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Molecular analysis of magnetotactic bacteria and development of functional bacterial magnetic particles for nano-biotechnology

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Biomineralization is an elaborate process that produces complex nano-structures consisting of organic and inorganic components of uniform size and highly ordered morphology that self-assemble into structures in a hierarchical manner. Magnetotactic bacteria synthesize nano-sized magnetite crystals that are highly consistent in size and morphology within bacterial species; each particle is surrounded by a thin organic membrane, which facilitates their use for various biotechnological applications. Recent molecular studies, including mutagenesis, whole genome, transcriptome and comprehensive proteome analyses, have elucidated the processes important to bacterial magnetite formation. Some of the genes and proteins identified from these studies have enabled us, through genetic engineering, to express proteins efficiently, with their activity preserved, onto bacterial magnetic particles, leading to the simple preparation of functional protein–magnetic particle complexes. This review describes the recent advances in the fundamental analysis of bacterial magnetic particles and the development of surface-protein-modified magnetic particles for biotechnological applications.

Magnetic nano-particles synthesized in magnetotactic bacteria

Magnetic particles are currently one of the most important materials in the industrial sector, where they have been widely used for biotechnological and biomedical applications [1–3]. The use of surface-modified magnetic particles in bioassays enables the separation of bound and free analytes by the application of a magnetic field.

Magnetotactic bacteria synthesize uniform, nano-sized magnetite (Fe_3O_4) particles, which are referred to as 'bacterial magnetic particles' (BacMPs) (Figure 1a). Each magnetic particle that is synthesized by magnetotactic bacteria possesses a magnetic dipole moment with a single magnetic domain [4,5]. The crystal size and morphology are considered to be precisely controlled by biological factors within the magnetotactic bacteria [6–8]. In contrary to artificial magnetic particles, BacMPs can be easily dispersed in aqueous solutions because of their enclosing membrane; therefore, BacMPs have potential for various technological applications, and the molecular mechanism of their formation is of particular interest [9]. Based on the molecular studies of BacMP biomineralization from genomics, proteomics and transcriptomics analyses of *Magnetospirillum magneticum* AMB-1 (Figure 1b), methods for the construction of functional BacMPs were established through genetic engineering. Furthermore, high-throughput immunoassays [10,11], SNP (single nucleotide polymorphism) discrimination [12,13] and DNA recovery systems [14] have been investigated for the application of BacMPs. Owing to their suitable size and magnetic properties, BacMPs have also been proposed as potential material for hyperthermia magnetotherapy [15].

This review describes the recent molecular studies on magnetite biomineralization in magnetotactic bacteria, new ideas for the development of functional BacMPs, and the applications of these unique biomaterials are also introduced.

Genome analysis of *Magnetorpirillm magneticum* AMB-1

During the past few years, the genomes of several magnetotactic bacteria have been sequenced, and the complete genome sequence of the magnetotactic coccus MC-1 has been compiled (http://genome.jgi-psf.org/microbial/). We have sequenced the genomic DNA of *M. magneticum* AMB-1 using a conventional whole genome shotgun strategy [16]: the genome consists of a single, circular chromosome of 4 967 148 bp (Figure 2). The genes were annotated, with an E value 1×10^{-1}. However, almost half of the 4559 open reading frames (ORFs) remained unknown with respect to functional predictions, whereas a remarkable number of sensor and response domains were identified. The inversions of GC content, or spikes, are observed mainly in IS insertion positions, of which the genome contains 33. Most of these are intensively localized in three specific regions, which have a lower GC content than the average of the whole genome, suggestive of gene transfer from other bacteria or phage.

