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New Technologies in Developing Recombinant Attenuated *Salmonella* Vaccine Vectors

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Abstract

Recombinant attenuated *Salmonella* vaccine (RASV) vectors producing recombinant gene-encoded protective antigens should have special traits. These features ensure that the vaccines survive stresses encountered in the gastrointestinal tract following oral vaccination to colonize lymphoid tissues without causing disease symptoms and to result in induction of long-lasting protective immune responses. We recently described ways to achieve these goals by using regulated delayed in vivo attenuation and regulated delayed in vivo antigen synthesis, enabling RASVs to efficiently colonize effector lymphoid tissues and to serve as factories to synthesize protective antigens that induce higher protective immune responses. We also developed some additional new strategies to increase vaccine safety and efficiency. Modification of lipid A can reduce the inflammatory responses without compromising the vaccine efficiency. Outer membrane vesicles (OMVs) from *Salmonella* containing heterologous protective antigens can be used to increase vaccine efficiency. A dual-plasmid system, possessing Asd⁺ and DadB⁺ selection markers, each specifying a different protective antigen, can be used to develop multivalent live vaccines. These new technologies have been adopted to develop a novel, low-cost RASV synthesizing multiple protective pneumococcal protein antigens that could be safe for newborns/infants and induce protective immunity to diverse *Streptococcus pneumoniae* serotypes after oral immunization.

Keywords

antigen synthesis; biological containment; delayed attenuation or attenuation; delayed recombinant vaccines; *Salmonella*; vaccine; lipid A; O-antigen

Introduction

In 1988, we first reported that a balanced-lethal vector-host system can be used in recombinant attenuated *Salmonella* vaccines (RASVs) [1, 2]. 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 discouraged for live vaccines by regulatory agencies [2–4]. During the last twenty years, fundamental

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discoveries have been made to elucidate the mechanisms by which *Salmonella* attach to, invade and colonize deep effector lymphoid tissues after mucosal delivery and therefore remodel the host cells that they target as well as promote immunomodulatory effects to induce immune responses in locations where *Salmonella* persist [5–9]. The key systems are the Type III secretion systems, including *Salmonella* pathogenicity islands 1 and 2 (SPI-1 and SPI-2). Details about this topic have been reviewed [10–13]. The SPI-1 encoded system ensures that *Salmonella* initiate the invasion by attaching to and invading into various mucosa-associated lymphoid tissues of the gut, nasopharynx, bronchi, etc. and induce mucosal, systemic and cellular immune responses with long-term protective immunity. Different reviews have covered topics related to means to generate the best candidate attenuated strains [14, 15], evaluation of *Salmonella* vaccines [16], application of *Salmonella* for use in cancer treatment [17–19], and especially *Salmonella enterica* serovar Typhi vaccine development [20–22].

In this review, we discuss some improved means of enhancing vaccine efficacy and safety by RASVs. Most of our improvements were achieved through the development of a safe RASV suitable for use in newborns/neonates and infants to induce protective immunity to the diversity of *Streptococcus pneumoniae* strains, which caused an estimated 14.5 million episodes of serious pneumococcal disease in 2000 and 826,000 deaths in children less than five years of age worldwide [23].

I. New strategies for live recombinant attenuated strain construction

In contrast to ways traditionally used to diminish the ability of *Salmonella* to withstand stresses encountered in the gastrointestinal (GI) tract after oral immunization or impair their ability to attach to, invade into and survive in the gut-associated lymphoid tissue (GALT) and deep lymphoid tissue, collectively leading to attenuation as well as diminished immunogenicity, we have developed multiple means to achieve regulated delayed attenuation in vivo [24]. At the time of immunization, the vaccine exhibits almost the same abilities as a fully virulent wild-type strain to contend with stresses encountered during transit through the GI tract and successfully colonizes effector lymphoid tissues before displaying attenuation to preclude onset of any disease symptoms. The mutations related to this strategy are summarized in Table 1 [14, 24]. These new strategies include three main ways: i) the reversible synthesis of lipopolysaccharide (LPS) O-antigen and/or parts of LPS core by mutations in the *pmi* or *galE* genes [25, 26]; ii) by the arabinose-regulated expression of *fur*, *crp*, *rpoS*, and *phoPQ* to result in regulated delayed attenuation in vivo [24], and iii) by regulated delayed lysis in vivo through regulation of the *murA* and *asd* genes as a means of antigen delivery and biological containment [27]. Details about these strategies have been discussed [14, 24]. We recently expanded our regulated delayed attenuation approaches by using arabinose-dependent regulated expression of the *rfaH* or *rfaH* genes to modify O-antigen synthesis [28, 29]. We also modified lipid A structure to reduce its reactogenicity while retaining its immunogenicity [30]. These different approaches can be combined to ensure achieving regulated delayed attenuation.

A. Sugar-dependent synthesis of LPS O-antigen and/or parts of the LPS core to result in the regulated delayed attenuation in vivo phenotype

After oral administration, *Salmonella* will encounter a succession of stresses, such as digestive enzymes, acid, bile, mucin, antimicrobial peptides, secreted IgA, anaerobiosis, osmolarity shifts, ion excess and innate immunity. In order to succeed in colonizing the intestinal tract, the presence of surface antigens, including LPS O-antigen side chains, a diversity of fimbrial adhesins, flagella and possibly certain outer membrane proteins, capsules, etc. are critical to facilitate the bacteria withstanding these stresses. Among these surface molecules, LPS is one of the critical components. It is required for *Salmonella* to

display resistance to killing by macrophages [31, 32] and serum [33, 34] and to multiply in blood. Rough mutants, arising due to loss of LPS O-antigen or parts of the LPS core, are defective in attaching to and invading intestinal cells and surviving in cells on the other side of the intestinal wall barrier [35–38], resulting in poor colonization in intestinal cells. For these reasons, rough mutants of *Salmonella* were not effective as live oral vaccines [39, 40]. To be safe and efficacious, an attenuated immunogenic live vaccine must display not only avirulence without induction of disease symptoms, but also be able to reach, multiply, and persist for a while in lymphoid organs to stimulate protective immune responses. Using a vaccine strain with a reversibly rough phenotype can achieve this goal. Two of the commonly used genes for this purpose are *galE* and *pmi* since supply of galactose and mannose, respectively, during growth of the vaccine strain prior to oral vaccination results in a smooth phenotype with synthesis of LPS O-antigen and core [14, 25, 26, 41]. In vivo, there is an absence of nonphosphorylated mannose and galactose so that synthesis of LPS O-antigen and outer core ceases to result in a rough phenotype and avirulence.

