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

**P. DasSarma**[a], **V.D. Negi**[b,c], **A. Balakrishnan**[c], **J.-M. Kim**[a,d], **R. Karan**[a], **D. Chakravortty**[c], and **S. DasSarma**[a,*]

[a]University of Maryland School of Medicine, USA

[b]National Institute of Technology, India

[c]Indian Institute of Science, India

[d]PuKyong National University, Republic of Korea

## 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 *sop*B4 and *sop*B5, 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 Δ*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.

*Corresponding author. Tel.: 410-234-8847; fax: 410-234-8896. sdassarma@som.umaryland.edu.

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**Keywords**

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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 countries[1–4]. 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 year[5]. Treatment is becoming more challenging due to increased prevalence of antibiotic resistance[6–8]. 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 2500 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 genomes[8]. 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 lifecycle[11]. 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 systems[12]. The importance of Th1 cells was shown by depletion of IFN-$\gamma$[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

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(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 epitopes[22]. In initial studies, a secreted inositol phosphate phosphatase protein, SopB, was selected and displayed on GVNPs 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 GVNPs 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 GVNPs, 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]. GVNPs are taken up by macrophages and processed slowly, producing strong long-lived humoral and cellular immune responses. GVNPs 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. GVNPs have a very long shelf life at room temperatures due to their high material strength, and the cost of producing large quantities of bioengineered GVNPs is low. As a result,

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GVNPs have great potential as an inexpensive, safe, and innovative antigen delivery and vaccine development system.

**1.3.1. Properties of gas vesicle nanoparticles (GVNPs)—**Our approach employs GVNPs (Figure 1) from the salt-loving haloarchaeal microbe, *Halobacterium* sp. NRC-1[21,28,34–36]. GVNPs consist of a thin (20 Å) lipid-free, rigid protein membrane surrounding a gas-filled space[37]. GVNPs 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. GVNPs 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 studies[38]. However, GVNPs have been extensively studied using genetic, proteomic and immunological approaches[20,26,29,39–43].

GVNPs 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 *gvp*C gene, which codes for the second-most abundant GVNP protein, displayed on the external surface of the nanoparticles. GvpC protein was observed in GVNPs by immunoblotting[26] and *gvp*C mutants produced smaller GVNPs 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 6$^{th}$ 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 GVNPs as adjuvant and antigen delivery system—**GVNPs have advantages over live-attenuated vectors, due to reduced risk, and over conventional purified antigens, through enhanced protection[48]. 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 reactogenicity[49]. Known desirable qualities of particulate delivery systems, including the size, charge, hydrophobicity, and shape of particles, all favor GVNPs as an effective adjuvant[50]. The GVNPs are in the nanometer range (virus-sized), found to be ideal for cytotoxic T cell responses, including cross-presentation of vaccine antigens. GVNPs have surface charges and hydrophobic properties which have been shown to increase adjuvanting properties due to better interactions with APCs and phagocytes. The non-spherical lemon-shape of GVNPs is also expected to result in more effective adjuvanting properties. The

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ability to control the structure and accessibility of the GVNP surface are also desirable properties for antigen display and vaccine development.

The effectiveness of GVNPs 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 GVNPs 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 GVNPs seems highly desirable from both the convenience and effectiveness standpoints.

## 2. Experimental approach and findings

### 2.1. Salmonella antigen display on GVNPs and immunological testing

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 codons[52]. The synthetic fragments were cloned in the expression plasmid (pSD104) into a unique *Afe*I site in the plasmid *gvp*C gene[25,30]. The resulting plasmids, pSD*sop*B4 and pSD*sop*B5, contained the respective SopB coding regions inserted in-frame into the *gvp*C gene (Figure 3). These plasmids were transformed into *Halobacterium* strain SD109, which has the entire *gvp* gene cluster deleted[53].

To determine if the *gvp*C-*sop*B4 and *gvp*C-*sop*B5 fusion genes were expressed in *Halobacterium*, transformed strains were grown under mevinolin selection on agar plates, and colonies examined for GVNP production. Transformants containing pSD*sop*B4 or pSD*sop*B5 were lysed by exposure to hypotonic conditions, and GVNPs 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 GVNPs from the *Halobacterium* expression strains, SD109 (pSD*sop*B4) and SD109 (pSD*sop*B5), but were absent in GVNPs from NRC-1, as expected, confirming production of the fusion proteins and their presence on the GVNPs.

