Review on Bacterial Ghost and its Application

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Abstract: Bacterial ghosts are empty cell envelopes of gram negative bacteria lacking cytoplasmic content yet retaining all unaltered morphological and structural features of their living counterparts. BGs (Bacterial ghosts) are produced by protein E-mediated lysis of Gram-negative bacteria. Lysis of bacteria by endogenous expression of the plasmid-encoded gene E is a method of inactivating bacteria without physical or chemical influences alternating the bacterial surface. Gene E expression leads to the formation of a trans-membrane tunnel structure through the inner and outer bacterial membranes. By releasing the high osmotic pressure of the cell, the cytoplasmatic contents are expelled into the surrounding medium resulting in ghosts that are pure envelope. Bacterial ghosts have been shown to be an innovative system to prepare vaccines of varies bacteria with all features of the intact bacterial cell envelopes of the ghost preparations to specific antigen presenting cells. The bacterial ghost system is a novel vaccine delivery system usually in that it combines excellent natural intrinsic adjuvant properties with versatile carrier functions for foreign antigens. The efficient tropism of bacterial ghosts for antigen presenting cells promotes the generation of both cellular and humoral responses to hetrologous antigens and carrier envelope structures. The simplicity of both BG production and packaging of multiple target antigens makes them particularly suitable for use of 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 and they are versatile with regards to DNA or protein antigen choice and size and as a delivery system they offer high bio availability.

Key words: Bacterial Ghost • Gene E • Vaccine Delivery System

INTRODUCTION

The most commonly used veterinary vaccines are killed microorganisms, inactivated either by heat, irradiation or chemical treatment. Unfortunately, during this inactivation process most of the essential structural components of the bacterial cell wall are denatured, resulting in impaired function and non efficient immune response. Vaccination with killed microorganisms enables the immune system to come into a riskless contact with an otherwise life-threatening pathogen. The use of killed pathogens as substitutes for living infectious agents has been widely used as a principle for vaccine development. Sub unit 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 system need to be developed which better activate mucosal immune response [1].

Adjuvants are molecules, compounds or macromolecular complexes that boost the potency and longevity of specific immune response to antigens, but cause minimal toxicity or long lasting immune effects on their own [2]. The addition of adjuvants to vaccines enhances, sustains and directs the immunogenicity of antigens, effectively modulating appropriate immune responses, reducing the amount of antigen or number of immunizations required and improving the efficacy of vaccines in newborns, elderly or immuno-compromised individuals. Adjuvants have limited or no efficacy unless properly formulated; therefore both adjuvant components and formulation (e.g. oil in water, particle size, charge, etc.) are crucial for enhancing vaccine potency [3].

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Recent advances have begun to shed light on the cellular and molecular nature of innate immunity and adjuvant activity [4]. The immune system recognizes pathogen associated molecular patterns (PAMPs) by means of pathogen-recognition receptors (PRRs), which include the Toll-like receptors (TLRs) [5] C-type lectin-like receptors [6], cytosolic nucleotide oligomerization domain-like receptors and retinoic acid inducible gene based-I-like receptors [7-8]. These receptors bind microbial ligands (including cell wall components, lipoproteins, proteins, lipopolysaccharides, DNA and RNA of bacteria, viruses, protozoa and fungi) to trigger different types of immune responses [9-10]. These PAMPs, specifically those binding the TLRs, are the basis of many adjuvants [11].

The application of new strategies to develop vaccine is essential in modern veterinary medicine. The bacterial ghost system is a novel vaccine delivery system endowed with adjuvant properties. Bacterial ghost are nonliving gram negative bacterial cell envelope devoid of cytoplasmic contents while maintaining their cellular morphology and the native surface antigenic structures including bio adhesive properties. Extended recombinant ghost systems are needed to develop alternative platform technologies which in the end create better qualities of combination vaccines [12].

The extended recombinant ghost system is currently evaluated to combining as many as possible candidate vaccines which are stable without the requirement of a cold chain and do not need any adjuvant. In the end, the vaccine candidates should be easily administered and should be effective early in life [1].