In addition to *galE* and *pmi*, we systematically evaluated additional genes involved in LPS core and O-antigen synthesis [42]. Although direct deletion of these genes is not a good option for live attenuated *Salmonella* vaccine strains due to reduced colonization ability and reduced immune responses against heterologous antigens [42], their expression can be regulated such that LPS core and/or O-antigen is/are synthesized at the time of oral vaccination but cease to be synthesized in vivo after colonization of internal lymphoid tissues. The vaccine strains with an arabinose-regulated promoter controlling LPS core or O-antigen synthesis, produce full-length LPS during immunization when arabinose is supplied during growth of strains in vitro, fight against stresses imposed by the host and then shut off O-antigen synthesis in vivo after the bacteria have colonized the host lymphoid tissues to achieve this goal. Thus, a tightly regulated *araC*P_{BAD} activator-promoter (P = promoter) [27, 43] was used to replace upstream regulatory and promoter sequences for two O-antigen synthesis genes, *rfc* and *rfaH*, so that the expression of these genes is dependent on arabinose provided during in vitro growth [28, 29]. The *Salmonella rfc* gene encodes the O-antigen polymerase. The *rfc* mutants exhibit a characteristic “semi-rough” phenotype, making lipid A and core capped with a single O-antigen subunit [28, 44]. RfaH acts as a transcriptional antiterminator that reduces the polarity of long operons required for the production of extracellular components of *S. Typhimurium*, including O-antigen and lipopolysaccharide core sugars [45]. The *rfaH* mutant strains produce truncated LPS and reduced amounts of O-antigen and core [46], rendering them sensitive to human serum [47] and hypersensitive to bile. Mutations in either of these genes cause significant attenuation in *Salmonella* strains [26, 42, 47–49].

We constructed different alleles of $\Delta P_{rfc}::TT\ araC P_{BAD}\ rfc$ and $\Delta P_{rfaH}::TT\ araC P_{BAD}\ rfaH$ to regulate *rfc* and *rfaH* expression levels by altering SD (Shine-Dalgarno ribosome binding site) and start codon sequences. However, only mutants with the $\Delta P_{rfc174}::TT\ araC P_{BAD}\ rfc$ or $\Delta P_{rfaH178}::TT\ araC P_{BAD}\ rfaH$ mutations synthesized complete O-antigen similar to wild type in the presence of arabinose and completely shut off O-antigen synthesis in the absence of arabinose. Thus, the optimization of *araC*P_{BAD} with different SD sequences and start codons to perfect the levels of expression of genes is necessary to achieve the best attenuating yet immunogenic phenotype. Vaccine strains with mutation $\Delta P_{rfc174}::TT\ araC P_{BAD}\ rfc$ are totally avirulent (doses $>10^9$ cfu can be used to immunize mice) and can induce protection against the wild-type parent strain challenge. The single deletion Δrfc is not as immunogenic as $\Delta P_{rfc174}::TT\ araC P_{BAD}\ rfc$ although a strain with Δrfc is totally attenuated [26, 28]. When $\Delta P_{rfc174}::TT\ araC P_{BAD}\ rfc$ was introduced into a vaccine strain attenuated by the presence of other mutations ($\Delta pabA$ and $\Delta pabB$), the strain displays similar colonization abilities in spleen and liver to the parent attenuated strain but induces higher antibody titers than the parent attenuated strain [28]. A strain with the

mutation $\Delta P_{rfaH178::TT\ araCP_{BAD}\ rfaH}$ is only partially attenuated (10^6 cfu). This is different from a strain with the single deletion $\Delta rfaH$, which is highly attenuated (10^8 cfu) [29, 47, 48]. When $\Delta P_{rfaH178::TT\ araCP_{BAD}\ rfaH}$ was introduced into same vaccine strain (as above) attenuated by presence of other mutations, the new strain also displays similar colonization abilities in spleen and liver to the parent attenuated strain but induces higher antibody titers than the parental strain as well as increased protection against *S. pneumoniae* challenge [29]. Moreover, use of the arabinose-regulated *rfaH* in vaccine strains can induce higher antibody responses against outer membrane proteins from other enteric bacteria. Thus, arabinose-regulated *rfaH* can be used to develop a vaccine against other enteric bacteria that share the same or similar core epitopes, such as other *Salmonella* serovars, *Shigella* spp. and pathogenic *Escherichia coli*. Arabinose-regulated *rfaH* may also help to reduce the immune responses against the *Salmonella* carrier while enhancing immune responses against heterologous antigens and outer membrane proteins cross-reactive with other enteric bacteria.

The results with arabinose-regulated expression of *rfaH* and/or *rfaH* provided evidence that the regulated delayed O-antigen synthesis by arabinose is an effective attenuation strategy. These mutations can also be used in combination with other mutations related to O-antigen synthesis, such as Δpmi and/or $\Delta gale$, as well as other regulated delayed in vivo attenuation strategies, to develop live vaccines to achieve an ideal balance between attenuation and immunogenicity. Combinations of different mutations, Δpmi , $\Delta gale$, $\Delta P_{rfc::TT\ araCP_{BAD}\ rfc}$ and/or $\Delta P_{rfaH::TT\ araCP_{BAD}\ rfaH}$, are currently under investigation in our lab.

