Biochemical and Biophysical Research Communications 445 (2014) 1–5

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Functional expression of an scFv on bacterial magnetic particles by *in vitro* docking

Yasuhiro Sugamata, Tsuyoshi Tanaka, Tadashi Matsunaga, Tomoko Yoshino *

Division of Biotechnology and Life Science, Institute of Engineering, Tokyo University of Agriculture and Technology, 2-24-16, Naka-cho, Koganei, Tokyo 184-8588, Japan

ARTICLE INFO

Article history:
Received 8 December 2013
Available online 25 January 2014

Keywords:
In vitro docking
Magnetospirillum magneticum AMB-1
Bacterial magnetic particles (BacMPs)
Single chain variable fragment (scFv)
Immunoglobulin Fc

ABSTRACT

A Gram-negative, magnetotactic bacterium, *Magnetospirillum magneticum* AMB-1 produces nano-sized magnetic particles (BacMPs) in the cytoplasm. Although various applications of genetically engineered BacMPs have been demonstrated, such as immunoassay, ligand–receptor interaction or cell separation, by expressing a target protein on BacMPs, it has been difficult to express disulfide-bonded proteins on BacMPs due to lack of disulfide-bond formation in the cytoplasm. Here, we propose a novel dual expression system, called *in vitro* docking, of a disulfide-bonded protein on BacMPs by directing an immunoglobulin Fc-fused target protein to the periplasm and its docking protein ZZ on BacMPs. By *in vitro* docking, an scFv–Fc fusion protein was functionally expressed on BacMPs in the dimeric or trimeric form. Our novel disulfide-bonded protein expression system on BacMPs will be useful for efficient screening of potential ligands or drugs, analyzing ligand–receptor interactions or as a magnetic carrier for affinity purification.

1. Introduction

Prokaryotes like *Escherichia coli* are often the first choice of the host for recombinant protein expression in terms of fast growth, high productivity, easy genetic manipulation. In Gram-negative bacteria, recombinant proteins cannot form disulfide bonds in reducing environment of the cytoplasm [1,2]. On the other hand, oxidative environment of periplasmic space in Gram-negative bacteria is suitable for expression of disulfide-bonded proteins [3]. To direct periplasmic expression of a protein of interest, signal sequence is added at the N-terminal of the protein. In *E. coli*, it is well known that unfolded protein precursors translocate to periplasm by either Sec (post-translational) or SRP (co-translational) system [4,5]. Although individual signal sequence varies from 15 to 30 amino acids and shows low degree of homology, they have overall similarities, and are effective over different types of bacteria and even between prokaryotes and eukaryotes in some cases [6].

Magnetospirillum sp. is a unique Gram-negative bacterium which synthesizes nano-sized magnetic particles arranged in chains in the cytoplasm. The bacterial magnetic particles (BacMPs) consist of pure magnetite (50–100 nm in diameter) surrounded by lipid bilayer membrane and exhibit strong ferrimagnetism. Mms13 protein is known as a major protein tightly bound to core magnetite, and be integrated in the membrane on the surface of the magnetite [7]. By using Mms13 protein as a fusion partner, recombinant protein expression on BacMPs has been established in *Magnetospirillum magneticum* AMB-1 [8–15]. The genetically engineered magnetic particles can be easily extracted and purified from cell lysate of *M. magneticum* AMB-1 transformant by magnetic separation using magnet. The recombinant protein–BacMP complexes have been applied to immunoassays, ligand screening assays and cell separation systems as magnetic carriers [16–18]. These expression techniques have an advantage over chemical conjugation of recombinant proteins on magnetic particles in terms of direct purification, and rapid and cost-effective preparation of bio-functionalized magnetic particles.

