

Bacterial ghosts and their potential future applications-A mini review

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ABSTRACT

Bacterial ghosts system represents an emerging novel platform for antigens, nucleic acids and drug delivery. Bacterial ghosts are non-living Gram-negative bacterial cell envelopes devoid of cytoplasmic contents while maintaining their structural and morphological properties of native bacterial cells including surface antigens and bio-adhesive properties. They are generally produced by PhiX174 protein E-mediated lysis of Gram-negative bacteria. The intrinsic adjuvant properties of bacterial ghost preparations enhance immune responses against envelope bound antigens, including T-cell activation and mucosal immunity. These particles have envisaged both medical and veterinary applications for vaccination and treatment of various infectious diseases and tumors. The advantages of bacterial ghosts include the simplicity of the production method, safety, independence from the cold chain, and their intrinsic cellular and tissue tropic abilities.

Key words: Adjuvant, Bacterial ghosts, Bio-adhesive, Carrier system, Multivalent vaccine, Safety, Tumor therapy

Bacterial ghosts (BGs) are Gram-negative bacterial cell envelopes, devoid of cytoplasmic contents, while retaining their structural and morphological features of the natural cells (Lubitz *et al.* 1999). They are produced by controlled expression of the cloned gene *E* of bacteriophage phiX174, which forms a lysis-tunnel structure within the envelope of the living bacteria (Szostak *et al.* 1996). When expressed in non-host range bacteria, *E* converts Gram-negative bacteria into BGs whereas, Gram-positive bacteria are killed without lysis (Langemann *et al.* 2010). This *E*-mediated lysis releases all cytoplasmic content to the environment while periplasmic components remain associated with the empty cell envelope. It maintains all surface proteins of the original bacterium in its original state and therefore, possesses all the structural, immunogenic and bio-adhesive properties of the original bacterium (Lubitz *et al.* 1999).

The lysis gene *E* encodes a membrane protein of 91 amino acids, which has the ability to oligomerize into a transmembrane tunnel structure spanning the inner (IM) and outer membrane (OM) that causes release of the cytoplasmic contents of the bacterium (Witte *et al.* 1992). The lysis tunnel diameter varies between 40 and 200 nm in *E. coli* and does not show any regular structure. The IM remains intact during expulsion of cytoplasmic material and electron micrographs clearly show a sealed periplasmic

space (PPS). The osmotic pressure difference between the cytoplasm and the surrounding medium seems to be the driving force for the rapid release of the cytoplasmic contents. However, the native structure of the peptidoglycan within the envelope complex remains intact. After the discovery of the process of *E*-mediated lysis in *E. coli*, BGs of numerous pathogenic Gram-negative bacteria were generated successfully (Hoffelner and Haas 2004). Recently, Amara *et al.* (2013) described a new method called “Sponge-Like Reduced Protocol (SLRP)” for BGs preparation using a defined concentration of chemical compounds, which could have effect on the bacterial cell wall and lead to the formation of BGs.

Generation of bacterial ghosts

The well established protocol for generation of bacterial ghosts is the controlled expression of cloned *E* gene from bacteriophage PhiX174 resulting cell lysis in Gram-negative bacteria as mentioned above. The gene *E* can be expressed under transcriptional control of either the temperature sensitive lpL/pR-cl857 promoter repressor, or chemical inducible promoter repressor systems, like lac promoter operator (lacPO) or the tol expression system (Kloos *et al.* 1994, Szostak *et al.* 1996, Ronchel *et al.* 1998). Jechlinger *et al.* (1999) developed an efficient expression systems by mutations in the lpR promoter/operator regions, 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. Alternatively, a cold-sensitive system for ghost formation has been developed

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by combining the lpR promoter/cI repressor system with the lacI/lacPO for control of gene *E* expression (Jechlinger *et al.* 1998). In this system, E mediated lysis is achieved by lowering the growth temperature of the bacteria from 37°C or higher to 28°C or lower. When recombinant proteins are expressed to become incorporated into the envelope complex (before E-mediated lysis) expression of the corresponding genes are induced chemically (e.g., lac-, arabinose induction system) before E-mediated lysis.

