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Characterization of WRSs2 and WRSs3, new second-generation *virG(icsA)*-based *Shigella sonnei* vaccine candidates with the potential for reduced reactogenicity

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Abstract

Live, attenuated *Shigella* vaccine candidates, such as *Shigella sonnei* strain WRSS1, *Shigella flexneri* 2a strain SC602, and *Shigella dysenteriae* 1 strain WRSd1, are attenuated principally by the loss of the VirG(IcsA) protein. These candidates have proven to be safe and immunogenic in volunteer trials and in one study, efficacious against shigellosis. One drawback of these candidate vaccines has been the reactogenic symptoms of fever and diarrhea experienced by the volunteers, that increased in a dose-dependent manner. New, second-generation *virG(icsA)*-based *S. sonnei* vaccine candidates, WRSs2 and WRSs3, are expected to be less reactogenic while retaining the ability to generate protective levels of immunogenicity seen with WRSS1. Besides the loss of VirG(IcsA), WRSs2 and WRSs3 also lack plasmid-encoded enterotoxin ShET2-1 and its paralog ShET2-2. WRSs3 further lacks MsbB2 that reduces the endotoxicity of the lipid A portion of the bacterial LPS. Studies in cell cultures and in gnotobiotic piglets demonstrate that WRSs2 and WRSs3 have the potential to cause less diarrhea due to loss of ShET2-1 and ShET2-2 as well as alleviate febrile symptoms by loss of MsbB2. In guinea pigs, WRSs2 and WRSs3 were as safe, immunogenic and efficacious as WRSS1.

Keywords

Shigella sonnei; Live attenuated vaccine candidates; WRSs2; WRSs3

1. Introduction

Shigella causes bacillary dysentery, an inflammatory gastrointestinal disease affecting the distal regions of the colon and the rectum. As few as 10–100 bacteria can cause disease in some volunteers that is characterized by acute abdominal pain, tenesmus, fever, nausea, vomiting, diarrhea and dysentery, which is small volume stools with blood and mucus. Human to human transmission of bacteria occurs via the fecal-oral route, but food-borne

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episodes have also been described. In healthy adults shigellosis is usually self-limiting and lasts for 7–10 days, but if left untreated, the disease can cause considerable morbidity and mortality in immunocompromised adults, in infants and children in developing countries where shigellosis is endemic [1–5]. All four serogroups, *S. flexneri*, *S. sonnei*, *S. boydii* and *S. dysenteriae* can cause dysentery, provided they contain a large, non-conjugative ~215 kb virulence plasmid that encodes critical factors necessary for epithelial cell invasion and spread of the bacterium within the host tissue. *S. sonnei*, also known as Group D *Shigella*, is the serogroup most frequently responsible for sporadic and epidemic enteritis in developed countries (CDC 2006) [6–9]. In the US, 70% of shigellosis cases are due to *S. sonnei* [10]. In recent years, *S. sonnei* has also emerged as the most prevalent *Shigella* species in newly industrialized countries [11–16]. An outbreak of *S. sonnei* in Taiwan during 2001–2003 was attributed to a foreign strain; however this strain has caused recurrent outbreaks [11]. *S. sonnei* has replaced *S. flexneri* as the predominant *Shigella* species in Taiwan. In 1996, 2002, 2003 and 2007 outbreaks of *S. sonnei* resistant to ampicillin and TMP-SM occurred in children in north Paris related to secondary transmission similar to an earlier outbreak in N. America [17–19]. In young children less than 5 years old, *S. sonnei* has been recently shown to infect more frequently than other species [20,21]. Compounding the problem is the isolation of multidrug resistant epidemic strains due to the spread of integrons [7,11,13,15,22–26].

Owing to the wide range of *Shigella* serotypes and subtypes, there is a need for a multivalent vaccine representing prevalent species and serotypes. However, apart from a live, noninvasive *S. flexneri* 2a–*S. sonnei* bivalent vaccine used in China, there are currently no licensed vaccines either in the US or elsewhere. Several different types of vaccines against *Shigella* have been experimentally tested in animal models and in volunteer trials. Each approach provides important information regarding the strategy used as well as safety, and immunogenicity data [27–38]. Based on protection data, it is generally believed that repeated exposure to *Shigella* from the environment eventually produces serotype-specific immunity. The primary antigen is the bacterial lipopolysaccharide (LPS), which is an integral component of the gram negative bacterial cell wall. The variable O-antigen repeat region of the LPS provides serotypic specificity to the immune response. WRSS1, a live attenuated *S. sonnei* vaccine, has recently completed Phase I trials in the US and in Israel and shown to be safe and highly immunogenic [39,40]. Although WRSS1 has not been tested for efficacy, the immune responses in vaccinated volunteers were comparable to the immune responses seen in SC602-vaccinated volunteers who were protected against challenge [28]. The principal attenuating feature of WRSS1, as well as SC602, is the loss of the virulence plasmid-encoded VirG(IcsA) function. VirG(IcsA) nucleates host cell actin which provides the motive force for the movement of the bacteria within the host cell. Loss of VirG(IcsA) results in a significantly attenuated bacterial strain whose phenotype can be scored in plaque assays, and in guinea pigs and monkey models of disease [28,32,41–44]. Safety, immunogenicity and efficacy studies with SC602 have provided proof of concept for this approach [45,46]. Further substantiation of this strategy has been obtained from clinical trials of WRSS1 and *S. dysenteriae* 1 vaccine candidate WRSd1 [39,40,47,48].

Around 15–20% of volunteers who were orally immunized with WRSS1 and SC602 at 10⁴ CFU developed short-term mild diarrhea and fever. These symptoms increased with higher doses. Since the initial trials with SC602, WRSS1 and WRSd1, novel iron-regulated enterotoxins (ShET1 and ShET2) have been reported to be present in *Shigella* [49–51]. It is believed that these enterotoxins elicit secretion early in the infection as the organisms pass thru the jejunum. The presence of these enterotoxins have also been implicated in the symptoms of watery diarrhea that often precede the onset of dysenteric stools during shigellosis and in mild cases of the disease, may represent the only symptom [52]. These

enterotoxins represent additional targets for the development of safer, second-generation vaccine candidates [32,53–55].

ShET1 is a chromosomally-encoded 55 kDa protein complex, predominantly seen in *S. flexneri* 2a strains and is responsible for 60–65% of the total enterotoxigenic activity in this strain [49]. ShET1 is encoded by two contiguous genes, *setIA* and *setIB*. The holotoxin is predicted to exist in an A1–B5 configuration. The enterotoxigenic nature of the activity was confirmed by demonstrating an electrical response in Ussing chambers and in rabbit ileal loops although its exact mechanism of action is unknown [49,51]. ShET2, encoded by *sen*, is present on the *Shigella* virulence plasmid and is therefore detected in all serotypes [50]. ShET2 (molecular weight 63.1 kDa) had initially been described as an iron-regulated enterotoxigenic activity in enteroinvasive *Escherichia coli* (and termed EIET for enteroinvasive enterotoxin) from where it was cloned and subsequently shown by hybridization to have a homolog in *Shigella* where it was named ShET2 [50]. Subsequently, sequencing of the virulence plasmid demonstrated a ShET2 paralog which was referred to as ShET2-2 [55]. The original plasmid-encoded ShET2 enterotoxin is now referred to as ShET2-1 (or SenA) to distinguish it from ShET2-2 (or SenB). ShET2-1 (568 aa) and ShET2-2 (533 aa) are similar in size and share 40% identity over the length of the protein, although the enterotoxigenic activity of ShET2-2 remains to be demonstrated. ShET2-1 and ShET2-2 are alternately referred to as OspD3 and OspD2, respectively [53]. The genes encoding ShET2-1 and ShET2-2 will be referred to here as *senA* and *senB*, respectively. In volunteer trials, a *S. flexneri* 2a vaccine candidate CVD1208, with deletions of *guaBA*, *setAB*, and *senA* genes eliminated diarrheal symptoms compared to the isogenic *guaBA* mutant alone CVD1204, demonstrating the combined functional activity of the ShET1 and ShET2-1 enterotoxins in a natural host [54].

