Microbiologically influenced corrosion (MIC) of metallic materials imposes a heavy economic burden. The mechanism of MIC of metallic iron (Fe⁰) under anaerobic conditions is usually explained as the consumption of cathodic hydrogen by hydrogenotrophic microorganisms that accelerates anodic Fe⁰ oxidation. In this study, we describe Fe⁰ corrosion induced by a nonhydrogenotrophic nitrate-reducing bacterium called MIC1-1, which was isolated from a crude-oil sample collected at an oil well in Akita, Japan. This strain requires specific electron donor-acceptor combinations and an organic carbon source to grow. For example, the strain grew anaerobically on nitrate as an sole electron acceptor with pyruvate as a carbon source and Fe⁰ as the sole electron donor. In addition, ferrous ion and L-cysteine served as electron donors, whereas molecular hydrogen did not. Phylogenetic analysis based on 16S rRNA gene sequences revealed that strain MIC1-1 was a member of the genus Prolixibacter in the order Bacteroidales. Thus, Prolixibacter sp. strain MIC1-1 is the first Fe⁰-corroding representative belonging to the phylum Bacteroidetes. Under anaerobic conditions, Prolixibacter sp. MIC1-1 corroded Fe⁰ concomitantly with nitrate reduction, and the amount of iron dissolved by the strain was six times higher than that in an aseptic control. Scanning electron microscopy analyses revealed that microscopic crystals of FePO₄ developed on the surface of the Fe⁰ foils, and a layer of FeCO₃ covered the FePO₄ crystals. We propose that cells of Prolixibacter sp. MIC1-1 accept electrons directly from Fe⁰ to reduce nitrate.

MATERIALS AND METHODS

Sampling of crude oil. Crude oil was sampled from an oil well in Akita Prefecture, Japan, on 16 December 2004. The oil sample was kept in a sealed nylon bag with an O₂-absorbing agent and a CO₂-generating agent (Anaero-Pack; Mitsubishi Gas Chemical, Tokyo, Japan) until inoculation on fresh culture medium.

Enrichment, isolation, and cultivation of a bacterial strain from a crude-oil sample. Sw medium was composed of (liter⁻¹) 0.54 g NH₄Cl, 0.14 g KH₂PO₄, 0.20 g MgCl₂·6H₂O, 0.15 g CaCl₂·2H₂O, 2.5 g NaHCO₃, 0.2 ml of vitamin solution (9) and 0.2 ml of a reductant solution containing 1 M sodium pyruvate, and 0.01% (wt/vol) yeast extract (Becton Dickinson, Franklin Lakes, NJ, USA) to prepare SPYSw medium. The SPYSw medium (20 ml) was inoculated with 0.5 ml of the crude oil sampled from an oil well in Akita Prefecture, Japan, on 16 December 2004. The oil sample was kept in a sealed nylon bag with an O₂-absorbing agent and a CO₂-generating agent (Anaero-Pack; Mitsubishi Gas Chemical, Tokyo, Japan) until inoculation on fresh culture medium.

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an oil well and cultivated at 25°C for 3 weeks. After the transfer of the culture into fresh medium, it was further cultivated for 3 weeks. This process was repeated several times, and strain MIC1-1 was purified on slants of SPSSw medium solidified with 1.5% (wt/vol) agar. Purification on agar slants was repeated several times until the cultures were deemed pure and a uniformly shaped axenic culture, designated strain MIC1-1, was obtained.

**Bacterial strain.** *Prolixibacter bellariivorans* JCM 13498T was obtained from the Japan Collection of Microorganisms of the RIKEN Bioresource Center (RIKEN-BRC JCM) and maintained in JCM medium no. 512 (http://www.jcm.riken.jp/JCM/catalogue.shtml).

