Nitrate reductase from the magnetotactic bacterium Magnetospirillum magnetotacticum MS-1: Purification and sequence analyses

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Nitrate reductase from the magnetotactic bacterium *Magnetospirillum magnetotacticum* MS-1: purification and sequence analyses[1]

Azuma Taoka, Katsuhiko Yoshimatsu, Masaaki Kanemori, and Yoshihiro Fukumori

Abstract: We purified the nitrate reductase from the soluble fraction of *Magnetospirillum magnetotacticum* MS-1. The enzyme was composed of 86- and 17-kDa subunits and contained molybdenum, non-heme iron, and heme *c*. These properties are very similar to those of the periplasmic nitrate reductase found in *Paracoccus pantotrophus*. The *M. magnetotacticum nap* locus was clustered in seven open reading frames, *napFDAGHBC*. The phylogenetic analyses of NapA, NapB, and NapC suggested a close relationship between *M. magnetotacticum nap* genes and *Escherichia coli nap* genes, which is not consistent with the 16S rDNA data. This is the first finding that the α subclass of *Proteobacteria* possesses a *napFDAGHBC*-type *nap* gene cluster. The *nap* gene cluster had putative fumarate and nitrate reduction regulatory protein (Fnr) and NarL protein binding sites. Furthermore, we investigated the effect of molybdate deficiency in medium on the total iron content of the magnetosome fraction and discussed the physiological function of nitrate reductase in relation to the magnetite synthesis in *M. magnetotacticum*.

Key words: nitrate reductase, magnetotactic bacteria, denitrification, horizontal gene transfer.

Résumé : Nous avons purifié une nitrate réductase de la fraction soluble de *Magnetospirillum magnetotacticum* MS-1. L'enzyme était composée de sous-unités de 86 et 17 kDa et renfermait du molybdène, du fer non héminique et de l'hème *c*. Ces caractéristiques s'apparentent à celles de la nitrate réductase périplasmique retrouvée chez *Paracoccus pantotrophus*. Le locus *nap* de *M. magnetotacticum* était regroupé dans plusieurs cadres de lecture ouverts, *napFDAGHBC*. Les analyses phylogénétiques de NapA, NapB et NapC ont mis à jour une parenté étroite entre les gènes *nap* de *M. magnetotacticum* et ceux de *Escherichia coli*, ce qui est en rupture avec les données d'ARNr 16s. Ceci est la première fois que nous rencontrons une sous-classe alpha de *Proteobacteria* possédant un groupe de gènes *nap* de type *napFDAGHBC*. Le groupe de gènes *nap* contenait des protéines régulatrices de la réduction du fumarate et du nitrate (Fnr) et des sites de liaison à la protéines NarL. D'autre part, nous avons évalué l'impact d'une carence en molybdate dans le milieu sur le contenu total de fer dans la fraction magnétosomale et avons traité des fonctions physiologiques de la nitrate réductase par rapport à la synthèse de la magnétite chez *M. magnetotacticum*.

Mots clés : nitrate réductase, bactéries magnétotactiques, dénitrification, transfert génique horizontal.

[Traduit par la Rédaction]

Introduction

Magnetospirillum (formerly *Aquaspirillum*) *magnetotacticum* MS-1 was isolated from the sediments of a freshwater swamp (Blakemore et al. 1979). One of the novel features of *M. magnetotacticum* is that this bacterium synthesizes intracellular particles, termed magnetosomes, which envelope single crystals of magnetite with lipid bilayers (Gorby et al. 1988). Recently, we purified a cytochrome *c*-550 (Yoshimatsu et al. 1995) and a cbb_3-type cytochrome *c* oxidase (Tamegai and Fukumori 1994) and found that these respiratory proteins have been constitutively synthesized in both magnetic and nonmagnetic cells. The structural features and enzymatic properties of *M. magnetotacticum* cbb_3-type cytochrome *c* oxidase are very similar to those of *Rhodobacter capsulatus* (Gray et al. 1994) and *Bradyrhizobium japonicum* (Preisig et al. 1996) cbb_3-type cytochrome *c* oxidases, which are specifically induced under an oxygen-limited growth condition. *Magnetospirillum magnetotacticum* locates several millimetres downward from the agar–air surface in the soft agar medium. Therefore, the physiological role of the magnetosome seems to be to cause the bacterium to orient itself along the lines of the geomagnetic field and to allow it to efficiently locate and maintain position in the microaerobic zone (Frankel et al. 1997).

Bazylinski and Blakemore (1983) investigated the optimum

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[1]The DNA sequences obtained in this study have been deposited and assigned accession No. AB055444 in the *DDBJ database*.
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growth conditions for magnetite production by *M. magnetotacticum* and found that the bacterium produces more magnetites under microaerobic conditions supplemented with nitrate, which is finally reduced to N_2O or N_2, than with NH_4. They also reported that the bacterium cannot grow under strict anaerobic conditions with nitrate (Bazylinski and Blakemore 1983). Therefore, it seems likely that the bacterium is a microaerobic denitrifier, using O_2 and nitrate simultaneously as final electron acceptors in the cell. Furthermore, Yamazaki et al. (1995) have reported that cd_1-type nitrite reductase is highly expressed in the periplasm of the magnetite-containing cells of *M. magnetotacticum* and shows a novel Fe(II)–nitrite oxidoreductase activity. These results suggest that denitrification may be required for magnetite synthesis in *M. magnetotacticum.* However, there are currently no reports on the characterization and function of other denitrifying enzymes of *M. magnetotacticum.*

In the present study, to better understand the relationship between denitrification and magnetite synthesis in *M. magnetotacticum*, we have, for the first time, purified the nitrate reductase. The purified enzyme is a soluble complex with two subunits and shows similar enzymatic and molecular features to periplasmic nitrate reductase (Berks et al. 1994). As well, we have cloned and sequenced the *nap* gene cluster and compared the organization and the deduced amino acid sequences with those of several denitrifying bacteria. Finally, we describe the experiment designed to examine the role of the nitrate reductase in intracellular production of magnetite by *M. magnetotacticum.*

Materials and methods

Microorganisms and cultivation

Magnetospirillum magnetotacticum MS-1 (ATCC 31632) was cultivated in a chemically defined liquid medium containing sodium nitrate as final electron acceptor under an O_2 (1%) – N_2 (99%) atmosphere at 25°C in the dark (Blakemore et al. 1979). To investigate the effect of Mo deficiency in the medium on magnetite synthesis, we subcultured the bacterium every week for 1–2 months in the medium in which $Na_2WO_4{\cdot}2H_2O$ (0.066 mg/L) was substituted for $Na_2MoO_4{\cdot}2H_2O$ (0.05 mg/L).

