Stable Chromosomal Expression of *Shigella flexneri* 2a and 3a O-Antigens in the Live *Salmonella* Oral Vaccine Vector Ty21a

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**ABSTRACT** We have been exploring the use of the live attenuated *Salmonella enterica* serovar Typhi Ty21a vaccine strain as a versatile oral vaccine vector for the expression and delivery of multiple foreign antigens, including *Shigella* O-antigens. In this study, we separately cloned genes necessary for the biosynthesis of the *Shigella flexneri* serotype 2a and 3a O-antigens, which have been shown to provide broad cross-protection to multiple disease-predominant *S. flexneri* serotypes. The cloned *S. flexneri* 2a *rfb* operon, along with *bgt* and *gtrI*, contained on the SfII bacteriophage, was sufficient in Ty21a to express the heterologous *S. flexneri* 2a O-antigen containing the 3,4 antigenic determinants. Further, this *rfb* operon, along with *gtrA, gtrB*, and *gtrX* contained on the Sfx bacteriophage and *oac* contained on the Sf6 bacteriophage, was sufficient to express *S. flexneri* 3a O-antigen containing the 6, 7, and 8 antigenic determinants. Ty21a, with these plasmid-carried or chromosomally inserted genes, demonstrated simultaneous and stable expression of homologous *S. Typhi* O-antigen plus the heterologous *S. flexneri* O-antigen. Candidate Ty21a vaccine strains expressing heterologous *S. flexneri* 2a or 3a lipopolysaccharide (LPS) elicited significant serum antibody responses against both homologous *S. Typhi* and heterologous *Shigella* LPS and protected mice against virulent *S. flexneri* 2a or 3a challenges. These new *S. flexneri* 2a and 3a O-antigen-expressing Ty21a vaccine strains, together with our previously constructed Ty21a strains expressing *Shigella sonnei* or *Shigella dysenteriae* 1 O-antigens, have the potential to be used together for simultaneous protection against the predominant causes of shigellosis worldwide as well as against typhoid fever.

**KEYWORDS** *Salmonella, Shigella, Ty21a, live vector vaccines*

*Shigella* spp. are facultative intracellular human pathogens that invade the Peyer’s patches of the intestinal mucosa to cause shigellosis. Shigellosis is typically characterized by limited diarrhea, fever, severe abdominal cramps, and frank dysentery, i.e., fever plus small volume fecal discharges containing blood, mucus, and cellular debris. Shigellosis is a major public health problem in resource-poor countries and continues to persist in many developed countries (1). The genus *Shigella* is divided into four species: *S. dysenteriae, S. flexneri, S. boydii*, and *S. sonnei*, also known as *Shigella* subgroups A, B, C, and D, respectively. The first three species are further divided into >45 serotypes on the basis of O-antigenic determinants. The annual shigellosis disease burden continues unabated, at an estimated 165 million cases worldwide, with an estimated 164,300 associated deaths annually (2, 3). The majority of shigellosis (60%) in developing countries is caused by *S. flexneri*. Among *S. flexneri* isolates, serotype 2a is the most predominant in these countries (32 to 58% of infections), followed by...
serotypes 1b (12 to 33%), 3a (4 to 11%), 4a (2 to 5%), and 6 (3 to 5%) (3, 4). Because of predicted serotype cross-protection, Noriega et al. (5) proposed that O-antigen-based vaccines against S. flexneri 2a and 3a could protect against all S. flexneri infections except serotype 6.

Lipopolysaccharide (LPS), a glycolipid found in the outer membrane of all Gram-negative bacteria, is composed of O-antigen linked to core oligosaccharide, which is linked to lipid A in the membrane. A large number of gene products are involved in the biosynthesis of LPS. As in other O-antigen gene clusters, genes involved in the biosynthesis of the S. flexneri O-antigen backbone are located in the chromosomal rfb operon (approximately 10 kb), which is flanked by the gnd and galF genes. There are 14 S. flexneri serotypes, and all of them, with the exception of serotype 6, have a common polysaccharide backbone that consists of repeating units of the tetrasaccharide N-acetylglucosamine-rhamnose-rhamnose-rhamnose (Fig. 1). The basic O-antigen backbone is termed serotype Y (6). In order to synthesize the O-antigen unit, individual sugars are sequentially transferred from their respective dinucleotide precursors to the carrier lipid, undecaprenyl pyrophosphate (UndPP), by specific sugar transferases. After the sugar transfers are completed, the tetrasaccharide O-antigen unit attached to UndPP is flipped to the periplasmic side of the cytoplasmic membrane, a process proposed to be mediated by the RfbX protein. An O-antigen polymerase, Rfc, then transfers the nascent O-antigen unit to a growing O-antigen chain, also anchored to the membrane by UndPP, with chain length being controlled by the Rol protein. The completed polymerized O-antigen chain is transferred to lipid A core by O-antigen ligase, Rfa, and the completed LPS molecule is then transported to the outer membrane (7).

Modification of the O-antigen sugar backbone by the addition of glucosyl and/or O-acetyl groups to different sugars in the tetrasaccharide, in order to give rise to different S. flexneri serotypes, occurs in the periplasm prior to O-antigen transfer to the lipid A core. Since adaptive host immunity to Shigella is largely serotype specific (8), O-antigen modification and antigenic variation enhance bacterial survival and have presumably been acquired and maintained due to selection for serotype variants that escape more general immune responses. In keeping with this view, genes involved in S. flexneri O-antigen modification have in many cases been acquired by horizontal gene transfer and are often carried on chromosomally integrated temperate bacteriophages, such as SfII, SfX, Sf6, and SfV. Although these phages are morphologically diverse, they share many features. In all of these phages, the O-antigen modification genes are found immediately adjacent to the phage attP site, which is proceed by the int and xis genes. The sequence homology of attP and int suggests that the phages SfII, SfX, and SfV integrate into the same position in the pro-lac region, and phage Sf6 integrates into the argW region of the S. flexneri chromosome. Phages SfII, Sf6, SfV, and SfX are responsible for the conversion of serotype Y LPS to serotypes 2a, 3b, 5a, and X, respectively. The S. flexneri serotyping scheme is based on the combination of type- and group-specific antigens, which have been defined both chemically and immunologically. A single O-acetyltransferase-encoding gene (oac) carried by the temperate bacteriophage Sf6 mediates O-acetylation to give rise to serotypes 1b, 3a, 3b, and 4b and confers the group 6 antigenic determinant. The phage SfX encodes the S. flexneri group 7,8 antigenic determinants, which occur in serotypes 3a, 2b, and 5b, that add a D-glucopyranosyl (X) on the first rhamnose of this O-antigen backbone (7). The antigenic determinants 3,4 occur in serotypes Y, 2a, 4a, and 5a, and antigenic determinant 4 occurs in serotype 1a. Further, immunization with an S. flexneri 2a strain expressing the 3 and 4 antigenic determinants was reported to elicit antibodies against S. flexneri serotypes Y, 4a, 5a, and 1a in guinea pigs, monkeys, and humans (9). Most importantly, a study by Noriega et al., (5) has shown that S. flexneri strains expressing serotype 2a and 3a LPS, which bear the S. flexneri individual antigenic determinants 3, 4, 6, 7, and 8, confer protection against all other S. flexneri serotypes, except S. flexneri serotype 6 in guinea pigs. Thus, although Shigella protection has long been considered to be serotype specific based upon limited comparisons between S. sonnei and S. flexneri 2a,
FIG 1 (A) Schematic representation of the different O-antigen chemical compositions of selected *S. flexneri* serotypes, modified from Allison and Verma (7). The common polysaccharide backbone, which represents serotype Y, consists of repeating tetrasaccharide units of N-acetylglucosamine-rhamnose-rhamnose-rhamnose. The addition of either glucosyl or O-acetyl groups to different sugars, within the tetrasaccharide repeat unit, gives rise to different serotypes and antigenic determinant groups. (B) The cloning of *S. flexneri* serotype 2a or 3a O-antigen biosynthetic genes into pMD-TV plasmid. In order to express *S. flexneri* 2a O-antigen, the bacteriophage SfII encoded bgt-gtrII genes were cloned into pMD-TV-Y upstream of the *S. flexneri* 2a rfb region. In order to express *S. flexneri* 3a O-antigen, bacteriophage SF6-carried oac and bacteriophage SFX-carried gtrX, gtra, and gtrB genes, with their cognate promoters, were tandemly cloned into pMD-TV-Y upstream of *S. flexneri* 2a rfb region.
which have totally different O-antigen sugars, there is evidence that considerable immune cross-protection can be afforded between *S. flexneri* serotypes (5, 9).

