Advances in the development of Salmonella-based vaccine strategies for protection against Salmonellosis in humans

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Abstract

Salmonella spp. are important human pathogens globally causing millions of cases of typhoid fever and non-typhoidal salmonellosis annually. There are only a few vaccines licensed for use in humans which all target Salmonella enterica serovar Typhi. Vaccine development is hampered by antigenic diversity between the thousands of serovars capable of causing infection in humans. However, a number of attenuated candidate vaccine strains are currently being developed. As facultative intracellular pathogens with multiple systems for transporting effector proteins to host cells, attenuated Salmonella strains can also serve as ideal tools for the delivery of foreign antigens to create multivalent live carrier vaccines for simultaneous immunization against several unrelated pathogens. Further, the ease with which Salmonella can be genetically modified and the extensive knowledge of the virulence mechanisms of this pathogen means that this bacterium has often served as a model organism to test new approaches. In this review we focus on (1) recent advances in live attenuated Salmonella vaccine development, (2) improvements in expression of foreign antigens in carrier vaccines and (3) adaptation of attenuated strains as sources of purified antigens and vesicles that can be used for subunit and conjugate vaccines or together with attenuated vaccine strains in heterologous prime-boosting immunization strategies. These advances have led to the development of new vaccines against Salmonella which have or will soon be tested in clinical trials.

Introduction

Salmonella spp. are Gram-negative, intracellular, facultative anaerobic and motile rods. They can be divided into two species: Salmonella enterica and Salmonella bongori. Salmonella enterica subspecies enterica is the primary species that causes infection in humans and animals. More than 2500 serovars have been identified based on their O-polysaccharide (OPS) and flagellin (O and H, respectively) antigens (Grimont 2007). Infection typically occurs by ingestion of contaminated food or water. Typhoidal serovars are human-restricted and cause enteric fever, whereas non-typhoidal Salmonella (NTS) serovars are zoonotic and typically produce a self-limiting gastroenteritis in humans. Certain NTS serovars also cause invasive NTS (iNTS) infections in infants and young children in sub-Saharan Africa (Feasey et al. 2012). In low- and middle-income countries (LMICs), typhoidal Salmonella is estimated to cause over 11 million infections and over 200 000 deaths every year; NTS is estimated to cause over 90 million cases and over 100 000 deaths annually (Mogasale et al. 2014; Majowicz et al. 2019).

The high morbidity and mortality due to typhoidal Salmonella and NTS as well as the increasing prevalence of multidrug resistant strains have spurred development of vaccines against these pathogens. The only licensed Salmonella vaccines are against S. Typhi, the primary cause of typhoid fever. This includes the orally administered live-attenuated Ty21a vaccine which protects against S. Typhi and offers some cross-protection against S. Paratyphi B but not S. Paratyphi A (Simanjuntak et al. 1991; Levine et al. 2007) and the injectable Vi capsular
polysaccharide and conjugate vaccines which are highly protective against S. Typhi infection but offer no cross-protection against other serovars unless these serovars also express Vi. This limited cross-reactivity underscores the need for vaccines against NTS in humans particularly in Asia where S. Paratyphi A infections are common.

While antibodies to O polysaccharide and Vi capsular polysaccharide are highly protective, antibodies to proteins such as flagellin also elicit protective antibody responses. Additionally, CD4+ Th1 cells and cytotoxic CD8+ cells are important for cytokine production and clearance of persistent Salmonella infections (Mastroeni et al. 2001; Gayet et al. 2017; Gal-Mor 2019). Furthermore, because live attenuated Salmonella vaccines stimulate humoral and cellular immune responses both systemically and at mucosal sites, many live vaccine candidates have been developed over the years. Due to the ease of genetically engineering Salmonella, they have been widely utilized as carriers to deliver heterologous antigens from a variety of pathogens and as a source of components for other vaccine formats.

In this review, we highlight the use of Salmonella as live attenuated vaccines, as live carrier vaccines to deliver foreign antigens or as reagent strains to purify components for subunit vaccines (summarized in Table 1).

**Live attenuated Salmonella vaccines**

Live oral attenuated Salmonella vaccines present several advantages over other vaccine formats. In addition to inducing local immune responses in the gut epithelium, live vaccines also stimulate systemic humoral and cellular immunity (Gayet et al. 2017). Oral administration also means that less hazardous waste is generated during delivery, and it also makes these vaccines easier to distribute to large populations. However, producing a live vaccine that is sufficiently attenuated and well tolerated while stimulating protective immune responses requires fine tuning to balance reactogenicity with immunogenicity (Galen and Curtiss 2014).

The only licensed live oral attenuated S. Typhi vaccine, Ty21a, was developed using chemical mutagenesis, and as a result, it carries multiple mutations including the deficiency in UDP-galactose-4-epimerase activity for which it was selected (Germanier and Fuer 1975; Kopecko et al. 2009). This highlights the lack of specificity in generating mutants using this method. Ty21a is given in four doses for optimal responses and requires boosters every 5 years. The goal for newer generation vaccines is to develop candidates that are effective at a single dose while having better defined mutations. Genetic engineering allows for site-directed mutagenesis and better characterization of mutants. Furthermore, incorporating multiple independent mutations reduces the likelihood that vaccine strains revert to wild type and become pathogenic. Several S. Typhi vaccine candidates have advanced to Phase 1 clinical trials and one such candidate is M01ZH09, a S. Typhi Ty2 ΔaroC ΔssaV mutant, which has limited intracellular replication due to the ssaV deletion in the Type 3 Secretion System (T3SS) and the aroC mutation which results in a requirement for aromatic amino acids for growth (Kirkpatrick et al. 2006). This vaccine did not cause bacteremia, fever or fecal shedding for an extended duration in volunteers but was able to induce antibody responses to lipopolysaccharide (LPS) and flagellin; however, when tested in a human challenge model, a single dose of M01ZH09 did not protect volunteers from developing typhoid fever when challenged with a virulent strain of S. Typhi (Darton et al. 2016). The Center for Vaccine Development and Global Health (CVD) has also produced several typhoid vaccine candidates including a series of S. Typhi Ty2 mutants that are able to elicit robust humoral and cellular immune responses (Tacket and Levine 2007). The leading candidate, CVD 909, has mutations in aroC and htrA and has been engineered to constitutively express Vi polysaccharide (Wang et al. 2000). CVD 909 elicited Vi-specific immune responses in addition to anti-LPS and anti-flagellin responses after a single dose making it an ideal typhoid vaccine candidate (Tacket et al. 2004).

