Inducible Expression of Transmembrane Proteins on Bacterial Magnetic Particles in *Magnetospirillum magneticum* AMB-1[▽]

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Received 24 July 2009/Accepted 12 December 2009

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.

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 ap-

proach 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.

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[▽] Published ahead of print on 28 December 2009.

TABLE 1. Plasmids used in this work

Plasmid	Description ^a	Source or reference
pUMG	BamHI-digested whole fragment of pMGT cloned in pUC19; Amp ^r	14
pUMGM13LC-Pmsp3	pUMG derivative, Amp ^r , <i>P_{msp3}-mms13-luc</i>	25
pUMP16M13LC	pUMG derivative, Amp ^r , <i>P_{mms16}-mms13-luc</i>	25
pUMtOR	pUMG derivative, Amp ^r , <i>P_{msp1}^{tetO}, P_{msp3}-TetR</i>	This study
pUMtORLC	pUMtOR derivative, Amp ^r , <i>P_{msp1}^{tetO}-luc, P_{msp3}-TetR</i>	This study
pUMtOR13GFP	pUMtOR derivative, Amp ^r , <i>P_{msp1}^{tetO}-mms13-GFP, P_{msp3}-TetR</i>	This study
pUMtOR13CD81'	pUMtOR derivative, Amp ^r , <i>P_{msp1}^{tetO}-mms13-FALG-CD81'-His₆, P_{msp3}-TetR</i>	This study
pUMtOR13CCR5	pUMtOR derivative, Amp ^r , <i>P_{msp1}^{tetO}-mms13-CCR5, P_{msp3}-TetR</i>	This study
pUMtOR13CXCR4	pUMtOR derivative, Amp ^r , <i>P_{msp1}^{tetO}-mms13-CXCR4, P_{msp3}-TetR</i>	This study
pUMtO13CD81'	pUMG derivative, Amp ^r , <i>P_{msp1}^{tetO}-mms13-FALG-CD81'-His₆</i>	This study
pUMtO13CCR5	pUMG derivative, Amp ^r , <i>P_{msp1}^{tetO}-mms13-CCR5</i>	This study
pUMtO13CXCR4	pUMG derivative, Amp ^r , <i>P_{msp1}^{tetO}-mms13-CXCR4</i>	This study

^a *luc*, luciferase gene.

MATERIALS AND METHODS

Materials. Anhydrotetracycline hydrochloride (ATc) was purchased from Cole-Parmer Instruments (Vernon Hills, IL). Lumi-Phos 530 was from Wako Pure Chemical Industries (Osaka, Japan). The bicinchoninic acid (BCA) protein assay kit was from Thermo Fisher Scientific Inc. (Rockford, IL). Alkaline phosphatase (ALP)-labeled anti-tetracycline repressor (TetR) antibody was from MoBiTec GmbH (Göttingen, Germany). ALP-labeled anti-FLAG antibody was from Sigma-Aldrich Inc. (St. Louis, MO). Recombinant hepatitis C virus (HCV) envelope protein E2 and anti-E2 antibody were from Abcam K. K. (Tokyo, Japan). ALP-labeled anti-goat IgG antibody was from Promega Corporation (Madison, WI). All other reagents were laboratory-grade, commercially available analytical reagents. Deionized distilled water was used in all procedures.

Bacterial strains and culture conditions. *E. coli* strain EPI300 (AR Brown Co., Ltd., Tokyo, Japan) was used as the host for gene cloning. Cells were cultured in LB medium containing 50 µg/ml ampicillin at 37°C. *M. magneticum* AMB-1 (ATCC 700264) was microaerobically cultured in magnetic spirillum growth medium (MSGM) at 25°C as previously described (12). Microaerobic conditions were established by purging the cultures with argon gas. AMB-1 transformants harboring each expression vector were cultured under the same conditions in medium containing 5 µg/ml ampicillin.

