



Published in final edited form as:

*Expert Rev Vaccines*. 2011 December ; 10(12): 1679–1682. doi:10.1586/erv.11.155.

## ***Salmonella* expressing detoxified lipopolysaccharide is immunogenic and protective both as an attenuated vaccine and for delivery of foreign antigens**

**James E Galen<sup>\*,1,2</sup>, Raphael Simon<sup>1,2</sup>, and Robert K Ernst<sup>3</sup>**

<sup>1</sup>Center for Vaccine Development, University of Maryland, Baltimore, MD 21201, USA

<sup>2</sup>Department of Medicine, University of Maryland School of Medicine, Baltimore MD 21201, USA

<sup>3</sup>Department of Microbial Pathogenesis, University of Maryland Dental School, Baltimore, MD 21201, USA

### **Keywords**

delayed attenuation; endotoxin; foreign antigen; monophosphoryl lipid A; protection; *Salmonella*; vaccine

---

The construction of safe and protective vaccines, derived from human pathogens that have been genetically modified to remove pathogenicity, is often easier to accomplish on paper than it is in the laboratory. Kong and colleagues have pursued a clever strategy to reduce the reactogenicity of attenuated *Salmonella* oral vaccines by genetically modifying the surface lipopolysaccharide to lower endotoxic activity. The resulting candidate vaccine strains were highly reduced in virulence yet were able to confer protection in a mouse model against challenge with virulent *Salmonella*. Remarkably, these strains could also be further modified to present foreign antigens from unrelated human pathogens and again confer protection against heterologous challenge. This work brings important new tools to bear on solving the problem of creating efficacious attenuated bacterial vaccines that maximize both safety and immunogenicity in clinical trials.

Success with live bacterial vaccines rests on a delicate balance between safety and immunogenicity. Construction of an attenuated bacterial vaccine is often accomplished by genetically engineering a minimum of two independent chromosomal deletions of genes involved in biochemical pathways essential to bacterial metabolism and proliferation. However, genetic inactivation of too many critical genes, or inappropriate selection of targets, can result in vaccine candidates that fail to colonize a host sufficiently to engage innate and acquired immunity and elicit durable protection. A systematic review of *Salmonella* live oral vaccines tested in clinical trials to date suggests that as pathogenicity and reactogenicity are minimized to improve safety, immunogenicity and protective efficacy often suffer [1]. This concept is clearly illustrated by a series of attenuated *Salmonella enterica* serovar Typhi candidate oral vaccines derived from the wild-type strain CDC10–80, all carrying a deletion in the *aroA* gene critical to the aromatic amino acid biosynthesis

---

\*Author for correspondence: Tel:+1 410 706 6995, Fax: +1 410 706 6205, jgalen@medicine.umaryland.edu.

**Financial & competing interests disclosure:** The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

pathway. When coupled with an additional mutation in *aroD*, the resulting  $\Delta aroA\Delta aroD$  strain proved to be insufficiently attenuated but highly immunogenic [2]. When the triple deletion mutant  $\Delta aroA\Delta aroD\Delta htrA$  was constructed (by further deletion of *htrA* encoding a heat shock serine protease), safety improved at lower oral dosage levels but immunogenicity declined; at higher oral doses, which improved immunogenicity, reactogenicity (i.e., fever and bacteremia) was unacceptably high [2]. Combining  $\Delta aroA$  with deletions in either *purA* (involved in the purine biosynthesis pathway), or *phoP/phoQ* (a two-component environmental regulator of virulence in *Salmonella*) dramatically reduced both reactogenicity and immunogenicity [3,4]. It was only when the *phoP/phoQ* deletion mutation was introduced into a different parent strain of *Salmonella* Typhi (Ty2) that it became possible to minimize reactogenicity while maximizing robust immunogenicity at high oral dosage levels [5]. The central hypothesis of the current report [6] is that the reactogenicity of a live oral *Salmonella* vaccine can be minimized using a novel approach to genetically modify the synthesis of *Salmonella* outer membrane lipopolysaccharide (LPS), such that when combined with additional deletions of metabolic functions, it will be possible to engineer a less reactogenic yet highly immunogenic mucosal vaccine strain administered at high doses.

