An adhesion protein of *Salmonella enterica* serovar Typhi is required for pathogenesis and potential target for vaccine development

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More than half of all *Salmonella enterica* serovar Typhi genes still remain unannotated. Although pathogenesis of *S. Typhi* is incompletely understood, treatment of typhoid fever is complicated by the emergence of drug resistance. Effectiveness of the currently available vaccines is also limited. In search of novel virulence proteins, we have identified several putative adhesins of *S. Typhi* through computational approaches. Our experiment shows that a 27-kDa outer membrane protein (T2544) plays a major role in bacterial adhesion to the host through high-affinity binding to laminin. Its role in bacterial pathogenesis is underscored by reduced systemic invasion and a 10-fold higher LD90 of the mutant bacteria in mice. T2544 is strongly immunogenic as revealed by the detection of sustained high titers of serum IgG and intestinal secretory IgA in the immunized mice. In vitro, T2544 antiserum enhanced uptake and clearance of *Salmonella* by macrophages and augmented complement-mediated lysis, indicating a contribution of T2544-specific antibodies to the killing process. This correlates well with the observed protection of mice immunized with recombinant T2544 or passively immunized with T2544 antiserum against subsequent bacterial challenge, suggesting that T2544-specific antibodies are involved in protection. The present study describes an adhesion protein of *S. Typhi* that contributes to bacterial pathogenesis. Protective antibodies in mice, rapid seroconversion of naturally infected individuals with increasing titers of anti-T2544 IgG from acute to convalescent sera suggesting antibody response in humans, and wide distribution and conservation of the cell-surface adhesion in the clinical isolates of different *Salmonella* serovars make T2544 a potential vaccine candidate.

Gram-negative pathogenic bacterium, *Salmonella enterica* Typhi (*S. Typhi*), remains a major threat to public health in the developing world. Approximately 21 million cases are estimated, resulting in 216,519 deaths in the year 2000 (1). The bacterium generally causes an acute febrile illness known as enteric fever, and a chronic carrier state may contribute to adenocarcinoma of the gallbladder (2). *Salmonella* spp. also remain the leading cause of septicaemia in the endemic population (3, 4).

Pathogenesis of *S. Typhi* is incompletely understood, and treatment failure is not uncommon in the era of multidrug resistance (3). The *Salmonella* genome contains clusters of virulence-associated genes called pathogenicity islands (PAIs). Of 17 PAIs identified so far (5), functions of only SFI 1, 2, and 7 are partially known. Functional characterization of other PAIs will help to identify new drug/vaccine targets.

Vaccination of a susceptible host may be most effective to protect the population living in the endemic zone (6). Currently available vaccines (live attenuated or killed) are at best moderately (50–70%) efficacious in older children and adults, but not suitable for children younger than 5 y (6–8), in whom a Vi-recombinant exoprotein A (rEPA) conjugate vaccine showed impressive results (protective efficacy of 91.1% at 27 mo) (9). The major shortcomings of the live vaccine is the cost and requirement of multiple (n = 3–4) doses (7). In contrast, boosters do not enhance protection and memory cells are not generated in case of T-cell-independent Vi polysaccharide, which also fails to induce intestinal secretory IgA (sIgA) response (6, 8).

Bacterial adhesion molecules for the host receptors (i.e., adhesins) play critical roles in pathogenesis (10). They may be divided into three broad groups: (i) large multimeric molecules called pili or fimbriae; (ii) non–pili-associated adhesins, which are monomeric or oligomeric proteins; and (iii) thin fibers known as curli (11). Adhesins may recognize host cell surface receptors and/or ECM components, such as collagen, laminin, fibronectin, and heparan sulfate (10) and their function is primarily blocked by sIgA. Laminin is a large (900 kDa), highly glycosylated, multidomain protein that forms a major component of the basement membrane. Laminin binding by many pilus and nonpilus bacterial adhesins is the starting point of invasion by many pathogenic microorganisms (12, 13).

Bacterial adhesins may induce strong protective immunity in the host and remain attractive vaccine targets. A FimH adhesion-based vaccine has been very successful to prevent infection with a wide range of uropathogenic *Escherichia coli* in mice (14), although it has failed in humans. However, large number of studies with bacterial adhesion molecules as candidate vaccines have shown considerable promise (15–19).

Here, we show that an outer membrane adhesion protein of *S. Typhi* contributes to pathogenesis through laminin binding. In addition, it is a strong immunogen in animals as well as in humans and induces protective antibody response.