Escherichia coli XL-1 Blue MRF′ (Stratagene, La Jolla, Calif.) was used for cloning studies and was grown at 37°C under aerobic conditions in Luria–Bertani medium (Sambrook et al. 1989). The antibiotic ampicillin was added to the medium to a final concentration of 50 μg/mL.

Purification of the nitrate reductase from *M. magnetotacticum*

All purification steps were conducted at 4°C, unless indicated otherwise. The frozen cells (about 25 g wet weight) cultivated in a Mo-supplemented medium were thawed, suspended in 100 mM Tris–HCl buffer (pH 8.0) containing 200 mM NaCl, and broken by three passages through a French pressure cell (100 MPa). After the unbroken cells and magnetosomes were both removed by centrifugation at *10 000 × g* for *15* min, the supernatant was recovered and further centrifuged at *187 000 × g* for 1 h. The supernatant obtained was treated with ammonium sulfate fractionation from 50 to 70% saturation. The resulting precipitates were suspended in 100 mM Tris–HCl buffer (pH 8.0) containing 40% saturated ammonium sulfate and were subjected to a first chromatography on a Butyl-Toyopearl (Fractogel TSK Butyl 650M, Tosoh, Tokyo, Japan) column (2.2 by 7 cm) equilibrated with 100 mM Tris–HCl buffer (pH 8.0) containing 40% saturated ammonium sulfate. The nitrate reductase adsorbed onto the column was eluted with a linear gradient of 40–0% saturation of ammonium sulfate. The two peaks of nitrate reductase were found on the Butyl-Toyopearl chromatography. The major peaks showing spectral features corresponding to heme *c* were pooled and adjusted to be about 40% saturation of ammonium sulfate by addition of solid ammonium sulfate. The solution was subjected to a second chromatography on a Butyl-Toyopearl column (1.0 by 5 cm) equilibrated with 100 mM Tris–HCl buffer (pH 8.0) containing 40% saturated ammonium sulfate. The enzyme was eluted with a linear gradient of 30–10% saturation of ammonium sulfate. For concentration, the enzyme fractions were precipitated by adding solid ammonium sulfate to 90% saturation. The resulting pellets were dissolved in a minimal volume of 100 mM Tris–HCl buffer (pH 8.0) containing 20% saturated ammonium sulfate and were subjected to gel filtration with a Sephacryl S-300 HR (Pharmacia Biotech, Piscataway, N.J.) column (2.9 by 100 cm) equilibrated with 100 mM Tris–HCl buffer (pH 8.0) containing 20% saturated ammonium sulfate. The enzyme fractions were pooled and adjusted to be about 50% saturation with the addition of solid ammonium sulfate. The suspension was subjected to chromatography using a Sepharose CL-6B (Pharmacia Biotech) column (1.2 by 3 cm) (Tschopp and Kirschner 1980) equilibrated with 100 mM Tris–HCl buffer (pH 8.0) containing 50% saturated ammonium sulfate. The nitrate reductase that eluted from the column in a gradient of 50–10% saturation of ammonium sulfate was collected and used as the final preparation.

Assay of the nitrate reductase activity

The nitrate reductase activity was assayed by the method of Fernández et al. (1982), with slight modifications. The standard reaction mixture contained 200 mM sodium phosphate buffer (pH 7.0), 0.05 mg/mL methyl viologen, and 10 mM sodium nitrate in a total volume of 1.0 mL. The reaction mixture was evacuated in a Thunberg-type cuvette, and then 50 μL of a sodium dithionite (8 mg/mL) plus sodium bicarbonate (8 mg/mL) mixture was added to the reaction mixture. The reaction was started by the addition of an aliquot of enzyme preparation, and after incubation at 25°C for 10 min, the reaction was stopped by rapid mixing with air. Nitrite concentration was determined by the diazocoupling procedure of Nicholas and Nason (1957). The kinetic parameters were determined by the following method. The reactions were performed under anaerobic conditions in 100 mM sodium phosphate buffer (pH 7.0) containing 0.05 mg/mL methyl viologen. Sodium dithionite was added to reduce methyl viologen to give an absorbance of about A_{604} = 1.0, and the reaction was started by the addition of 10 mM sodium nitrate and enzyme preparation. The absorbance at 604 nm was followed over time. The molar coefficient ε_{604} = 14 000 $M^{-1}cm^{-1}$ for reduced methyl viologen (Fernández et al. 1982) was used.

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Physical and chemical measurements

The protein content was determined by the method of Bradford (1976), with bovine serum albumin as a standard. The heme content was determined based on the molar coefficient of pyridine ferrohemochrome; ε_{550} = 29 100 $M^{-1}cm^{-1}$ (Falk 1964) for the ferrohemochrome of heme *c*. The contents of iron and Mo in the purified nitrate reductase preparation were determined by inductively coupled plasma atomic emission spectrometry measurements with an SPS 1500 VR Plasma spectrometer (Seiko Instruments Inc., Chiba, Japan). These measurements were performed after dialysis against a 10 mM Tris–HCl buffer (pH 8.0) containing 20% saturated ammonium sulphate and 1 mM EDTA for 12 h. Spectrophotometric measurements were carried out with a Shimadzu spectrophotometer MPS-2000, using a 1-cm light-path cuvette at 25°C. SDS–PAGE was performed by the method of Schägger and von Jagow (1987) or Laemmli (1970). The protein bands were stained by Coomassie brilliant blue R-250 or Silver Stain kit (Wako Pure Chemical Industries Ltd., Osaka, Japan).

