

8th Vaccine & ISV Congress, Philadelphia, USA, 2015

Haloarchaeal gas vesicle nanoparticles displaying *Salmonella* antigens as a novel approach to vaccine development

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Abstract

A safe, effective, and inexpensive vaccine against typhoid and other *Salmonella* diseases is urgently needed. In order to address this need, we are developing a novel vaccine platform employing buoyant, self-adjuvanting gas vesicle nanoparticles (GVNPs) from the halophilic archaeon *Halobacterium* sp. NRC-1, bioengineered to display highly conserved *Salmonella enterica* antigens. As the initial antigen for testing, we selected SopB, a secreted inosine phosphate effector protein injected by pathogenic *S. enterica* bacteria during infection into the host cells. Two highly conserved *sopB* gene segments near the 3'- region, named *sopB4* and *sopB5*, were each fused to the *gvpC* gene, and resulting SopB-GVNPs were purified by centrifugally accelerated flotation. Display of SopB4 and SopB5 antigenic epitopes on GVNPs was established by Western blotting analysis using antisera raised against short synthetic peptides of SopB. Immunostimulatory activities of the SopB4 and B5 nanoparticles were tested by intraperitoneal administration of SopB-GVNPs to BALB/c mice which had been immunized with *S. enterica* serovar Typhimurium 14028 $\Delta pmrG$ -HM-D (DV-STM-07), a live attenuated vaccine strain. Proinflammatory cytokines IFN- γ , IL-2, and IL-9 were significantly induced in mice boosted with SopB5-GVNPs, consistent with a robust Th1 response. After challenge with virulent *S. enterica* serovar Typhimurium 14028, bacterial burden was found to be diminished in spleen of mice boosted with SopB4-GVNPs and absent or significantly diminished in liver, mesenteric lymph node, and spleen of mice boosted with SopB5-GVNPs, indicating that the C-terminal portions of SopB displayed on GVNPs elicit a protective response to *Salmonella* infection in mice. SopB antigen-GVNPs were also found to be stable at elevated temperatures for extended periods without refrigeration. The results show that bioengineered GVNPs are likely to represent a valuable platform for antigen delivery and development of improved vaccines against *Salmonella* and other diseases.

Keywords: *Salmonella*; *Halobacterium*; vaccine; nanoparticle; gas vesicle

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Selection and peer-review under responsibility of the 8th Vaccine Conference Organizing Committee.

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1. Introduction

1.1. *Salmonella* diseases

Salmonella enterica, a Gram-negative intracellular pathogenic bacterium, infects humans and many warm blooded animals. Salmonellosis remains a serious problem in most developing countries and outbreaks are regularly seen in developed and industrialized countries¹⁻⁴. Typhoid fever is caused by *S. Typhi* or *S. Paratyphi*, with global incidence of 21.7 million cases of Typhi and an additional 5.4 million Paratyphi cases resulting in 217,000 deaths per year⁵. Treatment is becoming more challenging due to increased prevalence of antibiotic resistance⁶⁻⁸. While two licensed vaccines for typhoid fever are commercially available, they have limited protection and/or may not be administered to immunocompromised people or children and infants. As a result, there is a critical need to develop more effective *Salmonella* vaccine candidates, which would also be safe and easily scalable, and inexpensive to produce and deliver. A versatile, particulate antigen delivery system, gas vesicle nanoparticles (GVNPs), which can be bioengineered to express multiple *Salmonella* antigens is likely to offer significant advantages over currently available vaccines, due to reduced risk and improved effectiveness.

1.2. *Salmonella* pathogenesis and vaccine status

Salmonella enterica includes 2,500 serovars infecting humans, and several are of public health importance, including *S. Typhi* and *S. Paratyphi*, the causative agents of typhoid and paratyphoid fever^{2,9,10}. Transmission occurs through the fecal-oral route, upon ingestion of contaminated water and food. Occurrence of the disease may be confirmed by isolation of the pathogen, detection of antibodies against *Salmonella* specific O (somatic) and H (flagellar) antigens in the serum, or most sensitively, PCR based methods which utilize specific primers designed against unique regions of their genomes⁸. Treatment however is becoming more challenging, due to increased prevalence of multiple drug resistant (MDR) strains^{6,7}. The need for improved vaccines against typhoid fever has been amply underscored by recent WHO reports^{3,4}.

