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Review Article

Novel Applications of Bacterial Ghost- A Review

Joycee Jogi[1]*, Anju Nayak[2], Poonam Shakya[1], Ajay Rai[1], Smita Bordoloi[3] and Himani K.[4]

[1]Assistant Professor, Department of Veterinary Microbiology, College of Veterinary Science and A.H., NDVSU, Jabalpur, INDIA
[2]Associate Professor, Department of Veterinary Microbiology, College of Veterinary Science and A.H., NDVSU, Jabalpur, INDIA
[3]PhD Scholar, Department of Veterinary Microbiology, College of Veterinary Science and A.H., NDVSU, Jabalpur, INDIA
[4]PG Scholar, Department of Veterinary Microbiology, College of Veterinary Science and A.H., NDVSU, Jabalpur INDIA

*Corresponding Author: drjoycee@gmail.com

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Abstract

The increased urbanization and changes in lifestyle has gifted us with an increasing spectrum of Infectious diseases. The traditional techniques used for combating the issues that threaten the health are becoming less efficient and showing no or very meagre effects. Hence, there is an urgent need to develop or adopt alternative strategies and modern approaches to conserve the health and to combat infections at global scale. In this race bacterial ghosts are considered to be a suitable option. Bacterial Ghosts are intact bacterial cell envelopes that were subjected to the evacuation of their cell content either gene based biological or chemical poring methods as a result of which they are empty shells acting as ghost. The safety as well as the efficacy of the killed vaccines along with the maintenance of their antigenicity due to the mild preparation procedures can be improved by these kinds of Ghost techniques, resulting in an excellent candidate availability for immunotherapy. This review provides a bird's eye view on Bacterial Ghost.

Keywords: Antigenicity, Bacterial Ghost, Immunotherapy, Killed Vaccines

Introduction

In the current era, the increase in the rate and number of infections have lead to a surge in the demand for reliable vaccines, biological carriers as well highly efficient drug delivery systems for combating the medical complications that threaten the health. Live attenuated as well as killed (Bergmann and Leitner, 2014) microorganisms have been traditionally employed so far for the purpose of invoking the protective defence against infections. The live attenuated vaccines are prepared by either serially passaging the organism in cell culture or by selectively disabling of the genes associated with virulence as well as survival of the pathogen (Kamble and Lee, 2016). Despite eliciting a potent immune response against the infection, the live attenuated organisms are not widely employed because of the inadvertent risk of infection that is associated due to the presence of viable organisms in them. The Killed whole-cell vaccines are considered to be the first-generation versions that have served in the immunoprophylaxis of the human-race during several epidemics as an alternative to the live attenuated vaccine. The Inactivation of the microbes for the production of vaccine usually depends on chemical or physical techniques that are capable of permanently denaturing the genetic content of the organism (Szostak *et al.,* 1996) and thereby are easy to produce and as safe to administer. Despite their feasibility, killed vaccines are often packaged with certain disadvantages due to harsh attenuation procedures such as Binary EthylenImine (BEI) treatment or due to the complete disruption of the pathogen leading to the loss of many essential structural as well as immunogenic components of microorganisms. This kind of vaccine results in the impaired function as well in the production of non-efficient immune responses (Ahmad, 2016). Thus, low cell-mediated immunity (CMI) and shorter immunity is produced in comparison to the live vaccines (Rodriguez and Grubman, 2009).

In order to avoid these kinds of complications, novel vaccines like the DNA based and subunit vaccines were invented and analysed over the last two decades but so far very little effectiveness has been observed (Lee *et al.*, 2012). These vaccines exhibited poor immunogenicity when compared to the traditional vaccines and as a result the requirement for the presence of an appropriate adjuvant aroused. Further, the DNA based vaccines were not targeted effectively to the cells like antigen presenting cells (APC) leading to the requirement for a better delivery system (Nanda *et al.*, 2014).

