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Involvement of a Gene Encoding Putative Acetate Kinase in Magnetosome Synthesis in *Magnetospirillum magneticum* AMB-1

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A nonmagnetic mutant of *Magnetospirillum magneticum* AMB-1, designated NMA40, was constructed by mini-Tn*5* transposon mutagenesis to identify genes involved in magnetosome synthesis. Transposon delivery was carried out through conjugation between *M. magneticum* AMB-1 as a recipient and *Escherichia coli* S17-1 (ë pir) carrying pUTmini-Tn*5*Km1 as a donor strain. NAM40 did not respond to the magnetic fields and completely lacked of magnetosome in the cell. DNA sequence/gen interrupted by transposon (called flanking DNA) was isolated by inverse PCR and cloned into pGEM-T Easy. Alignment of the DNA sequence of the flanking DNA allowed the isolation of an open reading frame (ORF2) within an operon consisting of three genes. The amino acid sequence deduced from ORF2 showed homology with acetate kinase from *Sinorhizobium meliloti* (50% identity and 67% similarity), which function for acetate metabolism. Further analysis revealed that upstream of ORF2 is ORF1, had homology with phosphotransacetylase of *S. meliloti* (67% identity, 77% similarity), and ORF3 located downstream of ORF2, had homology with hypothetical protein of *Thermotoga maritima* (30% identity, 60% similarity). ORF2 was subsequently isolated, cloned, and overexpressed in *Escherichia coli* BL21 (DE3) pLysS as an ORF2-Histag fusion polypeptide.

Key words: *Magnetospirillum magneticum* AMB-1, magnetosome synthesis, transposon mutagenesis, cloning, overexpression

INTRODUCTION

Magnetospirillum magneticum strain AMB-1 discovered by Matsunaga *et al*. (1991) synthesizes single domain magnetic crystals of the iron mineral magnetite (Fe_3O_4) which are membrane-bound, called magnetosome (Matsunaga & Okamura 2003). The crystals remain within the bacterium, organized in long chain which directs their north-south orientation, therefore, *M. magneticum* AMB-1 is sensitive to the earth's magnetic field. This bacterium is microaerophilic and widely distributed in freshwater sediment. Like most magnetic bacteria producing magnetite so far described, AMB-1 has been classified as belong to the α-subdivision of the proteobacteria (Burgess *et al*. 1993). *Magnetospirillum magneticum* AMB-1 capable of growing on agar plate facilitates the gene manipulation techniques of this bacterium. The only this strain gene manipulation techniques are successfully applied (Matsunaga *et al*. 1992; Wahyudi *et al*. 2001).

To elucidate magnetosome synthesis in magnetic bacteria, genetic analysis of the protein specifically associated with magnetosome membrane, Mam22 in *M. magnetotacticum* MS-1 (Okuda & Fukumori 2001), MpsA (Matsunaga *et al*. 2000), Mms16 (Okamura *et al*. 2001), Mms6 (Arakaki *et al.* 2003) in *M. magneticum* AMB-1 have been reported. A gene encoding protein, Aor21, which function in cytoplasm has also been reported to be related to magnetosome formation in *M. magneticum* AMB-1 (Wahyudi *et al*. 2003). However, biomineralization of magnetosome formation in magnetic bacteria remain mostly unknown.

Manipulation and genetic analysis of bacterial genes have been greatly facilitated by the use of transposon carrying antibiotic resistance. Transposition of transposon into a gene usually leads to inactivation of that gene and the transposon insertion mutation represent a new genetic and physical marker at the site of insertion (DeBruijn & Lupski 1984). One of the transposon, mini-Tn*5*, has been widely used in many gram negative bacteria for genetic analysis. This transposon is derived from transposon Tn*5* flanked by 19-bp inverted repeats of Tn*5*, with the transposases gene located outside of the transposon. Therefore, insertion caused by mini-Tn*5* is more stable (De Lorenzo *et al*. 1990).

