Supporting Information

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SI Materials and Methods

Cells, Bacterial Strains, and Reagents. HT-29 and INT407 cells were maintained in DMEM supplemented with 10% FBS. THP-1 cells were grown in RPMI-1640 supplemented with 10% FBS and 0.05 mM 2-mercaptoethanol. All cells were purchased from American Type Culture Collection. *S.* Typhi Ty2 and CT18 and *S.* Typhimurium LT2 were gifts from J. Parkhill (Sanger Institute, Hinxton, United Kingdom). Other bacterial strains were provided by T. Ramamurty, S. Datta, and S. K. Niyogi (National Institute of Cholera and Enteric Diseases, Calcutta, India; Table S3). *Salmonella* and *Vibrio* strains were grown in Hektoen enteric agar and thiosulfate citrate bile salts sucrose agar (BD Difco), respectively, whereas other bacterial strains were maintained in Luria–Bertani agar at 37 °C. Liquid cultures were grown in Luria–Bertani broth (BD Difco). Plasmid pSIM6 and pQE60 were gifts from S. Datta (Institute of Post-Graduate Medical Education and Research, Calcutta, India) and D. Chakravorty (Indian Institute of Science, Bangalore, India), respectively. Oligonucleotides (Dataset S1) were custom-synthesized from IDT.

Construction of *S. Typhi* Ty2-t2544 Mutant, Plasmid Curing, and Complementation. Mutant bacteria were constructed by a technique called recombineering (1). Briefly, kanamycin-resistant cassette flanked by 50-bp regions of *t1831*, *t2544*, *t2769*, *tsaC*, and *pilS* genes of Ty2 and *STM0306* gene of LT2 were PCR-amplified from *E. coli* DY411 strain and transformed into the bacteria carrying pSIM6 plasmid that contains an ampicillin resistance cassette. Bacteria were incubated at 42 °C for 15 min to induce the expression of viral recombinase encoded by pSIM6. Isogenic mutants were selected from ampicillin–kanamycin plates. Mutant bacteria were cured of pSIM6 plasmid by growing 1,000-fold diluted cultures on LA plates without antibiotics. Ty2Δ*t2544* strain was complemented with *t2544* by transforming competent bacteria with pQE60-*t2544* plasmid.

ELISA. Microtiter wells containing bacteria or purified protein were incubated with the primary antibody PAS (Sigma) or RAS, respectively, followed by HRP-conjugated secondary antibody. Wells were developed by adding o-phenylenediamine dihydrochloride substrate (Sigma) and the enzyme reaction was analyzed by measuring OD at 492 nm.

Adhesion Assay. Adhesion of live bacteria or recombinant T2544 to cells and ECM was studied according to a method originally described by O'Ferrelly et al. (2). Briefly, monolayer cultures of HT-29/INT407/THP1 cells prefixed with 0.25% glutaraldehyde or ECM components coated on microtiter wells were blocked with 0.5% BSA and incubated with log-phase cultures of bacterial cells (10^6 to 10^8) or purified T2544 for 1 h. Although cell monolayers were prefixed with glutaraldehyde, which is known to cross-link proteins, it did not contribute to T2544-mediated adhesion as cells were thoroughly washed before addition of bacteria. Specificity of the adhesion reaction and absence of glutaraldehyde during ECM attachment, as reflected by subsequent experiments, also rule out this possibility. For competitive binding assay, wells were preincubated with T2544 protein before addition of bacteria or the bacteria were preincubated with RAS before being added to the wells. After thorough washing, adherent bacteria were fixed with 0.3% formaldehyde. Cell or ECM binding by the bacteria or purified T2544 was studied by ELISA as described earlier.

Confocal Microscopy. Monolayer cultures of HT-29 cells (70% confluent) were grown on collagen-coated coverslips, fixed with glutaraldehyde, and incubated with GFP-tagged *S.* Typhi for 10 to 15 min. Cells were washed vigorously, fixed with 4% paraformaldehyde, mounted on glass slides, and viewed under a confocal microscope.