## Methods & results

To reduce reactogenicity, these authors focused on modification and detoxification of the endotoxic lipid A moiety of LPS in *S. Typhimurium*. Three deletion mutations were engineered to inactivate *lpxR* and *pagL*, involved in catalyzing the deacylation of lipid A at the 3' and 3 position respectively, as well as inactivation of *pagP*, which modifies lipid A present in the outer leaflet of the outer membrane by introduction of an acyloxyacylpalmitate (C16:0) fatty acid at the 2 position of the glucosamine backbone. To reduce the reactogenicity of lipid A, the authors chose a particularly novel approach of introducing a foreign gene cassette into the deleted *pagP* chromosomal locus that encodes LpxE, an inner membrane phosphatase from *Francisella tularensis* subspecies *novicida* that specifically removes the 1-phosphate group of lipid A. The resulting lipid A molecule is monophosphorylated, reminiscent of the monophosphoryl lipid A (MPL) adjuvant approved for use as a vaccine adjuvant in Europe, Australia and the USA. Integration of *lpxE* into a chromosomal location already chosen for deletion (i.e.,  $\Delta pagP::lpxE$ ) was done to avoid unintentional attenuation of the strain by inappropriate integration elsewhere that might interrupt regional gene regulation. Interestingly, integration of this cassette into either of the other deleted *lpxR* or *pagL* loci did not result in the complete dephosphorylation of lipid A observed when *lpxE* replaced *pagP* (as judged by mass spectrometry of lipid A species purified from individual mutant strains). Mass spectrometry suggested that engineered *S. Typhimurium* carrying all three chromosomal mutations  $\Delta pagL\Delta pagP::lpxE\Delta lpxR$  synthesized mainly a modified 4'-monophosphoryl-hexa-acylated lipid A species.

A panel of *S. Typhimurium* strains was constructed from a wild-type parent, carrying either the single deletion  $\Delta pagP$ , the insertion  $\Delta pagP::lpxE$ , the triple deletion  $\Delta pagL\Delta pagP\Delta lpxR$ , or the triple deletion  $\Delta pagL\Delta pagP::lpxE\Delta lpxR$  expressing LpxE. These five strains were then examined for attenuation by determining the 50% lethal dose (LD<sub>50</sub>) in BALB/c mice immunized orally with doses increasing from  $1 \times 10^3$  colony forming units (CFU) to  $1 \times 10^9$  CFU. While deletion *pagP* alone had no effect on the LD<sub>50</sub> when compared with the wild-type parent, insertion of *lpxE* into this locus reduced virulence by markedly increasing the LD<sub>50</sub> by approximately 10<sup>5</sup>-fold. Introduction of the triple deletion alone reduced virulence approximately sixfold but introduction of *lpxE* into this triple mutant resulted in a completely avirulent strain, with no deaths or disease symptoms observed in mice receiving the highest dose of  $1 \times 10^9$  CFU. This reduction in virulence was not associated with either any general growth defect identified by culturing *in vitro* or with any gross anomalies with

LPS synthesis identified by characterization of LPS profiles using silver-stained SDS-PAGE gels. When these inoculated mice were examined for colonization of deep tissues, it was noted that while all strains were able to colonize both the spleen and liver, no increase in viable counts was observed for any mutant expressing MPL at 6 days postinfection. Mice that survived inoculation with  $1 \times 10^6$  to  $1 \times 10^9$  CFU of engineered *S. Typhimurium* were then challenged at 30 days postinoculation with  $1 \times 10^5$  LD of the virulent wild-type *S. Typhimurium* parent strain, and all mice survived. These experiments confirmed that *S. Typhimurium* strains engineered to express a monophosphorylated hexa-acylated lipid A are highly attenuated yet retain their immunogenicity and ability to colonize mouse lymphoid tissues.