B. Detoxification of lipid A to make safer and immunogenic vaccines

The delicate balance between attenuation and immunogenicity is critical for the success of a live *Salmonella* vaccine. There are many virulence factors causing reactogenicity to live oral *Salmonella* vaccines. How to minimize this reactogenicity but still retain the vaccines' immunogenicity is still a challenge [50]. Previous work has found that a *S. Typhi* strain with $\Delta aroA\ \Delta aroD$ is highly immunogenic but is insufficiently attenuated in humans [51], as was true for *S. Typhi* strains with $\Delta cya\ \Delta crp$ [52, 53] or with $\Delta gale$ [54] mutations. These results were unexpected since these mutations fully attenuated *S. Typhimurium* for mice [25, 55–58]. When combined with $\Delta htrA$, the $\Delta aroA\ \Delta aroD$ strain became safe but was less immunogenic. Similar problems were also observed when $\Delta aroA$ was combined with $\Delta purA$ or $\Delta phoPQ$ mutations [59, 60]. Considering more safety features and the increasing importance of biological containment, we are also commencing to add the regulated delayed lysis in vivo system to our RASV constructions [27, 61, 62]. However, the lysis of the strain will lead to reactogenicity. One of the key mediators for this reactogenicity is the lipid A portion of LPS, which is also known as endotoxin [63].

LPS, the major surface membrane component present in almost all Gram-negative bacteria, consists of lipid A, a core oligosaccharide, and a highly variable and immunogenic O-antigen polysaccharide. Lipid A is mainly responsible for the toxicity of LPS [64, 65]. It is detected by the Toll-like receptor 4 (TLR4)/ myeloid differentiation factor 2 (MD-2) receptor complex of the mammalian innate immune system. Lipid A in *Salmonella*, containing two phosphate groups and six acyl chains, is most efficient in activating pro-inflammatory responses through the TLR4-MD2-CD14 pathway [66–72], which is divided into a myeloid differentiation factor (MyD) 88-dependent pathway contributing to reactogenicity and a TIR-domain-containing adapter-inducing interferon- β (TRIF)-dependent pathway contributing to immunogenicity. Different enzymes can modify lipid A by altering the number or length of the acyl chains or reducing the number of phosphate groups (Fig. 1). Native *Salmonella* lipid A triggers an inflammatory response, whereas modified lipid A induces structural rearrangement of the TLR4/MD-2 multimer resulting in induction of diverse innate immune responses [73–75]. Lipid A lacking the optimal

configuration for inducing inflammation can be used as an adjuvant to enhance acquired immune responses. Modified lipid A with adjuvant activity will induce less inflammatory cytokines dependent on the MyD88 molecule, and up-regulate or maintain co-stimulatory molecules dependent on TRIF factors. It has been demonstrated that commercially used 3-*O*-deacylated-4'-monophosphoryl lipid A (3D-MPL) [76], which was extracted from *S. Minnesota* strain R595 by a chemical method, exhibited the characteristic of a good adjuvant with low endotoxic activity and enhanced immunostimulatory activity.

Our final goal is to develop a safe and effective live attenuated bacterial vaccine. Modification of lipid A will enhance safety of live vaccines for newborn, elderly, malnourished, pregnant and immunocompromised individuals [27]. The strain exhibiting regulated delayed in vivo lysis provides a new technology beneficial to these targeted groups noted above. However, lysis of live *Salmonella* vaccines will release a lot of lipid A [77, 78], leading to toxic shock, sepsis, and even death [79, 80]. Fortunately, lipid A can be modified to reduce its endotoxic activity (Fig. 1). Rendering lipid A with low or nontoxic activity may provide a means to safely increase the dose and/or reduce the level of attenuation to facilitate induction of a robust response to the vectored antigen and use with the regulated delayed lysis strategy for antigen or DNA vaccine delivery, as well as conferring biological containment. Removal of the 1-phosphate group of lipid A is sufficient to reduce its toxic effects [70, 81]. An inner membrane phosphatase LpxE from *Francisella tularensis* can selectively remove the 1-phosphate group of lipid A in living cells of *E. coli* and *Salmonella* [82] and generate a close analog of monophosphoryl lipid A (MPL) that remains covalently linked to LPS. We thus engineered a less reactogenic, but still very immunogenic strain, by insertion of a P_{lpp} *lpxE* cassette into the chromosome to remove the 1-phosphate group from *Salmonella* lipid A [30] (Fig. 1). Considering the potential stability issues and the protective antigen genes typically expressed from multi-copy plasmids, codon-optimized *lpxE* for high-level expression in *Salmonella*, transcription controlled by a strong *E. coli* promoter P_{lpp} and expressed from a unique chromosome location can provide a sufficient level of LpxE for nearly complete 1-dephosphorylation of the lipid A in *Salmonella*. The cassette P_{lpp} *lpxE* was inserted into different sites in the chromosome, in place of *pagL*, *pagP*, or *lpxR*, each of which were deleted. Only the $\Delta pagP81::P_{lpp}$ *lpxE* insertion effectively produced complete 1-dephosphorylated lipid A [30]. When combined with $\Delta pagL7 \Delta lpxR9$, the $\Delta pagP81::P_{lpp}$ *lpxE* *S. Typhimurium* strain can synthesize mainly a modified 4'-monophosphoryl-hexa-acylated lipid A species under the range of conditions experienced in the host. This was an important consideration for this modification to perform as we expected in vivo. The *lpxE* expression from this chromosomal location (*pagP*) also attenuated *Salmonella* about five orders of magnitude without compromising the wild type-like ability of the strain to transiently colonize lymphoid tissues nor the strain's immunogenicity. Therefore, the optimal *lpxE* construction in an attenuated *Salmonella* strain to produce dephosphorylated lipid A provides an additional safety feature. Evaluation of this triple mutation strain demonstrated that it induced a decreased inflammatory reaction, so that fewer inflammatory cytokines were produced and less tissue damage occurred. Further evaluation in a vaccine strain background showed that the strain with the triple mutation generated similar levels of antibody titers against heterologous pneumococcal antigen PspA compared to the parent strain and similar levels of protection against *S. pneumoniae* challenge. Thus, the chromosomal *lpxE* expression in a vaccine strain carrying additional attenuating mutations does not result in loss of efficacy when used to deliver a heterologous antigen. Detoxified lipid A provided the vaccine strain more safety but the strain still retained immunogenicity for induction of a robust response to the vectored antigen. This innovation enables us to reduce the reactogenicity of live oral *Salmonella* vaccines, especially those that undergo regulated delayed lysis in vivo.

