Design and Application of a New Cryptic-Plasmid-Based Shuttle Vector for *Magnetospirillum magneticum*

Yoshiko Okamura,¹ Haruko Takeyama,¹ Takumi Sekine,¹ Toshifumi Sakaguchi,¹
 Aris Tri Wahyudi,¹ Rika Sato,² Shinji Kamiya,² and Tadashi Matsunaga^{1*}

Department of Biotechnology, Tokyo University of Agriculture and Technology, Koganei, Tokyo,¹
 and TDK Akita Laboratory Corporation, Akita,² Japan

Received 10 October 2002/Accepted 23 April 2003

A 3.7-kb cryptic plasmid designated pMGT was found in *Magnetospirillum magneticum* MGT-1. It was characterized and used for the development of an improved expression system in strain AMB-1 through the construction of a shuttle vector, pUMG. An electroporation method for magnetic bacteria that uses the cryptic plasmid was also developed.

Magnetic bacteria synthesize intracellular, single-domain, nanometer-sized particles of magnetite (1) enveloped by lipid membranes that include numerous proteins. Aqueous dispersion of bacterial magnetic particles (BMPs) allows the development of highly sensitive chemiluminescence-based enzyme immunoassays by the chemical coupling of antibodies onto BMP surfaces (4). We have also previously reported applications of recombinant BMPs as novel bioassay platforms for protein display (12) and diagnosis of diabetes (19, 20). The enormous potential of BMPs for nanobiotechnological applications calls for the development of cloning vectors for magnetic bacteria to allow convenient gene transfer and maximum expression.

We have previously isolated two facultatively anaerobic *Magnetospirillum magneticum* strains, AMB-1 (ATCC 700264) (7) and MGT-1 (FERM P-16617) (8). The gene transfer system in *M. magneticum* AMB-1 magnetic bacteria was reported by our group (5). We have performed transposon mutagenesis in order to elucidate the molecular mechanism of BMP formation (11, 22). Electroporation has not been successfully applied in magnetic bacteria. Conjugative gene transfer was achieved (9) by using the broad-host-range vector pRK415 (3), but a low copy number was obtained. Replicons in the cryptic plasmids of magnetic bacteria may provide stable vectors yielding high copy numbers.

In the photosynthetic bacterium *Rhodospirillum rubrum*, which is genetically close to *Magnetospirillum* sp., broad-host-range vectors were unstable for replication (14). We previously screened small cryptic plasmids from marine *Rhodobacter* isolates and reported the construction of stable shuttle vectors containing the minimal replicon from a marine *Rhodobacter* isolate (10). In this study, a new cryptic plasmid designated pMGT was also found in *M. magneticum* MGT-1. This is the first report of a small-sized cryptic plasmid in magnetic bacteria. pMGT is extremely stable, even under nonselective conditions. This characteristic is valuable for the construction of shuttle vectors.

The strains and plasmids used in this study are described in Table 1. The complete nucleotide sequence of pMGT (submitted to the DDBJ database) was 3,741 bp, with a G+C content of 59.2%, which is lower than that of the *Magnetospirillum* genome (62 to 65%). Two potential open reading frames (ORF1 and ORF2) were found. ORF1 (positions 1727 to 2554 relative to position 1 at the *Bam*HI site) is 828 bp in size and encodes a predicted protein of 275 amino acids (30.4 kDa). The Shine-Dalgarno (AAG, AGG, or GGA) sequences (16) were at positions 1711 to 1713. The deduced amino acid sequence showed significant homologies to the sequences of the Rep proteins in

