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Live Attenuated Human *Salmonella* Vaccine Candidates: Tracking the Pathogen in Natural Infection and Stimulation of Host Immunity

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ABSTRACT Salmonellosis, caused by members of the genus *Salmonella*, is responsible for considerable global morbidity and mortality in both animals and humans. In this review, we will discuss the pathogenesis of *Salmonella enterica* serovar Typhi and *Salmonella enterica* serovar Typhimurium, focusing on human *Salmonella* infections. We will trace the path of *Salmonella* through the body, including host entry sites, tissues and organs affected, and mechanisms involved in both pathogenesis and stimulation of host immunity. Careful consideration of the natural progression of disease provides an important context in which attenuated live oral vaccines can be rationally designed and developed. With this in mind, we will describe a series of attenuated live oral vaccines that have been successfully tested in clinical trials and demonstrated to be both safe and highly immunogenic. The attenuation strategies summarized in this review offer important insights into further development of attenuated vaccines against other *Salmonella* for which live oral candidates are currently unavailable.

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INTRODUCTION

Salmonellosis, caused by oral infection with members of the genus *Salmonella*, is responsible for considerable global morbidity and mortality, in both animals and humans (1, 2). The genus *Salmonella* contains two species, *Salmonella enterica* and *Salmonella bongori*, each of which contains multiple serotypes of genetically distinct organisms (3). There are currently over 2,500 serotypes (referred to as serovars) of *Salmonella* as defined by immunologic identification of somatic O and flagellar H antigens. In turn, *S. enterica* is divided into six subspecies (subsp.), of which only one, *S. enterica* subsp. *enterica* colonizes warm-blooded animals; the remaining subspecies are typically isolated from cold-blooded animals and the environment (3). It was recently reported that, worldwide, the highest morbidities from human

infections resulting from food-borne diseases involved food contaminated with *S. enterica* (1, 2).

In this review, we will focus on *S. enterica* subsp. *enterica*, encompassing the majority of serovars identified thus far, and limit our discussion to vaccine candidates developed to prevent human disease that have been tested in human clinical trials. We will first describe the features of *S. enterica* infections in humans, including the host entry sites, tissues and organs affected, and mechanisms involved in pathogenesis; this information will provide a context for later description of vaccine candidates and the genetic strategies employed to rationally attenuate *Salmonella*. We will then describe general immune responses elicited by natural oral infection with *Salmonella* and discuss how these responses originate in relation to immunologically primed tissue. Finally, we will present clinical data derived from studies involving *S. enterica* serovars Typhi (the causative agent of typhoid fever) and Typhimurium (which typically causes gastroenteritis in humans). The pathogenic mechanisms explored here for two clinically relevant serovars, and genetic strategies employed to create attenuated but highly immunogenic live oral mucosal vaccines, can be applied to other *Salmonella* serovars for which vaccines are urgently needed (4).

PATHOBIOLOGY OF *SALMONELLA* INFECTIONS IN HUMANS

Overcoming the Gastric Acid Barrier

Infection with *Salmonella* is initiated following oral ingestion of contaminated food or water (see Fig. 1A). The minimum infectious dose required for establishing a productive infection depends on several key microbiological factors including the strain and serovar of *Salmonella* involved, as well as important host factors including the host mammalian species to be colonized and various gastrointestinal barriers to infection.

In humans, one significant physiological barrier strongly influencing the infectious dose is gastrointestinal acidity. *Salmonella* must overcome potentially lethal levels of inorganic acid (H+) which produce a pH as low as 2 in the stomach of healthy adults (Fig. 1B) (5). In human challenge studies conducted in the early 1970s at the University of Maryland, ~10^7 colony forming units (CFUs) was the minimum infectious dose required to establish infection in >50% of volunteers orally challenged with a

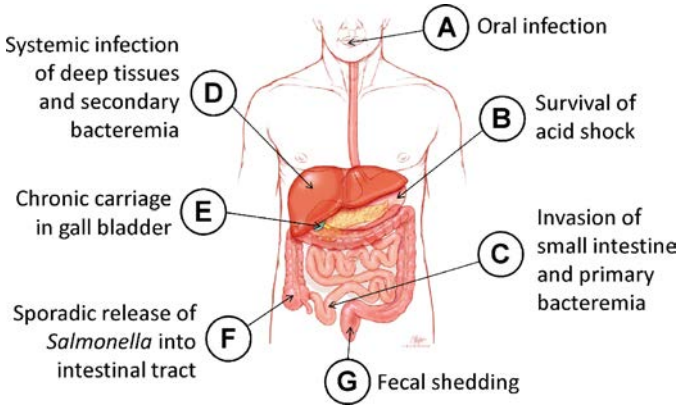

Figure 1 Pathobiology of human *Salmonella* infections. Infection with *Salmonella* is initiated following oral ingestion of contaminated food or water (A). *Salmonella* must then overcome potentially lethal levels of inorganic acid (H+) which produce pHs as low as 2 in the stomach of healthy adults (B). *Salmonella* organisms surviving the extreme acidic conditions of the stomach eventually drain into the small intestine, the portal for invasion into deeper tissues and development of systemic disease (C). *Salmonella* invade tissues of both villus epithelial tissue as well as lymphoid Peyer's patches. Following transit of invading *Salmonella* out of the lumen and across the epithelial barrier of the small intestine, bacteria eventually gain transient access to the bloodstream to eventually colonize deep tissues including the liver (D), spleen, and bone marrow. It is at this stage that infection with *S.* Typhimurium is typically halted and does not progress to systemic disease in immunologically competent humans. However, *S.* Typhi can be released from deep tissues back into the bloodstream, triggering a more substantial secondary bacteremia which precedes the onset of classic typhoid fever. In rare cases, typhoid fever can progress to an asymptomatic chronic infection in which *S.* Typhi can migrate down the hepatic ducts of the liver and into the gallbladder (E), setting up a convalescent carrier state in which very high levels of organisms can be intermittently released back into the small intestine, passing through the large intestine (F) and being released in the feces (G).

fully virulent *S.* Typhi Quailes strain given with milk (6); however, in recent challenge studies conducted at the University of Oxford, using the identical Quailes strain administered to volunteers who first ingested bicarbonate solution to neutralize stomach acidity, the dose required to establish infection in >50% of volunteers was reduced by a factor of 4 logs to 10^3 CFUs (7).

Encountering low pH is believed to provide an important environmental signal to *Salmonella* for deploying a cascade of virulence factors necessary for host cell invasion (8). *Salmonella* is equipped with a variety of genetic strategies that contribute to their survival and growth. Two key proteins involved in reallocation of metabolic resources to survive acid stress (called the acid tolerance response [ATR]) are RpoS and OmpR. RpoS is an al-

ternate sigma factor used by RNA polymerase to enhance cell survival under acidic conditions by switching RNA transcription and subsequent protein synthesis during exponential growth in nutrient-rich conditions to a much slower stationary phase growth in which a variety of acid resistance genes are induced (8). OmpR is also involved in this ATR, and is the effector component of a two-component environmental sensor, in which the sensor EnvZ activates OmpR upon exposure to both acidity and osmolarity (8). In addition to osmoregulation and acid tolerance, OmpR is also involved in the induction of intracellular survival factors required for survival of *Salmonella* within intracellular vacuoles, following invasion of permissive eukaryotic cells such as macrophages and intestinal epithelial cells (9).