**Microscopy.** Routine microscopic observation was performed with an IX-81 microscope (Olympus Co., Tokyo, Japan). Transmission electron micrographs were made with an H-7600 electron microscope (Hitachi High-Technologies Co., Tokyo, Japan) operated at 80 kV. Cells were stained with 1% (wt/vol) phosphotungstic acid (pH 6.5 to 7.0) and 0.04% (wt/vol) lead citrate.

**Growth tests with possible electron donors and acceptors.** The use of various compounds as electron acceptors or donors by strain MIC1-1 was determined by growth tests and measurement of the consumption of the compounds. For the identification of electron acceptors, the growth of strain MIC1-1 was tested in a screw-cap tube (18 by 180 mm; Sanshin Industrial Co., Yokohama, Japan) containing 10 ml of Sw medium (pH 7.0) lacking NH4Cl and supplemented with 0.1 ml of the vitamin solution (9), 2 mM l-cysteine-HCl, 10 mM sodium pyruvate, 0.01% (wt/vol) yeast extract, and various concentrations of potential electron acceptors, including sulfate (10 mM), sulfite (2 mM), thiosulfate (5 mM), elemental sulfur (1%, wt/vol), nitrate (10 mM), nitrite (2 mM), ferric chloride (2 mM), molecular oxygen (5% [vol/vol]), and fumarate (10 mM). Potential electron donors, including H2-CO3 (1%, 5%, and 10% [vol/vol]), sulfite (2 mM), elemental sulfur (1% [vol/vol]), thiosulfate (5 mM), sulfite (2 mM), ammonium (10 mM), nitrite (2 mM), Fe0 granules (diameter, 1 to 2 mm; Fe purity, 99.98%; Alfa Aesar, Lancaster, United Kingdom) (10% [wt/vol]), ferrous chloride (2 mM), and l-cysteine–HCl (2 mM), were also screened by growth tests in 10 ml of Sw medium (pH 7.0) lacking NH4Cl and supplemented with 0.1 ml of the vitamin solution (9), 10 mM sodium nitrate, 10 mM sodium pyruvate, and 0.01% (wt/vol) yeast extract. To a 10-ml volume of each of these Sw-based media, 0.01 ml of a preculture of strain MIC1-1 was added, followed by incubation at 25°C for 30 days. Bacterial growth was determined by measurement of the optical density at 660 nm with a Genesys 20 spectrophotometer (Thermo Scientific, MA, USA). The concentrations of electron acceptors and donors and their oxidoreduction products in each of the cultures were quantified with a high-performance liquid chromatography (HPLC) system (model HIC-20Asuper; Shimadzu Corp., Kyoto, Japan) equipped with a conductivity detector (model CDD-10ADsp), a Shim-Pack cation column (IC-C4), and a Shim-Pack anion column (IC-SA2).

**Preparation of DNA, PCR amplification of the 16S rRNA gene, and DNA sequencing.** Harvested cells (approximately 1 mg fresh weight) of strain MIC1-1 were lysed using 0.1 ml of a cell lysis solution (10 mg of lysozyme [Wako Pure Chemical Industries, Ltd., Osaka, Japan]/ml of Tris-EDTA [TE] buffer [pH 8.0]) at 37°C for 1 h, and the genomic DNA was extracted and purified by the method of Saito and Miura (10). The 16S rRNA gene was amplified by PCR with a forward primer (27F, 5'-AGAGTTTGATCCTGTCAG-3') and a reverse primer (920R, 5'-GTTAGCTTCTTGGA-3') containing a sequencing primer (positions 1510 to 1492). The PCR mixture (50 µl) contained 1× PCR buffer, 3.5 mM MgCl2, 10 mM deoxynucleoside triphosphates (dNTPs), 1.25 U AmpliTaq Gold (Applied Biosystems, CA, USA), and 0.4 µM of each forward and reverse primers. Approximately 100 ng of genomic DNA was used as a template under the following cycling conditions: initial AmpliTaq Gold activation at 95°C for 9 min, followed by 25 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 30 s, extension at 72°C for 60 s, and a final extension step at 72°C for 10 min. The PCR product was purified with the QIAquick PCR purification kit (Qiagen, Venlo, Netherlands), and an almost-complete 16S RNA gene sequence (1,444 bp) was determined by a 3130xl genetic analyzer (Applied Biosystems), the BigDye Terminator v3.1 cycle-sequencing kit, and one of the following six primers: 27F, 5'-GTGCCCAGCACCGCCGG-3', 920F (5'-AAACTCAAGGAATGAC-3', 520R (5'-ACGGCGGCTGCTGAC-3', 920R (5'-GCTAATCCCTTGAGTTT-3'), and 1492R.

