

CLONING OF A GENE ENCODING PROTEIN BELONGING TO ABC TRANSPORTER INVOLVED IN BACTERIAL MAGNETIC PARTICLE SYNTHESIS IN *MAGNETOSPIRILLUM MAGNETICUM* AMB-1

ARIS TRI WAHYUDI

*Department of Biology, Faculty of Mathematics and Natural Sciences,
Bogor Agricultural University, Bogor, Indonesia*

ABSTRACT

Magnetospirillum magneticum AMB-1 synthesizes intracellular magnetic particles, magnetite (Fe_3O_4), enveloped by membrane called magnetosome under micro-aerobic conditions. Initial study of random transposon-based mutagenesis generated 62 nonmagnetic mutants of AMB-1 in a mini-Tn5 library. In order to identify a gene involved in bacterial magnetic particle (BMP) synthesis in the magnetic bacterium *M. magneticum* AMB-1, a nonmagnetic mutant from the library designated as NMA38-4, was analyzed. The amino acid sequence deduced from the gene directly interrupted by transposon, ORF4 (1482 bp), showed homology to ATP binding cassette (ABC) transporter of *Mesorhizobium loti* with 62 % identity and 74 % similarity. It was strongly indicated by the occurrence of putative consensus sequence of ATP-binding motifs (ATP-binding protein). The ORF4 was subsequently cloned in pET-15b and the recombinant ORF4-Histag fusion protein was heterologously expressed in *Escherichia coli* BL21 (DE3) pLysS. A 55 kDa protein corresponding to the ORF4-Histag fusion protein was obtained after purification using Ni-NTA column. This is the first report describing a gene cluster containing gene encoding protein belonging to ABC transporter organized in an operon which is involved in BMP synthesis.

Key words: *Magnetospirillum magneticum* AMB-1, Bacterial Magnetic Particle (BMP), ATP Binding Cassette (ABC) Transporter, transposon mutagenesis.

INTRODUCTION

Magnetospirillum magneticum AMB-1 isolated from fresh water sediment (Matsunaga 1991) synthesizes bacterial magnetic particles (BMPs) of the iron mineral magnetite (Fe_3O_4) enveloped by membrane called magnetosome, under microaerobic conditions. These intracellular single domain magnetic particles are aligned in chains with each individual crystal having a diameter of 50-100 nm enveloped by an organic membrane (Gorby *et al.* 1988; Matsunaga 1991). It is postulated that BMPs confer sensitivity of the bacterium to the earth's magnetic field lines for migration along oxygen and iron gradients.

So far, only few magnetic bacterial strains can be cultivated under laboratory conditions in pure culture, these include *M. magnetotacticum* MS-1 (Blakemore *et al.* 1979) and *M. magneticum* AMB-1 (Matsunaga *et al.* 1991), and *M. gryphiswaldense* (Schleifer *et al.* 1991). These strains are usually used as model systems for the analysis of BMP synthesis. The capability of *M. magneticum* AMB-1 to grow on agar plate and liquid medium both under microaerobic and aerobic conditions (Matsunaga *et al.* 1992) makes the bacterium amenable for genetic manipulations to elucidate important metabolic mechanisms most especially for the complex process of BMP synthesis.

Since magnetic bacteria were first discovered (Blakemore 1975), only few genes were isolated namely *recA* (Berson *et al.* 1989), *aroD* (Berson *et al.* 1991), and *mam22* (Okuda *et al.* 1996) from *M. magneticum* MS-1. In the previous studies, *magA* (Nakamura *et al.* 1995a) and *mms16* (Okamura *et al.* 2001) genes were isolated from *M. magneticum* AMB-1. The *magA* gene functions for the iron transport across the BMP membrane. Mutation of this gene rendered the cells defective in iron uptake. The Mms16 protein has a GTPase activity for the invagination of the cytoplasmic membrane for the formation of the BMP membrane. Cells with inhibited GTPase activity showed that they produced disrupted BMPs.