While many NTS vaccine candidates have been developed and preclinically assessed, few have been tested in clinical trials. At the CVD, we have developed S. Typhimurium, S. Enteritidis and S. Newport vaccines with guaBA (encodes guanine biosynthesis), clpPX (encodes a regulatory protease), htrA (encodes heat-shock protein) and pipA (part of Salmonella pathogenicity island [SPI] 5 which contributes to fluid accumulation in the intestine) deletions (Tennent et al. 2015; Higginson et al. 2018; Fuche et al. 2019). Live attenuated NTS vaccines from other groups have included deletions in aroA, ssaV, crp, cdh, phoPQ, purB, dam, Lon protease and hfg among many others (Matsui et al. 2003; Allam et al. 2011; Tennant and Levine 2015; Galen et al. 2016). Candidates that have advanced to clinical trials include a S. Typhimurium LH1160 vaccine engineered with deletions in the virulence genes phoP and phoQ and purB to eliminate purine biosynthesis and which expressed Helicobacter pylori urease from a plasmid. Volunteers receiving a single dose of this vaccine-generated IgA and IgG antibody responses to Salmonella LPS and flagellin as well as detectable responses to H. pylori urease (Angelakopoulos and Hohmann 2000) indicating that attenuated NTS vaccines can be used to immunize against heterologous antigens. Another candidate that advanced to a Phase 1 clinical trial combined mutations in aroA
Table 1: List of selected recombinant Salmonella strains developed as vaccine candidates or as reagent strains for other vaccine formats

| Type of vaccine | Strain name | Characteristics | References |
|-----------------|-------------|-----------------|------------|
| Live attenuated vaccines | Ty21a-Gad (MD297) | S. Typhi Ty21a with S. flexneri gad (glutamate decarboxylase) genes integrated into tvd-vexA locus | Dharmasena et al. (2016a) |
| Reduced fecal shedding | AH9 | S. Typhimurium IR715 tshdA mutant | Kingsley et al. (2003) |
| | AH12 | S. Typhimurium IR715 tshatB mutant | Kingsley et al. (2003) |
| | CWD9 | S. Typhimurium 14028 mis::pGPT04 NalR tshdA::aph | Dorsey et al. (2005) |
| | MFA17 | S. Typhimurium ΔmraA Δmstl::cat ΔshdA::aph | Abd El Ghany et al. (2007) |
| Programmed attenuation and delayed lysis | χ8937 | S. Typhimurium UK-1 ΔsasA19::araC PBAD c2 ΔΔmuraA::araC PBAD murA Δ(gmd-fcd)-26. Plasmid pYA3685 expresses pneumococcal surface protein A (PspA) | Kong et al. (2008) |
| Live carrier vaccines | Ty21a-PA-1 | S. Typhi Ty21a expressing Bacillus anthracis PA83-HlyA fusion integrated into tvd-vexA locus | Sim et al. (2017) |
| | MD149 | S. Typhi Ty21a with Shigella dysenteriae serotype 1 O-antigen genes integrated into the tvd-vexA locus (expressed from native promoter) | Dharmasena et al. (2016b) |
| | MD174 | S. Typhi Ty21a with S. dysenteriae serotype 1 O-antigen genes integrated into the tvd-vexA locus (expressed from the lpp promoter) | Dharmasena et al. (2016a) |
| | MD194 | S. Typhi Ty21a with S. flexneri 2a O-antigen genes integrated into tvd-vexA locus (expressed from native promoter) | Dharmasena et al. (2017) |
| | MD196 | S. Typhi Ty21a with S. flexneri 3a O-antigen genes integrated into tvd-vexA locus (expressed from native promoter) | Dharmasena et al. (2017) |
| | Ty21a-AR-Ss | S. Typhi Ty21a with S. sonnei form I O-antigen gene cluster and gad genes integrated into tvd-vexA locus | Wu et al. (2017) |
| Use of secretion systems to export foreign antigens | LH1160 | S. Typhimurium ATCC 14028 ΔphoPΔphoQ ΔpurB; expresses H. pylori ureA and ureB | Angelakopoulos and Hohmann (2000) |
| | CKS257 | S. Typhimurium SL1344 ΔphoP ΔphoQ ΔaroA Δasd ΔstrA ΔstrB (pSB2131); expresses HIV Gag | Kotton et al. (2006) |
| | MpPS25 | S. Typhimurium NCTC 12023 ΔlhrA PΔgadA::GFP ΔpurD PpurD::OVA, KanR (carrier strain MpPS25); pWKS29 SseF1::los51-363::HA (designated as p2810); T3SS effector SseF fused to L. monocytogenes Llo | Husseiny et al. (2007) |
| | MpPS728 | S. Typhimurium NCTC 12023 ΔlhrA PΔgadA::GFP ΔpurD PpurD::OVA, KanR (carrier strain MpPS728); pWKS29 SseF1::los51-363::HA (designated as p3635); T3SS effector SseF fused to L. monocytogenes Llo | Hegazy et al. (2012) |
| | MpPS728 | S. Typhimurium NCTC 12023 ΔlhrA PΔgadA::GFP ΔpurD PpurD::OVA, KanR (carrier strain MpPS728); pWKS29 SseF1::los51-363::HA (designated as p3635); T3SS effector SseF fused to L. monocytogenes Llo | Hegazy et al. (2012) |
| | SV9699 | S. Typhimurium 14028 araAS51::Tn10 ΔaroB::KmR, pWKS29- PpurD::SseF-PrvV-FLAG; expresses P. aeruginosa V antigen (PrvV) | Aguiller-Herce et al. (2019) |
| | CVD908ssb-TXSVN | S. Typhi Ty2 with survivin-SseF fusion; candidate in multiple myeloma clinical trial | ClinicalTrials.gov Identifier: NCT03762291 |
| Recombinant Salmonella as reagent strains; As a source of antigen for conjugate vaccines | CVD1925 (pSEC10-wwzB) | S. Typhimurium 177 ΔguaBA Δc[pΔflID ΔflfB overexpressing wzzB; produces medium to long chain COPs; secretes large amounts of FlIC into supernatant; reagent strain for O:4 OPS and flagellin purification | Tennant et al. (2011); Balbiani et al. (2017); Hegerle et al. (2018) |
| | CVD1943 | S. Enteritidis R11 ΔguaBA Δc[pΔflID ΔflfB; secretes large amounts of FlIC into supernatant; reagent strain for O:9 OPS and FlIC purification | Tennant et al. (2011) |
and ssaV in an early Typhimurium version of the Typhi vaccine strain M01ZH09 discussed above. This vaccine, designated WT05, was well-tolerated and elicited immune responses in most volunteers but was shed for 23 days (Hindle et al. 2002) which raises concerns about possible transmission to vulnerable populations. Below, we describe new developments to improve the safety and immunogenicity of live attenuated Salmonella vaccines with an emphasis on strategies to enhance acid resistance and reduce the potential for transmission.

**Live attenuated vaccines enhanced for acid resistance**

To induce a robust and strongly efficacious immune response, vaccine organisms delivered orally must successfully transit the gastric acid barrier, passing into the intestinal tract in sufficient numbers to reach immune inductive sites and elicit both innate and adaptive responses. It has been hypothesized that passage through the gastric acid barrier induces critical virulence factors in Salmonella that prepare vaccine organisms for the eventual invasion and persistence within immune tissues (Galen et al. 2016). Historically, oral administration of live attenuated Salmonella vaccines has been carried out after administration of bicarbonate to fasting subjects to neutralize stomach acidity; an alternate method involves oral administration of vaccine contained within enteric coated acid resistant capsules which transit the stomach and subsequently deliver vaccine organisms directly into the intestinal tract. However, it has been postulated that bypassing the gastric acid barrier may also abort induction of SPI 1 and 2, potentially reducing the number of vaccine organisms able to invade intestinal tissues and trigger immunity (Brenneman et al. 2013, 2014). Several groups have therefore re-engineered candidate vaccine strains such that acid resistance can be induced on demand in vitro during vaccine preparation under routine growth conditions such that organisms are immediately prepared for exposure to acid prior to oral administration, and more organisms can successfully pass through the gastrointestinal tract to reach immune inductive sites (Brenneman et al. 2013, 2014; Dharmasena et al. 2016a). Two approaches have been used to enhance the acid resistance of live attenuated Salmonella candidate vaccines. The first strategy involves re-engineering endogenous extreme acid resistance pathways to enable induction of resistance on demand during vaccine production (Brenneman et al. 2013, 2014); the second method introduces exogenous acid resistance systems from other more acid-resistant enteric pathogens such as Shigella (Dharmasena et al. 2016a).

