Biomineralization in Magnetospirillum magnetotacticum

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Biomineralization in *Magnetospirillum magnetotacticum*

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Abstract ***M .magnetotacticum*** **mediated the uptake of Fe(III) by using iron transport system and reduced Fe(III) with cytoplasmic NADH-Fe(III) oxidoreductase to produce magnetites. On the other hand, the bacteria which were cultivated under microaerobic denitrifying condition had much more magnetites. In this paper we investigated the effects of Mo-deficiency in the culture medium on denitrification and magnetites synthesis. The experimental results indicate that the Mo-deficient cells contained the cytochrome cd_1 with Fe(II)-nitrite oxidoreductase activity and synthesized magnetosomes, although nitrate reductase activity was not detected in the cell-free extracts.**

I Introduction

Magnetospirillum magnetotacticum possesses interesting particles, termed magnetosome, with ferrimagnetic iron oxide magnetite in the cell. By using the magnetosomes as magnetic sensors, the bacterium orients itself along the lines of the earth's magnetic field. The magnetite crystal in the magnetosome occurs with almost the same size of 50-100nm in the cytoplasm and enclosed by lipid bilayers with some characteristic proteins. Bazylinski and Blakemore reported that under microaerobic conditions supplemented with nitrate, which is finally reduced to N_2O (N_2), the bacterium produces more magnetites than under aerobic condition [1]. On the other hand, Yamazaki, Oyanagi, Fujiwara and Fukumori have found that the cytochrome cd_1 is specifically expressed in the magnetic cells and shows interesting Fe(II)-nitrite oxidoreductase activity[2]. These results suggest that the denitrifying enzymes may be required for magnetite synthesis in *M. magnetotacticum.* Here, to elucidate the function of the denitrifying enzymes in magnetite biomineralization, we have investigated the effects of Mo–deficiency on magnetite synthesis. Although the cell-free extracts prepared from the Mo-deficient cells showed little nitrate reductase activity, the cytochrome cd_1 was highly expressed and interestingly, much magnetites were produced in the cell.

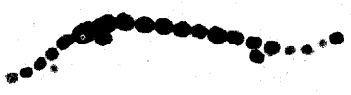

Fig.1 Magnetomes prepared from *M.magnetotacticum.*

II Materials and methods

A. Cultivation of M. magnetotacticum

M. magnetotacticum MS-1 (ATCC 31632) was cultivated in a chemically defined liquid medium containing sodium nitrate as the final electron acceptor under an O_2 (1%) - N_2 (99%) atmosphere at 25 °C in the dark [3]. Cells were harvested at the stationary phase by continuous centrifugation (21,150 x g) at 400 ml/min of flow speed. About 25 g of cells (wet weight) were obtained from 100 L cultivation and stored at -80 °C. The Mo-deficient cells were prepared by cultivation with the Mo-deficient metal mixture under the same growth conditions as described above.

B. Purification of nitrate reductase from M. magnetotacticum

Frozen cells 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 (1,000 fkg/cm^2). 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 obtained by the methods of ammonium sulfate fractionation, Butyl-Toyopearl column chromatography and gel filtration with a Sephacryl S-300 [unpublished [4]].

C. Assay of the nitrate reductase activity

The nitrate reductase activity was assayed by the method of Fernández, Gutiérrez and Ballesteros [5] with slight modifications. The standard reaction mixture contained 200 mM sodium-phosphate buffer (pH 7.0), 0.05mg/ml methyl viologen and 10 mM sodium nitrate in a total volume of 1.0 ml. The reaction was started by the addition of aliquot of enzyme and after incubation at 25 °C for 10 min, the reaction was stopped by mixing rapidly with air. Nitrite concentration was determined by the diazocoupling procedure of Nicholas and Nason [6].

D. One- and two-dimensional polyacrylamide gel electrophoresis and protein sequencing techniques

One- and two-dimensional electrophoresis (2-DE) were performed according to O'Farrell [7]. The first dimension was isoelectric focusing on a 5.0-8.0 Ampholine pH gradient. A 10% Tris-glycine polyacrylamide/SDS slab gel electrophoresis was used as second dimension [8]. The proteins were separated by 2-DE, and the spots were transferred to a polyvinylidene fluoride membrane (Sequi-Blot™ PVDF membrane, Bio-Rad). Selected spots were sequenced in an Applied Biosystems model 476A sequencer operating as recommended by the manufacturer.

E. Search and analysis of sequences

The sequence data were analyzed using the genome data base of *M. magnetotacticum* (http://www.jgi.gov/).

