0273-2289/01/91–93/0147/$12.00

Isolation of *Magnetospirillum magneticum* AMB-1 Mutants Defective in Bacterial Magnetic Particle Synthesis by Transposon Mutagenesis

Aris Tri Wahyudi, Haruko Takeyama, and Tadashi Matsunaga*

Department of Biotechnology,
Tokyo University of Agriculture and Technology,
2-24-16 Naka-Cho, Koganei, Tokyo 184-8588, Japan,
E-mail: tmatsuna@cc.tuat.ac.jp

Abstract

Nonmagnetic mutants of *Magnetospirillum magneticum* AMB-1 were recovered following mini-Tn*5* transposon mutagenesis. Transconjugants with kanamycin resistance were obtained at a frequency of 2.7×10^{-7} per recipient. Of 3327 transconjugants, 62 were defective for bacterial magnetic particle (BMP) synthesis. The frequency of independent transposition events for nonmagnetic mutants was about 1.4% in transconjugants. Further analysis of DNA sequences flanking transposon by inverted polymerase chain reaction allowed isolation of at least 10 genes or DNA sequences involved in BMP synthesis in *M. magneticum* AMB-1.

Index Entries: Transposon mutagenesis; mini-Tn*5*; *Magnetospirillum magneticum* AMB-1; inverse polymerase chain reaction; bacterial magnetic particles; sequence analysis.

Introduction

Magnetic bacteria are a diverse group of Gram-negative prokaryotes and synthesize membrane-bound intracellular particles of either magnetite (Fe_3O_4) or greigite (Fe_3S_4) that are aligned in chains of 10–30 along the length of the cell. Formation of bacterial magnetic particles (BMPs) is achieved by a biomineralization process regulated at the genetic level. Accumulation of iron and the deposition of specific particle sizes occur within membrane vesicles at specific locations in the cell *(1)*. Studies on

*Author to whom all correspondence and reprint requests should be addressed.

BMP synthesis usually use *Magnetospirillum magnetotacticum* MS-1 *(2)* and *M. magneticum* AMB-1 *(3)* as model systems. Little is known about the biosynthetic ability and molecular genetics of BMP formation in these bacteria. *M. magneticum* AMB-1 is the only strain with an established transformation system that can form colonies on laboratory medium. This feature facilitated the genetic analysis of BMP synthesis in *M. magneticum* AMB-1 *(4)*.

Transposon Tn*5* is one of the best-studied and most versatile transposons *(5,6)*. Tn*5* exhibits a relatively high transposition frequency and is stably inserted into the genome *(7)*. Mini-Tn*5* derivatives have proven to be extremely useful tools in bacterial genetics for mutagenesis or insertion of cloned DNA into the chromosome of Gram-negative bacteria *(8–10)*. These derivatives consist of an antibiotic-resistance marker flanked by 19-bp inverted repeats of Tn*5* that are essential for transposition *(11)*. The transposase gene is located outside the inverted repeats on the vector. Therefore, insertions produced by mini-Tn*5* are generally more stable because of the absence of transposase-mediated secondary transpositions *(8)*. Characterization, cloning, and sequencing of transposon-inserted genes require the isolation of flanking DNA sequences. This is generally achieved by cloning DNA fragments containing the transposon or by inverse polymerase chain reaction (IPCR) *(12)*. The *magA* gene encoding an iron-transporter protein located on BMP membrane and involved in BMP synthesis was isolated from *M. magneticum* AMB-1 by Tn*5* transposon mutagenesis *(13)*.

In the present study, we isolated numerous *M. magneticum* AMB-1 mutants that are defective in BMP synthesis by mini-Tn*5*-mediated mutagenesis and analyzed the DNA sequences interrupted with mini-Tn*5*.