Salmonella possess at least three acid resistance pathways, all of which rely on a two-component decarboxylase-antiporter system that acts upon cognate-specific amino acid substrates as shown in Fig. 1 (Zhao and Houry 2010). These pathways increase acid survival of bacteria under extreme acidic conditions as low as pH 2-5. Acid resistance systems are typically induced under low pH conditions in the presence of the specific amino acid required for decarboxylation; induction is also controlled by anaerobiosis. Upon induction under acid stress conditions, the decarboxylase raises intracellular pH by consuming one cytoplasmic proton per decarboxylation reaction to liberate CO₂. The product of this enzymatic reaction is then transported out of the cytoplasm through binding to an inner membrane substrate-specific antiporter that exchanges the modified substrate with a new amino acid to continue the cycle (Zhao and Houry 2010).

The endogenous Salmonella arginine decarboxylase acid resistance system was re-engineered in a candidate typhoid vaccine strain of S. Typhi by Brenneman et al. (2013), such that acid resistance became inducible on demand in the presence of the sugar rhamnose and the amino acid arginine. To accomplish this, the adiA gene encoding arginine decarboxylase acid antiporter system that acts upon cognate-specific amino acid substrates as shown in Fig. 1 (Zhao and Houry 2010).

**Table 1 (Continued)**

| Type of vaccine | Strain name | Characteristics | References |
|-----------------|-------------|-----------------|------------|
| As a source of outer membrane vesicles | 618 ΔtolR ΔmsbB ΔpagP | S. Enteritidis with enhanced shedding of GMMAs due to tolR deletion; reduced reactogenicity due to deletion of msbB and pagP | De Benedetto et al. (2017) |
| | 1418 ΔtolR ΔhtnR | S. Typhimurium with enhanced shedding of GMMAs due to tolR deletion; reduced reactogenicity due to deletion of htnR | De Benedetto et al. (2017) |

HIV, human immunodeficiency virus; T3SS, Type 3 Secretion System; OPS, O polysaccharide; GMMA, generalized modules for membrane antigens.
exceed those conferred by the unmodified native decarboxylase system when organisms were induced under the more restrictive anaerobic growth conditions (Brenneman et al. 2013). To test the acid resistance of these organisms in vivo, strains were administered orally using a low gastric pH histamine mouse model (Brenneman et al. 2014) and surviving organisms enumerated from murine gastrointestinal tissue 1-h post-inoculation; acid resistance was assessed using a competition assay in which acid-sensitive unmodified S. Typhi candidate vaccines were mixed 1 : 1 with acid-resistant isogenic S. Typhi strains and competitive indices quantitated post inoculation. In vivo survival of S. Typhi acid-resistant strains increased by ~10-fold when carrying the inducible arginine decarboxylase system (Brenneman et al. 2014).

Introduction of exogenous acid resistance systems has resulted in more robust improvement of acid resistance in candidate Salmonella vaccines. Dharmasena et al. (2016a) integrated a cassette encoding the glutamine decarboxylase system from Shigella flexneri 2a into the chromosome of the licensed S. Typhi typhoid vaccine Ty21a. This strategy was based on the observation that Shigella is substantially more resistant to acid stress than Salmonella, possibly due to the presence of a glutamine decarboxylase resistance pathway, not present in Salmonella, that confers the strongest resistance to extreme acid stress conditions (Zhao and Houry 2010). Therefore, a synthetic operon was constructed encoding the two paralogous GadA and GadB decarboxylases and the inner membrane antiporter GadC under the transcriptional control of the arabinose-inducible AraC-PBAD promoter; expression was tested for low copy number plasmid-based expression of this cassette versus expression when stably integrated into the chromosome. As expected, when acid survival was tested in vitro, plasmid-based acid resistance increased by 5 log_{10} versus unmodified Ty21a after 3 h at pH 2.5 (Dharmasena et al. 2016a); when Ty21a carrying a chromosomally integrated glutamine acid resistance system was exposed to pH 2.5 for 3 h, survival was still increased by 4 log_{10} although survival depended on pre-induction with cultures grown at pH 5.5 for 24 h prior to acid challenge (Dharmasena et al. 2016a).

Figure 1 Endogenous and exogenous acid resistance pathways exploited in Salmonella vaccines. The diagram illustrates the two-component amino acid decarboxylase-antiporter resistance (AR) systems that have been utilized in candidate Salmonella vaccines to increase acid resistance. These systems are from left to right—the glutamic acid-dependent acid resistance system (GDAR, yellow) comprised of the GadC glutamate/-aminobutyric acid (GABA) antiporter and glutamate decarboxylases, GadA/GadB; the arginine-dependent acid resistance system (ADAR, purple) comprised of the AdiC arginine/agmatine antiporter (Agm) and the inducible arginine decarboxylase, AdiA; and the lysine-dependent acid resistance system (LDAR, green) comprised of the CadB lysine/cadaverine antiporter (Cad) and the cytoplasmic inducible lysine decarboxylase, CadA. The ADAR and LDAR systems are native to Salmonella, while the GDAR system has been imported from Shigella into Salmonella strains to further enhance acid resistance. In low pH conditions (deep pink colour extracellular to the bacteria, large H^+) and in the presence of their cognate amino acid, the decarboxylase raises intracellular pH (indicated by the light-coloured cytoplasm) by decarboxylating the amino acid substrate (indicated by the orange square, purple triangle and green rectangle) in a proton-dependent manner releasing CO2 in the process. The product binds the substrate-specific antiporter in the inner membrane and exchanges the decarboxylated substrate with a new amino acid. IM, inner membrane; OM, outer membrane. Figure created with BioRender.com.
Optimization of vaccines for reduced fecal shedding and transmissibility

In the development of live attenuated oral vaccines, significant consideration must be given to limiting the length of time that vaccine strains are shed into the feces. Long periods of shedding into the environment can increase the opportunities for transfer of genetic elements or transmission of vaccine strains to unvaccinated, immunocompromised individuals (Davison 2002; Anderson 2008). Balancing tolerability with immunogenicity can be challenging as overattenuation may result in strains with limited shedding but that transit the gut so quickly that they fail to elicit protective immune responses. Among the approaches that have been advanced to develop live attenuated, Salmonella strains with limited fecal shedding and hence reduced transmissibility to unintended targets are gene mutations that limit colonization and survival, and strains engineered to self-destruct after several rounds of replication.

