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54 Titre : Modified strain of salmonella enterica typhi

57 Abrégé :

The present invention relates to the modification of a live attenuated strain of *Salmonella enterica* serovar Typhi, wherein its natural surface-exposed polysaccharide and flagellin antigens may be converted to, or augmented by, those from other strains of *Salmonella*, including *S. enterica* serovars Paratyphi, Typhimurium and Enteritidis. The present invention also relates to modified strains of *Salmonella enterica* serovar Typhi being suitable for use as components of a vaccine for enteric fever and salmonellosis.

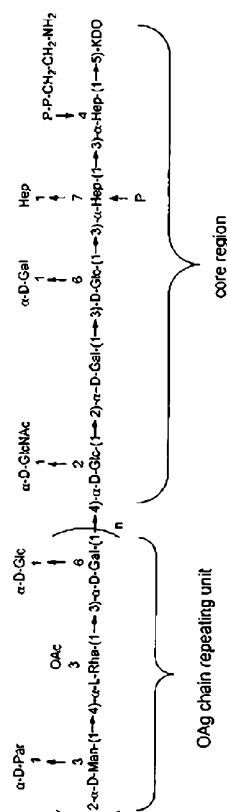


Figure 1

Fig. 1

MODIFIED STRAIN OF *SALMONELLA ENTERICA* TYPHI**Field of Invention**

The present invention relates to the modification of an attenuated strain of *Salmonella enterica* serovar Typhi, such that its natural surface-exposed polysaccharide and flagellin antigens are converted to, or augmented by, those from other strains of *Salmonella*, including *S. enterica* serovars Paratyphi, Typhimurium and Enteritidis. Such a modification utilises the long history of safe use of strains of *S. Typhi* in humans as a typhoid vaccine, to deliver homologous antigens from other members of the genus *Salmonella* as components of vaccines for enteric fever and Salmonellosis.

Background

Enteric fever is exclusive to humans and is caused by two serovars of *Salmonella enterica*: Typhi and Paratyphi, the latter comprising serovars A, B and C. Typhoid fever is estimated to have caused 21.7 million illnesses leading to 217,000 deaths in 2000, with 5.4 million cases of paratyphoid fever annually (Crump *et al.* 2004, Bull. World Health Organ. 82: 346-353). Typhoid and paratyphoid fevers are very similar infections of the reticuloendothelial system, intestinal lymphoid tissue and gallbladder, leading to acute febrile illnesses. Blood culture or serological tests are required to differentiate them. Outbreaks of typhoid fever are frequent in sub-Saharan Africa and Asia, with *S. Paratyphi* A responsible for up to 50% of enteric fever cases in Asia; enteric fever is also endemic in Latin America (Crump & Mintz 2010, Clin. Infectious Dis. 50: 241-246). *S. Paratyphi* A is the most abundant strain causing paratyphoid fever globally, with several reports showing it causing an increasing number of the total enteric fever cases (Fangtham & Wilde 2008, Int. J. Travel Med. 15: 344-350).

All licensed injected typhoid vaccines use the Vi capsular polysaccharide antigen purified from *S. Typhi*, and are single-dose with boosting recommended every 2-3 years (Martin 2012, Curr. Opin. Infect. Dis. 25: 489-499). The main adverse event is pain at the injection site. The only live attenuated typhoid vaccine is *S. Typhi* Ty21a (Vivotif®), developed by chemical mutagenesis of *S. Typhi* Ty2 and administered orally in 3-4 doses, with boosting required after 5-7 years (Martin 2012, Curr. Opin. Infect. Dis. 25: 489-499). Ty21a is very safe and well tolerated. In a comparative clinical study of injected vaccine Typherix® versus Vivotif®, only the latter was found to generate immune responses that mimic the natural infection (Kantele *et al.* 2013, Plos One 8: e60583).

The Vi antigen is not present in *S. Paratyphi* A or B (but is expressed by *S. Paratyphi* C), so injected Vi vaccines are ineffective against the two most prevalent *S. Paratyphi* strains. Ty21a has been proven to confer cross-protection against *S. Paratyphi* B in field studies (Levine *et al.* 2007, Clin. Infectious Dis. 45: S24-S28). However, field studies using Ty21a showed little or no cross-protection against *S. Paratyphi* A, despite the generation of cross-reactive antibody responses (Wahid *et al.* 2012, Clin. & Vaccine Immunol. 19: 825-834).

To try to address the short duration of protection and lack of memory response of Vi vaccines, Vi polysaccharide has been conjugated to carrier proteins in a new generation of Vi glycoconjugate vaccines. Carrier proteins include *Pseudomonas aeruginosa* exotoxin, tetanus and diphtheria toxoids (Martin 2012, Curr. Opin. Infect. Dis. 25: 489–499). Injectable conjugates of O-antigens purified from *S. Paratyphi* A have also been developed, primarily O2 conjugated to tetanus toxoid (O2-TT), to diphtheria toxoid (O2-DT) and to a detoxified mutant of the diphtheria toxin (O2-CRM₁₉₇), co-administered with Vi conjugated to the same carrier protein as enteric fever vaccines targeting *S. Typhi* and *S. Paratyphi* A (Martin *et al.* 2016, Vaccine 34: 2900-2902).

The live attenuated approach to enteric fever vaccine development has significant advantages over injectable Vi vaccines: longer duration of protection, generation of immunological memory, closer immunological profile to the natural infection and elimination of needles. In addition to the licensed chemically mutagenised typhoid vaccine strain Ty21a, other specifically mutated live vaccine strains of *S. Typhi* have been evaluated in clinical trials: CVD 906 and CVD 908 ($\Delta aroC$, $\Delta aroD$); CVD 906-*htrA* and CVD 908-*htrA* ($\Delta aroC$, $\Delta aroD$, $\Delta htrA$); CVD 909 ($\Delta aroC$, $\Delta aroD$, $\Delta htrA$ and constitutive expression of Vi); M01ZH09 ($\Delta aroC$, $\Delta ssaV$); Ty800 ($\Delta phoP$, $\Delta phoQ$); χ 3927 (Δcya , Δcrp) (Tennant & Levine 2015, Vaccine 33: C36-C41) and χ 4073 (Δcya , Δcrp , Δcdt) (Paterson & Maskell 2010, Hum. Vaccines 6: 379-384). It is reasonable to expect a degree of cross-protection from these specifically mutated *S. Typhi* strains to *S. Paratyphi* B as is the case for Ty21a.

Attenuated strains of *S. Paratyphi* A have also been produced, including $\Delta phoPQ$ mutants tested pre-clinically (Roland *et al.* 2010, Vaccine 28: 3679-3687), and CVD 1902 ($\Delta guaBA$, $\Delta clpX$) which has been evaluated in a clinical trial (Tennant & Levine 2015, Vaccine 33:

C36-C41). A combination of CVD 909 and CVD 1902 is in clinical development as a vaccine targeting *S. Typhi* and *S. Paratyphi A* (Martin *et al.* 2016, Vaccine 34: 2900-2902). However, this strategy requires the clinical evaluation of *S. Paratyphi A*, which does not have the long history of safe use of *S. Typhi*.

5

Non-typhoidal *Salmonella* (NTS) cause gastroenteritis, with symptoms including diarrhoea and fever. The increase in cases of an invasive form of non-typhoidal *Salmonella* (iNTS), predominantly in Africa, is an important public health issue. The strains responsible for the vast majority of iNTS cases are *S. enterica* serovars Typhimurium and Enteritidis, and multidrug resistant isolates are of particular concern (MacLennan & Levine 2013, Expert Rev. Anti Infect. Ther. 11:443-446). iNTS strains cause a significantly more severe form of the disease, with prolonged symptoms and shedding of bacteria lasting for several weeks. There are currently no vaccines for NTS approved for human use.

10

15 The benefits of live attenuated vaccines include the induction of mucosal and cell-mediated immune responses, in addition to systemic antibody responses, and the duration of these responses can be longer than those from injected subunit vaccines as described above for typhoid. Attenuated *S. Typhi* strains have been administered to millions of people as experimental and licensed vaccines with an excellent record of safety and immunogenicity.

20 This serovar also lacks the ability to persist in environmental reservoirs due to its exclusivity to humans, thus increasing its biosafety. Therefore, there are several reasons why it is advantageous to use live attenuated *S. Typhi* as a vector for delivery of homologous antigens from other serovars of *S. enterica*, rather than attenuating the wild-type strains where the effect of the attenuating mutations may not be predictable. For example, the *S.*

25 *Typhi* vaccine candidate ZH9 carrying mutations in the genes *aroC* and *ssaV* has been shown to be safe and well tolerated in multiple clinical trials (Lyon *et al.* 2010, Vaccine 28: 3602-3608), whereas the same mutations introduced into *S. Typhimurium* resulted in prolonged shedding in stools (Hindle *et al.* 2002, Infect. Immun. 70: 3457-3467).

30 The three most important surface antigens of the *S. enterica* serovars for the induction of protective immunity are lipopolysaccharide O-antigens, flagella (H-antigens) and Vi. The table below summarises the antigenic compositions of the principle enteric fever and iNTS strains following the Kauffmann-White-Le Minor scheme classification scheme (Grimont & Weill 2007, Antigenic formulae of the *Salmonella* serovars, 9th Edition).

S. <i>enterica</i> serovar	O-antigens	Vi	Flagella (H-antigens)	
			Phase 1	Phase 2
Typhi	9, 12	Vi	d	-
Paratyphi A	1, 2, 12	-	a	-
Paratyphi B	1, 4, [5], 12	-	b	1,2
Paratyphi C	6, 7	Vi	c	1,5
Typhimurium	1, 4, [5], 12	-	i	1,2
Enteritidis	1, 9, 12	-	g,m	-

[] indicates antigens exceptionally found in wild-type strains.

Salmonella lipopolysaccharides consists of lipid A linked to the KDO (3-deoxy-D-manno-
 5 octulosonic acid) terminus of a conserved core region, which is then linked to a variable, repeated O-antigen trisaccharide. In *S. Typhi*, *S. Paratyphi A*, *S. Paratyphi B*, *S. Typhimurium* and *S. Enteritidis* this repeated O-antigen is O12, a triglyceride of mannose (Man), rhamnose (Rha) and galactose (Gal). In *S. Paratyphi A*, a branch of paratose (Par; 3,6-dideoxy-D-ribo-hexose) from the C-3 of Man confers serogroup specificity: O2 (Figure
 10 1). In *S. Paratyphi B* and *S. Typhimurium* the C-3 Man has a diglyceride of abequose (Abe; 3,6-dideoxy-D-xylo-hexose) conferring the specificity O4. *S. Typhi* and *S. Enteritidis* have tyvelose (Tyv; 3,6-dideoxy-D-arabino-hexose) on the C-3 Man, conferring the specificity O9. Figure 2 shows the biosynthetic pathway resulting in either Abe, Par or Tyv and representing the O-antigen differences between the serovars. *S. Typhi* and *S. Paratyphi C*
 15 additionally express the Vi capsular polysaccharide antigen.

Except for the flagella produced by *S. Typhi* Ty21a, flagellin is not a component of any current licensed vaccine for an *S. enterica* infection. Flagellin is an important pathogen-associated molecular pattern (PAMP) that is recognised by toll-like receptor 5 (TLR5) and is
 20 highly immunogenic, making it an important component of a live vaccine for *S. enterica*. The flagella filament of *S. enterica* is composed of approximately 20,000 flagellin (FliC or FliB) proteins with a terminal cap encoded by *fliD* (Haiko & Westerlund-Wikström 2013, Biology 2: 1242-1267). *S. Typhi* and *S. Paratyphi A* are generally monotypic for flagellin, expressing only FliC.

There is a particular need in the art for improved vaccines directed toward *S. enterica* serovars Paratyphi A, B, C, Typhimurium and Enteritidis.

Summary of Invention

5 In a first aspect, the present invention provides a live attenuated strain of *Salmonella enterica* serovar Typhi wherein said strain is modified to express the lipopolysaccharide O2 O-antigens and the flagella proteins of *Salmonella enterica* serovar Paratyphi A.

10 In a second aspect, the present invention provides a live attenuated strain of *Salmonella enterica* serovar Typhi wherein said strain is modified to express the lipopolysaccharide O4 O-antigens and the flagella proteins of *Salmonella enterica* serovar Paratyphi B and *Salmonella enterica* serovar Typhimurium.

15 In a third aspect, the present invention provides a live attenuated strain of *Salmonella enterica* serovar Typhi wherein said strain is further modified to contain a functional *fepE* gene, such that long O-antigen chains are generated, preferably wherein the O-antigen chains are 100 repeated units of the trisaccharide backbone in length.

20 In a fourth aspect, the present invention provides a live attenuated strain of *Salmonella enterica* serovar Typhi wherein said strain is modified to either constitutively express the *gtrC* gene (encoding rhamnose acetyltransferase), or alternatively, wherein said strain is modified to express the *gtrC* gene in *trans*.

25 In a fifth aspect, the present invention provides a live attenuated strain of *Salmonella enterica* serovar Typhi wherein said strain has its native *fliC* gene (SEQ ID NO: 1) substituted with the *fliC* gene of *Salmonella enterica* serovar Paratyphi A (SEQ ID NO: 2), *Salmonella enterica* serovar Paratyphi B (SEQ ID NO: 3), *Salmonella enterica* serovar Paratyphi C (SEQ ID NO: 5), *Salmonella enterica* serovar Typhimurium (SEQ ID NO: 7) and *Salmonella enterica* serovar Enteritidis (SEQ ID NO: 9), such that the conferred serotype is
30 altered from an Hd serotype to a Ha, Hb, Hc, Hi and Hg,m serotype respectively.

In a sixth aspect, the present invention provides a live attenuated strain of *Salmonella enterica* serovar Typhi wherein the *fljBA* locus, controlling expression of the *fljB* gene of *Salmonella enterica* serovar Paratyphi B (SEQ ID NO: 4), *Salmonella enterica* serovar

Paratyphi C (SEQ ID NO: 6) and *Salmonella enterica* serovar Typhimurium (SEQ ID NO: 8) are inserted into the chromosome of *Salmonella enterica* serovar Typhi or expressed in *trans*.

5 In a seventh aspect, the present invention provides a live attenuated strain of *Salmonella enterica* serovar Typhi wherein said strain expresses the atypical variants of flagellin of *Salmonella enterica* serovar Paratyphi A, *Salmonella enterica* serovar Paratyphi B, *Salmonella enterica* serovar Paratyphi C, *Salmonella enterica* serovar Typhimurium and *Salmonella enterica* serovar Enteritidis.

10 In an eighth aspect, the present invention provides a live attenuated strain of *Salmonella enterica* serovar Typhi wherein said strain has inserted a second copy of the *tviA* gene (SEQ ID NO: 10).