**Phylogenetic analyses.** According to the method previously described (11), the 16S RNA gene sequences of 55 phylogenetically related bacteria in the order *Bacteroidales* were selected. Phylogenetic trees were constructed by the neighbor-joining (NJ) method with CLUSTAL X (12,13) and the maximum-likelihood (ML) method with MORPHY version 2.3b3 (14,15).

**Fe\(^{2+}\) corrosion test.** For Fe\(^{2+}\) corrosion tests, 20 ml of sulfide-free artificial-seawater medium supplemented with 100 mM HEPES buffer (pH 7.0) was prepared according to the method of Uchiyama et al. (7) and added anaerobically to anaerobic serum bottles containing either 1.5 g of Fe0 granules or an Fe0 foil (purity, >99.99%, 10 by 0.1 mm; Sigma-Aldrich, St. Louis, MO, USA). Air in the medium was removed by flushing with N2-CO2 (4:1 [vol/vol]), and the bottle was sealed with a butyl rubber stopper. If necessary, filtered solutions of 10 mM nitrate, 10 mM acetate, 10 mM citrate, 10 mM lactate, and/or 10 mM pyruvate were added to the medium as described above before inoculation with 0.2 ml of a preculture of strain MIC1-1. The culture was incubated at 25°C for 30 days.

**Chemical cultures.** Culture fluids (100 µl) containing dissolved iron were either acidified with 50 µl of 6 M HCl and reduced with 100 µl of 1 M ascorbic acid for the quantification of total iron (ferrous and ferric ions) or acidified with 150 µl of sulfuric acid, pH 2.5, for the quantification of ferrous ions. The iron ion concentration in each of the acidified solutions was determined colorimetrically using o-phenanthroline, as described by Sandell (16). The concentration of ferric ions in the culture fluid was calculated by subtraction of the concentration of ferrous ions from that of total iron ions. Molecular hydrogen concentrations in the headspaces of serum bottles were quantified on a gas chromatograph equipped with a thermal conductivity detector and a molecular-sieve 60/80 mesh column (Shimadzu Corp., Kyoto, Japan). The column, injector, and detector temperatures were set at 95°C, 150°C, and 120°C, respectively. Nitrate, nitrite, and ammonium ions in the culture were quantified with a TRAACS 2000 autoanalyzer (BLTEC K.K., Osaka, Japan). Organic acids in culture fluids were quantified on an ICS-2000 ion chromatography system ( Dionex, CA, USA).

**Scanning electron microscopy of the surfaces and cross sections of Fe0 foils.** Scanning electron microscopy with energy-dispersive X-ray spectroscopy (SEM/EDX) was used for surface analyses of corroded Fe0 foils, as previously described (7).

**Accession numbers.** *Prolixibacter* sp. MIC1-1 has been deposited in the RIKEN-BRC JCM and National Institute of Technology and Evaluation (NITE) Biological Resource Center (NBRC) of NITE under the culture collection accession numbers JCM 18694 and NBRC 102688, respectively. The 16S rRNA gene sequence of *Prolixibacter* sp. MIC1-1 has been deposited in the DDBJ/EMBL/GenBank nucleotide sequence database under accession number AB986195.