Persistence in the intestine has been attributed to many factors including adhesins required for attachment and colonization. However, few genes have been directly implicated in fecal shedding (Gal-Mor 2019). Two factors, \textit{shdA} and \textit{ratB}, were identified on a genetic island unique to serovars within \textit{S. enterica} subspecies I, which infect avian and mammalian species, but absent in subspecies II-VII (Kingsley et al. 2000, 2003). \textit{ShdA} encodes a fibronectin-binding protein belonging to the autotransporter Type 5 secretion system (T5SS) (Kingsley et al. 2004); an \textit{S. Typhimurium} strain with \textit{shdA} and \textit{aroA} mutations was shed in the feces of BALB/c mice intermittently and overall for a shorter time compared to a control \textit{aroA} mutant strain in a reduced virulence model of infection (Kingsley et al. 2000, 2002). Directly upstream of \textit{shdA} is \textit{ratB} and in similar experiments \textit{ΔratB} mutants were shed for a shorter period from stool of BALB/c mice and CBA/J mice which are more resistant to lethal \textit{S. Typhimurium} infection. In contrast, deletion of the \textit{svh} gene from this island did not limit shedding (Kingsley et al. 2003). An additional factor contributing to fecal shedding is the \textit{misL} gene which, like \textit{shdA}, encodes a T5SS protein that binds fibronectin (Dorsey et al. 2005). Unlike \textit{shdA} and \textit{ratB}, \textit{misL} is located on SPI3. A \textit{misL} mutant, MCL1, was not deficient in colonization but was recovered from cecum, spleen and mesenteric lymph nodes at much lower numbers than a competing \textit{phoN} mutant strain which was not deficient in colonization. Further, a \textit{shdA misL} double mutant, CWD9, was recovered in lower amounts than a \textit{shdA} mutant in competition experiments; however, this difference was not statistically significant (Dorsey et al. 2005). In parallel development towards a live vaccine with reduced shedding, \textit{shdA} and \textit{misL} mutations were introduced into candidate \textit{S. Typhimurium} vaccine SL3261 (an \textit{aroA} mutant), either singly or combined. Significantly fewer bacteria were detected in feces of mice 14 days after oral delivery of either the single or double mutant compared to a control strain (Abd El Ghany et al. 2007). Additionally, introduction of these mutations into a vaccine strain carrying a plasmid for expression of a heterologous antigen, tetanus toxin fragment C (TetC), did not diminish or alter systemic IgA and IgG responses or seem to negatively impact cytokine production while also stimulating anti-TetC antibody production. Most interestingly, a single dose of vaccine carrying either TetC alone or TetC in the presence of \textit{shdA} and \textit{misL} mutations was able to protect 90% and 100% of mice, respectively, from lethal \textit{S. Typhimurium} challenge (Abd El Ghany et al. 2007).

Reducing transmissibility by programmed attenuation and delayed lysis

Programmed bacterial cell lysis or delayed lysis is a methodology that ensures death of a candidate vaccine strain after colonization of immune tissues. This novel system was developed by Roy Curtiss III’s group to reduce the shedding of recombinant-attenuated \textit{Salmonella} vaccine (RASV) strains that have been exquisitely engineered to deliver homologous and heterologous antigens to immune system tissues (Kong et al. 2008). The system tightly controls expression of enzymes required for synthesis of two components of the peptidoglycan layer of the cell wall, diaminopimelic acid (DAP) and muramic acid. Synthesis of DAP is controlled by aspartate-semialdehyde dehydrogenase (encoded by \textit{asd}), and UDP-N-acetylgalactosamine enolpyruvyl transferase (encoded by \textit{murA}) is the first enzyme involved in muramic acid synthesis; placing both of the genes encoding these two enzymes under the transcriptional control of the arabinose inducible AraC-P\textit{BAD} promoter ensures that in the absence of exogenously supplied arabinose vaccine strains engineered using this approach lyse due to the inability to synthesize the cell wall. Additional mutations introduced into this strain to promote bacterial lysis include deletion of genes encoding enzymes for GDP-fucose synthesis (\textit{A(gmd-fcl)-26}) which prevents synthesis of colonic acid which can rescue \textit{asd} mutants and a mutation in \textit{relA} (\textit{ArelA1123}) which renders the bacteria incapable of responding to amino acid starvation and ensures death in vivo (Torok and Kari 1980; Whitfield 2006; Kong et al. 2008). These RASV strains exhibit no lethality in mouse models when delivered at considerably high doses and disseminate to and colonize lymphoid tissues. In the absence of arabinose during in vivo infection, these strains are engineered to lyse after several rounds of
replication and are eventually cleared from the liver, spleen and Peyer’s patches. Moreover, they have been shown to elicit serum IgG and mucosal IgA antibodies and stimulate antigen-specific cytokine production from T-cells.

**Salmonella live carrier vaccines**

The use of live attenuated bacterial vaccines as carriers for mucosal delivery of foreign antigens to stimulate the mucosal immune system was first proposed over three decades ago. This novel strategy aimed to induce immunity against at least two distinct pathogens using a single bivalent carrier vaccine. It was first tested using a live attenuated S. Typhi Ty21a strain (5076-1C) in clinical trials in 1984 (Tramont et al. 1984), with modest but promising humoral immune responses elicited against the foreign antigen. However, this candidate live attenuated vaccine strain proved to be genetically unstable, and promising initial clinical results could not be consistently repeated in later studies (Black et al. 1987; Herrington et al. 1990). Since then, clinical trials with additional *Salmonella*-based carrier vaccines have been conducted, with most vaccines derived from attenuated S. Typhi. As with the original trial, only modest foreign antigen-specific immunity has been achieved in most cases, despite incorporation of incremental improvements in antigen expression technologies and carrier design over the years.

Several critically important factors influence the quality of an adaptive immune response against carrier vaccines and against the foreign antigen(s) they present to the immune system. Based on data from both murine intranasal animal models (Galen et al. 1997; Pasetti et al. 2000; Pickett et al. 2000) and clinical trials (Bumann et al. 2001; Metzger et al. 2004; Khan et al. 2007; Bumann et al. 2010), several key factors influence carrier-and foreign antigen-specific immunity, including carrier strain genetics, antigen expression strategies, immunization protocols and human host factors (Galen and Curtiss 2014; Clark-Curtiss and Curtiss 2018). Clearly, several different strategies can be either engineered or carefully chosen to minimize any detrimental influence that each of these factors may exert on carrier vaccine immunogenicity. Here, we will focus our discussion on key concepts relevant to recent advances in *Salmonella*-based carrier vaccine development.

Since the first clinical trials conducted with carrier vaccines in the 1980s (Tramont et al. 1984; Black et al. 1987; Herrington et al. 1990), significant efforts have been invested in expression technologies for optimizing production of foreign antigens to stimulate biologically relevant immunity including either mucosal, humoral or cellular immune responses (Roland and Brenneman 2013; Galen and Curtiss 2014; Clark-Curtiss and Curtiss 2018). It is well appreciated that carrier vaccines derived from attenuated S. Typhi are fully capable of eliciting mucosal, humoral and cellular responses after oral immunization in humans (Levine et al. 2001; Sztein 2007; Booth et al. 2017). However, the design of carrier vaccines presents unique challenges posed directly by expression of the foreign antigen that are not encountered in the engineering of attenuated parental vaccines per se. In addition to the potential toxicity of a given foreign antigen when expressed in a carrier vaccine, which can directly interfere with the viability of the strain, inappropriate expression of high levels of an otherwise tolerable foreign antigen can stress the metabolic fitness of a carrier vaccine (Boe et al. 1987; Bailey 1993; Glick 1995; Zahn 1996; Corchero and Villaverde 1998). We and others have written extensively on this topic and will not recapitulate detailed arguments herein but rather refer the reader to more extensive discussions of mechanisms published elsewhere (Roland and Brenneman 2013; Galen and Curtiss 2014; Clark-Curtiss and Curtiss 2018). Here, we emphasize that inappropriately high expression levels of foreign antigens create a physiological burden on a vaccine strain. This reduction in fitness is functionally equivalent to overattenuation of the vaccine strain, reducing both the replication of these organisms and their ability to reach immune inductive sites at sufficient levels to elicit both innate and adaptive immunity.

