Noncovalent Immobilization of Streptavidin on In Vitro- and In Vivo-Biotinylated Bacterial Magnetic Particles[▽]

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Biotinylated magnetic nanoparticles were constructed by displaying biotin acceptor peptide (BAP) or biotin carboxyl carrier protein (BCCP) on the surface of bacterial magnetic particles (BacMPs) synthesized by *Magnetospirillum magneticum* AMB-1. BAP-displaying BacMPs (BAP-BacMPs) were extracted from bacterial cells and incubated with biotin and *Escherichia coli* biotin ligase. Then the in vitro biotinylation of BAP-BacMPs was confirmed using alkaline phosphatase-labeled anti-biotin antibody. In contrast, BacMPs displaying the intact 149 residues of AMB-1 BCCP (BCCP-BacMPs) and displaying the COOH-terminal 78 residues of BCCP (BCCP78-BacMPs) were biotinylated in AMB-1 cells. The in vivo biotinylation of BCCP-BacMPs and BCCP78-BacMPs was thought to be performed by endogenous AMB-1 biotin ligase. Streptavidin was introduced onto biotinylated BacMPs by simple mixing. In an analysis using tetramethyl rhodamine isocyanate-labeled streptavidin, approximately 15 streptavidin molecules were shown to be immobilized on a single BCCP-BacMP. Furthermore, gold nanoparticle-BacMP composites were constructed via the biotin-streptavidin interaction. The conjugation system developed in this work provides a simple, low-cost method for producing biotin- or streptavidin-labeled magnetic nanoparticles. Various functional materials can be site selectively immobilized on these specially designed BacMPs. By combining the site-selective biotinylation technology and the protein display technology, more innovative and attractive magnetic nanomaterials can be constructed.

Magnetic particles are widely used in various biomedical and environmental applications. A major advantage of magnetic particles is the ease of handling, which allows simple separation of target molecules from reaction mixtures. Magnetic particles are increasingly used as tools for cell separation (6, 12), DNA extraction (4, 19), and antigen detection (7, 17). Among the various magnetic particle composites developed to date, streptavidin-immobilized magnetic particles have shown great potential due to the stability of the biotin-streptavidin interaction and the resulting ability to separate target molecules specifically and efficiently (5, 14, 15, 21, 35). However, the synthesis of streptavidin-immobilized magnetic particles involves multiple steps, including synthesis of magnetic core particles, polymer coating, and immobilization of streptavidin, and each step increases the production time and cost.

Although various methods for immobilizing streptavidin on solid surfaces have been reported, the streptavidin-biotin interaction is the most commonly used method (8, 13, 32). In this method, the solid surface is first biotinylated using a cross-linker reagent, and this is followed by the addition of streptavidin. Streptavidin is easily immobilized on various surfaces and retains its biotin-binding capacity.

Site-selective biotinylation of proteins is achieved using biotin ligase, which catalyzes the posttranslational biotinylation of biotin enzymes, such as acetyl coenzyme A (acetyl-CoA) carboxylase. Biotin ligase introduces biotin into a specific ly-

sine residue of biotin carboxyl carrier protein (BCCP), a subunit of biotin enzymes. *Escherichia coli* biotin ligase is known to also biotinylate short peptides called biotin acceptor peptides (BAPs). BAPs in a peptide library have been screened (31) and are widely used as peptide tags for site-selective biotinylation (5, 10, 24). Using the BCCP or BAP sequences and biotin ligase, site-selective biotinylation of protein is easily performed.