Materials and Methods

Bacterial Strains, Culture Conditions, and Plasmids

Escherichia coli S17-1 λ *pir (11)* harboring plasmid pUTmini-Tn5Km1 *(5,9)* was grown on Luria Bertani (LB) broth (5.0 g/L of tryptone, 10 g/L of NaCl, 5.0 g/L of yeast extract) containing ampicillin (50 µg/mL) and kanamycin (25 µg/mL) at 37°C. *E. coli* DH5α was grown in LB at 37°C. *M. magneticum* AMB-1 (ATCC 700264) was cultured anaerobically with magnetic spirillum growth medium (MSGM) *(2)* at 25°C. Transconjugants of AMB-1 with mini-Tn*5* transposon were anaerobically cultured with MSGM supplemented with kanamycin (5 µg/mL). The pGEM-T Easy vector (Promega, Madison, WI) was used for cloning of IPCR products.

Transposon Mutagenesis of M. magneticum *AMB-1*

Transposon mini-Tn*5*Km1 (pUT-miniTn*5*Km1) was transferred from *E. coli* S17-1 λ *pir* into AMB-1 by conjugation. The donor, *E. coli* S17-1 λ *pir*, was grown in LB medium containing ampicillin and kanamycin. AMB-1 cells responsive for magnetic field were magnetically collected and used for cultivation. AMB-1 and *E. coli* S17-1 λ *pir* cells in logarithmic phase

(approx 1×10^8 cells/mL) were centrifuged, washed three times with 0.85% NaCl, and resuspended in MSGM at a concentration of about 10^{11} cells/mL. Fifty microliters of the donor and recipient were mixed in varying ratios of *E. coli* to AMB-1 (1:1, 1:10, and 10:1) and placed on a sterile nitrocellulose filter on MSGM agar, and mating was performed aerobically for 6 h at 25°C. The filters were then transferred into 300 µL of MSGM in a microcentrifuge tube and vortexed. One hundred microliters of each cell suspension was plated on MSGM agar supplemented with kanamycin (5 µg/mL) and incubated for 10–14 d anaerobically. Nonmagnetic mutants were defined by microscopic observation under magnetic fields.

Southern Hybridization Analysis

Isolation of genomic DNA from nonmagnetic mutants of AMB-1 was performed as described by Wilson *(14)*. The genomic DNA digested with *Pst*I or *Eco*RI was used for Southern hybridization analysis using 1.7-kb DNA fragments containing the kanamycin resistance gene from plasmid pUTmini-Tn*5*Km1 labeled as a probe with a digoxigenin (DIG) chemiluminescence kit (Boehringer Mannheim GmbH Biochemica). Hybridization was performed using a DIG DNA Detection Kit (Boehringer Mannheim).

IPCR Amplification and DNA Sequencing

Figure 1 shows the strategy for IPCR. IPCR was performed against DNA sequences flanking mini-Tn*5* in genomic DNA isolated from nonmagnetic mutants. The genomic DNA digested with *Eco*RV or *Bst*XI and *Apa*I was circularized and used as templates for IPCR. Amplification was performed using primers designed from the mini-Tn*5* sequence near the insertion sequence: primer 1, 5'-ACACTGATGAATGTTCCGTTG-3'; primer 2, 5'-ACCTGCAGGCATGCAAGCTTC-3'. A GeneAmp PCR System 2400 (Perkin-Elmer) was used to amplify DNA by denaturation at 95°C for 2 min, primer annealing at 60°C for 1 min, and primer extension at 72°C for 1 min for 30 cycles and 10 min for the last cycle.

The IPCR products were purified by gel electrophoresis and cloned using pGEM-T Easy vector. Recombinant plasmids were extracted from *E. coli* DH5α by standard methods *(15)* and used for DNA sequencing utilizing an automatic DNA sequencer, DSQ-2000L (Shimadzu, Japan). LASERGENE (DNASTAR, Madison, WI) was used for analysis of DNA homology and for detection of transposon insertion sites in the genome.

