RESEARCH ARTICLE

A Highly Effective Component Vaccine against Nontyphoidal *Salmonella enterica* Infections

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ABSTRACT **Nontyphoidal *Salmonella enterica* (NTS) infections are a major burden to global public health, as they lead to diseases ranging from gastroenteritis to systemic infections and there is currently no vaccine available. Here, we describe a highly effective component vaccine against *S. enterica* serovar Typhimurium in both gastroenteritis and systemic murine infection models. We devised an approach to generate supernatants of *S. enterica* serovar Typhimurium, an organism that is highly abundant in virulence factors. Immunization of mice with this supernatant resulted in dramatic protection against a challenge with serovar Typhimurium, showing increased survival in the systemic model and decreased intestinal pathology in the gastrointestinal model. Protection correlated with specific IgA and IgG levels in the serum and specific secretory IgA levels in the feces of immunized mice. Initial characterization of the protective antigens in the bacterial culture supernatants revealed a subset of antigens that exhibited remarkable stability, a highly desirable characteristic of an effective vaccine to be used under suboptimal environmental conditions in developing countries. We were able to purify a subset of the peptides present in the supernatants and show their potential for immunization of mice against serovar Typhimurium resulting in a decreased level of colonization. This component vaccine shows promise with regard to protecting against NTS, and further work should significantly help to establish vaccines against these prevalent infections.**

IMPORTANCE ***Salmonella enterica* infections other than typhoid and paratyphoid fever are a major global health burden, as they cause high morbidity and mortality worldwide. Strategies that prevent *Salmonella*-related diseases are greatly needed, and there is a significant push for the development of vaccines against nontyphoidal *Salmonella enterica* serovars. In this work, we describe an *S.* Typhimurium supernatant-derived vaccine that is effective in reducing bacterial colonization in mouse models of gastroenteritis as well as invasive disease. This is a component vaccine that shows high stability to heat, a feature that is important for use under suboptimal conditions, such as those found in sub-Saharan Africa.**

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Nontyphoidal *Salmonella enterica* (NTS) infections cause major morbidity and mortality worldwide, with diseases ranging from localized, self-limiting gastroenteritis with symptoms such as nausea, vomiting, and diarrhea to more serious typhoid-like systemic infections, including bacteremia, meningitis, and pneumonia (1). NTS infections are caused by *Salmonella enterica* serovars other than Typhi and Paratyphi, primarily serovars Typhimurium and Enteritidis (2–4). It is estimated that 93.8 million cases of gastroenteritis due to *S. enterica* occur worldwide and cause 155,000 deaths per year (5). In the United States, NTS infections have been reported as the leading cause of death among foodborne bacterial infections, with elderly people and young children being more susceptible to death (6). Invasive NTS infections present a significant challenge in developing countries, particularly in sub-Saharan Africa (3, 4), where NTS can be isolated from up to 50% of all patients with bacteremia, with mortality rates as high as 45% (7–9). NTS infections are associated with malnutrition, severe anemia, malaria, and concomitant HIV infection (4). Although there are two commercially available vaccines against *S.* Typhi (parenteral Vi polysaccharide and oral at-

tenuated *S.* Typhi strain Ty21a) (10), there is currently no vaccine available to prevent NTS infections. The lack of an NTS vaccine is exacerbated by the widespread presence of multiresistant clinical isolates, making the treatment of NTS infections even more difficult (5, 11, 12).

In this study report, we describe the development of an effective component vaccine against NTS infections by use of both local and systemic murine infection models. We show that mice immunized with "virulence factor-enriched supernatants" from *S.* Typhimurium cultures are dramatically protected from subsequent *S.* Typhimurium challenge, with a significant decrease in bacterial loads in intestinal and systemic sites in both models of NTS infection. Furthermore, the supernatants also protect mice generally resistant to NTS infections, a previously unreported achievement when using a component vaccine (13). We also describe the nature of the protective antigens and characterize the immune response to the vaccine; and these findings have allowed us to define a subset of potential *S.* Typhimurium antigens responsible for the protective response. Furthermore, we expressed and purified a subset of the aforementioned antigens to immunize mice in the systemic model. We were able to show significantly lower colonization in the ceca and spleens of mice immunized with the peptide mix. These proteins, together with other molecules present in the supernatant, could act as an effective component vaccine against NTS.

RESULTS AND DISCUSSION

Bacterial cell wall extracts, purified outer membrane proteins, flagella, lipopolysaccharide (LPS), and many surface molecules have been tested as antigens, with very limited success, against nontyphoidal *S. enterica* infections (13–18). This is probably because *S. enterica* is a facultative intracellular pathogen and requires both B and T cell responses for successful clearance. Previously, we were successful in developing an animal vaccine against *Escherichia coli* O157:H7 by using secreted proteins from that bacterium (19). From that work with secreted proteins from *E. coli* O157:H7 that produced an effective vaccine (19), we examined whether a similar method could be used to create a vaccine against NTS infections. We reasoned that the lack of efficacy in previous attempts to develop a component vaccine against *S.* Typhimurium may have been due to this pathogen's ability to modify its surface components, including LPS, and many membrane constituents once inside host cells (e.g., macrophages) (20, 21). *S.* Typhimurium contains two type III secretion systems: one involved in initial invasion into nonphagocytic host cells (*Salmonella* pathogenicity island 1 [SPI-1]) and the other critical for survival inside phagocytic cells (SPI-2). In the laboratory, different media conditions can be used to selectively activate these two systems (22). By harvesting supernatants from *S.* Typhimurium grown under conditions that selectively induce SPI-2 secretion, many of the modifications that occur when *S.* Typhimurium is inside host cells will occur.