Currently, no licensed vaccine is available for the prevention of shigellosis. Many health organizations, such as the World Health Organization and International Vaccine Institute, consider the development of a vaccine against shigellosis a priority due to a high disease burden in developing countries (3). It has long been known that protection against *Shigella* in humans is serotype specific and based upon LPS. This thought, plus the existence of a large number of known *Shigella* serotypes, has hindered anti-*Shigella* vaccine development efforts. Although the goal of cross-serotype protection mediated by immunization with conserved *Shigella* protein antigens has some experimental support, epidemiological surveillance and volunteer challenge studies suggest a relatively minor role for those antigens in the protection of human subjects (8). This further highlights the importance of O-antigen epitopes in vaccine development. Although it is impractical to include all known *Shigella* O-antigen epitopes in a vaccine, the Global Enteric Multicenter Study (GEMS) based on serotype prevalence suggested that a quadrivalent vaccine containing O-antigens of *S. sonnei*, *S. flexneri* 2a, *S. flexneri* 3a, and *S. flexneri* 6 could protect against the majority of shigellosis worldwide (10). Furthermore, a vaccine containing *S. flexneri* 2a and 3a O-antigens could protect against the majority of shigellosis episodes due to *S. flexneri*, since such a vaccine could provide broad direct coverage against most common serotypes and indirect coverage against all remaining *S. flexneri* subserotypes, except *S. flexneri* 6 through shared *S. flexneri* group antigens (5, 10).

We and others have explored the possibility of utilizing *Salmonella enterica* serovar Typhi Ty21a, the licensed oral vaccine for typhoid fever, as a vector for the delivery of foreign antigens, including *Shigella* O-antigens (11–14). Ty21a is a live attenuated bacterial strain that provides high-level durable protection against endemic typhoid fever. Additionally, there is evidence that Ty21a confers cross-protection against enteric fevers caused by *Salmonella enterica* serovar Paratyphi B (15) and provides cross-reactive immune responses against *S. enterica* serovar Paratyphi A (16, 17). Previously, we showed that Ty21a expressing *S. sonnei* or *S. dysenteriae* O-antigens provided robust immunity against virulent *Shigella* challenge in mice (13, 18). In this study, we cloned genomic regions directing the expression of *S. flexneri* 2a or 3a O-antigens, integrated these genes into a targeted site in the Ty21a chromosome, and showed both immunogenicity and protection against virulent challenge in a mouse model of infection.

**RESULTS**

**Analysis of *S. flexneri* 2a and 3a O-antigen expression in Ty21a from recombinant plasmids.** Like many O-antigen biosynthetic gene clusters in *Enterobacteriaceae*, the *S. flexneri* 2a *rfb* operon is flanked by the *galf* and *gnd* genes. Previous studies have shown that this locus can be expressed in a heterologous organism (*E. coli*) when cloned in a cosmid or other plasmid vector. However, in those studies, the minimum genetic region needed for O-antigen backbone biosynthesis was not determined (19, 20). In the current study, we cloned a chromosomal segment including the genes *rfbB*, *rfbD*, *rfbA*, *rfbC*, *rfbE*, *rfbF*, *rfbG*, *rfc*, *rfbl*, *rfbJ*, open reading frame (ORF) S2215, and ORF S2216, along with the region upstream of *rfbB* that includes the cognate *rfbB* promoter, into the multicopy-number plasmid pMD-TV to create pMD-TV-Y (Fig. 1B). Sequence analysis of this plasmid revealed an additional A residue in the intergenic region upstream of *rfbC*, compared to the published *S. flexneri* 2457T genomic sequence (AE14073.1). This change did not eliminate the expression of *S. flexneri* 2a O-antigen expression in either *E. coli* or Ty21a, as demonstrated by slide agglutination (data not shown) and Western blot analysis with anti-*S. flexneri* 2a antibody (Fig. 2).

As shown schematically in Fig. 1A, glycosylation of rhamnose III of the Y serotype O-antigenic repeat unit gives rise to serotype 2a. Two genes on the SflI serotype-converting bacteriophage, *bgt*, which encodes a bactoprenol glucosyl transferase, and *gtrII*, which encodes the type II antigen determining glucosyl transferase, were found to be necessary for 2a serotype conversion by generating the 3,4 group antigens. Up-
stream of bgt-gtrII are two other open reading frames (ORFs), the integrase gene (int) and another ORF that encodes a highly hydrophobic protein; both are transcribed in the same direction as bgt-gtrII. However, no obvious \( \sigma^{\text{54}} \) or \( \sigma^{\text{70}} \) promoter was discernible upstream of these genes (21). Therefore, in our construct, we provided an exogenous promoter, that of the gene encoding the type III-modifying enzyme O-acetyltransferase (oac), from the temperate bacteriophage Sf6, to promote transcription of bgt and gtrII in our construct. In this way, genes bgt, gtrII, and the rfb operon were cloned in tandem into pMD-TV to construct pMD-TV-2a. Expression of the S. flexneri 2a O-antigen from pMD-TV-2a in E. coli and Ty21a was detected by slide agglutination with S. flexneri type II and 3,4 epitope-specific antisera (data not shown). Serotype 2a O-antigen expression was further confirmed by Western blotting with S. flexneri type II-specific antiserum (Fig. 2A). The type II-specific antiserum recognized LPS extracted from S. flexneri 2a 2457T or from E. coli or Ty21a harboring pMD-TV-2a. These data suggest that pMD-TV-2a contains all genes required for stable S. flexneri 2a O-antigen expression. LPS from the negative-control strains E. coli, Ty21a(pMD-TV), S. flexneri 3a, E. coli(pMD-TV-3a), or Ty21a(pMD-TV-3a) did not react with S. flexneri type II-specific antiserum (Fig. 2A). Additionally, LPS from E. coli(pMD-TV-2a) or Ty21a(pMD-TV-2a) did not react with S. flexneri type III-specific antiserum (Fig. 2B). These results demonstrate that S. flexneri type II-specific antiserum binding to O-antigen expressed from pMD-TV-2a in E. coli or Ty21a is highly specific.

The oac gene carried by the temperate S. flexneri bacteriophage Sf6 encodes the O-acetyltransferase that mediates the transfer of an acetyl group onto the rhamnose III residue of the O-antigen backbone by an as-yet-uncharacterized mechanism (22, 23). This acetyl transfer results in the appearance of the group antigen 6 and conversion of the Y serotype to X. The oac gene and the region between int and oac that includes the oac native promoter were cloned upstream of the rfb operon in pMD-TV-Y to construct pMD-TV-3b. The expression of group antigen 6 was determined by slide agglutination with S. flexneri group antigen 6 epitope-specific antiserum (data not shown).

