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Isolation and genomic analysis of "*Metallumcola ferriviriculae*" MK1, a Gram-positive, Fe(III)-reducing bacterium from the Soudan Underground Mine, an iron-rich Martian analog site

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ABSTRACT The Soudan Underground Mine State Park, found in the Vermilion Iron Range in northern Minnesota, provides access to a ~ 2.7 billion-year-old banded iron formation. Exploratory boreholes drilled between 1958 and 1962 on the 27th level (713 m underground) of the mine intersect calcium and iron-rich brines that have recently been subject to metagenomic analysis and microbial enrichments. Using concentrated brine samples pumped from a borehole depth of up to 55 m, a novel Gram-positive bacterium was enriched under anaerobic, acetate-oxidizing, and Fe(III) citrate-reducing conditions. The isolated bacterium, designated strain MK1, is non-motile, rod-shaped, spore-forming, anaerobic, and mesophilic, with a growth range between 24°C and 30°C. The complete circular MK1 genome was found to be 3,720,236 bp and encodes 25 putative multiheme cytochromes, including homologs to inner membrane cytochromes in the Gram-negative bacterium *Geobacter sulfurreducens* and cytoplasmic membrane and periplasmic cytochromes in the Gram-positive bacterium *Thermincola potens*. However, MK1 does not encode homologs of the peptidoglycan (CwcA) and cell surface-associated (OcwA) multiheme cytochromes proposed to be required by *T. potens* to perform extracellular electron transfer. The 16S rRNA gene sequence of MK1 indicates that its closest related isolate is *Desulfitibacter alkalitolerans* strain sk.kt5 (91% sequence identity), which places MK1 in a novel genus within the *Desulfitibacteraceae* family and *Moorellales* order. Within the *Moorellales* order, only *Calderihabitans maritimus* strain KKC1 has been reported to reduce Fe(III), and only *D. alkalitolerans* can also grow in temperatures below 40°C. Thus, MK1 represents a novel species within a novel genus, for which we propose the name "*Metallumcola ferriviriculae*" strain MK1, and provides a unique opportunity to study a cytochrome-rich, mesophilic, Gram-positive, spore-forming Fe(III)-reducing bacterium.

IMPORTANCE The Soudan Underground Mine State Park gives access to understudied regions of the deep terrestrial subsurface that potentially predate the Great Oxidation Event. Studying organisms that have been relatively unperturbed by surface conditions for as long as 2.7 billion years may give us a window into ancient life before oxygen dominated the planet. Additionally, studying microbes from anoxic and iron-rich environments can help us better understand the requirements of life in analogous environments, such as on Mars. The isolation and characterization of "*Metallumcola ferriviriculae*" strain MK1 give us insights into a novel genus and species that is distinct both from its closest related isolates and from iron reducers characterized to date. "*M. ferriviriculae*" strain MK1 may also act as a model organism to study how the processes of sporulation and germination are affected by insoluble extracellular acceptors, as well as the impact of spores in the deep terrestrial biosphere.

KEYWORDS iron reduction, subsurface microbiology, deep biosphere

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Bacteria capable of respiring metals are found in various environments including freshwater and marine sediments, the deep terrestrial subsurface, and human intestinal microbiomes (1–4). Metal-reducing bacteria play important roles in these ecosystems by facilitating the cycling of iron and manganese. Within the deep terrestrial biosphere, the Soudan Underground Mine in northern Minnesota provides an ideal site to study the biogeochemical impacts of metal-reducing organisms in an environment abundant in metal oxide minerals. The mine's 27th and lowest level (713 m below the surface) provides access to calcium-dominated brine waters flowing from exploratory boreholes drilled within a ~ 2.7 billion-year-old banded iron formation within the Canadian Shield (5, 6). Drilled between 1958 and 1962, the downward boreholes in the West Drift of the 27th level have actively flowing brine waters (~1.1–3.4 mL/s) that are between two and three times saltier than seawater (102–174 vs ~50 mS/cm seawater conductivity), anoxic, and reduced (−200 to −500 mV) (6–8). Analysis of oxygen and hydrogen isotope ratios in water taken from these boreholes suggests long-term isolation from meteoric inputs that could allow for a secluded microbial community to develop (6).

Previous *in situ* electrode enrichments in the Soudan Mine isolated the metal- and electrode-respiring bacterium "*Desulfuromonas soudanensis* WTL" (3). Community analysis comparing the electrode enrichments to borehole brines identified bacteria from the *Deltaproteobacteria*, *Acidobacteria*, and *Firmicutes* lineages that are linked to metal reduction, but of these, only the *Deltaproteobacteria* were found to be enriched within the electrode-associated communities (3). Metagenomic analyses of filtered brine waters across several boreholes have since identified that the brine microbial communities are dominated by the *Proteobacteria* and *Firmicutes* phyla (8). Additionally, several metagenomic-assembled genomes (MAGs) have been generated and analyzed for potential metabolic capabilities, in which two MAGs were reported to contain genes from separate metal-reducing pathways (8).

Within the Soudan Mine boreholes, oxidized, insoluble metals are an abundant electron acceptor that microbes could potentially utilize as a respiratory substrate. To efficiently reduce these metals, microorganisms must find a way to transfer electrons from within the cytoplasm across the cellular membrane(s) and onto an extracellular acceptor, a process termed extracellular electron transfer (9). While the organisms capable of this process are diverse, most mechanistic studies on extracellular electron transfer focus on the model Gram-negative Proteobacteria *Shewanella oneidensis* and *Geobacter sulfurreducens* (1, 10, 11). While both organisms rely on multiheme cytochromes to transport electrons, *S. oneidensis* utilizes the inner membrane tetraheme cytochrome CymA (12), the periplasmic fumarate reductase FccA or tetraheme cytochrome CctA (13, 14), and the outer membrane complex MtrCAB (15) to create a pathway that transfers electrons to an extracellular acceptor. *G. sulfurreducens*, on the other hand, has multiple cytochromes located within the inner membrane, periplasm, outer membrane, and extracellularly, which provides a variety of potential pathways for extracellular electron transfer. Specifically, the inner membrane contains at least three menaquinone oxidases (CbcBA, CbcL, and ImcH) that demonstrate activities tuned to different redox potentials (16–18); the periplasm has at least five triheme cytochromes (PpcABCDE) that do not show specificity for specific electron acceptors (19); and the outer membrane may contain at least five cytochrome complexes (OmbB-OmaB-OmcB, OmbC-OmaC-OmcC, ExtABCD, ExtEFG, and ExtHIJKL) that do (20–22). Additionally, *G. sulfurreducens* produces at least three extracellular cytochrome filaments (OmcS, OmcE, and OmcZ) that appear to facilitate long-range (>10 µm) electron transport (23–25). While a basic understanding of electron flow in these Gram-negative bacteria is well studied, the mechanisms for electron transfer in metal-reducing Gram-positive bacteria have only recently received attention.

Evidence of metal reduction in Gram-positive organisms was first reported in 1947 for the iron-reducing bacterium *Bacillus polymyxa* (26), while the first manganese-reducing bacterium was *Bacillus* 29 reported in 1966 (27). Later studies proposed that these

organisms utilize metal reduction as a sink for excess electrons rather than respiration-linked microbial growth (28, 29). Additionally, it was discovered that *Bacillus* 29 indirectly reduced Mn(IV) through the production of hydrogen peroxide, rather than through a direct protein-mediated pathway (29, 30). The recent discovery of the flavin-based pathway in the pathogens *Listeria monocytogenes* (4) and *Enterococcus faecalis* (31, 32) and the proposed multiheme cytochrome-dependent pathway in *Thermincola potens* strain JR (33–36) provided some insight into protein-mediated pathways for metal reduction in Gram-positive bacteria. In *L. monocytogenes*, flavin adenine dinucleotide is used by a flavin mononucleotide (FMN) transferase (FmnB) to post-translationally modify an extracellular membrane-bound lipoprotein (PplA) (4). Electrons from a specialized NADH dehydrogenase (Ndh2) are transferred via demethylmenaquinone and/or membrane proteins to the FMNylated PplA, which then donates the electrons to the terminal electron acceptor (4, 37). In *Thermincola* species, a series of multiheme cytochromes have been proposed to facilitate electron transfer from the cytoplasmic membrane (ImdcA), through the periplasm (PdcA), across the peptidoglycan layer (CwcA), and then to the electron acceptor by the cell surface-associated OcwA (35, 36, 38). The MAGs generated from Soudan Mine brines identified organisms capable of both the cytochrome- and flavin-dependent pathways, as well as Gram-positive *Firmicutes* abundant in multiheme cytochromes (8).