Protein sequencing techniques

The enzyme with an 86-kDa subunit (about 200 μg) was dissolved in 0.1 M HCl and cleaved with 1 mg CNBr by the method of Gross and Witkop (1962), with slight modifications. After the solution was incubated for 20 h at room temperature, the digests were separated by Tricine SDS–PAGE (Schägger and von Jagow 1987) and transferred to a polyvinylidene fluoride membrane (Sequi-Blot™ PVDF membrane, Bio-Rad, Calif.). The N-terminal amino acid sequences of the CNBr peptides were determined by using the protein sequencer (model 476A) (Applied Biosystems, Foster City, Calif.).

Cloning and sequencing

All general DNA manipulations were carried out according to Sambrook et al. (1989). The chromosomal DNA was isolated from *M. magnetotacticum* by the methods of Blin and Stafford (1976). Based on (*i*) the N-terminal amino acid sequence of the CNBr peptide (8.0 kDa), prepared by the method described above (H-P-N-D-A-A-K-R-N-L-R-N-G-D-V-V), and (*ii*) the consensus amino acid sequence of NapA (G-H-G-H-D-L-A), two sets of degenerate oligonucleotides (primer I, 5′ TT-(ACGT)GC-(ACGT)GC-(AG)TC-(AG)TT-(ACGT)GG-(AG)TG-CAT 3′; primer II, 5′ GG(ACGT)-CA(CT)-GG(ACGT)-CA(CT)-GA(CT)-(CT)T(ACGT)-GC 3′) were synthesized by Amersham Pharmacia Biotech. By using primers I and II, a 0.3-kb DNA fragment was amplified by PCR from *M. magnetotacticum* chromosomal DNA. The PCR product was ligated into pGEM-T vector (Promega Corporation, Madison, Wis.). The recombinant plasmids obtained were then introduced into *E. coli* XL-1 Blue MRF′ and sequenced with a Thermo Sequenase core sequencing kit with 7-deaza-dGTP (Amersham Life Science). It was confirmed that the PCR product encoded a part of *napA* by the sequence, and this DNA fragment was used as a screening probe for cloning the *nap* genes.

The chromosomal DNA isolated from *M. magnetotacticum* was digested with several restriction enzymes, separated by electrophoresis on agarose gel, and blotted onto a nylon transfer membrane (Hybond-N$^+$, Amersham Biosciences Corp., Piscataway, N.J.). DNA probe labeling and Southern hybridization were performed with an Alkphos Direct kit (Amersham Pharmacia Biotech). The 1.4-kb *Hin*dIII fragment of the chromosomal DNA hybridized with the labeled 0.3-kb PCR product. For cloning of the hybridizing 1.4-kb *Hin*dIII fragment, the PCR product was used to screen a pUC119 genomic library prepared from *Hin*dIII fragments containing 1.0–2.0 kb of *M. magnetotacticum* genomic DNA, and two positive colonies were then purified. Two colonies carried the 1.4-kb *Hin*dIII fragment insert that encodes a part of *napA*. To clone the entire *nap* gene clusters, the 1.4-kb *Hin*dIII fragment was used as a probe for the Southern hybridization experiment. The 3.2-kb *Pst*I and 6.0-kb *Eco*RI fragments hybridized with the labeled 1.4-kb *Hin*dIII fragment. These fragments were also cloned by the same method as described above.

The cloned DNA fragments were sequenced on both strands by dideoxy chain termination method after subcloning the restriction fragments onto pUC119 vector. DNA sequences were determined by a Hitachi DNA sequencer SQ5500 (Hitachi Instruments Service Co., Ltd., Tokyo, Japan).

Search and analysis of sequences

The sequence data obtained in the present study were analyzed using the BLAST program (Altschul et al. 1990). GenBank, EMBL, and DDBJ nucleic acid sequences and SWISS-PROT protein sequence databases were used for homology searches. For motif search, the sequence data were analyzed using MOTIF (Bioinformatics Center 2001), and for signal sequence search, data were analyzed using PSORT (Nakai 1991) or SignalP (Nielsen 1997). The phylogenetic tree was constructed with CLUSTAL W software (Thompson et al. 1994). The amino acid sequence of *E. coli* DmsA (DMSO reductase) was defined as an out-group.

Determination of iron content in magnetosome fraction

The cells (wet weight 0.1–0.2 g) were suspended in 10 mM Tris–HCl buffer (pH 8.0) and completely disrupted with a sonic oscillator (20 kHz, 100 W) for 10 min. The lysate was centrifuged at 8000 × *g* for 20 min. The resulting pellet and supernatant were used as the magnetosome fraction and cell-free extract fraction, respectively.

The total iron content of the magnetosome fraction prepared from Mo-supplemented cells and Mo-deficient cells of *M. magnetotacticum* were chemically determined as follows. After the organic components in the magnetosome fraction were decomposed by dry-ashing, the resulting brown ash was dissolved in 1 M HCl and diluted into a known volume with distilled and deionized water. The iron concentration was measured by photometric determination with 1,10-phenanthroline (Vydra and Kopanica 1963). A calibration curve was prepared with aqueous standard solutions of Fe (Wako).

Results

Purification of *M. magnetotacticum* nitrate reductase

As summarized in Table 1, the nitrate reductase has been purified from the soluble fraction of Mo-supplemented cells of *M. magnetotacticum* by ammonium sulfate fractionation, hydrophobic chromatography, and gel filtration. The enzyme showed absorption peaks at 551, 521, and 419 nm in the reduced form and was composed of 86- and 17-kDa proteins

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Table 1. Purification of nitrate reductase from *Magnetospirillum magnetotacticum*.