The reactogenicity of *S. Typhimurium* expressing modified LPS was examined both in BALB/c mice and in rabbit ligated ileal loops exposed to these strains. Measurement of inflammatory cytokines in mice immunized with the  $\Delta pagL\Delta pagP::lpxE\Delta lpxR$  triple mutant showed significantly lower levels of IL-6, IL-10 and TNF- $\alpha$  when compared with mice immunized with any other construct including the wild-type parent. In addition, when the histopathology of rabbit ligated ileal loops exposed to *S. Typhimurium* was examined, extensive destruction of the brush border and massive infiltration of polymorphonuclear lymphocytes was noted in loops injected with wild-type *S. Typhimurium*, while loops injected with strains expressing monophosphorylated LPS remained pristine with no infiltration of polymorphonuclear lymphocytes. These observations confirmed that *S. Typhimurium* strains engineered to express MPL are not only highly attenuated and immunogenic but also less reactogenic, resulting in significantly reduced inflammatory responses.

Interestingly, these authors were able to introduce the  $\Delta pagL\Delta pagP::lpxE\Delta lpxR$  triple mutant strategy into a vaccine strain previously shown to be safe and immunogenic in mice. Rather than resulting in an over-attenuated construct, the resulting strain could then be used as a recombinant attenuated *Salmonella* vaccine (RASV) to secrete the *Streptococcus pneumoniae* surface protein PspA encoded by genetically stabilized multicopy plasmids. In BALB/c mice orally immunized with two doses of  $1-2 \times 10^9$  CFU, 5 weeks apart, the triple mutant RASV strain expressing modified lipid A elicited both anti-PspA and anti-LPS serum IgG titers comparable to the parent vaccine strain, and also achieved protection against intraperitoneal challenge with 200 LD<sub>50</sub>s of wild-type *S. pneumoniae* equivalent to that observed in mice immunized with the unmodified RASV strain expressing PspA.

### Expert commentary & five-year view

In its fully active form, *Salmonella* LPS is highly toxic, can cause mortality due to toxic shock, and is a key mediator of the cytokine storm that exacerbates the pathogenesis of invasive *Salmonella* infections *in vivo* [7]. On the other hand, activation of the host immune system by LPS is necessary for protection from mortality caused by *Salmonella* infection; numerous studies utilizing mice that are hyporesponsive to LPS, including the spontaneous mutant C3H/HEJ mouse line that contains a nonfunctional Toll-like receptor (TLR)4 receptor as well as mice with targeted ablation in TLR4 signaling components, display greatly increased susceptibility to lethal *Salmonella* infection [8]. Here, Kong and colleagues provide evidence that partial activation of the host innate immune system by reducing the signaling capacity of LPS in an otherwise virulent *Salmonella* strain is attenuating, and furthermore that the resulting graded innate immune response is sufficient for generating functional immunity to both homologous pathogen antigens, as well as to a heterologous coexpressed foreign pathogen antigen [6].

The signaling capacity of LPS was reduced by engineering targeted deletion mutations into the chromosome of *S. Typhimurium*, leading to dephosphorylation of lipid A and synthesis

of a LPS species similar to the clinically relevant MPL molecule. MPL is generated through a chemical modification of *Salmonella minnesota* R595 LPS and displays a diminished capacity to signal through TLR4, resulting in lower levels of inflammatory cytokine production [9]. The reduced toxicity of MPL extends its use as an adjuvant for coadministered protein antigens, as it retains immunogenic properties, while diminishing the occurrence of vaccine-related adverse events. Studies designed to assess safety and efficacy in several animal models as well as in human clinical trials have demonstrated that it is >1000-fold less active than fully functional LPS, and can increase the levels of specific antibody to coadministered antigens [9]. Based on these results, MPL is now being tested clinically in several GlaxoSmithKline adjuvant formulations including AS04, which is used in the commercially available GlaxoSmithKline human papillomavirus (Cervarix) and hepatitis B (Fendrix) vaccines [10,11].

