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Anaerobic Nitrate-Dependent Iron (II) Oxidation by a Novel Autotrophic Bacterium, *Citrobacter freundii* Strain PXL1

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Anaerobic Fe(II) Oxidizing Denitrifiers (AFODN), a type of newly found Fe(II)-oxidizing bacteria, play an important role in iron and nitrogen cycling. In the present study, a novel AFODN strain PXL1 was isolated from anaerobic activated sludge. Phylogenetic analysis of 16S rRNA gene sequence revealed similarity between this strain and *Citrobactor freundii*. The strain reduced 30% of nitrate and oxidized 85% of Fe(II) over 72 h with an initial Fe(II) concentration of 3.4 mM and nitrate concentration of 9.5 mM. Oxidation of iron was dependent on the reduction of nitrate to nitrite in the absence of other electron donors or acceptors. Nitrate reduction and Fe(II) oxidation followed first-order reaction kinetics. Iron oxides accumulated in the culture were analyzed by Fourier transform infrared (FTIR) spectroscopy, X-ray diffraction (XRD) spectroscopy and scanning electron microscopy-energy dispersive X-ray spectroscopy (SEM-EDS). The strain recovered deposited oxidized Fe in the form of amorphous Fe oxides.

Keywords: anaerobic oxidation, Fe(II) bio-oxidation, nitrate reduction, PCR, XRD

Introduction

Iron (Fe) is the dominant redox-active metal in organisms, soil and sediment (Cornell and Schwertmann 2003). Microbe-mediated anaerobic oxidation of ferrous iron (Fe(II)) is a widespread process among prokaryotes (Benz et al. 1998; Finneran et al. 2002; Senko et al. 2005), and has been observed in various types of ecosystems including activated sewage sludge (Nielsen and Nielsen 1998), anoxic subsurface aquifer sediments (Elias et al. 2003) and marine sediments (Schippers and Jørgensen 2002).

Widdel et al. (1993) reported the O_2-independent light-driven bacterial oxidation of ferrous iron by *Rhodomicrobium vannielii* BS-1with reduction of CO_2. Other anoxygenic Fe(II)-oxidizing phototrophic bacteria belonging to three major phylogenetic lineages of purple sulfur bacteria (Croal et al. 2004), purple nonsulfur bacteria (Ehrenreich and Widdel. 1994; Heising and Schink 1998; Straub et al. 1999) and green bacteria (Heising et al. 1999) were also reported.

Nitrate-dependent Fe(II) oxidation to Fe(III) by bacteria has been only recently described. Straub et al. (1996) obtained the first Fe(II)-oxidizing, nitrate-reducing (enrichment) culture from brackish water lagoon sediment. This culture grew fully autotrophically, using ferrous iron as the only electron donor and nitrate as the electron acceptor, according to the reaction: $5Fe^{2+} + NO_3^- + 12H_2O = 5Fe(OH)_3 + 0.5N_2 + 9H^+$ (Blöthe et al. 2009). The same process has also been demonstrated with *Acidovorax* BrG1, *Aquabacterium* BrG2 (Straub et al. 1996), the hyperthermophilic archaeon *Ferroglobus placidus* (Hafenbradl et al. 1996), the *Thermomonas* BrG3 (Straub et al. 2004), the mesophilic proteobacteria *Chromobacterium violacens* strain 2002 (Weber et al. 2006) and the *Paracoccus ferrooxidans* strain BDN-1 (Kumaraswamy et al. 2006). Because this process is not restricted to habitats that are exposed to light, it may be more important in nature than light-dependent ferrous iron oxidation by anoxygenic bacteria (Kappler and Straub 2005). The organisms capable of coupling Fe(II) oxidation to nitrate reduction can be referred to as anaerobic Fe(II) oxidation denitrifiers. A variety of anaerobic Fe(II) oxidation denitrifier strains were isolated from marine, brackish and freshwater sediment samples (Kappler and Straub 2005).

