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Cloning and Overexpression of a Gene Encoding Protein Belong to Tetratricopeptide Involved in Magnetosome Synthesis in *Magnetospirillum magneticum* AMB-1

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A genomic DNA fragment involved in magnetosome synthesis was isolated from *Magnetospirillum magneticum* AMB-1 through mini-Tn*5* transposon mutagenesis. The DNA fragment flanking transposon from the genomic DNA of a non-magnetic mutant, designated as NMA42, was isolated by inverse polymerase chain reaction (inverse PCR), sequenced and aligned against the whole genome sequence of AMB-1. A 6020 bp genomic DNA sequence consisting of four open reading frames (ORFs) organized in an operon was determined. The deduced amino acid sequence of 1977 base pair from ORF4 directly interrupted by transposon homolog with tetratricopeptide repeat domain protein (TPR) from *Chlamydophila pneumoniae* (30% identity, 46% similarity). The presence of four repeats of a degenerate 34 amino acids consensus sequence indicate that the protein may interact with proteins in the cytoplasm to function during magnetosome synthesis. The ORF4 was subsequently isolated from AMB-1 genome by polymerase chain reaction (PCR), cloned, and overexpressed in *Escherichia coli* as a 70.8 kDa His-tagged polypeptide.

Key words: *Magnetospirillum magneticum* AMB-1, cloning, overexpression, tetratricopeptide repeat protein, magnetosome

Magnetic bacteria orient and swim along geomagnetic fields and are widely distributed in freshwater and marine habitats. These bacteria respond to magnetic fields due to the presence of magnetosomes synthesized under microaerobic conditions. Magnetosomes are membrane-bound (Gorby *et al.* 1988; Matsunaga 1991), intracellular single domain magnetic particles of either an iron oxide, magnetite (Fe_3O_4) (Bazylinski *et al.* 1988; Matsunaga *et al.* 1991), or iron sulfide, greigite (Fe_3S_4) (Bazylinski *et al.* 1993). The magnetic bacterium, *Magnetospirillum magneticum* AMB-1 was isolated from freshwater sediments and is capable of growing on agar plates and in liquid medium under microaerobic conditions (Matsunaga *et al.* 1991). This makes *M. magneticum* AMB-1 the only magnetic bacterial strain ammenable to genetic manipulation (Matsunaga *et al.* 1992; Wahyudi *et al.* 2001). Therefore, *M. magneticum* AMB-1 was used as a model system to elucidate the mechanisms involved in magnetosome synthesis at the molecular genetic level. Analysis of the genes involved in magnetosome synthesis first requires the isolation of nonmagnetic mutants which can be generated by transposon mutagenesis.

To elucidate magnetosome synthesis in magnetic bacteria, genetic analysis of proteins specifically associated with the magnetosome membrane including, MpsA which is believed to be acyl-CoA transferase (Matsunaga *et al.* 2000), Mms16 which functions as GTP-binding protein (Okamura *et al.* 2001) in *M. magneticum* AMB-1, and tetratricopeptide repeat (TPR) which is speculated to function as a receptor for the cytoplasmic protein in *M. magnetotacticum* MS-1 (Okuda & Fukumori 2001) have been reported. However, only the *mag*A gene has been reported to function as an iron transporter in *M. magneticum* AMB-1 (Nakamura *et al.* 1995). Recently, non-magnetic mutants were generated using mini-Tn*5* transposon mutagenesis of *M. magneticum* AMB-1 and sequence analyses of the DNA flanking the transposon reveals at least 10 genes are required for magnetosome synthesis (Wahyudi *et al.* 2001). Thus, magnetosome synthesis in *M. magneticum* AMB-1 is a complex system involving many genes.