Invasion Assay. Growing cultures of HT-29 cells were infected with log-phase culture of Ty2 or Ty2Δ*t2544* (10^8 cfu/mL) for 1 h at 37 °C. After vigorous washing, cultures were grown for 2 h in presence of gentamycin (200 μg/mL) to inhibit the growth of extracellular bacteria. Cells were lysed with 0.1% Triton X-100 and number of viable intracellular bacteria were determined by cfu count on LA plates with appropriate selection.

Recombinant Protein Purification. Recombinant N-terminally His-tagged T2544 protein was induced in *E. coli* BL21 (DE3) C41 transformed with the pET28a-t2544 constructs upon treatment with 1 mM isopropyl β-D-1-thiogalactopyranoside. Protein from insoluble inclusion bodies were recovered by solubilizing with 6M guanidine hydrochloride, purified by using Ni^{2+}-NTA agarose (Qiagen), and refolded by dialysis. LPS contamination of the purified protein was found to be 0.1 EU/μg as determined by *Limulus amebocyte* assay. Proper folding of recombinant protein was confirmed by spectrophotometric analysis at 340 nm. Further confirmation regarding refolding of protein was obtained by using naturally folded recombinant protein, which was affinity-purified by using Ni^{2+}-NTA agarose in a low amount when produced in *E. coli* BL21(DE3)Plys S. Adhesion assays performed with naturally folded T2544 and refolded T2544 were identical.

Preparation of Antiserum. Male New Zealand White rabbits were injected intramuscularly with 100 μg of recombinant T2544 emulsified with Freund complete adjuvant followed by three booster doses of 150 μg of protein emulsified with incomplete Freund adjuvant administered at intervals of 7 d. The animals were bled 7 d after the last injection and antiserum was separated from the blood.

Outer Membrane Fraction Preparation. Overnight cultures of bacteria were harvested, washed with 20 mM Tris (pH 7.5), lysed by sonication after adding DNase (0.1 mg) and RNase (0.5 mg), and incubated at room temperature for 1 h. Unbroken cells were removed by centrifugation at 5,000 × *g* for 10 min. Membrane fractions were collected by centrifugation of the supernatant at 40,000 × *g* for 60 min at 4 °C and resuspended in 20 mM Tris containing 2% (wt/vol) sodium lauryl sarcosine. The mixture was incubated at room temperature for 30 min and the outer membrane fractions were collected by centrifugation as described earlier (3).

Quantitative Analysis of Laminin Binding of T2544. K_a of the equilibrium between T2544 and laminin was determined by Scatchard plot (4). Briefly, laminin-coated microtiter wells were incubated with increasing concentrations of purified T2544 at 37 °C followed by addition of RAS. Bound T2544 was determined as described in *Adhesion Assay* and a saturation–binding curve was generated. Free T2544 was obtained from the difference of the total and the bound protein. The K_a value was calculated by using GraphPad Prism software.

In Vitro Bactericidal Assay. Sera collected from typhoid and non-typhoid patients were preincubated with Ty2Δ*t2544* before use. A serial dilution of the heat-inactivated sera (50 μL) was prepared in a microtiter plate. Early log-phase culture (OD of 0.2 at 600 nm) of the target bacterial cells along with 25% guinea pig comple-

ment was added to appropriate wells, bringing the total volume to 100 μL and incubated at 37 °C for 1 h. Brain–heart infusion medium 100 μL was added to each well and the bacteria were allowed to grow for 4 h. Optical density was measured in an ELISA reader at 600 nm. Neither the heat-inactivated sera nor the complement alone killed the target bacteria.

