Development of a live attenuated bivalent oral vaccine against *Shigella sonnei* shigellosis and typhoid fever

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FOOTNOTE

CONFLICT OF INTEREST STATEMENT

The authors are owners or full time employees of Protein Potential, LLC and Sanaria Inc. that seek to commercialize Ty21-AR-Ss vaccine under the trade name TyOraSs.

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MEETING PRESENTATIONS

This work has been presented at the Maryland Tech Council (2015, North Bethesda, MD, USA), Vaccines of Enteric Diseases (2015, Edinburgh, Scotland, UK), American Society of Tropical Medicine and Hygiene (2015, Philadelphia, PA, USA), Vaccine R&D (2015, Baltimore, MD, USA), and Vaccines for Shigellosis and ETEC (2016, Washington, DC, USA) conferences.

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ABSTRACT

Shigella sonnei (Ss) and *Salmonella* Typhi cause significant morbidity and mortality. We exploited the safety record of the oral, attenuated *S.* Typhi vaccine (Ty21a) by utilizing it as a vector to develop a bivalent oral vaccine to protect against Ss shigellosis and typhoid fever. We recombineered the Ss form I O-antigen gene cluster into the Ty21a chromosome to create Ty21a-Ss, which stably expresses Ss form I O-antigen. To enhance survivability in the acid environment of the stomach, we created an acid resistant strain, Ty21a-AR-Ss, by inserting *Shigella* glutaminase-glutamate decarboxylase systems co-expressed with Ss form I O-antigen gene. Mice immunized intranasally (IN) with Ty21a-AR-Ss produced antibodies against Ss and *S.* Typhi, and survived lethal IN Ss challenge. This paves the way for proposed GMP manufacture and clinical trials intended to test the clinical effectiveness of Ty21a-AR-Ss in protecting against Ss shigellosis and typhoid fever as compared to the current Ty21a vaccine.

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INTRODUCTION

Shigellosis (bacterial dysentery) is caused by *Shigella spp.* bacteria. There were 188 million human *Shigella* infections in 2010 [1], affecting mainly children and causing 65,000 deaths. There are more than 40 serotypes of *Shigella*, but only a few responsible for the majority of shigellosis. In developing countries, *S. flexineri* accounts for most cases in children under age 5, while *S. sonnei* (Ss) is the second leading cause, at ~24% [2]. In developed countries Ss is the leading cause of shigellosis, with 75% of the estimated ~500,000 annual U.S. cases caused by Ss [3, 4]. Moreover, drug-resistant Ss infections associated with international travel have been increasingly reported [5, 6]. There is no safe and efficacious licensed vaccine against shigellosis [7].

Protection against shigellosis is believed to be mediated primarily by antibodies to O polysaccharide (O-antigen) [8]. Genes encoding Ss O-antigen are uniquely located on the virulence plasmid so that only cells that express O-antigen (form I) are virulent [9]. Our strategy is to generate a bivalent vaccine against typhoid fever and Ss shigellosis by administering the *Salmonella* Typhi Ty21a vaccine expressing the Ss form I O-antigen (Ss-f1-Oag).

The typhoid vaccine Ty21a (Vivotif®) [10] administered orally during one week, affords sustained protection for 7 years, with efficacies ranging from 42-96% in Indonesian, Chilean, and Egyptian field trials [11-13], and has had an excellent safety record in more than 200 million

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recipients [10, 14-17]. Ty21a is nonpathogenic even when given at 100 times the standard dose [12].

Ty21a expressing Ss-f1-Oag from a plasmid was shown to be protective in clinical trials [18]. However, the plasmid was unstable [19] and associated with lot-to-lot variation [18, 20]. Because of this instability, development of this Ty21a-based shigellosis vaccine was halted.

The genetic instability associated with plasmid-borne immunogens has been overcome. A recombinant Ty21a strain with a genome-integrated Ss-f1-Oag gene cluster induced serum antibodies against Ss-f1-Oag and *Salmonella* O-9, 12 antigens, and protected mice against lethal challenge [21]. However, the immunization and infection routes were through intraperitoneal (IP) injection, which is not the natural oral, mucosal route for Ty21a immunization and *S*. Typhi and Ss infection. It is unknown if the vaccine strain will be effective when immunization and challenge is through a mucosal route.

To induce protective immune responses, Ty21a must pass from the mouth to the ileum, where Ty21a invades M cells. The major barrier for a live, oral *S.* Typhi vaccine is the low pH in the stomach. *Salmonella* does not survive well at pH <3. Most *E. coli* strains and *Shigella spp.* remain viable at low pH for several hours [22, 23]. This difference likely explains why only 10-100 *Shigella* cells are sufficient to cause infection, while ~10^5 CFU of *Salmonella* are needed. To

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facilitate the journey from mouth to ileum without being eliminated in the stomach, Ty21a is placed in enteric-coated capsules meant to withstand gastric low pH.

The ability of *E. coli* and *Shigella* to withstand pH < 2.5 primarily relies on an acid resistant (AR) system known as the glutamate-dependent acid resistance pathway [22, 24] consisting of the enzyme glutamate decarboxylase (GAD) and a membrane bound antiporter. GAD consumes an intracellular proton to decarboxylate glutamate to produce γ-amino butyric acid (GABA) [25-29]. There are two isoforms of GAD, encoded by *gadA* and *gadB*, which are 98.7% identical in amino acid sequence and functionally redundant. The antiporter, encoded by *gadC*, pumps glutamate and GABA in and out of the cell [27].

Recently, a newly discovered glutamine-dependent AR system was reported [30]. A previously uncharacterized bacterial glutaminase, encoded by *ybaS*, converts glutamine to glutamate in acidic conditions, releasing an ammonium that neutralizes an intracellular proton [30]. The antiporter for transporting substrate and product across cell membrane is also GadC [30]. Because the product of glutaminase is the substrate for GAD, we hypothesized that the glutaminase-GadC and GAD-GadC systems work in concert to convert a glutamine molecule into GABA, neutralizing two protons in the cell (Figure s1 and [30]). This concerted AR systems should function more efficiently than the GAD-GadC system alone [31] to enhance Ty21a viability in an acid environment.

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We report the construction and characterization of Ty21a strains expressing Ss-f1-Oag alone or with the concerted glutaminase-GAD AR systems. Expression of the AR genes rendered our vaccine construct acid resistant *in vitro*. The strains induced antibodies to *Shigella* and *Salmonella* O-antigens when administered intranasally to mice, and the immunized mice survived intranasal lethal challenge with Ss. These results provide the foundation for development of a cost-effective and easy-to-administer vaccine against shigellosis and typhoid fever.