15 The present invention further includes a vaccine comprising one or more said modified strains for use in enhancing immunogenicity against *Salmonella enterica* serovar Paratyphi A, Paratyphi B, Paratyphi C, Typhimurium and Enteritidis.

Description of Figures

20 **Figure 1** illustrates the structure of the *S. Paratyphi* O-antigen chain linked to the core region (Micoli *et al.* 2012, Plos One 7: e47039). Par (α -D-Par) is replaced by Tyv in *S. Typhi* and *S. Enteritidis*, and by Abe in *S. Paratyphi B* and *S. Typhimurium*.

25 **Figure 2** illustrates the biosynthetic pathways of O-antigen-related CDP-linked sugars in *Salmonella enterica* serovars: Typhi and Enteritidis (terminating with CDP-tyvelose); Paratyphi A (terminating with CDP-paratose); Paratyphi B and Typhimurium (terminating with CDP-abequose). Enzymes involved at each step are indicated. Adapted from Reeves *et al.* 2013 Plos One 8: e69306.

30 **Figure 3** illustrates part of the wild-type O-antigen locus from *S. Typhi* ZH9 that has been modified by mutation of the wild-type *rfbE* cistron (SEQ ID NO: 12): either by deletion of the majority of the *rfbE* cistron to generate *S. Typhi* ZH9PL2 or replacement of the *rfbE* cistron with spacer DNA comprising the *wbdR* cistron (SEQ ID NO: 13) maintaining the original reading frame in *S. Typhi* ZH9W.

Figure 4 shows micrographs of *S. Typhi* ZH9 and derivative strains ZH9PL2 and ZH9W probed with A. an anti-*S. Typhi* LPS mAb, and B. an anti-*S. Paratyphi A* LPS mAb; left column images are phase contrast and right column images are immuno-fluorescence micrographs.

Figure 5 shows a silver-stained polyacrylamide gel of LPS preparations from *S. Typhi* ZH9 and derivative strains ZH9PL2 and ZH9W, indicating the short and long O-antigen chains.

Figure 6 illustrates part of the wild-type O-antigen locus from *S. Typhi* ZH9 modified by replacement of the *rfbS* (SEQ ID NO: 14) and *rfbE* cistrons with *rfbJ* (SEQ ID NO: 15).

Figure 7 illustrates the plasmid pBAD2fepE with the P_{araBAD} promoter (SEQ ID NO: 17) used to express the *fepE* cistron.

Figure 8 shows a silver-stained polyacrylamide gel of LPS preparations from *S. Typhi* ZH9 and ZH9(pBAD2fepE) induced by the addition of arabinose, indicating the short, long and very long O-antigen chains.

Figure 9 shows micrographs of *S. Typhi* ZH9 and derivative strains ZH9PF probed with A. an anti-*S. Typhi* flagellin antiserum, and B. an anti-*S. Paratyphi A* LPS flagellin antiserum; left column images are phase contrast and right column images are immuno-fluorescence micrographs.

Figure 10 illustrates the flagella phase variation in *S. enterica* (from Bonifield and Hughes 2003, J. Bacteriol. 185: 567–3574).

Figure 11 illustrates the plasmid pBRT4tviA with the P_{ssaG} promoter (SEQ ID NO: 16) used to express the *tviA* cistron.

Figure 12 shows micrographs of *S. Typhi* ZH9 derivative strain ZH9PA probed with A. anti-*S. Typhi* and anti-*S. Paratyphi A* LPS mAbs, and B. anti-*S. Typhi* and anti-*S. Paratyphi A* flagellin antisera; left images in each pair are phase contrast and right images are immuno-fluorescence micrographs.

Figure 13 shows endpoint titers of antibodies specific for Typhi LPS (O:9), Paratyphi LPs (O:2), Typhi flagellin (H:d) and Paratyphi flagellin (H:a) in mouse sera following a single subcutaneous immunization of mice with ZH9 alone (1×10^8 cfu/mouse), ZH9PA alone (1×10^8 cfu/mouse) or a combination of ZH9 and ZH9PA (0.5×10^8 cfu + 0.5×10^8 cfu ZH9PA/mouse).

Detailed Description

The following description is presented to enable any person skilled in the art to make and use the present invention. Various modifications to the disclosed embodiments will be readily apparent to those skilled in the art.

In a first aspect, the present invention provides a live attenuated strain of *Salmonella enterica* serovar Typhi wherein said strain may be modified to express the lipopolysaccharide O2 O-antigens and the flagella proteins of *Salmonella enterica* serovar Paratyphi A.

The term 'live attenuated strain' in the context of the present invention refers to the alteration of said strain to reduce its pathogenicity, rendering it harmless to the host, whilst maintaining its viability. This method is commonly used in the development of vaccines due to its ability to elicit a highly specific immune response whilst maintaining an acceptable safety profile. Development of attenuated live bacterial vaccines may involve a number of methods, examples include, but are not limited to; passing the pathogens under *in vitro* conditions until virulence is lost, chemical mutagenesis and genetic engineering techniques.

It is envisaged that the lipopolysaccharide O9 O-antigens of *Salmonella enterica* serovar Typhi may be replaced with the O2 O-antigens of *Salmonella enterica* serovar Paratyphi A.

The *S. Paratyphi A* O-antigen biosynthetic pathway involves the precursor CDP-4-keto-3,6-dideoxy-D-glucose being converted to CDP-Par by the CDP-paratose synthase, RfbS. In addition to *rfbS* (previously called *prt*), *S. Typhi* has a functional *rfbE* gene (previously called *tyv*) encoding CDP-paratose 2-epimerase, which converts CDP-Par to CDP-Tyv (Figure 2).

The present invention involves the inactivation of the chromosomal *rfbE* in *Salmonella enterica* serovar Typhi (Figure 3). It was found, surprisingly, that inactivation of *rfbE* resulted in the standard long LPS of *S. Typhi*, known to be highly immunogenic from clinical studies, being altered to instead have the chemical composition of that of *S. Paratyphi A*. It is a surprising finding that such a biochemical modification should result in the microorganism having the ability to produce long LPS of the *S. Paratyphi* type. In one embodiment, the inactivation may result from the deletion of *rfbE*. The deletion may be either a partial or full deletion.

- 10 The *rfbE* inactivation prevents Tyv from being synthesised, resulting in Par being attached to Man. This alteration in the biochemical pathway introduces the *Salmonella enterica* serovar Paratyphi A O2 O-antigen (Figures 1 and 4).

- 15 In a preferred embodiment, the modification to inactivate the *rfbE* gene retains non-coding DNA without disrupting the expression of downstream (non-*rfbE*) coding sequences.

In a preferred embodiment, the deletion of the *rfbE* cistron is accompanied by the insertion of a non-coding spacer region intended to maintain the correct reading frame.

- 20 The spacer region may be any suitable non-coding DNA sequence which retains the correct reading frame when inserted. Preferably, the spacer region of DNA is the cistron of the *Escherichia coli* gene *wbdR* which results in the production of long LPS.

- 25 It is a surprising finding that the modifications disclosed herein result in a long LPS with the *S. Paratyphi A* O2 O-antigen characteristic. This has benefits in vaccine production, allowing live attenuated strains of *S. Typhi*, and/or derivatives thereof, to be produced, offering additional protection against *S. Paratyphi A*. The vaccine therefore has benefits over conventional vaccines which protect only against *S. Typhi*.

- 30 The term 'spacer region of DNA' in the context of the present invention refers to a region of non-coding DNA located between genes. The term 'cistron' refers to a section of DNA which encodes for a specific polypeptide in protein synthesis. The insertion of a spacer region of DNA may involve the transformation of an electrocompetent plasmid with a replacement cassette. See Example 1 for further details.

Where the methods herein described involve the use of a plasmid, said plasmid will ideally have an origin of replication selected from pMB1, ColEI, p15A, pSC101 and RK2. The plasmid may contain an antibiotic resistance gene selected from β -lactamase (*bla*),
 5 kanamycin phosphotransferase (*kan*), tetracycline efflux protein (*tetA*) or chloramphenicol acetyltransferase (*cat*). Ideally the antibiotic resistance gene will be excised prior to or shortly after transformation into the live bacterial vector strain, for example by a mechanism such as 'X-mark' (Cranenburgh & Leckenby 2012, WO2012/001352). A plasmid maintenance system may be required to prevent plasmid loss. These may include
 10 mechanisms to place a native chromosomal gene under a heterologous promoter such as the 'Operator-Repressor Titration for Vaccines' (ORT-VAC; Garmory *et al.* 2005, Infect. Immun. 73: 2005-2011) or 'oriSELECT' (Cranenburgh 2005, WO 2005/052167) systems, neither of which require an additional selectable marker gene to be present on the plasmid. Alternatively, a selectable marker gene will be used that is not an antibiotic resistance gene,
 15 such as a gene to complement a host cell mutation (Degryse 1991, Mol. Gen. Genet. 227: 49-51).

Preferably, the spacer region of DNA is the cistron of the *Escherichia coli* gene *wbdR*. Other non-functional genes of *Salmonella enterica* serovar Typhi of approximately the same
 20 length as the *rfbE* cistron may also be used for this purpose. It is preferable that the chosen spacer DNA used for this purpose will be approximately 50-2000 base pairs in length as well as lacking a terminator sequence. The use of this spacer region results in the inactivation of *rfbE* without causing any downstream effects (SEQ ID NO: 20) and effectively changing *Salmonella enterica* serovar Typhi LPS to *Salmonella enterica* serovar Paratyphi
 25 A.

The inventors have shown that deletion of *rfbE* whilst maintaining the original reading frame (via the use of a spacer region of DNA) is a crucial requirement of the above process.

30 Preferably, the resulting lipopolysaccharide O2 O-antigens of *Salmonella enterica* serovar Paratyphi A are at least equivalent in length to the lipopolysaccharide O9 O-antigens of *Salmonella enterica* serovar Typhi. It is preferable that the resulting lipopolysaccharide will be 16-35 O-antigen repeat units in length, a range which constitutes a 'long'

lipopolysaccharide species. A person skilled in the art will understand the desirability of the presence of O-antigen repeat units in triggering an immunogenic reaction.

It is envisaged that the present invention may also include the live attenuated strain, according to above, wherein said strain may have its native *fliC* gene replaced with the *fliC* gene of *Salmonella enterica* serovar Paratyphi A, such that the conferred serotype is altered from an Hd serotype to a Ha serotype, where 'serotype' refers to a distinct variation within the bacterial species.

The Phase 1 flagellum of *S. Typhi* is essential for motility and invasion, and confers the serotype Hd. The filament consists of the flagellum protein FliC, with a FliD cap. The inventors have discovered that replacing the *fliC* on the *S. Typhi* chromosome with that of *S. Paratyphi A* results in the conversion from the Hd to the Ha serotype of functional flagella.

Chromosomal replacement may be used to achieve the above substitution. The substitution may be a full or partial replacement. In the context of a partial replacement, it is preferable that the replacement of the amino acids in positions 176-414 is carried out. The latter may involve the transformation of an electrocompetent plasmid with a replacement cassette. See Example 2 for further details. Alternatively, the substituted *fliC* gene may be expressed in *trans* from a plasmid or additional chromosomal location.

An additional embodiment of the present invention is the live attenuated strain described above wherein the strain may be further modified to contain a functional *fepE* gene, such that long O-antigen chains are generated, preferably wherein the O-antigen chains are 100 repeated units of the trisaccharide backbone in length.

The *fepE* gene encodes the length regulator of very long O-antigen chains, wherein 'very long' is taken to mean more than 100 repeated units of the trisaccharide backbone. *S. Typhi* does not possess these long O-antigen chains due to a mutation introducing a stop codon into the gene (SEQ ID NO: 21). *S. Typhi* may be manipulated into expressing these long O-antigen chains via a number of methods; the natural promoter of *fepE* may be replaced with an alternative promoter, for example P_{araBAD} , the chromosomal mutation of *fepE* in *S. Typhi* may be repaired or a functional copy of *fepE* (SEQ ID NO: 11) may be inserted elsewhere in the *S. Typhi* chromosome. For vaccine applications, an *in vivo*-

induced promoter or a constitutive promoter may be utilised, examples of such promoters include P_{pagC} , P_{nirB} , P_{ssaG} , P_{sifA} , P_{sifB} , P_{sseA} , P_{sseG} , P_{sseJ} , P_{lac} , P_{lac} , P_{trc} and lambda P_L/P_R .

A 'promoter' refers to a DNA regulatory region capable of binding RNA polymerase and initiating transcription of a downstream (3' direction) coding or non-coding sequence. A promoter may also be a regulatory DNA sequence that affects the binding of RNA polymerase at the transcription initiation site. For the purposes of defining the present invention, the promoter sequence is bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence may be found a transcription initiation site, as well as protein binding domains responsible for the binding of RNA polymerase.

Promoters can be constitutively active (wherein 'active' means transcription is 'on'), spatially restricted or inducible. As used herein 'spatially restricted' refers to a promoter that is only active in a specific subset of cells or cellular compartment of a multicellular organism. A spatially restricted promoter can thus be used to activate the expression of a nucleic acid in a particular tissue or cell type of a multicellular organism.

As used herein an 'inducible promoter' refers to a promoter that enables the temporal and/or spatial activation of transcription in response to external physical or environmental stimuli. Inducible promoters include those activated by the presence of specific small molecules that alleviate transcriptional repression. For example, transcription from such an inducible promoter may be regulated by a 'repressor protein'. As used herein, 'repressor protein' refers to a polypeptide that binds to and occupies the inducible promoter to prevent transcription initiation. When bound to the promoter, said repressor protein can prevent binding or recruitment of RNA polymerase or associated co-factors to the transcription initiation site to prevent the activation of transcription. However, upon binding its relevant small molecule, or encountering its relevant physical or environmental stimulus, the repressor protein can no longer bind to the promoter sequence, and thus transcriptional repression is relieved. Where the above examples include *in vivo*-induced promoters for expression of cistrons encoding enzymes involved in O-antigen biosynthesis, or for expression of alternative *fliC* cistrons or *TviA*, such promoters include but are not limited to: P_{pagC} , P_{nirB} , P_{ssaG} , P_{sifA} , P_{sifB} , P_{sseA} , P_{sseG} and P_{sseJ} (Dunstan *et al.* 1999, Infect. Immun. 67:

5133-5141; Xu *et al.* 2010, Infect. Immun. 78: 4828-4838; Kröger *et al.* 2013, Cell Host & Microbe 14: 683-695). Other promoters of use include lambda P_L and P_R the temperature-induced lambda repressor cI including its thermo-labile mutant repressor cI857 (Love *et al.* 1996, Gene 176:49-53; SEQ ID NO: 24 & 25) and promoters that are constitutive in *Salmonella* in the absence of the LacI repressor such as P_{lac} , P_{tac} and P_{trc} (Terpe 2006, Appl. Microbiol. Biotechnol. 72: 211–222). In some embodiments, the functional variants include those having similar or modified sequences to P_{pagC} , P_{nirB} , P_{ssaG} , P_{sifA} , P_{sifB} , P_{sseA} , P_{sseG} , P_{sseJ} and lambda P_L/P_R , and similar or substantially identical promoter activity as the wild-type promoter from which the variant is derived, particularly with respect to its ability to induce expression *in vivo*. Similar modified sequences may include having at least about 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% identity to the wild-type sequence of any of P_{pagC} , P_{nirB} , P_{ssaG} , P_{sifA} , P_{sifB} , P_{sseA} , P_{sseG} , P_{sseJ} and lambda P_L/P_R .