The central role of RpoS in the survival of *Salmonella* has been exploited for the development of live attenuated vaccines against typhoid fever. Several candidate vaccines tested in clinical trials have been engineered from wild-type *S.* Typhi Ty2 strains in which the *rpoS* gene has been naturally inactivated (10). When attenuated vaccine candidates derived from Ty2 were compared with identically attenuated candidates derived from wild-type *S.* Typhi ISP1820 strains, in which *rpoS* was intact, these RpoS+ strains were unacceptably reactogenic (11). It has also been shown that the only licensed and well-tolerated live oral vaccine against typhoid fever, Ty21a, which was created by chemical mutagenesis of Ty2 resulting in multiple attenuating chromosomal lesions, is also deficient in synthesis of RpoS; consequently, this vaccine requires multiple doses to confer protection against disease and displays no acid tolerance response (12).

Invasion of the Small Intestine

Salmonella organisms that can survive the extreme acidic conditions of the stomach eventually drain into the small intestine, the portal for invasion into deeper tissues and development of systemic disease (Fig. 1C). It is at this stage of infection that differences in the serovar-specific progression of disease most clearly manifest themselves. *S.* Typhi possesses several essential clusters of pathogenicity genes, grouped into distinctly regulated chromosomal locations called *Salmonella* pathogenicity islands (SPIs), which enable invading *S.* Typhi organisms to reach deeper tissues of the human liver, spleen, and bone marrow, and bypass innate immunity clearance mechanisms (13, 14). In contrast, *S.* Typhimurium is not equipped with several of these essential intracellular survival mechanisms required for deep penetration of human tissues; therefore, infection of immunocompetent individuals with *S.* Typhimurium typically results only in local tissue invasion and a self-limiting gastroenteritis (13).

It is believed that Peyer's patches are important sites involved in transepithelial migration for *Salmonella* across the luminal surface of the small intestine, based on murine experimental challenge studies with *S.* Typhimurium (15). While direct *in vivo* evidence supporting invasion of Peyer's patches by *S.* Typhi in humans is lacking, several observations support this notion. Examination of intestinal mucosal biopsies from volunteers orally challenged with 10^9 CFUs of the Quailes strain showed granulomatous lesions throughout the small intestine including the duodenum, jejunum, and ileum, accompanied by infiltration of inflammatory cells, and coinciding with systemic clinical symptoms including fever and positive blood cultures (16). Peyer's patches are present throughout the human small intestine, with densities increasing through the jejunum and the largest patches typically residing in the terminal ileum in both children and adults (17, 18), and *Salmonella* is believed to take advantage of this antigen-sampling compartment of the gastrointestinal tract to invade deeper tissues (19).

In addition to invading Peyer's patches, *Salmonella* is also likely to invade villous epithelial tissue of the small intestine. *Salmonella* are equipped with a molecular arsenal of pathogenicity factors, some of which are common to both *S.* Typhimurium and *S.* Typhi, enabling rapid and efficient invasion of intestinal epithelial tissues. One critical environmental signal orchestrating a cascade of virulence factors that participate in the invasion process at precisely the right time is osmolarity. Villi of the small intestine possess a gradient of osmolarity that is highest at the luminal surface of villi (~700 mOsm kg^{-1} H_2O) and decreases to physiological levels (~300 mOsm kg^{-1} H_2O) in the lower crypts of the villus (20, 21). Incoming luminal *Salmonella* sense the high osmolarity of villus surfaces, which in turn signals induction of a pathogenicity island common to all *S. enterica* serovars capable of infecting mammals, called SPI-1. This locus encodes a type III secretion apparatus that injects effector proteins into target eukaryotic cells, resulting in ruffling of the outer membrane and engulfment of invading bacteria (22).

The transition from extracellular to intracellular pathogen induces an extensive reorganization of both bacterial

metabolism and virulence factors. Upon transitioning across the epithelial barrier, the osmolarity of the surrounding tissue drops to physiological levels, providing a critical signal for *Salmonella* to downregulate SPI-1 while inducing synthesis of intracellular survival proteins injected into infected cells by a separate and distinct SPI-2 type III secretion system (23). At this stage of infection, crucial genetic differences between invading *S.* Typhimurium and *S.* Typhi begin to strongly influence the course and manifestation of disease (24). Although both serovars possess fully functional SPI-2 and ancillary effector virulence proteins, *S.* Typhi is also equipped with several additional genomic modifications, allowing it to avoid the host natural inflammatory response to invading organisms, which is characterized by a massive and rapid influx of neutrophils; this is not the case for *S.* Typhimurium, which causes acute gut inflammation.

S. Typhi has been characterized as an excellent example of "reductive genomic evolution" of a human pathogen (25). In contrast to *S.* Typhimurium, *S.* Typhi has evolved to become an exclusive human pathogen, incapable of establishing a productive natural infection in any other mammalian species, and relying on the host to provide multiple essential factors required for survival and growth. In adapting exclusively to infection of humans, *S.* Typhi has naturally accumulated a series of genetic disruptions and inactivations involving approximately 5% of its genome (26). Such inactivations include both loss of metabolic capacity and modifications to the bacterial outer membrane surface that reduce interaction and signaling via Toll-like Receptors (TLRs) expressed by innate immune cells. In contrast, recent data suggest that *S.* Typhimurium may actually benefit from initiation of an inflammatory response because incoming innate immune cells inadvertently generate a critical metabolite called tetrathionate from the oxidative burst; tetrathionate is used exclusively by *S.* Typhimurium as an alternate electron acceptor in anaerobic respiration, and therefore becomes available for enhanced growth prior to excretion from the colon (27, 28). Given that *S.* Typhi spends relatively little time in the intestine prior to invasion and systemic infection, it comes as no surprise that *S.* Typhi has lost the ability to utilize tetrathionate for anaerobic respiration (29).

Transepithelial migration and the accompanying drop in osmolarity induces yet another pathogenicity island, exclusive to *S.* Typhi, called SPI-7 (30, 31, 32), which encodes an outer polysaccharide capsule called Vi (26). The Vi capsule shields lipopolysaccharide (LPS) in the bacterial outer membrane from signaling an inflammatory sensor on the surface of phagocytic cells called Toll-like receptor 4 (TLR4) (33). Signaling of TLR4 induces the secretion of cytokines tumor necrosis factor alpha (TNF-α) and interleukin (IL)-6, which are responsible for recruitment of neutrophils and other inflammatory cells to the site of infection (13). To further reduce TLR4 interaction and signaling by invading organisms progressing to systemic tissues, *S.* Typhi has acquired an inactivating mutation in *fepE*, a gene controlling the length of the repeating O antigen comprising *S.* Typhi LPS. The result of losing *fepE* is that synthesis of extremely long LPS (>200 repeat units) is now blocked, and LPS is no longer able to protrude through the protective layer of the Vi capsule (34). Surface expression of the Vi capsule also interferes with neutrophil chemotaxis and phagocytic killing of invading organisms by blocking complement deposition onto the surface of *S.* Typhi; the Vi capsule is composed of a homopolymer of saccharides devoid of free hydroxyl groups required for deposition of the complement component C3b, which in turn activates the complement cascade through the alternative pathway, thereby generating the chemoattractant C5a which attracts neutrophils (35).