METHODS

Bacterial strains and media. Bacterial strains used or generated are listed in Table S1. Ty21a (Vivotif, Crucell Vaccines Inc, Miami Lakes, FL) was purchased. Seed banks were made in CY medium (1.2% yeast extract, 2% Hy-Case, 1.2% pepticase, 0.125% NaH_2PO_4, 0.33% NaCl, pH 7.2, with 0.2% glucose and 0.005% galactose), which is used for Vivotif production [10]. Ss 53G was a gift from Dr. Dennis Kopecko [9]. Form I Ss 53G were distinguished from form II based on colony morphology. Unless indicated, Ty21a and derivatives were grown on TSA or in TSB supplemented with 0.02% galactose. Ss strains were grown in TSA or TSB.

Recombinant Ty21a strains were generated using the λ Red recombineering technology [21, 32]. PCR fragments were amplified from a donor plasmid (Table S1) by Phusion high-fidelity polymerase NEB (Ipswich, MA) using upstream primers TviD-2004F (5'-TGATTGCTAA CGTCATGAGC-3') or GadC-1F (5'-ATGGCTACAT CAGTACAGAC-3') and downstream primer VexA-

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1066R (5'-AGAAAGAATT AGTGCCGCGG-3') and integrated in Ty21a harboring pKD46 [21, 32]. The KanR selectable marker was deleted from the chromosomal integrants by transformation with pCP20 and selecting for Kan^s transformants as described [21, 33]. Chromosomal integration and selection marker eviction were confirmed by genomic PCR analysis and antibiotics sensitivity tests.

Plasmids. Plasmids are listed in Table S1 and details of plasmid construction using standard techniques and enzymes (NEB) are described in Supplementary data. Integrity of all plasmids generated in this study was confirmed by DNA sequencing.

Assays. Methods for assays used in this study are described in detail in Supplementary data.

Animal immunization. 4-8-week old female BALB/c (Jackson Laboratory, Bar Harbor, Maine) were maintained at Bioqual (Rockville, MD). They were immunized and assessed according to a protocol [34] approved by the Bioqual Laboratory Animal Care and Use Committee. On day of immunization, overnight cultures of bacterial strains grown in CY or CY supplemented with 1% trehalose and 0.75% arabinose (AR activated) were harvested and resuspended in PBS to a target concentration of 1×10^{11} CFU/mL. Mice anesthetized by isoflurane were intranasally inoculated with 5 µL of bacterial suspension at the anterior of each naris. Sera were collected a day before 1st immunization, and 2 weeks after each immunization.

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Ss challenge. 3-6 single colonies of form I Ss 53G were pooled to inoculate into TSB and allowed to grow until mid-log phase (OD_{600} ~1.0). Cells were resuspended in PBS to a targeted concentration of 1×10^{11} CFU/mL and 10 μL used to inoculate each mouse intranasally. Mice were monitored daily for 14 days post-infection.

Statistical analyses. All tests were two-sided and performed using R (version 3.2.3) with stat and survival (2.38-3) packages.

RESULTS

Stable integration and expression from Ty21a chromosome

We constructed a recombinant Ty21a with stable chromosomally integrated Ss-f1-Oag gene cluster between the *tviD* and *vexA* ORFs (Figure 1A) [21, 32, 33]. This construct though similar to previously described [21] is different in that the gene cluster was amplified from genomic DNA of Ss 53G form I and initiated from the conserved 5' border of O-antigen clusters [35] (Figure 1B). We included the *wzz* ORF that was not previously expressed [21], because *wzz* expression facilitated uniform distribution of Ss-f1-Oag on Ty21a similar to that of native Ss (Figure S2). Our final vaccine strain, designated Ty21a-Ss, did not have the antibiotic resistance marker. Chromosomal integration and integrity of the Ss-f1-Oag gene cluster was confirmed by DNA sequencing and only 3 non-synonymous single nucleotide polymorphisms were identified

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in comparison to the published sequence pSs046 [35]. Sequencing of independently amplified PCR fragments from Ss 53G genomic DNA demonstrated these polymorphisms were present in Ss 53G; we believe they arose from natural variation between laboratory isolates.

We hypothesized that expression of the AR genes in Ty21a would make a better vaccine. Because regulation of AR gene expression is not fully understood, we put these genes under the regulation of the arabinose-controlled promoter, P_{ara}, which responds quickly to the arabinose-bound transcription factor, AraC, which activates robust gene transcription. We PCR-amplified *Shigella ybaS* and the *gadBC* coding sequences, fused and placed them under P_{ara} to create a polycistronic AraC-P_{ara}-YbaS-GadBC expression cassette. This cassette was integrated into the Ty21a chromosome to replace the *tviE* ORF. The resulting strain was then used for stable integration of the Ss-f1-Oag expression cassette using *gadC* and *vexA* as homologous sequences for recombination (Figure 1C) and the KanR marker evicted. The resulting final, marker-less, strain was designated Ty21a-AR-Ss.

Cell surface expression

We examined Ss-f1-Oag expression by Western blots (Figures 2A, 2B) and immunofluorescence assays (Figure 2C). Because the arabinose-controlled AR genes in Ty21a-AR-Ss are located 5' to the Ss-f1-Oag gene cluster, we also examined form I O-antigen expression in the presence of 0.75% arabinose in the culture medium, mimicking AR activating conditions. Ss-f1-Oag in Ss 53G is expressed as a ladder of two-sugar repeats that is LPS core-linked and a smear at higher

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molecular weight, which is linked to group 4 capsule (Figure 2A). Ty21a did not and Ty21a-Ss did express Ss-f1-Oag (Figures 2A, 2C). As described [21, 36], most Ss-f1-Oag was expressed in the form that is capsule-linked in Ty21a-Ss. Ty21a-Ss expressed the Ss-f1-Oag uniformly on the cell surface, similar to the native form expressed on Ss 53G form I (Figure S2C(2)). Ty21a-AR-Ss expressed Ss-f1-Oag of similar levels and morphology to that from Ty21a-Ss regardless of presence of arabinose (Figure 2A, lanes 3, 4, and C (3, 4)). The expression levels of *Salmonella* groups O9, 12-antigen from Ty21a-Ss and Ty21a-AR-Ss strains were comparable to those of Ty21a (Figure 2B), the Ss and *S.* Typhi O-antigens co-existed on the cell surface (Figures 2C).