Bacterial ghost (empty cell envelopes) have an intact outer surface make-up which provides them with the original targeting functions of the pathogen they are derived from and are thus able to induce strong immunity. Ghosts are produced by genetic inactivation of pathogenic bacteria due to the controlled expression of cloned bacteriophage PhiX174 lysis gene E. Expression of plasmid-encoded gene E leads to the formation of a transmembrane tunnel structure through the cell envelope of Gram-negative bacteria. The resulting bacterial ghosts share functional and antigenic determinants of the envelope with their living counterparts. Ghosts are also excellent carriers of foreign proteins and have properties for targeting antigen. In addition, ghosts have excellent adjuvant properties [1]. Therefore the objectives of this paper were to describe bacterial ghost system and its production and to introduce briefly the basic principles of application of bacterial ghost system.

Production of Bacterial Ghost: Bacterial ghosts are produced by expression of cloned gene E from bacteriophage PhiX174 resulting in cell lysis in Gram negative bacteria. Lysis gene E codes for a protein of 91 amino acids and exerts its lytic function in Gram-negative bacteria by the fusion of inner and outer membranes and transmembrane tunnel formation [13]. Through this tunnel the cytoplasmic content of the bacteria is expelled [14], leaving an empty internal space devoid of the bacterial nucleic acids, ribosomes or other higher or lower molecular weight constituents, whereas the IM (intramuscular) and outer membrane structures of the Bacterial ghost (BG) are preserved [15]. The driving force for the release of cytoplasmic material is the osmotic pressure difference between the cytoplasm and the medium created by the opening of the tunnel structure. The current working model of E-mediated lysis divides the process into three phases: Phase 1:- is characterized by integration of protein E into the inner membrane, Phase 2:- is initiated by a conformational change of protein E transferring its C-terminus across the inner membrane, Phase 3:- a local fusion of C-terminal domain of protein E towards the surface of the outer membrane of the bacterium [12].

Except for the lysis hole, the morphology of the bacteria, including all cell surface structures are not affected by the lysis event. This hypothesis was reinvestigated by comparing the murine composition before and during protein E induced lysis of E. coli. The analysis showed that the overall composition of murine is not changed after induction of protein E-mediated lysis. Nevertheless, murine degradation seems to be stimulated by the action of protein E as shown by an increase in the total amount of murine turnover products by about 10%. Since the peptidoglycan is not degraded, E-lysed bacteria resemble structurally their living counterparts. For the production of ghosts, bacteria are grown at 28°C until mid log phase, before lysis is induced by temperature up shift to 42°C. Because the antigenic determinants on the surface of bacteria can change under different environmental conditions, it seemed favorable to allow pathogens to grow at 37°C before induction of lysis [12].

E-mediated lysis has been achieved in various Escherichia coli strains, Salmonella typhimurium, Salmonella enteritidis, Klebsiella pneumoniae, Bordetella bronchiseptica, Helicobacter pylori, Vibrio cholerae, Actinobacillus pleurapneumoniae, Haemophilus influenzae, Pasteurella haemolytica, Pasteurella multocida, Pseudomonas aeruginosa, Pseudomonas putida, Ralstonia eutropha and...
Fig. 1: Gene E mediated lysis of bacteria and expulsion of the bacterial nucleic acids, ribosomes and other higher or lower molecular weight constituents.
Source: [12]

Fig. 2: A. Scanning Electro-micrograph of *P. hemolytica* (pSON2) grown at 28 B. Scanning Electro-micrograph of *P. hemolytica* (pSON2) ghosts shifted to 42 °C lysis- hole is indicated by arrow.
Source: [16]

*Ervinia cypripedii*. This broad spectrum of bacteria shows that E-mediated lysis might work in every Gram-negative bacterium provided that the E lysis cassette can be introduced in the new recipient by an appropriate vector allowing proper gene E expression. In Gram-positive bacteria gene E expression brings cell killing without lysis of the bacteria as the proper mechanism of E-mediated lysis depends on the fusion of inner and outer membranes [1].

