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学位授与の題目	Studies on Biosynthesis System of Magnetite in Magnetic Bacterium *Magnetospirillum magnetotacticum* MS-1 （磁性細菌 *Magnetospirillum magnetotacticum* MS-1におけるマグネタイト生合成経路に関する研究）
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学位論文要旨

Abstract: I purified the nitrate reductase from *Magnetospirillum magnetotacticum* MS-1. The enzyme was composed of 86 kDa- and 17 kDa-subunits and contained molybdopterin guanine dinucleotide and heme *c* as cofactors. *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 *E. coli nap* genes, which is not consistent with the 16S rDNA data. Furthermore, I investigated the effects of molybdate-deficiency in the medium on the total iron contents of the magnetosome fraction. The result suggested that the periplasmic nitrate reductase activity is not essential for magnetite biosynthesis in *M. magnetotacticum* but necessary for optimum synthesis of magnetite.

Secondary, I tried developing the method of the targeted gene disruption for *M. magnetotacticum*. Broad-host-range plasmids of the IncP group were transferred by conjugation and replicated in this bacterium. Studies are under way to construct of *nap* genes disruption mutant of *M. magnetotacticum*.

Finally, I found that two Fe^{3+}-siderophore receptors were highly expressed in the magnetic cells. These receptors were also induced when the cells were cultivated with the Fe^{2+} rich medium. These results suggest that the bacteria transport and utilize Fe^{3+} for magnetite synthesis even under the Fe^{2+} rich growth condition.

Introduction

Magnetospirillum magnetotacticum MS-1 was isolated from the sediments of the freshwater swamp [1]. One of the novel features of *M. magnetotacticum* is that this

bacterium synthesizes intracellular particles, termed magnetosomes, which are enveloped single crystals of magnetite with lipid bilayers.

Bazylinski and Blakemore investigated the optimum 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 (N_2) [2]. They also reported that the bacterium cannot grow under strict anaerobic conditions with nitrate [2]. Therefore, it seems likely that the bacterium is a microaerobic denitrifier: the aerobic respiration with O_2 and the anaerobic respiration with nitrate as the final electron acceptors occur simultaneously in the cell. Furthermore, Yamazaki et al. have reported that the cd_1-type nitrite reductase is highly expressed in the periplasm of the magnetic cells of *M. magnetotacticum* and shows a novel Fe(II):nitrite oxidoreductase activity [3]. These results suggest that the 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.*

Materials and Methods

Microorganisms and growth conditions

M. magnetotacticum was cultivated in a chemically defined liquid medium [1]. The Mo-deficient cells were prepared by cultivation with the Mo-deficient metal mixture under the same growth conditions as described above. 1.4 mM NH_4Cl was added to the medium as N source for the growth of *nap* deletion strain.

Escherichia coli XL-1 Blue MRF' was used for cloning studies. *E. coli* S17-1 [4] was used for the donor of diparental conjugation. *E. coli* HB101(pRK2013) and *E. coli* HI1006 were used as helper strain and donor strain of triparental conjugation studies, respectively. *E. coli* strains were grown at 37 °C under aerobic conditions in Luria-Bertani (LB) medium [5].

Purification of nitrate reductase from M. magnetotacticum

M. magnetotacticum cells were broken by three passages through the French pressure cell (100 MPa). After the unbroken cells and magnetosomes were removed by a centrifugation at 10,000 x *g* for 15 min, the supernatant was recovered and further centrifuged at 187,000 x g for 1 h. The nitrate reductase was purified from the supernatant by anion exchange-, cation exchange- and hydroxylapatite-chromatographies.

Assay of the nitrate reductase activity

The nitrate reductase activity was assayed by the method of Fernández et al. with slight modifications [6]. Nitrite concentration was determined by the diazocoupling procedure of Nicholas and Nason [7].

One- and two-dimensional polyacrylamide gel electrophoreses (2-DE)

One- and two-dimensional electrophoreses were performed according to methods of Laemmli [8] and O'Farrell [9], respectively.