Remarkably, the SPI-7 locus also encodes a regulatory protein called TviA, which, while upregulating expression of Vi, also downregulates expression of flagellin (31), another powerful innate signaling protein that binds to and activates TLR5 in the basolateral membrane of intestinal epithelial cells (36, 37). Activation of TLR5 induces secretion of another potent cytokine, IL-8, which is also a powerful recruiter of neutrophils and other inflammatory cells to the site of invading pathogens. Importantly, flagellin is not expressed once *S.* Typhi has invaded macrophages, thereby reducing pyroptosis of infected cells and further recruitment of neutrophils (38). This strategy of reducing pyroptosis also prevents release of *S.* Typhi from its protected intracellular niche, thus facilitating systemic dissemination.

Transient Primary Bacteremia

Following transit of invading *Salmonella* out of the lumen and across the epithelial barrier of the small intestine, bacteria eventually gain access to the bloodstream for a brief period of time (39), facilitating a more sustained infection of the liver, spleen, and bone marrow (Fig. 1D). The passage of bacteria into the bloodstream

involves intracellular persistence within human macrophages. The ability of *Salmonella* to survive and replicate within these cells, thereby facilitating systemic infection, clearly differentiates *S.* Typhi from *S.* Typhimurium. Compared with *S.* Typhimurium, *S.* Typhi has up to 100-fold higher survival rates in elutriated primary human macrophages from peripheral blood, with intracellular replication resulting in very little cell death (40); interestingly, the *rpoS*+ strain ISP1820 survives better in human macrophages than the *rpoS*– strain Ty2 (40), again suggesting a role for *rpoS* in host survival.

It has been reported that bacteremia can be detected by PCR in volunteers challenged with the *S.* Typhi Quailes strain within 12 hours after oral ingestion of organisms (41). Culture positive detection of *S.* Typhi is observed in the monocyte fraction of the peripheral blood cells recovered from naturally infected typhoid patients (42). *S.* Typhi is present at very low levels of ~2 CFU/ml in blood from patients with uncomplicated enteric fever during the first week of illness. This level drops to ~1 CFU/ml during the second and third weeks, and declines to ~0.3 CFU/ml during the fourth week; bloodborne *S.* Typhi is not usually present as extracellular organisms, with approximately 63% of viable bacteria residing intracellularly in peripheral blood cells (43). Much higher levels of *S.* Typhi can be detected in the bone marrow of patients with confirmed uncomplicated typhoid fever, with ~5 CFU/ml in bone marrow aspirate recovered in the first week of illness and increasing to ~160 CFU/ml during the third week; again most bacteria are intracellular and therefore less susceptible to antibiotic treatment (44).

Establishment of the Carrier State and Shedding

Invading *S.* Typhi reaching the liver, spleen, and bone marrow can replicate within resident macrophages, and can then be released back into the bloodstream, triggering a more substantial secondary bacteremia that precedes the onset of classic typhoid fever (45, 46). However, 2 to 5% of typhoid cases (47, 48) eventually progress to an asymptomatic chronic infection in which *S.* Typhi can migrate through the hepatic ducts of the liver and into the gallbladder (Fig. 1E), setting up a convalescent carrier state in which very high levels of organisms can be intermittently released back into the small intestine, passing through the large intestine (Fig. 1F), and being shed in the feces at levels as high as 10^6 to 10^{10} viable organisms per gram of stool (Fig. 1G) (49). This intermittent shedding facilitates further spread of the disease to susceptible individuals through contaminated food or water. Chronic infection of the gallbladder is often accompanied by the presence of gallstones, upon which *S.* Typhi are able to establish robust colonization through the formation of biofilms. These biofilms are composed of polysaccharides including Vi and LPS but only minor amounts of flagellar proteins (50). *S.* Typhi residing in biofilms is recalcitrant to antibiotic treatment regardless of the inherent susceptibility of the pathogen. Further direct infection of gallbladder epithelium by free *S.* Typhi growing in the gallbladder lumen can take place via osmotic activation of the SPI-1 invasion locus, resulting in tissue damage and sloughing of epithelial cells down the bile duct and back into the duodenum of the small intestine (51, 52). Interestingly, although acute typhoid fever does not elicit appreciable antibody responses against Vi antigen, chronic carriers are able to mount a very high Vi-specific serum antibody response, which has been shown to be an excellent diagnostic marker for the carrier state (53, 54).

Late-Stage Complications and Death

Throughout the discussion of *S.* Typhi infections, we have described the pathobiology of acute typhoid fever, without the involvement or influence of life-threatening extraintestinal complications. However, while the vast majority of typhoid cases resolve without life-threatening sequelae, up to 10% of typhoid patients can develop serious complications, including death from gastrointestinal hemorrhages and peritonitis from intestinal perforation (39). The rate of intestinal perforations in patients with typhoid fever worldwide has been estimated to be 3% (55). The average case fatality rate for intestinal perforation was reported to be 15%, with rates as high as 40% depending on geographic location (56). Of note, intestinal perforations usually occur within 45 cm of the ileocecal valve (57), an area with the highest density of Peyer's patches and an important portal of invasion for *S.* Typhi (17, 18).

STRATEGIES FOR ATTENUATION AND DESIGN OF HUMAN LIVE ORAL *SALMONELLA* VACCINES

Immune Responses to Natural Infection

Understanding the progression of human *Salmonella* infections and immune mechanisms that can control infection greatly facilitates the rational design of efficacious live attenuated vaccines against these organisms.

Unlike many other enteric pathogens, infection with *Salmonella* does not typically induce a long-lasting protective immunity. Pathogen-specific antibodies, used as readouts of pathogen exposure and immunological priming, are important for clearance only while *Salmonella* is extracellular or within the lumen of the intestines. These antibodies block motility and facilitate complement-mediated lysis or phagocytic killing. Studies in typhoid patients from Bangladesh, where *Salmonella* is endemic, have shown elevated serum IgA and IgG antibodies against LPS, whole-cell extract, and membrane preparations, during infection (58), although membrane preparation-specific IgA antibodies were more prevalent than IgG. These responses are highest in adults, yet children also develop IgA antibodies specific for *S.* Typhi membrane antigens which decline during late convalescence (59).