Hence, in this complicated scenario, Bacterial Ghost (BG) technology is emerged to rescue. The Bacterial Ghost technology is an innovative product that can be used for the purpose of vaccine, drug delivery, active substance transport as well as for technical applications in the field of biotechnology. The Ghosts are the microbial cells who were subjected to evacuation of content but still retain the three-dimensional cellular structure and adhesion capabilities. In simple terms, the bacterial ghost is an empty envelope of Gram-negative bacteria whose surface as well as virulence characters are conserved and are devoid of the internal components (Langemann *et al.*, 2010). Apart from being employed as an alternative to the killed vaccines (Szostak *et al.*,1996), the bacterial ghost technology also confers service such as, being an efficient carrier with the capability to enhance the low immunogenic property of the protein subunits as well as DNA-encoded antigens, in simultaneously expressing diversified antigens (Walcher *et al.*, 2004) or in carrying mixtures of antigens or lysates (c; Michalek *et al.*, 2017), they can also act as appropriate adjuvant (Riedmann *et al.*, 2007; Hajam *et al.*, 2017), or can even express the receptors for antitoxins (Paton *et al.*,2015). Bacterial ghost technology is an explorative field with the ability to provide abundant beneficial services. Further research is required to promote this trend for the maximization of the productivity as well as the safety of the ghost vaccines.

1. Bacterial Ghost Production

The Bacterial ghosts (BGs) can be synthesized by the genetic methods or on chemical basis. Mostly, the ghosts are produced from Gram-negative bacterial cells that are subjected to the controlled expression procedure of cloned phage lysis-gene, along with their quantitative as well as qualitative immunogenic property being conserved (Huter *et al.,* 2000). The requirement for the product of ghosts from the microbes other than the Gram-negative ones led the advent of less inhibitory-chemicalized protocols to induce the pore formation in the cell envelope thereby laying down the basis for chemical production of bacterial ghosts (Amara *et al.*, 2014). Some of the methodologies used for the production of Bacterial Ghosts are as follows.

1.1: Phage-Mediated Lysis Protocol in Gram-Negative Bacteria

The bacteriophages are also host specific and infect only their target hosts or the bacterial strains that are closely

related to each other. The role played by the gene *E* in the lysis of Gram-negative bacteria, *Escherichia coli*, was reported first in the study by Hutchison and Sinsheimer (1966) and this gene was subsequently identified in the *E. coli* cells that were heavily UV-irradiated. The gene *E* codes for about 91 amino acids capable of lytic action but they do not possess any inherent enzymatic activity. The membrane protein encoded has hydrophobic moieties at its N-terminal region capable of oligomerizing into a transmembrane tunnel structure (Bläsi *et al.*, 1989). The E-specific tunnel structure is responsible for spanning the inner and outer membrane and is located at the membrane adhesion sites within the host cell. Electron microscopic analysis has revealed that the tunnel formation is associated with the fusion of the inner and outer membrane which seals the periplasmic space leading to high osmotic pressure as a result of which the cytoplasmic contents are expelled through the tunnel leaving behind empty cell envelopes known as BG. The lysis of E-gene is accomplished in actively growing cells (Witte *et al.*, 1998) by the fusion of the inner as well as the outer membrane which thereby leads to the formation of transmembrane tunnel that can vary in size (Wang X. and Lu C. ,2009 and Wang *et al.*,2016). Subjecting the host cell to osmotic pressure leads to the voiding of its contents through the formation of initial few completed pore while the normal cell-shape is conserved (Young *et al.*, 1992; Mu *et al.*, 2011). This results in the formation of Bacterial Ghost. The *Escherichia coli* was the first and most extensively studied bacterial strains for the purpose of application in ghost vaccines as well as platform production, as ΦX174 is mainly a dedicated phage for the *E coli*.

