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Siderophore production of a periplasmic transport binding protein kinase gene defective mutant of *Magnetospirillum magneticum* AMB-1

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Abstract

A non-magnetic mutant, NMA61, of the magnetic bacterium *Magnetospirillum magneticum* AMB-1 was generated by transposon mutagenesis to identify genes involved in magnetosome synthesis. The genomic region of NMA61 interrupted by a Mini-Tn5 transposon was analyzed. The transposon was inserted in an open reading frame (ORF) coding for a periplasmic transport binding protein kinase gene homologue. Three adjacent ORFs and a promoter were identified upstream, indicating that the sequences comprised an operon. Phenotype characterizations showed that the growth inhibition imposed by the exogenous non-assimilable iron chelator nitrilotriacetate was relieved in wild type but not in NMA61, by the addition of the isolated wild type siderophore. Higher concentration of siderophores accumulated in the culture medium of NMA61 than in wild type. These data suggest that the interrupted periplasmic transport binding protein kinase gene homologue is required for siderophore transport into *M. magneticum* AMB-1.

Keywords: *Magnetospirillum magneticum* AMB-1; Siderophores; Magnetosomes; Non-magnetic mutant

In magnetic bacteria, iron is assimilated in large amounts for the biosynthesis of intracellular magnetosomes which are membrane-bound crystals of the magnetic iron mineral magnetite (Fe_3O_4) or greigite (Fe_3S_4) [1–4]. These highly organized structures, aligned in chains, are proposed to function as biomagnetic antennae to orient the bacterium along iron and oxygen gradients through the Earth's magnetic field lines [5,6]. A clear-cut biochemical pathway of iron assimilation leading to magnetosome biomineralization has yet to be fully elucidated. In *Magnetospirillum magneticum* strain AMB-1, genes and their respective expressed proteins involved in magnetite synthesis were cloned and sequenced. The proteins Mms16, MagA, and Mms6 were shown to function for magnetosome membrane biogenesis, iron translocation, and magnetic crystal nucleation, respectively [7–9]. An open reading frame with an amino acid sequence homology to aldehyde ferredoxin oxidoreductase was also identified to be involved in ferric reduction in the cytoplasm during magnetosome formation [10].

Although iron is abundant in nature, it is converted to its trivalent state, Fe(III), in oxic environments at neutral pH which is very insoluble and hence not biologically available [11]. One of the alternative strategies of bacteria in assimilating insoluble iron is the synthesis and excretion of low molecular weight, high affinity Fe(III)-specific ligands termed siderophores. Although

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siderophore-mediated iron acquisition system has been extensively described in diverse groups of microorganisms, this is virtually an unexplored field in the complex iron metabolism of magnetic bacteria. In *Magnetospirillum gryphiswaldense* MSR-1, spent culture fluids stimulated the uptake of ferric ion at a high rate in iron-depleted cells but no siderophore-like compounds were detected [12]. An unusual result was obtained from *Magnetospirillum magnetotacticum* MS-1 in which more siderophores were detected in spent culture fluids of cells grown under sufficient to high iron conditions than in those grown under deficient iron condition [13]. It was however demonstrated in *M. magneticum* AMB-1 that the initial high concentration of iron was rapidly assimilated from the medium within only 4 h after inoculation reaching levels comparable to iron-deficient cultures thereby triggering siderophore production [14]. It was proposed that this rapid and profligate assimilation of iron may most probably give the answer to the unusual siderophore production observed in *M. magnetotacticum* MS-1.

Previously, 3327 mutants of *M. magneticum* AMB-1 generated by transposon mutagenesis were screened for defective magnetosome synthesis [10]. One of the non-magnetic mutants, NMA61, produced more siderophore than wild type. In this study, the genomic region of NMA61 interrupted by mini-Tn5 and its upstream sequence was analyzed. The phenotype of NMA61 with defective periplasmic transport binding protein kinase gene homologue was investigated.

Materials and methods

Cells and culture conditions. Magnetospirillum magneticum AMB-1 (ATCC700264) [15] was grown microaerobically at 26 °C in modified magnetic spirillum growth medium (MSGM) at pH 6.75, previously described [16]. The mutant strain, NMA61, generated by Mini-Tn5-transposon mutagenesis previously described [10], was maintained with 5 μg/ml kanamycin under the same conditions.