To obtain isolates of iron-reducing bacteria from the Soudan Mine, brine waters were pumped from a depth of up to 55 m from within a legacy borehole found at the mine's bottom level (713 m below the surface). From enrichment cultures of these samples, we describe the isolation of "*Metallumcola ferriviriculae*' strain MK1, a Gram-positive, rod-shaped bacterium that forms terminal endospores. "*M. ferriviriculae*" strain MK1 was unable to reduce Fe(III) at 37°C or above, and only has a diminished ability to reduce Fe(III) in a tenth of the salinity of Soudan Mine brines. Of the tested growth substrates, it was only able to utilize Fe(III) citrate and AQDS as electron acceptors when coupled with acetate oxidation.

Genomic and physiological characterization identified "*M. ferriviriculae*" as a novel genus within the *Desulfitibacteraceae* family in the newly reclassified *Moorellales* order in the *Firmicutes* phylum (39, 40). Additionally, we identified three homologs (two *cbcBA* and one *imcH*) of *G. sulfurreducens* inner-membrane cytochromes involved in extracellular electron transfer, as well as homologs associated with the proposed *T. potens* electron transfer pathway (*imdcA* and *pdcA*) (16, 35, 36). The isolation of "*M. ferriviriculae*" strain MK1 represents one of the only reported mesophilic, multiheme cytochrome-rich, metal-reducing, Gram-positive bacteria and can provide insights into a potentially unique iron reduction pathway in Gram-positive organisms.

The further characterization of microbes from an anoxic, saline, and iron-rich environment could also give potential biological context to analogous systems, such as the subsurface of Mars. Compositional models based on Martian meteorite samples and Mars Odyssey gamma-ray observations predict that the iron content (reported as FeO) of the bulk silicate Mars (representative of the Martian crust and mantle) is between 14.7% and 18.1% by weight, compared to terrestrial basalt having FeO content of ~10% by weight (41, 42). This abundant iron has been found primarily as the iron silicate olivine and the iron oxides hematite and magnetite and secondarily as other iron oxides (goethite and akaganeite) and iron sulfur minerals (jarosite and pyrite) (43–46). Deposits of the iron oxide hematite (α-Fe_2O_3) on Mars likely required large amounts of water to form, which suggests these sites may have been habitable (44, 47). Additionally, it has been proposed that iron oxide mineralization could allow for the potential preservation of microfossil specimens within these hematite deposits (47). Therefore, a better understanding of microbial life associated with terrestrial iron oxide-rich environments could inform our search for microbial life in the subsurface of Mars or other iron-rich planetary bodies.

MATERIALS AND METHODS

Media and culture conditions

Soudan Mine medium (SM100) used as the base media for "*M. ferriviriculae*" contained the following per liter: $CaCl_2 \cdot 2H_2O$, 22.1 g; $MgCl_2 \cdot 6H_2O$, 15.3 g; NaCl, 15.8 g; $MgSO_4 \cdot 7H_2O$, 0.01 g; NH_4Cl, 1 g; KH_2PO_4, 0.05 g; sodium acetate, 1.64 g; $NaHCO_3$, 1.8 g; non-chelated minerals, 10 mL (48); and Wolfe's vitamins, 10 mL (3). For enrichment and isolation, Soudan Mine medium was amended with Fe(III)-citrate (55 mM) as the sole electron acceptor (SMFC100), while solid medium used for isolation was prepared by adding 0.9% (wt/vol) agar as well as 0.5 mM cysteine to act as a reducing agent. For characterization and routine cultivation, SMFC50 or SMFC25 was used, which contained half or a quarter, respectively, of the concentration of chloride salts. For the preparation of all SM media, all ingredients except $CaCl_2 \cdot 2H_2O$, $MgCl_2 \cdot 6H_2O$, NaCl, and $NaHCO_3$ were first combined, adjusted to pH 6.8, brought to 0.7 times the final volume before adding $NaHCO_3$, purged with N_2:CO_2 (80:20) gas, and autoclaved in butyl rubber-stoppered Balch tubes or serum bottles. An SM media chloride salt solution was purged with argon gas and autoclaved separately, which was then added aseptically and anaerobically to achieve the desired final volume. The chloride salt solution contained 73.5 g $CaCl_2 \cdot 2H_2O$, 50.8 g $MgCl_2 \cdot 6H_2O$, and 52.6 g NaCl per liter for SM100 salt concentrations and was appropriately diluted prior to purging and autoclaving for other desired salt concentrations.

Isolation and cultivation

Brine and sediment were collected from diamond drill hole (DDH) 951, located in the West Drift of the 27th level of the Soudan Underground Mine State Park (Soudan, MN, USA; 47.8168°N, 92.2489°W). DDH951 has a length of 144 m and intersects with both banded iron formations and high purity hematite ore deposits (6). Brine waters from DDH951 are more than twice as salty as seawater (110 vs ~55 mS/cm seawater conductivity) and flow from the borehole at a rate of 3.4 mL/s (6, 7). Brine and the accompanying sediment were collected at a depth of up to 55 m utilizing a peristaltic pump connected to 70% EtOH-sterilized tremie pipes that were sequentially added to extend down the borehole. Only previously collected brine waters were used for any flushing to loosen sediment, and no external water was added to the borehole brines to prevent contamination. The sediment was concentrated by allowing it to settle overnight with an N_2-flushed headspace. After settling, excess brine water was removed by siphoning. Both fresh brine and concentrated sediment were collected in pre-autoclaved and Ar-purged butyl rubber-stoppered serum bottles to keep them anaerobic during transport. Upon returning to the laboratory, Balch tubes containing 10 mL SMFC100 were anaerobically inoculated with 1 mL DDH951 brine water and concentrated sediment and left to incubate at 24°C in the dark. After visually noticeable Fe(III) reduction, enrichment cultures were transferred (1% inoculum) into liquid SMFC100 media, as well as spotted (5 µL) and streaked onto solid SMFC100 plates in a vinyl anaerobic chamber (Coy Laboratory Products, Grass Lake, MI, USA). The inoculated plates were then transferred to an anaerobic jar (Almore International, Beaverton, OR, USA) containing Pt catalyst recharge pellets (Microbiology International, Frederick, MD, USA) under a N_2:CO_2:H_2 (75:20:5) atmosphere and incubated in the dark at 24°C. Growth on plates was repeatedly streaked on SMFC100 under similar conditions until isolation. Isolated colonies were then inoculated into 0.5 mL SMFC100 medium and incubated in an anaerobic jar under the aforementioned conditions, before storing as axenic cultures at −80°C in 20% (vol/vol) dimethyl sulfoxide (DMSO). Routine cultivation of "*M. ferriviriculae*" has since been carried out at 24°C in SMFC25 media.

Physiological and biochemical characterization

Basic cell morphology and swimming motility were examined by brightfield and phase contrast microscopy with a Revolve Fluorescence Hybrid Microscope (Discover Echo Inc., San Diego, CA, USA). Gram-staining reaction was determined by using a standard

protocol (49). Scanning electron microscope (SEM) imaging was also performed using a JEOL 6500 field-emission gun scanning electron microscope. Cells were prepared for SEM imaging by anaerobically fixing with 3.5% (vol/vol) glutaraldehyde in 0.1 M HEPES for 24 h. The cells were then collected on filter paper and rinsed three times with N_2-purged MilliQ water to prevent iron oxide formation during the washing process. After this, the cells were dehydrated using a series of ethanol solutions with increasing concentration [30%, 50%, 70%, 80%, 90%, 95%, 100%, 100%, and 100% (vol/vol)], with the solutions changed in 10-min intervals. The filter was sectioned and coated with 7.5 nm Pt before imaging.