Development of effective vaccines against *Salmonella* diseases is challenging due to its complex lifecycle¹¹. The facultative intracellular pathogen enters the host by crossing intestinal epithelial cells via Peyer's patches and infecting monocytes, macrophages, and dendritic cells. The bacteria can then travel to the mesenteric lymph nodes (MLN), spleen, and liver via circulating phagocytes. The host defense mechanisms for clearing *Salmonella* involve stimulation of both the adaptive and innate immune systems¹². The importance of Th1 cells was shown by depletion of IFN- γ ^{13,14} and CD4 $^{+}$ T cells have been shown to play a significant role in immunity induced by *Salmonella* flagellin^{15,16} and live attenuated *Salmonella*¹⁷. B cell depletion studies and immunization studies in Ig-deficient mice also indicate the importance of B cells for immunity against *Salmonella*^{18,19}. *Salmonella* during its intracellular life in macrophages also induces a modification of lipopolysaccharides (LPS) recognized by TLR-4 and triggers a downstream signaling cascade to evoke host immune response. As a result of these complexities, it is clear that potent antigens as well as adjuvants are important in potentiating the immune response and modulating its quality.

Early efforts to develop an effective vaccine against *Salmonella* began with whole, killed cells, which were shown to be moderately effective in field trials in the 1960s, but most countries have eliminated its use due to undesired side effects. Currently, two licensed commercial vaccines for typhoid fever, a subunit (Vi polysaccharide or Vi PS) and a live attenuated *S. Typhi* strain (Ty21a), are commercially available. However, the use of these vaccines is limited because of the short period of protection and lack of effectiveness in small children. The Vi PS vaccine given in a single dose provides protection for only 3 years, while the live oral vaccine Ty21a requires 3-4 doses for ~7 years of protection. For Ty21a, the limitations include requirement of large numbers (10^9) of bacteria, its inactivation by stomach acidity, and limited period of protection. The Ty21a vaccine cannot be used by children under the age of 6 or immunocompromised individuals. The Vi PS does not induce a switched antibody response, requires frequent boosts, and also cannot be used to immunize infants under the age of 2. Additionally, there are no licensed vaccines against *S. Paratyphi* A or B.

In order to address these limitations, we are developing an improved vaccine utilizing an innovative new adjuvant and antigen delivery system, gas vesicle nanoparticles (GVNPs)^{20,21}. To select antigens for display, we conducted

bioinformatic analysis of secreted proteins of *S. enterica* serovar Typhimurium (*S. typhimurium*) and *S. enterica* serovar Typhi (*S. typhi*) in pathogenicity islands 1 and 2 to map MHC-I and MHC-II binding epitopes²². In initial studies, a secreted inositol phosphate phosphatase protein, SopB, was selected and displayed on GVNP and elicited strong immunogenic responses, partially protecting animals challenged with virulent *Salmonella*²³.

1.3. Gas Vesicle Nanoparticles

GVNPs offer a novel antigen delivery system with powerful adjuvanting properties that is likely to enhance the prospect of an improved *Salmonella* vaccine. The ability of GVNP to deliver multiple antigens, and their stability, scalability and safety, represent significant innovations. These nanoparticles are buoyant, hollow, and lemon-shaped, and are naturally produced by salt-loving nonpathogenic microorganisms, called Haloarchaea, a group of the Archaea (3rd Domain of life) lacking lipopolysaccharides (LPS)²⁴. A large gene cluster (*gvp*) coding these nanoparticles has been cloned and extensively characterized^{20,25-29}. In previous work, antigenic proteins and peptides have been successfully displayed on GVNP, and in all cases, they were highly immunogenic in mice, with no toxic effects at either at the site of administration or systemically^{23,30-33}. GVNP are taken up by macrophages and processed slowly, producing strong long-lived humoral and cellular immune responses. GVNP act as adjuvants when administered intraperitoneally or subcutaneously in mice. The nanoparticles are easily purified as a result of their buoyancy after simple osmotic cell lysis. GVNP have a very long shelf life at room temperatures due to their high material strength, and the cost of producing large quantities of bioengineered GVNP is low. As a result, GVNP have great potential as an inexpensive, safe, and innovative antigen delivery and vaccine development system.

