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Laser Raman Spectroscopic Study on Magnetite Formation in Magnetotactic Bacteria

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Magnetotactic bacteria have one or more chains of magnetosome, consisting of nano-sized magnetic crystal covered with a phospholipid bilayer and use it to sense the geomagnetic fields. In order to elucidate the molecular process to make magnetosome from the iron compounds found in the bacteria, laser Raman spectroscopic measurements were performed with the magnetotactic bacterium, *Magnetospirillum magnetotacticum* MS-1 and the fractions separated from it. The major Raman signals at 662 cm^{-1} and 740 cm^{-1} were observed. The former was assigned to the Raman signal of magnetite and the latter, to that of ferrihydrite. The Raman signal of ferrihydrite was observed not only in the membrane fraction, but also in the cytoplasmic fraction. Based on the results, the role of ferrihydrite in magnetosome synthesis in the magnetotactic bacteria was discussed. [doi:10.2320/matertrans.MER2007333]

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1. Introduction

Magnetotactic bacteria are the microbes that synthesize nano-sized magnetic particles designated as magnetosome. These particles are composed of single crystals of magnetite (Fe_3O_4) or greigite (Fe_3S_4) covered with phospholipid bilayer. Magnetosomes have narrow size distribution, species-specific crystal shapes and high chemical purity.[1)] In the bacteria magnetosomes are fixed within the cell to form a chain structure (Fig. 1). The magnetotactic bacteria utilize the magnetosome-chain to orient themselves in geomagnetic fields and swim to micro-aerobic environments by sensing of oxygen or redox gradients.[2)]

To date only a few strains of magnetotactic bacteria were isolated and can be pure-cultured, mainly due to the fact that they love the micro-aerobic environment. Thus, the studies on the mechanism of magnetosome formation were mainly proceeds in the pure-cultured magnetotactic bacteria, such as *M. magnetotacticum* MS-1, *M. magneticum* AMB-1, and *M. gryphiswaldense*, with the magnetosome consisting of magnetite. With the aid of recent progress in molecular biological studies on the magnetotactic bacteria, the genomic structure[3,4)] and function of several proteins[1,5)] involved in the formation of the chain structure of magnetosome were elucidated.

However, the process of magnetite formation in magnetotactic bacteria is not clarified yet. Under alkaline aqueous conditions, magnetite is formed through four different pathways; by oxidation of green rust which formed from ferrous ion via white rust ($Fe(OH)_2$), by interaction of ferrous iron with ferrihydrite ($5Fe_2O_3 \cdot 9H_2O$), and by co-precipitation of ferrous and ferric ions from the mixture.[6)] In another pathway, ferrous ion is adsorbed at the surface of lepidocrocite (γ-FeOOH) and the resultant $(\gamma\text{-FeOOH})_2FeOH^+$ changes into magnetite.[7)] These pathways of oxidation of iron compounds are summarized comprehensively,[8)] and gathered into the so-called "Misawa diagram".[9)]

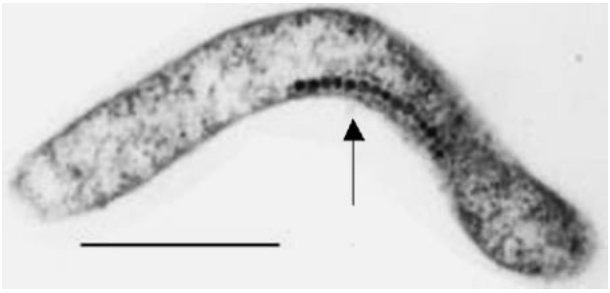

Fig. 1 TEM image of *M. magnetotacticum* MS-1 kindly provided by Dr. Y. Shimazaki. Arrow indicates a chain of magnetosomes. Bar; 1 μm.