Although it is evident that both innate and humoral immune responses are required for control of *Salmonella* infections, cellular immunity is believed to play a crucial role in the clearance of *Salmonella* residing intracellularly. However, the exact roles of individual cell types and mechanisms underlying protective immunity remain to be elucidated. Upon infection of phagocytic and professional antigen-presenting cells (i.e., macrophages and dendritic cells) *Salmonella* antigens are processed and presented for stimulation of CD4 and CD8 T cells, resulting in activation and differentiation of T helper (Th) 1, Th2, and Th17 cells (60, 61, 62), as well as T cytotoxic (Tc) (63, 64) and T regulatory (Treg) cells (65), which are believed to be essential for protection against disease (66). Natural infection also results in the induction of Th1 type CD4+ and CD8+ T cells that produce high levels of interferon gamma (IFN-γ) during acute and convalescent phases of infection (60). Peripheral blood cells isolated from typhoid patients and stimulated with bacterial antigens produced higher levels of other cytokines, including IFN-γ, macrophage inflammatory protein-1β, soluble CD40 ligand, TNF-β, IL-13, and IL-9 during convalescence, which are likely required for an effective immune response that leads to bacterial clearance (62). Recent studies have also shown that natural infection results in the upregulation of the gut-homing integrin α4β7 on T regulatory cells (65), which may play a role in downregulating proinflammatory CD4 and CD8 T-cell responses.

General Strategies for Live Oral Vaccine Design

As discussed above, *Salmonella* possess specialized virulence mechanisms that allow them to reach permissive niches within the human host, establishing reservoirs for subsequent replication. Therefore, genetically inactivating one or more of these essential virulence factors constitutes one highly successful approach for weakening fully virulent organisms and constructing attenuated live *Salmonella* vaccines intended for oral immunization of humans. A more subtle but equally effective strategy for attenuating pathogens targets metabolic pathways required to sustain infection in the host, but this approach must be accomplished in such a way that the resulting live vaccine remains metabolically fit enough to reach immune inductive sites and elicit biologically relevant protective immunity in the absence of overt disease. Overattenuation of a candidate vaccine, while resulting in a highly safe and nonreactogenic organism, will require the administration of multiple oral doses to achieve protective immunity, a requirement that complicates deployment of the vaccine into the field. These key concepts for designing live oral vaccine candidates are particularly well illustrated with a select group of attenuated candidate live oral vaccines that are summarized in Table 1 and individually examined in detail below.

Chemical Mutagenesis and Ty21a

The only licensed vaccine against human infections caused by *Salmonella* is Ty21a, a typhoid fever vaccine derived from the parental wild-type *S.* Typhi strain Ty2 by chemical mutagenesis using *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) (67). MNNG is an alkylating agent that induces transition mutations (purine to purine or pyrimidine to pyrimidine base transitions) in replicating DNA, causing pleiotropic mutations to arise in multiple genes that can affect a wide variety of unrelated cell functions (68). Consequently, sequencing of the chromosome of Ty21a has revealed that this vaccine contains multiple mutations in a metabolic pathway controlling the incorporation of galactose into properly synthesized LPS (69). Ty21a requires growth in the presence of trace amounts (0.001%) of galactose to synthesize full-length LPS, but growth in the presence of higher concentrations (0.1%) leads to the intracellular accumulation of toxic intermediates that causes premature lysis of the vaccine strain (69). Ty21a contains additional metabolic mutations in amino acid synthesis pathways, leading to a requirement for isoleucine and valine (in addition to tryptophan and cysteine inherited from the parent strain Ty2) for growth under nutrient limiting conditions (69).

Table 1 Selected candidate live oral *Salmonella* vaccines against human disease tested in phase 1/phase 2 clinical trials

Attenuation strategy	Vaccine strain	Serovar; strain; relevant genotype	Relevant phenotype	References
Chemical mutagenesis	Ty21a	Typhi; Ty2; Δ*rpoS* Δ*galE* Δ*galK* Δ*ilvD* Δ*vexD*	Natural RpoS-dependent sensitivity to environmental stressors; defective synthesis of LPS; auxotrophic for isoleucine and valine; defective synthesis of Vi	69
Engineered deletions in *rpoS*	χ9639[a]	Typhi; Ty2; Δ*rpoS*	Natural RpoS-dependent sensitivity to environmental stressors	90
	χ9640[a]	Typhi; Ty2; *rpoS*+	Engineered RpoS-mediated resistance to environmental stressors	90
	χ9633[a]	Typhi; ISP1820; *rpoS*+	Natural RpoS-mediated resistance to environmental stressors	90
Engineered deletions in *phoP/phoQ*	Ty800	Typhi; Ty2; Δ*rpoS* Δ*phoP* Δ*phoQ*	Natural RpoS-dependent sensitivity to environmental stressors; defective in pH and osmolarity induction of invasion virulence factors	94
	Ty1033[b]	Typhi; Ty2; Δ*rpoS* Δ*phoP* Δ*phoQ*	Natural RpoS-dependent sensitivity to environmental stressors; defective in pH and osmolarity induction of invasion virulence factors	96
	LH1160[b]	**Typhimurium; ATCC 14028;** Δ*phoP* Δ*phoQ*	Defective in pH and osmolarity induction of invasion virulence factors	95
Engineered deletions in *ssaV*	M01ZH09	Typhi; Ty2; Δ*rpoS* Δ*aroC* Δ*ssaV*	Natural RpoS-dependent sensitivity to environmental stressors; auxotrophic for aromatic amino acids; defective for proper secretion of SPI-2 effector proteins	97, 99, 100 101, 102
	WT05	**Typhimurium; TML;** Δ*aroC* Δ*ssaV*	Auxotrophic for aromatic amino acids; defective for proper secretion of SPI-2 effector proteins	97
Engineered deletions in *aroC*, *aroD*, and *htrA*	CVD 908	Typhi; Ty2; Δ*rpoS* Δ*aroC* Δ*aroD*	Natural RpoS-dependent sensitivity to environmental stressors; auxotrophic for aromatic amino acids	105
	CVD 908-*htrA*	Typhi; Ty2; Δ*rpoS* Δ*aroC* Δ*aroD* Δ*htrA*	Natural RpoS-dependent sensitivity to environmental stressors; auxotrophic for aromatic amino acids; sensitive to environmental heat shock	107
	CVD 909	Typhi; Ty2; Δ*rpoS* P_{tac}-*tviA* Δ*aroC* Δ*aroD* Δ*htrA*	Natural RpoS-dependent sensitivity to environmental stressors; constitutive expression of TviA regulator and Vi antigen; auxotrophic for aromatic amino acids; sensitive to environmental heat shock	77, 108, 110

[a]These vaccines were engineered as live vector vaccines, carrying additional chromosomal mutations, as well as carrying multicopy plasmids expressing a pneumococcal foreign antigen. However, all candidate vaccines carried the same attenuating lesions and differed only with respect to parental strain used (Ty2 versus ISP1820) and the presence of *rpoS*.

