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Bacterial ghosts as antigen delivery vehicles

Ulrike Beate Mayr[a], Petra Walcher[a], Chakameh Azimpour[a], Eva Riedmann[a,b], Christoph Haller[a], Werner Lubitz[a,b,*]

[a]*Department of Medical/Pharmaceutical Chemistry, University of Vienna, A-1090 Vienna, Austria*
[b]*BIRD-C GmbH and CoKEG, Schoenborngasse 12, A-1080 Vienna, Austria*

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Abstract

The bacterial ghost system is a novel vaccine delivery system unusual in that it combines excellent natural intrinsic adjuvant properties with versatile carrier functions for foreign antigens. The efficient tropism of bacterial ghosts (BG) for antigen presenting cells promotes the generation of both cellular and humoral responses to heterologous antigens and carrier envelope structures. The simplicity of both BG production and packaging of (multiple) target antigens makes them particularly suitable for use as combination vaccines. Further advantages of BG vaccines include a long shelf-life without the need of cold-chain storage due to their freeze-dried status, they are safe as they do not involve host DNA or live organisms, they exhibit improved potency with regard to target antigens compared to conventional approaches, they are versatile with regards to DNA or protein antigen choice and size, and as a delivery system they offer high bioavailability.

Keywords: Target antigen; Gram-negative bacterial envelope; Particle presentation technology; DNA vaccine; Adjuvant; Delivery system; Bacterial ghosts

Contents

Abbreviations: APC, antigen presenting cells; APP, *Actinobacillus pleuropneumoniae*; BG, bacterial ghosts; CPS, cytoplasmic space; DC, dendritic cells; GFP, green fluorescent protein; IM, inner membrane; LPS, lipopolysaccharide; MBP, maltose binding protein; OM, outer membrane; OMP, outer membrane protein; PBMC, peripheral blood derived monocytic cells; PPS, periplasmic space; StrpA, streptavidin; TA, target antigen; TCP, toxin-co-regulated pili; VCG, *Vibrio cholerae* ghosts.

* Corresponding author. Department of Medical/Pharmaceutical Chemistry, University of Vienna, A-1090 Vienna, Austria. Tel.: +43 1 4277 55115; fax: +43 1 406 50 93.
E-mail addresses: Werner.Lubitz@univie.ac.at, Lubitz@bird-c.com (W. Lubitz).

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1. Introduction

Subunit vaccines composed of purified components can be produced from many microorganisms; however, they are often poorly immunogenic necessitating an appropriate adjuvant in the vaccine formulation. Similarly, for DNA vaccines to reach their full potential, new vaccine delivery systems need to be developed which better activate mucosal immune responses.

The bacterial ghost system is one such vaccine delivery system, combining targeting of antigen components to antigen presenting cells (APC) and providing the required adjuvant activity without the need for further additions. BG are produced by protein E-mediated lysis of Gram-negative bacteria. They are non-living bacterial envelopes, which maintain the cellular morphology and native surface antigenic structures including bioadhesive properties of the natural cell. Lipopolysaccharide (LPS) present in the outer membrane (OM) does not limit the use of BG as vaccine candidates because of the minimal toxicity of cell-associated LPS compared to free LPS. The intrinsic adjuvant properties of BG enhances T-cell activation and systemic, mucosal and cellular immunity to target antigens.

E-mediated lysis has been achieved in various Gram-negative bacteria, including *Escherichia coli* K12, enterohaemorrhagic (EHEC) and entertoxigenic (ETEC) strains, *Actinobacillus pleuropneumoniae*, *Bordetella bronchiseptica*, *Erwinia cypripedii*, *Helicobacter pylori*, *Klebsiella pneumoniae*, *Mannheimia haemolytica*, *Pasteurella multocida*, *Pseudomonas putida*, *Ralstonia eutropha*, *Salmonella typhimurium* and *enteritidis* strains, and *Vibrio cholerae*. This broad spectrum of bacteria shows that E-mediated lysis most probably works in every Gram-negative bacterium, provided that the E specific lysis cassette can be introduced into the new recipient by an appropriate vector allowing tight repression and induction control of lethal gene E. Although BG have been used as vaccine candidates against their own envelope structures, a more practical use remains versatile carrier and adjuvant vehicles for foreign target antigens of bacterial or viral origin. As described in more detail in the following sections, BG have the capacity for pre-lysis localization of target antigens in or a combination of the OM, the inner membrane (IM), the periplasmic space (PPS) and the internal lumen of the cytoplasmic space (CPS). The choice of antigen compartmentalization gives the bacterial ghost system significant potential for the challenges of constructing subunit or DNA human and veterinary vaccines.