Preferably, the introduction of these long O-antigen chains may be beneficial in inducing an LPS-specific immune response. There may be an additional benefit where the LPS is naturally very long such as from expression of *fepE*.

It is further envisaged that the live attenuated strain described above may be modified to constitutively express *gtrC* or to express *gtrC* *in trans*.

Particular *S. enterica* serovars are acetylated on the rhamnose on the O-antigen, a feature which has been demonstrated as important for O2 O-antigen specificity in *S. Paratyphi A*. The family 2 *gtr* operon (SEQ ID NO: 22) encodes the rhamnose acetyltransferase GtrC in *S. Typhi* and *S. Paratyphi A*. To achieve a greater and more consistent level of rhamnose acetylation it may be desirable to make *gtrC* constitutively expressed, for example, either on a plasmid or from an additional chromosomal locus. Alternatively, the native family 2 *gtr* operon promoter responsible for phase variation can be replaced with a constitutive promoter or one that is conditionally expressed *in vivo*.

It is further envisaged that the live attenuated strain described above may be further modified to contain an additional copy of the *tviA* gene under the control of a phagosomally induced promoter.

The Vi capsular polysaccharide antigen contributes to the virulence of *S. Typhi* but is naturally down-regulated upon invasion of the liver and spleen (Janis *et al.* 2011, Infect. Immun. 79: 2481-2488). Regulation of Vi expression is carried out by the positive transcriptional regulator TviA.

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The insertion of a second copy of the *tvfA* gene into *S. Typhi* may induce immune responses against Vi and enhance the anti-flagellin response. The second copy may be inserted into the *S. Typhi* in *trans*, either on a plasmid (Figure 11) or integrated into the *S. Typhi* chromosome, such that it is under the control of a phagosomally induced promoter.

10 Examples of appropriate phagosomally induced promoters include; P_{pagC} , P_{nirB} , P_{ssaG} , P_{sifA} , P_{sifB} , P_{sseA} , P_{sseG} , P_{sseJ} .

A further embodiment of the present invention may be a vaccine comprising the live attenuated strains herein disclosed, for use in enhancing immunogenicity against *S. Paratyphi A* and for use in the treatment or prevention of enteric fever and salmonellosis. The vaccine may contain a single live attenuated strain or combine more than one live attenuated strain, for example, the vaccine may contain ZH9 and/or one of its derivative strains; ZH9PA, ZH9PL2, ZH9W or ZH9PF. For example, combinations may include ZH9 + ZH9PL2, ZH9 + ZH9W, ZH9 + ZH9PF, preferably the combination is ZH9 + ZH9PA.

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The term 'immunogenicity' refers to the ability of a particular substance to provoke an immune response.

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The term 'vaccine' may be taken to comprise a number of additional elements in addition to the attenuated live strain herein disclosed. The attenuated live strain may be present in a composition together with any other suitable adjuvant, diluent or excipient. Examples of suitable adjuvants, diluents or excipients include, but are not limited to; disodium hydrogen phosphate, soya peptone, potassium dihydrogen phosphate, ammonium chloride, sodium chloride, magnesium sulphate, calcium chloride, sucrose, sterile saline and sterile water.

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The vaccine may be administered by any appropriate route, preferably orally or intranasal routes; however the former is the preferred route of administration. The vaccine strain or strains will preferentially be lyophilised by a process such as freeze-drying and will be stored in sachets for later rehydration and oral administration to young children.

Alternatively, they may be dispensed into enterically coated capsules for oral administration to older children and adults. For the encapsulated formulation, the lyophilised *Salmonella* will ideally be mixed with a bile-adsorbing resin such as cholestyramine to enhance survival when released from the capsule into the small intestine (Edwards and Slater 2009, Vaccine 27: 3897-3903).

The skilled person will appreciate that the vaccine may contain the aforementioned live attenuated strains (e.g. ZH9 and ZH9PA) of *Salmonella enterica* serovar Typhi at a density of 10^8 , 10^9 , 10^{10} , 10^{11} or 10^{12} colony-forming units per dose. The dosing regime may involve a single dose or multiple doses, ideally the vaccine will be administered in 1-3 doses.

In a second aspect, the present invention provides for a live attenuated strain of *Salmonella enterica* serovar Typhi wherein said strain is modified to express the lipopolysaccharide O4 O-antigens and the flagella proteins of *Salmonella enterica* serovar Paratyphi B and *Salmonella enterica* serovar Typhimurium.

To achieve the above modification, the gene *rfbJ* (previously called *abe*), encoding CDP-abequose synthase from *S. Paratyphi B* or *S. Typhimurium*, can be inserted to replace *rfbS* with or without the simultaneous replacement of *rfbE* (Figure 6), as *RfbJ* is at the equivalent step in the *S. Typhi/Paratyphi A* biosynthetic pathway as CDP-paratose synthase and enables the conversion of CDP-4-keto-3,6-dideoxy-D-glucose to CDP-Abequose (Figure 2). This would result in the O-antigen repeats containing Abe instead of Tyv, giving the O4 specificity of *S. Paratyphi B* and *S. Typhimurium*. Preferably, *rfbS* is inactivated via a mutation resulting in a partial or total deletion of the cistron, for example, using Xer-cise wherein a lambda-Red-mediated recombineering approach is used to inactivate the *rfbS* cistron, followed by Xer recombination to remove the selectable marker gene.

Alternatively to the replacement method described above, and *rfbJ* may be expressed in *trans*, either from a plasmid or alternative chromosomal locus, leading to a mixture of O4 and O9 O-antigens, designed to induce antibody responses to *S. Typhi*, *S. Paratyphi B* and *S. Typhimurium*.

The invention further intends the live attenuated strain, according to the second aspect of the present invention, may have its native *fliC* gene replaced with the *fliC* gene of *Salmonella enterica* serovar Paratyphi B and/or *Salmonella enterica* serovar Typhimurium, such that the conferred serotype is altered from an Hd serotype to a Hb and Hi serotype respectively.

It is further envisaged that the live attenuated strain, according to the second aspect of the present invention, may have the *fljBA* locus of *Salmonella enterica* serovar Paratyphi B and *Salmonella enterica* serovar Typhimurium inserted into the chromosome of *Salmonella enterica* serovar Typhi or expressed in *trans*.

Several serovars of *S. enterica* (including *S. Paratyphi* B and C, and *S. Typhimurium*) have an additional antigenically distinct flagellin gene *fljB*, which is subject to phase variation such that flagella composed of either FliC or FljB is produced (Figure 10). Co-transcribed with *fljB* is *fljA*, a flagellum-specific sigma factor that represses the *fliC* gene (Bonifield and Hughes 2003, J. Bacteriol. 185: 567–3574). The *fljBA* promoter is flanked by the Hin recombinase recognition sites *hixL* and *hixR*. Together with enhancer proteins Fis and HU, Hin mediates a reversible DNA inversion between the *hix* sites such that in one orientation the *fljBA* promoter transcribes the *fljBA* operon (SEQ ID NO: 23) producing FljB flagellin and repressing *fliC* expression via FljA, thus generating flagella filaments composed of FljB. In the opposite orientation there is no expression of the *fljBA* operon, enabling the production of flagellin consisting of *fliC*. *S. Typhi* does not express Phase 2 flagella due to deletion of *fljB* and *hin*, so has flagella comprised of FliC only (McClelland *et al.* 2004, Nature Genetics 36: 1268–1274).

It is envisaged that the *fljBA* locus of *S. Paratyphi* B, *S. Paratyphi* C or *S. Typhimurium* may be inserted into the chromosome of *S. Typhi* or expressed in *trans* from a plasmid, thus introducing the phase-variable flagella phenotype of the desired serovar. Alternatively, one or both of the *hix* sites flanking the native promoter of the *fljBA* operon, or the *hin* recombinase gene, may be mutated to prevent DNA inversion, leading to constitutive expression such that flagella filaments are comprised of only FljB. The latter approach may be coupled with the pre-described modification of the *S. Typhi fliC*.

It is further envisaged that the live attenuated strain, according to the second aspect of the present invention, may be further modified to include the additional modifications previously described regarding *fepE*, *gtrC* and *tviA* expression.

- 5 A further intended application of the present invention is a vaccine comprising the live attenuated strain, according to the second aspect of the present invention, for use in enhancing immunogenicity against *Salmonella enterica* serovar Paratyphi B and/or *Salmonella enterica* serovar Typhimurium.
- 10 In a third aspect, the present invention provides a live attenuated strain of *Salmonella enterica* serovar Typhi wherein said strain is further modified to contain a functional *fepE* gene, such that long O-antigen chains are generated, preferably wherein the O-antigen chains are 100 repeated units of the trisaccharide backbone in length. The method by which this effect may be achieved, and further details regarding this aspect of the invention,
- 15 have been previously outlined on pages 12 and 13 of the present application.

- In a fourth aspect, the present invention provides a live attenuated strain of *Salmonella enterica* serovar Typhi wherein said strain is modified to either constitutively express the *gtrC* gene, or alternatively, wherein said strain is modified to express the *gtrC* gene in *trans*.
- 20 The method by which this effect may be achieved, and further details regarding this aspect of the invention, have been previously outlined on page 14 and 15 of the present application.

- In a fifth aspect, the present invention provides a live attenuated strain of *Salmonella enterica* serovar Typhi wherein said strain has its native *fliC* gene substituted with the *fliC* gene of *Salmonella enterica* serovar Paratyphi A, *Salmonella enterica* serovar Paratyphi B, *Salmonella enterica* serovar Paratyphi C, *Salmonella enterica* serovar Typhimurium and *Salmonella enterica* serovar Enteritidis, such that the conferred serotype is altered from an Hd serotype to a Ha, Hb, Hc, Hi and Hg,m serotype respectively. The method by which this
- 25 effect may be achieved, and further details regarding this aspect of the invention, have been previously outlined on page 12 and 17 of the present application.
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In a sixth aspect, the present invention provides a live attenuated strain of *Salmonella enterica* serovar Typhi wherein the *fliBA* locus of *Salmonella enterica* serovar Paratyphi B,

Salmonella enterica serovar Paratyphi C and *Salmonella enterica* serovar Typhimurium are inserted into the chromosome of *Salmonella enterica* serovar Typhi or expressed in *trans*. The method by which this effect may be achieved, and further details regarding this aspect of the invention, have been previously outlined on pages 17 and 18 of the present application.

In a seventh aspect, the present invention provides a live attenuated strain of *Salmonella enterica* serovar Typhi wherein said strain expresses the atypical variants of flagellin of *Salmonella enterica* serovar Paratyphi A, *Salmonella enterica* serovar Paratyphi B, *Salmonella enterica* serovar Paratyphi C, *Salmonella enterica* serovar Typhimurium and *Salmonella enterica* serovar Enteritidis.

S. Typhi isolates are subject to phase variation: one such isolate expresses a variant of FljB called Hz66 from a linear plasmid called pBSSB1 (Baker *et al.* 2007, Plos Pathogens 3: e59), another flagella variant is Hj which has a 261 bp in-frame deletion of the central region of the Hd *fliC* gene (Frankel *et al.* 1989, EMBO J. 8: 3149–3152).

For Hz66 this can be achieved by the inclusion of the pBSSB1 plasmid in the *S. Typhi*-derived vaccine strain. Alternatively, FljB^{Hz66} or Hj may be expressed from a chromosomal location which may be the location of the deleted *fliB* gene, the native chromosomal location of *fliC* (thereby replacing it with a variant), or expressed on a plasmid from its native promoter or from a phagosomally induced promoter such as *P_{ssaG}*. The amino acid sequences of the Hz66 and Hj are described in Schreiber *et al.* 2015, Nature 5: 7947.

In an eighth aspect, the present invention provides a live attenuated strain of *Salmonella enterica* serovar Typhi wherein said strain has inserted a second copy of the *tvfA* gene. The method by which this effect may be achieved, and further details regarding this aspect of the invention, have been previously outlined on page 15 of the present application.

It is envisaged that the live attenuated strains herein disclosed may be administered in isolation or in combination (e.g. ZH9 and ZH9PA), in the form of a vaccine, to give the subject a broad protection against a variety of *S. enterica* serovars, specifically, *Salmonella enterica* serovar Paratyphi A and B, and *Salmonella enterica* serovar Typhimurium.

The table lists *S. Typhi* ZH9 and its derivative strains altered for LPS and flagellin:

Strain name	O-antigen	H-antigen
ZH9	O9	Hd
ZH9PL2	O2 (short)	Hd
ZH9W	O2	Hd
ZH9PF	O9	Ha
ZH9PA	O2	Ha

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The invention will now be illustrated in the following examples with reference to the accompanying drawings.

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Example 1

To construct *S. Typhi* ZH9 expressing *S. Paratyphi* A LPS, the *rfbE* gene was deleted in two different ways. In one method of deletion, a spacer cistron *wbdR* was synthesised flanked with ~700 bp of DNA homologous to *rfbE* upstream gene *rfbS* and downstream gene *rfbX* to create a deletion cassette. A *NotI* restriction site at the 3' end of the spacer cistron was included to clone a *dif*-flanked antibiotic resistance marker gene *cat* gene amplified with primers designed with *NotI* restriction site. The *cat* gene was amplified from pBRT1N plasmid synthetically generated with 5NotI_{dif}cat and 3NotI_{dif}cat primers designed with *dif* sequences. *E. coli* TOP10 cells were used for cloning operations to generate the pUCpW_*dif*CAT plasmid. Chromosomal replacement of the *rfbE* gene with the spacer gene was carried out as described in Bloor and Cranenburgh 2006 (Appl. Environ. Microbiol. 72: 2520–2525). Briefly, *S. Typhi* ZH9 was first transformed with a pLGBEK plasmid coding for λ Red gene functions for integration of linear DNA. Electrocompetent ZH9(pLGBEK) was then transformed with the deletion cassette linearised using *Sall* and *SacI* restriction enzymes (SEQ ID NO: 26), and transformants were selected on LB-aro (LB medium containing aromatic amino acids and precursors of aromatic amino acid biosynthesis) agar plates supplemented with 20 µg/ml chloramphenicol. Single colonies were isolated and

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cultured overnight in LB-aro broth in the absence of antibiotics. Xer recombination deleted the *cat* gene to generate chloramphenicol-sensitive colonies of ZH9W.