[b]These vaccines were engineered as live vector vaccines, carrying one additional chromosomal deletion in the *purB* gene, as well as carrying multicopy plasmids encoding both *purB* and the foreign antigen urease from *H. pylori*. However, both *S.* Typhi and *S.* Typhimurium candidate vaccines were isogenic for the attenuating lesions tested in clinical trials.

As a result of multiple mutations in critical metabolic pathways, Ty21a grows slower than the wild-type Ty2 *in vitro* (67), and cannot be recovered from the small intestine of orally vaccinated humans regardless of the number of organisms or vaccine doses administered (70). For this reason, successful oral immunization of humans requires a minimum of 3 doses to provide durable protection against challenge, with efficacy also depending on growth conditions and specific formulation of the vaccine. Ty21a grown in the absence of galactose results in a rough vaccine strain lacking O antigen that was poorly immunogenic and failed to protect volunteers challenged with 10^5 CFUs of the Quailes strain (70). In contrast, 3 oral doses of O-expressing Ty21a, administered orally every other day in enteric-coated capsules, conferred 62% protection in field trials over a 7-year period; 3 doses of a liquid formulation, reconstituted from buffered lyophilized sachets, elicited 78% protection over a 5-year period (71). These data support the hypothesis that O-antigen-specific immunity plays an important role in protection against typhoid fever. Interestingly, the recurrence rate for exposed individuals who recovered from a previous episode of typhoid fever ranged from 20% in an endemic region (72) to 23% in convalescent volunteers challenged with 10^5 CFUs of the Quailes strain (73), suggesting that natural infection does not necessarily elicit the robust

protective immune effector mechanisms against reinfection as observed in vaccinees receiving multiple oral doses of Ty21a.

Both serum antibody titers and the frequency of circulating antibody-secreting cells (ASCs) have historically been the primary method to ascertain immunogenicity of orally delivered live *Salmonella* vaccines (74). The B cell responses induced by Ty21a include LPS-specific serum IgG and IgA antibodies (75) and mucosally primed ASCs bearing the α4β7 gut homing integrin (76). Among individuals who received the routine 4 doses of Ty21a in the United States, half of the recipients developed strong anti-LPS IgA B memory responses and 30 to 40% developed antiflagella IgG and IgA B memory responses (77). Ty21a-induced antibodies have been shown to bind to *S.* Typhi and enhance phagocytosis and bactericidal activity (75, 78).

Ty21a has also been shown to induce IFN-γ-producing CD4 and CD8 T terminal effector memory cells expressing α4β7 (79). Peripheral blood mononuclear cells from Ty21a-vaccinated volunteers stimulated with *S.* Typhi flagella produced cytokines required for clearance of intracellular pathogens and cell-mediated cytotoxicity including IFN-γ, TNF-α, IL-1β, IL-6, and IL-10 (80). In an early study, >90% of Ty21a-vaccinated volunteers developed LPS- and flagella-specific antibodies (78). Interestingly, when *Salmonella*-specific IgA antibodies were incubated together with CD4 T cells, antibody-dependent cellular cytotoxicity was observed against infected cells (81). Ty21a vaccination has also been show to elicit IFN-γ-secreting CD8 T cells that exhibited cytotoxic activity against infected cells (82, 83, 84). Further analysis showed that the vaccine-induced CD8 T cells were long-lived memory cells with the capacity to produce multiple cytokines including IFN-γ, TNF-α, IL-2, and IL-17 (64, 85). Taken together, these data suggest that, in contrast to natural infection, repeated vaccination with Ty21a appears more effective than wild-type organisms at presenting a variety of otherwise immunologically muted antigens such as LPS, flagella, and other outer membrane proteins to the immune system, thereby resulting in robust pathogen-specific humoral and cellular immunity.

Genetically Engineered Vaccines with Mutations in *rpoS*

In addition to mutations in genes controlling the synthesis of tryptophan and cysteine, the parent strain Ty2 used to create the vaccine Ty21a also contains a naturally occurring mutation inactivating *rpoS* (10, 86). The role of *rpoS* in adapting to environmental stresses, including its role in the acid tolerance response, was described previously in the pathobiology of *Salmonella* infections. However, *rpoS* also regulates the synthesis of Vi capsular polysaccharide (87, 88), which plays a critical role in the systemic survival of invading *S.* Typhi in natural disease. 99.5% of 2,222 *S.* Typhi blood-borne clinical isolates express the Vi polysaccharide (89), and human clinical trials clearly demonstrate that Vi^+ wild-type *S.* Typhi challenge strains are more virulent than Vi^- strains (6). However, in other studies, 36% (15/41) of *S.* Typhi clinical isolates were proven to carry defective *rpoS* alleles; interestingly, no inactivating mutations were identified in *S.* Typhimurium clinical isolates (86), suggesting different roles for *rpoS* in the physiology and resulting pathogenic potential of *S.* Typhimurium versus *S.* Typhi in humans. It has also been shown *in vitro* that RpoS– strains such as Ty2 express more Vi capsular antigen than wild-type RpoS+ strains under inducing osmolarity conditions (87, 88). The picture that emerges is that, under the high-osmolarity conditions of the intestinal lumen, *rpoS* is upregulated, which represses synthesis of Vi; this negative control is further strengthened by the osmotic repression of *tviA*, which is a positive activator of Vi synthesis. Since these conditions induce SPI-1 and the invasion of the small intestine, transepithelial migration results in a reduction of osmolarity to physiological levels, thereby repressing *rpoS* and activating *tviA* for induction of Vi synthesis (Fig. 2).

It is therefore plausible that although RpoS– strains are attenuated with respect to survival in environmentally stressful conditions such as intragastric acidity (controlling successful passage into the small intestine), vaccines derived from the RpoS– Ty2 parent might nonetheless be more immunogenic versus isogenic RpoS+ strains because of better intracellular survival within macrophages, allowing invading organisms to penetrate deeper into the host and reach immune inductive sites at levels high enough to elicit protective immunity. However, this hypothesis was recently shown to be incorrect in a phase 1 clinical trial in which volunteers were orally immunized with a single dose of 10^{10} CFUs of two isogenic vaccine strains derived from Ty2 (χ9639 and χ9640, Table 1) (61, 90), one carrying the original mutated *rpoS* allele and the other carrying a genetically engineered wild-type *rpoS* allele. Although not achieving statistical significance, a trend for higher serum IgA and IgA ASCs, specific for

