Published in final edited form as:

Crit Rev Immunol. 2010 ; 30(3): 255–270.

New Technologies in Using Recombinant Attenuated *Salmonella* Vaccine Vectors

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Abstract

Recombinant attenuated *Salmonella* vaccines (RASVs) have been constructed to deliver antigens from other pathogens to induce immunity to those pathogens in vaccinated hosts. The attenuation means should ensure that the vaccine survives following vaccination to colonize lymphoid tissues without causing disease symptoms. This necessitates that attenuation and synthesis of recombinant gene encoded protective antigens do not diminish the ability of orally administered vaccines to survive stresses encountered in the gastrointestinal tract. We have eliminated these problems by using RASVs with regulated delayed expression of attenuation and regulated delayed synthesis of recombinant antigens. These changes result in RASVs that colonize effector lymphoid tissues efficiently to serve as “factories” to synthesize protective antigens that induce higher protective immune responses than achieved when using previously constructed RASVs. We have devised a biological containment system with regulated delayed lysis to preclude RASV persistence in vivo and survival if excreted. Attributes were added to reduce the mild diarrhea sometimes experienced with oral live RASVs and to ensure complete safety in newborns. These collective technologies have been used to develop a novel, low-cost, RASV-synthesizing, multiple-protective *Streptococcus pneumoniae* antigens that will be safe for newborns/infants and will induce protective immunity to diverse *S. pneumoniae* serotypes after oral immunization.

Keywords

antigen synthesis; biological containment; delayed attenuation or attenuation; delayed; recombinant vaccines; *Salmonella*; vaccine

I. INTRODUCTION

In 1989, at this symposium held at Lake Louise in the Banff National Park of Canada, I reported¹ on our success in developing a balanced-lethal vectorhost system for use with recombinant attenuated *Salmonella* vaccines (RASVs). This system ensured the stability of plasmid vectors encoding protective antigens in vivo after immunization of animal hosts, and eliminated the use of drug-resistance markers, which were being discouraged for live vaccines.^{2–4} Much has transpired in these past 20 years,^{5–8} but a still frequently asked question is why use *Salmonella* as a vaccine vector for delivery of antigens to the immune system rather than more benign pathogens or commensals? The most critical issue is invasion and colonization of deep effector lymphoid tissues after mucosal delivery. In other words, getting to regional lymph nodes plus the spleen is of critical importance in inducing

memory T cells in locations where they persist. *Salmonella* is the most proficient pathogen in accomplishing this at the lowest mucosally delivered immunizing dose, and initiates this deep invasion by first attaching to and invading various mucosa-associated lymphoid tissues of the gut, nasopharynx, bronchi, etc. This results in the induction of mucosal, systemic, and cellular immune responses with induction of long-term protective immunity. Orally administered attenuated pathogens that are not invasive, such as *Escherichia coli* and *Vibrio cholerae*, and rough mutants of *Salmonella* lacking lipopolysaccharide (LPS) components or that are only invasive to mucosal tissues (i.e., *Shigella*) seldom induce lasting immunity. In addition, *Salmonella* has exceptional adjuvant qualities and can even induce mucosal and systemic antibody titers in MyD88^{-/-} (myeloid differentiation factor 88 deficient) and TRIF^{-/-} (Toll-interacting receptor-domain-containing adaptor inducing interferon deficient) mice that are equal to or higher than those induced in wildtype mice.^{9,10} In addition, we know a great deal about the pathogenicity, physiology, and genomics of *Salmonella enterica* serotypes and can genetically manipulate these strains using all of the molecular genetic tools developed for the study of gram-negative enteric bacteria since the 1940s.

The use of live bacterial vaccines for oral delivery eliminates the need for needles, with their high cost and associated risks, but is associated with problems not fully appreciated during the early development of these technologies. Indeed, we all have endeavored to design and construct RASVs with maximal abilities to colonize internal effector lymphoid tissues to induce maximal protective mucosal, systemic, and cellular immunities against the pathogen of choice. However, the sojourn of a RASV from a liquid suspension at ambient temperature to entry into the mouth leads to a succession of stresses imposed by the changing environments of the gastrointestinal (GI) tract. The responses to some of these stresses, such as exposure to acid,^{11,12} bile,^{13,14} and defensins,¹⁵ have been well studied, whereas other stresses and combinations of stresses have not. Thus, even wild-type *Salmonella* strains need a reasonably high oral inoculum (10⁵ to 10⁶ colony-forming units [CFU]) to cause disease even though they are capable of turning on a succession of different genes to cope with this succession of different encountered stresses. This capability often does not exist, or only partially exists, in many *Salmonella* vaccine strains attenuated by defined deletion mutations inactivating global regulators or imposing nutrient deprivation that are much more susceptible to not surviving one or more stresses encountered in the GI tract. Our recognition of this^{16,17} led us to develop a means to genetically engineer vaccine strains to display a regulated delayed attenuation such that strains would have attributes of a wild-type strain at the time of oral inoculation and then, after colonization of mucosa-associated lymphoid tissues and internal lymphoid tissues, would gradually lose virulence traits as a function of cell division and do so in a timely manner so as to not cause disease symptoms. Also, as has been recognized for some time,¹⁸ high-level expression of the protective protein antigens needed to induce maximal mucosal and serum antibody titers was often somewhat attenuating and reduced the ability of RASV strains to efficiently colonize lymphoid tissues.¹⁹ We have therefore developed a means of regulated delayed synthesis of recombinant protective antigens²⁰ that also contributes to improved RASV colonization and immune responses.²¹