Previously, a non-magnetic mutant of *M. magneticum* AMB-1, designated NMA38-4, was generated by mini-Tn5 transposon mutagenesis (Wahyudi *et al.* 2001). In the present study, we have identified a gene (ORF4) involved in BMP synthesis in *M. magneticum* AMB-1. This gene encoding protein which belongs to ABC transporter was cloned and heterologously expressed in *E. coli* as an ORF4-Histag fusion polypeptide. Mutation within this gene rendered *M. magneticum* AMB-1 was unable to synthesize BMPs. The aim of this study was to isolate and clone a gene (ORF4) from *M. magneticum* AMB-1 genome and express it in *Escherichia coli* BL21 (DE3) pLysS.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

Escherichia coli DH5 α was routinely cultured in Luria broth (LB) (tryptone 5.0 g l⁻¹, NaCl 10 g l⁻¹, yeast extract 5.0 g l⁻¹) and *E. coli* BL21 (DE3) pLysS was cultured in LB supplemented with chloramphenicol 34 μ g ml⁻¹ at 37°C. *Magnetospirillum magneticum* AMB-1 (ATCC 700264) was cultured micro-aerobically in MSGM at 25°C (Blakemore *et al.* 1979). A nonmagnetic mutant, NMA38-4 (Wahyudi *et al.* 2001), was micro-aerobically cultured in MSGM supplemented with kanamycin (5 μ g ml⁻¹).

Observation of NMA38-4

A nonmagnetic mutant, NMA38-4 cells (Wahyudi *et al.* 2001) was cultured until logarithmic phase and cells of this phase were observed under light microscopy (Olympus BH2, Tokyo, Japan). Samarium-cobalt magnet was moved in different directions near the glass slide to determine the cells magnetic response.

Isolation of Flanking DNA and Sequence Analysis

Mutant DNA fragments flanking transposon in this work was isolated by inverse PCR after *EcoRV* digestion and circularization, using primers designed from the mini-Tn5Km1 sequence (Primer 1: 5'-GTA CCG AGC TCG AAT TC-3' and Primer 2: 5'-GAT CCT CTA GAG TCG AC-3'). The primers were directed outward from the transposon. A 1.3 kb inverse PCR product (Fig. 1) was purified from the gel by Gene Clean III Kit (Bio-101, Carlsbad, CA.) and sub-cloned in pCR2.1 (TA Cloning, Invitrogen, USA), designed pCR2.1-38.4. This recombinant plasmid was transformed into *E. coli* DH5 α . The recombinant plasmid was subsequently isolated from *E. coli* by Qiaprep Miniprep (Qiagen, Gmbh, Germany) and used as a template for DNA sequencing. The DNA sequencing was performed using an automatic DNA sequencer ABI 377 (Perkin Elmer, USA). The DNA sequence was subsequently aligned against the complete genome sequence of *M. magneticum* AMB-1 (Matsunaga *et al.* 2005). A computer software package, LASERGENE (DNASTAR, Madison, WI) was used for DNA and protein sequence analysis. The sequence was further analyzed by performing homology searches using program of BLAST (Altschul *et al.* 1997) against the GenBank and EMBL DNA databases.

Gene Cloning and Expression in *Escherichia coli*

Based on the sequence of ORF4 (Fig. 1), two oligonucleotide primers (Primer P1: 5'-GGG GGA CAT ATG AGC GAC GTC GTC GAA-3' and Primer P2: 5'-GGG GGA TCC AAA TCA CGT GTC GTC CCC CCA-3') were designed (underlined nucleotides indicate *NdeI* and *BamHI* sites, respectively). The recombinant plasmid pET15b-ORF4 was constructed by cloning of the PCR product amplified from ORF4 into the *NdeI/BamHI* site of expression vector pET15b (Novagen, USA). To amplify ORF4, a primer P1 with *NdeI* site introduced at a start codon ATG, and primer P2 with the *BamHI* site introduced downstream of the stop codon (TGA) of the gene were used. The 50 μ l PCR reaction mixture contained 100 ng *M. magneticum* AMB-1 genomic DNA, 2.5 U LA Taq (Takara, Tokyo, Japan), 400 μ M dNTPs, 2.5 mM MgCl₂, and 0.2 μ M 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, 60 °C, and 72 °C, respectively, 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). A 1.5 kb purified fragment was sub-cloned in pCR2.1 vector (Invitrogen, USA), designed pCR2.1-ORF4, and was subsequently transformed to *E. coli* DH5 α . The recombinant plasmid was isolated and digested with *NdeI* and *BamHI*. The fragment corresponding to the ORF4 was ligated into pET15b expression vector (Novagen, Madison, WI) linearized with *NdeI* and *BamHI*, to yield a recombinant plasmid, designed as pET15b-ORF4 (Fig. 1). This recombinant plasmid was introduced into *E. coli* DH5 α and subsequently isolated and then transformed into *E. coli* BL21 (DE3) pLysS as a host strain for gene expression. Transformants were plated on LB plate containing ampicillin (50 μ g ml⁻¹) and chloramphenicol (25 μ g ml⁻¹).