Isolation of flanking DNA and sequence analysis. Target sequence of mini-Tn5 in the genome was GGC CAG GGC. The DNA sequences flanking the transposon-interrupted region were obtained by inverse PCR [10] using primers with (R): 5 · ACA CTG ATG AAT GTT CCG TTG-3 and (F): 5 · ACC TGC AGG CAT GCA AGC TTC-3 sequences. The PCR product was cloned into the vector pGEM-T-easy (pGEM-T-easy Vector System, PROMEGA, WI, USA) and sequenced using automatic DNA sequencers, DSQ-2000L (Shimadzu, Kyoto, Japan) and ABI PRISM 377 (Perkin–Elmer, CA, USA). The obtained sequences were aligned against the whole genome sequence of *M. magneticum* AMB-1 (in preparation) database. A computer software package, LASERGENE (DNASTAR, Madison, USA), was used for DNA and protein sequence analyses. GENETYX (Software Development, Tokyo, Japan) was used for promoter analysis. The sequence was further analyzed by performing homology searches using programs of FASTA [17,18] and BLAST [19] against the GenBank and EMBL DNA databases.

Transmission electron microscopy. Approximately 1×10^8 cells/ml from each growth phase of NMA61 were placed on electron microscopy copper grid and allowed to settle for 1–2 h at room temperature. Excess liquid was removed with a piece of filter paper and the grid was stained with 2% phosphotungstic acid for 15 min, air-dried overnight, and observed by transmission electron microscopy (TEM) (model H-700H, Hitachi, Japan).

Measurement of iron-uptake. The iron concentration from cell-free culture supernatants was determined by using 3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4 triazine, also referred to as ferrozine, a spectrophotometric reagent for iron [20]. A modified method [8] was used in which 50 μl of 60% hydroxylamine hydrochloride was added to 100 μl of the samples as a reductant and argon gas was sealed into the sample tubes. After a 24-h incubation, 100 μl of glacial acetic acid–sodium acetate buffer, pH 6.0 (4 g sodium acetate, dissolved with a small amount of distilled water, plus 3.5 ml concentrated glacial acetic acid, diluted to 100 ml) and 200 μl of 1% ferrozine were added. The sample solutions were then measured at Abs_{562} spectrophotometrically in triplicate samples.

Isolation of siderophore. Siderophore was isolated from a 20-L volume of cell-free wild type *M. magneticum* AMB-1 culture supernatant by adsorption into adsorbent resin (52 g/100 ml) (Diaion HP20, Mitsubishi Chemical, Tokyo). The supernatant was discarded and the resin was mixed vigorously with 100% methanol for 2 h to desorb low molecular mass molecules. The methanol was collected and the resin was washed with 70% methanol to retrieve residual adsorbed molecules. Samples in batches of 500 ml were rotoevaporated at 37 °C, freeze-dried and resuspended in 10 ml sterile Milli-Q water, and injected in size-exclusion and desalting chromatography columns using Sephacryl S-100 and Sephadex G-25 (Amersham–Pharmacia, Biotech, Uppsala, Sweden) connected to fast protein, peptide, and polynucleotide liquid chromatography (FPLC) system (Amersham–Pharmacia, Biotech, Uppsala, Sweden) monitored at Abs_{280}. Sterile Milli-Q water was used as the mobile phase at a flowrate of 1 ml/min. The chrome azurol sulfonate (CAS) assay by Schwyn and Neilands [21] was used to screen the collected fractions for siderophore activity. The pooled siderophore fractions were deferrated with 5% of 8-hydroxyquinoline in chloroform as described by Meyer and Abdallah [22].

Siderophore bioassay. Bioactivity of the isolated siderophore was determined by testing its ability to abolish the growth inhibition imposed by the addition of non-assimilable synthetic iron chelator nitrilotriacetate (NTA) (Wako Chemical, Japan) to *M. magneticum* AMB-1 or NMA61 cultures. Cells were cultured in 40 ml of (1) standard MSGM, (2) MSGM with 100 μM NTA or (3) MSGM with 100 μM NTA plus 10% (v/v) isolated siderophore from wild type. Cell growth was measured by counting the cells with a hemocytometer every 4 h within a 24-h growth period. Determinations were done in triplicate.