2. Production of bacterial ghosts

BG are produced by expression of cloned gene *E* from bacteriophage PhiX174 resulting cell lysis in Gram-negative bacteria. Expression of gene *E* can be placed under transcriptional control of either the thermosensitive λpL/pR-cI857 promoter, or under chemical inducible promoter repressor systems, like lacPO or the tol expression system [1–3]. Mutations to the λpR promoter/operator regions have resulted in new expression systems, which stably repress gene *E* expression at temperatures of up to 37 °C, but still allowed induction of cell lysis at a temperature range of 39–42 °C [4]. Alternatively, by combining the λpR promoter/cI repressor system with the lacI/lacPO for control of gene *E* expression, a cold-sensitive system for ghost formation by lowering the growth temperature of the bacteria from 37 °C or higher to 28 °C or lower has been obtained [5].

Gene *E* codes for a membrane protein of 91 amino acids, which is able to fuse inner and outer membranes of Gram-negative bacteria [6,7], forming an E-specific lysis tunnel through which the cytoplasmic content is expelled [8]. The remaining empty CPS (internal lumen) of the bacteria is largely devoid of

nucleic acids, ribosomes or other constituents, whereas the IM and OM structures of the BG are well preserved [6,9]. The IM remains intact during expulsion of cytoplasmic material and electron micrographs clearly show a sealed PPS [6,8]. The protein E-induced occurrence of lysophosphatidylethanol-amine in the host cell membrane most probably facilitates the IM and OM fusion [10]. Although E-mediated lysis is dependent on activities of the autolytic system of the bacteria, peptidoglycan sacculi prepared from E-lysed cells remain intact emphasizing that the overall composition of this rigid layer is not changed by the E-mediated process. The diameter of the E-specific transmembrane tunnel structure ranges between 40 and 200 nm and is determined by the mesh size of the surrounding murein [11]. Electron microscopic studies emphasize that the E-specific transmembrane tunnel structure is not randomly distributed over the cell envelope but is restricted to areas of potential division sites, predominantly in the middle of the cell or at polar sites [6,8]. Analysis of E-mediated lysis in bacterial mutant strains with defects in cell division suggest that initiation of cell division rather than specific functions of the septosome plays an essential role in protein E-mediated lysis [9,11].

The E-specific membrane fusion process can be divided into three phases including the integration of protein E into the IM, followed by a conformational change of protein E and assembly into multimers at potential cell division sites [12]. The mechanism for the conformational change is most probably a *cis–trans* isomerization of the proline 21 residue within the first membrane-embedded α-helix of protein E [13]. The local fusion of the IM and OM is achieved by a transfer of the C-terminal domain of protein E towards the surface of the OM of the bacterium.

BG have been developed for envelope and/or heterologous antigen presentation from a range of important Gram-negative bacterial pathogens including *Francisella tularensis*, *Brucella melitensis*, enterotoxigenic and enterohemaorrhagic *E. coli* (EHEC, ETEC), and *V. cholerae*. To date, immune responses against *P. multocida*, *Mannheimia* (former *Pasteurella*) *haemolytica*, *A. pleuropneumoniae* and *V. cholerae* have been assessed in several animal models for parenteral, oral and aerogenic modes of delivery, in view of human and veterinary applications. The BG particle presentation technology for target antigens to induce an immune response against the target antigens has also been studied extensively by our group (reviews in [14–17]) and promising results of recent studies will be presented below.

3. Bacterial ghosts as candidate vaccines

3.1. Parenteral immunization with bacterial ghosts

Bovine pneumonic pasteurellosis caused by *M. haemolytica* is a serious disease leading to death in cattle if it remains untreated. Pilot subcutaneous BG immunization studies of mice and rabbits with either *P. multocida*- or *M. haemolytica*-ghosts induced antibodies cross-reactive to heterologous *Pasteurella* strains. The number of proteins in *Pasteurella* whole-cell protein extracts recognized by the sera constantly increased during the observation period of 50 days. More importantly, dose-dependent protection against homologous nasal challenge was observed in mice immunized with *P. multocida* ghosts [18].

Following on from this work, *M. haemolytica* ghosts cattle immunization studies were performed using a cattle lung challenge model. Protective immunity of cattle against homologous challenge was induced by alum-adjuvanted *M. haemolytica* ghosts. It is important to note that BG do not need additional adjuvants to induce protective immunity. However, alum was added to the ghost vaccine preparation in this study to compare its antigenicity with a commercially available vaccine [19].