The whole process of production of BGs from the inoculation of the pre-culture to the purified BGs concentrate ready for lyophilization can be accomplished within 24 h (Langemann *et al.* 2010) and thus rapid production of bacterial ghosts is feasible. Flow cytometry has been established as a reliable real-time tool for the assessment of E-lysis onset and the progress of BGs formation (Haidinger *et al.* 2001, Haidinger *et al.* 2003a). The presence of protein E in the envelope complex of bacteria does not necessarily kill all bacteria by E-lysis. In applications where nucleic acid-free BGs are produced, inactivation can be accomplished by the expression of an additional “kill gene” such as staphylococcal nuclease A (SNUC), which reduces the DNA content below the detection limit of real-time PCR (Haidinger *et al.* 2003b). Addition of the beta-propiolactone to BGs suspension was effective in fully inactivating all viable cells either in combination with or as an alternative to SNUC (Langemann *et al.* 2010). However, protein E in the membrane renders all bacteria more acutely sensitive to killing by lyophilization and no living cell counts could be detected in the lyophilized BGs samples.

In the newly described SLRP or chemically induced method, BGs are produced by defined concentrations of chemicals that remove the bacterial cytoplasmic constituents. The minimum inhibition concentration (MIC) and the minimum growth concentration (MGC) of the chemicals were the main guide for the BGs preparation. The critical concentrations of the chemicals in different steps of the protocol lead to killing of the bacterial cells with the release of their cytoplasmic constituents without deformation of the cell 3D structure. In this protocol, no genetically modified or recombinant elements were used and hence, it is considered safe for the preparation of the BGs (Amara *et al.* 2013).

Usefulness of bacterial ghosts

The idea of utilizing BGs derived from different gram-negative bacteria as candidate vaccines emerged due to the demand for both potent and safe new vaccines. The BGs system offers many advantages over traditional vaccination techniques including targeting and the inherent adjuvant properties of the BGs particles. In addition, recombinant DNA technology facilitates the development of multivalent protein or DNA vaccines. BGs may be used either in their most primal form as empty envelope structures or in their modified form as carriers for antigens or DNA. Antigens can be anchored within the inner and outer membrane, in

the periplasmic space as well as in the inner lumen of bacterial ghosts (Lubitz *et al.* 1999). Another great feature of BGs is the fact that no denaturing effects occur during E-lysis and hence all antigenic determinants are preserved throughout BGs generation. Bacterial ghosts can be loaded with biologically active compounds and DNA molecules representing ideal, target oriented delivery vehicles (Jalava *et al.* 2003, Ebensen *et al.* 2004).

As candidate vaccines: Bacterial ghosts of different species of bacteria have been tested in different animal models as candidate vaccines (Hensel *et al.* 1996, Marchart *et al.* 2003, Vinod *et al.* 2014). They can be used as vaccine candidates as such with intrinsic adjuvant properties. For example, BGs have been tested as a vaccine against swine pleuropneumonia, a disease with a high mortality rate in pigs. Intramuscular immunization of pigs with *Actinobacillus pleuropneumoniae* (APP) ghosts or formalin-inactivated APP whole-cell bacteria protected clinical disease in both vaccination groups (Hensel *et al.* 2000). However, colonization of the respiratory tract with APP was prevented by BGs immunization alone. In this study, APP ghosts were found to be more efficacious in protecting pigs against colonization and infection than the inactivated whole-cell vaccine. Similarly, immunization studies on mice and rabbits with either *Pasteurella multocida* or *Manheimia haemolytica* ghosts induced antibodies cross-reactive to heterologous *Pasteurella* strains (Marchart *et al.* 2003). In another experiment, rabbits immunized with *V. cholerae* ghosts which was positive for toxin-co-regulated pili (TCP) induced protection against heterologous challenge (Eko *et al.* 2000). Very recently, Jawale *et al.* (2014), demonstrated effectiveness of *Salmonella* Enteritidis (SE) ghosts in chickens against virulent challenge. Besides humoral immune response, BGs stimulate the activation of cellular Th1 immune response and endotoxicity does not limit the use of BGs as candidate vaccine (Mader *et al.* 1997). In addition, exposure of dendritic cells (DC) to BGs resulted in a marked increase in their ability to activate T cells. Thus, BGs is a promising vaccine candidate with intrinsic adjuvant properties.

The application of BGs via mucosal inoculation was found to be superior to parental inoculation. The mucosal application of APP ghosts as aerosol or oral immunization induced sterile immunity and cross-protection against other serotypes in pigs (Hensel *et al.* 1996, Huter *et al.* 2000), whereas intra-muscular immunization fully prevented the vaccinated pigs against the disease after lethal challenge but did not confer sterile immunity because the challenged bacteria could be re-isolated from the tonsils of the vaccinated pigs (Hensel *et al.* 2000). Likewise, immunization of rabbits with *V. cholerae* ghosts induced humoral and cellular immune response against cell envelope constituents including protective immunity against challenge infections (Eko *et al.* 2003a). Recently, Vinod *et al.* (2014) showed chemically induced *Salmonella* Enteritidis ghost as effective vaccine candidate against virulent challenge in rats.