Detection of siderophores. The CAS assay [21] was used as a universal chemical assay for the detection of siderophores in samples aliquoted at 4-h intervals from 40 ml cultures of wild type and NMA61 within a 24-h growth period. Detected siderophores were expressed as micromolar equivalents of the iron chelator deferrioxamine (DFX) (Sigma, USA). Detections were done in triplicate.

Results

Genetic organization of the gene interrupted by transposon

Inverse PCR amplification of the sequences flanking the inserted Mini-Tn5 allowed the isolation of an 800 bp DNA sequence. This was aligned against the whole genome sequence of *M. magneticum* AMB-1. A promoter was found 2 kb upstream of an open reading frame (ORF) directly interrupted by the transposon.

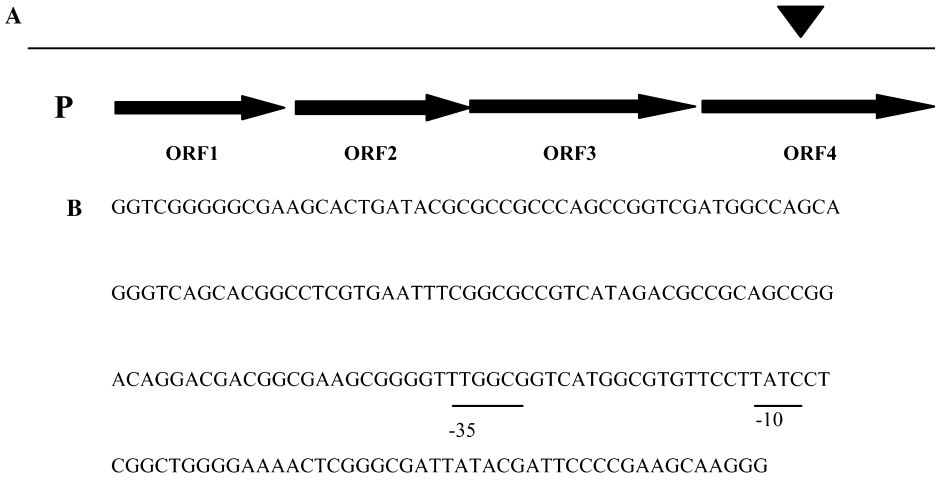

Fig. 1. Genetic organization of the gene cluster consisting of four open reading frames (ORF) in *M. magneticum* AMB-1 genome inserted with transposon Mini-Tn5. Interrupted ORF is indicated by an inverted triangle. Putative promoter is indicated by P. ORF homology: (1) carboxypeptidase, (2) transcriptional regulator, (3) periplasmic protein TonB, and (4) periplasmic transport binding protein kinase. (B) DNA sequence containing a putative promoter upstream of ORF1.

Three other adjacent ORFs upstream of the interrupted ORF were organized under the same promoter, indicating that all four ORFs comprise an operon (Accession No. AB126694) (Fig. 1).

Analysis of amino acid sequence homology deduced from the ORFs

The results of homology analysis using BLAST search of deduced amino acid sequences are shown in Table 1. ORF1 has high homology with a carboxypeptidase from *Yersinia pestis*. Carboxypeptidase is a metalloprotease and requires zinc for activity. Domain search revealed a predicted domain Pfam:Peptidase M32. The start of the sequence is at position 15 and ends at 510 within a total sequence of 512 residues (Sanger Institute://www.sanger.ac.uk/Software/Pfam/). The *E* value, 3.50e−111, and the protein size are equal to other similar proteins.

ORF2 encodes a protein consisting of 101 amino acids and shows homology with a transcriptional regulator. Its *E* value however is high. Results from the domain search showed that the basic region is a leucin zipper (SMART Accession No. SM00338) which starts at position 8 and ends at position 82 with an *E* value of 473. Another predicted domain is a helix–loop–helix domain (SMART Accession No. SM00353) which starts at position 35 and ends at position 79 with 4195 *E* value. These high values suggest that this protein might not actually function as a transcriptional regulator.

ORF3 encodes a protein of 90 amino acids which show homology with TonB. Its *E* value however is also high. Domain search led only to a domain which is a high mobility group HMG17 (SMART Accession No. SM00527) responsible for binding proteins to nucleosomes in the chromatin. The domain starts at position 6 and ends at position 72 with 1623 *E* value. BLAST top hit indicates that only a very short region of ORF3 from position 12 to 38 showed 62% high similarity to TonB. These data indicate that ORF3 may be a pseudogene.