1.3.1. Properties of gas vesicle nanoparticles (GVNP)

Our approach employs GVNP (Figure 1) from the salt-loving haloarchaeal microbe, *Halobacterium* sp. NRC-1^{21,28,34-36}. GVNP consist of a thin (20 Å) lipid-free, rigid protein membrane surrounding a gas-filled space³⁷.

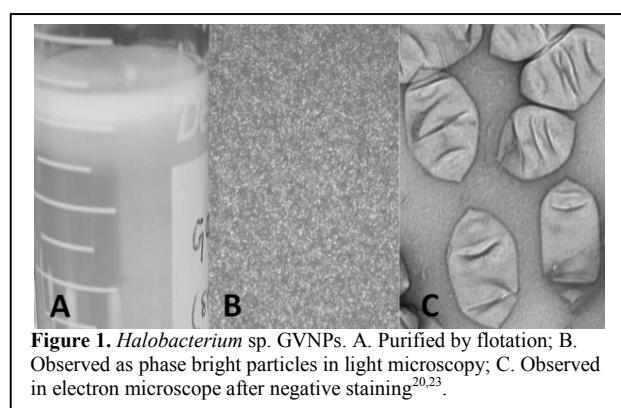


Figure 1. *Halobacterium* sp. GVNP. A. Purified by flotation; B. Observed as phase bright particles in light microscopy; C. Observed in electron microscope after negative staining^{20,23}.

GVNP are lemon-shaped, about 300 nm long, and 150 nm in diameter, and grow from small bicones to progressively larger structures. During this process, water is excluded by hydrophobicity at the inner surface of the membrane, while ambient gases accumulate through passive diffusion. GVNP are released by osmotic cell lysis with hypotonic conditions and may be easily purified in large quantities by centrifugally accelerated flotation (Figure 1)²⁵. Extreme resistance of the GVNP membrane to solubilization by detergents and chaotropic agents has precluded detailed biochemical studies³⁸. However, GVNP have been extensively studied using genetic, proteomic and immunological approaches^{20,26,29,39-43}.

GVNP have ideal properties as an adjuvant and antigen delivery system. They are extremely stable, yet non-toxic, and genetically engineerable for displaying proteins on their surface. Their protein composition has been studied in considerable detail using immunological probes and proteomic studies, which has shown that at least 7 proteins are present in the nanoparticles^{25,29,44-46}. Genetic analysis has shown the involvement of 14 genes (*gvpMLKJIHGFEDACNO*) in GVNP biosynthesis^{21,27,36}. Within the *gvp* gene cluster is the *gvpC* gene, which codes for the second-most abundant GVNP protein, displayed on the external surface of the nanoparticles. GvpC protein was observed in GVNP by immunoblotting²⁶ and *gvpC* mutants produced smaller GVNP than wild-type^{20,27}, indicating their function in strengthening the nanoparticles^{44,47} (our unpublished data). The NRC-1 GvpC protein contains a novel protein motif which is repeated 8 times (Figure 2), and it is likely that GvpC functions in binding to the major GvpA protein on the GVNP surface. Insertions of sequences coding ≥ 250 amino acids after the 6th GvpC repeat are tolerated without disrupting GVNP formation, providing a site for insertion of foreign antigenic sequences^{20,23,30-31}.