Organic carbon source, environmental pH and Fe(II) source may affect the enrichment and isolation of AFODN, and the efficiency of Fe(II) oxidation and nitrate reduction. The forms of Fe(II) oxidation products can also be affected by these factors (Chaudhuri et al. 2001; Kumaraswamy et al. 2006; Weber et al. 2006). Chaudhuri et al. (2001) isolated *Dechlorosoma suillum* strain PS from a swine waste lagoon. This organism was capable of rapidly oxidizing Fe(II) to magnetite with nitrate as the electron acceptor.

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Kumaraswamy et al. (2006) isolated a nitrate-reducing *Paracoccus ferrooxidans* strain BDN-1, which can grow using $[Fe(II)EDTA]^{2-}$ as the electron donor. However, Fe(II) oxidation products generated by this organism were not examined. *Chromobacterium violaceum* has been reported to form mixed-phase Fe(II)-Fe(III) minerals such as green rust and/or magnetite by oxidizing $FeCl_2$ (Chaudhuri et al. 2001; Weber et al. 2006). Nielsen and Nielsen (1998) observed biological Fe(II) oxidation associated with reduction of nitrate and nitrite in activated sludge. However, no microbial species was isolated from the activated sludge in their study.

In the present study, we report the isolation and characterization of the *Citrobacter freundii* strain PXL1 capable of nitrate-dependent Fe oxidation, its nitrate reduction and Fe(II) oxidation kinetics and the mineralogy of Fe(III) oxides produced by this microorganism.

Materials and Methods

Culture and Isolation of Microorganisms

Anoxic activated sludge samples were collected with anaerobic sterilized sealed bottles from the Hedong sewage treatment plant of Urumqi, China. The samples were quickly transferred to a DG250 anaerobic workstation (Don Whitley Scientific, West Yorkshire Shipley, England). Ten mM $FeCl_2{\cdot}4H_2O$ were added to the samples as electron donor and 20 mM KNO_3 were added to the samples as acceptor. After 7 days of incubation, the samples were transferred to the fresh liquid medium containing the following components (per liter): 20 g of potassium sodium tartrate ($C_4H_4O_6KNa{\cdot}4H_2O$), 1 g of K_2HPO_4, 1 g of $MgSO_4{\cdot}7H_2O$, 4 g of KNO_3 and 0.3976 g of $FeCl_2{\cdot}4H_2O$. The solid medium was prepared by mixing 2% agar with the liquid medium. MPN enumeration series were selected for the isolation of microorganisms.

Ten-fold serial dilution was performed to 10^{-7} stage. The plates were incubated in the anaerobic workstation at 30°C for 7 days. Colonies showing Fe(II) oxidation ability, as identified by the development of brownish-red Fe(III) oxide precipitates on or around colonies, were selected and transferred into the liquid medium. After 3 days of incubation in anaerobic atmosphere at 30°C, nitrite (NO_2^-) concentration in the medium was measured by the Griess method (Kleinbongard et al. 2002). One isolated strain (named PXL1) showing Fe(II) oxidation ability was selected for further characterization.

16S rRNA Gene Sequencing and Similarity Analysis

Genomic DNA was extracted from the isolated bacterial cultures by alkaline lysis. Using genomic DNA as a template, the 16S rRNA gene was amplified by a polymerase chain reaction (PCR). The forward and reverse primers were 5′-AGAGTTTGATCCTGGCTCAG-3′ and 5′-AAGGAGGTGATCCAGCCGCA-3′, respectively (Hall et al. 1999). The PCR program was described by Achal and Pan (2011), starting with an initial 5 min denaturation step at 94°C, 36 cycles at 94°C for 1 min, annealing of primers to the DNA template at 48°C for 30s, extension at 72°C for 2 min, and final extension at 72°C for 7 min. The sequences were generated by dideoxynucleotide chain termination using an Applied Biosystem automated sequencer (Biorad, USA). The detected sequence was submitted to GenBank. The retrieved sequences from GenBank were added to alignments. The possible sister groups were found by BLAST research. These sequences were edited and a phylogenetic tree was constructed by the neighbor-joining method using MEGA 4.0 software (Tamura et al. 2007).

