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Effective expression of human proteins on bacterial magnetic particles in an anchor gene deletion mutant of *Magnetospirillum magneticum* AMB-1

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ABSTRACT

Biologically synthesized magnetic particles by magnetotactic bacteria (BacMPs) have promising potential in the area of functional protein display technology for various biotechnological and biomedical applications. Functional proteins fused with an anchor protein, Mms13, have been demonstrated to be an effective and stable method for the display of functional proteins on BacMPs. However, the expression of some human proteins is relatively low. Useful host strains of *Escherichia coli* have been developed for the enhanced expression of recombinant proteins using a genetic engineering approach. To improve human protein expression level on BacMPs in *Magnetospirillum magneticum* AMB-1, a mutant strain with a deleted native *mms13* gene (*Δmms13* strain) was established and evaluated for effective functional protein display technology. As a result, the *Δmms13* strain synthesized BacMPs with significantly improved expression of two human proteins, thyroid-stimulating hormone receptor (TSHR) and the class II major histocompatibility complex (MHC II) molecules. The *Δmms13* strain could therefore be an effective strain for the display of other important human proteins on BacMPs and may be useful for further applications.

1. Introduction

Magnetic particles are increasingly used in various applications across biomedical and environmental fields, and progressively have been incorporated as support materials in areas such as enzyme immobilization [1,2], drug delivery [3], and cell separation [4,5]. The greatest advantage of magnetic particles is the ability to manipulate them with an external magnetic field. This characteristic makes it possible to easily recover functionalized magnetic particle-bound target molecules from complex heterogeneous reaction solutions. For the effective binding of a range of targets in various reaction conditions, methodologies for magnetic particle synthesis and surface functionalization have been developed [4,6–8].

Magnetotactic bacteria synthesize nano-sized (50–100 nm), uniform, and strong ferrimagnetic bacterial magnetic particles (BacMPs). The BacMPs are covered with a lipid membrane, derived from cytoplasmic membrane through the invagination process [9,10]. Because the lipids on BacMPs are mainly comprised of phospholipids, the purified BacMPs show high dispersibility based on the negative-charged electrostatic interaction. Previous molecular studies have documented that BacMP synthesis is regulated by a unique set of membrane proteins on the BacMPs [10–12]. Genome sequencing and identification of BacMP membrane proteins opened the door for the novel protein display technology on BacMPs to functionalize its surface using genetic engineering in *Magnetospirillum magneticum* AMB-1 (*M. magneticum* AMB-1).

A fusion protein expression system involving an anchor protein identified from the BacMP membrane and a functional protein have allowed various functionalized BacMPs to be produced thus far [13,14]. The expression plasmid, pUMG, is a stable and high copy number plasmid which has been used for the expression of fusion genes containing an anchor protein gene and target protein gene [15]. Transformation using the plasmid vector with selective integration of the promoter region and anchor protein gene allows for efficient functional protein expression onto BacMPs. Among the anchor proteins, Mms13, a major BacMP membrane protein tightly bound to core magnetite particles, has been successfully demonstrated to stably display target proteins on BacMPs [16]. In addition, highly active promoters in *M. magneticum* AMB-1 were identified and an inducible protein expression system was recently developed to avoid the toxic effects of transmembrane protein expression in bacterial cells [17]. Various expression systems for protein display on BacMPs have been developed based on the techniques of other host cells, such as *Escherichia coli*(*E. coli*). However, the amount of displayed proteins on BacMPs depends on the

Abbreviations: *M. magneticum* AMB-1, *Magnetospirillum magneticum* AMB-1; BacMP, bacterial magnetic particle; *Δmms13*, *mms13* deletion mutant; TSHR, thyroid-stimulating hormone receptor; MHC, major histocompatibility complex; MSGM, magnetic spirillum growth medium; GPCR, G protein-coupled receptor.

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properties of the human protein. In particular, the amount of expressed human protein or membrane protein on BacMPs is quite low. Therefore, the establishment of techniques for protein displays on BacMPs is still in demand.