1.2: Plasmid Vector based Bacterial Ghosts Preparation

The lysis genes are initially cloned into the plasmid vectors for the purpose of ensuring a regulated expression as well as appropriate lysis effect (Figure 1). The plasmids usually harbour an antibiotic resistance gene marker (Witte *et al.*, 1992) or aspartate semialdehyde dehydrogenase (asd) (Nakayama *et al.*, 1988) for ensuring the stability of the plasmid which results in the successful formation of the Bacterial ghosts with an expressed protein.

In general, the gene *E* mediated lysis leads to the complete inactivation of almost all the Gram-negative bacteria (Table 1) except in the case of *E. coli*. In this scenario, for the purpose of achieving a complete killing, H_2O_2 is added to the bacterial culture after an 4 h induction of lysis gene *E*. This leads to the complete killing as well as simultaneous genomic DNA inactivation of the *E. coli* cells (Paton *et al.*, 2015). Another alternative to this kind of inactivation of *E. coli* is the expression of *staphylococcal nuclease* (SNUC) gene in association with the E-mediated lysis gene (Ahmad *et al.*, 2012).

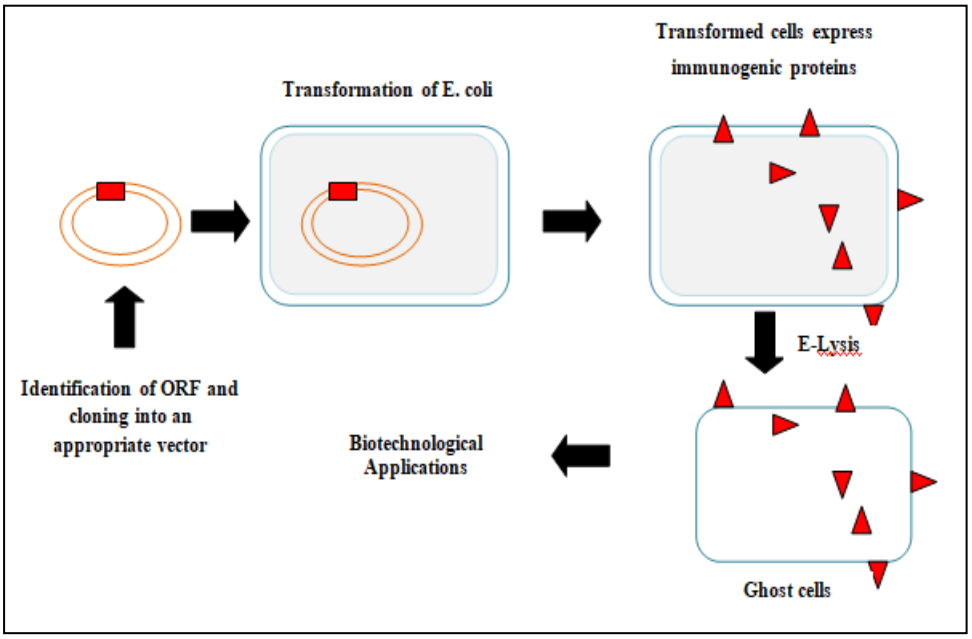

Figure 1: Representation of Gene based Bacterial Ghost production

Other approaches for the inactivation of viable cells include, addition of beta-propiolactone or the addition of antibiotics such as gentamycin or chloramphenicol (Young *et al.*, 2000; Ganeshpurkar *et al.*, 2014). However, the technique involving the expression of the *SNUC* gene or the addition of H_2O_2 to the culture is considered to be more advantageous than the use of antibiotics as the former completely inactivates even existing residuals of genomic

DNA thereby restricting the introduction of resistance genes or pathogenic islands which can potentially influence the gut commensals. The Bacterial Ghosts (BG) of a number of Gram-negative bacteria have been synthesized through the process of E mediated lysis and have been evaluated as an efficient candidate vaccines and adjuvants in a wide range of animal models. Apart from the E gene mediated lysis, the BG of *E. coli* has also been produced by the application of high hydrostatic pressure (HP) in which, the cells are initially sensitized to conditions of high pressure shock through the over-expression of E. *coli* K12 Mrr protein (Young *et al.*, 1992). In another method, holin-endolysins were expressed along with the gene E in *Salmonella* (mention species also) and this study reported complete inactivation much faster than the current BG production techniques (Schroll *et al.*,1998).