Acid resistance

To confirm the integrated AR genes expressed upon induction and were enzymatically active, we subjected Ty21a-AR-Ss to glutaminase and GAD assays [37] with slight modifications. Triton X-100 was omitted from the original GAD and glutaminase reagents. Therefore, only when a functional GadC transporter was present, could there be transportation of glutamine or glutamate into the cell for enzyme utilization. Bromocresol green was pH indicator. Ty21a and Ty21a-Ss were unable to utilize glutamine or glutamate to increase pH in the test reagent; the reactions remained yellow after incubation at 37°C for 30 min (Figure 3A, lanes 1, 2). Reactions containing Ty21a-AR-Ss grown in absence of arabinose turned slightly bluish-green, indicating a low level leaky expression of the AR enzymes (Figure 3A, lane 3). When grown in the presence of arabinose, Ty21a-AR-Ss turned the assay reagents strongly bluish-green, indicating robust

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expression of enzymatically active AR genes (Figure 3A, lane 4); Ty21a-AR-Ss expressed functional AR genes in a transcriptionally controlled manner.

We next tested the ability of Ty21a-AR-Ss to survive at pH 2.5. Because *Salmonella* needs acidic culture conditions to induce acid tolerance response, a prerequisite for AR [38], 1% trehalose was included for acid fermentation in addition to 0.75% arabinose. Bacterial strains were grown to stationary phase and cultures diluted 1:20 in acid medium at pH 2.5 in the presence of 1.5 mM glutamine. Reactions were incubated at 37°C with agitation and viability examined at indicated time points (Figure 3B). Ss 53G form II maintained ~100% viability after 1 hr incubation at pH 2.5 (gray squares). In contrast, Ty21a survived poorly at this condition: viability was reduced by >10^2-fold at 15 min and >10^7-fold by 30 min (black circles). Cell viability was further reduced in Ty21a-Ss (blue triangles), with no viable colony recovered at 15 min, equivalent to a >10^8-fold reduction. Ty21a-AR, the parental strain for Ty21a-AR-Ss, maintained >50% viability at 30 min and ~10% at 45 min (red inverted triangles). Ty21a-AR-Ss maintained ~50% viability at 30 min. However, viability quickly deteriorated, with ~1% at 45 min (closed green diamonds). Increasing glutamine concentration to 6 mM improved survival for Ty21a-AR-Ss; cell survival at 45 min improved to ~50% (open green diamonds). Ty21a recovered from the acid exposure was slow-growing and lost expression of the *Salmonella* O-antigen, while Ty21a-AR and Ty21a-AR-Ss maintained levels of O-antigen expression comparable to before acid exposure (Figure 3C). Expression of the concerted glutaminase-GAD AR systems improved acid resistance and cell viability of recombinant Ty21a strains.

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Stability

We generated genetic seedbanks for Ty21a-Ss and Ty21a-AR-Ss. All microbiological, biochemical, immunological, genetic, and molecular properties were as expected (Figure 4A). Cells were grown under non-selective conditions for 200 generations. 100 colonies from each culture were subjected to colony immunoblot analysis for Ss-f1-Oag expression. All of 100 colonies of Ty21a-Ss (Figure 4B) and Ty21a-AR-Ss (Figure 4C) tested retained Ss-f1-Oag expression, demonstrating 100% stability of the chromosomally integrated genes after 200 generations of growth.

Immunogenicity

To assess if Ty21a-Ss and Ty21a-AR-Ss were immunogenic when administered via the mucosal route, we immunized four groups of 10 mice intranasally with 4 doses of 1×10^9 CFU Ty21a, Ty21a-Ss, Ty21a-AR-Ss (AR gene not expressed), and Ty21a-AR-Ss grown in the presence of trehalose and arabinose (AR genes activated; referred to as Ty21a-AR-Ss+ARA) at 2-week intervals. Serum IgG antibody responses against Ss-f1-Oag at 2 weeks after doses 2, 3, and 4 were assessed by ELISA. Results are reported as the geometric mean (GM) OD 1.0 (serum dilution at which the optical density was 1.0, Figure 5A). Mice immunized with Ty21a-Ss (blue symbols), Ty21a-AR-Ss (red symbols), and Ty21a-AR-Ss+ARA (green symbols) produced higher serum IgG antibodies against Ss-f1-Oag than the Ty21a control (black symbols) or naïve mice (gray symbols), and antibodies increased with increased numbers of immunizations ($p < 0.05$ by

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Wilcoxon Rank test). At 2 weeks after dose 4, the GM OD 1.0 to Ss-f1-Oag of mice immunized with Ty21a-Ss, Ty21a-AR-Ss, and Ty21a-AR-Ss+ARA were 7,909.5 (range 2,560 – 21,700), 7,202.4 (range 3,363 – 15,910), and 23,020.8 (range 8,521 – 71,575), respectively.

We also assessed antibodies to *Salmonella* groups O 9, 12-antigens, the native O-antigens expressed on Ty21a surface that induce protective immunity against typhoid fever (Figure 5B). For mice immunized with Ty21a, the GM OD 1.0 of serum IgG antibodies to *Salmonella* O 9,12-antigens were 53.9 (range 1 - 864), 54.5 (range 1 - 545), and 328.3 (range 72 - 2,295) at 2 weeks after doses 2, 3, 4, respectively. Ty21a-Ss, Ty21a-AR-Ss, and Ty21a-AR-Ss+ARA induced anti-*Salmonella* O 9, 12 -antigen serum IgG at comparable levels. At 2 weeks after dose 4, the GM OD 1.0 was 421.6 (range 98 – 1,241), 182.3 (range (74 – 1,030), and 426.8 (range 78 – 2,539) for Ty21a-Ss, Ty21a-AR-Ss, and Ty21a-AR-Ss+ARA. Naïve mice had a GM OD 1.0 of 13.6 (range 1 - 38).

Protective efficacy

We challenged the immunized mice six weeks after dose 4 with Ss 53G form I by intranasal instillation. Three of 9 control mice immunized with Ty21a survived the challenge, while 7/10, 9/10, and 9/10 mice immunized with Ty21a-Ss, Ty21a-AR-Ss, and Ty21a-AR-Ss+ARA respectively survived and remained healthy throughout the 14-day monitoring period (Figure 6). The protective efficacies were 55.2%, 85.1%, and 85.1% for Ty21a-Ss, Ty21a-AR-Ss, and Ty21a-AR-Ss+ARA, respectively. For the two groups that received the Ty21a-AR-Ss constructs, the

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difference between Ty21a control and immunized mice was significant ($p = 0.02$, Fishers Exact Test, 2-tailed).

DISCUSSION

Oral immunization with Ty21a expressing Ss-f1-Oag from a plasmid protected humans against Ss diarrhea and dysentery [18]. However, the plasmid-based construct was unstable with DNA rearrangements and deletions of plasmid material probably causing lot-to-lot variation [19]. Chromosomal integration using bacterial recombineering techniques addressed this issue [21]. Indeed, our recombinant vaccine strains maintained stable and robust expression of Ss-f1-Oag for a minimum of 200 generations (Figure 4B, 4C).