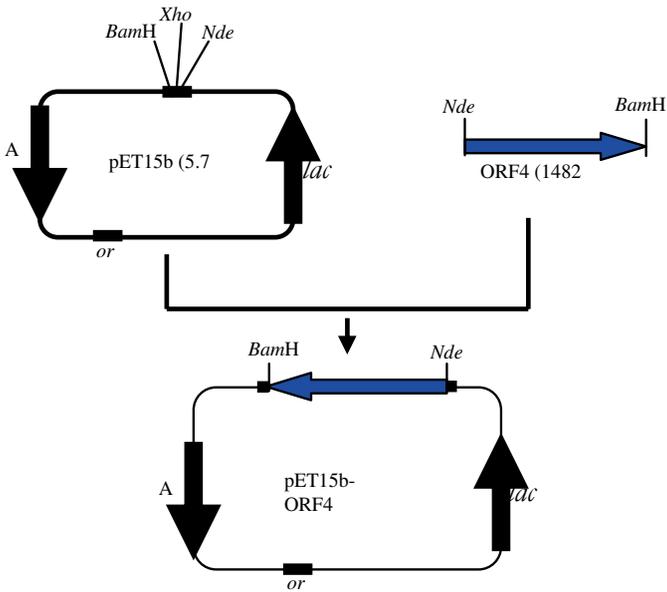


Figure 1. Construction of a recombinant plasmid pET15b-ORF4 (~7.2 kb).

Purification of ORF4-Histag Fusion Protein

Ten milliliter culture of *E. coli* BL21 (DE3) pLysS carrying pET15-ORF4 was induced by 0.1 mM IPTG at OD₆₀₀ of 0.6 for 3 h. by shaking at 37 °C. The culture was then centrifuged, and pellet was frozen at -70 °C until use. The protein was purified under denatured condition using Ni-NTA column (Qiagen, GmbH, Germany).

SDS-PAGE and Western Blotting

Pellets of uninduced cells (1 ml) and induced cells (0.5 ml), solubilized lysate, or purified -ORF4-Histag fusion protein, were mixed with 2 x sample buffer (Tris. HCl, glycerol, SDS, 2-mercaptoethanol, and bromophenol blue) and denatured by boiling for 5 minutes. SDS-PAGE was performed at 12.5 % (w/v) acrylamide gel, and protein was stained with comassie brilliant blue. For Western blotting, the ORF4-Histag fusion protein polyacrylamide gel was blotted onto a PVDF membrane by electroblotting. The Western blot was stained using monoclonal mouse anti-Histag antibody at 1:5000 dilution. A secondary goat anti-mouse IgG antibody conjugated to alkaline phosphatase was used for imaging (Zymed Laboratories Inc.).

RESULTS

Observation of NMA38-4 Cells and Colony

Colony of this mutant, designated as NMA38-4, was grown on MSGM plate. The color was white indicating the BMP was not synthesized. Whereas the color of the AMB-1 wild type grown on the same media was brown-black. The brown-black color indicated that BMP was synthesized. Observation of NMA38-4 cells under light microscopy showed that cells did not respond to the magnetic fields applied. This indicates BMP may have not been synthesized due presumably to transposon insertion into the genome, especially in the gene involved in BMP synthesis. To confirm this result, NMA38-4 cells were observed by transmission electron microscopy which showed that they did not contain BMPs in the cell (data not shown), indicating that BMPs were not synthesized completely.

DNA Sequence Analysis of ORF4

Identification of the gene interrupted by mini-Tn5 transposon in NMA38-4 genome was accomplished by isolation of flanking DNA by inverse PCR, sequencing of the flanking DNA, and sequence analysis through homologous searches of major databases. Figure 2 shows a gel electrophoresis of 1.3 kb inverse PCR product amplified from DNA flanking the transposon of NMA38-4 genome. To characterize this locus, we used sequence of DNA flanking the transposon aligned against the whole genome sequence of *M. magneticum* AMB-1 (Matsunaga *et al.* 2005) and assembled one contig which contained mini-Tn5-interrupted gene, ORF4. Homology search of ORF4 sequence by BLASTX program revealed its homology with ABC transporter from *Mesorhizobium loti* (62% identity, 74% similarity). An ORF directly interrupted by transposon, ORF4, with location of transposon insertion site, is shown in Fig. 3. Putative ribosomal binding site (RBS) was found at the position of 7 bp upstream of start codon (ATG).