Table 1: Table representing Bacterial Ghosts produced from various bacterial cells

Microorganism	Examples of Bacterial Ghost
Escherichia coli	*E. coli* (O101) ghost of avian pathogenic *E. coli* serotype O78:K80 (Ebrahimi *et al.*, 2018; Lv *et al.*, 2010)
	E. coli (O101) ghost serotype O2:K1 (Shahidi *et al.*, 2019; Shahidi *et al.*, 2020; Tabar *et al.*, 2020)
Salmonella spp.	*S. Enteritidis* ghosts containing ampicillin marker (Jawale *et al.*, 2012)
	Ghosts of *S. enterica serovar gallinarum biovar pullorum* (Guo *et al.*, 2016)
	S. typhi ghost harboring LTB (Kim *et al.*, 2017)
	S. typhimurium ghost carrying *Helicobacter pylori* outer inflammatory protein gene (Chen *et al.*,2014)
	S. typhimurium ghost carrying pcDNA-HA plasmid encoding for the H1N1 hyaluronidase of *Influenza virus* (1 Kamble *et al.*, 2019)
Vibrio spp.	*Vibrio cholerae* (O1) ghost (VCG)(Eko *et al.*,, 1994)
	Ghosts of *Vibrio mimicus* (Cao *et al.*,2018).
Pasteurella spp.	*P. haemolytica* (Marchart *et al.*, 2003).
	P. multocida and *P. haemolytica* combined ghost (Marchart *et al.*, 2003)
Edwardsiella spp.	*E. tarda* ghost vaccine (Wang *et al.*, 2009)
	E. ictaluri ghost (Wang, 2016)
Aeromonas spp.	*A.hydrophila* ghost (Chu *et al.*, 2008; Tu *et al.*, 2010)
	A. veronii ghost (Jiang *et al.*, 2019)
Helicobacter pylori	*H. pylori* ghost loaded with rOmp18 (Talebkhan *et al.*, 2010)

1.3: Chemical Evacuation Methods

The chemical evacuation is the process employed for the production of Bacterial Ghosts from other cells apart from the conventionally employed Gram-negative bacteria. Various gentle chemical-based protocols are available for the induction of the evacuation pores at the cell wall of the host microbe. The chemical-based methods has an advantage of overcoming the risk associated with the vector plasmids that can harbour native pathogenic or antibiotics resistance genes and can possibly transfer it to the commensals in the human or animal host (Amara *et al.*, 2013a; 2014). The chemical-based approach involves minimal concentrations of chemicals such as NaOH, SDS and H_2O_2 which results in the production of sponge like structures (Witte A. *et al.*, 1992). This type of method can be applied for both the Gram-negative as well as Gram-positive bacteria. Mu *et al.*, 2011 prepared BG of *Listeria monocytogenes* employing chemical method which is, suggestive of the possibility of *Listeria monocytogenes* as candidate for future vaccine development against the important Gram-positive food-borne pathogens. The important approaches are mentioned as follows:

1.3.1: Sponge-like protocol

In an experiment conducted by the Egyptian-Saudi research group at King Saud University in Saudi Arabia 2013, the Plackett-Burman optimization and randomization protocol (Plachett and Burman, 1946; Amara *et al.*, 2013b;2014) was applied for the analysis of minimum inhibitory concentration (MIC) of a NaOH in combination with other chemical and physical conditions for stimulation to form pores within the in the microbial cells (Amara, 2015). Lower MIC concentration of the NaOH was employed for pore formation and Sodium Dodecyl Sulphate was added to wash the "sponge-like" content of the cell in the presence of $CaCO_3$ that had the potential to increase the permeability of the cell. As a final step H_2O_2 was applied for the hydrolysis of the residual nucleic acids in the

ghosts and inactivation of the remaining non-lysed cells was done using ethanol (El-Baky *et al.,* 2014; Amara.,2016).

