Bacterial Ghosts as Adjuvant Particles

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Abstract

The development of more advanced and effective vaccines is of great interest in modern medicine. These new generation vaccines, based on recombinant proteins or DNA, are often less reactogenic and immunogenic than traditional vaccines. Thus there is an urgent need for the development of new and improved adjuvants. Besides many other immunostimulatory components, the bacterial ghost system is currently under investigation as a potent vaccine delivery system with intrinsic adjuvant properties. Bacterial ghosts are non-living cell envelope preparations from Gram-negative cells, devoid of cytoplasmic contents while their cellular morphology and native surface antigenic structures remain preserved. Due to the particulate nature of bacterial ghosts and the fact that they contain many well known immune stimulating compounds, bacterial ghosts have the potential to enhance immune responses against ghost delivered target antigens.

Key Words

Vaccines, bacterial ghosts, adjuvants, immunology, antigen delivery,

1. Adjuvants

Immunological adjuvants were originally described as “substances used in combination with a specific antigen that produced a more robust immune response than the antigen alone” (Ramon 1924). This broad definition includes a wide range of materials. Despite extensive research in this field, the only adjuvants currently approved for routine use in humans are aluminum based mineral salts, generically called alum. Alum has been used as an adjuvant for human and veterinary vaccines for many years but its mechanism of action is still poorly understood. Recent studies have disproved the original theory that alum does establish an antigen depot at the injection site [1]. It has been indicated that alum upregulates co-stimulatory signals to human monocytes and promotes the release of IL-4 *in vitro* [2]. Alum adsorption may also contribute to a reduction in toxicity for some vaccines, due to the adsorption of contaminating endotoxin [3]. Alum has a good safety record, but comparative studies show that it is a weak adjuvant for antibody production to protein subunits and a poor adjuvant for cell mediated immunity, especially cytotoxic T-cell responses [4].

New generation vaccines, based on DNA or recombinant proteins, are often less reactogenic and immunogenic than traditional vaccines. As a consequence, combinations of antigens with adjuvants which are effective and cost-efficient are necessary to improve vaccine performance. Based on their principal mechanism of action, adjuvants can broadly be separated into two classes; immunostimulatory adjuvants and vaccine carrier systems, some of which have adjuvant properties. While vaccine carrier systems are generally particulate, for example microparticles, iscoms, liposomes and emulsions, immunostimulatory adjuvants are predominantly molecular structures derived from pathogens, for example lipopolysaccharide (LPS), monophosphoryl lipid A (MPL) or CpG DNA. Vaccine carrier systems mainly function to target associated antigens to antigen presenting cells (APC). In contrast, immunostimulatory adjuvants can activate cells of the innate immune system, which once they are activated will drive and focus the acquired immune response. Few examples of the

two different classes are given, and bacterial ghosts which belong to the group of particulate adjuvants with targeting functions for APC are the main focus of this review.

1.1. Immunostimulatory Adjuvants

Immunostimulatory adjuvants often represent pathogen associated molecular patterns (PAMPs) like LPS, MPL or bacterial DNA. Understanding of the mechanism behind this potentiation of immune response has improved since some of the pattern-recognition receptors (PRRs) involved in the innate immune response to PAMPs have been identified [5].

One class of immunostimulatory adjuvants are derived from LPS of gram-negative bacteria. The most extensively evaluated member of this family, MPL, is obtained from *Salmonella minnesota* and interacts with toll-like receptor 4 [6]. MPL induces the synthesis and release of cytokines, particularly IL-2 and IFN- γ , which promotes the generation of Th1 responses [7]. In addition, MPL appears to increase the migration and maturation of dendritic cells (DC) [8]. MPL has often been used in complex formulations, including liposomes and emulsions, making it difficult to determine the contribution of MPL to the overall adjuvant effect [9]. It has also been claimed that MPL may be used as an adjuvant for DNA vaccines [10] and for mucosal delivery of vaccines [11, 12].

Bacterial DNA, but not vertebrate DNA, has been found to have a direct immunostimulatory effect on immune cells *in vitro* [13]. This effect is due to the presence of unmethylated CpG dinucleotides [14], which are under-represented and methylated in vertebrate DNA. Vertebrate immune systems appear to have evolved a specific toll-like receptor, TLR9, that distinguishes bacterial DNA from self-DNA [15]. Bacterial DNA containing CpG oligonucleotides activates cells of the innate immune system, including macrophages and DCs [16], and may preferentially elicit Th1 type responses [17]. The potential of CpG as a mucosally delivered adjuvant was shown in a recent study where the adjuvanticity of CpG was evaluated with intranasal and oral delivery of tetanus toxoid or the

hepatitis B surface antigen in mice [18]. The use of synthetic CpG oligos as adjuvants to boost the immune response to DNA- and protein-based immunogens in rodents led to an increase in antigen specific serum antibody levels and IFN γ production. The adjuvant effect could be maximized by physically linking the CpG containing motifs to the immunogen [19]. CpG oligonucleotides have mainly been evaluated in rodent models, but recent studies have described sequences that are active in primates, including humans [20].

Another group of immunostimulatory adjuvants known as saponins are derived from the bark of a tree native to Chile (*Quillaja saponaria*). Saponins have been widely used as adjuvants for many years and have been included in veterinary vaccines. Although not pathogen-derived, saponins function mainly through the induction of cytokines. The surface active saponins cause haemolysis of red blood cells *in vitro*, although haemolysis does not appear to correlate with adjuvant activity [21]. QS21, a pure fraction of Quil A saponin with low toxicity, has been shown to be a potent adjuvant for CTL induction, and induces the Th1 related cytokines IL-2 and IFN- γ [21]. A number of clinical trials have been conducted using QS21 as an adjuvant for a number of infectious diseases, including HIV-1, influenzae, herpes, malaria and hepatitis B [6]. QS21 has also been claimed to perform as an adjuvant for DNA vaccines, following both systemic and mucosal immunization [22].