1.3.2. Bioengineered GVNP as adjuvant and antigen delivery system

GVNPs have advantages over live-attenuated vectors, due to reduced risk, and over conventional purified antigens, through enhanced protection⁴⁸. Bioengineered GVNPs function as both adjuvant and delivery system, via presentation of antigens to the antigen presentation cells (APCs), and as immunopotentiators, increasing immunogenicity without adverse reactogenicity⁴⁹. Known desirable qualities of particulate delivery systems, including the size, charge, hydrophobicity, and shape of particles, all favor GVNP as an effective adjuvant⁵⁰. The GVNP are in the nanometer range (virus-sized), found to be ideal for cytotoxic T cell responses, including cross-presentation of vaccine antigens. GVNP have surface charges and hydrophobic properties which have been shown to increase

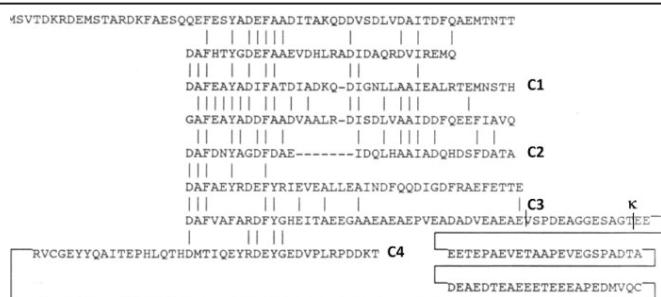


Figure 2. The amino acid sequence of GvpC of *Halobacterium* sp. NRC-1 is shown with conserved residues (vertical bars) in the eight imperfect repeats. The GvpC segments used in this study are labelled C1, C2, C3 and C4 at the C-terminal end. The position of the κ site is labelled 'κ²³'.

adjuvanting properties due to better interactions with APCs and phagocytes. The non-spherical lemon-shape of GVNP is also expected to result in more effective adjuvanting properties. The ability to control the structure and accessibility of the GVNP surface are also desirable properties for antigen display and vaccine development.

The effectiveness of GVNP as an adjuvant and antigen delivery system is also favored by their ability for administration via multiple routes. While traditional human vaccines have been administered by needle injection, parenteral delivery mainly induces circulating antibodies^{49,50}. The ability to administer GVNP via alternate administration routes, particularly mucosal administration, may reduce cost and improve compliance, while simultaneously increasing mucosal immunity without reducing systemic immunity. In the case of preventing illness resulting from the *Salmonella* pathogen, oral administration employing GVNP seems highly desirable from both the convenience and effectiveness standpoints.

2. Experimental approach and findings

2.1. *Salmonella* antigen display on GVNP and immunological testing

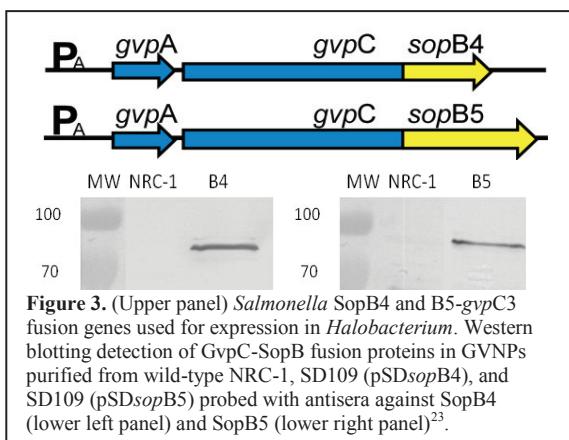


Figure 3. (Upper panel) *Salmonella* SopB4 and B5-gvpC3 fusion genes used for expression in *Halobacterium*. Western blotting detection of GvpC-SopB fusion proteins in GVNP purified from wild-type NRC-1, SD109 (pSDsopB4), and SD109 (pSDsopB5) probed with antisera against SopB4 (lower left panel) and SopB5 (lower right panel)²³.

The *Salmonella enterica* antigen SopB was selected for GVNP-display and testing^{8,20,23,51}. For this work, SopB4 and B5 (amino acids 300-400 and 395-561, respectively) were synthesized as codon optimized-gene fragments, using information from the fully sequenced *Halobacterium* sp. NRC-1 genome to replace rare codons with frequently used codons⁵². The synthetic fragments were cloned in the expression plasmid (pSD104) into a unique *Af*el site in the plasmid gvpC gene^{25,30}. The resulting plasmids, pSDsopB4 and pSDsopB5, contained the respective SopB coding regions inserted in-frame into the gvpC gene (Figure 3). These plasmids were transformed into *Halobacterium* strain SD109, which has the entire gvp gene cluster deleted⁵³.

