Inducible Expression of Transmembrane Proteins on Bacterial Magnetic Particles in Magnetospirillum magneticum AMB-1

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Bacterial magnetic particles (BacMPs) produced by the magnetotactic bacterium Magnetospirillum magneticum AMB-1 are used for a variety of biomedical applications. In particular, the lipid bilayer surrounding BacMPs has been reported to be amenable to the insertion of recombinant transmembrane proteins; however, the display of transmembrane proteins in BacMP membranes remains a technical challenge due to the cytotoxic effects of the proteins when they are overexpressed in bacterial cells. In this study, a tetracycline-inducible expression system was developed to display transmembrane proteins on BacMPs. The expression and localization of the target proteins were confirmed using luciferase and green fluorescent protein as reporter proteins. Gene expression was suppressed in the absence of anhydrotetracycline, and the level of protein expression could be controlled by modulating the concentration of the inducer molecule. This system was implemented to obtain the expression of the tetraspanin CD81. The truncated form of CD81 including the ligand binding site was successfully displayed at the surface of BacMPs by using Mms13 as an anchor protein and was shown to bind the hepatitis C virus envelope protein E2. These results suggest that the tetracycline-inducible expression system described here will be a useful tool for the expression and display of transmembrane proteins in the membranes of BacMPs.

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Transmembrane proteins play critical roles in cellular metabolism, participating in processes such as ion transport, nutrient uptake, signal transduction, and intercellular communication. As evidence of the essential functions of these proteins, more than half of all drug targets have been shown to be transmembrane proteins, and the analysis of the interactions of transmembrane proteins and their ligands is one of the most promising avenues for the discovery of new drug candidates. As a means of producing sufficient amounts of transmembrane proteins for binding analyses, heterologous protein expression systems have been developed using Escherichia coli (10), yeast (16), insect, and mammalian (4) cells as hosts. Transmembrane proteins generally are expressed at low levels and are extremely hydrophobic, rendering the analysis of interactions with ligands very difficult. In all cases, the analysis of membrane proteins requires a lipid or similar synthetic environment to maintain the native structure and function of the proteins. The purification of transmembrane proteins from cells frequently is time-consuming and typically results in the loss of the proteins’ native conformation.

Magnetospirillum magneticum AMB-1 synthesizes intracellular nanosized bacterial magnetic particles (BacMPs; 50 to 100 nm); these are surrounded by a lipid bilayer membrane and exhibit strong ferrimagnetism. Functional soluble proteins have been expressed on BacMP surfaces through gene fusion techniques (11, 21, 24, 27) using BacMP membrane proteins (MagA, Mms16, and Mms13) as anchor proteins; this approach permits heterologous proteins to be localized efficiently and oriented appropriately on BacMPs. In a previous report, we demonstrated the successful display of the D1 dopamine receptor, a G protein-coupled receptor possessing seven transmembrane domains, on BacMPs. Mms16-D1, an dopamine receptor fusion protein, was expressed under the mms16 promoter, and a ligand-binding assay was performed (28). The assembly of transmembrane proteins on magnetic particles provides significant advantages for binding assays, including the easing of the purification of target proteins from bacterial cells without the loss of native conformation and the availability of a fully automated bioassay using robotic magnetic separation. Despite these advantages, there are not enough studies for the overexpression of transmembrane proteins other than the D1 dopamine receptor in M. magneticum AMB-1 because of its difficulty. In other host cells, a system for controlling gene expression has been employed to overcome its difficulty, and some successful efforts had achieved this for crystal structure analysis (5, 15, 18). The lack of these systems for M. magneticum has hampered the extension of this application to other transmembrane proteins.

In this study, the tetracycline-inducible expression system was adapted for displaying transmembrane proteins on BacMPs in M. magneticum AMB-1. Expression vectors carrying the tetracycline repressor gene (tetR) and the target gene under the control of a strong promoter and the tetracycline operator (tetO) sequence were constructed, and the function of the system was evaluated using reporter genes. Finally, this system was applied to the overexpression of the transmembrane protein, tetraspanin CD81. This is the first report of an inducible expression system in M. magneticum, and it demonstrates efficient display of a transmembrane protein at the surface of BacMPs.
TABLE 1. Plasmids used in this work