Besides the enterotoxins, another target for improving the reactogenicity of live *Shigella* vaccines, is the highly endotoxic lipid A moiety of the bacterial LPS. Lipid A is a known pyrogen and most likely contributes to the febrile symptoms seen in some volunteers [56]. Lipid A consists of a mono- or bi phosphorylated glucosamine disaccharide backbone that is variably acylated with C12–C14-fatty acids. The 4 + 2 hexacylated form of lipid A, normally present in *Shigella*, is the most endotoxic, while pentacylated and tetracylated forms of lipid A are less endotoxic [57,58]. The *msbB* gene, normally encoded on the bacterial chromosome, encodes a fatty acyl transferase that catalyzes the acyl-oxyacyl linkage of myristate to a hydroxymyristate that is linked to the 3' position of the glucosamine disaccharide [59–61]. *E. coli* *msbB* mutants show lower levels of hexacylated lipid A resulting in a dramatic decrease in the endotoxicity of the molecule, as demonstrated by reduced TNF- α production by infected macrophages and lower induction of selectin expression by endothelial cells [61]. Unlike *E. coli*, *Shigella* has two *msbB* genes, one on the chromosome, *msbB1*, and the second on the virulence plasmid, *msbB2*, a feature *Shigella* shares with EHEC O157:H7 [62]. Loss of either gene in *Shigella* results in lesser abundance of hexacylated lipid A [58]. The purpose of having two *msbB* genes is presumably to ensure the hexacylation of lipid A, although the two genes are differentially regulated, a feature that might hold some physiological significance [63]. In a rabbit ileal loop model of infection, *S. flexneri* 2a *msbB1* or *msbB2* mutant show lesser pathology of the intestinal epithelium with no evidence of rupture of the epithelial lining characteristically seen with wild-type strains. The *msbB1* *msbB2* double mutant demonstrated even further attenuation [58]. Mice immunized intranasally with 2457T *msbB1* or *msbB2* mutants show significant reduction in morbidity and expression of proinflammatory cytokines in lung washes confirming previous observations [85].

S. sonnei live vaccine candidates WRSs2 and WRSs3 have been constructed that, in addition to VirG(IcsA), also lack the plasmid-encoded enterotoxin ShET2-1 and its paralog ShET2-2.

WRSS3 further lacks MsbB2. In healthy adult volunteers WRSS1, attenuated by the loss of VirG(IcsA) alone, was generally safe and highly immunogenic at 10^4 colony forming units (CFU) dose, but more reactogenic at higher doses. Based on the attenuating mutations and the behaviour in animal models, WRSS2 and WRSS3 are expected to be significantly less reactogenic, thereby providing a wider window of safety while retaining the immunogenicity of WRSS1.

2. Materials and methods

2.1. Bacterial strains, growth conditions, and cell culture

WRSS2 and WRSS3 were constructed from the parent *S. sonnei* strain Moseley, which was isolated in 1975 from an infected laboratory worker (bacterial strains and plasmids used are listed in Table 1). The strain was the source for a GMP (Good Manufacturing Procedures) product developed at the Walter Reed Army Institute of Research Pilot Bioproduction Facility (WRAIR-PBF) in June 2000. A vial of the Production Cell Bank (PCB) of Moseley (lot # 0794) was the source for the construction of WRSS2 and WRSS3 (Table 1). WRSS1 represents the first generation *S. sonnei* live, vaccine candidate with a 212 bp deletion of the *virG(icsA)* gene [47]. WRSS1 vials for this study were obtained from the WRAIR-PBF (lot # 0433). *S. sonnei* strain 53G (lot # 0593) and *S. flexneri* 2a strain 2457T (lot # 0590) were obtained from individual Master Cell Bank (MCB) and PCB vials obtained from the WRAIR-PBF. BS103 is a plasmid-cured 2457T strain, which gives white colonies on Congo Red (CR) plates and is noninvasive in epithelial cells. *Shigella* strains were routinely propagated in Luria-Bertani (LB) Miller or Tryptic Soy Broth (TSB) media and plated on LB agar or Tryptic Soy agar (TSA) plates or TSA containing 0.2% galactose and 0.05% (v/w) Congo Red (CR plates).

2.2. Cell lines, antigens and antibodies

HeLa (CCL-2) cells, Baby Hamster Kidney (BHK-21[C-13]) cells, RAW 264.7 murine macrophages were from ATCC (Virginia, USA). Monoclonal antibodies to IpaB (2F1) and to IpaC (2G2) have been previously described [68] and were obtained from Dr. Ed Oaks at WRAIR. *S. sonnei* antiserum (Group D) was obtained from Difco (Difco Laboratories, MI) and Denka Seiken (Denka Seiken Co. Tokyo, Japan). Purified *S. sonnei* LPS and *S. sonnei* Invaplex (Invaplex 50) were obtained from Dr. Edwin Oaks at WRAIR [31]. Invaplex is a complex purified from water extracts of *Shigella* and is composed mainly of bacterial LPS and the Ipa proteins IpaB and IpaC.

2.3. Plasmids

Plasmids pKD3, pKM208, and pCP20 were vectors used for lambda red recombineering in *Shigella* strains and have been previously described (see Table 1) [30,64]. Plasmid pKM208 is a low-copy number, temperature-sensitive replicon that carries bacteriophage λ red genes (γ , β , and *exo*) (Table 1). Plasmids pKD3 is a π -dependent plasmid carrying chloramphenicol-resistance gene (*cam*), flanked by the recognition sites (FRT sites) of the yeast FLP recombinase in direct repeats. Plasmid pCP20 is a temperature-sensitive replicon expressing the FLP gene. Plasmid pSUB11 carries a kanamycin resistance gene (KmR) flanked by FRT sites and a 24 nucleotide (nt) 3XFLAG sequence [65].

2.4. Construction of FLAG-tagged ShET2-1 and ShET2-2

The 3XFLAG epitope tags were introduced into the C-terminal ends of ShET2-1 and ShET2-2, using pSUB11 and the method of recombination tagging protocol using the lambda red recombination system [30,65]. A DNA cassette that begins with the 3XFLAG sequence and includes an antibiotic-resistance cassette for kanamycin (KmR) flanked by

FRT sites was amplified using pSUB11 as template and with primers carrying extensions (36–40 nt, see supplemental Table S1 for primer sequences used for construction) homologous to the region immediately preceding the translation stop signal of the target gene (*senA* and *senB*) and to a region downstream from it. Linear DNA fragments containing the 3XFLAG epitope and a KmR cassette were PCR-amplified from the plasmid pSUB11 and electroporated into 2457T and Moseley containing pKM208. Recombinant bacteria synthesize the target protein with the epitope sequence fused to its carboxy terminus. Putative FLAG-tagged recombinants were analysed by PCR and by carrying out secretion assays.

