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Iron Corrosion Induced by Nonhydrogenotrophic Nitrate-Reducing *Prolixibacter* sp. Strain MIC1-1

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Microbiologically influenced corrosion (MIC) of metallic materials imposes a heavy economic burden. The mechanism of MIC of metallic iron (Fe^0) under anaerobic conditions is usually explained as the consumption of cathodic hydrogen by hydrogenotrophic microorganisms that accelerates anodic Fe^0 oxidation. In this study, we describe Fe^0 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 a sole electron acceptor with pyruvate as a carbon source and Fe^0 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 Fe^0-corroding representative belonging to the phylum *Bacteroidetes*. Under anaerobic conditions, *Prolixibacter* sp. MIC1-1 corroded Fe^0 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 $FePO_4$ developed on the surface of the Fe^0 foils, and a layer of $FeCO_3$ covered the $FePO_4$ crystals. We propose that cells of *Prolixibacter* sp. MIC1-1 accept electrons directly from Fe^0 to reduce nitrate.

Metallic iron (Fe^0) and stainless steel corrosion causes a severe economic burden. The annual cost of corrosion worldwide has been estimated to exceed 3% of the world's GDP (http://www.nace.org/uploadedFiles/Publications/ccsupp.pdf). Under aerobic conditions, molecular-oxygen-mediated corrosion may be predominant, whereas under anaerobic conditions, microbiologically influenced corrosion (MIC) is believed to be a major cause of corrosion-related failures (1). Specifically, sulfate-reducing bacteria (SRB) are considered to be major causative microorganisms of MIC in anaerobic environments because FeS has frequently been observed as a major corrosion product (2).

The mechanism underlying Fe^0 corrosion by SRB has been explained on the basis of the cathodic-depolarization theory: Fe^0 oxidation occurs at the anode ($Fe^0 \rightarrow Fe^{2+} + 2e^-$), while protons are reduced to molecular hydrogen at the cathode ($2e^- + 2H^+ \rightarrow H_2$). In the presence of SRB, a hydrogenase of the organism consumes either molecular hydrogen or electrons at the cathode, thereby accelerating the anodic Fe^0 oxidation (3, 4). Recently, a methanogenic archaeon and an iron-oxidizing bacterium were shown to cause Fe^0 corrosion (5–7). These reports suggest that diverse microorganisms contribute to metal corrosion.

MIC of metallic materials imposes a heavy economic burden, and technologies for MIC control are being actively investigated. However, the process of MIC is still poorly understood, and further research is required. The characterization of Fe^0-corroding microorganisms and a clear understanding of the mechanism of MIC are required for effective MIC prevention and control. In this study, we isolated a novel Fe^0-corroding bacterium from a crude-oil sample collected at an oil well and investigated its Fe^0 corrosion activity under a variety of culture conditions.

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 O_2-absorbing agent and a CO_2-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 NH_4Cl, 0.14 g KH_2PO_4, 0.20 g $MgCl_2 \cdot 6H_2O$, 0.15 g $CaCl_2 \cdot 2H_2O$, 2.5 g $NaHCO_3$, and 1.0 ml of a modified trace element solution used by Touzel and Albagnac (8) lacking NaCl and supplemented with 4.0 mg/liter of $Na_2WO_4 \cdot H_2O$. The pH of the medium was adjusted to 7.0 with 6 N HCl, and 20 ml of the medium was dispensed into each 50-ml serum bottle. Dissolved air was removed by flushing with N_2-CO_2 (4:1 [vol/vol]), and the bottles were sealed with butyl rubber stoppers. Prior to inoculation, 0.2 ml of vitamin solution (9) and 0.2 ml of a reductant solution containing 0.5 g/liter of Na_2S and 0.5 g/liter of L-cysteine–HCl were added to each bottle after the filtration of the solutions through 0.2-μm-pore-size membrane filters.