Magnetospirillum magneticum AMB-1 synthesizes intracellular nano-size bacterial magnetic particles (BacMPs) (50 to 100 nm) that are surrounded by a lipid bilayer membrane, possess a single magnetite magnetic domain, and exhibit strong ferromagnetism (1, 16). Display of foreign proteins on BacMPs has been achieved by expression of a fusion gene combining the target gene and a gene for an anchor protein isolated from the BacMP membrane of AMB-1 (23). In previous reports, the MagA and Mms16 proteins found on the surface of BacMPs were used as anchor proteins for displaying various functional proteins, such as the immunoglobulin G-binding domain of protein A, the estrogen receptor hormone-binding domain, and G protein-coupled receptor (19). Recently, an efficient and stable protein display technology was developed using a novel anchor protein, Mms13, which is bound to magnetite directly and tightly (36). The introduction of streptavidin onto BacMPs was previously demonstrated via cross-linking, and many applications of streptavidin-immobilized BacMPs have been reported by our laboratory (15, 21, 22). However, streptavidin display on BacMPs by gene fusion techniques has not been successfully demonstrated until now.

In this study, streptavidin was noncovalently immobilized on enzymatically biotinylated BacMPs without cross-linking. After

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screening of the BCCP gene from the AMB-1 genomic sequence, BAP and BCCP were displayed on the surface of BacMPs individually. Initial biotinylation of BAP-displaying BacMPs (BAP-BacMPs) was demonstrated with *E. coli* biotin ligase in vitro, showing that enzymatic modification of proteins displayed on BacMPs is possible. Furthermore, in vivo biotinylation of BCCP-displaying BacMPs (BCCP-BacMPs) was confirmed, and fluorescence-, alkaline phosphatase (ALP)-, or gold nanoparticle-labeled streptavidin was introduced onto biotinylated BacMPs. BCCP displayed on BacMPs was biotinylated in AMB-1 cells by endogenous biotin ligase in the first study to examine the in vivo modification of functional proteins displayed on BacMPs.

MATERIALS AND METHODS

Materials. *E. coli* biotin ligase was purchased from Avidity L.L.C. (Aurora, CO). (+)-Biotin, magnesium chloride hexahydrate, Lumi-Phos 530, and Tween 20 were purchased from Wako Pure Chemical Industries (Osaka, Japan). ATP (disodium salt) was purchased from Sigma (St. Louis, MO). ALP-labeled anti-biotin antibody was purchased from Rockland Immunochemicals, Inc. (Gilbertsville, PA). ALP-labeled streptavidin was purchased from CHEMICON International, Inc. (Silenus, Melbourne, Australia). Streptavidin labeled with gold nanoparticles (5 nm) was purchased from Nanocs, Inc. (New York, NY). Tetramethyl rhodamine isocyanate (TRITC)-labeled streptavidin was purchased from Beckman Coulter (Fullerton, CA). All other reagents were commercially available analytical reagents and were laboratory grade. Deionized, distilled water was used in all procedures.

Bacterial strains and culture conditions. *E. coli* strains DH5 α and BL21(DE3) were used as hosts for gene cloning. Cells were cultured in LB medium containing 50 μ g/ml ampicillin at 37°C. *M. magneticum* AMB-1 was microaerobically cultured in magnetic spirillum growth medium at 25°C as previously described (19). Microaerobic conditions were established by purging the cultures with argon gas. An AMB-1 transformant with pUM13BAP was cultured under the same conditions in medium containing 5 μ g/ml ampicillin. Transformants with pUM13BCCP and pUM13BCCP78 were individually cultured under the same conditions in medium containing 5 μ g/ml ampicillin and 50 μ M additional biotin.

Preparation of BacMPs. Cells of *M. magneticum* AMB-1 (= ATCC 700264) were collected by centrifugation ($11,344 \times g$ for 10 min at 4°C), resuspended in 40 ml of 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 cell fraction using a columnar neodymium-boron (Nd-B) magnet. The BacMPs were then 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 at 660 nm of the solution using a spectrophotometer (UV-2200; Shimadzu, Kyoto, Japan). A value of 1.0 corresponded to 172 μ g (dry weight) BacMPs/ml.