Results and Discussion

Isolation of Transposon Mutants Defective in BMP Synthesis

The highest transconjugation frequency was 2.7×10^{-7} when a 10:1 ratio of *E. coli* and AMB-1 was employed (Table 1). AMB-1 with BMPs form brown-black colonies. A total of 3327 transconjugants were screened, of

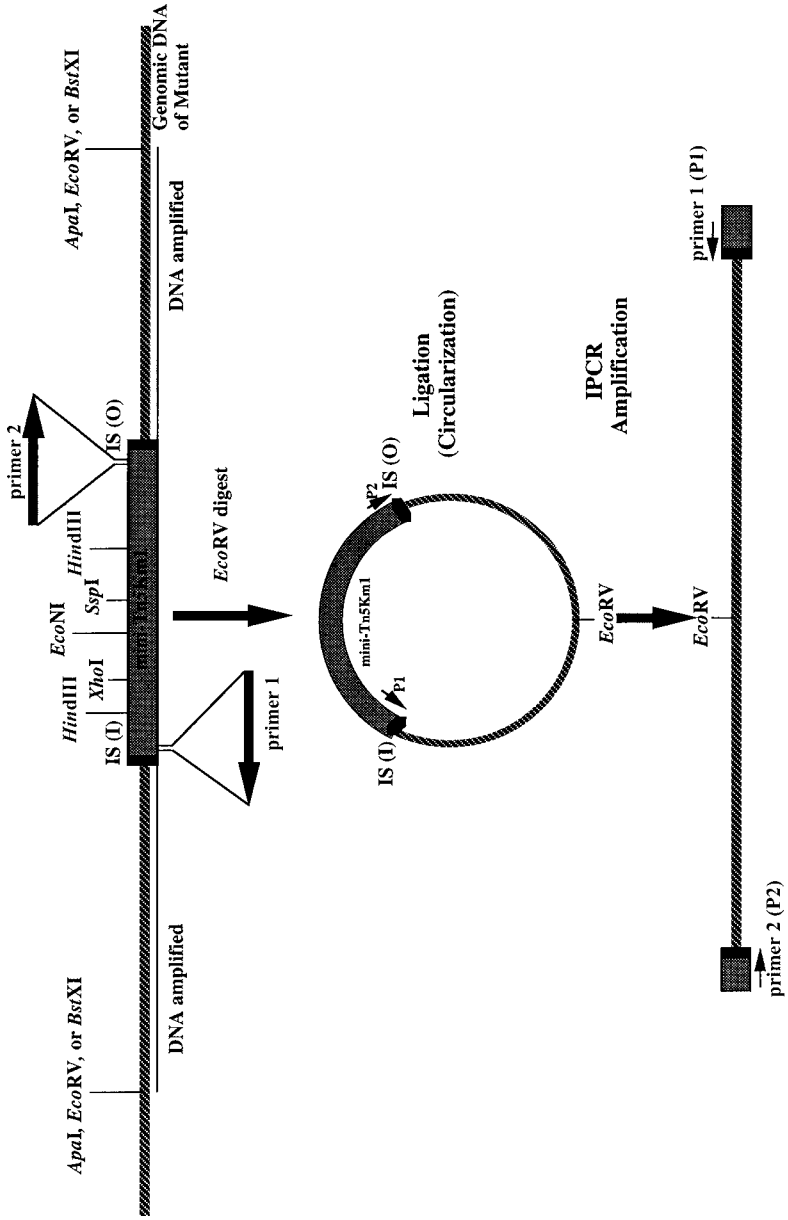

Fig. 1. The strategy for IPCR. Genomic DNA of AMB-1 containing inserted transposon was digested with restriction enzyme *ApaI*, *BstX*I, or *Eco*RV. IS, insertion sequence of the transposon, 19 bp (inner and outer).