2.5. Secretion of ShET2-1 and ShET2-2

Bacterial cultures containing FLAG-tagged enterotoxins were grown to log phase in DMEM at 37 °C, treated with Congo Red (0.2 mg/ml final concentration) and incubated for another 3 h at 37 °C. Culture supernatants were filtered to remove bacteria, proteins precipitated with trichloroacetic acid (final concentration 10%), washed with acetone and electrophoresed on SDS-PAGE gels. The proteins were transferred to nitrocellulose and blotted with anti-FLAG M2 monoclonal antibody (Sigma–Aldrich). Alkaline-phosphatase-conjugated anti-mouse IgG (Sigma–Aldrich) was used as the secondary antibody and the blots were developed with Fast Red TR/Naphthol AS-MX (Sigma–Aldrich). The lambda red recombineering technique was also used to delete *ipaB* from 2457T to yield 2457TΔ*ipaB*. Secretion assays were also carried out with 2457TΔ*ipaB* mutants containing FLAG-tagged ShET2-1 and ShET2-2 to observe constitutive secretion of bacterial proteins regulated by the TTSS.

2.6. Construction of WRSs2 and WRSs3

The construction of WRSs2 and WRSs3 schematically represented in Fig. 1 involved a series of gene deletions using the technique lambda red recombineering described in detail elsewhere [30,64]. Briefly, *S. sonnei* Moseley was transformed with the temperature sensitive plasmid pKM208, expressing the lambda red genes (*gam*, *bet*, *exo*). Linear DNA was prepared by PCR amplification of the chloramphenicol-resistant gene (*cam*) flanked by FRT sites from plasmid pKD3 with primers containing ≥50 bp sequence homology to the 5' and 3' sequences flanking the gene to be deleted (see supplemental Table S1 for lambda red recombination primers used for individual gene deletions). Electroporation of the linear DNA into Moseley(pKM208) resulted in replacement of the targeted gene with the *cam* cassette. In the next step the *cam* cassette was removed by transient expression of the yeast FLP recombinase using the temperature sensitive plasmid pCP20. PCR analysis with gene-specific primers was used to confirm the loss of each gene and PCR reactions with sequences flanking the deleted genes were used to ensure that the flanking sequences remained intact after the recombination events. This protocol was used sequentially to delete *senA*, *senB* and *virG(icsA)* to generate WRSs2-tet^R (Fig. 1). The *tet* resistance, originally present in the parent strain Moseley was removed from WRSs2-tet^R by growing it in fusaric acid as previously described [47]. To replace the *msbB2* gene from WRSs2, a longer linear DNA fragment was amplified from a 2457T mutant that had its *msbB2* gene replaced by a *cam* cassette. PCR-amplified linear DNA bearing 50 bp homology to sequences flanking the Moseley *msbB2* gene did not yield transformants when electroporated into WRSs2(pKM208). The set of primers used for the amplification of the longer linear DNA (using *msbB2* extended homology primers in Table S1) had homology to sequences ~500 bp upstream and ~100 bp downstream of the *msbB2* gene on the 2457T plasmid. This longer ~1.75 kb PCR-amplified DNA construct was electroporated into WRSs2(pKM208) and successfully replaced its *msbB2* gene to generate WRSs3. Template DNA for PCR analysis to confirm individual gene deletions, determine presence of 5' and 3' open reading frames (ORFs) flanking the deleted genes as well as epitope tagging were done using lysates made

from single colonies of individual strains. Single colonies were suspended in 100–200 μ l of sterile water, boiled for 10 min, centrifuged and the supernatant used in PCR reactions. Supplemental Table S2 lists the primers used for confirming gene deletions and for integrity of 5' and 3' ORFs flanking the deleted genes.

2.7. HeLa cell invasion assay

The HeLa cell invasion assay was performed as previously described [32]. Cultures of *Shigella* were grown aerobically at 37°C in LB broth overnight (12–16 h), then subcultured until an OD₆₀₀ of 0.3–0.6 was reached. *Shigella* cultures at a multiplicity of infection (moi) of 10 were added directly to the HeLa cells in 24-well tissue culture plates, the plates centrifuged for 5 min at 3000 rpm and incubated initially for 1.5 h in a CO₂ incubator at 37 °C followed by a washing procedure and further 2 h incubation in the presence of 50 μ g/ml gentamicin. The HeLa cells were washed again and lysed with 0.1% Triton X-100 (v/v) and dilutions of the lysate were plated for recovery of intracellular bacteria.

2.8. Plaque assay

The plaque assay was performed as previously described using BHK cells [32]. The plaques were visualized under a microscope.

2.9. LDH and cytokine release assays

RAW 264.7 murine macrophages (ATCC TIB-71) were maintained and passaged in DMEM containing 10% heat-inactivated FBS (Gibco). For the experiments described herein, macrophages were between passage 3 and 20 from the original ATCC stock. For *Shigella* infections, macrophages were seeded into 24-well plates in DMEM/10% FBS at $\sim 10^6$ cells/well and incubated overnight at 37°C in a humidified, 5% CO₂ atmosphere for infection the following day. Cultures of *Shigella* were grown aerobically at 37 °C in LB Miller broth overnight (12–16 h), then subcultured until an OD₆₀₀ of 0.3–0.6 was reached. Macrophages were infected at an MOI of 10 by adding 10^7 *Shigella* directly to appropriate wells, then centrifuging the plates at $\sim 250 \times g$ for 5 min at ambient temperature to synchronize cell contact. The plates were then incubated at 37 °C in a humidified 5% CO₂ atmosphere for 25 min. Subsequently, all wells were washed three times with pre-warmed PBS to remove extracellular bacteria, then incubated as above with fresh, pre-warmed DMEM/10% FBS containing 100 μ g/ml gentamicin to inhibit re-infection; this was defined as the start of infection. 30 min later, the medium was replaced with fresh, pre-warmed DMEM/10% FBS containing 10 μ g/ml gentamicin to reduce bactericidal effects on intracellular bacteria. At 2 and 4 h post-infection, culture supernatants were removed for subsequent analysis and stored at 4 °C (LDH assay) or –35 °C (ELISA for TNF α).

Shigella-induced cytotoxicity on RAW 264.7 macrophages was assessed using the Cytotox 96 Non-Radioactive Cytotoxicity Assay kit (Promega) to quantify lactate dehydrogenase (LDH) activity in culture supernatants. The assay was performed according to the manufacturer's protocol. The effect of *Shigella* infection on TNF α release from RAW 264.7 macrophages was assessed by sandwich ELISA using the DuoSet ELISA Development System (R&D Systems). The assay was performed according to the manufacturer's protocol, except that the capture antibody was used at 1.2 μ g/ml.

2.10. LPS extraction and mass spectroscopic analysis of lipid A

LPS was extracted from bacterial strains by previously described methods as well as using LPS extraction kits from Commonwealth Biotechnologies Inc., Richmond, VA [66]. To carry out lipid A mass spectral analyses, bacterial strains were grown in LB medium overnight at 37 °C, the cells pelleted by centrifugation, washed in cold acetone, and frozen

on dry ice. A portion of the dry pellet (10 mg) was used to extract lipid A as described ([67]). Briefly, pellets were suspended in isobutyric acid–ammonium hydroxide 1 M (5:3, v/v; 400 μ l) and kept for 2 h at 100 °C in a screw-cap (Teflon-lined) glass vial with magnetic stirring. The mixture was cooled on ice, transferred to a tube (0.6 ml) and centrifuged (2000 $\times$ g for 15 min). The supernatant was subsequently transferred to another tube, diluted with water (1:1, v/v), and lyophilized. The resulting sample was washed twice with methanol (400 μ l) and extracted with chloroform–methanol–water (3:1.5:0.25, v/v; 200 μ l). After desalting with ion-exchange resin (Dowex 50W-X8, H⁺-form), an aliquot was spotted onto a MALDI plate with either dihydroxybenzoic acid (DHB) or 5-chloro-2-mercaptobenzothiazole as matrix. Mass spectral analyses were performed in the negative ion mode utilizing an Applied Biosystems/MDS-Sciex 4800 MALDI-TOF/TOF analyzer.