Techniques for heterologous protein expression using *E. coli*, yeast, insect, and mammalian cells have been developed to enhance expression levels. The varieties of available expression plasmids, inducible expression systems, recombinant fusion partners, and mutant strains have advanced the possibilities in *E. coli*. Various mutant *E. coli* host strains have been developed by genetic engineering and widely used for many applications. For example, expression strains should be deficient in the most harmful natural proteases because one of the major problems associated with the expression of heterologous proteins in *E. coli* is the degradation of cloned gene products by host-specific proteases. In addition, *recA* negative strains and *trxB/gor* negative mutants have been used for the stabilization of target plasmids and the enhancement of cytoplasmic disulfide bond formation [18]. Thus, the genomic engineering of host strains is a useful strategy for expression of recombinant proteins.

In this study, we focused on improving the amount of displayed human protein using an anchor protein gene deletion mutant (*Δmms13*) strain, which eliminates the expression of the *mms13* gene in the genome. During expression of human proteins on BacMPs in *M. magneticum* AMB-1, the anchor protein, Mms13, appears to be expressed both from the plasmid vector as a fusion protein and from the genome as a native protein. The competitive expression of these proteins might restrict the number of functional fusion proteins on BacMPs.

The objectives here were to: (1) establish the *mms13* gene deletion mutant strain, (2) evaluate BacMP production within the *Δmms13* strain, and (3) express and evaluate production of thyroid-stimulating hormone receptor (TSHR) and class II major histocompatibility complex (MHC II) molecules on BacMPs in the *Δmms13* strain. The results reported here suggest that the novel *Δmms13* strain may be an efficient strain for the display of various human proteins on BacMPs, and a useful tool for a number of applications. This is the first report of a mutant *M. magneticum* host strain for protein display on BacMPs and it demonstrates efficient display of human proteins at the surface of BacMPs.

2. Materials and methods

2.1. Bacterial strains and culture conditions

E. coli strain S17-1 was used as the host cell for conjugation [19] and *E. coli* DH5α was used as the host for gene cloning. Cells were cultured in LB medium containing 25 μg/ml kanamycin or 50 μg/ml ampicillin at 37 °C. *M. magneticum* AMB-1 (ATCC 700264) [20] was microaerobically cultured in magnetic spirillum growth medium (MSGM) at 28 °C as previously described [20,21]. Microaerobic conditions were established by purging the cultures with argon gas. *M. magneticum* AMB-1 transformants harboring each expression vector were cultured under the same conditions in medium containing 5 μg/ml ampicillin. All other reagents were laboratory-grade, commercially available analytical reagents. Deionized distilled water was used in all procedures.

2.2. Preparation of BacMPs from M. magneticum AMB-1

Cultured *M. magneticum* AMB-1 cells were collected by centrifugation at 11,344 *g* for 10 min at 4 °C, resuspended in 10 mM phosphate buffered saline (PBS, 40 ml, pH 7.4) and disrupted by three passes through a French press cell at 1500 kg/cm^2 (Ohtake Works Co. Ltd., Tokyo, Japan). BacMPs were collected from the disrupted cells using a columnar neodymium-boron (Nd-B) magnet and washed 10 times with 10 mM HEPES buffer (pH 7.4). The washed BacMPs were suspended in PBS and stored at 4 °C. The concentration of BacMPs in suspension was determined by measuring the optical density (660 nm) using a spectrophotometer (UV-2200; Shimadzu, Kyoto, Japan). A value of 1.0 corresponded to 172 μg (dry weight) BacMPs/ml.

2.3. Construction of the Δmms13 strain by homologous recombination

The plasmids and primers used in this study are described in Table S1. The *mms13* sequence (0.4 kb) was obtained from the NCBI (Gene ID: 3805263). The DNA fragment was amplified from *mms13* upstream (0.8 kb) and downstream (0.8 kb) sequences by PCR with primer sets M13F5-800 and M13R3-800, which contained the restriction sites for *Bam*HI. The resulting fragment was cloned into the *Ssp*I site of pUC19, and the resulting plasmid was defined as pUC19M13. After confirming the DNA sequence, the outside region of the *mms13* fragment within pUC19M13 was amplified with primers M13F3-ad and M13R5-ad. These primers had an additional 24 bp sequences for In-Fusion PCR cloning (Takara). The gentamicin-resistance gene was amplified primer set Gm5-ad and Gm3-ad. These primers also had an additional 24 bp sequences, corresponding to the additional sequences of primer set M13F3-ad and M13R5-ad (Table S1). The purified PCR product of the gentamicin-resistance gene was fused to the amplified fragment of the outside region of the *mms13* using the In-Fusion system, which generated pUC19M13updownGm^r. To construct the plasmid for homologous recombination, the sub-cloned DNA fragment M13updownGm^r in pUC19M13updownGm^r was integrated into the *Bam*HI site within pK19*mobsacB* [22], which contains *sacB* as a counter-selectable suicide marker [23], to create pK19*mobsacB*M13updownGm^r (pK19*mobsacB*M13Gm^r).