To determine if the gvpC-sopB4 and gvpC-sopB5 fusion genes were expressed in *Halobacterium*, transformed strains were grown under mevinolin selection on agar plates, and colonies examined for GVNP production. Transformants containing pSDsopB4 or pSDsopB5 were lysed by exposure to hypotonic conditions, and GVNP

were isolated by centrifugally accelerated flotation. The nanoparticle proteins were fractionated by SDS-PAGE and antigenic proteins were identified by Western blotting analysis using rabbit polyclonal antisera prepared against synthetic peptides corresponding to SopB4 and B5 (Figure 3) or GvpC^{8,23,29}. Proteins of expected sizes were observed in the Western blots of purified GVNP s from the *Halobacterium* expression strains, SD109 (pSDsopB4) and SD109 (pSDsopB5), but were absent in GVNP s from NRC-1, as expected, confirming production of the fusion proteins and their presence on the GVNP s.

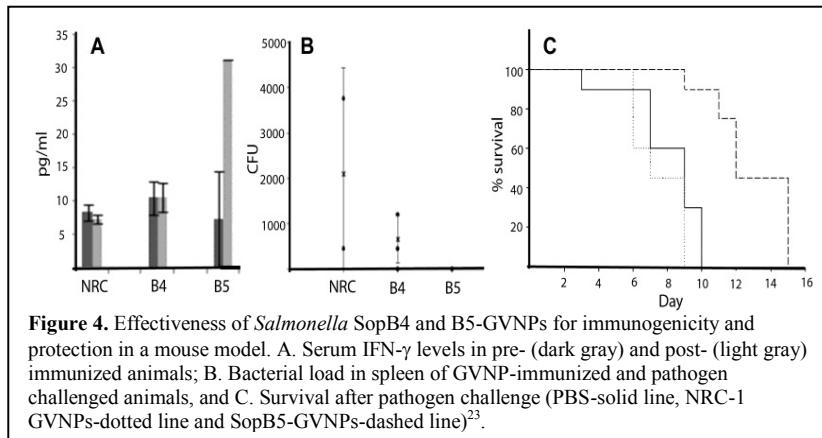


Figure 4. Effectiveness of *Salmonella* SopB4 and B5-GVNP s for immunogenicity and protection in a mouse model. A. Serum IFN- γ levels in pre- (dark gray) and post- (light gray) immunized animals; B. Bacterial load in spleen of GVNP-immunized and pathogen challenged animals, and C. Survival after pathogen challenge (PBS-solid line, NRC-1 GVNP-dotted line and SopB5-GVNP-dashed line)²³.

of IFN- γ , GM-CSF, and IL-2, all of which are Th1 cytokines, were found to be significantly increased in post-SopB5-GVNP-immunization sera compared to pre-immunization sera, indicating that the Th1 pathway involved in response to intracellular bacterial pathogens is induced by these GVNP s²³ (Figure 4A).

Following immunizations and boosting, mice were challenged orally with 10^7 cfu of wild-type *Salmonella*, and the MLN, liver, and spleen were isolated one week post-challenge. Bacterial loads were determined for each organ by plating dilutions and counting cfu. In each of the three organs, bacterial cfu were lower by at least two orders of magnitude for mice immunized with SopB5-GVNP²³ (Figure 4B). Bacterial load in the spleen was also significantly reduced in SopB4-GVNP immunized mice. CD4 $^+$ T-cells were also found to be elevated by 2-4-fold in the spleen of mice immunized with SopB5-GVNP s compared to wild-type²³. Further challenge experiments were conducted to determine protection and mice immunized with SopB5-GVNP survived 3-5 days longer than those immunized with wild-type NRC-1 (Figure 4C).