The genes gtrA, gtrB, and gtrX carried on another temperate bacteriophage, Sfx, have been found to be responsible for full O-antigen conversion of serotype Y to X by glycosylation of rhamnose I of the O-antigen backbone. The first gene in the cluster, gtrA, encodes a small highly hydrophobic protein that mediates the translocation of lipid-linked glucose across the cytoplasmic membrane. The second gene in the cluster,
gtrB, catalyzes the transfer of the glucose residue from UDP-glucose to a lipid carrier. The third gene, gtrX, encodes a glucosyltransferase that catalyzes the transfer of glucosyl moieties onto a specific sugar residue of the O-antigen repeating unit. GtrX in the absence of GtrA and GtrB is only able to mediate a partial conversion of serotype X to serotype Y to serotype X (24). Therefore, in order to completely glycosylate rhamnose I of the O-antigen backbone and convert 3b to 3a, the entire gtrA-gtrB-gtrX gene cluster, along with the intergenic region between int and gtrA that includes the gtrA promoter, was cloned immediately upstream of the S. flexneri 2a rfb operon and downstream of oac on pMD-TV-3b to construct pMD-TV-3a. The expression of group 7,8 antigen in E. coli or Ty21a by pMD-TV-3a was determined by slide agglutination with S. flexneri group 7,8 epitope-specific antiserum (data not shown). Expression of the 3a O-antigen, which includes group antigens 6, 7, and 8, was further confirmed by Western blotting with S. flexneri type III-specific antiserum. The type III-specific antiserum reacted with LPS from S. flexneri 3a J99, E. coli(pMD-TV-3a), and Ty21a(pMD-TV-3a). However, S. flexneri type III-specific antiserum did not react with negative-control strains S. flexneri 2a, E. coli, E. coli(pMD-TV-2a), Ty21a(pMD-TV), or Ty21a(pMD-TV-2a) (Fig. 2B). Furthermore, LPS extracted from E. coli or Ty21a harboring pMD-TV-3a did not react with S. flexneri type II-specific antiserum (Fig. 2A). Thus, these results demonstrate that the S. flexneri type III-specific antiserum binding to O-antigen expressed by pMD-TV-3a in E. coli or Ty21a is highly specific. Sequencing of all plasmids listed in Table 1 detected no additional mutations, only the single-nucleotide polymorphism (SNP) in the rfb operon noted above.

**Stable integration of S. flexneri 2a and 3a O-antigen genes into the Ty21a chromosome.** The S. flexneri O-antigen Y serotype backbone genes in pMD-TV-Y were PCR amplified with tviD forward (pMD133.tviDF) and vexA reverse (pMD87.vexA.R.32bp) primers to produce a 13,653-bp fragment encompassing the entire operon together with flanking homologous sequences. This fragment was introduced by transformation into electrocompetent Ty21a cells expressing λ Red proteins from pKD46. Recombinants in which the entire construct was integrated into the Ty21a chromosome were selected on media containing kanamycin, as described in reference 13. Integrants were

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**TABLE 1 Bacterial strains and plasmids**

| Strain or plasmid | Genotype or description | Reference/source |
|-------------------|-------------------------|------------------|
| **Strains**        |                         |                  |
| E. coli DH5α      | supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1 | NEB              |
| S. enterica serovar Typhi Ty21a | galE ivd viaB (Vi⁻) H₂S⁻ | 26               |
| S. flexneri 2a 2457 |                         | Lab stock        |
| S. flexneri 3a J99 |                         | Lab stock        |
| Ty21a-Y (MD114)   | S. flexneri rfb operon integrated into tviD-vexA on the chromosome, expressed from native promoter; Kan⁺ Amp⁺ Cm⁺ | This study       |
| Ty21a-2a (MD194)  | S. flexneri 2a O-antigen genes integrated into tviD-vexA on the chromosome, expressed from native promoter; Kan⁺ Amp⁺ Cm⁺ | This study       |
| Ty21a-3a (MD196)  | S. flexneri 3a O-antigen genes integrated into tviD-vexA on the chromosome, expressed from native promoter; Kan⁺ Amp⁺ Cm⁺ | This study       |
| Ty21a-2al-Kan (MD212) | S. flexneri 2a O-antigen genes integrated into tviD-vexA on the chromosome, expressed from lpp promoter; Kan⁺ Amp⁺ Cm⁺ | This study       |
| Ty21a-2al (MD268) | S. flexneri 2a O-antigen genes integrated into tviD-vexA on the chromosome, expressed from lpp promoter; Kan⁺ Amp⁺ Cm⁺ | This study       |
| Ty21a-2aol (MD270) | S. flexneri 2a O-antigen genes integrated into tviD-vexA on the chromosome, all genes in an operon, expressed from lpp promoter; Kan⁺ Amp⁺ Cm⁺ | This study       |
| Ty21a-Sdl-kan (MD166) | S. dysenteriae O-antigen genes integrated into tviD-vexA on the chromosome, expressed from lpp promoter; Kan⁺ Amp⁺ Cm⁺ | 18              |
| **Plasmids**       |                         |                  |
| pMD-TV            | pSC101 derivative, multicopy plasmid, Kan⁺ flanked by FRT, Ty21a tviD and vexA genes | 13              |
| pMD-TV-Y          | S. flexneri 2a rfb operon cloned into pMD-TV | This study       |
| pMD-TV-2a         | S. flexneri 2a O-antigen genes cloned into pMD-TV | This study       |
| pMD-TV-3b         | S. flexneri 3b O-antigen genes cloned into pMD-TV | This study       |
| pMD-TV-3a         | S. flexneri 3a O-antigen genes cloned into pMD-TV | This study       |

*Kan⁺, kanamycin sensitive; Amp⁺, ampicillin sensitive; Cm⁺, chloramphenicol sensitive.*
verified by PCR with primers prMD92 and prMD124 having targets upstream and downstream of the site of integration, respectively, producing an approximately 14.5-kb band. After removing the kanamycin resistance (Kanr) cassette by site-specific recombination between the FLP recombination target (FRT) sites, as described in reference 13, antibiotic-sensitive chromosomal integrants were further analyzed by PCR for deletion of the Kan cassette and by sequencing of the resulting PCR product (strain MD114/Ty21a-Y, ~13-kb band) obtained using primers prMD92 and prMD124. Sequencing of this PCR product detected no sequence changes relative to pMD-TV-Y.

In order to integrate the genes encoding \textit{S. flexneri} 2a O-antigen-modifying enzymes, \textit{bgt} and \textit{gtrII}, upstream of the \textit{rfb} operon in Ty21a-Y, a 5,132-bp PCR product that contains part of the \textit{tviD} gene, the Kan cassette flanked by FRT sites, the genes encoding \textit{S. flexneri} 2a-modifying enzymes, \textit{bgt} and \textit{gtrII}, and the first ~500 bp of the \textit{rfb} operon were used. After integrating the serotype 2a-modifying enzymes upstream and downstream of the site of integration, prMD92 and prMD124, resulted in an ~17-kb band. After removing the Kanr cassette, antibiotic-sensitive chromosomal integrants were further analyzed by PCR for deletion of the Kan cassette by sequencing the PCR product (strain MD194 [Ty21a-2a], ~15.5-kb band) obtained from primers prMD92 and prMD124. The sequence of this PCR product was compared to the \textit{bgt-gtrII} region carried by SfII and the \textit{rfb} region of \textit{S. flexneri} 2a 2457, and no changes were found. Strains harboring chromosomally inserted \textit{S. flexneri} 2a LPS genes, as examined by Western blotting with \textit{S. flexneri} type II-specific antiserum, demonstrated only weak...
expression compared to strains harboring the corresponding plasmid clones (Fig. 4). It is likely that multiple copies of the plasmid (~10 per cell) provide for more \textit{S. flexneri} 2a O-antigen than a single copy of \textit{S. flexneri} 2a O-antigen biosynthetic genes integrated into the Ty21a chromosome.