F. Determination of iron contents in the magnetosome fraction

The cells (wet weight 0.1-0.2 g) were suspended in the 10mM Tris-HCl buffer (pH 8.0) and completely disrupted with a sonic oscillator (20kHz, 100W) for a total period of 10 min. The lysate was centrifuged at 8,000 x *g* for 20min. The resulting pellet was used as the magnetosome fraction.

The total iron contents of the magnetosome fraction prepared from Mo-supplement 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 1N HCl and diluted into a known volume with distilled and deionized water. The iron concentration was measured by photometric determination with 1,10-phenanthroline [9]. Calibration was performed by using a calibration curve prepared with aqueous standard solutions of Fe.

III Results

A. Ferric Iron uptake by M. magnetotacticum

We have compared the protein profiles of the non-magnetic cells and the magnetic cells of *M. magnetotacticum* by two-dimensional polyacyrlamide gel electrophoresis. Fig. 2 shows the 2-DE pattern of the cell-free extracts prepared from the magnetic cells of *M. magnetotacticum*. One of two abundant proteins(circles in Fig.2), with an apparent molecular mass of 75kDa, estimated by gel electrophoresis, was present in the cell-free extracts prepared from the magnetic cells. The 75kDa-protein was specificallys expressed in the magnetic cells. The N-terminal amino acid sequence of the 75kDa-protein was determined to be ADLATDMPEVVIRGK by the method as described in Materials and methods. This was identified as exogenous ferric siderophore receptor in the genome sequences of *M. magnetotacticum*. Therefore, it seems likely that *M. magnetotacticum* utilizes ferric iron-uptake system for magnetite synthesis.

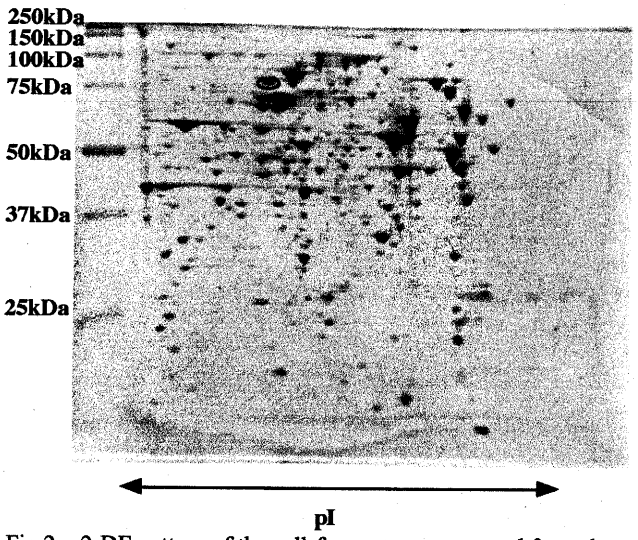

Fig.2 2-DE pattern of the cell-free extracts prepared from the magnetic cells of *M. magnetotacticum*.

B. Ferric iron reduction by M. magnetotacticum

Ferric iron reductase was purified from *M. magnetotacticum* to an electrophoretically homogeneous state [10]. The enzyme was loosely bound on the cytoplasmic face of the cytoplasmic membrane and was found more frequently in magnetic cells than in non-magnetic cells. The molecular mass of the purified enzyme was calculated to be about 36 kDa on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, which is almost the same mass as that calibrated by gel filtration analysis. The enzyme required NADH and FMN as optimal electron donors and cofactors, respectively, and the activity was strongly inhibited by Zn^{2+} acting as a partial mixed-type inhibitor. The *K*m values for NADH and FMN were 4.3 μM and 0.035 μM, respectively, and the *K*i values for Zn^{2+} were 19.2 μM and 23.9 μM for NADH and FMN, respectively. When the bacterium was grown in the presence of $ZnSO_4$, the magnetosome number in the cells and the ferric iron reductase activity declined in parallel with an increase in the $ZnSO_4$ concentration of the medium, suggesting that the ferric iron reductase may participate in magnetite synthesis[10].

C. Denitrification and magnetite synthesis in M. magnetotacticum

Bazylinski and Blakemore investigated the optimal growth conditions for magnetite synthesis by *M. magnetotacticum* and found that the bacterium produces much magnetite under microaerobic denitrifying conditions[1]. The bacterium scarcely synthesizes magnetites under aerobic conditions and, furthermore, does not grow using nitrate respiration (denitrification) under strictly anaerobic conditions. Therefore, the denitrification is not required for the growth in *M.magnetotacticum*. Denitrification involves four distinct enzyme systems, nitrate reductase, nitrite reductase, nitric oxide reductase and nitrous oxide reductase which catalyze the following reaction, $NO_3 \Rightarrow NO_2 \Rightarrow NO \Rightarrow N_2O \Rightarrow N_2$. In the present study, we have studied the involvement of denitrifying enzymes in the magnetite synthesis.