However, as noted above, the cytoplasm, where BacMPs are synthesized, is in a reduced state. Therefore, disulfide bond formation of recombinant proteins on BacMPs relies on air oxidation during extraction and purification process of BacMPs. However, the rate and yield of disulfide bond formation by air oxidation are much slower than that *in vivo* [19]. In addition, concerns about protein aggregation or degradation due to misfolding or unfolding are raised. From these reasons, the conventional expression system on BacMPs is not suitable for disulfide-bonded proteins. In fact, it was difficult to functionally express disulfide-bonded secretory or multi-transmembrane proteins in a satisfactory level [12,15], while soluble cytoplasmic or non-disulfide-bonded proteins such as green fluorescent protein (GFP) [20], firefly luciferase [21] or

* Corresponding author.
E-mail address: y-tomoko@cc.tuat.ac.jp (T. Yoshino).

http://dx.doi.org/10.1016/j.bbrc.2013.12.102
0006-291X/

ZZ domain of protein A from *Staphylococcus aureus* [22] can be easily expressed on BacMPs [9,12].

In this study, we aimed to establish a novel, efficient expression system of disulfide-bonded proteins on BacMPs. The expression system, here we call "*in vitro* docking", consists of a dual expression vector directing one for periplasm and another for cytoplasmic BacMPs. The diagram of *in vitro* docking is illustrated in Fig. 1. In newly proposed method, an Fc-fused disulfide-bonded protein is expressed and folded in the periplasm whereas its binding partner, Mms13–ZZ is expressed on BacMPs. Since both Mms13 and ZZ have no cysteines, Mms13–ZZ is expressed and folded on BacMPs without the need of disulfide bond formation. The Fc-fused disulfide-bonded protein can interact with the Mms13–ZZ on BacMPs (ZZ-BacMPs) during the extraction process of BacMPs. Using this system, an Fc-fused scFv was functionally bound on ZZ-BacMPs. The proposed method will expand the possibilities for the development of antibody and BacMP complexes.

2. Materials and methods

2.1. Bacterial strains and cultures

E. coli strain TOP10 (Life technologies, Carlsbad, CA, USA) was used as a host for gene cloning. *E. coli* transformants were cultured at 37 °C in Luria–Bertani (LB) broth with ampicillin (100 μg/ml). *M. magneticum* AMB-1 (ATCC 700264) was microaerobically cultured in magnetic spirillum growth medium (MSGM) at 28 °C as described previously [23]. *M. magneticum* AMB-1 transformants were cultured under the same conditions with 5 μg/ml ampicillin.

2.2. Construction of vectors and transformants

Plasmids were constructed by standard cloning techniques [24] or In-Fusion cloning technology (Clontech, Mountain View, CA, USA). The fusion gene for single chain variable fragment (scFv) for anti-β-galactosidase (scFv13R4) [25] and constant fragment (Fc) of human immunoglobulin G (IgG) (GenBank: AAA02914.1) was generated by artificial gene synthesis (Integrated DNA Technologies, Coralville, IA, USA). A mutant form of scFv was used, which is well-characterized for high-level soluble expression in the cytoplasm of *E. coli* [25]. The scFv–Fc fusion protein was cloned into *NsiI* site of pUM13ZZ (Fig. 2(A)) [9] with Shine–Dalgano (SD) sequence followed by DsbC signal sequence (DsbCss) [26] and FLAG tag at the N-terminal, yielding pUM13ZZ/scFvFc (Fig. 2(C)). In pUM13ZZ/scFvFc, both DsbCss–FLAG–scFv–Fc and the IgG binding ZZ domain of protein A from *S. aureus* fused with Mms13 (Mms13–ZZ) are expressed as a single operon under the control of *mms16* promoter (P_{mms16}). The expression vector for Mms13–scFv fusion protein (pUM13scFv) (Fig. 2(B)) was constructed by ligation of PCR-amplified N-terminal FLAG-tagged scFv to *Ssp*I site of pUMP16M13 [9]. Each expression plasmid (pUM13ZZ, pUM13scFv and pUM13ZZ/scFvFc) was transformed into *M. magneticum* AMB-1 by electroporation as described previously [27]. The transformants were cultured in MSGM as described above.