Construction of expression vectors. Plasmids pUM13BAP, pUM13BCCP, and pUM13BCCP78 were derived from pUMGP16M13 (Ap^r; 6.4 kbp) (27, 36). For pUM13BAP construction, the gene encoding BAP (5'-ATGGCTCAGCGTCT GTTCCACATTCTGGACGCTCAGAAAATTGAATGGCACGGTCCGAA AGGTGGTTCT-3') was generated by PCR amplification using a BAP gene-containing plasmid as the template with primers BAP-F (5'-ATGGCTCAGCG TCTGTCC-3') and BAP-R (5'-TTAAGAACCACCTTTCCGAC-3'). For construction of pUM13BCCP and pUM13BCCP78, the genes encoding intact BCCP and truncated BCCP consisting of the COOH-terminal 78 residues of BCCP (BCCP78) were generated by PCR amplification using AMB-1 genomic DNA as the template with primer BCCP-R (5'-CTATTCGATGATCAGCAA GGGC-3') and either BCCP-F (5'-ATGGGCAACAAGACTCCCATC-3') or BCCP78-F (5'-CATCCCGGCGCGGTG-3'). Each PCR fragment was cloned into SspI-digested pUMGP16M13.

The plasmids described above were transferred into wild-type *M. magneticum* AMB-1 by electroporation as previously described (27).

Biotinylation of BAP-BacMPs using *E. coli* biotin ligase. BacMPs (50 μ g) were magnetically collected using a columnar Nd-B magnet, suspended in 100 μ l of PBS containing 10 μ g/ml *E. coli* biotin ligase, 2.44 μ g/ml (+)-biotin, 10.2 mg/ml magnesium chloride hexahydrate, and 55.1 mg/ml ATP (disodium salt), and incubated for 60 min at room temperature with vortexing every 20 min. BacMPs

were magnetically separated from the reaction mixture using an Nb-B magnet and washed once with 100 μ l of PBS containing 0.05% Tween 20 (PBST) using sonication.

Observation of BacMP membrane fusion proteins. BacMP membrane proteins were extracted by boiling 2 mg of BacMPs with 1% sodium dodecyl sulfate (SDS) in an aqueous solution for 30 min. Magnetite was removed by centrifugation and magnetic separation, and the membrane proteins were mixed with SDS sample buffer containing (final concentrations) 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 15% (wt/vol) polyacrylamide gel. The gel was stained with Coomassie brilliant blue R-250.

Introduction of antibody and streptavidin to BacMPs. BacMPs (50 μ g) were magnetically collected and added to a solution of ALP-labeled anti-biotin antibody (10 μ g/ml) or ALP-labeled streptavidin (1/100 dilution of a stock solution) dissolved in 50 μ l of PBST. The mixture was incubated for 60 min at room temperature with pulsed sonication every 5 min. The BacMPs were then magnetically separated, washed three times with 100 μ l of PBST using sonication, and resuspended in 50 μ l of PBS. Then 50 μ l of Lumi-Phos 530 was added as the luminescence substrate. After 5 min of incubation, the luminescence intensity was measured with a luminometer (Aloka, Tokyo, Japan).

BacMPs (100 μ g) were magnetically collected and added to 200 μ l of a solution of TRITC-labeled streptavidin in PBST. The mixture was incubated for 60 min at room temperature with pulsed sonication every 5 min. The BacMPs were magnetically separated and washed three times with 200 μ l of PBST. Then 50 μ g of the resulting BacMPs was suspended in 500 μ l of PBS, and the fluorescence intensity of the suspension was measured (excitation wavelength, 550 nm; emission wavelength, 580 nm) using a spectrofluorometer (Horiba Iteck, Tokyo, Japan). To estimate the amount of streptavidin on BacMPs, a calibration curve was constructed by measuring the fluorescence intensities of 500- μ l PBS solutions containing 50 μ g of BacMPs and various concentrations of TRITC-labeled streptavidin.