The alteration of lipid A by other mutations was further investigated [83, 84]. The *msbB* gene encodes an enzyme for adding myristic acid to the lipid A precursor [85]. The $\Delta aroA$ vaccine strain with a $\Delta msbB$ mutation is equally effective as the parent strain at conferring protection against wild-type *Salmonella* challenge and enhances immune responses towards heterologous antigens [86]. Incorporation of an *msbB* mutation into a tumor-targeting *Salmonella* strain resulted in reduced production of TNF- α , which mediates septic shock [87, 88], and reduced virulence [89, 90] without compromising the tumor-targeting ability of the parental strain [89]. The $\Delta msbB$ mutation, when combined with $\Delta lppB$ or $\Delta purI$, can suppress tumor growth in mice [91, 92]. However, a phase I clinical trial showed that the strain with $\Delta msbB \Delta purI$ still induced a dose-related increase in serum proinflammatory cytokines [90]. We found that the palmitoylation state of lipid A in the *msbB* mutant had dramatic impacts on the inflammatory responses following oral administration [83, 84, 86]. The $\Delta pagP \Delta msbB$ mutant, which lacks palmitate and myristate chains on lipid A, can induce lower levels of pro-inflammatory cytokines, while retaining the ability to elicit humoral and mucosal immune responses, which endow this particular combination of mutations additional advantages when designing anticancer vaccines [83]. Thus, this combination of mutations should also be beneficial when developing a vaccine against mucosal infecting pathogens. Lipid A modification caused by the *msbB* mutation alone and in combination with *pagL*, *pagP*, and *lpxR* mutations led to higher IgA production in the vaginal tract but still retained the same IgG titer level in serum to pneumococcal PspA antigen and to outer membrane proteins from *Salmonella* [83]. Thus, modification of lipid A will provide another means for increasing vaccine safety without compromising its efficacy. The strategy can be used with other attenuation strategies, such as mutations for modifying O-antigen and regulated delayed in vivo attenuation. The combining of lipid A modification mutations with the regulated delayed in vivo lysis strategy is under investigation in our lab.

C. Combined use of multiple means to develop safe and effective vaccine

Attenuated live bacterial vaccines with two or more means to confer attenuation will ensure safety. *S. Typhi* strain Ty21a, the only licensed oral live bacteria vaccine against typhoid fever, has multiple mutations include *galE*, *rpoS*, *ilvD* and *tvi* [41, 93, 94]. Other candidate live attenuated *S. Typhi* vaccines tested in clinical trials also included at least two mutations [20–22, 95, 96]. Our current vaccine strains have combined different regulated delayed attenuation mutations, $\Delta P_{fur81}::TT \text{ araC } P_{BAD} \text{ fur}$ and $\Delta P_{crp527}::TT \text{ araC } P_{BAD} \text{ crp}$ deletion-insertion mutations and the $\Delta pmi-2426$ deletion mutation [97, 98], since each of these three mutations contributes significantly to high immunogenicity [24, 97, 99]. They also have a constellation of mutations that aid in enhancing strain tolerability [100] and efficacy, including the $\Delta sopB$ mutation [101], and in regulating protective antigen synthesis by the $\Delta relA::araC P_{BAD} \text{ lacI } TT$ deletion-insertion mutation [98, 102–104]. This vaccine genotype has proven to be safe in a phase I clinical trial with live *S. Typhi* strains (unpublished results). Although our current strains are safe [98, 100], further optimization of the strain will probably be needed. As we mentioned above, optimization of the vaccine with different alleles of regulated delayed attenuation mutations, similar to what we found for the mutations $\Delta P_{rfc}::TT \text{ araC } P_{BAD} \text{ rfc}$ and $\Delta P_{rfaH}::TT \text{ araC } P_{BAD} \text{ rfaH}$, and optimization of the chromosome site for insertion, as in the case for the $P_{lpp} \text{ lpxE}$, are critical to further increase vaccine efficacy and reduce reactogenicity. We can expect that careful comparison of combinations of different means of attenuation will result in an optimal safe and effective vaccine.

II. Recombinant antigen synthesis and delivery

A. Plasmid vector properties for plasmid maintenance

Although plasmids based on auxotrophic selection markers such as *purA* (purine biosynthesis), *purB* (adenylosuccinate lyase) [105], *thyA* (thymidylate synthase gene), *glnA* (glutamine synthetase gene) [105–107], *trpS* (tryptophanyl-tRNA synthetase gene) [108], *infA* (translation initiation factor 1) [109], and *supD* (amber suppressor gene) were tested successfully, they either suffered from limited areas of application (i.e., only used in food or industrial applications) or encountered certain technical impediments or side effects [110, 111], for example, the presence of *supD* on plasmids suppresses expression of approximately 10% of *Lactococcus lactis* genes [112]. Moreover, poor plasmid stability or the incompatibility of the selection markers has limited wide-ranging applications.

Diaminopimelic acid (DAP) is a unique essential component of peptidoglycan of *Salmonella* and cell lysis will occur in its absence. We developed a balanced-lethal vector-host system using a vaccine strain with deletion of the *asdA* gene on the chromosome to impose an obligate requirement for DAP and a plasmid vector with the wild-type *asdA* gene [1, 2, 113] to establish a complementation heterozygote. Absence of DAP in mammalian tissues forces *Salmonella* vaccine strains carrying a Δasd mutation to stably maintain Asd^+ plasmids to prevent death by lysis due to defective peptidoglycan structure. This system confers a greater safety advantage by eliminating drug-resistance gene markers in live vaccines and provides enough selective pressure for the maintenance of the plasmid vector in all live vaccine cells in vivo. The technology and its optimization have been recently reviewed [14, 114]. Current data demonstrated that the *asd*-based system was more robust in all these balanced-lethal systems because it led to rapid “Dapless” death of the bacterium [2]. However, the bacteria with auxotrophic selection markers may only cease to grow, but not die, resulting in accumulation of bacteria without antigen expression, thus increasing the needed dose of vaccine to be effective [105].

