Published in final edited form as:
Expert Rev Vaccines. 2013 September ; 12(9): 1033–1045. doi:10.1586/14760584.2013.825454.

Salmonella as a vaccine delivery vehicle

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Abstract

Attenuated *Salmonella* vaccines can be administered orally to deliver recombinant antigens to mucosal surfaces inducing a protective immune response against a variety of targeted pathogens. A number of exciting new approaches and technologies for attenuated *Salmonella* vaccines have been developed recently. However, there remains a disconnect between results obtained with mice in preclinical studies and results obtained in human clinical trials. This is due to an incomplete understanding of *S.* Typhi interactions with human hosts and inadequate animal models available for study. In this review, we describe recent progress in identifying important differences underlying *S.* Typhi-host interactions, the development of novel approaches to vaccine design and six recent clinical trials evaluating *Salmonella*-vectored vaccines.

Keywords

Salmonella-vectored vaccine; clinical trials; murine typhoid models; attenuation strategies; recombinant vaccine

Introduction

Live recombinant attenuated *Salmonella*-vectored vaccines hold great promise as a means to improve human health by achieving long-lasting mucosal, humoral and cellular immunity against a variety of non-*Salmonella* pathogens at a low cost. The approach is fairly straightforward. Heterologous protective antigen genes from a pathogen are cloned and expressed in attenuated *Salmonella enterica*. The recombinant *Salmonella* strain expressing the heterologous gene can then be orally administered to elicit an immune response against the pathogen from which the heterologous gene was derived. While strains of other bacteria such as *Escherichia coli*, *Listeria*, *Shigella* and *Vibrio* have been and continue to be evaluated as vaccines, the invasive nature of *Salmonella* makes it adept at eliciting B and T cell memory responses and gives *Salmonella* the greatest potential to elicit long lasting immunity.

The *Salmonella* serovar of choice for human vaccines is the human-restricted *Salmonella enterica* serovar Typhi. As the causative agent of typhoid fever, *S.* Typhi is fully capable of invading human mucosal tissues and entering systemic sites, ultimately targeting the host immune system to stimulate strong mucosal, humoral and cellular responses. Although many other pathogenic serovars of *Salmonella*, such as *Salmonella enterica* serovar Typhimurium, cause disease in humans, they have not been pursued as actively as *S.* Typhi. This is because S. Typhimurium, though capable of causing acute gastroenteritis, is unable to invade beyond the gut mucosa in healthy, immunocompetent humans and is thus less equipped to stimulate long-lasting immunity [1]. Because *S.* Typhi is host-restricted, small

animal models are of limited value. Fortunately, *S*. Typhimurium causes a disease in mice similar to typhoid fever in humans. Therefore, the behavior of *S*. Typhimurium in mice is used to evaluate new or novel attenuation and heterologous gene expression strategies. Results from hundreds of animal experiments have demonstrated the potential and utility of this approach. However, while quite useful in some respects, reliance on the *S*. Typhimurium mouse model to work out attenuation strategies has also been somewhat misleading. First, mutations that result in suitable attenuation and immunogenicity of *S*. Typhimurium are not necessarily adequate for *S*. Typhi vaccines. While there are mouse models to evaluate virulence and immunogenicity of *S*. Typhi-vectored vaccines, none utilize the oral route due to the inability of Typhi to establish an active infection in orally inoculated mice [2]. Second, the mouse model provides little insight into an *S*. Typhi vaccine's reactogenicity. As a result, the few clinical trials evaluating attenuated *S*. Typhi vectoring a heterologous antigen (eight trials reported by Galen [3] and five additional trials reported here) have been hampered by strains that are either over attenuated and poorly immunogenic or too reactogenic [3].

What is the disconnect between successful *S*. Typhimurium animal studies and unsuccessful *S*. Typhi human trials? The answer is likely the combination of a number of factors. First, *S*. Typhimurium and *S*. Typhi interact differently with their respective hosts. *S*. Typhimurium has evolved to elicit strong inflammatory responses in the gut, characterized by a massive influx of neutrophils into the terminal ileum and colon. This is an important survival strategy as it leads to the production of alternative terminal electron acceptors, particularly tetrathionate and nitrate. *S*. Typhimurium is uniquely suited to utilize these molecules, and gains a growth advantage in the inflamed gut due to its ability to respire on tetrathionate [4] and to grow anaerobically on ethanolamine [5]. In contrast, *S*. Typhi does not cause a diarrheal disease. In the gut it elicits intestinal inflammatory infiltrates consisting primarily of mononuclear cells. *S*. Typhi also actively suppresses the development of pro-inflammatory cytokines by production of the Vi capsule. Second, *S*. Typhi produces colonization factors, i.e. fimbriae that are not present in *S*. Typhimurium, and vice versa [6–10]. While many of these have been studied, their in vivo functions are not always fully understood. Finally, many of the differences in lifestyle result from the expression of different sets of virulence genes. There are significant differences in the chromosomal structures between the two organisms, exemplified by the presence of >200 pseudo-genes in *S*. Typhi [11], a common occurrence in host-restricted organisms. S. Typhi also contains a number of unique pathogenicity island-associated virulence factors, including the Vi capsular antigen, bundle forming pilus and typhoid toxin (*cdtB*).

Thus it is likely that failures in the clinic are due, at least in part, to a lack of understanding of *S*. Typhi pathogenesis. The good news is that many laboratories have made significant progress in the last five years toward closing this gap in our understanding. In addition, there has been significant progress in developing new and novel strategies for vaccine design, some of which have made their way into the clinic. We note that there have been a number of recent reviews describing *Salmonella* vaccine technologies with regard to vaccine design and preclinical studies [12–17]. We refer our readers to those reviews for additional information. Our focus here is to describe important insights into typhoid pathogenesis, highlight new and novel strategies for vaccine design and review recent clinical trials.

Recent insights into *S*. Typhi pathogenesis

The past five years have seen some significant breakthroughs in our understanding of *S*. Typhi pathogenesis. This is important because, at present, the greatest hindrance to developing *S*. Typhi-based vaccines stems from the fact that *S*. Typhi is not *S*. Typhimurium, and mice are not humans. Recent research in the field has begun to break

through these barriers to better understand the specific abilities and interactions of *S*. Typhi with its host. A great deal of work has been done to explore the properties of the Vi polysaccharide capsule. Not present in *S*. Typhimurium, the Vi capsule has a large impact on both the pathogenesis of *S*. Typhi and the development of the host immune response. Capsule is produced immediately following invasion into the intestinal epithelial mucosa [18]. Here, it suppresses the production of pro-inflammatory cytokines and IL-8 by masking critical toll-like receptor (TLR) ligands on the surface of the bacterium such as LPS and flagella and by down-regulating the expression of such ligands [19, 20]. However, Vi capsule production does not continue throughout the course of infection, most likely because the presence of Vi decreases the ability of the bacteria to attach to host cells [21]. Instead, it appears that Vi capsule is produced only after initial invasion into the host, and then is down regulated in the deeper lymphatic tissues [22]. This is in stark contrast to the behavior of *S*. Typhimurium, which promotes rapid pro-inflammatory cytokine release and neutrophil influx into the gastrointestinal mucosa immediately after infection.

Some interesting research has been done with the effectors of the Salmonella pathogenicity islands (SPIs) SPI-1, SPI-2 and SPI-3. All three pathogenicity islands are present in both *S*. Typhimurium and *S*. Typhi, but there are substantial differences in the ways the two serovars deploy these systems during infection. SPI-1 and SPI-2 are well-studied pathogenicity islands that encode type 3 secretion systems. In *S*. Typhimurium, SPI-1 is responsible for the initial invasion of the bacteria into the host, while SPI-2 is essential for survival inside macrophages [23]. The same does not appear to be true for *S*. Typhi, however, as SPI-2 is not essential for *S*. Typhi survival in the host [24]. There are also differences in the specific effector proteins secreted by these two systems. *S*. Typhi lacks some of the effector proteins that have been associated with gastric inflammation and fluid secretion (e.g. SopA and SopD2) [25, 26]. Another inflammation-associated effector, SopE2, is present in Ty2, but absent in CT18 [27]. The absence of these effectors contributes to the significantly lower level of inflammation associated with *S*. Typhi infections compared to *S*. Typhimurium infections. In addition, these missing proteins also appear to be detrimental to the natural course of typhoid pathogenesis, as introduction of the *sopD2* gene from *S*. Typhimurium results in a decreased ability of *S*. Typhi to invade human epithelial cells [28]. Similar results are observed when the *sseJ* gene is reintroduced into *S*. Typhi [29].

The SPI-3 pathogenicity island is involved with the colonization and persistence of *S*. Typhimurium on the intestinal mucosa [30]. While the magnesium transport system of SPI-3 (*mgtBC*) is upregulated by *S*. Typhi inside macrophages [31], the remaining functions conferred by this system appear to interfere with the success of typhoid infection. Several genes in this system are pseudogenes in *S*. Typhi and re-introduction of a functional SPI-3 system into *S*. Typhi results in a dramatic decrease in the ability of the bacteria to survive in human macrophages [32]. These studies and continued research into the molecular basis for *S*. Typhi pathogenesis could provide crucial information leading to improved vaccine approaches.