In the present study, the genomic region of *M. magneticum* AMB-1 interrupted by transposon mini-Tn*5* and its upstream and downstream sequences was analyzed. The gene that directly interrupted by transposon was isolated, cloned and heterologously expressed in *E. coli* as a Histag-fusion protein.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Culture Conditions. *Escherichia coli* S17-1 ë pir harboring plasmid pUTmini-Tn*5*Km1 (DeLorenzo *et al*. 1990) used for transposon mutagenesis was grown in Luria Broth (LB) (tryptone 5.0 g l^{-1}, NaCl 10 g l^{-1}, yeast extract 5.0 g l^{-1}) containing ampicillin (50 ìg ml^{-1}) and kanamycin (25 ìg ml^{-1}) at 37 °C. *Escherichia coli* DH5á used as a host strain for cloning experiment was routinely grown in LB at 37 °C, whereas *E. coli* BL21 (DE3) pLysS was cultured in LB suplemented with chloramphenicol (25 µg ml^{-1}). *Magnetospirillum magneticum* AMB-1 (ATCC

700264) was cultured microaerobically with magnetic spirillum growth medium (MSGM) (Blakemore *et al*. 1979). A plasmid pGEM-T Easy (Promega, USA) was used as a cloning vector of inverse PCR product, whereas pET15b (Novagen, USA) was used as a cloning vector for gene expression.

Construction of a Nonmagnetic Mutant. Transposon mini-Tn*5*Km1 (pUTmini- Tn*5*Km1, 7055 bp) from *E. coli* S17-1 λ pir was transferred into *M. magneticum* AMB-1 by conjugation (Wahyudi *et al*. 2001). Bacterial cells in logarithmic phase (approximately, 1×10^8 cells ml^{-1}) were centrifuged and pellets were washed with 0.85% NaCl and resuspended in MSGM at a concentration of about 1×10^{10} cells/ml. A 50 ì l of the donor and recipient mixture was placed on a strerile nitrocellulose filter on MSGM agar. Conjugation was performed aerobically for 6 hours at 25 °C. Cells suspension was plated on MSGM agar plate supplemented with kanamycin (5 ìg ml^{-1}) and incubated for 10-14 days microaerobically at 25 °C in anaerobic jars. Nonmagnetic mutant was observed under light microscopy (Olympus BH2, Tokyo, Japan).

Southern Hybridization Analysis. Genomic DNA of a nonmagnetic mutant, designated as NMA40, was extracted according to the method described by Wilson (1994). The DNA digested with *Eco*RI was electrophoresed on 1% agarose gel. Blotting of the DNA from the gel onto nitrocellulose membranes (Hibond TM-N, Life Sciences, England) and fixing of the DNA onto the membrane by UV cross-linking were performed according to the manufacturer's instruction (Boehringer Mannheim, Biochemica, Germany). A 1.8 kb *Eco*RI/*Pst*I DNA fragment containing kanamycin resistance gene from plasmid pUTmini-Tn*5*Km1 was used as a probe which was labeled with a DIG chemiluminescence Kit (Boehringer Mannheim, Biochemica, Germany). Prehybridization for 6 hours followed by hybridization with mini-Tn*5*Km1 probe was incubated overnight at 42 °C for 6 hours. X-ray film was used for detection of the hybridization (Boehringer Mannheim, Biochemica, Germany).

Isolation of Flanking DNA, Cloning, and Sequencing. The DNA fragments flanking transposon from a nonmagnetic mutant genome was isolated by inverse polymerase chain reaction (inverse PCR) (Wahyudi *et al*. 2001), using primers designed from mini-Tn*5*Km1 sequence near the insertion sequences directed outward from the transposon. Primers used for inverse PCR were 5'-GAT CCT CTA GAG TCG AC-3' (sense primer) and 5'-GTA CCG AGC TCG AAT TC-3'(antisense primer). The inverse PCR product was purified from the gel by Gene Clean III Kit (Bio 101, Carlsbad, CA), cloned in pGEM-T Easy (Promega, Madison, WI, USA), and transformed into *E. coli* DH5α by electrophoration. The flanking DNA fragment in pGEM-T was sequenced using an automatic DNA sequencer ABI 377 (Perkin Elmer, USA).