As an alternative to the use of cytokine inducing adjuvants, cytokines may also be used themselves as an adjuvant. Most cytokines have the ability to modify and re-direct the immune response; the ones most extensively evaluated as adjuvants including IL-1, IL-2, IFN- γ , IL-12 and GM-CSF. The dose related toxicity of systemically applied cytokines may be overcome by a more precise delivery through the use of microparticle carriers such as liposomes [23]. However, due to their protein nature, cytokines have stability problems and are relatively expensive to produce in large quantities. It is therefore unlikely that they will find widespread application as adjuvants in routine vaccination [6].

The toxins of *Vibrio cholerae* (CT) and *E. coli* heat-labile enterotoxin (LT) have been described as very potent mucosal adjuvants, however they are too toxic for use in humans. They will be dealt with in more detail in the section on mucosal adjuvants.

1.2. Delivery Systems

Vaccine delivery systems are generally particulate and have comparable dimensions to pathogens, which the immune system has evolved to combat. The principal mode of action of many particulate adjuvants or "antigen delivery systems" may be to promote the uptake of the antigen by APC at the site of injection. The most important APC involved in antigen capture are the DCs. Following antigen uptake and cell activation, DCs undergo maturation and migrate to lymph nodes where they present the antigen to naive T cells. However, some of these delivery systems might also be capable of moving away from the injection site in lymph and directly delivering antigen to a lymph node. Nevertheless, the successful delivery of antigen to a lymph node alone will not necessarily result in the induction of an immune response, as the support of co-stimulatory molecules and cytokines is essential. Immunostimulatory adjuvants may also be included in particulate delivery systems to enhance the level of response, or to drive it towards a desired type of response, for example a Th1 or Th2 dominant response.

A number of lipid formulations have been evaluated as adjuvants. Widely used emulsions in animal immunization studies are the Freund's adjuvants. These are potent but toxic water-in-mineral oil formulations, which may also contain killed mycobacteria. While the oil creates a depot and enables slow release of the antigen, the mycobacteria stimulate the immune system. A squalane oil-in-water emulsion, MF59, represents a potent adjuvant with an acceptable safety profile [24]. The safety and immunogenicity of MF59-formulated influenza vaccine has been confirmed in clinical trials with elderly subjects [25, 26] and infants [27]. When compared to the commercial alum adjuvanted hepatitis B vaccine (HBV),

MF59 has been shown to be 100-fold more potent [28]. MF59 is an adjuvant that is approved for human use [9]. This adjuvant formulation contains squalene (2,6,10,15,19,23-hexamethyl-2,6,10,14,18,22-tetracosahexane) which can induce inflammatory arthritis in susceptible rats and mice, although to date, the only report of possible adverse reactions in humans was restricted to some individuals with Gulf War Syndrome symptoms that had received certain lots of an anthrax vaccine [29]. A recent review by Kurodo et al reported that autoantibodies in mice appear to be associated with the hydrocarbon's adjuvanticity, however, these authors indicate that the significance of this for human use is complex and unclear at present [30]. Emulsions can also be used as delivery systems for immunostimulatory adjuvants, including MPL and QS21, allowing them to be targeted for enhanced uptake by APC. In a HIV-1 human trial, this formulation induced high antibody titers and proliferative T-cell responses, but no CTL activity [31].

Liposomes are phospholipid vesicles that have been evaluated both as adjuvants and as delivery systems for antigens and adjuvants [32]. Several liposomal vaccines based on viral membrane proteins (viroosomes) without additional immune stimulators, have been evaluated in the clinic and are approved as products in Europe for hepatitis A and influenza [33]. Modified liposomal structures termed "choleates" have been evaluated in small animal models as systemic and mucosal adjuvants [34]. Choleates can be used to trap different agents and are formed as a precipitate when calcium cations are added to negatively charged liposomes [35]. These are a versatile delivery system reported to be highly effective for the delivery of DNA- and protein-based vaccine formulations via mucosal and parenteral routes resulting in long lasting efficacious immune responses [36]. In addition, liposomes can be prepared so as to modify their specific properties. For example, preparation of liposomes with cationic lipids can be advantageous for the absorption of certain antigens [37]. Enhanced stability of liposomes in the gut has been described for polymerized liposomes [38].

In the immunostimulating complex (ISCOM), multiple copies of an antigen are attached to a matrix built up by the immunostimulatory fraction of *Quillaja saponaria*, cholesterol and phospholipids [39]. A study in macaques has shown that an influenza ISCOM vaccine was more immunogenic than a classical subunit vaccine [40], and a similar formulation induced a CTL response in human clinical trials [41]. The principal advantage of the ISCOM preparation is the dose reduction of the hemolytic Quil A component and its association with cholesterol. This prevents the interaction with cell membranes and significantly reduces the hemolytic activity of Quil A [39]. However, a potential problem with ISCOMs is that the inclusion of antigens into the adjuvant is often difficult, sometimes requiring extensive antigen modification [6]. While hydrophobic and amphipathic antigens are spontaneously incorporated into ISCOMs by hydrophobic interactions, hydrophilic soluble proteins, such as the nucleoprotein of influenza A virus, are not. These proteins may be linked to hydrophobic carrier molecules, such as palmitic acid [42] or bacterial LPS [43]. Another approach uses treatment of the antigen with low pH in order to expose hydrophobic stretches in the protein molecule that are normally hidden [44, 45]. However, such treatments may result in altering the protective immunogenicity of the antigen.