To determine the effect of *Salmonella* SopB4 and B5 antigens displayed on GVNPs, 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 μg each of SopB4, B5, or wild-type NRC-1 GVNPs respectively, 1 and 2 weeks post immunization. Cytokine responses were determined for each cohort of mice, and the most notable changes found were found for mice immunized with SopB5-GVNPs. Serum levels 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

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Th1 pathway involved in response to intracellular bacterial pathogens is induced by these GVNPs[23] (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-GVNP[23] (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-GVNPs compared to wild-type[23]. 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).

### 2.2. Stability of GVNPs in Salmonella vaccine producing strains

*Halobacterium* strains containing SopB-GVNPs were tested over several months for stability of the GvpC-SopB proteins using Western blotting assays. The recombinant GVNPs 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 nanoparticles[23] (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.

### 2.3. Improvement of the GVNP bioengineering system

The genetic system used for bioengineering of SopB-GVNPs employed a relatively large plasmid, pSD104, containing the entire *gvp* gene[28]. In order to facilitate future bioengineering of nanoparticles, we constructed a series of smaller, more versatile plasmid expression vectors containing *gvp*C, as well as a *gvp*C deletion host strain[20](Figure 6). The *Halobacterium* sp. NRC-1Δ*ura*3Δ*gvp*C deletion strain, constructed using the *ura*3-based gene deletion method[54], showed a partially gas vesicle-deficient phenotype with small, mainly lemon-shaped gas vesicles. We next constructed a *gvp*C expression vector series using pMC2, an expression plasmid with the cold-inducible *csp*D2 promoter, and pDRK, an expression plasmid incorporating the higher-level *gvpA* promoter[55,56]. A series of PCR amplified *gvp*C gene fragments (C-series) were then inserted into the expression plasmids to test their ability to bind to GVNPs and complement nanoparticle production. The plasmids were transformed into the Δ*gvp*C deletion strain and the results showed correlation between progressively longer *gvpC* fragments and larger size GVNPs[20].

Next, a codon-optimized synthetic gene coding luciferase from the marine copepod *Gaussia princeps* was fused to the *gvp*C gene fragments on the expression plasmid and resulted in production of the chemiluminescence protein on GVNPs[20] (Figure 7). When inserted together with a wild-type *gvpC* gene, we obtained GVNPs displaying both of GvpC and GvpC-luciferase fusion proteins. This finding confirmed that multiple GvpC protein types may be expressed simultaneously on the surface of GVNPs, providing the capability to express multiple antigenic proteins and delivering multivalent GVNP vaccines in a single

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strain. This system also opens the potential for expression of other therapeutic proteins on GVNPs 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 nanoparticles (GVNPs) produced by extremophilic *Halobacterium* sp. NRC-1 are being used to develop an improved vaccine against *Salmonella* pathogens that is both inexpensive and effective[57]. When administered to animals, GVNPs 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 proteins, and the bioengineered SopB-GVNPs were found to be highly immunogenic in mice. Our results showed that animals boosted with SopB-GVNPs stimulated IgGs, proinflammatory cytokines, and CD4+ T cells, and 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.

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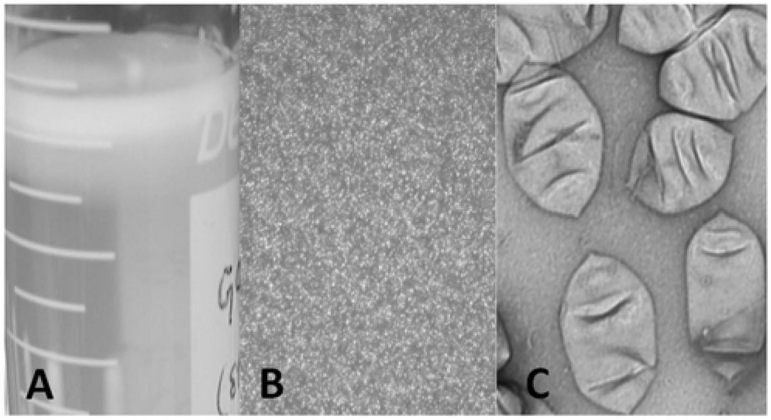


**Figure 1.**

*Halobacterium* sp. GVNPs. A. Purified by flotation; B. Observed as phase bright particles in light microscopy; C. Observed in electron microscope after negative staining[20,23].