**FIG 4** Analyses of SDS-PAGE-separated polysaccharide isolated from various Ty21a isolates by Western immunoblotting with anti-\textit{S. flexneri} 2a and anti-\textit{S. Typhi} O-specific 9 factor antibodies (A) and with anti-\textit{S. flexneri} 3a and anti-\textit{S. Typhi} O-9 antibodies (B). (A) Vaccine strain Ty21a-2a, in which \textit{S. flexneri} 2a O-antigen biosynthetic genes were integrated into the Ty21a chromosome, shows a very low level of serotype 2a O-antigen expression compared to that of plasmid p2a. Replacing the \textit{oac} promoter upstream of the O-antigen-modifying enzymes with the highly transcribed \textit{lpp} promoter increased the serotype 2a O-antigen expression in strain Ty21a-2al. Additionally, removing the intergenic region between \textit{gtrII} and \textit{rfbB} and placing all \textit{S. flexneri} 2a O-antigen biosynthetic genes under the control of an upstream \textit{lpp} promoter in strain Ty21a-2aol increased \textit{S. flexneri} 2a O-antigen expression. All candidate vaccine strains expressed \textit{S. Typhi} O-antigen, determined by anti-\textit{S. Typhi} O-specific 9 factor antibody. (B) Chromosomally inserted \textit{S. flexneri} 3a LPS expression, determined with \textit{S. flexneri} type III-specific antisera, was reduced compared to the plasmid recombinant. These vaccine strains also express \textit{S. Typhi} O-antigen, determined by anti-\textit{S. Typhi} O-specific 9 factor antibody.
A 6,448-bp PCR product that contains part of the \textit{tviD} gene, the Kan cassette flanked by FRT sites, the \textit{S. flexneri} 3a O-antigen-modifying genes (the \textit{gtrX}, \textit{gtrA}, and \textit{gtrB} gene cluster, along with \textit{oac}), and the first 500 bp of the \textit{rfb} operon was transformed into Ty21a-Y competent cells expressing \textit{Red} to construct Ty21a-3a. After integration, PCR with primers upstream and downstream of the site of integration, prMD92 and prMD124, resulted in an 18.5-kb band and after removing the Kanr cassette (strain MD194 [Ty21a-3a]) resulted in an 17-kb band. The sequence of this PCR product was compared to those of bacteriophage Sf6 \textit{oac} (X59553), bacteriophage SfX \textit{gtrX}, \textit{gtrA}, and \textit{gtrB} (AF056939), and \textit{S. flexneri} 2a 2457, and no changes were found. As with \textit{S. flexneri} 2a LPS expression, \textit{S. flexneri} 3a LPS expression in this strain, as determined by Western blotting with \textit{S. flexneri} type III-specific antiserum, was reduced compared to that from plasmid-bearing strains (Fig. 4B). All of the above-mentioned Ty21a derivative vaccine strains express \textit{S. Typhi} O-antigen, determined by anti-\textit{S. Typhi} O-specific 9 factor antibody.

### Enhancing \textit{S. flexneri} 2a O-antigen expression in Ty21a

Since \textit{S. flexneri} 2a O-antigen expression from a single chromosomal copy was reduced compared to plasmid-mediated expression, we sought to increase this LPS expression by replacing the native \textit{bgt} promoter with the \textit{lpp} promoter, a highly transcribed constitutively active promoter. Ty21a-sdl (in which the \textit{S. dysenteriae} serotype 1 O-antigen biosynthesis genes are expressed from the \textit{lpp} promoter) (18) was used as the template for PCR, utilizing a \textit{tviD} forward primer and an \textit{lpp} promoter reverse primer with a 150-bp \textit{bgt} homology extension (Table 2). The PCR-amplified region comprised part of the \textit{tviD} gene, the \textit{S. flexneri} 3a O-antigen cassette followed by FRT sites, and the 200-bp insert containing the \textit{lpp} promoter linked to the first 150 bp of the \textit{bgt} gene. This PCR product was transformed into Ty21a-2a (MD194) competent cells expressing \textit{Red} proteins and selected for Kanr, followed by removal of the Kan cassette, to construct Ty21a-2aL (MD268), which was verified by sequence analysis. Replacing the native upstream promoter with \textit{lpp} promoter resulted in enhanced \textit{S. flexneri} 2a O-antigen expression; however, heterologous O-antigen expression was still notably less than that engendered by the Ty21a p2a plasmid, as determined by Western blotting with \textit{S. flexneri} type I-specific antiserum (Fig. 4). Therefore, we attempted to further enhance heterologous \textit{S. flexneri} 2a

**TABLE 2 Primers used in this study**

| Primer name | Sequence 5’ to 3’a | bgt homology extension is in bold. |
|-------------|---------------------|-----------------------------------|
| prMD3-2A.RFB.F | GATCGGATCTCAATGAAAAATCTGACCGGAGTTAACGGTTG   |                        |
| prMD16.5042.R | GATCACTTCGAGGAAATATCTACGGAG   |                        |
| prMD18.rfb.5021.F | CCCCTCGCTAGGATTTCTGGCTGGAG   |                        |
| prMD4-2A.RFB.R | GATCGGGTACCTTGTTTTCTGAGCAATATATATAAG   |                        |
| prMD1-2AGTR-F | GATCGGCGGCCGAGCCTAAATGAGAAGATGCTATTTTTCTCGGAAAATAGAAAAATATCTCCTTGTCTGTCGTCTC   |                        |
| prMD47.3A.OAC.NOTI | ATAGAAGATGGCAAGCATGAAATAACAGATAC   |                        |
| prMD30.3A.BAMHI.R | GATCAAGATTCAAGGAAATATAGCTTCTAG   |                        |
| prMD120.SFX.BAMHI.F | GATCGGATCTGACTGAGATGCTATGCTCTTCTCGGAGGAGCAAGAGACGGAGAGACGAGAGAGATTTATATGTATTCCTTTCTTTCTT   |                        |
| prMD121.SFX.BAMHI.R | GATCGGATCTGACTGAGATGCTATGCTCTTCTCGGAGGAGCAAGAGACGGAGAGACGAGAGAGATTTATATGTATTCCTTTCTT   |                        |
| prMD87.VEK.328P | TTAGAAGAATATGTCGGCCGCGTCAAAAGACG   |                        |
| prMD133.TVID.F | CCGTCCTCTATGGATATGTGCTAC   |                        |
| prMD123.Y.R | CGGCACAATAAGTATTTGCTCACG   |                        |
| prMD124.F.TVID | CAGCAATATTTTCAATGATGGCAGAC   |                        |
| prMD92.VEX.B.17800 | GATCGGATCTGACTGAGATGCTATGCTCTTCTCGGAGGAGCAAGAGACGGAGAGACGAGAGAGATTTATATGTATTCCTTTCTT   |                        |
| prMD152.2ALPP.R | GATCGGATCTGACTGAGATGCTATGCTCTTCTCGGAGGAGCAAGAGACGGAGAGACGAGAGAGATTTATATGTATTCCTTTCTT   |                        |
| prMD178.2aO.R | GATCGGATCTGACTGAGATGCTATGCTCTTCTCGGAGGAGCAAGAGACGGAGAGACGAGAGAGATTTATATGTATTCCTTTCTT   |                        |
| **Restriction sites are underlined.** | **Promoter of 3a O-antigen-modifying gene oac is in bold.** | **bgt homology extension is in bold.** |
| **cbgt homology extension is in bold.** | **drfbB homology extension is in bold.** | **d**

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O-antigen expression. Although transcriptional/translational terminator sequences were not found in the region of the native rfb promoter downstream of gtrII (7), this intergenic region was removed, so that genes encoding S. flexneri 2a O-antigen-modifying enzymes and the rfb genes were all transcribed from the lpp promoter. Removal of the intergenic region (Ty21a-2aol) resulted in marginally enhanced S. flexneri 2a O-antigen expression (Fig. 4A). Our efforts to enhance S. flexneri 3a O-antigen expression by replacing the native upstream promoter with lpp promoter were unsuccessful.