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference(s) or source
Strains		
<i>Magnetospirillum magneticum</i>		
MGT-1	Wild type	6,7
AMB-1	Wild type	6,8
<i>Escherichia coli</i>		
DH5 α MCR	F [−] <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) ϕ 80/ <i>lacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>) U169 <i>deoR recA1 endA1 phoA supE44</i> λ [−] <i>thi-1</i>	Gibco BRL
S17-1	Pro r [−] m ⁺ RecA [−] Tp ^r Sm ^r Ω RP4-Tc::Mu-Kn::Tn7	17, 18
JG112	<i>polA lac thy str</i>	21
Plasmids		
pMGT	Cryptic plasmid from <i>M. magneticum</i> MGT-1	This study
pUC19	Cloning vector; Ap ^r	Pharmacia
pUMG	<i>Bam</i> HI-digested whole fragment of pMGT cloned in pUC19; Ap ^r	This study
pMGTKm	Km ^r cassette cloned in pMGT	This study
pACYC184	Cloning vector; Cm ^r	Nippon Gene
pSUP202	Cloning vector; Ap ^r <i>mob</i>	17
pGV-CS	<i>luc</i> as reporter gene cloned vector; Ap ^r	Toyo Inki
pGV-magA	<i>magA::luc</i> cloned in pGV-CS	This study
pMCL	<i>PmagA-magA-luc</i> fragment cloned in pUMG; Ap ^r	This study
pKML	<i>PmagA-magA-luc</i> fragment cloned in pRK415; Tc ^r	12

* Corresponding author. Mailing address: Department of Biotechnology, Tokyo University of Agriculture and Technology, 2-24-16 Koganei, Tokyo 184-8588, Japan. Phone: 81-423-88-7020. Fax: 81-423-85-7713. E-mail: tmatsuna@cc.tuat.ac.jp.

FIG. 1. Determination of the minimum replication region of pMGT. The digested fragments were inserted into pSUP202. The replication ability of each recombinant plasmid in *M. magneticum* AMB-1 is shown at the right.

plasmid pNU73 from *Pseudomonas fulva* (percent identity and percent similarity, 33.0 and 55.8%, respectively; accession no. AB032348-1) and pLME108 from *Propionibacterium freudenreichii* (percent identity and percent similarity, 32.5 and 62.0%, respectively; accession no. AJ006662-1). ORF1 also has many amino acids that are conserved in all Rep proteins. ORF2 is 1,110 bp in size and is located at positions 2700 to 3741 and 1 to 64, corresponding to 369 amino acids with an estimated molecular mass of 40.3 kDa. The Shine-Dalgarno sequences were at positions 2694 to 2697. The deduced amino acid sequence showed high homology with the sequence of the N-terminal 170 amino acids of the Mob protein in plasmid pIP823 from *Listeria monocytogenes* (percent identity and percent similarity, 31.5 and 58.6%, respectively; accession no. U40997-4) (2) and of the whole Mob protein in plasmid pTB19 from *Bacillus* sp. (percent identity and percent similarity, 31.5 and 60.5%, respectively; accession no. JQ1212) (13). Alignment of the deduced amino acid sequence of ORF2 with those of the Mob proteins from other plasmids showed weak but significant sequence identities; hence, ORF2 was putatively designated the *mob* gene. Thus, plasmid pMGT should be mobilizable.

To identify the minimum replication region of pMGT, several segments of pMGT were inserted into the *Eco*RI site of pSUP202 (17) and transferred into *M. magneticum* AMB-1 by conjugation (Fig. 1). The *Xho*I-*Sma*I region of pMGT can replicate in *M. magneticum* AMB-1, but the *Hinc*II-*Sma*I region cannot. A typical plasmid replicon contains an origin

sequence (*ori*) and a *rep* gene. The structural features of *ori* include the AT-rich region (15). Positions 58 to 97 show a high A+T content of 70.7%. In addition, many repeated sequences consist of nucleotide sequences more than 10 bp long within the *Xho*I-*Hinc*II region. Two hairpin structures were found at positions 266 to 298, with a 15-bp stem ($\Delta G = -28.40$ kcal/mol) and a 5-bp loop, and at positions 350 to 388, with an 11-bp stem ($\Delta G = -16.60$ kcal/mol) and a 17-bp loop. The 3.0-kb *Xho*I-*Sma*I fragment containing the AT-rich region, and the putative Rep protein may therefore be the replication region.