The Sponge like protocol was reduced to be more reliable and given a name "Sponge Like Reduced Protocol" (SLRP). In a study, SLRP was employed to prepare *Klebsiella pneumoniae* (KPGs) with correct 3D structure and surface antigens. The produced KPGs were able to immunize rats subjected to different routes of administration against viable K. pneumonia (Menisy *et al.,* 2017). This protocol was proved to cause the degradation of RNA in the *Newcastle disease virus* ghosts (El-Baky *et al.,* 2014), and was also employed for the production of *Saccharomyces cerevisiae* ghosts (Amara,2015).

1.3.2: Minimum inhibitory concentration of sodium hydroxide

In another study at Pai Chai University in Korea, NaOH-MIC when applied alone was capable of producing bacterial ghosts. In 2014, protective vaccine against *Salmonella enteritidis* was successfully produced by this method (Vinod *et al.*,2014), similarly vaccines against *Staphylococcus aureus* (Vinod *et al.*,2015) and *Salmonella typhimurium* (Vinod *et al.,* 2017). This study proved that the NaOH alone was capable of providing both a complete cell lysis and complete DNA removal (Park H. *et al.,* cell lysis and complete DNA removal (Park H. *et al.,* 2016).

In a study, Vibrio parahaemolyticus ghosts (VPGs) were generated by chemically-induced lysis and the method was based on minimum inhibitory concentration (MIC) of sodium hydroxide (NaOH), acetic acid, boric acid, citric acid, maleic acid, hydrochloric acid, and sulfuric acid. Among those chemicals, NaOH-induced VPGs appeared completely DNA-free, which was confirmed by quantitative real-time PCR. Scanning electron microscopy showed the formation of trans-membrane lysis tunnel structures in the NaOH-induced VPGs. SDS-PAGE and agarose gel electrophoresis also confirmed that cytoplasmic proteins and genomic DNA released from the VPGs to culture medium through the lysis tunnel structures. Taken together, all these data indicate that the NaOH-induced VPGs show the potency of a safe, economical, and effective inactivated bacterial vaccine candidate (Park *et al.,* I2016).

1.3.3: Bacterial ghosts' preparation using Tween 80

In a study conducted in Egypt, BGs were prepared by a novel protocol by exposing the bacterial cells to tween 80 for an extended period of time followed by sudden reduction of the surrounding pH. *Salmonella enterica serovar typhimurium* ATCC 13311 was used for this purpose (Rabea *et al.,* 2019). High quality BGs were visualized by scanning electron microscopy (SEM) revealing punctured cells with intact outer shells and at least one intramembranous tunnel. The integrity of cells was proved by visualization of Gram-stained cells using light microscope and this new protocol was simple, economic and feasible for BGs preparation

2. Applications of Bacterial Ghost

2.1: As Vaccines

The BG vaccines are a fascinating invention and are comprised of freeze-dried BG particles devoid of any kinds of artificial stabilizers or adjuvants. Intrinsically the BG possess abilities to attract the immune cells, such as the dendritic cells, macrophages or the monocytes thereby achieving recognition via toll-like receptors or by the process of opsonisation (Riedmann *et al.*,2007). The various advantages associated with BG vaccines are:

1. They are devoid of hazards of horizontal gene transfer
2. They do not belong to genetic manipulated organisms (GMOs)
3. Do not require addition of adjuvants
4. Stable as Lyophillized powder
5. Cold chain independent
6. Can be administered via various routes
7. Amendable for mucosal administration
8. Highly versatile

2.2: As Carrier of Subunit Vaccines

Genetic engineering when applied to the host bacteria can lead to the modification of their cell envelope in order to

facilitate the carrying of foreign protein. This leads to the anchoring or the entrapment of a foreign protein within the bacterial envelop after *E* Gene mediated lysis (Mayr *et al.,* 2005).