Sw medium was supplemented with filter-sterilized 10 mM sulfate, 10 mM 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

Received 13 November 2014 Accepted 22 December 2014
Accepted manuscript posted online 29 December 2014
Citation Iino T, Ito K, Wakai S, Tsurumaru H, Ohkuma M, Harayama S. 2015. Iron corrosion induced by nonhydrogenotrophic nitrate-reducing *Prolixibacter* sp. strain MIC1-1. Appl Environ Microbiol 81:1839–1846. doi:10.1128/AEM.03741-14.
Editor: S.-J. Liu
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doi:10.1128/AEM.03741-14

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 SPYSw 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 13498^T 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 NH_4Cl 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 oxide (2 mM), ferric chloride (2 mM), molecular oxygen (5% [vol/vol]), and fumarate (10 mM). Potential electron donors, including H_2-CO_2 (1%, 5%, and 10% [vol/vol]), sulfide (2 mM), elemental sulfur (1% [wt/vol]), thiosulfate (5 mM), sulfite (2 mM), ammonium (10 mM), nitrite (2 mM), Fe^0 granules (diameter, 1 to 2 mm; Fe^0 purity, 99.98%; Alfa Aesar, Lancashire, 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 NH_4Cl 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′-AGAG TTTGATCCTGGCTCAG-3′; positions 8 to 27 in the *Escherichia coli* numbering system) and a reverse primer (1492R, 5′-GGTTACCTTGTT ACGACTT-3′; positions 1510 to 1492). The PCR mixture (50 μl) contained 1× PCR buffer, 3.5 mM $MgCl_2$, 10 mM deoxynucleoside triphosphates (dNTPs), 1.25 U AmpliTaq Gold (Applied Biosystems, CA, USA), and 0.4 μM (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, Venro, Netherlands), and an almost-complete 16S rRNA gene sequence (1,444 bp) was determined with a 3130*xl* genetic analyzer (Applied Biosystems), the BigDye Terminator v3.1 cycle-sequencing kit, and one of the following six primers: 27F, 520F (5′-GTGCCAGCAGCCGCGG-3′), 920F (5′-AAACTCAAAGGAATTGAC-3′), 520R (5′-ACCGCGGCTGC TGGC-3′), 920R (5′-GTCAATTCCTTTGAGTTT-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^0 corrosion test. For Fe^0 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 a 50-ml serum bottle containing either 1.5 g of Fe^0 granules or an Fe^0 foil (purity, >99.99%; 10 by 10 by 0.1 mm; Sigma-Aldrich, St. Louis, MO, USA). Air in the medium was removed by flushing with N_2-CO_2 (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 analyses. 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 Fe^0 foils. Scanning electron microscopy with energy-dispersive X-ray spectroscopy (SEM/EDX) was used for surface analyses of corroded Fe^0 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, Fe^0, ferrous ion, and L-cysteine served as sole electron donors, whereas molecular hydrogen (Fig. 1), sulfide, elemental sulfur, thiosulfate, sulfite, 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 Fe^0 and ferrous ion as electron do-

TABLE 1 Growth of *Prolixibacter* sp. MIC1-1 on various media under anaerobic and aerobic conditions[a]

	Component or characteristic										
	Anaerobic conditions (condition no.)								Aerobic conditions (condition no.)		
Parameter	1	2	3	4	5	6	7	8	1	2	3
Electron acceptor	Nitrate	Nitrate	Nitrate	Nitrate	Nitrate	Nitrate	Nitrate	Others[b]	Molecular oxygen	Molecular oxygen	Molecular oxygen
Electron donor	Iron(0)	Iron(II)	L-Cysteine	Iron(0)	Iron(II)	L-Cysteine	Others[c]	L-Cysteine	L-Cysteine	L-Cysteine	Others[c]
Carbon source	Pyruvate	Pyruvate	Pyruvate	None	None	None	Pyruvate	Pyruvate	Pyruvate	None	Pyruvate
Growth[d]	+	+	+	−	−	−	−	−	+	−	−