	Volume (mL)	Protein concn. (mg/mL)	Total protein (mg)	Total activity (nmol/s)	Specific activity ($nmol \cdot s^{-1} \cdot mg^{-1}$)	Purification	% recovery
Soluble fraction	130	4.02	522	185	0.353	1.00	100
$(NH_4)_2SO_4$ precipitation	30.0	5.20	156	174	1.12	3.17	94.4
First Butyl-Toyopearl chromatography	20.9	1.77	37.0	84.2	2.28	6.45	45.6
Second Butyl-Toyopearl chromatography	29.7	0.486	14.4	70.7	4.90	13.9	38.3
Sephacryl S-300 gel filtration	26.2	0.128	3.35	47.2	14.1	39.8	25.5
Sepharose CL-6B	12.8	0.088	1.13	24.6	21.8	61.7	13.3

on SDS–PAGE, although the 17-kDa protein band was less markedly stained with Coomassie brilliant blue (Fig. 1, lanes 1 and 3). These properties bear some resemblance to those of the periplasmic nitrate reductase of *Paracoccus pantotrophus* (Berks et al. 1994). Assuming that the enzyme is composed of one molecule of each of the 86- and 17-kDa proteins, the Fe and Mo contents were 5.4 (mol/mol of enzyme) and 0.83 (mol/mol of enzyme), respectively. The heme *c* content was determined to be 2.2 (mol/mol of enzyme). When reduced methyl viologen was used as electron donor, K_m and V_{max} values of the enzyme for nitrate were 3.2 μM and 2.5 s^{-1}, respectively.

On the other hand, the minor fractions with nitrate reductase activity (about 25% of total activities) were obtained after the first column chromatography. Although the minor enzyme exhibited nitrate reductase activity that used methyl viologen (reduced form) as electron donor, the enzyme showed no spectral features corresponding to heme *c*. Furthermore, the enzyme was composed of an 86-kDa protein (Fig. 1, lanes 4 and 6), and its Fe and Mo contents were 2.4 (mol/mol of enzyme) and 0.65 (mol/mol of enzyme), respectively. These results suggest that the minor nitrate reductase may be a catalytic subunit of the two-subunits enzyme, which contains Mo and non-heme iron. It should be noted that the NapB subunit is easily lost during the purification of the *R. capsulatus* enzyme (McEwan et al. 1987).

Cloning and sequencing of the gene encoding *M. magnetotacticum* nitrate reductase

The primers regenerated from the N-terminal amino acid sequence of the 8.0-kDa CNBr peptide of the 86-kDa protein and from the consensus sequence of NapA successfully amplified a 0.3-kb fragment from *M. magnetotacticum* chromosomal DNA. According to the Southern hybridization experiment, the 1.4-kb *Hin*dIII, 3.2-kb *Pst*I, and 6.0-kb *Eco*RI fragments were identified and cloned (see Materials and methods). These fragments were subcloned and sequenced. Nucleotide sequence analysis of these DNA fragments revealed the presence of seven major open reading frames (ORFs) in the same direction (Fig. 2). These seven ORFs were identified and designated *napF*, *napD*, *napA*, *napG*, *napH*, *napB*, and *napC* (DDBJ accession No. AB055444). These seven ORFs identified in the 6-kb region are closely connected, and thus support the idea of polycistronic organization.

Analysis of protein sequences

The deduced primary structures of the NapF, NapD, NapA, NapG, NapH, NapB, and NapC consist of 171, 104, 827, 264, 279, 154, and 213 amino acids, respectively, with molecular masses of 17 418, 11 544, 92 948, 27 993, 29 610, 17 165, and 24 418 Da, respectively, and isoelectric points of 7.79, 4.93, 8.56, 8.22, 9.06, 9.13, and 8.87, respectively,.

The N-terminal sequence (H-P-N-D-A-A-K-R-N-L-R-N-G-D-V-V) of the CNBr peptide prepared from the purified 86-kDa protein was identical to that from H748 to V763 of NapA. The computer-assisted sequence analysis strongly suggested that NapA has an N-terminal signal peptide of 29 residues, characteristic of periplasmic-exported proteins (Pugsley 1993). Furthermore, it has the T-R-R-D-F-I-K motif for a twin-arginine translocation system (Weiner et al. 1998). Therefore, *M. magnetotacticum* NapA may be transported from cytoplasm to periplasm and may result in a mature protein of about 90 095 Da, corresponding to the molecular mass of the large subunit of the purified nitrate reductase. On the other hand, non-heme irons and Mo atoms were detected in the purified nitrate reductase. The biochemical and spectroscopic studies of periplasmic nitrate reductase from *P. pantotrophus* clearly show that the enzyme contained a molybdopterin guanine dinucleotide (MGD) cofactor (Bennett et al. 1994) and a [4Fe-4S] cluster (Breton et al. 1994). In addition, C177 (cystein, position 177 of the amino acid sequence deduced from *M. magnetotacticum* NapA) for the ligand of the Mo atom in the MGD cofactor of *Desulfovibrio desulfuricans* NapA (Dias et al. 1999) and one [4Fe-4S] cluster binding motif in the N-terminal region were conserved in *M. magnetotacticum* NapA. Therefore, the NapA of *M. magnetotacticum* nitrate reductase seems to have one [4Fe-4S] cluster and an MGD cofactor like those of *P. pantotrophus* and *D. desulfuricans* periplasmic nitrate reductases.

N-terminal amino acid sequence of the 17-kDa protein was determined to be E-E-V-K-X-L-R-P-X-A (X was not determined) by protein sequencing. This sequence corresponds to amino acid positions 31–40 of the amino acid sequence deduced from the *napB* sequence. Furthermore, sequence analysis strongly suggested the existence of a long signal sequence for translocation to periplasm in the N-terminal amino acid sequence of NapB (positions 1–30). These results indicate that the molecular mass of the mature NapB can be estimated to be 14 287 Da, although this is much lower than that calculated using SDS–PAGE. On the