We have now developed a second balanced-lethal system using the *dadB* gene as a selective marker (Fig. 2) [115]. In *S. Typhimurium*, two separate genes (*alr* and *dadB*) encode alanine racemases, which are key enzymes that catalyze the interconversion of D-alanine (D-Ala) and L-alanine [116, 117]. D-alanine is an essential building block of the peptidoglycan layer of cell walls in Gram-negative bacteria [118, 119]. We took advantage of the obligate requirement of the enzyme alanine racemase to develop a new *S. Typhimurium* balanced-lethal alanine racemase vector system. Due to the redundant functions of *Alr* and *DadB*, deletion of both genes is required to generate a D-alanine auxotroph. As we expected, a strain with the $\Delta alr \Delta dadB$ mutations together has an obligate requirement for exogenous D-alanine for survival or requires complementation with a wild-type copy of either gene in the form of an Alr^+ or a $DadB^+$ plasmid. The latter component forms the vector part of the *alr dadB* balanced-lethal host-vector system. Initially we used *alr* as the selective marker because it is constitutively expressed for peptidoglycan biosynthesis and used to overproduce the vitamin D-pantothenic acid [120]. However, we found that *DadB*, induced when cells are grown with high concentrations of L- or D-alanine, is a better choice than Alr^+ and thus developed a $DadB^+$ balanced-lethal vector. The $DadB^+$ plasmid can complement the $\Delta alr \Delta dadB$ mutations of *E. coli* and *S. Typhimurium*. The vaccine strain containing these mutations does not survive in medium devoid of D-alanine without the $DadB^+$ plasmid; this ensures both stable maintenance of the plasmid and biocontainment of the vaccine strain. The $DadB^+$ vector can be used either alone (in a $\Delta alr \Delta dadB$ mutant) or in conjunction with an Asd^+ plasmid (in a $\Delta asdA \Delta alr \Delta dadB$ triple-mutant strain) in *S. Typhimurium* to deliver multiple antigens (see below) [115].

Our initial constructions showed that there was an inverse correlation between the amount of DadB produced for complementation of the $\Delta alr \Delta dadB$ mutant when using DadB⁺ vectors with different copy numbers and this attenuated the virulence of vaccine strains [115]. It was reported that low-copy-number plasmids are relatively more stable and induce greater levels of antigen-specific immune responses than their high-copy-number versions [121].

However, we had already reported that the problem of instability of high-copy number plasmids can easily be addressed by decreasing the amount of Asd produced by high copy number plasmid vectors [122]. We therefore deleted the SD sequence for the *asd* gene on a pBR *ori* vectors [122] and in addition changed the start codon from ATG to GTG on pUC *ori* vectors [27] or changed the sequence in the SD or altered the distance between the SD sequence and the start codon. After all these modifications, *S. Typhimurium* strains with any Asd⁺ plasmid in a Δasd mutant had the same LD₅₀ as did the wild-type *S. Typhimurium* parent strain. In this regard, it should be noted that overexpression of a selective marker gene, such as *asd*, or a gene encoding a protective antigen act to attenuate *S. Typhimurium* and also reduce immunogenicity in the vaccine strain by decreasing colonization efficacies. Based on the above considerations, we therefore modified the upstream region of the native *dadB* gene by increasing the space between the ribosome binding site (SD sequence) and the start codon (by placing additional nucleotides in the sequence) and changing the start codon from ATG to GTG, to reduce the level of DadB synthesis. Finally, complementation of the $\Delta alr \Delta dadB$ mutant with the DadB⁺ vector restores the virulence of the mutant to the wild-type levels (partially in the case of pBR *ori* and pUC *ori* derivatives). The DadB⁺ vector with pBR *ori* can be stably maintained under nonselective conditions for ~50 generations in a vaccine strain with a complex genotype. For further optimizing antigen synthesis, we adopted the promoter P_{lpp} with the pBR *ori* followed by a strong 5ST1T2 transcription terminator that prevents read-through transcription, which may lead to a reduced level of antigen synthesis. We also constructed a series of plasmids with various copy numbers to provide flexibility in designing the expression vector based on the nature of the heterologous antigen.

For stable maintenance of plasmid vectors in vaccine host strains, an alternate strategy of a post-segregational killing system based on the noncatalytic *hok-sok* plasmid addiction system was developed for incorporation into plasmid vectors [123, 124]. In these cases, all viable recombinant vaccine cells with the plasmid vector encoding the protective antigen(s) can be maintained in the immunized individual. It is possible to use other post-segregational killing systems, complementation of auxotrophic mutations, toxin-antitoxin systems, operator-repressor titration, RNA markers, or the overexpression of a growth essential gene to develop new plasmid maintenance systems [125–128].

B. Dual-plasmid system to deliver multiple protective antigens by a recombinant attenuated *Salmonella* vaccine

Salmonella vaccine delivery of multiple antigens is preferred to confer complete protection against a pathogen. We tested a fusion construction of pneumococcal surface protein PspA representing two PspA families (Rx1 and EF5668) constituting an effective anti-pneumococcal vaccine, extending and enhancing protection against multiple strains of *S. pneumoniae* [129]. However, it may not be convenient to create fusion constructs of multiple protective antigens whenever a multivalent vaccine is desired. In some cases, a multivalent *Salmonella* vaccine that can deliver multiple protective antigens by expressing two or more heterologous antigen genes on separate vectors may be necessary to confer maximal protection against a target pathogen. The major challenge to achieve this is whether or not a recombinant vaccine strain is capable of stably maintaining two or more expression vectors simultaneously, each carrying a unique selectable marker. To facilitate cloning of multiple antigen genes in a single bacterium, we explored the use of DadB⁺ vectors

complementing $\Delta alr \Delta dadB$ mutations, eliminating two alanine racemases. The DadB⁺ vector, together with an Asd⁺ plasmid, can thus be used to deliver multiple antigens in a vaccine strain with $\Delta alr \Delta dadB$ and Δasd mutations.