Fe(II) Oxidation and Nitrate Reduction Experiments

Three tubes of medium inoculated with AFODN culture were used to determine the stoichiometry of Fe(II) oxidation and nitrate reduction. The isolated AFODN strain was inoculated at a cell density of around 5×10^6 cells per milliliter. All experiments were carried out in the DG250 anaerobic workstation. Nitrate, nitrite and Fe(II) concentrations were measured at specified time intervals as described later. The initial concentrations of $FeCl_2{\cdot}4H_2O$ and KNO_3 were 3.4 mM and 9.5 mM, respectively.

Analytical Methods

Concentrations of nitrate, nitrite and Fe(II) were measured using a UV/Vis spectrophotometer (UV-2800, Unico, Shanghai, China). Nitrate concentration was determined by UV spectrophotometry at 220 nm and 275 nm (Slanina et al. 1976). Nitrite concentration was monitored spectrophotometrically at 540 nm by the formation of purple azo compounds (Griess-Romijn-van 1966). Fe(II) concentration was measured spectrophotometrically with phenanthroline at 510 nm (Gendel and Lahav 2008). For measurement of Fe(II) concentration, the samples were immediately transferred to 25% (vol/vol) HCl solution to avoid Fe(II) oxidation. All analysis was finished within 2 h after sampling.

Microbially induced Fe(II) oxidation sediments were characterized by scanning electron microscopy coupled with energy dispersive X-ray spectroscopy (SEM-EDS). Sediment samples for SEM-EDS analysis were prepared as follows: the sample was centrifuged for 15 min at 12000 rpm; the supernatant was then discarded; samples dehydrated with absolute alcohol; and dried at 60°C for 24 h. The samples were examined using a scanning electron microscope (Zeiss Super 55VP, Germany) at accelerating voltages from 15 to 35 kV. The elemental composition of the sediments was determined by an energy dispersive X-ray spectrometer (Bruker XFlash 5010, Germany).

The Fe(II) oxidation products were further analyzed with a FTIR spectrometer (Vertex 70, Bruker, Germany). The samples were treated as follows: centrifuged for 15 min at 12000 rpm; refrigerated for 3 h at −80°C and then freeze-dried for 48 h at −60°C. About 1 mg of sediment was ground with 100 mg of KBr in an agate mortar. The FTIR spectra obtained at 4000–400 cm^{-1} were used to examine the main functional groups. XRD spectra from 5 to 80°of the freeze-dried and

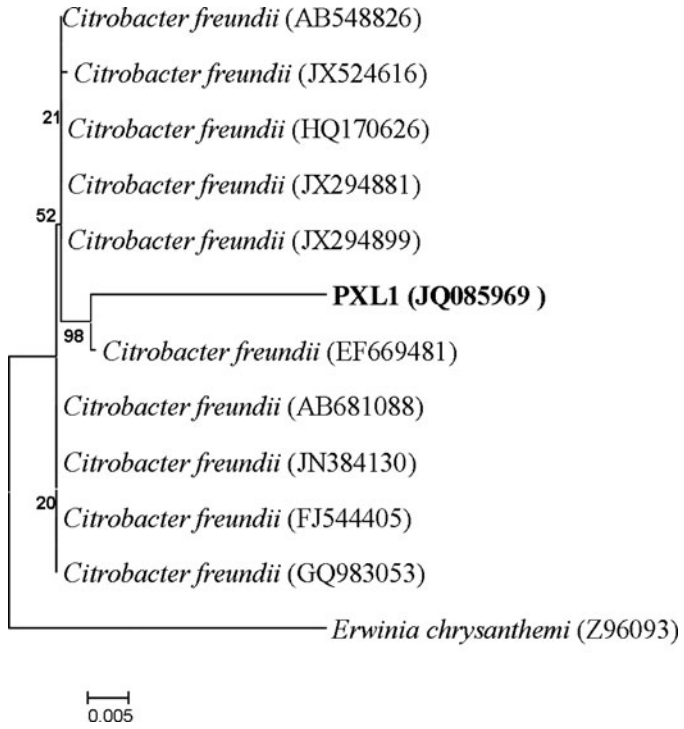

Fig. 1. Neighbor-joining tree based on 16S rRNA sequences showing the phylogenetic affiliation of strain PXL1. Bootstrap values were determined on the basis of results of 1,000 replicates. The numbers in parentheses are accession numbers. *Erwinia chrysanthemi* was used as out-group. The scale bar indicates a 0.5% sequence difference.

ground sediment samples were also obtained using a Bruker D8 diffractometer with a Cu anode (Bruker, Germany).