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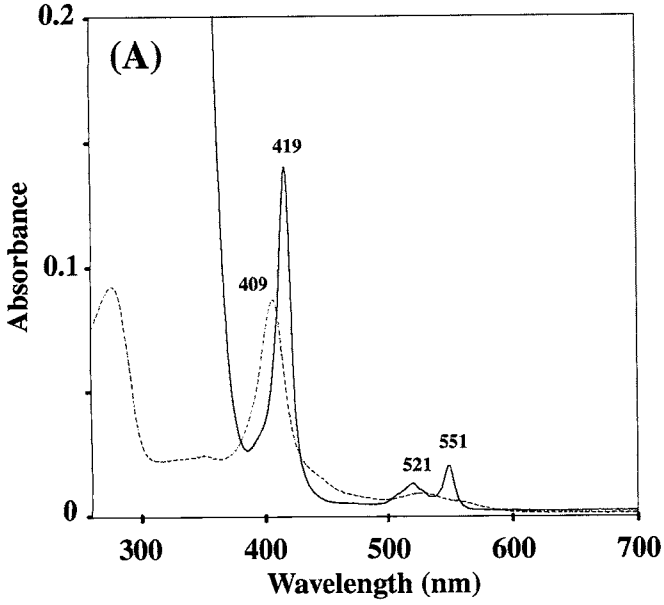
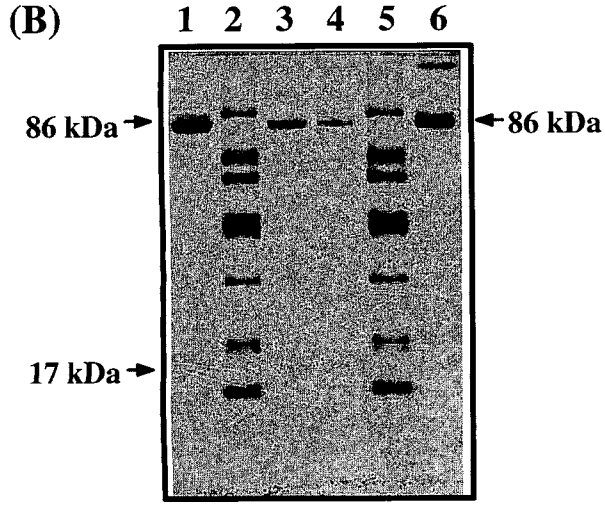
Fig. 1. (A) Absorption spectra of *Magnetospirillum magnetotacticum* nitrate reductase (broken line), air-oxidized; (solid line), reduced with dithionite. (B) Tricine SDS–PAGE of *M. magnetotacticum* nitrate reductase. Lanes 1 and 3, major nitrate reductase; lanes 4 and 6, minor nitrate reductase. Lanes 2 and 5 are marker proteins: phosphorylase *b* (97 kDa), bovine serum albumin (66 kDa), L-glutamate dehydrogenase (55 kDa), ovalbumin (43 kDa), aldolase (40 kDa), carbonic anhydolase (31 kDa), soybean trypsin inhibitor (22 kDa), and lysozyme (14 kDa).





other hand, the sequence indicates the two heme *c* binding motifs. These motifs are consistent with the heme *c* content determined by pyridine ferrohemochrome of the native enzyme, a finding that suggests that NapB is a di-heme cytochrome *c*. The NapC has one potential transmembrane helix and four heme *c* binding motifs. Recently, Simon et al. (2000) reported that a NapC–NirT-type cytochrome *c* has quinol oxidase activity. Therefore, NapB may be localized at the periplasmic space and may function as an electron transfer protein between the catalytic subunit NapA and the membrane-bound component NapC, which accepts electrons from the quinone pool in the membrane.

NapF, NapG, and NapH of periplasmic nitrate reductase are iron–sulfur proteins. *Magnetospirillum magnetotacticum* NapF, NapG, and NapH have the same [4Fe-4S] cluster binding motifs as those of *E. coli* NapF, NapG, and NapH. Recently, Brondijk et al. (2002) reported that NapG and NapH but not NapF, of *E. coli* periplasmic nitrate reductase, are essential for electron transfer from ubiquinol to NapAB. Potter and Cole (1999) reported that the mutation of *napD* resulted in the total loss of methyl-viologen-dependent nitrate reductase activity of *E. coli*. Although the function of NapD in *M. magnetotacticum* has not been revealed, *M. magnetotacticum* NapD showed sequence similarity to *E. coli* NapD (33% identity and 52% similarity).

Comparison and phylogenetic analyses of *nap* operons

According to phylogenetic analyses of *M. magnetotacticum* 16S rDNA sequences (Eden et al. 1991) and cytochrome *c* sequences (Yoshimatsu et al. 1995), the bacterium belongs to the α subclass of *Proteobacteria*. However, a phylogenetic tree of NapA amino acid sequences, as shown in Fig. 3, indicated that *M. magnetotacticum* NapA bore a closer relationship to NapA of the γ subclass of *Proteobacteria* than to NapA of the α subclass of *Proteobacteria*. The phylogenetic trees of NapB and NapC amino acid sequences also indicate a close relationship between the γ subclass of *Proteobacteria* and *M. magnetotacticum* (data not shown). Furthermore, the organization of the *M. magnetotacticum nap* gene cluster was exactly the same as that of *E. coli* and *Haemophilus influenzae*, which belong to the γ subclass of *Proteobacteria*.

Upstream region of *M. magnetotacticum nap* operon

Darwin et al. (1998) have investigated the regulation of the *E. coli* K-12 *napF* operon and found that the expression is atypically regulated via Fnr and NarL–NarP proteins. Figure 4 shows the 5′ end of the *napF* region of the *M. magnetotacticum nap* operon. The binding sites of two putative transcriptional factors, Fnr and NarL–NarP proteins, were found in this region. Fnr protein is a global regulator that controls transcription of the genes whose functions facilitate adaptation to growth under O_2-limiting conditions (Kiley and Beinert 1999). Although TTGAcCAACgTCAA, located 86 bases upstream of the putative start codon of *napF*, is not the same as the consensus sequence of the Fnr binding site, TTGATNNNNATCAA, it seems to be a potential Fnr binding site of the *M. magnetotacticum nap* operon. On the other hand, NarL–NarP is a transmitter component of the two-component system and controls gene expression in response to nitrate and nitrite (Rabin and Stewart 1993). Several putative NarL–NarP binding sites, heptermers (TAC(C/T)(C/T) (A/C)T), were found upstream of *M. magnetotacticum napF*, as shown in Fig. 4. Although Darwin et al. (1998) have reported that the NarL–NarP binding site of the *E. coli* K-12 *napF* operon is located between the Fnr binding site and the initiation site of the *napF*, the NarL–NarP binding site could not be found in the same region of the *M. magnetotacticum napF* operon. Furthermore, the putative NarL–NarP binding sites found upstream of *M. magnetotacticum napF* were not organized as inverted repeats with 2-bp spacing. Therefore, it seems likely that the expressions of the genes encoding the periplasmic nitrate