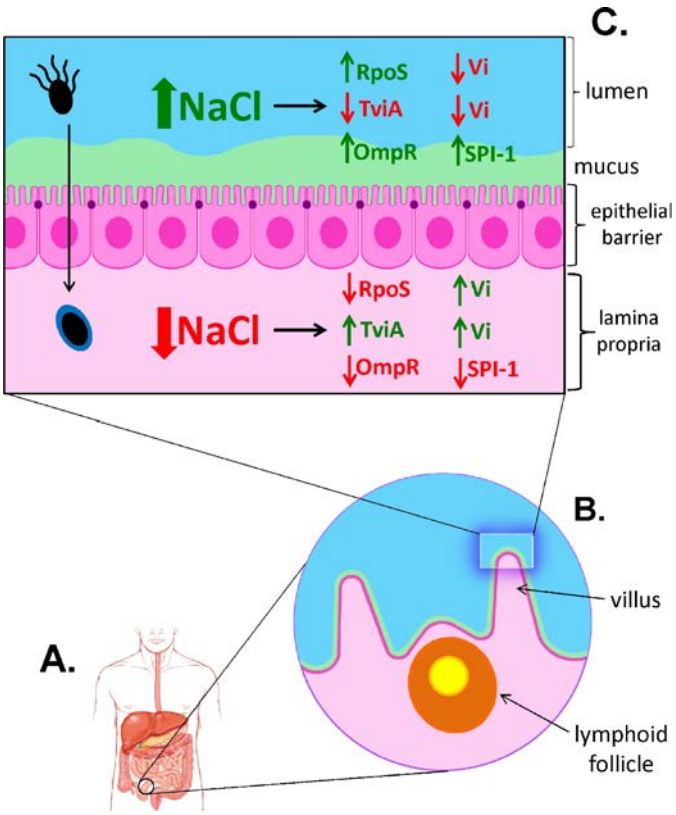

Figure 2 Induction of villus invasion by *Salmonella*. Invasion of *Salmonella* into deeper tissues of the human host occurs primarily in the small intestine (A) and is triggered by environmental signals including osmolarity. Villi of the small intestine possess a gradient of osmolarity, which is highest at the luminal surface of villi and decreases to physiological osmolarity in the lower crypts of the villus. Incoming luminal *Salmonella* sense the high osmolarity of villus surfaces that induce invasion (B). High osmolarity in the lumen upregulates *rpoS*, which in turn represses synthesis of Vi in *S.* Typhi to enhance invasion; this negative control is further strengthened by the osmotic repression of *tviA*, which is a positive activator of Vi synthesis (C). In addition, high osmolarity signals OmpR to upregulate *Salmonella* Pathogenicity Island 1 (SPI-1) to inject effector proteins into target eukaryotic cells, resulting in ruffling of the outer membrane and engulfment of invading bacteria. Transepithelial migration then reduces the osmolarity to physiological levels, repressing *rpoS* and activating *tviA* for induction of Vi synthesis. TviA is also a repressor of flagellar synthesis; therefore, *S.* Typhi is motile in the intestinal lumen when TviA is repressed (C, top left), but replaces flagella with the Vi capsule upon entry into host tissue (C, bottom left).

S. Typhi surface antigens, was observed for RpoS+ vaccines versus RpoS– strains (90). Interestingly, a third arm of this study was included in which volunteers were orally vaccinated with 10^{10} CFUs of an identically attenuated *S.* Typhi vaccine strain, derived from ISP1820 and designated χ9633, which carried the endogenous wild-type allele of RpoS. One subject from this group had a single positive blood culture 5 days following vaccination that spontaneously resolved without clinical intervention (90). These results seem to suggest an important role for RpoS in the early stages of vaccination with attenuated strains of *S.* Typhi, in which limited invasion of the host must be carefully balanced by additional chromosomal mutations in engineered vaccine strains to ensure both safety and immunogenicity. It is likely that the candidate vaccines included in this study may have proven more immunogenic if given in more than a single dose. Nonetheless, it was concluded from this trial that future vaccine development by this group would focus on RpoS+ Ty2 derivatives.

Engineered Vaccines with Deletions Blocking Systemic Infection: *phoPQ*

The licensed and experimental vaccines discussed thus far have relied on interference of essential metabolic pathways for construction of safe live oral vaccines. However, careful selection of virulence factors for inactivation can also yield safe vaccines that are highly immunogenic. PhoP/PhoQ is a pleiotropic two-component signal transduction system in which the environmental sensor PhoQ (activated either by low pH or low concentrations of extracellular Mg^{2+}) triggers the transcriptional regulator PhoP to induce the synthesis of the SPI-2 locus controlling intracellular survival functions (91). In addition, PhoP/PhoQ regulates expression of other genes within unlinked pathogenicity islands such as SPI-11, in which the *pagC* gene is involved with intracellular survival of *Salmonella* within macrophages (92, 93). Therefore, inactivation of PhoP/PhoQ would be expected to interrupt the systemic phase of *Salmonella* infection. When this attenuating strategy was used as the sole means for construction of a single dose of live oral typhoid vaccine, again derived from the parent strain Ty2 and designated Ty800 (Table 1), the resulting candidate vaccine was shown in phase 1 clinical trials to be safe, well-tolerated, and immunogenic after single oral doses of up to 10^{10} CFUs (94). Humoral immunity was comparable to responses from a second cohort of the study receiving 4 doses of Ty21a, and robust dose-dependent *S.* Typhi LPS-specific IgA-secreting cell responses were detected in 10 of 11 subjects (94).

Given that the PhoP/PhoQ regulon is highly conserved between *S.* Typhi and *S.* Typhimurium, construction and initial clinical testing of Ty800 offered an intriguing opportunity to specifically investigate the immunogenicity in humans of two closely related serovars of *Salmonella*

(i.e., *S.* Typhi versus *S.* Typhimurium) in which systemic dissemination of the organism was now blocked in both cases, and observed immunogenicity would therefore theoretically depend only on local immune induction sites. Two parallel phase 1 studies were performed in which *S.* Typhi Ty1033 and *S.* Typhimurium LH1160 candidate vaccine strains (Table 1), similarly deleted for PhoP/PhoQ, were administered to volunteers, with subjects vaccinated orally with *S.* Typhi Ty1033 receiving $\geq 10^{10}$ CFUs (95) and those vaccinated with *S.* Typhimurium LH1160 receiving an oral dose of 5 to 8 × 10^7 CFUs (96). As expected, no bacteremia was observed for either serovar, and both vaccines were highly immunogenic with no adverse reactions. However, despite the fact that subjects immunized with the attenuated *S.* Typhimurium vaccine received a 3 log unit lower dose of vaccine than those vaccinated with attenuated *S.* Typhi, 3 of 6 volunteers were durably colonized and excreted vaccine organisms for up to 10 days (96). Despite the absence of clinical symptoms, volunteers vaccinated with LH1160 were treated prophylactically with antibiotics to hasten complete elimination of this *S.* Typhimurium vaccine. In contrast, shedding of *S.* Typhi Ty1033 vaccine organisms was limited to no longer than 4 days in 9 of 9 volunteers, requiring no therapeutic intervention with antibiotics. Interestingly, volunteers more durably colonized with the *S.* Typhimurium vaccine mounted the most robust vaccine-specific humoral (anti-LPS serum IgA and IgG) and mucosal (anti-LPS ASCs) immune responses. Only 1 of 6 subjects immunized with *S.* Typhimurium LH1160 failed to mount significant mucosal or serological responses against any *S.* Typhimurium antigens, but this subject only excreted vaccine organisms for 2 days, suggesting that prolonged intestinal colonization can enhance immunogenicity (96). This surprising difference in fecal shedding of vaccine organisms seems to underscore the fact that *S.* Typhimurium is metabolically adapted to survival and growth within the human intestinal tract, whereas *S.* Typhi spends relatively little time in this environment because it quickly invades into deeper and more permissive tissues of the human host.