pK19*mobsacB*M13Gm^r was introduced into *M. magneticum* AMB-1 cells using *E. coli* S17-1 as the donor strain for the conjugation. The cells were incubated in MSGM solid medium containing 2.5 μg/ml gentamicin at 28 °C as previously described [24]. Individual colonies were picked and grown in MSGM containing 2.5 μg/ml gentamicin. The cells were then incubated on plate medium in the presence of 2.5 μg/ml gentamicin and filtered 1% sucrose under the same conditions. Resulting colonies were analyzed by PCR and sequenced to confirm that *mms13* was replaced with the gentamicin-resistance gene (Fig. 1).

2.4. Protein profiling of BacMP membrane from Δmms13

Quantitation of protein on the BacMPs from the wild-type and *Δmms13* strain was performed by the Lowry method. BacMP membrane protein (1 mg) was extracted by treatment with 0.02% SDS and boiling. BSA was used as a standard for protein quantification. Extracted and purified BacMPs (3 mg) from the wild-type and *Δmms13* strain were treated by boiling in 1% SDS for 30 min to obtain the membrane proteins. BacMPs were removed by centrifugation and magnetic separation, and the supernatant (BacMP membrane protein fraction) was mixed with SDS sample buffer containing 6.25 mM Tris–HCl (pH 6.8), 5% 2-mercaptoethanol, 2% SDS, 5% sucrose, and 0.002% bromophenol blue. The membrane proteins were denatured and subjected to SDS–polyacrylamide gel electrophoresis (SDS–PAGE) in a 12.5% (wt/vol) polyacrylamide gel. The gel was stained with Coomassie brilliant blue.

2.5. Expression of Mms13 fusion proteins in the Δmms13 strain

Each protein expression vector was derived from pUMG (Apr; 6.4 kb) [15] and pUMGP16M13 [16], which includes the Mms16 promoter and the coding sequence for Mms13 in plasmid pUMG.