An 80 kb cluster encoding proteins involved in BacMP formation, such as *mms6*, *mamA* and *mamK*, was identified from a spontaneous, non-magnetic mutant

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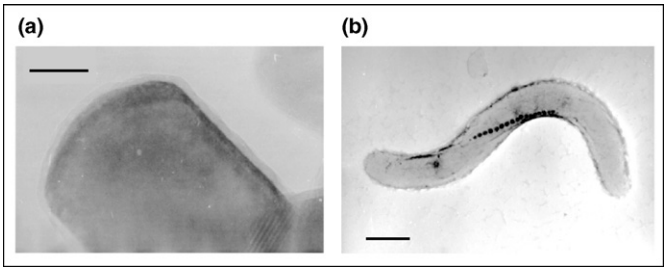

Figure 1. Transmission electron micrographs of **(a)** bacterial magnetic particle (BacMP) (Scale bar = 20 nm) and **(b)** *Magnetospirillum magneticum* AMB-1 cell (Scale bar = 500 nm). Reproduced from [55], with permission from Elsevier.

of *Magnetospirillum gryphyswaldense* MSR-1 [17]: a 35 kb region of this cluster contains one of a pair of IS 66 elements. Furthermore, multiple deletions of this genomic region in non-magnetic mutants of MSR-1 were reported [18]. During the genome analysis of *M. magneticum* AMB-1 we have also obtained a spontaneous non-magnetic mutant that lacks a 98 kb genomic region [19], which has the characteristics of a genomic island: it has a low GC content; it is located between two 1.1 kb repetitive sequences; and an integrase is present in the flanking region of the first repetitive sequence. The spontaneous deletion of this 98 kb genomic region and the circular form after excision from the chromosome were detected by PCR amplification. The island appears to be derived from lateral gene transfer, considering the typical structure of a genomic island and the dynamics after excision from the chromosome.

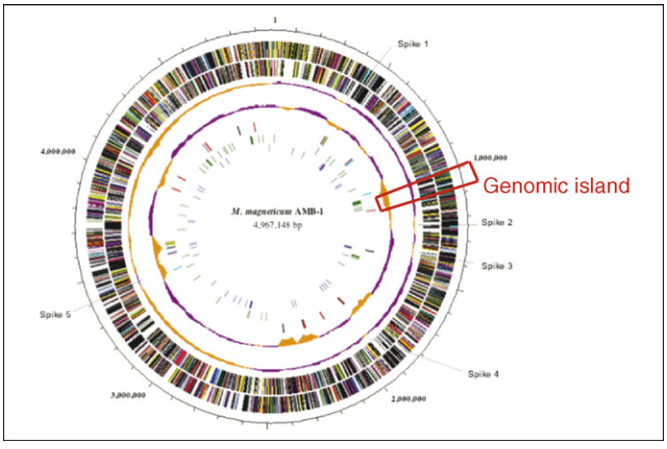

Figure 2. Genome map of *Magnetospirillum magneticum* AMB-1. The outer and first inner circles represent predicted ORFs on the plus and minus strands, respectively (salmon: translation, ribosomal structure and biogenesis; light blue: transcription; cyan: DNA replication, recombination and repair; turquoise: cell division; deep pink: post-translational modification, protein turnover and chaperones; olive drab: cell envelope biogenesis; purple: cell motility and secretion; forest green: inorganic ion transport and metabolism; magenta: signal transduction; red: energy production; sienna: carbohydrate transport and metabolism; yellow: amino acid transport; orange: nucleotide transport and metabolism; gold: co-enzyme transport and metabolism; dark blue: lipid metabolism; blue: secondary metabolites, transport and catabolism; gray: general function prediction only; black: function unclassified or unknown). The second inner circle represents GC skew: purple indicates >0, orange indicates <0. The third inner circle further represents GC content: purple indicates higher than average, orange indicates less than average. The fourth inner circle represents insertion sequence (IS) elements (black: ISmag1; orange: ISmag2; pink: ISmag3; purple: ISmag4; blue: ISmag5; red: ISmag6; light blue: ISmag7; light green: ISmag8; green: ISmag9; brown: ISmag10; yellow: ISmag11). The fifth and sixth inner circles indicate the genes encoding magnetosome membrane proteins and loci of genes disrupted by transposons, respectively. Figure reproduced from [16] with permission from Oxford University Press.

