An Iron-Corroding Methanogen Isolated from a Crude-Oil Storage Tank

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Microbiologically influenced corrosion of steel in anaerobic environments has been attributed to hydrogenotrophic microorganisms. A sludge sample collected from the bottom plate of a crude-oil storage tank was used to inoculate a medium containing iron (Fe\(^0\)) granules, which was then incubated anaerobically at 37°C under a N\(_2)/CO\(_2\) atmosphere to enrich for microorganisms capable of using iron as the sole source of electrons. A methanogen, designated as strain KA1, was isolated from the enrichment culture. An analysis of its 16S rRNA gene sequence revealed that strain KA1 is a *Methanococcus maripaludis* strain. Strain KA1 produced methane and oxidized iron much faster than did the type strain of *M. maripaludis*, strain JJ\(^T\), which produced methane at a rate expected from the abiotic H\(_2\) production rate from iron. Scanning electron micrographs of iron coupons that had been immersed in either a KA1 culture, a JJ\(^T\) culture, or an aseptic medium showed that only coupons from the KA1 culture had corroded substantially and were covered with crystalline deposits that consisted mainly of FeCO\(_3\).
Iron ($\text{Fe}^0$) is an inexpensive metal and is widely used in many industrial processes and industrial/commercial products. When iron contacts an aqueous electrolyte, it readily corrodes. This happens because, as a result of metallurgical and environmental heterogeneities, the electrolytes are not evenly distributed across the surface of the metal and consequently the electric potential is also unevenly distributed. Therefore, electrons flow within the metal from an area of higher electrical potential (the anode) to an area of lower electrical potential (the cathode). At the anode, iron atoms lose electrons and dissolve into ferrous ions ($\text{Fe}^{2+}$), whereas cations or elements dissolved in solution (e.g., $\text{H}^+$ under anaerobic conditions or $\text{O}_2$ under aerobic conditions) are reduced by electrons at the cathode.

The corrosion of structures that contain iron is economically devastating. It has been estimated that in the United States alone, the cost of corrosion is 276 billion dollars annually (17). Iron is corroded not only by physiochemical processes but also by the metabolic activity of microorganisms; this metabolic process is termed microbiologically influenced corrosion (MIC). Some 10% of all corrosion damage may be the result of microbial activity (15), and sulfate-reducing bacteria (SRB) are widely regarded as the causative agents of MIC in anaerobic environments (11, 12, 18, 21). The mechanism by which SRB stimulate iron corrosion may occur via the uptake of electrons at the cathodic surface of iron (cathodic depolarization) in conjunction with sulfate reduction: $8\text{e}^- +$
SO₄²⁻ + 10H⁺ → H₂S + 4H₂O (27), while at the anionic surface, iron atoms are oxidized to ferrous ions (Fe → Fe²⁺ + 2e⁻). In fact, certain SRB use not only hydrogen but also iron as a source of electrons for sulfate reduction (1, 9, 22). Because not all SRB grow as fast in the presence of iron as they do in the presence of hydrogen (9), fast-growing SRB on iron may have a specific enzyme(s) that removes electrons from iron. Because some methanogens are viable in a hydrogen atmosphere, as are most SRBs, these methanogens may also cause iron corrosion under anaerobic conditions. Several methanogens have been shown to grow and produce methane in medium containing iron as the sole source of electrons (5). The extent of the corrosion by these methanogens was, however, not substantial (2). Others have reported that methanogens do not increase the rate of iron corrosion in comparison with aseptic solutions (6, 7). Recently, Dinh and colleagues (9) isolated a methanogen (strain IM1) that produces methane more rapidly than does *Methanococcus maripaludis* (DSMZ 2771) when cultured with iron granules. Although the rate of iron oxidation was not measured in their experiments, their results suggest that strain IMI oxidizes iron more rapidly than does strain DSMZ 2771. We report herein that a methanogen that was isolated from the sludge of an oil storage tank can, unequivocally, oxidize iron.