In the other method of deletion, the *dif*-flanked antibiotic resistance gene *cat* was amplified with the primers *rfbEdelF* and *rfbEdelR* designed with homologous sequences to *rfbS* and *rfbX* genes respectively. The *difCAT* cassette was amplified from the synthetic pMKtetORTDAP plasmid to generate the PL deletion cassette (SEQ ID NO: 27). Electrocompetent ZH9(pLGBEK) were then transformed with the amplified DNA sequence and transformants were selected on LB-aro agar plates supplemented with 20 µg/ml chloramphenicol. Single colonies were isolated and cultured overnight in LB-aro broth in the absence of antibiotics. Xer recombination deleted the *cat* gene to generate chloramphenicol-sensitive colonies of ZH9PL2.

Example 2

To construct *S. Typhi* ZH9 expressing *S. Paratyphi A* flagellin, the native *fliC* gene was replaced with the *S. Paratyphi A fliC*. The replacement cassette was synthesised with *S. Paratyphi A fliC* flanked with ~700 bp of DNA homologous to the gene *fliD* upstream of *fliC*, and a pseudogene downstream of *fliC*. A *NotI* restriction site at the 3' end of *S. Paratyphi A fliC* was included to clone a *dif*-flanked antibiotic resistance marker gene *cat* gene amplified with primers designed with *NotI* restriction site. The *cat* gene was amplified from pBRT1N plasmid synthetically generated with 5*NotI*difcat and 3*NotI*difcat primers designed with *dif* sequences. TOP10 *E. coli* cells were used for cloning operations to generate pUCpF_*difCAT* plasmid. Chromosomal replacement of *S. Typhi* *FliC* gene with *S. Paratyphi A FliC* was carried out as described in Example 1: electrocompetent ZH9(pLGBEK) was transformed with the pUCpF_*difCAT* replacement cassette excised using *Sall* and *SacI* restriction enzymes (SEQ ID NO: 28), and transformants were selected on LB-aro agar plates supplemented with 20 µg/ml chloramphenicol. Single colonies were isolated and cultured overnight in LB-aro mix broth in the absence of antibiotic. Xer recombination resulted in the deletion of the *cat* gene to generate chloramphenicol-sensitive colonies of ZH9PF.

To construct *S. Typhi* ZH9 expressing both *S. Paratyphi A* LPS and Flagellin, electrocompetent ZH9PF (pLGBEK) was then transformed with the replacement cassette generated from the pUCpW_*difCAT* cassette excised using *Sall* and *SacI* restriction

enzymes, and transformants were selected on LB-aro agar plates supplemented with 20 µg/ml chloramphenicol. Single colonies were isolated and cultured overnight in LB-aro broth in the absence of antibiotic. Xer recombination resulted in the deletion of the *cat* gene to generate chloramphenicol-sensitive colonies of ZH9PA.

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Example 3

To construct the medium copy-number expression plasmid pBRT4*tviA* (Figure 11; SEQ ID NO: 29), primers 5*tviA*NdeI and 3*tviA*Sall were used to amplify the *tviA* gene of *S. Typhi* ZH9 from chromosomal DNA. The PCR product and expression plasmid pBRT4c were digested using NdeI and Sall and ligated to generate the precursor plasmid pBRT4*tviA*c. An *E. coli pepA* mutant strain was used for cloning operations. The pBRT4*tviA*c plasmid was transformed into *S. Typhi* ZH9, and transformants were selected on LB-aro agar plates supplemented with 20 µg/ml chloramphenicol. Single colonies were isolated and cultured overnight in LB-aro mix broth in the absence of antibiotic. Xer recombination resulted in the deletion of the *cat* gene to generate chloramphenicol-sensitive colonies of ZH9(pBRT4*tviA*). pBRT4*tviA* has *tviA* under the control of the phagosome-induced SPI-2 promoter P_{ssaG} , which is not active in nutrient broths such as LB or TB. PCN (Phosphate Carbon Nitrogen minimal medium) with a low concentration of inorganic phosphate was used to induce P_{ssaG} .

20 Example 4

To construct the medium copy number expression plasmid pBAD2*fepE* (SEQ ID NO: 30), primers *fepE*5_pBAD and *fepE*3_pBAD_pSC were used to amplify the *fepE* gene of *S. Typhimurium* WT05 (*aroC*, *ssaV*) from chromosomal DNA. The PCR product and expression plasmid pBAD2 were digested using NdeI and Sall and ligated to generate the plasmid pBAD2*fepE*. pBAD2*fepE* has *fepE* under the control of arabinose promoter, which is not active in nutrient broths such as LB or TB and require the addition of arabinose at 0.02% to induce the expression of the length regulator of very long (VL) O antigen chains. *E. coli* NEB5- α was used for cloning operations. The pBAD2*fepE* plasmid was transformed into *S. Typhi* ZH9, and transformants were selected on LB-aro agar plates supplemented with 50 µg/ml kanamycin. Single colonies of ZH9 (pBAD2*fepE*) were isolated and cultured overnight in LB-aro broth supplemented with 50 µg/ml kanamycin and 1:1000 of 20% arabinose to induce expression of LPS with very long (VL) O-antigen chains.

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Example 5

For immunofluorescence microscopy, *S. Typhi* ZH9 and its derivative strains ZH9W, ZH9PL, ZH9PF and ZH9PA were cultured for 18 hours in LB-aro broth at 37°C and 200 r.p.m. A volume of each culture equivalent to an optical density of $A_{600} = 1$ was collected and washed in PBS. Pellets were resuspended in 10 μ l of PBS with 1 μ l of primary antibody and incubated for 10 minutes at ambient temperature. LPS analysis was carried out by staining ZH9, ZH9W, ZH9PL and ZH9PA with one of the following primary antibodies: anti-*S. Typhi* LPS monoclonal antibody B348M (Genetex), anti-*S. Paratyphi A* LPS monoclonal antibody (Bio-rad), O:9 antiserum (SSI) and O:2 antiserum (SSI). Flagellin analysis was carried out by staining ZH9, ZH9PF and ZH9PA with the following primary antibodies: H:d antiserum (SSI) and H:a antiserum (SSI). Bacterial cells primary stained were then washed in PBS and pellets were resuspended in 10 μ l of PBS with 1 μ l of secondary antibody conjugated to Dylight 488 fluorochrome. After 10 minutes incubation at room temperature, cells were washed in PBS and a small volume were applied on a microscope slide to be visualised using a fluorescent microscope (Zeiss Axiophot) with attached Zeiss Axiocam camera. Fluorescence imaging demonstrated the conversion of the O9 to the O2 serotype of LPS in ZH9W and ZH9PL (Figure 4), and the conversion of the Hd flagellin serotype to Ha in ZH9PF (Figure 9), with both modifications introduced into ZH9PA (Figure 12).

To analyse LPS O-antigen length, ZH9 and ZH9(pBAD2fepE) pre-cultures were used to inoculate LB-aro broth and grown at 37°C and 200 r.p.m., with ZH9(pBAD2fepE) supplemented with 50 μ g/ml kanamycin and induced by adding 0.02% arabinose. When exponential phase was reached, 4 mL of each culture was lysed to prepare LPS using an LPS extraction kit (Intron Biotechnology). LPS samples were run on an SDS-PAGE gel and silver-stained. Figure 5 shows that the replacement of *rfbE* gene by the spacer gene *wbdR* is required to express LPS with long O-antigen chains. ZH9(pBAD2fepE) expressed LPS with the very long O-antigen chains not seen in ZH9 (Figure 8).

Example 6

To assess the immunogenicity of the ZH9PA strain and to confirm retained immunogenicity of both ZH9 and ZH9PA strains when co-administered, an immunogenicity study was conducted in mice. Balb/c animals were immunized via a single subcutaneous immunization with ZH9 alone (1×10^8 cfu/ mouse), ZH9PA alone (1×10^8 cfu/mouse) or combination of the two ZH9+ZH9PA (0.5×10^8 cfu ZH9 + 0.5×10^8 cfu ZH9PA / mouse). Pre-

immune serum samples were collected prior to immunization and terminal serum samples were collected 35 days after immunization. All samples were centrifuged for serum isolation and serum stored at -80°C.

- 5 The sera were used to run in house standardized ELISA assays aimed at assessing the titers specific against *Salmonella* Typhi LPS (O:9) and flagellin (H:d), *S. Paratyphi* A LPS (O:2) and flagellin (H:a). Briefly, half-area ELISA plates (Corning) were coated with the following specific antigens (all provided by The Native Antigen Company), diluted in 50 mM carbonate/ bicarbonate buffer (pH 9.6):

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Antigen	Coating dilution/ concentration
<i>S. Typhi</i> LPS	1:500
<i>S. Paratyphi</i> SLAV LPS	1:500
<i>S. Typhi</i> flagellin	0.2 µg/ mL
<i>S. Paratyphi</i> SLAV flagellin	0.5 µg/ mL

Plates were incubated at 4°C overnight (~16h). The day after, plates were washed with PBS+0.05 % Tween-20 before blocking with Pierce™ Protein-Free (PBS) blocking buffer for 1 hour at 37°C. After washing plates as before, mouse sera from the oral immunogenicity study were added for 1 additional hour at 37°C. A standard curve was also generated for each antigen by using serovar-specific reagents as follows:

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Reagent	Working dilution
Monoclonal antibody anti- <i>S. Typhi</i> LPS	1:1000
Monoclonal antibody anti- <i>S. Paratyphi</i> A LPS	1:250
Rabbit antiserum <i>S. Typhi</i> flagellin	1:50
Rabbit antiserum <i>S. Paratyphi</i> A flagellin	1:200

Reacting sera and standard curve for *S. Typhi* and *Paratyphi* A LPS were detected using a secondary goat anti-mouse antibody conjugated directly to horse radish peroxidase (HRP), whilst standard curve for *S. Typhi* and *Paratyphi* A flagellin with HRP-conjugated goat anti-rabbit antibody. After washing plates as above, secondary antibodies were added at

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1:2000 dilution in Pierce™ Protein-Free (PBS) blocking buffer and incubated at room temperature for 1 hour. Positive sera were revealed using 3,3',5,5'-Tetramethylbenzidine (TMB) substrate and measured using a spectrophotometer at 450nm. Absorbance (OD) was plotted using a 4 parametric logistic curve of the positive control; then end point titers were determined as logarithm of the dilution value at which the serial dilution curve of each mouse serum met OD equal 1.

Figure 13 shows that the endpoint titers of antibodies specific for Typhi LPS (O:9), Paratyphi LPS (O:2), Typhi flagellin (H:d) and Paratyphi A flagellin (H:a) display no significant differences following immunization with ZH9/ZH9PA alone and the combination of the two. Accordingly, this demonstrates that when the strains are co-administered that immunogenicity is retained.

Sequences used throughout the specification and forming part of the description:

SEQ ID NO: 1 (S. Typhi *fliC* cistron)

atggcacaagtcattaatacaaacagcctgtcgcgtgtgaccagaataacctgaacaaatcccagtcgcgactgggcactgct
 atcgagcgtttgtcttccggctgcggtatcaacagcgcgaaagacgatgcggcaggacaggcgattgctaaccggtttaccgcg
 aacatcaaaggtctgactcaggcttcccgtaacgtaacgacggtatctccattgcgcagaccactgaaggcgcgctgaacga
 aatcaacaacaacctgcagcgtgtgcgtgaactggcgggttcagctgcgaatggactaactcccagtcgtacctcgactccatc
 caggctgaaatcaccagcgcctgaacgaaatcgaccgtgtatccggccagactcagttcaacggcggtgaaagtctggcgc
 aggacaacaccctgaccatccagggtggtgccaacgacggtgaaactatcgatatgtattaaaagaaatcagctctaaaaca
 ctgggacttgataagcttaattgtccaagatgcctacaccccgaaagaaactgctgtaaccgttgataaaactacataaaaaatg
 gtacagatcctattacagcccagagcaatactgatalccaaactgcaattggcgggtggtgcaacgggggttactggggctgata
 tcaaatttaagatggtcaatactatttagatgttaaaggcgggtgcttctgctggtgtttataaagccacttatgatgaaactacaaa
 gaaagttaattatgatacgactgataaaactccgttggcaactgcggaagctacagctattcggggaacggccactataaccca
 caaccaaattgctgaagtaacaaaagaggggtgttgatacgaccacagttgcggctcaacttgctgcagcaggggttactggcg
 ccgataaggacaatactagccttgtaaaactatcgtttgaggataaaaaacggtaagggtattgatggtggctatgcagtgaaaat
 gggcgacgatttctatgccgctacatatgatgagaaaacaggtgcaattactgctaaaaccactacttatacagatggtactggc
 gttgctcaaactggagctgtgaaattggtggcgcaaattggtaaatctgaagttgttactgctaccgatggttaagacttacttagca
 agcgacctgacaaacataacttcagaacaggcgggtgagcttaagagggttaatacagataagactgaaaaccactgcag
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5 **SEQ ID NO: 2 (S. Paratyphi A *fliC* cistron)**

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25 **SEQ ID NO: 3 (S. Paratyphi B *fliC* cistron)**

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26

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10

SEQ ID NO: 4 (*S. Paratyphi B fljB* cistron)

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SEQ ID NO: 5 (*S. Paratyphi C fliC* cistron)

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SEQ ID NO: 6 (*S. Paratyphi C fljB* cistron)

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5 **SEQ ID NO: 7 (*S. Typhimurium fliC* cistron)**

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25 **SEQ ID NO: 8 (*S. Typhimurium fljB* cistron)**

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29

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SEQ ID NO: 9 (*S. Enteritidis fliC* cistron)

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SEQ ID NO: 10 (*S. Typhi tviA* cistron)

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SEQ ID NO: 11 (*S. Typhimurium fepE*)

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SEQ ID NO: 12 (*S. Typhi rfbE* cistron)

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 taccattgtcaacagcctatcattactgaattattcaaattgcttgaagattattgcaacatagatatgaggttactaatttacctgta

agggaaagtgtatcagcgtgttttgttcagatattaaaaaatcactaatgcaattgactggagcccgaaagtctcggcaaaa
gatggtgtccagaaaatgtatgattggactagttctatatga

SEQ ID NO: 13 (*E. coli* O157:H7 *wbdR* cistron)

5 Atgaattgtatggtatttttgggtgctggaagttatggtagagaaacaatacccattctaaatcaacaaataaagcaagaatgtggt
tctgactatgctctggttttgggatgatgttttggcaggaaagaaagttaatggtttgaagtgtttcaaccaactgctttctaaaag
ccccttatttaaaaaaglatttaatgttgctattgctaataagatacgacagagagtgctgagtgcaatattattacacgggggtt
gaaccaataactataaaacatccaaatagcgtttttatgatcactatgataggtagtggtgctattatttctcccttgttacaata
tctactaatactcatatagggagggtttttcatgcaaacatatactcatacgttgcacatgatgtgcaaataggagactatgttacattt
10 gtcctggtgggctaaatgtaatggatagttgttattgaagacaatgcatatataggctcgggtgcagtaattaagcagggtgttcct
aatcgcccacttattattggcgcgaggccattataggtatgggggctgtgtcactaaaagtgttcctgccggtataactgtgtgc
ggaaatccagcaagagaaaatgaaaagatcgccaacatctatttaa