2.3. Extraction of BacMPs

Recombinant AMB-1 from 250 ml culture were collected by centrifugation at 9000*g* for 10 min at 4 °C, and re-suspended in 2.5 ml of Extraction buffer A (50 mM Tris–HCl, pH 8.0, 5 mM EDTA). Then, lysozyme and a protease inhibitor, phenylmethylsulfonyl fluoride were added to a final concentration of 0.4 mg/ml and 2 mM, respectively. After 5 min incubation at room temperature (RT), 500 μl of Extraction buffer B (1.5 M NaCl, 0.1 M $CaCl_2$, 0.1 M $MgCl_2$ and 20 μg/ml DNase I) was added and incubated for 5 min at RT. Then, Triton X-100 was added to a final concentration of 0.2% and incubated at RT. BacMPs were magnetically collected from the cell lysate using a columnar neodymium–boron (Nd–B) magnet and washed 5 times with 10 mM phosphate buffered saline with 0.05% Tween 20 (PBST). The concentration of BacMPs in suspension was determined by measuring the optimal density at 660 nm. A value of 1.0 corresponded to 172 μg (dry weight) BacMPs/ml.

2.4. Western blot analysis of fusion proteins onto BacMPs

Membrane proteins from BacMPs were extracted by heat treatment (95 °C, 15 min) of BacMPs in 1% sodium dodecyl sulfate (SDS)

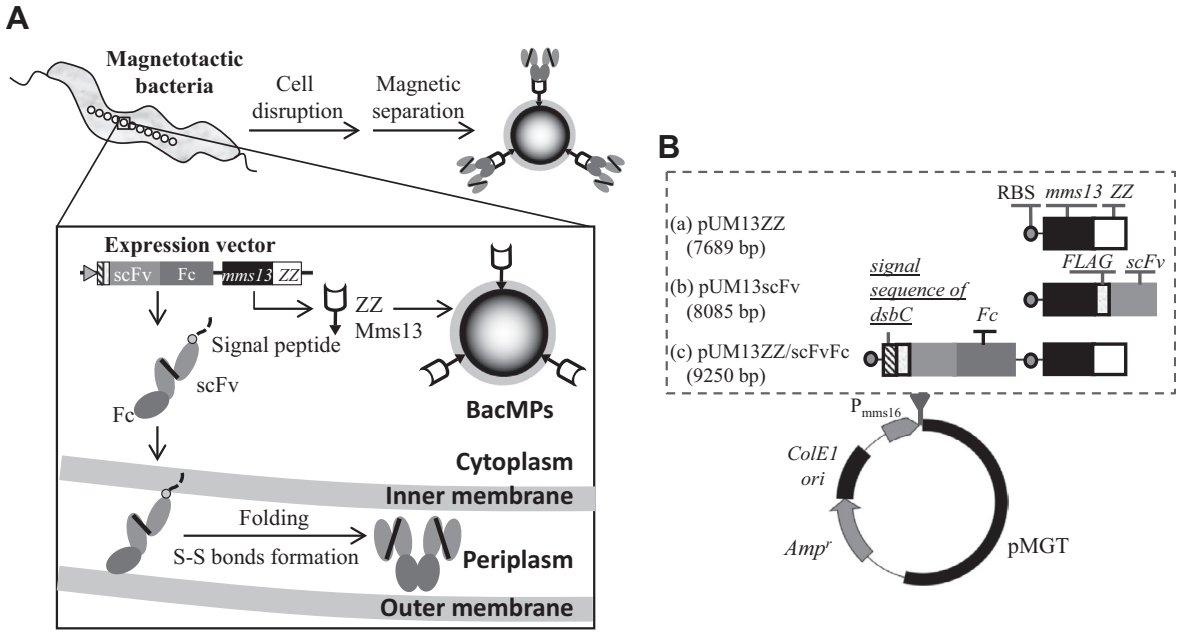

Fig. 1. Diagram of *in vitro* docking. (A) Disulfide-bonded target protein is translocated to the periplasm, whereas its binding protein is expressed on the cytoplasmic BacMPs. The target protein docks to the binding protein on BacMPs during magnetic separation process of BacMPs from the cell lysate. (B) Expression vectors. All the fusion genes shown here are expressed under *mms16* promoter derived from *M. Magneticum* AMB-1.