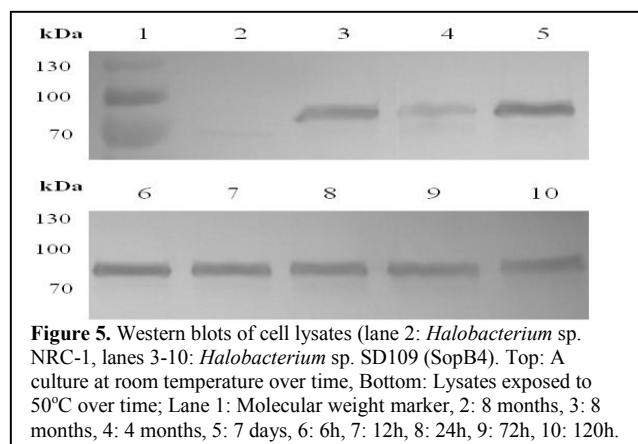
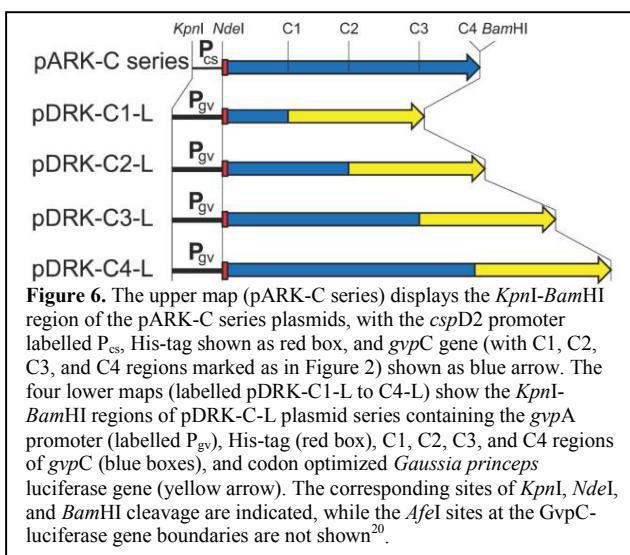


Figure 5. Western blots of cell lysates (lane 2: *Halobacterium* sp. NRC-1, lanes 3-10: *Halobacterium* sp. SD109 (SopB4). Top: A culture at room temperature over time, Bottom: Lysates exposed to 50°C over time; Lane 1: Molecular weight marker, 2: 8 months, 3: 8 months, 4: 4 months, 5: 7 days, 6: 6h, 7: 12h, 8: 24h, 9: 72h, 10: 120h.

To determine the effect of *Salmonella* SopB4 and B5 antigens displayed on GVNP s, we immunized mice intraperitoneally (i.p.) with 10^3 colony forming units (cfu) of the *S. Typhimurium* attenuated strain followed by two boosts with 100 μ g each of SopB4, B5, or wild-type NRC-1 GVNP s respectively, 1 and 2 weeks post immunization. Cytokine responses were determined for each cohort of mice, and the most notable changes were found for mice immunized with SopB5-GVNP s. Serum levels

2.2. Stability of GVNP s in *Salmonella* vaccine producing strains

Halobacterium strains containing SopB-GVNP s were tested over several months for stability of the GvpC-SopB proteins using Western blotting assays. The recombinant GVNP s were found to be stable over several months at room temperature, as well as when exposed to high temperatures of 50°C, with little to no degradation of the *Salmonella* antigen displayed on the nanoparticles²³ (Figure 5). Thus, our results clearly showed that the GVNP-antigen display system is shelf-stable without the need for refrigeration, even under tropical summer conditions.



amplified *gvpC* gene fragments (C-series) were then inserted into the expression plasmids to test their ability to bind to GVNP s and complement nanoparticle production. The plasmids were transformed into the $\Delta gvpC$ deletion strain and the results showed correlation between progressively longer *gvpC* fragments and larger size GVNP s²⁰.