Protection against typhoid fever by immunization with Ty21a was excellent when the vaccine was administered as a buffered liquid [13] and higher as compared to enteric coated capsules [16, 39]. This increased efficacy was probably due to more viable Ty21a making it through the acid environment of the stomach into the small intestine when administered in a buffered liquid. Conferring acid resistance to Ty21a should allow for even more viable bacteria reaching the small intestine leading to improved immunogenicity and protective efficacy at the same dose of Ty21a. Ty21a-AR-Ss maintained ~50% viability at pH 2.5 for 30-45 min *in vitro*. In comparison, viability of Ty21a was reduced by >10^2-fold at 15 min and >10^7-fold by 30 min. The half-life for gastric emptying time in humans is about 26-45 min [40, 41]. Importantly, co-expression of the AR genes did not affect expression of Ss-f1-Oag and native Ty21a O-antigen *in*

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vitro (Figure 2), or immunogenicity of the bacterial strain *in vivo* (Figures 5 and 6). Ty21a quickly lost viability and O-antigen expression upon exposure to acid, while Ty21a-AR-Ss maintained the same levels of O-antigen expression (Figure 3C). Moreover, we observed slightly better antibody responses and higher protective efficacy in mice immunized with Ty21a-AR-Ss compared to Ty21a-Ss, especially when the AR genes were activated (Ty21a-AR-Ss+ARA). This was probably because culture conditions for AR gene induction favored cell viability. Even though we immunized mice with similar amounts of bacterial cells (determined by $OD_{600\ nm}$), the viable counts for the Ty21a-AR-Ss+ARA were always higher, but no more than 2-fold higher than that of the other three groups. By rendering Ty21a acid-resistant, we expect increased bioavailability, which should increase immunogenicity and protective efficacy.

Humans become infected with *S.* Typhi by ingestion of the bacteria. Ty21a is an orally administered vaccine. However, *S.* Typhi does not infect mice, making it difficult to assess immunization by oral administration. Thus, testing immunogenicity and protective efficacy in mice after IP administration was undertaken [21]. This could be misleading. LPS is highly immunogenic when administered IP, but not when administered by mucosal routes [42]. Furthermore, deaths from IP inoculation of gram-negative bacteria are related to the toxic effects of endotoxin [43], which may be distinct from that of invasive *Shigella* infection in human intestinal epithelium. The route for immunization and challenge of a Ty21a-based vaccine is ideally oral. However, because Ty21a, *S.* Typhi, [44], and *Shigella* spp do not infect normal mice when administered orally, an intranasal route has been established in mice for the evaluation of mucosal immune responses to live attenuated bacterial vaccines [34, 45]. The

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serum antibody responses in mice were about 10-fold lower using the intranasal as compared to the IP route (unpublished). Nevertheless, using this approach, we demonstrated that Ty21a-Ss and Ty21a-AR-Ss stimulated antibodies against both Ss and *Salmonella* LPS.

Most importantly, 9/10 mice immunized intranasally with Ty21a-AR-Ss or Ty21a-AR-Ss+ARA vaccine as compared to 3/9 immunized with Ty21a alone survived a lethal intranasal infection with Ss 53G (protective efficacy for each was 85.1%).

Ty21a protects for up to 7 years [12]. We expect similar protection with the typhoid-shigellosis combination vaccine. Ty21a can be foam-dried, providing for temperature stabilization and a potential shelf life of 5-10 years [46], which will facilitate storage and distribution. Most importantly, Ty21a is extremely safe. There is no report of bacteremia or Ty21a-assoicated post-vaccination reactive arthritis, a potential problem with other live attenuated vectors including non-typhoid *Salmonella*, *Shigella*, and *Yersinia*. We do not foresee safety concerns in regard to inclusion of the AR genes. The AR genes are present and functional in avirulent strains of *E. coli* and *Shigella spp.*, such as Ss 53G form II (Figure 3B). Therefore, they are insufficient to cause disease on their own. The AR genes are stably integrated into the Ty21a genome and placed under an artificially inducible promoter. These properties make horizontal transfer to and gene activation in another bacterium extremely unlikely. Finally, except for the expression of the AR and Ss-f1-Oag transgenes, Ty21a-AR-Ss displays the microbiological, biochemical, immunological, and genetic properties of Ty21a (Figure 4), demonstrating that expression of

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the AR genes has no effect on Ty21a attenuation. This finding is similar to a recently reported study with the GAD-GadC AR system, but not the glutaminase-GadC system we also included [31]. We cannot directly compare our results because the viability of the Ty21a control after acid challenge was lower in our experiments.

The recent Global Enteric Multicenter Study (GEMS) field study of 1130 *Shigella spp.* concluded, "A quadrivalent vaccine with O-antigens from *S. sonnei*, *S. flexneri* 2a, *S. flexneri* 3a, and *S. flexneri* 6 can provide broad direct coverage against these most common serotypes and indirect coverage …through shared *S. flexneri* group antigens…" (up to 88% coverage) [2]. Our goal is to develop such a quadrivalent vaccine.

However, Ss is responsible for 75% of all cases of shigellosis in the U.S. [4], and most cases in Israel [47], Thailand [48], and Southern Vietnam [49]. While developing the three other constructs for the quadrivalent vaccine, we plan to move forward in parallel to GMP manufacture of our acid-resistant Ty21-AR-Ss vaccine (TyOraSs). The next step will be to prove it is safe and immunogenic and protective against shigellosis and typhoid in humans.

CONFLICT OF INTEREST STATEMENT

The authors are owners or full time employees of Protein Potential LLC and Sanaria Inc that seek to commercialize Ty21-AR-Ss vaccine under the trade name TyOraSs.

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FIGURE LEGENDS

Figure 1. Schematic illustration the chromosomal gene organization of (A) Ty21a vector, **(B)** Ty21a-Ss, a recombinant Ty21a strain with the *tviE* locus replaced by a stably integrated *S. sonnei* form I O-antigen (Ss-f1-Oag) gene cluster cloned from *S. sonnei* 53G form I, and **(C)** Ty21a-AR-Ss, a recombinant Ty21a strain with stable integration of both arabinose-controlled acid resistant genes and the Ss-f1-Oag gene cluster at the *tviE* locus.