**MATERIALS AND METHODS**
Enrichment, isolation, and cultivation of *Methanococcus maripaludis* strain KA1.

Sludge deposited on the bottom plate of a crude-oil storage tank was collected, and ~1 g of the sludge was used to inoculate 40 ml of anoxic seawater medium (AS medium) (27) that contained 3 g of iron granules (1–2 mm in diameter and 99.8% purity; Alfa Aesar) in a 122-ml serum bottle. The culture was incubated at 37°C under a N₂/CO₂ (80:20, vol/vol) atmosphere for two weeks, then, it was diluted 20-fold into the same medium and recultivated as described above. A culture, termed “enrichment culture” was established after five passages. This culture was serially diluted 10-fold 8 times into the same medium, and the diluted cultures were then incubated as described above. The most-dilute sample that still showed methanogenesis activity was again serially diluted 10-fold 8 times into the same medium. The dilution procedure was repeated a total of three times, each time using the most-dilute culture with methanogenesis activity. The last culture was plated onto AS medium with 2% (w/v) agar and cultivated under a H₂/CO₂ (80:20, vol/vol) atmosphere to obtain isolated colonies. Strain KA1 was thus isolated. The strain was cultivated in sulfide-free AS medium supplemented with 3 g of iron and 100 mM

N-2-hydroxyethylpiperazine- N'-ethanesulfonic acid (HEPES; pH 7.0). The ability of KA1 to grow on various substrates was tested at 37°C under a N₂ atmosphere and in bicarbonate-free AS medium (27) supplemented with 100 mM HEPES (pH 7.0) and
filter-sterilized substrates.

Source and cultivation of \textit{M. maripaludis} strain JJ\textsuperscript{T}. \textit{M. maripaludis} strain JJ\textsuperscript{T} (NBRC 101831) was purchased from the NITE Biological Resource Center (NBRC), National Institute of Technology and Evaluation (NBRC) and maintained in AS medium under a H\textsubscript{2}/CO\textsubscript{2} (80:20, vol/vol) atmosphere.

Preparation of DNA and PCR amplification of the 16S rRNA gene. Cells and iron granules from 200-ml cultures were collected aerobically by centrifugation at 20,000 \textit{x} g for 20 min at 4\textdegree C. The procedure of Zhou and colleagues (28) was used for DNA extraction. Partial sequences of 16S rRNA genes were obtained using either a primer set that was specific for bacteria (341F: 5\textsuperscript{\prime}-GGTTACCTTGTTACGACTT-3\textsuperscript{\prime}; \textit{Escherichia coli} positions 341–357 (19) and 1491R: 5\textsuperscript{\prime}-GGTTACCTTGTTACGACTT-3\textsuperscript{\prime}; \textit{E. coli} positions 1491–1509 (26)) or one that was specific for archaea (ARC344F: 5\textsuperscript{\prime}-ACGGGGYGCAGCAGGCGCGA-3\textsuperscript{\prime}; \textit{E. coli} positions 344–363 and ARC915R: 5\textsuperscript{\prime}-GTGCTCCCCCGCCAATTCCT-3\textsuperscript{\prime}; \textit{E. coli} positions 915–934) (4). The primer set ARC8F (5\textsuperscript{\prime}-TCYGGTTGATCCTGCC-3\textsuperscript{\prime}; \textit{E. coli} positions 8–23) and ARC1406R (5\textsuperscript{\prime}-ACGGGCGGTGTGTRCAA-3\textsuperscript{\prime}; \textit{E. coli} positions 1406–1422) was used for amplification of the almost full-length archaeal 16S rRNA gene (13).

PCR was performed in a T-3000 Thermocycler (Biometra). Each 50-\textmu l PCR
reaction contained 0.05 U of LA Taq (TaKaRa), 0.4 mM each of the dNTPs, 2.5 mM MgCl₂, 1× LA buffer II (TaKaRa), 0.4 µM of each primer, and 10 ng of template DNA. The PCR conditions were as follows: 94°C for 1 min, followed by 32 cycles of 98°C for 20 s, 50°C for 20 s, and 72°C for 1 min. The PCR-amplified fragments were purified using the QIAquick PCR Purification Kit (QIAGEN). The purified PCR products were ligated into a pGEM-T vector (Promega) according to the manufacturer’s instructions, and the ligation products were introduced into competent E. coli cells that were supplied with the pGEM-T vector cloning kit. Fifty clones were randomly chosen and sequenced using the primers T7W (5′-TAATACGACTCACTATAGGGC-3′) and SP6W (5′-ATTTAGGTGACACTATAGAATACTC-3′).

DNA sequencing and sequence analyses. Sequencing reactions were carried out using an ABI PRISM BigDye terminator cycle sequencing ready reaction kit (Applied Biosystems). The reaction products were purified according to the manufacturer’s instructions and sequenced using an ABI 3730 automatic sequencer (Applied Biosystems). The 16S rRNA gene sequences were analyzed using GENETX software (Software Kaihatsu), and sequence comparisons were made with the BLAST server (http://blast.ncbi.nlm.nih.gov/Blast.cgi; National Center for Biotechnology Information, National Institutes of Health).
Chemical analyses. Methane, hydrogen, and nitrogen that were produced by the cultures were collected in the headspaces of serum bottles, and the amounts were quantified using a gas chromatograph (GC-14B; Shimadzu, Japan) equipped with a thermal conductivity detector and a molecular sieve (60/80 mesh) column (Shimadzu). The column, injection, and detector temperatures were 95°C, 150°C, and 120°C, respectively. Ferrous iron in the culture media and in whole cultures including iron granules was extracted with 7 N HCl and quantified colorimetrically using the o-phenanthroline method (23), with FeS as the standard.