Animal models for host-restricted serovars

Some of the most exciting research in the past five years has come in the area of typhoid host restriction. Recent studies have identified the phagosome of monocyte-derived cells (monocytes, macrophages and dendritic cells) as a key location driving host specificity. *S*. Typhi is able to manipulate the phagosomes of human (but not murine) cells to promote its own survival and to block antigen presentation, thereby initiating a successful infection [33, 34]. The SPI-1 effector protein GtgE, present in *S*. Typhimurium, is absent in *S*. Typhi. The lack of this protein has a profound effect on pathogenesis and appears to be at least partially

responsible for the restriction of *S*. Typhi to human hosts [35, 36]. The GtgE protein of *S*. Typhimurium (and other broad host-range serovars) proteolytically inactivates the Rab29 GTPase recruited to the *Salmonella*-containing vacuole, allowing bacterial replication inside the cell [36]. The absence of this effector results in the rapid killing of *S*. Typhi inside non-human cells [35]. The identification of specific molecules that are responsible for the permissiveness of the host is an important breakthrough that will hopefully allow for the creation of accurate animal models for typhoid.

The lack of robust, reliable animal models has been one factor hampering the progress of vaccine development. Vaccine immunogenicity can be tested by intranasal administration of Typhi vaccine strains into BALB/c mice [37]. In a preclinical setting, this model is the current gold standard for assessing the immunogenicity of candidate strains. Despite its widespread use, this model suffers from the fact that it bypasses the oral compartment and provides no reliable measurement of strain reactogenicity or invasiveness in humans. However, several new animal models have been reported in recent years that may provide needed insight into developing vaccines from host-restricted *Salmonella* serovars such as Typhi and Paratyphi A.

Three humanized mouse models sensitive to *S*. Typhi infection have been reported [38–40]. In these models, immunodeficient mice (either $Rag2^{-/-}\ \gamma c^{-/-}$ or nonobese diabetic *scid* $IL2r\gamma^{null}$) are engrafted with human hematopoietic stem and progenitor cells (from fetal liver or cord blood). The resulting mice are susceptible to infection with wild-type *S*. Typhi and the resulting infection resembles the pathogenesis of typhoid in humans. These models will provide much-needed new avenues for increasing our understanding of important *S*. Typhi virulence factors, and they may also be useful for evaluating vaccines, since a *phoPQ*-attenuated S. Typhi stimulated serum antibody production against *Salmonella* in some of the infected mice, but was unable to cause disease [39]. The downside to these models is that they are expensive and labor-intensive, which will no doubt limit their use. In addition, there have thus far been no studies examining what happens when a host-restricted *Salmonella* serovar is administered orally. The establishment of a systemic infection after oral administration of *S*. Typhi would enhance the value of any small animal model.

A second murine typhoid model capitalizes on the differences between the murine and human innate immune systems to allow infection of mice by *S*. Typhi. Toll-like receptors (TLRs) play an important role in the innate immune system as sensors for pathogen associated molecular patterns as typified for gram-negative pathogens by lipid A (TLR4) and flagellin (TLR5) [41]. A recent report demonstrated that TLR11 is also a flagellin sensor [42]. TLR11 is produced in mice, but not in humans. Interestingly, mice deficient in TLR11 are susceptible to oral *S*. Typhi infection, leading to the hypothesis that TLR11 is a primary host factor responsible for the host-restricted phenotype of *S*. Typhi [42]. These TR11-deficient mice are otherwise immunocompetent and can develop protective immunity against *S*. Typhi infection by direct or passive immunization. In addition, *S*. Typhi pathogenesis was dependent on production of flagellin, consistent with the role of TLR11 as a flagellin sensor. One drawback of this model is that currently, TR11-deficient mice are not available commercially. While all of the mouse models we have mentioned have their weaknesses, this model, if commercialized, should be much less expensive than the humanized mouse models and is compatible with studies to evaluate orally administered *Salmonella* vaccines vectored by Typhi or Paratyphi A.

Finally, a rabbit model for evaluating immunogenicity and reactogenicity was described [43]. In that study, attenuated *S*. Paratyphi A strains were orally administered to New Zealand white rabbits. Interestingly, some of the strains elicited symptoms in the rabbit similar to those reported by human subjects in clinical trials, namely lethargy and loss of

appetite. No fevers or diarrhea were reported for the rabbits. Rabbits inoculated with non-reactogenic strains such as Ty21a or the Δ*phoPQ* mutant Ty800, did not display any symptoms of reactogenicity. In addition, all strains elicited dose-dependent serum titers of anti-LPS IgG. Importantly, elevation of anti-LPS IgG serum titers required administration of live cells. While this model needs further development and characterization, it represents another useful tool for evaluating *S*. Typhi and *S*. Paratyphi A vaccine strains.

Recently described technologies for enhancing the immunogenicity and safety of *Salmonella* vaccines

In an effort to enhance the ability of *Salmonella* to survive within the host long enough to elicit an optimal immune response, Roy Curtiss and colleagues have developed several new approaches for vaccine development including regulated delayed attenuation, regulated delayed antigen synthesis and regulated delayed lysis (for a detailed review of these approaches, see [44]).

Regulated delayed attenuation

Vaccine strains with regulated delayed attenuation display wild-type characteristics when they are orally administered, providing them with a full complement of genes required to survive transit through the gastrointestinal tract and to carry out the initial stages of infection. Once inside host tissues, expression of specific virulence genes or attributes is turned off, resulting in a fully attenuated strain. Vaccine strains utilizing this strategy have been demonstrated to be safe and immunogenic in mice [45, 46]. One approach to this technology has been to utilize the arabinose-responsive *araC* P_{BAD} promoter to drive expression of *Salmonella* virulence genes, including *crp, phoP, rpoS* and *fur* [45]. When the vaccine is grown prior to administration, arabinose is added to the culture medium and the arabinose-regulated virulence gene(s) is expressed. After immunization, when the vaccine strain reaches host tissues where free arabinose is not present, the virulence genes cease to be expressed and their protein products are lost by dilution as the bacteria divides. Alternatively, deletion of the *pmi* or *galE* genes results in strains that are dependent on exogenous mannose or galactose, respectively, for the synthesis of O-antigen, a virulence factor critical for the survival of *Salmonella* in the host (reviewed in [13]). Cells are grown with the appropriate sugar prior to administration. Free mannose and free galactose are not present in host tissues and the cells gradually lose their O-antigen and become attenuated.

Regulated delayed antigen synthesis

A second technology, regulated delayed antigen synthesis, is designed to preclude or reduce the synthesis of the heterologous antigen until the vaccine has invaded host tissues. This is important because heterologous antigen synthesis consumes cellular resources, thus reducing the ability of the vaccine strain to grow and to cope with host defenses. There have been previous efforts by others in this area. Most approaches have placed the heterologous antigen under the control of a promoter that is typically turned on only in vivo, such as the *ssaG* or *pagC* promoters [47]. The strong, constitutive P_{trc} promoter was shown to be a good, but not ideal choice for driving antigen gene expression in vivo [48]. One interpretation of this result is that P_{trc} is suboptimal is because it is unregulated in *Salmonella*. To address this, Wang and colleagues introduced an arabinose-regulated *lacI* gene into the *Salmonella* chromosome, resulting in a strain that can reduce or eliminate antigen production when the strain is grown in the presence of arabinose [16]. When arabinose is absent (as in host tissues), synthesis of heterologous antigen begins and reaches maximum levels after approximately 9 cell divisions due to dilution of LacI [16]. This system can improve the host immune response to a heterologous antigen when the gene is expressed from the P_{trc} promoter. It provides the same level of immunogenicity as the in

vivo-regulated promoter strategy, but is more flexible overall, since the P_{trc} promoter is compatible with a wider variety of attenuation strategies [47]. One drawback of this technology is that overproduction of LacI reduces *Salmonella* virulence [49]. Despite this, LacI does enhance the immunogenicity of an RASV carrying a P_{trc}-driven heterologous antigen gene, but reduces immunogenicity in strains carrying other promoters that do not bind LacI [47]. Thus, it is likely that the presence of the P_{trc} promoter on a multicopy plasmid titrates the LacI so that its negative effect on virulence and immunogenicity is suppressed.

Live attenuated *Salmonella* vaccine strains have been constructed that utilize both regulated delayed attenuation and regulated delayed antigen synthesis. For example, *S*. Typhimurium strain χ9558 has been designed to include arabinose-regulated *crp* and *fur* genes in addition to a Δ*pmi* mutation [46]. This strain also carries a Δ*sopB* mutation to enhance Th2 responses [50], an arabinose-regulated *lacI* gene to control heterologous antigen gene expression, a Δ*asdA* mutation to allow use of the AsdA-based balanced-lethal plasmid maintenance system, as well as some additional mutations designed to reduce biofilm formation to enhance biocontainment. Immunization of mice with strain χ9558 carrying a protective antigen from *Streptococcus pneumoniae*, resulted in a greater level of protection against lethal *S. pneumoniae* challenge than a traditionally attenuated *S*. Typhimurium strain carrying the same antigen [46]. Strain χ9558 derivatives carrying *Yersinia* antigens have also been shown protect mice against lethal challenge with *Yersinia pestis*. A set of *S*. Typhi strains with a similar constellation of mutations was constructed [51] for evaluation in a clinical trial (Table 1).