**RESULTS**

**Growth properties of strain MIC1-1.** As shown in Table 1, an appropriate electron donor, electron acceptor, and organic carbon compound must be supplied for growth of strain MIC1-1. When nitrate and pyruvate were provided under anaerobic conditions as an electron acceptor and a carbon source, respectively, Fe0, ferric ion, and l-cysteine served as sole electron donors, whereas molecular hydrogen (Fig. 1), sulfide, elemental sulfur, thiosulfate, sulfate, ammonium, and nitrite did not (Table 1). Pyruvate did not serve as an electron donor, indicating that the strain is lithotrophic.

The strain grew with molecular oxygen as an electron acceptor in the presence of l-cysteine as an electron donor and pyruvate as a carbon source. The roles of Fe0 and ferrous ion as electron do-
nons could not be confirmed under aerobic conditions because of insoluble ferric compound formation that interfered with growth measurement. Aside from nitrate and molecular oxygen, other compounds, including sulfate, sulfite, thiosulfate, elemental sulfur, nitrite, ferric oxide, and fumarate, did not serve as sole electron acceptors.

The strain did not use CO₂ as a sole carbon source, even in the presence of l-cysteine and nitrate (Table 2), but used pyruvate (10 mM) as a carbon source under aerobic (with molecular oxygen as an electron acceptor) or anaerobic (with nitrate as an electron acceptor) conditions in the presence of l-cysteine (Table 2), indicating that the bacterium is lithoheterotrophic.

**Taxonomic characterization of strain MIC1-1.** Phylogenetic analysis based on the 16S rRNA gene sequence showed that strain MIC1-1 belonged to the family *Prolixibacteraceae* in the order *Bacteroidales* and formed an independent phylogenetic lineage with *P. bellariivorans* JCM 13498T with high bootstrap values by two phylogenetic analysis methods (Fig. 2). The 16S rRNA gene sequence similarity of strain MIC1-1 to *P. bellariivorans* JCM 13498T was 97.5%. Therefore, strain MIC1-1 was provisionally identified as *Prolixibacter* sp.

Cells of *Prolixibacter* sp. MIC1-1 were rods 0.3 to 0.5 μm wide and 3.4 to 6.3 μm long (Fig. 3). Motility, flagellation, and spore formation were not observed under phase-contrast and electron microscopy. In summary, *Prolixibacter* sp. MIC1-1 is a facultatively aerobic, obligately heterotrophic, iron-oxidizing and nitrate-reducing bacterium (NRB) belonging to the order *Bacteroidales*.

**Fe⁰ corrosion by the bacterial isolate.** *Prolixibacter* sp. MIC1-1 oxidized Fe⁰ granules in the presence of 10 mM nitrate when artificial-seawater medium was supplemented with 10 mM acetate, 10 mM lactate, or 10 mM pyruvate as a carbon source (Table 3). The surfaces of the Fe⁰ granules in these cultures turned dull gray to grayish black, and the culture fluids assumed a light-yellow color. The ratio of ferrous ion to ferric ion in these cultures ranged from approximately 3:2 to 2:3, whereas it was approximately 4:1 in the aseptic control. Slight Fe⁰ granule oxidation by *Prolixibacter* sp. MIC1-1 was detected in the presence of nitrate or in the absence of an electron donor, but the metal surface did not lose its luster. *Prolixibacter* sp. MIC1-1 did not oxidize Fe³⁺ granules in the absence of nitrate (Table 3).

Importantly, *P. bellariivorans* JCM 13498T, a close relative of *Prolixibacter* sp. MIC1-1, did not oxidize Fe³⁺ granules under any of the culture conditions described above (data not shown). Thus, the ability of *Prolixibacter* sp. MIC1-1 to use Fe³⁺ as an electron donor is not a common trait of the genus *Prolixibacter*.