DNA Sequence Analysis. The DNA sequence flanking transposon was used for alignment against the whole genome sequence of *M. magneticum* AMB-1 (Matsunaga *et al*. 2005), to obtain large fragment containing the complete open reading frames (ORF) or gene interrupted by transposon. Assembly of DNA sequence, identification and translation of open reading frames (ORFs), and calculation of molecular masses of the proteins were done by the LASERGENE (DNASTAR, Madison, Wisconsin, USA). Sequence alignment was carried out using the program Seqman II or ClustalW, which is part of the same software. Protein sequence was compared to the GenBank, EMBL, and SwissProt databases by using the BLAST program (Altschul *et al*. 1997).

Cloning and Overexpression of ORF2 in *E. coli.* Based on the sequence of ORF2 (GenBank Accession Number AP007255; locus: amb2554) (Figure 2), two oligonucleotide primers, Primer 1 (P1): 5'-GGG GGA CAT ATG CGT GAA GGT ATC CTG -3' and Primer 2 (P2): 5'-GGG GGA TCC AAA CTA GTC GCG CAA GCT CCG -3' (underlined nucleotides indicate *Nde*I and *Bam*HI sites, respectively) were used for amplification of ORF2. The recombinant plasmid pET15b containing ORF2 was constructed by cloning of the PCR product amplified from ORF2 into the *Nde*I/*Bam*HI site of expression vector, pET15b. To amplify the ORF2, a primer P1 with *Nde*I site introduced at a start codon ATG, and primer P2 with the *Bam*HI site introduced downstream of the stop codon (TGA) of the ORF2 were used. The 50 ìl PCR reaction mixture contained 100 ng *M. magneticum* AMB-1 genomic DNA, 2.5 U LA Taq (Takara, Tokyo, Japan), 400 ìM dNTPs, 2.5 mM $MgCl_2$, and 0.2 ìM of each of the two primers. The temperature program for PCR was one cycle of 3 min at 95 °C, 30 cycles of 1 min at 95 °C, 1 min at 62 °C, 1 min at 72 °C, and one cycle for 10 min at 72 °C. The amplified fragment was subsequently isolated from the gel and purified by Gene Clean III Kit (Bio 101). The 1.2 kb amplified fragment was cloned in pGEM-T Easy vector (Promega, Tokyo, Japan) designated pGEM-T-ORF2 and transformed into *E. coli* DH5α. This plasmid was subsequently isolated from DH5α and digested with *Nde*I and *Bam*HI, and 1.2 kb fragment was ligated with pET15b expression vector (Novagen, USA) liniarized with *Nde*I and *Bam*HI, to give a recombinant plasmid pET15b-ORF2. This recombinant plasmid was introduced into *E. coli* DH5á by electrophoration. This recombinant plasmid extracted from DH5á was subsequently transformed into a host for expression, *E. coli* BL21 (DE3) pLysS, by electrophoration and plated on LB plate containing ampicillin (50 ìg ml^{-1}) and chloramphenicol (25 ìg ml^{-1}).

For expression of the ORF2, *Escherichia coli* BL21 (DE3) pLysS carrying pET15b-ORF2 was grown aerobically at 37 °C in 10 ml of LB containing ampicillin and chloramphenicol to 2×10^8 cells ml^{-1} (OD_{600} of 0.6). The culture was then induced by adding 0.1 mM IPTG and incubated at 37 °C for 3 hours with good agitation. Uninduced-and induced-cells by IPTG were analyzed by 12.5% SDS-PAGE.

Purification of Histag-Fused Protein from *E. coli*. Ten milliliter culture of *E. coli* BL21 (DE3) pLysS carrying pET15b-ORF2 was induced by 0.1 mM IPTG at OD_{600} of 0.6 for 3 h at 37 °C by shaking, centrifuged, and pellet was frozen at -70 °C until use. ORF2 protein was purified under denatured condition using Ni-NTA column (Qiagen, GmbH, Germany).