Results and Discussion

16S rRNA Gene Sequencing and Similarity Analysis

The complete 16S rRNA gene sequence of strain PLX1 has been submitted to GenBank and the accession number JQ085969 was assigned. Phylogenetic analysis of 16S rRNA gene sequence of strain PXL1 showed that strain PXL1 was most closely related to *Citrobacter freundii* (EF669481), a facultative Gram-negative bacillus (Figure 1) with 98% similarity index. Strain PXL1 was not closely related to other groups of microorganisms capable of nitrate-dependent Fe(II) oxidation, implying that Fe(II)-oxidizing organisms are more diverse than previously anticipated (Senko et al. 2005; Straub et al. 2004).

Biological Nitrate-Dependent Fe (II) Oxidation by AFODN

Figure 2a shows changes of nitrate and nitrite concentrations with incubation time. Nitrate concentration decreased over the entire experimental period. It declined dramatically from 9.5 mM to about 6.6 mM after 72 h, with 30% of the nitrate reduced. Stoichiometrically, 2.9 mM nitrite should be

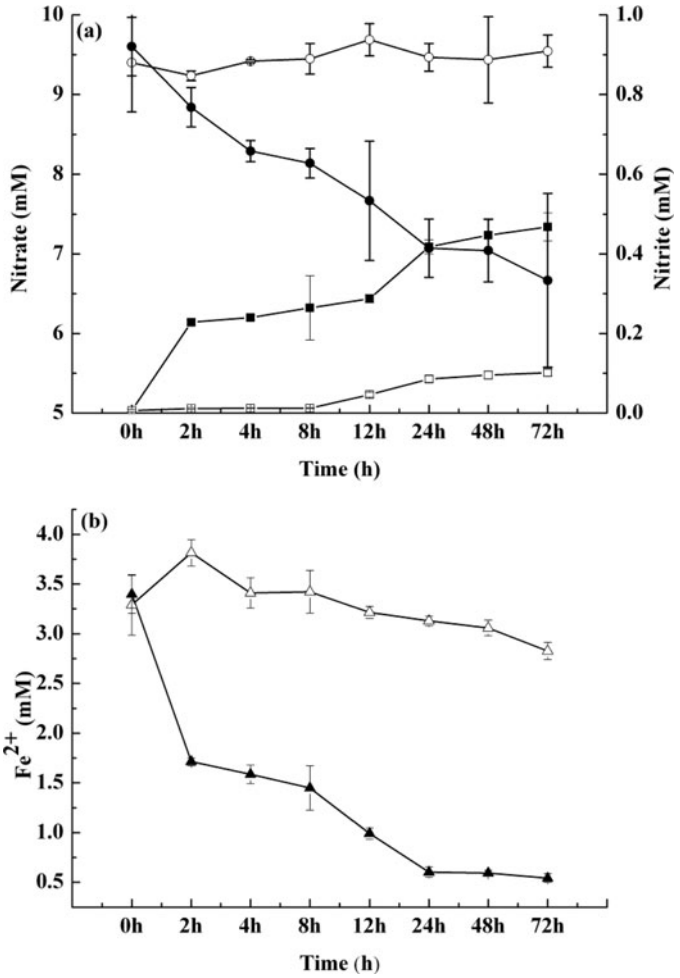

Fig. 2. Change of nitrate (a), nitrite (a) and Fe(II) ion (b) concentrations with time in the *C. freundii* strain PXL1 culture. Data were presented as means ± standard deviations (SD) of triplicate. Filled and open symbols represent data of the inoculated and the non-inoculated cultures, respectively. ● and ○, Nitrate; ■ and □, Nitrite; ▲ and □, Fe(II).

produced, while 2.9 mM nitrate was reduced. However, nitrite concentration was only 0.47 mM at 72 h, which was far below the expected value of the amount of nitrite converted from nitrate. This suggests that nitrite might be further converted to N_2O or N_2 (g) or NH_4^+. Fe(II) was oxidized concurrently with nitrate reduction (Figure 2b). At 72 h, Fe(II) concentration decreased by 85% from 3.4 mM to about 0.5 mM. In the noninoculated control, almost no nitrate reduction, nitrite formation and Fe(II) oxidation were observed, confirming that it was *C. freundii* strain PXL1 that oxidized Fe(II) and reduced nitrate.