**Expression of foreign antigens from the chromosome**

Overattenuation of carrier vaccines due to the metabolic burden associated with inappropriate expression of foreign antigens often occurs when these antigens are expressed from plasmids of high copy number. Plasmid-based expression of foreign antigens exerts metabolic pressure on the carrier vaccine that can induce the spontaneous loss of the plasmid in vivo to improve growth rate (Galen and Curtiss 2014). This point is clearly illustrated by the early efforts by Osorio et al. (2009) to develop a live oral Ty21a-based vaccine against anthrax focused on targeting the 83-kDa full-length Protective Antigen (PA83) of anthrax toxin, expressed from a multicopy expression plasmid. Placing the gene encoding PA83 under the transcriptional control of a strong constitutive P_{lpp} promoter induced loss of the plasmid in 98% of passage colonies after 50 generations of propagation in the antibiotic selection for the plasmid. Combining transcriptional control using an environmentally regulated P_{traA} promoter with an HlyA-derived antigen export system significantly improved plasmid stability and antigen expression, eliciting high levels of PA83-specific serum IgG responses and toxin neutralizing antibody (TNA)
titres in immunized mice and 100% protection against a lethal aerosol challenge with 20 times the 50% lethal dose (LD$_{50}$) of purified anthrax spores (Osorio et al. 2009). After considering both the risk of plasmid instability in vivo, as well as the need for plasmid selection using antibiotics (currently discouraged by the US Food and Drug Administration for live vaccines intended for human oral immunization), the candidate Ty21a-derived anthrax vaccine described above was re-engineered to express PA83 from a genetic cassette integrated into the chromosome (Sim et al. 2017). To accommodate the loss of copy number after removal from plasmids, the integrated cassette was placed under the transcriptional control of the strong constitutive P$_{pp}$ promoter; however, subsequent passaging of the resulting candidate vaccine strain resulted in 100% genetic stability and excellent expression of PA83 after >100 generations of propagation. Despite a significant drop in TNA titres elicited in mice by chromosomally encoded antigen (9942, Sim et al. 2017) versus plasmid encoded antigen (16241, Osorio et al. 2009), 100% protection was observed after aerosol challenge with 5 LD$_{50}$ of purified spores; interestingly 70% protection was also observed in vaccinated rabbits when aerosol challenged with 200 LD$_{50}$ of purified fully virulent Ames spores. These data clearly demonstrate the feasibility of overcoming many of the potential toxicity, genetic stability and antibiotic resistance problems associated with plasmid-based expression of foreign antigens in live carrier strains while still maintaining robust immunogenicity and protective efficacy against challenge.

The pace of engineering chromosomal expression of foreign antigens in Ty21a typhoid carrier vaccines has significantly increased in the past 5 years with efforts to develop bivalent vaccines against both typhoid fever and shigellosis caused by multiple serotypes of Shigella. These efforts have focused on the expression of protective Shigella O polysaccharide antigens on the surface of Ty21a. Since the operons encoding these antigens are typically >10 kilobases (kb) in length, it would be unlikely that such large cassettes would be genetically stable on multi-copy expression plasmids. Therefore, chromosomal expression of such large foreign antigen cassettes was hypothesized to ensure stable expression that would potentially engender protection. This hypothesis was confirmed by Dharmasena et al. (2013) in a study that compared chromosomal versus plasmid-based expression and immunogenicity of a large synthetic operon encoding the form I O-antigen of Shigella sonnei in Ty21a. When an ~12-kb genetic cassette encoding the S. sonnei O-antigen biosynthetic operon was inserted into a low copy expression plasmid and introduced into Ty21a, expression of the heterologous O antigen was observed in Ty21a but attempts to remove the antibiotic resistance marker to improve clinical acceptability resulted in significant plasmid instability. After integration into the chromosome, excellent expression of form I O-antigen was observed, and 100% genetic stability was demonstrated over 75 generations of propagation. Robust titres of serum IgG against S. sonnei were observed after two intraperitoneal immunizations, and 100% protection against an intraperitoneal challenge with 100 LD$_{50}$ of fully virulent S. sonnei was also observed (Dharmasena et al. 2013). These observations support the early observations documented in clinical trials with Ty21a strain 5076-IC in which a chromosomally integrated cassette encoding S. sonnei engendered antigen-specific serum immunity in volunteers and showed protection against severe dysentery in human challenge studies, despite the genetic instability of the construct (Black et al. 1987). Dharmasena et al. (2016b) have gone on to construct and test similar Ty21a bivalent vaccines stably expressing heterologous O antigens from S. dysenteriae, S. flexneri 2a and S. flexneri 3a (Dharmasena et al. 2017). Interestingly, attempts to elevate expression levels of the foreign O antigen by placing the operons under the transcriptional control of the strong constitutive P$_{pp}$ promoter were not required to confer 100% protection against homologous challenge with Shigella, despite significantly enhancing O antigen expression versus native Shigella promoters.

Bivalent Ty21a vaccines against shigellosis have also been constructed by incorporating chromosomally encoded acid resistance operons and assessed by Wu et al. (2017) for immunogenicity and protective efficacy in mouse models. In contrast to studies mentioned above, in which mice were both immunized and challenged by the intraperitoneal route, mice in these experiments were immunized and challenged intranasally to more closely approximate the oral route of immunization intended for human use. This group successfully integrated both the acid resistance and S. sonnei O antigen operons tandemly into the same chromosomal locus employed by previous investigators (the inactive Vi locus of Ty21a). This tandem set of chromosomal insertions resulted in robust expression of acid resistance, cell surface expression of the heterologous O antigen, and high levels of LPS-specific IgG responses. Most importantly, protective efficacy was 55% for mice immunized with an isogenic strain without the acid resistance cassette, but efficacy improved to 85% for mice receiving isogenic vaccines encoding for acid resistance (Wu et al. 2017). These results suggest that a single chromosomal locus can be effectively exploited for the simultaneous expression of immunogenic levels of multiple vaccine antigens that could not otherwise be stably expressed either on a single expression plasmid or multiple expression plasmids contained within a single carrier strain.
**Exploiting *Salmonella* secretion systems in live carrier vaccines**

The T3SS of *Salmonella* provides an additional mechanism for the development of live vector vaccines against *Salmonella* and other pathogens. T3SS translocate effector proteins directly to the host cell cytoplasm and SPI1 and SPI2 translocate effectors at different phases of infection. These systems in *Salmonella* are among the best studied systems in Gram-negative bacteria, and their composition and functions have been thoroughly reviewed (Pinaud et al. 2018; De Souza Santos and Orth 2019; Miletic et al. 2020).

Fusion of heterologous antigens to T3SS effectors has been a successful strategy for development of *Salmonella* as live vector vaccines and fusion to various effector proteins have mediated export to the endosomal compartment and elicited protective immune responses. This is particularly advantageous for immunization against non-*Salmonella* diseases. Using Typhimurium strain 12023 with mutations in a variety of genes including htrA, purD, galE or htrA/purD as carrier strains, fragments of *Listeria monocytogenes* listeriolysin (*lisA*, Llo) and p60 (*iap*) were fused to the N-terminal translocation signal of the effector SseF (Husseiny et al. 2007). Translocation of fusion proteins was detected in bone-marrow derived dendritic cells and a macrophage-like cell line. Importantly, mice immunized with these constructs had reduced organ burden 8 weeks after challenge with a sublethal dose of *L. monocytogenes* (Husseiny et al. 2007).

