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Characterization of Iron Uptake in the Magnetic Bacterium *Aquaspirillum* sp. AMB-1

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ABSTRACT

The aim of this work is to elucidate the iron uptake mechanism in the magnetic bacterium *Aquaspirillum* sp. AMB-1, which can utilize a wide variety of iron chelates. However, no siderophore could be detected in spent culture. A 400-bp DNA fragment was amplified by polymerase chain reaction (PCR) from the genomic DNA of AMB-1, using primers designed from the *sfuC* gene of *Serratia marcescens*. This gene encodes a nucleotide binding protein in the periplasmic binding protein (PBP) dependent iron transport system. The amplified fragment was homologous with the conserved sequence of *sfuC*. Our results suggest that a nucleotide binding protein mediated iron transport system similar to that observed in *Serratia marcescens* is present in *Aquaspirillum* sp. AMB-1.

Index Entries: Biomineralization; magnetite; *Aquaspirillum*; iron uptake; nucleotide binding protein.

INTRODUCTION

Magnetic bacteria synthesize intracellular magnetic particles, which are covered with an organic membrane and are aligned in chains (*1*). The authors have reported the application of bacterial magnetite (*1–3*). A large amount of iron is required for the synthesis of magnetite (Fe_3O_4) particles,

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and 100 times the amount of iron is present in a magnetic bacterium than is present in *Escherichia coli* cell (*4,5*). Although iron transport is important for magnetite synthesis, the mechanism of iron transport across the outer membrane and the cytoplasmic membrane in magnetic bacteria is unknown.

Since Fe^{3+} is insoluble at neutral pH, the supply of iron to microbes requires chelating agents, such as siderophores (*6,7*). However, no siderophore could be detected in *Aquaspirillum* sp. strain AMB-1. Fe^{3+} quinate, Fe^{3+} malate, Fe^{3+} citrate, Fe^{3+} gallate, and various other iron sources are also utilized by AMB-1 for growth and magnetite synthesis (*5*). This suggests that AMB-1 does not synthesize siderophores, but can utilize any of a broad range of iron chelators. One possible explanation for these observations is that iron transport across the outer and cytoplasmic membranes in AMB-1 takes place in the same way as in *Serratia marcescens* (*8–10*). *S. marcescens* has a periplasmic binding protein (PBP) dependent iron transport system that involves three proteins encoded by the *sfu* operon. The periplasmic substrate binding protein (SfuA) binds Fe^{3+} directly (*10*). The *sfuC* gene encodes a nucleotide binding protein (*9*). SfuC is thought to hydrolyze nucleotide for energy coupling during translocation of iron across the cytoplasmic membrane (*10*). Since amino acid sequences of nucleotide binding proteins are conserved (*9,11*), PCR primers were designed from the *sfuC* nucleotide sequence. PCR was performed using genomic DNA of AMB-1 as template DNA. A PCR fragment of the correct size (400 bp) was obtained.

In this article, the iron uptake ability of *Aquaspirillum* sp. AMB-1 has been characterized. PCR has been used to amplify a DNA fragment containing a sequence that is homologous to the *sfuC* iron transport protein from *Serratia marcescens*.

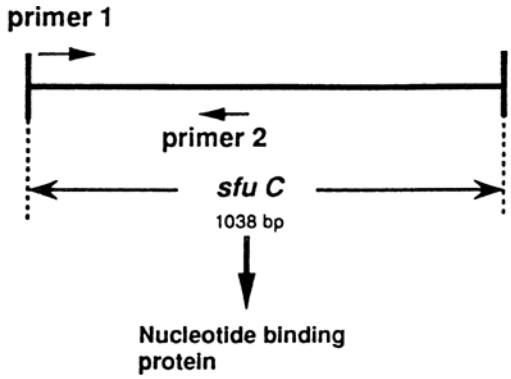
MATERIALS AND METHODS

Strains and Growth Conditions

Aquaspirillum sp. strain AMB-1 (*5*) and *Aquaspirillum magnetotacticum* MS-1 ATCC 31632 (*4*) were grown at 25°C in MSGM (*4*). *Serratia marcescens* IFO3046 and IFO3736 were cultured in medium containing 1.0% polypeptone, 0.2% yeast extract, and 0.1% $MgSO_4 \cdot 7H_4O$ (pH 7.0) at 30°C. *Escherichia coli* DH5α (*12*) was grown in Luria broth at 37°C.