[a] The medium composition and detailed growth conditions are described in Materials and Methods.
[b] Ten millimolar sulfate, 2 mM sulfite, 5 mM thiosulfate, 1% (wt/vol) elemental sulfur, 2 mM nitrite, 2 mM ferric oxide, 2 mM ferric chloride, 10 mM fumarate, and none.
[c] H_2-CO_2 (4:1 [vol/vol], N_2-CO_2-H_2 (79:20:1 [vol/vol/vol]; 75:20:5 [vol/vol/vol]; 70:20:10 [vol/vol/vol]), 2 mM sulfide, 1% (wt/vol) elemental sulfur, 5 mM thiosulfate, 2 mM sulfite, 10 mM ammonium, 2 mM nitrite, and none.
[d] +, growth; −, no growth.

nors 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, ferric chloride, and fumarate, did not serve as sole electron acceptors.

The strain did not use CO_2 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 13498^T 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 13498^T 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*.

Fe^0 corrosion by the bacterial isolate. *Prolixibacter* sp. MIC1-1 oxidized Fe^0 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 Fe^0 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 Fe^0 granule oxidation by *Prolixibacter* sp. MIC1-1 was detected in the presence of citrate or in the absence of an electron donor, but the metal surface did not lose its luster. *Prolixibacter* sp. MIC1-1 did not oxidize Fe^0 granules in the absence of nitrate (Table 3).

Importantly, *P. bellariivorans* JCM 13498^T, a close relative of *Prolixibacter* sp. MIC1-1, did not oxidize Fe^0 granules under any of the culture conditions described above (data not shown). Thus, the ability of *Prolixibacter* sp. MIC1-1 to use Fe^0 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

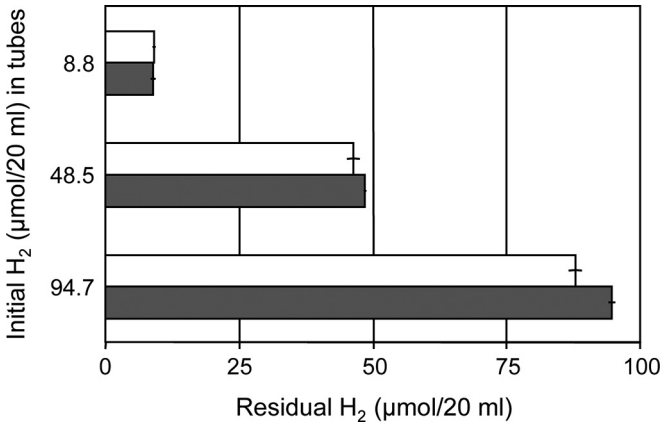

FIG 1 Use of molecular hydrogen as a sole electron donor by *Prolixibacter* sp. MIC1-1. Experiments were performed under three different initial concentrations of molecular hydrogen (8.8, 48.5, and 94.7 μmol/20 ml) with nitrate as the electron acceptor, and the concentration of residual molecular hydrogen 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 ($n = 3$).

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

	Component or characteristic			
	Anaerobic conditions with indicated carbon source		Aerobic conditions with indicated carbon source	
Parameter	CO_2	Pyruvate	CO_2	Pyruvate
Electron acceptor	Nitrate	Nitrate	Molecular oxygen	Molecular oxygen
Electron donor	L-Cysteine	L-Cysteine	L-Cysteine	L-Cysteine
Growth[a]	−	+	−	+

[a] +, growth; −, no growth.