Determination of iron contents in the magnetosome fraction

The cells were completely disrupted with a sonic oscillator (20kHz, 100W). The lysate was centrifuged at 8,000 x *g* for 20min. The resulting pellet was used as the crude magnetosome fraction. After the organic components in the fraction were decomposed by dry ashing, the resulting brown ash was dissolved in 1N HCl and diluted into distilled and deionized water. The iron concentration of resulting solution was measured by photometric determination with 1,10-phenanthroline [10].

Mating experiments

The mating experiments were conducted by the methods of Simon et al. [11] and de Bruijn and Rossbach [12] with some modifications.

Results and Discussion

Periplasmic nitrate reductase and magnetite biosynthesis

I purified the periplasmic nitrate reductase, the first enzyme of denitrification, from *M. magnetotacticum*. The enzyme shows absorption peaks at 551, 521 and 419 nm in the reduced form and was composed of 86 kDa- and 17 kDa-proteins on SDS-PAGE, although the 17 kDa-protein band was less markedly stained with Coomassie Brilliant Blue (Fig.1). Further, the enzyme contained molybdopterin guanine dinucleotide and heme *c* as cofactors.

The *M. magnetotacticum nap* locus was clustered in seven open reading frames, *napFDAGHBC*. The organization of the *M. magnetotacticum nap* gene cluster is exactly the same as that of the gamma subclass of *Proteobacteria* as *E. coli*, which is not consistent with the phylogenetic analyses of the 16S rDNA sequences [13] and the cytochrome *c* sequences [14]. The phylogenetic analyses of NapA, NapB and NapC suggested a close relationship between *M. magnetotacticum nap* genes and *E. coli nap* genes (Fig.2). Therefore, the *nap* gene cluster of *M. magnetotacticum* might be transferred by horizontal gene transfer within the bacterial community. Furthermore, two binding sites of the putative transcriptional factors, Fnr and NarL/NarP, were found in upstream of *napF* region.

To investigate the physiological function of the periplasmic nitrate reductase in *M. magnetotacticum*, I cultivated the bacterium in the absence of Mo and compared the nitrate reductase activities of the cell-free extracts and the total iron contents of the

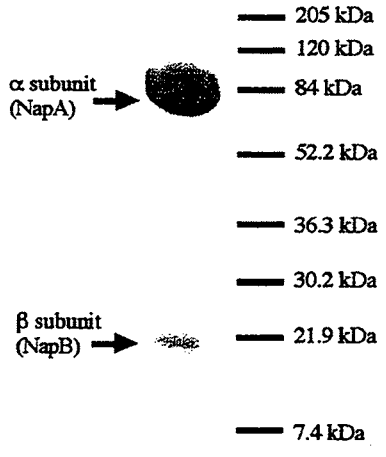

Fig. 1 SDS-PAGE of purified *M. magnetotacticum* periplasmic nitrate reductase. The gels were stained with Coomassie Brilliant Blue R-250.

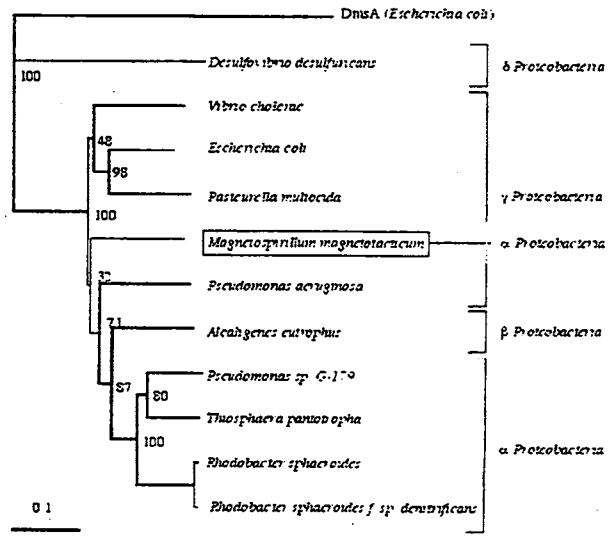

Fig.2. Phylogenetic tree of the NapA. *E. coli* DMSO reductase DmsA was defined as out-group.