ORF4 encodes a protein consisting of 329 amino acid residues. It has high homology with a periplasmic transporter binding protein (PBP) kinase, ArgK. Domain search supports the sequence start at position 22 and ends at position 295 corresponding to Pfam:ArgK domain with 2.9e−155 *E* value (Sanger Institute://www.sanger.ac.uk/Software/Pfam/). Bacterial periplasmic transport systems require the function of a specific substrate-binding protein located in the periplasm and several cytoplasmic membrane transport components. Periplasmic proteins can be phosphorylated by a single kinase, ArgK, [23] which facilitates transport across the periplasm.

Table 1
Homology search of open reading frames (ORFs) comprising the operon in *M. magneticum* AMB-1 genome inserted with transposon Mini-Tn5

ORF	Amino acid residue (aa)	Accession No.	Protein homologue	Microorganism	Identity/ similarity (%)	*E* value
1	512	AJ414151-309	Carboxypeptidase protein	*Yersinia pestis* CO92	48/66	e−143
2	101	AJ248284-11	Transcriptional regulator protein	*Pyrococcus abyssi*	32/58	0.48
3	91	JHP1260	Periplasmic protein TonB	*Helicobacter pylori*	62/65	0.006
4	329	AE005917-3	LAO transporter binding protein kinase	*Caulobacter crescentus* CB15	70/83	e−110

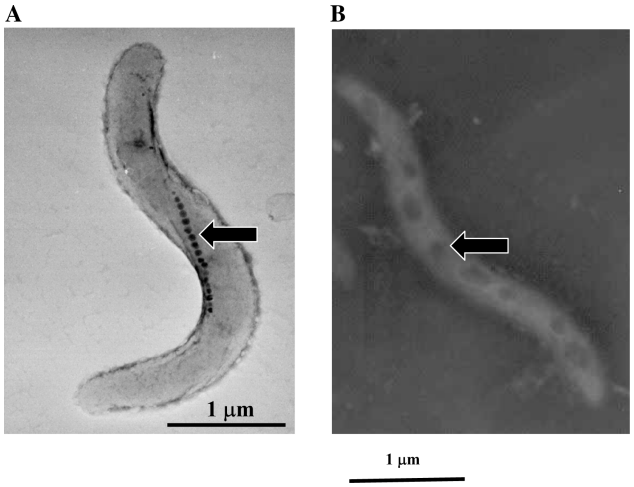

Fig. 2. Transmission electron photomicrograph of *M. magneticum* AMB-1. (A) Wild type with magnetosomes aligned in a chain (arrow). (B) Non-magnetic mutant without highly organized typical magnetosomes but polyhydroxybutyrate-like structures were present (arrow). Scale = 1 μM.

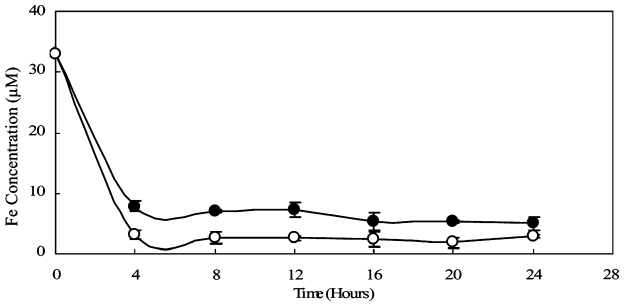

Fig. 3. Iron-uptake of wild type *M. magneticum* AMB-1 (○) and non-magnetic mutant, NMA61 (●). Cells were grown in standard magnetic spirillum growth medium (MSGM) with 33 μM initial iron concentration. Supernatants were aliquoted at 4-h intervals within a 24-h growth period. Iron concentrations were measured using ferrozine. Data are mean values of triplicate measurements.

Electron microscopy of non-magnetic mutant NMA61

Observations under the light microscope showed that NMA61 cells from each growth phase did not exhibit magnetic response when a samarium–cobalt magnet was moved in different directions near the glass slide. TEM revealed that the cells did not contain the typical highly organized magnetosomes aligned in chains observed in wild type (Fig. 2A). NMA61 cells contained polyhydroxybutyrate (PHB)-like granules (Fig. 2B) similar to those observed by Balkwill et al. [24] in *M. magnetotacticum* MS-1 spontaneous mutants and in *M. magneticum* AMB-1 non-magnetic mutant, NMA21 [10].