As adjuvants: To elicit their full immunological potential, new generation subunit and DNA vaccines commonly need to be combined with adjuvants. However, the currently used adjuvants have various limitations and the adjuvant options available to any recombinant vaccine manufacturer are very limited. Due to the presence of well-known immune-stimulating compounds such as LPS, lipid A, peptidoglycan or flagella, which are also known as pathogen-associated molecular patterns (PAMPs), BGs elicit an innate immune reaction as first response (Langemann *et al.* 2010). The adjuvant properties of the ghosts are further enhanced by opsonization of the envelopes and thus recognized by the macrophages and dendritic cells. Uptake of bacterial ghosts by dendritic cells and macrophages and the induction of inflammatory mediators have been studied in a human macrophage cell line. The ghosts stimulated significantly increased secretion of cytokines TNF α , IF γ , IL-12 and IL-18. Thus, bacterial ghosts can excite cellular Th1 immune responses. Furthermore, DC exposed to ghosts had an increased ability to activate T-cells (Haslberger *et al.* 2000).

As delivery systems: Bacterial ghosts can be used as a delivery system for antigens, nucleic acids, drugs and soluble compounds for various applications. Not only can they act as delivery vehicles for inner and/or outer membrane anchored antigens, but they can also deliver water-soluble drugs or antigens (Jalava *et al.* 2003, Paukner *et al.* 2003). The cellular and tissue tropism of bacterial ghosts in combination with excellent carrier capacity in several cellular compartments offers much potential for antigen, nucleic acid and drug delivery. Bacterial ghosts are taken up very effectively by antigen-presenting cells (APCs) such as macrophages and dendritic cells and are particularly suited as vaccines for mucosal administration by oral, intranasal or aerogenic routes, resulting in the induction of humoral and cellular immune responses (Jalava *et al.* 2003).

As carrier of foreign antigens: Among the various known technical approaches towards vaccination against infectious diseases, ranging from attenuated living microorganisms and physically or chemically inactivated pathogens or part of a pathogen to peptide and nucleic-acid based vaccines, bacterial ghosts represent a truly distinctive approach towards vaccination. Unlike attenuated bacteria, they are non-living and are devoid of any potentially harmful cell content. In contrast to subunit vaccines, they do not need adjuvant and are highly stable. As such, they mimic a natural bacterial infection and thus elicit immune responses superior to the ones of existing vaccine approaches. Since BGs can be created from pathogenic or non-pathogenic gram-negative bacteria expressing one or more recombinant antigens in various cell membrane compartments, the technology platform can also be applied to vaccine development against gram-positive bacteria, viruses and eukaryotic pathogens.

Foreign target antigens (TA) 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 (Szostak *et al.* 1993, Hobom *et al.* 1995, Truppe *et al.* 1997). Outer membrane TA expression exploits outer membrane proteins (OMP), which can be modified to incorporate foreign sequences. In a study, the gene encoding the major OMP, *omp1*, of *Chlamydia trachomatis* was expressed in *V. cholerae*, as an IM-anchored protein. Intranasal and intramuscular immunization of naive mice with *V. cholerae* ghosts (VCG) expressing OMP1 induced a strong Th1 immune response in the genital mucosa. In addition, immune T cells from immunized mice could transfer partial protection against a *C. trachomatis* genital challenge to naive 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 (Eko *et al.* 2003b).

Likewise, soluble target antigens can be expressed in the PPS of BGs as the E-lysis tunnel seals the IM and OM. This can be achieved by fusion of the protein with MalE or PPS signal sequences. Localization of TA in the PPS is not only protected from external degradation processes but the sugar-rich environment of membrane-derived oligosaccharides also protects TA during lyophilization (Mayr *et al.* 2005). Bacterial ghosts were also produced to express antigens in the CPS as fusion protein with surface layer (S-layer) genes such as *sbsA* and *sbsB* (Truppe *et al.* 1997). Since S-layers are made up of several hundred thousand monomers per cell, they are not expelled with the cytoplasm during E-lysis. Linking MalE to SbsA, the protein subunits can also be exported to the PPS prior to S-layer formation (Riedmann *et al.* 2003). On the other hand, the CPS of BGs can be filled either with water soluble 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 BGs. For example, BGs with streptavidin anchored on the IM can be filled by resuspending lyophilized BGs in solutions carrying biotinylated TA (Szostak and Lubitz 1991).