Microscopy. Phase-contrast and fluorescence micrographs were taken with a camera attached to a fluorescence microscope (model AX70, Olympus). Cells in liquid culture were placed on a black Isopore membrane (pore size, 0.22 µm; Millipore) and fixed with PBS (10 mM sodium phosphate [pH 7.0], 130 mM NaCl) containing 4% (v/v) formaldehyde. They were then stained with 4′,6-diamidino-2-phenylindole (DAPI) and counted with the aid of an epifluorescence microscope.

Scanning electron microscopy. Iron coupons (1 mm × 1 mm × 0.1 mm), after being withdrawn from the culture media, were rinsed with anoxic water, dried, and then stored under a N₂ atmosphere. The surface of the dried coupons were examined with a Hitachi S800 scanning electron microscope by using an accelerated voltage of 15 kV. The coupons were sectioned using a cross-section polisher (JEOL), and the cross-sections
were examined with a scanning electron microscope coupled to an energy-dispersive spectrometer (JEOL JSM7001F) at an acceleration voltage of 15 kV. Minerals on the surface of the iron coupons were analyzed with an X-ray photoelectron spectrometer equipped with a monochromated 450 W Al Kα source (ESCA5500; ULVAC-PHI), and an X-ray diffraction analyzer with CuKα radiation ranging from 2θ = 5 to 100° at a scanning rate of 1°/min (RINT1500; Rigaku).

**Nucleotide sequence and accession number.** The 16S rRNA gene sequence of strain KA1 has been deposited in the DDBJ, EMBL and GenBank nucleotide sequence databases under accession number AB264796. A sample of the strain was provided to the NITE Biological Resource Center (NBRC) of the National Institute of Technology and Evaluation (NITE) under the culture collection accession number NBRC 102054.
RESULTS

Enrichment of microorganisms that use iron as the sole source of electrons.

Enrichment of microorganisms was performed anaerobically in AS medium that contained iron as the sole source of electrons. The medium was inoculated with oil sludge obtained from a crude-oil storage tank. Microbial growth and methane formation were observed in the culture 2 weeks after the inoculation. After five successive transfers of the culture into fresh medium every 2 weeks [5% (v/v) inoculum], methane production increased. Two days after the final inoculation in sulfite-free AS medium, the presence of a white precipitate was noted, and the outer surface of the iron granules became a dull gray color. Fluorescence micrographs of microorganisms found in the enrichment culture showed at least two morphologically distinct microorganisms—an F420-autofluorescent coccoid-shaped one and a rod-shaped one (Fig. 1A and B). The F420-autofluorescent coccoid-shaped microorganism was also found on the surface of the iron debris that formed during cultivation (Fig. 1C and D).

During cultivation of microbes in unbuffered, sulfite-free AS medium, the pH of the medium increased to >8.0 before the methane production and the dissolution of iron ceased, which was concurrent with the cessation of microbial growth. To avoid such a pH change, sulfite-free AS media buffered with 0.1 M HEPES buffer (pH 7.0) were used for
all subsequent experiments.

To identify the microbes in the enrichment culture, partial 16S rRNA gene sequences were PCR-amplified using either bacteria-specific or archaea-specific primers and the extracted DNA from the enrichment culture. DNA fragments that had been amplified using the two primer sets were cloned, and 50 bacteria-specific clones and 50 archaea-specific clones were sequenced. Forty-nine of the fifty bacteria-specific clones had the same sequence, which was most closely related to that of Desulfofustis glycorlcus (97% similarity), whereas the sequence from the remaining clone was most closely related to Pelobacter venetianus (98% similarity). The nucleotide sequences of the 50 archaea-specific clones were all identical to that of M. maripaludis strain JJ\textsuperscript{T}.