All growth tests were performed anaerobically in triplicate Balch tubes containing 10 mL of medium. Additionally, for all growth tests in SMFC media, Fe(III) reduction was used as a stand-in for growth and/or metabolic activity due to the color and precipitation of salts from the media hindering standard optical density (OD) measurements. Fe(II) was quantified using the ferrozine assay (50). The temperature range for the growth of "*M. ferriviriculae*" was tested by incubating SMFC50 cultures (5% inoculum) at 24°C, 30°C, 37°C, and 56°C, along with triplicate uninoculated SMFC50 tubes. This temperature range was chosen to reflect the optimal growth temperatures of several related strains and the enrichment temperature. The chloride salt tolerance of "*M. ferriviriculae*" was tested by diluting the SM media chloride salt solution (26.24 g/L total chlorides) prior to its addition to SMFC media to chloride concentrations of 100% (26.24 g/L), 75% (19.95 g/L), 67% (17.80 g/L), 50% (13.45 g/L), 33% (9.10 g/L), 25% (7.06 g/L), and 10% (3.22 g/L) of enrichment conditions (referred to as SM100, SM75, SM67, SM50, SM33, SM25, and SM10, respectively). The various SMFC media were then inoculated with 5% of "*M. ferriviriculae*" grown in SMFC50 media. The utilization of various growth substrates was tested using SM25 media and amended with the following substrates: anthraquinone-2,6-disulfonate (AQDS, 2.5 mM), citrate (10 mM), Fe(III) oxide (~50 mM), fructose (25 mM), fumarate (40 mM), methanol (25 mM), monomethylamine (25 mM), sulfate (20 mM), and thiosulfate (20 mM). Additionally, utilization of H_2/CO_2 was tested by sparging the headspace with filtered H_2 for 1 min after inoculation. Fe(III) oxide was prepared as akaganeite (β-FeOOH) by the dropwise addition of 25% NaOH to stirring 0.4 M $FeCl_3$ until pH 7, held at pH 7 for 1 h and then washed with ddH_2O via centrifugation, before adding 1 mL to 9 mL SM medium prior to autoclaving (51, 52). Substrate utilization was determined for all conditions by an increase in cell growth determined by a measured increase of OD_{600}, except for in media supplemented with AQDS and Fe(III) oxide in which the accumulation of reduced products was measured by OD_{450} and the ferrozine assay, respectively (50, 53). In conditions where low growth yield was anticipated, microscopy was also done to qualitatively determine if growth had occurred. Cultures that indicated a utilization of the substrate were subcultured into similar media to verify that growth or substrate reduction was not due to carryover from the original inoculum.

Genomic DNA extraction, sequencing, and analysis

Initial genomic DNA extractions of "*M. ferriviriculae*" for short-read sequencing were performed using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) on 10 mL SMFC50 cultures. Due to high iron and salt concentrations from the samples, sequencing of 16S rRNA genes was performed by first diluting gDNA samples 1/100 in nuclease-free water before PCR amplification using the universal bacterial primers 27F and 1492R as previously reported (54). Amplified PCR products were then submitted for Sanger sequencing (ACGT, Inc., Wheeling, IL, USA) using the 27F and 1492R primers. For long-read sequencing, the gDNA samples were extracted from 10 mL SMFC50 grown cultures of "*M. ferriviriculae*" and resuspended bacterial growth from solid SMFC50 plates using phenol-chloroform extraction as described in Bouillaut et al. (55). Samples were then cleaned of excess iron and salt through drop dialysis as described by Silhavy et al. (56). Briefly, gDNA samples were placed on a 25 mm diameter, Type-VS membrane (Millipore, Burlington, MA, USA) floating on 1× TE buffer (10 mM

Tris-HCl and 1 mM EDTA, pH 8) for up to 4 h. The gDNA sample was then carefully removed by micropipette, and the membrane was rinsed with 1× TE buffer to extract any remaining DNA. Cleaned gDNA was quantified with a Qubit instrument (Invitrogen, Waltham, MA, USA) and then sent for both short-read Illumina and long-read Nanopore sequencing through SeqCenter (Pittsburgh, PA, USA), which also assembled a closed, circular genome of "*M. ferriviriculae*." The 3,720,236 bp completed genome was initially annotated using Prokka version 1.14.6 (57). The bioinformatics tool FeGenie was also used to identify genes potentially involved in iron acquisition, storage, oxidation, and reduction (58). Additionally, putative multiheme *c*-type cytochromes were identified utilizing a Python script (single_genome_multihemes.py) searching for three or more CxxCH motifs within protein sequences, which can be found in the following GitHub repository: https://github.com/bondlab/scripts (3). The GenBank submitted annotation was manually curated using a combination of annotations from the NCBI Prokaryotic Genome Annotation Pipeline version 5.3 (build5742), Prokka version 1.14.6, and FeGenie amended with *Geobacter*-related multiheme *c*-type cytochrome Hidden Markov Models (HMMs) (57–59). The cytochrome HMMs utilized by this study can be found in the following GitHub repository: https://github.com/davhsu776/Fe_Genie_HMMs. Genome average nucleotide identity (ANI) comparison of the genome to Soudan Mine MAGs was performed using the online enveomics ANI calculator tool (http://enve-omics.ce.gatech.edu/ani/) (60, 61).

Phylogenomic classification

The 16S rRNA gene from the sequenced genome was used to identify the closest related isolates by NCBI BLAST search. FASTA nucleotide sequences for 22 representative genomes from the *Clostridia* class were then downloaded from NCBI and analyzed against "*M. ferriviriculae*" using Anvi'o version 7.1 (62). A concatenated alignment of 71 conserved single-copy core genes was used to generate an unrooted maximum-likelihood phylogenomic tree visualized using the Anvi'o interactive interface and finalized for publication using Inkscape version 1.1.

RESULTS AND DISCUSSION

Enrichment and isolation of MK1

Down-borehole brine samples from the Soudan Mine were collected at a depth of up to 55 m within DDH951 (Fig. 1A). Within the pumped sample was an insoluble black magnetic material (Fig. 1B), which was later identified as the poorly crystalline iron sulfide mineral mackinawite (63). The brine and mineral samples were concentrated and anaerobically transported to the laboratory, where they were used to inoculate brine-mimicking Fe(III)-reducing media (SMFC100). After 46 days of incubation at 24°C in the dark, the SMFC100 cultures containing Fe(III) citrate as the sole electron acceptor indicated iron reduction through the darkening of the medium, and an eventual clearing of color was first noted after 59 days of incubation (Fig. 2A). The culture was then used to both streak and spot SMFC100 agar plates and incubated in anaerobic jars in the dark at 24°C. After 43 days of incubation, bacterial growth was observed on the plates as indicated by salt precipitation on top of dense bacterial growth regions, zones of clearing caused by iron reduction, and isolated pink colonies (Fig. 2B and C). After re-streaking the pink colonies for further isolation, they were then picked to be grown in SMFC100 liquid medium and saved at −80°C in 20% (vol/vol) DMSO.