The amount of dissolved iron in *Prolixibacter* sp. MIC1-1 cultures in the presence of nitrate and lactate was approximately six

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### Table 1: Growth of *Prolixibacter* sp. MIC1-1 on various media under anaerobic and aerobic conditions

<table>
<thead>
<tr>
<th>Component or characteristic</th>
<th>Anaerobic conditions (condition no.)</th>
<th>Aerobic conditions (condition no.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parameter</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Electron acceptor</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nitrate</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Nitrate</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Nitrate</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Molecular oxygen</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Molecular oxygen</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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### Table 2: Growth of *Prolixibacter* sp. MIC1-1 on various carbon sources under anaerobic and aerobic conditions

<table>
<thead>
<tr>
<th>Component or characteristic</th>
<th>Anaerobic conditions with indicated carbon source</th>
<th>Aerobic conditions with indicated carbon source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parameter</td>
<td>CO₂</td>
<td>Pyruvate</td>
</tr>
<tr>
<td>Electron acceptor</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Electron donor</td>
<td>L-Cysteine</td>
<td>+</td>
</tr>
</tbody>
</table>

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* a, growth; −, no growth.

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**FIG 1** Use of molecular hydrogen as a sole electron donor by *Prolixibacter* sp. MIC1-1. Experiments were performed under three different initial concentrations of molecular hydrogen (8.8, 48.5, and 94.7 μmol/20 ml) with nitrate as the electron acceptor, and the concentration of residual molecular hydrogen in the headspace (20 ml) of each bottle was determined at the end of the experiment. Solid bars, *Prolixibacter* sp. MIC1-1 cultures; open bars, aseptic control. The data points and error bars represent means and standard deviations, respectively (n = 3).
times greater than that in the aseptic control (Fig. 4A). Molecular hydrogen was detected in the aseptic control, but not in the culture of Prolixibacter sp. MIC1-1 grown in the presence of nitrate and lactate (Fig. 4B). A significant amount of nitrate was transformed to nitrite or ammonium in the Prolixibacter sp. MIC1-1 culture. The reduction of nitrate to nitrite or ammonium, but in much smaller amounts, was also observed in the aseptic control (Fig. 4C, D, and E). Nitrite was the sole metabolic product of nitrate in Prolixibacter sp. MIC1-1 when L-cysteine was used as the sole electron donor (data not shown). This result indicated that ammonium was formed by a chemical reaction between nitrate/nitrite and Fe⁰, as reported previously (17, 18). The amount of lactate in the Prolixibacter sp. MIC1-1 culture decreased compared with that in the aseptic control (Fig. 4F).

**Electron microscopic analyses of corroded Fe⁰ foils.** Prolixibacter sp. MIC1-1 was cultivated in a sulfide-free artificial seawater medium supplemented with nitrate and lactate in which an Fe⁰ foil was submerged. After incubation for 30 days, the surfaces of the Fe⁰ foils were observed with a scanning electron microscope, together with Fe⁰ foils incubated in aseptic media. As shown in Fig. 5A and B, crystal structures developed on the surfaces of Fe⁰ foils that had been submerged in a culture of Prolixibacter sp. MIC1-1. Rod-like extrusions 3 to 6 μm long were also observed (Fig. 5B), with the size corresponding to that of Prolixibacter sp. MIC1-1. On the surfaces of Fe⁰ foils incubated under aseptic conditions, smaller crystal structures developed (Fig. 5D and E). X-ray photoelectron spectroscopy and X-ray diffraction analyses of cross-sectional samples revealed that the crystals that developed on the Fe⁰ foil surface mainly consisted of FePO₄ (Fig. 5C and F). The crystals on the Fe⁰ foils from a Prolixibacter sp. MIC1-1 culture were covered by a layer of FeCO₃ (Fig. 5C). Such a layer was not observed in Fe⁰ foils from aseptic controls (Fig. 5F).