Regulated delayed lysis

Another recently described technology is the design of vaccine strains that undergo regulated delayed lysis [52]. This system was developed to address a number of goals, including biocontainment, release of non-secreted protein antigens and DNA vaccine delivery. In this system, the vaccine strain features strict arabinose-controlled expression of two genes required for peptidoglycan synthesis, *asdA* and *murA*. The two genes are effectively complemented by a plasmid that carries both genes, along with an antigen gene of interest. The P_{trc} promoter drives antigen gene expression, for delivery of protein antigens, while eukaryotic promoters such as P_{CMV} can be used for DNA vaccine delivery. For DNA vaccines, the plasmids have been further modified to include nuclear targeting sequences and to enhance stability inside host cells and a number of additional mutations are included in the *Salmonella* strain to facilitate efficient DNA delivery [53]. Results from preclinical trials demonstrate the utility of this system, but it has not yet been evaluated in a clinical setting.

Reducing reactogenicity by lipid A modification

Kong and associates have taken a unique approach to enhancing RASV safety my modifying the lipid A (endotoxin) in living cells to a less toxic form, while retaining its adjuvant properties [54]. The significance and impact of this strategy was recently reviewed [55].

Sugar-inducible acid resistance

Another novel system is focused on increasing the acid resistance of *S*. Typhi vaccine strains. The basis of this approach is that, to be effective, a live attenuated *Salmonella* vaccine must first survive its encounter with the low pH environment of the stomach. While wild type salmonellae have evolved mechanisms to survive this environmental insult long enough to pass into the intestinal tract, many of the means used to attenuate *Salmonella* for virulence have a secondary effect of increasing sensitivity to acid. Additional problems arise because the model organism, *S*. Typhimurium, is inherently more acid resistant than *S*.

Typhi [56]. Also, the gastric pH in a fasted mouse is around 4.0 [57], while the gastric pH in a fasted human is around 2.0 [58]. This difference could account, at least in part, for the discrepancies between results obtained in clinical studies and results obtained using the *S*. Typhimurium mouse model system. It may also help to explain why very high doses of *S*. Typhi vaccines are required to achieve an efficacious immune response, while lower doses of *S*. Typhimurium work well in mice. Because of the low gastric pH in humans, oral *Salmonella* vaccines are typically given with an agent designed to increase the gastric pH, such as bicarbonate. While this approach is helpful, it precludes the *Salmonella* vaccine from sensing an important environmental signal, low pH, signifying its entry into a host.

Enteric bacteria utilize a number of means to deal with low pH, including several amino acid decarboxylase systems [59]. However, these systems are often induced only by anaerobic growth at low pH. These growth conditions are not typically used for vaccine preparation, so *S*. Typhi cells are physiologically unprepared for passage through the stomach. To address this problem, we have constructed *S*. Typhi vaccine strains such that expression of *adiA* and *adiC*, encoding arginine decarboxylase and the arginine-agmatine antiporter, is driven by a sugar-inducible promoter [60]. Addition of the appropriate sugar results in expression of *adiA* and *adiC* and a concomitant increase in acid resistance. Our in vitro results demonstrated that this system results in significant increases in survival for Δ*aroD* and Δ*phoPQ S*. Typhi strains at both pH 3.0 and pH 2.5 [60]. We are currently examining the effects of other amino acid decarboxylases using a similar approach. Preliminary animal data indicates that inclusion of these systems into attenuated *S*. Typhimurium results in a significant enhancement of immunogenicity in mice (Brenneman and Roland, unpublished).

Plasmid stabilization systems

In most attenuated *Salmonella* vectored vaccines, heterologous antigens are carried on multicopy plasmids (but not all – see TSB7, Table 1). This is done to ensure that a high level of antigen synthesis is achieved to adequately stimulate the desired immune response. Stable maintenance of plasmids in vaccine strains is required to assure that every cell in the population is capable of producing and delivering the heterologous antigen. To obviate the need for antibiotic selection, a number of non-antibiotic stabilization systems were developed, beginning in 1990 with the $AsdA^+$ balanced-lethal system [61], which has been extensively used in preclinical and clinical trials. Others include the $ThyA^+$ system [62], used in some recent trials (Table 1), $PurB^+$ [63], $GlnA^+$ [64] and the ORT balanced-lethal system [65]. Although all of these systems have not been directly compared, in a recent preclinical study, the $AsdA^+$ $GlnA^+$ and $PurB^+$ selection systems were evaluated in isogenic Δ*phoPQ S*. Typhimurium strains. The strains carried plasmids encoding the *Yersinia pestis* F1-V antigen that differed only in the gene used for plasmid selection [66]. Mice were given two doses of vaccine (days 0 and 14) and anti-F1-V serum IgG responses were determined. On days 28 and 42, the IgG titers elicited by immunization with the strain using the $AsdA^+$ system were >3-fold higher than mice in the other two groups (ca. 2,000 vs. 650 and 380). These results indicate that the $AsdA^+$ system led to more rapid serum responses than the other two. This is not necessarily surprising, since loss of a $GlnA^+$ or $PurB^+$ plasmids, as well as a $ThyA^+$ plasmid, does not necessarily lead to cell death, only a cessation of growth. In fact, *glnA* mutants of *S*. Typhimurium are not attenuated in mice [67]. Thus it is not clear what impact the loss of a $GlnA^+$ plasmid would have in vivo. It would be interesting to determine how the $AsdA^+$ system compares to the $ThyA^+$ system, since this system was used to stabilize plasmids in several recent clinical trials (Table 1).

A novel plasmid stabilization system was recently described by Galen and associates, which relies on complementation of a deletion in the gene *ssb* that encodes single strand binding protein [68]. Because *ssb* is an essential gene, loss of the antigen-encoding plasmid results in

cell death. This system results in 100% plasmid retention. However, antigen load can still be a problem, resulting in strains that retain the plasmid, but lose the ability to synthesize the heterologous antigen. The authors demonstrated that antigen synthesis from lower copy number plasmids eliminated this problem, resulting in a strain with highly stable antigen expression. In their study, they evaluated the PA83 antigen from *Bacillus anthracis*. The antigen gene was fused to *clyA* to facilitate protein secretion [69] and expressed from *ssb* plasmids that also encoded a partition function to further enhance plasmid stability. The plasmids were introduced into *S*. Typhi strains derived from CVD908 and CVD908-*htrA*. Mice were intranasally immunized with two doses and serum IgG responses against PA83 were measured. Strains carrying plasmids with the lowest copy number (pSC101) elicited significantly higher anti-PA83 titers than strains carrying a higher copy number plasmid (p15a). For strains carrying the pSC101 plasmid, on day 41, prior to parenteral boosting with purified antigen, mice immunized with CVD908-*htrA* produced GMTs of 2,322 and mice immunized with CVD908 produced GMTs of 4,929. Six days after parenteral boosting on day 42, mice primed with CVD908 and CVD908-*htrA* produced remarkably high titers of 500,970 and 156,330, respectively, while the titers achieved with the higher copy number p15a plasmids were 59,247 and 13,806, respectively. These data support the testing of this system in a clinical trial.

Recent clinical trials - vectors

We have identified six clinical trials, published or in progress since 2007 that investigate twelve candidate *Salmonella* vaccines delivering heterologous antigens (Table 1). Before we discuss heterologous antigen delivery, we will first describe a few interesting studies focused on enhancing our understanding of host-restricted *Salmonella* delivery vectors. The group at the Center for Vaccine Development (CVD) in Maryland, USA has taken a methodical approach to *Salmonella* vector development, carefully assessing their attenuated strains in clinical trials and making minor, but rational strain modifications along the way. They began by evaluating strains with *aro* mutations and later added a mutation in *htrA* to achieve their current safe and immunogenic vector strain, CVD908-*htrA* (see ref [70] for a complete review of these strains).

In general, live *S*. Typhi vaccines do not elicit antibodies against the Vi antigen, most likely due to the tight regulation of Vi synthesis in *S*. Typhi (reviewed in [71]). However, high titers of anti-Vi IgG protect against infection. To overcome this problem, Levine and associates genetically modified CVD908-*htrA* to constitutively produce Vi antigen, resulting in strain CVD909 [72] (Table 2). While this strain is does not vector a heterologous antigen, we include it in our discussion here because the strain was manipulated to bypass natural Vi regulation so that capsule is constitutively synthesized in order to quickly stimulate the host immune system.

When administered to volunteers, this strain elicited antibody-secreting cells against Vi, but only 2 of 32 volunteers produced anti-Vi serum IgG [73]. Importantly, the constitutive production of Vi did not diminish the ability of CVD909 to elicit T cell responses against Typhi-specific antigens, although these responses were not boosted by a second dose [74]. Building on a number of preclinical studies showing the priming capacity of *Salmonella*-vectored vaccine strains (e.g. [75, 76]), CVD909 was used to immunize volunteers who then received a parenteral boost with purified Vi [77]. While bacterially primed subjects produced higher levels of anti-Vi IgG and IgA, statistical significance was not achieved. However, priming did significantly enhance the production of memory B cells, indicating that this approach may lead to a longer-lived, more robust protection against typhoid fever than current vaccines.