FIG. 2. Effect of electric field strength on transformation efficiency in *M. magneticum* AMB-1.

FIG. 3. Strategy for cloning and construction of expression vector pMCML.

Recombinant plasmid pUMG was constructed by ligating pUC19 at the *Bam*HI site of pMGT. In addition, plasmid pMGTKm was constructed by inserting a kanamycin resistance cassette into the *Bam*HI site of pMGT. Both of the constructed plasmids were transformed into *Escherichia coli* DH5 α MCR and JG112. Plasmid pUC19, containing pMB1 *ori*, was also used as a control. The replicon derived from pUC19 is unable to replicate in the *polA* mutant *E. coli* JG112, which lacks the *polA* gene, which encodes DNA polymerase I and is required for replication of *ColE1* and pMB1 *ori* (21). Therefore, replication of pUMG, which contains pMGT and pUC19 replicons, depended on pMGT *ori* in *E. coli* JG112. In addition, pMGTKm, containing only pMGT *ori*, was able to replicate in both *E. coli* strains. Thus, pUMG and pMGTKm containing the pMGT replicon are capable of replicating both in *Magnetospirillum* species and in *E. coli*.

Molecular genetic analysis of the mechanisms of BMP synthesis requires DNA transfer to the cell since homologous recombination of target genes is essential for this purpose. Several preliminary experiments demonstrated that the existence of intracellular BMPs caused cell death when electroporation was applied. Intracellular BMPs are not produced when strain AMB-1 is grown under aerobic conditions. Even though electroporation was performed with cells grown aerobically, transformants screened under anaerobic conditions retained the ability to synthesize BMPs. Electroporation was performed with a Gene Pulser (Bio-Rad Laboratories, Richmond, Calif.), at a capacitance of 25 μ F and a resistance of 200 Ω , and 0.1-cm cuvettes. Aerobically grown *M. magneticum* AMB-1 was harvested and washed with 10 mM TES [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid] buffer containing 272 mM sucrose (pH 7.5) and resuspended in the same buffer at 10^9

cells/ml. A 50- μ l cell suspension was aliquoted as electrocompetent cells. The cells were subjected to single-pulse electroporation and immediately transferred to 500 μ l of magnetic *Spirillum* growth medium (MSGM) supplemented with 20 mM Mg^{2+} and incubated at 27°C overnight with shaking at 100 rpm. Cells were diluted in 5 ml of MSGM containing 0.7% agar and plated on 1% agar in MSGM containing 5 μ g of ampicillin per ml or 2.5 μ g of kanamycin per ml and incubated under anaerobic conditions. *M. magneticum* cells containing magnetic particles did not survive after the application of an electric pulse. On the other hand, the survival percentage of aerobically cultured cells, which do not produce intracellular magnetic particles, after application of a pulse was 10% of the number of CFU of aerobically cultured cells that had not been pulsed. Furthermore, the maximum transformation efficiency of 9.6×10^5 colonies/ μ g of DNA was obtained at 10 kV/cm (Fig. 2). Transformation efficiency increased 10-fold after addition of Mg^{2+} under aerobic conditions with gentle shaking to allow the cells to recover.

The copy number of plasmid pUMG in *M. magneticum* AMB-1 was measured. Five nanograms of pACYC184 (Cm^r , 4,244 bp), which was used as the standard for plasmid extraction efficiency, was added to harvested transformant cells harboring pUMG (Ap^r , 6,327 bp) or pRK415 (Tc^r , 10.5 kbp). After extraction, the plasmid mixture was transformed into *E. coli* DH5 α MCR. The copy number was calculated from the following equation: copy number of pUMG = (number of Ap^r or Tc^r colonies/number of Cm^r colonies) $\times$ (molecular number of 5 ng of pACYC184/cell number). The copy number of pUMG containing the replicon of pMGT in AMB-1 was 39 ± 10 copies/cell, whereas the vector pRK415 yielded only 3 ± 1 copies/cell.