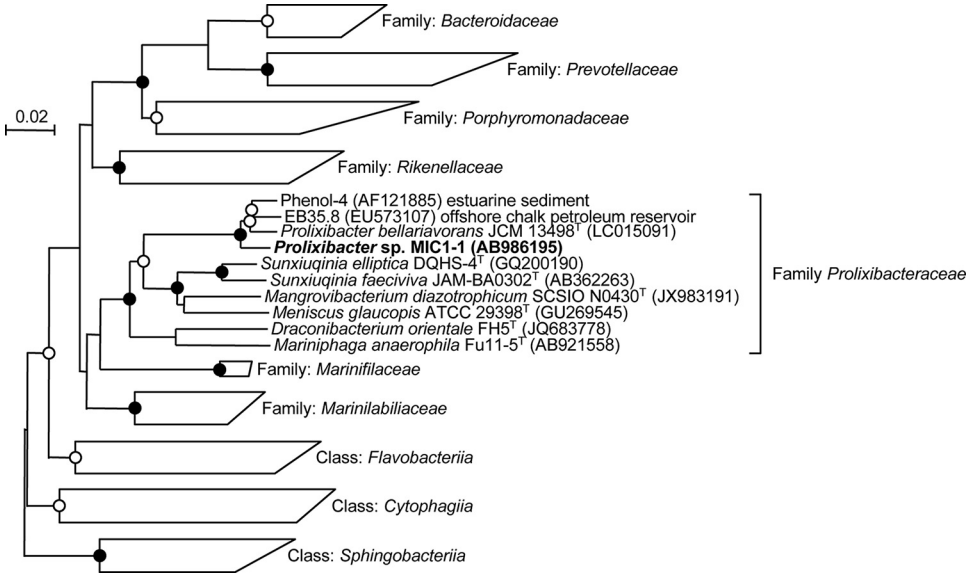

FIG 2 Phylogenetic tree of *Prolixibacter* sp. MIC1-1, related bacteria, and environmental clones based on the 16S rRNA gene sequences. The tree was inferred from an alignment of 1,308 bp of 16S rRNA gene sequences and constructed by NJ. The solid circles at branching nodes indicate supporting probabilities of >95% by bootstrap analyses of both the NJ and ML methods, whereas the open circles indicate probabilities of >85% by either of the analyses. The numbers at nodes are bootstrap percentages derived from 1,000 replications (NJ/ML methods). Bar, 0.02 substitution per nucleotide position.

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 Fe^0, 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 Fe^0 foils. *Prolixibacter* sp. MIC1-1 was cultivated in a sulfide-free artificial seawater medium supplemented with nitrate and lactate in which an Fe^0 foil was submerged. After incubation for 30 days, the surfaces of the Fe^0 foils were observed with a scanning electron microscope, together with Fe^0 foils incubated in aseptic media. As shown in Fig. 5A and B, crystal structures developed on the surfaces of Fe^0 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 Fe^0 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 Fe^0 foil surface mainly consisted of $FePO_4$ (Fig. 5C and F). The crystals on the Fe^0 foils from a *Prolixibacter* sp. MIC1-1 culture were covered by a layer of $FeCO_3$ (Fig. 5C). Such a layer was not observed in Fe^0 foils from aseptic controls (Fig. 5F).

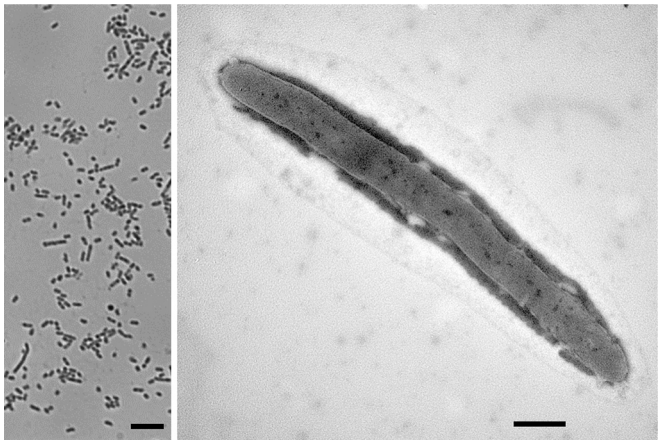

FIG 3 Phase-contrast (left) and transmission electron (right) micrographs of cells of *Prolixibacter* sp. MIC1-1. Bars, 5.0 μm (left) and 0.5 μm (right).