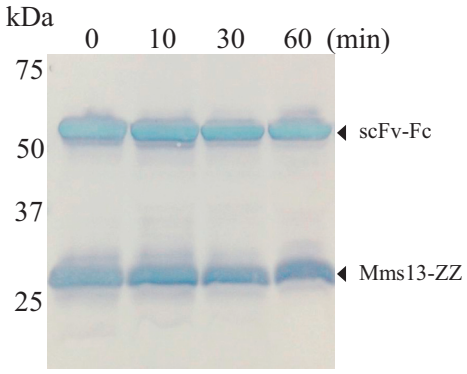

Fig. 2. Time course of scFv–Fc binding on ZZ-BacMPs. BacMPs were magnetically collected from the cell lysate at 0, 10, 30 and 60 min after cell disruption. Then, the membrane proteins extracted from BacMPs were subjected to the Western blotting with mouse anti-FLAG antibody and donkey anti-mouse IgG-AP.

solution in the presence (reduced condition) or absence (non-reduced condition) of 0.1 M dithiothreitol (DTT). The membrane proteins were separated by SDS–polyacrylamide gel electrophoresis (PAGE) through 10–20% (*w*/*v*) gradient gel, then transferred to a polyvinylidene difluoride (PVDF) membrane. scFv–Fc or Mms13–scFv fusion proteins were detected with 1 μg/ml each of either mouse anti-FLAG M2 momoclonal IgG (anti-FLAG IgG; Sigma–Aldrich, St. Louis, MO, USA), mouse anti-FLAG IgG-AP conjugated with alkaline phosphatase (anti-FLAG IgG-AP; Sigma–Aldrich, St. Louis, MO, USA) or AP-conjugated donkey anti-mouse IgG (anti-mouse IgG-AP; Novus Biologicals, Littleton, CO, USA), or rabbit anti-human λ light chain IgG (anti-λ IgG; Bethyl Laboratories, Montgomery, TX, USA) and goat anti-rabbit IgG conjugated with AP (anti-rabbit IgG-AP; Life technologies, Carlsbad, CA, USA). The Mms13–ZZ fusion protein was detected with 1 μg/ml each of rabbit anti-human IgG and anti-rabbit IgG-AP, or anti-mouse IgG-AP through the interaction between Fc and ZZ. PBST was used for washing, and PBST with 2% bovine serum albumin (BSA) was used for dilution of all the antibodies.

2.5. The antigen-binding assay for recombinant scFv

BacMPs from transformants (50 μg each) were mixed with 1 μg/ml of antigen; biotin-conjugated β-galactosidase (biotin-β-galactosidase; Aviva Systems Biology, San Diego, CA, USA) and incubated at RT for 15 min. BacMPs were then magnetically separated and washed once with PBST. Then, 0.5 μg/ml of AP-conjugated streptavidin (AP-SA) (Vector Laboratories, Burlingame, CA, USA) was added and incubated at RT for 10 min. The BacMPs were washed 3 times, and mixed with Lumi-Phos 530 (Wako Pure Chemical Industries, Osaka, Japan) as a luminescence substrate for AP. The luminescence intensity was measured using a SH9000 luminometer (CORONA, Ibaraki, Japan) after addition of the substrate.

3. Results

3.1. Evaluation of in vitro docking of scFv–Fc fusion protein onto ZZ-BacMPs

The proof of concept (Fig. 1(A)) study was performed by the confirmation of scFv–Fc binding to Mms13–ZZ on BacMPs (ZZ-BacMPs) using AMB-1 transformant with co-expression vector (pUM13ZZ/scFvFc) of scFv–Fc fusion protein and Mms13–ZZ fusion protein (Fig. 1(B)). Cells were partially lysed with lysozyme and DNase I for 10 min, and BacMPs were magnetically separated from the cell lysate at 0, 10, 30 and 60 min after the addition of Triton X-100 to complete the cell disruption. Then, the membrane proteins extracted from the BacMPs were subjected to the Western blotting with mouse anti-FLAG IgG (primary antibody) and donkey anti-mouse IgG-AP (secondary antibody). The Fc domain of donkey anti-mouse IgG-AP can also interact with Mms13–ZZ. The scFv–Fc fusion protein (52 kDa) was detected with mouse anti-FLAG IgG and anti-mouse IgG-AP in the expected sizes (Fig. 2, upper bands). In addition, the expression of the Mms13–ZZ fusion protein (27 kDa) was also confirmed with anti-mouse IgG-AP (Fig. 2, lower bands). These results indicate that the scFv–Fc fusion protein was successfully bound onto BacMPs via the interaction with ZZ in the process of BacMPs extraction. Unexpectedly, most of the binding of scFv–Fc onto ZZ-BacMPs completed at 0 min, and reached plateau at more than 10 min (Fig. 2, upper bands). These results indicate that 10 min after the complete disruption of cells is enough to magnetically separate BacMPs from the cell lysate of AMB-1 transformant harboring pUM13ZZ/scFvFc.