Besides the aforementioned lipid based adjuvants, microparticles are being evaluated as delivery systems. Antigen uptake by APC is enhanced by association of antigen with polymeric microparticles, or by the use of proteins which self-assemble into particles. Both, physical particles such as polylactide-co-glycolides (PLG), and biological particles including virus-like particles (VLP) and bacteria derived particles (bacterial ghosts), present interesting approaches in adjuvant and vaccine delivery developments.

Poly(lactide-co-glycolide) (PLG), biodegradable and biocompatible polyesters, are the primary candidates for the development of microparticles as adjuvants. They have been used in humans for many years as suture material and as controlled release drug delivery systems [46]. Their adjuvant effect, achieved through the encapsulation of antigen into PLG, has been

demonstrated in several animal studies [47-49]. In contrast to alum, PLG microparticles can effectively elicit CTL responses against entrapped antigens in rodents [50], but so far this has not been demonstrated in primates [6]. Microparticles have also shown potential as adjuvants for DNA vaccines [51]. This novel approach was demonstrated using cationic microparticles with adsorbed plasmids which further enhanced both antibody and CTL responses when compared with that following immunization with naked DNA in different animal models [52]. Preparation of anionic microparticles was effectively used for delivery of adsorbed proteins and resulted in induction of potent CTL and antibody responses to the HIV-1 p55 gag protein in mice [53]. In another HIV-1 vaccine study, a better immune response was obtained by formulating the antigen p24 into PLG microparticles and MF59 emulsion than with MF59 plus p24 alone, hence demonstrating the enhancing adjuvant capacity of microparticle formulation [54].

Recombinant proteins that naturally self-assemble into particles may also enhance delivery of antigens to APC. The first recombinant protein vaccine was developed against hepatitis B virus, based on hepatitis B surface antigen (HBsAg) expressed in yeast as a particulate protein [55]. Nucleic acid vaccination of mice with HBsAg showed to be highly immunogenic and can be used to prime CTL responses *in vivo* [56]. Furthermore HBsAg and other virus-like particles (VLP's) can be used as adjuvants for co-expressed proteins. A semi-synthetic system in which the particle forming *S. cerevisiae* p1 protein, encoded by the *tya* gene of the yeast retrotransposon Ty, self assembles into VLP, has shown potential as an adjuvant [57]. Recombinant Ty VLPs, carrying a number of CTL epitopes from *Plasmodium* species, have been shown to prime protective CTL responses in mice [58]. Ty VLPs have also induced CTL activity in macaques against a co-expressed SIV antigen [59], and in clinical trials Ty VLPs have been shown to be safe and immunogenic in humans [60].

The potential of bacteria-derived particles as adjuvants and delivery systems has been well demonstrated. One of the most extensively studied bacterial carriers for vaccination is

Salmonella, but many other bacteria, including *V. cholerae*, *Shigella* sp., enterotoxigenic *Escherichia coli*, *Listeria monocytogenes*, *Mycobacterium tuberculosis* and *Lactobacillus* sp. have been studied [61-63]. Live vaccines, however, may themselves carry some risk of causing illness or disease, especially in immune deficient individuals. Non-replicating vaccines, consisting of killed microbes or particles made from bacterial outer membranes, may represent an alternative to attenuated live organisms.

The use of outer membrane vesicles (OMVs) from group B meningococci is an approach to delivering vaccine candidates from this bacterium that has been shown to be immunogenic in mice following intranasal immunization [64]. Such vaccines when delivered nasally appeared to induce antibody responses of better functional quality than those resulting from a parenterally delivered vaccine. In addition, it would appear that the concept of "self-adjuvant" that is possible with such bacterial formulations may have certain immunological advantages for mucosal vaccines. In taking this further, a formulation of formalin-inactivated influenza virus particles with the group B meningococci OMVs, was reported to induce higher virus-specific immune responses compared with formalin-inactivated influenza virus particles on their own [64].

The use of empty bacterial cell envelopes (bacterial ghosts), derived from a variety of Gram-negative bacteria, as providing both an adjuvant and a delivery system has been extensively studied by our group [65] and promising results of recent studies will be presented below.

1.3. Mucosal Adjuvants

Most vaccines have traditionally been administered by intramuscular or subcutaneous injection, which often leads to systemic immune responses only. Since most diseases are caused by live microbes entering the host through mucosal membranes it seems reasonable to develop vaccine strategies that induce mucosal immunity. Thus, immunity would be

stimulated at the sites where most pathogens initially infect the host, and this has the potential for improved vaccine efficacy. Induction of mucosal immunity might be particularly advantageous in the very young and in the elderly, since mucosal immunity seems to develop earlier than systemic immunity, and does not appear to be subject to age-associated immune dysfunction [66].