Isolation of a microorganism that uses iron as its sole source of electrons. For the isolation of methanogens from the enrichment culture, a serial 10-fold dilution method was used. Substantial methane formation was detected in cultures diluted 10\textsuperscript{5}-fold or more. After having increased the methanogenesis activity by performing three rounds of serial dilutions, further purification was achieved by growing individual colonies on solid agar under a H\textsubscript{2}/CO\textsubscript{2} atmosphere (80:20, vol/vol). After a 7-day incubation at 37°C, colonies developed that were 0.5 mm in diameter. They were white in color and lens shaped. One of the colonies was isolated and named strain KA1. Strain KA1 was classified as a M. maripaludis because its rRNA gene sequence was identical to the
sequence of the type strain of that species. Strain KA1 was weakly motile and did not form spores. The cells were Gram negative and grew to 0.5–1.2 µm in length. Among the potential growth substrates tested at a concentration of 20 mM, strain KA1 did not grow under a N₂ atmosphere in the presence of acetate, lactate, methanol, ethanol, 2-propanol, methylamine, pyruvate, or dimethylsulfide within a 14-day period at 37°C. Conversely, strain KA1 grew in the presence of formate under a N₂ atmosphere and in the presence of iron under either a N₂/CO₂ atmosphere (80:20, vol/vol) or a H₂/CO₂ atmosphere (80:20, vol/vol).

Enhancement of iron corrosion in microbial cultures. The extent of methane production, hydrogen production, iron dissolution, and the increase in the cell count for an enrichment culture, for a strain KA1 culture, and for a strain JT culture (16) were measured in sulfite-free medium containing iron granules as the sole source of electrons (Fig. 2). Hydrogen was produced on the surface of the iron granules in the control medium, as has been observed previously (24). Although the cell count of strain JT increased slightly and produced methane, the rate of dissolution of iron to ferrous ion by this strain was similar to that found for the control. Thus, low levels of methane production and a slight population increase were observed for strain JT, which may have been the result of strain JT using H₂ generated by the abiotic oxidation of iron. Strain KA1 produced methane to a much greater extent than did strain JT (Fig. 2D). The cell
count of strain KA1 was 40 times greater than that of strain JJT; whereas the dissolution
of iron to ferrous ion by strain KA1 was almost eight-fold greater than those of the JJT
culture and the control.

The rates of methane production and iron dissolution by strain KA1 were very
similar to those found for the enrichment culture from which strain KA1 had been
isolated. When 2-bromoethanesulfonate (BESA; Sigma), a methanogen inhibitor (10),
was added at a concentration of 20 mM to the enrichment culture immediately after the
start of cultivation, the increase in the cell count and methane production were completely
inhibited, and iron dissolution was reduced to the level found for the aseptic control (Fig.
2). These results strongly indicate that strain KA1 was mainly responsible for the iron
corrosion in the enrichment culture. As was found for the enrichment culture, the strain
KA1 culture contained a white precipitate, and the outer surface of the iron granules in
the culture were a dull gray color. Such phenomena were not observed in the strain JJT
culture or in the aseptic control.

The reaction $8\text{H}^+ + 4\text{Fe} + \text{CO}_2 \rightarrow \text{CH}_4 + 4\text{Fe}^{2+} + 2\text{H}_2\text{O}$ is expected to occur during
methanogenesis when iron is the sole electron donor (25). The expected stoichiometric
ratio of ferrous ion to CH$_4$ was, however, found only for strain JJT; for strain KA1, the
molar amount of ferrous ion released from the iron granules was only half of what was
expected when the cultural fluid was assayed. The smaller amount of ferrous ion may
have resulted from precipitation of ferrous ion (as FeCO$_3$) on the surface of the iron granules. In fact, when the whole culture was extracted with 6 N HCl, a stoichiometry of 3.8:1 was found for HCl-extracted iron (1,710 µmol) to CH$_4$ (451 µmol).

**Surface analyses of iron coupons.** Iron coupons that had been submerged in the KA1 or JJ$^T$ cultures or in an aseptic culture for 2 weeks were analyzed by scanning electron microscopy (Fig. 3). The surfaces of the coupons in the aseptic control and in the strain JJ$^T$ cultures were covered with small crystalline deposits. The surfaces of the coupons in the strain KA1 culture were quite different in appearance, with plate-shaped crystals that flaked off of the coupons. The cross-sections of the KA1 coupons showed that the surfaces had corroded to a much greater extent than did those of the other coupons. X-ray photoelectron spectroscopy and X-ray-diffraction analyses revealed that the crystals on these coupons consisted mainly of FeCO$_3$.

**DISCUSSION**

The great iron-corroding ability that was found for strain KA1 is strain specific, because the type strain of *M. maripaludis*, JJ$^T$, was not corrosive. The family Methanococcaceae contains two genera, *Methanococcus* and *Methanothermococcus*. 
Methanothermococcus thermolithotrophicus strains have been isolated from the reservoir water of a North Sea oilfield (strain ST22) and from coastal geothermally heated sediments near Naples, Italy (strain SN-1) (14). Strain SN-1 grows and produces methane when iron is the sole electron donor (5). More recently, Dinh and colleagues (9) isolated a methanogen (strain IM1) that produces methane more rapidly than *M. maripaludis* when iron is the sole source of electrons. The analysis of the 16S rRNA gene sequence of strain IM1 indicates that the strain is not a member of the *Methanococcaceae* family. Therefore, the ability of methanogens to extract electrons from iron may not be confined to any specific phylogenetic group.