As part of their ongoing work to develop a live attenuated *Salmonella* vaccine against enteric fever, the group at CVD has recently initiated a clinical trial to evaluate attenuated *S.* Paratyphi A strain CVD1902 [101] (Table 2). Although S. Paratyphi A is closely related to S. Typhi, immune responses to *S.* Typhi vaccine Ty21a are not cross protective [78]. Prior to this study, no clinical trials have been conducted using S. Paratyphi A, so it will be interesting to see how this serovar behaves in humans when attenuated.

Recent clinical trials – heterologous antigen delivery

S. Typhi strain M01ZH09 (previously ZH9) has been evaluated in Phase I and Phase II clinical trials for use as a typhoid vaccine and was found to be safe and immunogenic (Table 2). Khan and associates described an *S.* Typhi M01ZH09 derivative that carries a chromosomally integrated *eltB* gene, encoding the B subunit of heat-labile toxin from enterotoxigenic *Escherichia coli* [79]. The *eltB* gene was inserted into the *aroC* gene site, under transcriptional control of the *ssaG* promoter, so that *eltB* expression was upregulated in vivo [80]. In their study, 12 volunteers received two doses of 10^8 CFU and 24 volunteers received two doses of 10^9 CFU. The vaccine was generally well tolerated, although most volunteers experienced mild to severe intestinal symptoms that resolved within a few days. The vaccine was shed for up to 3 days, with a single volunteer shedding for 6 days. Importantly, 67% of volunteers produced an IgG or IgA immune response against heat labile toxin (LT) as determined by ELISA (IgG) or ELISPOT (IgA), with most seropositive volunteers producing anti-LT IgG. 97% of volunteers produced IgG and/or IgA responses to *S.* Typhi lipopolysaccharide (LPS), with most volunteers responding to both. In addition, there was evidence of first dose priming of LT responses. These data indicate that M01ZH109 is a strong candidate as both a live typhoid vaccine and as an antigen delivery vector.

As described above, the Curtiss group has developed a number of new technologies for enhancing the effectiveness and safety of *Salmonella* vaccines To assess the impact of some of these technologies in the clinic, they compared three *S.* Typhi strains that carry nine or ten mutations each to evaluate their ability to deliver the pneumococcal protein PspA [102] (Table 1). Many of the attenuating mutations in these strains are based on their regulated, delayed attenuation strategy [45, 51] (see below) and *pspA* was carried on an $AsdA^+$ plasmid. In their trial, volunteers were given escalating doses from 10^7 to 10^{10} CFU. Their study was also designed to evaluate the impact of RpoS on immunogenicity [45, 51]. This is an important question, since most *S.* Typhi vaccine strains are derived from strain Ty2, an *rpoS* mutant and *rpoS* is a virulence factor for *S.* Typhimurium [81]. The outcome of this completed trial has not yet been published.

Ty21a – new uses for an old vaccine

Recently, there has been interest in expanding the uses of the licensed vaccine strain, Ty21a. Despite the fact that the immunogenicity of Ty21a is less than ideal, a number of groups have been looking more closely at it for a number of different purposes. While there are a number of demonstrably more immunogenic live strains (e.g. CVD908-*htrA*), we suspect that the focus on Ty21a is driven by market forces (time to licensure, cost of licensing, Phase III trials, etc. for a new vaccine vs. potential profits, availability to target populations). Thus, finding new uses for Ty21a may represent the most rapid, least expensive path toward reaping potential health benefits. Several reports have examined the potential of Ty21a to elicit cross protective immune responses against *S.* Paratyphi A, *S.* Paratyphi B [78, 82] and non-typhoid salmonellae [83]. In addition, several groups have modified Ty21a to deliver heterologous antigens in clinical trials that are described below (see Table 1). A preclinical study demonstrated the ability of Ty21a to secrete and deliver the *Bacillus anthracis* protective antigen, PA [84]. A low copy number plasmid was constructed in which the gene

encoding PA was transcribed from an in vivo-inducible promoter and secreted via the HlyA type I secretion system. Mice immunized intranasally produced high serum IgG titers against PA and were protected from an aerosol spore challenge.

Several groups have used the clinic to compare multiple vaccine candidates in a single study. Aebeischer and associates utilized a *thyA* derivative of Ty21a to deliver two different *Helicobacter pylori* antigens, UreAB (urease) and HP0231, a protective antigen related to *E. coli* DsbG [85], whose genes were carried on plasmids utilizing a $ThyA^+$ selection system [86]. The study was set up in two parts, a preliminary trial with 20 subjects and a secondary study using 47 subjects. Volunteers received three doses (first trial) or four doses (second trial), with two days between doses. In the first trial, volunteers were challenged with *H. pylori* five months later. By seven days post-challenge, all volunteers were positive for *H. pylori* as determined using the [^{14}C] urea breath test (UBT). By week six, three of nine vaccinated volunteers were *H. pylori* negative (33%) as judged by the UBT and three other diagnostic tests including histological analysis of biopsy samples. All four control individuals were positive by one or more test, indicating that vaccination provided some degree of protection. In the second trial, volunteers were challenged 42 days after the primary immunization. Although *H. pylori* clearance by all four diagnostic tests was not demonstrated in any volunteer, some evidence of protection using the UBT was achieved with both vaccine candidates. With regard to immune responses, on day 5 post-vaccination, urease-specific $CD4^+$ T cells were detected in 25% of volunteers in the first study and 8% of volunteers in the second study. No antigen-specific $CD8^+$ cells were detected. The authors also reported that PBMC isolated from some volunteers could be stimulated with purified antigen to produce IFNγ. However, no evidence of antigen-specific B cell responses was detected, although anti-*Salmonella* responses were detected in some volunteers. Taken together, these data indicate that the antigens were detected and processed by the host immune system, though efficacy remains in question.

Bumann and associates tested the potential of *Salmonella*–based vaccines to protect against airway infections by *Pseudomonas aeruginosa* (Table 1). They compared the ability of two antigen delivery strains, $ThyA^-$ derivatives of CVD908-*htrA* and Ty21a, to deliver an OprF-OprI fusion protein [87] shown to protective against *P. aeruginosa* infection in animal studies [88]. The genes for the OprF-OprI antigen were carried on a $ThyA^+$ plasmid. There were two plasmid variants that differed only in the promoter driving transcription of the *Pseudomonas* gene. The antigen gene was transcribed from either an artificial, constitutive promoter (designated Pyz), or from the in vivo-inducible *pagC* promoter. Volunteers were inoculated with either a single dose of the CVD908-*htrA*-vectored vaccines (10^8 CFU/dose) or 3 doses of the Ty21a-vectored vaccines (10^{10} CFU/dose), following the standard schedule for Ty21a, days 0, 2 and 4. The study also included control groups that received the purified OprF-OprI antigen, delivered either by injection with an aluminum adjuvant or intranasally in a gel without adjuvant. Twenty-eight days after the primary inoculation, all groups received an injection of OprF-OprI in adjuvant. The groups given the *Salmonella*–vectored vaccines failed to mount a serum antibody response against the *P. aeruginosa* antigen after the initial immunization, while both groups receiving purified antigen developed a 1000-fold increase in IgG and a 100-fold increase in IgA titers to OprF-OprI on day 28. After the booster injection, all groups produced similar high titers of serum IgG and IgA against the antigen. Interestingly, both *Salmonella*–vectored vaccines elicited similar, significant anti-OprF-OprI IgG and IgA responses in induced sputum samples, a measure of the mucosal responses in the lower airways. Similar mucosal responses were observed in subjects immunized intranasally, but not in those immunized systemically by injection. Systemic boosting did not boost the mucosal responses in any group. Surprisingly, all four *Salmonella* vaccines elicited similar immune responses, regardless of differences in attenuation strategies and promoters driving antigen gene expression. However, volunteers in the

CVD908-*htrA* group received only a single dose compared to 3 doses administered in the Ty21a group, indicating that CVD908-*htrA* was more immunogenic. These results are consistent with previous animal studies indicating that *Salmonella*–vectored vaccines induce strong mucosal responses, but weak systemic responses. While cellular responses were not directly measured, no differences were observed in IgG1/IgG2a ratios. These results are encouraging as they demonstrate that it is possible to generate a mucosal antibody response in the respiratory tract after oral administration of a *Salmonella* vaccine.

In a unique application, Ty21a is being investigated as a DNA vaccine delivery vector. In this scheme, Ty21a carries a plasmid encoding a humanized, full length VEGFR-2, which encodes vascular endothelial growth factor receptor 2 [89] for use as an anti-angiogenic cancer therapeutic vaccine (Table 1). In preclinical studies using an *S*. Typhimurium *aroA* mutant as the delivery vector, a similar construct, using a mouse-derived gene, was effective at protecting mice against challenge with melanoma, colon carcinoma and lung carcinoma cells [90]. Importantly, it reduced the growth of established metastases. In the clinical trial, the vaccine was administered to patients with stage IV and advanced pancreatic cancer in a Phase I trial. The results of this study are not yet available, but it represents the first human trial to evaluate DNA delivery by *Salmonella*.