Opsonization Assay. Mouse peritoneal macrophages were harvested by flushing the peritoneal cavity with ice-cold PBS solution. Collected cells were centrifuged and resuspended in prewarmed RPMI supplemented with 10% serum. Approximately 5×10^5 cells were added to each well of a 24-well tissue culture plate and incubated for 2 h at 37 °C. Nonadherent cells were removed, medium was replaced with fresh medium, and cells were incubated overnight at 37 °C. Before inoculation, log-phase culture of bacteria were mixed with anti-T2544 antisera or preimmune sera and each well was inoculated with 10^6 opsonized bacteria. After 30 min, wells were washed with PBS solution and medium was replaced by gentamycin containing RPMI. Samples were incubated for 0, 60, 90, and 120 min. Cells were lysed with 0.1% Triton X-100 and number of viable intracellular bacteria were determined by cfu count on LA plates with appropriate selection (5).

Measurement of Bacterial Load and sIgA in Mice Feces. Fecal mater was collected form immunized and nonimmunized mice before and after the challenge with bacteria. Equal amounts of fecal matter was dissolved in PBS solution and supernatant was collected after centrifugation at $400 \times g$ for 10 min. Number of viable bacteria was determined by cfu count on LA plates with appropriate selection. sIgA titer was determined by using an ELISA-based assay.

Mice Immunization Assay. Inbred Swiss Albino mice ($n = 50$) were immunized with four subcutaneous injections of recombinant T2544 (0.5 μg) administered at intervals of 7 d. Antisera and intestinal lavage ($n = 5$) were collected at the indicated time points, and the titers of T2544-specific antibody isotypes were measured by ELISA in the pooled samples (sIgA, secretory IgA).

Passive Immunization Assay. Passive protection assay was performed as described by Iankov et al. (5). Briefly, groups of 10 iron-overload mice were injected i.v. with 200 μg of heat-inactivated mouse anti-T2544 immune sera or normal mouse serum. After 1 h, animals were challenged intraperitoneally with 10^4, 10^5, 5×10^5, and 10^6 Ty2 bacteria. Protection was evaluated as percent survival during the next 7 d.

Western Blot Analysis. Endogenous and recombinant T2544 run in SDS/PAGE and transferred to a PVDF membrane were probed with RAS and mouse anti-His antibody, respectively. In a separate experiment, the blot was probed with human antisera collected from typhoid-positive and -negative individuals. Following addition of HRP-conjugated secondary antibody, the blot was developed by chemiluminescence and autoradiographed.

Statistical Analysis. Statistical significance was analyzed by the Student t test using GraphPad QuickCalcs software. The results were considered significant at $P \leq 0.01$.

1. Datta S, Costantino N, Court DL (2006) A set of recombineering plasmids for gram-negative bacteria. *Gene* 379:109–115.
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4. Saha N, Banerjee KK (1995) Carbohydrate-dependent binding of the cell-free hemagglutinin of Vibrio cholerae to glycoprotein and glycolipid. *J Bacteriol* 177: 758–764.
5. Iankov ID, et al. (2004) Protective efficacy of IgA monoclonal antibodies to O and H antigens in a mouse model of intranasal challenge with *Salmonella enterica* serotype Enteritidis. *Microbes Infect* 6:901–910.

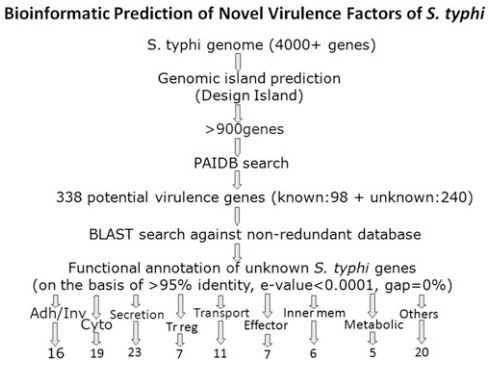

Fig. S1. Scheme followed for the computational prediction of novel virulence factors of *S.* Typhi.