BacMPs (10 μ g) were magnetically collected and added to 200 μ l of PBST containing streptavidin-labeled gold nanoparticles (1/2 dilution of a stock preparation). The mixture was incubated for 60 min at room temperature with pulsed sonication every 10 min. The BacMPs were magnetically separated and washed three times with 100 μ l of PBST and once with 100 μ l of distilled water. After washing, 4 μ l of the gold nanoparticle-labeled streptavidin-BacMP conjugate solution was added to grids and incubated for 30 min. The immobilization of gold nanoparticles on BacMPs was observed by transmission electron microscopy (TEM) (H700H; Hitachi, Tokyo, Japan) at an accelerating voltage of 150 kV.

RESULTS

In vitro biotinylation of BAP-BacMPs. Initially, a BAP with the sequence MAQRLFHILDAQKIEWHGPCKGGS was displayed on BacMPs, and whether BAP-BacMPs could be biotinylated in AMB-1 cells by endogenous biotin ligase was analyzed. After extraction of BAP-BacMPs and wild-type BacMPs (WT-BacMPs), ALP-labeled anti-biotin antibody and the luminescence substrate were added, and the luminescence intensity was measured. BAP-BacMPs showed a luminescence intensity similar to that of WT-BacMPs without *E. coli* biotin ligase (Fig. 1B), indicating that BAP-BacMPs were not biotinylated in AMB-1 cells. Next, BAP-BacMPs and WT-BacMPs were treated with *E. coli* biotin ligase in vitro. They were incubated with biotin, ATP, MgCl₂, and purified *E. coli* biotin ligase, and biotinylation of BAP-BacMPs was confirmed using ALP-labeled anti-biotin antibody. High luminescence intensity from BAP-BacMPs treated with *E. coli* biotin ligase was observed (Fig. 1B), indicating that BAP displayed on the surface of BacMPs could be biotinylated by *E. coli* biotin ligase.

Although enzymatic and site-selective biotinylation of BacMPs was achieved without cross-linking, this method was still complicated and expensive due to the required in vitro biotinylation step with purified *E. coli* biotin ligase. In addition, the efficiency of biotinylation was not increased even with

FIG. 1. In vitro biotinylation of BAP-BacMPs. (A) Schematic diagram of the preparation and in vitro biotinylation of BAP-BacMPs. Plasmid *pUM13BAP* containing the *Mms13*-BAP fusion gene was used to transform wild-type AMB-1 (step a), and the AMB-1 transformant harboring *pUM13BAP* was broken open to release BAP-BacMPs (step b). Then BAP-BacMPs were magnetically separated and purified by stringent washing (step c), and BAP-BacMPs were incubated with biotin, MgCl_2 , ATP, and *E. coli* biotin ligase (step d). (B) The biotinylation of BAP-BacMPs treated with or without *E. coli* biotin ligase was evaluated using ALP-labeled anti-biotin antibody and a method derived from an enzyme-linked immunosorbent assay.

changes in biotinylation conditions, including increased incubation time and increased concentrations of biotin ligase, biotin, and ATP (results not shown). Therefore, we attempted to develop a simpler and more efficient in vivo biotinylation method.

Amino acid sequence of AMB-1 BCCP. To develop a simple method for biotinylating BacMPs, amino acid sequences that are biotinylated in AMB-1 cells should be displayed on the surface of BacMPs. Here, we focused on the BCCP sequence. BCCP is the biotin-binding subunit of acetyl-CoA carboxylase and is thought to be present in all organisms. The entire genome of *M. magneticum* AMB-1 was sequenced, annotated, and analyzed in our laboratory (18). Therefore, the AMB-1 BCCP gene could be identified in the AMB-1 genome by similarity searches with known eukaryotic, eubacterial, and archaeal counterparts. The AMB-1 BCCP is encoded by the *amb2707* gene. In addition, the AMB-1 biotin ligase gene, *amb2773*, was also identified. The amino acid sequence of AMB-1 BCCP (accession number YP_422070) was compared with the amino acid sequences of BCCPs from *Magnetospirillum magnetotacticum* MS-1 (accession number ZP_00051435), *Magnetospirillum gryphiswaldense* MSR-1 (accession number CAM75821), and *Rhodospirillum rubrum* (accession number YP_427521) (28). Based on this comparison, the COOH-terminal 78 residues are largely consistent in these species. Therefore, the intact 149 residues of AMB-1 BCCP and the truncated form containing the COOH-terminal 78 residues (BCCP78) were fused to *Mms13* and displayed on BacMPs.