S. Typhimurium vaccines

Saltzman's group has been evaluating an attenuated *S*. Typhimurium strain expressing a fragment of human IL-2 (*SalpIL2*) as a therapeutic cancer vaccine [91, 92]. The gene for IL-2 is carried on a low copy number plasmid stabilized using the $AsdA^+$ balanced-lethal system. In preclinical trials, this strain has been shown to home to tumor cells, to be highly immunogenic and to reduce tumor burden and tumor-associated systemic inflammation, while increasing proliferation of tumor-targeting cytotoxic NK cells [91]. Due to the success of this novel approach, *SalpIL2* is currently being evaluated in a dose escalation study in humans. The outcome of this trial will be of interest for several reasons. This strain may provide a new tool to be used for treating a particularly devastating form of cancer. However, as mentioned before, *S*. Typhimurium is not well adapted to achieve systemic spread, so it is not clear whether the vaccine will be able to reach the tumor in a human host. In addition, only two trials using a Typhimurium vector have been reported. The strains in these trials were attenuated by different means, Δ*phoPQ* [63], Δ*aroC Δssa*V [93] and, in the current trial, Δ*cya Δcrp* [101]. While the first two attenuation strategies were shown to be attenuating in *S*. Typhi, a Δ*cya Δcrp S*. Typhi strain was too reactogenic to be considered for further development [94]. Thus, it will be of interesting to see how effective the Δ*cya Δcrp* strategy is for attenuating *S*. Typhimurium in human volunteers.

Acknowledgments

This work was supported by grants from the National Institutes of Health - R01 AI60557, R01 AI093348, R21 AI092307 and R21 AI105479.

Appendix

Expert Commentary

The need for inexpensive, mucosally applied vaccines to serve the underdeveloped world has been a driving force in our laboratory. *Salmonella*-vectored vaccines are ideally suited for this task. Many of the vaccines for the underdeveloped world must be suitable for administration to children. Thus, efforts toward development of a protein-antigen-based *Salmonella*–vectored pneumococcal vaccine has led the Curtiss group to develop a number of new strategies to enhance safety and immunogenicity. The need to understand the safety

and immunogenicity of RASVs in infants and children has led a number of groups to carefully examine the impact of their vaccines in infant and baby mice. An expansion of our knowledge of mucosal immunology in the young, malnourished and immunocompromised has supported efforts to develop vaccines for the developing world.

While there are still crucial problems yet to be solved before RASVs are effective enough to be commercially viable, recent clinical trials have served to expand our knowledge base of *S*. Typhi vectored vaccines and to demonstrate their many potential applications. As outlined in Table 1, RASV technology has been applied to vaccines against traveller's diarrhea, infant pneumonia, *Pseudomonas aeruginosa* infections in cystic fibrosis patients, *Helicobacter pylori* infections and cancer. These trials demonstrate the ability of RASVs to elicit mucosal responses and to serve as effective immunological priming agents in human volunteers. Careful analyses of mucosal and cellular responses in vaccinees will drive our understanding of how these vaccines may best be used to unlock their enormous potential.

5-year view

The biggest advances in the field will come as the new animal models are applied to the existing pool of potential *Salmonella*-based vaccines. Right now, the biggest restriction on the field is the amount of time and effort it takes to evaluate a single candidate vaccine. Researchers spend years to gather pre-clinical data, apply to the FDA and then perform a relatively straightforward experiment, the clinical trial, to see if their original hypothesis about vaccine design was correct. This is the main reason that there are so few clinical *Salmonella* vaccine trials. The use of humanized mouse or rabbit models will allow researchers to rapidly generate data on the actual vaccine strain, as opposed to a *S*. Typhimurium surrogate, to serve as a relevant indicator of what will happen in the clinic. That will allow the entire field to move much more quickly and have the potential to explore other long-standing problems in the field, such as how to direct the attention of the host immune response to the heterologous antigen and ignore *Salmonella*. Research conducted in the next five years should provide us with long-awaited solutions to many of the fundamental problems facing the field.

References

1. de Jong HK, Parry CM, van der Poll T, Wiersinga WJ. Host-pathogen interaction in invasive Salmonellosis. PLoS Pathog. 2012; 8(10):e1002933. [PubMed: 23055923]
2. Pasetti MF, Levine MM, Sztein MB. Animal models paving the way for clinical trials of attenuated *Salmonella enterica* serovar Typhi live oral vaccines and live vectors. Vaccine. 2003; 21(5–6):401–418. [PubMed: 12531639]
3. Galen JE, Pasetti MF, Tennant S, Ruiz-Olvera P, Sztein MB, Levine MM. *Salmonella enterica* serovar Typhi live vector vaccines finally come of age. Immunol Cell Biol. 2009; 87(5):400–412. [PubMed: 19417771]
4. Winter SE, Thiennimitr P, Winter MG, et al. Gut inflammation provides a respiratory electron acceptor for *Salmonella*. Nature. 2010; 467(7314):426–429. [PubMed: 20864996]
5. Thiennimitr P, Winter SE, Winter MG, et al. Intestinal inflammation allows *Salmonella* to use ethanolamine to compete with the microbiota. Proc Natl Acad Sci U S A. 2011; 108(42):17480–17485. [PubMed: 21969563]
6. Baumler AJ, Gilde AJ, Tsolis RM, van der Velden AW, Ahmer BM, Heffron F. Contribution of horizontal gene transfer and deletion events to development of distinctive patterns of fimbrial operons during evolution of *Salmonella* serotypes. J Bacteriol. 1997; 179(2):317–322. [PubMed: 8990281]
7. Baumler AJ, Tsolis RM, Heffron F. Contribution of fimbrial operons to attachment to and invasion of epithelial cell lines by *Salmonella typhimurium*. Infect Immun. 1996; 64(5):1862–1865. [PubMed: 8613405]

8. Folkesson A, Advani A, Sukupolvi S, Pfeifer JD, Normark S, Lofdahl S. Multiple insertions of fimbrial operons correlate with the evolution of *Salmonella* serovars responsible for human disease. Mol Microbiol. 1999; 33(3):612–622. [PubMed: 10417651]
9. Forest C, Faucher SP, Poirier K, Houle S, Dozois CM, Daigle F. Contribution of the *stg* fimbrial operon of *Salmonella enterica* serovar Typhi during interaction with human cells. Infect Immun. 2007; 75(11):5264–5271. [PubMed: 17709421]
10. Townsend SM, Kramer NE, Edwards R, et al. *Salmonella enterica* serovar Typhi possesses a unique repertoire of fimbrial gene sequences. Infect Immun. 2001; 69(5):2894–2901. [PubMed: 11292704]
11. Parkhill J, Dougan G, James KD, et al. Complete genome sequence of a multiple drug resistant *Salmonella enterica* serovar Typhi CT18. Nature. 2001; 413(6858):848–852. [PubMed: 11677608]
12. Bonilla, CG.; Mera, EP.; Quinonez, AD.; Yepez, SH. *Salmonella* as live carrier of antigens in vaccine development. In: Kumar, Y., editor. Salmonella - A Diversified Superbug. Croatia: InTech, Rijeka; 2012. p. 309-326.
13. Curtiss R 3rd, Xin W, Li Y, et al. New technologies in using recombinant attenuated *Salmonella* vaccine vectors. Crit Rev Immunol. 2010; 30(3):255–270. [PubMed: 20370633]
14. Hegazy WA, Hensel M. *Salmonella enterica* as a vaccine carrier. Future Microbiol. 2012; 7(1): 111–127. [PubMed: 22191450]
15. Kong W, Clark-Curtiss J, Curtiss R 3rd. Utilizing Salmonella for antigen delivery: the aims and benefits of bacterial delivered vaccination. Expert Rev Vaccines. 2013; 12(4):345–347. [PubMed: 23560914]
16. Wang S, Li Y, Scarpellini G, et al. *Salmonella* vaccine vectors displaying delayed antigen synthesis in vivo to enhance immunogenicity. Infect Immun. 2010; 78(9):3969–3980. [PubMed: 20605977] *This paper provides preclinical data evaluating antigen delivery in strains where heterologous antigen gene transcription is regulated by the novel LacI-mediated regulation of the P_{trc} promoter compared to strains in which transcription is driven by traditional in vivo-regulated promoters.
17. Zhang S, Walters N, Cao L, Robison A, Yang X. Recombinant *Salmonella* vaccination technology and its application to human bacterial pathogens. Curr Pharm Biotechnol. 2013; 14(2):209–219. [PubMed: 23360265]
18. Tran QT, Gomez G, Khare S, et al. The *Salmonella enterica* serotype Typhi Vi capsular antigen is expressed after the bacterium enters the ileal mucosa. Infect Immun. 2010; 78(1):527–535. [PubMed: 19901065]
19. Wilson RP, Raffatellu M, Chessa D, Winter SE, Tukel C, Baumler AJ. The Vi-capsule prevents Toll-like receptor 4 recognition of *Salmonella*. Cell Microbiol. 2008; 10(4):876–890. [PubMed: 18034866]
20. Winter SE, Raffatellu M, Wilson RP, Russmann H, Baumler AJ. The *Salmonella enterica* serotype Typhi regulator TviA reduces interleukin-8 production in intestinal epithelial cells by repressing flagellin secretion. Cell Microbiol. 2008; 10(1):247–261. [PubMed: 17725646]
21. Bishop A, House D, Perkins T, Baker S, Kingsley RA, Dougan G. Interaction of *Salmonella enterica* serovar Typhi with cultured epithelial cells: roles of surface structures in adhesion and invasion. Microbiology. 2008; 154(Pt 7):1914–1926. [PubMed: 18599820]
22. Janis C, Grant AJ, McKinley TJ, et al. In vivo regulation of the Vi antigen in *Salmonella* and induction of immune responses with an in vivo-inducible promoter. Infect Immun. 2011; 79(6): 2481–2488. [PubMed: 21402763]
23. Buckner MM, Croxen MA, Arena ET, Finlay BB. A comprehensive study of the contribution of *Salmonella enterica* serovar Typhimurium SPI2 effectors to bacterial colonization, survival, and replication in typhoid fever, macrophage, and epithelial cell infection models. Virulence. 2011; 2(3):208–216. [PubMed: 21540636]
24. Forest CG, Ferraro E, Sabbagh SC, Daigle F. Intracellular survival of *Salmonella enterica* serovar Typhi in human macrophages is independent of Salmonella pathogenicity island (SPI)-2. Microbiology. 2010; 156(Pt 12):3689–3698. [PubMed: 20817644]