The DadB⁺ plasmids described above are compatible with Asd⁺ vectors, a critical feature to develop a dual-plasmid delivery system. Thus in our dual-plasmid system (Fig. 2), the first component of this delivery system is a vaccine strain of *S. Typhimurium* carrying genomic deletions in *alr*, *dadB*, and *asd*, resulting in obligate requirements for D-alanine and DAP for growth. The second component is an Asd⁺ and DadB⁺ plasmid pair, each carrying the wild-type *asdA* and *dadB* gene, respectively, to complement the mutations. The presence of two plasmids in a single vaccine strain did not decrease synthesis of individual antigens. Although the Asd⁺ plasmid was more stable than the DadB⁺ plasmid, the stability of both plasmids was ~95% over 50 generations of growth and did not differ significantly, suggesting that antigen synthesis and delivery *in vivo* were not compromised in this system [115].

The commonly used promoter sequences, transcriptional terminator sequences, and other plasmid backbone elements on expression plasmids are good target sequences for homologous recombination. This is a problem that the dual-plasmid system encounters. In *S. Typhimurium*, RecF mediates intra- and interplasmid recombination [130–132]. Deletion of *recF* reduces recombination frequency between plasmids at least 10 times without compromising the virulence of the bacterium [132]. Furthermore, including a *recF* deletion mutation improved the stability of two balanced-lethal vectors, especially of the DadB⁺ vector in a *Salmonella* vaccine strain with Asd⁺ and DadB⁺ vectors each specifying a different protective antigen [115]. The *Salmonella* vaccine strain carrying PspA and PspC by Asd⁺ and DadB⁺ vectors, respectively, induced higher serum and secretory antibody responses than the strain delivering a single antigen or than a mixture of two vaccine strains each specifying one protective antigen and offered superior protection against challenge with different serotypes of *S. pneumoniae* [115]. The DadB⁺-Asd⁺ dual-plasmid system represents an important tool to develop multivalent live recombinant vaccines [115].

C. Means for enhanced protective antigen synthesis

High levels of protective antigen synthesis, which are important to the induction of higher mucosal and systemic antibody responses, are normally achieved by expression of the gene on a plasmid, codon optimization of the gene sequence and adopting a strong promoter. However, high levels of antigen synthesis decrease vaccine fitness and result in increased attenuation and decreased immunogenicity. The problem can be addressed by different approaches, such as exporting the antigen to an extracellular compartment [133], using *in vivo* inducible promoters, including those for the *pagC* [134], *nirB* [135], *spv* and *dps* genes [136], and regulated delayed antigen synthesis [102, 104]. Another option is to use the MazF–MazE toxin-antitoxin system. In this system, the toxin, MazF, is stabilized by the antitoxin, MazE. MazE is labile to be degraded by an ATP-dependent serine protease, either ClpA–ClpP [137] or Lon [138]. MazF is an ACA-specific endoribonuclease and can be induced to cleave almost all cellular mRNAs, inhibiting cell growth. The ribosomal RNAs and tRNAs are protected from MazF by ribosomal proteins and secondary structures [139]. MazF-induced cells are still fully active in various metabolic pathways and retain the capacity to produce RNAs and proteins. Thus in a MazF-induced cell, if the gene of a protein does not contain any ACA sequence, it will be translated efficiently and only the protein from this mRNA will be produced at a high yield even without the production of any other cellular proteins [140]. This idea has been used to develop a single protein production system in *E. coli* [140–142]. When used in live *Salmonella* vaccines, the transcription of *mazE* can be controlled by an arabinose regulated promoter, the MazE will be synthesized with arabinose supplied *in vitro*. *In vivo*, there is no arabinose available, the transcription of

mazE stops, the MazF will be gradually released and inhibit cell growth in a vaccine strain to reduce its persistence. The protective antigen encoding gene in this live vaccine should be modified by eliminating all ACA sequences without amino acids alterations. In the MazF-induction condition, only the modified antigen gene can be expressed to synthesize a large amount of protective antigen without synthesis of *Salmonella* immunodominant self antigens. However, the function of MazF should be tested in *Salmonella* since *Salmonella* does not have this toxin-antitoxin system. MazF can cut mammalian cellular mRNA and induced apoptosis in mammalian cells [143]. This should be considered to avoid unwanted side effects when using this system in humans.

D. Using OMV delivery of heterologous antigens to increase immunogenicity

We now know that export of antigens to the periplasm of vaccine strains yielded superior antibody titers [122] compared to when the protective antigen was retained in the cytoplasm of the vaccine strain [144]. Different secretion systems, such as the *E. coli* hemolysin secretion system (Type I secretion system) [145, 146], the *clyA* secretion system [147], Type V secretion systems [148, 149], Type III secretion systems to stimulate CD8 T-cell responses [150–152], and the regulated delayed lysis strategy that confers biological containment while being able to deliver a bolus of protective antigen at the time of in vivo vaccine strain cell lysis [27, 61, 62] have been investigated. We extensively compared the delivery of protective antigens by different Type II secretion systems (T2SS) [115, 122], including the *phoA*, *ompA*, *lpp*, *eltB* (Type II) and *dsbA* signal sequences with equally good results [153, 154].