Preparation of Nonmagnetic Mutants

Mutants of the magnetic bacterium AMB-1 that do not synthesize magnetite were obtained by using *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG). Log-phase cells were washed with and resuspended in phosphate buffer (pH 7.0), and NTG was added to the cell suspension at a final concentration of 2 $\mu g \cdot mL^{-1}$ and incubated for 1 h at 25°C. Cells were



primer 1 : 5' GGCCCTTCCGGCTCCGGCAA 3'
primer 2 : 5' GAGAACGGTTCATCCAGCAG 3'

Fig. 1. Designed PCR primers from the DNA sequence of the periplasmic iron transport protein of *Serratia marcescens* (8).

harvested by centrifugation, washed twice with liquid MSGM, and then cultured on MSGM agar plates in an anaerobic jar for 10 d.

Measurement of Iron Uptake Ability

Iron uptake ability of magnetic bacteria was assayed by measuring the decrease of iron concentration in liquid cluture with the growth of strain AMB-1 and the nonmagnetic mutant. Iron concentration was measured using ferrozine, which is a spectrophotometric reagent for iron (*13*). Cell growth was determined using a hemocytometer.

Siderophore Assay

The chrome azrol S (CAS) method was used for the detection of siderophore synthesis in magnetic bacteria (*14*). Magnetic bacteria were cultured in iron-enriched (40 μM), iron-limited (<1 μM), and normal iron conditions (30 μM) in MSGM. Cell-free supernatant (0.5 mL) was separated from the stationary culture by centrifugation, and CAS Fe^{3+}-dye complex solution was added. The absorbance spectrum (630 nm) of the CAS solution mixture was measured with spectrophotometer. Presence of siderophores was detected by the decrease of absorbance at 630 nm and the shift of absorbance toward shorter wavelengths.

PCR and Southern Hybridization

PCR primers were designed from *S. marcescens sfu* genes (*9*) and synthesized using a DNA synthesizer (Applied Biosystems Co. Ltd.) (Fig. 1). Primers 1 and 2 encode GSGK and DEP amino acid sequences, respectively, which are conserved in nucleotide binding protein sequences belonging to PBP system (*9*). Template genomic DNA was prepared from

magnetic bacteria, *S. marcescens*, and *E. coli* (*15*). Polymerase chain reaction was performed using *Taq* polymerase (Promega) with the thermal cycle repeated 30 times—95°C for 1 min, 45°C for 2 min (annealing), and 72°C for 2 min (*13*).

After ethanol precipitation, PCR products were subjected to agarose gel electrophoresis and transferred to a nylon membrane. Southern hybridization was carried out by using the nonradioactive DIG Luminescent Detection Kit (Boehringer Mannheim Biochemica Co. Ltd.). The *sfuC* gene amplified by PCR of *S. marcescens* was used as a probe.

RESULTS AND DISCUSSION

Iron Uptake Ability

Wild-type AMB-1 forms black colonies on MSGM agar plates in a microaerobic atmosphere, but colonies of nonmagnetic mutants are light brown. Nonmagnetic mutants could therefore be isolated from plates incubated after NTG treatment. One mutant, MG-10, was selected for the measurement of iron uptake. MG-10 had lower iron uptake ability than the other mutants (data not shown). Figure 2 shows the decrease in iron concentration with increasing cell numbers of the wild-type, AMB-1, and the mutant, MG-10. Thirty percent of the iron in liquid MSGM was taken up by wild-type cells before the stationary phase. Almost all of the iron that was taken up into wild-type cells was converted into intracellular magnetic particles, which made up approx 2% of cellular dry weight (approx 180 μmol/g dry wt cells). MG-10 was defective in iron uptake. Removal of iron by MG-10 was $< 40\%$ of that removed by the wild type. This result suggests that the genes associated with the functional iron uptake were damaged by NTG treatment.