**Application Areas of Bacterial Ghost:** BG have been developed for envelop and or heterologous antigen presentation from a range of important Gram negative bacterial pathogens including *Francisella tularensis, Brucella melitensis*, enterotoxigenic and enterohemorrhagic *E.coli* (EHEC, ETEC) and *V.cholerae*. To date, immune responses against *P. multocida, Mannheimia haemolytica, A. pleuropneumoniae* and *V. cholerae* have been assessed in several animal modelso
Fig. 3: Scanning electron micrograph of an E. coli bacterial ghost produced using protein E lysis procedure. A large opening enables the bacterial ghost envelope complex to be revealed.

Source:[17]

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 [18].

**Bacterial Ghosts as Candidate Vaccine**

**Parenteral Immunization with Bacterial Ghosts:** Bacterial ghosts have been tested as a vaccine against different disease conditions. *Bovine pneumonia pasteurellosis* is a series disease leading to death in cattle if it remains untreated. Subcutaneous immunization studies of mice and rabbits with either *P. maltocida* or *P. hemolytica* ghosts induced antibodies cross reactive to heterologous pasteurella strains [1].

Bacterial ghosts have also been tested as a vaccine against swine pleura-pneumonia, a disease with a high mortality rate in pigs. Intramuscular immunization of pigs with *APP* ghosts or formalin inactivated *APP* whole cell bacteria protected for clinical disease in both vaccination groups and the protective efficacy was evaluated. Immunization with BG did not cause clinical side effects. The control group of pigs developed fever and pleuro-pneumoniae. In both vaccination groups animals were fully protected against clinical disease and lung lesions, where as colonization of the respiratory tract with *APP* was prevented by BG immunization alone [1].

Cholera is another disease causing significant mortality and morbidity and vaccine is very much needed. *V. cholera* ghosts were produced and assessed in rabbit model. Rabbits were immunized subcutaneously or intramuscularly with ghosts prepared from *V. cholera* strains of O1 and O139 and induced protection against heterologous challenge [19].

**Induction of Cytokines by Bacterial Ghosts:** *In vivo* experiments in rabbits with intravenous administration of *E. coli* BG below a threshold dose reveals no toxic effects whilst the dose used stimulated significant humoral immune responses [20]. Significant production of IL-12 in dendritic cell (DC) was induced by *E. coli* BG. Secretion in DC of cytokines TNF-α and IL-12 was increased 37 and 18 fold, respectively, where as in peripheral blood monocytes the secretion of TNF-α and IL-12 increased only by two fold. These results suggest that BG stimulate the activation of cellular Th1 immune responses. In addition, maturation of DC is pre requisite for efficient stimulation of T cells and exposure of DC to BG resulted in marked increasing of their ability to activate T cells. Thus, BGs are promising carrier and adjuvant for targeting antigens [1].

**Mucosal Immunization with BG:** Binding and up take of BG into APC is dependent on surface structures of the envelope being recognized by toll like receptors on human or animal cells. *APP* BG assessed in an aerosol immunization model has been shown to induce complete protection against pleura-pneumoniae in pigs [21]. The capacity of BG to induce a T-cell mediated immune response was studied following up take of *APP* ghosts by APC of pigs. Specific T-cell responses were detected after in vitro re stimulation of T cells with *APP* ghosts. 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. BG effectively stimulates monocytes and macrophages to induce Th1 type cytokine directed immune responses [17].

In the investigation of the immunological and protective efficacy of *V. cholera* ghosts in intragastrically immunized pigs, serum vibriocidal antibodies were observed in all immunized animals and it could be shown that adult rabbits were protected against diarrhea and death following intra lumen challenge with cholera sero groups O1 and O139. The investigation indicates that the *Vibrio cholera* ghost (VCG) induce humoral and cellular immune responses against cell envelope constituents including protective immunity against infections [17]. All oral ghost vaccination experiments were carried out with freeze dried ghosts re suspended in saline without the addition of adjuvant, stabilizers or other substances [22].
Bacterial Ghost as a Carrier and Delivery System: Alternatively, BGS can be employed as a delivery system for proteins/antigens, nucleic acids, drugs and soluble compounds for various medical applications [23-26].

BGs as Delivery System for Antigens: Because new vaccines based on recombinant proteins are less immunogenic than traditional vaccines they require specific choices of particles and adjuvant to improve their presentation and targeting to thus induce appropriate immune response. This is particularly important at the mucosa, the most effective site for immune stimulation. The choice of carriers and/or adjuvant and the antigen itself have the potential to modulate the immune response, predominantly the T cell and the B cell based, appropriate for a particular pathogen [17].