Abe have reported that nitrite can be used as an effective oxidizing reagent for the chemical synthesis of spinel-type ferrites in aqueous solution [11]. In this model, nitrite functions as oxidizing reagent for chemical synthesis of magnetite, suggesting that the similar phenomenon, i.e., enzymatic oxidation of ferrous iron by nitrite might be occurred in *M. magnetotacticum*. Recently, Yamazaki, Oyanagi, Fujiwara and Fukumori have purified nitrite reductase from *M. magnetotacticum* and found that the enzyme has high Fe (II)-nitrite oxidoreductase activity [2]. Furthermore, the enzyme is situated on the periplasmic face of the cytoplasmic membrane. O'Brien, Paoletti and

Blakemore analyzed spectrophotometrically cytochrome composition in the soluble fractions and membranes prepared from *M.magnetotacticum* and found that the cytochrome cd_1 was highly expressed in the denitrifying cells which have much amounts of magnetites[12]. Therefore, the oxidation of ferrous iron produced by cytoplasmic iron reductase seems to be occurred at the periplasm, in neither of the cytoplasm nor the magnetosome vesicle.

These results strongly suggest that "denitrification" in *M. magnetotacticum* is closely related with magnetite synthesis. In the present study, to better understand the relationship between "denitrification" and magnetite synthesis in *M. magnetotacticum*, we first purified the nitrate reductase, the first enzyme of denitrification, from *M. magnetotacticum* and characterized the enzymatic and molecular features. Furthermore, we cultivated the bacterium in the absence of Mo and compared the iron contents of the magnetosomes and the nitrate reductase activity.

Fig.3 shows the absorption spectra of nitrate reductase purified 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 (Data not shown). These properties are very similar to those of the periplasmic nitrate reductase of *Thiosphaera pantotropha* [13]. Assuming that the enzyme is composed of one molecule of each of the 86 kDa- 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 an electron donor, the *Km* and *V*max values of the enzyme for nitrate were 3.2μM and 2.5 s^{-1}, respectively.

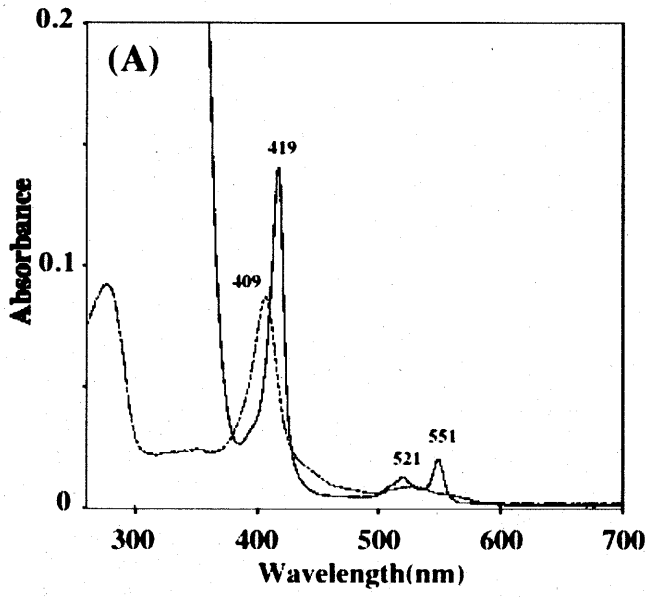

Fig.3 Absorption spectra of *M. magnetotacticum* nitrate reductase.

To investigate the 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 the nitrate reductase activity of the cell-free extract and the total iron contents of the magnetosome fractions prepared from the Mo-deficient cells with those of Mo-supplemented cells of *M. magnetotacticum*, respectively. As shown in Fig4, 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 70% (3.24 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 periplasmic nitrate reductase. However, surprisingly, the cytochrome cd_1 was highly expressed in the Mo-deficient cells because the broad absorption peak at *ca.* 670nm, which is contributed from heme d_1, was predominantly observed in the [reduced with dithionite] minus [oxidized with $K_3Fe(CN)_6$] difference spectrum of the cell-free extract prepared from the Mo-deficient cells (Fig.5).