For the initial identification of the organism, genomic DNA was extracted and diluted to preclude the inhibition of polymerase activity by high salt and iron concentrations. Sequencing of 16S rRNA genes using universal bacterial primers 27F/1492R identified its closest isolate as *Desulfitibacter alkalitolerans* strain sk.kt5 (91% sequence identity) (40). This 16S rRNA gene similarity falls between the proposed minimum taxonomic thresholds of a novel genus (94.5%) and novel family (86.5%) as proposed by Yarza et al. (64), indicating our isolate belongs to a novel genus within the recently defined

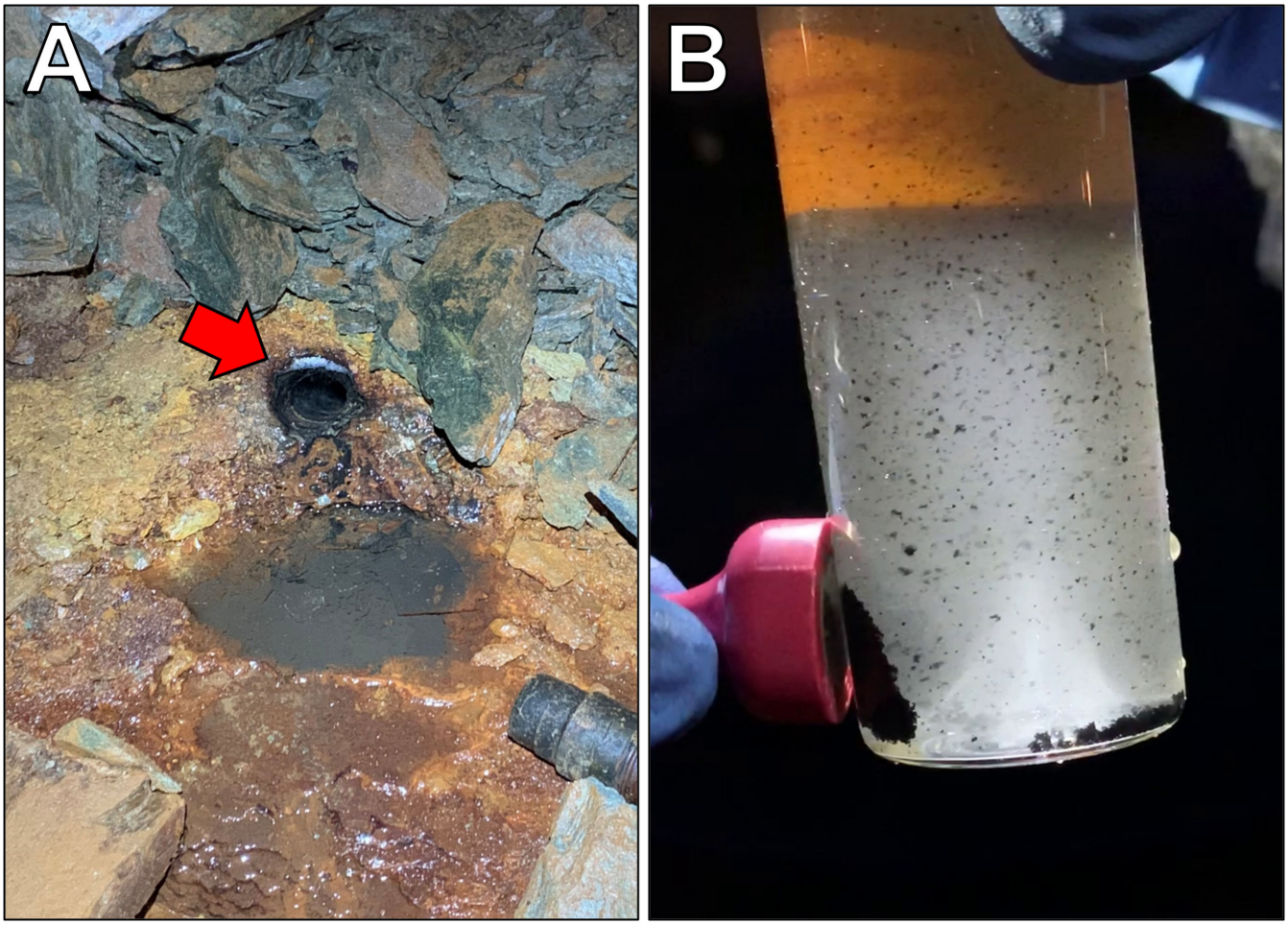

FIG 1 Brine and sediment from diamond drill hole 951. (A) Photograph of DDH951 (red arrow) in the West Drift of the 27th level (713 m below the surface) of the Soudan Underground Mine State Park. Visible in the borehole waters are small, methane-dominated (72.6%) gas bubbles coming from within the brine water (7). (**B**) Photograph of brine water sample pumped up from DDH951. Fine black sediment within the brine water was magnetic and was later identified through X-ray diffraction as the poorly crystalline iron monosulfide mackinawite (FeS) (63).

Desulfitibacteraceae family (39). The isolate was given the proposed name "*Metallumcola ferriviriculae*" strain MK1, which was derived as follows: *Metallumcola* (metallum: mine or place where metals are found, -cola: one who inhabits; *Metallumcola*: one who inhabits a mine) *ferriviriculae* (ferri: iron, viriculae: small force; *ferriviriculae*: a small force of iron).

Morphological and physiological characterization of strain MK1

Initial morphological characterization of strain MK1 cells grown on SMFC100 media plates under light microscopy identified it as a straight rod-shaped bacterium with terminal endospores (Fig. 3A). Gram stain testing of strain MK1 showed a Gram-positive staining reaction, appearing as a mixture of straight and slightly curved rod cell morphologies (Fig. 3B). Based on the Gram stain reaction results strain MK1 is likely a Gram-positive bacterium; however, further physical characterization of the cellular structure would be required to confirm a monoderm cell wall and peptidoglycan layer thickness. Scanning electron microscopy verified that strain MK1 is a straight rod bacterium with terminal endospores (Fig. 3C and D). From the SEM micrographs, cells were measured to be between 1.75 and 2.3 µm in length and 0.2–0.25 µm in width without endospores (Fig. 3C), while the cells with endospores were measured to be between 2.5 and 3 µm by 0.18–0.22 µm in size (Fig. 3D). Endospores were measured to a