AMB-1 is nonselective as an iron chelator. Even nonchelated Fe^{3+} or Fe^{3+} EDTA, which is a very stable chelation, could be utilized as the iron source for growth and magnetite synthesis (*5*). This suggests that AMB-1 can synthesize strong iron-chelating siderophores or could maintain an unusual iron transport system different from the systems of other microaerophiles.

Measurement of Siderophore Production

The results of the CAS assay are shown in Table 1. If siderophores are secreted into the medium, the supernatant and CAS solution mixtures indicate change of absorbance at 630 nm. No absorbance change was measured in sample fluid from AMB-1 culture at any Fe^{3+} concentration. Magnetite synthesis did not occur under iron-limited conditions, which had no effect on cell growth. Hydroxamate siderophore production by *Aquaspirillum magnetotacticum* MS-1 under iron-rich conditions has been

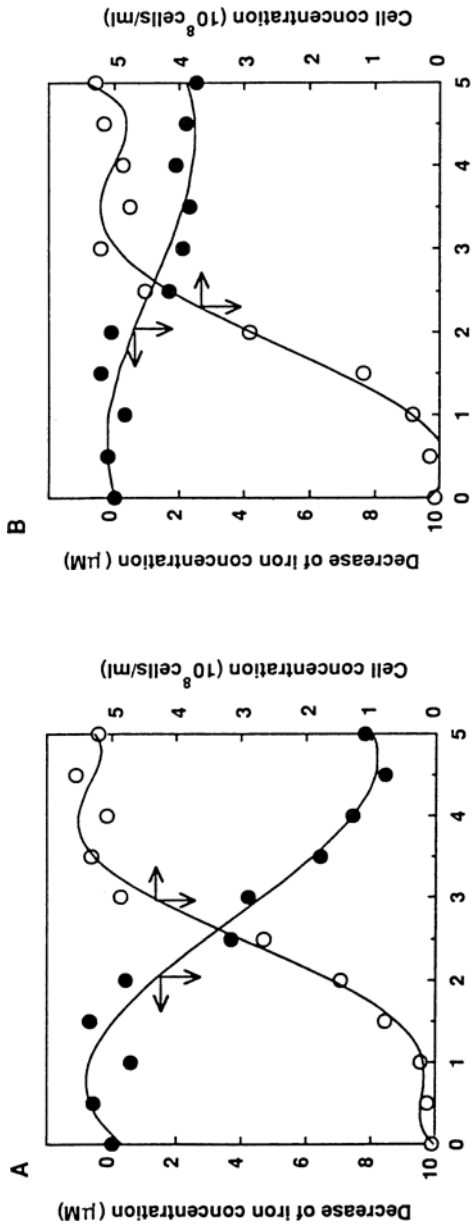


Fig. 2. Relationship between decrease of iron concentration and growth of wild type AMB-1 and nonmagnetic mutant MG-10. Graph A and B respectively show AMB-1 and MG-10. Open circles indicate the cell concentration and closed circles indicate the total decrease in iron concentration from the initial iron concentration.

Table 1
Detection of Siderophores of Magnetic Bacteria Using CAS Assay

Strains	Iron limited condition[a]	Normal iron condition[b]	Iron rich condition[c]
MS-1	–	–	+
AMB-1	–	–	–

Absence (–) or presence (+) of siderophore was indicated by a color change in the indicator iron-dye complex.
[a]Iron concentration was less than 1 μM.
[b]30 μM.
[c]40 μM.

reported (*17*). Siderophore production in *Aquaspirillum* sp. AMB-1 is different from that of *Aquaspirillum magnetotacticum* MS-1. Iron transport systems without siderophore production have been reported in *Neisseria gonorrhoea* (*18*), and siderophore-independent iron uptake is possible for AMB-1.