Transcriptome analysis of iron-uptake genes

M. magneticum AMB-1 takes up approximately a hundred times more iron ions than non-magnetic bacteria, to synthesize intracellular BacMPs. To identify global gene expression profiles, particularly those implicated in iron-uptake, transcriptome analysis through microarrays imprinted with DNA from the whole genome sequence of AMB-1 was investigated. The global expression analysis revealed five gene expression profile patterns [20]: three ferrous and two ferric iron-uptake systems encoded by 16 genes were identified from the genome sequence. High-affinity ferrous iron transport genes were up-regulated in BacMP-forming cells grown under iron-rich conditions, and ferric iron transport genes were down-regulated under these conditions. Under iron-rich conditions, wherein BacMP synthesis occurs, the ferrous-iron-uptake system is dominant, whereas ferric iron or siderophore transport genes were only expressed after the initial high iron concentrations were taken up by the magnetic cells. This is consistent with our previous report on the siderophore production of *M. magneticum* AMB-1 grown in media containing different initial iron ions [21,22]. Furthermore, enhancement of BacMP production was observed when ferrous iron was used as the iron source [23]. These results suggest that ferrous-iron-uptake systems mainly serve as iron supply lines for BacMP formation. The results also indicate that despite the unusually high iron requirement of *M. magneticum* AMB-1, it uses robust but simple iron-uptake systems, similar to those of other Gram-negative bacteria. It was also shown that AMB-1 possesses unique regulation systems for the uptake of iron.

Proteome analysis of BacMP membrane

Proteins associated with biominerals are known to have a direct role in the BacMP formation process [24,25]. To elucidate the molecular mechanism of BacMP formation, comprehensive proteome analyses of BacMP membrane proteins have been conducted in *M. magneticum* AMB-1 [26–29], *M. gryphiswaldense* MSR-1 [30,31] and *M. magnetotacticum* MS-1 [32]; from these, many novel proteins showing potentially crucial roles in BacMP formation have been discovered. In the analysis of BacMP membrane proteins from *M. gryphiswaldense* MSR-1, ~30 proteins were identified; many of these were found to be assigned in gene clusters located within the genomic island [30,31]. A similar examination was attempted with *M. magneticum* AMB-1, where a total of 78 proteins were identified from the surface of its BacMP [29]. To investigate the origin of BacMP membrane proteins acquired from *M. magneticum* AMB-1, cells were separated into outer membrane, cytoplasmic membrane, BacMP membrane and cytoplasm–periplasmic fractions, accordingly. Although apparent contaminants were also observed in the BacMP membrane protein fraction of AMB-1, some of these, such as cytochromes, dehydrogenases and ATPase subunits, might possibly function in the formation of BacMPs. A high degree of similarity was observed between the protein profiles of the BacMP membrane and the cytoplasmic membrane of *M. magneticum* AMB-1. One surprising observation was the identification of the Mms13 protein, which is associated with the BacMP surface, within the

cytoplasmic membrane fraction as well as in the BacMP membrane fraction [29] – comparative analysis of the fatty acids also indicated that both these fractions showed similar profiles. These results suggest that the BacMP membrane could have been derived from the cytoplasmic membrane. Direct evidence of this phenomenon was recently reported by Komeili *et al.* [33], who clearly observed, using electron cryotomography, invagination from the cytoplasmic membrane to form vesicles.

Further functional analysis related to BacMP formation was performed on several key proteins from the BacMP membrane fraction. One of the dominant proteins in the BacMP membrane is a 24 kDa protein designated as Mms24 (identical to Mam22 and MamA) [26,30,32]. An AMB-1 mutant strain harboring a defect in the gene encoding this protein formed fewer BacMPs in comparison with the wild type, suggesting that the protein might be required for the activation of BacMP vesicles [34]. Another key protein, Mms16, is a small GTPase that is speculated to prime the invagination of the cytoplasmic membrane for vesicle formation in *M. magneticum* AMB-1 [35]. A protein with high sequence similarity to Mms16 was also discovered in *M.*

Box 1. Potential applications of bacterial magnetic particles (BacMPs).

