reduces iron when \(H_2\) is added but does not when \(H_2\) is replaced by one of the fermentation products (formate, ethanol, lactate, acetate, and \(CO_2\)) (Fig. 4); (ii) a much smaller amount of \(H_2\) accumulates during glucose fermentation in the presence of ferric iron oxide compared to that during glucose fermentation alone (see Table S4 in the supplemental material); and (iii) hydrogenases were annotated in the genome of strain Z6 (Fig. 7). The alignment shows that one gene cluster in the genome of strain Z6 contains 4 protein-coding genes that share 46 to 65% identity in amino acids with four subunits of the electron-bifurcating dehydrogenases of Acetobacterium woodii, which catalyze \(NAD^+\)-dependent reduction of ferredoxin (Fd) with \(H_2 (2H_2 + NAD^+ + Fd = 3H^+ + NADH + Fd_{red}^{-2})\) and build up an electrochemical proton ion potential for this ex-ergonic reaction (76). Hydrogenase has been reported to be directly associated with dissimilatory reduction of metalloids (e.g., Tc(VII)) (77). 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 (69–71), which suggests catalysis of the reactions of production and consumption of molecular \(H_2\) (78, 79). This type of electron transfer process has been identified in phylogenetically diverse fermentative iron reducers (e.g., Bacillus, Clostridium, Thermoterrabacterium, Thermotoga, Therm ana, Thermoaerobacter, and Thermococcus spp.) (1, 10, 80–82). 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, 82). This is consistent with the calculated 2.4 to 4.7% of the electron equivalents transferred to ferrihydrite during fermentative iron reduction by strain Z6 in the presence of different mono- and disaccharides (see equation 1 and Fig. 4). However, consumption of fermentation products, such as \(H_2\), by means of their use as an electron donor(s) for iron reduction, creates more thermodynamically favorable conditions that result in better carbohydrate decomposition and elevated biomass production (5, 82–84). This may be important in natural habitats where available organic matter and other electron donors are limited.

The kinetics of microbial iron oxide reduction are influenced by many factors, including mineral surface area, the extent of particle aggregation, crystal structure, solubility, and the amount of usable energy available for microbial metabolism (26, 62, 68, 85). In the present study, strain Z6 follows the general trend that less crystalline ferric iron minerals are more rapidly reduced and to a greater extent than the more crystalline iron oxides (Fig. 5). Further analyses indicate a generally linear relationship between total initial surface area for the different ferric oxides under investigation and iron reduction rates for strain Z6 (Fig. 5b). This observation is similar to that reported for Shewanella alga strain BrY (63). It was suggested that surface area positively correlates with the concentration of surface sites present for enzymatic contact and/or solubility of iron oxides (63). The relationship between surface area and solubility has been shown for different ferric iron minerals, whereby smaller minerals exhibited higher solubility due to greater structural disorder and surface tension effects (86). Thus, the available active sites associated with ferric iron oxide surface area might be the most significant factor for iron reduction by strain Z6.

Conclusions. In this study, strain Z6 was isolated from groundwater sampled from the Cambrian-aged Mt. Simon sandstone, 2.02 km beneath the Earth’s surface in the Illinois Basin, USA (27). The capacity of this species to reduce a diverse array of ferric iron minerals expands our understanding of the metabolic
capabilities of members of the genus *Orenia*, which have typically been characterized solely as fermenters (38, 59). It underscores the significance of understanding environmental backgrounds of the habitats and comprehensive physiological characterizations of novel organisms beyond the characteristics for their known phylogenetic relatives (e.g., iron reduction by strain Z6). The capacity of strain Z6 to tolerate a broad range of physical and geochemical conditions in terms of temperature, pH, and salinity and its capacity to reduce both amorphous and crystalline ferric iron oxides suggest that this organism is indeed indigenous to the deep subsurface of the Illinois Basin. Moreover, strain Z6 also broadens the phylogenetic affiliation and iron reduction mechanisms that have been demonstrated for the previously known iron reducers (2, 56). The observations of phylogenetically and physiologically diversified iron-reducing organisms from the geographically distant deep-subsurface environments support the claim that metal reduction may be a widespread characteristic in the domain *Bacteria* (1). In addition, it also suggests the adaptive evolution of these iron-reducing organisms in response to a broad range of physical and geochemical conditions that enable them to survive and carry out biogeochemical processes in extreme environments that control the states, forms, and conversion of iron minerals.

**Description of *Orenia metallireducens* sp. nov.** *Orenia metallireducens* (met.alli.re-du.cens. L. n. *metallum* metal; L. part. adj. *reduces*, in chemistry, converting to a different oxidation state; N.L. part. adj. *metalli*reducens, reducing metal).

Cells are rod shaped, ca. 0.5 μm wide by 2 to 20 μm long. They contain pili and are motile in liquid medium. Colonies are formed in agar shake amended with Fe(III) citrate. Growth is obligately anaerobic. Cellulose, fructose, glucose, glycine, glycerol, maltose, mannose, starch, sucrose, trehalose, and yeast extract support growth. They contain H2, trimethylamine, and some fermentable substrates (e.g., galactose, glutamate, glucosamine, mannitol, lactose, or peptone. Only H2, trimethylamine, and some fermentable substrates (e.g., H2) act as the electron donor or supply electron equivalents (e.g., H2) for iron reduction. Ferric iron oxides with different crystallinity, including ferrihydrite, lepidocrocite, goethite, and hematite, can be actively reduced by this organism. Other electron acceptors include Co(III) [Co(III)-EDTA], Cr(VI) (chromate), and Mn(IV) (MnO2). The isolate tolerates a broad range of pH 6 to 9.6, salinity of 0.4 to 3.5 M NaCl, and temperature of 20 to 60°C. 16S rRNA gene analysis indicates that this isolate is affiliated with the *Orenia* cluster in the *Halobacteriaceae 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 31419).

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