In this review, we discuss these improved means of enhancing the induction of protective immune responses by RASVs, and discuss other means to enhance vaccine safety, tolerability, and immunogenicity. Our focus has been on developing safe RASVs suitable for use in newborns/neonates and infants and, more specifically, to develop a vaccine to induce protective immunity to the diversity of *Streptococcus pneumoniae* strains encountered throughout the world that cause some two million annual deaths, mostly in children less than five years of age.²²

II. MEANS FOR ACHIEVING REGULATED DELAYED ATTENUATION OF RASV STRAINS FOR MUCOSAL IMMUNIZATION

During the past seven years, our group has endeavored to develop a vastly improved array of means to enhance the safety, efficacy, and utility of *Salmonella* antigen-delivery technologies. Many well-known means for attenuation of *Salmonella* (Table 1, Part A) diminish its ability to withstand stresses encountered in the GI tract after oral immunization or impair its ability to attach to, invade into and survive in the gastric mucosa-associated lymphoid tissues, collectively leading to diminished immunogenicity. Therefore, we have developed three classes of usable means to achieve regulated delayed attenuation in vivo such that the vaccine at the time of immunization exhibits almost the same abilities as a fully virulent wild-type strain to contend with stresses and successfully reach effector lymphoid tissues before display of attenuation to preclude onset of any disease symptoms.¹⁶

A. Reversible Synthesis of LPS O-Antigen and/or Parts of the LPS Core With Regulated Delayed Attenuation In Vivo Phenotype

Colonization of the intestinal tract by *Salmonella* following oral administration is dependent upon/facilitated by the expression of a number of surface antigens, including LPS O-antigen side chains, a diversity of fimbrial adhesins, flagella, and possibly certain outer membrane proteins, as well by the expression of a succession of gene products that enable survival against a succession of stresses (acid, bile, defensins, anaerobiosis, osmolarity shifts, ion excess, etc.) (Table 1, Part B). Thus, rough mutants that have mutational lesions precluding synthesis of LPS O-antigen or parts of the LPS core do not colonize the intestinal tract^{23,24} and are defective in attaching to and invading intestinal cells and surviving in cells on the other side of the intestinal wall barrier.^{25,26} This latter phenotype is undoubtedly due to the fact that LPS is needed for *Salmonella* to display resistance to killing by macrophages^{27,28} and serum^{29,30} and to multiply in blood. In accord with these observations, rough *Salmonella* mutants defective in LPS synthesis, and thus defective in infection, are among the most frequently isolated mutants using signature-tagged mutagenesis,³¹ and genes for LPS biosynthesis are often up-regulated during infection, as revealed using in vivo expression technology.³² For these reasons, rough mutants of *Salmonella* have not been effective when used as live oral vaccines.^{33,34} It follows that to be safe and efficacious, an attenuated, immunogenic live vaccine must not only display avirulence and not induce disease symptomology, but also be able to reach, multiply, and persist for a while in those lymphoid organs necessary to stimulate a protective immune response. Obviously, permanently rough *Salmonella* mutants cannot achieve the latter. As a potential way around this, the use of *Salmonella* strains with mutations in the *galE* locus encoding uridine diphosphate (UDP)-galactose epimerase, an enzyme that interconverts UDP-glucose and UDP-galactose,³⁵ had previously been considered.

UDP-galactose is needed for the synthesis of both the LPS core and the O antigen in many *Salmonella* serotypes.³⁶ When *Salmonella galE* mutants are provided low levels of galactose, they make normal LPS, but in an environment devoid of free (nonphosphorylated) galactose, they rapidly lose the ability to synthesize a complete LPS O-antigen and core.³⁷ One of the difficulties with *galE* mutants is that they are sensitive to galactose^{38,39} and accumulate galactose-resistant mutants in their population, which are permanently rough and therefore not only avirulent but also nonimmunogenic. Also, because of the LPS core defect, these *galE* mutants, even when administered parenterally, are somewhat hyper-attenuated (in animals but not humans) and do not induce high-level protective immunity.^{40,41} Another alternative to generate a reversibly rough phenotype is to make use of *pmi* mutants that have a mutation in the gene for phosphomannose isomerase,⁴² which interconverts mannose 6-phosphate and fructose 6-phosphate. Mannose 6-phosphate is then converted to GDP-

mannose and used for the synthesis of O-antigen side chains.⁴³ Fortunately, *pmi* mutants are not mannose sensitive and, as shown by Collins et al.,⁴⁴ are partially attenuated. We have demonstrated that *pmi* mutants, when grown in media containing mannose, synthesize wild-type levels of LPS O-antigen side chains, are reasonably attenuated but highly immunogenic.¹⁶

We have extensively investigated *Salmonella typhimurium* strains with the Δpmi -2426 mutation (Δ = deletion mutation) to demonstrate its attenuation (not complete), high immunogenicity, efficacy in enhancing induction of high-antibody titers to cross-protective outer membrane proteins,¹⁶ and ability to enhance production of outer membrane vesicles that can also deliver recombinant protective antigens to enhance induction of protective immunity.^{45,46} These results, obtained with cultures grown in the presence of mannose, imply that the LPS O-antigen side chains persist long enough while the vaccine strain is in the intestinal tract to enable subsequent successful colonization of lymphoid tissues, and/or that sufficient concentrations of free mannose exist in the intestinal tract to sustain LPS O-antigen side chain synthesis. Our results, however, in accord with those of others,⁴⁴ indicate that free mannose (nonphosphorylated) is not available in sufficient quantity in animal tissues to support a level of LPS O-antigen synthesis for display of a wild-type level of virulence. An additional consequence of the loss of O-antigen side chains by *pmi* mutants in vivo is the heightened induced antibody titers against the LPS core^{47,48} that is common to most *S. enterica* subspecies 1 serotypes^{49,50} (except *S. arizonae*⁵¹), as well as to exposed outer membrane proteins. It is also possible to combine the $\Delta galE$ and Δpmi mutations in the same vaccine strain so that LPS synthesis is dependent on the presence of both nonphosphorylated galactose and nonphosphorylated mannose, and therefore the synthesis of the LPS O-antigen and complete core ceases in vivo due to the unavailability of these nonphosphorylated sugars.