In vivo biotinylation of BCCP-BacMPs and BCCP78-BacMPs. Expression of the fusion proteins *Mms13*-BCCP (31

kDa) and *Mms13*-BCCP78 (23 kDa) was confirmed by SDS-PAGE (Fig. 2). Fusion proteins were observed in the BacMP membrane fraction at the predicted mass, and the major bands from the BacMP membrane proteins were observed. These results indicated that *Mms13*-BCCP and *Mms13*-BCCP78 were expressed on BacMPs extracted from the *pUM13BCCP* and *pUM13BCCP78* transformants, respectively.

Furthermore, biotinylation of BCCP and BCCP78 on BacMPs was confirmed using ALP-labeled anti-biotin antibody and the same protocol that was used to confirm in vitro biotinylation (Fig. 3). BacMPs extracted from both the *pUM13BCCP* and *pUM13BCCP78* transformants were biotinylated in vivo, indicating that both the intact and truncated forms of BCCP displayed on the surface of BacMPs were recognized by AMB-1 biotin ligase in the cells. The luminescence intensities measured from in vivo biotinylated BCCP-BacMPs and BCCP78-BacMPs were 10 times higher than that measured from an equal concentration of in vitro-biotinylated BAP-BacMPs, suggesting that in vivo biotinylation is more efficient than in vitro biotinylation.

Construction of streptavidin-immobilized BacMPs. Immobilization of streptavidin on biotinylated BacMPs was conducted by simply mixing ALP-labeled streptavidin and BCCP-BacMPs or BCCP78-BacMPs. After BacMPs were washed and the luminescence substrate was added, greater luminescence intensity was observed from BCCP-BacMPs and BCCP78-BacMPs than from WT-BacMPs (Fig. 4A). These results confirm that streptavidin was easily introduced onto BCCP-BacMPs and BCCP78-BacMPs in the absence of covalent binding. Truncation of BCCP to 78 amino acid residues did not affect

FIG. 2. SDS-PAGE of BacMP membrane proteins. The positions of the Mms13-BCCP and Mms13-BCCP78 fusion proteins are indicated by arrowheads.

the amount of streptavidin immobilized on the surface of BacMPs. Immobilization of streptavidin on in vitro-biotinylated BAP-BacMPs was also confirmed using ALP-labeled streptavidin (data not shown). However, the luminescence intensity from BAP-BacMPs was less than that from BCCP-BacMPs and BCCP78-BacMPs, similar to the findings for ALP-labeled antibiotin antibody binding.

Furthermore, the amount of streptavidin immobilized on BacMPs was evaluated using TRITC-labeled streptavidin. Saturation binding analysis was performed by adding TRITC-labeled streptavidin to BCCP-BacMPs. The fluorescence intensity from TRITC-labeled streptavidin and BCCP-BacMPs conjugates was measured after three wash steps, and approximately 1,300 ng of streptavidin was introduced onto 1 mg of BCCP-BacMPs (Fig. 4B). This amount corresponded to 15 streptavidin molecules immobilized on a single BCCP-BacMP (molecular mass of streptavidin, 60 kDa; diameter of BacMPs, 75 nm; density of BacMPs, 5.2 g/cm³).

Various functional streptavidin-labeled materials can be immobilized on biotinylated BacMPs. Here, the immobilization of streptavidin-labeled gold nanoparticles on biotinylated BCCP-BacMPs was demonstrated. Streptavidin-labeled gold nanoparticles were mixed with BCCP-BacMPs and observed by TEM. Little nonspecific adsorption of gold nanoparticles to WT-BacMPs was detected, while immobilization of gold nanoparticles on the surface of BCCP-BacMPs was observed (Fig. 4C). This result suggests that nano-size materials labeled with streptavidin can be immobilized on BacMPs displaying BCCP via the biotin moiety. The approximate number of gold nanoparticles immobilized on a single BCCP-BacMP corresponded to the value determined by the TRITC-labeled streptavidin assay.