C. freundii can reduce nitrate or nitrite to ammonia (Rehr and Klemme 1989). In anaerobic environments, *C. freundii* may compete successfully with denitrifying microorganisms (Puchenkova 1996). In the present study, accumulation of nitrite was due to reduction of nitrate by *C. freundii* strain PXL1.

The molar ratios of reduced nitrate to oxidized ferrous iron were calculated. The minimum ratio of 0.41 was obtained at 2 h

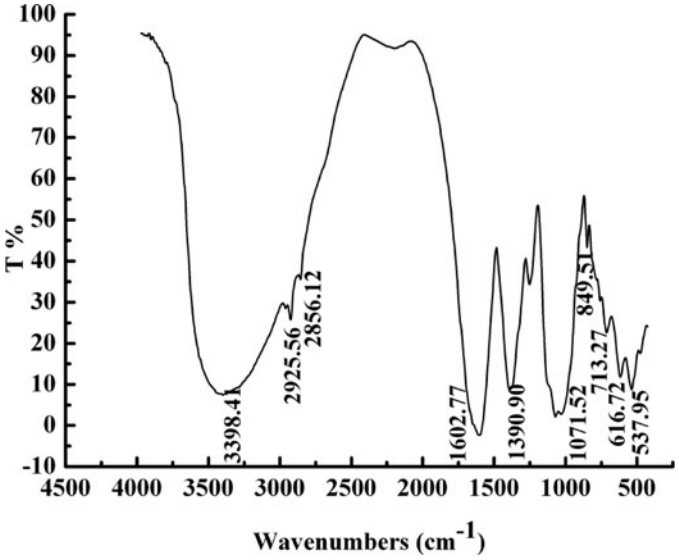

Fig. 3. Infrared spectrum of sediment from the *C. freundii* strain PXL1 culture. The characteristic bands from 1071 cm^{-1} to 537 cm^{-1} were related to iron oxides.

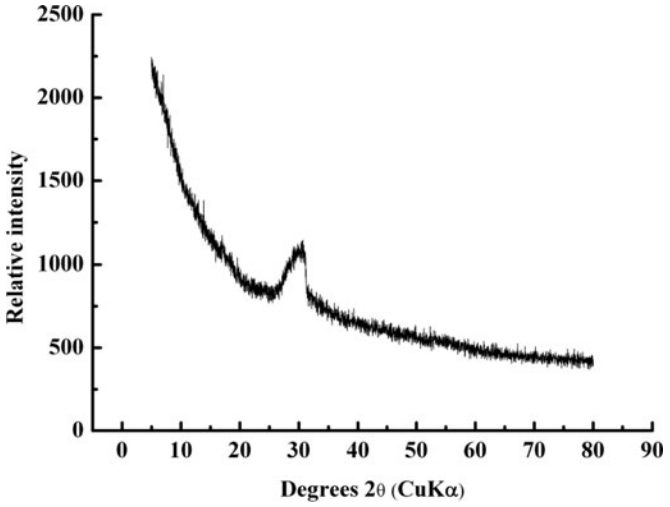

Fig. 4. X-ray diffraction spectrum of sediment from the *C. freundii* strain PXL1 culture. No distinct diffractive acuti-apices implies the formation of amorphous iron oxides.