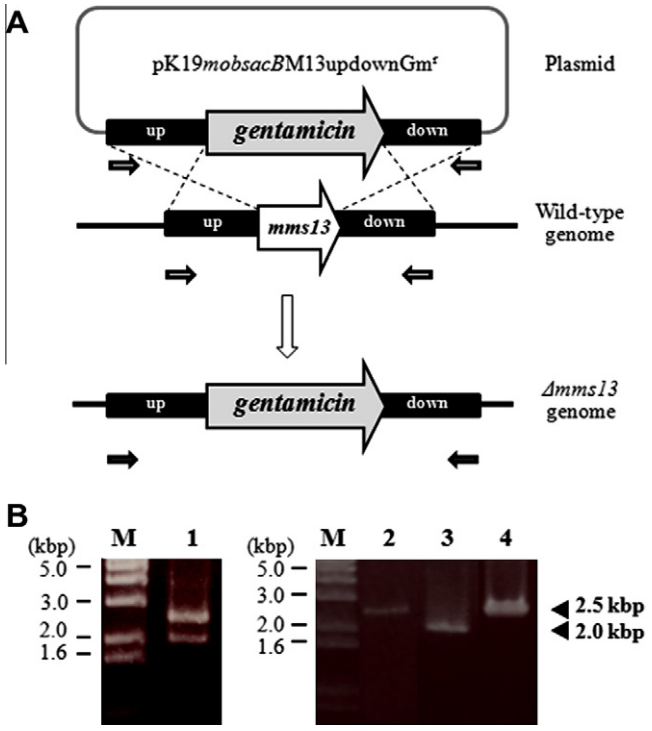

Fig. 1. Construction and confirmation of the *Δmms13* strain of *M. magneticum* AMB-1. Schematic overview of homologous recombination to create the *mms13* deletion from *M. magneticum* AMB-1 genome (A). Arrows indicate primer sets. Primers: m13_upstream, m13_downstream. Confirmation of the integrated plasmid and replacement of *mms13* by the gentamicin-resistance gene were analyzed by direct colony PCR (B). The gel image shows the results from cells in single cross over status (lane 1) and double cross over status (lane 2). To confirm the fragment sizes of single cross over and double cross over, the wild-type (lane 3) and pK19*mobsacB*M13Gm^r (lane 4) strains were analyzed as controls.

For the pUMGP16M13NFlag-TSHR construction, the gene encoding the FLAG tag and extracellular domain of TSHR (amino acids 1-165) was generated by PCR amplification using primers Flag-TSHR-F and TSHR-R (Table S1). The PCR products were cloned into *Ssp*I-digested pUM16M13 (pUMGP16M13NFlag-TSHR). Vector pUM13L(100) HLA-DR4 included the Mms13-NS polypeptide linker and the extracellular domain of HLA-DR4 α chain (amino acids 26-206)-$(G_4S)_5$-HLA-DR4 β chain (amino acids 30-221) fusion proteins. The synthesized gene HLA-DR4 α chain-$(G_4S)_5$-HLA-DR4 β chain was cloned into pUM13L(100) encoding the synthetic polypeptide ($[N_4S]_{18}$LVPRGSN$_4$S) [25], creating pUM13L(100) HLA-DR4. For detection of the Mms13-fusion proteins from the BacMP membrane fraction, the extracted proteins were analyzed by western blotting. The extracted proteins from BacMPs were detected using the FLAG tag with alkaline phosphatase (ALP)-labeled anti-FLAG tag antibody (1/1000 dilution of 1% PBST), purchased from Sigma–Aldrich Inc. BCIP-NBT (Sigma, St. Louis, MO) was used as the ALP substrate for visualization. The expression levels were measured by image processing with ImageJ, and compared between the wild-type and *Δmms13* strain.

3. Results and discussion

3.1. Construction of the *Δmms13* strain

Various target proteins have been displayed on BacMPs in *M. magneticum* AMB-1 using the anchor protein, Mms13. In this work, an *mms13* gene deletion mutant strain of *M. magneticum* AMB-1 was established for the efficient display of human proteins. Fig. 1 shows a schematic view of the homologous recombination that consisted of two important genetic modifications within the genome. First, the transformed plasmid (pK19*mobsacB*M13Gm^r) was entirely integrated into the *mms13* gene locus within the genome (single cross over). Because the integrated plasmid encodes the gentamicin-resistance gene, the transformants demonstrate resistance to gentamicin. To confirm the integration of this plasmid into the *M. magneticum* AMB-1 genome, colony direct PCR was conducted for amplification of *mms13* flanking regions using upstream and downstream primer sets (Fig. 1A). Because the plasmid was integrated in the genome, two sets of *mms13* upstream and downstream regions should exist in the genomes of the transformants, and the two amplified DNA fragments (2.0 kb, 2.5 kb) were successfully found. Then, single cross over status of *M. magneticum* AMB-1 was confirmed as shown in Fig. 1B, lane 1. Second, during the multiple cultivation of the plasmid integrated-strain in the absence of antibiotic, the integrated plasmid was removed from the genome due to homologous recombination (double cross over). As a result, the *mms13* gene was replaced by the gentamicin-resistance gene (Fig. 1A). To confirm the deletion of *mms13*, cells were examined whether the colonies harboring the gentamycin-resistance gene were in single cross over or double cross over status using the same primers. The amplified single band (2.5 kb) indicates double cross over status (Fig. 1B, lane 2), demonstrating that the *mms13* gene was successfully replaced by the gentamicin-resistance gene (the *Δmms13* strain). These amplified fragments were compared to the PCR product using control templates, wild type cell (lane 3) and plasmid (pK19*mobsacB*M13Gm^r) (lane 4). The homologous recombination event was also confirmed with PCR and sequencing.