Table 1
Frequency of Transconjugation
of Transposon mini-Tn*5*Km1
from *E. coli* to *M. magneticum* AMB-1

Mating cell ratio of *E. coli* and AMB-1	Frequency[a]
1:10	2.3×10^{-8}
1:1	4.6×10^{-8}
10:1	2.7×10^{-7}

[a]Frequency of transconjugation is calculated per recipient. Values are the mean of 10 experiments.

which 62 colonies were white and thus defective for BMP synthesis. All nonmagnetic mutants grew well in MSGM supplemented with kanamycin under anaerobic conditions, similar to the AMB-1. Because spontaneous mutants with kanamycin resistance did not occur on MSGM plates supplemented with kanamycin, AMB-1 mutants defective in BMP synthesis were owing presumably to the transposon insertion into the genome.

Southern Hybridization Analyses of Nonmagnetic Mutants by IPCR

Thirty-four among 62 nonmagnetic mutants were arbitrarily selected for further analysis. Southern hybridization analysis of nonmagnetic mutants revealed the presence of the transposon in genomic DNA. Furthermore, 26 nonmagnetic mutants were derived from independent transposition events (11 of 34 nonmagnetic mutants are shown in Fig. 2). The frequency of transposition events for nonmagnetic mutants was about 1.9% in total transconjugants (62 nonmagnetic mutants of 3327 transconjugants), and 76% of nonmagnetic mutants analyzed were independent mutants (26 of 34 nonmagnetic mutants). Assuming that AMB-1 has a typical genome size of ~4000 genes like *E. coli*, these data suggest that as many as 60 genes may be involved in BMP synthesis. Biosynthesis of BMPs in AMB-1 is a complex system involving many genes. Jiang et al. *(10)* speculated that 200 genes in *Rhodospirillum centenum* (assuming ~4000 genes in the genome) might be involved in swarm cell response to light because the frequency of transposition events was 5% of the screened 23,000 mutants.

IPCR Amplification of DNA Fragments Flanking Transposon and Their Sequence Analysis

DNA fragments flanking the mini-Tn*5* transposon were amplified by IPCR using primers designed from mini-Tn*5* sequence. Single bands of 1.3–4.7 kb were amplified from 14 nonmagnetic mutants. Sequence analysis of IPCR products from these nonmagnetic mutants showed that 10 mutants were derived from independent transposition events. This suggested that at least 10 genes or DNA sequences are required for BMP synthesis in AMB-1.