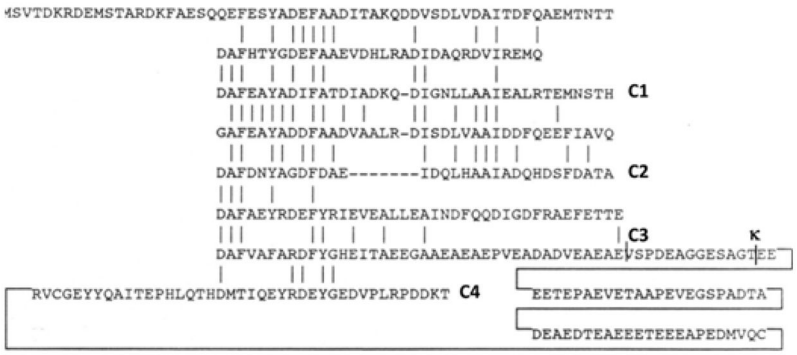


**Figure 2.**

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 'κ'[23].



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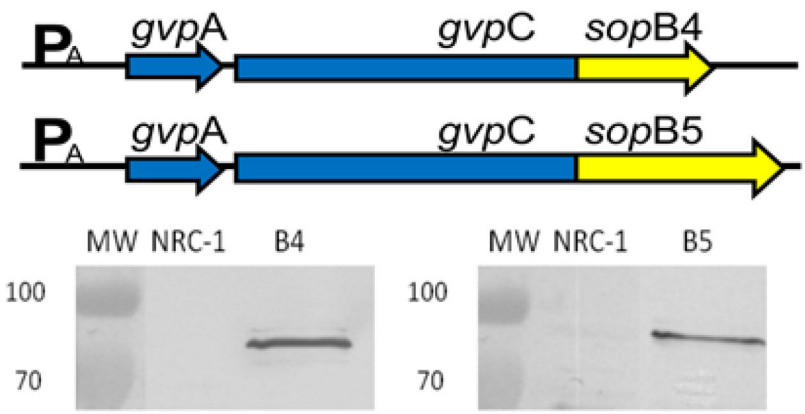


**Figure 3.**

(Upper panel) *Salmonella* SopB4 and B5-*gvp*C3 fusion genes used for expression in *Halobacterium*. Western blotting detection of GvpC-SopB fusion proteins in GVNPs purified from wild-type NRC-1, SD109 (pSD*sop*B4), and SD109 (pSD*sop*B5) probed with antisera against SopB4 (lower left panel) and SopB5 (lower right panel)[23].

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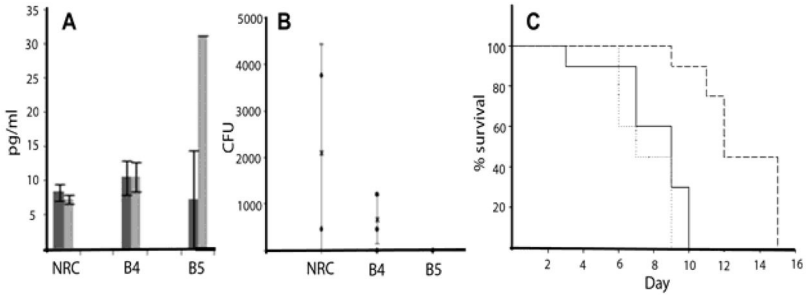


**Figure 4.**

Effectiveness of *Salmonella* SopB4 and B5-GVNPs 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 GVNPs-dotted line and SopB5-GVNPs-dashed line)[23].

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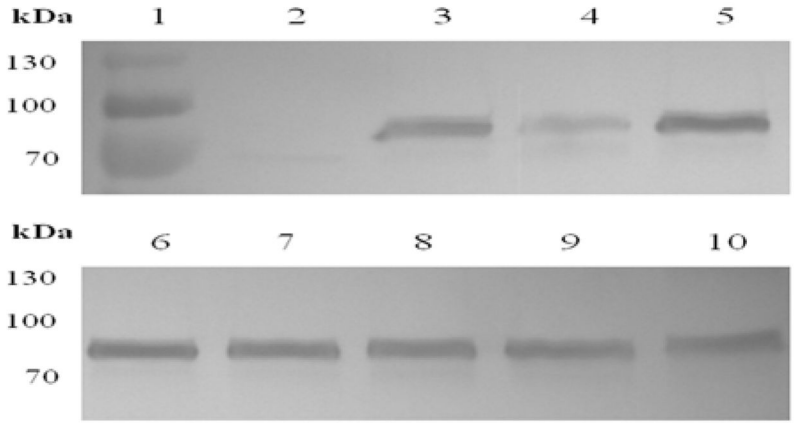


**Figure 5.**

NRC-1, lanes 2–10: *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.