SEQ ID NO: 14 (*S. Typhi* *rfbS* cistron)

15 Atgaaaattctaataatgggagcgtttgggttccttggatcacgacttacatcctacttcgaaagtcgacatactgtgattggcttag
caaggaagaggaacaatgaagctaccataaataatattattacacgacagaaaataattggatcgaaaaaatactagaattt
gaaccgaatattattattaacactattgcttgcattggaagacataacgaacctgcaacagctttaatagaaagcaatattcttatg
cctatcagagtattagaatctatctcatcacttgatgcagtattcalaaattgtggaacatcactgccaccaataacgagtttatatgc
atatactaaacaaaaagcaaatgaactcgccgccgcatfatagataaagtttgcggtaaatatatagagttaaaattggagca
20 tttctatggagcttttgatggagacgataagtttaccagtatggttattagacgttgtttaagtaaccagccagtaaaagttaacatctg
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attgaatttggcgtggtcaaagaaagagtaaatgaattgatgtatagttgtgctgatatagcagaacttgaaaaaattaggatgga
aaagagagttctctcttgtgatgcattaactgaaataattgaagaggaaggggaaatga

25

SEQ ID NO: 15 (*S. Typhimurium* *rfbJ* cistron)

Atgaccttttgaaagaatatgtaattgtcagtggggcttccggcttattggttaagcatttactcgaagcgctaaaaaatcgggg
atttcagttgtcgaatcactcgagatgtaataaaaaataatagtaatgcattagctaattgttagatggtgcagttgggataatatcg
aattattagtcgaggagtatcaattgattctgcattaattggatcattcatttggcaacagaalatgggcataaaacatcatctctca
30 taaatattgaagatgcaaatgtataaaaccattaaagcttcttgatttggcaataaaatatcgggcggtatcttttaaacagat
agtttttggcaagaaagatttaattatcaacatatcgggccttatataattactaaaagacactttgatgaaattgggcattattatg
ctaataatgcatgacatttcatitgtaaacatgcgattagagcatgtatatgggcctggggatggtgaaaataaatttattccatacatt
atcgactgttaataaaaaaacagagttgcgtgaaatgtacaacaggcgaacagataagagactttattttgtagatgatgtggt
aaatgcttatttaactatattagaaaatagaaaagaagfaccttcatatactgagtatcaagttggaactgggtgctggggtaagtttg

aaagattttctggtttatttgcaaaatactatgatgccaggttcacgagtagatattgaatttggcgatagagcaaagagataatga
aataatgttctctgtagcaaataataaaaaatttaaaagcaatgggctggaaaccaaatttcgattataaaaaaggaattgaaga
actactgaaacgggttatga

5 **SEQ ID NO: 16 (*S. Typhimurium* P_{ssaG} promoter region)**

Tattgccatcgcggtatgcgcctgtcttattctaccatcataaacatcatttgcctatggctcacgacagtaggcaatgccgttttt
ataattgctaattgtttcgccaatcaacgcaaaagtagggcgattgctaaagccgtctccctgggcggttagattagccttaaccgcg
acggtaatgactcattcacttgagtggttagttgggactacagcctcatttattagagcgtcttaatatgattacctatggactaat
gagtttactcgcttcggtatggatgggtaggcaatgaccggtatgcaggtcagcagcccattatcgtttgctggctcaggtaac
10 gccagaacaacgtgcgcccggagtaatcgtttcaggtatataccggatgttcattgctttctaaattttgctatgttgccagtatcctta
cgatgtatttttaaggaaaagc

SEQ ID NO: 17 (*E. coli* *araC* repressor and P_{araBAD} promoter)

ttatgacaacttgacggctacatcattcactttttcttcacaaccggcacggaactcgctcgggctggccccgggtgcatttttaata
15 cccgcgagaaatagagttgatcgtaaaaaccaacattgcgaccgacggggcgataggcatccgggtgggtgctcaaaagca
gcttcgctggctgatacgttggctcctcgcgccagcttaagacgctaataccctaactgctggcggaagagatgtgacagacgcg
acggcgacaagcaaacatgctgtgacgctggcgatatcaaaattgctgtctgccagggtgatcgctgatgtactgacaagcct
cgcgtaaccgattatccatcggtggatggagcgactcgttaatcgcttccatgcgcccagtaacaattgctcaagcagatttattc
gccagcagctccgaatagcgcccttccccttggccggcgtaataatttgccaaacaggtcgctgaaatgcggctgggtgcgctt
20 catccggggcgaaagaacccccgatttggaataattgacggccagtaagccattcatgccagtaggcgcgaggacgaaagta
aaccactgggtgataccattcgcgagcctccggatgacgaccgtagtgatgaatctctcctggcggaacagcaaaatatcac
ccggctgggcaaaacaaattctcgtccctgatttttaccacccccctgaccgcgaatgggtgagattgagaatataacctttcattcca
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cccggcagcaggggatcattttgcgcttcagccatactttcactcctccgcccattcagagaagaaaccaattgtccatattgcac
25 agacattgccgtcactgcgtcttttactggctcttctcgtaacaaaccggtaaccccgcttattaaaagcattctgtaacaaagc
gggaccaaagccatgacaaaaacgcgtaacaaaagtgtctataatcacggcagaaaagtccacattgattattgcacggcg
tcacactttgctatgcatagcattttatccataagattagcggatcctacctgacgcttttatcgcaactctctactgtttctccatac
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30 **SEQ ID NO: 18 (*S. Paratyphi A* *rfaE* pseudogene)**

Ttgaagaggaagagaaatgaagcttaattaccgggtggatgtggcttcttgggagtaatttgcctcctttgctttaagtcaaggg
attgatttaattgtattcgataatctatcacgtaaaaggtgcaacagataatttacattgggtatcctccttaggaaactttgagttgtac
atgggtgatattcgcaacaaaaatgatgttacaagattaataactaagtatatgcctgatagctgttttcatcttcagggtcaagtggc
aatgactacatctattgacaatccttgatggattttgaaattaatgtagggtggaactttaaatftactgaggcagtagcggcagtagata

33

attcaaattgtaataataattatcatcaacaaataaagtatacggcgatcttgagcaataataacaatgaaacagaaactaga
 tacacttgtagataagcctaattgatatgatgagagcacacaattagattccactcaccatatgggtgtcaaaagggtgctgca
 gaccaatacatgcttgattatgcaaggatttttgggttgaatacagtggtgttcaggcattcatcaatgtatgggtgggagacagtttgc
 tactatgatcaaggctgggtagggttgggttgcacaaaagcgggtgaaattaaaaatgggtatataaaccttcactatttctggta
 5 atggtaagcaagttagggtatgtttgcatgctgaagatatgatttcgttatatttcactgccttggcaaatgtatcaaaaaattaggggg
 aacgcttttaatatgttggttaccattgtcaacagcctatcattacttgaattatcacaattgcttgaagattatgcaacatagatatga
 ggttactaatttacctglaagggaagtgatcagcgtgttttgttcagatatataaaaaaatcactaatgcaattgactggagccc
 gaaagtcctcggcaaaagatgggtgtccagaaaatgtatgattggactagtctatatga

10 **SEQ ID NO: 19 (S. Typhi *rfbE* locus with a partial deletion of *rfbE*)**

tcacgacttacatcctacttcgaaagtcgacatactgtgattggcttagcaaggaagaggaacaatgaagctaccataaataat
 attatttacacgacagaaaaataattggatcgaaaaaatactagaatttgaaccgaatattattttaacactattgcttctatggaa
 gacataacgaacctgcaacagcttaataagaaagcaatattcttatgcctatcagagtattagaatctatctcatcacttgatgcag
 tattcataaattgtggaacatcactgccaccaaatacagagtttatgcatataactaaacaaaaagcaaatgaactcgccgccgc
 15 cattatagataaagtttgcggtaaatatataagagttaaaattggagcatttctatggagcttttgatggagacgataagttaccagt
 atgggtattagacgttgttaagtaaccagccagtaaaagttaacatctggttgcacagagagatttcttctatataaaagatctact
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 gtgaatatgtagatactgttaaaaaatcacaaaaagcaattctataattgaatttggcgtggcacaagaaagagtaaatgaattg
 atgtalagttgtgctgatatagcagaactgaaaaaataaggatggaaaagagagttctctcttcttgatgcattaactgaaataatt
 20 gaagaggaagggaaatgaaaagcttggtagcagctcgatccactagtaacggccgccagtgctggaattcgcccttta
 agcggccgcatttaacataataacattatgcgcaccgcccgcggaaagggcggaattctgcagatalccatcacactggcg
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 aagataataattgccggtgttcagttagtaagtgtaaatcttcttagaaattcttggcgaagaatcatacgctgtatttactctttaac
 tggattattggctctggttagcattgcagatatgggattggtagtagtctacaaaattatatactgagttgaaagctgatagaaaat
 25 catatgatgcataatcaaggccgcagttcatattctattcgcacctaataatcaatttaagctctacattatcttctatcagataaattat
 cgtcactatatcttacttcatttagcgatgaattgaaaaacaactcaggaagttatttttatagcaagtatattttatattcatcggc
 gttgggagtggtgtctataaaatattatttgcggaactgttaggggtgaaagctaataataatgcattatcttctttaggttttta
 gatgtagttcgatccattatttaatgccagattcgagfataccttcgcttagtagcatgtatgctccggtagcaatactgccatta
 tatafatatcgtttcggtatataatgttcttaagcgaaagtaaaactttaacacctataaattattactatcacgttcatcagggttctg
 30 atttttctgcttctatcgataatagttttacaaactgattatattgtgatgtctcagaaattatctgctgcagatatataaaaataactgta
 acgatgaaaatatttgggttaattgttttataactgcggtattacaagcattatggccagtaigtgct

SEQ ID NO: 20 (S. Typhi *rfbE* locus with *rfbE* disrupted by *wbdR*)

aggccttgactacagagcatttagattatgtagttagcaagtttgaagagttcttgggttgaatttctaatttgaggggggggattcc
 cctctatgatttcatgaaaattctaataatgggagcgttgggttcccttgatcacgacttacatcctacttcgaaagtcgacatactgt
 gattggccttagcaaggaagaggaacaatgaagctaccataaataatattttacacgacagaaaataattggatcgaaaaaa
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 5 atattcttatgcctatcagagtattagaatctatctcatcacttgatgcagttatcataaatttggaacatcactgccaccaaatacg
 agtttatatgcatataactaaacaaaaagcaaatgaactcgccgcccattatagataaagtttgcggtaaatatataagagttaa
 aattggagcatttctatggagcttttgatggagacgataagttaccagttatggttattagacgttggtaagtaaccagccagtaaa
 gttaacatctgggttgaacagagagatttcttgatataaaagatctactaacagcgttcgattgtattataagtaattgtaataatttc
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 10 caattctataattgaatttggcgtggtcaaagaaaagagtaaatgaattgatgtatagttgtgctgatatagcagaacttgaaaaat
 aggatggaaaagagagttctctcttggatgcattaactgaaataattgaagaggaagggaaatgaattgtatggtatttttggtg
 ctggaagtattggtagagaaacaataccatttctaatacaaaaaaagcaagaatgtggttctgactatgctctggttttttggtg
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 atgttgctattgctaataagatagacagagagtgctgagtcataattattacacgggggtgaaccaataactataaaacat
 15 ccaaalagcgttggttatgatcatactatgataggtagtggcgctatttctcccttggtaacaatactactatcatatagggga
 ggtttttcatgcaaacatatactcatagcttgcacatgattgtcaaataaggagactatgttacatttgctcctggggctaaatgtaatg
 gatattgttattgaagacaatgcataatagggctcgggtgcagtaattaagcaggggttccctaactgcccacttattatggcgc
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 aaaagatcgccaacatctattaatgcggccgcatttaacataataacattatgcgcaccgcccggccagtgtaggatcctg
 20 tttctgcccgcgaaagcgggcataattaaagaatgaaatattttataaataaaagatgaagctgacgtgaggaaactgagggtg
 gttagaattccaagacatcttattattgccgttctcttggctttcaaagataataatggcgggtgttcagttagtaagtgttaaattctt
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 ccttaatcattttaagctctacattattcttctatcagataaattatcgtcactatatcttacttcatttagcgaatgaaaaacaact
 25 caggaagtattttttatagcaagtataattttatattcatcggcgttgggagtggtgtataaaaatatttgcggaactgttaggg
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 tcgctttagtagcattgtatgctccggtagcaactgcccattatataatcggttcggtatataatgttcttaaagcgaaagtaaa
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 tgtctcagaaattatctgctgcagatattataaaatatactgtaacgatgaaaatatttggttaattgtttttatttatactgcggtattac
 30 aagcattatggccagttatgtgctgaattacgagtgaaaatgcagtgagaaagctgcatagaatcatttcttaaatattattggtg
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 gactaattttatcgttctcgctaactgtttttggggattgccagtgattatattgataagagtaaaaggctagcataatatgaagggtat

cattttgtatcccaacgtataatcgagtaaaatcattgaagaccttcttgaaagtattaataatcaatcttctcactccttaattgtaga
agtatgt

SEQ ID NO: 21 (*S. Typhi* *fepE* pseudogene)

5 Atgccatctcttaatgtaaaacaagagaaaaatcagtcatttgcaggttactcactgccgcccgccaacagtcagaaatcgattt
gtttagccttatagagggtgttatggcaggcgaaacgtcgatcttctgclaccgtttcgctttgctgctgggttgcttctgctcttct
gctgccgcaaaaatggaccagccaggcgattgtcacaccggcgaggtagcagtagcaggggctggagagaacgttga
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10 attgtcgtgctgagcgaaaaaatgaaagcgggtggacagcaatgccggcaagaaaaatgaaacgtcgttattcacgtcgtgga
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15 aaaggggtaacggacgtggccgagatcgacggtgatttgcgtaaccgtcaatactatgttgaaacaactggcggaatgaatgt
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acgcgctggcgatagatgaacgggttagtctga