Mucosal administration of vaccines offers a number of advantages that include easier administration, reduced adverse side effects and the potential for frequent boosting. The most attractive routes for mucosal immunization are oral and intranasal, due to the ease and acceptability of administration through these routes [67]. Alternative, less attractive routes of mucosal immunization include pulmonary inhalation, rectal and ocular immunization. Several orally administered vaccines, based on live-attenuated organisms, are commercially available, the most prominent and successful being the live oral polio vaccine. However, many important pathogens cannot be successfully attenuated, are difficult or impossible to grow in culture and in some cases cannot be easily manipulated using existing techniques in molecular biology [68]. Consequently, there is considerable interest in the development of novel oral delivery systems, which can be used to package and deliver a variety of antigens, and, for safety reasons, these should be based on non-living carrier systems. The high acidity in the stomach and a protective coating of mucus, which limits access to the mucosal epithelium, have proven extremely difficult for effective oral immunization with non-living antigens [6]. Thus, non-living mucosal vaccines need some special qualities, or mucosal adjuvants, to make them more effective.

Cholera toxin (CT) and the *E. coli* heat-labile toxin (LT), are the most potent mucosal adjuvants currently available. Because of their toxic effect they are not considered sufficiently safe for routine use in humans. However, genetically modified forms of CT and LT may prove to be useful as mucosal adjuvants even in humans [69]. For example, protection against *Helicobacter pylori* could be achieved in the mouse model using selected antigens of *H.*

pylori with non-toxic LT mutants [70]. In addition, LT mutants have been shown to be potent oral adjuvants for co-administered model antigens, such as keyhole limpet hemocyanin (KLH) [71]. While many of the mutant holotoxins retain their promiscuous binding to the GM1-ganglioside receptor, newer strategies may overcome this while still retaining the adjuvanticity. For example, construction of a gene fusion protein between the enzymically active CTA1 and a dimer of the Ig-binding D moiety derived from *Staphylococcus aureus* protein A, results in a fusion product that selectively targets B cells and has no systemic toxicity [72]. However, this may have limited adjuvant effects for oral immunization in the absence of additional formulation. Replacement of specific amino acids to produce mutant LT enterotoxins that are non toxic and have no binding activities to their specific gangliosides have been reported to also retain immunomodulatory activities [73].

It is well established that particulate antigens are more effective for oral immunization than soluble antigens. The ability of microparticles to perform as effective adjuvants following mucosal administration is largely a consequence of their uptake into the specialized mucosal associated lymphoid tissue (MALT). For uptake by the MALT of the gut or respiratory tract, microparticles need to have appropriate dimensions (i.e. < 10µm) and hydrophobic particles are taken up to a greater extent than hydrophilic particles [74]. The optimal size for particle uptake by the gut MALT does vary with liposomes ranging from approximately 100 to 350 nm being successfully delivered through M cells to bacteria with sizes greater than 5 µm also being effectively transported [75]. The specific physical size of the microparticles may not be the limiting factor in uptake across the gut for all approaches involving encapsulation of antigens into microparticles and oral delivery [76]. Whether or not the uptake in humans is sufficient to allow the development of an effective oral vaccine is currently unknown. In rodents, however, the extent of uptake can be enhanced by designing the microparticles to target M cells or the MALT of the Peyer's patches [77]. While it has been demonstrated that specific vaccine formulations, including bacterial formulations, can be

preferentially taken up by the MALT, specific cellular receptor mechanisms associated with M cells are still relatively undefined.

Certain microparticle formulations, such as PLG microparticles, have been shown to induce potent immune responses following oral immunization [78]. In addition, mucosal immunization with PLG microparticles induces protection against challenge with *Bordetella pertussis* [48], *Chlamydia trachomatis* [79], and *Salmonella typhimurium* [80]. Microparticles have also been used for the mucosal delivery of DNA vaccines [81]. Comparative studies indicate that microparticles are one of the most potent adjuvants available for mucosal delivery of vaccines [82].

While microparticles have significant potential for mucosal delivery of vaccines, their potency may be improved by their use in combination with additional adjuvants. Such favourable composition is naturally found in BG. On one hand, bacterial ghosts represent a particulate delivery vehicle and on the other hand they contain many immune stimulating compounds [83, 84].

2. Bacterial ghost platform technology

The Bacterial Ghost-Vaccine Platform Technology (BG-VPT) delivers antigens to APCs by its particulate nature derived from the cell envelope of gram-negative bacteria. Their natural outer surface make-up provides the BG with the original antigenic structures and bioadhesive properties of the bacteria they are derived from and endows them with intrinsic adjuvant properties. Model investigations with a series of different envelopes (*Escherichia coli* K12 strains, EHEC, ETEC, *Vibrio cholerae*, *Salmonella typhimurium*, *Shigella flexneri*, *Actinobacillus pleuropneumoniae*, *Mannheimia haemolytica*, *Pasteurella multocida*, *Helicobacter pylori*) and target antigens (protein: HIV-1 gp41/RT, *C. trachomatis* MOMP/PorB, *H. influenzae* Omp26, HBcAg-149, *B. anthrax* PA, possum ZP2/3; DNA: egfp, lacZ, PA, cafI) have shown that the BG-VPT is a highly potent, flexible and safe

vaccine platform for subunit antigens. BGs provide ample space for such target antigens in different compartments of the BG, such as membrane anchored to the inner membrane, exported to the periplasmic space, embedded into the outer membrane as fusion protein, or as part of pili protruding from the surface. In addition, the inner lumen of the BG which is free of cytoplasm can be decorated with plasmids or membrane attached minicircle DNA, or can be filled with protein antigens either bound to a membrane-anchored matrix or fused into a self-assembling structure built up by multitude repetitive monomers (Fig. 1) [85-91].