Further development of this system demonstrated that fusion of ovalbumin (OVA) and listeriolysin to the effector SseJ stimulated OVA-specific T-cells in vitro and Llo-specific cytotoxic T-cells which are important for clearance of *L. monocytogenes* (Hegazy et al. 2012).

This strategy has also been applied to development of an anti-cancer vaccine. The tumour-associated antigen, survivin, optimally expressed as a fusion protein with SseJ was translocated to the cytoplasm of bone marrow-derived dendritic cells demonstrating efficient export of a tumour antigen. Oral delivery of this vaccine strain resulted in CD8 T-cell infiltration and shrinking of tumours (Xu et al. 2014). This engineering has been adapted to CVD 908-htrA, and this vaccine candidate will be assessed in a Phase 1 clinical trial for treatment of multiple myeloma (ClinicalTrials.gov Identifier: NCT03762291). Most recently, fusion of *Pseudomonas aeruginosa* V antigen (PcrV) to SseJ was successfully exported into RAW264.7 macrophage-like cells when expressed by Typhimurium strain 14028 (Aguilera-Herce et al. 2019). The PcrV-SseJ fusion protein was exported from a Typhimurium vaccine strain SV9699 (*aroA551::Tn10 ΔaroB::Km*) and induced high titres of PcrV-specific IgG in mice after a single intraperitoneal dose. Mice subsequently challenged with a lethal dose of *P. aeruginosa* PAO1 had reduced bacterial loads in the lung and spleen and were significantly protected from challenge in comparison to immunization with vector only (Aguilera-Herce et al. 2019).

One of the few live *Salmonella* vaccines expressing a foreign antigen to be tested in clinical trials includes an HIV-1 vaccine developed by engineering a plasmid to encode the first 104 amino acids of SopE fused to a monoclonal antibody tag (M45), Influenza A nucleoprotein (NP), and a mutant HIV Gag protein codon optimized for T3SS. This plasmid was introduced into S. Typhimurium strain SL1344 carrying multiple mutations, *AphoP/AphoQ aroA, strA/strB* and *asd* to produce vaccine strain CKS257 (Kotton et al. 2006). Preclinical studies using a simian immunodeficiency virus (SIV)-specific construct demonstrated Gag epitope-specific CD4+ and CD8+ T-lymphocytes in immunized rhesus macaques but animals were not protected from challenge with SIV (Evans et al. 2003). In a Phase 1 safety trial, volunteers developed *Salmonella*-specific IgA and IgG titers but no antibodies against the HIV Gag protein suggesting that insufficient protein was expressed and/or translocated.

**Use of recombinant *Salmonella* as reagent strains for the production of antigens used in other vaccine formats**

*Salmonella* strains engineered using the strategies discussed above have additional value in their capacity as sources of bacterial surface components for parenteral vaccines. Attenuation allows for them to be grown to high concentration for high yield of products with reduced risk to personnel. These bacterial components can subsequently be used as purified antigens for subunit or conjugate vaccines. Conjugate vaccines are composed of polysaccharides covalently linked to a protein, and while polysaccharides alone are poor immunogens, particularly in children, the addition of protein carriers boosts humoral and cellular immune responses (Rappuoli 2018). Tybar-TCV, a vaccine composed of the Vi polysaccharide of *S. Typhi* conjugated to tetanus toxoid, had an efficacy of over 80% in Phase 3 trials in children aged 9 months to 16 years in Nepal (Shakya et al. 2019). It remains to be seen how long this protection will last, but its efficacy in children makes it an important tool in reducing typhoidial *Salmonella* infections in LMICs.

NTS do not express capsular polysaccharides like Vi; however, OPS does elicit antibody responses that mediate clearance of bacteria (Baliban et al. 2017; Micoli et al. 2018; Schuster et al. 2019). The diversity in O-antigens
presents a challenge in developing a broadly protective vaccine but the ability to produce large quantities of specific O-antigens and proteins from corresponding serogroups make the production of a multivalent glycoconjugate vaccine more feasible. Outer membrane vesicles (OMVs) represent a parallel strategy as the polysaccharide and protein components are already closely associated with the bacterial membrane. Below, we discuss progress in production of glycoconjugate and vesicular vaccines against NTS.

**Salmonella as a source of antigens for conjugate vaccines**

The immune response to natural infection with *Salmonella* includes elevated antibody titres to the core and OPS (COPS). OPS alone is a poor immunogen and does not elicit helper T cell memory, but this can be overcome by chemical linkage to carrier proteins. Early studies using saccharides derived from phage-mediated hydrolysis of an *S. Typhimurium* OPS demonstrated that mice were protected from homologous lethal challenge after active immunization with purified saccharides alone, saccharides conjugated to diphtheria toxoid, tetanus toxoid or outer membrane proteins (OMPs) (Jorbeck et al. 1979; Watson et al. 1992). Jorbeck et al. (1981) also raised antibodies in rabbits against similar vaccines and passively transferred that sera to mice prior to lethal challenge; transfer of these antibodies alone was sufficient to protect mice from infection suggesting that antibodies to OPS are important for limiting disease. In Phase 1 and 2 clinical trials, conjugates of *S. Paratyphi* A OPS linked to tetanus toxoid increased LPS-specific IgG titers in volunteers, and these antibodies exhibited bactericidal activity that was abrogated by absorption with *S. Paratyphi* A LPS (Konadu et al. 2000).

The linkage of OPS to a variety of carrier proteins to produce glycoconjugate vaccines has been a major focus in the development of vaccines against *Salmonella*. While the protein partners in these glycoconjugates have standard sizes and share homology, OPS structure varies widely across and within species of Gram-negative bacteria. Even within a single strain, multiple O antigen chain lengths are produced and vary depending on the growth phase. Controlling the chain length is important as the size of the polysaccharide can influence the immune response. Additionally, conjugate vaccines are highly defined and having reagent strains that produce O polysaccharide within a certain size range means that less effort needs to be expended to purify O polysaccharide of a specific molecular weight thereby enabling more economical production.

One of the major pathways controlling the synthesis of OPS in *Salmonella* is the Wzx/Wzy-dependent pathway, a family of integral inner membrane proteins comprised of a Wzx flippase, Wzy polymerase and several other related Wzz polysaccharide chain-length regulators (Islam and Lam 2014). The first step of the pathway involves a series of glycosyltransferase reactions which synthesize O-antigen repeats on a lipid carrier, undecaprenyl pyrophosphate (UndPP). The Wzx flippase then translocates the UndPP-linked O-antigen across the inner membrane to the periplasmic side and transfers it to the Wzy polymerase for addition to a growing polymer. The Wzz regulator protein controls the number of OPS repeats in this polymer thereby controlling the length of LPS. *Salmonella* can encode more than one Wzz protein and each produces OPS of a different modal length. WzzB produces long-chain OPS with lengths of 16–35 O-antigen repeat units while the *Escherichia coli* Wzz homolog FepE produces very long OPS with lengths of >100 O-antigen repeating units (Murray et al. 2003).