20 SEQ ID NO: 22 (*S. Typhi* family 2 *gtr* operon)

actgcactgacggtggcggttgaaacgctgaaggcataaagatcttcactctcccgccgatgtcctgataactcatctccagcg
caaacagtcgaatgatattgcgctcgatctcgctacagggggtctgaagcttctcaccaattatggctcaaaagtgccgttac
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25 atgcctggccggacctgaagggtttaaagtgttctgcgaaatcgaagggatgggacatgtgccattctttttatgttactaaa
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30 ttgctttctgtatgtatgggatgcatacgcacatcaggcgctgacgaactttccgattttgtatcgctglatcgctcagcttctatgcta
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5 cttgtatcatgtcttattgttgggttgatttatttccaggatgattacaaatfacttggttaagcatgttttagtggctcattctttatatca
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15 gacgtgtacaattattataaatalggagaactactccgtggaggatgtcactcagtacaactactgctgccatatccaatgg
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20 gattgtttcattggaccagtcccggaatggaatgcaaatttagttaaaaaataatctaaactacctgagtgagtttaaaaaaactcca
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gggacatttaacaaagcctgttctgatttcttttaataaaaatlgaaataaaaataatcaaatagataggctgttactattacatat
aaatccaatalggaacatgccagtcatactgtgtaactgccactatattaacggtgatcgctcaggcggtcaccgaactcgataa
25 taaagcgaa

SEQ ID NO: 23 (S. Typhimurium *hin-fljBA* locus)

tgttgtaatttttatttaattcattcggttttttatgcggttgcgggaaaatatctgtataaggtagatacgccaataccaaaaataata
gctagttgctgccgaggatggcctttcttaatagccgactaatctgttctgttcaigtgttgatcgcccgagggcgccctcccag
30 tctgcttgcgctctggcggcagccagtcgggcaagggttgcctcgacaattaattctcgctccatctcgccagtgctgacattac
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30

SEQ ID NO: 24 (Bacteriophage lambda tandem P_R and P_L promoters)

Acgttaaatctatcaccgcaagggtataaatatctaacaccgtgcgtgtgactattttacctctggcggtgalaatggttgcattgac
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5 **SEQ ID NO: 25 (Bacteriophage lambda thermo-labile repressor *cI857* cistron)**

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15

SEQ ID NO: 26 (pUCpW_difCAT *rfbE* deletion cassette)

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 aatagaaagcaataattcttatgcctatcagagtattagaatctatctcatcacttgatgcagatttcataaatttggaacatcactgc
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 25

SEQ ID NO: 27 (PL *rfbE* deletion cassette)

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10 **SEQ ID NO: 28 (pUCpF_difCAT *fliC* replacement cassette)**

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SEQ ID NO: 29 (*tvfA* expression plasmid pBRT4*tvfA*)

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Claims

1. A vaccine for use in enhancing immunogenicity against *Salmonella enterica* serovar Paratyphi A and for use in the treatment or prevention of enteric fever and salmonellosis, said vaccine comprising a live attenuated strain of *Salmonella enterica* serovar Typhi, wherein said strain is modified to express the lipopolysaccharide O2 O-antigens and the flagella proteins of *Salmonella enterica* serovar Paratyphi A.
2. The vaccine for use of claim 1, wherein the modification of said strain includes where the native *rfbE* gene is inactivated.
3. The vaccine for use of claim 1 or 2, wherein the modification of said strain includes where the native *rfbE* gene is deleted.
4. The vaccine for use of any one of claims 1-3, wherein the native *rfbE* gene is inactivated by the insertion of a spacer region of DNA.
5. The vaccine for use of claim 4, wherein the spacer region of DNA is the cistron of the *Escherichia coli* gene *wbdR*.
6. The vaccine for use of any one of claims 1-5, wherein the resulting lipopolysaccharide O2 O-antigens are at least equivalent in length to the lipopolysaccharide O9 O-antigens of *Salmonella enterica* serovar Typhi.
7. The vaccine for use of any one of claims 1-6, wherein said strain has its native *fliC* gene substituted with the *fliC* gene of *Salmonella enterica* serovar Paratyphi A, or wherein the substituted *fliC* gene is expressed in *trans*, such that the conferred serotype is altered from an Hd serotype to an Ha serotype.
8. The vaccine for use of any one of claims 1-7, wherein said strain is modified to contain a functional *fepE* gene, such that long O-antigen chains are generated.
9. The vaccine for use of claim 8, wherein the O-antigen chains are 100 repeated units of the trisaccharide backbone in length and/or wherein said strain is further modified

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to constitutively express *gtrC*, or wherein the *gtrC* gene is expressed in *trans*, and/or wherein said strain is further modified to contain an additional copy of the *tviA* gene under the control of a phagosomally induced promoter.

- 5 10. The vaccine for use of any one of claims 1-9, wherein said strain is ZH9 or ZH9PA.
11. The vaccine for use of claim 10, wherein ZH9 or ZH9PA is a single component of said vaccine or wherein ZH9 and ZH9PA are present in combination.

10

Abstract

- 5 The present invention relates to the modification of a live attenuated strain of *Salmonella enterica* serovar Typhi, wherein its natural surface-exposed polysaccharide and flagellin antigens may be converted to, or augmented by, those from other strains of *Salmonella*, including *S. enterica* serovars Paratyphi, Typhimurium and Enteritidis. The present invention also relates to modified strains of *Salmonella enterica* serovar Typhi being
- 10 suitable for use as components of a vaccine for enteric fever and salmonellosis.

Abstract Drawing

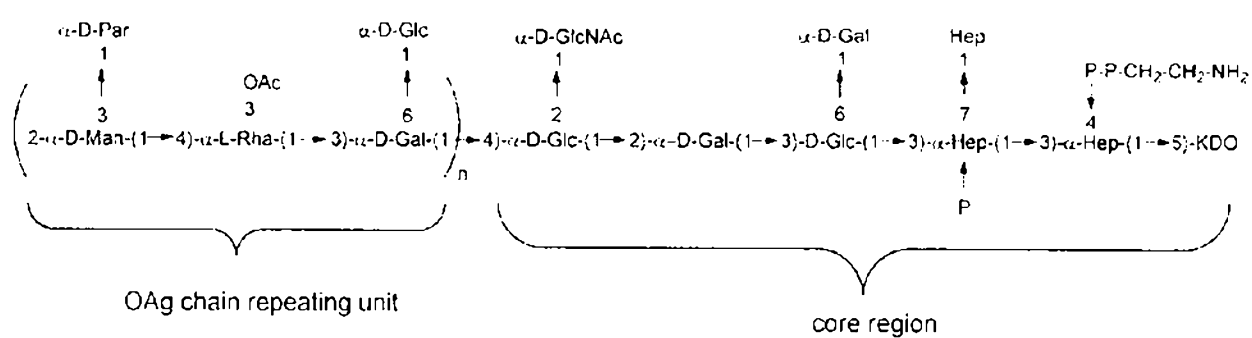


Figure 1

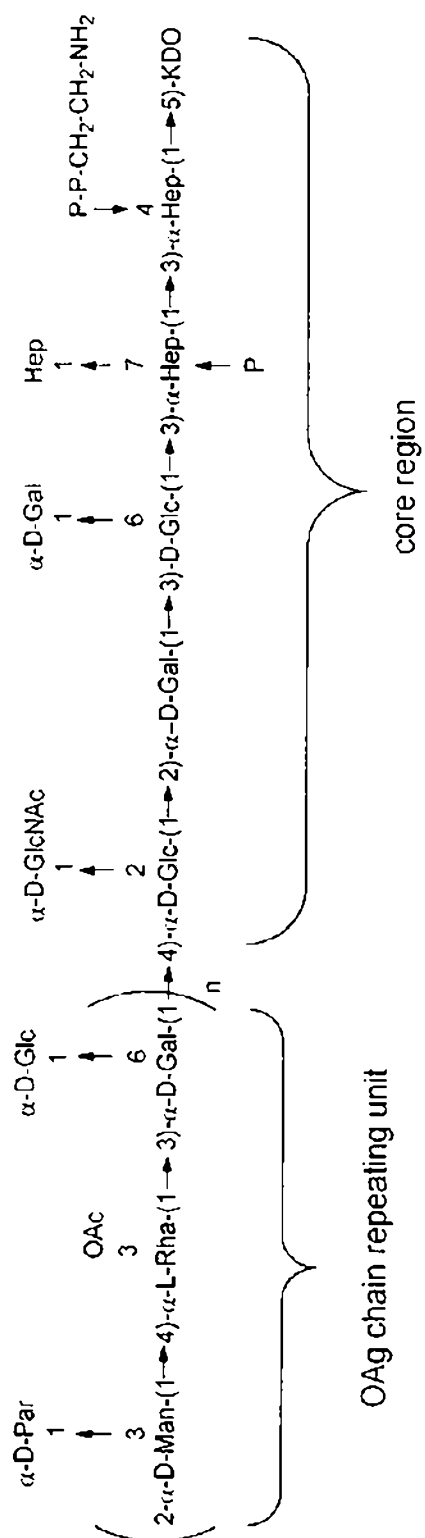


Figure 1

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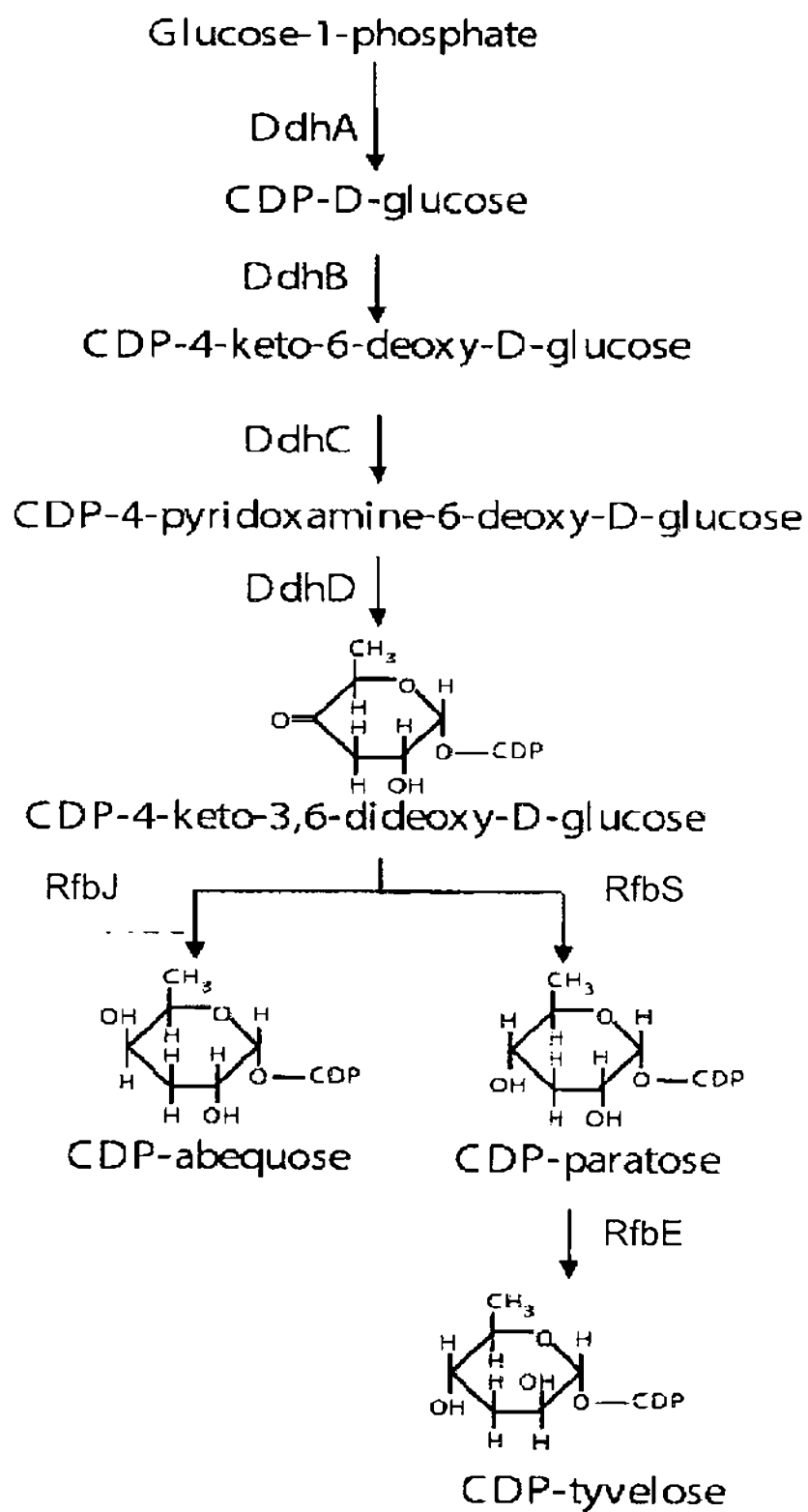


Figure 2

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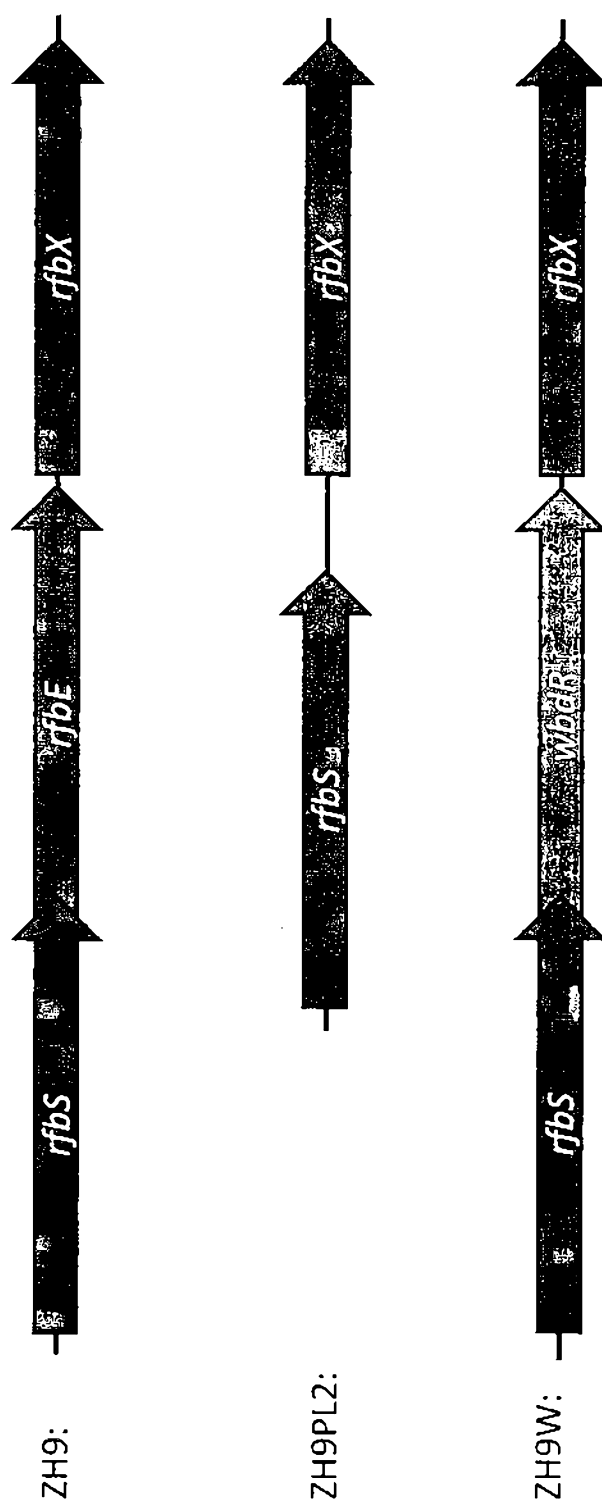
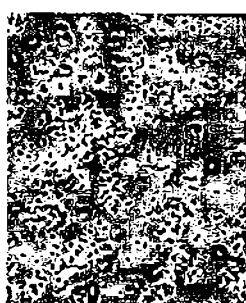
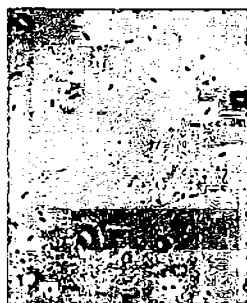
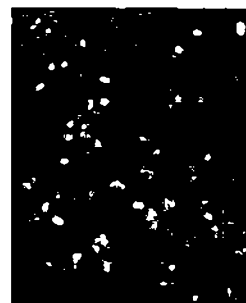
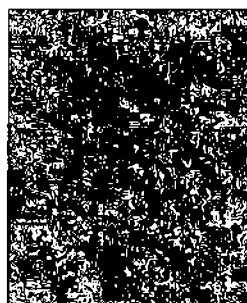