SDS-PAGE and Western Blot Analysis. Pellets of uninduced-cells (1 ml) and induced-cells (0.5 ml), solubilized lysate, and purified Histag-ORF2 fused protein were analyzed by SDS-PAGE and performed at 12.5% (w/v) acrylamide gel, and protein was stained with Silver Staining Kit (Pharmacia). Western blotting was performed as follows. Polyacrylamide

gel of purified Histag-ORF2 protein was blotted onto PVDF membrane by electroblotting. For staining of Western blot, monoclonal antibody mouse anti-Histag antibody was used at 1:5000 dilution and developed with goat anti-mouse IgG secondary antibody conjugated to alkaline phosphatase (Zymed Laboratories).

RESULTS

Transposon Mutagenesis and Southern Hybridization. Frequency of transconjugation of Mini-Tn5Km1 from *E. coli* S17-1 (λ pir) to *M. magneticum* AMB-1 occurred was about 1.5×10^7 cell per recipients. Observation of one white colony of transconjugant, designated as NMA40, under light microscopy showed that cells did not respond to magnetic fields. This indicates that magnetosome was not synthesized as observed by electron microscopy (data not shown). Spontaneous mutation did not occur on MSGM plate supplemented with kanamycin (5 ìg ml^{-1}). Southern hybridization analysis of this mutant genome revealed the presence of a single mini-Tn*5* transposon insertion into the NMA40 genome as indicated by a single hybridization band (Figure 1a).

Molecular Organization of Gene Interrupted by Transposon. Inverse PCR amplification of upstream and downstream sequence of the DNA flanking the transposon from NMA40 allowed the isolation of 1.4 kb DNA fragment (Figure 1b). To characterize this locus, I obtained its upstream and downstream sequences through alignment against the whole genome sequence of AMB-1 (Matsunaga *et al.* 2005). An ORF directly interrupted by transposon, ORF2, consisting of 1192 bp was identified. Target sequence of the transposon was GCCCAGAGC located at 485 bp position from the start codon (ATG). Putative ribosomal binding site (RBS) was found at 11 bp position upstream of the start codon of ORF2. Putative promoter sequence was found upstream of the start codon of ORF1-ORF3. Therefore, ORF1-ORF3 might be organized in an operon (Figure 2).

Homology Analysis of Amino Acid Sequences Deduced from ORFs. The results of homology analysis of amino acid sequence deduced from ORFs are shown in Table 1. The first ORF, ORF1 consisting of 1410 bp, encodes 470 amino acids, has high homology with phosphotransacetylase from *Sinorhizobium meliloti* (67% identity and 77% similarity) with predicted molecular mass of 50.2 kDa. ORF2 is 1194 bp, encodes a protein of 398 amino acids with predicted molecular mass of about 43 kDa and has high degree of homology with acetate kinase from *Sinorhizobium meliloti* (50% identity, 67% similarity) (Finan *et al*. 2001). Immediately downstream from the ORF2 is an ORF3 that encodes an inferred peptide with 34% identity and 60% similarity to hypothetical protein from *Thermotoga maritima* (Table 1).

Cloning, Expression, and Western Blot. The recombinant plasmid pET15b-ORF2 containing gene encodes putative acetate kinase (ORF2) with Histag was transformed and expressed in *E. coli* BL21 (DE3) pLysS. After the recombinant plasmid was transformed to *E. coli* BL21 (DE3) pLysS, expression of the ORF2 was induced by addition of 0.1 mM IPTG and under the control of promoter T7 *lac*. For expression of the Histag-ORF2 fusion protein, the total protein profile of the whole cells were analyzed by SDS-PAGE using silver staining for visualization. As shown in Figure 3, the protein band approximately 34 kDa of the Histag-ORF2 fusion protein was highly overexpressed in *E. coli*, and pure protein was obtained after purification. It was confirmed to be the ORF2-Histag fusion protein by Western blot analysis using anti-Histag antibody (Figure 3).