Previously, a nonmagnetic mutant, designated as NMA42, was generated by introduction of transposon Mini-Tn*5* into the genome of *M. magneticum* AMB-1 (Wahyudi *et al.* 2001). In this study, the genomic region interrupted by transposon was analyzed. The gene directly interrupted by transposon which is involved in magnetosome synthesis was isolated, clone and heterologously overexpressed in *Escherichia coli* as a Histag fusion protein.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Culture Conditions. *Escherichia coli* DH5α was routinely cultured on 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 routinely cultured on LB supplemented with chloramphenicol (25 μg ml^{-1}) at 37 °C. *Magnetospirillum magneticum* AMB-1 (ATCC 700264) was cultured microaerobically in magnetospirillum growth medium (MSGM) (Blakemore *et al.* 1979) at 25 °C, while NMA42 (Wahyudi *et al.* 2001) was cultured in MSGM supplemented with kanamycin (5 μg ml^{-1}).

Isolation of Flanking DNA and Sequence Analysis. Genomic DNA of NMA42 was isolated using method described by Wilson (1994). The DNA fragments flanking the transposon

were isolated by inverse PCR after *Eco*RV digestion (Wahyudi *et al.* 2001), using primers designed from the mini-Tn*5*Km1 sequence near the insertion sequences oriented outward from the transposon (Primer F: 5'-GATCCTCTAGAGTCGAC-3'; Primer R: 5'-GTACCGAGCTCGAATTC-3'). The inverse PCR products were purified from the gel using a Gene Clean III Kit (Bio-101, Carlsbad, CA.) and sub-cloned in pGEM-T Easy (Promega, Madison, WI, USA) to produce the recombinant plasmid, pGEMT-42. This plasmid was transformed into *E. coli* DH5α by electrophoration. The recombinant plasmid was isolated from *E. coli* using Qiaprep Miniprep (Qiagen, Gmbh, Germany) and used as a template for DNA sequencing. Both strands of DNA flanking the transposon were sequenced using an automatic DNA sequencer ABI 377 (Perkin Elmer, USA) with universal primers M13 used for cycle sequencing. The DNA sequence was subsequently aligned against the whole genome sequence of *M. magneticum* AMB-1 (Matsunaga *et al.* 2005). The DNA and protein sequences were analyzed using Lasergene (DNASTAR, Madison, WI). The sequence was further analyzed by performing homology searches using the BLAST program (Altschul *et al.* 1997) with GenBank and EMBL DNA databases.

Analysis of TPR Motif of the Protein. The deduced amino acid sequence of the ORF4/gene was analyzed for the presence of TPR motifs. InterPro Search (http://oban.ebi.ac.uk:6600/pub/ipsearch.html) and ProfileScan server (http://www.isrec.isb-sib.ch/sofware/PFSCAN_form.html) were used to search the TPR motifs for the TPR protein sequence.

Cloning and Overexpression of ORF4. Based on the sequence of the ORF4, two oligonucleotide primers (Primer 1, 5'-GGG GGA CAT ATG AAC ATC GAT CAG GAC-3' and Primer 2, 5'-GGG GGA TCC AAA TCA AAC GTC TAT ATT TTC-3') were designed. The recombinant plasmid, designated as pET-ORF4, containing ORF4 was constructed by cloning the PCR product amplified from the gene into the *Nde*I/*Bam*HI site of expression vector pET15b. To amplify this gene, primer P1 with an *Nde*I site was introduced at a start codon ATG, and primer P2 with a *Bam*HI site was introduced downstream of the stop codon (TGA). The 50 µl PCR reaction mixture contained 100 ng *M. magneticum* AMB-1 genomic DNA, 2.5 U LA Taq (Takara, Tokyo, Japan), 400 µM dNTPs, 2.5 mM $MgCl_2$, and 0.2 µM of each of the two primers. The temperature program for PCR was one cycle of 2 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 for the last cycle. The amplified fragment was subsequently isolated from the gel and purified using a Gene Clean III Kit (Bio 101). The 2.0 kb purified fragment was sub-cloned in pGEM-T Easy vector (Promega, USA) and transformed into *E. coli* DH5α. The recombinant plasmid was isolated and digested with *Nde*I and *Bam*HI. The fragment corresponding to the gene was ligated into pET15b expression vector (Novagen, Madison, WI, USA) linierized with *Nde*I and *Bam*HI, to yield the recombinant plasmid pET15b-ORF4.