The development of efficacious vaccines against intracellular bacteria, parasites and virus requires induction of T cell mediated response. One ingenious delivery system makes use of antigen fusion with the listeriolysine enzyme of Listeria monocytogenes to help induce a CD8 T cell response against phagosomal and cytosolic antigen [27]. Listeriolysine expression disrupts a phagosomal membrane releasing the target antigen to the cytoplasm for MHC I and T cell activation. Similarly, other groups have employed Escherichia coli hemolysine secretion system for delivery of heterologous antigen and large number of hybrid proteins has been generated by gene fusion with the terminal end of Escherichia coli hemolysine [17].

Another intra cellular antigen delivery system has been developed with virus like particles as well as virosomes. Peptide vaccination with virosome carriers has been investigated in several disease models including malaria, melanoma and hepatitis [28].

Compared with simple virus like particle, the bacterial cell offers several compartments for the delivery of immunogenic antigens and has a greater capacity. Expression of an antigen in the cytosol, periplasm or outermembrane of the carrier bacteria can have a profound impact on the elicited immune response. For instance surface- exposed expression or secretion of antigens leads to a better induction of specific antibodies [17]. Antigenic epitopes have also been inserted into flageline, fimбриae or the outer membrane or periplasmic proteins. An alternative to conventional bacterial delivery system approaches has been reported in the development of surface- display system based on the use of the spore coat of Bacillus subtilis. This has an interesting advantage in that bacterial spores can survive extremes of temperature, desiccation and exposure to solvents and other noxious chemicals [29].

Bacterial ghosts offer a safe, easy to manipulate and straight forward to produce alternative to traditional antigen bacterial carrier system, with all of the advantages of the latter. Foreign protein localization within bacterial ghosts performed by fusion with specific anchor sequences for attachment on the inside of the inner membrane, export in to the periplasmic space by fusion to the MalE signal sequence or attachment to the outer membrane as fusion proteins with OmpA or pili [17].

The suitability of bacterial ghost technology for designing antichlamydial vaccine was evaluated by constructing a candidate vaccine based on Vibrio cholerae vector expressing major outer membrane. The efficacy of the vaccine was assessed in a murine model of Chlamydia trachomatis genital infection [30]. Intramuscular delivery of the vaccine candidate induced local genital mucosal as well as systemic TH1 responses. In addition, immune T cells from immunized mice could transfer partial protection against a C. trachomatis genital challenge. These result suggested that V. cholerae ghosts expressing chlamydial proteins might constitute a suitable sub unit vaccine for inducing an efficient mucosal T cell response that protects against C.trachomatis infection [17].

BGs as Delivery System for Nucleic Acid: The development of bacterial carriers extends the application of DNA vaccines for mucosal immunization [21]. Significant humoral and cellular immune responses against bacterial, viral and other antigens have been induced by in vivo delivery of DNA vaccines in small animal models. A delivery system based on bacterial ghosts has been proven effective for DNA vaccines. In vitro studies showed that Mannheimia hemolytica ghosts loaded with a plasmid carrying the gene encoding green florescent protein are efficiently taken up by APCs with high transfection rates [30]. Subsequent in vivo vaccination studies in mice demonstrated that M. hemolytica ghost mediated DNA delivery by intradermal or intramuscular routes of eukaryotic expression of plasmid encoding for beta galactosidase under the control of cytomegalovirus promoter, stimulated more efficient antigen specific humoral and cellular immune responses than naked DNA. Bacterial ghosts not only target the DNA vaccine construct to APCs, but also provide a strong danger signal by acting as a natural
adjuvant, thereby promoting efficient maturation and activation of DCs. Thus, BGs constitute a promising technology platform for the development of more efficient DNA vaccines. More recently, a new delivery system based on bacterial ghosts has been developed in which, following E mediated lysis, DNA tethered via a DNA binding membrane-anchored protein to the ghost inner membrane [17].