Time course of iron-uptake in wild type and NMA61

Iron-uptake ability of both wild type and NMA61 under microaerobic conditions was almost similar (Fig. 3). A rapid decrease in iron was observed in both wild type and NMA61. This iron uptake pattern was also observed in non-magnetic mutant, NMA21 [10].

Growth inhibition of the iron chelator NTA on wild type and NMA61

The activities of synthetic chelators are essential in establishing the basis of iron deprivation in biological systems. NTA, like ethylenediamine-di(*o*-hydroxyphenyl acetic acid) (EDDA, EDDHA or EHPG) and 2,2′-dipyridyl, is a bacteriostatic agent which predominantly chelates Fe(II) and also tenaciously binds Fe(III). NTA addition causes inhibition of iron uptake by cells since the available free iron is chelated by an excess concentration of NTA which lacks specific outer membrane receptors unlike siderophores and other genetically regulated iron transport molecules. In this study, the growth of both wild type and NMA61 cells was inhibited by NTA. Addition of the isolated siderophore sample from the spent culture medium of wild type relieved the growth inhibition imposed by NTA to wild type cells (Fig. 4A). On the other hand, the growth inhibition of NMA61 cells was not abolished by the added siderophore (Fig. 4B). These results indicate that the added siderophore was able to sequester iron from the NTA–iron complex and transported into the cells via the siderophore uptake system in wild type, but not in NMA61. The results also suggest that NMA61 produced siderophores and sequestered iron from the NTA–iron complex but may have failed to pass the iron to iron translocating proteins across the cytoplasmic membrane because of the interrupted ORF4 which codes for a transporter protein binding kinase, hence, the growth inhibition was not relieved by the addition of the isolated siderophore.

Detection of siderophore in wild type and NMA61

Siderophore detection experiments were conducted to confirm if NMA61 indeed had the ability to produce siderophores although the mutant could not assimilate siderophore–iron complexes. In wild type, siderophore production was observed within 4 h (Fig. 5) which corresponds to the significant decrease of the iron concentration in the medium during the same period of time (Fig. 3). NMA61 on the other hand accumulated higher siderophore concentration in the culture supernatant during 4–8 h (Fig. 5). This phenomenon indicates that the ORF4 mutation has blocked the transport of the siderophore–iron complex into the cell, hence the siderophores released by NMA61 accumulated extracellularly. The almost similar rapid iron uptake of both strains also indicates that *M. magneticum* AMB-1 utilizes other iron transport systems aside from a siderophore-mediated

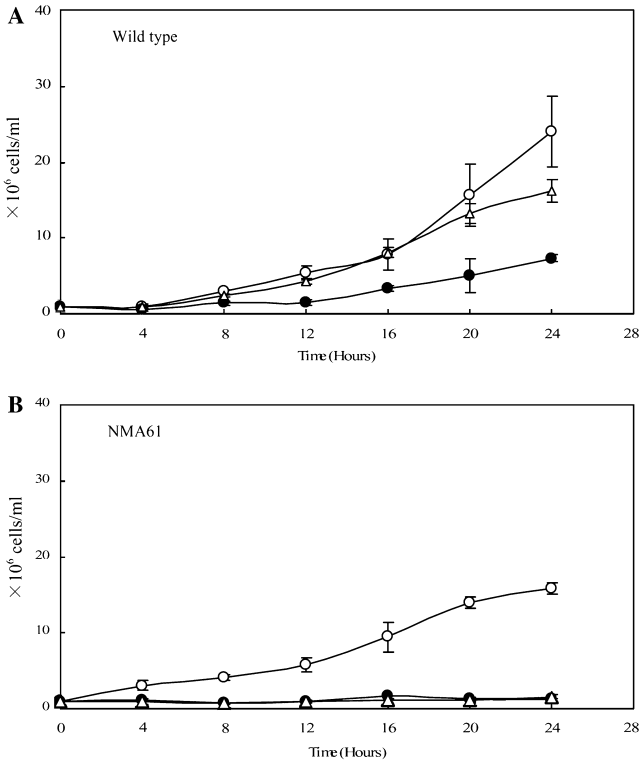

Fig. 4. Biological acitivity of the isolated siderophore from *M. magneticum* AMB-1 culture supernatant. Activity of the isolated siderophore from wild type was determined by testing its ability to abolish the growth inhibition imposed by the non-assimilable synthetic iron-chelator nitrilotriacetate (NTA) to (A) *M. magneticum* AMB-1 or (B) non-magnetic mutant, NMA61. Cells were counted with hemocytometer every 4 h within a 24-h growth period. Cells were cultured in 40 ml of standard magnetic spirillum medium (MSGM) (○), MSGM with 100 μM NTA (●) or MSGM with 100 μM NTA plus 10% (v/v) isolated siderophore sample (△). Data are mean values of triplicate determinations.