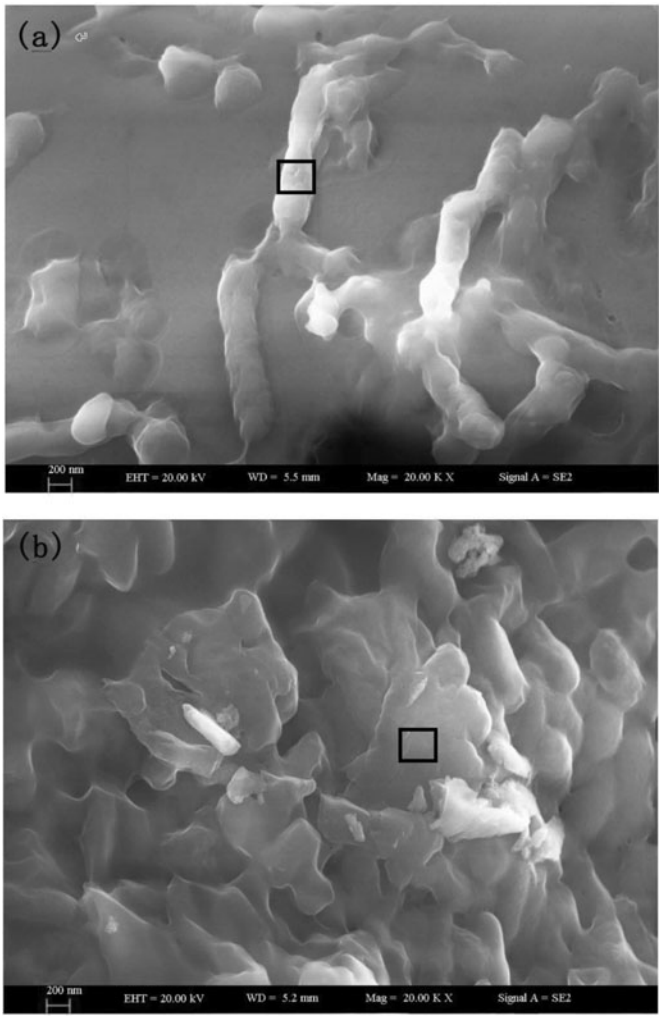

Fig. 5. SEM images of sediment from the *C. freundii* strain PXL1 cultures. (a) topography of bacterial cells, magnification: ×20,000; (b) topography of bacterial cells, magnification: ×20,000.

and the maximum ratio of 1.00 at 72 h with an average ratio of 0.76, which was higher than those reported in the literature. In a mixed culture of nitrate-dependent Fe(II)-oxidizing bacteria, Nielsen and Nielsen (1998) observed that the lowest molar ratio of reduced nitrate to oxidized ferrous iron was 0.20 and the highest ratio was 0.44. The molar ratios of reduced nitrate to oxidized ferrous iron for three different AFODN strains were in the range of 0.34–0.58 (Straub et al. 1996).

Ratio of reduced nitrate to oxidized Fe(II) around 0.2 indicates nitrate reduction to N_2 (Blöthe and Roden 2009). Stoichiometric calculation shows that ratios of 1, 0.5, 0.25, and 0.13 indicate reduction to nitrogen dioxide, nitrite, nitrous oxide and ammonia, respectively. Higher ratio obtained in this study may be due to the conversion of nitrate to nitrogen dioxide or nitrite. Furthermore, N is essential element for microbial growth.and assimilatory processes including synthesis of microbial cell components will affect the ratio of reduced nitrate to oxidized Fe(II).

The nitrate reduction data was fitted with the first-order kinetics equation (1):

$$\ln C_0 - \ln C_t = kt \quad (1)$$

where C_0 and C_t are initial nitrate/Fe(II) concentration and nitrate/Fe(II) concentration at time t, respectively. k is the kinetics rate constant. The nitrate reduction data were satisfactorily described by the first-order kinetics equation ($R^2 = 0.9769$). The nitrate reduction rate constant and half time were 0.050 h^{-1} and 13.75 h, respectively. Fe(II) oxidation kinetics also followed the first order reaction kinetics ($R^2 = 0.9202$), with a Fe(II) oxidation rate of 0.299 h^{-1} and a half-time of 2.34 h.