- Immunoassays for human disease diagnosis or environmental assessment
- Cell separation
- DNA and/or RNA recovery
- DNA discrimination within species (e.g. bacteria)
- Detection of single nucleotide polymorphisms (SNPs) related to human disease
- Receptor-binding assay for drug screening
- Drug delivery
- Hyperthermia
- Magnetic probes
- Material for magnetic force microscopy (MFM) cantilever

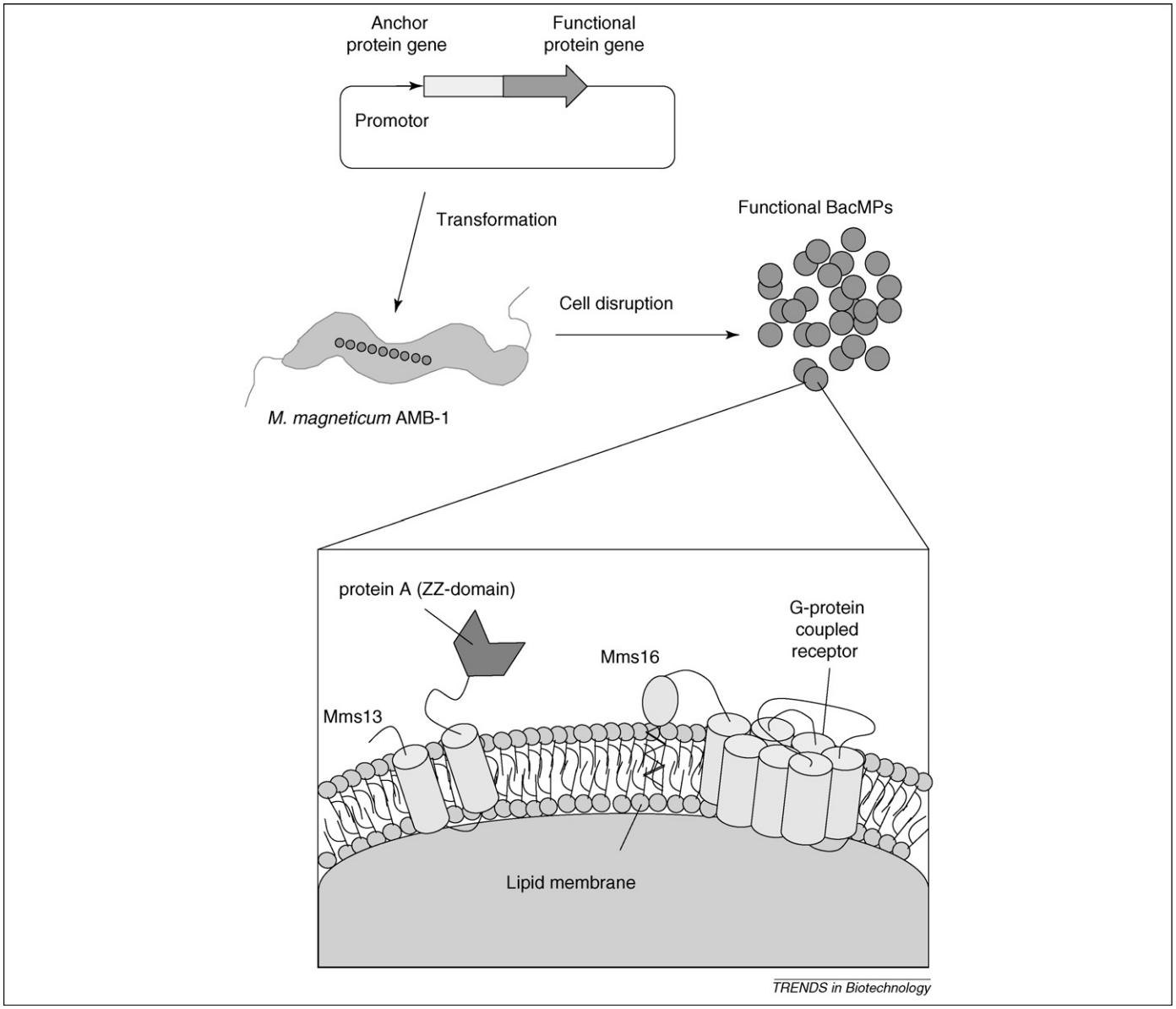

Figure 3. Schematic diagram for the preparation of functional bacterial magnetic particles (BacMP) using the protein display technique. To express a functional protein onto BacMPs, the functional protein gene was fused to an anchor protein gene. A plasmid containing the fusion gene was transformed into *M. magneticum* AMB-1. Expression efficiency is strongly dependent on the expression level of the anchor protein. The functional BacMPs produced were extracted by disrupting cells.