Bacterial ghosts have been tested as a vaccine against swine pleuropneumonia, a disease with a high mortality rate in pigs. Intramuscular immunization of pigs with *A. pleuropneumoniae* (APP) ghosts or formalin-inactivated APP whole-cell bacteria protected for clinical disease in both vaccination groups. The protective efficacy was evaluated by clinical, bacteriological, serological and post-mortem examinations. Immunization with BG did not cause clinical side-effects. After aerosol challenge, the control group of pigs developed fever and pleuropneumonia. In both vaccination groups, animals were fully protected against clinical disease and lung lesions, whereas colonization of the respiratory tract with APP was prevented by BG immunization alone. The induction of specific mucosal antibodies as detected in the

bronchoalveolar lavage suggests that immunization with BG induces antibody populations specific to non-denaturated surface antigens. In this study at least, APP-BG are more efficacious in protecting pigs against colonization and infection than the inactivated whole-cell vaccine [20]. Indications for a cross-protective potential of the ghost vaccine were supported by studies on rabbit hyperimmune sera [21].

Cholera is a significant cause of morbidity and mortality in humans and a vaccine is very much needed. To this end, *V. cholerae* ghosts were produced and assessed in a rabbit model. Rabbits were immunized s.c./i.m. with ghosts prepared from *V. cholerae* strains of O1 or O139 serogroup following growth under culture conditions which favour or repress the production of toxin-co-regulated pili (TCP). Immunoblotting confirmed the TCP status of *V. cholerae* ghosts (VCG), which retained the cellular morphology and surface component profile of viable bacteria. Sera from immunized rabbits was assayed for antibodies to lipopolysaccharide (LPS) and to TCP. Regardless of the TCP status of the VCG preparations used for immunization, all animals produced antibodies to LPS as demonstrated in bactericidal assays. Anti-LPS antibodies were likely responsible for conferring passive immunity in the infant mouse cholera model to challenge with the homologous O139 strain. Cross-protective anti-TCP antibody was generated only in rabbits immunized with TCP-positive VCG. This sera induced protection against heterologous challenge [22].

3.2. Induction of cytokines by bacterial ghosts

To investigate the activation of APC by BG we studied the in vitro uptake of VCG and *E. coli* BG in dendritic cells (DC) and RAW macrophages and the induction of inflammatory mediators in the THP-1 human macrophage cell line. The synthesis of inflammatory mediators such as TNF-α in the THP-1 cell line was stimulated by a hundred-fold higher dose of VCG than the corresponding amount of free LPS [23,24]. These results support in vivo experiments in rabbits with intravenous administration of *E. coli* BG. Below a threshold dose, no toxic effects of BG administration could be detected whilst the doses used stimulated significant humoral immune responses [25]. Significant production of IL-12 in DC was induced by *E. coli* BG. Secretion in DC of cytokines TNFα and IL-12 was increased 37 and 18-fold, respectively, whereas in peripheral blood monocytes the secretion of TNFα and IL-12 increased only twofold. These results suggest that BG stimulate the activation of cellular Th1 immune responses. In addition, maturation of DC is a prerequisite for efficient stimulation of T cells and exposure of DC to BG resulted in a marked increase in their ability to activate T cells. Thus, BG are promising carrier and adjuvants for target antigens.

3.3. Mucosal immunizations with bacterial ghosts

Different routes of mucosal immunizations with BG (aerogen, oral, intranasal, intravaginal, intraocular or rectal) have been assessed in various animal models. Binding and uptake of BG into APC is dependent on surface structures of the envelope being recognized by toll like receptors on human or animal cells.

Inhalation and deposition of BG within the airways are the initial steps preceding adherence of the vaccine candidates to the respiratory tract. Once BG are deposited in the lung lining fluids (mucosa), they are rapidly cleared by alveolar macrophages and translocation of deposited particles in the mucus also lead to clearance via the gastrointestinal tract [26].

APP-BG, after evaluation in the pig lung infection model was then further assessed in an aerosol immunization model [27]. The model utilized computer-controlled standardized inhalation conditions for the recipient pigs. APP-BG aerosol immunization has been shown to induce complete protection against pleuropneumonia in pigs [28].