B. Arabinose-Dependent Regulated Synthesis of Virulence Traits to Result in Regulated Delayed Attenuation In Vivo

The second means with which to achieve regulated delayed attenuation relies on using a more tightly regulated *araC* P_{BAD} activator-promoter (P = promoter)⁵² than the original sequence from *E. coli* B/r^{53,54} in place of upstream regulatory and promoter sequences for four genes needed for full display of virulence (Table 1, Part B). We deleted the promoter, including sequences for activator or repressor protein binding, for the following genes: *fur*, encoding the Fur protein, which represses all genes involved in iron acquisition and its absence attenuates *Salmonella*, presumably due to iron overload¹⁶; *phoPQ*, encoding a two-component regulatory system essential for virulence⁵⁵; *crp*, encoding a cAMP receptor protein that is necessary for virulence of *Salmonella* and is also needed for maximal transcription from the P_{BAD} promoter^{56,57}; and *rpoS*, encoding a sigma factor needed to express genes required for survival in stationary phase, to respond to stresses, and for virulence.^{58,59} The absence of each gene by deletion attenuates *S. typhi* for mice and chickens. We then substituted the improved *araC* P_{BAD} cassette to yield *Salmonella* strains with the $\Delta P_{fur}::TT\ araC\ P_{BAD}\ fur$, $\Delta P_{phoPQ}::TT\ araC\ P_{BAD}\ phoPQ$, $\Delta P_{crp}::TT\ araC\ P_{BAD}\ crp$ and $\Delta P_{rpoS}::TT\ araC\ P_{BAD}\ rpoS$ deletion-insertion mutations (TT = transcription terminator, which is needed to preclude transcription of the *araC* gene from continuing into adjacent genes). In the case of the *fur* and *phoPQ* genes, we down-regulated their expression levels by altering SD (Shine-Dalgarno ribosome binding site) and start codon sequences. This was necessary because growth of vaccine strains in Luria broth plus 0.2% arabinose necessary to achieve high levels of repressor synthesis to enable the regulated delayed antigen synthesis phenotype as described below, leads to high concentrations of Fur and PhoP when expressed from wild-type sequences. Overexpression of Fur leads to iron starvation during growth and immunization that can be deleterious to maximal colonization

of lymphoid tissues, and overproduction of PhoP also causes hyperattenuation to diminish immunogenicity. The various deletion-insertion mutations constructed with the best combination of modifications for transcription and translation are diagrammed in our recent paper.¹⁷ Growth of strains with these deletion-insertion mutations in the presence of arabinose leads to transcription of the *fur*, *phoPQ*, *crp*, and *rpoS* genes, but expression ceases in internal tissues because there is no free arabinose.⁵² Attenuation develops as the products of these genes decrease due to the dilution resulting from cell division. In the case of RpoS, the decrease is also due to proteolytic instability depending on growth stage and other stress factors,⁶⁰ although predicting these states in vivo is difficult.

We can delay onset of attenuation by including Δ *araBAD23* (which deletes structural genes for catabolism of arabinose) to prevent use of arabinose retained in the cell cytoplasm at the time of oral immunization and/or Δ *araE25* (deletes gene for arabinose transport), which enhances the retention of arabinose (Table 1, Part E). Inclusion of the Δ *araBAD23* mutation also prevents acidification of the medium during growth of the vaccine strain in the presence of arabinose, an occurrence that can be detrimental to vaccine performance. Strains with the Δ P_{fur81}::TT *araC* P_{BAD} *fur*, Δ P_{phoPQ175}::TT *araC* P_{BAD} *phoPQ*, Δ P_{rpoS183}::TT *araC* P_{BAD} *rpoS* and Δ P_{crp527}::TT *araC* P_{BAD} *crp* deletion-insertion mutations are totally attenuated at oral doses of 10⁹ CFU in mice, although strains with the original Δ P_{fur33}::TT *araC* P_{BAD} *fur* mutation retain some virulence.¹⁷ This is undoubtedly due to the fact that the high concentration of Fur at the time of immunization delays onset of attenuation such that some mice inoculated with high doses do not survive. This observation served as the basis for decreasing Fur synthesis by altering SD and the start codon for the *fur* gene. However, mice surviving infection with strains having the Δ P_{fur81}::TT *araC* P_{BAD} *fur*, Δ P_{fur33}::TT *araC* P_{BAD} *fur* and Δ P_{crp527}::TT *araC* P_{BAD} *crp* mutations all survive challenge with 10⁹ CFU of the wild-type *S. typhi* UK-1 parent. In contrast, strains with the Δ P_{phoPQ107}::TT *araC* P_{BAD} *phoPQ* and Δ P_{rpoS183}::TT *araC* P_{BAD} *rpoS* mutations, although completely attenuated, are not quite as immunogenic, presumably because of overattenuation.¹⁷ Again, we altered the SD and the start codon for the *phoP* gene to improve the immunogenicity of strains with the regulated delayed *phoPQ* attenuation. Transcription of the P_{BAD} promoter is dependent on both interaction of arabinose with the AraC protein and the Crp protein with the promoter sequence. We therefore include the Δ P_{crp527}::TT *araC* P_{BAD} *crp* mutation as an added safety feature in vaccine strains, because Crp synthesis will also cease and serve as a second means to shut off expression of the *fur*, *phoPQ*, and/or *rpoS* genes fused to P_{BAD}.¹⁷ This has yielded strains with three means of regulated delayed attenuation that have exhibited very high immunogenicity.²¹ It is likely that a combination of additional means for achieving regulated delayed attenuation might require additional tinkering to perfect the levels of expression of genes contributing to the attenuating phenotype.