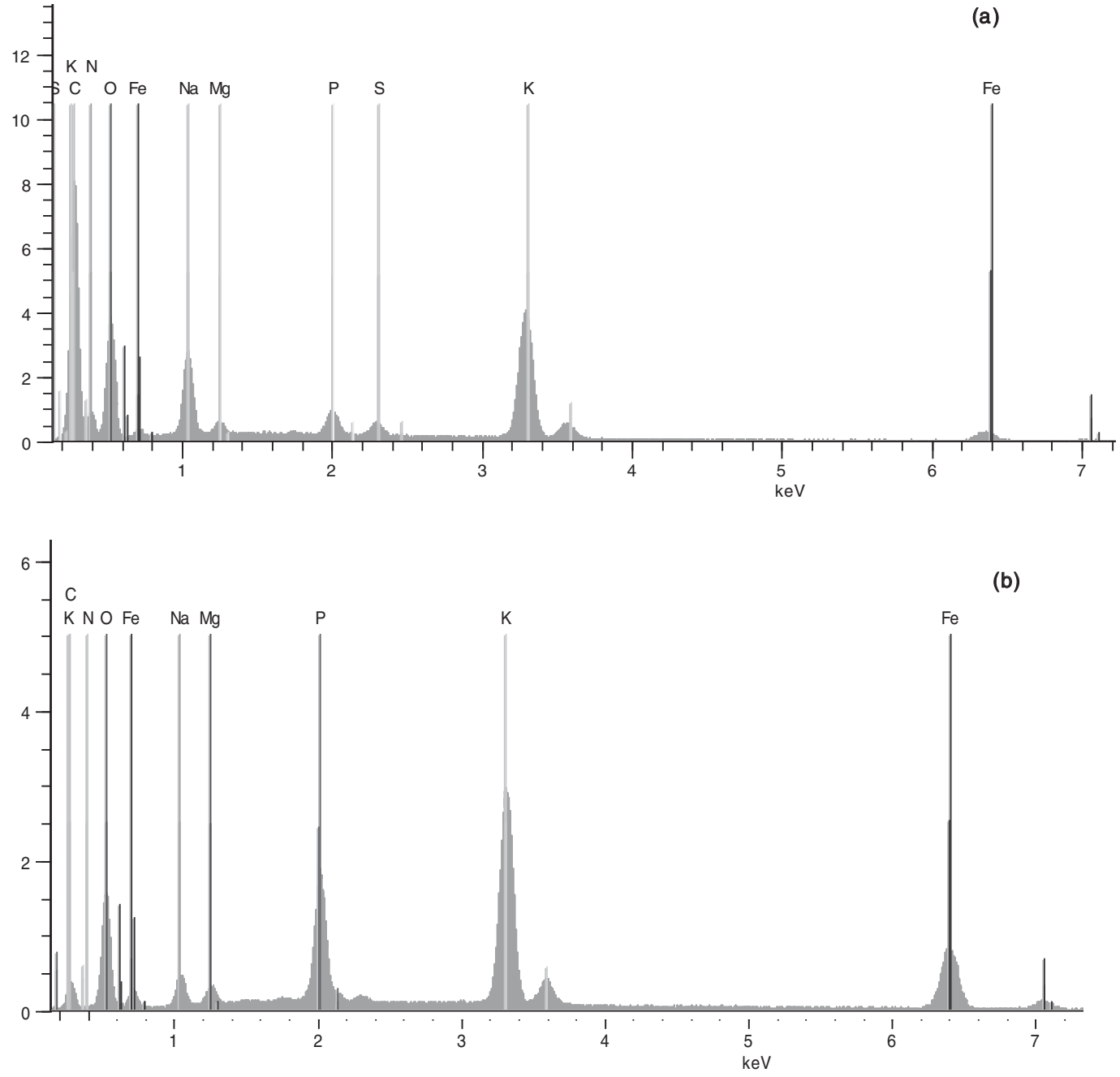

Fig. 6. EDS analysis of the *C. freundii* strain PXL1 sediment: (a) microorganisms and (b) microorganisms. Vertical line indicates the characteristic peak position of the element. Peak area represents the content of the element.