**DISCUSSION**

This study reports the isolation and characterization of the Fe⁰-corroding facultatively aerobic bacterium Prolixibacter sp. MIC1-1, which is a member of the order Bacteroidales (Fig. 2). The genus Prolixibacter may be widespread in the marine environment, because *P. bellariivorans* and its phylogenetic relatives have previously been cultured from estuarine and marine sediments and an offshore oil reservoir (19–21). To date, methanogens belonging to the phylum Euryarchaeota and SRB and iron-oxidizing bacteria, both belonging to the phylum Proteobacteria, have frequently been studied as causative microorganisms of MIC (3, 6, 7, 22, 23). To our knowledge, Prolixibacter sp. MIC1-1 is the first Fe⁰-corroding representative belonging to the phylum Bacteroidetes.

The molar amount of iron dissolved by Prolixibacter sp. MIC1-1 cultivated for 30 days corresponded to 2/3 of that dis-
solved by *Methanococcus maripaludis* KA1 (7) and 1.8 times that dissolved by *Mariprofundus* sp. strain GSB2 (6).

The finding that the major corrosion products of Fe\(^0\) formed in anaerobic cultures of *Prolixibacter* sp. MIC1-1 were FePO\(_3\) and FeCO\(_3\) indicates that Fe\(^0\) was oxidized to both ferrous and ferric ions. The oxidation to ferric ion by MIC under anaerobic conditions was unusual because ferrous compounds have generally been detected as corrosion products in SRB- and methanogen-mediated MIC (7,22). We propose the following mechanisms for the production of ferric ion from Fe\(^0\) by *Prolixibacter* sp. MIC1-1.

The strain may oxidize Fe\(^0\) primarily to ferrous ion, similarly to other corrosive microorganisms:

\[
\text{Fe}^0 + \text{NO}_3^- + 2\text{H}^+ \rightarrow \text{Fe}^{2+} + \text{NO}_2^- + \text{H}_2\text{O} \quad (1)
\]

Given that *Prolixibacter* sp. MIC1-1 was able to use ferrous ion as an electron donor, ferrous ion formed by equation 1 may be further oxidized to ferric ion:

\[
2\text{Fe}^{2+} + \text{NO}_3^- + 2\text{H}^+ \rightarrow 2\text{Fe}^{3+} + \text{NO}_2^- + \text{H}_2\text{O} \quad (2)
\]

In addition, chemical reactions that oxidize Fe\(^0\) to ferrous ion

\[
\text{Fe}^0 + \text{H}_2\text{O} \rightarrow \text{Fe}^{2+} + 2\text{H}^+ + \text{e}^{-} \\
\text{Fe}^{2+} + \text{H}_2\text{O} \rightarrow \text{Fe}^{3+} + \text{H}_2 + \text{e}^{-}
\]

### TABLE 3 Fe\(^0\) corrosion by *Prolixibacter* sp. MIC1-1 in the presence of various carbon sources

<table>
<thead>
<tr>
<th>Strain</th>
<th>Electron acceptor</th>
<th>Carbon source</th>
<th>Dissolved iron ((\mu\text{mol}/20 \text{ ml}))(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Fe(^2+)</td>
<td>Fe(^3+)</td>
</tr>
<tr>
<td><em>Prolixibacter</em> sp. MIC1-1</td>
<td>Nitrate</td>
<td>Acetate</td>
<td>156.7 ± 3.6</td>
</tr>
<tr>
<td></td>
<td>Nitrate</td>
<td>Citrate</td>
<td>99.5 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>Nitrate</td>
<td>Lactate</td>
<td>235.3 ± 4.1</td>
</tr>
<tr>
<td></td>
<td>Nitrate</td>
<td>Pyruvate</td>
<td>224.3 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>Nitrate</td>
<td>None</td>
<td>60.0 ± 1.4</td>
</tr>
<tr>
<td><em>Prolixibacter</em> sp. MIC1-1</td>
<td>None</td>
<td>Lactate</td>
<td>27.8 ± 4.2</td>
</tr>
<tr>
<td>Aseptic control</td>
<td>Nitrate</td>
<td>Lactate</td>
<td>64.8 ± 9.5</td>
</tr>
</tbody>
</table>

\(^a\) ND, not determined.