25. Hoffmann C, Galle M, Dilling S, et al. In macrophages, caspase-1 activation by SopE and the type III secretion system-1 of *S. typhimurium* can proceed in the absence of flagellin. PLoS One. 2010; 5(8):e12477. [PubMed: 20814576]
26. Zhang S, Santos RL, Tsolis RM, et al. The *Salmonella enterica* serotype Typhimurium effector proteins SipA, SopA, SopB, SopD, and SopE2 act in concert to induce diarrhea in calves. Infect Immun. 2002; 70(7):3843–3855. [PubMed: 12065528]
27. Deng W, Liou SR, Plunkett G 3rd, et al. Comparative genomics of *Salmonella enterica* serovar Typhi strains Ty2 and CT18. J Bacteriol. 2003; 185(7):2330–2337. [PubMed: 12644504]
28. Trombert AN, Rodas PI, Mora GC. Reduced invasion to human epithelial cell lines of *Salmonella enterica* serovar Typhi carrying *S*. Typhimurium *sopD2*. FEMS Microbiol Lett. 2011; 322(2):150–156. [PubMed: 21707735]
29. Trombert AN, Berrocal L, Fuentes JA, Mora GC. *S*. Typhimurium *sseJ* gene decreases the *S*. Typhi cytotoxicity toward cultured epithelial cells. BMC Microbiol. 2010; 10:312. [PubMed: 21138562]
30. Morgan E, Campbell JD, Rowe SC, et al. Identification of host-specific colonization factors of *Salmonella enterica* serovar Typhimurium. Mol Microbiol. 2004; 54(4):994–1010. [PubMed: 15522082]
31. Retamal P, Castillo-Ruiz M, Mora GC. Characterization of MgtC, a virulence factor of *Salmonella enterica* Serovar Typhi. PLoS One. 2009; 4(5):e5551. [PubMed: 19436747]
32. Retamal P, Castillo-Ruiz M, Villagra NA, Morgado J, Mora GC. Modified intracellular-associated phenotypes in a recombinant *Salmonella* Typhi expressing *S*. Typhimurium SPI-3 sequences. PLoS One. 2010; 5(2):e9394. [PubMed: 20195364]
33. Bueno SM, Gonzalez PA, Carreno LJ, et al. The capacity of *Salmonella* to survive inside dendritic cells and prevent antigen presentation to T cells is host specific. Immunology. 2008; 124(4):522–533. [PubMed: 18266715]
34. Xu T, Maloy S, McGuire KL. Macrophages influence *Salmonella* host-specificity in vivo. Microb Pathog. 2009; 47(4):212–222. [PubMed: 19660538]
35. Spano S, Galan JE. A Rab32-dependent pathway contributes to *Salmonella* Typhi host restriction. Science. 2012; 338(6109):960–963. [PubMed: 23162001] ** Identifies the key player in typhoid host specificity as the Rab32 protein found in the membrane of mouse phagosomes. Lack of the GtgE protease, present in *S*. Typhimurium, but absent in *S*. Typhi, renders *S*. Typhi susceptible to killing by mouse macrophages.
36. Spano S, Liu X, Galan JE. Proteolytic targeting of Rab29 by an effector protein distinguishes the intracellular compartments of human-adapted and broad-host *Salmonella*. Proc Natl Acad Sci U S A. 2011; 108(45):18418–18423. [PubMed: 22042847]
37. Galen JE, Gomez-Duarte OG, Losonsky GA, et al. A murine model of intranasal immunization to assess the immunogenicity of attenuated *Salmonella typhi* live vector vaccines in stimulating serum antibody responses to expressed foreign antigens. Vaccine. 1997; 15(6–7):700–708. [PubMed: 9178472]
38. Libby SJ, Brehm MA, Greiner DL, et al. Humanized nonobese diabetic-scid *IL2rγnull* mice are susceptible to lethal *Salmonella* Typhi infection. Proc Natl Acad Sci U S A. 2010; 107(35):15589–15594. [PubMed: 20713716]
39. Song J, Willinger T, Rongvaux A, et al. A mouse model for the human pathogen *Salmonella* Typhi. Cell Host Microbe. 2010; 8(4):369–376. [PubMed: 20951970] ** Describes a humanized murine model for typhoid. The model demonstrates potential to provide attenuation and safety information about S. Typhi vaccine strains prior to initiating clinical trials in humans.
40. Mian MF, Pek EA, Chenoweth MJ, Coombes BK, Ashkar AA. Humanized mice for *Salmonella typhi* infection: new tools for an old problem. Virulence. 2011; 2(3):248–252. [PubMed: 21623167]
41. Duthie MS, Windish HP, Fox CB, Reed SG. Use of defined TLR ligands as adjuvants within human vaccines. Immunol Rev. 2011; 239(1):178–196. [PubMed: 21198672]
42. Mathur R, Oh H, Zhang D, et al. A mouse model of *Salmonella* Typhi infection. Cell. 2012; 151(3):590–602. [PubMed: 23101627] ** Describes a TLR-11 knockout murine model for typhoid pathogenesis and demonstrates a role for TLR-11 in host restriction. Importantly, this paper

demonstrates that mice develop a protective immune response against *S*. Typhi after oral administration

43. Roland KL, Tinge SA, Kochi SK, Thomas LJ, Killeen KP. Reactogenicity and immunogenicity of live attenuated *Salmonella enterica* serovar Paratyphi A enteric fever vaccine candidates. Vaccine. 2010; 28(21):3679–3687. [PubMed: 20338215]
44. Wang S, Kong Q, Curtiss R 3rd. New technologies in developing recombinant attenuated *Salmonella* vaccine vectors. Microb Pathog. 2012
45. Curtiss R 3rd, Wanda SY, Gunn BM, et al. *Salmonella* strains with regulated delayed attenuation in vivo. Infect Immun. 2009 **This paper provides the basis for the novel regulated delayed attenuation strategy, demonstrating safety and immunogenicity in a preclinical setting.
46. Li Y, Wang S, Scarpellini G, et al. Evaluation of new generation *Salmonella enterica* serovar Typhimurium vaccines with regulated delayed attenuation to induce immune responses against PspA. Proc Natl Acad Sci U S A. 2009; 106(2):593–598. [PubMed: 19114649]
47. Wang S, Li Y, Shi H, Sun W, Roland KL, Curtiss R 3rd. Comparison of a regulated delayed antigen synthesis system with in vivo-inducible promoters for antigen delivery by live attenuated *Salmonella* vaccines. Infect Immun. 2011; 79(2):937–949. [PubMed: 21134969]
48. Dunstan SJ, Simmons CP, Strugnell RA. Use of in vivo-regulated promoters to deliver antigens from attenuated *Salmonella enterica* var. Typhimurium. Infect Immun. 1999; 67(10):5133–5141. [PubMed: 10496887]
49. Eswarappa SM, Karnam G, Nagarajan AG, Chakraborty S, Chakravortty D. *lac* repressor is an antivirulence factor of *Salmonella enterica* : its role in the evolution of virulence in *Salmonella*. PLoS One. 2009; 4(6):e5789. [PubMed: 19495420]
50. Li Y, Wang S, Xin W, et al. A *sopB* deletion mutation enhances the immunogenicity and protective efficacy of a heterologous antigen delivered by live attenuated *Salmonella enterica* vaccines. Infect Immun. 2008; 76(11):5238–5246. [PubMed: 18765737]
51. Shi H, Santander J, Brenneman KE, et al. Live recombinant *Salmonella* Typhi vaccines constructed to investigate the role of *rpoS* in eliciting immunity to a heterologous antigen. PLoS One. 2010; 5(6):e11142. [PubMed: 20585446]
52. Kong W, Wanda SY, Zhang X, et al. Regulated programmed lysis of recombinant *Salmonella* in host tissues to release protective antigens and confer biological containment. Proc Natl Acad Sci U S A. 2008; 105(27):9361–9366. [PubMed: 18607005]
53. Kong W, Brovold M, Koeneman BA, Clark-Curtiss J, Curtiss R 3rd. Turning self-destructing *Salmonella* into a universal DNA vaccine delivery platform. Proc Natl Acad Sci U S A. 2012; 109(47):19414–19419. [PubMed: 23129620]
54. Kong Q, Six DA, Roland KL, et al. *Salmonella* synthesizing 1-dephosphorylated [corrected] lipopolysaccharide exhibits low endotoxic activity while retaining its immunogenicity. J Immunol. 2011; 187(1):412–423. [PubMed: 21632711]
55. Galen JE, Simon R, Ernst RK. *Salmonella* expressing detoxified lipopolysaccharide is immunogenic and protective both as an attenuated vaccine and for delivery of foreign antigens. Expert Rev Vaccines. 2011; 10(12):1679–1682. [PubMed: 22085171]
56. Hone DM, Harris AM, Levine MM. Adaptive acid tolerance response by *Salmonella typhi* and candidate live oral typhoid vaccine strains. Vaccine. 1994; 12(10):895–898. [PubMed: 7975830]
57. McConnell EL, Basit AW, Murdan S. Measurements of rat and mouse gastrointestinal pH, fluid and lymphoid tissue, and implications for in-vivo experiments. J Pharm Pharmacol. 2008; 60(1): 63–70. [PubMed: 18088506]
58. Verdu EF, Fraser R, Armstrong D, Blum AL. Effects of omeprazole and lansoprazole on 24-hour intragastric pH in *Helicobacter pylori* -positive volunteers. Scand J Gastroenterol. 1994; 29(12): 1065–1069. [PubMed: 7886393]
59. Viala JP, Meresse S, Pocachard B, Guilhon AA, Aussel L, Barras F. Sensing and adaptation to low pH mediated by inducible amino acid decarboxylases in *Salmonella*. PLoS One. 2011; 6(7):e22397. [PubMed: 21799843]
60. Brenneman KE, Willingham C, Kong W, Curtiss R 3rd, Roland KL. Low-pH rescue of acid-sensitive *Salmonella enterica* serovar Typhi strains by a rhamnose-regulated arginine decarboxylase aystem. J Bacteriol. 2013; 195(13):3062–3072. [PubMed: 23645603]