Figure 2. Expression of O-antigens in recombinant Ty21a-Ss and Ty21a-AR-Ss candidate vaccine strains by Western blots (A, B) and immunofluorescence assay (C). O-antigens were extracted from 5 × 10^8 CFU of *S. sonnei* 53G form I (1), Ty21a (2), Ty21a-Ss (3), Ty21a-AR-Ss (4), and Ty21a-AR-Ss grown in the presence of arabinose (5, hereafter referred to as Ty21a-AR-Ss+ARA), resolved on a 4-20% Tris-glycine SDS-PAGE gel, transferred to a PVDF membrane, and probed with rabbit polyclonal antibodies against *S. sonnei* form I **(A)** or Ty21a **(B)**. Sizes of the molecular weight markers in kDa are indicated to the left of the gel. **(C)** Immunofluorescence staining pattern for each of the four strains (C1) Ty21a, (C2) Ty21a-Ss, (C3) Ty21a-AR-Ss, and (C4) Ty21a-AR-Ss grown in the presence of arabinose. A single representative immunofluorescence image is shown triple-stained with Ss form I (green), Ty21a (red), and DNA inside the cell counter-stained with DAPI (blue). Experimental details are described in Supplementary information.

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Figure 3. Chromosomally integrated acid resistance genes are active *in vivo*. (A) Enzymatic assays of glutaminase (GLNase) and glutamate decarboxylase (GAD) activities. Cells from saturating cultures of Ty21a (1), Ty21a-Ss (2), Ty21a-AR-Ss (3), and Ty21a-AR-Ss+ARA (4) were harvested and assays as described in materials and methods. After 30-minute incubation at 37°C, a yellow color indicates no activity and a blue-greenish color indicates positive for the indicated enzymatic activity. **(B)** Viability assay at pH 2.5. Cells from indicated saturating cultures grown in TSB supplemented with 1% trehalose and 0.75% arabinose were diluted 1:20 into acid medium and viability measured as described in materials and methods. **(C)** Colonies recovered from acid challenge were grown in TSB at 37°C for overnight. O-antigens were extracted from the overnight cultures for Western blot analyses using anti-Ty21a and anti-*S. sonnei* form I antisera. Experimental details are described in Supplementary information.

Figure 4. Characterization of Ty21a-Ss and Ty21a-AR-Ss seed banks. A. Two random vials from each of the seed banks were characterized as previously published [10] with additional assays designed specifically for the integrated genes. Ty21a (Vivotif), *S. enterica* serovar Typhi Ty2 were controls. A representative anti-Ss form I O antigen colony blot of Ty21a-Ss **(B)** and Ty21a-AR-Ss **(C)** after 200 generations of growth is shown, demonstrating 100% stability of the transgene.

Figure 5. Ty21a-Ss and Ty21a-AR-Ss immunized through mucosal route generated high antibody responses to *S. sonnei* form I Ty21a O-antigens. Mice were immunized intranasally with 1×10^9 CFU of Ty21a (black), Ty21a-Ss (blue), Ty21a-AR-Ss (red), or Ty21a-AR-Ss+ARA

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(green) at 2-week interval for 4 times. Sera were collected 2 weeks after doses 2, 3, 4 and antibody responses to extracted **(A)** *S. sonnei* LPS or **(B)** Ty21a LPS (*Salmonella* groups 9, 12 O-antigens) were measured by enzyme-linked immunosorbent assay (ELISA) OD 1.0. Data were collected using Softmax 5.0 and were fit to a 4-parameter logistic curve. Sera from 5 un-immunized, naïve mice (gray) of the same age were also collected and measured as negative controls. Each point represents an individual mouse and the intensity of the point indicates whether the mouse was protected (dark) or unprotected (light) from a lethal *S. sonnei* 53G form I infection. The geometric mean for each group is indicated by a horizontal bar.

Figure 6. Mice immunized with Ty21a-Ss vaccine strains were protected from lethal *S. sonnei* 53G form I infection. All mice were challenged 6 weeks after vaccine dose 4 with a lethal infection of *S. sonnei* 53G form I by nasal instillation and monitored daily for 14 days. Each mouse was calculated to have received 1.2×10^9 CFU, corresponding to 24 50% lethal doses determined by pilot infection experiments with naïve mice. Only 3 out of 9 mice immunized with Ty21a survived, while 7 out of 10, 9 out of 10, and 9 out of 10 mice immunized with Ty21a expressing Ss-f1-Oag (Ty21a-Ss), Ty21a expressing Ss-f1-Oag but not the AR genes (Ty21a-AR-Ss), and Ty21a expressing Ss-f1-Oag and AR genes (Ty21a-AR-Ss+ARA). The difference in survival rates among all four groups was analyzed by Fishers Exact Test, 2-tailed ($p = 0.021$). The difference in survival rates for all immunized vs. controls was 25/30 vs. 3/9 ($p = 0.0078$), for Ty21a-AR-Ss or Ty21a-AR-Ss+ARA vs. Ty21a control was 9/10 vs. 3/9 ($p=0.0198$), and for Ty21a-Ss vs. naïve control was 7/10 vs. 3/9 ($p=0.1789$).

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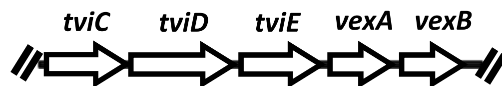
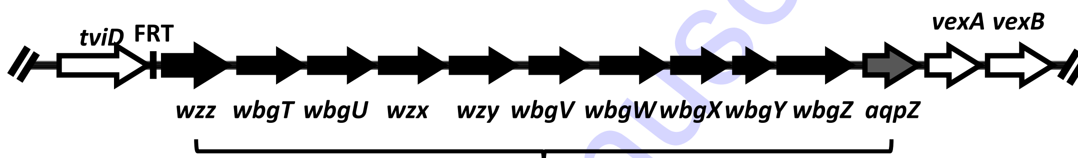
A.

Ty21a



B.

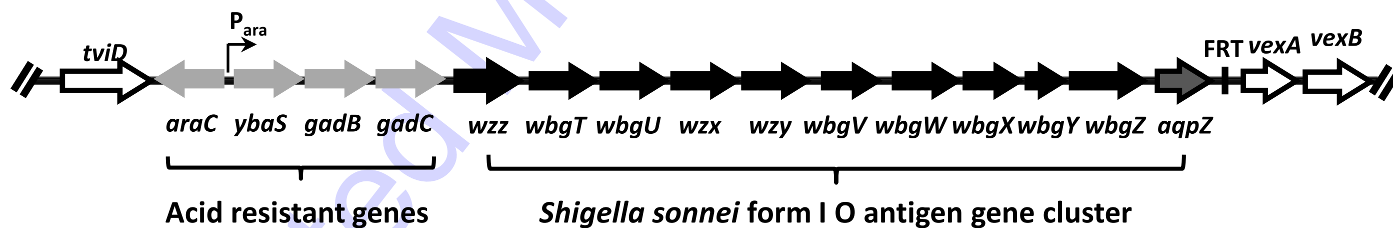
Ty21a-Ss



Shigella sonnei form I O antigen gene cluster

C.