The recombinant plasmid, pET15-ORF4, was introduced into *E. coli* DH5α and subsequently extracted and transformed into a host strain for expression, *E. coli* BL21 (DE3) pLysS. Transformants were plated on LB plates containing ampicillin (50 µg ml^{-1}) and chloramphenicol (25 µg ml^{-1}). For expression of the gene, *Escherichia coli* BL21 (DE3) pLysS carrying pET15-ORF4 was grown aerobically at 37 °C in 10 ml of LB containing ampicillin and chloramphenicol to 2 x 10^8 cells ml^{-1} (OD_{600} of 0.6). The culture was induced by the addition of 0.1 mM IPTG and incubated at 37 °C for 3 h with sufficient aeration and then analyzed by sodium dodecyl sulfate polyacrylamid gel electrophoresis (SDS-PAGE).

Protein Purification and SDS-PAGE. Ten milliliter culture of *E. coli* BL21 (DE3) pLysS carrying pET15b-ORF4 was induced by 0.1 mM IPTG at an OD_{600} of 0.6 for 3 h by shaking at 37 °C. The culture was then centrifuged, and the pellets were frozen at -70 °C until use. Histag-ORF4 fusion protein was purified under denatured conditions using Ni-NTA column (Qiagen, GmbH, Germany). Pellets were thawed and resuspended in buffer A (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris.HCl, pH 8.0), incubated at room temperature for 1 h under agitation, and centrifuged for 20 min at 15 000 rpm. Soluble fractions were loaded onto the Ni-NTA column equilibrated with buffer A and centrifuged at 2000 rpm for 2 min. The column was washed with buffer B (buffer A pH 6.3), and Histag-ORF4 fusion protein was eluted with 200 µl buffer E (buffer A pH 4.5).

Pellets of uninduced cells (1 ml) and induced cells (0.5 ml), solubilized lysate, and purified Histag-ORF4 fusion protein were mixed with 2 x sample buffer (0.13 M Tris-HCl, pH 6.8, 20% glycerol, 4% SDS, 0.2% 2-mercaptoethanol, 0.001% bromophenol blue), and denatured. SDS-PAGE was performed with a 12.5% (w/v) acrylamide gel, and the proteins were stained with commassie brilliant blue G-250.

RESULTS

Molecular Organization of ORF4. Inverse PCR amplification of upstream and downstream sequence of the DNA flanking the transposon from NMA42 allowed the isolation of a 900 bp DNA fragment. To characterize this locus, we 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, ORF4 (Figure 1) consisting of 1977 bp, was identified. Target sequence of the transposon was GCCCAGAGC located at 145 bp position from the start codon (ATG). Putative ribosomal binding site (RBS) was found at 5 bp position upstream of the start codon of ORF4. Putative promoter like-sequence was found upstream of the start codon of ORF1-ORF4. Therefore, ORF1-ORF4 might be organized in an operon (Figure 1). These genes organized in an operon may also function during magnetosome synthesis in *M. magneticum* AMB-1 under microaerobic conditions.

Analysis of Protein Sequence Deduced from ORFs. Homology searches of the deduced amino acid sequences of each ORF are shown in Table 1. The analyses revealed that the transposon was directly inserted into ORF4 and had a high degree of homology with the TPR domain protein from *Chlamydophila pneumoniae* (30% identity and 46% similarity). The complete DNA sequence and deduced amino acid

sequence of this gene as well as hydrophilicity plot (Kite-Doolittle) analysis indicated that the protein contains 20-22 transmembrane alpha helices (Figure 2a). The TPR protein deduced from ORF4 is characterized by the presence of several repeats of a degenerate 34 amino acids consensus sequence arranged as tandem arrays. As shown in Figure 2b, c, this protein contains four tandem TPR repeats in its N-terminal amino acid sequence. This indicates that 70.8 kDa of this protein is in the TPR protein family and is proposed as a membrane protein.