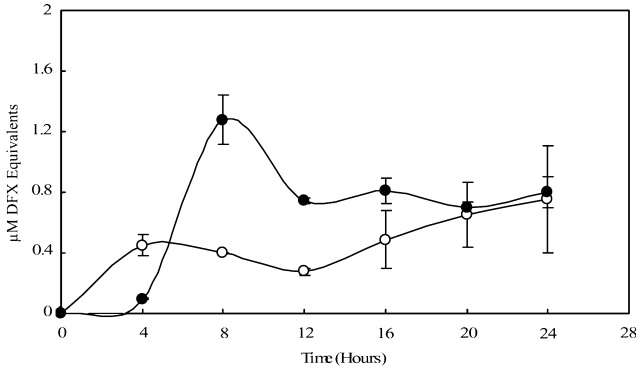

Fig. 5. Siderophore production of wild type *M. magneticum* AMB-1 (○) and non-magnetic mutant, NMA61 (●). Cells were grown in standard magnetic spirillum growth medium (MSGM) with 33 μM initial iron concentration. Supernatants were aliquoted at 4-h intervals within a 24-h growth period. Siderophores were detected using CAS assay and expressed as micromolar equivalents of the iron-chelator deferrioxamine (DFX).

process. The gene product therefore of ORF4 has a crucial function in molecular transport similar to its gene homologue which codes for a phosphorylating kinase for periplasmic binding proteins which transports amino acids across the periplasmic space in *Escherichia coli* [23].

Discussion

Periplasmic transport systems in bacteria require the function of a periplasmic specific-binding protein and several cytoplasmic membrane components. In *E. coli* K-12, two periplasmic transport systems exist: (1) the arginine–ornithine transport system which requires an arginine–ornithine-binding protein and (2) the lysine–arginine–ornithine (LAO) transport system which includes an LAO-binding protein [25,26]. Both periplasmic proteins can be phosphorylated by a single kinase, ArgK. The ArgK protein functions as an ATPase enzyme and as a kinase.

In this study, although superficial iron uptake of NMA61 cells was observed similar to that of wild type, the inability of the added siderophore to relieve the growth inhibition imposed by NTA and the accumulation of siderophores in the culture medium reflects the inactivation of the transposon-inserted ORF. FhuD is the best characterized bacterial ferri-siderophore periplasmic binding protein in *E. coli* which interacts with several ferri-hydroxamates. The crystal strucure of FhuD is similar to those of other binding proteins like the ferric binding protein (Fbp) in *Haemophilus influenzae* [27,28]. FhuD might be regulated by periplasmic transport binding protein kinase.

Although *M. magneticum* AMB-1 may posses several routes for iron acquisition, the impairment of ORF4 and the consequent interrupted operon have greatly contributed to the generation of a non-magnetic phenotype. ATP hydrolysis and phosphorylation by protein kinases drive the functional conformations of numerous proteins and are therefore global regulators which may include those for magnetosome synthesis. The interrupted operon in this study, and the other Fe-uptake systems in this bacterium might be tightly coupled and form a complicated cascade of interactions to generate the highly organized magnetosomes. The operon described in this study therefore is one of the Fe-uptake systems in *M. magneticum* AMB-1 and the transposon interruption in NMA61 has resulted in a non-magnetic phenotype.

The formation of intracellular membrane-bound magnetites in magnetic bacteria is an example of biomineralization which entails a complex system of iron acquisition. This study presents an interesting case of inconsistency of behaviors between iron uptake and siderophore production, and is the first to describe the involvement of an element homologous to a periplasmic transporter binding protein kinase during siderophore utilization and magnetosome formation in magnetic

bacteria. The non-magnetosome forming mutant generated in this study is a valuable tool in facilitating comprehensive proteome analysis and other succeeding studies on the elucidation of iron trafficking in the complex iron metabolism of magnetic bacteria.

Acknowledgments

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