FIG. 3. In vivo biotinylation of BCCP-BacMPs and BCCP78-BacMPs. (A) Schematic diagram of the preparation and in vivo biotinylation of BCCP-BacMPs. Plasmid *pUM13BCCP* containing an *Mms13*-BCCP fusion gene was used to transform wild-type AMB-1 (step a), and BCCP-BacMPs were biotinylated by endogenous AMB-1 biotin ligase (step b). Then the AMB-1 transformant harboring *pUM13BCCP* was broken open to release BCCP-BacMPs (step c), and BCCP-BacMPs were magnetically separated and purified by stringent washing (step d). (B) The in vivo biotinylation of BCCP-BacMPs and BCCP78-BacMPs was evaluated using ALP-labeled antibiotin antibody and a method derived from an enzyme-linked immunosorbent assay.

FIG. 4. Immobilization of streptavidin on the surface of biotinylated BCCP-BacMPs and BCCP78-BacMPs. (A) Immobilization of ALP-labeled streptavidin on WT-BacMPs, BCCP-BacMPs, and BCCP78-BacMPs as determined by luminescence intensity. (B) Saturation binding curve of TRITC-labeled streptavidin bound to BCCP-BacMPs. (C) TEM images of gold nanoparticles bound to BCCP-BacMPs via streptavidin-biotin interaction.

DISCUSSION

Biotin enzymes are composed of an apoenzyme and a biotin moiety. Biotin is attached to the apoprotein through an amide linkage to the ϵ -amino group of a unique lysine residue by biotin ligase (11). Biotin enzymes are critical to the carboxylation of important metabolites, such as acetyl-CoA, propionyl-CoA, and pyruvate, and to the decarboxylation of metabolites, such as oxaloacetate and glutamyl-CoA (30). Because biotin enzymes play essential roles in metabolism, they and their synthetase (biotin ligase) are thought to be present in all organisms. When the sequences around the biotinylated lysine are aligned, some residues are well conserved in various biotin enzymes (30). The short amino acid sequences biotinylated by *E. coli* biotin ligase were screened from peptide libraries and designated BAPs (31). They were used for site-selective biotin labeling of proteins.

In this study, BAP, intact AMB-1 BCCP, and a truncated form of AMB-1 BCCP (BCCP78) were displayed on the surface of BacMPs. Although BAP-BacMPs were not biotinylated in vivo, the biotinylation of BAP-BacMPs in vitro by *E. coli* biotin ligase was confirmed by analysis with ALP-labeled anti-biotin antibody. Recognition of BAP by endogenous AMB-1 biotin ligase did not occur in the manner previously observed for *Saccharomyces cerevisiae* (yeast), *Bacillus subtilis*, and *Methanococcus jannaschii* (2). In contrast, BCCP-BacMPs were biotinylated in vivo, indicating that intracellular AMB-1 biotin ligase successfully recognized AMB-1 BCCP displayed on the surface of BacMPs. The in vivo biotinylation of BacMPs expressing Mms13-BCCP was found to be more efficient than the in vitro biotinylation of BacMPs expressing Mms13-BAP, as the amount of anti-biotin antibody bound to in vivo-biotinylated BCCP-BacMPs was 10 times greater and more uniform than the amount on BAP-BacMPs biotinylated in vitro by *E. coli*

biotin ligase. In magnetotactic bacteria, BacMP membrane vesicles are thought to originate from invagination of the cytoplasmic membrane (9, 26, 33). BacMP membrane proteins, including Mms13, are thought to immigrate from the cytoplasmic membrane with the invagination of the cytoplasmic membrane and the formation of vesicles. In this study, Mms13, which is tightly bound on the magnetite surface, was used as an anchor protein. Because the Mms13-BAP fusion protein was biotinylated in vitro, interaction between Mms13-BAP and *E. coli* biotin ligase was limited to the surface of the BacMP membrane. In contrast, Mms13-BCCP and Mms13-BCCP78 can interact with AMB-1 biotin ligase not only on the surface of BacMPs but also in the cytoplasm or cytoplasmic membrane. Mms13-BCCP and, in particular, Mms13-BCCP78 are assumed to have high mobility in the cytoplasm prior to insertion into the cytoplasmic membrane, allowing more efficient biotinylation compared to Mms13-BAP.