Cloning and Overexpression of ORF4. We have isolated, cloned and over expressed a 1977 bp of the ORF4 (Figure 1) in *E. coli* BL21 (DE3) pLysS. Gene expression after cloning in plasmid pET15b was controlled by the T7 *lac* promoter. To determine expression of the Histag-ORF4 fusion gene, the total protein from IPTG induced and uninduced cells, including soluble lysates, and purified Histag-ORF4 fusion proteins, were analyzed by SDS-PAGE. As shown in Figure 3a, the presence of a 70.8 kDa protein band, representative of the molecular mass of Histag-ORF4 fusion protein was highly over expressed in *E. coli*. The soluble fraction was purified by Ni-NTA affinity chromatography and analyzed by SDS-PAGE. Analysis showed the presence of a major 70.8 kDa species, which is the expected size of the Histag-ORF4 fusion protein, and minor species of 45 kDa, and 33 kDa indicated other proteins that may be contaminate Histag-ORF4 fusion protein (Figure 3b). Confirmation by Western blot analysis (Wahyudi *et al.* 2003) reveals a single band hybridization of the protein at a Histag-ORF4 fusion protein at the position of 70.8 kDa (data not shown).

DISCUSSION

In this study, ORF4 encoding protein homolog with tetratricopeptide (TPR) was directly interrupted by mini-Tn*5* transposon rendered *M. magneticum* AMB-1 defective in complete magnetosome synthesis. Similar TPR proteins were also found in other bacteria such as *M. magnetotacticum* MS-1, *Caulobacter crescenthus, Chlamidophyla pneumoniae,* and yeast, *Sacharomyces cerevisiae*. Cloning of ORF4 TPR protein in *E. coli* BL21 (DE3) pLysS using pET15b as an expression vector revealed that the ORF4 protein was overexpressed well in *E. coli* (Figure 3a,b). Expression of this gene cloned in pET15b was transcribed by T7 promoter RNA polymerase. This promoter can be induced by isoprophyl thiogalactopyranoside (IPTG) added to the medium. The predicted molecular mass of Histag-ORF4 fusion protein was about 70.8 kDa as determined by SDS-PAGE.

The tetratricopeptide repeat is a 34 amino acid sequence motif, typically found in tandem clusters, that occurs in a wide variety of proteins in prokaryotes and eukaryotes (Hirano *et al.* 1990; Okuda & Fukumori 2001). TPR protein isolated from the magnetosome membrane of the magnetic bacterium, *M. magnetotacticum* MS-1, the gene encoding TPR protein, *mam*22, is 662 bp and encodes for 220 amino acids. The deduced amino acid sequence of Mam22 consists of three TPR repeats (Okuda *et al.* 1996). The *mam*22 homolog is also found in *M. gryphiswaldense* MSR-1 and *Magnetococcus* MC-1 (Grunberg *et al.* 2001). However, alignment of TPR gene sequence isolated from AMB-1, with *mam*22 from *M. magnetotacticum* MS-1 showed them to be non-homologous,