**BGs as Delivery System for Drugs:** Many diseases require the systematic administration of highly aggressive drugs to already immune compromised patients. Deleterious and often severe side effects result from a lack of cellular and tissue selectivity. Another major issue is the poor solubility of some drugs used in treatment. Considering this a development of a safer and more efficient drug delivery system [DDS] is the priority for future prophylactic treatments. The DDS has three major goals: enhancement of drug permeability, targeting of drugs to the point of action and controlled release of drugs [31]. The use of erythrocytes in drug targeting has many advantages including biocompatibility, complete biodegradability, lack of toxic products, longer life span. Another DDS that exploits host constituents is the use of fusion with the plasma proteins, albumin. Albumin meets several requirements of a drug carrier and shows accumulation in tumors and inflamed joints [32].

More recently it has been shown that bacteria offer a solution for drug delivery, particularly through their tropic capacity. As a naturally tissue tropic delivery system, bacterial ghosts have shown early promise as a DDS. Bacterial ghosts made from *M. hemolytica* have been used for invitro delivery of Doxorubicin [DOX] to human colorectal adenocarcinoma cells. Adherence studies showed that the *M. hemolytica* ghosts targeted Caco2 cells and released the loaded DOX within the cells. Cytotoxicity assays showed a two long enhancement in cytotoxic and anti proliferative activity in cells incubated with DOX loaded ghosts compared with DOX directly added to the culture media [17].

Current work with bacterial ghosts lies in the investigation of carrier capacity of the cytoplasmic lumen. This intra cellular space of bacterial ghosts can be filled either with water soluble substances or emulsions such that the drug of interest can be coupled to *streptavidin* anchored on the inside of the inner membrane [22].

It is also advantageous to fill the internal space of the ghost with a substituted matrix which then binds the drug(s) of interest. In model experiments biotinylated florescence -labeled dextran has been used to completely fill the internal space of *streptavidin*-ghosts [33]. As substituted dextran has a high capacity for binding peptides, drugs or other substances, therapy and prevention might yet prove feasible with BGs as tropic carriers. In the study conducted by Paukner and his colleagues, *E.coli* ghosts were filled with the reporter substances calcein and sealed by fusion with membrane vesicles to maintain inner membrane integrity. Adherence and uptake studies showed that *murine* macrophages and human Caco-2 cells took up the bacterial ghosts and calcein was released within the cell [22].

![Fig. 4: Schematic of localization of different molecules in bacterial ghosts. Source: [34]](source)
Bacterial Ghost Application in Veterinary Vaccines: The mucosal routes of immunization has several advantages over other routes because of easy administration, reduced side effects and the high potential for frequent boosting [35]. Mucosal applied killed bacterial vaccines very often induce immune response that is often not sufficient for full protection against the disease and mucosal adjuvant, such as detoxified E.coli heat labial toxin or cholera toxin have to be added for immune potentiation [36]. BG application to mucosal surfaces by oral, intranasal, eye drop or aerogenic delivery at doses that induce a relevant immune response did not show any reactogenecity in experimental animals [18-21]. Recent tissue culture studies also showed that BGs even in high doses do not induce Cytotoxicity or genotoxicity [37].

Aerogenic application of A. pleuropneumonia BGs to pigs induced sterile mucosal immunity, whereas intra muscularly delivered A. pleuropneumonia Bgs also induced protection against the challenge but not against the potential carrier state of the vaccinated animals [38-39]. There are no limitations in BG vaccine approaches observed using mucosal or parentrally routs as it was detected in several cases that protective immunity against carrier bacteria [38-41] or target antigens [42-43] could be induced in experimental animals.

Immunizations with BGs produced from vary pathogenic Gram negative bacteria strains have been studied using different animal models [44] BG prepared from A. pleuropneumonia was used to protect pigs against Actinobacillosis. Animals vaccinated intramuscularly with A. pleuropneumonia BG were not only protected against colonization of the lungs by A. pleuropneumonia up on exposure [33]. Aerosal immunization of pigs with BGs derived from the lung pathogen A.pleuropneumonia lead to a significant increase in specific IgG titers together with increased ratios of CD4+:CD8+ lymphocytes [39].