BGs elicit both humoral and cellular immune responses in animals and have been proposed as an alternative to conventionally inactivated vaccines [92]. In contrast to heat- or chemical inactivation procedures, all native cell wall components are preserved during the formation of BGs [85, 86]. Bacterial cell envelopes contain immune stimulating compounds, such as LPS, lipid A and peptidoglycan and, therefore, have the potential to enhance the immune response to the same extent as traditionally used adjuvants [93]. BGs are the subject of current studies to investigate their potential as vaccine candidates or delivery systems for recombinant proteins, foreign DNA or other pharmacologically relevant drugs.

2.1. Protein E-mediated lysis

The process of protein E-mediated lysis has been investigated in detail in a variety of Gram-negative bacteria over the past 2 decades and the model proposed has nicely been reviewed by Jechlinger *et al.* [65]. Electron micrographs of lysed cells show the fusion of the inner and outer membranes at distinct zones on the bacteria. Usually, only one transmembrane tunnel structure with a diameter of 40-200 nm is observed per cell and it is restricted to areas of potential cell division sites. This indicates that no regular structure, such as a defined cylinder, is formed during lysis. The size and shape of the lysis tunnel is influenced by the osmotic pressure inside the cell, local autolytic effects and the particular mesh size of the peptidoglycan [65]. Electron micrographs of a typical BG are shown in Figure 1.

349

350 2.2. Bacterial ghosts as carriers

351 In the extended BG system, the cell envelopes can be utilized as carriers for foreign
352 DNA, soluble drugs or recombinant proteins [94].

353 BGs are under investigation as DNA delivery vectors. *In vitro* studies showed that
354 BGs filled with plasmid DNA encoding the green fluorescence protein (GFP) were taken up
355 very efficiently by DCs [95] and macrophages [96], and the marker protein GFP was
356 expressed at high levels. Recently, DNA-loaded BGs have been successfully tested *in vivo* for
357 their potential as vaccine candidates [97].

358 Loading of BGs with DNA could recently be simplified by the development of a self-
359 immobilizing plasmid (SIP) that is retained in the host envelope complex due to specific
360 protein/DNA interaction during and after protein E-mediated lysis [98]. Combination of a
361 novel site-specific recombination system with the above mentioned SIP system to create
362 membrane-bound minicircle DNA allows removal of plasmid sequences dispensable for
363 vaccination, thereby further refining this technology [99].

364 BGs have been plugged in order to use them as drug delivery systems for soluble, non-
365 attached, hydrophilic substances. The sealing process of BGs is based on a fusion of the ghost
366 membrane and inside-out membrane vesicles, prepared from the bacterium used to produce
367 ghosts [100]. BGs successfully filled and sealed with the reporter substance calcein were
368 taken up efficiently by murine macrophages, and therefore represent alternative drug delivery
369 and release vehicles for advanced cell targeting [100]. In addition, BGs can be filled with
370 substances without sealing them. These substances are then slowly released from the ghosts to
371 the environment. This potential slow release system has been studied using the cytostatic drug
372 doxorubicin [101].

373 A membrane anchoring system has been developed which allows the attachment of
374 foreign proteins to the inner side of the cytoplasmic membrane [102]. Different HIV-1

antigens have been anchored in *E. coli* [88] and *V. cholerae* ghosts [103]. Furthermore, the enzymes alkaline phosphatase [104], β -galactosidase and polyhydroxybutyrate-synthase have been bound to the inner membrane of *E. coli* ghosts [105]. The enzymatic activities of membrane attached β -galactosidase and polyhydroxybutyrate-synthase was not impaired [105].

Construction of fusion proteins with the outer membrane protein (Omp) A allows the anchoring of foreign proteins in the outer membrane. Model proteins, such as the green fluorescent protein [106] or beta-galactosidase [107], have been successfully displayed on the surface of *E. coli*. Further, relevant foot-and-mouth disease viral antigens [108] as well as malarial antigens [109] have been fused to OmpA and these fusions elicited antigen-specific immune responses. This principle has previously been employed for the production of recombinant bacterial ghosts displaying the hepatitis B core antigen on their surface [110].

The periplasmic space of a ghost represents a sealed compartment, so soluble proteins become part of the ghost envelope when they are exported to the periplasm prior to lysis. This can be achieved by fusion of the protein of interest to the *malE* signal sequence of maltose binding protein (MBP), which directs the resulting fusion protein to the periplasm [105]. The possibility of filling the cytoplasmic or periplasmic space of a ghost with crystalline S-layer protein arrays further extends the potential of bacterial ghosts as a vehicle for delivering foreign proteins [105]. The S-layer genes *sbsA* and *sbsB* from *Bacillus stearothermophilus* PV72 have been cloned and sequenced [111, 112] and heterologous expression of the S-layer proteins in *E. coli* leads to accumulation of self-assembled sheet-like arrays of protein in the cytoplasm [112, 113]. It has been shown that the insertion of up to 600 amino acids encoding a foreign protein component into the S-layer proteins SbsA and SbsB does not inhibit the self-assembly [114]. These studies have demonstrated the potential diversity for design of BG delivery vehicles.

2.3. Immune responses to bacterial ghosts

BGs have been tested in different animal models for their ability to induce an immune response [115, 116]. Since all native cell wall components are preserved during the formation of ghosts, BGs derived from pathogens provide an alternative to conventionally inactivated vaccines. The following studies provide evidence that BGs have significant potential as a combined adjuvant and vaccine delivery system using both systemic and mucosal routes.