We found that culture supernatant from *S.* Typhimurium grown under SPI-2-inducing conditions dramatically protected immunized mice from subsequent challenge, as it significantly decreased bacterial loads in the cecum, spleen, and liver (Fig. 1A, B, and C). Moreover, the supernatant provided protection against disease, preventing a decrease in cecal weight, a hallmark of infection (Fig. 1D) (23). Furthermore, immunization of these mice with the supernatant led to a significant increase in the survival rate of vaccinated mice compared to control mice immunized with saline and adjuvant (Fig. 1E). Mice vaccinated with culture supernatant from *S.* Typhimurium grown under SPI-1-inducing conditions (LB medium), however, did not show signs of protection in this model (data not shown).

Next, we analyzed the immune response responsible for the supernatant-elicited protection. We tested if the supernatant required both B and T cells to confer protection. Mice deficient in B cells and $CD4^+$ and $CD8^+$ T cells were immunized with the supernatant, and the effects on *S.* Typhimurium colonization of the spleen were recorded subsequent to challenge with *S.* Typhimurium. While the burden in $CD4^+$ and $CD8^+$ T cell-deficient mice was still reduced in vaccinated mice, protection was totally lost in mice that were deficient in B cells (Fig. 1F). These results indicate that B cells are critically required for a protective immune response elicited by the supernatant. Antibody responses were also measured from serum of immunized mice and compared to those from control mice (Fig. 1G and H). Both specific IgG and IgA were significantly increased in immunized mice, confirming elicitation of the humoral immune response.

The ability of a component vaccine to protect against gastrointestinal infections commonly caused by *S.* Typhimurium (as opposed to systemic infections) has never been reported in a mouse model. Therefore, we sought to determine if this vaccine could also protect mice from *S.* Typhimurium-induced gastroenteritis. To do so, mice were immunized with wild-type (WT) supernatant and infected using the *S.* Typhimurium gastroenteritis model (24). In mice, *S.* Typhimurium does not cause gastroenteritis, as is commonly seen in humans. To model the disease, mice were pretreated with streptomycin 24 h prior to the infection with *S.* Typhimurium, a step that is not performed in the systemic model. This step ensures that, similarly to gastroenteritis in humans, bacterial counts and histopathology changes in the ceca of the mice are observed. Oral delivery of the supernatant of *S.* Typhimurium grown under SPI-2-inducing conditions with the adjuvant was able to significantly protect against gastroenteritis in C57BL/6J mice (Fig. 2A). Intestinal pathology decreased with immunization of these mice, although the differences were not statistically significant (Fig. 2B).

C57BL/6J mice are highly susceptible to *S.* Typhimurium infection, as they display high colonization rates and a severe intestinal pathology that cannot be overcome by the mouse immune system and leads to death (25). However, C57BL/6J mice have a mutation in the gene for NRAMP, which is significantly responsible for the accelerated fatality of these mice. More resistant mouse strains, such as strain 129S1/SvImJ, that are $NRAMP^{+/+}$ display significantly lower colonization rates and are able to recover from infection, similar to what occurs in humans (25). We therefore tested whether oral immunization conferred protection in this resistant mouse model, as well as in 129S1/SvImJ $NRAMP^{-/-}$ mice, which are more susceptible to infection. Significant protection was observed in both mouse strains, with $NRAMP^{+/+}$ mice showing no colonization by *S.* Typhimurium (sterility) when immunized with the supernatant (Fig. 2C). Intestinal pathology was also significantly decreased in both mouse strains (Fig. 2D). Overall, we found that immunization with WT supernatant was able to strongly decrease, and even eliminate (as in the case of 129S1/SvImJ $NRAMP^{+/+}$ mice), colonization by *S.* Typhimurium and prevent intestinal pathology (24). Similar to the systemic model, antibody levels were examined in mice im-