2.11. Plasmid stability tests of *S. sonnei* strains

10 Form I colonies of each *S. sonnei* strain were isolated from a TSA plate and inoculated into 2.5 ml TSB at 30 °C and subcultured (1:100 dilution in fresh media) every 24 h up to 72 h. During each subculture, aliquots were diluted and plated on three TSA plates that were incubated at 37 °C for 16 h. The colonies on the plates were counted and then scored for Form I and II phenotypes by observing the colonies under a light microscope. Form I colonies were small and round with a smooth edge while Form II colonies represented variant colonies which were larger and flat with irregular edges. For evaluating the conversion of Form I colonies to Form II after plating on TSA plates, five isolated Form I colonies of each strain were inoculated into TSB and grown at 30 °C to an OD₆₀₀ of 1.0. Appropriate dilutions (10⁻⁶) were plated on TSA plates for growth at 37 °C. After 12, 14, 16, and 18 h of growth on the plates, the colonies were counted and scored microscopically. The colonies on each plate were then lifted onto nitrocellulose filters for colony immunoblot assay with MAb to IpaB as previously described [32].

2.12. Oral challenge of gnotobiotic (GB) piglets with *S. sonnei* strain Moseley and Moseley2 Δ ShET2

The gnotobiotic (GB) piglet model [68] is being developed as a small animal model to study shigellosis since oral challenge of these animals with *Shigella* produces diarrhea (Jeong and Tzipori, unpublished data). Briefly, 3-day-old piglets, maintained throughout the experiment in sterile microbiological isolators, were orally inoculated with 3 ml of a bacterial suspension in phosphate-buffered saline (PBS) containing either 5 $\times$ 10⁹ CFU of Moseley (starting with n = 13) or the Moseley2 Δ ShET2 strain (starting with n = 11). Fecal consistency was scored daily for 7 days after inoculation and on some post-inoculation days (PID) one to four animals were sacrificed for other determinations (data not included in this study). Daily mean fecal scores were recorded based on the fecal consistency from PID1 to PID7. Fecal scoring standards are: 0, normal; 1, pasty; 2, semi-liquid; 3, watery. Scores above 2 were considered diarrheic. Each piglet was observed three times daily to assess fecal scores (diarrhea) and average score was kept for each piglet. Later those respective daily scores were averaged for daily group scores. Pigs that were sacrificed for other determinations were given a numeric score that represented the average group score for that day. The data was a composite of two individual experiments and the number of piglets that were observed from PID1 to PID7 were 13, 10, 6, 5, 5, 5, 5 for Moseley and 11, 9, 9, 7, 7, 7, 6 for Moseley2 Δ ShET2. Statistical analysis of the data was carried out using a mixed model procedure with the aid of the SAS statistical software. The treatment (Moseley vs. Moseley2 Δ ShET2) and PID were set as fixed effects and the individual pigs were treated as a random effect. The analysis looked for 2-way interactions between pig ID and the treatment.

2.13. Safety, immunogenicity and efficacy studies in guinea pigs

S. sonnei strains used for immunization and challenge assays were prepared by spreading 8–10 Form I colonies obtained from an invasion assay, uniformly onto a LB agar plate. The plated bacteria were grown to confluence for 24 h at 37 °C. 4 ml of normal saline was added to each plate and the bacteria were harvested by gently scraping the plates with a 10 µl loop. The concentration of the suspension was adjusted to about 2×10^{10} CFU/ml using OD₆₀₀ measurements of serially diluted samples. Male Hartley guinea pigs (200–250 g) were sedated with 2:1 mixture of ketamine and xylazine, and 25 µl of the bacterial suspension in normal saline (2×10^8 CFU) were administered to each eye of the guinea pig. Animals were immunized twice, on days 0 and 14. The eyes were observed for disease and after each inoculation a safety evaluation was done (Sereny test). On day 42 the animals were sedated and $1\text{--}2 \times 10^8$ CFU of virulent *S. sonnei* strain 53G in 25 µl of normal saline was inoculated into the conjunctival sac of each eye of the immunized guinea pigs and only one eye of unimmunized control animals. The guinea pig eyes were observed for disease over several days and the level of inflammation observed after challenge was scored as follows: 0, no inflammation or mild irritation; 1, mild keratoconjunctivitis or clearing; 2, keratoconjunctivitis without purulence; and 3, severe keratoconjunctivitis with purulence. Percent protection was calculated by the following formula: $[\% \text{ disease in controls} - \% \text{ disease in vaccines}] / (\% \text{ disease in controls}) \times 100$. *Animal research was conducted under a WRAIR IACUC-approved protocol in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adhered to the principles stated in the Guide for the Care and Use of Laboratory Animals.*

2.14. Immune response measurements in guinea pigs

Blood and eye washes were collected before and after immunization (day 0, 14, 28) and after challenge (CLG) for analysis of humoral and mucosal immune responses. Eye washes were obtained by washing the conjunctival sacs of guinea pigs eyes with 100 µl of cold PBS. Serum and eye wash antibody levels against purified *S. sonnei* LPS and *S. sonnei* Invaplex were determined using an enzyme-linked immunosorbent assay (ELISA) as previously described [32]. End-point titers for each animal were defined as the reciprocal of the dilution at which the average OD₆₅₀ value was greater than the mean plus 10 standard deviations of values from preimmune samples or 0.1, whichever was greater.

2.15. Statistical analysis

Statistical analyses of individual experiments are described in the text or in the figure legends where appropriate. For analysis of immunogenicity in guinea pigs, the geometric mean titers (GMTs) for each group of guinea pigs was calculated using Prism 5 (Graph-pad Software, Inc) with 95% confidence intervals. Different groups were compared with one-way analysis of variance (ANOVA) using Bonferroni's multiple comparison test with significance level of $P < 0.05$. A statistical comparison of the guinea pig protection data was determined using a Fisher's exact test. P values of <0.05 were considered significant. For the plasmid stability tests statistical significance calculation was carried out using ANOVA Bonferroni multiple test with $P < 0.05$ or confidence of 95%.

3. Results

3.1. Secretion of ShET2-1 and ShET2-2 in Shigella culture supernatants

ShET2-2 was recognized as a paralog of ShET2-1 during invasion plasmid sequencing efforts [53,55]. In order to evaluate ShET2-2 expression and secretion and to compare it to ShET2-1, the genes for both proteins were tagged with 3XFLAG in Moseley and 2457T (see Table 1). FLAG-tagged 2457T and Moseley strains were grown in DMEM media at 37 °C

and the culture supernatants were examined for the FLAG-tagged proteins in the absence and presence of Congo Red, a dye that induces TTSS-based secretion. Culture supernatants revealed the presence of both ShET2-1 and ShET2-2 in 2457T and Moseley strains where the individual enterotoxins were tagged with the 3XFLAG epitope, indicating that both proteins are secreted (Fig. 2A and C). Addition of Congo Red induced the secretion of both enterotoxins. In a $\Delta ipaB$ background, such as seen in 2457T $\Delta ipaB$, both ShET2-1 and ShET2-2 were secreted constitutively, as previously demonstrated for other TTSS-based *Shigella* effector proteins (Fig. 2B). In these studies, ShET2-2 appeared less abundant than ShET2-1 in both Moseley and 2457T culture supernatants.