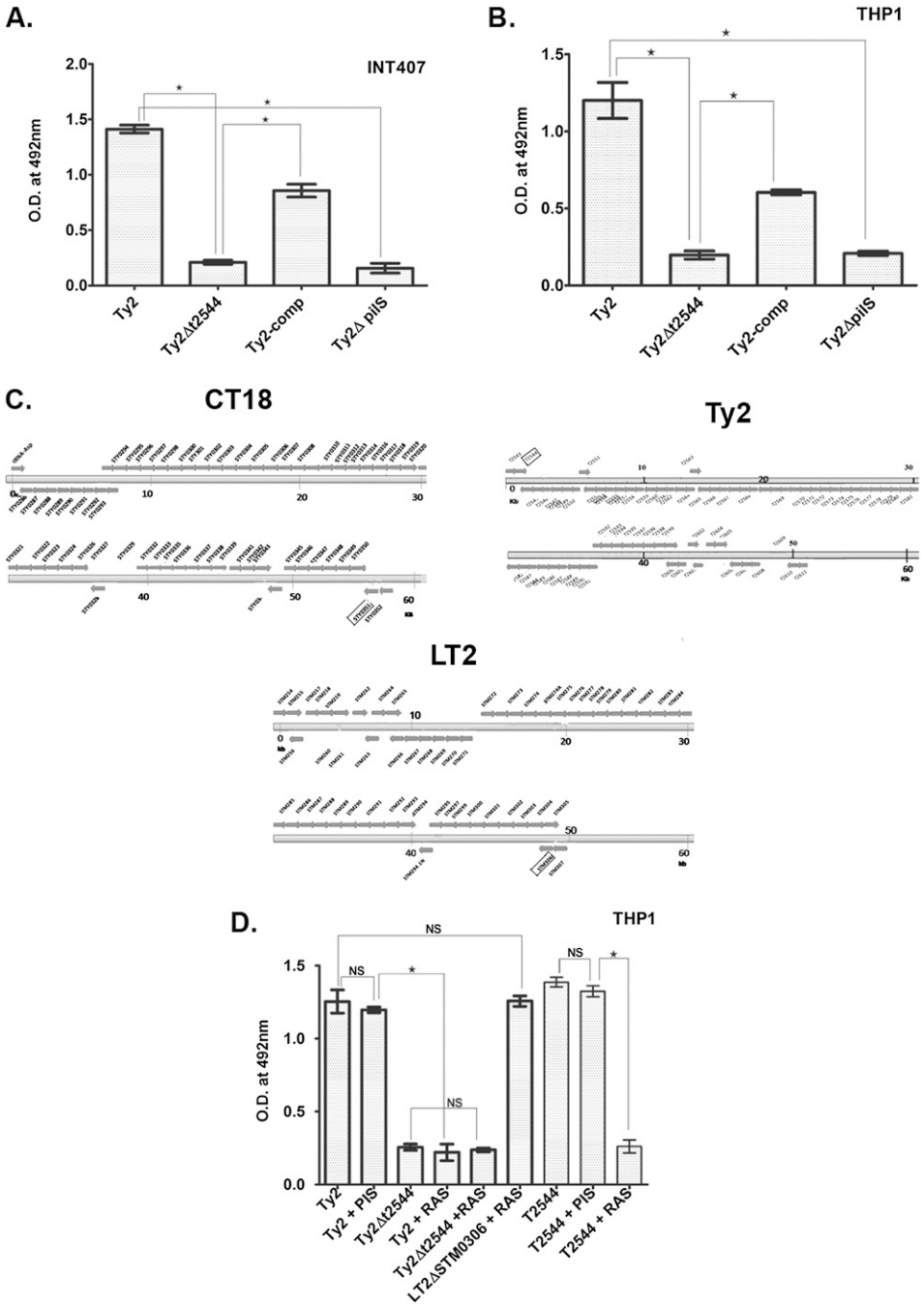

Fig. S2. (*A* and *B*) Bacterial adhesion to cell monolayers as done in Fig. 1*A*. (*C*) Gene array of SPI-VI in different *Salmonella* strains. The number of protein-coding genes is equal in CT18 and LT2 ($n = 52$ in each) and somewhat less than that of Ty2 ($n = 60$). A majority of the genes located in this pathogenicity island of Ty2 are annotated as hypothetical in GenBank, and putative functions have been designated to most of the corresponding LT2 genes. *t2544* and its homologues are boxed and none of them is part of an operon system. (*D*) Cell monolayer (THP-1) adhesion of live bacteria or recombinant