3.2. Structure of scFv–Fc on BacMPs

In general, Fc–scFvs are known to form an antibody-like dimerized structure via disulfide bonds between hinge regions and interaction between CH3 regions [28–30]. To investigate the structure of recombinant scFv–Fc, the Western blot analysis was performed using anti-FLAG antibodies. The protein samples were prepared in the presence (reduced condition) and absence (non-reduced condition) of a reducing agent (DTT) to maintain the S–S bond formation of proteins. In reduced condition, the expression of recombinant proteins (Mms13–ZZ, scFv–Fc and Mms13–scFv) was confirmed in the expected sizes (Fig. 3(A)). On the other hand, in non-reduced condition, two bands corresponding to dimeric and trimeric form of scFv–Fc (105 or 157 kDa) as well as monomeric scFv–Fc (52 kDa) were detected (Fig. 3(B), middle lane), while the Mms13–scFv was detected as a single band of the expected size (41 kDa) (Fig. 3(B), right lane).

To further investigate the folding of scFv–Fc, the Western blot analysis was performed using anti-human λ chain antibody against conformational epitope. Two bands corresponding to dimeric or trimeric form of scFv–Fc were detected with the antibody in non-reduced condition (Fig. 3(C), left lane), while no band was observed in Mms13–scFv in the same conditions (Fig. 3(C), right lane). Considering the amino acid variability in the variable region of immunoglobulin molecules, the anti-human λ chain antibody probably recognized conformational epitopes of the V_L-λ domain of scFv13R4. Therefore, we concluded from these results that periplasmically expressed scFv–Fc was correctly folded in a dimeric form with disulfide bridges and also in a trimeric form whereas Mms13–scFv was not correctly folded on BacMPs in the cytoplasm. Although the reason for the preference of the trimeric form of scFv–Fc is unknown, multimerization of scFv–Fc is also reported elsewhere [9,31].

3.3. Confirmation of the antigen binding ability of scFv–Fc bound on ZZ-BacMPs

The antigen–antibody interaction of scFv–Fc bound onto ZZ-BacMPs and β-galactosidase (antigen) was confirmed by ELISA. Biotinylated β-galactosidase was reacted with scFv–Fc/ZZ-BacMP complexes, and subsequently streptavidin-conjugated alkaline phosphatase (SA-AP) was reacted to detect the antigens. Biotin-β-galactosidase was detected on BacMPs of the scFvFc and Mms13–ZZ co-transformant but not of Mms13–ZZ single transformant (Fig. 4). In addition, biotin-β-galactosidase was not detected on BacMPs of Mms13–scFv transformant (Fig. 4). These