**Simultaneous expression of heterologous and homologous LPSs in Ty21a verified by confocal microscopy.** Dual fluorescence staining of recombinant Ty21a combination vaccine strains with specific antibodies against heterologous or homologous LPS antigens provided direct evidence that the hybrid Ty21a vaccine strains [i.e., Ty21a-2a, Ty21a-2al, Ty21a-2aol, Ty21a(p2a), Ty21a-3a, and Ty21a(p3a)] simultaneously express S. flexneri 2a or 3a LPS in addition to homologous S. Typhi LPS, shown in green and red, respectively, in Fig. 5. Furthermore, both LPS types appear to be distributed over the cell surface. Although the amount of O-antigen cannot be quantitatively assessed by this method, the plasmid constructs Ty21a(p2a) and Ty21a(p3a), which express more S. flexneri 2a/3a O-antigen than chromosomal constructs, as assessed by Western blotting, generally appeared to have greater staining intensity for the heterologous antigens than the chromosomal constructs. Control strains, such as Ty21a alone or specific S. flexneri strains, stained only red or green, as expected. Under the conditions employed, Ty21a cells typically appeared as elongated rods, whereas S. flexneri appeared to be more coccobacillary in form (Fig. 5).

**Stable heterologous O-antigen expression in Ty21a.** The new vaccine strains Ty21a-2a, Ty21a-2al, Ty21a-2aol, and Ty21a-3a, which are antibiotic sensitive and contain chromosomally integrated heterologous O-antigen genes, were tested for stability of S. flexneri 2a/3a O-antigen expression after ~75 generations of growth by colony immunoblotting with O-antigen-specific antibodies (see Materials and Methods). All of the 300 colonies tested for each strain retained S. flexneri 2a/3a O-antigen expression, demonstrating 100% stability of the chromosomally integrated genes (data not shown).

**Growth and phenotypic characteristics.** Limited growth and phenotypic characteristics of Ty21a-2a, Ty21a-2al, Ty21a-2aol, and Ty21a-3a were studied to verify equivalence with Ty21a. API 20E (bioMérieux, Inc.) biochemical assays, carried out according to the manufacturer’s instructions, showed that Ty21a and all S. flexneri LPS-expressing derivatives retained the same biochemical characteristics as Ty21a (data not shown). Furthermore, these strains showed essentially identical growth curves in tryptic soy broth (TSB) or casein-yeast extract (CY) medium, with all strains reaching equivalent maximal density (see Fig. S1 in the supplemental material).

**Antibodies elicited following mouse immunization with Ty21a expressing heterologous S. flexneri 2a or 3a O-antigens.** Mice were immunized intraperitoneally with the engineered vaccine strains Ty21a-2a, Ty21a-2al, Ty21a-2aol, and Ty21a-3a, the parent strain Ty21a, or control phosphate-buffered saline (PBS). Mice were bled 2 weeks after the last injection, and the serum antibodies elicited against S. flexneri 2a, S. flexneri 3a, or Ty21a LPS were determined by enzyme-linked immunosorbent assay (ELISA). As shown in Fig. 6A, immunization with recombinant S. flexneri 2a strains Ty21a-2a, Ty21a-2al, Ty21a-2aol, and Ty21a(p2a) resulted in significantly higher anti-LPS antibody titers against S. flexneri 2a LPS than the Ty21a parent strain. Both Ty21a-2a and Ty21a-2al elicited modest antibody mean endpoint titers of 13,760. Ty21a-2aol, with enhanced heterologous LPS, expressed a significantly higher mean antibody titer (58,880; P = 0.0088) than Ty21a-2al. The strain Ty21a(p2a), which expresses more S. flexneri 2a LPS than any of the chromosomally integrated strains, elicited the highest antibody titer of 102,400 (Fig. 6A). These immunogenicity studies demonstrated simultaneous immune responses against both heterologous S. flexneri 2a O-antigen and homologous Ty21a LPS, generated by Ty21a-2a, Ty21a-2al, Ty21a-2aol, and Ty21a(p2a).
Moreover, titers against Ty21a LPS elicited by the recombinant vaccine strains Ty21a-2a, Ty21a-2al, Ty21a-2aol, and Ty21a(p2a) were similar to those elicited by the Ty21a parent (Fig. 6E).

Similarly, mice were immunized with the *S. flexneri* 3a vaccine strain Ty21a-3a or Ty21a(p3a), the parent strain Ty21a, or control PBS. Mice were bled 2 weeks after the last injection, and serum antibodies elicited against either *S. flexneri* 3a or Ty21a LPSs...
were determined by ELISA. Both the chromosomal integrant Ty21a-3a and the plasmid-bearing Ty21a(p3a) elicited modest antibody responses, approaching endpoint titers of ∼12,000 (Fig. 6B). Equivalent to the *S. flexneri* 2a O-antigen-expressing vaccine stains, the anti-Ty21a LPS titers elicited by the *S. flexneri* 3a O-antigen-expressing recombinant strains were comparable to those elicited by the parent strain Ty21a (Fig. 6F). It has been reported previously that anti-*S. Typhi* antibodies, produced by immunizing mice with Ty21a alone or a prototype Ty21a expressing both homologous *S. Typhi* and heterologous *Shigella* O-antigens, protect against virulent *S. Typhi* Ty2 challenge in the mouse intraperitoneal (i.p.) challenge model (25).

*S. flexneri* 2a and 3a O-antigens share a polysaccharide backbone comprising repeating units of the tetrasaccharide N-acetylglucosamine-rhamnose-rhamnose-rhamnose. Despite this O-antigen backbone sugar similarity, serum antibodies elicited by the *S. flexneri* 2a O-antigen-expressing vaccine strains Ty21a-2a, Ty21a-2al, Ty21a-2aol, and Ty21a(p2a)
FIG 6 Mouse serum IgG responses, measured by ELISA, to *S. flexneri* 2a LPS (A and D), *S. flexneri* 3a LPS (B and C), and Ty21a LPS (C and D) after intraperitoneal immunization of mice with PBS, Ty21a, or candidate vaccine strains simultaneously expressing both Ty21a LPS and *S. flexneri* 2a or 3a LPS. Error bars represent the means ± SEM of the results, with 10 mice per group. (A) Immunization with *S. flexneri* 2a O-antigen-expressing recombinant vaccine strains Ty21a-2a, Ty21a-2al, Ty21a-2aol, and Ty21a(p2a) resulted in increased anti-LPS antibody titers against *S. flexneri* 2a LPS. Both Ty21a-2a and Ty21a-2al elicited a modest antibody response of approximately 15,000. Ty21a-2aol elicited a significantly higher antibody titer of 58,880 (*P* = 0.0088) than Ty21a-2al, and the plasmid-containing strain Ty21a(p2a) elicited the highest mean antibody titer of 99,200. (B) Immunization with *S. flexneri* 3a LPS-expressing recombinant vaccine strains, chromosomal integrant Ty21a-3a, or plasmid-expressing strain Ty21a(p3a) elicited modest antibody titers of 7,335 and 12,292, respectively, compared to Ty21a. (C) Antibodies elicited by *S. flexneri* 2a O-antigen-expressing vaccine strains [Ty21a-2a, Ty21a-2al, Ty21a-2aol, and Ty21a(p2a)] did not show (Continued on next page)
did not cross-react with *S. flexneri* 3a LPS. Serum antibodies elicited by *S. flexneri* 3a O-antigen-expressing vaccine strains Ty21a-3a and Ty21a(p3a) did not cross-react with *S. flexneri* 2a LPS (Fig. 6C and D). These results support the conclusion that key serotype-specific epitopes of these O-antigen subunits, due to the serotype-specific modifications of their shared backbone structure, are responsible for major immune protection.