Overexpression of wzzB cloned from *S. Typhimurium* I77, a clinical isolate from blood culture, resulted in increased expression of long relative to short and medium length OPS in *S. Typhimurium* CVD 1925, a reagent strain engineered for OPS purification for conjugate vaccines (Hegerle et al. 2018). Importantly, overexpression of *S. Typhimurium* wzzB in the candidate live attenuated vaccine strain *S. Paratyphi* A CVD 1902 also led to the production of OPS with chain lengths comparable to that of *S. Typhimurium*. Additionally, overexpression of a second *S. Typhimurium* wzz family member, fepE, produced very long OPS in heterologous systems.

At the CVD, we have developed conjugate vaccines against *S. Typhimurium* and *S. Enteritidis* which consist of COPS chemically linked to phase 1 flagellin proteins (FlIC) from the homologous serovar (Baliban et al. 2017). We have constructed reagent strains to make purification of COPS and FlIC safer, simpler, and more economical (Fig. 2). Deletion of *guaBA* and *clpP* (encodes a protease which regulates flagella expression) from *S. Typhimurium* I77 and *S. Enteritidis* R11 resulted in CVD 1921 and CVD 1941, respectively (Tennant et al. 2011). These strains require guanine for growth and are hyperflagellated and hyper motile. Subsequent deletion of *flID* eliminates the cap protein of the flagellin and results in large quantities of monomeric FlIC being released into the supernatant. The resulting strains CVD 1923 (*S. Typhimurium ΔguaBA ΔclpP ΔflID*) and CVD 1943 (*S. Enteritidis ΔguaBA ΔclpP ΔflID*) are highly attenuated and have an LD$_{50}$ greater than 5 log$_{10}$ above their parental strains (Tennant et al. 2011). Deletion of the gene that encodes Phase 2 flagella, FliB, in *S. Typhimurium* ensures that only Phase 1 flagellin is produced by our reagent strain, CVD 1925. Since *S. Typhimurium* produces mostly short length OPS, we overexpressed wzzB from a...
plasmid to ensure that medium to long chain OPS was produced consistently (Baliban et al. 2017; Hegerle et al. 2018). Importantly, because these strains are highly attenuated, they can be grown to high concentration by fermentation, and flagellin monomers can be purified directly from the culture supernatant (Simon et al. 2014) while OPS can be extracted from the bacterial cell mass. Conjugate vaccines produced using COPS and FliC from S. Typhimurium CVD 1925 (pSEC10-wzzB) and S. Enteritidis CVD 1943 were immunogenic and able to protect mice against lethal challenge with wild-type NTS (Simon et al. 2011; Baliban et al. 2017). These vaccines are currently being evaluated in a Phase 1 clinical trial (ClinicalTrials.gov Identifier: NCT03981952).

Outer membrane vesicles

In addition to their use for the synthesis of conjugate vaccines, reagent strains derived from Salmonella have also recently been used for production of OMVs (Fig. 3). OMVs are small approximately spherical exosomes that are spontaneously shed from Gram-negative bacteria. They range in size from ~25 to 250 nm in diameter (approximately one tenth the size of the originating bacterium) and are comprised predominantly of the endogenous proteins, lipoproteins and LPS found in the bacterial outer membrane, in addition to other smaller amounts of peptidoglycan, periplasmic proteins and other cellular constituents (Schwechheimer and Kuehn 2015; van der Pol et al. 2015; Rossi et al. 2016; De Benedetto et al. 2017). Due to the robust immunogenicity of these vesicles and their relative ease of manufacture, significant interest in the use of OMVs as vaccines against a variety of human pathogens has rapidly expanded the genetic and pharmaceutical technologies involved in development of this novel vaccination strategy.

Purified OMVs from S. Typhimurium have been reported to be immunogenic facsimiles of wild-type S. Typhimurium, capable of inducing activation and maturation of murine macrophages and dendritic cells in vitro, as well as antigen-specific B cell and T cell CD4+ responses in vivo that resulted in protection against homologous challenge with virulent S. Typhimurium (Alaniz et al. 2007). Schetters et al. (2019) have also reported that purified S. Typhimurium OMVs are capable of antigen-specific dendritic cell-mediated cross-presentation to CD8+ T cells. Interestingly, in these experiments, OMVs were engineered for expression of a heterologous ovalbumin model 98-residue peptide. Such observations support the use of purified OMVs as an immunologically flexible vaccine modality, capable of inducing pathogen-specific humoral and cellular immunity that potentially confers robust protection against disease.

Among the full array of biological molecules contained on OMVs are pathogen-associated molecular patterns which act as powerful agonists to host innate pattern-recognition receptors including toll-like receptors (TLRs), responsible for triggering initial inflammatory responses.
to the presence of potential pathogens. The success of a candidate vaccine often rests on the balance between stimulating maximum protective immunity while at the same time minimizing clinically unacceptable inflammatory responses (i.e., systemic reactogenicity). For Gram-negative bacteria, strong reactogenicity is often associated with the interaction of LPS with TLR4 complexed with myeloid differentiation factor 2 (TLR4/MD2); the principle LPS domain responsible for this interaction is the lipid A membrane anchoring portion of the molecule (Bertani and Ruiz 2018; Mancini et al. 2020). It has been demonstrated that using various combinations of naturally occurring bacterial acylating and deacylating enzymes involved in lipid A synthesis, it is possible to genetically engineer the structure of lipid A to generate a broad spectrum of immunostimulatory LPS variants ranging from fully reactogenic to non-reactogenic entities (Needham et al. 2013).

Although production of OMVs from Salmonella and other Gram-negative bacteria occurs naturally, release of these vesicles is strongly influenced by growth conditions and bacterial metabolism and is therefore not amenable to scale-up for vaccine production. However, efficient production of OMVs from Salmonella can be easily facilitated by genetic deletion of the chromosomal tolR gene which disrupts the stable anchoring of the outer membrane to the inner membrane (Egan 2018). This strategy has been applied to the development of an OMV-based vaccine against shigellosis derived from S. sonnei (1790GAHB); these Generalized Modules for Membrane Antigens (GMMAs) are highly immunogenic and have rapidly progressed through Phase 1 and 2 clinical trials (Launay et al. 2017, 2019; Obiero et al. 2017). The same strategy has also been applied to the development of a bivalent GMMMA-based vaccine against iNTS derived from invasive strains of both S. Typhimurium and S. Enteritidis, the two most prominent serovars in endemic areas of sub-Saharan Africa (Gilchrist and MacLennan 2019).