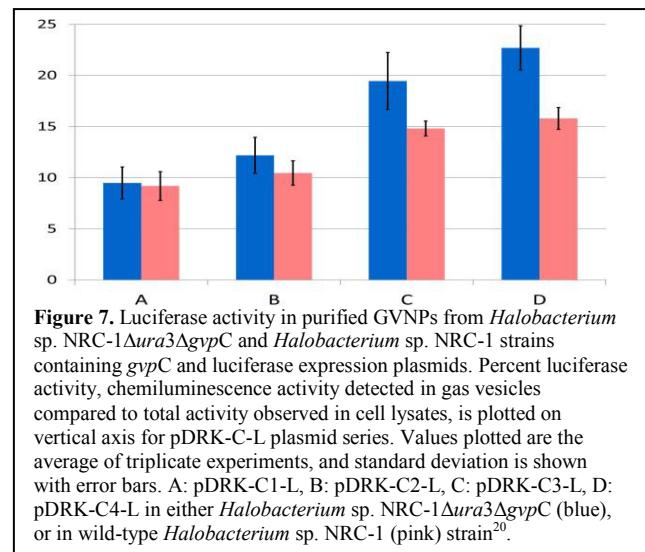
Next, a codon-optimized synthetic gene coding luciferase from the marine copepod *Gaussia princeps* was fused to the *gvpC* gene fragments on the expression plasmid and resulted in production of the chemiluminescence protein on GVNP s²⁰ (Figure 7). When inserted together with a wild-type *gvpC* gene, we obtained GVNP s displaying both of the GvpC and GvpC-luciferase fusion proteins. This finding confirmed that multiple GvpC protein types may be expressed simultaneously on the surface of GVNP s, providing the capability to express multiple antigenic proteins and delivering multivalent GVNP vaccines in a single strain. This system also opens the potential for expression of other therapeutic proteins on GVNP s for applications to drug delivery.

3. Conclusion

Salmonella pathogens remain important causes of morbidity and mortality due to the lack of an effective, long-lasting vaccine. Novel gas vesicle nanoparticle (GVNP) s produced by extremophilic *Halobacterium* sp. NRC-1 are being used to develop an improved vaccine against *Salmonella* pathogens that is both inexpensive and effective⁵⁷. When administered to animals, GVNP s have previously been shown to produce strong long-lived humoral and cellular immune responses against surface-displayed antigens. In this study, a secreted protein conserved in *Salmonella enterica* pathogens, SopB, was displayed by genetic fusion to GvpC, a GVNP surface protein, and the bioengineered SopB-GVNP s were found to be highly immunogenic in mice. Our results showed that animals boosted with SopB-GVNP s stimulated production of IgGs, proinflammatory cytokines, and CD4+ T cells, and

2.3. Improvement of the GVNP bioengineering system

The genetic system used for bioengineering of SopB-GVNP s employed a relatively large plasmid, pSD104, containing the entire *gvp* gene²⁸. In order to facilitate future bioengineering of nanoparticles, we constructed a series of smaller, more versatile plasmid expression vectors containing *gvpC*, as well as a *gvpC* deletion host strain²⁰ (Figure 6). The *Halobacterium* sp. NRC-1 $\Delta ura3\Delta gvpC$ deletion strain, constructed using the *ura3*-based gene deletion method³⁴, showed a partially gas vesicle-deficient phenotype with small, mainly lemon-shaped gas vesicles. We next constructed a *gvpC* expression vector series using pARK, an expression plasmid with the cold-inducible *cspD2* promoter, and pDRK, an expression plasmid incorporating the higher-level *gvpA* promoter^{55,56}. A series of PCR



resulted in reduced bacterial load in key organs. Additionally, the vaccine-producing strains were found to be shelf-stable over months when stored at room temperature and at elevated temperatures. We also developed an improved GVNP expression host and plasmid series and demonstrated its potential by displaying the *Gaussia princeps* luciferase reporter. This work opens the possibility of delivery of therapeutic proteins displayed on GVNPs.

4. Acknowledgements

This work was supported by National Institutes of Health grant R03 AI107634 and Bill & Melinda Gates Foundation grant OPP1061509 to SD, PKNU Research Abroad Fund CD-2013-0914 to JK, and by the Director of the Indian Institute of Science, Bangalore, India and the Department of Biotechnology (DBT 311, DBT197 and DBT 172), Life Science Research Board (LSRB0008) and DBT-IISc partnership program for advanced research in biological sciences and bioengineering to DC.

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