<table>
<thead>
<tr>
<th>Plasmid Description</th>
<th>Source or reference</th>
</tr>
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<tr>
<td>pUMG</td>
<td>BamH-digested whole fragment of pMGT cloned in pUC19; Amp′</td>
</tr>
<tr>
<td>pUGM13LC-Pmsp3</td>
<td>pUMG derivative, Amp′, Pmmsp1-mms13-luc</td>
</tr>
<tr>
<td>pUML13LC</td>
<td>pUMG derivative, Amp′, Pmmsp1-mms13-luc</td>
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<tr>
<td>pUMtOR</td>
<td>pUMG derivative, Amp′, Pmmsp1-mms13-luc, TetR</td>
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<td>pUMtORLC</td>
<td>pUMG derivative, Amp′, Pmmsp1-mms13-GFP, Pmmsp1-mms13-CCR5, Pmmsp1-mms13-CCR5</td>
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<tr>
<td>pUMtOR13GFp</td>
<td>pUMG derivative, Amp′, Pmmsp1-mms13-CCR5, Pmmsp1-mms13-CCR5</td>
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<tr>
<td>pUMtOR13CD81′</td>
<td>pUMG derivative, Amp′, Pmmsp1-mms13-CD81′-His6, Pmmsp1-mms13-CD81′-His6</td>
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<td>pUMtOR13CXCR4</td>
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<td>pUMtOR13CXCR4</td>
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<tr>
<td>pUMtOR13CD81′</td>
<td>pUMG derivative, Amp′, Pmmsp1-mms13-FALG-CD81′-His6, Pmmsp1-mms13-FALG-CD81′-His6</td>
</tr>
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<td>pUMtOR13CXCR5</td>
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<td>pUMG derivative, Amp′, Pmmsp1-mms13-CXCR4, Pmmsp1-mms13-CXCR4, Pmmsp1-mms13-CXCR4</td>
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a luc, luciferase gene.
absence of ATc until stationary phase and then were observed by fluorescence microscopy (Olympus Co., Tokyo, Japan). BacMPs (50 μg) extracted from AMB-1 grown under each set of culture conditions were suspended in 500 μl HEPES buffer. The fluorescence intensity of the suspension was measured (excitation wavelength, 489 nm; emission wavelength, 508 nm) using a spectrophotometer (Horiba Itech, Tokyo, Japan).

Antibody-binding assay on BacMPs. BacMPs (100 μg) were magnetically collected and added to a solution of ALP-labeled anti-FLAG tag antibody (100 ng/ml) dissolved in 100 μl PBS containing 0.05% Tween 20 (PBST). The mixture was incubated for 60 min at room temperature with pulsed sonication every 5 min. The BacMPs then were magnetically separated, washed three times with 100 μl PBST using sonication, and resuspended in 100 μl PBS, followed by the addition of 100 μl Lumi-Phos 530 as the luciferase substrate. After 5 min of incubation, the luminescence intensity was measured with a luminescence meter.

Western blot analysis. For the detection of TetR expression in M. magneticum AMB-1, the cytoplasm was fractionated by a method similar to that described in a previous report (13). Cytoplasmic proteins were quantified using the BCA protein assay kit, and 40 μg protein was mixed with SDS sample buffer (final concentration, 62.5 mM Tris-HCl, pH 6.8, 5% 2-mercaptoethanol, 2% SDS, 5% sucrose, and 0.002% bromophenol blue), denatured, separated by SDS-PAGE through a 12.5% (wt/vol) gel, and transferred to a polyvinylidene difluoride membrane. TetR was detected using mouse anti-TetR antibody (10 μg/ml in PBST) and ALP-labeled goat anti-mouse IgG antibody (1/10,000 in PBST) using sonication, and resuspended in 100 μl PBST containing 0.05% Tween 20 (PBST). The mixture was incubated for 60 min at room temperature with pulsed sonication every 5 min. The BacMPs then were magnetically separated, washed three times with 100 μl PBST using sonication, and resuspended in 100 μl PBS, followed by the addition of 100 μl Lumi-Phos 530 as the luciferase substrate. After 5 min of incubation, the luminescence intensity was measured with a luminescence meter.