Construction of expression vectors. The plasmids and primers used in this study are described in Table 1 and Table 2. Each expression vector was derived from pUMG (Ap^r; 6.4 kbp) (14). For the construction of pUMtORLC, pUMtOR13GFP, and pUMtOR13CD81', the gene encoding the *E. coli* tetracycline repressor (TetR) was generated by PCR amplification using a primer set consisting of TetR-F and TetR-R with pcDNA6/TR (Invitrogen, Carlsbad, CA)

as the template. The sequence encoding the *msp3* promoter (*P_{msp3}*) was amplified using the primers Pmsp3-F and Pmsp3-R with pUMGLC-Pmsp3 (25) as the template. Both PCR fragments containing the *tetR* gene and *P_{msp3}* were cloned in series into pUMG treated with appropriate restriction enzymes. This plasmid construct was designated pUMtR (Ap^r; 7.5 kbp). The sequence encoding luciferase was amplified using the primers LC-F and LC-R with pUMLC (26) as the template. The sequence encoding the fusion protein Mms13-green fluorescent protein (GFP) was amplified using the primers M13-SF and GFP-R with pUM13GFP as the template. The sequence encoding Mms13 and the N-terminal FLAG-tagged region spanning the second and fourth transmembrane domains of CD81 (CD81') was amplified using the primers M13-SF and CD81'-R with pUMP16M13-FLAG-CD81' as the template. The PCR-amplified fragments of luciferase, Mms13-GFP, and Mms13-FLAG-CD81' were cloned into SspI-digested pUMtR. The sequences (17 bp) upstream of the putative -35 and the -10 promoter consensus sequences of *P_{msp1}* were replaced with tetracycline operator elements (6). This *P_{msp1}* derivative, named *P_{msp1}^{tetO}*, was amplified using the primers MspI(TetO)-F and MspI(TetO)-R, with artificial synthetic DNA as a template. The amplified *P_{msp1}^{tetO}* fragment was cloned into the plasmid upstream of the site of the luciferase, Mms13-GFP, or Mms13-FLAG-CD81' gene.

For the construction of pUMtOR13CCR5 and pUMtOR13CXCR4, the sequence encoding *P_{msp1}^{tetO}* and the SspI digestion site was amplified using the primers Pmsp1(TetO)-F and Pmsp1(TetO)-R with the synthetic DNA described above as the template. The PCR-amplified fragment was cloned into SspI-digested pUMtR, and the construct was designated pUMtOR (Ap^r; 7.8 kbp). The sequences encoding CCR5 and CXCR4 were amplified using a HepG2 cDNA library as the template and cloned into SspI-digested pUMG16M13. The fusion genes *mms13-CCR5* and *mms13-CXCR4* were amplified using the primers M13-F and CCR5-R or CXCR4-R with plasmids containing each gene as templates. The PCR-amplified sequences carrying the *mms13-CCR5* or *mms13-CXCR4* gene were ligated into SspI-digested pUMtOR.

Plasmids were transformed into wild-type *M. magneticum* AMB-1 by electroporation, and a colony formation experiment was conducted as previously described (14).

Preparation of BacMPs. Cultured *M. magneticum* AMB-1 cells were collected by centrifugation at 11,344 × *g* for 10 min at 4°C, resuspended in 40 ml 10 mM phosphate-buffered saline (PBS), pH 7.4, and disrupted by three passes through a French press cell at 1,500 kg/cm² (Ohtake Works Co. Ltd., Tokyo, Japan). BacMPs were collected from the disrupted cells using a columnar neodymium-boron (Nd-B) magnet, washed 10 times with 10 mM HEPES buffer, pH 7.4, by dispersion using sonication, and collected using an Nd-B magnet. 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 of the suspension at 660 nm using a spectrophotometer (UV-2200; Shimadzu, Kyoto, Japan). A value of 1.0 corresponded to 172 µg (dry weight) BacMPs/ml.

Measurement of luciferase activity in whole AMB-1 cells. Wild-type AMB-1 cells and transformants harboring pUMtORLC were cultured in MSGM containing 0 to 500 ng/ml ATc until stationary phase. One hundred microliters of the cultures was mixed with an equal volume of substrate solution (luciferase assay system; Promega Corporation, Madison, WI). After 5 min of incubation, luminescence was measured with a luminometer (Aloka, Tokyo, Japan).