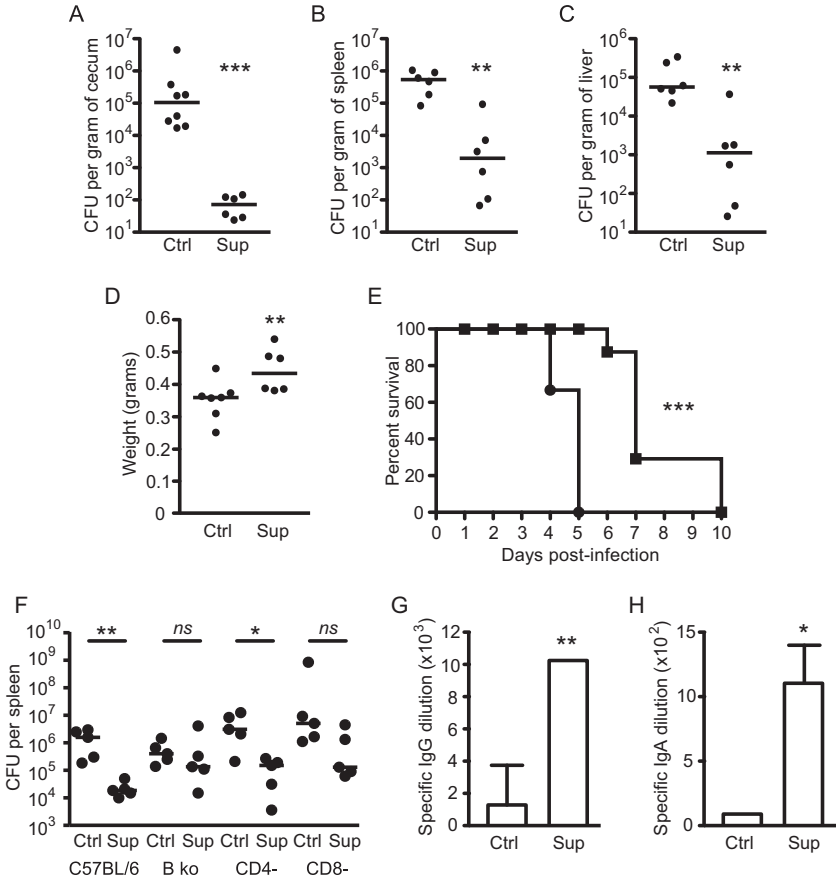

FIG 1 Supernatant of *S.* Typhimurium grown under SPI-2-inducing conditions decreases the bacterial load in systemic NTS infection. C57BL/6J mice ($n = 6$ to 8 mice per group) were immunized subcutaneously with *S.* Typhimurium supernatant and TiterMax (as adjuvant), challenged with 3×10^6 CFU *S.* Typhimurium, and euthanized 3 days later to obtain CFU counts from cecal (A), spleen (B), and liver (C) samples. (D) Cecal weights. (E) Survival curve. (F) Spleen *S.* Typhimurium counts of C57BL/6J, B6.129S2-*Ighmtm1Cgn*/J (B cell-deficient), B6.129S2-*Cd4tm1Mak*/J (CD4 cell-deficient), or B6.129S2-*Cd8atm1Mak*/J (CD8 cell-deficient) mice ($n = 5$ per group) immunized against systemic salmonellosis. (G) Specific IgG levels in the serum of C57BL/6J mice immunized against systemic salmonellosis. (H) Specific IgA levels in the serum of C57BL/6J mice immunized against systemic salmonellosis. Ctrl, saline-plus-adjuvant control; Sup, supernatant from SL1344 plus adjuvant. Bars indicate medians. Bars in the ELISA graphs show standard errors of the means. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not statistically significant.

munized orally (gastrointestinal model) (Fig. 2E to G). Significantly higher specific IgG and IgA titers were detected in serum of immunized mice than in control mice, and higher secretory IgA levels were also detected in feces of immunized mice, indicating that the immunization elicited the expected antibody response to the vaccine.

In order to characterize the protective antigen(s) present in the supernatant and responsible for the protection observed in mice, we performed studies using *S.* Typhimurium mutants as well as biochemical approaches. First, we examined whether the SPI-2 secreted proteins were responsible for the protection effect. Surprisingly, we found that although the supernatant was from cells grown under SPI-2-inducing conditions, mice immunized with the supernatant from a mutant *S.* Typhimurium strain incapable of secreting SPI-2 proteins (*ssaR*), grown under the same conditions, provided the same level of protection as the WT supernatant (Fig. 3A; see also Fig. S1A in the supplemental material). Dramatic decreases were seen in bacterial counts in the spleen, cecum, and liver (see Fig. S1A) when mice were immunized with supernatants from the *ssaR* mutant strain and challenged with *S.* Typhimurium. This suggests that the protective antigens are probably regulated under SPI-2 conditions but are not secreted by the SPI-2 type III secretion system. Since the growth conditions that were used might also affect the expression of PhoP, we tested whether PhoP was needed for protection. PhoP is a transcriptional regulator that is activated by PhoQ under conditions of low extracytoplasmic Mg^{2+} concentrations (26) or acidic pH (21) and regulates the transcription of more than 50 genes (27). However, the supernatant from a *phoP* mutant strain was found to still be protective, decreasing *S.* Typhimurium colonization to levels similar to those after immunization with the WT supernatant (Fig. 3A; see also Fig. S1A), indicating that the protective antigen(s) is not PhoP regulated. Flagellin is another major secreted antigen of *S. enterica* that is known to induce an immune response against *S. enterica* (18). Therefore, we examined whether a genetic mutant strain incapable of producing flagella (Δ*fliF*) was able to provide protection. We found that immunization with a supernatant obtained from the *fliF* mutant strain gave protection similar to the WT supernatant (Fig. 3A; see also Fig. S1A), indicating that flagellin is not responsible for the protection of mice against *S. enterica* infection. The conditions used to obtain the protective supernatant were similar to those shown to induce modifications of the lipid A