C. Regulated Delayed Lysis In Vivo as a Means of Attenuation and Biological Containment

We have devised a host-vector system with arabinose-regulated expression of the chromosomal *murA* gene that encodes the first enzyme in muramic acid synthesis (due to an arabinose-dependent conditional-lethal Δ P_{murA}::TT *araC* P_{BAD} *murA* deletion-insertion mutation) and a deletion-insertion Δ *asdA*::TT *araC* P_{BAD} *c2* mutation imposing a requirement for diaminopimelic acid (DAP) (Table 1, Part C). The complementing plasmid vector pYA3681 (Fig. 1) contains, as a cassette flanked by TT sequences, a tightly arabinose-regulated *araC* P_{BAD} GTG-*murA* GTG-*asdA* followed by a C2-repressible P₂₂ P_R in the opposite orientation.⁵² The change in start codon from ATG to GTG for both the *asdA* and *murA* genes is important and reduces translation efficiency 10-fold. In the absence of these changes, cell division continues too long, often resulting in animal death. Another cassette contains the LacI-repressible P_{trc}, followed by a cloning site for insertion of recombinant sequences encoding protective antigens, followed by another TT. This enables

the lysis system to deliver a bolus of protective antigen at the time of cell lysis to stimulate the induction of a strong immune response. The third cassette contains the pBR *ori*, although we have versions with pSC101 *ori*, p15A *ori* and pUC *ori*. When this host-vector strain is grown in Luria broth plus 0.2% arabinose, synthesis of MurA and Asd enables cell wall synthesis, and production of C2 (from the chromosomal $\Delta asdA27::TT araC P_{BAD} c2$ mutation) will prevent transcription from P_R to preclude synthesis of antisense *asdA* and *murA* mRNA (Fig. 1).

In vivo, where there is an absence of arabinose,⁵² transcription of the *murA* and *asdA* genes cease and synthesis of antisense *asdA* and *murA* mRNA from P_R commences as a means to shut down translation of any residual *murA* and/or *asdA* mRNA. This host-vector is totally avirulent at oral doses in excess of 10^9 CFU to BALB/c mice, and we have been so far unable to recover any arabinose-independent mutants, even in the presence of DAP. Strains with this regulated delayed lysis in vivo system are not recovered from orally immunized mice two to three weeks after oral immunization, although the vaccine strain can achieve colonization levels of about 10^5 CFU in spleens about one week after immunization. Any bacteria excreted are also unable to survive and undergo rapid, cell wall-less death. This is facilitated by the inclusion of a *relA* mutation (such as $\Delta relA198::araC P_{BAD} lacI TT$, Table 1, Part D) that uncouples growth from a continuance of protein synthesis.

D. Combined Use of Multiple Means to Achieve Regulated Delayed Attenuation

It has been generally accepted as good practice in the attenuation of live bacterial vaccines to have two or more means to confer attenuation and thus ensure safety. Therefore, we have used two of the above-described strategies for achieving regulated delayed attenuation. We are now routinely using the $\Delta P_{fur81}::TT araC P_{BAD} fur$ and $\Delta P_{crp527}::TT araC P_{BAD} crp$ deletion-insertion mutations in conjunction with the $\Delta pmi-2426$ mutation,²¹ because each of these three mutations contributes significantly to high immunogenicity.^{16,17,21} As an added safety feature and because of the increasing importance of biological containment, we are also beginning to add the regulated delayed lysis in vivo system for future RASV constructions.

III. RECOMBINANT ANTIGEN SYNTHESIS AND DELIVERY

A. Plasmid Vector Properties

RASVs should be sensitive to all antibiotics that might be useful for therapeutic use in treating individuals with unexpected consequences of immunization with live vaccines, especially if derived from *S. typhi* or *S. paratyphi* A. The use of traditional cloning vectors with selective drug-resistance genes to encode protective antigens is unacceptable from a safety perspective, and also cannot provide a means for selective maintenance of the plasmid vector in all live vaccine cells in vivo. To eliminate the use of plasmid vectors with drug-resistance genes and to stabilize plasmid vectors in RASVs in vivo, many years ago we developed a balanced-lethal vector-host system using a vaccine host strain with deletion of the *asdA* gene to impose an obligate requirement for DAP, and a plasmid vector with the wild-type *asdA* gene^{2,61} to establish a complementation heterozygote.

DAP is a unique constituent of the peptidoglycan layer of the bacterial cell wall necessary to maintain cell shape and stability. It is only synthesized by bacteria and is unavailable in tissues in the immunized host. An alternate strategy for stable maintenance of plasmid vectors in vaccine host strains can be accomplished⁶² through incorporation into plasmid vectors of a post-segregational killing system based on the noncatalytic *hok-sok* plasmid addiction system.⁶³ In both cases, all viable recombinant vaccine cells in the immunized individual maintain the plasmid vector encoding the protective antigen(s).