Engineered Vaccines with Deletions Blocking Systemic Infection: *ssaV*

A similar study design was pursued in a separate investigation, this time involving a direct comparison in a single phase 1 clinical trial of identically attenuated *S.* Typhi versus *S.* Typhimurium vaccines, in which a more narrowly focused deletion in *ssaV* targeted only the SPI-2 secretion apparatus to again interrupt systemic dissemination (Table 1). In this study, 18 volunteers were randomly assigned to two groups and orally immunized with a single escalating dose of either *S.* Typhi vaccine M01ZH09 or *S.* Typhimurium vaccine WT05, in doses ranging from 10^7 to 10^9 CFUs (97). Importantly, both vaccines also carried an additional metabolic deletion mutation in the *aroC* gene encoding chorismate synthase, rendering both strains auxotrophic for the biosynthesis of aromatic amino acids (98). As with the PhoP/PhoQ vaccines, no bacteremia was observed after vaccination with either serovar, and both vaccines were highly immunogenic with no adverse reactions. However, prolonged excretion of vaccine organisms for 12 to 23 days was again observed in 5 of 6 subjects receiving either 10^8 or 10^9 CFUs of the *S.* Typhimurium vaccine, regardless of the engineered auxotrophy for aromatic amino acids (97). Despite the limitation of these amino acids within the tissues of human hosts, enough of these nutrients are freely available within the lumen of the intestinal tract to support extended growth and excretion of *S.* Typhimurium vaccines. Therefore, this strategy for metabolic attenuation does not by itself ensure sufficient attenuation for *S.* Typhimurium vaccines.

However, the *S.* Typhi vaccine M01ZH09, which carries only these two deletion mutations in *aroC* and *ssaV*, has been shown to elicit excellent mucosal and humoral immunity. Next to Ty21a, M01ZH09 is the most extensively evaluated typhoid vaccine to date, having successfully completed four phase 1 clinical trials and two phase 2 clinical trials, involving a total of 356 orally immunized subjects from North American, Europe, and endemic Asian populations (97, 99, 100, 101, 102). The vaccine given orally as a single dose of up to 10^{10} CFUs is safe, causes no bacteremia, and engenders both mucosal and serum antibody responses comparable to those observed in individuals immunized with 3 doses of Ty21a, with aggregate *S.* Typhi LPS-specific seroconversions between 50 and 92% for M01ZH09 versus 50 to 64% for Ty21a and LPS-specific ASCs in 90 to 100% of vaccinees receiving M01ZH09 versus 63 to 96% for Ty21a (102).

Engineered Vaccines with Deletions Blocking Systemic Infection: *htrA*

Engineered auxotrophic dependence on supplementation with aromatic amino acids for growth was also exploited in another successful live oral typhoid vaccine that underwent three iterations of refinements, each

tested in phase1 or phase 2 clinical trials to guide further development (Table 1). CVD 908 was first engineered from wild-type Ty2 to carry deletion mutations in *aroC* (encoding chorismate synthase) and *aroD* (encoding 3-dehydroxoquinate dehydrogenase), two independent nonreverting mutations in the essential aromatic amino acid biosynthesis pathway (11, 98). Following a single oral dose of CVD 908, the majority of vaccine recipients responded with LPS-specific serum IgG (83%) and all of them with LPS-specific IgA ASCs (103). In the presence of *S.* Typhi flagella and killed organisms, peripheral blood mononuclear cells from vaccinated individuals proliferated and produced IFN-γ and IL-6 indicative of a Th1 type/proinflammatory response (104). However, despite the presence of two distinct deletion mutations in *aroC* and *aroD* (plus the *rpoS* mutation from Ty2), vaccine organisms were still able to cross the intestinal epithelial barrier and were detected in the blood of vaccinees receiving oral doses as low as 5×10^7 CFUs (105). Therefore, it was deemed prudent to engineer additional chromosomal deletions to prevent this self-limiting bacteremia, even though no symptoms were documented in any of these volunteers and no therapeutic intervention was required.

The resulting candidate vaccine, CVD 908-*htrA*, contained a new deletion in *htrA* encoding a heat shock protease (105); previous data obtained *in vitro* with *S.* Typhimurium suggested that *htrA* enhanced survival within macrophages (106), and thus deletion of this gene might limit systemic spread of the vaccine. Indeed, phase 1 clinical trials of CVD 908-*htrA* conclusively demonstrated that the desired balance between reactogenicity and immunogenicity had been achieved. No vaccine organisms were detected in the blood, and limited shedding of vaccine organisms for less than 3 days was seen in volunteers orally vaccinated with up to 5×10^9 CFUs of freshly harvested organisms. In addition, excellent mucosal, humoral, and cellular immune responses were observed. One hundred percent (15/15) of volunteers vaccinated with 5×10^8 or 5×10^9 CFUs seroconverted to serum IgG against *S.* Typhi LPS and 73% (11/15) against flagella. IgA anti-LPS ASCs were detected in all vaccinees receiving 5×10^8 or 5×10^9 CFUs as well, and lymphoproliferative responses against flagella or inactivated whole-cell antigen were detected in 69% (9/13) and 77% (10/13) of subjects, respectively (105). Interestingly, when lyophilized vaccine was further tested in phase 2 clinical trials, mucosal ASC responses were maintained (94 to 100% LPS-specific ASCs and 50 to 82% flagella-specific ASCs) at doses of 4.5×10^8 CFUs (the highest dose given orally), but serum antibody responses declined slightly with only 49% of volunteers mounting anti-LPS IgG responses and 41% generating antiflagella responses; 63% of vaccinees had lymphoproliferative responses to flagella, and 44% responded to particulate inactivated whole cell (107).

CVD 908-*htrA* was then further genetically modified to constitutively express Vi polysaccharide (108). This novel approach was based on a hypothesis proposed by Levine et al., who observed that Ty21a live oral vaccine and a subunit parenteral vaccine composed of purified Vi polysaccharide both confer substantial protection against typhoid disease after multiple doses, despite the fact that Ty21a does not synthesize Vi polysaccharide and that the purified Vi vaccine is a monovalent vaccine lacking other surface antigens from *S.* Typhi (108). This suggested that immunity to typhoid disease may be manifested by at least two distinct immune mechanisms, one involving targeted antibody responses against Vi and the other involving more broad humoral and cell-mediated immunity against *S.* Typhi surface antigens other than Vi. Given that all genetically engineered live oral vaccines tested in clinical trials to date have elicited very poor serum immunity to Vi, Levine et al. proposed that perhaps a more broadly immunogenic vaccine could be developed, eliciting immunity against surface antigens including Vi, by further engineering constitutive expression of Vi in CVD 908-*htrA*.