On the biomineralization of magnetite in magnetotactic bacteria, several pathways were suggested previously. Frankel *et al.* suggested that magnetite was formed by reduction of ferrihydrite based on their Mössbauer spectroscopic observation.[10)] Matsunaga first suggested that magnetite was formed via lepidocrocite[11)] and recently that magnetite was formed by co-precipitation of ferrous and ferric ions in the magnetosome vesicle,[12)] which is kept alkaline by the iron/proton anti-transporter protein, MagA.[13)]

In this study, we investigated the iron compounds in a magnetotactic bacterium, *Magnetospirillum magnetotacticum* MS-1 by laser Raman microscopy and revealed their distribution inside the bacterial cell, supposing that these iron oxides and/or oxihydroxides would be detected as intermediates of magnetite formation. We observed two major Raman signals at 662 and 740 cm^{-1}. The former was assigned to that of magnetite and the latter, to that of ferrihydrite. This is the first evidence by the Raman spectroscopic measurements for the presence of ferrihydrite and magnetite in the magnetotactic bacteria.

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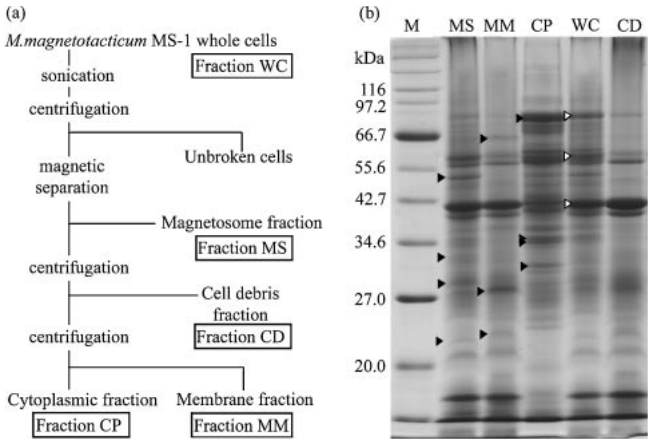

Fig. 2 (a) The flow chart of fractionation of *M. magnetotacticum* MS-1 cell. (b) SDS gel electrophoresis of cell fractions. M; molecular weight marker, MS; magnetosome fraction, MM; membrane fraction, CP; cytoplasmic fraction, WC; whole cell, CD; cell debris fraction. The size of molecular weight marker is shown in the left of the lane M. White arrowheads show the major protein bands of whole cell of *M. magnetotacticum* MS-1. Black arrowheads show the protein bands characteristic to the each fraction.

2. Experimental Methods

The magnetotactic bacterium *Magnetospirillum magnetotacticum* MS-1 (ATCC31632) was grown micro-aerobically in chemically defined medium (MSGM medium) at 25°C.[14] Cells were harvested at stationary phase by centrifuging at $6000 \times g$ at 4°C and subjected to experiments immediately.

All the procedures for cell disruption and fractionation were performed on ice or at 4°C and summarized in Fig. 2a. The cells were suspended in 50 mM Tris-HCl pH 6.8, and disrupted by ultrasonic homogenizer (Sonoplus HD2070, Bandelin, Berlin, Germany) with 20 kHz at 70 W (Amplitude; 30%) for a total period of 10 min. After the centrifugation at $600 \times g$ for 15 min to remove unbroken cells, a magnetosome (MS) fraction was isolated from the disrupted cell suspension by a magnet. After the following centrifugation of the suspension at $20000 \times g$ for 30 min, the pellet was used as a cell debris (CD) fraction. The supernatant was further ultracentrifuged for 60 min at $100000 \times g$ to separate a membrane (MM) and a cytoplasmic (CP) fraction. Each fraction was lyophilized and pressed to form a pellet with a diameter of 3 mm for laser Raman spectroscopic measurement. Magnetite and lepidocrocite used as the standard of Raman measurements were gift from T. Misawa.

The proteins in the fractionated samples were analyzed by SDS-PAGE. The samples were mixed with gel-electrophoresis sample buffer (50 mM Tris-HCl pH 6.8, 1% SDS, 1% 2-mercaptoethanol, 20% glycerol, and 0.02% bromophenol blue) and heated at 95°C for 5 min. Samples were loaded on 4.5% stacking gel and 12.5% separating gel. The gels were stained with CBB-R250. The amount of the total protein in the each fraction was determined by BCA Protein Assay Reagent Kit (Pierce, Rockford).