The capability of BG to induce a T-cell-mediated immune response was studied following uptake of APP ghosts by primary APC of pigs. Specific T-cell responses were detected after in vitro re-stimulation of primed blood T cells with APP ghosts. In addition, we investigated uptake of APP BG by DC and subsequent DC activation. DC are known to be phagocytic in specific immature stages of development. Following the internalization and processing of the antigens, increased expression of MHC class II molecules in APC was shown 12 h after their exposure to BG. Together with the specific T-cell response to the antigen processed by the APC, it could be demonstrated that porcine APC have the capacity to stimulate antigen-specific T cells after internalization

and processing of the antigen. The data suggest that BG effectively stimulate monocytes and macrophages to induce TH1-type cytokine directed immune responses. DC stimulated by BG can be used for active immunization and immunotherapy in situ [24].

The immunological and protective efficacy of *V. cholerae* ghosts (VCG) expressing TCP (VCG-TCP) from *V. cholerae* serogroups O1 and O139 has been investigated in the reversible intestinal tie adult rabbit diarrhea (RITARD) model. Rabbits were immunized 3 times intragastrically with a mixture of lyophilized VCG-TCP from serogroup O1 and serogroup O139 and were challenged 30 days after the first immunization with virulent *V. cholerae* O1 and *V. cholerae* O139 strains.

Serum vibriocidal antibodies were observed in all immunized animals and it could be shown that adult rabbits were protected against diarrhea and death following intralumen challenge with fully virulent *V. cholerae* serogroups O1 and O139 [29]. Animal models indicate that VCG induce humoral and cellular immune responses against cell envelope constituents including protective immunity against challenge infections. All oral ghost vaccination experiments were carried out with freeze-dried ghosts resuspended in saline without the addition of adjuvants, stabilizers or other substances [29]. VCG have the advantage of ease of production by simple fermentation under conditions, which favour the expression of TCP.

4. Bacterial ghost system as carrier of foreign target antigens

Foreign target antigens can be tethered to the OM or IM, exported into the PPS or can be expressed as S-layer fusion proteins, which form shell-like self assembly structures filling either the PPS or CPS (Fig. 1). The OM (Fig. 1) is an asymmetric lipid bilayer with LPS in the outer leaflet and phospholipids in the inner leaflet. The polysaccharide moieties of LPS, filaments and pili extend from the OM to the environment. The role of TCP to confer cross-protective immunity in VCG has been mentioned earlier.

Outer membrane target antigen expression exploits outer membrane proteins (OMP), which can be modified to incorporate unrelated sequences [30]. In a recent study, hepatitis B virus core 149 antigen was incorporated into OMP-A as fusion protein and displayed on the surface of *E. coli* BG. These ghosts induced a significant immune response against the HBC 149 core antigen in mice [31].

Localization of target antigens in the PPS offers several advantages. Target antigens exported to this compartment are not only protected from external degradation processes but are also immersed in a sugar-rich environment of membrane-derived oligosaccharides, which protect TA during lyophilization. Furthermore, soluble target antigens can be expressed in the PPS of BG as the E-lysis tunnel seals the IM and OM.

MalE fusion proteins have been constructed which secrete the target antigens into the PPS either as a soluble protein or as part of a S-layer self-assembly structure (Fig. 1). Site-directed mutagenesis of the S-layer genes *sbsA* and *sbsB* and structural/functional analysis of S-layer domains essential for intra- and/or inter-molecular interactions [32–34] revealed flexible surface loops in both proteins that accept foreign target antigens sequences coding for up to 600 aa [35]. Such recombinant S-layer fusion proteins within a BG consist of several hundred thousand monomers per cell and because of their ability to assemble into a superstructure, they do not form inclusion bodies.

Depending on the specific aim, multiple presentation of target antigens within the S-layer structure could have beneficial effects compared to the soluble form of the corresponding antigen (Fig. 1).

Electron microscopic pictures show sheet like self-assembly structures of recombinant SbsA–Omp26 subunits in the PPS of *E. coli* ghosts [36]. The Omp26 of non-typeable *Haemophilus influenzae* (NTHi) was carried within the superstructure and *E. coli* BG harboring this construct were highly immunogenic for Omp26 when administered intraperitoneally to mice [36].