T2544 as in Fig. 1*E*. All data represent one of three independent experiments; bar diagrams show mean ± SD. $^*P \leq 0.01$; NS, not significant.

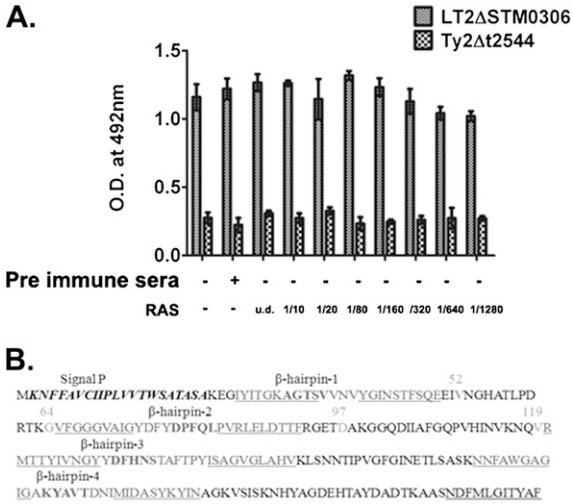

Fig. S3. (*A*) Dose-dependent inhibition by RAS of bacterial adhesion to laminin as described in Fig. 2*A*, *3*. (*B*) Predicted topology of T2544 protein: signal peptide at the N terminus, nine-stranded (underlined) β-barrel, and two loop like structures (aa 52–64 and 97–119). All data represent one of three independent experiments; bar diagrams show mean ± SD. The difference in adhesion between the two strains was statistically significant ($P \leq 0.01$)