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Fig. 2. Physical map of the *Magnetospirillum magnetotacticum nap* operon and sequencing strategy. The arrows show the positions, directions, and extent of sequences obtained. The bars show cloned DNA fragments from genomic DNA libraries. Restriction sites: A, *Acc*I; Bg, *Bgl*I; Bs, *Bst*XI; E, *Eco*RI; Hc, *Hinc*II; Hd, *Hind*III; N, *Nco*I; P, *Pst*I; Sc, *Sac*I; Sl, *Sal*I; Sp, *Sph*I; and X, *Xho*I.

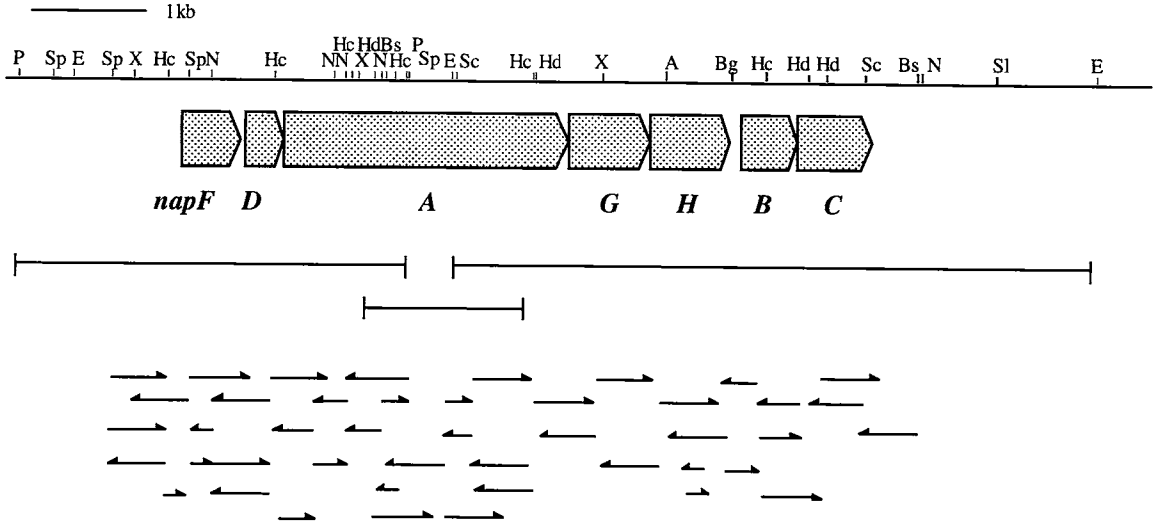

Fig. 3. Phylogenetic tree of prokaryotic nitrate reductase NapA. *Escherichia coli* DMSO reductase DmsA was defined as an out-group. The tree was constructed with CLUSTAL W software (Thompson et al. 1994) using the neighbor-joining method. Figures represent bootstrap confidence percentages from 1000 bootstrap samples for grouping. The scale bar represents 0.1 substitutions per position.