Mutual co incubation of APCs and A.pleuropneumonia BGs with T cells isolated from the blood of animals immunized with A. pleuropneumonia BGs resulted in significantly higher proliferation of specific T cells compared to proliferation of T cells stimulated with APCs but with no BGs. Observed florescence intensities of MHC class II molecules expressed on the surface of DCs incubated with BGs were at least one magnitude higher compared to non-stimulated DCs [45]. The capacity of BGs to activate the immune system of the host and protect the host from lethal challenge with the bacterial pathogen was also detected after intragastric immunization of mice with BGs prepared from EHEC. BGs induced humoral immune responses and stimulated Ag specific IFN-ã producing T cells which were able to protect 86% of animals immunized with a single dose of BGs after challenge with a heterologous EHEC strain. Furthermore additional booster with BGs increased the level of survival up to 90% [40].

The capacity of P.multocida and M. hemolytica BGs to stimulate specific immune responses against the examined pathogen was also tested in rabbits and mice. Administration of P.multocida and M. hemolytica led to development of specific antibodies against the strains used for generation of BGs. Furthermore, study results showed that the induced antibodies exhibited cross-reactivity against other pastewrella serotypes. Cattle immunized with M.hemolytica BGs develop better protective immunity compared to commercially available vaccines [46]. BGs from E. coli O78:K80 and other serotypes provided protection to one day old chicks against colibacillosis and induce no acute infection after administration when compared with the conventional vaccine. Furthermore immunization of piglets with K.pneumonia Kpn-3 BGs stimulated an antigen specific humoral immunity, with levels of antibody titers similar to the levels detected after infection with virulent strains. Moreover the results obtained showed that antibodies induced after immunization with K.pneumonia Kpn-3 BGs possessed cross reactivity against a heterologous strain, including cross protective immunity stimulated by vaccination with BGs. V.cholera BGs prepared from O1 and O139 strains were evaluated. Rabbits orally immunized with different doses of VCG formulations were fully protected against the disease [30].

Bacterial Ghost: Future Vaccine Candidate for Aquaculture: Aquaculture has been growing in importance, in particular fish farming and has experienced an industrial boom due to an increased demand for marine food in the nutrition market. The majority of industrial food animal, including fish, suffer from physical stress because of their growth in environment which is usually over crowded. These stress full conditions make them more prone to bacterial infections such as Edwardsielliosis and vibrosis, which in most cases result in massive economic losses. Continuous treatment of these diseases with antibiotics leads to the development of antibiotic resistance bacterial strains. Recently there has been a growing concern among end consumers regarding drug residues in the meat of industrial farm animal including fish [34].
E. trada is a Gram negative bacterium causing Edwardseilosis in both fresh and marine fish (e.g. cat fish, tilapia and Chinook salmon). This disease is characterized by septicemia with extensive skin lesion and leads to massive economic losses. So far, there is no effective vaccine available against this disease owing to the wide range of E. trada serotypes [47]. BGs prepared from E. trada represent a novel potential system for the design.

Intraperitoneal immunization of tilapia fish (Oreochromis mosambicus) with E. trada BGs provided a higher degree of protection against Edwardseilosis compared with the group injected with formalin killed E. trada [47]. Furthermore, E. trada BGs administered orally to Olive flounder (Paralichthys olivaceus) proved to be an ideal vaccine candidate eliciting both systemic and mucosal immune responses. Moreover, both studies confirmed that immunization with BGs is simple and less stressful to vaccinate fish of any size [48-50].

Recently BG from another fish pathogen, V. anguilarum, was produced for the future animal studies to counter the most serious fish disease named Vibrosis [51]. Furthermore, the BG system has been used to design a novel type of attenuated fish vaccine by combining live-attenuated V. anguilarum. This was successfully used to induce cross protective immunity against Vibrio pathogens through the use of BG technology.

In this novel approach, the attenuated bacteria carrying an in vivo inducible lysis gene E will be administered orally to the target fish population; this will lead to the production of BGs from attenuated bacteria in vivo. This new vaccine system provides two major benefits. First the BG technology applied to the selected attenuated pathogen will guarantee no reversal to the native pathogenic form and second the target expression of foreign recombinant antigens in the cytoplasm or their incorporation in to the membrane of the host pathogen selected for immunization might serve as a multivalent vaccine and stimulate immune responses against both delivered antigen and the pathogen. These results indicate the future exploitable potential of the novel vaccination system combining the features of the BG technology and live bacterial vectors [34].