Measurement of GFP fluorescence on BacMPs. Wild-type AMB-1 and transformants harboring pUMtOR13GFP were cultured in MSGM in the presence or

TABLE 2. PCR primers used in this work

Name of primer	DNA sequence ^a
Msp3-F	5'- <u>ACTAGTACGTTGAATCCCAGCGCCCC</u> -3'
Msp3-R	5'- <u>ACTAGTTAGAAAGGCTCCCTCATAGGG</u> CTTGG-3'
TetR-F	5'- <u>ACTAGTATGTCTAGATTAGATAAAA</u> G-3'
TetR-R	5'-TTAAGACCCACTTTCACATTTAAG-3'
MspI(TetO)-F	5'-GCGGTAACGCCGCTCGCCG-3'
MspI(TetO)-R	5'-GGTATCTCCTCTCTTTGGCTTTGC-3'
MspI(TetO)-SR	5'- <u>AATATTGGTATCTCCTCTCTTTGGCTTT</u> G-3'
LC-F	5'- <u>AATATTATGGAAGACGCCAAAAAAC</u> -3'
LC-R	5'-TTACAATTTGGACTTTCCGCC-3'
M13-SF	5'- <u>AATATTATGCCCTTTCACCTTGCCCCCT</u> ATC-3'
M13-F	5'-ATGCCCTTTCACCTTGCCCCCTATCTG-3'
GFP-R	5'-TCACTTGTAACAGCTCATCCATGCCG-3'
CD81'-R	5'-TTAATGGTGGTGGTGGTGGTGGTAC-3'
CXCR4-R	5'-TTAGCTGGAGTGAAAACCTGAAGACT C-3'
CCR5-R	5'-TCACAAGCCCACAGATATTTCTCTGC-3'

^a Underlining indicates endonuclease recognition sequences.

absence of ATc until stationary phase and then were observed by fluorescence microscopy (Olympus Co., Tokyo, Japan). BacMPs (50 μ g) extracted from AMB-1 grown under each set of culture conditions were suspended in 500 μ l HEPES buffer. The fluorescence intensity of the suspension was measured (excitation wavelength, 489 nm; emission wavelength, 508 nm) using a spectrofluorometer (Horiba Iteck, Tokyo, Japan).

Antibody-binding assay on BacMPs. BacMPs (100 μ g) were magnetically collected and added to a solution of ALP-labeled anti-FLAG tag antibody (100 ng/ml) dissolved in 100 μ l 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 μ l PBST using sonication, and resuspended in 100 μ l PBS, followed by the addition of 100 μ l Lumi-Phos 530 as the luciferase substrate. After 5 min of incubation, the luminescence intensity was measured with a luminometer.

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 μ g 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 μ g/ml in PBST) and ALP-labeled goat anti-mouse IgG (750 ng/ml in PBST). 5-Bromo-4-chloro-3-indolylphosphate nitroblue tetrazolium (BCIP-NBT) (Sigma, St. Louis, MO) was used as the ALP substrate for visualization.

For the detection of recombinant proteins on the surface of BacMPs, membrane proteins were extracted from 2.5 to 3 mg BacMPs as described previously (1) and separated by SDS-PAGE as described above. FLAG tag was detected with ALP-labeled anti-FLAG tag antibody (1/1,000 in PBST). BCIP-NBT (Sigma, St. Louis, MO) was used as the ALP substrate for visualization.

Ligand binding to CD81'-BacMPs. Ligand-binding experiments using Mms13-FLAG-CD81'-displaying BacMPs (CD81'-BacMPs) extracted from AMB-1 transformants harboring pUMtOR13CD81' were performed using the HCV envelope protein E2. BacMPs (2 mg) were suspended in a total volume of 2 ml PBST containing 2 μ g E2 and incubated for 2.5 h at room temperature. BacMPs then were magnetically separated and washed twice in 2 ml PBST with sonication to remove free E2. After being washed, BacMP proteins were extracted and subjected to Western blot analysis as described above. For immunostaining, goat anti-E2 antibody (1 μ g/ml) and ALP-labeled anti-goat IgG antibody (1/10,000 in PBST) were used.

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 ($K_a = 2.8 \times 10^9$ 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_{msp3}) had been identified previously using the *M. magneticum* AMB-1 genome and proteome databases (25). The TetR proteins are continuously expressed under 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

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.

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 cultured in MSGM containing 0 to 500 ng/ml ATc, 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. 2. Inducible expression of GFP on BacMPs in *M. magneticum* AMB-1. (A) Microscopic images of wild-type AMB-1 and 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 μ m).