The high immunogenicity of protective antigens secreted by the T2SS may be due to the formation of outer membrane vesicles (OMVs) that incorporate antigens secreted into the periplasmic space. The production of such vesicles leads to targeting of antigens to antigen-presenting dendritic cells [155]. Research with OMVs from different bacteria, including *Neisseria meningitidis* [156–158], *Porphyromonas gingivalis* [159], *Haemophilus influenzae* [160], *Brucella melitensis* [161], *Vibrio cholerae* [162, 163], *Shigella flexneri* [164], *Burkholderia pseudomallei* [165], *Edwardsiella tarda* [166] and *Francisella novicida* [167] have proven OMVs as new vaccine candidates [168]. The results of a phase I clinical trial of OMVs from *N. lactamica* proved it is safe and demonstrated it induced a weak but broad humoral immune response to *N. meningitidis* [157]. Unlike other investigations, our recent results established that a protective immune response could be induced by mucosal delivery of *Salmonella* OMVs with a heterologous antigen from *S. pneumoniae* [169]. Vesicles isolated from a live recombinant attenuated *Salmonella* strain that contained PspA engineered to be secreted into the periplasmic space were capable of inducing serum antibody responses against PspA, *Salmonella*-derived lipopolysaccharide and outer membrane proteins in mice after intranasal immunization with isolated OMVs. Although the response to PspA was low, mice immunized with the vesicle preparation were completely protected against a 10× median lethal dose (LD₅₀) challenge of *S. pneumoniae* and significantly protected against a 200× LD₅₀ challenge [169]. We also noticed that there were no detectable responses developed in mice immunized with an equivalent dose of purified PspA. This result demonstrated that OMVs have adjuvant characters [170–172], which is a benefit of using *Salmonella*. Thus, any mutation that can increase OMV release may help to increase immune responses induced by a live vaccine vector [171]. To further optimize this system, generation of low endotoxic OMVs by introducing *msbB* mutations or other lipid A modifications as mentioned above will contribute to developing a better vaccine [173–175]. Currently, OMVs used for immunization research are obtained by multiple steps of filtration, centrifugation and/or column purification. Using live vaccines with controlled release technologies to deliver OMVs will help to reduce such expenses.

III. Other important RASV attributes

Salmonella has evolved many mechanisms to contend with adverse environments imposed by the host and to suppress or modulate induction of immune responses mainly by SPI-2 effectors [176]. Although many mechanisms still need to be elucidated, much progress has been made to facilitate attenuated vaccine development. RASV strains need to be modified to inactivate these *Salmonella* defense strategies without compromising its ability to induce immune responses. Inactivation of SPI-2 effector SopB results in improved humoral and cellular immunity [177] and especially mucosal immunity [101] as well as reducing fluid secretion [178] to lessen the mild diarrhea experienced by a small percentage of individuals receiving live *Salmonella* vaccines [51, 179]. In *Salmonella*, the gene *sifA* is one of the major factors in maintaining the *Salmonella*-containing vacuoles (SCVs) once bacteria are inside host cells. SifA mimics GTPase [180], one of the master regulators of multiple cell functions. The *sifA* mutants are unable to maintain the integrity of the SCVs and as a consequence are found in the host cell cytosol [181, 182]. Thus the ability of this mutant to access the cytosol may enhance its ability to deliver antigens. The *sifA* mutant can efficiently deliver a eukaryotic promoter-driven green fluorescent protein reporter gene for expression in dendritic cells, but did not induce enhanced levels of cytotoxic T cell or interferon-gamma production [183]. This mutation has been used to increase the delivery ability for DNA vaccines [184]. Our recent results show that a *sifA* mutation in a regulated delayed lysis system can significantly increase the protection level against influenza challenge [62].

While striving to enhance the immunogenicity of RASV strains, it is equally important to enhance safety for animals as well as humans. With regard to humans, the increased number of individuals with immunosuppression due to infection, genetic factors and increasing age is a concern in using live attenuated bacterial vaccines. Preferably these live vaccines should be safe for such individuals as well as for infants, pregnant women and malnourished individuals. Due to lack of an animal model, progress to develop safe *S. Typhi* vaccines for humans has not progressed rapidly. Considering the differences between the human and mouse mucosal lymphoid system [185, 186], several humanized immune system mouse models were developed [187–190]. These mice displayed classical manifestations of human typhoid fever including meningitis, liver pathology and mortality after intravenous challenge with *S. Typhi* [188–190]. However, infection of these humanized mouse models by *S. Typhi* still used intravenous or intraperitoneal routes, which are different from the natural oral route of *S. Typhi* infection. These models also have problems with variation and challenge techniques. These disadvantages increase its cost, an important factor for vaccine development, and thus have limited use of these humanized mice. We believe further research and improvement of these humanized mouse models will aid in clarifying questions on *S. Typhi* virulence, pathogenesis and immune responses [187]. Thus, the development of *S. Typhi* animal models will further accelerate the live vaccine development and clinical evaluation in humans.

Conclusion

Much progress has been made in improving the technologies to design, construct and evaluate RASVs for oral immunizations. These improvements enhanced safety, tolerability and effectiveness in inducing protective immunity. These technologies are now being used to develop vaccines to protect against pathogens of agriculturally important animals as well as *S. Typhi* vaccines with genetic modification to protect against a number of important human pathogens. In this regard, we have succeeded in constructing *S. Typhimurium*-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 [97]. Evaluation of *S. Typhi* strains with similar properties in human volunteers is ongoing and will reveal the value of these technologies in development of cost-effective safe vaccines for humans. With emergence of knowledge about *Salmonella* interacting with hosts and the by using new technologies, we finally should be able to achieve our goal to develop needle-free vaccines with safety, efficiency and low production costs.

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Figure 1.

Lipid A structure and modification in *Salmonella* [30, 65, 83, 84]. *Salmonella* lipid A is a β -1', 6-linked disaccharide of glucosamine, with phosphates at the 1 and 4' positions and acylated at the 2, 3, 2', and 3' positions with *R*-3-hydroxymyristic acid (3-OH C₁₄:0) by LpxA or LpxD, respectively. The structure is further acylated with secondary laurate (C₁₂:0) and myristate (C₁₄:0) chains in acyloxyacyl linkage at the 2' and 3' positions by HtrB (LpxL) and MsbB (WaaN, LpxM), respectively. Lipid A of *Salmonella* can be modified by other genes including *pagL*, *pagP*, *lpxR*, *arnT*, *eptA* and *lpxT*. PagL and LpxR catalyze 3-O-deacylation and removal of the 3'-acyloxyacyl moiety from the *Salmonella* lipid A, respectively. The addition of palmitate (C₁₆) to position 2 of the *R*-3-hydroxymyristoyl chain is catalyzed by PagP (pink). The L-Ara4N or pEtN decoration at the 1- and 4' phosphate is catalyzed by ArnT (green) or EptA (PmrC) (brown), respectively. Other covalent lipid A modifications include the hydroxylation of the 3' secondary myristoyl chain by LpxO (+O). The addition of phosphate group at the 1' phosphate is catalyzed by LpxT (YeiU) (red) [191]. Heterologous expression of *lpxE* (blue) derived from *Francisella* leads to nearly complete 1-dephosphorylation of all *Salmonella* lipid A species, resulting in monophosphorated lipid A, which will reduce the toxicity but still retain ability to activate

TLR4 via TRIF but not MyD88. LpxF (Cyan) derived from *Francisella* can remove the 4'-phosphate group only from penta-acylated lipid A (*msbB* mutant) or tetra-acylated lipid A (*msbB htrB* mutant).