3.2. Clinical symptoms in gnotobiotic (GB) piglets challenged with *S. sonnei* strains

In order to demonstrate the contribution of the enterotoxins to *Shigella*-induced diarrhea in an *in vivo* model, Moseley and Moseley deleted of ShET2-1 and ShET2-2 (Moseley2 Δ ShET2) were tested in GB piglets. GB piglets orally infected with virulent *Shigella* get diarrhea, mimicking features of human shigellosis. These animals are therefore being evaluated for use as a small animal model for *Shigella*-induced diarrhea. When 3-day-old piglets were orally administered 5×10^9 CFU of Moseley, piglets showed moderate anorexia and dehydration from PID1 to PID3, and moderate to severe diarrhea (fecal scores ≥ 2) at PID2–PID4, followed by a recovery stage (Fig. 3). Anorexia was determined by a significant decrease in milk volume taken in by the animals and dehydration was evaluated by a shabby/coarse skin condition. The fecal scores obtained with Moseley2 Δ ShET2 were significantly lower than Moseley over the course of the study ($P < 0.0001$) indicating that the double mutant is less diarrheagenic in this model compared to the wild-type strain (Fig. 3).

3.3. Construction of *S. sonnei* vaccine candidate WRSs2 and WRSs3

WRSs2 and WRSs3 were constructed through a series of sequential gene deletions in Moseley using lambda red recombineering (Fig. 1, strains and primers used shown in Table 1 and Table S1). Since the parent strain Moseley is resistant to tetracycline, WRSs2-tet was plated on fusaric acid plates for removal of tetracycline resistance [69]. Care was taken to ensure that primers used to determine the presence of upstream and downstream ORFs flanking the deleted genes were not within IS elements, since these were multiply repeated in the virulence plasmid and often were the immediate sequences present on either side of the four genes that were deleted. Thus, *virA* and *ushA*, *ospC1* and *ipaH7.8*, *phoN2* and *mkaD* were the upstream and downstream ORFs for *virG(icsA)*, *senA*, and *senB* genes respectively, whose presence was confirmed by PCR and used to demonstrate that the sequences flanking the deleted genes were present and remained unaltered in size (supplemental Fig. S1). The *msbB2* gene in *Shigella* is situated within the *shf-wabB-virK/ecf3-msbB2* locus on the virulence plasmid. The primer sequences corresponding to the upstream and downstream ORFs in this case were in the gene *virK* and in the gene SSOP183 [70]. PCR analysis with the appropriate primers (supplemental Fig. S1, panel D) clearly show the loss of the *msbB2* gene in WRSs3.

3.4. Characterization of WRSs2 and WRSs3

Epithelial cell invasiveness is a critical feature of *virG(icsA)*-based live vaccine candidates. Both WRSs2 and WRSs3 were positive in a HeLa cell gentamicin-based invasion assay (Fig. 4A). The number of CFU of WRSs2 and WRSs3 recovered after epithelial cell lysis was consistently higher than the CFU obtained after infection with either Moseley or WRSs1 (Fig. 4A). As expected for strains lacking the *virG(icsA)* gene, both WRSs2 and WRSs3 were plaque negative in BHK cells while 2457T and Moseley form well-developed normal sized clear plaques, indicating VirG(IcsA)-based intercellular dissemination (data not shown).

The profile of LPS extracted from Moseley, WRSS1, WRSs2 and WRSs3 were identical on silver stained SDS-PAGE gels indicating that no observable changes occurred to the LPS during the construction of the new strains (data not shown). The lipid A profile from WRSs3 was compared to the parent strain to determine the effect of MsbB2 on its acylation status. MS characterization of lipid A was carried out with cultures grown in LB medium at 37 °C and frozen in dry ice. Lipid A was extracted by a previously published method [67]. The ions between 1300 and 1900 m/z are lipid A (Fig. 4B). The ions above 2400 m/z are probably LPS (not shown in figure). The MS data revealed that both Moseley and WRSs3 were predominantly hexaacylated (1797.8 m/z). However, the WRSs3 lipid A profile differed from the Moseley profile in that it contained a pen-taacylated product consisting of four 3-hydroxymyristate residues, one laurate, and two phosphates. Fragmentation data supported the presence of a pyrophosphate moiety on this structure as well (data not shown) [71].

In tissue culture experiments with RAW 264.7 mouse macrophages, WRSs2 and WRSs3 as compared to Moseley (moi of 10) induced higher levels of cytotoxicity at 2 h post-infection, although by 4 h post-infection these differences were not statistically significant (Fig. 4C). However, as expected for a strain with lesser endotoxic lipid A, the levels of TNF α in culture supernatants of WRSs3-infected macrophages were significantly lower than levels seen in Moseley and WRSs2 infected cells, although the differences with WRSs2 was not statistically significant (Fig. 4D). At lower moi of 2 and 0.2, the differences in TNF α secretion between Moseley and WRSs3 were less evident (data not shown).

3.5. Safety and immunogenicity of WRSs2 and WRSs3 in guinea pigs

Wild-type *Shigella* strains such as Moseley, when administered ocularly to guinea pigs, induce an acute keratoconjunctivitis reaction. This reaction, known as the Sereny reaction or Sereny test, is used as a measure of virulence of the bacterial strain [72]. The Sereny test measures the capability of the bacteria to invade, multiply and disseminate within the epithelium, causing an intense inflammatory reaction. Loss of VirG(IcsA) results in a Sereny negative reaction as was seen with WRSS1 and other VirG(IcsA)-mutants of *Shigella*. WRSs2 and WRSs3 are also negative in the Sereny test. Unlike Moseley infected eyes, no sign of irritation or inflammation of the conjunctiva or cornea was seen in guinea pig eyes infected with WRSs2 or WRSs3 (data not shown).

In an efficacy study, guinea pigs were immunized ocularly and challenged 4 weeks later with virulent *S. sonnei* strain 53G (53G is used as a *S. sonnei* challenge strain in human trials). The guinea pig eyes were monitored daily after challenge for the development of a reaction, the intensity of which was monitored for 7 days and scored according to a pre-established grading method (ranging from 0 to 3) described in Section 2 (Fig. 5, Table 2). In the control group of unimmunized animals, full blown keratoconjunctivitis was observed within 2–3 days after challenge with 53G (Fig. 5 and Table 2). In contrast, in animals immunized with WRSS1, WRSs2, or WRSs3, very low levels of disease was seen over the 7-day duration period (Fig. 5 and Table 2). The protective efficacy for the three vaccine strains was calculated to be >90% (Table 2). Only two eyes in the WRSS1 immunized group received a score of 2 after challenge during the observation period (Table 2).

Blood was collected for evaluation of serum antibody responses to *S. sonnei* LPS and Invaplex. Invaplex is a complex of LPS and predominantly IpaB and IpaC proteins isolated from water extracts of *S. sonnei*, major antigens recognized after natural infection with *Shigella* providing enhanced sensitivity with ELISAs [31]. Serum GMTs of LPS-specific and Invaplex-specific IgG and IgA were very similar across the three vaccine candidates, indicating that WRSs2 and WRSs3 elicited comparable levels of humoral immune responses

in guinea pigs as WRSS1 (Fig. 6). After challenge, the serum antibody responses of immunized animals were even higher indicating a booster response (Fig. 6).