The potential of *E. coli* ghosts carrying MalE–Omp26 or MalE–SbsA–Omp26 fusion proteins in the PPS was assessed as a delivery system for mucosal immunization in a rat model and different routes of immunization were evaluated. Animals were mucosally immunized targeting either gut only or gut and lung mucosal sites. In the gut/lung regime, two initial gut targeted inoculations with BG were followed by an intratracheal (IT) boost with purified Omp26. The gut only immunization regime showed a moderate enhancement of bacterial clearance following pulmo-

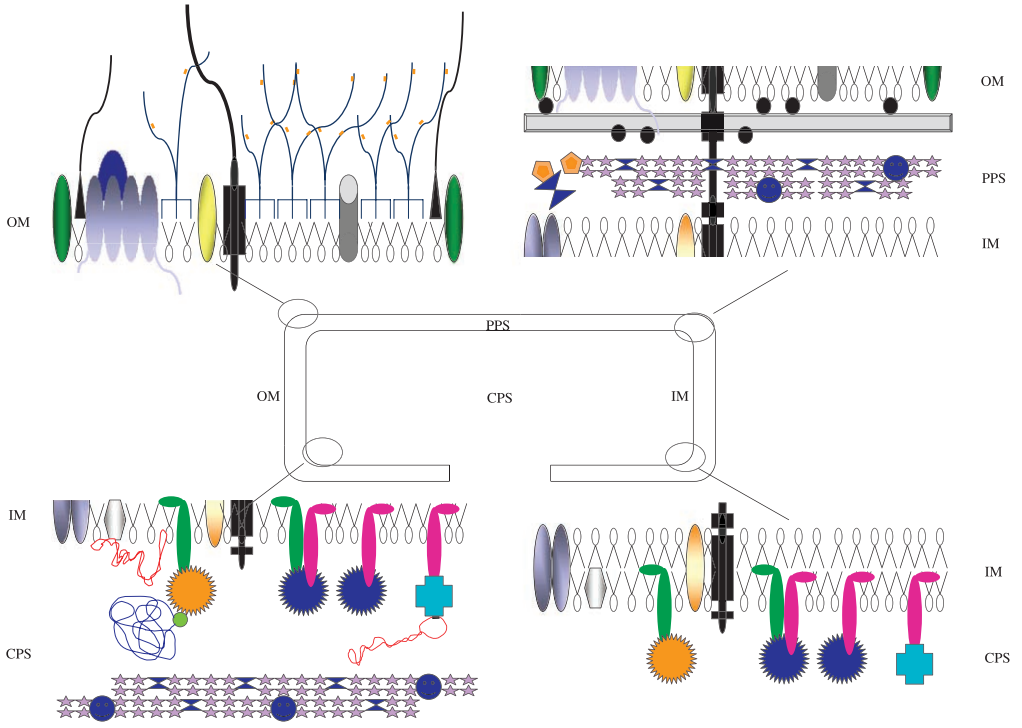
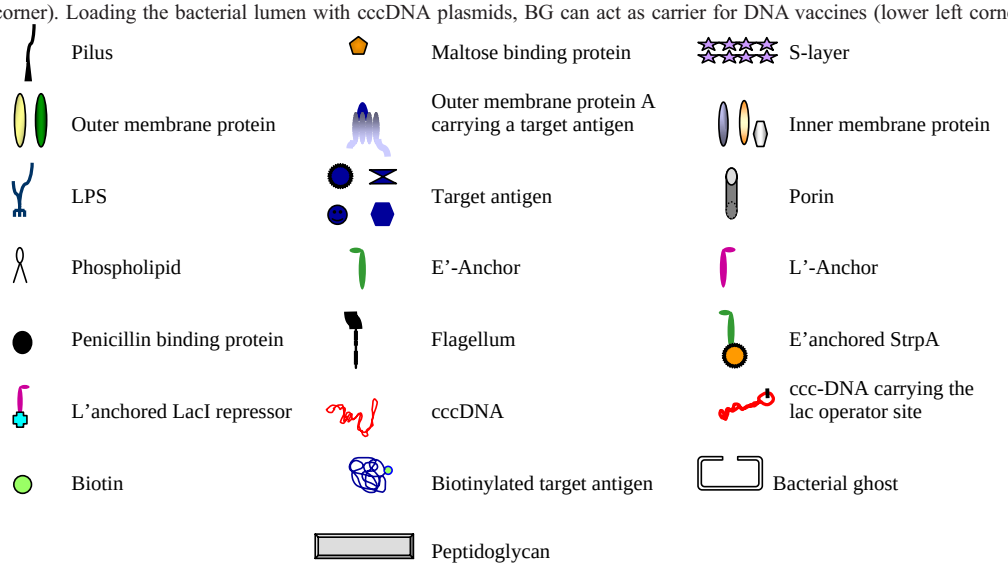