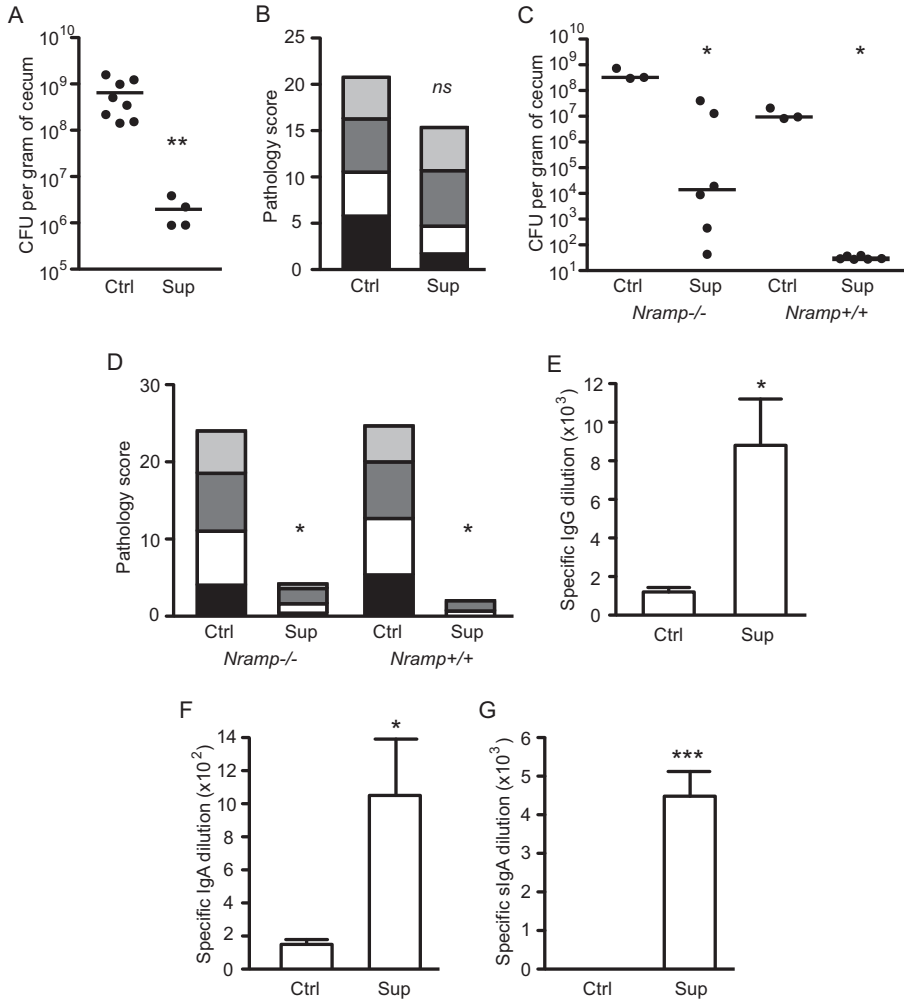

FIG 2 Supernatant is protective against *S.* Typhimurium gastrointestinal infection. C57BL/6J, 129S1/SvImJ Nramp$^{+/+}$ and Nramp$^{-/-}$ mice (n = 3 to 8 mice per group) were immunized orally with supernatant and CpG as adjuvant and challenged with 3×10^6 CFU *S.* Typhimurium after streptomycin treatment. (A) Cecum *S.* Typhimurium counts in C57BL/6J mice after 3 days of infection; (B) intestinal pathology scores for the ceca of C57BL/6J mice after infection. Black bars represent pathology scores of the intestinal lumen, white bars represent scores of the surface epithelium, dark gray bars represent scores of the mucosa, and light gray bars represent scores of the submucosa of the tissue. (C) Cecum *S.* Typhimurium counts in 129S1/SvImJ Nramp$^{+/+}$ and Nramp$^{-/-}$ mice. (D) Intestinal pathology scores of the ceca of Nramp$^{+/+}$ and Nramp$^{-/-}$ mice after infection. (E) Specific IgG levels in the serum of C57BL/6J mice immunized against salmonella gastroenteritis. (F) Specific IgA levels in the serum of C57BL/6J mice immunized against salmonella gastroenteritis. (G) Specific secretory IgA (sIgA) levels in feces of mice immunized against salmonella gastroenteritis. Ctrl, saline-plus-adjuvant control; Sup, supernatant from SL1344 plus adjuvant. Bars show medians. Bars in the ELISA graphs shown standard errors of the means. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant.

component of *S.* Typhimurium LPS (28). As LPS is known to elicit a potent immune response, we therefore tested whether the LPS present in the supernatant was responsible for protection. LPS was specifically removed from the supernatant by using polymixin B columns, and LPS-free supernatant was used to immunize the mice. The LPS-free supernatant retained the protection observed in mice (Fig. 3B; see also Fig. S1B). In addition, when LPS was purified under SPI-2-inducing conditions and used as a vaccine, no protection was observed (see Fig. S2 in the supplemental material), further confirming that LPS is not responsible for the observed protection.

To further characterize the immune-activating compound(s) present in the supernatant, we performed heat and proteinase K treatments, as well as ammonium sulfate fractionation. We found that mice immunized with a supernatant that was boiled for 20 min and then digested with proteinase K exhibited higher colonization with *S.* Typhimurium than did mice immunized with WT supernatant, but the counts were still lower than in control mice (Fig. 3B; see also Fig. S1B in the supplemental material). We also found that the protective component(s) was precipitated by 50% ammonium sulfate treatment (Fig. 3B; see also Fig. S1B and C), whereas the remaining solution (50% sup in Fig. 3B) failed to confer protection against *S.* Typhimurium systemic infection.

In an effort to identify specific antigens responsible for the protection conferred by the supernatant, we separated the components that precipitated with 50% ammonium sulfate by using reversed-phase high-performance liquid chromatography (RP-HPLC). A total of 30 fractions were collected and analyzed by Western blotting using the serum of immunized mice (see Fig. S1D in the supplemental material). We observed several proteins that reacted with serum in fractions F13 to F30. Fractions F9, F13, F14, F18, F19, and F22 were chosen to immunize mice, based