61. Galan JE, Nakayama K, Curtiss R 3rd. Cloning and characterization of the *asd* gene of *Salmonella typhimurium*: use in stable maintenance of recombinant plasmids in *Salmonella* vaccine strains. Gene. 1990; 94(1):29–35. [PubMed: 2227450]
62. Morona R, Yeadon J, Considine A, Morona JK, Manning PA. Construction of plasmid vectors with a non-antibiotic selection system based on the *Escherichia coli thyA* + gene: application to cholera vaccine development. Gene. 1991; 107(1):139–144. [PubMed: 1720753]
63. Angelakopoulos H, Hohmann EL. Pilot study of *phoP/phoQ* -deleted *Salmonella enterica* serovar Typhimurium expressing *Helicobacter pylori* urease in adult volunteers. Infect Immun. 2000; 68(4):2135–2141. [PubMed: 10722611]
64. Ryan ET, Crean TI, Kochi SK, et al. Development of a Δ*glnA* balanced lethal plasmid system for expression of heterologous antigens by attenuated vaccine vector strains of *Vibrio cholerae*. Infect Immun. 2000; 68(1):221–226. [PubMed: 10603391]
65. Garmory HS, Leckenby MW, Griffin KF, et al. Antibiotic-free plasmid stabilization by operator-repressor titration for vaccine delivery by using live *Salmonella enterica* serovar Typhimurium. Infect Immun. 2005; 73(4):2005–2011. [PubMed: 15784541]
66. Sizemore DR, Warner EA, Lawrence JA, Thomas LJ, Roland KL, Killeen KP. Construction and screening of attenuated Δ*phoP/Q Salmonella typhimurium* vectored plague vaccine candidates. Hum Vaccin Immunother. 2012; 8(3):371–383. [PubMed: 22327496]
67. Klose KE, Mekalanos JJ. Simultaneous prevention of glutamine synthesis and high-affinity transport attenuates *Salmonella typhimurium* virulence. Infect Immun. 1997; 65(2):587–596. [PubMed: 9009317]
68. Galen JE, Wang JY, Chinchilla M, et al. A new generation of stable, nonantibiotic, low-copy-number plasmids improves immune responses to foreign antigens in *Salmonella enterica* serovar Typhi live vectors. Infect Immun. 2010; 78(1):337–347. [PubMed: 19884333] **The authors describe a novel method for antibiotic-free plasmid selection and maintenance that does not depend on the catalytic activity of a gene product. The paper also demonstrates and inverse relationship between plasmid copy number and plasmid stability.
69. Galen JE, Zhao L, Chinchilla M, et al. Adaptation of the endogenous *Salmonella enterica* serovar Typhi *clyA* -encoded hemolysin for antigen export enhances the immunogenicity of anthrax protective antigen domain 4 expressed by the attenuated live-vector vaccine strain CVD 908-*htrA*. Infect Immun. 2004; 72(12):7096–7106. [PubMed: 15557633]
70. Tacket CO, Levine MM. CVD 908, CVD 908-*htrA* and CVD 909 live oral typhoid vaccines: a logical progression. Clin Infect Dis. 2007; 45(Suppl 1):S20–S23. [PubMed: 17582563] *This paper provides a detailed history of the CVD 908 series of vaccine strains and vectors, including the rationale for attenuation and clinical trials results.
71. Santander J, Roland KL, Curtiss R 3rd. Regulation of Vi capsular polysaccharide synthesis in *Salmonella enterica* serotype Typhi. J Infect Dev Ctries. 2008; 2(6):412–420. [PubMed: 19745516]
72. Wang JY, Noriega FR, Galen JE, Barry E, Levine MM. Constitutive expression of the Vi polysaccharide capsular antigen in attenuated *Salmonella enterica* serovar typhi oral vaccine strain CVD 909. Infect Immun. 2000; 68(8):4647–4652. [PubMed: 10899868]
73. Tacket CO, Pasetti MF, Sztein MB, Livio S, Levine MM. Immune responses to an oral typhoid vaccine strain that is modified to constitutively express Vi capsular polysaccharide. J Infect Dis. 2004; 190(3):565–570. [PubMed: 15243933]
74. Wahid R, Salerno-Goncalves R, Tacket CO, Levine MM, Sztein MB. Cell-mediated immune responses in humans after immunization with one or two doses of oral live attenuated typhoid vaccine CVD 909. Vaccine. 2007; 25(8):1416–1425. [PubMed: 17182155]
75. Galen JE, Chinchilla M, Pasetti MF, et al. Mucosal immunization with attenuated *Salmonella enterica* serovar Typhi expressing protective antigen of anthrax toxin (PA83) primes monkeys for accelerated serum antibody responses to parenteral PA83 vaccine. J Infect Dis. 2009; 199(3):326–335. [PubMed: 19099487]
76. Baillie LW, Rodriguez AL, Moore S, et al. Towards a human oral vaccine for anthrax: the utility of a *Salmonella* Typhi Ty21a-based prime-boost immunization strategy. Vaccine. 2008; 26(48): 6083–6091. [PubMed: 18805452]

77. Wahid R, Pasetti MF, Maciel M Jr, et al. Oral priming with *Salmonella* Typhi vaccine strain CVD 909 followed by parenteral boost with the *S*. Typhi Vi capsular polysaccharide vaccine induces CD27+IgD- *S*. Typhi-specific IgA and IgG B memory cells in humans. Clin Immunol. 2011; 138(2):187–200. [PubMed: 21146460]

78. Wahid R, Simon R, Zafar SJ, Levine MM, Sztein MB. Live oral typhoid vaccine Ty21a induces cross-reactive humoral immune responses against *Salmonella enterica* serovar Paratyphi A and S Paratyphi B in humans. Clin Vaccine Immunol. 2012; 19(6):825–834. [PubMed: 22492745] * Evaluates the ability of the licensed typhoid vaccine Ty21a to provide protection against other forms of enteric fever. Demonstrates that the immune response that develops against Ty21a homes to the gut mucosal tissues and includes the generation of memory B cells.

79. Khan S, Chatfield S, Stratford R, et al. Ability of SPI2 mutant of *S. typhi* to effectively induce antibody responses to the mucosal antigen enterotoxigenic *E. coli* heat labile toxin B subunit after oral delivery to humans. Vaccine. 2007; 25(21):4175–4182. [PubMed: 17412462]

80. Stratford R, McKelvie ND, Hughes NJ, et al. Optimization of *Salmonella enterica* serovar Typhi Δ*aroC Δ*ssaV* derivatives as vehicles for delivering heterologous antigens by chromosomal integration and in vivo inducible promoters. Infect Immun. 2005; 73(1):362–368. [PubMed: 15618173]

81. Fang FC, Libby SJ, Buchmeier NA, et al. The alternative sigma factor *katF* (*rpoS*) regulates *Salmonella* virulence. Proc Natl Acad Sci U S A. 1992; 89(24):11978–11982. [PubMed: 1465428]

82. Pakkanen SH, Kantele JM, Kantele A. Cross-reactive gut-directed immune response against *Salmonella enterica* serovar Paratyphi A and B in typhoid fever and after oral Ty21a typhoid vaccination. Vaccine. 2012; 30(42):6047–6053. [PubMed: 22858557]

83. Kantele A, Pakkanen SH, Siitonen A, Karttunen R, Kantele JM. Live oral typhoid vaccine *Salmonella* Typhi Ty21a - a surrogate vaccine against non-typhoid salmonella? Vaccine. 2012; 30(50):7238–7245. [PubMed: 23084770]

84. Osorio M, Wu Y, Singh S, et al. Anthrax protective antigen delivered by *Salmonella enterica* serovar Typhi Ty21a protects mice from a lethal anthrax spore challenge. Infect Immun. 2009; 77(4):1475–1482. [PubMed: 19179420]