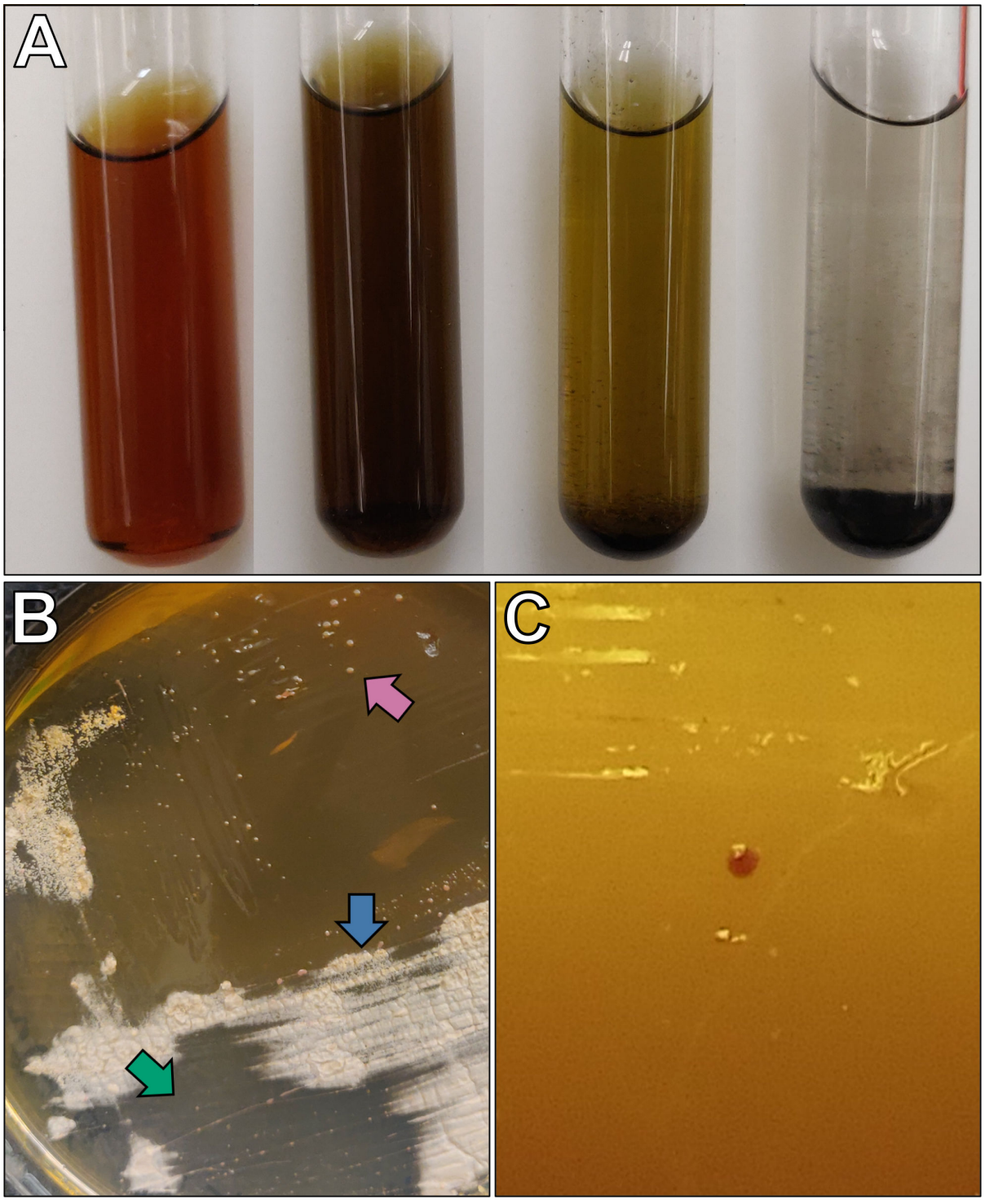

FIG 2 Fe(III)-citrate reduction enrichments of novel Soudan Mine isolate. (A) Time-lapse photos showing the visual progression of Fe(III)-citrate reduction of enrichments inoculated with DDH951 brine and sediment sample. From left to right: uninoculated Fe(III)-citrate SMFC100 medium; preliminary Fe(III) reduction represented by the darkening of the media (46 days post-inoculation); further Fe(III) reduction seen by the brightening of the media (59 days post-inoculation); complete Fe(III) reduction shown by clearing of the media (93 days post-inoculation). (B) Photograph of the initial isolation of strain MK1 on an SMFC100 medium plate. Zones of clearing caused by Fe(III) reduction (green arrow) and salt precipitation (blue arrow) are shown in regions of dense bacterial growth, and isolated colonies are shown in regions without salt precipitation (pink arrow). (C) Photograph of strain MK1 streaked on an SMFC50 plate highlighting the pinkish-red color of isolated colonies.

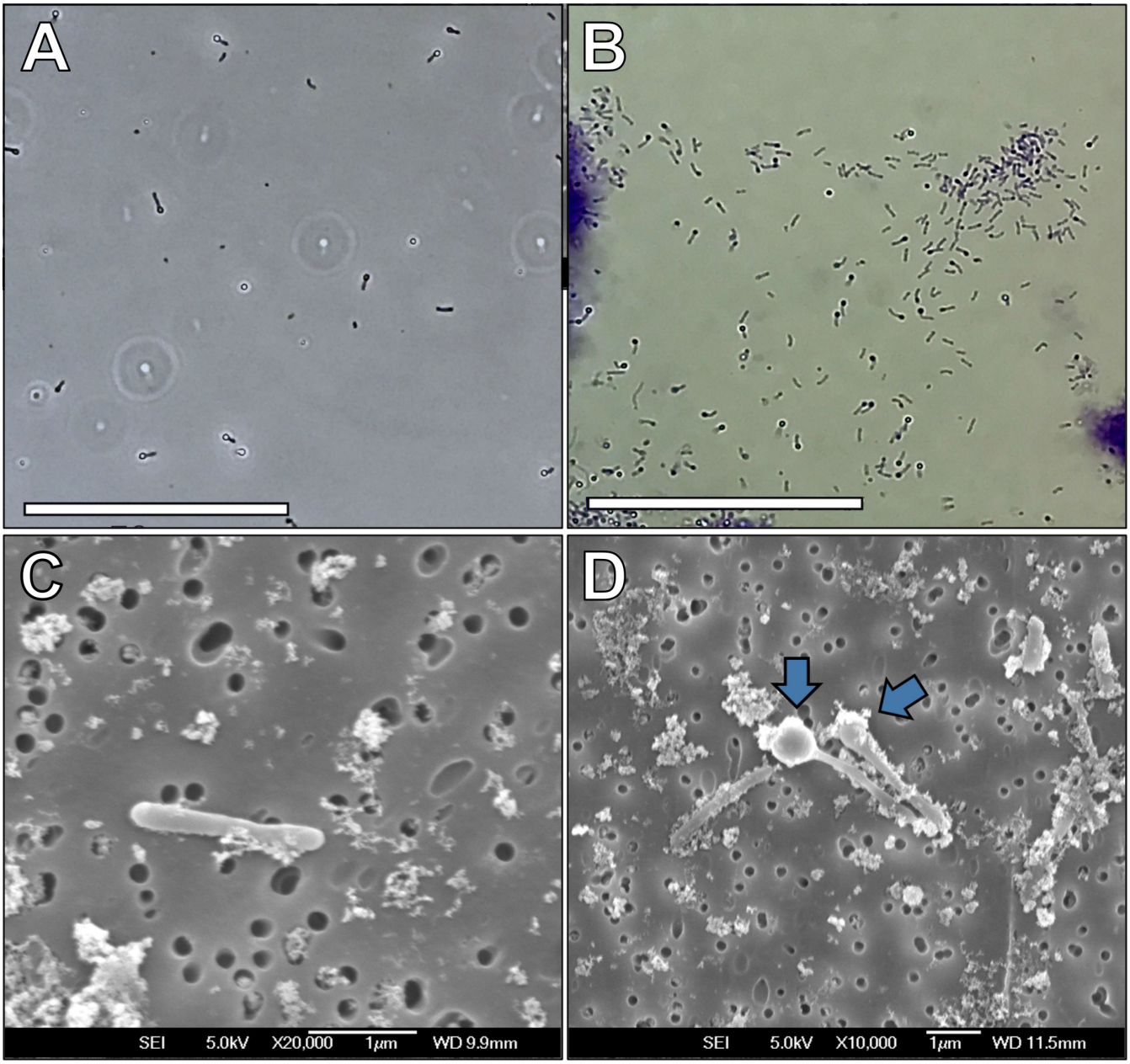

FIG 3 Light and scanning electron microscopy of "*Metallumcola ferriviriculae*" MK1 cells. "*M. ferriviriculae*" viewed under 40× showing (A) phase contrast micrograph of a wet mount and (B) brightfield micrograph of a Gram-positive stain reaction. Both images show rod-shaped cells with terminal endospores. (C and D) Scanning electron micrographs of "*M. ferriviriculae*" with terminal endospores highlighted by blue arrows in panel D. The SEM micrographs were used to measure the size of cells with and without endospores, as well as the endospores themselves. Scale bars: (A and B) 50 µm and (C and D) 1 µm.

diameter of 0.5–0.78 µm, while free spores were found to be 0.4–0.7 µm in diameter (Fig. 3D).