One of the key issues remaining in the use of attenuated *Salmonella* strains as human vaccines is achieving immunogenicity and protective efficacy, not only against the *Salmonella* vaccine itself, but also to foreign antigens delivered by the vaccine strain. To date, only modest immune responses have been achieved against foreign antigen delivery [12,13], and ultimate success will undoubtedly be attained through the engineering of live-attenuated mucosal vaccines able to present multiple protective foreign antigens by oral delivery at high doses. Over the years, the current group has been refining yet another novel strategy of simultaneous *de novo* attenuation and antigen delivery by *Salmonella* vaccine strains, accomplished *in vivo* through the genetic construction of an artificial arabinose-controlled network of critical metabolic functions [14–16]. The remarkable aspect of this approach is that fully virulent organisms are administered orally and as these organisms begin to proliferate in the host, intracellular supplies of arabinose run out and the vaccine organisms can no longer synthesize the critical enzymes required for survival; among the proteins no longer synthesized is a repressor molecule that when no longer made unmasks strong synthesis of foreign antigens for delivery to the host. Eventual lysis of vaccine organisms *in situ* then facilitates efficient delivery of these antigens to the immune system. Incorporation of lipid A mutations into such strains may dramatically reduce the reactogenicity of these delayed-attenuation strains when administered at high doses, while eliciting significant protective immune responses against multiple foreign antigens.

## Acknowledgments

Preparation of this manuscript was funded by the Middle Atlantic RCE Program NIAID/NIH 2 U54 AI057168 grant (MM Levine, principal investigator). R Simon was also supported by NIH T32 AI07524, Fellowship Training Program in Vaccinology (MM Levine, principal investigator)

## References

Papers of special note have been highlighted as:

- of interest
- of considerable interest

1. Levine, MM.; Galen, JE.; Pasetti, MF.; Sztein, MB. Attenuated strains of *Salmonella enterica* serovars Typhi and Paratyphi as live oral vaccines against enteric fever. In: Levine, MM.; Dougan, G.; Good, MF., et al., editors. *New Generation Vaccines*. 4th. Informa Healthcare; NY, USA: 2010. p. 497-505.
2. Dilts DA, Riesenfeld-Orn I, Fulginiti JP, et al. Phase 1 clinical trials of *aroAaroD* and *aroAaroDhtrA* attenuated *S typhi* vaccines; effect of formulation on safety and immunogenicity. *Vaccine*. 2000; 18(15):1473–1484. [PubMed: 10618545]