DISCUSSION

This study reports the isolation and characterization of the Fe^0-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 Fe^0-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-

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

Strain	Electron acceptor (10 mM)	Carbon source (10 mM)	Dissolved iron (μmol/20 ml)[a]		
			Total	Fe^{2+}	Fe^{3+}
Prolixibacter sp. MIC-1	Nitrate	Acetate	156.7 ± 3.6	85.9 ± 2.0	70.7 ± 1.6
	Nitrate	Citrate	99.5 ± 0.3	ND	ND
	Nitrate	Lactate	235.3 ± 4.1	106.9 ± 1.9	128.5 ± 2.3
	Nitrate	Pyruvate	224.3 ± 0.1	92.9 ± 0.1	131.4 ± 0.1
	Nitrate	None	60.0 ± 1.4	ND	ND
	None	Lactate	27.8 ± 4.2	ND	ND
Aseptic control	Nitrate	Lactate	64.8 ± 9.5	49.6 ± 7.3	15.3 ± 2.2

[a] ND, not determined.

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:

$$Fe^0 + NO_3^- + 2H^+ \rightarrow Fe^{2+} + NO_2^- + H_2O \quad (1)$$

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:

$$2\,Fe^{2+} + NO_3^- + 2\,H^+ \rightarrow 2\,Fe^{3+} + NO_2^- + H_2O \quad (2)$$

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

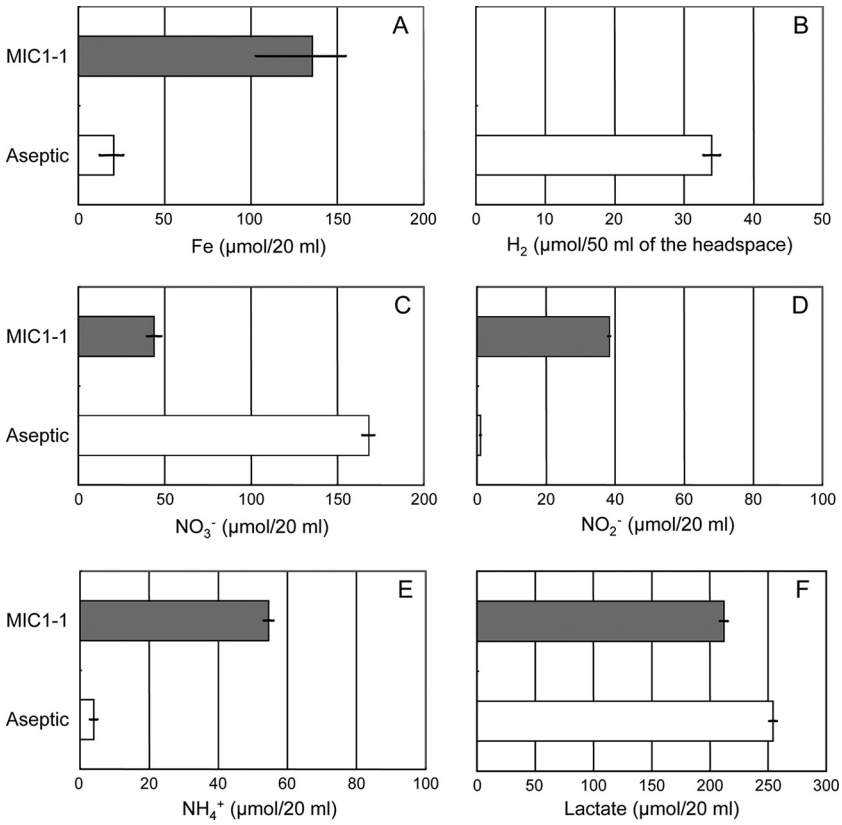

FIG 4 Fe^0 dissolution (A), molecular-hydrogen production (B), nitrate consumption (C), nitrite (D) and ammonium (E) production, and lactate consumption (F) in *Prolixibacter* sp. MIC1-1 cultures and in aseptic controls. All experiments were performed with Fe^0 granules as the sole source of electrons. 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.