The cellular fractions and the whole cells of *M. magnetotacticum* MS-1 were lyophilized and pressed into a disk for the laser Raman spectroscopic measurements. Laser Raman spectra were measured using micro-probed Raman scattering system with a triple grating monochromator (NRS-2100, JASCO, Tokyo, Japan) in the backscattering configuration. The 532 nm beam line from Nd:YVO_4 laser was used for excitation and focused to about 2 μm in spot size on the surface of the sample. The laser power was controlled to 0.7×10^{-1} mW at the sample surface to avoid damaging to sample by rising temperature. The measurement in which samples were exposed to laser for 120 sec was repeated 100 times and the data were accumulated by the kaleidagragh (Hulinks, Tokyo, Japan)

3. Results and Discussion

3.1 Characterization of cell fractions

Cell components of the magnetotactic bacteria were separated according to the procedures shown in Fig. 2a. The proteins contained in the each fraction were compared by SDS-PAGE (Fig. 2b). In the whole cell fraction of *M. magnetotacticum* MS-1 (lane WC in Fig. 2b), three major bands were observed at molecular weight 84, 59 and 42 kDa (white arrowheads). The 84 kDa protein was also observed in the CP fraction (lane CP in Fig. 2b), but not in the other fractions. The large part of 59 kDa protein was found in MS fraction (lane MS in Fig. 2b), but not in MM fraction (lane MM in Fig. 2b). The 42 kDa protein was found in both MS and MM fractions and that in CP fraction was in smaller amount. The each fraction has its specific bands at 50, 31, 28 and 22 kDa in MS fraction, at 70, 27, and 23 kDa in MM fraction, and at 82, 34, 33, and 30 kDa in CP fraction (black arrowheads in each lane). The many protein bands of MS fraction were also observed in MM fraction, probably due to the fact that MS membrane is made from the inner membrane of the bacterial cell.[15] However, the characteristic profiles of each fraction shown in Fig. 2b indicate that the cell fractions were well-separated by our procedures.

3.2 The Raman spectroscopic measurements

As far as we know, this is the first application of the laser Raman microscopy to analyze the magnetotactic bacteria and their intracellular components. From the whole cell fraction, two major Raman peaks were observed at 662 and 740 cm^{-1} (Fig. 3a). To reveal intracellular distribution of the origin of these Raman peaks, the Raman spectra of the cell fractions were measured (Figs. 3b–3e). The wave numbers of Raman peaks observed from MS and CP fraction were listed in the Table 1 with those of iron oxide compounds.[16–20] The peak at 662 cm^{-1} observed in MS and CD fractions (Fig. 3b and 3e, respectively) was assigned to magnetite by comparing with that of the standard magnetite measured with our system (upper spectrum in Fig. 3f). The Raman spectra of magnetite were reported by several groups.[16,17,21] It has a main peak at 660–680 cm^{-1} and two broad peaks between 532–550 cm^{-1} and 298–301.6 cm^{-1},[16,21] although the peak value at the wave number was slightly different depend on the reports. Three additional weak peaks nearby 420, 320 cm^{-1} and sharp 300 cm^{-1} peak were also observed.[17,21] Our standard spectrum of magnetite (Fig. 3f) belongs to the former type and that of MS fraction (Fig. 3b) was similar to the latter. These weak peaks observed in the latter spectrum would be