3. Levine MM, Herrington D, Murphy JR, et al. Safety, infectivity, immunogenicity, and *in vivo* stability of two attenuated auxotrophic mutant strains of *Salmonella typhi*, 541Ty and 543Ty, as live oral vaccines in humans. *J Clin Invest*. 1987; 79(3):888–902. [PubMed: 3818953]
4. Hohmann EL, Oletta CA, Miller SI. Evaluation of a *phoP/phoQ*-deleted *aroA*-deleted live oral *Salmonella typhi* vaccine strain in human volunteers. *Vaccine*. 1996; 14(1):19–24. [PubMed: 8821644]
5. Hohmann EL, Oletta CA, Killeen KP, Miller SI. *phoP/phoQ*-deleted *Salmonella typhi* (Ty800) is a safe and immunogenic single-dose typhoid fever vaccine in volunteers. *J Infect Dis*. 1996; 173(6): 1408–1414. [PubMed: 8648213]
6. Kong Q, Six DA, Roland KL, et al. *Salmonella* synthesizing 1-monophosphorylated lipopolysaccharide exhibits low endotoxic activity while retaining its immunogenicity. *J Immunol*. 2011; 187(1):412–423. [PubMed: 21632711]
7. Roy MF, Lariviere L, Wilkinson R, et al. Incremental expression of TLR4 correlates with mouse resistance to *Salmonella* infection and fine regulation of relevant immune genes. *Genes Immun*. 2006; 7(5):372–383. [PubMed: 16738669]
8. Nagai Y, Akashi S, Nagafuku M, et al. Essential role of MD-2 in LPS responsiveness and TLR4 distribution. *Nat Immunol*. 2002; 3(7):667–672. [PubMed: 12055629]
9. Casella CR, Mitchell TC. Putting endotoxin to work for us: monophosphoryl lipid A as a safe and effective vaccine adjuvant. *Cell Mol Life Sci*. 2008; 65(20):3231–3240. [PubMed: 18668203]
10. Didierlaurent AM, Morel S, Lockman L, et al. AS04, an aluminum salt- and TLR4 agonist-based adjuvant system, induces a transient localized innate immune response leading to enhanced adaptive immunity. *J Immunol*. 2009; 183(10):6186–6197. • Provides mechanistic insight into the contribution of monophosphoryl lipid A as part of a coformulation with aluminum salt in the commercial GlaxoSmithKline adjuvant AS04. [PubMed: 19864596]
11. Garcon N, Segal L, Tavares F, Van MM. The safety evaluation of adjuvants during vaccine development: the AS04 experience. *Vaccine*. 2011; 29(27):4453–4459. [PubMed: 21527299]
12. Khan S, Chatfield S, Stratford R, et al. Ability of SPI2 mutant of *S. typhi* to effectively induce antibody responses to the mucosal antigen enterotoxigenic *E. coli* heat labile toxin B subunit after oral delivery to humans. *Vaccine*. 2007; 25(21):4175–4182. [PubMed: 17412462]
13. Tacket CO, Galen J, Sztein MB, et al. Safety and immune responses to attenuated *Salmonella enterica* serovar Typhi oral live vector vaccines expressing tetanus toxin fragment C. *Clin Immunol*. 2000; 97(2):146–153. [PubMed: 11027455]
14. Curtiss R 3rd, Xin W, Li Y, et al. New technologies in using recombinant attenuated *Salmonella* vaccine vectors. *Crit Rev Immunol*. 2010; 30(3):255–270. [PubMed: 20370633]
15. Li Y, Wang S, Scarpellini G, et al. Evaluation of new generation *Salmonella enterica* serovar Typhimurium vaccines with regulated delayed attenuation to induce immune responses against PspA. *Proc Natl Acad Sci USA*. 2009; 106(2):593–598. •• Paradigm shift in live-attenuated bacterial vaccine strategies in which fully virulent vaccine organisms become attenuated after immunization. [PubMed: 19114649]
16. Wang S, Li Y, Scarpellini G, et al. *Salmonella* vaccine vectors displaying delayed antigen synthesis *in vivo* to enhance immunogenicity. *Infect Immun*. 2010; 78(9):3969–3980. [PubMed: 20605977]

### Key issues

- Success with live *Salmonella* bacterial vaccines rests on a delicate balance between safety and immunogenicity.
- To reduce reactogenicity, Kong and colleagues focused on modification and detoxification of the endotoxic lipid A to generate candidate live vaccine strains presenting surface lipopolysaccharide that resembled the clinically acceptable adjuvant monophosphoryl lipid A.
- The resulting candidate vaccine strains were reduced in virulence by five orders of magnitude, elicited reduced inflammatory cytokine responses in mice and induced no obvious histopathology in rabbit ligated ileal loops.
- Vaccine strains elicited excellent lipopolyaccharide-specific serum IgG and mucosal IgA responses in orally immunized mice, and mice were protected against homologous challenge with  $10^5$  CFUs of virulent *Salmonella*.
- When candidate vaccines were used to orally deliver the foreign antigen PspA from *Streptococcus pneumoniae*, PspA-specific serum IgG and mucosal IgA responses were observed, and mice were protected against lethal challenge with virulent *S. pneumoniae*.
- Future incorporation of lipid A mutations into strains exhibiting regulated delayed attenuation may dramatically reduce the reactogenicity of these strains when administered at high doses, while eliciting significant protective immune responses against multiple foreign antigens.