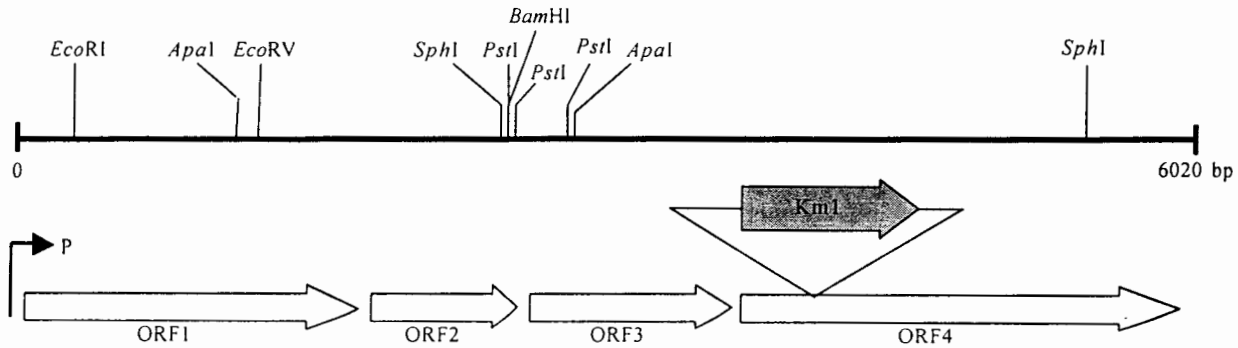
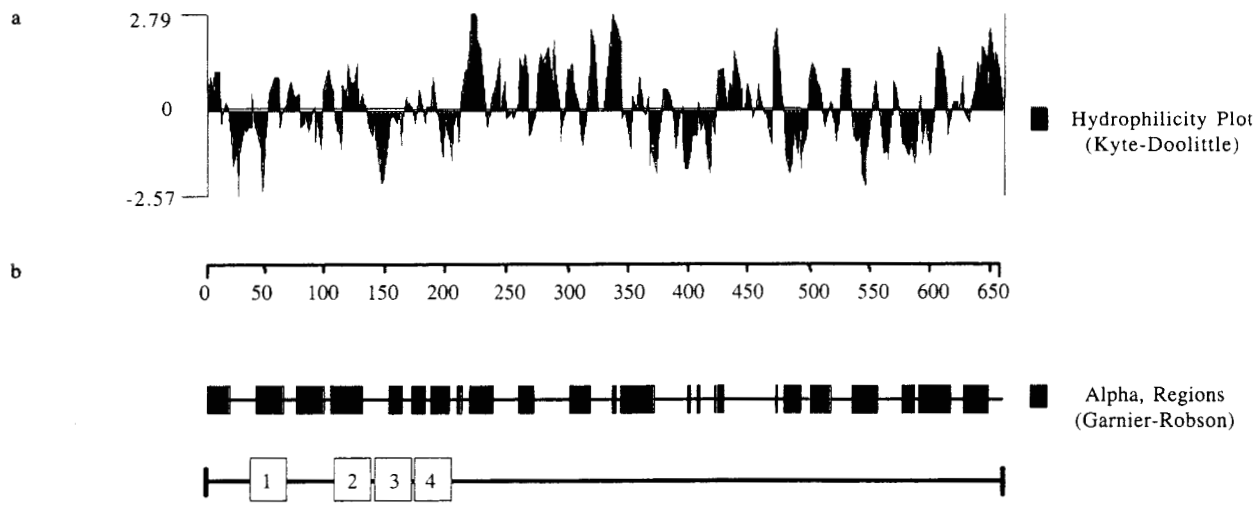


Figure 1. Physical map of an operon required for magnetosome synthesis in *M. magneticum* AMB-1. ORF4 is directly interrupted by transposon as indicated by arrowhead with Km1. P indicates promoter sequence identified upstream of ORF1. Restriction enzymes that digest this DNA fragment is also shown.

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

ORF	Size (bp)	Amino acid residue	Homologous protein	Accesion No.	Id/pos (%)	Microorganism
1	1362	554	Diguanylate cyclase/phosphodiesterase	PA2870	45/56	*Pseudomonas aeruginosa*
2	927	309	Uncharacterized ACR	mlr3686	33/49	*Mesorhizobium loti*
3	1005	335	Predicted phosphohydrolase	mll9676	34/53	*Mesorhizobium loti*
4	1977	659	TPR-repeat-containing protein	CPn0693	30/46	*Chlamydophila pneumoniae*