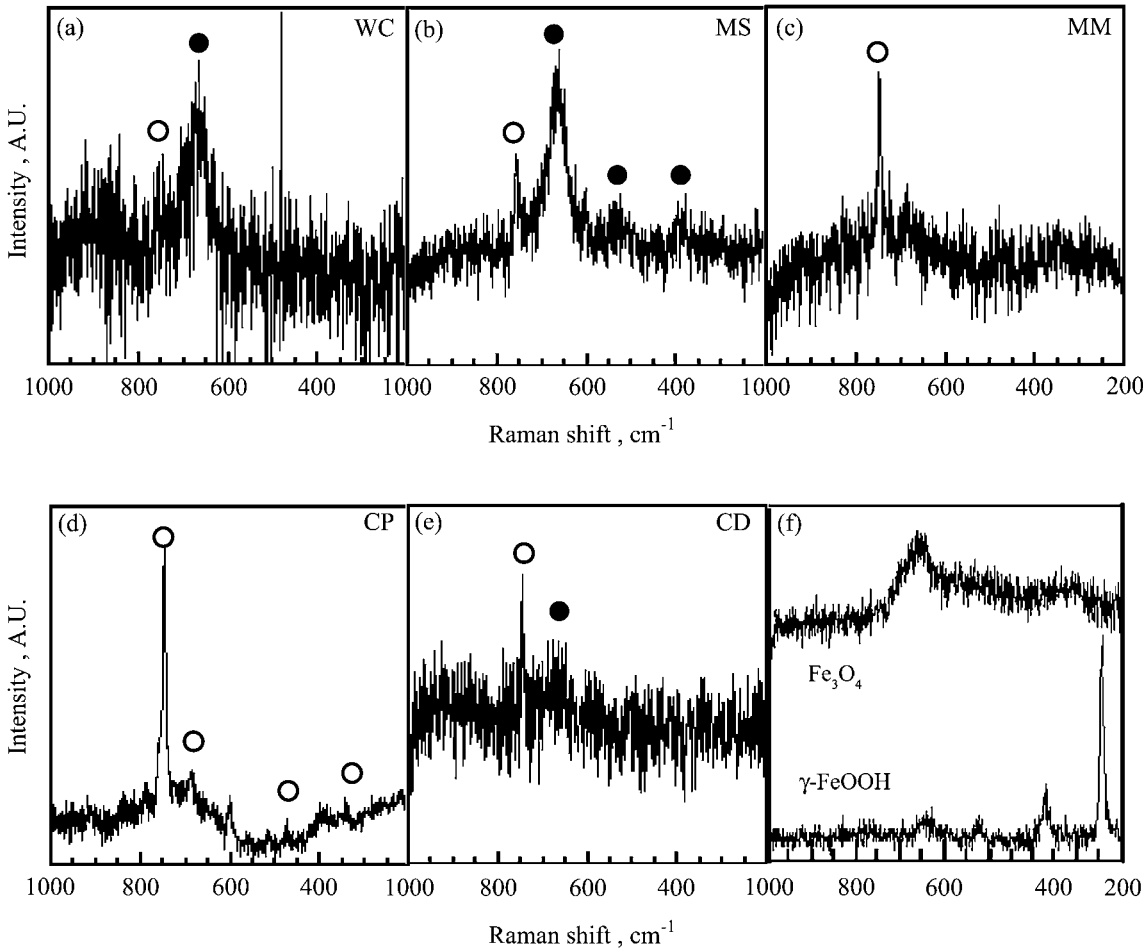

Fig. 3 Raman spectra of the whole cell and the each cell fraction of *M. magnetotacticum* MS-1. (a–e) Solid circle; the peak derived from magnetite. Open circle: the peaks correspond to ferrihydrite. WC, MS, MM, CP and CD are same as in Fig. 2b. (f) The Raman spectra of standard samples of magnetite (Fe_3O_4) and lepidocrocite (γ-FeOOH) measured by our system.

derived from hematite (α-Fe_2O_3) as the conversion of magnetite into hematite is sometimes caused by laser irradiation.[21] Thus, the Raman peak at 662 cm^{-1} observed in MS and CD fraction was assigned to magnetite. This 662 cm^{-1} peak of the MS fraction (Fig. 3b) was sharper than that of the standard spectrum (Fig. 3f), suggesting that magnetite in magnetosome has better crystallinity than that of the artificial magnetite used as standard.