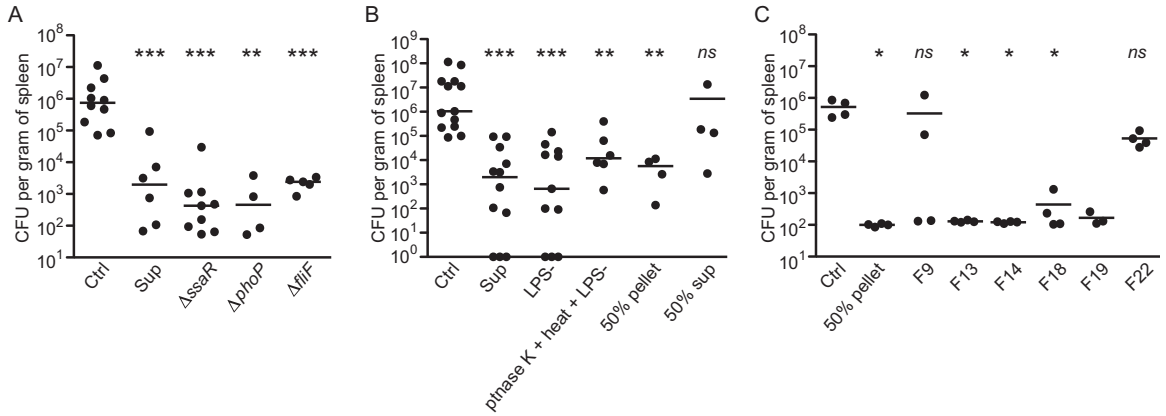

FIG 3 Characterization of the protective molecule. C57BL/6J mice ($n = 4$ to 13 mice per group) were immunized subcutaneously with different supernatants as described in the text, and the spleens were harvested 3 days postinfection to determine *S.* Typhimurium counts. (A) Spleen *S.* Typhimurium counts of mice vaccinated with supernatant harvested from either WT *S.* Typhimurium (Sup) or Δ*ssaR*, Δ*phoP*, or Δ*fliF S.* Typhimurium strains. (B) Spleen *S.* Typhimurium counts from mice vaccinated with Sup, LPS-free supernatant (LPS−); proteinase K and heat-treated LPS-free supernatant (ptnase K + heat + LPS−); supernatant contents that precipitated with 50% ammonium sulfate (50% pellet), and remaining supernatant (50% sup). (C) Spleen counts of 50% ammonium sulfate-precipitated supernatant and fractions after HPLC fractionation of the 50% pellet (fractions F9, F13. F14, F18, F19, and F22).

on the unique set of bands they exhibited. Four fractions (F13, F14, F18, and F19) conferred similar levels of protection as the pellet obtained after 50% ammonium sulfate precipitation (Fig. 3C; see also Fig. S1C). However, fractions F9 and F22 showed no protection compared to control mice. We analyzed the proteins present in all six fractions by mass spectrometry and found 10 proteins that were present in one or more of the protective fractions (Table 1). Three of these proteins have been previously described to react with antibodies present in the serum of *S. enterica*-infected mice and/or humans (29–32). These proteins were SL4489 (OsmY), SL1010 (OmpA), and SL0731 (Pal [peptidoglycan-associated lipoprotein]). To our knowledge, the other 7 proteins have never been associated with *S. enterica*-induced immune responses. They are SL2251 (GlpQ), SL1780 (hypothetical protein), SL0866 (Art I), SL4363 (CybC), SL1061 (putative secreted protein), SL4109 (HupA), and SL1492 (HdeB).

Six of the peptides present in the protective supernatant were cloned and expressed in *E. coli* (SL0866, SL1492, SL1780, SL2251, SL4109, and SL4489). These proteins were purified and mixed to immunize mice under the same conditions used for the systemic infection model immunization. Mice immunized with the protein mix exhibited lower *S. enterica* colonization than saline-immunized mice (Fig. 4). Significantly decreased *S. enterica* counts were observed in the ceca and spleens of immunized mice, while liver counts were lower, yet not significantly so. These results suggested that the tested proteins offer protection in this model; however, full protection was only observed with the full *S. enterica* supernatant.

Our data suggest that the protective components are very stable, as they were partially resistant to proteinase K treatment and were able to confer protection even after extended heating. The implications of such a stable vaccine are critical for use in developing countries, where proper refrigeration during transport and storage may be difficult (33). We were able to express and purify 6 proteins present in the supernatant that decreased *S. enterica* colonization in mice, suggesting a way to establish a component vaccine against NTS. Further studies could test more components of the supernatant to obtain the same level of protection observed with the full supernatant. Component vaccines in general are safer than live attenuated vaccines, and killed bacteria vaccines are usu-

TABLE 1 Proteins detected in selected HPLC fractions of the 50% ammonium sulfate pellet

Protein[a]	Comment (reference[s])	Predicted gene[b]	Peptide detected in fraction[c]					
			F9	F13	F14	F18	F19	F22
Protection			−	+	+	+	+	−
SL4489*	Targeted in mice (29)	*osmY*	<	+	+	+	+	<
SL0866*		*art I*	<	+	+	+	<	<
SL1010	Targeted in mice and humans (29–32)	*ompA*	<	+	+	+	+	+
SL0731	Targeted in mice (29)	*pal*	−	+	+	+	+	+
SL1780*		Hypo ptn	−	+	+	+	+	−
SL2251*		*glpQ*	<	+	+	<	<	<
SL4369		*cybC*	−	+	−	−	−	−
SL1061		Put sec ptn	−	+	−	−	−	−
SL4109*		*hupA*	<	+	+	+	+	+
SL1492*		*hdeB*	−	+	+	−	+	−

[a] Asterisks (*) indicate proteins used in the protein mix experiments.
[b] Hypo ptn, hypothetical protein; Put sec ptn, putative secreted protein.
[c] −, absence of protein; +, presence of protein; <, protein present in a relatively smaller amount.