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 were observed by fluorescence microscopy after culture in the presence or absence of ATc. Significant fluorescence was observed in cells cultured in the presence of ATc (ATc⁺), while transformant cultured without ATc (ATc⁻) showed a level of fluorescence similar to that of wild-type AMB-1 (Fig. 2A). BacMPs then were extracted from each culture and assayed for fluorescence. Higher fluorescence intensity was detected in BacMPs of the transformants grown under ATc⁺ conditions than BacMPs of either transformants grown without ATc or wild-type cells (Fig. 2B). These results indicate that the Mms13-GFP expressed by ATc induction was successfully localized on the surface of BacMPs. Furthermore, Mms13-GFP could be observed on BacMPs when ATc was added even at 66 h after inoculation (Fig. 2C). ATc addition at mid- to late log phase could induce the expression and assembly of Mms13 fusion protein onto BacMPs; therefore, the tetracycline-inducible system could be applied effectively to the expression of high-toxicity proteins in *M. magneticum* AMB-1.

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 tetraspanin 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 1×10^8 cells), and the bacteria were incubated on MSGM-agar 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 tetraspanin 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). This

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

Plasmid	CFU/100 μ l
pUMtOR13CD81'	108 $\pm$ 20
pUMtO13CD81'	0
pUMtOR13CXCR4	48 $\pm$ 12
pUMtO13CXCR4	0
pUMtOR13CCR5	62 $\pm$ 2
pUMtO13CCR5	0

^a Each expression vector was transformed into wild-type *M. magneticum* AMB-1 by electroporation. Transformant cells (1×10^8) were diluted in 5 ml of MSGM containing 0.7% agar and plated on 1% agar in MSGM containing 0.5 μ g of ampicillin per ml and incubated under microaerobic conditions.

FIG. 3. Growth curves of AMB-1 transformant harboring pUMTOR13CD81'. The transformant was grown in MSGM in the absence or presence of 500 ng/ml ATc. ATc was added to the medium at the time of inoculation (filled circle) of culture or at mid-log phase (open triangles). The solid arrow indicates the stage for the addition of ATc.

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 using ALP-conjugated anti-FLAG tag antibody (Fig. 4A). Mms13-FLAG-CD81' fusion proteins were observed in the CD81'-BacMP membrane fraction at the predicted mass (35 kDa) (Fig. 4B).

Furthermore, the topology of the fusion protein consisting of Mms13, FLAG tag, and the truncated form of CD81 (CD81') on BacMPs was confirmed by an antibody-binding assay of BacMPs. When CD81 is displayed on BacMPs with Mms13 as an anchor protein, the orientation of the CD81 becomes a problem. Both terminals of CD81 have been shown to be exposed on the inside of the plasma membrane (17). In contrast, the C terminus of Mms13 is exposed on the outer, cytoplasmic surface of the BacMP membrane. To fuse the C terminus of Mms13 and the N terminus of CD81, we truncated the first intracellular domain and the first transmembrane domain of CD81 to maintain the normal orientation of the CD81 LEL. An antibody-binding assay to CD81'-BacMPs was performed, and anti-FLAG antibody was able to bind directly to the surface of CD81'-BacMPs, where the FLAG tag was present between Mms13 and CD81' in the fusion protein. These findings indicate that the N-terminal site of CD81' was accessible on the surface of BacMPs and in its normal orientation.

Finally, the activity of CD81' expressed on the surface of BacMPs was confirmed by the assay of binding to HCV envelope protein E2. Following the incubation of E2 with BacMPs expressing Mms13-FLAG-CD81', proteins were extracted and analyzed by Western blotting using anti-E2 antibody (Fig. 4C). The specific binding of E2 to the CD81'-BacMPs is shown, indicating that the CD81' expressed in the BacMP membrane retained its affinity for E2. These results suggest that CD81'-

FIG. 4. Assembly of the truncated form (consisting of the second to fourth transmembrane domains) of tetraspanin CD81 on BacMPs. (A) Schematic of the gene construct and CD81'-BacMPs. (B) Expression analysis of CD81' on BacMPs by ELISA and Western blotting using ALP-conjugated anti-FLAG tag antibody. Wild-type BacMPs (WT-BacMPs) served as a control. (C) Binding of the HCV envelope protein E2 to the surface of CD81'-BacMPs. CD81'-BacMPs were incubated with E2, and after being washed, proteins were removed from the surface of the BacMPs by treatment with 1% SDS and analyzed by Western blotting. Cells were cultured in the absence (ATc⁻) or presence (ATc⁺) of ATc added at mid-log phase.

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. magneticum* 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.

ACKNOWLEDGMENTS

This research was supported by the Ministry of Education, Culture, Sports, Science, and Technology in Japan under a grant-in-aid for the Division of Young Researchers and in part by the Industrial Technology Research Grant Program in 2009 from the New Energy and Industrial Technology Development Organization of Japan.

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