Eye washes from guinea pigs were used to evaluate mucosal antibody responses (Fig. 7). Day 14 titers indicate that WRSS1 induced a robust mucosal antibody response against LPS and Invaplex (GMT of 224 and 64, respectively), and this response was significantly higher than WRSs2 against both antigens (GMT of 49 and 30, respectively) and higher than WRSs3 for Invaplex antigen (GMT = 35). However, no significant differences in titers were observed between the WRSS1 and WRSs3 immunized animals against LPS (WRSs3 GMT = 79) and between WRSs2 and WRSs3 immunized guinea pigs (Fig. 7). Day 28 levels were similar across all three strains.

3.6. Stability of vaccine strains

Starting from isolated Form I colonies, overnight cultures of WRSS1, WRSs2 and WRSs3 were grown in TSB and subcultured every 24 h for 72 h. During each subculture, an aliquot was plated on TSA plates to determine the stability of the Form I phenotype (Fig. 8A). In these studies, the percentage of Form II colonies over 72 h was determined to be ~11% and 8% for WRSs2 and WRSs3, respectively (with no significant differences), while the corresponding values for Moseley and WRSS1 were ~4% (significantly lower than WRSs2 and WRSs3). The ratio of Form I to Form II colonies within each culture remained constant over the 72 h of subculture and growth (Fig. 8A).

Previous studies have indicated that the conversion of Form I to Form II occurred after plating on solid agar media, and that in liquid culture, the predominant phenotype is Form I [73]. To examine this, cultures of Moseley, WRSS1, WRSs2 and WRSs3, were diluted and plated on TSA plates, and these plates were incubated at 37 °C for different lengths of time (Fig. 8B). With increasing times of incubation, the number of Form II colonies on plates with WRSs2 and WRSs3 appeared 2–3-fold higher than plates containing Moseley and WRSS1. No significant differences were seen between Moseley and WRSS1, while the differences between Moseley and WRSS1 strains versus WRSs2 and WRSs3 strains were significant. However, colony immunoblots with IpaB monoclonal antibody of the colonies on the same plates showed that, the number of colonies positive for IpaB remained more or less constant for each type of culture over the length of the incubation period and no significant differences were seen between the different strains at 12 and 18 h of incubations (Fig. 8C).

4. Discussion

WRSs2 and WRSs3 represent second-generation live, oral *S. sonnei* vaccine candidates primarily attenuated by the loss of the *virG(icsA)* gene. These two candidates have been manufactured under cGMP conditions and will be tested in Phase I trials in the near future. Other *S. sonnei* vaccine candidates that have undergone clinical trials include an orally delivered, formalin-inactivated whole-cell vaccine that was given as a 3-dose or a 5-dose regimen, each dose containing 2.0×10^{10} inactivated cells [74]. The vaccine was safe and immunogenic, and expanded studies are being planned to evaluate this strategy. *S. sonnei* conjugate vaccines composed of purified O-antigens conjugated to mutant exoprotein A of *P. aeruginosa* or *C. diphtheriae* toxin mutant have demonstrated safety and immunogenicity in adults and children in Israel [36,75,76]. Further modifications of the product are being examined to increase the potency in infants and children. The licensed *Shigella* vaccine used in China is a live, oral, noninvasive, bivalent vaccine expressing O-antigens of *S. sonnei* and *S. flexneri* 2a, was designed to provide protection against both serotypes and based on passive surveillance studies reportedly provides ~60% protection in adults [34].

WRSS1 was constructed during the mid-1990s and continues to be evaluated in clinical trials in populations outside of the US. In two inpatient trials in the US, volunteers received a single oral dose of the vaccine ranging from 10^3 to 10^5 CFU. The vaccine was generally well tolerated, however, 22% showed mild short-term diarrhea and/or fever. The vaccine was remarkably immunogenic with substantial serum antibody responses and the median anti-LPS IgA ASC responses in the groups receiving 10^3 , 10^4 , 10^5 or 10^6 CFU being 150, 58, 345, and 265, respectively. These responses were comparable to better than those observed with *S. flexneri* 2a vaccine candidate SC602 which proved to be efficacious [45]. An outpatient Phase I trial was conducted in 45 adult Israeli volunteers and household contacts to determine the risk of adventitious spread of WRSS1. Groups of 15 volunteers were administered single dose regimens of 5×10^3 , 2×10^4 and 4×10^5 CFU of WRSS1. The vaccine was well tolerated at the lower two doses with a 12% rate of short-term diarrhea or fever (5%). At the highest dose, the rate of febrile reactions and moderate diarrhea increased to 28%, indicating that the safe dose for any future trials with WRSS1 would be 10^4 CFU. In this community-based trial, WRSS1 was excreted for an average of 5 days, and most importantly, from a regulatory point of view, there was no microbiological evidence of vaccine spread to household contacts. The immune responses were robust and reached levels similar to that observed in US volunteers.

The mild and transient symptoms of diarrhea and fever in volunteers immunized with SC602 and WRSS1 indicated that, lesser reactogenicity and a wider window of safety would be a desirable feature for *virG(icsA)*-based *Shigella* vaccines. The contributing role of the enterotoxins to diarrhea suggested that deleting the corresponding genes would result in alleviation of these symptoms [54]. Although the role of ShET2-2 as an enterotoxin remains to be demonstrated, its size-similarity and 40% identity to ShET2-1 suggest, that the two proteins may have a similar function. The presence of multiple enterotoxin genes in a bacterial pathogen is not unique to *Shigella* and has been described for other organisms such as *V. cholera* [77]. Studies described here with FLAG-tagged proteins have clearly shown that both ShET2-1 and ShET2-2 are expressed in *Shigella* and both are secreted via the TTSS. While ShET2-2 appears to be present in significantly lesser amounts than ShET2-1 in culture supernatants, the optimal growth conditions for maximal production and secretion of ShET2-2 have not been investigated and could be different from ShET2-1. As compared to Moseley, the reduced fecal scores of gnotobiotic piglets administered Moseley 2ΔShET2 indicate that the loss of these two proteins in a live vaccine candidate predict lesser diarrheal symptoms in human volunteers. Furthermore, in the guinea pig ocular model, the loss of ShET2-1 and ShET2-2 did not affect immunogenicity or efficacy suggesting that, like WRSS1, both WRSs2 and WRSs3 will elicit a robust protective immune response in spite of the loss of both enterotoxins.

WRSs3 lacks MsbB2, a plasmid-encoded MsbB protein that has also been described in the plasmid of EHEC O157:H7 [62]. More recent observations have indicated that contrary to MsbB1, the expression of *Shigella* MsbB2 is regulated by Mg and PhoP/Q [63] suggesting that MsbB1 and MsbB2 are differentially expressed within the host. Loss of MsbB2 reduces the hexacylated form of lipid A resulting in reduced endotoxicity. Lipid A is the bioactive component of LPS and is responsible for the majority of IL-1 induction and immunoregulation in human mononuclear cells [59,78,79]. Lipid A recognition and subsequent innate host responses occur through a series of binding and transfer reactions among different host response proteins such as lipid A binding protein (LBP), CD14, and the MD-2/TLR4 complex [80]. Lipid A engages host membrane and cytosolic receptors activating signaling pathways that eventually generate immune and inflammatory responses that destroy the invading pathogen [81]. Modifications to the lipid A structure have been shown to alter the specific binding affinity and transfer properties of the lipid A species that result in reduced inflammatory responses [82,83]. This was seen in RAW 264.7

macrophages infected with WRSs3 as described in this study. In J774 macrophage infected with *S. flexneri* 2a *msbB1* and *msbB2* mutants, specific proinflammatory cytokines (IL-1 β , MIP-1 α , and TNF- α) are also significantly reduced [85]. *In vivo*, reduced endotoxicity of *S. flexneri* 2a *msbB* mutants have been described in a rabbit ileal loop model of infection [58]. In an acute mouse pulmonary challenge model, attenuation of *msbB* mutants correlated with a decrease in the production of proinflammatory cytokine and chemokine release without significant changes in lung histopathology or overall immunogenicity [85]. These observations lead to the conclusion that WRSs3 with reduced endotoxinic lipid A will contribute to lower febrile responses due to release of less proinflammatory cytokines, but the immunogenicity generated by this strain will remain robust and will not be affected by the loss of the *msbB2* gene.