Amino Acid Sequence Analysis Deduced from the Genes

The ORF4 of *M. magneticum* AMB-1 encodes a protein of 494 amino acids with molecular mass of 52.8 kDa, as shown in Fig. 4. Examination of the deduced amino acid sequence revealed a high degree of homology with ABC transporter. Further analysis indicated that the sequence contains ATP-binding sites in the ORF4 and the presence of two segment Walker motifs, walker A and B was identified (Walker *et al.* 1982). The two sequences, GSKKEGKLTCDTMLAL (Walker A) and KVAKPGHRLLMVS (Walker B) were localized in ORF4 at position 170-185 and 309-321, respectively (Fig. 2). The multi alignment of the amino acid sequences revealed a highly conserved region in segment A, whereas the consensus in segment

B was less restrictive, permitting various amino acid substitutions. Analysis of hydrophilicity plot of the protein deduced from ORF4 using an algorithm Kite-Doolittle showed that ORF4 protein possessed 10 putative hydrophobic transmembrane α helices (data not shown).

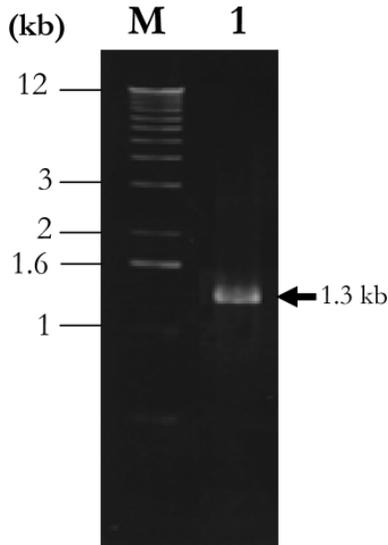


Figure 2. 1.3 kb DNA fragment flanking transposon mini-Tn5Km1 amplified by inverse PCR method (1) and DNA Marker 1 kb ladder (M).

Expression and Purification of Histag fusion protein

After recombinant plasmid pET15b-ORF4 was transformed to *E. coli* BL21 (DE3) pLysS, expression of the ORF4 was induced by addition of 0.1 mM IPTG and under the control of promoter T7 *lac*. The total protein profile of the whole cells was analyzed by SDS-PAGE to express the Histag-ORF4 fusion protein. As shown in Figure 4A, the protein band ~55 kDa corresponding to the molecular mass of the Histag-ORF4 fusion protein was highly expressed in *E. coli*, and pure protein was obtained after denatured purification. It was confirmed to be the Histag-ORF4 fusion protein by Western blot analysis using anti-Histag antibody (Fig. 4B).

DISCUSSIONS

In this study, we demonstrated that the gene encoding protein which belongs to ATP binding protein is linked to BMPs synthesis. Mutation of this gene in NMA38-4 generated non-magnetic cell. The mutant did not respond to the magnetic fields and BMPs were not completely synthesized. Inverse PCR method allowed us to amplify 1.3 kb genomic flanking DNA from NMA38-4 genome and align the sequence against the whole genome sequence of *M. magneticum* AMB-1. The contig containing the sequence of the gene directly interrupted by transposon comprised 1482 bp (Fig. 3). Although this gene did not contain a native promoter, it could be highly expressed in *E. coli* under T7 *lac* promoter (Fig. 4). This indicates ORF4 isolated from *M. magneticum* AMB-1 genome involved in BMPs synthesis could be expressed in *Escherichia coli*.

Effect of the transposon, not only inactivated the gene directly interrupted, but also the genes located downstream of the transposon insertion (De Bruijn & Lupski 1984;

Kleckner *et al.* 1977). Therefore, ORF4 may most probably have important roles during BMP synthesis. Interestingly, ORF encodes majority of the protein with functions which is related to ATP-binding cassette (ABC) transporter. Involvement of ABC transporter in iron transport has been reported in Gram negative and Gram positive bacteria such as *Yersinia pestis* (Gong *et al.* 2001; Fetherston *et al.* 1999; Bearden *et al.* 1998), *Streptococcus pneumoniae* (Brown *et al.* 2001a; Brown *et al.* 2001b), *Neisseria meningitidis* (Khun *et al.* 1998), *S. pyogenes* (Janulczyk *et al.* 1999), and *Brachyspira hyodysenteriae* (Dugourd *et al.* 1999). Taken these all together, it is therefore possible that genes with close homology to ATP-binding protein identified in this study may have a significant role in iron transport during BMPs synthesis after the iron passed across the outer cell membrane.