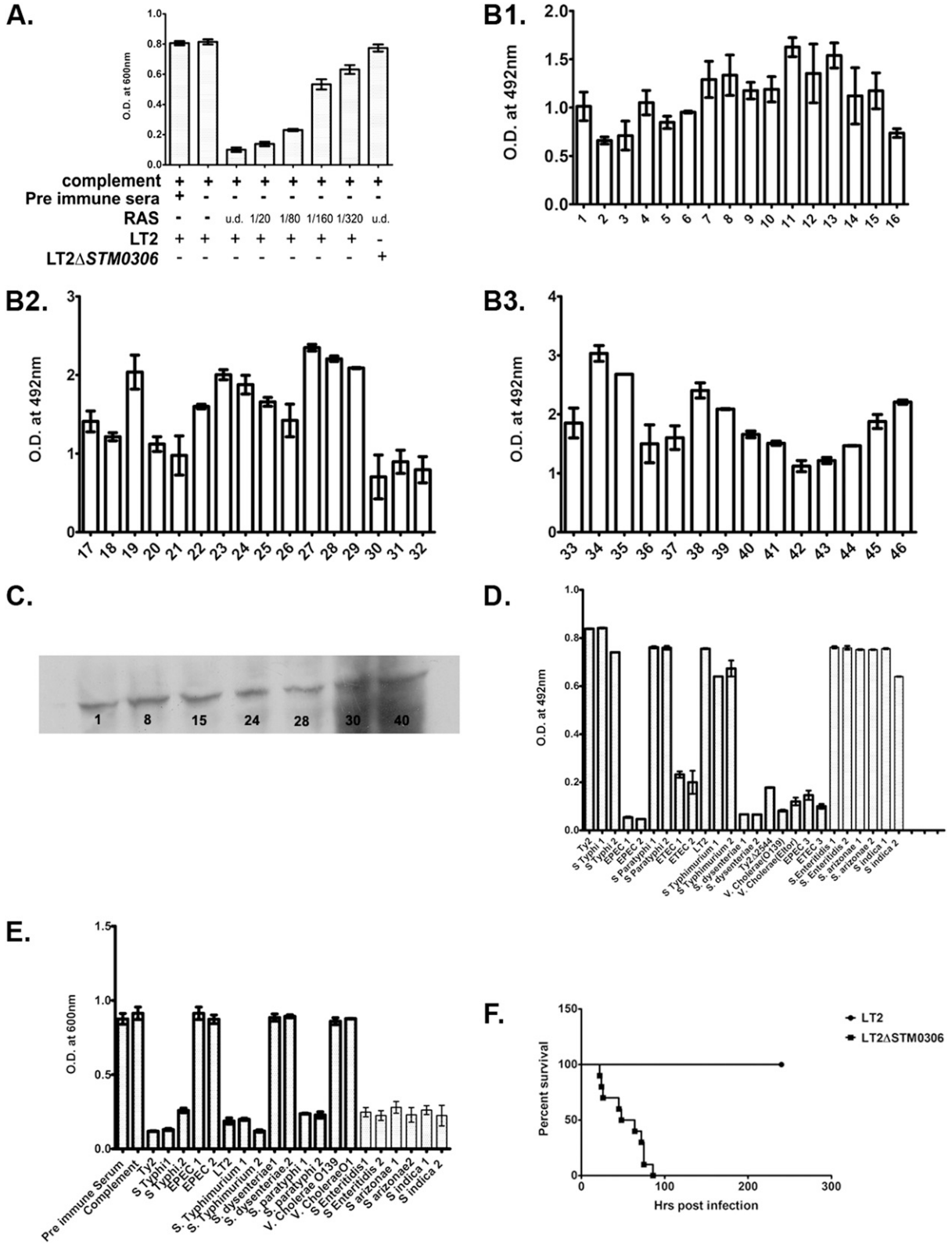

Fig. S4. (*A*) In vitro bactericidal assay as performed in Fig. 4*A*, *2*. (*B*) *1–3*: Expression of T2544 protein by clinical *Salmonella* isolates recovered during 2003 to 2010 from different geographical locations (east, west, and south) in India. Log-phase cultures of bacteria were fixed on microtiter wells, incubated with RAS followed by HRP-conjugated secondary antibody. OD was measured at 492 nm after addition of OPD substrate. 1–35 = *S.* Typhi; 36–45 = *S.* paratyphi. (*C*) T2544 protein detected by Western blot in the indicated clinical isolates of *B*, *1–3*. (*D*) Expression of T2544 by different enteric pathogens of clinical origin. Experiment done as in *B*, *1–3*. (*E*) In vitro bactericidal assay as performed in *A* with the clinical strains mentioned in *D*. (*F*) Kaplan–Meier plot of the survival assay performed as in Fig. 4*C*, *1*. Mice were immunized with recombinant T2544 and subsequently challenged with LT2 or LT2Δ*STM0306*. All data represent one of three independent experiments; bar diagrams show mean ± SD.

Table S1. Systemic invasion of mice

Organ/DPI	Mean difference in bacterial count log10 values	*t* Statistic	Significance	95% CI of difference
Blood				
2	0.59	7.54	<0.001	0.41–0.77
4	0.69	17.14	<0.001	0.59–0.78
6	0.69	11.88	<0.001	0.56–0.83
Liver				
2	0.42	3.07	<0.01	0.10–0.74
4	0.52	5.09	<0.001	0.28–0.76
6	0.43	11.25	<0.001	0.34–0.52
Spleen				
2	0.80	15.56	<0.001	0.68–0.92
4	0.66	10.03	<0.001	0.51–0.82
6	0.78	23.90	<0.001	0.70–0.86

Unpaired *t* test results showing the significance of difference between the numbers of live organisms recovered from the blood, liver, and spleen of mice infected with Ty2 or Ty2Δt2544. DPI, days postinfection.