Overexpression of the selective marker on plasmid vectors can contribute to the attenuation of the vaccine strain and lessen colonization efficiency and thus immunogenicity. We first observed this in regard to synthesis of the Asd enzyme on high-copy-number vectors with the pUC *ori*, in which the LD₅₀ of a strain only possessing a $\Delta asdA$ mutation and the pUC *ori* Asd⁺ vector was increased about 10-fold over that of the wild-type parental strain.⁶⁴ We therefore eliminated the P_{asdA} sequence but not the SD sequence from Asd⁺ plasmids with the pBR *ori* (Fig. 2). Alternatively, or in addition as we use with pUC *ori* plasmids, changing the *asdA* gene start codon from ATG to GTG or TTG and/or with modification of the SD sequence for ribosome binding from AGGA to AAGG decreases the amount of Asd enzyme synthesized to an amount specified by a single-copy chromosomal gene, and thus restores the efficiency of colonization of lymphoid tissues to enhance immunogenicity.

The choice of plasmid copy number is important in regard to the nature of the protective antigen to be synthesized and delivered and the type of immune response desired. Thus, synthesis of a surface-localized pilus antigen is often best using low-copy-number plasmids with pIncI *ori* (single copy number) or pSC101 *ori* (low copy number). In contrast, for partial or total secretion of an antigen, it is often advantageous to use plasmids with p15A *ori* (moderate copy number), pBR *ori* (high copy number), and/or pUC *ori* (very high copy number). In general, the induction of significant mucosal and serum antibody responses is favored by maximizing the amount of antigen delivered to the immunized individual by using vectors with the p15A *ori*, pBR *ori* or pUC *ori*, whereas induction of cellular immunities is usually better achieved by delivering lower amounts of the protective antigen. Thus, the induction of CD8 T-cell responses using the *Salmonella* Type III secretion system (T3SS) is often better achieved by using low-copy-number plasmids with pSC101 *ori* or p15A *ori*,^{65,66} or even by incorporating the expression cassette into the chromosome.⁶⁷

B. In Vivo and Regulated Delayed Synthesis of Protective Antigens

Overexpression of protective antigens by RASV strains (as well as overexpression of the selective marker) can reduce colonizing ability and thus immunogenicity. It was for this and other reasons that Chatfield et al.⁶⁸ proposed the use of the *nirB* promoter (P_{nirB}) that is more active anaerobically than aerobically in accord with a more likely in vivo anaerobic environment. The P_{trc} that we have used,^{2,64,69} as well as P_{tac}, is constitutive under most environments but is more transcriptionally active both anaerobically and aerobically than the P_{nirB}⁶⁸ (see Fig. 1, lanes 2 and 5 vs. lanes 1 and 4 in Chatfield et al.⁶⁸). We therefore developed a system in which transcription from P_{trc} could be repressed during in vitro cultivation and following immunization with a gradual de-repression as a consequence of cell divisions occurring during colonization of internal lymphoid tissues. We thus generated the $\Delta relA198::araC$ P_{BAD} *lacI* TT deletion-insertion mutation so that recombinant vaccine strains growing in the presence of arabinose synthesize the LacI repressor to repress transcription from P_{trc} on the plasmid expression vector until after vaccination, when the vaccine strain is already colonizing internal lymphoid tissues^{20,21} where arabinose is absent.⁵²

This technology was incrementally improved to ultimately increase expression of the *lacI* gene 40-fold by changing the SD sequence from AGGG to AGGA, the wild-type *lacI* start codon from GTG to ATG, and by changing *lacI* codons to maximize translation efficiency in *Salmonella*. A similar strategy has been used to make synthesis of the phage P22 C2 repressor dependent on presence of arabinose in the growth medium to delay transcription of DNA sequences under the control of P22 P_L and P_R promoters to exhibit a regulated delayed in vivo expression. This was accomplished by the $\Delta asdA27::TT araC$ P_{BAD} *c2* deletion-insertion mutation.^{21,52} Other investigators have evaluated other promoters for in vivo expression of genetic information encoding protective antigens to be delivered by attenuated

Salmonella vaccines. These include the PhoP-PhoQ regulated *pagC* promoter P_{pagC} ,^{70,71} the anaerobically induced promoter of the *dmsA* gene P_{dmsA} ,⁷² the *ssrB*-regulated P_{ssaG} promoter,⁷³ and the stringent starvation *sspA* gene promoter P_{sspA} .⁷⁴ It is likely that further research to modify the DNA sequences for promoters and the sequences that interact with regulatory molecules will enable further improvements to modulate the synthesis of protective antigens in a manner that maximizes the induction of the desired immune responses. Many of these modifications are likely to be vaccine specific.