**Protection conferred by recombinant vaccine strains against lethal challenge.** The mouse i.p. challenge model has previously been used for preliminary screening of Ty21a and Ty21a bifunctional vaccine strains expressing *Shigella* LPS. Although this is not a natural disease model for typhoid fever or shigellosis, it can be employed to assess immunogenicity following vaccination and to measure protection against endotoxin-mediated death following virulent i.p. challenge (11–13, 25, 26). As described in Materials and methods, BALB/c mice were immunized with vaccine strains Ty21a-2a, Ty21a-2al, Ty21a-2aol, and Ty21a(p2a), Ty21a alone, or PBS by the i.p. route and then i.p. challenged 4 weeks following the last dose with virulent *S. flexneri* 2a 2457T at a dose of approximately $5 \times 10^5$ CFU. This challenge resulted in 100% mortality in PBS-immunized and Ty21a-immunized mice. However, immunization with Ty21a-2a, Ty21a-2al, Ty21a-2aol, and Ty21a(p2a) resulted in 90 to 100% protection. (B) Mice immunized with 3 vaccine doses of *S. flexneri* 3a O-antigen-expressing recombinant vaccine strains Ty21a-3a and Ty21a(p3a) (n = 10 mice/group) were challenged i.p. with virulent *S. flexneri* 3a at a dose of $5 \times 10^5$ CFU. This challenge resulted in 90 to 100% mortality in saline-immunized mice and mice immunized with Ty21a alone. However, immunization with Ty21a-3a and Ty21a(p3a) resulted in 100% protection.

**FIG 7** Mouse protection against virulent *S. flexneri* 2a (A) and 3a (B) challenges. Mice immunized with 3 vaccine doses of with *S. flexneri* 2a O-antigen-expressing recombinant vaccine strain Ty21a-2a, Ty21a-2al, Ty21a-2aol, or Ty21a(p2a) (n = 10 mice/group) were challenged i.p. with virulent *S. flexneri* 2a strain 2457T at a dose of approximately $5 \times 10^5$ CFU. This challenge resulted in 100% mortality in PBS-immunized and Ty21a-immunized mice. However, immunization with Ty21a-2a, Ty21a-2al, Ty21a-2aol, and Ty21a(p2a) resulted in 90 to 100% protection. (B) Mice immunized with 3 vaccine doses of *S. flexneri* 3a O-antigen-expressing recombinant vaccine strains Ty21a-3a and Ty21a(p3a) (n = 10 mice/group) were challenged i.p. with virulent *S. flexneri* 3a at a dose of $5 \times 10^5$ CFU. This challenge resulted in 90 to 100% mortality in saline-immunized mice and mice immunized with Ty21a alone. However, immunization with Ty21a-3a and Ty21a(p3a) resulted in 100% protection.

**DISCUSSION**

We report here the construction and initial characterization of two hybrid bacterial strains with the potential to serve as mixed live vaccine components to provide...
simultaneous protection against both typhoid fever and shigellosis caused by the predominant strains of *Shigella flexneri*. Using the licensed *Salmonella* Typhi Ty21a live vaccine strain as a platform, we have cloned, chromosomally integrated, and maximized expression of the essential *S. flexneri* genes required for expression of serotype 2a or 3a O-antigens. Expression of these heterologous antigens was 100% stable over 75 generations of broth growth, and the candidate strains retained the biochemical and growth properties of the original Ty21a parent strain (Fig. 7).

Together, *S. flexneri* serotype 2a and 3a LPSs bear the immunodominant antigenic group factors found in all *S. flexneri* strains except serotype 6. The vaccine candidates elicited high antibody titers in mice against each specific serotype LPS. Interestingly, no cross-reactivity was observed between serotypes 2a and 3a. These results suggest that the O-antigen sugar backbone modifications comprise the immunodominant epitopes, and these O-antigen modifications may block the antigenicity of the tetrasaccharide backbone. Note that Van De Verg et al. (9) reported that vaccination of humans or guinea pigs with a hybrid *E. coli* K-12-*S. flexneri* 2a oral vaccine resulted in only a very low level of cross-reactivity with *S. flexneri* 3a LPS. The fact that we did not detect any cross-reactivity between mouse antibodies elicited by Ty21a-*S. flexneri* 2a or 3a vaccine candidates could be due to the high level of stringency in our ELISA. Alternatively, a high level of expression of the polysaccharide backbone-modifying enzymes in our vaccine strains may lead to more complete O-antigen modification, thereby more completely eliminating cross-reactive epitopes.

The mice immunized with hybrid Ty21a vaccine strains expressing either *S. flexneri* 2a or 3a O-antigen showed solid protection against challenge with a lethal dose of virulent *S. flexneri* 2a or 3a, respectively. As reported in previously published work, we have already constructed Ty21a candidate vaccine strains expressing *S. sonnei* and *S. dysenteriae* serotype 1 O-antigens and have demonstrated immunogenicity and protection against challenge with these organisms in mice. Now, together with the new *S. flexneri* 2a O-antigen-expressing and 3a O-antigen-expressing candidate vaccine strains, we have completed construction and initial preclinical studies on four potential components of a multivalent oral vaccine that has the potential to provide protection against ~90% of shigellosis worldwide (4, 10), as well as against typhoid fever (with some cross-protection against paratyphoid strains).

Bacillary dysentery and enteric fevers continue to be major causes of morbidity and mortality in developing countries due to poor sanitation and hygienic conditions, as well as malnutrition. Since improving sanitary infrastructure is a distant goal for many of these countries, effective vaccination could significantly reduce both disease burden and mortality. In addition, the increasing incidence of multiply antibiotic-resistant enteric bacteria makes antibiotic therapy in resource-poor areas even more difficult. A bifunctional Ty21a-*Shigella* vaccine would offer many advantages. In resource-poor countries, the cost-benefit ratio is the most predominant deciding factor for mass immunizations. In addition, a bifunctional Ty21a-*Shigella* vaccine could simultaneously protect against two major causes of enteric disease morbidity and mortality in developing countries, thereby enhancing cost-effectiveness. Since such a vaccine (after licensure) would be administered orally, it could be self-administered, thus reducing the need for and cost of skilled health care professionals. Also, there would be no requirement for sterile needles and syringes as needed to deliver parenteral vaccines (27). The technique of foam-drying live vaccine candidates, such as Ty21a, greatly enhances their temperature stability, allowing them to survive for extended periods without refrigeration (28). Such an approach could further facilitate the deployment of Ty21a-vectored vaccines in less-developed regions and in emergency mass vaccination campaigns. Additionally, needle-free administration would increase acceptability (and thus compliance) and enhance the occupational safety of vaccinators and other health care providers (29).