**FIG 4** Fe\(^0\) dissolution (A), molecular-hydrogen production (B), nitrate consumption (C), nitrite (D) and ammonium (E) production, and lactate consumption (F) in *Prolixibacter* sp. MIC1-1 cultures and in aseptic controls. All experiments were performed with Fe\(^0\) granules as the sole source of electrons. Solid bars, *Prolixibacter* sp. MIC1-1; open bars, aseptic control. The data points and error bars represent means and standard deviations, respectively (\(n = 3\)). The ammonium produced was calculated by subtracting the amount of ammonium initially added from the amount of ammonium detected at the end of the experiment.
concomitant with the reduction of nitrate to either nitrite (equation 1) or ammonium (equation 3) are known (17, 18).

\[
4.5 \text{Fe}^0 + \text{NO}_3^- + 10 \text{H}^+ \rightarrow \text{Fe}^{2+} + \text{NH}_4^+ + 3 \text{H}_2 \text{O} \quad (3)
\]

Chemical reactions that oxidize ferrous ion to ferric ion concomitant with the reduction of nitrate either to nitrite (equation 2) or to ammonium (equation 4) have also been reported (24).

\[
9 \text{Fe}^{2+} + \text{NO}_3^- + 10 \text{H}^+ \rightarrow 9 \text{Fe}^{3+} + \text{NH}_4^+ + 3 \text{H}_2 \text{O} \quad (4)
\]

Thus, the reduction of nitrate to either nitrite or ammonium coupled with the oxidation of Fe$^0$ to Fe$^{3+}$ is described as follows:

\[
2/3 \text{Fe}^0 + \text{NO}_3^- + 2 \text{H}^+ \rightarrow 2/3 \text{Fe}^{3+} + \text{NO}_2^- + \text{H}_2 \text{O} \quad (5)
\]

\[
3 \text{Fe}^0 + \text{NO}_3^- + 10 \text{H}^+ \rightarrow 3 \text{Fe}^{3+} + \text{NH}_4^+ + 3 \text{H}_2 \text{O} \quad (6)
\]

The stoichiometric relationship between Fe$^0$ oxidation and nitrate reduction was investigated in an aseptic medium (Fig. 4). In this experiment, 21 μmol of dissolved iron was detected in the culture fluid, 34 μmol of molecular hydrogen was produced in the headspace, and 1 μmol of nitrite and 4 μmol of ammonium were detected in the culture fluid.

Two-step chemical reactions that generate molecular hydrogen from Fe$^0$ are known. As shown in equation 7, Fe$^0$ undergoes oxidation at the anode by a reaction whose rate is low under anaerobic conditions at neutral pH:

\[
\text{Fe}^0 \rightarrow \text{Fe}^{2+} + 2e^- \quad (7)
\]

At the anode, electrons generated in reaction 7 are consumed to produce molecular hydrogen if no other electron acceptor is present:

\[
2\text{H}^+ + 2e^- \rightarrow \text{H}_2 \quad (8)
\]

The generation of 34 μmol of molecular hydrogen, 1 μmol of nitrite, and 4 μmol of ammonium in the aseptic medium required 106 μmol of electrons. Given that the ratio of ferrous and ferric ions present in the aseptic culture fluid was approximately 4:1, 1 μmol of Fe$^0$ was estimated to generate 2.2 μmol of electrons. Thus, 48 μmol of Fe$^0$ was calculated to be dissolved, whereas the experimentally determined value was 21 μmol. Given that the acid extraction of iron ions from crystal structures of FePO$_4$, developed on the surface of Fe$^0$ (Fig. 5) may be inefficient, the experimentally determined iron ion concentrations may be underestimated.