Ty21a-AR-Ss



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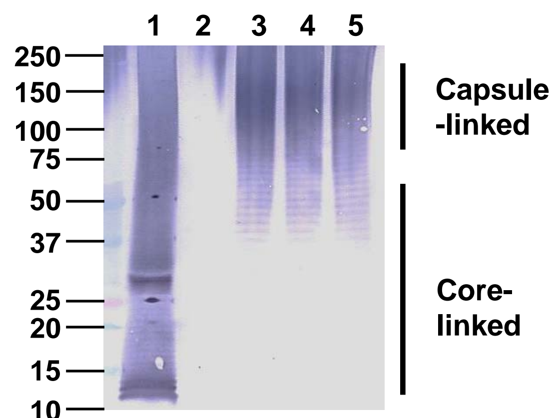
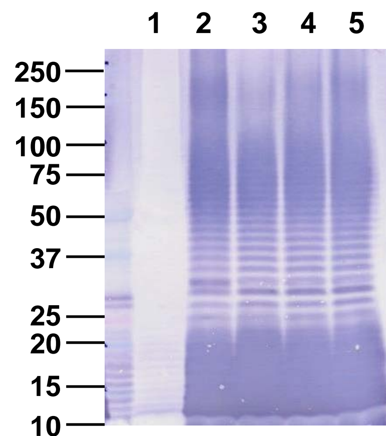
A.

Anti-*S. sonnei* form I

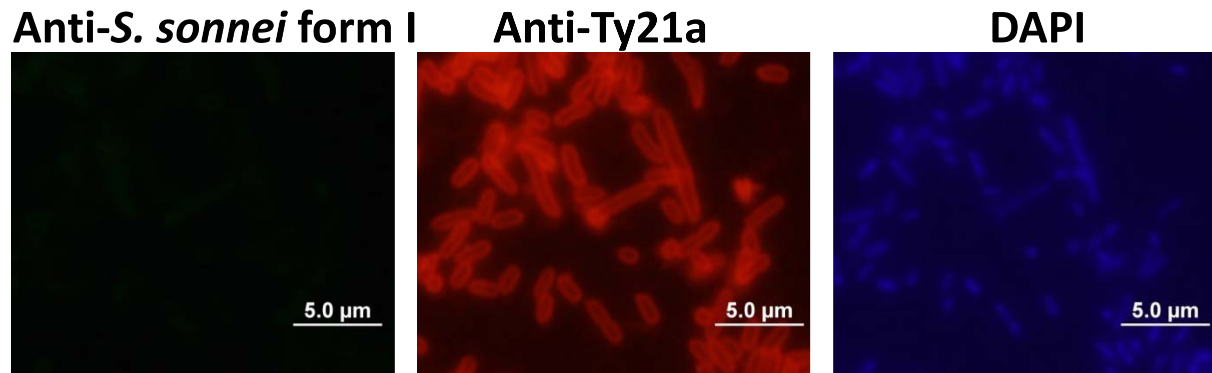


B.

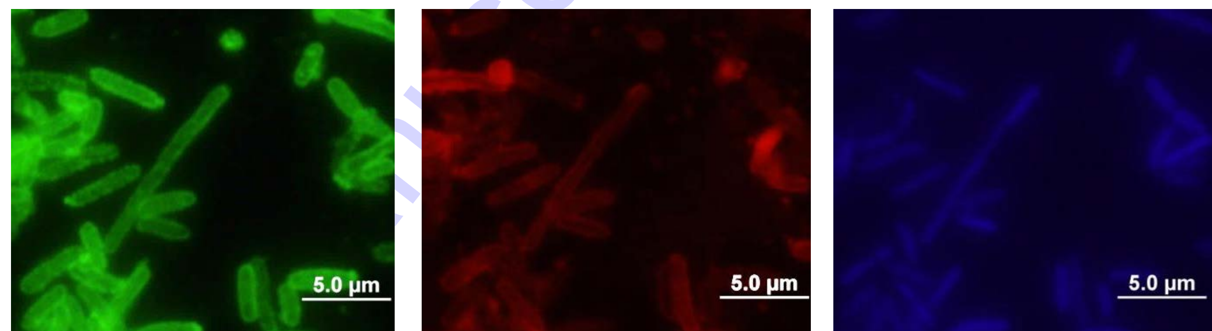
Anti-Ty21a



C. (1)



(2)



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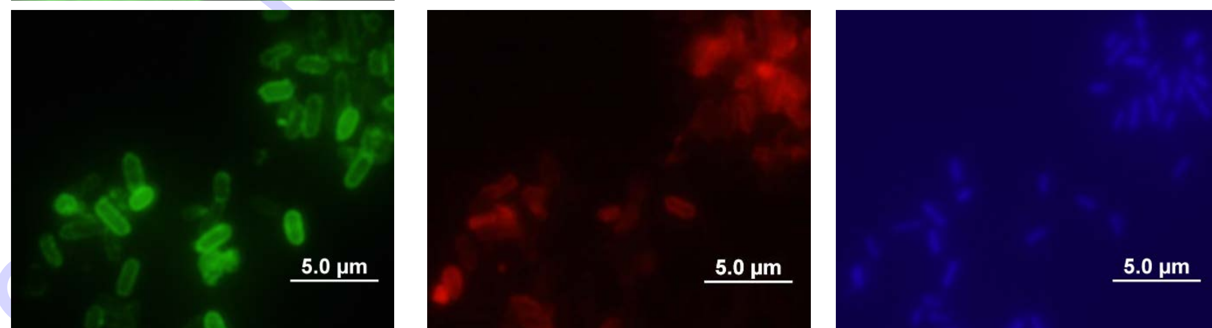