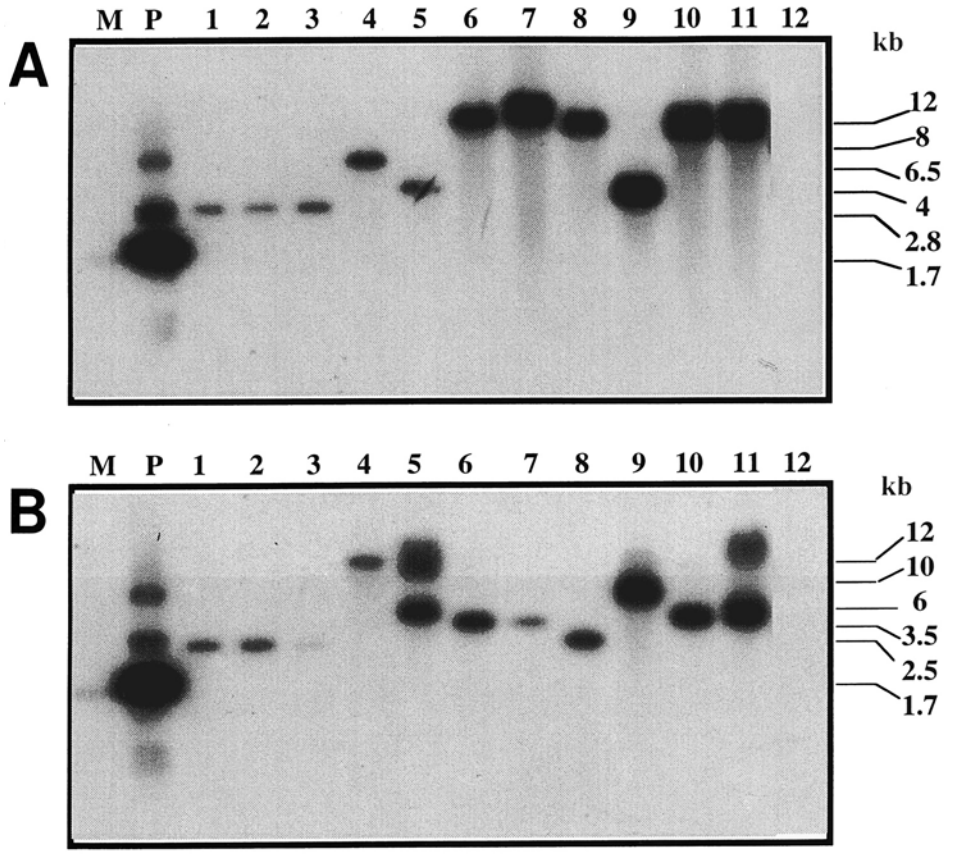

Fig. 2. Southern hybridization analysis of transconjugants using kanamycin-resistant gene as a probe. **(A)** Genomic DNA digested with *Eco*RI, and **(B)** *Pst*I. Lanes 1–11, nonmagnetic mutant genomes no. 1–11; lane 12, wild-type AMB-1 genome (negative control); lane M, 1-kb DNA ladder; lane P, pUTmini-Tn*5*Km1 digested with *Eco*RI and *Pst*I (positive control).

Table 2 shows the results of homology searches on the IPCR products from these 10 mutants. The amino acid sequence derived from the DNA sequence flanking the inserted mini-Tn*5* in mutant 9 had 51% identity with cytochrome C–type SHP in *R. sphaeroides*, which functions as an oxygen-binding heme protein during autooxidation *(16)*.

Further studies are currently being conducted to clarify the role of these genes. Complementation analysis using wild-type genome will help further the understanding of BMP synthesis in *M. magneticum* AMB-1. Elucidation of the remaining mutants will also give more information about the genes involved in BMP synthesis.

Acknowledgment

This work was supported in part by Grant-in-Aid for Scientific Research on Priority Area (A) no. 10145102, The Ministry of Education, Science, Sport and Culture.

Table 2
DNA Sequences Interrupted by Transposon and Homolog Search Analysis

NMA[a] no.	IPCR product (bp)	Insertion site	Homolog		
			Name	Identity/positive (%)	Comment/reference
6=7	2285	GCGC ATCC	Inositol polyphosphate phosphatase	39/45	From *Caenorhabditis elegans (17)*
9	2089	GTCC GGCC	Cytochrome C–type protein SHP	51/62	Oxygen-binding heme protein from Rhodobacter sphaeroides *(16)*
10	1159	GGGC GGCC	Y4XK protein	30/44	Hypothetical lypoprotein Y4XK precursor from *Rhizobium meliloti*, membrane protein; unknown function *(18)*
14	1012	GGGC CTCC	Regulatory component of sensor transduction	49/64	From *Synechocystis* sp. *(19)*
15	1180	GGGG GTCC	Putative mobilization protein	47/49	Uncultured eubacterium; putative role in conjugative DNA replication *(20)*
17	1000	AGGC ATCC	gra-ORF30	34/44	From *Streptomyces violaceoruber (21)*
19	1100	TGCG GGAC	Flagellar M ring protein	29/51	From *Caulobacter crescentus*; for energy transduction *(22)*
20=27	1006	GGAT GTTC	Hypothetical protein	38/68	From *Aquifex aelolicus (23)*
33	850	TCAT GTCC	Hypothetical protein	54/60	From *Pseudomonas*; SDSB region *(24)*
34	890	CATC GTCC	Growth factor receptor–bound protein	27/43	From *Mus musculus (25)*

[a]NMA, non-magnetic mutant of AMB-1.