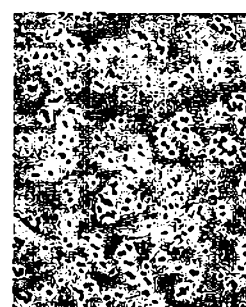
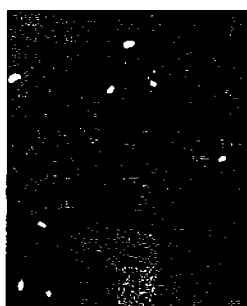
Figure 3

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B Anti-S. Paratyphi A (O2) LPS mAb



A Anti-S. Typhi (O9) LPS mAb



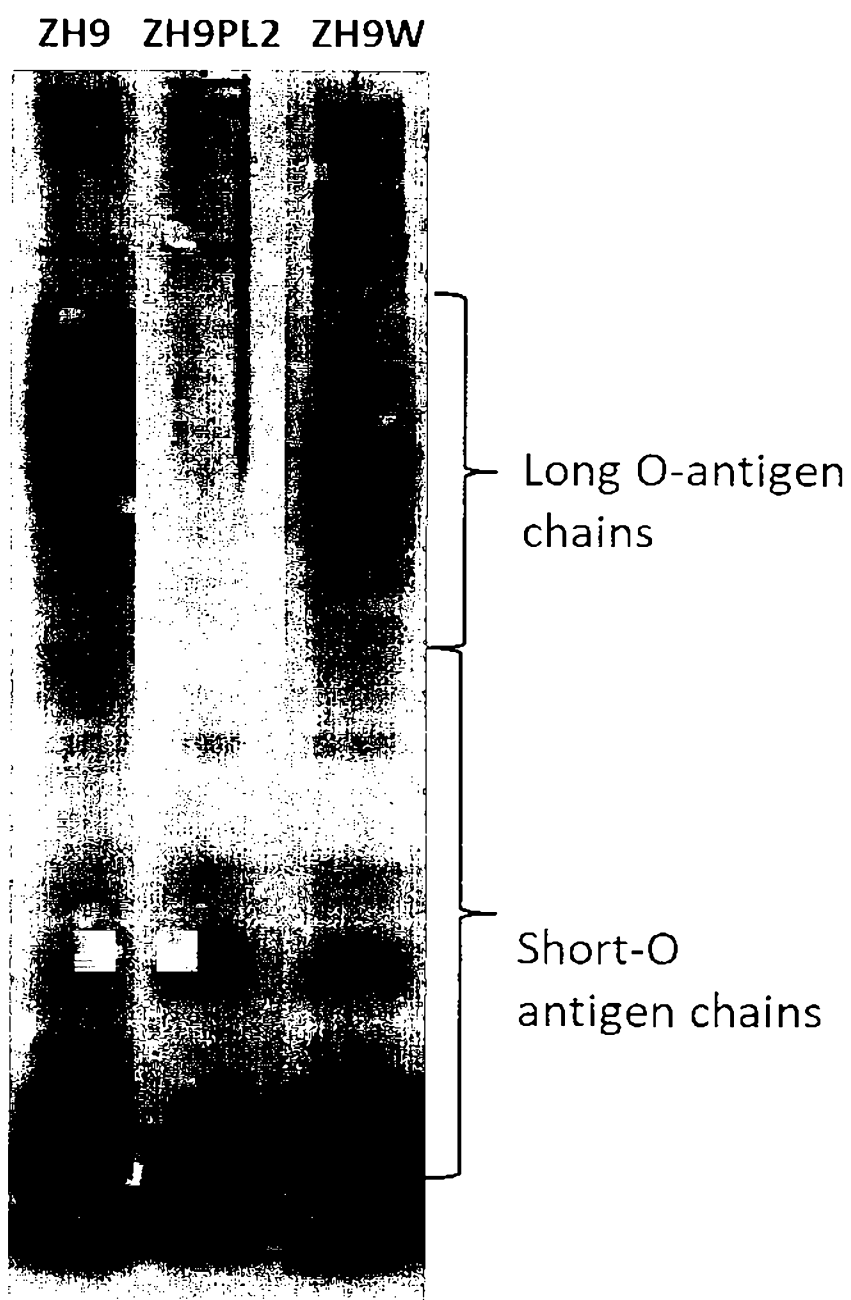
ZH9

ZH9PL2

ZH9W

Figure 4

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**Figure 5**

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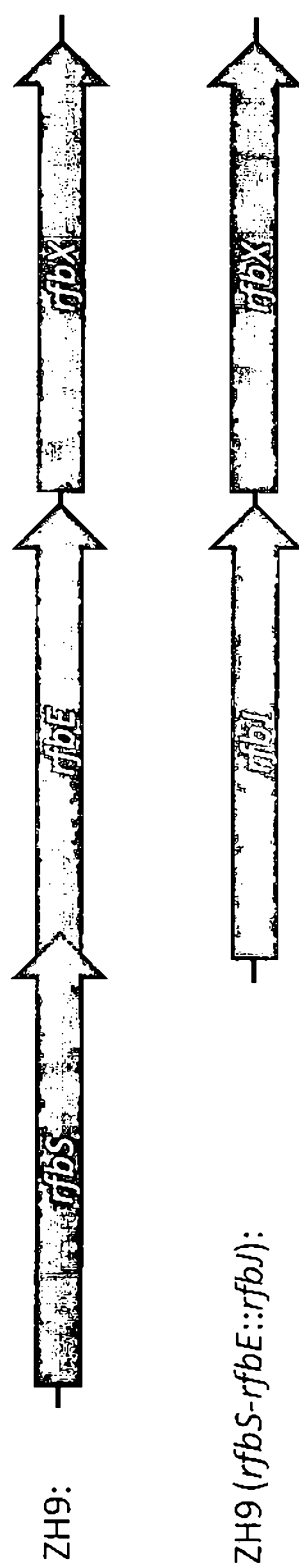


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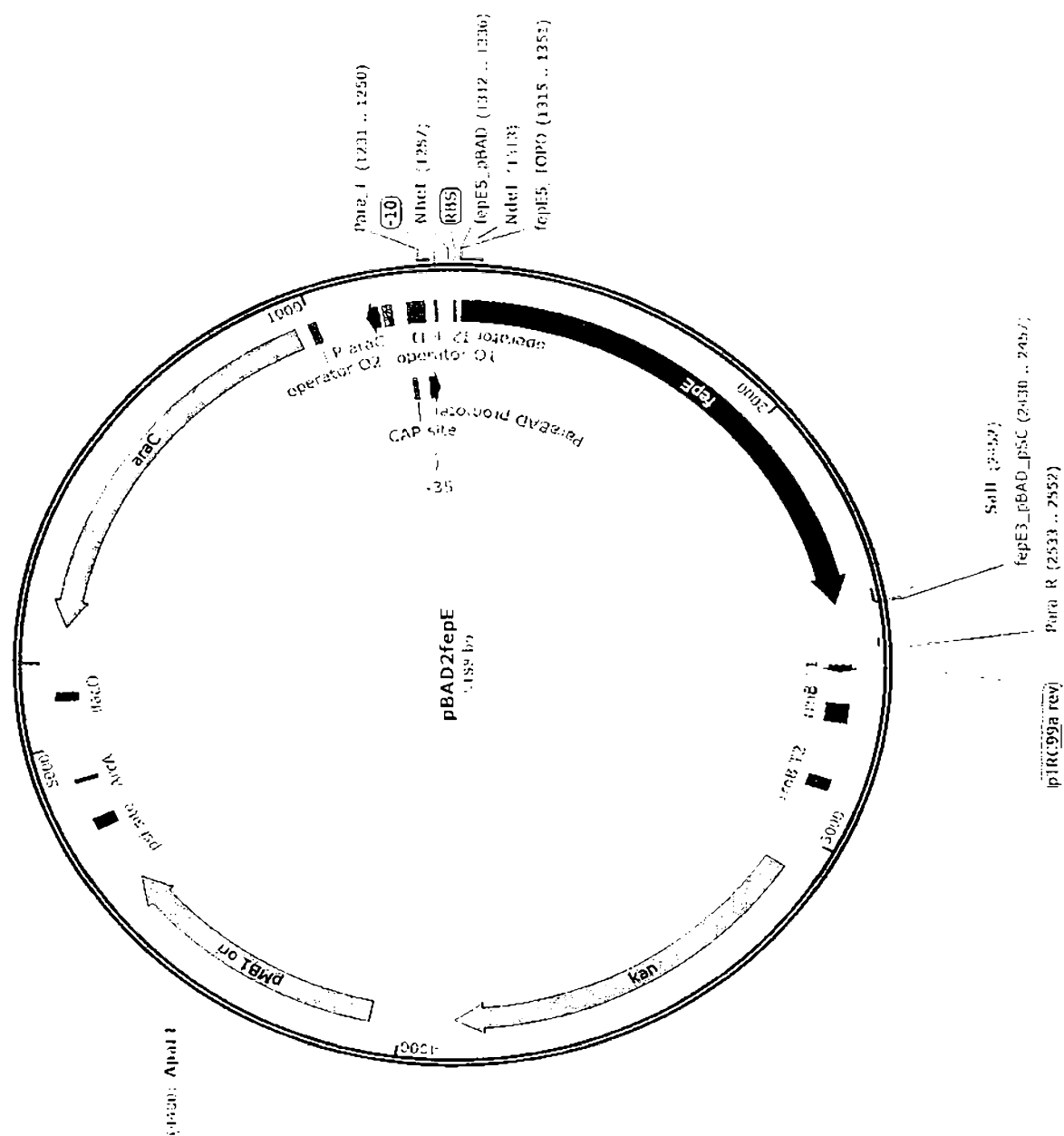


Figure 7

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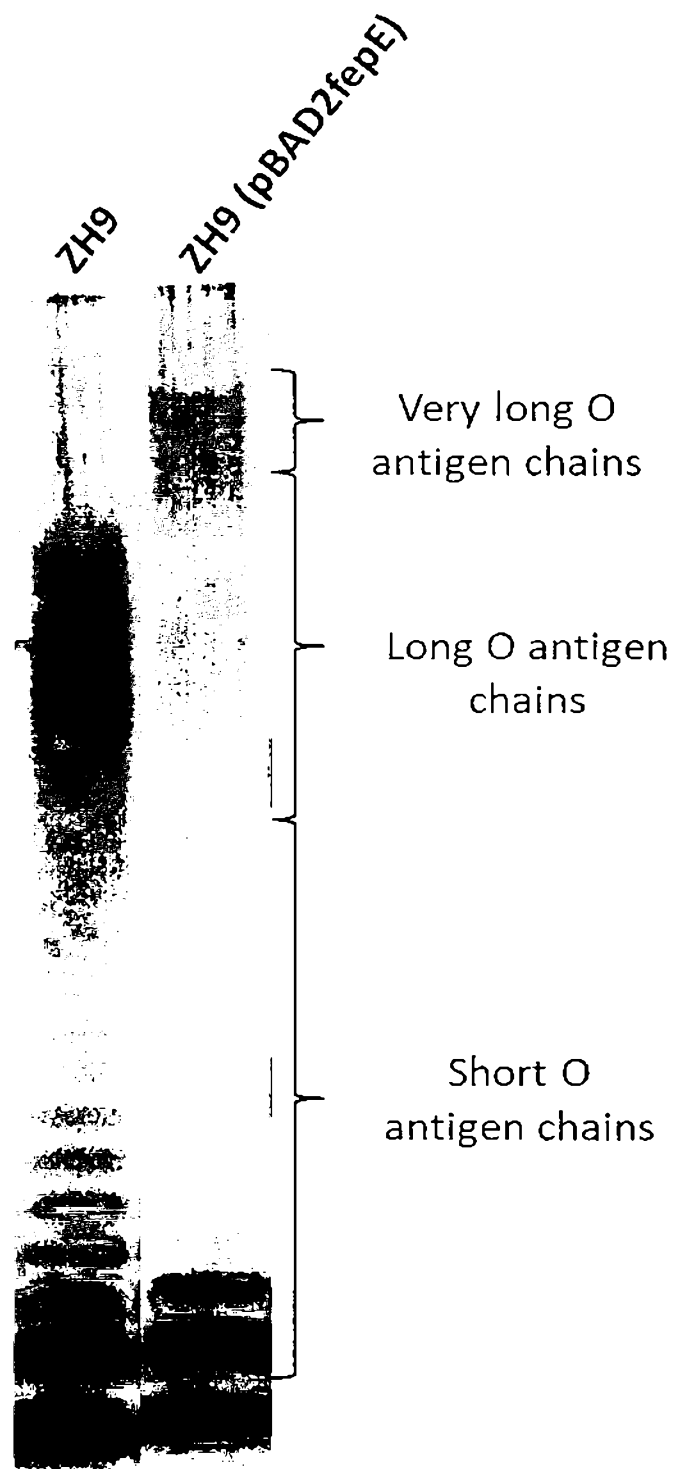
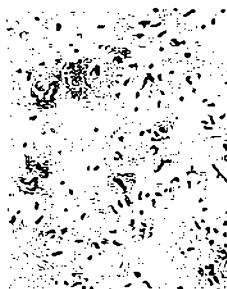
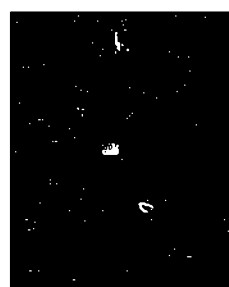