Despite the significant impact of BMPs to the physiological functions of the cell, information on the mechanisms and the factors affecting their formation is still very limited. Our data suggests that a gene for ATP binding cassette protein and the other genes within the same cluster may most probably be linked to BMPs synthesis in *M. magneticum* AMB-1. This finding may significantly contribute to the complete elucidation of the complex process of BMP synthesis, especially in *M. magneticum* AMB-1.

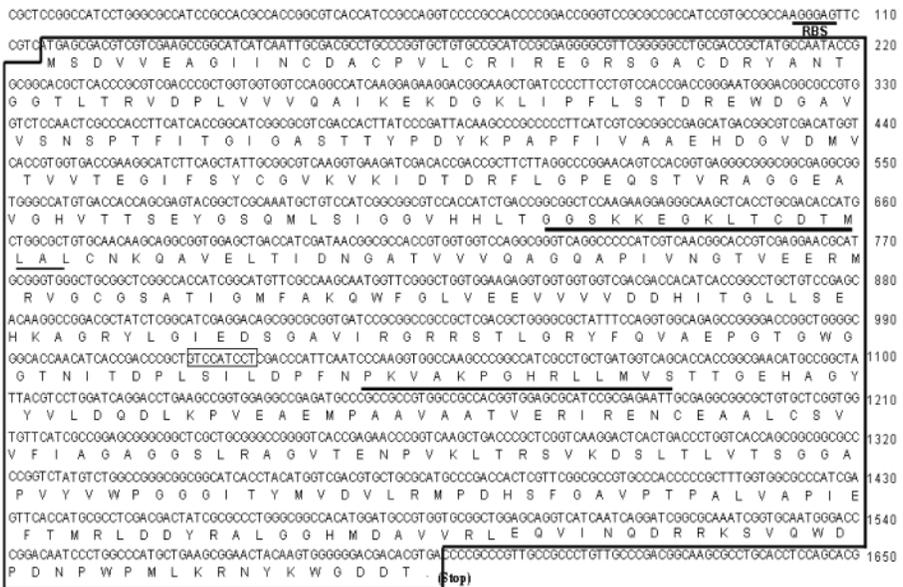


Figure 3. DNA sequence of ORF4 (boxed) started with ATG (start codon) and ended by TGA (stop codon). 9 bases boxed show mini-Tn5 insertion site. Walker A (GGSKKEGKLTCDTMLAL), Walker B (PKVAKPGHRLLMVS), and ribosomal binding site (RBS) are also indicated by underline.

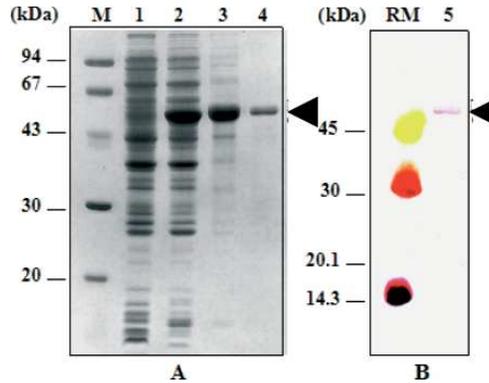


Figure 4. (A). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of protein profile of ORF4 expressed in *Escherichia coli*. Lane M: marker protein. Lane 1: Total cell proteins from uninduced-cells of *E. coli* BL 21 (DE3) pLysS harboring pET15-ORF4. Lane 2: Total cell proteins from induced-cells of *E. coli* BL 21 (DE3) harboring pET15-ORF4 induced by 0.1 mM IPTG. Lane 3: Total protein cell lysate (15 μ g). Lane 4: Purified Histag-ORF4 protein (6 μ g). (B) Lane RM: Rainbow Marker protein. Lane 5: Western blot analysis of purified Histag-ORF4 fusion protein (6 μ g) corresponding to lane 4.

CONCLUSION

A gene involved in bacterial magnetic particle synthesis has been isolated from *M. magneticum* AMB-1 genome, cloned and over-expressed in *E. coli* BL21 (DE3) pLysS. This gene encoded protein had homology with ABC transporter. A 55 kDa protein corresponding to the gene (ORF4)-Histag fusion protein resulted from the gene expression was detected by SDS-PAGE. The purified protein was confirmed to be ORF4-Histag fusion protein by Western blot analysis. Mutation within this gene (ORF4) rendered *M. magneticum* AMB-1 defective in bacterial magnetic particle synthesis.

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