To test the impacts of brine conditions on iron reduction rates, strain MK1 was inoculated into SMFC media ranging from 10% to 100% Soudan Mine brine conditions (SM10–SM100, respectively). Iron reduction activity was observed by MK1 in all brine concentrations tested but exhibited an extended lag prior to the initiation of iron reduction at the lowest brine condition (SM10) while demonstrating slightly shorter lag times at the SM25 and SM33 brine conditions (Fig. 4A). Strain MK1 reduced iron between 24°C and 30°C and not at temperatures above 37°C (Fig. 4B). These temperature ranges are similar to its closest related isolate within the *Desulfitibacteraceae* family, but atypical

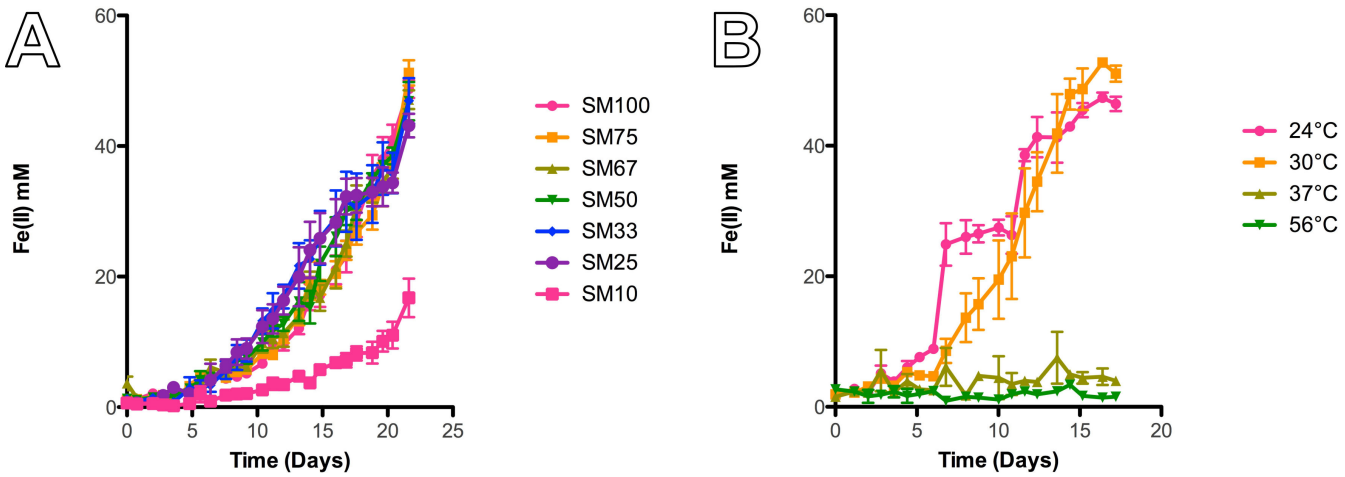

FIG 4 Fe(III) reduction of pure cultures of "*Metallumcola ferriviriculae*" MK1 under varying physiological conditions. (A) Fe(III) citrate reduction over time in SMFC media with chloride salt concentrations varying from calculated *in situ* brine conditions (SM100) to a tenth (SM10) brine conditions. Rates of Fe(III) reduction by "*M. ferriviriculae*" were consistent in all conditions down to 25% brine concentrations and slowed significantly at 10% brine concentration. (B) Fe(III) citrate reduction over time in SMFC50 medium incubated at 24°C, 30°C, 37°C, or 56°C. "*M. ferriviriculae*" cultures are unable to reduce Fe(III) citrate at 37°C or higher. All experiments were conducted in triplicate, and results were plotted as mean values ± SD.

of the other characterized members of the *Moorellales* order, which are thermophiles with preferred growth ranges above 55°C (Table 1) (39, 40, 65, 66).

To identify iron-independent growth strategies of strain MK1, SM25 media was prepared with various substrates to test different carbon sources and electron acceptors. Strain MK1 was unable to ferment citrate and unable to grow acetogenically with H_2:CO_2, methanol, or monomethylamine (Table 1). Unlike its close relative *Moorella thermoautotrophica* (67), strain MK1 was unable to ferment fructose. Strain MK1 was able to utilize AQDS as an electron acceptor, but not fumarate, sulfate, or thiosulfate. The utilization of AQDS is unsurprising considering that it is a redox-active molecule that can also be used by many iron-reducing microbes as an electron acceptor (68, 69). The ability to reduce Fe(III) citrate but not the iron oxide akaganeite may indicate that MK1 can only reduce soluble forms of ferric iron or that the ability to reduce insoluble substrates was lost during cultivation. Alternatively, strain MK1 may have selectivity between the different forms of insoluble Fe(III) oxides that can be reduced, which may be based on the variations of physical characteristics or reducing potentials between different Fe(III) oxide minerals (70, 71). A more comprehensive test of various soluble (such as ferric nitrilotriacetate) and insoluble (such as ferric hydroxide, schwertmannite, or ferrihydrite) ferric electron acceptors, along with manganese oxide, is required to better understand metal reduction in MK1.

Genomic DNA extraction and analysis

Through a combination of short-read and long-read sequencing, a closed, circular genome of strain MK1 was generated. The "*Metallumcola ferriviriculae*" strain MK1 genome contained 3,720,236 bp with a GC content of 43.7%. ANI comparisons of the genome to previously generated Soudan Mine MAGs found a two-way ANI of 100% (standard deviation of 0.09%) to the Soudan-20 MAG, which marks the isolation of a Soudan Mine bacterium identified in a previous metagenomic study (8). The annotated genome encodes 3,527 genes, 2,978 with predicted functions, as well as 49 tRNAs and 4 16S-23S-5S rRNA operon copies. The central metabolism of strain MK1 predicted by the genome includes a non-oxidative pentose phosphate pathway and an Embden-Meyerhof-Parnas glycolysis/gluconeogenesis pathway. A pyruvate:ferrodoxin oxidoreductase connects a TCA cycle lacking an annotated malate dehydrogenase. Additionally, the genome encodes for the complete Wood-Ljungdahl pathway, as seen in many *Moorella*

TABLE 1 Physiological and metabolic properties of *Metallumcola ferriviriculae* strain MK1 and related strains[c]

Strain (reference)	*Metallumcola ferriviriculae* MK1	*Desulfitibacter alkalitolerans* sk.kt5 (40)	*Zhaonella formicivorans* K32 (39)	*Moorella thermoacetica* DSM521 (39, 67)	*Calderihabitans maritimus* KKC1 (66)	*Thermincola potens* JR (33)
16S RNA identity (%)	100	91	88.4	88.09	87.2	85.5
Source of isolation	Underground iron mine	District heating plant	Oilfield	Horse feces	Marine caldera sediment	Anaerobic digester
Size (µm)	1.75–2.3 × 0.2–0.25	2.5–9.6 × 0.4–0.6	2.5–8.0 × 0.4–0.8	2.8 × 0.4	1.0–3.0 × 0.5–1.0	ND
Gram reaction	+	+	–	+	–	+
Motility	–	+	+	–	–	–
Spore formation	+	+	–	+	+	–
Growth temperature (°C)	30–37	35–37	55	55–60	65	56
G+C (mol%)	43.7	41.6	46	56	50.6	45.9
Fermentation						
Citrate	–	ND	ND	ND	–	ND
Fructose	–	–	+	+	+	ND
Acetogenesis						
H_2/CO_2	–	–	–	+	–	+
Methanol	–	–	–	+	–	ND
Monomethylamine	–	ND	ND	ND	ND	ND
Electron donors[a]						
Acetate	+	–	–	–	–	+
H_2/CO_2	ND	–	–	+	–	+
Methanol	ND	+	–	+	–	ND
Electron acceptors[b]						
Fumarate	–	–	–	–	+	ND
Sulfate	–	–	–	ND	+	ND
Thiosulfate	–	+	–	+	+	ND
AQDS	+	ND	ND	ND	+	+
Fe(III) citrate	+	–	–	ND	+	+
Fe(III) oxide	–	ND	ND	ND	+	+

[a]For strain MK1, Fe(III) citrate was used as an electron acceptor.
[b]For strain MK1, acetate was used as an electron donor.
[c]+, positive reaction; –, negative reaction; ND, not reported.

species (65, 67, 72). Although strain MK1 was unable to grow acetogenically with the addition of hydrogen gas (Table 1), strain MK1 is predicted to contain three carbon monoxide dehydrogenases, suggesting this donor may be used for acetogenesis or metal reduction (73, 74). Genes for assimilatory sulfate reduction are also predicted in the genome, though strain MK1 showed no growth when provided sulfate as the sole electron acceptor for dissimilatory sulfate reduction (Table 1).