Table S2. Opsonization assay

	Mean ± SD, CFU × 10^4/well			
Opsonized with *S.* Typhi Ty2	0 min	60 min	90 min	120 min
T2544 antisera	25.9 ± 5.7	23.4 ± 5.07	42.3 ± 7.8	82.8 ± 11.6
Preimmune sera	20.5 ± 1.2	93.4 ± 11.5	109 ± 11.2	110 ± 11
t Statistic	1.60	9.52	8.37	2.88
Significance	NS	<0.001	<0.001	NS

Unpaired *t* test results show significance of differences between numbers of live Ty2 recovered from mouse peritoneal macrophages after opsonization with preimmune or immune sera.

Table S3. Bacterial strains used in this study

Strain name	Phenotype	Genotype	Source	Reference
S. Typhi Ty2	—	—	Commercial	ATCC 700931
S. Typhi	O9, 12, (Vi) Hd	—	Clinical	1
S. Paratyphi A	O1, 2, 12, Ha:1, 5	—	Clinical	1
S. Typhimurium	01, 04, 05, Hi, 1, 2	—	Clinical	Unpublished
S. Typhimurium LT2	—	—	Commercial	ATCC 25870
Salmonella enteritidis	01, 09, 12, Hg	—	Clinical	Unpublished
Salmonella arizonae	18, z4, z23, 2	—	Clinical	Unpublished
Salmonella indica	11, Hb, e, n, x	—	Clinical	Unpublished
ETEC (1)	O6:H16	LT+	Clinical	Unpublished
ETEC (2)	OUT	LT+	Clinical	Unpublished
ETEC (3)	OUT	LT+	Clinical	Unpublished
EPEC (1)	O126	bfp+	Clinical	Unpublished
EPEC (2)	O86	bfp+	Clinical	Unpublished
EPEC (3)	O115	bfp+	Clinical	Unpublished
Shigella dysenteriae type I	Congo red (+), Sereny (+)	—	Clinical	2
V. cholerae (Eltor)	Ogawa	*Ctx*(+),*tcp*(+)	Clinical	3
V. cholerae O139 Bengal (SG24)	—	*Ctx*(+),*tcp*(+)	Clinical	3
S. Typhi Ty2Δ*t2544*	—	*t2544*(-),*PilS*(+)	Laboratory	Present study
S. Typhi Ty2Δ*pilS*	—	*t2544*(+),*PilS*(-)	Laboratory	Present study
E. coli BL21(DE3)	—	—	Commercial	Novagen 69387

OUT, O-untypable antigen.

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3. Saha PK, et al. (1996) Nontoxigenic Vibrio cholerae 01 serotype Inaba biotype El Tor associated with a cluster of cases of cholera in southern India. *J Clin Microbiol* 34:1114–1117.

Dataset S1.

Dataset S1

Bioinformatic analysis and additional information. Sheet 1, Correspondence analysis of the putative adhesions/invasions. Sheet 2, Genes subjected to correspondence analysis. Sheet 3, Functionally annotated genes in the PAI of S. Typhi. Sheet 4, Putative/hypothetical genes in the PAIs. Sheet 5, Secretion-related proteins. Sheet 6, Cytoplasmic proteins. Sheet 7, Transcriptional regulator. Sheet 8, Adhesion/invasion–related protein. Sheet 9, Effector proteins. Sheet 10, Transporters. Sheet 11, Metabolic protein. Sheet 12, Inner membrane protein. Sheet 13, Other genes. Sheet 14, Patient data. Sheet 15, Primers used in this study. Sheet 16, sIgA and bacterial count in the feces of immunized mice challenged with Ty2. Sheet 17, Detailed statistical analysis for Figs. S2*A*, *1* and S4*B*, *3*.