In another method, any gene of interest can be expressed as a hybrid protein with N-, C- or N-/C-terminal membrane anchors (EV-, LV-, EV-LV) directing and attaching the fusion proteins to the cytoplasmic side of the IM of the bacteria prior to E-mediated lysis (Szostak *et al.* 1993). It was shown that the enzymatic activities of beta-galactosidase, PHB-synthase or alkaline phosphatase which were expressed with membrane anchors 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 (e.g. b-galactosidase) is possible (Szostak *et al.* 1996).

As a vehicle for DNA vaccines: Practical use of DNA vaccines is hindered by the requirement of high plasmid dosages and poor immunogenicity. Bacterial ghosts constitute a promising technology platform for the development of more efficient DNA vaccines. It offers a novel and efficient targeting vehicles for DNA delivery to the monocyte-derived dendritic cells (Kudela *et al.* 2005).

Recent *in vitro* investigations proved that BGs have no cytotoxic or genotoxic impact on different types of human cells after mutual co-incubation. This observation was independent of the BGs species used (Langemann *et al.* 2010). Ghost-mediated delivery of DNA vaccine consisting of an eukaryotic expression plasmid containing the gene coding for β -galactosidase under the control of the CMV immediate early gene promoter (pCMVb) by intradermal or i.m. route stimulated more efficient antigen-specific humoral and cellular ($CD4^+$ and $CD8^+$) immune responses than naked DNA in BALB/c mice (Ebensen *et al.* 2004).

The internal space of BGs can be filled with a matrix (e.g. biotinylated dextran or polylysine), which can bind to the target antigens. Plasmid DNA complexed with polylysine can be efficiently packaged into BGs (Szostak *et al.* 1991). Plasmids can also be retained inside BGs, by anchoring lac repressor proteins (LacI) in the IM. It can bind to the lac operator sequences carried on plasmid DNA. Plasmids bound to the membrane by this specific interaction are retained in BGs (self immobilized plasmid) and are not expelled to the culture medium following E-mediated lysis (Mayrhofer *et al.* 2005). Plasmid DNA also attaches unspecifically with the inside of the IM. Purified plasmid DNA can be loaded to BGs by resuspension of freeze dried BGs in DNA solutions and up to 6,000 midsize plasmid copies per BGs can be loaded. (Paukner *et al.* 2005). When BGs carrying plasmids encoding the green fluorescent protein (GFP) were exposed under tissue culture conditions to Caco-2 cells, DC or macrophages, the cells successfully expressed high levels of GFP (Paukner *et al.* 2004).

As vehicle for biologically active substances: Number of serious toxic side effects including increased risk of cardiovascular diseases caused by highly cytotoxic chemical agents in the treatment of different types of tumors decreases the usefulness of chemotherapeutics to inhibit fast tumor cell replication and to efficiently cure patients suffering from cancer. Many of these toxic effects limit repeated drug application in the treatment of cancer patients. Development of new delivery systems with no cytotoxicity and high efficiency of drug and/or gene delivery as alternatives to current methods is very much needed. Bacterial ghosts offer such an alternative as it can be plugged in order to use them as carrier and adjuvant systems for soluble and hydrophilic TA and drugs. 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. Ortho-nitrophenyl-galactoside (ONPG), calcein and fluorescein-labeled DNA were used as reporter substances to test that BGs can be sealed by restoring membrane integrity (Paukner *et al.* 2003). Besides, many strategies have been worked out to seal the BGs using streptavidin and biotin interaction for controlled release of loaded drugs (Paukner *et al.* 2003).

Targeted tumor therapy: Stimulation of tumor-associated antigen (TAA)-specific T cells possessing high affinity T cell receptors capable of recognizing tumor cells by DCs stimulated with interferons might lead to a more

efficient immune response and hence bypass regulatory elements of tumor microenvironment. Bacterial ghosts combine features of the ideal vaccine candidate and tumor therapy vehicle. Recent investigations confirm the recognition of BGs by various types of tumor cells and the capacity of BGs to efficiently target and internalized by melanoma, leukemia and colorectal carcinoma cells (Paukner *et al.* 2004, Kudela *et al.* 2008). Applications of drug or antigen loaded BGs directly into the tumor microenvironment might lead to induction and/or to an increase of an antigen-specific immune response and cytokine milieu alteration resulting in the attraction of immunocompetent cells participating in the immune response against the tumor cells.