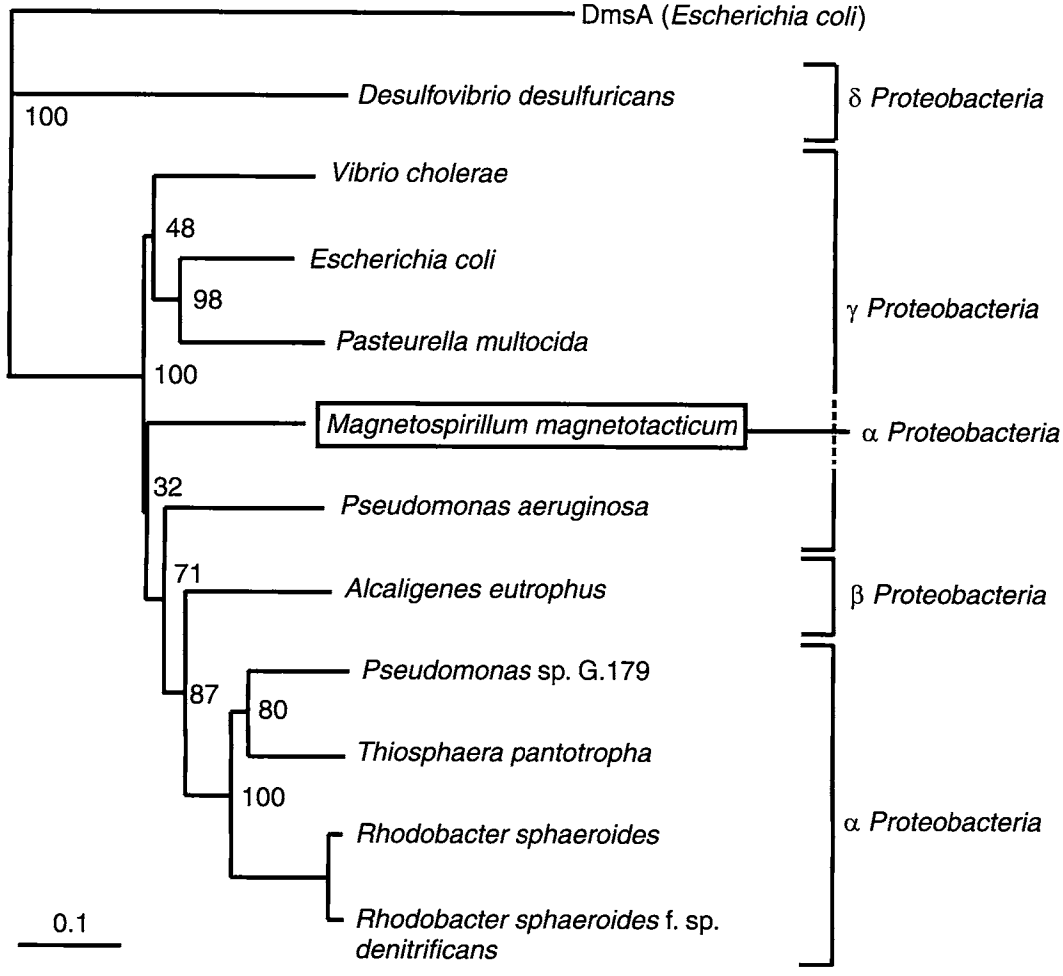

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Fig. 4. The upstream region of *Magnetospirillum magnetotacticum napF*. Putative Fnr and putative NarL binding sites are underlined and double-underlined, respectively. The first few N-terminal amino acids of the NapF are shown in one-letter code. The putative ribosome binding site of *napF* is overlined.

```
GCATGCTGATGAACGATGtGGATACGGCGGTGCGCAAGATGAGGgCGCTG
CGCGATCTCGGAATCGGACTgTCCATCGACGATTTCGGCACAGGATATTC
GTCGCTTAGCTATCTCGGGCGCTTTCCCATCACCACGCTGAAGATCGATC
GCGcCTTCATCGCCGATGtGGACACCAATCCCAAGACCGcCGAGATCGCC
AGGGCCATCATCGGCCTGTCGCGCGGCCTCAATCTCGAGGTTGTGGCGGA
AGGGGCGGAAATCGCCGCCCATATCGCCTTCCTGCGCAGCAACGGATGCG
ACACCGTGCAAGGGTTCTTCTACTCCcGGCCCGTCCCGGCTGACGAGTTC
GAACAGATGATGCGCCAGCGGATCATGGCCCACGCCTGACCGCGTCTTCC
CCCTCTCTCTTTTGAcCAACgTCAAGGCGCCAGTCTGGGCGCGGTGACAC
CTTTCCGCCATCTGGCGTCATTCCTGGGGAGAAGGAATGGCCTGCCGCGG
GAGGAAATCCGATGTCTGGGCTGCTGTCCCGACGGACGTTGTTGACGAGA
          M  S  G  L  L  S  R  R  T  L  L  T  R
```

reductase of *M. magnetotacticum* are regulated with oxygen but not with nitrate and (or) nitrite.

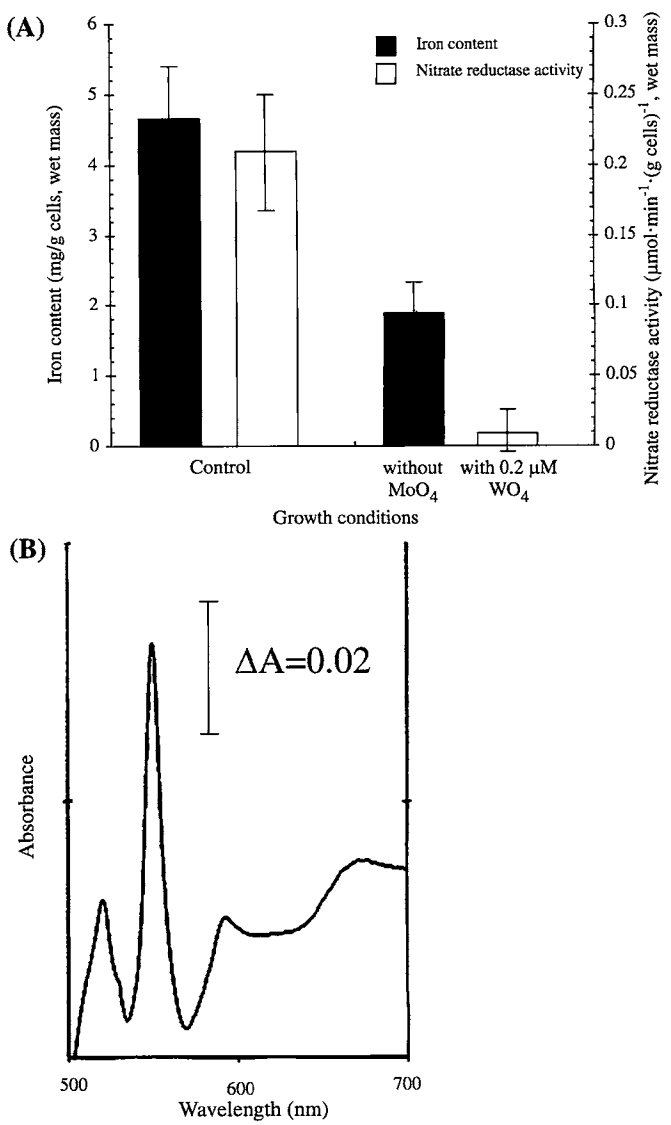
Effects of Mo deficiency in the medium on magnetite synthesis

To investigate physiological function of the periplasmic nitrate reductase in *M. magnetotacticum*, we cultivated the bacterium in the absence of Mo for 1–2 months, as described in Materials and methods, and compared (*i*) nitrate reductase activity of the cell-free extract and (*ii*) total iron content of the magnetosome fractions prepared from the Mo-deficient cells with those prepared from Mo-supplemented cells of *M. magnetotacticum*. To achieve complete Mo depletion, Mo-deficient cells were grown with excess amount of sodium tungstate (0.066 mg/L). As shown in Fig. 5A, the nitrate reductase activity of Mo-deficient cells of *M. magnetotacticum* was almost undetectable, while the iron content in the magnetosome fractions was about 40% (1.88 mg/g cells) of the control contents of the magnetosome fraction prepared from Mo-supplemented cells (4.65 mg/g cells). These results show that the bacterial magnetites could be synthesized in the absence of the active periplasmic nitrate reductase. However, surprisingly, cd_1-type nitrite reductase was highly expressed in Mo-deficient cells because the broad absorption peak at ca. 670 nm, which is contributed from heme d_1, was predominantly observed in the cell-free extracts difference spectrum, reduced with dithionite minus oxidized with $K_3Fe(CN)_6$, prepared from Mo-deficient cells (Fig. 5B).