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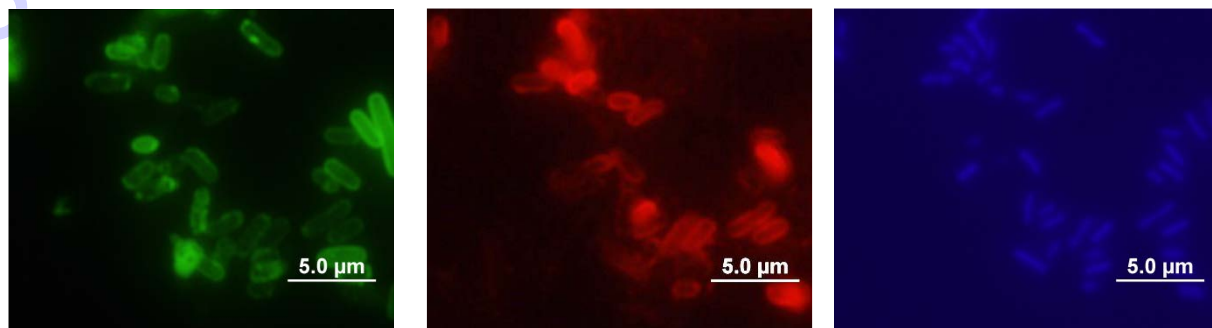
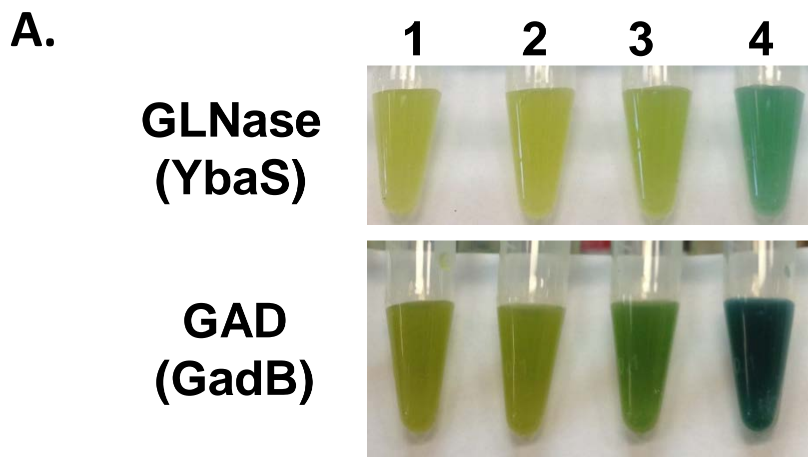
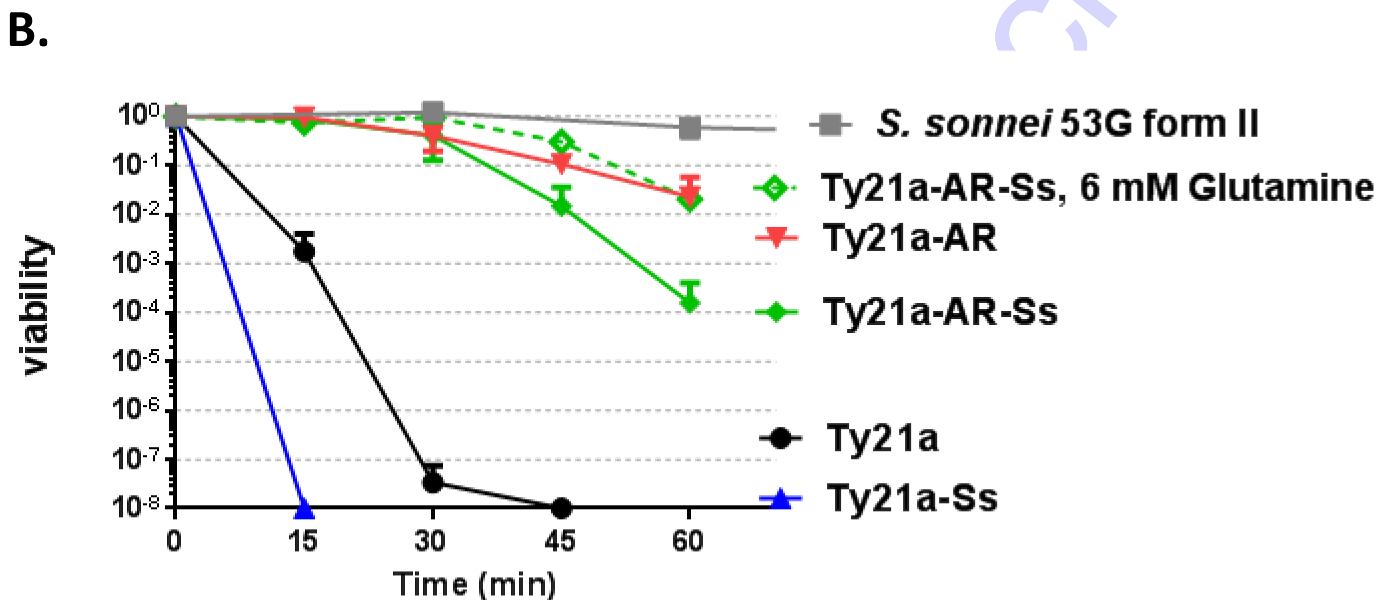
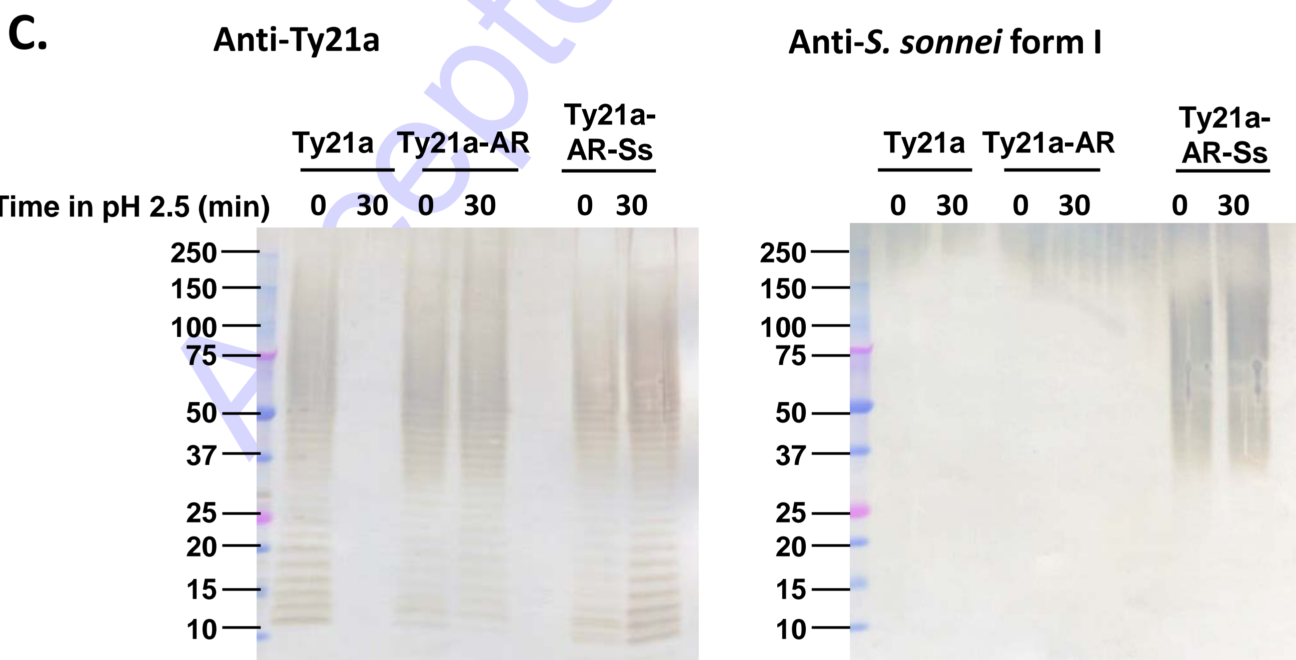