85. Roszczenko P, Radomska KA, Wywial E, Collet JF, Jagusztyn-Krynicka EK. A novel insight into the oxidoreductase activity of *Helicobacter pylori* HP0231 protein. PLoS One. 2012; 7(10):e46563. [PubMed: 23056345]

86. Aebischer T, Bumann D, Epple HJ, et al. Correlation of T cell response and bacterial clearance in human volunteers challenged with *Helicobacter pylori* revealed by randomised controlled vaccination with Ty21a-based *Salmonella* vaccines. Gut. 2008; 57(8):1065–1072. [PubMed: 18417532]

87. Bumann D, Behre C, Behre K, et al. Systemic, nasal and oral live vaccines against *Pseudomonas aeruginosa*: a clinical trial of immunogenicity in lower airways of human volunteers. Vaccine. 2010; 28(3):707–713. [PubMed: 19887136]

88. Knapp B, Hundt E, Lenz U, et al. A recombinant hybrid outer membrane protein for vaccination against *Pseudomonas aeruginosa*. Vaccine. 1999; 17(13–14):1663–1666. [PubMed: 10194820]

89. Niethammer AG, Lubenau H, Mikus G, et al. Double-blind, placebo-controlled first in human study to investigate an oral vaccine aimed to elicit an immune reaction against the VEGF-Receptor 2 in patients with stage IV and locally advanced pancreatic cancer. BMC Cancer. 2012; 12:361. [PubMed: 22906006]

90. Niethammer AG, Xiang R, Becker JC, et al. A DNA vaccine against VEGF receptor 2 prevents effective angiogenesis and inhibits tumor growth. Nat Med. 2002; 8(12):1369–1375. [PubMed: 12415261]

91. Sorenson B, Banton K, Augustin L, et al. Safety and immunogenicity of *Salmonella typhimurium* expressing C-terminal truncated human IL-2 in a murine model. Biologics. 2010; 4:61–73. [PubMed: 20376175]

92. Sorenson BS, Banton KL, Augustin LB, Leonard AS, Saltzman DA. Antioxidant oils and *Salmonella enterica* Typhimurium reduce tumor in an experimental model of hepatic metastasis. Onco Targets Ther. 2011; 4:59–69. [PubMed: 21691578]

93. Hindle Z, Chatfield SN, Phillimore J, et al. Characterization of *Salmonella enterica* derivatives harboring defined *aroC* and *Salmonella* pathogenicity island 2 type III secretion system (*ssaV*) mutations by immunization of healthy volunteers. Infect Immun. 2002; 70(7):3457–3467. [PubMed: 12065485]

94. Tacket CO, Hone DM, Curtiss R 3rd, et al. Comparison of the safety and immunogenicity of Δ*aroC Δ aroD* and Δ*cya Δcrp Salmonella typhi* strains in adult volunteers. Infect Immun. 1992; 60(2):536–541. [PubMed: 1730487]

95. Kirkpatrick BD, McKenzie R, O'Neill JP, et al. Evaluation of *Salmonella enterica* serovar Typhi (Ty2 *aroC-ssaV*-) M01ZH09, with a defined mutation in the *Salmonella* pathogenicity island 2, as a live, oral typhoid vaccine in human volunteers. Vaccine. 2006; 24(2):116–123. [PubMed: 16140433]

96. Lyon CE, Sadigh KS, Carmolli MP, et al. In a randomized, double-blinded, placebo-controlled trial, the single oral dose typhoid vaccine, M01ZH09, is safe and immunogenic at doses up to 1.7 x 10(10) colony-forming units. Vaccine. 2010; 28(20):3602–3608. [PubMed: 20188175]

97. Tran TH, Nguyen TD, Nguyen TT, et al. A randomised trial evaluating the safety and immunogenicity of the novel single oral dose typhoid vaccine M01ZH09 in healthy Vietnamese children. PLoS One. 2010; 5(7):e11778. [PubMed: 20668668]

Websites

101. [accessed March 7, 2013] IL-2 expressing attenuated *Salmonella* Typhimurium in unresectable hepatic spread. http://www.clinicaltrials.gov/ct2/show/NCT01099631?term=salmonella+cancer&rank=1

102. [accessed March 7, 2013] Recombinant attenuated *Salmonella* Typhi vaccine vectors producing *Streptococcus pneumoniae* PspA. http://www.clinicaltrials.gov/ct2/show/NCT01033409?term=salmonella+pneumonia&rank=1

103. [accessed March 7, 2013] Safety and immunogenicity of CVD 1902 oral attenuated vaccine to prevent *S*. Paratyphi A infection. http://www.clinicaltrials.gov/ct2/show/NCT01129453?term=paratyphi+a&rank=1

Key Issues

1. *S*. Typhi vaccine vectors do not produce strong immune responses in humans. This is true not only for typhoid antigens, but especially for vectored heterologous antigens.
2. A broader understanding of *S*. Typhi pathogenesis allows researchers to better understand how to attenuate vaccine strains.
3. Predictive correlates for human immunity and reactogenicity must be established to provide a basis for interpreting preclinical data.
4. Development of alternative, relevant animal models will lead to more immunogenic, safer *Salmonella*-vectored vaccines. Use of these new models will allow more effective screening of vaccine candidates, which should improve the chances for successful clinical trials and development of effective vaccines.
5. Recent research has introduced a variety of new vaccine approaches, including attenuation strategies, antigen synthesis and antigen delivery mechanisms.
6. Recent research has also elucidated some of the key differences between *S*. Typhi and *S*. Typhimurium, allowing researchers to design vaccines that take these differences into account.

Table 1

Clinical trials evaluating *Salmonella*–vectored vaccine strains.

Serovar	Strain	Attenuating mutations	Antigen	Plasmid stabilization	Antigen gene promoter	Study type	Reference
Typhi	TSB7	*ΔaroC ΔssaV*	LT-B	Not applicable	P_{ssaG}	Open label	[79]
Typhi	χ9639(pYA4088)	$RpoS^-$ and footnote [1]	PspA	$AsdA^+$	P_{trc} regulated	Double blind	[102]
Typhi	χ9640(pYA4088)	$RpoS^+$ and footnote [1]	PspA	$AsdA^+$	P_{trc} regulated	Double blind	[102]
Typhi	χ9633(pYA4088)	$RpoS^+$ and footnote [2]	PspA	$AsdA^+$	P_{trc} regulated	Double blind	[102]
Typhi	CVD908-*htrA*	*ΔaroC ΔaroD htrA thyA*	OprF-OprI	$ThyA^+$	Pyz	Not reported	[87]
Typhi	CVD908-*htrA*	*ΔaroC ΔaroD htrA thyA*	OprF-OprI	$ThyA^+$	P_{pagC}	Not reported	[87]
Typhi	Ty21a	*galE thyA* Vi^- Multiple mutations	OprF-OprI	$ThyA^+$	Pyz	Not reported	[87]
Typhi	Ty21a	*galE thyA* Vi^- Multiple mutations	OprF-OprI	$ThyA^+$	P_{pagC}	Not reported	[87]
Typhi	Ty21a	*galE thyA* Vi^- Multiple mutations	Urease	$ThyA^+$	Undescribed constitutive promoter	Double blind	[86]
Typhi	Ty21a	*galE thyA* Vi^- Multiple mutations	HP0231	$ThyA^+$	Undescribed constitutive promoter	Double blind	[86]
Typhi	VXM01 (Ty21a)	*galE thyA* Vi^- Multiple mutations	VEGFR-2	Not described	CMV	Double blind	[89]
Typhimurium	*SalpIL2*	*Δcya Δcrp ΔasdA*	Human IL-2	$AsdA^+$	P_{trc} constitutive	Open label	[103]

[1] ΔP_{crp527}:TT *araC* P_{BAD}*crp* ΔP_{fur81}:TT *araC* P_{BAD}*fur* Δ*pmi-2426* Δ (*gmd-fcl*)*-26* Δ*sopB1925* Δ*relA198*:*araC* P_{BAD}*lacI* TT Δ*araE25* Δ*tviABCDE10* Δ*agfBAC811* Δ*asdA33*

[2] ΔP_{crp527}:TT *araC* P_{BAD}*crp* ΔP_{fur81}:TT *araC* P_{BAD}*fur* Δ*pmi-2426* Δ*(gmd-fcl)-26* Δ*sopB1925* Δ*relA198*:*araC* P_{BAD}*lacI* TT Δ*araE25* Δ*araBAD* 23 Δ*tviABCDE10* Δ*agfBAC811* Δ*asdA33*

Table 2

Clinical trials to evaluate live attenuated strains as paratyphoid or typhoid fever vaccines.

Serovar	Strain	Mutations	Comments	Reference
Paratyphi A	CVD1902	*guaBA clpX*		[103]
Typhi	CVD909	*aroC aroD htrA* P_{tac} *tviA*	prime boost	[72, 75]
Typhi	M01ZH09 (ZH9)	*ΔaroC ΔssaV*	Phase I, US volunteers	[91]
Typhi	M01ZH09	*aroC ssaV*	Phase I, US volunteers	[95]
Typhi	M01ZH09	*aroC ssaV*	Phase II, US volunteers	[96]
Typhi	M01ZH09	*aroC ssaV*	Phase II, Vietnamese children	[97]