The sharp 740 cm^{-1} peak was observed in the all fractions. This peak would be assigned to ferrihydrite, because the other iron compounds do not have a strong Raman signal at around 740 cm^{-1} as shown in Table 1. In CP fraction (Fig. 3d) almost all peaks corresponded to those of ferrihydrite reported before.[18]

In the living cells iron is usually stored as ferrihydrite by the iron storage protein.[22] Two types of iron storage protein were found in the living organism. Ferritin is widely present in the three kingdoms of life, and bacterioferritin is observed only in bacteria.[23]

The assignment of the 740 cm^{-1} Raman peak as ferrihydrite stored in ferritin or the similar protein of MS-1 is not contradict the result that the peak was observed in all cell fractions, because ferritin can be observed in both membrane and soluble fraction. Doig *et al.* reported that approximately 20% of the total amount of ferritin-like protein was detected in membrane fraction and the majority of the protein was localized in the cytosolic fraction in *Helicobacter pylori*.[24] If the distribution of ferritin-like protein in MS-1 cell is similar to that observed in *H. pylori*, the Raman spectrum of

Table 1 Raman wave numbers for cell fractions and those of iron oxides. Main peak was represented in bold type.

Iron oxides	Raman peak(cm^{-1})	Reference
Fraction MS	**662**, 532, 403	Fig. 3b
Fraction CP	**740**, 685, 600, 470, 341	Fig. 3d
	660, 580, 300	Fig. 3f
Fe_3O_4	667, 532	16)
Magnetite	1322, **676**, 550, 418, 319, 298	17)
$5Fe_2O_3 \cdot 9H_2O$	**725**, 692, 493, 347	18)
Ferrihydrite	**730**, 500, 375	19)
γ-FeOOH	1054, 654, 528, 380, **255**	Fig. 3f
Lepidocrocite	1307, 1054, 654, 528, 380, **255**	17)
$Fe(OH)_2$	**3570**, **407**, 260	19)
White rust		
$[Fe^{2+}_{(1-x)}Fe^{3+}_{x}(OH)_2]^{x+} \cdot [x/nA^{n-} \cdot m/nH_2O]^{x-}$	**528**, **427**	20)
Green rust		

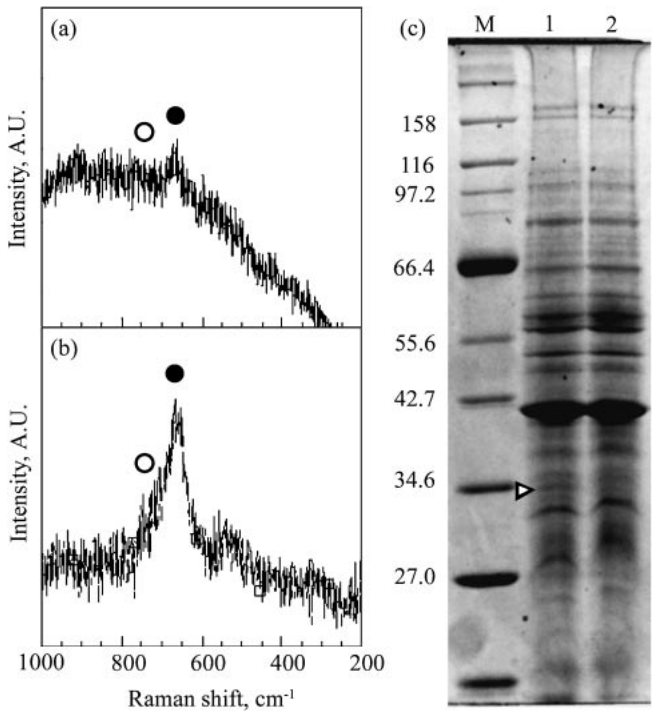

Fig. 4 (a) Raman spectrum of the MS fraction after incubated with 1% SDS at room temperature. (b) Raman spectrum of the MS fraction prepared from the bacterial cells stored at −80°C. Only the Raman peak of magnetite was observed in (a) and (b). (c) Protein profiles from MS fraction. Lane 1, proteins eluted by boiling the MS fraction in 2% SDS; lane 2, proteins washed out from MS fraction with 1% SDS. The marked band at 33.5 kDa is probably the protein tightly bound to magnetite particle.

ferrihydrite would be so strong in CP fraction that the small peaks could be clear and only the strong peak could be observed in MM fraction.