crude magnetosome fractions prepared from the Mo-deficient cells with those of Mo-supplemented cells of *M. magnetotacticum*, respectively. As shown in Figure 3A, the nitrate reductase activity of the Mo-deficient cells of *M. magnetotacticum* was almost undetectable, while the iron contents in the magnetosome fractions was about 40% of the control experiment. These results show that the bacterial magnetites could be synthesized in the absence of the periplasmic nitrate reductase. Neither of nitrate nor nitrite might be essentially required for biosynthesis of magnetite in contrast to the previously results. However, it should be noted that the cytochrome cd_1 was highly expressed in the molybdate-deficient cells (Fig.3B). Therefore, the bacterium might utilize oxygen as 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 [3].

***Development of a targeted gene disruption method for* M. magnetotacticum**

At first, I established the method for plasmid transfer by conjugation in *M. magnetotacticum*. The two RK2 derivative plasmids pRK415 and pKS800 were transferred to recipient strain, *M. magnetotacticum*, by diparetal and triparetal conjugations, respectively. This is the first report on plasmid transfer by conjugation in *M. magnetotacticum* MS-1.

Secondly, I tried the targeted disruption of *napFDAG* genes in *M. magnetotacticum*. The gene disruption plasmid, namely, pKF205, was constructed and transferred into *M. magnetotacticum*. To force recombination, substitution plasmid, pKS807, was further transferred into resultant transconjugant [15]. The occurrences of recombination events were verified by Southern hybridization analysis, suggesting that the recombination events occurred between the disruption plasmid and genomic DNA.

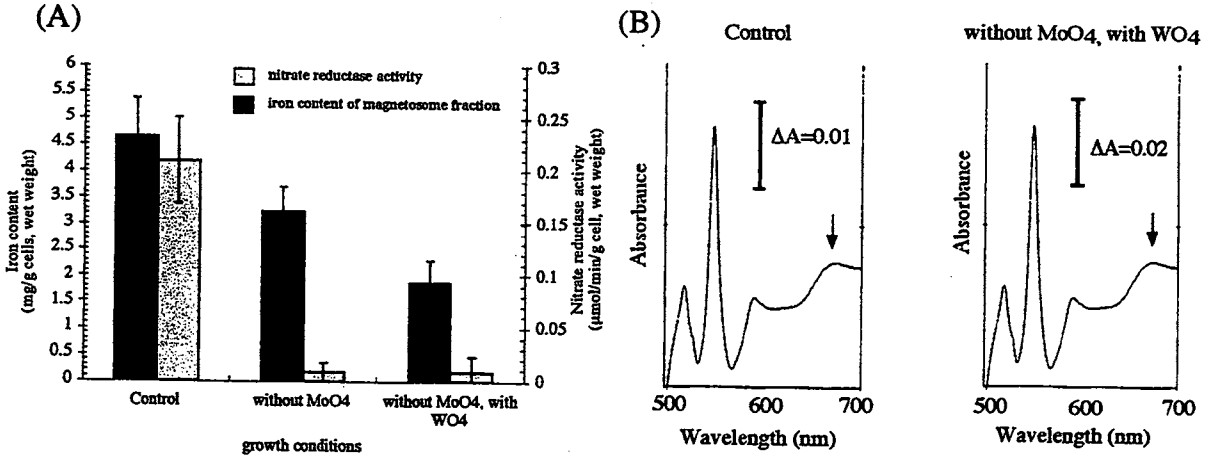

Fig 3. (A) Comparison of the iron content and nitrate reductase activity between Mo-deficient cells and Mo-supplemented cells. (B) The difference spectra of [reduced with $Na_2S_2O_4$] minus [oxidized with $K_3Fe(CN)_6$] of the cell-free extracts prepared from Mo-supplemented cells and Mo-deficient cells. The broad absorption peak at 670nm is attributed to cytochrome cd_1.