In the culture of Prolixibacter sp. MIC1-1, approximately 124 μmol of nitrate was transformed and 38 μmol of nitrite and 55 μmol of ammonium were produced. The inconsistency between

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**FIG 5** Scanning electron micrographs of the surfaces and cross sections of Fe$^0$ foils submerged in a Prolixibacter sp. MIC1-1 culture and an aseptic medium. (A and B) Surface of an Fe$^0$ foil incubated in the Prolixibacter sp. MIC1-1 culture. (C) Cross section of an Fe$^0$ foil incubated in the Prolixibacter sp. MIC1-1 culture. (D and E) Surface of an Fe$^0$ foil incubated in the aseptic medium. (F) Cross section of an Fe$^0$ foil incubated in the aseptic medium.
the amount of nitrate reduced and the amounts of nitrite and ammonium formed could be the result of experimental error. For the production of 38 μmol of nitrate and 55 μmol of ammonium, 571 μmol of electrons was required. Given that the ratio of ferrous and ferric ions present in Prolixibacter sp. MIC1-1 culture was approximately 1:1, 1 μmol of Fe⁰ was estimated to generate 2.5 μmol of electrons. Thus, 228 μmol of Fe⁰ was calculated to have been dissolved, whereas the experimentally determined value was 136 μmol (Fig. 4). As proposed above, the discrepancy could be due to the inefficiency of extraction of iron ions from crystal structures of FePO₄ developed on the surface of Fe⁰ (Fig. 5).

Molecular hydrogen, which accumulated in the aseptic control, was not detected in the Prolixibacter sp. MIC1-1 culture. We speculate, but did not show, that electrons and molecular hydrogen generated in the reactions shown in equations 7 and 8 were largely consumed by the chemical reduction of nitrite to ammonium. In the aseptic medium, molecular hydrogen accumulated because the speed of nitrite formation was lower than that of molecular hydrogen formation, whereas in the Prolixibacter sp. MIC1-1 culture, the speed of nitrite formation greatly exceeded that of molecular hydrogen formation.

Given that Prolixibacter sp. MIC1-1 reduced nitrate only to nitrite when the electron donor was L-cysteine, we infer that the contribution of biotic activity in Fe⁰ corrosion was limited to Fe⁰ oxidation coupled to the reduction of nitrate to nitrite. Assuming that the amount of biologically reduced nitrate was 88 [38 + 55 − (1 + 4)], it was approximately 18 times [88/(4 + 1)] higher than that of chemically reduced nitrate in the aseptic control. As described above, the Fe⁰ corrosion activity in the Prolixibacter sp. MIC1-1 culture was expressed as 571 μmol of electrons, whereas the biological activity generated only 176 μmol of electrons, or 30% of the total corrosion activity.

NRB-assisted Fe⁰ corrosion has been reported mainly in association with the remediation of nitrate-contaminated groundwater using granular Fe⁰. The biological denitrification of groundwater by a combination of a hydrogenotrophic NRB and Fe⁰ granules has been studied, and the addition of an NRB, such as Paracoccus denitrificans ATCC 17741, or an NRB-containing microbial consortium generally resulted in faster nitrate removal and faster Fe⁰ oxidation than in aseptic controls (25–30). The biocorrosive mechanisms of the NRB-assisted Fe⁰ corrosion in these studies are largely unknown. One explanation of the Fe⁰ corrosion by hydrogenophilic NRB is the classic cathodic depolarization mechanism. However, given that nitrite was shown to be much more corrosive than nitrate (31), another possibility is that NRB indirectly stimulate Fe⁰ corrosion through the production of nitrite.

Unlike these NRB, Prolixibacter sp. MIC1-1 was not hydrogenophilic and thus may directly stimulate Fe⁰ corrosion by abstracting cathodic electrons from the surface of Fe⁰. The model of Fe⁰ corrosion by Prolixibacter sp. MIC1-1 discussed above is summarized in Fig. 6.

Nitrification injection into oil reservoirs has been used to mitigate SRB-assisted MIC, with the idea that it promotes NRB growth and in turn inhibits SRB growth (32–34). However, it should be realized that amendment with nitrate can lead to either hydrogenophilic-NRB-assisted or MIC1-1-like bacterium-assisted MIC by the mechanisms described above.

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