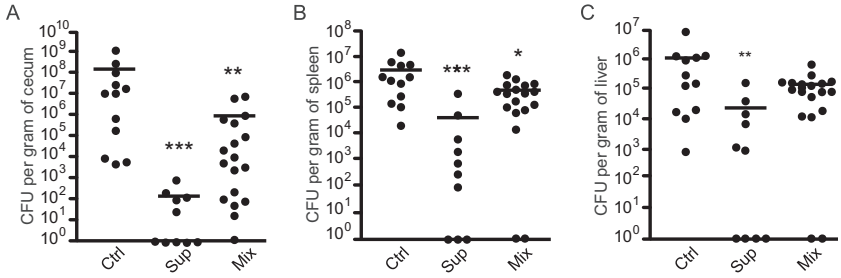

FIG 4 A mix of 6 heterologous expressed and purified proteins present in the supernatant of the *S.* Typhimurium culture decreased bacterial loads in systemic NTS infections. C57BL/6J mice ($n = 10$ to 18 mice per group) were immunized subcutaneously with a mix containing purified proteins (SL0866, SL1492, SL1780, SL2251, SL4109, and SL4489) and TiterMax as adjuvant, challenged with 3×10^6 CFU *S.* Typhimurium, and euthanized 4 days later to obtain cecal (A), spleen (B), and liver (C) *S.* Typhimurium counts. Ctrl, saline-plus-adjuvant control; Sup, supernatant from SL1344 plus adjuvant; mix, purified proteins (SL0866, SL1492, SL1780, SL2251, SL4109, and SL4489) mixed, plus adjuvant. Bars indicate medians. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

ally nonimmunogenic. There is significant concern regarding a live attenuated *S. enterica* vaccine for animals as well as humans, due to environmental release concerns and general health concerns. Immunization with component vaccines would promote less antigenic competition and offer the ability to deliver the vaccine to the site where immunity is required (34). The approach presented here is economical, as large batches of supernatants can be produced in fermentors under SPI-2-inducing conditions. Future studies will focus on analysis of the protection elicited by the peptides described here for use in mice, pigs, and humans.

MATERIALS AND METHODS

Bacterial strains and supernatant preparations. We harvested supernatants from *S.* Typhimurium (SL1344) grown under conditions that selectively induce SPI-2 secretion (22). *S.* Typhimurium was grown overnight in LB broth, washed twice in low-phosphate, low-magnesium (LPM) medium, and then inoculated at a 1:50 dilution in 3 ml of LPM medium at pH 5.8. Cultures were grown at 37°C with shaking for 4 to 6 h. *S.* Typhimurium was then grown in the same medium in a 30-liter MBR fermentor at the National Research Council Canada for approximately 23 h. The culture was concentrated using a Millipore Pellicon tangential flow unit powered by a peristaltic pump (0.22-μm-pore filter). The filtrate collected was further concentrated by using a 5-kDa membrane (Biomax-5; Millipore). Samples from the final steps of supernatant preparation were grown in LB for 24 h at 37°C to check for sterility, and no growth was observed.

Generation of chromosomal mutations. Chromosomal mutations were generated as previously described. The *ssaR* (35) and *fliF* mutations were generated by allelic exchange using a counterselectable suicide vector containing the levansucrase-encoding *sacB* gene (36). The PhoP mutant strain was constructed by P22 transduction of 14028s from *phoP*::Tn*10*d Tc (38).

Mice. Six- to 8-week-old *S. enterica*-susceptible C57BL/6, B6.129S2-*Ighmtm1Cgn*/J (B cell-deficient), B6.129S2-*Cd4tm1Mak*/J (CD4 T cell-deficient), and B6.129S2-*Cd8atm1Mak*/J (CD8 T cell-deficient) mice (Jackson Laboratories) were used in the immunization experiments. Also, 6- to 8-week-old 129S1/SvImJ Nramp$^{+/+}$ and Nramp$^{-/-}$ mice were bred and housed at the University of British Columbia (UBC) Animal Facility. The protocols used in the experiments were in accordance with animal care guidelines as outlined by the UBC Animal Care Committee and the Canadian Council on the Use of Laboratory Animals.

Immunization of mice against systemic salmonellosis. Groups of 4 or 8 female C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME), 6 to 8 weeks old, were injected subcutaneously (s.c.) with supernatant containing 40 μg of total protein and 1 volume of TiterMax Gold adjuvant (Sigma, St. Louis, MO, USA). Thirty days later, mice were boosted s.c. with supernatant containing 25 μg of total protein and 1 volume of TiterMax. Mice were infected 30 days after the booster with 3×10^6 CFU of *S.* Typhimurium orally. Three days postinfection, CFU were determined for the cecum, spleen, and liver.

Immunization of mice against *S. enterica* gastroenteritis infection. Mice were administered supernatant containing 90 μg of total protein with 1 μg CpG (InvivoGen, CA, USA) orally. The mice were boosted 21 days later with supernatant containing 45 μg of total protein with 1 μg CpG, orally. Thirteen days later, the mice were orally gavaged with 100 μl of streptomycin (20 mg; Sigma), 1 day prior to oral infection with 3×10^6 CFU of *S.* Typhimurium. Three days later, the number of CFU per gram was determined for the cecum, liver, and spleen.

LPS studies. The supernatant was run over Detoxy gel AffinityPack prepacked columns (Pierce, Rockford, IL, USA) to remove LPS. The removal of LPS was verified in an LAL assay (*Limilus* amebocyte lysate assay; Pyrogen Plus; Cambrex, NJ, USA) following the manufacturer's instructions. To extract LPS, overnight bacterial cultures were inoculated in LB (SPI-1) or LPM (SPI-2) and grown to an optical density at 600 nm (OD_{600}) of 0.4. The LPS extraction kit (iNtRON Biotechnology, South Korea) was used to extract the LPS from the cultures. The LPS concentration was determined using the LAL assay (Cambrex), and the same concentration determined to be present in the wild-type supernatant was used to vaccinate the mice (184.6 μg for the prime and 92.3 μg for the booster).