The majority of shigellosis cases (~70%), and of deaths (~60%), occur among children less than 5 years of age (3). While the current capsular formulation of Ty21a is licensed only for adults and children over the age of 6, the Ty21a liquid formulation was
recently tested in children age 2 to 5 (30). Our data provide an incentive for further exploration of additional formulations, including those for bifunctional shigellosis-typhoid vaccines that would be safe and effective in younger children and protect against both diseases.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids utilized herein are described in Table 1. *E. coli* strains were grown at 37°C in Luria-Bertani (LB) broth or on LB agar from Difco. *Shigella* strains were grown in tryptic soy broth (TSB) or tryptic soy agar ( TSA) from Difco. *Salmonella* strain Ty21a or derivatives were grown in TSB or on TSA. For growth comparisons, Ty21a and derivative strains were grown at 37°C in TSB with 0.01% galactose supplementation or a casein-yeast extract (CY) medium with 0.01% galactose supplementation (CY medium is currently used to manufacture Vivotif; Kopecko et al. [28]). Plasmid-containing strains were selected in growth medium containing ampicillin (Amp; 100 μg/ml), spectinomycin (Spc; 100 μg/ml), chloramphenicol (Cm; 35 μg/ml), or kanamycin (Kan; 30 μg/ml). All constructed plasmids were sequenced, and the sequences were assembled and analyzed using the Vector NTI suite 9.0 software (Invitrogen).

Cloning *S. flexneri* 2a and 3a O-antigen genes into pMD-TV. Standard molecular biology techniques were used for cloning. The restriction endonucleases and DNA ligase were purchased from New England BioLabs (NEB) or Fermentas, and Phusion polymerase (Fisher) was used for all PCRs. Since cloning the entire rfb operon in one step into multicopy plasmid pMD-TV (13) was challenging, the cloning was carried out in two steps. The primer MD18.rfb.5021.F and MD4-2a.rfb.R primer pair was used to amplify rfb to oriT2 (Fig. 1A) of the rfb operon from *S. flexneri* 2a strain 2457T (GenBank accession AF021347) genomic DNA. The resulting PCR product (~6 kb) and pMD-TV were digested with Xhol and KpnI, PCR purified (Qiagen), and ligated using T4 ligase to construct pMD-TV.2a. Similarly, the MD3-2a.rfb.F and MD16.5042.R primer pair was used to amplify 4 kb of the rfb operon, upstream of the rfb promoter to rbc. The resulting PCR product and pMD-TV.6kb plasmid (6-kb PCR product cloned into pMD-TV) were digested with Xhol and BamHI, PCR purified, and ligated to construct pMD-TV.Y.

In order to express *S. flexneri* 2a O-antigen, the bgt-gtrII region of bacteriophage SfiI was cloned into pMD-TV-Y upstream of the *S. flexneri* 2a rfb region. First, the bgt-gtrII region was PCR amplified from *S. flexneri* 2a 2457T genomic DNA with primers MD133.tviD.F (contains tviD promoter in primer extension) and MD118.R.2aY. The resulting PCR product and pMD-TV-Y were digested with NotI and BamHI, PCR purified, and ligated to construct pMD-TV-2a.

In order to express *S. flexneri* 3a O-antigen, bacteriophage Sfi-carried oac and bacteriophage SF6-carried gtrX, gtrA, and gtrB genes, with their cognate promoters, were tandemly cloned into pMD-TV-Y upstream of the *S. flexneri* 2a rfb region. The Sfi-carried oac was PCR amplified from *S. flexneri* 3a strain J99 genomic DNA with primers MD47.3a.OAC.NotI and MD30.3a.BamHI.R. The resulting PCR product and pMD-TV-3a were digested with NotI and BamHI, PCR purified, and ligated to construct pMD-TV-3a.

Integrating *S. flexneri* O-antigen genes into the Ty21a chromosome. The *Shigella* O-antigen genes were integrated into the Ty21a chromosome using λRed recombination, as previously described (13), in a stepwise manner. First, the *S. flexneri* O-antigen Y serotype backbone genes were integrated into the Ty21a chromosome. The rfb backbone genes that were cloned into pMD-TV (pMD-TV-Y) were used as the template for PCR with tviD forward (pMD133.tviD.F) and vexA reverse (pMD87.vexA.R.32bp) primers. The resulting PCR product was digested with DpnI to remove circular plasmid DNA, and was purified, and ~1 to 2 μg was transformed into freshly grown Ty21a competent cells expressing λ Red proteins and selected for Kan resistance (Kan), as previously described (13). The Kan' chromosomal integrants were transformed with pCP20 (31), and Cm' transformants were selected at 30°C, after which a few isolates were nonselectively colony purified at 37°C and then tested for loss of all antibiotic resistances.

Next, the genes encoding *S. flexneri* 2a-modifying enzymes, bgt and gtrII, were integrated upstream of the rfb operon in Ty21a-Y. The plasmid pMD-TV-2a was used as the template for PCR with the tviD forward primer (pMD133.tviD.F) and rfb operon reverse primer (pMD118.R.2aY). The resulting PCR products were transformed into Ty21a-Y competent cells expressing λ Red proteins and selected for Kan resistance, and the Kan' cassette was eliminated as described before to construct Ty21a-2a (MD194). Similarly, genes encoding *S. flexneri* 3a-modifying enzymes, the gtrX, gtrA, and gtrB gene cluster and oac, were integrated upstream of the rfb operon in the Ty21a-Y strain, as described above. The pMD-TV-3a was used as the template for PCR with the prMD133.tviD.F and prMD118.R.2aY primer pair. Finally, this PCR product was transformed into Ty21a-Y competent cells expressing λ Red and selected initially for Kan', and then the Kan' cassette was eliminated as described above to construct Ty21a-3a (MD196).

Replacing oac promoter upstream of bgt with lpp. Ty21a-sdi-Kan (MD166, *S. dysenteriae* O-antigen biosynthesis genes expressed from the lpp promoter) (18) was used as the template for PCR with the tviD forward primer (pMD133.tviD.F) and the lpp promoter reverse primer containing a 150-bp bgt homology extension (pMD152.2AL.lpp.R). The PCR products were transformed into Ty21a-2a (MD194) competent cells expressing λ Red proteins and selected for Kan resistance (Ty21a-2a-Kan [MD212]). Next, the Kan' cassette was removed as described above to construct Ty21a-2a (MD268).

Removing the intergenic region between gtrII and rfbB. Ty21a-2a-Kan (MD212) was used as the template for PCR with the tviD forward primer (pMD133.tviD.F) and gtrII reverse primer with a 150-bp
rfB homology extension (prMNID178.2aO.R). The resulting PCR products were transformed into Ty21a-2al (MD268) competent cells expressing 1 RecE proteins and selected for Kan resistance; the Kan cassette was then removed, as described before, to construct Ty21a-2aol (MD270).