RESULTS AND DISCUSSION

Construction of tetracycline-inducible vectors for use in M. magneticum AMB-1. We have developed a tetracycline-inducible protein expression system in Magnetospirillum magneticum AMB-1 to prevent the toxic effects of transmembrane protein expression in bacterial cells. The tetracycline-inducible expression system is based on the tetracycline resistance operon of E. coli transposon Tn10 (8). In this system, the tetracycline analog anhydrotetracycline is used as an inducer of gene expression. These molecules can pass through the phospholipid bilayer by simple diffusion without transporter proteins (2), bind to the tetracycline repressor with high affinity ($K_d = 2.8 \times 10^{-9} M^{-1}$) (20), and induce gene expression in the absence of activator proteins (8). Due to these properties, the tetracycline-inducible expression system has been successfully adapted for various organisms (3).

pUMtOR was constructed as a basic vector in this study. Several strong promoters ($P_{msp1}$ and $P_{msp2}$) had been identified previously using the M. magneticum AMB-1 genome and proteome databases (25). The TetR proteins are continuously expressed in the control of $P_{msp3}$, which is one of the strongest promoters in AMB-1 (25). Target proteins expressed under the controlled of $P_{msp1}$ $tetO$, the $P_{msp1}$ derivative containing $tetO$ elements. TetR proteins bind to the $tetO$ elements integrated in $P_{msp1}$ $tetO$, resulting in the suppression of target gene transcription. The addition of ATc results in a conformational change in TetR, followed by the dissociation of TetR from $tetO$ and, finally, the expression of the target gene.

Evaluation of the tetracycline-inducible expression system in AMB-1. For the evaluation of ATc toxicity, wild-type M. magneticum AMB-1 cells were grown in MSGM under different concentrations of ATc. (B) Luminescence intensity of AMB-1 transformants harboring pUMtORLC cultured under different concentrations of ATc.

The functional evaluation of the expression system was carried out using the luciferase gene as a reporter. AMB-1 transformants harboring pUMtORLC were incubated until the stationary phase in the presence of concentrations of ATc ranging from 0 to 500 ng/ml, and the cell number was calculated with a direct cell count using a microscope. The growth curves were very similar for all concentrations of ATc (Fig. 1A), demonstrating that ATc did not have a significant impact on the growth of AMB-1 cells.

The functional evaluation of the expression system was carried out using the luciferase gene as a reporter. AMB-1 transformants harboring pUMtORLC were incubated until the stationary phase in the presence of concentrations of ATc ranging from 0 to 500 ng/ml, and the luminescence intensity of each transformant was measured. Little luminescence was detected in the absence of ATc, while the luminescence intensity increased as the concentration of ATc increased (Fig. 1B). A high concentration of TetR (22 kDa) in the AMB-1 transformant harboring the vectors was confirmed by Western blot analysis using anti-TetR antibody (data not shown). These results indicate that gene expression was almost completely suppressed by TetR in the absence of the inducer molecule ATc, and the target gene expression level could be controlled by the concentration of ATc.

Inducible expression of GFP on BacMPs. The evaluation of the display of target proteins on BacMPs was performed by the expression of an Mms13-GFP fusion protein. Mms13 was used

FIG. 1. Evaluation of the inducible expression system in the presence or absence of the inducer anhydrotetracycline hydrochloride (ATc). (A) Growth curve in the presence of ATc. Wild-type AMB-1 cells were grown in MSGM under different concentrations of ATc. (B) Luminescence intensity of AMB-1 transformants harboring pUMtORLC cultured under different concentrations of ATc.
as the anchor protein because it bound tightly to the surface of the magnetite and was, therefore, able to maintain a more stable protein display than Mms16, which had been used for dopamine receptor display in a previous study (28). AMB-1 transformants harboring pUMtOR13GFP and cultured in the absence (ATc−) or presence (ATc+) of 500 ng/ml ATc. The images at the top are GFP fluorescence images. The images at the bottom are differential interference contrast (DIC) microscopic images. (B) Fluorescence intensity of GFP expressed on BacMPs extracted from wild-type AMB-1 (WT-BacMPs) and AMB-1 transformants harboring pUMtOR13GFP (GFP-BacMPs). (C) Fluorescence images of GFP-BacMPs extracted from transformants. ATc was added at different stages after inoculation. The images at the top are GFP fluorescence images. The images at the bottom are differential interference contrast (DIC) microscopic images. (Scale bar, 10 μm).