GMMAs purified from S. Typhimurium and S. Enteritidis have been examined in detail for reactogenicity; interestingly, unmodified lipid A is present as a mixture

![Figure 3](https://example.com/figure3.png)

**Figure 3** Production of outer membrane vesicles (OMVs) and lipid A modifications. OMVs are spherical vesicles enriched with proteins. Their production can be manipulated to express antigens from unrelated pathogens as shown in this figure. Black dashed arrows indicate the movement of the antigen from cytoplasm to outer membrane (OM). (1) An unfolded heterologous antigen (HA) in the periplasm (yellow line with an N-terminal secretion signal in red and a C-terminal β-signal in blue) is translocated from the cytoplasm across the inner membrane (IM) via the Sec translocase and the chaperone protein SurA directs HA to the BAM complex. (2) The BAM complex facilitates proper folding of HA and its insertion into the OM. (3) Vesicles subsequently bud from the OM and contain OM proteins, LPS, periplasmic proteins, and the foreign antigen. (4) The inset shows lipid A modifications used to reduce the reactogenicity of OMVs. Black solid arrows indicate the position at which the acyl chain (zigzag lines) modifications are made. These include deletion of HtrB which blocks the addition of a 12-carbon secondary chain to the 2’ position; deletion of MsbB which blocks the addition of a 14-carbon secondary chain to the 3’ position; deletion of PagP which blocks the addition of a 16-carbon secondary chain to the existing acyl chain at the 2’ position. PagL is a deacylase that removes the β-hydroxyacyl group at carbon 3 position and LpxE dephosphorylates lipid A at the carbon 1 position. COPS, core O polysaccharide; IM, inner membrane. Figure created with BioRender.com.
of hepta-acylated and hexa-acylated species and as such was expected to be unacceptably reactogenic in monocyte activation test (MAT) assays. Therefore, gene deletions were introduced into parent strains targeting either PagP (catalyzes the addition of a 16 carbon secondary acyl chain to the existing acyl chain at position 2 of lipid A), MsbB (catalyzes the addition of a 14 carbon secondary chain to the 3’ position of lipid A), HtrB (catalyzes the addition of a 12 carbon secondary chain to the 2’ position of lipid A), or combinations of these deletions (Rossi et al. 2016). Construction of the modified S. Enteritidis strain ΔtolR ΔmsbB ΔpagP resulted in purified OMVs containing LPS with uniformly penta-acylated lipid A and a 30-fold reduction in reactivity in the MAT assay (a level comparable to that of similarly engineered S. sonnei GMMA tested in clinical trials), while the same engineered deletions in S. Typhimurium resulted in a penta-acylated LPS with a 200-fold reduction in reactivity (Rossi et al. 2016). Surprisingly, it was also reported that the engineering of less reactogenic lipid A can result in the shortening of O-antigen repeats in the resulting LPS (De Benedetto et al. 2017). Although this effect was not observed for vesicles derived from S. Enteritidis, the effect was dramatic for engineered vesicles from S. Typhimurium, with the molar percent of O-antigen to total LPS being reduced from 10% for ΔtolR vesicles to levels less than 1% for vesicles purified from ΔtolR ΔmsbB and ΔtolR ΔhtrB engineered strains; a triple ΔtolR ΔmsbB ΔpagP S. Typhimurium mutant was not tested in these experiments. When used to immunize CD1 mice subcutaneously with two doses of adjuvanted S. Typhimurium OMVs (~1 mg per dose) spaced 4 weeks apart, LPS-specific serum IgG responses were comparable to unmodified vesicles from ΔtolR strains, but functional serum bactericidal titres dropped 3–5-fold relative to unmodified vesicles (De Benedetto et al. 2017).

In an elegant study recently reported by Micoli et al. (2018), the immunogenicity and efficacy of unmodified OMVs from ΔtolR strains of S. Typhimurium and S. Enteritidis were compared to glycoconjugate vaccines in C57BL/6 mice immunized subcutaneously with two doses spaced 28 days apart and challenged intraperitoneally 17 days later with fully virulent S. Typhimurium or S. Enteritidis. Both OMV-based and glycoconjugate vaccines were tested with or without the adjuvant Alhydrogel. OMVs were found to induce excellent O-antigen-specific humoral responses and serum bactericidal responses comparable to the conjugates, but without the use of adjuvant; unadjuvanted glycoconjugate vaccines were not immunogenic. However, the OMVs used in this study were not engineered for reduction in the reactivity of lipid A. Therefore, adsorption of OMVs to aluminium-based adjuvants may still be necessary to improve the safety of these vaccines, based on the observation that the reactivity of LPS in purified OMVs can be reduced relative to free LPS by adsorption to aluminium-based adjuvants (Rosenqvist et al. 1998). Such a solution may resolve the issue of reduced O-antigen concentrations observed in OMVs purified from strains of S. Typhimurium engineered for reduction of lipid A reactivity.

New directions for OMV-based vaccines

The purified OMVs described above were all generated from ΔtolR mutants of S. Typhimurium and S. Enteritidis, further engineered by deletion of endogenous acylating enzymes to create less reactogenic vaccine candidates. However, it has been recently reported by Elhenawy et al. (2016) that overexpression (rather than deletion) of an endogenous lipid A modifying enzyme PagL in S. Typhimurium results in overproduction of OMVs by an unknown mechanism. PagL is a deacetylase that removes the β-hydroxymyristoyl chain at the carbon 3 position of lipid A. When overexpressed from a multicopy expression plasmid in S. Typhimurium, OMV production increased four fold (Elhenawy et al. 2016). This remarkable observation raises the intriguing possibility of easily introducing plasmids encoding PagL into other Gram-negative strains to induce the formation of vesicles that could potentially be used as vaccines similar to the GMMA strategy.

Over the years we and others have developed attenuated strains of S. Typhi, several of which have been successfully tested in clinical trials (Galen et al. 2016). Our group has also developed genetically stabilized low copy number plasmids for expression of foreign antigens from unrelated human pathogens as candidate vaccines (Galen et al. 2010), as well as surface expression systems capable of moving such foreign antigens out to the surface of our attenuated vaccine strains (Galen et al. 2004, 2009). Expression of PagL in such attenuated strains raises the hypothesis of being able to produce recombinant OMVs (rOMVs) expressing foreign vaccine antigens on their surface in the context of TLR-specific agonists capable of efficiently stimulating innate immunity and more robust adaptive responses. Through integration into the chromosome or further addition of other lipid A modifying enzymes such as LpxE from Francisella tularensis (Needham et al. 2013), which dephosphorylates lipid A at the carbon 1 position, it becomes theoretically possible to engineer S. Typhi candidate vaccine strains capable of producing naturally adjuvanted OMVs similar in reactivity profile to the clinically acceptable monophosphoryl lipid A (MPL) adjuvant. It has also been reported that vaccine antigen administered by two separate routes or
formulations (i.e., a heterologous prime-boosting strategy) results in more robust immunogenicity when compared to immunity elicited by either individual modality alone (Vindurampulle et al. 2004; Chinchilla et al. 2007; Ramirez et al. 2010). It may therefore be possible to use attenuated S. Typhi vaccine strains engineered for hyperexpression of rOMVs in combination with purified rOMVs in a novel heterologous prime-boost immunization regimen to elicit protective immunity against a wide variety of human pathogens.

**Conclusions**

The global burden of *Salmonella* remains a concern given increasing antimicrobial resistance. However, the variety of *Salmonella* vaccine approaches under development is a promising sign of a future where this burden is minimized. Although live attenuated *Salmonella* vaccines are highly immunogenic and easy to administer, further research is needed to reduce fecal shedding of NTS vaccine strains to address regulatory concerns. Additional studies will be needed to determine whether live attenuated *Salmonella* vaccines will be safe and immunogenic in HIV-infected or other immunocompromised individuals. Notably, *Salmonella* are also ideal vectors for the expression of heterologous antigens and can provide a flexible platform in the development of vaccines against other pathogens. Finally, the ease with which *Salmonella* can be manipulated has also allowed them to serve as reagent strains for safe and economical production of subunit, OMV-based and conjugate vaccines.

**Conflict of Interest**

Drs Galen and Tennant have multiple patents on *Salmonella* vaccines.

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