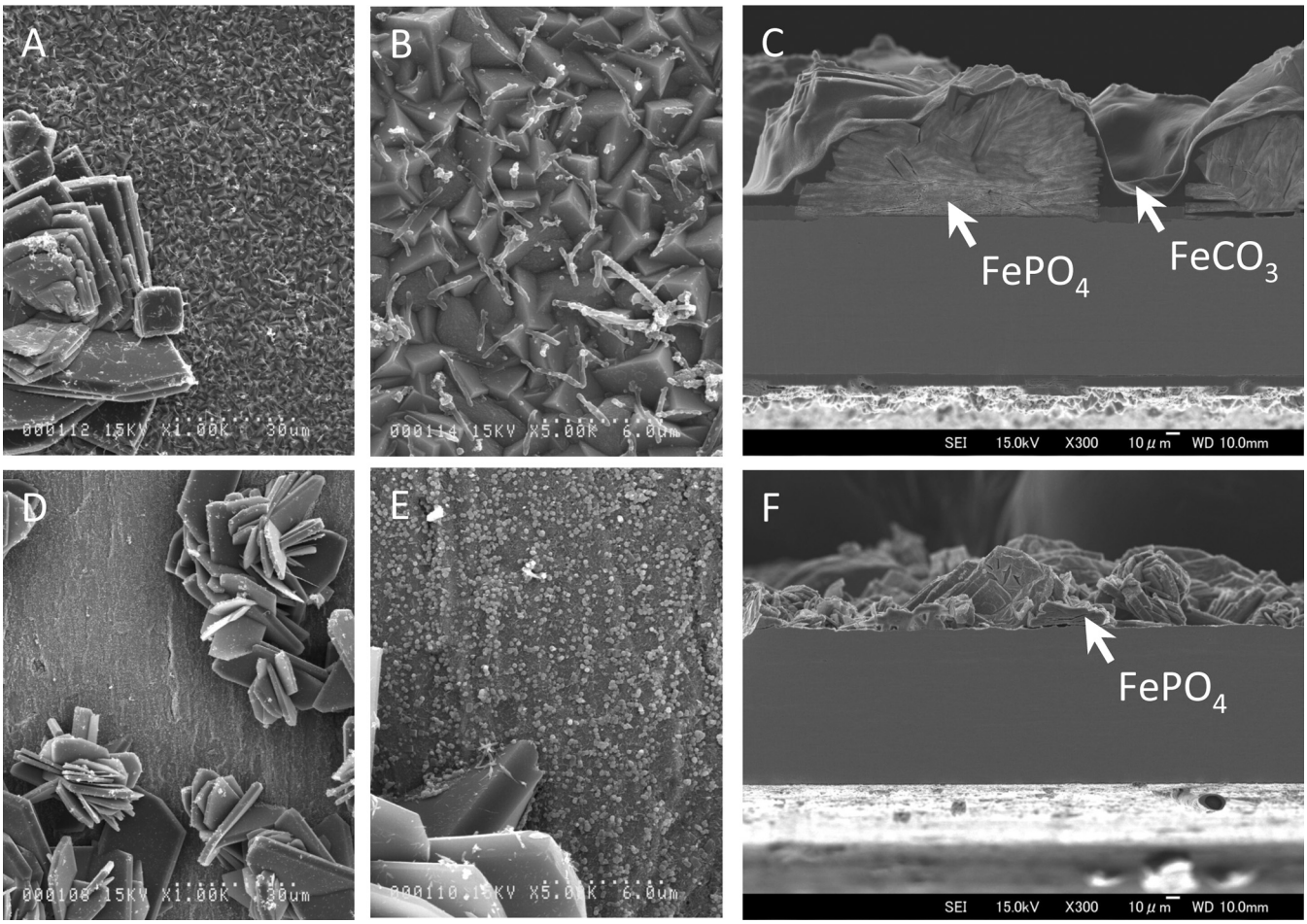

FIG 5 Scanning electron micrographs of the surfaces and cross sections of Fe^0 foils submerged in a *Prolixibacter* sp. MIC1-1 culture and an aseptic medium. (A and B) Surface of an Fe^0 foil incubated in the *Prolixibacter* sp. MIC1-1 culture. (C) Cross section of an Fe^0 foil incubated in the *Prolixibacter* 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.