V. cholerae ghosts (VCG) evoke a specific antibody response as well as vibriocidal antibody activity in mice [117]. Intraperitoneal immunization of mice with VCG elicits significantly higher antibody responses than immunization with heat-inactivated whole-cell *V. cholerae* [117]. The protective efficacy of VCG candidate vaccines following oral immunization has recently been shown in the reversible intestinal tie adult rabbit diarrhea (RITARD) model [118]. Rabbits immunized with VCG preparations from *V. cholerae* serogroups O1 and O139 show significantly increased titers of serum vibriocidal antibodies, which is known to correlate with protection from cholera. Serum from these immunized animals mediated complement-dependent killing of homologous and heterologous strains. In contrast to a previous report, where the toxin-coregulated pilus (TCP) was found to be essential for cross strain protection [119], the TCP status of the ghost preparation made no difference in this study. Protective immunity against intraduodenal challenge appeared to be dose dependent and was associated with marked inhibition of colonization. In addition to their potential as non-living candidate vaccines, VCG have been used as carriers of heterologous antigens such as HIV-1 reverse transcriptase [103] or the major outer membrane protein MOmp of *Chlamydia trachomatis* [120]. Intranasal and intramuscular immunization of mice with *V. cholerae* ghosts expressing MOmp induced elevated local genital mucosal as well as systemic Th1 type responses, which provided protective immunity. In addition immune T cells from immunized mice transferred partial protection against a *C. trachomatis* genital challenge to naive mice [120]. Compared to this single subunit construct, immunization with

a multiple subunit vaccine consisting of VCG expressing both MOmp and Omp2 induced a higher frequency of Th1 cells and a relatively greater ability to confer protective immunity [121]. These results support the current opinion that protective immunity against *Chlamydia* is a function of the level of Th1 response elicited.

The feasibility of designing an efficacious single-dose oral enterohemorrhagic *E. coli* (EHEC) BG vaccine was recently shown in mice. Intragastric immunization with EHEC ghosts induced both cellular and humoral immunity, and more than 85% of the vaccinated animals survived a lethal challenge with a heterologous EHEC strain after a single-dose oral immunization [122].

The use of *Actinobacillus pleuropneumoniae* ghosts has been studied in pigs. Aerosol immunization with *A. pleuropneumoniae* ghosts induced complete protection against a homologous aerosol challenge [123] and the same result has been shown following intramuscular immunization [124]. In another study, immunization with *A. pleuropneumoniae* ghosts and formalin-inactivated whole-cells was compared. While both vaccination groups were protected against clinical disease, colonization of the lungs with *A. pleuropneumoniae* was only prevented in BG-vaccinated pigs. The sera of BG-vaccinated pigs contained antibodies, which may be related to prevention of lung colonization [125]. A recent *in vitro* study found that DCs were highly effective in the uptake of *A. pleuropneumoniae* BGs, as well as in the activation of cellular immune responses. Felnerova and colleagues showed that APCs were not only able to internalize BGs, but were also able to process and present them to T cells. The process of internalization was related to increased expression of MHC II molecules on the cell surface [126].

The production of *H. pylori* BGs and their successful use for oral immunization of BALB/c mice has recently been reported [127]. Immunization with *H. pylori* ghosts resulted in a significant reduction of the bacterial load in the BG vaccinated group and the addition of a mucosal adjuvant resulted in complete protection [127].

454 In addition to their potential as non-living candidate vaccines, BG, especially ones
455 derived from the model organism *E. coli* K12, have been extensively studied as carrier and
456 delivery systems for a great variety of heterologous protein antigens. Different antigens from
457 HIV-1 have been expressed in *E. coli* prior to protein E-mediated lysis [88]. Immunization of
458 mice and rabbits with recombinant *E. coli* BGs carrying HIV-1 gp41 or gp120 fusion proteins,
459 led to strong humoral and cellular immune responses to both the carrier bacterial envelope
460 components as well as to the viral target proteins [88, 102]. Furthermore, immunization with
461 HIV-1 reverse transcriptase anchored in the inner membrane of *E. coli* ghosts induced a
462 reverse transcriptase-specific humoral immune response [93]. As mentioned above, HIV-1
463 reverse transcriptase has also been anchored to the inner membrane of VCGs [103], providing
464 a different composite vaccine. The BG system is also being applied to the development of
465 contraceptive vaccines with *E. coli* BG carrying the ZP3 (zona pellucida protein 3) as a fusion
466 with SbsA currently being evaluated as an immunocontraceptive candidate vaccine in
467 possums [201].

468 Expression of recombinant S-layer proteins in *E. coli* prior to induction of protein E-
469 mediated lysis, results in sheet-like self-assembly products that are not released to the external
470 medium but are retained within the cytoplasmic space [128]. Fusion of the S-layer gene *sbsA*
471 to the *malE* signal sequence allows the transport of SbsA protein subunits to the periplasm
472 and self-assembly in this sealed compartment has been observed [128]. This immobilization
473 strategy has been applied in the development of candidate vaccines against the human
474 pathogen nontypeable *Haemophilus influenzae* (NTHi). The recently identified NTHi antigen
475 Omp26 [129] has been inserted at different positions into the S-layer protein SbsA [128].
476 Successful expression and S-layer self-assembly of the resulting SbsA/Omp26 fusion proteins
477 in the cytoplasm or periplasm of *E. coli* could be demonstrated. Mice immunized
478 intraperitoneally with bacterial ghosts harboring selected SbsA/Omp26 fusions showed a