Immunotherapy and tumor vaccine: Many tumor cells may not be recognized by the immune system. This immune evasion might be caused by the down regulation or loss of relevant TAA expressions, and/or defects in antigen processing and presentation machinery. Dendritic cells are the most potent professional APCs as well as potent initiators and modulators of the T cell immune responses *in vivo* including sensitization of MHC-restricted T cells, development of T cell-dependent antibody production, and induction of immunological tolerance. Intact surface structures of BGs including LPS enhance maturation of DC, affect endosomal acidification of DC, and also improve cross-presentation of antigen (Trombetta and Mellman 2005). Cross-presentation of antigens delivered to DCs by BGs could activate both $CD4^+$ and $CD8^+$ T cells and stimulates the immune system to enhance immune response against antigens expressed by target cells. An increase of IFN- γ producing antigen-specific $CD8^+$ T cells was observed in animals vaccinated with DNA loaded BGs in response to restimulation by APCs pulsed with peptide containing the immunodominant MHC class I epitope (Ebensen *et al.* 2004). BGs also enhanced expression of MHC class I molecules and costimulatory molecules on DCs. Intradermal and intramuscular immunizations of Balb/c mice with antigen loaded BGs stimulated more efficiently both humoral and cellular antigen-specific immune responses than the immunization with naked DNA (Ebensen *et al.* 2004). Likewise, intravenous immunization with dendritic cells loaded *ex vivo* with pCMVb containing ghosts also resulted in β -galactosidase-specific immune responses (Ebensen *et al.* 2004). Using BGs loaded with lysates of autologous and allogeneic tumor cells for *ex vivo* TAA delivery to the immunocompetent cells, in particular DC, followed by their effectual maturation and return to the patient would start or restore the immune response against delivered TAA, lead to elimination of tumor cells, and reduce the chance for tumor evasion. Similarly, immunization of cancer patients using BGs loaded with autologous and allogeneic tumor cell lysates might lead to cross-presentation of TAA delivered to DC by BGs, activation of both $CD4^+$ and $CD8^+$ T cells, and stimulate immune system to enhance immune response against TAA expressed by tumors

Drug delivery to tumors: A delivery system that transports chemotherapeutic drugs directly to the cytosol and nuclear area of target cells at levels sufficient to inhibit tumor cell proliferation would allow the use of decreased drug dosages, and thus lessen the negative impacts on people already challenged with serious diseases. Doxorubicin loaded BGs made by simple resuspension and incubation of lyophilized BGs in doxorubicin solution, were used as a model system for BGs and drug delivery (Paukner *et al.* 2004). Incubation of doxorubicin loaded BGs with colon carcinoma cells led to efficient internalization of BGs, their degradation, the release of the content of BGs to the cytoplasm of target cells and accumulation in the nuclear area. Mutual co-incubation of doxorubicin loaded BGs and tumor cells led to significantly higher inhibition of target cell proliferation (at least two orders of magnitude) in comparison to results obtained after incubation with pure doxorubicin at the same concentration levels. Analysis of doxorubicin loaded BGs cytotoxicity showed that doxorubicin-loaded BGs reduced cell proliferation up to 300 times more effectively than when free drug was added to the cell culture system. These facts support the potential of the BGs system in tumor therapy to provide a desirable reduction in the toxic side effects of currently used chemotherapeutic agents.

Future perspectives

Bacterial ghosts are very useful non-living delivery system for foreign antigens, nucleic acids and drugs in one or more cellular compartments simultaneously. Their ease of production, stability above sub-zero temperature, inherent adjuvant properties, ideally suited for needle-free mucosal application and safety profiles are important considerations for a wide range of applications in biomedicine. The identical surface structures of bacterial ghosts with their living counterparts are being exploited for specific cellular and tissue targeting. The inner space of BGs can be loaded with a combination of peptides, drugs or foreign DNA which gives us an opportunity to design new types of multivalent vaccines. Production of purified BGs ready for lyophilization does not take longer than a day and thus meets modern criteria of rapid vaccine production rather than keeping large stocks of vaccines. As such, 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 and in the treatment of various cancers. Bacterial ghosts may also be exploited as enzyme carriers, another possible application for future that needs optimization for various purposes.

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