Id: Identity, Pos: Positive



c

1	VPVRYNLALFLLMAGRHTEALPHLDRILAAQPGH	(37-70)	TPR 1
2	PESLLALGNALRTLSRMEEAAQAFHRLTRVAPGF	(103-136)	TPR 2
3	VGGHINLGILLVSMADTQAALSALDDAVRLHPGV	(137-170)	TPR 3
4	GELHALRGQALLRLGRHSEAIAALEKALAINPAL	(171-204)	TPR 4

Figure 2. Amino acid sequence analysis ORF4. a. Hydrophiliity plot analysis using Kyte-Doolittle, b: Occurance 4 repeats of 34 degenerate amino acids (TPR) as indicated by number in the box, and c: Position of four amino acid sequences showed TPR motif in the protein.

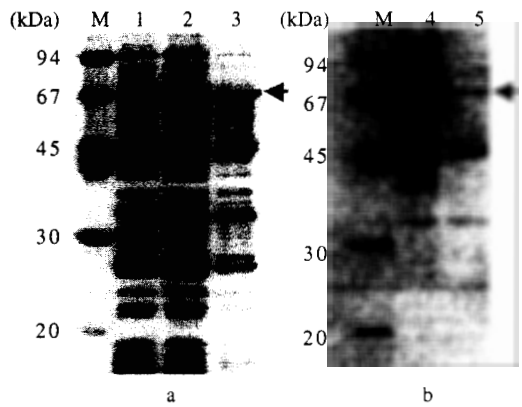


Figure 3. SDS-PAGE of protein profile extracted from *E. coli* BL21 (DE3) pLysS carrying pET15-42. 1: uninduced-cell, 2: induced-cell by IPTG, 3: Soluble lysate, 4: Soluble lysate (2X), 5: purified protein. a: Gel is stained by silver, and b: Gel is stained by Coomassie Blue.

indicating the TPR gene reported here is indeed different compared with *mam*22. Therefore, we conclude that the TPR protein isolated from AMB-1 is a new protein belonging to the TPR domain protein family, and that in *M. magneticum* AMB-1 it is involved in magnetosome synthesis. TPR protein family analysis of the whole genome sequence of *M. magneticum* AMB-1 reveals that this bacterium has 42 genes encoding protein belong to TPR protein family (data not shown).

A general function of TPR domains is to bind other protein or to mediate protein-protein interaction (Goebl & Yanagida 1991; Lambl *et al.* 1995). The TPR motif was first identified in the cell division cycle genes cdc16, cdc23, and cdc27 which encode subunits of the anaphase promoting complex (APC) (King *et al.* 1995). The role of the APC is to target cell cycle proteins for ubiquity-dependent degradation at both the onset of anaphase and at the exit of mitosis. Mutations within the TPR motifs of these protein cause mitotic arrest at the metaphase to anaphase transition. The TPRs occur in over 25 proteins of diverse biological functions present in organisms as diverse as bacteria and humans contain TPR motifs. In addition to cell cycle regulation, processes such as transcription control, mitochondrial and peroxisomal protein transport, neurogenesis, protein kinase inhibition, Rac-mediated activation of NADPH oxidase, and protein folding involve TPR motifs (Lambl *et al.* 1995). The molecular chaperone machinery contains multiple protein components that have one or more structural domains composed of tetratricopeptide repeat (TPR) motifs have also been reported (Smith 2004).

Taken the data all together, it can be speculated that the operon identified containing TPR gene may contribute to protein-protein interaction during magnetosome synthesis under microaerobic respiration. The ORF4 encodes for a TPR protein that may be transported to the magnetosome membrane and function during magnetosome synthesis cooperatively with other proteins in the cytoplasm. Mutation within this operon generated cells lacking highly organized magnetosome aligned in chains for the cell's magnetic response. The future analysis is functional characterization and location of TPR protein transported into the cell during magnetosome synthesis in *M. magneticum* AMB-1

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