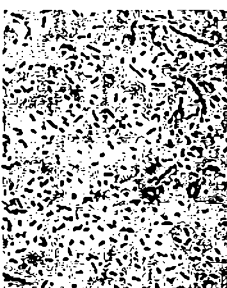
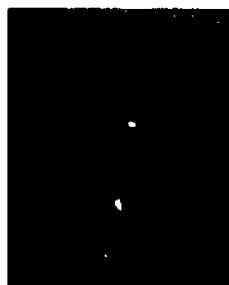
Figure 8

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B Anti-S. Paratyphi A (Ha)
flagellin antiserum



A Anti-S. Typhi (Hd)
flagellin antiserum



ZH9

ZH9PF

Figure 9

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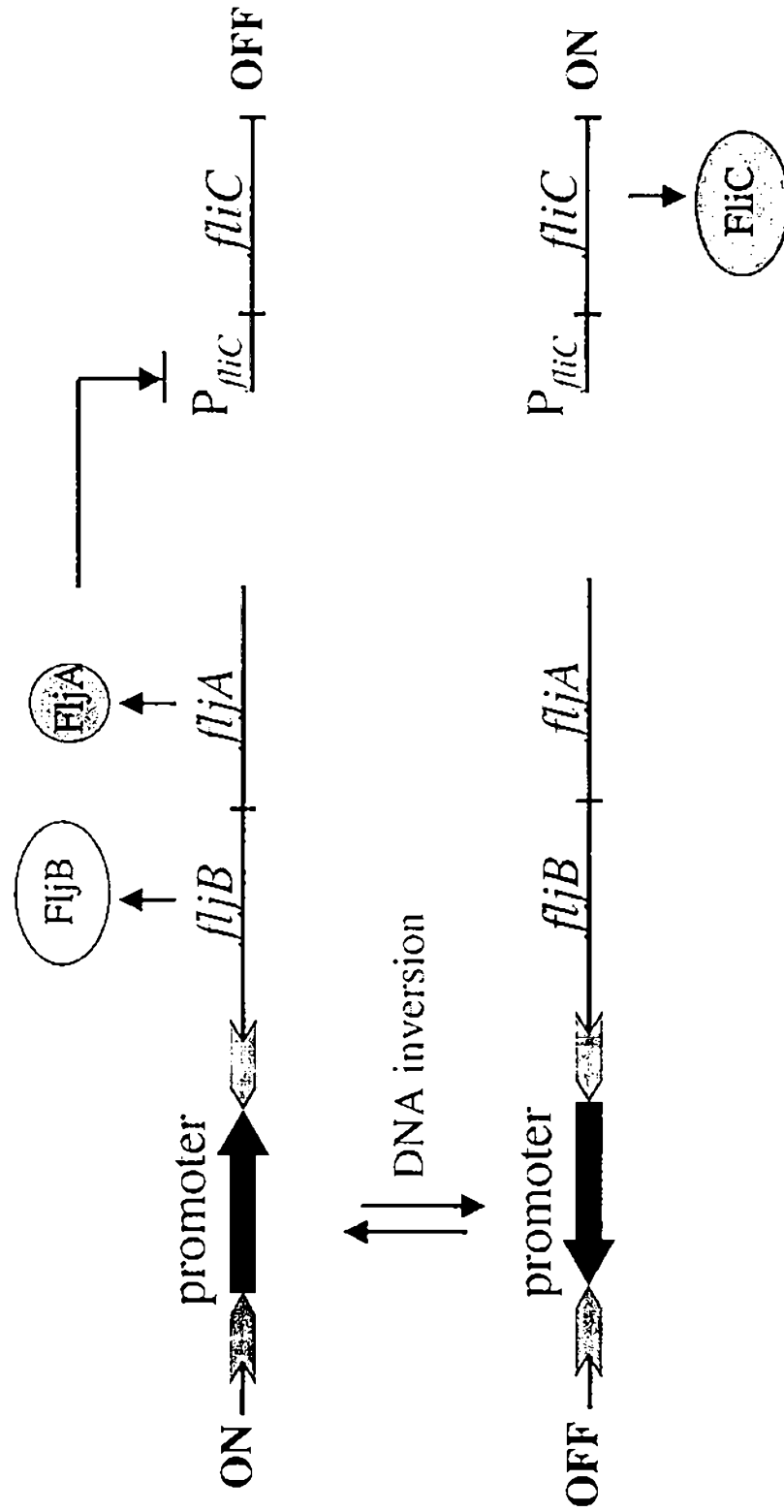


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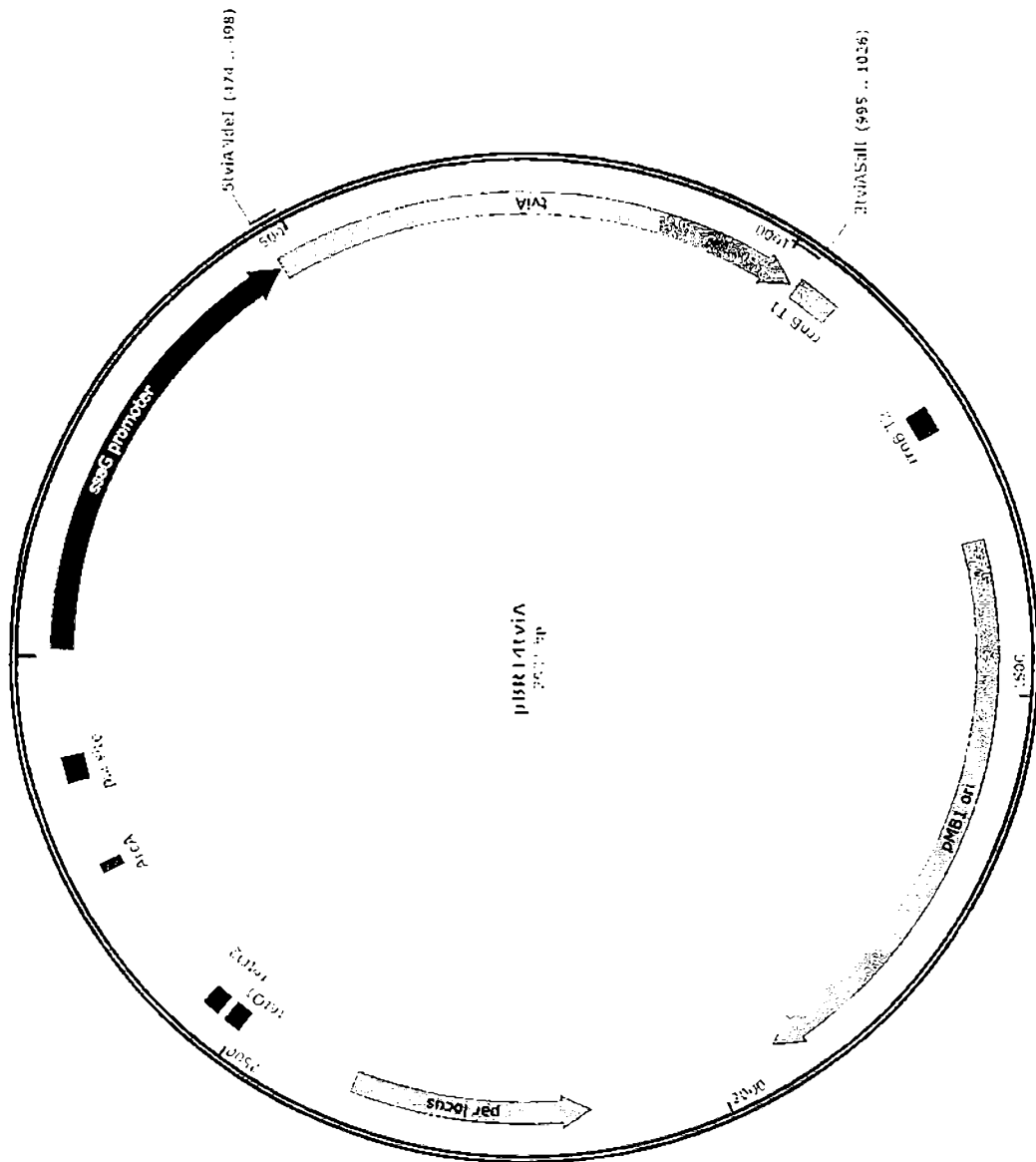


Figure 11

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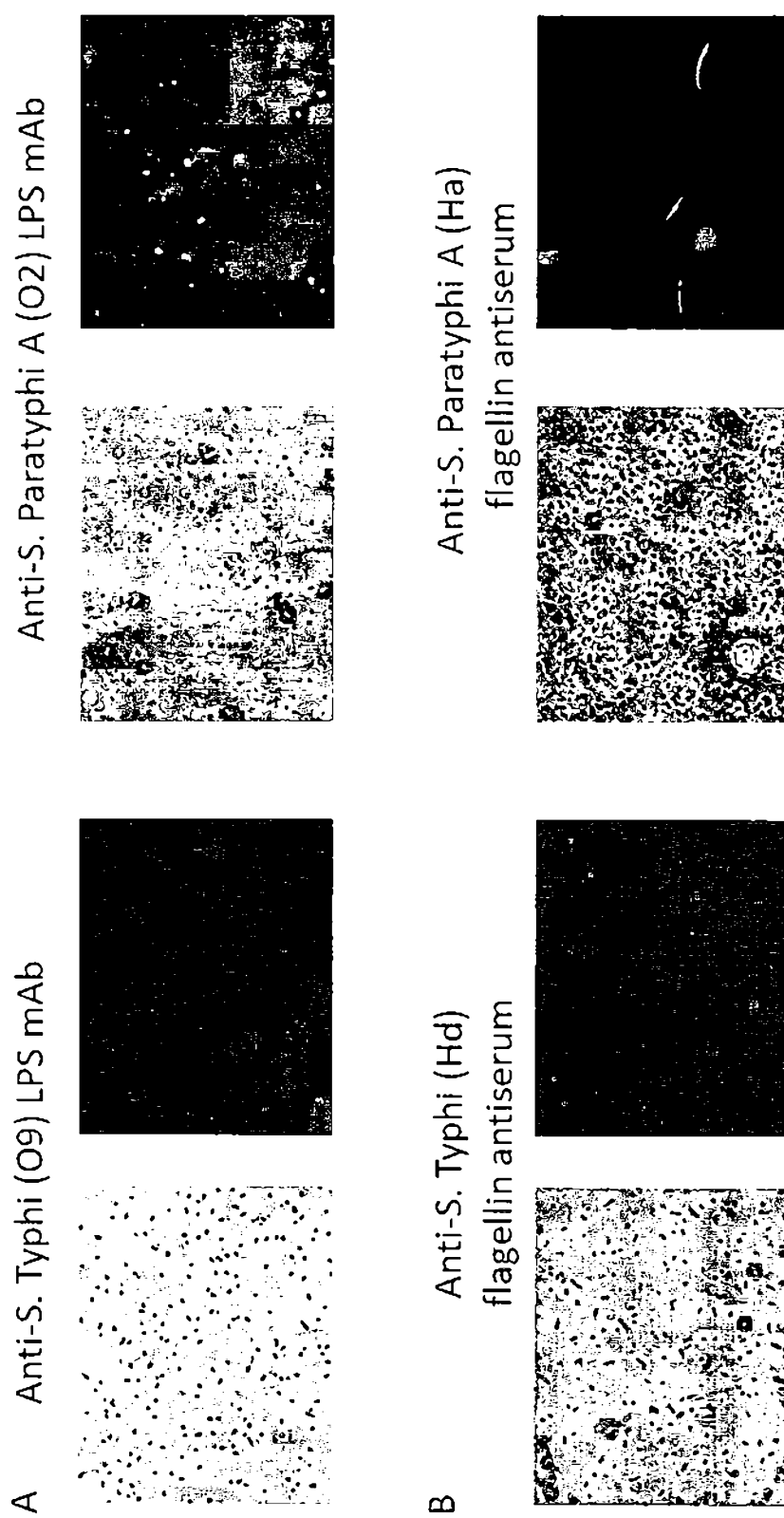


Figure 12

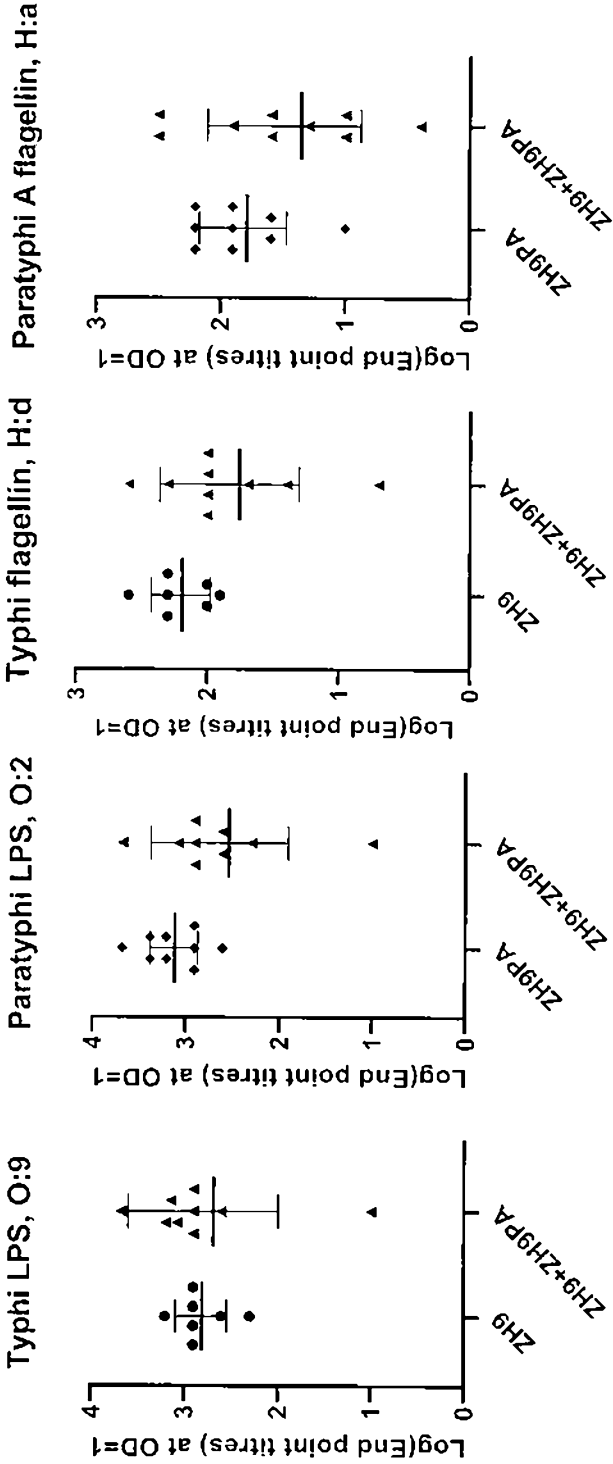


Figure 13