A truncated form of AMB-1 BCCP (BCCP78) displayed on BacMPs was also biotinylated in vivo. Previously, the COOH-terminal 75 amino acid residues in the 1.3S subunit of *Propionibacterium shermanii* transcarboxylase (3), also a member of biotin enzyme family, and the 87 residues of BCCP in *E. coli* acetyl-CoA carboxylase (25) have been biotinylated by biotin ligase. These reports suggested that the COOH-terminal domain of a biotin-binding protein is important for recognition by biotin ligase. In this study, the amino acid sequence of AMB-1 BCCP was identified using the AMB-1 genome and compared with the sequences of other bacterial BCCPs. In this comparison, the COOH-terminal 78 residues showed high similarity to residues in organisms closely related to AMB-1, including *M. magnetotacticum* MS-1, *M. gryphiswaldense* MSR-1, and *R. rubrum*. Therefore, we constructed pUM13BCCP78, which expresses the Mms13-BCCP78 fusion protein, and obtained

BCCP78-BacMPs. Analysis of BCCP78-BacMPs using ALP-labeled antibiotin antibody showed that the truncated BCCP78 molecule was biotinylated in AMB-1 cells as well as intact BCCP was (Fig. 3).

After extraction of biotinylated BacMPs, streptavidin was immobilized on BacMPs by simply mixing streptavidin molecules and biotinylated BacMPs (Fig. 4). Previously, we attempted to use two approaches to immobilize streptavidin on BacMPs. First, fusion proteins with streptavidin and BacMP membrane proteins were created. However, the overexpressed streptavidin fusion protein was too toxic to allow growth of the AMB-1 transformant. Second, streptavidin was immobilized on BacMPs using cross-linker reagents (15, 21, 22). In this method, the cross-linker Sulfo-NHS-LC-LC-biotin, which reacts with the amine group, was initially bound to phosphatidylethanolamine in the BacMP membrane. Following biotin modification of BacMPs, streptavidin was successfully immobilized. However, the cross-linker reagents are expensive, and when functional protein-displaying BacMPs were biotinylated, excess cross-linker reagents also bound to these proteins indiscriminately and inhibited their function (17). In this study, in vitro and in vivo biotinylation of BacMPs was demonstrated. With these novel methods biotin modification of BacMPs was achieved simply, site selectively, and inexpensively, because the biotin moiety was enzymatically introduced onto the surface of BacMPs without cross-linking.

Enzymatic biotinylation methods also can be used for assembly of nanomaterial composites. Recently, composites of magnetic nanoparticles and other functional materials, such as quantum dots (29, 34) and metal nanoparticles (20), have been developed. Although these composites are useful in various fields, the construction processes are complicated. In this study, construction of a gold nanoparticle and BCCP-BacMP composite was accomplished via a stable biotin-streptavidin interaction. This method is simple and widely available for construction of composites of magnetic nanoparticles and various functional materials.

In conclusion, BAP-BacMPs were biotinylated by *E. coli* biotin ligase in vitro, and BCCP-BacMPs and BCCP78-BacMPs were biotinylated in vivo. Streptavidin was readily immobilized on biotinylated BacMPs. The conjugation system developed in this work provides a simple, low-cost method for producing biotin- and streptavidin-immobilized magnetic nanoparticles. By combining the site-selective biotinylation technology developed in this study and the protein display technology, more innovative and attractive magnetic nanomaterials can be constructed.

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