After the incubation with 1% SDS at room temperature, the Raman spectrum of the MS fraction did not represent the 740 cm^{-1} peak (Fig. 4a). If the 740 cm^{-1} peak observed in the MS fraction was derived from the protein containing ferrihydrite, it would be washed out by the 1% SDS and the 740 cm^{-1} Raman peak would disappear. Fig. 4c showed the proteins contained in the magnetosome membrane (lane 1 in Fig. 4c) and those washed out by 1% SDS (lane 2 in Fig. 4c), indicating that almost all proteins were washed out by 1% SDS. The marked band at 33.5 kDa is probably the protein tightly bound to magnetite particle. Based on the quantification of the amount of the total protein, 91% of the protein (42 ± 5 μg out of 46 ± 5 μg/mg MS fraction) was washed out. As shown in Fig. 4c, both protein profiles were almost identical except the band at 33.5 kDa. The results indicate that the origin of the 740 cm^{-1} Raman peak (ferritin-like protein) would be washed out by 1% SDS from the MS fraction (magnetosome).

Furthermore this 740 cm^{-1} Raman peak was not observed after the cell were stored at −80°C (Fig. 4b). It was shown by Mössbauer measurements that the ferrihydrite stored in the ferritin was changed into high-spin ferrous iron when it was frozen and thawed.[10,25] All the observations mentioned above do not contradict to the assignment of the 740 cm^{-1} Raman peak.

In our laser Raman measurements magnetite and ferrihydrite were detected in MS-1 cell, but not lepidocrocite and the other iron compounds. Lepidocrocite is a candidate for a precursor of magnetite as suggested before.[11] Furthermore, white rust and green rust easily transform into lepidocrocite by rapid oxidization.[8] If the bacterial cell contains white rust or green rust, it would easily transform into lepidocrocite under our conditions. As shown in Fig. 3f the Raman spectrum of lepidocrocite showed a very sharp peak at 255 cm^{-1}, but was not observed in the all fractions of MS-1 cell. Magnetite can be formed from lepidocrocite or green rust or white rust or ferryhydrite as mentioned before. Based on our results, ferrihydrite would be a most plausible precursor of magnetite and/or storage of iron in MS-1 cell. It would be still possible, however, that magnetite is formed via ferrihydrite, lepidocrocite, white rust or green rust when the life time of the precursor of magnetite is very short as reported in *M. gryphiswaldense*[26] and Raman signals of them cannot be detected.

In some bacteria, the expression of the gene encoding ferritin or bacterioferritin was regulated by ferric-uptake-regulator (fur) protein,[27] and that encoding a receptor protein of siderophore is also regulated by fur.[28] In MS-1 cell ferritin-like iron storage protein might provide iron as ferrihydrite to magnetosome vesicles after receiving iron from siderophore on the inner membrane. This model is well coincident with our results that the 740 cm^{-1} Raman peak was observed in both MS and MM fractions.

4. Conclusion

Iron compounds in a magnetotactic bacterium *Magnetospirillum magnetotacticum* MS-1 was studied by laser Raman microscopy. Our Raman measurements detected magnetite and ferrihydrite in MS-1 cell. The MS-1 cells were fractionated and the distribution of the iron compounds was studied. Magnetite was found in only magnetosome fraction, but ferrihydrite was detected in magnetosome membrane, cytoplasmic fraction, cell debris fraction, and membrane fraction. This is the first Raman measurement of magnetotactic bacteria and the results suggest that ferrihydirite may be a precursor of magnetite and/or storage of iron in MS-1 cell.

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