Discussion

This report described the first purification of the nitrate reductase that catalyzes the first step of denitrification of the magnetotactic bacterium *M. magnetotacticum*. The enzyme is a complex consisting of 86- and 17-kDa subunits and has 5.4 Fe (mol/mol of enzyme) and 0.83 Mo (mol/mol of enzyme), respectively. During purification, we obtained an 86-kDa subunit that has 2.4 Fe (mol/mol of enzyme) and 0.65 Mo (mol/mol of enzyme), indicating that the complex possibly has a Mo cofactor, an iron–sulfur center, and two c

Fig. 5. (A) Comparison of iron content and nitrate reductase activity among Mo-deficient and Mo-supplemented cells. Iron content of the magnetosome fraction was determined by the method described in Materials and methods. The nitrate reductase activity of the cell-free extracts was measured using formate as electron donor. The reaction mixture contained 10 mM sodium nitrate, 250 mM sodium formate, and 50 mM sodium phosphate buffer (pH 7.0) (Siddiqui et al. 1993). The reaction was started by addition of an aliquot of enzyme preparation. After incubation at 25°C for 10 min, the reaction was stopped by addition of chemical regents used for nitrite analysis (Nicholas and Nason 1957). Values are averages of four independent cultures. The error bars indicate the standard deviation of the mean. (B) The difference spectrum of cell-free extracts, reduced with $Na_2S_2O_4$ minus oxidized with $K_3Fe(CN)_6$, prepared from Mo-deficient cells of *Magnetospirillum magnetotacticum*. The spectrum was measured at room temperature. The broad absorption peak at 670 nm is attributed to cytochrome cd_1.



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hemes in the molecule. These properties are very similar to those of the periplasmic nitrate reductase from *P. pantotrophus* (Berks et al. 1994). In general, two respiratory nitrate reductions occur in bacterial cells. One is catalyzed by a membrane-bound nitrate reductase and another by a periplasmic nitrate reductase. The former is predominantly expressed under anaerobic growth conditions, while the latter is predominantly expressed under aerobic growth conditions. Furthermore, the cytoplasmic membranes prepared from *M. magnetotacticum* did not show any nitrite reductase activity (data not shown) and currently available genome sequencing data on *M. magnetotacticum* (Bertani and Simon 2001) do not clarify the presence of the genes encoding a membrane-bound nitrate reductase. Therefore, *M. magnetotacticum* utilizes a sole nitrate reductase in the periplasmic space under microaerobic conditions. The periplasmic location is also logical, considering that oxygen primarily inhibits denitrification at the level of nitrate transport (Denis et al. 1990), which is not required for the Nap system.

Magnetospirillum magnetotacticum has constitutively a cbb_3-type cytochrome *c* oxidase that has similar spectral properties and genes (DDBJ accession No. AB024290) to those of *R. capsulatus* (Thöny-Meyer et al. 1994) (DDBJ/ EMBL/GenBank acc. No. X80134) and *B. japonicum* (Preisig et al. 1993) (DDBJ/EMBL/GenBank acc. No. L07487). The cbb_3-type cytochrome *c* oxidase shows a proton pumping activity (Toledo-Cuevas et al. 1998). Therefore, it seems likely that denitrification of *M. magnetotacticum* in the periplasmic space is not essential for energy production under microaerobic conditions. However, the bacterium optimally produces magnetites under microaerobic growth conditions in the presence of nitrate (Blakemore et al. 1985). Furthermore, Yamazaki et al. (1995) reported that the periplasmic nitrite reductase (cytochrome cd_1) may function as a Fe(II)–nitrite oxidoreductase for magnetite synthesis in *M. magnetotacticum*. These suggest a possible involvement of denitrification in magnetite synthesis in *M. magnetotacticum*. However, the cell-free extract prepared from Mo-deficient cells of *M. magnetotacticum* has little periplasmic nitrate reductase activity, while the magnetosome fraction has approximately 40% iron of that prepared from Mo-supplemented cells (Fig. 5). Neither nitrate nor nitrite might be essential for biosynthesis of magnetite in contrast to the previous results. However, it should be noted that the cytochrome cd_1 was highly expressed in Mo-deficient cells (Fig. 5). Therefore, the bacterium might utilize oxygen as an alternative electron acceptor for Fe(II) oxidation by cytochrome cd_1 in the absence of Mo because *M. magnetotacticum* cytochrome cd_1 shows *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine–O_2 oxidoreductase activity (Yamazaki et al. 1995).

The *M. magnetotacticum* periplasmic nitrate reductase was encoded in *napFDAGHBC*. The organization of the *M. magnetotacticum nap* gene cluster is exactly the same as that of the γ subclass of *Proteobacteria*, such as *E. coli*. Recently, Brondijk et al. (2002) reported that *E. coli* NapG and NapH are essential for electron transfer from ubiquinol to NapAB. It is of interest that *M. magnetotacticum* uses only ubiquinol as electron mediator in the membrane (Sakane and Yokota 1994). On the other hand, the phylogenetic analyses of NapA, NapB, and NapC suggest a close relationship between the γ subclass of *Proteobacteria* and *M. magnetotacticum*, which is not consistent with phylogenetic analyses of the 16S rDNA sequences (Eden et al. 1991) and the cytochrome *c* sequences (Yoshimatsu et al. 1995) (Fig. 3). In the course of evolution, genomes are shaped by processes like gene loss, gene duplication, and horizontal gene transfer. Therefore, the *nap* gene clusters of *M. magnetotacticum* might be transferred by horizontal gene transfer within the bacterial community, although transposable elements, differences of GC contents, and codon usage could not be found around the *nap* operon. It is generally accepted that magnetotactic bacteria have multiple evolutionary origins (Spring and Schleifer 1995). Although more study is needed to elucidate the evolution of magnetotactic bacteria, the genetic information for biosynthesis of the magnetite system might be transferred by horizontal gene transfer among physiological groups, such as the *nap* genes.

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