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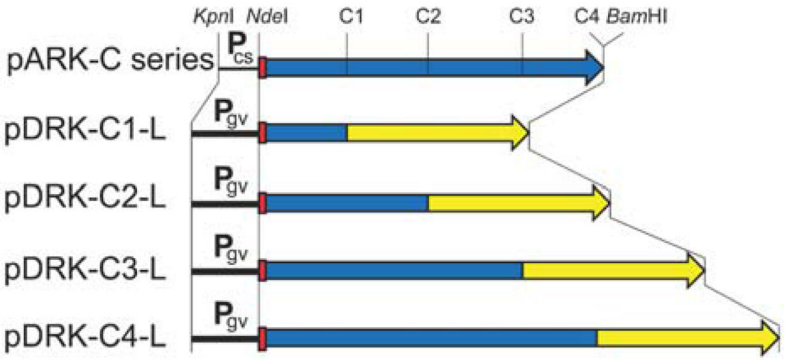


**Figure 6.**

The upper map (pARK-C series) displays the *Kpn*I-*Bam*HI region of the pARK-C series plasmids, with the *csp*D2 promoter labelled $P_{cs}$, His-tag shown as red box, and *gvp*C gene (with C1, C2, C3, and C4 regions marked as in Figure 2) shown as blue arrow. The four lower maps (labelled pDRK-C1-L to C4-L) show the *Kpn*I-*Bam*HI regions of pDRK-C-L plasmid series containing the *gvp*A promoter (labelled $P_{gv}$), His-tag (red box), C1, C2, C3, and C4 regions of *gvp*C (blue boxes), and codon optimized *Gaussia princeps* luciferase gene (yellow arrow). The corresponding sites of *Kpn*I, *Nde*I, and *Bam*HI cleavage are indicated, while the *Afe*I sites at the GvpC-luciferase gene boundaries are not shown[20].

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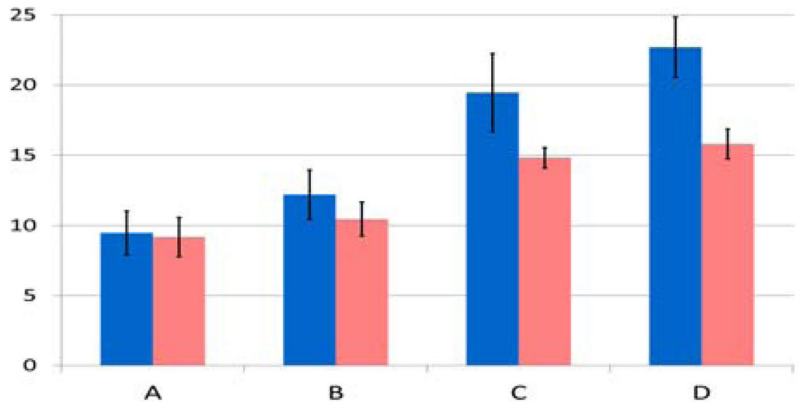


**Figure 7.**

Luciferase activity in purified GVNPs from *Halobacterium* sp. NRC-1Δ*ura*3Δ*gvp*C and *Halobacterium* sp. NRC-1 strains containing *gvp*C and luciferase expression plasmids. Percent luciferase activity, chemiluminescence activity detected in gas vesicles compared to total activity observed in cell lysates, is plotted on vertical axis for pDRK-C-L plasmid series. Values plotted are the average of triplicate experiments, and standard deviation is shown with error bars. **A**: pDRK-C1-L, **B**: pDRK-C2-L, **C**: pDRK-C3-L, **D**: pDRK-C4-L in either *Halobacterium* sp. NRC-1Δ*ura*3Δ*gvp*C (blue), or in wild-type *Halobacterium* sp. NRC-1 (pink) strain[20].