significant increase in Omp26-specific IgG titers in serum following immunization with a combination of recombinant S-layer proteins [128]. The potential of these candidate vaccines for mucosal immunization was further assessed in a rat model. Different routes of immunization including inoculation via the Peyer's patches (IPP), the duodenum (ID) and the trachea (IT) were evaluated. While a gut immunization regime (IPP/ID) showed only moderate bacterial clearance following pulmonary challenge with the homologous NTHi strain, the gut/lung immunization regime (IPP/ID/IT) showed significantly enhanced clearance. Both immunization regimes induced high levels of Omp26-specific antibodies in the serum of immunized rats, with higher levels in the group that received the IT boost. Analysis of IgG isotypes present in serum suggested that predominantly a Th1 type response was induced. Lymphocytes from animals vaccinated using the gut/lung regime responded significantly to Omp26 when compared to control groups. These results show the feasibility of a combination of recombinant S-layer proteins and BGs to mucosally deliver the NTHi vaccine candidate Omp26 to the immune system [Riedmann EM, Lubitz W and Kyd JM, unpublished data].

Based on results from several *in vitro* studies, showing that plasmid-loaded BGs are efficiently taken up by different APCs, BGs have been suggested as a potential delivery system for DNA vaccines. First immunization experiments in mice using *Mannheimia haemolytica* BGs loaded with plasmid DNA were promising. When using intradermal or intramuscular routes of immunization, BG-mediated DNA delivery stimulated more efficient humoral and cellular (CD4+ and CD8+) immune responses than naked DNA. The use of BGs also allowed modulation of the T helper response from a mixed Th1/Th2 to a dominant Th2 pattern [97]. Accompanying *in vitro* studies showed that BGs not only targeted the DNA vaccine construct to APC, but also acted as natural adjuvants. They most likely provided a strong 'danger signal' and thereby promoted efficient maturation and activation of DCs.

The above mentioned immunization studies have all been performed using freeze dried BGs resuspended in saline or water, without additional adjuvants, stabilizers or any other additives. The particulate nature of BGs promotes the uptake of the antigen by APC at the site of administration. *In vitro* internalisation of *A. pleuropneumoniae* BGs and the effects on the immune response have recently been studied in DCs, the most important APC involved in antigen capture. About 80% of the porcine APC population exhibited phagocytic activity which, following uptake and processing of the bacterial ghosts, have the capacity to stimulate specific T-cells [126]. Furthermore BGs mediated the maturation of human DC as indicated by the decrease in expression levels of CD83. Macrophages can also be targeted very efficiently by BGs, with demonstrated internalization rates over 80% [100].

The endotoxin content of Gram-negative bacteria has been suggested as a potential problem for non-living vaccines. However, the synthesis of inflammatory macrophage mediators, such as TNF- α , needs a 100-fold higher dose of ghosts from *V. cholerae* than the corresponding amount of free LPS [130]. These results confirm previous experiments indicating no toxic effects of BGs in rabbits even after intravenous administration in doses stimulating significant humoral responses [131]. Considering the extreme sensitivity of rabbits to endotoxins and that maximal endotoxic fever reactions will result from intravenous application, effective immune responses to vaccination can be expected by administration of appropriate doses in both animals and humans without significant side effects [131]. Aerosol vaccination of pigs with *A. pleuropneumoniae* BGs provided protective immunity following doses which did not elicit clinical side-effects [132].

The application of new strategies to develop effective vaccines is essential to preventative therapeutic approaches in modern medicine. New generation vaccines, based on DNA or recombinant proteins, are often less reactogenic and immunogenic than traditional vaccines. Thus there is an urgent need for inexpensive new and improved adjuvants and delivery systems. The BG system has been extensively studied over the past few years and

shows great strategic potential for vaccine development. As BGs retain their natural outer surface make-up along with their original target functions they present an alternative to conventionally inactivated vaccines. To target foreign antigens BGs act as perfect carriers and intrinsic adjuvant particles. Results of immunization studies using BGs derived from a variety of Gram-negative bacteria in different animal models are promising. BGs can enhance immune responses to the same extent as traditionally used adjuvants and the easy manufacturing, low production costs, stability without cold chain, and indicative safety profile of BGs constitute additional advantages of this system.

4. Expert commentary

The BG-VPT represents a particulate carrier system for protein subunit or DNA-encoded antigens endowed with intrinsic adjuvant properties. By all its biological background BG-V alert the immune system with signals for a bacterial infection and induce innate and adaptive immune responses against the antigens. Presentation of subunit vaccines within the BG complex is of advantage for the recognition of the target antigens by the immune system. Such antigens in purified form are normally not very immunogenic and need the addition of adjuvants to be efficacious in the vaccinee. Delivered as particles, to facilitate the uptake by professional APCs, BG-vaccines satisfy the requirement of naturally furnished adjuvant particles for submit vaccine candidates. Such BG particles have a surface make-up which is not denatured and their surface adhesins are fully functional for the interaction with cellular receptors of APCs to induce the release of natural danger signals and cytokines characteristic of infections with real pathogens to a certain degree.