Fig. 1. Bacterial ghost as carrier of autologous or foreign target antigens. The BG can be divided into four different compartments carrying TA, namely, the OM, the PPS, the IM and the CPS which are presented in detail on the corners of the schematic BG in the centre. TA can be BG components themselves (e.g. pili, LPS, OMP, IMP and flagella) or represent foreign TA displayed on the BG surface as fusion protein with the OMP-A (upper left corner) or are exported to the sealed PPS as maltose-binding protein (male) or malE-sbsA/sbsB S-layer fusion proteins (upper right corner). TA can be anchored into the IM via E′, L′ or E′ and L′ anchor sequences (lower right corner). Membrane-anchored StrpA (E′-StrpA) can bind any biotinylated TA to the inner membrane (lower left corner). DNA carrying a lac operator sequence can bind to a membrane anchored lacI (LacI-L′) repressor molecule (lower left corner). Recombinant S-layer proteins carrying foreign TA can fill up the CPS of the BG (lower left corner). Loading the bacterial lumen with cccDNA plasmids, BG can act as carrier for DNA vaccines (lower left corner).



nary challenge whereas the gut/lung immunization regime resulted in significantly enhanced pulmonary clearance of NTHi. Both immunization regimes induced high levels of Omp26 specific antibodies in the serum of immunized rats, with higher levels in the groups that received the IT boost with purified Omp26. Analysis of IgG isotypes present in serum suggest that the immune response was predominantly of a T-helper1 type. Additionally, immunization induced a significant cellular immune response with lymphocytes from animals vaccinated using the gut/lung regime responding significantly to Omp26 when compared to control groups. In summary, mucosal immunization with recombinant Omp26 in *E. coli* ghosts followed by a boost with purified Omp26 induced a specific and protective immune response.

Bacterial ghosts have also been produced to express target antigens in the CPS. Expression of SbsA or SbsB fusion proteins in the CPS followed by E-mediated lysis of the bacteria results in crystalline planar arrays of S-layer proteins not released to the surrounding medium (Fig. 1).

The CPS of BG can be filled either with water soluble subunit antigens or emulsions such that the target antigen itself or a matrix can be coupled to appropriate anchors on the inside of the IM of BG (Fig. 2). For example, BG with streptavidin anchored on the inside of the IM can be filled by resuspending lyophilized BG in solutions carrying biotinylated TA [37].

For membrane anchoring of target antigens or of acceptor proteins like streptavidin to the cytoplasmic side of the IM, a membrane targeting system was developed [38]. By cloning foreign DNA sequences into the membrane targeting vector pMTV5, any gene of interest can be expressed as a hybrid protein with N-, C- or N-/C-terminal (E′-, L′-, E′-L′; Figs. 1 and 2) membrane anchors directing and attaching the fusion proteins to the cytoplasmic side of the IM of the bacteria prior to E-mediated lysis. The current list of membrane anchored target proteins comprises various viral core or envelope proteins and bacterial target antigens or enzymes. For the latter, it could be shown that the enzymatic activities of β-galactosidase, PHB-synthase or alkaline phosphatase were not impaired indicating that the membrane anchors do not interfere with the proper folding of the target proteins and that clustering and self-assembly (for example for β-galactosidase) is possible. The IM anchored HIV1-RT and HIV1-gp41 target antigens carried by BG induced humoral as well as a cellular immune responses in animal models [39,40].

Any BG can be used as carrier for foreign antigens. In a recent study [41], VCG have been successful used to immunize against *Chlamydia trachomatis*. In accordance with the new paradigm for vaccine design, an efficacious anti-chlamydial vaccine should elicit a genital mucosal Th1 response. To design a candidate vaccine against *Chlamydia* based on the BGS, the gene encoding the major OMP, *omp1*, of *C. trachomatis* was expressed in *V. cholerae*, as an IM-anchored protein. Intranasal and intramuscular immunization of naive mice with *V. cholerae* ghosts expressing OMP1 induced a strong Th1 immune response in the genital mucosa. The ability of this vaccine delivery system to protect susceptible animals from chlamydial infection offers potential for the future development of efficacious vaccines capable of protecting human against pathogens causing intracellular infections. In addition, immune T cells from immunized mice could transfer partial protection against a *C. trachomatis* genital challenge to naïve mice. These results suggest that VCG expressing chlamydial proteins may constitute a suitable subunit vaccine for inducing an efficient mucosal T-cell response that protects against *C. trachomatis* infection [41]. In this example, VCG offer the opportunity for designing TA vaccines within the context of a cell envelope which is also able to induce protective immunity against cholera.