2.3: As Carrier of DNA

DNA either in the linear or circular covalent closed form incorporated back to BG serves as carrier for immediate gene transfer experiments or for freeze drying (Paukner *et al.,* 2005).

2.4: As Carrier Vehicles for Active Substances in Tumour Therapy

DNA as well as the drugs can be employed as an active substance for tumour treatment and various investigations have reported that human tumour cells can be targeted with BG for the delivery of DNA or drugs (Curiel-Lewandrowski and Demierre,2000).

2.5: As mucosal vaccines

At present there is a dire need for the development of Bacterial vaccines as well as delivery vehicles for obtaining optimal mucosal immune responses. For DNA vaccines to exhibit their maximum potential, novel delivery systems activating mucosal immune responses are required. The bacterial ghost platform provides an alternative delivery system capable of targeting the mucosal tissues as the ghost cells are devoid of cytoplasmic contents but retain cellular morphology and native antigenic structures with bioadhesive properties (Jalava *et al.,* 2003).

2.6: As an adjuvant

Several considerable studies have so far demonstrated the effectiveness of BG as adjuvants and on their ability to induce proinflammatory cytokine production by a range of immune and non-immune cell types. These proinflammatory cytokines trigger a generalized recruitment of T and B lymphocytes to lymph nodes maximizing the chances of encounter with their cognate antigen, and subsequent elicitation of potent immune responses (Hajam *et al*., 2017).

2.7: As carrier for drug delivery

BGs are devoid of cytoplasmic content and possess all bacterial bio-adhesive surface properties in their original state while not posing any infectious threat. They are ideally suited as an advanced drug delivery system (ADDS) for toxic substances in tumor therapy as well as other infections. The inner space of BGs can be loaded with either single components or combinations of peptides, drugs or DNA which provides an opportunity to design new types of (polyvalent) drug delivery vehicles (Kudela *et al.,* 2010).

A summarization of the importance of various applications of Bacterial Ghosts has been depicted in Table 2.

Table 1: Applications of bacterial ghosts

Application	Importance
DNA Vaccines	Increased DNA transfection effciencies, increased immunogenicity, enhanced protective efficacy (Riedmann *et al.,*2007).
Delivery systems	Effective delivery of drugs into cancerous cells, enhanced cytotoxic potential (Curiel-Lewandrowski and Demierre, 2000).
	As carriers of immunocontraceptives evoked humoral and cell-mediated immune responses against ova proteins (Walcher *et al.,* 2008)
	As carriers of foreign antigens for treating infectious diseases (Montanaro *et al.,2015)*
	As carriers of enzymes, antibiotics and vitamins (Tabrizi *et al.,*2004).
Adjuvants	BG contain well-known innate immune stimulating components, and have thus tremendous potential to act as efficient adjuvants (Hajam *et al*., 2017).
Immunomodulatory agents in cancer immunotherapy	Significant increase in survival rate and circulating CD8a+ T cells, signifcant decrease in metastasis foci area and incidence (Krasko *et al*., 2016)

Conclusion

The technology of Bacterial Ghost has the potential to revolutionize the field of Biotechnology with special emphasizes on the Immunotherapy. The Ghosts are considered to be highly qualified to serve as immune-presentable platforms. They can be considered as an intermediate state in the development that can potentially overcome the several defects of the killed vaccines. So far, this technology has provided success as an alternative to the killed, enveloped, recombinant as well as DNA ghost-vaccines, as well as adjuvants, the antidotes, contraceptive, therapeutic as well as the cellular vaccines. Furthermore, explorative research is required to expand the beneficial landscape of the Bacterial Ghost.

Conflict of Interests

There is no conflict of interest.

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