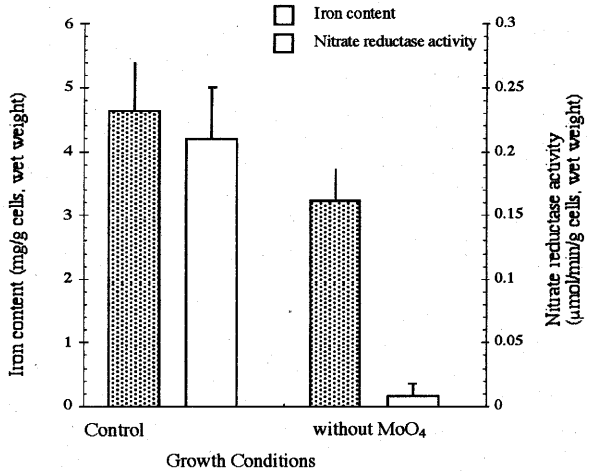

Fig.4 Effects of Mo-deficiency in the culture medium on nitrate reductase activity and iron contents of magnetosomes.

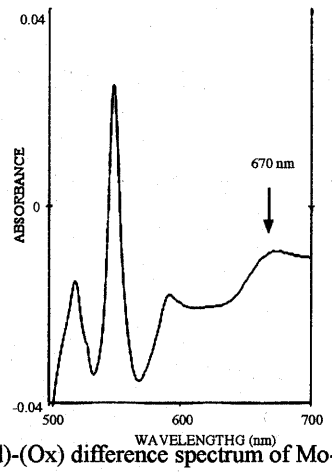

Fig.5 (Red)-(Ox) difference spectrum of Mo-deficient cells.

IV Discussion

How *M.magnetotacticum* produces magnetite crystals in the cell at room temperature? First, the bacterium transports ferric quinate across the membrane into cytoplasm via a poorly characterized system. Paoletti and Blakemore reported that the magnetic cells of *M. magnetotacticum* produced siderophore as hydroxamate material when cultured with high concentration of ferric quinate and three outer membrane proteins with 72kDa-85kDa were coordinately produced at iron concentrations conductive to hydroxamate production [14]. In the present study, we have found that the ferric siderophore receptor is specifically induced in the magnetic cells. Therefore, it seems likely that *M. magnetotacticum* utilizes ferric iron complexed with siderophore for magnetite synthesis.

Once in the cell, ferric iron is reduced by iron reductase using NADH as reducing power in the presence of FMN in the cytoplasm [10]. In general, ferrous ion is rapidly oxidized at neutral pH in the presence of oxygen to be insoluble Fe(III) oxides. However, *M. magnetotacticum* has a microaerobic respiratory chain and then, oxygen is reduced to H_2O by cytochrome *c* oxidase [15]. Therefore, it is possible that most of ferrous ion is present as free ion at neutral pH even under microaerobic condition. However, it is not clear whether ferrous iron is storaged as intracellular Fe-binding components or not.

Recently, we have proposed that the periplasmic cytochrome cd_1 nitrite reductase may function as ferrous ion oxidase for magnetite synthesis. In the present study, to elucidate the function of the denitrifying enzymes in magnetite biomineralization, we have characterized another denitrifying enzyme, the nitrate reductase of *M. magnetotacticum*. The enzyme was composed of 86 kDa- and 17 kDa-subunits and contained Mo, non-heme iron and heme *c*. Therefore, in *M. magnetotacticum* the denitrification may occur in the periplasm, not in the cytoplasm, suggesting a possible involvement of the denitrification in the magnetite synthesis. However, the cell-free extract prepared from the Mo-deficient cells has little nitrate reductase activity, while the magnetosome fraction has approximately 70% iron of that prepared from the Mo-supplemented cells. 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. 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 [2].

On the basis of the high-resolution transmission electron microscopy studies, the magnetite produced by *M. magnetotacticum* is reported to be a well ordered single-domain octahedral crystal [16]. Therefore, the nucleation of magnetite occurs at one primary nucleation site, suggesting one possibility that protein molecules, active in nucleation, are spatially organized at an unique site in the magnetosome membrane or cytoplasmic membrane. Recently, we have found that the 38-kDa protein in the cytoplasmic membrane has high affinity for ferric ions (unpublished [17]). The molecular mechanism of single magnetite crystal formation at room temperature will be a problem of central interest in *M.magnetotacticum*. Genetic systems in the bacterium have only recently begun, and little is known at the enzyme level. Improvement of genetic systems will be critical for obtaining knowledge of regulatory mechanism of crystallization of magnetite.

V References

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