O-antigen expression analyses. Slide agglutination reactions were performed with the following serotype-specific rabbit polyclonal antisera: S. flexneri type 1, 2, 3, 4, 5, 6 and antisera, S. flexneri serotype 2-specific antisera, S. flexneri serotype 3, 4, 5, and 6 and S. flexneri serotype 6-specific antisera, S. flexneri serotype 7, 8 and 9-specific antisera, S. flexneri serotype 10-specific antisera, or S. Typhi O-specific 9 factor antisera (Difco). For immunoblotting, Salmonella, Shigella, and E. coli strains with or without various recombinant plasmids were grown overnight with aeration at 37°C in medium containing appropriate antibiotics, and the bacterial cultures were standardized by optical density. LPS was purified using an LPS extraction kit (Boca Scientific, FL), according to the manufacturer’s instructions. Purified LPS was separated by 14% Tris-glycine PAGE gels (Life Technologies, NY). Standard Western blotting procedures were carried out with the above-mentioned antibodies for the identification of specific LPS types.

The stability of recombinant clones of Ty21a-2a, Ty21a-2al, Ty21a-2aol, and Ty21a-J99 (i.e., expressing S. flexneri 2a or 3a O-antigens) was tested by immunoblotting of colonies plated from an overnight culture (i.e., 24 h culture following high dilution = ~25 generations). Subsequent similar serial dilution and regrowth were conducted, for a total of ~75 generations. The resulting colonies from agar platings were transferred to a nitrocellulose membrane and analyzed by standard Western blotting procedures using the LPS-specific antibodies specified above. More than 300 colonies were examined from each strain after ~75 generations of growth to assess genetic stability.

Confocal microscopy. Dual fluorescence staining was used to demonstrate coexpression of either heterologous S. flexneri serotype 2a or 3a O-antigens in addition to the homologous S. Typhi LPS by a combination of monoclonal and rabbit polyclonal antibodies against LPS antigens. Different Ty21a candidate vaccine strains and S. flexneri strains were cultured overnight, diluted 1:100, and grown for 4 h at 37°C. Two milliliters of the log-phase cultures was centrifuged, resuspended in 100 µl of sterile PBS, added to each well of the µ-Slide VI 0.4 Collagen IV (Eibidi, WI, USA), and incubated at 4°C overnight. The unattached cells were removed, and 100 µl of 4% paraformaldehyde was added to each well to fix the cells. After 1 h of incubation at 4°C, paraformaldehyde was removed, 100 µl of PBS was added to each well, and the slide was placed on a platform rocker for 5 min to wash the wells. After 3 washes, the wells were blocked with serum-free block (Dako, CA) for 1 h at room temperature. Subsequently, 100 µl of primary rabbit antibodies against Shigella O-antigen, S. flexneri type II-specific antiseraum or S. flexneri type III-specific antiseraum and S. Typhi LPS-specific mouse antiseraum was diluted 1:500 in antibody diluent (Dako) and added to each well. To prepare anti-S. Typhi LPS of mouse origin, mice were immunized with Ty21a, and antibodies against non-LPS antigens were removed from the resulting immune sera. The antibodies against non-LPS antigens were removed by absorption with acetone powder (32) that was prepared from a Ty21a derivative (Ty21a-sdl [MD174] [18]), which normally expresses a very low level of S. Typhi LPS and then was purposely grown in M9 minimal medium without added galactose to eliminate LPS expression.

The slides with primary antibody were incubated overnight at 4°C, and the wells were washed with TBST (Tris-buffered saline with 0.5% Tween 20; Dako) 3 times as described before. The secondary antibodies conjugated with either Alexa Fluor 488 goat anti-rabbit IgG or Alexa Fluor 594 goat anti-mouse IgG (Life Technologies) diluted 1:500 in antibody diluent (Dako) and added to each well. To prepare anti-S. flexneri O-specific antibody, mice were immunized with Ty21a, and antibodies against non-LPS antigens were removed from the resulting immune sera. The antibodies against non-LPS antigens were removed by absorption with acetone powder (32) that was prepared from a Ty21a derivative (Ty21a-sdl [MD174] [18]), which normally expresses a very low level of S. Typhi LPS and then was purposely grown in M9 minimal medium without added galactose to eliminate LPS expression. The slides with primary antibody were incubated overnight at 4°C, and the wells were washed with TBST (Tris-buffered saline with 0.5% Tween 20; Dako) 3 times as described before. The secondary antibodies conjugated with either Alexa Fluor 488 goat anti-rabbit IgG or Alexa Fluor 594 goat anti-mouse IgG (Life Technologies) diluted 1:500 in antibody diluent (Dako) and added to each well. To prepare anti-S. flexneri O-specific antibody, mice were immunized with Ty21a, and antibodies against non-LPS antigens were removed from the resulting immune sera. The antibodies against non-LPS antigens were removed by absorption with acetone powder (32) that was prepared from a Ty21a derivative (Ty21a-sdl [MD174] [18]), which normally expresses a very low level of S. Typhi LPS and then was purposely grown in M9 minimal medium without added galactose to eliminate LPS expression.

Animal immunization and challenge studies. Eight-week-old female BALB/c mice, with 10 per group, were immunized with vaccine candidate strains Ty21a(p2a), Ty21a(p3a), Ty21a-2a, Ty21a-2al, Ty21a-2aol, and Ty21a-J99 or negative controls of Ty21a alone or PBS. All strains were grown overnight in TSB, washed, and suspended in sterile PBS to a concentration of ~0.4 x 10^9 to 1 x 10^10 CFU per ml. Mice were inoculated intraperitoneally with a 0.5-ml dose containing ~2 x 10^7 to 5 x 10^7 CFU per mouse of either candidate vaccine, control Ty21a cells, or 0.5 ml of sterile PBS, for three total doses spaced 2 weeks apart. Mice were tail-bled 2 weeks after the last injection. Immunized and control mice were challenged intraperitoneally, 4 weeks after final immunization, with 1 x 10^9 CFU/ml of freshly grown mid-log-phase virulent S. flexneri 2a 2457 or S. flexneri 3a J99 in 0.5 ml of 5% hog gastric mucin (Sigma) dissolved in sterile saline. Survival was monitored for 7 days.

Detection of anti-LPS antibodies by ELISA. S. flexneri 2a strain 2457, S. flexneri 3a strain J99, or Ty21a was grown overnight at 37°C with aeration in TSB, and LPS was purified using an LPS extraction kit (Boca Scientific), according to the manufacturer’s instructions. Microtiter plates were coated with S. flexneri 2a 2457, S. flexneri 3a J99, or S. Typhi Ty21a purified LPS in 0.1 M NaCO3-NaHCO3 (pH 9.5). Coated microtiter plates were blocked with blocking buffer containing 1% bovine serum albumin (BSA; Sigma) in TBST (TBST with 0.3% Tween 20) for 2 h. Serial serum dilutions were added to each plate and incubated at 4°C overnight. After washing six times with TBST, bound antibodies were detected with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (SouthernBiotech). Endpoint titers were defined as the reciprocal of the antibody dilution for the last well in a column with a positive optical density (OD) for each sample after subtracting for background. Background values were determined with preimmunization sera, where the OD values of the preimmunization sera were averaged and then doubled. This value was subtracted from the OD of all the wells containing titrations of every mouse serum sample. Each data point represents the mean of the endpoint titers of two independent ELISAs performed for every mouse...
serum sample. The individual sample titers and the mean ± standard error of the mean (SEM) for each group of 10 mice are shown in Fig. 6. Statistical analyses were performed by means of an unequal t test using GraphPad Prism version 5. A P value of <0.05 (two-tailed) was considered to be statistically significant (**).

Accession numbers. The sequences are available under GenBank accession numbers KT988055 for Ty21a.2a (MD194), KT988054 for Ty21a.2al (MD268), KT988056 for Ty21a.2aol (MD270), KT988057 for Ty21a.3a (MD196), KT988058 for pMD.TV.2a, and KT988059 for pMD.TV.3a.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/CVI.00181-17.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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