gryphiswaldense MSR-1, but this protein was shown to activate poly(3-hydroxybutyrate) depolymerization [36]. Both the proteins have high similarity, but functional differences between them were observed: there was no P-loop structure and GTPase activity from the protein discovered in the MSR-1 strain. Other key proteins relevant to BacMP formation include: the MpsA protein, which is a homologue of acyl-CoA carboxylase (transferase) and possibly functions as a mediator of the invagination process [27]; the transmembrane transporter protein MagA, which is the most probable candidate in iron uptake [37]; and the MamB and MamM proteins, which are also speculated to be responsible for iron transportation into the BacMP-formation vesicles [17]. The Mms6 protein is a small iron-binding protein that was isolated from the magnetite crystal surface and is postulated to initiate magnetite crystal formation and/or regulate morphology by producing a self-assembled framework structure [28]. Crucial proteins for BacMP alignment in the cell have also been reported. MamK is a homolog of a bacterial actin-like protein that is proposed to form filaments to establish the specific localization of BacMPs [33]. The acidic protein MamJ is thought to be associated with the filamentous structure and the direction of the assembly of BacMP chain [38], indicating the presence of a highly organized ultrastructural system in magnetotactic bacteria that is similar to eukaryotic cells.

The results obtained from these studies with regards to the *Magnetospirillum* strain enabled us to postulate that BacMP formation comprises several stages, such as vesicle formation, iron uptake, and crystallization [7,8]. Further studies, including comparative analyses of genes and proteins in different magnetotactic bacterium, will elucidate the detail of this highly controlled magnetite biomineralization mechanism.

Molecular design of functional BacMPs by bioengineering

The particulate materials produced by assembling functional molecules on nanoparticles can be used for various biotechnological applications (Box 1). The extensive genome and proteome research outlined in the previous sections enables the development of functional BacMPs.

The activity and stability of assembled proteins are strongly dependent on the environment surrounding them [39,40]. Functional proteins were displayed on BacMPs through the development of a fusion technique involving anchor proteins isolated from *M. magneticum* AMB-1 [41–44]. The fusion proteins usually become highly dependent on the structural properties of the inserted foreign protein domain because the protein will be more constrained when inserted into the permissive site of an anchor molecule [45].

A transmembrane protein, MagA, was used as an anchor molecule for the expression of foreign proteins on the surface of BacMPs. Soluble proteins, such as luciferase [41], protein A [42] and acetate kinase [46], were expressed on BacMPs using this anchor. However, Mms16, a novel small BacMP membrane protein, was also considered to be useful for the expression of large transmembrane proteins because it has less effect on the expression of foreign proteins through dimensional obstruction. For example, efficient expression of a seven-transmembrane protein on BacMPs was successfully achieved using Mms16 [46] (Figure 3). A newly found integral BacMP membrane protein, Mms13 (identical to the MamC in *M. gryphiswaldense*) [30], was also investigated for its efficiency as an anchor molecule to display functional proteins on BacMPs [28]. The luminescence intensity observed from BacMPs that used Mms13 as an anchor molecule was 400 to 1000 times higher than those that used Mms16 or MagA as anchor molecules [44]. To establish highly expressed protein displays on BacMPs, strong promoters were also identified, based on the AMB-1 genome database and proteome analysis [47].

As an alternative method for protein display on BacMPs, *in vitro* reconstitution of fusion proteins with integral membrane proteins or peptides into lipid vesicles was also developed [48]. A biotin-modified antimicrobial peptide (temporin L) was used as an anchor peptide to immobilize streptavidin on BacMPs. Temporin L forms an amphiphilic structure, with the hydrophobic part organized in a helix when associated with the membrane. This