Analysis of PCR Product

Primers 1 and 2 were designed from the nucleotide binding protein specific conserved sequence (ideal size approx 400 bp). PCR using these primers successfully amplified the expected size of DNA fragment from all tested chromosomal DNA (Fig. 3A). Panel B shows Southern hybridization using the PCR product of *S. marcescens* as a probe. This probe hybridized strongly to the 400-bp DNA fragment from AMB-1. However, the 1.4-kbp nonspecific PCR product showed no hybridization. In the same way, 400-bp products from *E. coli* did not hybridize to probe DNA. Chromosomal DNA of *E. coli* encodes many nucleotide binding proteins in periplasmic binding protein substrate transport systems, for example *fecE*, *fhuC* (iron siderophore, *19,20*), and *btuC* (vitamin B_{12}, *21*). However, the amplified fragments of *E. coli* were not hybridized with *sfuC* gene. The polymerase chain reaction has probably failed in the amplification of such genes as *E. coli* encoding the nucleotide binding proteins, because maximum homologies between *sfuC* and these genes are $< 60\%$ in the PCR-primer-designed domain. Therefore, strong hybridization suggests that the amplified fragment of AMB-1 DNA codes a region highly homologous with *sfuC*, which is expected to be a nucleotide binding protein in the iron transport system. This system is thought to work as a siderophore-independent system in the magnetic bacterium AMB-1.

In addition, a 500-bp DNA fragment amplified from the chromosomal DNA of MS-1 also showed homology to *sfuC*. MS-1 can synthesize siderophore only in iron-rich conditions. No siderophore was detected in limited and normal iron condition (Table 1). Hydroxamate siderophore

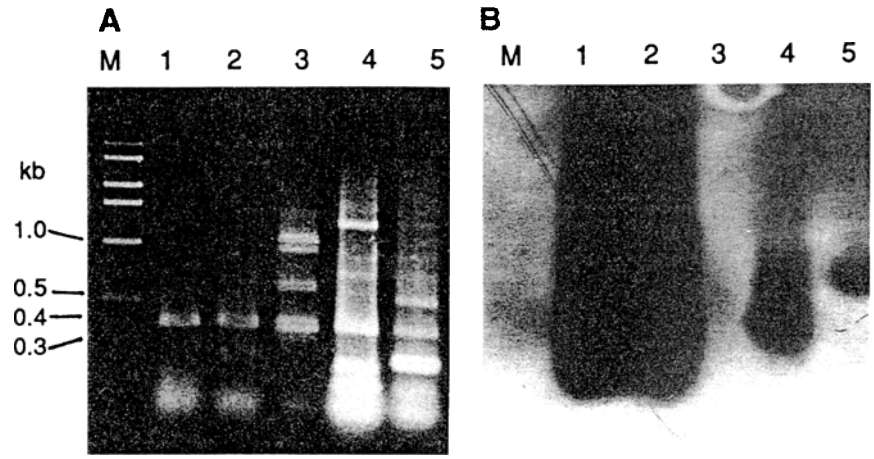


Fig. 3. Agarose gel electrophoresis and Southern hybridization analysis of PCR products amplified using primers from *sfuC* sequence of *Serratia marcescens*. Lane M, 1kb DNA ladder (GIBCO BRL); lane 1, PCR product of *S. marcescens* IFO3046; lane 2, PCR product of *S. marcescens* IFO3736; lane 3, PCR product of *E. coli;* lane 4, PCR product of *Aquaspirillum* sp. AMB-1; lane 5, PCR product of *A. magnetotacticum* MS-1.

production by MS-1 is unusual, because siderophores are usually synthesized in low-iron condition by microbes (*7*). A hypothesis that could be proposed from the siderophore assay result and the hybridization analysis is that similar iron transport systems are functioning in *Aquaspirillum* sp. AMB-1 and MS-1 at low iron concentrations.

In conclusion, the basic characterization of the iron uptake ability of the magnetic bacterium *Aquaspirillum* sp. AMB-1 was completed, and a possible iron transport system of magnetic bacteria has been proposed in this work.

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