The annotated genome also predicts genes encoding a full set of flagella component proteins, even though motility in strain MK1 was not observed under light microscopy in wet mounts prepared anaerobically (data not shown). Many major genes associated with sporulation are also predicted, including the master sporulation regulator protein *spo0A*, the peptidoglycan remodeling enzymes *spoIID*, *spoIIP*, and *spoIIM*, the spore morphogenesis protein *spoIVA*, and the small, acid-soluble spore proteins *sspA*, *sspB*, *sspC*, *sspD*, and *sspF*, consistent with microscopic observations (Fig. 3). Strain MK1 is likely monoderm as the genome does not carry genes associated with outer membrane formation; however, there are predicted genes associated with S-layer formation (75). Additionally, the genome has a ~35 kb region with genes encoding for many prophage-like proteins, as well as a predicted CRISPR array with 73 repeat regions near several genes encoding CRISPR-associated proteins.

MK1 carries *Geobacter*-like multiheme cytochromes

The bioinformatics tool FeGenie (58) was unable to identify genes associated with the commonly studied Fe(III)-reduction pathways. However, when FeGenie was augmented to include HMMs specific to known *Geobacter sulfurreducens* cytochromes associated with Fe(III) reduction, two gene clusters were identified (MFMK1_2258–2259 and MFMK1_2264–2265) with characteristics similar to *G. sulfurreducens* inner membrane multiheme cytochrome complexes composed of a co-expressed di-heme *b*-type and seven-heme *c*-type cytochrome domains (16). Both clusters in strain MK1 have predicted *b*-type cytochrome (MFMK1_2258 and MFMK1_2264) and seven-heme *c*-type cytochrome (MFMK1_2259 and MFMK1_2265) domains. While the *b*- and *c*-type cytochromes were in two separate reading frames, similar to *cbcBA* of *G. sulfurreducens,* protein alignments of MFMK1_2258–2259 and MFMK1_2264–2265 showed higher protein identity and similarity with the *G. sulfurreducens* CbcL inner membrane cytochrome (Fig. S1). CbcL is similar to CbcBA, except that it is a single fused protein that contains both the *b*- and *c*-type cytochrome domains and is responsible for reduction at mid-range potentials (17) (Fig. S1). Potential Cbc-like gene clusters have also been reported in two Gram-positive *Carboxydocella thermautotrophica* strains that cannot (type strain 41; CTH_2221–2222 and CTH_2327–2328) and can (strain 019; CFE_2192–2193 and CFE_2225–2226) reduce iron, but their functional roles in iron reduction have yet to be reported (76). Interestingly, each MK1 Cbc-like gene cluster is flanked upstream by three small hypothetical proteins and downstream by an 11-heme NapC/NirT family cytochrome. These regions are right next to each other but show low nucleotide sequence identity to one another (only ~30% of the region has >50% identity). The *C. thermautotrophica* Cbc-like gene clusters also share this similar genomic pattern, but each region is further separated on their respective genomes. It is unclear if these surrounding genes are important for iron reduction, but the shared genomic contexts could indicate a potential conserved region between Gram-positive iron reducers.

Further analysis of the strain MK1 genome identified 23 additional putative multiheme cytochromes, defined as proteins containing three or more CxxCH heme-binding motifs. Of these, a putative *imcH* gene (MFMK1_1670) was also identified (Fig. S2). ImcH is another characterized inner membrane menaquinone oxidase needed for the reduction of Fe(III) citrate and other high-potential electron acceptors in *G. sulfurreducens* (18). Both *C. thermautotrophica* strains 41 and 019 have also been reported to have ImcH-like multiheme cytochromes (CTH_1728 and CFE_1714, respectively), but these both share more sequence identity with the strain MK1 multiheme cytochromes MFMK1_2376 and MFMK1_2377 (Fig. S2) (76). These two cytochromes were originally predicted to also be

ImcH-like, but recent characterizations of ImcH suggest that they, as well as CTH_1728 and CFE_1714, are more likely to be homologs of the nitrite reduction-related menoquinone oxidase NrfH (77). Aside from poor sequence identity to ImcH, MFMK1_2377, CTH_1728, and CFE_1714 were found to share the first heme-binding motif of CxxCHxM found in NrfH rather than the characteristic first heme-binding CxxxCH motif found in ImcH and MFMK1_1670 (Fig. S2) (77). Interestingly, MFMK1_2376 and MFMK1_2377 also showed homology to the *T. potens* strain JR cytoplasmic membrane multiheme cytochrome ImdcA (31.5% and 35.5% sequence identity), which has been proposed to coordinate electrons from the quinone pool for extracellular electron transfer in *T. ferriacetica* (36). Furthermore, a homolog (MFMK1_2500) to the *T. potens* strain JR periplasmic decaheme cytochrome PdcA (33.7% sequence identity) was also found. In tandem, ImdcA and PdcA are proposed to pass electrons from the quinone pool to a polymeric CwcA cytochrome chain that transects the peptidoglycan layer to the cell surface terminal metal reductase OcwA; however, no homologs to either *cwcA* or *ocwA* were found in strain MK1 (35, 36, 38). These findings suggest that strain MK1 may use a *G. sulfurreducens*-like inner membrane cytochromes and *Thermincola*-like periplasmic cytochromes to coordinate electron transfer to the periplasmic space, but crossing the peptidoglycan layer would require an unidentified mechanism. Cytochrome functionality is notoriously difficult to predict through bioinformatic approaches (78, 79); therefore, these similarities would need to be validated physiologically through the creation of knockout mutants once a genetic system is developed in MK1.

Phylogenomic analysis and comparisons of related isolates

Based on 16S rRNA gene sequence identity, strain MK1 falls into the *Clostridia* class within the *Firmicutes* phylum, and based on the cutoffs [novel genus (94.5%) and novel family (86.5%)] proposed by Yarza et al. (64), strain MK1 belongs to a novel genus and species in the newly classified *Desulfitibacteraceae* family (39, 40). The closest related isolates to strain MK1 are *Desulfitibacter alkalitolerans* strain sk.kt5^T (91%) (40), *Zhaonella formicivorans* strain K32^T (88.4%) (39), multiple strains of *Moorella,* including *Moorella thermoacetica* strain DSM521^T (88.09%) (67) and *Calderihabitans maritmus* strain KKC1^T (87.2%) (66) (Table 1). These strains all fall into the recently proposed *Moorellales* order, which consists of the *Desulfitibacteraceae*, *Zhaonellaceae*, *Moorellaceae*, and *Calderihabitantaceae* families, respectively (39). Utilizing an average nucleotide identity approach that compared a concatenated set of 71 conserved genes, a phylogenomic tree was created of representative members of the *Clostridia* class that supported the 16S rRNA gene sequence identity analysis (Fig. 5).

Among the members of the *Moorellales* order, the *Desulfitibacteraceae* family isolates are unique in that they are the only mesophilic members (30°C–37°C vs 55°C–65°C temperature optimums, respectively) (Table 1). While the current temperatures of the Soudan brine water (10°C–12°C) (6) would not support thermophilic life, this banded iron formation was proposed to have developed over 2.5 billion years ago through a combination of seafloor deposition and hydrothermal alterations at temperatures ranging from 150°C to >300°C (80). As these iron formations originated near hydrothermal vents on the seafloor, it is possible that strain MK1 is distantly descended from thermophilic marine bacteria, like *Calderihabitans maritimus* strain KKC1, that diverged and evolved separately.