concomitant with the reduction of nitrate to either nitrite (equation 1) or ammonium (equation 3) are known (17, 18).

$$4.5\,Fe^0 + NO_3^- + 10\,H^+ \rightarrow Fe^{2+} + NH_4^+ + 3\,H_2O \quad (3)$$

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).

$$9\,Fe^{2+} + NO_3^- + 10\,H^+ \rightarrow 9\,Fe^{3+} + NH_4^+ + 3\,H_2O \quad (4)$$

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

$$2/3\,Fe^0 + NO_3^- + 2\,H^+ \rightarrow 2/3\,Fe^{3+} + NO_2^- + H_2O \quad (5)$$

$$3\,Fe^0 + NO_3^- + 10\,H^+ \rightarrow 3\,Fe^{3+} + NH_4^+ + 3\,H_2O \quad (6)$$

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 ionization at the anode by a reaction whose rate is low under anaerobic conditions at neutral pH:

$$Fe^0 \rightarrow Fe^{2+} + 2e^- \quad (7)$$

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

$$2H^+ + 2e^- \rightarrow H_2 \quad (8)$$

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

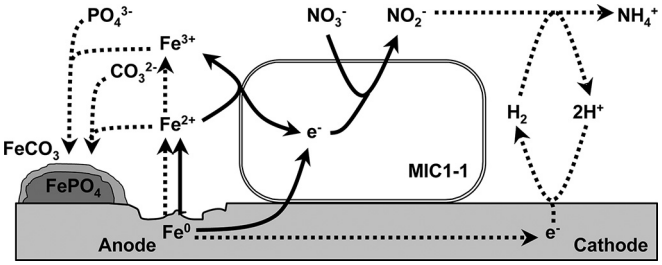

FIG 6 Hypothetical model of Fe^0 corrosion by the nitrate-reducing *Prolixibacter* sp. MIC1-1. Solid arrows, biotic steps; dotted arrows, abiotic chemical steps.

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 nitrite 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 Fe^0 was estimated to generate 2.5 μmol of electrons. Thus, 228 μmol of Fe^0 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 $FePO_4$ developed on the surface of Fe^0 (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 Fe^0 corrosion was limited to Fe^0 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 Fe^0 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 Fe^0 corrosion has been reported mainly in association with the remediation of nitrate-contaminated groundwater using granular Fe^0. The biological denitrification of groundwater by a combination of a hydrogenotrophic NRB and Fe^0 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 Fe^0 oxidation than in aseptic controls (25–30). The bioelectrochemical mechanisms of the NRB-assisted Fe^0 corrosion in these studies are largely unknown. One explanation of the Fe^0 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 Fe^0 corrosion through the production of nitrite.

Unlike these NRB, *Prolixibacter* sp. MIC1-1 was not hydrogenophilic and thus may directly stimulate Fe^0 corrosion by abstracting cathodic electrons from the surface of Fe^0. The model of Fe^0 corrosion by *Prolixibacter* sp. MIC1-1 discussed above is summarized in Fig. 6.

Nitrate 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.

ACKNOWLEDGMENTS

We thank Takahiro Iwami (National Institute of Technology and Evaluation) for technical support and Koji Mori (NITE) for helpful discussions of several aspects of the paper.

This study was partly supported by the New Energy and Industrial Technology Development Organization (NEDO) (grant no. P05032).

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