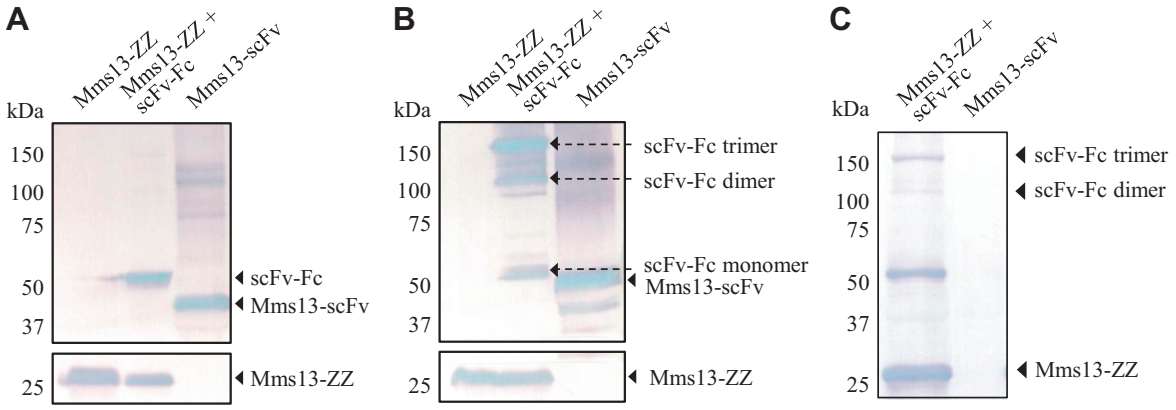

Fig. 3. Western blot analysis of Mms13–ZZ, Mms13–scFv and scFv–Fc on BacMPs prepared under reduced (A) or non-reduced (B) and (C) conditions. (A) and (B) scFv fusion proteins were detected with anti-FLAG IgG-AP, and Mms13–ZZ was detected with donkey anti-mouse IgG-AP. (C) Mms13–ZZ and scFv fusion proteins were detected with rabbit anti-human λ light chain polyclonal antibody and AP-conjugated anti-rabbit IgG. Mms13–ZZ: BacMP membrane proteins from transformant harboring pUM13ZZ; Mms13–ZZ + scFv–Fc: BacMP membrane proteins from transformant harboring pUM13ZZ/scFvFc; Mms13–scFv: BacMP membrane proteins from transformant harboring pUM13scFv.

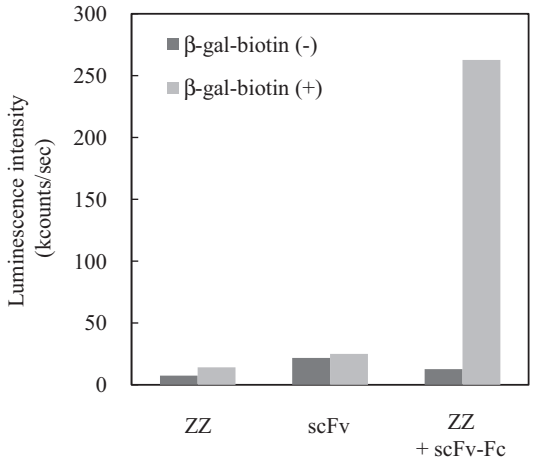

Fig. 4. β-Galactosidase binding assay on BacMPs. ZZ: BacMPs extracted from transformant harboring pUM13ZZ; scFv: BacMPs extracted from transformant harboring pUM13scFv; ZZ + scFv–Fc: BacMPs extracted from transformant harboring pUM13ZZ/scFvFc.

results indicate that periplasmically expressed scFv–Fc was correctly folded and functional.

4. Discussion

In this paper, we proposed a novel method for efficient expression of a disulfide-bonded protein on BacMPs of *M. magneticum* AMB-1 by *in vitro* docking periplasmically expressed target protein with its binding partner on the BacMPs. We chose a protein A-derived ZZ domain and an Fc-fused scFv as interaction molecules. An alternative approach for expressing antibody fragment on BacMPs has been reported; i.e. the single-domain antibody fragment (referred to as sdAb, VHH or nanobody) was functionally expressed on BacMPs in the cytoplasm [32]. sdAbs are better suited for intrabody applications because of their rigid folding and stability [33], however, they are prepared by immunization of *camelids or* sharks, thus the availability is limited. In contrast, scFvs are commonly available, and Fc-fused scFvs are known to form an antibody-like dimerized structure via disulfide bonds between hinge regions and interaction between CH3 regions. This provides an avidity effect of bivalent binding. Therefore, our proposed approach based on *in vitro* docking will expand the possibilities for the development of antibody and magnetic particle complexes.

The scFv (scFv13R4) against β-galactosidase used in this study is artificially engineered to improve the solubility and *in vivo* folding in the *E. coli* cytoplasm. However, even highly-soluble scFv13R4 could not be functionally expressed on BacMPs in the cytoplasm of *M. magneticum*, although Mms13-scFv13R4 was expressed on BacMPs in the expected size (Fig. 3(A)). Possible reasons for this could be explained as followings; first, Mms13-scFv13R4 may not have had enough time to be folded correctly in *M. magneticum* AMB-1. Secondly, N-terminally fused Mms13 may have completely blocked the binding to β-galactosidase.