To accomplish this, the powerful constitutive promoter P_{tac} was used to replace the highly regulated and osmotically controlled P_{tviA} promoter controlling expression of the Vi operon *viaB* encoded within the SPI-7 pathogenicity island. It was then confirmed *in vitro* that excellent expression of Vi antigen in the resulting vaccine candidate CVD 909 (Table 1) was now independent of osmotic induction. Interestingly, it was also demonstrated that CVD 909 was less invasive for Henle 407 cells, a human embryonic intestinal epithelial cell line, than the parent CVD 908-*htrA* at low osmolarity (108); this observation agrees with previously published *in vitro* data in which induced high-level expression of Vi polysaccharide by osmotic induction of wild-type *S.* Typhi Ty2 significantly reduced invasion of intestinal epithelial cells (109).

CVD 909 proved to be safe and immunogenic in phase 1 clinical trials despite overexpression of the Vi polysaccharide virulence factor (77, 110). However, serum

antibody responses against *S.* Typhi LPS and flagella were lower in comparison with the parent vaccine CVD 908-*htrA*. Only 2 of 6 (33%) volunteers orally vaccinated with a single dose of 2.5×10^9 freshly harvested vaccine organisms mounted anti-LPS IgG serum antibody responses (110) versus 8 of 8 vaccinated with a 10-fold lower dose of 5×10^8 CFUs of CVD 908-*htrA* (105); similarly, only 1 of 6 CVD 909 vaccinated subjects had antiflagellar serum IgG antibody responses versus 6 of 8 for CVD 908-*htrA*. This reduction in surface antigen-specific antibody responses is consistent with strong expression of the regulator TviA, which downregulates flagellar expression while upregulating synthesis of Vi capsule to mask surface LPS (31, 38). Curiously, despite overexpression of the capsular polysaccharide in CVD 909, Vi-specific serum immune responses were not observed (110).

In a subsequent clinical trial, volunteers primed with a single oral dose of 5×10^9 CFUs of CVD 909 and boosted intramuscularly with 25 μg of the licensed Vi polysaccharide vaccine Typhim Vi did not have Vi-specific IgM, IgG, or IgA antibodies significantly elevated above volunteers who received a placebo prime and boost with Typhim Vi (77). Sixty-four percent of the CVD909 recipients developed anti-LPS serum IgG and IgA while only 18 to 27% developed antiflagella serum antibodies (77). Importantly, these antibodies exhibited functional opsonophagocytic activity against wild-type *S.* Typhi (75). Over half (55%) of CVD909 primed individuals developed anti-Vi IgA B memory cells, compared with 12.5% in the placebo-primed group. This vaccine strain also resulted in the production of antiflagellar IgA B memory cells that remained for at least 1 year postvaccination (77). It remains puzzling that no Vi-specific antibody responses were observed in subjects primed with a Vi overexpressing live vaccine prior to boosting with a purified Vi subunit vaccine. The fact that robust Vi-specific antibody responses are observed in asymptomatic human carriers chronically colonized with *S.* Typhi underscores the fact that much still remains to be elucidated regarding induction of protective immunity against *Salmonella* and how to exploit this information in the rational design of efficacious live oral vaccines.

CONCLUSIONS AND FUTURE DIRECTIONS

Herein, we have summarized the pathogenesis of human *Salmonella* infections, contrasting *S.* Typhi and *S.* Typhimurium with regard to niches colonized and immune responses elicited by wild-type organisms. Understanding the natural progression of disease provides an important context in which attenuated live vaccines can be rationally designed and developed. With this in mind, we have reviewed a series of attenuated live vaccines that have been tested in clinical trials, and demonstrated to be both safe and highly immunogenic in the case of *S.* Typhi typhoid vaccines. However, we have also pointed out that correlates of protection against enteric fever have yet to be adequately defined. Therefore, at this point, immune responses elicited by candidate vaccines remain only suggestive of protective efficacy in the absence of challenge studies conducted with vaccinated volunteers. However, it is encouraging that a human typhoid challenge model has now been reestablished (7), offering the opportunity to compare the various attenuation strategies for currently available *S.* Typhi vaccine candidates, and their ability to induce immune responses that can protect against disease. Given the paucity of data relevant to mechanisms involved in disease clearance, it is entirely possible that some vaccines clinically proven to be safe and highly immunogenic will nonetheless fail to offer significant protection when administered orally as a single dose. Such future challenge studies may specifically inform the development of more efficacious vaccine schedules involving two or more doses, without having to reengineer further vaccine candidates, to elicit durable protective immunity.

Although there are now multiple examples of attenuated oral *S.* Typhi vaccines that have been clinically demonstrated to be safe and immunogenic, all *S.* Typhimurium vaccine candidates tested to date have displayed unacceptable safety profiles, with prolonged colonization of human volunteers leading to unacceptable shedding of viable vaccine organisms over several weeks (96, 97). It became evident from these important studies that strategies proven successful for attenuation of *S.* Typhi do not necessarily guarantee success when applied to *S.* Typhimurium. This is undoubtedly related, at least in part, to the differences in metabolic niches exploited by these two serovars, with *S.* Typhimurium typically thriving extracellularly in the gastrointestinal lumen, while *S.* Typhi proliferates intracellularly in deeper tissues of the host. However, the pathogenicity of *S.* Typhimurium has recently been changing, with more invasive and multidrug-resistant strains being increasingly isolated from the blood of malnourished children and immunocompromised adults living in sub-Saharan Africa (111, 112, 113). These newly emerging strains have developed an improved ability to replicate within human macrophages

while downregulating production of flagella to reduce innate immune recognition (114). With this unsettling rise in unconventional invasive nontyphoidal *Salmonella* (iNTS) strains, it may therefore be appropriate to revisit available attenuated *S.* Typhimurium vaccines with the goal of improving the safety of existing vaccine candidates. We recognize, however, that, while development of improved second-generation *S.* Typhimurium live vaccines might prove to be safe and highly immunogenic in developed countries, they could nonetheless prove to be far less immunogenic in endemic regions of developing countries where malnourished children and immunocompromised adults suffering from coinfection with malaria parasites or HIV would be severely compromised for humoral and cell-mediated immunity (112). Notwithstanding this caveat, it would be intriguing to target the intestinal proliferation of *S.* Typhimurium to reduce or eliminate the unacceptable shedding of vaccine organisms. In theory, this might be accomplished by inactivating the ability to exploit tetrathionate as an alternate electron acceptor for anaerobic respiration, thereby eliminating the metabolic advantage of *S.* Typhimurium over competing flora to enhance clearance and reduce unacceptably high levels of shedding (27, 28). Another possible approach could involve introduction of proven attenuation strategies into an iNTS isolate of *S.* Typhimurium, with further deletion of a novel invasion gene called *st313-td*, recently reported to enhance systemic invasion in experimental animal models of infection (115). We conclude that the attenuation strategies we have summarized offer important insights into further development of attenuated *S.* Typhimurium vaccines, as well as for other serovars for which vaccines are currently unavailable.

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