Characterizing Biogenic Iron Oxides

The characteristics of biogenic iron oxidation products were analyzed by FTIR, SEM-EDS and XRD techniques. The FTIR spectrum of the microbially produced iron oxides is shown in Figure 3. The peaks in the 3500–2000–^{1} and 1700–1000 cm^{-1} regions indicate the presence of organic compounds (Chua-anusorn and Webb 2000). The broad absorbance band around 3400 cm^{-1} in spectra of all samples was due to O—H stretching and H-bonding, which is a common feature of some molecular water and/or —OH groups (Chua-anusorn and Webb 2000). Two prominent FTIR bands at 849 cm^{-1} and 713 cm^{-1} could be assigned to the Fe—O—H bending vibrations, and the FTIR bands at 1071 cm^{-1}, 616 cm^{-1} and 537 cm^{-1} could be attributed to Fe—O stretching vibrations (Gotić and Musić 2007; Predoi 2007). The characteristic bands related to iron indicate that the *C. freundii* strain PXL1 produced iron oxides.

No distinct diffractive acuti-apices was found in the XRD spectrum (Figure 4), implying that the poorly crystalline

Fe(III) oxides were produced by the *C. freundii* strain PXL1 (Tuhela et al. 1993). The formation of poorly crystalline iron oxides was in accordance with previous XRD analysis of Fe-precipitates generated by some AFODN species (Kappler et al. 2005) and microaerophilic oxygen-dependent Fe(II)-oxidizing bacteria (Emerson and Moyer 1997). However, some Fe(II) oxidizing denitrifiers could form magnetite (Chaudhuri et al. 2001). Many factors, such as chemical forms of Fe(II) and Fe(II) oxidation rate, may affect the biogenic Fe(III) mineralogy (Senko et al. 2005).

The *C. freundii* strain PXL1 was an approximately 1 μm long bacillus (Figure 5a). Amorphous iron oxides were observed on the cell surface of *C. freundii* strain PXL1 and no crystalline iron oxides were observed by SEM (Figure 5b). The bacterial cells were mainly composed of C (34.93%), O (29.01%) and N (15.10%) with a low proportion of Fe (2.82%) by weight (Figure 6a). The amorphous iron oxides produced by the bacteria mainly consisted of Fe, O, K, P, C, Na, N and Mg. Fe accounted for the highest proportion of the precipitates at about 28.57%, followed by oxygen at about 24.59% by weight. K also had a high proportion (23.69% by weight) because of high contents of KNO_3, K_2HPO_4, and potassium sodium tartrate in the medium. All FTIR, XRD and SEM-EDS results confirmed that *C. freundii* strain PXL1 produced amorphous iron oxides.

Environmental Implications

The *Citrobacter* genus was discovered in 1932 by Werkman and Gillen. *C. freundii* was originally isolated from soil. Later, the same organism was recovered from water and sludge (Wang et al. 2000). It is capable of reducing nitrate to nitrite and then to N_2O or dinitrogen gas or ammonium. Therefore, *C. freundii* plays an important role in the nitrogen cycle. Our research shows that *C. freundii* strain PXL1 can oxidize Fe(II) in the form of $FeCl_2$ with nitrate as the sole electron acceptor under anaerobic condition. This reveals that the *C. freunfii* plays an important role not only in the nitrogen cycle but also in the geomicrobiological cycling of iron in environment. This process can also be important in pollution control. Generally, biogenic Fe(III) oxides by microbes have a specific surface area of 8–400 $m^2\ g^{-1}$, resulting in their high capacity to adsorb heavy metal ions (Wu et al. 2010). This specific metabolic pathway of AFODN provides a new promising way to simultaneously remove nitrate and heavy metals from water in anaerobic aquatic environment.

Conclusions

A novel AFODN strain PXL1, which could oxidize Fe(II) using nitrate as the sole electron acceptor, was isolated from anoxic activated sludge. The microorganism was most closely related to *C. freundii* and not closely related phylogenetically to other groups of microorganisms capable of nitrate-dependent Fe(II) oxidation. The *C. freundii* strain PXL1 produced poorly crystalline Fe (III) oxides during its anaerobic Fe(II) oxidation and nitrate reduction.

Acknowledgments

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