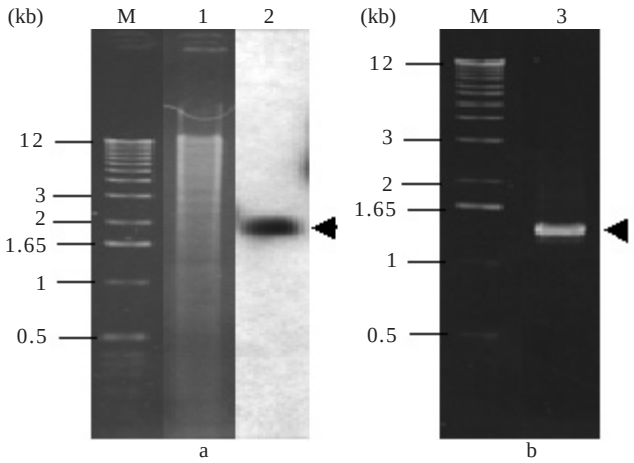

Figure 1. a. Southern hybridization analysis of genomic DNA of *M. magneticum* mutant NMA40. The genomic DNA was digested with *Eco*RI (1) and result of Southern hybridization (2); b. 1.4 kb of flanking DNA amplified by inverse PCR (3).

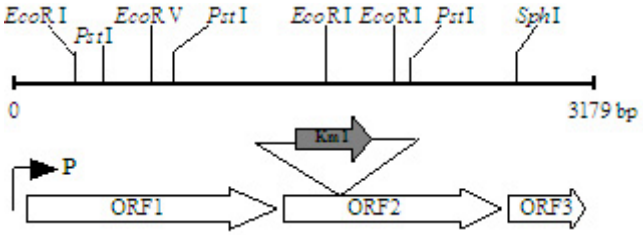

Figure 2. An operon consisting of three genes required for magnetosome synthesis identified from *M. magneticum* AMB-1 genome. ORF2 is an ORF directly interrupted by transposon (as indicated by Km1 arrowhead). P indicates promoter identified upstream from ORF1. Some restriction enzymes digesting the sequence are also performed.

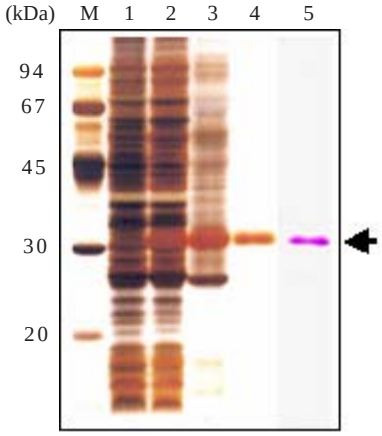

Figure 3. Profil protein anlyzed by SDS-PAGE. Whole cells proteins of uninduced cells (1), induced cells (2), Lysate (3), pure protein (4), and Western blot analysis of purified protein (5) as indicated by arrow. M: Molecular marker of protein.

Table 1. Homologous searches of an operon required for magnetosome synthesis in *Magnetospirillum magneticum* AMB-1

ORF	Size (bp)	Amino acid residue	Homologous protein	Id/pos (%)	Microorganism
1	1410	470	Phosphotransacetylase	67/77	*Sinorhizobium meliloti*
2	1194	398	Acetate kinase	50/67	*Sinorhizobium meliloti*
3	378	126	Hypothetical protein	30/60	*Thermotoga maritima*

DISCUSSION

Of 525 transconjugant colonies, one colony was screened to be defective in magnetosome synthesis based on the white color of the colony and its magnetic response. Wild-type *M. magneticum* AMB-1 cells form brown-black colonies. This color come from iron metabolism indicating magnetosome production of AMB-1 cultured on MSGM agar plate under microaerobic condition (Sakaguchi *et al*. 1996). Therefore, white colony of a nonmagnetic mutant, designated NMA40, was classified to be defective in magnetosome synthesis. It was due presumably to the transposon insertion into the genome, especially in the DNA sequence or gene required for magnetosome synthesis.