Figure 2.

Diagram of dual-plasmid system for antigen synthesis [115, 122]. (A) Asd⁺ vector system with pBR *ori*. The system has a chromosome deletion of the *asd* gene complemented with a plasmid encoded wild-type *asd* gene. (B) DadB⁺ vector system with pBR *ori*. The system has two chromosome deletions of *alr* and *dadB* genes complemented with a plasmid encoded wild-type *dadB* gene. 5ST1T2 is a transcriptional terminator. Slash line, gene deleted.

Table 1Mutations and associated phenotypes used to modify *Salmonella* vaccine strains^a

Genotype	Phenotype
A. Deletion mutations used to achieve attenuation	
$\Delta aroA$, $\Delta aroC$ and $\Delta aroD$	imposes 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.
$\Delta ssaV$	component of the SPI-2 secretion apparatus for SPI-2 effector secretion. Mutation results in reduced replication and survival in host and reduced spreading
$\Delta htrA$	encodes a heat-shock protein; mutation results in susceptibility to oxidative stress and impaired ability to survive and replicate in host tissues
B. Deletion and deletion-insertion mutations to confer a regulated delayed attenuation phenotype	
$\Delta galE$	encodes UDP-galactose epimerase needed to synthesize LPS outer 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.
$\Delta P_{crp::TT} araCP_{BAD} crp$	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 and since it is needed for high-level transcription of P_{BAD} , acts as a double shut-off along with absence of arabinose for all $araCP_{BAD}$ regulated genes.
$\Delta P_{phoPQ::TT} araCP_{BAD} phoPQ$	makes synthesis of PhoP and PhoQ dependent on presence of arabinose in growth medium and cease 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.
$\Delta P_{fur::TT} araCP_{BAD} fur$	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.
$\Delta P_{rpoS::TT} araCP_{BAD} rpoS$	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.
$\Delta P_{rfaH::TT} araCP_{BAD} rfaH$	makes synthesis of RfaH dependent on presence of arabinose in growth medium and ceases to be synthesized in vivo due to the absence of arabinose. RfaH decreases very rapidly in vivo, resulting in incomplete O-antigen synthesis and attenuation.
$\Delta P_{rfc::TT} araCP_{BAD} rfc$	makes synthesis of Rfc dependent on presence of arabinose in growth medium and ceases to be synthesized in vivo due to the absence of arabinose. Rfc decreases very rapidly in vivo resulting in semi-rough O-antigen synthesis and attenuation.
C. Deletion mutations to facilitate balanced-lethal vector host systems	
$\Delta asdA$	essential for the synthesis of diaminopimelic acid required for peptidoglycan synthesis. The $\Delta asdA$ mutation is complemented by an Asd^+ plasmid vector.
$\Delta alr \Delta dadB$	alanine racemases to produce D-alanine required for peptidoglycan synthesis. The $\Delta alr \Delta dadB$ mutations are complemented by a $DadB^+$ plasmid vector.
$\Delta purA \Delta purB \Delta thyA$	imposes a nutritional requirement for purine or thymidine. The $\Delta purA$, $\Delta purB$ or $\Delta thyA$ mutation can be complemented by a $PurA^+$, $PurB^+$ or $ThyA^+$ plasmid vector.
$\Delta glnA$	imposes a nutritional requirement for glutamine. The $\Delta glnA$ mutation is complemented by a $GlnA^+$ plasmid vector.
D. Deletion and deletion-insertion mutations to facilitate regulated delayed lysis in vivo	
$\Delta P_{murA::TT} araCP_{BAD} murA$	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. The mutation is complemented by a $MurA^+ AsdA^+$ lysis vector.

Genotype	Phenotype
$\Delta asdA::TT \ araCP_{BAD} c2$	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 or aMurA ⁺ AsdA ⁺ lysis 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.
$\Delta(wza-wcaM)$	eliminates operon for colanic acid synthesis, which can protect cells undergoing cell wall-less death from lysing.
E. Deletion and deletion-insertion mutations to modify lipid A in vivo	
$\Delta pagP \Delta pagL \Delta lpxR$	PagP catalyzes the addition of palmitate (+C ₁₆) to the 2 position of R-3-hydroxymyristoyl chain of lipid A. LpxR and PagL catalyzes the removal of the 3'-acyloxyacyl and the 3-hydroxymyristoyl chains from lipid A, respectively. This mutant will produce hexa-acylated lipid A in vivo and in vitro.
$\Delta pagP::P_{lpp} lpxE$	LpxE from <i>Francisella</i> modifies <i>Salmonella</i> lipid A to form 1-dephosphorylated lipid A with low toxicity.
$\Delta msbB (\Delta waaN, \Delta lpxM)$	MsbB adds myristate (C ₁₄ :0) chains in acyloxyacyl linkage at 3' positions. If combined with $\Delta pagP \Delta pagL \Delta lpxR$, it makes a penta-acylated lipid A.
F. Promoters and deletion-insertion mutations for regulated delayed in vivo synthesis of antigen	
P _{nirB}	a promoter expressed at high level under anaerobic conditions.
$\Delta relA::araCP_{BAD} lacI TT$	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} .
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.
G. Other contributing mutations	
$\Delta araBAD$	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.
$\Delta sifA$	impairs the integrity of the <i>Salmonella</i> -containing vacuoles (SCVs) to enhance the ability to deliver antigens into cytosol.

^a Δ = deletion; TT = transcription terminator; P = promoter. The table was modified from ref [14].