Bacterial ghosts have been more recently developed for delivery of antigens such as DNA. The internal space of BG can be filled with a substituted matrix, e.g. biotinylated dextran or polylysine which then binds the target antigens of interest (Fig. 2a). For DNA vaccines, it has been shown that plasmid DNA complexed with polylysine can be efficiently packaged into BG [37]. If the lac repressor proteins (LacI) is membrane anchored (Fig. 1), it is still able to bind lac operator sequences carried on plasmid DNA. Plasmids bound to the membrane by this specific interaction are retained in BG and are not expelled to the culture medium following induction of E-mediated lysis [42].

It has also been observed that plasmid DNA associates unspecifically with the inside of the IM. Purified covalent closed circular DNA (cccDNA) can be loaded to BG by resuspension of freeze dried BG

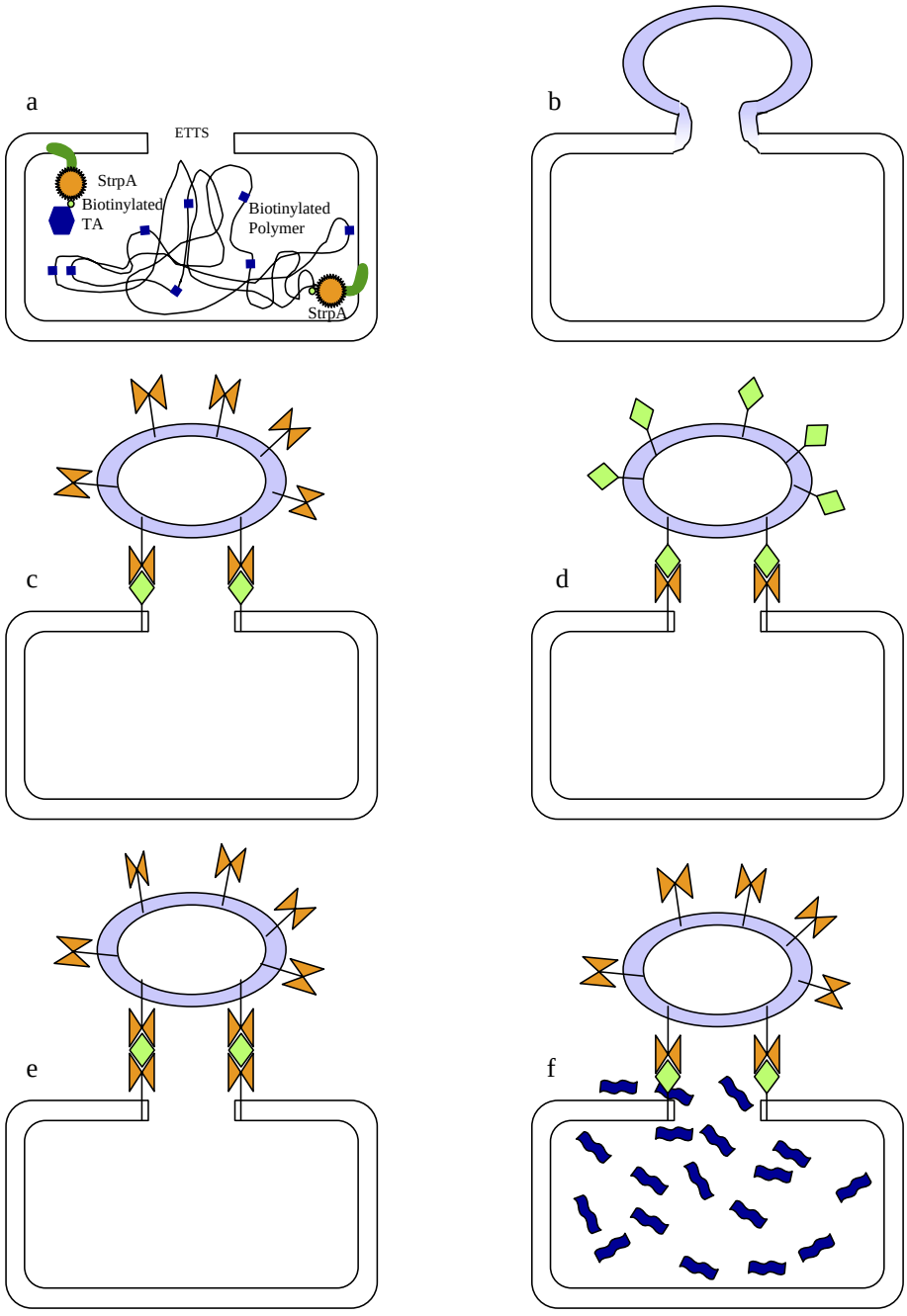


Fig. 2. Targeting membrane vesicles on top of the E-specific transmembrane tunnel (ETTS) structure of bacterial ghosts. (a) BG with streptavidin-biotin coupled target antigens (TA) or biotinylated polymer in the CPS with open E-specific transmembrane tunnel structure. (b) Sealing of inside-out vesicles of Gram-negative bacteria to the E-specific transmembrane tunnel structure of BG. Protein E fusion proteins (c) in vivo biotinylated ( ) or (d) extended with streptavidin ( ) using the specific biotin–streptavidin interactions ( ) to position the membrane vesicle of the E-specific transmembrane tunnel structure. (e) Multimers of streptavidin–biotin molecules (for simplicity only one construct is shown) can form chimney like structures between the BG and the targeted vesicle. (f) Soluble target antigens of other substances ( ) can be carried in the CPS of BG.

in DNA solutions. Depending on the DNA concentration roughly 2000–4000 plasmids per single BG can be attached to the IM.

When BG carrying plasmids encoding the green fluorescent protein (GFP) were exposed under tissue culture conditions to Caco-2 cells, DC or macrophages the cells expressed high levels of GFP [43].

As the latter two cell types are well known for their high capacity to degrade phagocytosed material, it is even more astonishing that GFP plasmids delivered by BG transferred the DNA from the endosome/lysosome to the nucleus without any help of endosomolytic agents or of nuclear localization signals. In an initial immunization study, plasmids encoding the TA LacZ loaded in BG revealed strong humoral as well as a Th1 help-mediated immune responses against β-galactosidase in mice [44].