3.2. Growth of the *Δmms13* strain and BacMPs production

Supplementary Fig. S1 shows the growth curve of the wild-type and *Δmms13* strain. There was no growth difference between the wild-type and *Δmms13* strain; the final cell concentration of the wild-type and *Δmms13* strain was 1.2×10^8 cells/ml and 1.0×10^8 cells/ml, respectively. In addition, there was little difference between the wild-type and *Δmms13* particle yields. The structure of BacMPs in the wild-type and *Δmms13* strain were compared using electron micrographs. The micrographs in supplementary Fig. S2 show that the formation of BacMPs was unaffected by the deletion of the *mms13* gene. In addition, the amount of total membrane protein from 1 mg BacMPs in the wild-type and *Δmms13* strain was 36.4 μg and 38.0 μg, respectively. The protein profiles of BacMPs were also investigated. While the profiles of BacMP membrane protein fractions were quite similar between the wild-type and *Δmms13* strain, the absence of a protein band at the theoretical size (13 kDa) of Mms13 confirmed the deletion of *mms13* gene from *M. magneticum* AMB-1 (Fig. 2).

3.3. Expression levels of the human protein fusions with Mms13 on BacMPs in the *Δmms13* strain

The expression levels of human proteins (TSHR and MHC II) on BacMPs in the *Δmms13* strain were evaluated. TSHR is a member of the G protein-coupled receptor (GPCR) family and expressed on the surface of thyroid follicular cells. Graves' disease is a common autoimmune disease caused by autoantibodies to the TSHR (TRAbs) [26,27]. The N-terminal 165 amino acids have an important role in binding to the TRAb, and this region was displayed on BacMPs. To compare the protein expression levels of the human protein, wild-type and *Δmms13* transformants harboring pUMGm13 TSHR were constructed. By western blotting, protein expression on BacMP membranes was confirmed in both strains and quantified (Fig. 3A). The expression level of TSHR on BacMPs was

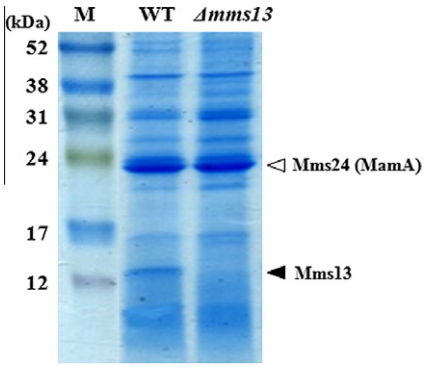

Fig. 2. Protein profiling of BacMPs from the wild-type (WT) and *Δmms13* strain (*Δmms13*). The theoretical position (13 kDa) of Mms13 is indicated by the black arrowhead. Mms24 (24 kDa) is indicated by an opened arrowhead. Lane M, molecular weight markers. Protein samples were obtained from the BacMPs membrane by treatment with boiling 1% SDS solution. The proteins were run on a 12% SDS polyacrylamide gel and stained with Coomassie Brilliant Blue.

significantly increased (fourfold) in the *Δmms13* strain compared with the wild-type (Fig. 3B). It is noteworthy that the efficient expression of TSHR in the *Δmms13* strain resulted in various degradation products as well. The mechanism of degradation is still unclear, but the optimization of codon usage from human to *M. magneticum* AMB-1 and/or further genetic modification, such as the deletion of protease genes from the genome, might allow effective expression without degradation. To further confirm the system for human protein display on BacMPs, another protein, MHC II, was evaluated for enhanced expression. The MHC locus is the most gene-dense region of the mammalian genome and is critical to immunity and reproductive success; there are two classes of MHC molecules that derive peptides from two intercellular compartments. Antigen presenting cells express MHC class II molecules bound to peptide fragments and are responsible for activating CD4(+) T cells that then broadly influence many branches of the immune response [28]. The expression of MHC II molecules using *Δmms13*-BacMPs showed an eightfold increase over that of wild type (Fig. 3B). The degradation of the recombinant protein was relatively low for MHC II. The expression levels of TSHR and MHC II on BacMPs were successfully improved in the *Δmms13* strain compared with the wild-type strain. In the wild-type transformants, genome-derived Mms13 and plasmid-derived Mms13 probably compete with each other to express and/or localize on BacMPs. By the deletion of the native *mms13* gene, much more plasmid-derived Mms13 fusion proteins might be successfully expressed and localized onto BacMPs. Furthermore, there was no substantial effect on BacMP synthesis after deletion of the *mms13* gene. In previous studies, Mms13 fused to various proteins has been overexpressed on BacMPs. In both cases, *mms13* gene deletion and Mms13 protein overexpression, no remarkable difference was observed for BacMP synthesis in *M. magneticum* AMB-1. Furthermore, in the closely related strain *M. gryphiswaldense*, MRS-1, a Mms13 (MamC) deletion mutant, also showed minor effects on magnetite crystal formation [29]. From these observations, it has been suggested that Mms13 does not have a crucial role in magnetite crystal formation, or other proteins expressed on BacMPs would compensate for the function for BacMP formation.