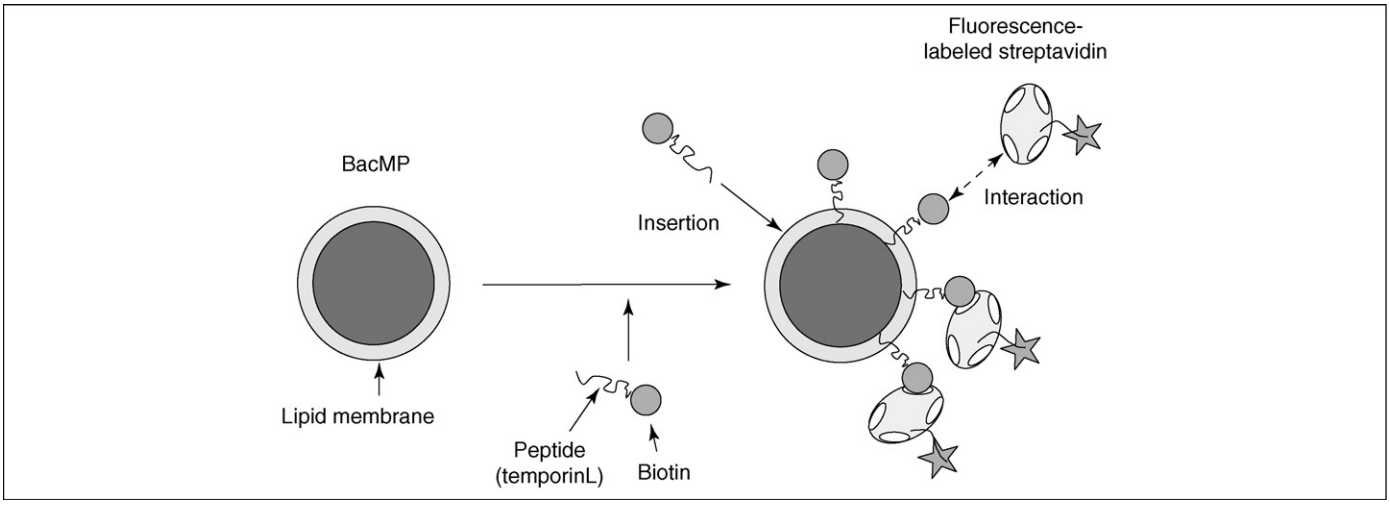

Figure 4. *In vitro* assembly of fluorescence-labeled streptavidin on bacterial magnetic particle (BacMP) using a transmembrane peptide. A short peptide, temporin L, conjugated to the biotin molecule was inserted into the BacMP membrane. Fluorescence-labeled streptavidin was then immobilized on the biotin-inserted BacMP through biotin–streptavidin interaction. Using the same principle, this technique can be used for the immobilization of antibodies and the other soluble proteins.

spontaneous integration mechanism was applied for protein assembly onto BacMPs [49]. The C-terminal of temporin L was incorporated into a BacMP membrane, and the N-terminus was located on the BacMP membrane surface to bind to the functional protein (Figure 4). Improved efficiency of integration of the functional proteins is obtained by selecting the most suitable anchor molecule under optimized conditions.

Application of functional BacMPs to immunoassay and receptor binding assay

The Z-domain of protein A (Box 2) was assembled on the BacMP surface by a gene fusion technique [42]. When a chemiluminescence sandwich immunoassay was carried out using antibody–protein A–BacMP complexes and BacMPs chemically immobilized with antibodies, the antigen-binding activity per microgram of antibodies on antibody–protein A–BacMP complexes was two times higher than that for antibody–BacMP conjugates prepared by chemical immobilization. Human insulin concentrations in blood serum were measured by a fully automated sandwich immunoassay using antibody–protein A–BacMP complexes and alkaline phosphatase-conjugated antibodies as primary and secondary immunoreactants, respectively [10].

BacMPs displaying G-protein-coupled receptors (GPCRs) (Box 2) were developed and used for receptor-binding assays: BacMPs displaying D1 dopamine receptor (D1R) [45] or estrogen receptor ligand-binding domain (ERLBD) [43] were used as models. Efficient assembly of D1R into the lipid membrane of nanosized BacMPs was accomplished, and this was used in a competitive dopamine-binding assay. This novel system provides advantages for studying membrane proteins that are usually difficult to assay.

Box 2. Functional proteins displayed on bacterial magnetic particle (BacMP).