In this study, ORF2 encoding putative acetate kinase (*ack*) was directly interrupted by mini-Tn*5* transposon rendered *M. magneticum* AMB-1 defective in complete magnetosome synthesis. Similar acetate kinase genes were also found in other bacteria such as *M. magnetotacticum* MS-1, *E. coli*, *S. meliloti*, and *Corynebacterium glutamicum*. Cloning of ORF2 encoding acetate kinase in *E. coli* BL21 (DE3) pLysS using pET15b as an expression vector revealed that the ORF2 protein was highly overexpressed (Figure 3). Expression of this gene cloned in pET15b was transcribed by T7 promoter RNA polymerase and is under the control of the *lac*UV5 promoter. This promoter can be induced by isoprophyl thiogalactopyranoside (IPTG) added to the medium. The predicted molecular mass of Histag-ORF2 fused protein deduced from ORF2 was about 43 kDa. However, result of expression of ORF2 cloned in pET15b from this study detected by SDS-PAGE, the molecular mass was about 34 kDa in size (Figure 3). This may be due to modification processes after translation (post translation) or other processes that have not been understood. Therefore, it needs further analysis to clarify it. The size of protein molecular mass was also the same when expression of this ORF2 using another plasmid vector pMAL-C2 for overexpression (data not shown).

Genes for phosphotransacetylase (*pta*) and acetate kinase (*ack*) found from this study were predicted that these genes were organized in an operon. This has olso been reported from many bacteria (Wanner & Wilmers-Reisenberg 1992; Reinscheid *et al*. 1999; Summers *et al*. 1999). The *pta* gene encodes the enzyme phosphotransacetylase, responsible for the reversible interconversion of acetyl-coA and acetyl phosphate. The *ack* gene encodes acetate kinase, responsible for the reversible interconversion of acetate and acetyl phosphate (Summers *et al*. 1999). Intracellular of acetyl phosphate is controlled by two reaction catalyzed by acetate kinase (*ack*) and phosphotransacetylase (*pta*). In *Sinorhizobium meliloti*, expression of *pta-ack* genes are under the control of the response-regulator protein PhoB (Bardin & Finan 1998) in respond to phosphate availability. Recent studies have shown that phosphorylation of regulator protein was come from small molecules phospho-donor acetyl phosphate (Lukat *et al*. 1992; McCleary *et al*. 1993; McCleary & Stock 1994). In the phosphate starvation will activate expression of *pta-ack* operon in *S. meliloti,* therefore, high Pi phosphate concentration in the cell will affect to inactivation the *pta-ack* operon (Bardin & Finan 1998; Summers *et al*. 1999). In this study, insertion of the transposon in *ack* gene resulted *M. magneticum* AMB-1 defective in magnetosome synthesis. Inactivation of *ack* gene should provide an opportunity for acetyl phosphate pools to accumulate. Consequently, intracellular phosphate concentration is also increase, it may be can inactivate *pta-ack* operon that links to another genes/operon required for magnetosome synthesis in AMB-1.

Acetate kinase in prokaryote such as *Geobacter sulfurreducens* and *Shewanella putrefaciens* have been reported to function as a substrate that is required in ferric iron (Fe^{3+}) reduction mechanisms. Ferric (Fe^{3+}) oxide is insoluble at neutral pH conditions. Reduction of insoluble Fe^{3+} can be taken placed either via direct contact with the surface of the cell or extracellular electron shuttle (Lloyd 2003). These statements support the hypothesis proposed by Nakamura *et al*. (1995) revealing that the first step in magnetosome synthesis in magnetic bacterium, *M. magneticum* AMB-1, is iron transport into the cell via reduction of Fe^{3+} to Fe^{2+}, subsequently transport Fe^{2+} into vesicle membrane of magnetosome to form the complete of magnetosome. Furthermore, Fe^{3+} reduction via iron chelating agent, siderophore, has also been reported in *M. magnetotacticum* MS-1 (Paoletti & Blakemore 1988) and *M. magneticum* AMB-1 (Calugay *et al*. 2004). It looks like that mechanisms of magnetosome synthesis in magnetic bacteria, especially *M. magneticum* AMB-1, involving ferric iron reduction is one of the important process under microaerobic condition. However, analysis of the protein activity and functions are needed to clarify the role of acetate kinase involved in magnetosome synthesis in *M. magneticum* AMB-1.

Taken the data all together, it can be speculated that this operon may be contribute to ferric iron reduction or the operon products link to other gene/operon functions during magnetosome synthesis under microaerobic respiration. Mutation within this operon in ORF2 generated cells lacking highly organized magnetosome aligned in chains for the cell's magnetotactic response.

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