C. Antigen Delivery Strategies

Most early investigations of RASV strains for the delivery of protective antigens used vaccine strains in which the protective antigen synthesized was retained in the cytoplasm of the attenuated *Salmonella* strain, thus necessitating that the immunized host disrupt the cells to release the protective antigen for processing. This probably explains the minimal successes in early studies using RASV strains to induce significant protective immunity to heterologous pathogens, whether in mice or humans. Several years ago,⁶⁴ we learned that export of antigens to the periplasm of vaccine strains yielded superior antibody titers than if the protective antigen was retained in the cytoplasm of the vaccine strain.⁷⁵ We initially used the β -lactamase Type II secretion system (T2SS),⁶⁴ but now have validated use of the *phoA*, *ompA*, *lpp*, *eltB* (Type II), and *dsbA* signal sequences with equally good results.^{20,76} In these various vectors, we use either P_{trc} or P_{22} P_L as promoters with expression levels controlled by the LacI and C2 repressors, respectively. Others have investigated use of the *E. coli* hemolysin secretion system (Type I secretion system) to export protective antigens.^{77,78} Also, results have been very positive using the *clyA* secretion system to externalize protective antigens from *Salmonella* vaccine strains.⁷⁹ It is likely that the high immunogenicity of protective antigens secreted by the T2SS is due to the formation of outer membrane vesicles that incorporate antigens secreted into the periplasmic space. It has now been established that the production of such vesicles leads to targeting of antigens to antigen-presenting dendritic cells.⁴⁶ As already noted above, the T3SS has been effectively used to deliver protective antigens to the cytosol of cells in the immunized host to enable MHC class I presentation to stimulate CD8 T-cell responses.^{65,80,81} Lastly, *E. coli*, *Salmonella*, and other enterics have Type V secretion systems, and the first such autotransporter in the Enterobacteriaceae was discovered and described by us.⁸² It is thus likely that α -helical domains of protective antigens could be delivered by such transporters; the AIDA autotransporter has been so evaluated.⁸³ The last type of antigen delivery is achieved by the regulated delayed lysis strategy, which confers biological containment while being able to deliver a bolus of protective antigen at the time of in vivo vaccine strain cell lysis.⁵²

D. Modification of Regulatory and Coding Sequences to Maximize Immunogenic Results

RASV design to enable induction of protective immunity to some heterologous pathogens requires the selection and delivery of one or more protective antigens from the targeted heterologous pathogen. A full discussion of the means for identification of such protective antigens is beyond the scope of this review. However, many of these antigens are surface-localized or secreted by pathogens, and the desired protective immune responses neutralize toxins, lysins, enzymes, viruses, etc., or block attachment (adherence), invasion, and/or survival to preclude colonization. Before cloning a DNA sequence encoding a putative protective protein antigen, one must decide whether the objective is to induce mucosal and/or serum antibodies or cellular immunity. This, of course, depends on the nature and life style of the pathogen. If the former, then delivery of the antigen in high amounts to the surface or periplasmic space of the RASV should be engineered. Achieving this is not always easy. The first step in designing the construction is to make a full examination of the amino acid sequence and structural properties of the protein, examine the DNA sequence for

G+C content and codons used, and the mRNA structure for potential hairpins. The presence of multiple Cys residues can be indicative of folding properties that would preclude secretion, and extensive hydrophobic sequences can also lead to toxicities and impairment in secretion. Deletion or modification of such sequences can often eliminate problems in synthesis and delivery.⁸⁴ If the protein is normally secreted, it may still be desirable to substitute the signal peptide sequence for a signal sequence from a protein efficiently secreted by *Salmonella*.⁶⁴ Efficient translation of mRNA can be achieved by changing all of the codons used less often than 5% to 10% of the time in *Salmonella* highly expressed genes.^{85–87}

We have also adjusted the G+C content to within approximately 4% of the *Salmonella* DNA G+C content during codon optimization because *Salmonella* expresses genes within islands of DNA acquired by horizontal gene transfer whose G+C content deviates by $\pm 4\%$ from the *Salmonella* average. Translation efficiency can also be improved by changing the SD sequence or its distance to the start codon and also by making the second and third codons A rich.⁸⁸ Lastly, because hairpins in mRNA might contribute to RNaseE attack to reduce mRNA half-life, base-pair changes that do not alter the amino acid sequence can be used to eliminate these targets for mRNA stability.⁸⁶ Once cloning vectors are constructed under the control of a repressible promoter, one examines growth rate and plasmid stability (over approximately 50 generations) with and without antigen synthesis and with and without selective pressure for maintenance of the plasmid vector. Slow growth and/or plasmid instability due to antigen synthesis often are indicative of a poor ability of the RASV to effectively colonize effector lymphoid tissues necessary to induce high-level immune responses. Such results warrant further modifications, which might include using a plasmid vector with a reduced plasmid copy number. We also examine antigen stability by following persistence of antigen amount after adding chloramphenicol to the culture maintained at 37°C.¹⁷ If degradation products are detected, we examine the amino acid sequence for protease cleavage sites and determine if any of these could possibly generate the fragment(s) detected. If so, amino acid substitutions are introduced to see if this enhances protein stability to enhance induction of protective immunity. Cell-fractionation studies,^{64,89} in conjunction with western blotting, reveal the proportion of synthesized antigens present in various cellular and extracellular compartments.

The complexities in the synthesis and delivery of protective antigens to stimulate T-cell immunities are less than detailed above for antigens to be synthesized at a high enough level to stimulate systemic antibody responses. However, not all proteins or peptides are efficiently transmitted through the T3SS needle, and therefore must be delivered to cells in the immunized host by other methods, such as by using a RASV with regulated delayed lysis in vivo. In these cases, lysis in the endosome or cytosol would be expected to yield predominantly CD4 and CD8 responses, respectively.