The growth properties of strain KA1 under a H\(_2\)/CO\(_2\) atmosphere (80:20, vol/vol) were similar to those of *M. maripaludis* strain JJ\(^T\) (data not shown). Conversely, the growth rate with iron as the sole source of electrons and the dissolution rate of iron into ferrous ion were much greater for strain KA1 than for strain JJ\(^T\); the rate of ferrous ion production in strain JJ\(^T\) was almost identical to that of the aseptic control (i.e., it was almost identical to the rate of the abiotic chemical reaction). Therefore, (i) the growth of strain JJ\(^T\) in the presence of iron seems to depend on using hydrogen generated by the abiotic chemical reaction as the source of electrons; and (ii) the metabolic consumption of hydrogen by strain JJ\(^T\) is not sufficient to cause the dissolution of iron. It also may be inferred that strain KA1 maybe equipped with a more efficient mechanism that allows it...
to withdraw either cathodic electrons or anodic ferrous ion (e.g., by precipitating ferrous iron as carbonate salt and consequently shift the equilibrium to favor $\text{Fe}^{2+}$).

Other methanogens that are viable when iron is the sole source of electrons have been studied (2, 5, 9). The large rate of iron corrosion by *M. thermolithotrophicus*, as originally estimated from the rate of methane production (5), was later found to be an overestimation. When the actual rate of iron corrosion was experimentally determined at a later date, it was found to be increased by, at most, two-fold (2). In contrast to the previous studies, strain KA1 enhanced the rate of iron corrosion (i.e., iron dissolution) almost 10-fold. Furthermore, both the X-ray photoelectron spectroscopy and X-ray-diffraction analyses revealed that the corrosion products consisted mainly of $\text{FeCO}_3$.

In the oil and gas industries, carbon dioxide corrosion of iron ("sweet corrosion") that forms pits in the iron (8) is a very serious problem. To date, methanogens have not been found to be responsible for such corrosion pits, only because attention has focused on SRBs, which have long been considered to be the major MIC microbe, and because FeS (generated by sour corrosion) has been used as the fingerprint for MIC (3). Our study highlights the possible involvement that some methanogens may have in both carbon dioxide–mediated corrosion and the pitting of steel. In conclusion, the present study provides data that MIC is generated not only by the presence of SRBs but also by
methanogens in anaerobic environments.

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FIGURE LEGENDS

FIG. 1. Photomicrographs of microorganisms present after five successive transfers in an enrichment culture for which iron was the sole source of electrons. (A) Phase-contrast micrograph of cells in the enrichment culture. (B) Fluorescence micrograph of the sample shown in (A). (C) Phase-contrast micrograph of a small corrosion particle. (D) Fluorescence micrograph of the particle shown in (C). The microbes that autofluorescence at 420 nm (F420) are methanogens. Bars are 5 µm.
FIG. 2. Cell count, iron dissolution, hydrogen production, and methane production. All experiments were carried out with iron granules as the sole source of electrons. (A) Cell count, (B) ferrous iron concentration in the supernatants of centrifuged cultures, (C) hydrogen production, and (D) methane production. Open squares, enrichment culture; filled squares, enrichment culture supplemented with 20 mM BESA; filled circles, strain KA1 culture; open circles, strain JJ\textsuperscript{T} culture; and filled diamond, aseptic control. Data points and bars are the means and standard deviations, respectively (n = 4).

FIG. 3. Scanning electron micrographs of the surface and cross-section of iron coupons present in strain JJ\textsuperscript{T} and strain KA1 cultures. (A) The surface of an iron coupon incubated for 2 weeks in a JJ\textsuperscript{T} culture. (B) The cross-section of an iron coupon incubated as in (A). (C) The surface of an iron coupon incubated for 2 weeks in a KA1 culture. (D) The cross-section of an iron coupon incubated as in (C).
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Fig. 2. Uchiyama et al.

Cell counts (Cells/ml)
Ferrous iron (umol/40 ml medium)

H₂ production (umol/82 ml head space)

CH₄ production (umol/82 ml head space)

0 5 10 15
600 400 200
0
600 400 200
0
600
900 300
0
1200

A B C D

Day

Day

Day

Day
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