The feasibility of plugging the E-lysis tunnel of BG to entrap target antigens in the CPS following loss of cellular cytoplasmic constituents has been assessed. Using a vesicle-to-ghost membrane fusion system, BG can be plugged in order to use BG as carrier and adjuvant systems for soluble, non-attached, hydrophilic TA. The sealing process of ghosts requires inside-out vesicles of gram-negative bacteria and fuses the vesicles to the inner membrane at the edges of the lysis tunnel of the ghost carrier. Orthonitrophenyl-galactoside (ONPG), calcein and fluorescein-labeled DNA were used as reporter substances to test that BG can be sealed by restoring membrane integrity (Fig. 2b) [45].

The technique of loosely closing BG is under optimization and, as can be seen in Fig. 2c–f, antigen carriers can be obtained by targeting a vesicle on top of the E-specific transmembrane tunnel. In the most simple model, vesicles can be targeted to the E-specific transmembrane tunnel by specific interaction of biotinylated protein E with membrane anchored streptavidin on the surface of inside out vesicles (Fig. 2c) or vice versa by using E-streptavidin fusion proteins for creation of the E-specific transmembrane tunnel and inside out vesicles with membrane anchored biotinylated receptor sequences (Fig. 2d). In an alternative model, both receptor sequences on the BG as well as on the inside out vesicle display streptavidin on the surface and free biotin is used as coupling agent (Fig. 2e). This traps the vesicle on top of the E-specific transmembrane tunnel and can be used to construct BG carrying back packed envelope fragments from other bacteria or viruses being either biotinylated or modified with streptavidin.

With such constructs soluble drugs or antigens filled into BG may be able to leak out through the tiny cleft between the vesicle and the BG carrier (Fig. 2f). The release rate of the enclosed substances can be regulated by the distance between the BG carrier and vesicle attached by adding various amounts of free biotin and streptavidin, forming chimney-like structures which can be constructed with different release properties.

Target antigens embedded in BG can be regarded as subunit vaccine candidates fully equipped with a whole bacterial cell adjuvant for better uptake by APC by pattern recognition. The bacterial ghost antigen presentation technology combines target antigen(s) on a carrier, which elicits an efficient immune response. The formulation of the target antigens packaged into the ghost envelope structures is dependent on their own physiochemical properties and it may be of benefit to either loosely package them into the inner space of the envelopes or to fix them to a matrix. Clearly, different combinations of substances may need to be located simultaneously in various compartments of the BGS for optimal formulation. The bacterial ghost technology warrants further investigation as it has great strategic potential in areas of vaccine development against viral and bacterial threats for which conventional vaccines do not exist or are not sufficiently efficient.

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