Within the *Moorellales* order, physiological features like motility, spore formation, and even Gram stain reaction are also inconsistent with each other (Table 1). Motility has been observed in both *D. alkalitolerans* strain sk.kt5 and *Z. formicivorans* strain K32 but not in strain MK1 or *C. maritimus* strain KKC1 (39, 40, 66). While various species of *Moorella* have shown motility, the type species of the genus *M. thermoacetica* strain DSM521 has not (65, 67). Spore formation is only absent in *Z. formicivorans* strain K32, while both *Z. formicivorans* strain K32 and *C. maritimus* strain KKC1 stain Gram-negative, while the rest of the order stains Gram-positive. The vast physiological differences

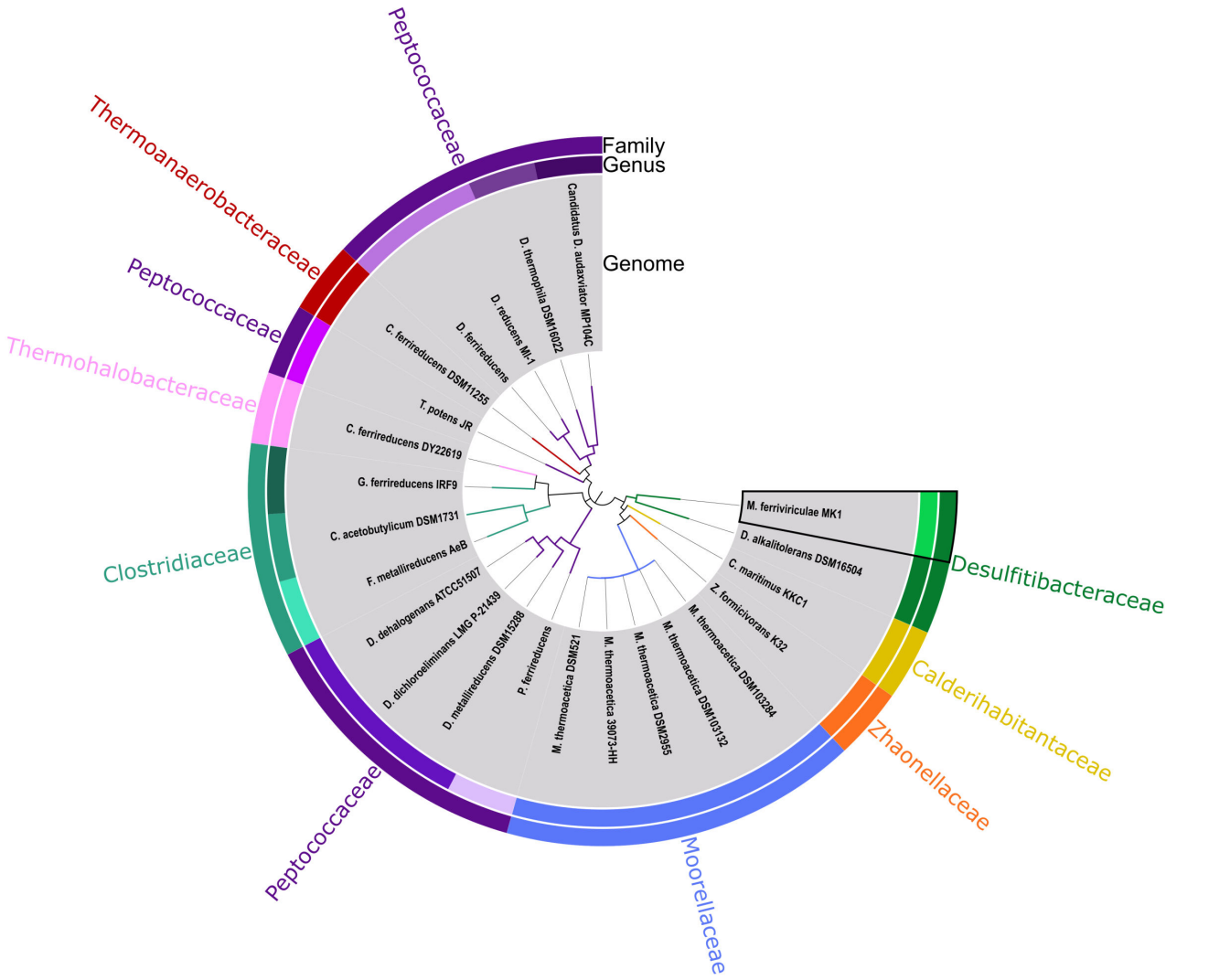

FIG 5 Maximum-likelihood phylogenomic tree of representative Clostridia class member genomes. The unrooted tree was constructed from an alignment of a concatenated set of 71 conserved single-copy core genes in Anvi'o (62). The tree incorporates 23 representative genomes across eight families colored in the outside ring: *Calderihabitantaceae* (yellow), *Clostridiaceae* (teal), *Desulfitibacteraceae* (green), *Moorellaceae* (blue), *Peptococcaceae* (purple), *Thermoanaerobacteraceae* (red), *Thermohalobacteraceae* (pink), and *Zhaonellaceae* (orange). The genus of each genome is colored in the middle ring with a varying shade of the corresponding family color, as shown in the embedded legend. The tree was visualized by the Anvi'o interactive interface and finalized for publication using Inkscape version 1.1.

between these closest isolates highlight the lack of cultured species representation within the *Moorellales* order.

Many of the representative strains utilized for the phylogenomic tree are proposed Fe(III)-reducing organisms, but only *C. maritimus* strain KKC1 has been shown to reduce iron among the *Moorellales* order (Table 1) (66) and is predicted to contain a homolog of *ocwA*. Of these representative strains within the *Moorellales* order, only strain MK1 has been reported to utilize acetate as an electron donor. Within the order, several members of the *Moorella* genus carry carbon monoxide dehydrogenases used for acetogenesis in the Wood-Ljungdahl pathway, highlighting the potential for strain MK1 to do the same (72, 74). Additionally, *D. alkalitolerans* strain sk.kt5, the closest related isolate to strain MK1, is known to be able to reduce sulfite but not sulfate as an electron acceptor (40), which may be the same case for strain MK1 as the genome predicts sulfur

metabolic capabilities. Further investigation of these predicted metabolic similarities of these related isolates could give insights into potential alternative growth strategies for strain MK1.

Conclusion

Through enrichment from mineral particulate from Soudan Underground Mine State Park brine waters, the novel Fe(III)-reducing, Gram-positive bacterium "*Metallumcola ferriviriculae*" strain MK1 was isolated. While other Fe(III)-reducing organisms have been isolated from the Soudan Mine (3), strain MK1 represents the first enriched from brine waters collected from deep within the boreholes and is the first Gram-positive Fe(III) reducer isolated from the mine. The cultivation of strain MK1 provides the ability to further study the mechanisms of metal reduction in Gram-positive organisms, which may rely both on *Geobacter*-like and *Thermincola*-like multiheme cytochrome components. Additionally, the study of the sporulation and germination of strain MK1 could aid in understanding the impact that spores could have on metal cycling within the mines and within the subsurface biosphere as a whole. While the full metabolic capabilities of strain MK1 still require further evaluation, its predicted Wood-Ljungdahl pathway and sulfur reduction pathways could provide an additional model for cycling of carbon and sulfur within the brine network, which has not been fully elucidated (8). Furthermore, the identification of strain MK1 as a new member of the newly classified *Moorellales* order helps give phylogenomic context to the order, as well as the *Desulfitibacteraceae* family (39, 40). The phylogenomic relation of strain MK1 to deep marine thermophiles may also support the geologic history of the Soudan Iron Formation, as well as help provide context to the source of the trapped brine waters within the deep terrestrial subsurface. Further evaluation of iron-reducing microbes trapped in ancient iron oxide formations can also provide insight into life in similar environments, such as Martian hematite deposits, as well as facilitate the development of better strategies and techniques for their study. The continued study of strain MK1 will help elucidate its role in the Soudan Mine brine and provide new insights into the diversity of metal reduction mechanisms.

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DATA AVAILABILITY

Genome sequence and annotation are available from GenBank via accession no. CP121694. Raw reads have been deposited to the NCBI Sequence Read Archive (SRA) under BioProject PRJNA949713.

ADDITIONAL FILES

The following material is available online.

Supplemental Material

Supplemental material (AEM00044-24-s0001.pdf). Figures S1 and S2.

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