Unlike the large invasion plasmids of other *Shigella* serogroups, the virulence plasmid of *S. sonnei* is intrinsically somewhat unstable. The frequency of plasmid loss in *S. sonnei* varies widely from 1% to about 50% depending upon the strain [47,73]. Plasmid-free strains grow faster on solid media that is reflected in the larger size of the Form II colonies. The ruffled edges of Form II colonies may reflect membrane alterations that accompany loss of the plasmid-encoded O-antigens. In *S. flexneri* 2a, there are four protein sets that maintain segregational stability of the virulence plasmid: the partition proteins *parA/parB*, the *stbA/stbB* system involved in post-segregation killing of bacteria, and two toxin-antitoxin systems *ccdA/ccdB* and *mvpA/mvpT*. The *S. sonnei* plasmid lacks *ccdA/ccdB* and in addition has a novel *stbDE* toxin-antitoxin system with homologs on plasmid R485 of *M. morganii* and pB171 of *E. coli* [70]. It is not clear whether these differences contribute to the overall instability of the *S. sonnei* plasmid. Although WRSs2 and WRSs3, like WRSS1, were derived from the same parent strain, the new strains appear to generate Form II colonies on solid media at a slightly higher rate, although cGMP manufactured products of WRSs2 and WRSs3 have maintained ~10% Form II phenotype over a 6-month period of time suggesting that stability, although critical, can be controlled if proper care is taken to store the strains (in this case at -80 °C), to initiate vaccine production starting with Form I colonies and to evaluate Form I stability on solid media using optimal parameters (12–14 h time of incubation at 37 °C, evaluating stability by colony immunoblots).

The guinea pig model for *Shigella* infections is used to measure virulence of a strain as well as its ability to induce immunogenicity and demonstrate efficacy against challenge. *virG(icsA)* mutants are negative for the Sereny test and as expected, like WRSS1, WRSs2 and WRSs3 are also Sereny negative. However, since WRSs2 and WRSs3 are further modified by additional gene deletions, it was necessary to evaluate in an animal model such as the guinea pig, whether these changes would alter the potential of these two strains to be as immunogenic and efficacious as WRSS1. The results were affirmative.

The humoral and mucosal responses to the O-antigen are critical in determining the outcome of *Shigella* vaccination. Natural infections that cause disease also provide immunity against homologous serotype by inducing high levels of O-antigen-specific immune response [45]. During SC602 and WRSS1 trials, although significant immunogenicity was attained, reactogenicity was also observed. This has led to a commonly held belief that the immunogenicity induced in a host after immunization is directly related to the intensity of symptoms that is induced by the vaccine. Furthermore, the level of reactogenicity observed with a particular vaccine candidate in a naïve population may prove not to be so in another group that is frequently exposed to the pathogen, such as in an endemic population. These considerations make it difficult to strike a balance between the two conflicting parameters of immunogenicity and reactogenicity and has been the major hurdle in the design of live attenuated *Shigella* vaccines. The stepwise design and testing of *virG(icsA)*-based first and second-generation live vaccine candidates is an ongoing effort in the direction of attaining

that balance. Both *in vitro* and *in vivo* studies as well as in volunteer trials, the loss of the *Shigella* enterotoxins have resulted in lower diarrheal output. While it is clear that deleting MsbB2 results in a less endotoxic lipid A, what is not yet proven in volunteer trials is whether a vaccine candidate with the potential to cause fewer reactions is also capable of maintaining the high levels of immunogenicity seen with less attenuated strains. In rhesus monkeys all three vaccine candidates, WRSS1, WRSS2 and WRSS3 successfully survived transit through the gastrointestinal tract and were excreted to similar extents with only minor clinical side effects [84]. This suggests that loss of MsbB2 will not affect colonization of the host, given that effective colonization is key to the elicitation of a significant immune response. Furthermore, immunogenicity data from guinea pigs (described here), mice and rhesus monkeys [85] indicate that loss of MsbB2 does not affect the immunogenicity of the vaccine candidate. This will be put to a test in a head-to-head trial of WRSS2 and WRSS3 that is being planned to determine which of these two new *S. sonnei* vaccine candidates has achieved the right balance of safety and immunogenicity.

Recent PCR analysis across the gene deletions in WRSS2 and WRSS3 has indicated that in WRSS2, the region between senB(shET2-2) and senA(shET2-1) on the large plasmid has inverted with no loss of ORFs or phenotype while in WRSS3, the region between msbB2 and virG(icsA) as well as the region between senA(shET2-1) and virG(icsA) has inverted with no loss of ORFs or phenotype. We believe this has occurred due to the activity of the FLP enzyme to bring about inversion between two FRT sites on the large plasmid that are left behind after lambda Red recombineering.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi: 10.1016/j.vaccine.2009.11.001.

Fig. 1.

Steps involved in the construction of *S. sonnei* vaccine candidates WRSs2 and WRSs3. The parental strain Moseley, which is tetracycline resistant, was also the source for WRSS1 [47]. WRSs2 and WRSs3 were constructed by successive deletions of *senA*, *senB*, *virG(icsA)* and *msbB2* using the lambda red recombineering technique. Tetracycline resistance was eliminated by selection on fusaric acid plates.

Fig. 2.

Secretion of ShET2-1 and ShET2-2 in *Shigella* culture supernatants. Cultures of Moseley, 2457T and 2457T Δ ipaB with FLAG-tagged ShET2-1 and FLAG-tagged ShET2-2 were grown to log phase and treated with or without Congo Red for TTSS-mediated secretion. Lanes 1–2 in each panel has strains without FLAG-tagged proteins. Lanes 3–4 in panel A and lane 3 in panel B refer to 2457T3XFLAG ShET2-1. Lanes 3–4 in panel C refer to Moseley3XFLAG ShET2-1. Lanes 5–6 in panel A and lane 4 in panel B refer to 2457T3XFLAG ShET2-2. Lanes 5–6 in panel C refer to Moseley3XFLAG ShET2-2. Western blots were developed with anti-FLAG MAbs.

Fig. 3. *Shigella* infections in gnotobiotic piglets. 3-Day-old gnotobiotic piglets were orally inoculated with either 5×10^9 CFU of Moseley or Moseley2ΔShET2 strain. Fecal consistency was scored daily for 7 days after inoculation. Daily mean fecal scores were recorded based on the fecal consistency from post-inoculation day (PID) 1 to PID7. Scores above 2 were considered diarrheic. Statistical analysis was carried out using a mixed model (SAS software) as described in Section 2. The scores for Moseley were significantly higher than Moseley2ΔShET2 over the course of the study ($P < 0.0001$).

Fig. 4.