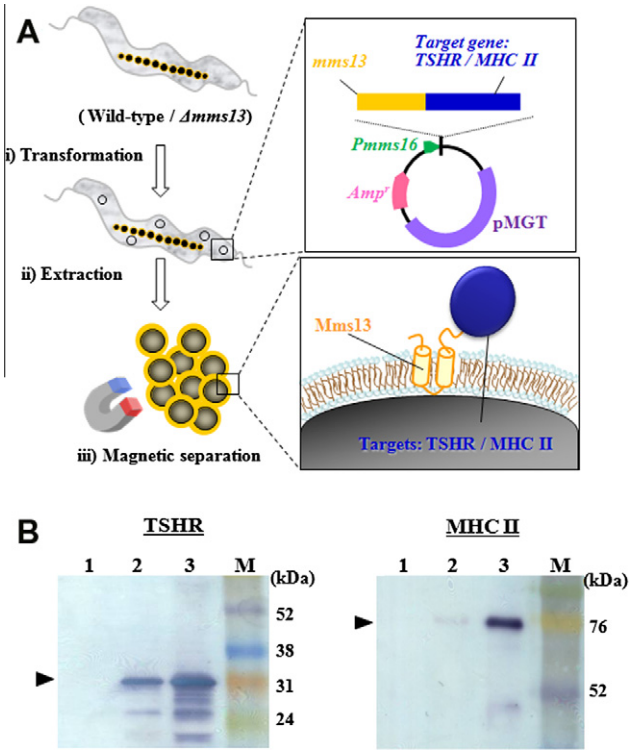

Fig. 3. Confirmation of effective expression of human proteins on BacMPs in the *Δmms13* strain by western blotting analysis. Target proteins were expressed on BacMPs in the wild-type and *Δmms13* strain. (A) Schematic diagram for preparation of BacMPs displaying target proteins. Plasmids pUM13 TSHR or pUM13MHC II were introduced into the wild-type and *Δmms13* strain for expression on BacMPs (step i). TSHR BacMPs or MHC II-BacMPs were magnetically separated and purified by stringent washing (step ii). (B) Western blotting using ALP-conjugated anti-FLAG tag antibody. Membrane protein fractions were extracted from BacMPs by boiling in 1% SDS. M: marker, lane 1: BacMPs from the wild-type of *M. magneticum* AMB-1, lane 2: TSHR-BacMPs expressed in the wild-type (left), MHC II-BacMPs expressed in the wild-type (right), Lane 3: TSHR-BacMPs expressed in the *Δmms13* strain (left), MHC II-BacMPs expressed in the *Δmms13* strain (right).

In this study, a *Δmms13* strain was established and demonstrated improved expression of two human proteins, TSHR and MHC II (fourfold and eightfold, respectively). We suggest that the *Δmms13* strain will be a useful host for the display of a number of human proteins on BacMPs. This advantageous strain can effectively display various Mms13 fusions on BacMPs and could be used for various biological and medical applications. By combining the use of the tetracycline-inducible protein expression system with the BacMPs, a more valuable BacMP system with highly functional displayed proteins might be easily produced in the future. Additionally, BacMP-expressed TSHR and MHC II could be powerful tools for the detection of TRAb autoantibodies or tumor antigen peptides from patient's serum more rapidly.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.07.116.

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