The display of heterologous proteins maintained in a biologically active form on the surface of supports is an important task, with potential applications in a variety of fields ranging from biosensors to diagnostics. Protein A is an immunoglobulin (IgG)-binding cell wall protein, which is known to be a virulence factor of *Staphylococcus aureus*. The IgG-binding site consists of a tandem repeat of five IgG-binding domains – E, D, A, B, and C. The Z domain is an engineered analogue of the IgG-binding domain B. Protein A can be used to immobilize antibody molecules in a highly oriented manner and maintain antibody molecular functional configuration on a solid-phase surface, unlike in immobilization by chemical conjugation, making it possible to construct highly sensitive immunoassays.

G-protein-coupled receptors (GPCRs) are located at the cell surface and are responsible for the transduction of an endogenous signal into an intracellular response. The natural ligands of this receptor superfamily are extremely diverse, comprising peptide and protein hormones, nucleotides, lipids and others. Binding of these specific ligands to the extracellular or transmembrane regions causes conformational changes in the receptor and, consequently, inhibition or stimulation of the production of intracellular secondary messengers. Therefore, molecules that bind to these receptors as agonists or antagonists might be candidate medicines for various diseases. However, these proteins are generally expressed at low levels in the cell and are extremely hydrophobic, rendering the analysis of ligand interaction difficult. In addition, purification of GPCRs from cells is time-consuming and usually results in the loss of the native conformation. By contrast, use of GPCRs displayed on BacMPs conveniently refines the native conformation of GPCRs without the need for detergent solubilization, purification and reconstitution after cell disruption. This also provides an advantage when applied to automated ligand-screening systems using magnetic separation.

Box 3. Advantages of magnetic nanoparticles for magnetic cell-separation.

Magnetic cell-separation using immunomagnetic particles has become a popular tool for the isolation of target cells from cell suspensions because of its rapid and simple methodology, compared with cell sorting using a flow cytometer [53,54]. The separation of target cells typically uses a combination of chemically synthesized magnetic particles and antibodies against the target cell-surface antigen. Commercially available magnetic particles are superparamagnetic and require the use of specially designed magnetic columns to produce a high magnetic field gradient for cell separation. In general, nano-sized magnetic particles, rather than micro-sized beads, are preferred for cell separation because of their advantages in assay sensitivity, rapidity, precision and lack of toxicity to the cells. However, it is difficult to synthesize nano-sized magnetic particles that are uniform in size and shape and possess good dispersion properties in aqueous solutions; therefore, novel techniques are required for their production.

Application of BacMPs to cell separation

Immunomagnetic particles have been used, preferentially, in target cell separation from leukocytes as an *in vitro* diagnostic because of the rapid and simple methodology compared with cell sorting using flow cytometer [50]. BacMPs consist of ferromagnetic iron oxide and are, thus, easily separated from cell suspensions using a permanent magnet (Box 3). We have applied BacMPs to develop a highly efficient magnetic cell-separation system. BacMPs expressing protein A (protein A–BacMPs) binding to the Fc fragment of an anti-mouse IgG was used to separate mononuclear cells from peripheral blood [51]. The procedure of positive selection involves incubation of mononuclear cells and mouse monoclonal antibodies against different cell surface antigens (CD8, CD14, CD19 and CD20) before treatment with protein A–BacMP binding to rabbit anti-mouse IgG secondary antibodies. The average purities of the separated mononuclear cells for $CD19^+$ and $CD20^+$ were 97.5% and 97.6%, respectively; and >95% of the recovery ratio was obtained for these cells. Furthermore, in the evaluation of the influence of protein A–BacMPs towards the separated cells, the $CD14^+$ cells separated using protein A–BacMPs were successfully differentiated into dendritic cells [52].

Conclusion

Comprehensive molecular studies on the genes and proteins involved in BacMP formation have led to a more complete postulation on the step-by-step events of magnetite biomineralization, although the details of some of these steps remains unclear. Some of the genes and proteins identified from these fundamental studies have enabled us to develop novel molecular constructs on the BacMP surface. The constructed functional BacMPs generated by both chemical processes and genetic manipulations are useful for various biotechnological applications. The use of functional BacMPs in a fully automated system

provides simple, precise, rapid and high-throughput analyses by using a magnetic separation technique. Further studies will facilitate the development of more advanced biomaterials for a wide array of nano-biotechnological applications.

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