Up to now, several investigations of BG vaccines in different animal models have shown that they can be applied with all routes of administration and that they are able to induce relevant immune responses. The most promising route of application is the mucosal route (intranasal, intraocular, oral, rectal, and aerosol). Despite the fact that BG contain LPS,

they are safe and studies according the US Pharmacopoeia have shown that even intravenous administration of BG in rabbits up to doses which were fully immunogenic did not induce adverse side effects. One recent focus of BG-VPT is the development of new antidiarrhoeal vaccines which can be administered via mucosal routes, and which should be able to substitute for live attenuated vaccines that are not always well tolerated in the young, the elderly or in the immunocompromised. Other major advantages of the BG-VPT can be summarized as such: (i) the production of the BGs is simple, (ii) scalability of BG production is given using conventional steel or disposable fermenters, (iii) depending on the fermentation capacity high dosis of BG-vaccines can be produced in rather short time from small working seeds, (iv) no need of extensive downstream processing for the final vaccine product except of washing and freeze drying, (v) BG are stable at ambient temperature and BG-vaccines can be stock piled due to expected long shelf-life, (vi) BG-VPT generates potent, safe and affordable veterinary and human vaccines

5. Five years view

The focus of recent applications of the BG-VPT is the use of the BG carrier with intrinsic adjuvant properties for the delivery of subunit vaccines preferentially to the MALT. The major advantage of the BG-VPT compared to most other particulate delivery systems is that the BG-platform allows the production of subunit antigens together with adjuvants in a one-step procedure. As the adjuvant particles are bacterial envelopes, the resources for their production depend only on medium for bacterial growth and fermentation capacities. Using conventional as well as disposable fermenters the fast production and scalability of the candidate vaccines is limited only by the volume and performance of the equipment. In other words, once a master/working seed exists, the production of the vaccine can be achieved locally with integrated equipment in varying quantities as needed. This strategy of

582 decentralized production possibilities is likewise tailored to fight against any pandemic
583 infectious diseases.

584 The BGs interact with surface molecules present on macrophages and dendritic cells
585 and the BG particulate antigen-adjuvant complex is taken up by mechanisms available to all
586 APC. In this context it is important that the natural BG carrier particle alerts the innate
587 immune system and induces signalling for a proper humoral and cellular immune response
588 against the target antigens. The stimulation of both arms of the adaptive immune system is
589 characteristic for bacterial infections and target antigens presented by BGs are more highly
590 responded against by this general induction system.

591 Additional progress can be achieved in the coming years by testing BGs from various
592 Gram-negative bacterial pathogens, e.g. enteropathogenic bacteria, which exhibit specific
593 surface ligands for MALT adhesion to select the most effective carriers for the different
594 routes of mucosal application of BG-vaccines, e.g. for the urogenital tract, lungs,
595 gastrointestinal tract, conjunctiva, inner ear or other sites. Depending on the selected BG
596 vaccine carrier BGs could meet specific requirements for self-administration (e.g., intranasal,
597 oral or combinations of BGs with suppositories) in the young, adult and elderly.

598 Mucosal surfaces are normally endowed with cleansing mechanisms that remove
599 incoming foreign material. Bacteria which withstand this natural body safeguard system can
600 be found among pathogens mentioned above and their natural engineered and highly specific
601 adhesion properties can be used to facilitate targeting of human or animal lymphoid tissues.
602 The potential hazard coming from the genome of such organism can be diminished by the use
603 of a nuclease incorporated during the BG production process to clean up the BG-vaccine from
604 any remaining residual chromosomal or plasmid DNA. This approach adds to the safety
605 profile of the non-living BG and takes away any risk from the BG serving as carrier of
606 pathogenic islands or antibiotic resistance cassettes. Being dead and free of pathogenic DNA

information is a major advantage for using BGs in comparison to live vaccine carriers of similar origin.

Using the above mentioned principle, multivalent vaccines against different bacterial pathogens or combinations of BG originating from pathogens as carriers of bacterial or viral antigens can be constructed. For the upcoming years we envisage great potential for such combinations in the group of class A and B biothreats. Diarrhoeal pathogens for human using the BG-VPT are already in the pipeline and we expect them to enter human clinical trials in the next 2 – 3 years.

The BG-VPT system is not only suitable for protein antigens but has recently been extended into a carrier for DNA vaccines. The transformation efficiency for macrophages or DC showed values of 70% and 80% for these professional APCs and are among the highest values found in literature for these cells. Here again, the system is as such that a one step production system for BG as carrier and adjuvants for DNA minicircle vaccines has been developed. More experiments in different animal models have to be performed to evaluate a recent finding that the BG-DNA adjuvant system induces a stronger Th2-mediated humoral immune response against the DNA-encoded antigens than a cellular Th1 response. This finding has a great future application potential as the non-living BG carrier is able to modulate the immune response for DNA vaccines. As a new adjuvant platform technology, it might take time for BGs to emerge in the range of alternative strategies considering the fact that many patents and patent applications have been filed on adjuvants including particulate ones.

6. Key issues

Particular advantages of the bacterial ghost particulate adjuvant system are:

- 632 • one-step production process for subunit antigens (protein and/or DNA) carried in
633 particulate adjuvants delivery vehicle for APC
- 634 • simplicity and scalability of production in conventional steel or disposable fermenters
635 and rapid expansion from small to large production lots in a short time
- 636 • no need of extensive downstream processing for final product
- 637 • no addition of external adjuvant in vaccine formulation
- 638 • BG are stable at ambient temperature as a freeze-dried powder
- 639 • BG delivery via mucosal routes generates robust specific humoral and cellular
640 immune responses
- 641 • BG-VPT has the potential to answer specific demands for generating potent, safe and
642 affordable veterinary and human vaccines.

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1021 **Figure Legends**

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1023 **Figure 1**

1024 Electron micrographs of *Escherichia coli* pop2135 cell envelope preparations. Ultrathin
1025 sections of lysed cells harboring recombinant S-layer self-assembly products in the periplasm
1026 (A) or the cytoplasm (B). Fusion of the inner and outer membrane, typical for protein E
1027 mediated lysis, is indicated by an arrow (A). Bars 200nm.

Figure 1