Supernatant characterization. For the proteinase K treatment, LPS-free supernatant was digested with proteinase K (0.2 mg/ml; Sigma) at 55°C overnight, following incubation in boiling water for 30 min. The supernatant was then centrifuged at 13,000 rpm for 1 min, and the supernatant was collected and used to vaccinate the mice. For the ammonium sulfate precipitation step, ammonium sulfate (Sigma) at 20% (final concentration) was added to the supernatant and incubated at 4°C with shaking for 1 h. The mixture was centrifuged at 13,000 rpm for 15 min, and the supernatant and pellet were collected. Ammonium sulfate at 50% (final concentration) was added to the supernatant fraction from the 20% precipitation, and the same procedure described above was performed. The pellets of the 20% and 50% fractions and the supernatant of the 50% fraction were dialyzed in PBS and used to vaccinate the mice.

Proteomic analysis of fractions. The pHs of both the pellet and supernatant were adjusted to 8.5 by using 3 M NaOH. Prior to digestion, proteins in both samples were reduced with 2 mM dithiothreiotol and alkylated with 4 mM iodoacetamide. A total of 4 μl trypsin (0.5 μg/μl) was added to the pellet fraction and 3 μl was added to the supernatant fraction, and both samples were incubated overnight at 37°C. After digestion, both samples were desalted by using a small plug of C_{18} material (3M Empore C_{18} extraction disk) packed into a 200-μl pipette tip (Eppendorf), similar to methods described previously (39). Samples were reconstituted in 40 μl of 0.1 M acetic acid in water.

RP-HPLC fractionation. Reversed-phase protein separation was performed using a Zorbax 300SB-C3 analytical column (4.6-mm inner di-

ameter [i.d.], 50-mm length, 3.5-μm pore size; Agilent Technologies), a Rheodyne 7725i injection valve, and an Agilent 1100/1200 HPLC system consisting of a G1376A capillary pump operated in normal flow mode, a G1315C diode array detector, and a G1364C analytical-scale fraction collector. The 50% ammonium sulfate pellet was dried completely, suspended in 50 μl 0.1% trifluoroacetic acid (TFA) in water, and injected onto the column at a flow rate of 1 ml/min in 100% solvent A (0.1% TFA in water). Proteins were separated in a 15-min gradient from 10% to 80% solvent B (0.1% TFA in 8/2 [vol/vol] acetonitrile-water), and a total of 30 fractions were collected.

Nanoflow HPLC-MS analysis of HPLC fractions. All samples were analyzed by nanoflow liquid chromatography using an Agilent 1200 HPLC system (Agilent Technologies) coupled online to an LTQ-Orbitrap XL mass spectrometer essentially as described previously (40). Aqua C_{18} resin (5 μm; Phenomenex) was used for the trap column, and Reprosil-Pur C_{18}-AQ resin (3 μm; Dr. Maisch GmbH) was used for the analytical column. Peptides were trapped at 5 μl/min in 100% solvent A (0.1 M acetic acid in water) on a 2-cm trap column (100-μm i.d.) and eluted into a 20-cm analytical column (50-μm i.d.) at 100 nl/min in a 90-min gradient from 10 to 40% solvent B (0.1 M acetic acid in 8:2 [vol/vol] acetonitrile-water). The eluent was sprayed via in-house-made emitter tips, butt-connected to the analytical column. The mass spectrometer was operated in data-dependent mode, automatically switching between mass spectrometry (MS) and tandem mass spectrometry (MS/MS). Full-scan MS spectra (from *m*/*z* 300 to 1,600) were acquired in the Orbitrap apparatus with a resolution of 60,000 at *m*/*z* 400 after accumulation to a target value of 1,000,000. The five most intense ions at a threshold above 500 were selected for collision-induced fragmentation in the linear ion trap at a normalized collision energy of 35% after accumulation to a target value of 10,000.

Database searching and label-free quantitation of HPLC fractions. All MS/MS (MS2) spectra were converted to single dta files (41) and merged into a Mascot generic format file, which was searched using an in-house licensed Mascot v2.3.01 search engine (Matrix Science) against a concatenated *Salmonella* database (containing 9,472 forward and reverse sequences). The mass tolerance of the precursor ion was set to 50 ppm and that of fragment ions was set to 0.6 Da. A peptide false-positive discovery rate (FDR) of $<$1% was estimated (42). A minimum of two peptides per protein and a protein cutoff score of 60 led to a protein FDR of $<$1%. Label-free relative quantitation was performed by calculating the exponentially modified protein abundance index (emPAI) (43), and protein ratios were obtained by comparing the emPAI values between different fractions.

ELISA. Vaccine-specific IgG and IgA levels were measured from serum and specific secretory IgA levels were measured from the feces of immunized and control mice from each model of vaccination by using an antibody enzyme-linked immunosorbent assay (ELISA). ELISA plates (Corning-Costar) coated at 4°C overnight with 100 μl of 0.5-μg/ml vaccine supernatant were washed three times with PBS–0.05% Tween 20 (PBS/T) prior to blocking with 100 μl PBS–2% bovine serum albumin (BSA)–0.05% Tween 20 (PBS/BSA/T) for 1 h at room temperature. Dilutions of mouse serum in PBS–0.5% BSA were added to the plates (100 μl) and incubated at 37°C for 2 h prior to washing three times with PBS/T. To detect total IgG or IgA, 100 μl of horseradish peroxidase (HRP)-labeled goat anti-mouse IgG or IgA (1:1,000; Santa Cruz Biotechnology) was added, and the plates were incubated for 1 h at 37°C. After three washes in PBS/T, the reaction mixture was developed with 100 μl of 3,3′,5,5′-tetramethylbenzidine substrate solution (BD Biosciences) for 20 min and stopped with 50 μl of 3 M H_2SO_4. The OD_{450} was measured.