A.
1
2
3
4
GLNase (YbaS)
GAD (GadB)



B.
viability
Time (min)
S. sonnei 53G form II
Ty21a-AR-Ss, 6 mM Glutamine
Ty21a-AR
Ty21a-AR-Ss
Ty21a
Ty21a-Ss

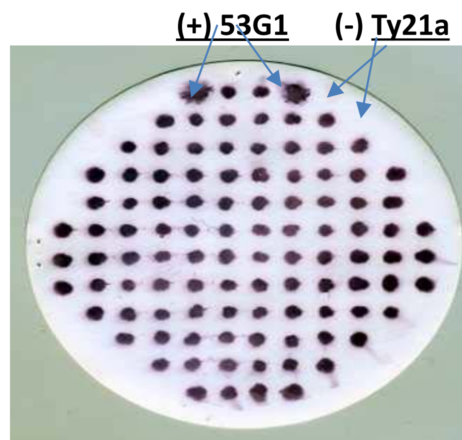


C.
Anti-Ty21a
Anti-S. sonnei form I
Ty21a
Ty21a-AR
Ty21a-AR-Ss
Time in pH 2.5 (min)
0
30
250
150
100
75
50
37
25
20
15
10

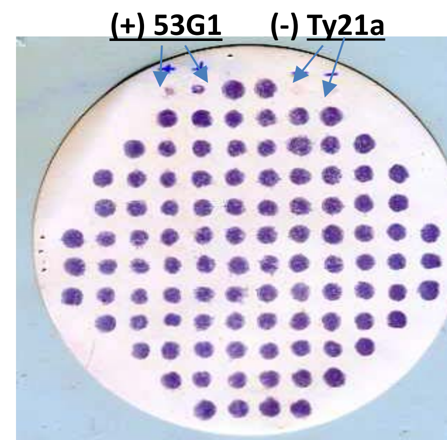
A.

Assay	Method	Ty21a-Ss	Ty21a-AR-Ss	Ty21a	Ty2
Microbiological	API 20 E	S. typhi	S. typhi	S. typhi	S. typhi
	Galactose fermentation (Colony appearance on Bromothymol Blue agar +1% galactose)	Blue	Blue	Blue	Yellow
Biochemical	Minimal media + cysteine + tryptophan	No growth	No growth	No growth	Growth
	Minimal media + cysteine + tryptophan + valine + isoleucine	Growth	Growth	Growth	Growth
	Heat stress at 55°C for 20 min	Sensitive	Sensitive	Sensitive	Resistant
	Oxidative stress in 0.3% H_2O_2 for 20 min	Sensitive	Sensitive	Sensitive	Resistant
	Galactose (1%)-induced bacteriolysis	Sensitive	Sensitive	Sensitive	Resistant
	Arabinose inducible Glutaminase/GAD activity	Negative	Positive	Negative	ND
	Viability at pH 2.5 for 30 min	$<10^{-5}$	>10%	$<10^{-5}$	ND
Immunological	*Salmonella* group 9,12 O-antigen agglutination	+	+	+	+
	Vi antigen agglutination	–	–	–	+
	S. sonnei O-Ag Expression (Colony and Western blot) at 200 generations	+	+	–	ND
Genetic	16S rDNA sequence	Identical to Ty21a	Identical to Ty21a	Ty21a	Ty2
	galE DNA sequence	Identical to Ty21a	Identical to Ty21a	Ty21a (T367C; C442Δ)	Ty2 (wild type)
	Chromosomally integrated S. sonnei form I O-Ag gene cluster (PCR)	+	+	–	–

B.



C.



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A.

Anti-*Shigella sonnei* form I O-Ag serum IgG

Dose2 | Dose 3 | Dose 4

Serum IgG by ELISA OD 1.0

100000
10000
1000
100
10
1

naive
Ty21a
Ty21a-Sso
Ty21a-YBC-Sso
Ty21a-YBC-Sso+ARA

Immunogen
naive
Ty21a
Ty21a-Sso
Ty21a-YBC-Sso
Ty21a-YBC-Sso+ARA

Status
Protected
Unprotected

B.

Anti-*Salmonella* group 9,12 O-Ag serum IgG

Dose2 | Dose 3 | Dose 4

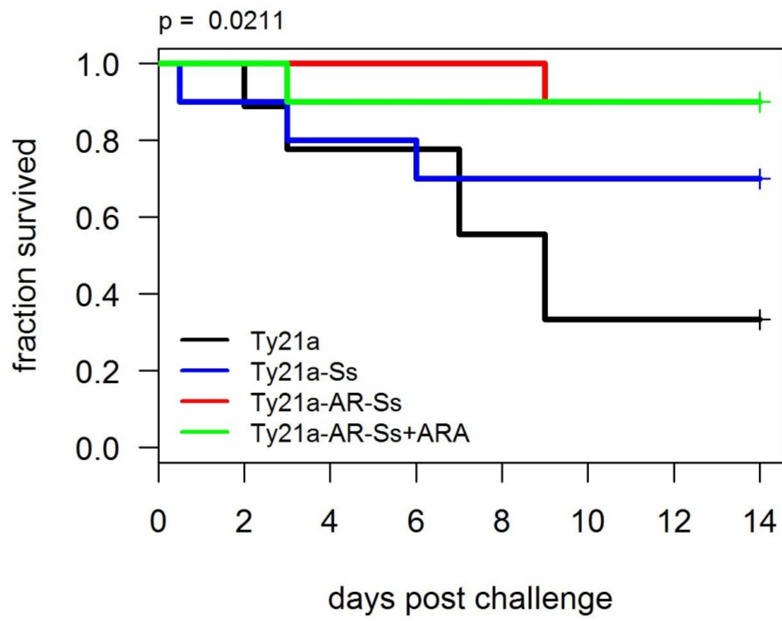
Serum IgG by ELISA OD 1.0

1000
100
10
1

naive
Ty21a
Ty21a-Sso
Ty21a-YBC-Sso
Ty21a-YBC-Sso+ARA

Immunogen
naive
Ty21a
Ty21a-Sso
Ty21a-YBC-Sso
Ty21a-YBC-Sso+ARA

Status
Protected
Unprotected



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