Evaluation of pUMG as an expression vector was carried out through a reporter-luciferase expression system. The plasmid designated pMCML (10.0 kb, Tc^r) was constructed by the following procedure (Fig. 3). pGV-magA was constructed by ligating the *magA* gene promoter at the 5' end of the luciferase gene (*luc*) in pGV-CS (Toyo Inki, Tokyo, Japan). pGV-magA was digested with *Hind*III and *Xho*I and ligated to pUMG at the same sites. Constructed plasmid pMCML and previously constructed plasmid pKML, both containing *PmagA-magA-luc* (12), were introduced into AMB-1 by conjugation (5). BMPs from transformants harboring pMCML or pKML were extracted and purified as described by Nakamura et al. (12). Luciferase activities on BMPs from cells harboring pMCML were five times higher than those on BMPs from cells harboring pKML (85,200 and 16,200 counts/mg of BMPs, respectively). These results indicated that a higher vector copy number resulted in higher protein expression. The plasmid containing the replicon of pMGT is more favorable for the production of useful proteins on BMPs.

Plasmid pUMG, containing the pMGT replicon, showed stability and a high copy number and allowed higher expression of the reporter gene. These results are useful in improving the mass production of BMPs and protein A displayed on BMPs in batch-fed cultures, as we have previously described (9, 23). The data obtained from this study may be used to meet other challenges in nanobiotechnology, like the manufacturing of more sophisticated and highly efficient biosensors and biomaterials at the nanoscale level that are useful in interdisciplinary fields.

This work was funded in part by Grant-in-Aid for Specially Promoted Research no. 13002005 from the Ministry of Education, Science, Sports and Culture of Japan.