In vitro characterization of WRSs2 and WRSs3. Panel A: HeLa cell invasion assay. Invasiveness is plotted as a percentage relative to *S. flexneri* 2a strain 2457T, which is set at 100%. Strain Δ IpaB is a Moseley strain lacking the *ipaB* gene and is included as a noninvasive control strain. The figure shown is a representative experiment in which three colonies of each strain were grown and each culture was inoculated in duplicate in a 24-well tissue culture plate containing HeLa cells. Each bar represents the average number of cfu recovered for each strain and the error bars represent the calculated standard deviations. Panel B: MS analysis of lipid A from Moseley and from WRSs3. The ions above 2400 m/z are probably LPS (not shown). The ions between 1300 and 1900 m/z are associated with lipid A. The 1769.8 peak is probably hexaacylated with two C12 chains instead of one. The composition of the peaks at 1568 m/z and 1587 m/z which represent pentacylated products are indicated in panel B. The 1360 m/z peak has four acyl chains. Panels C and D: Phenotype of WRSs3 in cultured macrophages. RAW 264.7 macrophages were infected with log-phase wild-type Moseley (white bars), WRSs2 (gray bars), or WRSs3 (black bars). Culture supernatants were analysed at 2 or 4 h post-infection for *Shigella*-induced cytotoxicity on macrophages, indicated by lactate dehydrogenase (LDH) activity (panel C), and inflammatory potential of *Shigella*-infected macrophages, indicated by TNF α release (panel D). Data presented are the means of two independent experiments, each done in triplicate. Each error bar represents one standard deviation from the mean. Differences between wild-type Moseley ($n = 12$) and WRSs2 or WRSs3 ($n = 6$) were assessed for statistical significance using a two-tailed Mann–Whitney U -test. * $P < 0.05$.

Fig. 5.

Efficacy of WRSs2 and WRSs3 tested in guinea pig eyes. Guinea pigs ocularly immunized with WRSS1, WRSs2 or WRSs3 and saline control guinea pigs were challenged 26 days after the final immunization with 1×10^8 CFU/eye of wild-type *S. sonnei* 53G. The reactogenicity in the eyes of each guinea pig were scored over 7 days (x-axis) by the Sereny test-based score (y-axis) as described in Section 2. The asterisks indicate significant difference in reactogenicity between the control animals and animals vaccinated with different *S. sonnei* vaccine candidates.

Fig. 6. Antibody titer responses in guinea pigs immunized with *S. sonnei* vaccine candidates. Serum IgG- and IgA-specific antibody titers against *S. sonnei* LPS and *S. sonnei* invasin-LPS complex (Invaplex) were measured in guinea pigs immunized with WRSS1, WRSs2 or WRSs3. The immune responses were determined for days 0 (D0), 14 (D14), and 28 (D28). The geometric mean titer (GMT) for each group of guinea pigs was calculated and is shown on the y-axis. Serum immune responses were also measured 2 weeks after challenge of immunized guinea pigs with 53G (CLG). 95% confidence intervals are indicated by error bars. No significant differences were found between the three vaccine candidates.

Fig. 7.

Mucosal responses measured in eye washes of guinea pigs vaccinated with *S. sonnei* vaccine candidates. Eyewashes from immunized animals were used to determine the IgA specific antibody against *S. sonnei* LPS and Invaplex. The GMT (y-axis) were determined for day 0 (D0), 14 (D14), and 28 (D28) for each group of guinea pigs. The error bars indicate 95% confidence intervals. The asterisk over the bar representing WRSS1 D14 GMT in the anti-Invaplex IgA indicates significance with respect to WRSS2 and WRSS3. The asterisk over the bar representing WRSS1 D14 GMT in the anti-LPS IgA panel indicates significance with respect to only WRSS2.

Fig. 8. Stability of the Form I phenotype in vaccine candidates. Panel A: Overnight cultures of 10 Form I isolates of each strain were subcultured every 24 h and at each subculture, aliquots of each culture were plated out on three TSA plates. The plates were incubated at 37 °C for 16 h. After determining the colony count on each plate, Form II colonies were scored under the microscope and indicated as a percentage of the total colony count (y-axis). Each bar represents the average of the ten isolates of each strain and the error bars represent the standard deviations for each strain. Panel B: Five colonies of each strain were grown to an $OD_{600} = 1.0$ and dilutions plated out on TSA plates that were incubated at 37 °C for 12, 14, 16, or 18 h. At each time point the plates were removed, total colony counts obtained and Form II percentage scored as described under panel A. Each bar represents the average of Form II colonies from the five TSA plates representing an individual strain at each time point and the error bars are the standard deviations. Panel C: The plates in panel B were subjected to colony blot assay with MAb to IpaB. The number of Form II colonies were determined from the total count (Form I and II colonies) minus the number of pink spots representing IpaB positive colonies (Form I only). Each bar represents the average percentage of Form II colonies from the five TSA plates representing an individual strain at each time point and the error bars are the standard deviations.

Table 1

Strains and plasmids used in study.

Strains	Description	Reference
2457T	<i>S. flexneri</i> 2a wild-type strain	WRAIR
2457TΔipaB	2457T with deletion of the <i>ipaB</i> gene	This study
2457T3XFLAG ShET2-1	3X FLAG ShET2-1 in 2457T	This study
2457T3XFLAG ShET2-2	3X FLAG ShET2-2 in 2457T	This study
Moseley	<i>S. sonnei</i> virulent strain, Tet ^R	WRAIR
53G	<i>S. sonnei</i> virulent strain	WRAIR
BS103	Plasmid-cured 2457T, noninvasive	WRAIR
MoseleyΔipaB	Moseley with deletion of the <i>ipaB</i> gene	This study
Moseley 2ΔShET2	Moseley with deletion of <i>senA</i> , <i>senB</i>	This study
Moseley3XFLAG ShET2-1	3X FLAG ShET2-1 in Moseley	This study
Moseley3XFLAG ShET2-2	3X FLAG ShET2-2 in Moseley	This study
WRSS1	Moseley with deletion of 212 bp of the <i>virG(icsA)</i> gene	[47], WRAIR
WRSS2	Moseley with deletion of <i>virG(icsA)</i> , <i>senA</i> , and <i>senB</i>	This study
WRSS3	Moseley with deletion of <i>virG(icsA)</i> , <i>senA</i> , <i>senB</i> and <i>msbB2</i> genes	This study
<i>Plasmids</i>		
pKD3	Expresses chloramphenicol resistance flanked by FRT sites	E. coli Genetic Center, Dept MCDB, Yale Univ
pKM208	Temperature sensitive replicon, carries the lambda Red genes expresses Beta, Gam and Exo from Ptac promoter under control of <i>lacI</i> repressor	[64]
pCP20	Temperature sensitive replicon, expresses FLP recombinase from λP _R promoter under control of λ cI857 repressor	E. coli Genetic Center, Dept MCDB, Yale Univ
pSUB11	Plasmid with a 24 nt FLAG sequence and FRT sites flanking a kanamycin cassette	[65]

Table 2

Protective efficacy of WRSs2 and WRSs3 in guinea pigs.

Immunizing strain (ocular)	# of eyes inoculated	# of eyes with the indicated rating ^a				Disease/total ^b	% protection ^c	<i>P</i> value ^d
		0	1	2	3			
Saline	10	0	0	0	10	10/10	0	
WRSs1	20	16	2	2	0	2/20	90	<0.0001
WRSs2	20	14	6	0	0	0/20	100	<0.0001
WRSs3	20	17	3	0	0	0/20	100	<0.0001

^aInflammation rating: 0, no inflammation or mild irritation; 1, mild keratoconjunctivitis; 2, keratoconjunctivitis without purulence; 3, severe keratoconjunctivitis with purulence.

^bGuinea pigs were considered diseased if they had a rating of 2 or 3.

^c% protection was calculated by the formula: [(% disease in control – % disease in vaccinees)/% disease in control] × 100.

^d*P* value was determined using Fisher's exact test.