Transformation of AMB-1 using tetracycline-inducible expression vectors harboring transmembrane protein genes. There are considerable challenges to the stable expression of transmembrane proteins because of their cytotoxic effects when overexpressed in cells (7, 23). Various strategies have been tested to address this issue and obtained optimum expression (9, 19, 22). Inducible expression systems are one of the most promising approaches for the stable expression of transmembrane proteins. To evaluate the general applicability of the tetracycline-inducible system to the expression of transmembrane proteins at the surface of BacMPs, we compared transformation efficiencies using two types of vectors, a tetracycline-inducible vector containing transmembrane protein genes and a vector lacking tetR, which cannot repress the gene expression of transmembrane protein. Each vector contains a gene encoding an Mms13 target fusion protein. The chemokine receptors CXCR4 and CCR5 and a truncated form of tetranspanin CD81 (CD81′) were used as model transmembrane proteins for the evaluation of transformation efficiency; these proteins are prime targets for drug discovery but have not yet been expressed in AMB-1 because of their toxicity. The tetracycline-inducible or tetR-lacking vectors containing the fusion protein genes were introduced into wild-type AMB-1 (400 ng expression vectors used to transform 1 × 10⁶ cells), and the bacteria were incubated on MSGMagar plates containing ampicillin. No transformant was produced in cells transformed with the vector lacking the tetR gene due to the absence of the repression of the target gene, while the tetracycline-inducible vector was able to transform wild-type AMB-1 (Table 3). This study clearly demonstrates the applicability of the inducible system for the effective transformation of AMB-1 cells with vectors encoding transmembrane protein.

E2-binding activity of CD81 displayed on BacMPs. Finally, the display of the truncated form of CD81, which is involved in HCV infection, on the surface of BacMPs was attempted with the inducible expression system. HCV is an enveloped, positive-strand RNA virus of the Flaviviridae family, and chronic HCV infection has been reported to result in liver disease. The envelope protein E2 binds to CD81 expressed on the surface of various cell types, including hepatocytes and B lymphocytes. CD81 belongs to the tetranspanin family and has four transmembrane domains, and the second extracellular loop is larger than the first. E2 binds this larger extracellular loop (LEL), and therefore an inhibitor of the human CD81-HCV E2 interaction would be expected to prevent HCV infection (17).

### TABLE 3. Quantitative analyses for colony formation of AMB-1 transformants harboring transmembrane protein expression vectors

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>CFU/100 μl</th>
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<tbody>
<tr>
<td>pUMtOR13CD81′</td>
<td>108 ± 20</td>
</tr>
<tr>
<td>pUMt013CD81′</td>
<td>0</td>
</tr>
<tr>
<td>pUMtOR13CXCR4</td>
<td>48 ± 12</td>
</tr>
<tr>
<td>pUMt013CXCR4</td>
<td>0</td>
</tr>
<tr>
<td>pUMtOR13CCR5</td>
<td>62 ± 2</td>
</tr>
<tr>
<td>pUMt013CCR5</td>
<td>0</td>
</tr>
</tbody>
</table>

*Each expression vector was transformed into wild-type M. magneticum AMB-1 by electroporation. Transformant cells (1 × 10⁶) were diluted in 5 ml of MSGM containing 0.7% agar and plated on 1% agar in MSGM containing 0.5 μg of ampicillin per ml and incubated under microaerobic conditions.
interaction was the motivation behind our efforts to produce CD81-displaying BacMPs as a research tool.

Expression of Mms13-FLAG-CD81′ in the AMB-1 transformants harboring pUMtOR13CD81′ was induced by the addition of ATc. Growth curves of the transformants expressing the induced proteins at each growth stage are shown in Fig. 3. When ATc was added at the time of inoculation, the growth rate of the transformants was very low because of the toxic effect of the Mms13-FLAG-CD81′ fusion protein. In an effort to minimize the toxic effects of the fusion protein, we added ATc at mid-log phase and incubated the cultures overnight. BacMPs were isolated from the transformants, and the transmembrane proteins expressed on the surface of CD81′-BacMPs were extracted and analyzed by Western blotting. Cells were cultured in the absence or presence of 500 ng/ml ATc. ATc was added to the medium at the time of inoculation (filled circle) or at mid-log phase (open triangles). The solid arrow indicates the stage for the addition of ATc.

BacMPs can be applied effectively in the search to identify inhibitors of the CD81-E2 interaction.

Conclusions. We have developed an efficient inducible expression system in M. magnetism that can be used to effectively express and display diverse transmembrane proteins, including toxic proteins; this has not been possible with conventional systems, which lack a component regulating gene expression. This novel system provides a useful tool for the investigation of a variety of membrane proteins, which often are refractory to analysis.

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REFERENCES