References

1. Schuler, D. and Frankel, R. B. (1999), *Appl. Microbiol. Biotechnol.* **52,** 464–473.
2. Blackemore, R. P., Maratea, D., and Wolfe, R. S. (1979), *J. Bacteriol.* **140,** 720–729.
3. Matsunaga, T., Sakaguchi, T., and Tadokoro, F. (1991), *Appl. Microbiol. Biotechnol.* **35,** 651–655.
4. Matsunaga, T., Nakamura, C., Burgess, J. G., and Sode, K. (1992), *J. Bacteriol.* **174,** 2748–2753.
5. DeBruijn, F. J. and Lupski, J. R. (1984), *Gene* **27,** 131–149.
6. Simon, R., Preifer, U., and Puhler, A. (1983), *Bio/Technology* **1,** 784–791.
7. Berg, D. E. (1989), in *Mobile DNA*, Berg, D. E and Howe, M. M., eds., American Society for Microbiology, Washington, DC, pp. 185–210.
8. Herrero, M., deLorenzo, V., and Timmis, K. N. (1990), *J. Bacteriol.* **172,** 6557–6567.
9. Holtwick, R., Meinhardt, F., and Keweloh, H. (1997), *Appl. Environ. Microbiol.* **63,** 4292–4297.
10. Jiang, Z. Y., Rushing, B. G., Bai, Y., Gest, H., and Bauer, C. E. (1998), *J. Bacteriol.* **180,** 1248–1255.
11. DeLorenzo, V., Herrero, M., Martinko, J. M., and Parker, J. (1990), *J. Bacteriol.* **172,** 6568–6572.
12. Martin, V. J. J. and Mohn, W. W. (1999), *J. Microbiol. Methods* **35,** 163–166.
13. Nakamura, C., Burgess, J. G., Sode, K., and Matsunaga, T. (1995), *J. Biol. Chem.* **270,** 28,392–28,396.
14. Wilson, K. (1994), in *Current Protocols in Molecular Biology*, vol. 1, Ausubel, F. M., Brent, R., and Kingston, R. E., eds., John Wiley & Sons, New York, pp. 2.4.1–2.4.5.
15. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989), *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, New York.
16. Klarskov, K., Van Driessche, G., Barkers, K., Dumortier, C., Meyer, T. E., Tollin, G., Cusanovich, M. A., and Beeumen, J. J. V. (1998), *Biochemistry* **37,** 5995–6002.
17. Wilson, R., Ainscough, R., Anderso, K., et al. (1994), *Nature* **368,** 32–38.
18. Freiberg, C., Fellay, R., Bairoch, A., Broughton, W. J., Rosenthal, A., and Parret, X. (1997), *Nature* **387,** 394–401.
19. Kaneko, T., Sato, S., Kotani, H., et al. (1996), *DNA Res.* **3,** 109–136.
20. Tietze, E. (1998), *Plasmid* **39,** 165–168.
21. Sherman, D. H., Malpartida, F., Bibb, M. J., Kieser, H. M., and Hopwood, D. A. (1989), *EMBO J.* **8,** 2717–2725.
22. Ramakrishnan, G., Zhao, J. L., and Newton, A. (1994), *J. Bacteriol.* **176,** 7587–7600.
23. Deckart, G. and Warren, P. V. (1998), *Nature* **392,** 353–358.
24. Davison, J., Brunel, F., Phanopoulos, A., Prozzi, D., and Terpstra, P. (1992), *Gene* **114,** 19–24.
25. Ooi, J., Yatnik, V., Immanuel, D., Gordon, M., Moskow, J. J., Buchberg, A. M., and Margolis, B. (1995), *Oncogene* **10,** 1621–1630.