In conclusion, we demonstrated a successful method of expressing disulfide-bonded proteins on BacMPs by *in vitro* docking. BacMPs produced by the proposed method may be useful for screening of potential ligands or drugs, analyses of ligand–receptor interactions or as magnetic carriers for affinity purification of binding partners. Further study will be necessary to see whether this system will be effective with various disulfide-bonded proteins.

References

[1] S. Pollitt, H. Zalkin, Role of primary structure and disulfide bond formation in beta-lactamase secretion, J. Bacteriol. 153 (1983) 27–32.
[2] A. Derman, J. Beckwith, *Escherichia coli* alkaline phosphatase fails to acquire disulfide bonds when retained in the cytoplasm, J. Bacteriol. 173 (1991) 7719–7722.
[3] A. de Marco, Strategies for successful recombinant expression of disulfide bond-dependent proteins in *Escherichia coli*, Microb. Cell Fact. 8 (2009) 26.
[4] T. den Blaauwen, A.J. Driessen, Sec-dependent preprotein translocation in bacteria, Arch. Microbiol. 165 (1996) 1–8.
[5] J. Luirink, I. Sinning, SRP-mediated protein targeting: structure and function revisited, Biochim. Biophys. Acta 1694 (2004) 17–35.
[6] J. Hall, G.P. Hazlewood, M.A. Surani, B.H. Hirst, H.J. Gilbert, Eukaryotic and prokaryotic signal peptides direct secretion of a bacterial endoglucanase by mammalian cells, J. Biol. Chem. 265 (1990) 19996–19999.
[7] A. Arakaki, J. Webb, T. Matsunaga, A novel protein tightly bound to bacterial magnetic particles in *Magnetospirillum magneticum* strain AMB-1, J. Biol. Chem. 278 (2003) 8745–8750.
[8] T. Yoshino, T. Matsunaga, Development of efficient expression system for protein display on bacterial magnetic particles, Biochem. Biophys. Res. Commun. 338 (2005) 1678–1681.
[9] T. Yoshino, T. Matsunaga, Efficient and stable display of functional proteins on bacterial magnetic particles using Mms13 as a novel anchor molecule, Appl. Environ. Microbiol. 72 (2006) 465–471.
[10] Y. Kanetsuki, M. Tanaka, T. Tanaka, T. Matsunaga, T. Yoshino, Effective expression of human proteins on bacterial magnetic particles in an anchor gene deletion mutant of *Magnetospirillum magneticum* AMB-1, Biochem. Biophys. Res. Commun. 426 (2012) 7–11.
[11] Y. Maeda, T. Yoshino, M. Takahashi, H. Ginya, J. Asahina, H. Tajima, et al., Noncovalent immobilization of streptavidin on in vitro- and in vivo-biotinylated bacterial magnetic particles, Appl. Environ. Microbiol. 74 (2008) 5139–5145.