IV. BIOLOGICAL CONTAINMENT OF RASV STRAINS

Live vaccines that are orally administered have the potential to be excreted in a viable form that might lead to persistence in the environment to lead to immunization (even if ineffective) of those unintended to be vaccinated. In this regard, vaccine strains with multiple mutations that might impair growth, due to the need for required nutrients or the inability to use available substrates, are likely to have reduced survivability in the environment; additional mutations that eliminate the synthesis of surface components needed to form biofilms would likely further reduce such survival. The RASV-regulated delayed lysis system dependent on the absence of arabinose⁵² affords another type of biological containment. However, arabinose present in complex polysaccharides present in foods of plant origin can be released by the actions of other microorganisms in intestinal

contents and possibly in other ex vivo environments. Thus, cessation of the synthesis of cell wall peptidoglycan would be more gradually achieved with resulting death by lysis. The inclusion of a *relA* mutation in these strains uncouples the occurrence of growth-dependent lysis on a need for continued protein synthesis. Because production of a colanic acid capsule can enable survival of cells undergoing cell wall-less death,⁹⁰ the inclusion of the $\Delta(gmd-fcl)$ mutation in these strains precludes synthesis of a colanic acid capsule and facilitates cell wall-less death. Undoubtedly, further improved methods for achieving biological containment will be discovered.

V. OTHER IMPORTANT RASV ATTRIBUTES

Salmonella, like many pathogens, has devised means to contend with innate immunity and to suppress or modulate induction of immune responses. Most of these means remain undiscovered, but as discoveries are made, RASV strains need to be modified to inactivate these *Salmonella* defense strategies. The SopB protein plays an immunosuppressive role,⁹¹ and its inactivation results in improved humoral and cellular immunity⁹¹—especially mucosal immunity.⁹² An added benefit of including a $\Delta sopB$ mutation in *S. typhi* vaccine strains is that it reduces fluid secretion⁹⁰ to lessen the mild diarrhea experienced by a small percentage of vaccinees.^{93,94}

While striving to enhance the immunogenicity of RASV strains, it is of critical importance to also enhance safety for animals as well as humans. In regard to humans, the increases in individuals with immunosuppression due to infection, genetic factors, and increasing age is a cause for concern in using live attenuated bacterial vaccines. Preferably, these vaccines would be safe for such individuals as well as for infants, pregnant women, and those who are malnourished. However, at this stage we are not sure whether such safety has been achieved, because results with RASV strains derived from *S. typhi* and tested in mice and other animals do not necessarily predict the behavior of RASV strains derived from *S. typhi* and evaluated in humans. In this regard, many of the previously studied means of attenuation, which render *S. typhi* totally attenuated and highly immunogenic in mice, fail to render *S. typhi* attenuated for humans.^{95,96} There is also the issue of whether a *Salmonella* vaccine vector used to deliver protective antigens from one heterologous pathogen to induce protective immunity to that pathogen can be used a second or even third time to effectively deliver protective antigens from another heterologous pathogen to the same individuals vaccinated with the first RASV. Certainly, vaccine strains attenuated by traditional means used in the past are disappointing in this regard and fail to have utility for repeated use.⁹⁷

VI. CONCLUSION

Much progress has been made in improving the technologies to design, construct, and evaluate RASVs for oral, needle-free immunizations. Many of these improvements have enhanced safety, tolerability, and effectiveness in inducing protective immunity. These technologies are now being used to develop vaccines to protect against important diseases of agriculturally important animals, as well as vaccines using genetically engineered strains of *S. typhi* to protect against a number of important human infectious diseases. In this regard, we have succeeded at least in constructing *S. typhi*-based RASV strains that induce equal or higher serum and mucosal immune responses to delivered protective antigens from *S. pneumoniae* than to *Salmonella* outer membrane protein and LPS surface antigens.²¹ Evaluation of *S. typhi* strains with similar properties in human volunteers will soon reveal whether these technologies have practical significance in the development of cost-effective and safe vaccines for humans.

Acknowledgments

We thank Erika Arch for help in assembling this article and Josephine Clark-Curtiss and Kenneth Roland for editorial and content advice. Research was supported by grants from the Bill & Melinda Gates Foundation, the National Institutes of Health, the US Department of Agriculture, and the Ellison Medical Foundation.

ABBREVIATIONS

RASV	recombinant attenuated <i>Salmonella</i> vaccine
LPS	lipopolysaccharide
GI	gastrointestinal
CFU	colony-forming units
UDP	uridine diphosphate
DAP	diaminopimelic acid

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FIGURE 1.

The regulated programmed lysis system. A, Map of plasmid pYA3681. Plasmid sequences include the *trpA*, *rrfG*, and 5S ribosomal RNA transcriptional terminators; the P_{BAD} , P_{trc} , and P22 P_R promoters; the *araC* gene; and the start codon-modified *murA* and *asdA* genes preceded by their SD sequences. B, Diagram of model illustrating the regulatory interactions in the programmed lysis system. Details of this system relative to expression of *murA* and *asdA* genes and repression of anti-mRNA and protective antigen gene transcription in vitro when arabinose is present and the opposite activities in vivo when arabinose is absent are detailed more fully in the text. (Slight modification of Figure 2 from Kong et al.,⁵² copyright 2008, National Academy of Sciences. Used with permission.)

FIGURE 2.

Asd⁺ secretion vectors pYA4102 and pYA4106 with omission of P_{asdA} but with retention of the SD sequence preceding the *asdA* gene start codon. The -35, -10, and SD sequences associated with P_{trc} are indicated, and the translation start codon is in boldface. An arrow within the sequence indicates the signal peptidase cleavage site. Unique restriction enzyme sites in the multi-cloning site are indicated. 5ST1T2 is a transcriptional terminator. Both plasmids contain the pBR ori. A, *ompA* SS vector pYA4102. The map of pYA4102 and the nucleotide sequences of the P_{trc} promoter region and multi-cloning sites are shown. B, *phoA* SS vector pYA4106. The map of pYA4106 and the nucleotide sequences of the P_{trc} promoter region and multi-cloning sites are shown. (Slight modification of Figure 1 from Xin et al.²⁰ and republished with permission from the American Society for Microbiology. Copyright 2008, American Society for Microbiology.)