REFERENCES

1. Bazylinski, D. A., R. B. Frankel, B. Heywood, S. Mann, J. W. King, P. L. Donaghay, and A. K. Hanson. 1995. Controlled biomineralization of magnetite (Fe_3O_4) and Greigite (Fe_3S_4) in a magnetotactic bacterium. *Appl. Environ. Microbiol.* **61**:3232–3239.
2. Charpentier, E., G. Gerbaud, and P. Courvalin. 1999. Conjugative mobilization of the rolling-circle plasmid pIP823 from *Listeria monocytogenes* BM4293 among gram-positive and gram-negative bacteria. *J. Bacteriol.* **181**:3368–3374.
3. Keen, N. T., S. Tamaki, D. Kobayashi, and D. Trollinger. 1988. Improved broad-host-range plasmids for DNA cloning in gram-negative bacteria. *Gene* **70**:191–197.
4. Matsunaga, T., M. Kawasaki, X. Yu, N. Tsujimura, and N. Nakamura. 1996. Chemiluminescence enzyme immunoassay using bacterial magnetic particles. *Anal. Chem.* **68**:3551–3554.
5. Matsunaga, T., C. Nakamura, J. G. Burgess, and K. Sode. 1992. Gene transfer in magnetic bacteria: transposon mutagenesis and cloning of genomic DNA fragments required for magnetosome synthesis. *J. Bacteriol.* **174**:2748–2753.
6. Matsunaga, T., and T. Sakaguchi. 1992. Production of biogenic magnetite by aerobic magnetic spirilla strains AMB-1 and MGT-1, p. 262–267. In T. Yamaguchi and M. Abe (ed.), *Proceedings of the Sixth International Conference on Ferrites (ICF6)*. Japan Society of Powder and Powder Metallurgy, Tokyo, Japan.
7. Matsunaga, T., T. Sakaguchi, and F. Tadokoro. 1991. Magnetite formation by a magnetic bacterium capable of growing aerobically. *Appl. Microb. Biotechnol.* **35**:651–655.
8. Matsunaga, T., F. Tadokoro, and N. Nakamura. 1991. Mass culture of magnetic bacteria and their application to flow type immunoassays. *IEEE (Inst. Electr. Electron. Eng.) Trans. Magn.* **26**:1557–1559.
9. Matsunaga, T., H. Togo, T. Kikuchi, and N. Nakamura. 2000. Production of luciferase-magnetic particle complex by recombinant *Magnetospirillum* sp. *Biotechnol. Bioeng.* **70**:704–709.
10. Matsunaga, T., K. Tsubaki, H. Miyashita, and J. G. Burgess. 1990. Chloramphenicol acetyltransferase expression in marine *Rhodobacter* sp. NKP80021 by use of shuttle vectors containing the minimal replicon of an endogenous plasmid. *Plasmid* **24**:90–99.
11. Nakamura, C., J. G. Burgess, K. Sode, and T. Matsunaga. 1995. An iron-regulated gene, *magA*, encoding an iron transport protein of *Magnetospirillum magneticum* strain AMB-1. *J. Biol. Chem.* **270**:28392–28396.
12. Nakamura, C., T. Kikuchi, J. G. Burgess, and T. Matsunaga. 1995. Iron regulated expression and membrane localization of the *magA* protein in *Magnetospirillum magneticum* strain AMB-1. *J. Biochem.* **118**:23–27.
13. Oskam, L., D. J. Hillenga, G. Venema, and S. Bron. 1991. The large *Bacillus* plasmid pTB19 contains two integrated rolling-circle plasmids carrying mobilization functions. *Plasmid* **26**:30–39.
14. Saegesser, R., R. Ghosh, and R. Bachofen. 1992. Stability of broad host range cloning vector in the phototrophic bacterium *Rhodospirillum rubrum*. *FEMS Microbiol. Lett.* **95**:7–12.
15. Scott, J. R. 1984. Regulation of plasmid replication. *Microbiol. Rev.* **48**:1–23.
16. Shine, J., and L. Dalgarno. 1974. The 3'-terminal sequence of *E. coli* 16S ribosomal RNA: complementarity to nonsense triplets and ribosomal binding sites. *Proc. Natl. Acad. Sci. USA* **71**:1342–1346.
17. Simon, R., M. O'Connell, M. Labes, and A. Pühler. 1986. Plasmid vectors for genetic analysis and manipulation of rhizobia and other gram-negative bacteria. *Methods Enzymol.* **118**:640–659.
18. Simon, R., U. Priefer, and A. Pühler. 1983. A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in gram-negative bacteria. *Bio/Technology* **1**:784–791.
19. Tanaka, T., and T. Matsunaga. 2000. Fully automated chemiluminescence immunoassay of insulin using antibody-protein A-bacterial magnetic particle complexes. *Anal. Chem.* **72**:3518–3522.
20. Tanaka, T., and T. Matsunaga. 2001. Detection of HbA1c by boronate affinity immunoassay using bacterial magnetic particles. *Biosens. Bioelectron.* **16**:1089–1094.
21. Terawaki, Y., Y. Itoh, H. Zeng, T. Hayashi, and A. Tabuchi. 1992. Function of the N-terminal half of RepA in activation of Rts1 *ori*. *J. Bacteriol.* **174**:6904–6910.
22. Wahyudi, A. T., H. Takeyama, and T. Matsunaga. 2001. Isolation of *Magnetospirillum magneticum* AMB-1 mutants defective in bacterial magnetic particle synthesis by transposon mutagenesis. *Appl. Biochem. Biotechnol.* **91**:147–154.
23. Yang, C.-D., H. Takeyama, and T. Matsunaga. 2001. Iron feeding optimization and plasmid stability in production of recombinant bacterial magnetic particles by *Magnetospirillum magneticum* AMB-1 in fed-batch culture. *J. Biosci. Bioeng.* **91**:213–216.