Western blotting. Purified proteins separated by denatured SDS-PAGE were transferred to methanol-activated polyvinylidene difluoride membranes (Bio-Rad). Membranes incubated in the blocking buffer (5% nonfat milk–PBS–0.1% Tween 20) for 1 h at room temperature were then incubated in a 1:1,000 dilution of vaccinated mouse sera at 4°C overnight with gentle rocking. After 3 washes with the blocking buffer, mixtures were incubated in goat anti-mouse IgG horseradish peroxidase-conjugated antibody (1:5,000) for 1 h at room temperature. After the washes with the blocking buffer, detection was carried out using an enhanced chemiluminescence (ECL) Western blotting substrate (Pierce).

Cloning. Proteins SL1780, SL0866, SL4109, SL2251, SL1492 and SL4489 were cloned into the pET-28a vector (Novagen, EMD Biosciences, Madison, WI) with an N-terminal His tag by using the following restriction enzymes: NheI and XhoI for SL2251, NdeI and XhoI for SL1780, SL0866, and SL4109, and NcoI and XhoI for SL1492 and SL4489. The primers used for cloning SL1780 were SlL1780NdeIfw (CAT ATG ATG AAA ACC AGT GTG CGC ATT GGC) and SL1780XhoIrev (CTC GAG TCA GGC AAG ACG CAT GAC CCA GG). For SL0866 the primers were SL0866NDelfw (CAT ATG AAA AAG TTC TGA TTG CCG CG) and SL0866nonstopXholrev (CTC GAG CTT CTG GAA CCA TTT GTT ATA GAT GGT CTC). For 4109, the primers were SL4109ndelfw (CAT ATG AAC AAG ACT CAA CTG ATT GAT GTA ATT G) and SL4109Xholrev (CTC GAG TTA CAT AAC TGC GTC TTT CAG AGC TTT ACC). For SL2251 the following primers were used: SL2251NheIfw (GCT AGC ATG AAA ACC ACA CTG AAA AAC CTT AGC GTG) and SL2251XhoIRev (CTC GAG TTA GTC ATT TTT TTG CAG GAA CAT GAC C). For SL1492, the primers were SL1492 fw (TGA CTG ACC ATG GGC AAT AAA TTC TCC CTT GCT ACA GCA GG) and SL1492rev (ACT GAC TCT CGA GGC TGC CTT TTG AGA GTT CTT TCT TGA TTT CGT C). For SL4489, the primers were SL4489fw (TGA CTG ACC ATG GGC ACT ATG ACA AGA CTG AAG ATT TCT AAA ACT C) and SL4489rev (ACT GAC TCT CGA GGC TGC CCT GAA CTT TCA GAT CGT TTT TAA CAC). All plasmids were verified by sequencing.

Protein expression and purification. Proteins were expressed in *E. coli* BL21(λDE3). Bacteria were grown in 0.5 liters of LB broth supplemented with 50 μg/ml kanamycin at 220 rpm and 37°C until mid-exponential phase. Protein expression was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), and cultures were further incubated at 220 rpm and 16°C overnight. Bacterial cells were harvested by centrifugation and lysed in buffer A (20 mM Tris, 500 mM NaCl, 2 mM $MgCl_2$; pH 8) for SL1344_1780 and SL1344_2251 or buffer B (40 mM Tris, 150 mM NaCl, 3 mM $MgCl_2$, 10 mM imidazole, 0.02% NaN_3; pH 8) for all proteins except for SL1780 and SL2251. Buffers A and B were supplemented with lysozyme (Sigma), benzonase endonuclease (Santa Cruz Biotechnology), Complete EDTA-free protease inhibitor cocktail (Roche), and 1% Triton X-100, and lysates were incubated for 20 min on ice. Cell lysis was completed after two French press cycles at 10^5 kPa. The lysate was cleared by centrifugation for 45 min at 16,000 $\times$ *g* and 4°C. The His-tagged proteins were purified by incubating the cleared lysate with nickel-Sepharose HP (GE Healthcare) for 2 h at 4°C. The beads were washed and proteins were eluted with buffer A (for SL1780 and SL2251) or buffer B (for all proteins except for SL1780 and SL2251), both of which were supplemented with 300 mM imidazole. To reduce the volume to less than 2 ml, eluted proteins were concentrated using centrifugal concentrators with a 3-kDa (for SL1492, SL1780, and SL4109) or 10-kDa (for all others) molecular mass cutoff. Size exclusion chromatography was performed using a 2-ml sample loop and a Superdex 200-pg HiLoad 16/600 column (GE Healthcare) equilibrated in PBS at room temperature and a flow rate of 1 ml/min. Fractions of 1.5 ml were collected, analyzed by SDS-PAGE, and concentrated as described above. Protein concentrations were determined using the Coomassie Plus protein assay (Thermo Scientific Pierce).

Immunizations with protein cocktails. For immunizations with a combination of proteins, groups of 5 to 10 mice were vaccinated subcutaneously with a mix of 6 proteins (SL0866, SL1780, SL1492, SL2251, SL4109, and SL4489; 10 μg of each) and an equal volume of the adjuvant (TiterMax). Mice were boosted 30 days after the primary immunization. Thirty days later, the mice were challenged with WT *S.* Typhimurium at 3×10^6 CFU/ml. Mice were euthanized 4 days post-*S.* Typhimurium infection, and the ceca, spleens, and livers were analyzed for the presence of the bacteria as described above.

Statistical analysis. Statistical significance was calculated using the Mann-Whitney test with a 95% confidence interval within the Prism program version 4.0 (GraphPad Software, San Diego, CA). The results were expressed as mean values with standard errors of the means.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01421-15/-/DCSupplemental.

Figure S1, PDF file, 0.4 MB.

Figure S2, PDF file, 0.02 MB.

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