Table 1**Mutations and Associated Phenotypes Used to Modify *Salmonella* Vaccine Strains**

Genotype	Phenotype
A. Deletion mutations used to achieve attenuation	
$\Delta aroA$, $\Delta aroC$, and $\Delta aroD$	Impose a nutritional requirement for aromatic amino acids and several essential vitamins such as <i>p</i> -aminobenzoic acid
Δcya and Δcrp	Eliminate synthesis of cyclic-AMP and the catabolite regulatory protein that are necessary for virulence
$\Delta phoP$ and $\Delta phoQ$	A two-component regulatory system essential for virulence
Δfur	Iron uptake regulatory protein that regulates genes needed to maintain adequate but not excessive concentrations of iron
$\Delta rpoS$	Sigma factor that regulates genes needed for stationary phase growth and to respond to many types of stress
B. Deletion and deletion-insertion mutations to confer a regulated delayed attenuation phenotype	
$\Delta galE$	Encodes UDP-galactose epimerase needed to synthesize LPS core and O-antigen and thus necessary for virulence
Δpmi	Encodes phosphomannose isomerase needed for synthesis of GDP-mannose for LPS O-antigen and thus necessary for virulence
ΔP_{crp} : :TT <i>araC</i> P_{BAD} <i>crp</i>	Makes synthesis of Crp dependent on presence of arabinose in growth medium and ceases to be synthesized in vivo due to the absence of arabinose; Crp decreases as a consequence of cell division in vivo to lead to attenuation
ΔP_{phoPQ} : :TT <i>araC</i> P_{BAD} <i>phoPQ</i>	Makes synthesis of PhoP and PhoQ dependent on the presence of arabinose in growth medium and ceases to be synthesized in vivo due to the absence of arabinose; PhoP and PhoQ decrease as a consequence of cell division in vivo to lead to attenuation
ΔP_{fur} : :TT <i>araC</i> P_{BAD} <i>fur</i>	Makes synthesis of Fur dependent on presence of arabinose in growth medium and ceases to be synthesized in vivo due to the absence of arabinose; Fur decreases as a consequence of cell division in vivo to lead to attenuation presumably due to an iron overload
ΔP_{rpoS} : :TT <i>araC</i> P_{BAD} <i>rpoS</i>	Makes synthesis of RpoS dependent on presence of arabinose in growth medium and ceases to be synthesized in vivo due to the absence of arabinose; RpoS decreases very rapidly in vivo to lead to attenuation
C. Deletion and deletion-insertion mutations to facilitate regulated delayed lysis in vivo	
ΔP_{murA} : :TT <i>araC</i> P_{BAD} <i>murA</i>	Makes synthesis of MurA, the first enzyme in the synthesis of muramic acid, dependent on presence of arabinose in growth medium and ceases to be synthesized in vivo due to the absence of arabinose; MurA decreases as a consequence of cell division in vivo to ultimately lead to cell lysis and death
$\Delta asdA$: :TT <i>araC</i> P_{BAD} <i>c2</i>	The Asd enzyme is essential for the synthesis of diaminopimelic acid required for peptidoglycan synthesis; the arabinose-dependent synthesis of the C2 repressor is to enable a regulated delayed expression of DNA sequences under the control of a promoter repressed by C2; the $\Delta asdA$ mutation is complemented by an Asd ⁺ plasmid vector
$\Delta(gmd-fcl)$	Eliminates two enzymes needed to synthesize GDP-fucose that is required for colanic acid synthesis, which can protect cells undergoing cell wall-less death from lysing
D. Promoters and deletion-insertion mutations for regulated delayed in vivo synthesis	
P_{nirB}	A promoter expressed at high level under anaerobic conditions
$\Delta relA198$: : <i>araC</i> P_{BAD} <i>lacI</i> TT	The <i>relA</i> mutation uncouples growth regulation from a dependence of protein synthesis, an important attribute in strains with regulated delayed lysis; the arabinose-dependent synthesis of the LacI repressor is to enable a regulated delayed expression of DNA sequences under the control of P_{trc}
P_{trc}	A promoter expressed at high level under both aerobic and anaerobic conditions and is repressible by the arabinose-dependent synthesis of the LacI repressor
Phage P22 P_L and P_R	These promoters are repressible by the arabinose-dependent synthesis of the C2 repressor
P_{pagC}	A promoter expressed in vivo after activation of PhoP
P_{dmsA}	A promoter expressed at high level under anaerobic conditions
P_{ssaG}	A promoter activated in vivo by the <i>Salmonella</i> Pathogenicity Island-2 <i>ssrB</i> gene
P_{sspA}	A promoter activated during starvation
E. Other contributing mutations	

Genotype	Phenotype
$\Delta araBAD$	Eliminates ability to use arabinose to prevent acid production during growth of strains in media with arabinose and extends delay in shut-off of arabinose-dependent gene expression for about one added cell division
$\Delta araE$	Enhances retention of arabinose after uptake
$\Delta sopB$	Reduces fluid secretion to lessen the mild diarrhea and enhances immunogenicity

Δ , deletion; TT, transcription terminator; P, promoter