Recombinant Bacterial Ghosts: For the production of combination vaccines against bacterial and viral pathogens or the use of bacterial ghosts as carrier systems for other antigens, a membrane targeting system was developed for the attachment of foreign protein entities to the inner side of the cytoplasmic membrane. By cloning the 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 membrane anchors directing and attaching the fusion protein to the envelope complex of the bacteria prior to E-mediated lysis. The list of membrane anchored target proteins is constantly growing and comprises various viral core or envelope proteins of HIV-1, SIVagm3, HBV, HSV-1 or PRV, bacterial target antigens like CTB of V. cholerae, StrpA of S. avidini and VpsA of M. bovis; or enzymes like β-galactosidase and PHB-synthase. In the latter examples the enzymatic activities of the membrane anchored enzymes (β-galactosidase requires quartomer formation to be active) are not impaired showing that the anchors do not interfere with the proper folding of the target proteins and that clustering and self assembly is possible. The membrane anchored target antigens carried by recombinant ghosts induced humoral as well as a cellular immune responses in animal models. It should be further emphasized that the system of membrane anchoring is not limited by the size of the foreign protein moieties attached to the inside of the inner membrane and that combinations of different antigens to be anchored are possible [16].

On the study that investigates the feasibility of a combination of recombinant surface layer (S-layer) proteins and empty bacterial cell envelopes (ghosts) to deliver candidate antigens for a vaccine against nontypeable Haemophilus influenzae (NTHi) infections. The S-layer gene sbsA from Bacillus stearothermophilus PV72 was used for the construction of fusion proteins. Fusion of maltose binding protein (MBP) to the N-terminus of SbsA allowed expression of the S-layer in the periplasm of Escherichia coli. The outer membrane protein (Omp) 26 of NTHi was inserted into the N-terminal and C-terminal regions of SbsA. Electron microscopy showed that the recombinant SbsA maintained the ability to self-assemble into sheet-like and cylindrical structures. Recombinant E. coli cell envelopes (ghosts) were produced by the expression of SbsA/Omp26 fusion proteins prior to gene E-mediated lysis. Intraperitoneal immunization with these recombinant bacterial ghosts induced an Omp26-specific antibody response in BALB/c mice. These results demonstrate that the NTHi antigen, Omp26, was expressed in the S-layer self-assembly product and this construct was immunogenic for Omp26 when administered to mice in bacterial cell envelopes [52].

In the recombinant bacterial ghost system, foreign proteins are attached on the inside of the inner membrane as fusions with specific anchor sequence, ghosts have a sealed periplasmic space and the export of proteins into
this space vastly extends the capacity of ghost or recombinant ghost to function as carriers of foreign antigens. In addition S-layer proteins forming shell-like self assembly structures can be expressed in candidate vaccine strains prior to E-mediated lysis. Such recombinant S-layer proteins carrying foreign epitopes further extend the possibilities of ghost as carriers of foreign epitopes (this make the ghosts have inherent adjuvant properties) [53].

**Conclusion and Recommendations:** Despite the exponential rate of discovery of new antigens and DNA vaccines resulting from modern molecular biology, the lack of effective delivery technology is a major limiting factor in their application. Bacterial ghosts are very useful nonliving carriers as they can carry foreign antigens, nucleic acid and drugs in one or more cellular locations. Their ease of manufacture and the fact that they can be stored and processed without the need for refrigeration and their excellent safety profile—even when administered at high doses—are important consideration for a broad spectrum of application. The identical surface receptors of the bacterial ghost and their living counterparts are being exploited for specific cellular and tissue targeting. Considering the above facts, the following recommendations were forwarded:

- BG is a new and on research technology, which needs much work and further investigation.
- Ethiopian Veterinary Biological production institutes should plan and adopt this new and innovative technology so as to increase the wide range of vaccine delivery systems.
- Further scientific improvements in application of BG as delivery system for vaccines, drugs and nucleic acids should be made.

**REFERENCES**