[12] T. Yoshino, A. Shimojo, Y. Maeda, T. Matsunaga, Inducible expression of transmembrane proteins on bacterial magnetic particles in *Magnetospirillum magneticum* AMB-1, Appl. Environ. Microbiol. 76 (2010) 1152–1157.
[13] Y. Maeda, T. Yoshino, T. Matsunaga, In vivo biotinylation of bacterial magnetic particles by a truncated form of *Escherichia coli* biotin ligase and biotin acceptor peptide, Appl. Environ. Microbiol. 76 (2010) 5785–5790.
[14] Y. Kanetsuki, T. Tanaka, T. Matsunaga, T. Yoshino, Enhanced heterologous protein display on bacterial magnetic particles using a lon protease gene deletion mutant in *Magnetospirillum magneticum* AMB-1, J. Biosci. Bioeng. 116 (2013) 65–70.
[15] Y. Sugamata, R. Uchiyama, T. Honda, T. Tanaka, T. Matsunaga, T. Yoshino, Functional expression of thyroid-stimulating hormone receptor on nano-sized bacterial magnetic particles in *Magnetospirillum magneticum* AMB-1, Int. J. Mol. Sci. 14 (2013) 14426–14438.
[16] T. Tanaka, T. Matsunaga, Fully automated chemiluminescence immunoassay of insulin using antibody-protein A-bacterial magnetic particle complexes, Anal. Chem. 72 (2000) 3518–3522.
[17] T. Yoshino, F. Kato, H. Takeyama, M. Nakai, Y. Yakabe, T. Matsunaga, Development of a novel method for screening of estrogenic compounds using nano-sized bacterial magnetic particles displaying estrogen receptor, Anal. Chim. Acta 532 (2005) 105–111.
[18] T. Yoshino, H. Hirabe, M. Takahashi, M. Kuhara, H. Takeyama, T. Matsunaga, Magnetic cell separation using nano-sized bacterial magnetic particles with reconstructed magnetosome membrane, Biotechnol. Bioeng. 101 (2008) 470–477.
[19] R. Rudolph, H. Lilie, In vitro folding of inclusion body proteins, FASEB J. 10 (1996) 49–56.
[20] F. Yang, L.G. Moss, G.N. Phillips, The molecular structure of green fluorescent protein, Nat. Biotechnol. 14 (1996) 1246–1251.
[21] Y. Ohmiya, F.I. Tsuji, Mutagenesis of firefly luciferase shows that cysteine residues are not required for bioluminescence activity, FEBS Lett. 404 (1997) 115–117.
[22] B. Nilsson, T. Moks, B. Jansson, L. Abrahmsén, A. Elmblad, E. Holmgren, et al., A synthetic IgG-binding domain based on staphylococcal protein A, Protein Eng. 1 (1987) 107–113.
[23] T. Matsunaga, T. Sakaguchi, F. Tadakoro, Magnetite formation by a magnetic bacterium capable of growing aerobically, Appl. Microbiol. Biotechnol. 35 (1991) 651–655.
[24] J. Sambrook, D.W. Russell, Molecular Cloning: A Laboratory Manual, Cold Spring Harbour Press, 2001.
[25] P. Martineau, P. Jones, G. Winter, Expression of an antibody fragment at high levels in the bacterial cytoplasm, J. Mol. Biol. 280 (1998) 117–127.
[26] X. Liu, C.C. Wang, Disulfide-dependent folding and export of *Escherichia coli* DsbC, J. Biol. Chem. 276 (2001) 1146–1151.
[27] Y. Okamura, H. Takeyama, T. Sekine, T. Sakaguchi, A.T. Wahyudi, R. Sato, et al., Design and application of a new cryptic-plasmid-based shuttle vector for *Magnetospirillum magneticum*, Society 69 (2003) 4274–4277.
[28] K.M. Lo, Y. Sudo, J. Chen, Y. Li, Y. Lan, S.M. Kong, et al., High level expression and secretion of Fc-X fusion proteins in mammalian cells, Protein Eng. 11 (1998) 495–500.
[29] A.J. Bitonti, J.A. Dumont, S.C. Low, R.T. Peters, K.E. Kropp, V.J. Palombella, et al., Pulmonary delivery of an erythropoietin Fc fusion protein in non-human primates through an immunoglobulin transport pathway, Proc. Natl. Acad. Sci. USA 101 (2004) 9763–9768.
[30] D.B. Powers, P. Amersdorfer, M. Poul, U.B. Nielsen, M.R. Shalaby, G.P. Adams, et al., Expression of single-chain Fv–Fc fusions in *Pichia pastoris*, J. Immunol. Methods 251 (2001) 123–135.
[31] H. Sonoda, Y. Kumada, T. Katsuda, H. Yamaji, Cytoplasmic production of soluble and functional single-chain Fv–Fc fusion protein in *Escherichia coli*, Biochem. Eng. J. 53 (2011) 253–259.
[32] A. Pollithy, T. Romer, C. Lang, F.D. Müller, J. Helma, H. Leonhardt, et al., Magnetosome expression of functional camelid antibody fragments (nanobodies) in *Magnetospirillum gryphiswaldense*, Appl. Environ. Microbiol. 77 (2011) 6165–6171.
[33] V. Alzogaray, W. Danquah, A. Aguirre, M. Urrutia, P. Berguer, E. García Véscovi, et al., Single-domain llama antibodies as specific intracellular inhibitors of SpvB, the actin ADP-ribosylating toxin of *Salmonella typhimurium*, FASEB J. 25 (2011) 526–534.