However, the culture was mixture of wild type cells and single and double crossover recombinant cells. Now I have tried isolation of double crossover homologues recombinant strain from the culture of *M. magnetotacticum* (pKF205, pKS807) by dilution method.

Uptake of iron by* M. magnetotacticum *-Ferric iron receptors-

To identify the proteins for magnetite biomineralization, I have compared the protein profile of the non-magnetic cells with that of the magnetic cells of *M. magnetotacticum* by 2-DE and found that two TonB-dependent outer membrane receptor homologues were highly expressed in magnetic cells (Fig.4). Furthermore, the expressions were enhanced in Fe^{2+} rich condition. Therefore, it seems likely that *M. magnetotacticum* transports and utilizes Fe^{3+} for magnetite synthesis from the culture by these receptors.

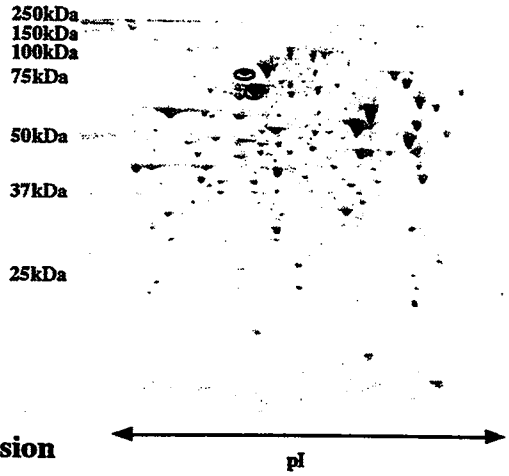

Fig.4 2-DE pattern of the cell-free extracts prepared from the magnetic cells of *M. magnetotacticum*. The circled proteins were magnetic cells specific ferric-siderophore receptor homologues.

Conclusion

I purified the periplasmic nitrate reductase and characterized the *nap* locus. Molybdenum starvation experiment suggested that the nitrate reductase activity is not essential for magnetite biosynthesis in *M. magnetotacticum* but necessary for optimum synthesis of magnetite. Furthermore, I tried developing the method of the targeted

gene disruption for *M. magnetotacticum*. Finally, I compared the protein profile of the non-magnetic cells with that of the magnetic cells and found that two TonB-dependent outer membrane receptor homologues were highly expressed in magnetic cells, suggesting that *M. magnetotacticum* transports Fe^{3+} for magnetite synthesis.

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学位論文審査結果の要旨

本論文は、磁性細菌 *M.magnetotacticum* の脱窒系の開始酵素であるペリプラズム型硝酸塩還元酵素（Nap）の生化学的性質と遺伝子塩基配列を解明するとともに、本酵素の発現制御機構および分子進化を考察することにより、磁気微粒子生合成機構と脱窒経路との関わりを明らかにし、さらに磁気微粒子生合成に関与する新規蛋白質を同定した論文である。

M.magnetotacticum の Nap を精製し、本酵素が２種類のサブユニットで構成され、補欠分子族としてモリブデンコファクター、鉄硫黄クラスター、ヘム *c* を持つ事を明らかにした。さらに、その遺伝子塩基配列から、本酵素が *napFDAGHBC* gene cluster にコードされていること、転写因子 FNR と NarL/P により発現制御されることを明らかにした。また、NapA,NapB,NapC アミノ酸配列による系統解析により、本細菌の *nap* 遺伝子群は *a* proteobacteria の *nap* 遺伝子群の水平伝搬によることを示唆した。一方、これまで同定されていない磁気微粒子生合成に関与する蛋白質として２種類の Fe（III）-siderophore レセプターを同定し、本細菌が磁気微粒子生合成にため Fe（III）を積極的に取り込んでいることを示唆した。

以上、本研究により、磁性細菌 *M.magnetotacticum* のマグネタイト合成に関して、多くのことを明らかにし、それらの知見は当該分野の研究発展に大いに寄与するものと考えられる。従って、審査委員会は、本論文が博士論文として妥当であると判断した。