Results

Bioinformatic Prediction of Pathogenicity-Associated Genes of *S. Typhi*. More than half of the genes of *S. Typhi* still remain uncharacterized. We took a bioinformatic approach to predict virulence properties of the hypothetical unannotated genes (Fig. S1). In a recently published analysis of the *S. Typhi* CT18 genome, more than 900 genes were detected as horizontally acquired and part of the “genomic islands” (20). Given that bacterial virulence genes are also horizontally acquired, we searched the Pathogenicity Island Database (http://www.gem.re.kr/paidb) and retrieved 338 of these genes that may be potentially important for pathogenesis. More than two thirds of them, designated as “hypothetical” or “putative” by GenBank (National Center for Biotechnology Information), were subjected to BLAST search against the nonredundant database, and finally, 114 genes were functionally annotated (Fig. S1). A multivariate statistical analysis called correspondence analysis was performed with 16 genes that were denoted as putative adhesins/invasins. This showed one major trend (first major axis) that accounts for almost half the total variations in amino acid use by the genes in the dataset. Position of them along this axis negatively correlates with conservation of the amino acid sequence (Fig. 2A).

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correlated with the hydrophobicity of the encoded proteins ($r = -0.623$, $P < 0.01$) (21), of which three (STY0351, STY1115, and STY2988) were found to be the most hydrophobic (Dataset S1).

**T2544 is a Major Outer Membrane Adhesion Protein of S. Typhi.** Proteins with hydrophobic regions are membrane-localized and may function as adhesion molecules. We studied the three most hydrophobic putative adhesins by generating isogenic mutants in $S$. Typhi. Ty2 background (deletion of $t2544$, $t833$, and $t2769$ genes that are 100% identical in the nucleotide sequence to STY0351, STY1115, and STY2988 genes of CT18, respectively), as CT18 carries several drug-resistance cassettes in the plasmid. Adhesion to human cell lines was significantly impaired for only one mutant (Ty2Δ$t2544$; Fig. 1 A and B and Fig. S2 A and B), whereas $t2544$ and STY0351 were found to be located in the SPI-VI (Fig. S2C). That impaired adhesion of Ty2Δ$t2544$ was directly related to protein expression rather than polar effect of mutation was proved by unaltered expression of downstream and upstream genes, namely $t2548$ and $t2543$, respectively. Deletion of the upstream usher $t2548$ (tsuC) did not impair adhesion of Ty2 to the cell monolayer, suggesting that $t2544$ may be a standalone adhesin. Ty2Δ$t2544$ was equally adhesion-impaired as Ty2spoIAB, the bacteria mutated of type IV pil that constitute the prototype adhesion apparatus of $S$. Typhi. In contrast, complemented $t2544$ mutant (Ty2-comp) significantly restored this function (Fig. 1A). Together these results indicate that $t2544$ is required for and may be directly involved in host cell adhesion of $S$. Typhi. Interestingly, mutant Salmonella Typhimurium LT2 lacking $T2544$ homologue (LT2ΔSTM0306) did not show impaired adhesion. Given that $S$. Typhi and $S$. Typhimurium predominantly use type IV and type I pili, respectively, the latter may use different host receptors or $S$. Typhimurium may have additional adhesion mechanism(s). In a cell invasion assay, the number of live, intracellular bacteria recovered from HT-29 cells directly correlated with their ability to adhere, suggesting that $T2544$ primarily contributes to cell adhesion (Fig. 1C). To study if $T2544$ is directly involved in adhesion or functions through other molecule(s), recombinant $T2544$ was purified from $E$. coli. The size of the purified protein in SDS/PAGE (27 kd) corresponded to the predicted size from the amino acid sequence and Western blot analysis detected a single band (Fig. 1D, 1). Proper reolding of the protein was confirmed by spectrophotometric analysis at 340 nm and comparing its adhesion function with the small amounts of naturally refolded fraction of the recombinant protein. Subcellular localization studies that used rabbit antiserum (RAS) raised against recombinant $T2544$ showed that it was more concentrated in the outer membrane fraction compared with the whole-cell lysate, whereas it was absent from the cytosolic fraction. As expected, the protein could be detected in both Ty2 and Ty2-comp, albeit to a lesser extent in the latter, but not in Ty2Δ$t2544$ (Fig. 1D, 2). Purified $T2544$ attached to HT-29 (Fig. 1E) and THP-1 (Fig. S2D) cell monolayers as robustly as live Ty2. RAS, but not preimmune serum (PIS), almost completely blocked adhesion of Ty2 and recombinant $T2544$, but not LT2ΔSTM0306, indicating specificity of the antibody effects. Further studies that used increasing doses of $T2544$ showed comparable adhesion to HT-29 and THP-1, which was substantially higher than that of BSA (Fig. 1F). Together these results suggest that $T2544$ is a standalone adhesin that performs a nonredundant function in $S$. Typhi.

**T2544 binds to laminin and is required for adhesion of $S$. Typhi to the host.** Host adhesion may be mediated through attachment to the cell surface receptor(s), ECM, or both. To investigate the host molecule to which $T2544$ binds, adhesion of different Ty2 strains to microtiter plates coated with various ECM components was studied. The results showed significantly greater adhesion of Ty2 compared with Ty2Δ$t2544$ to the solid-phase laminin only. Ty2-comp considerably restored adhesion, suggesting that $T2544$ contributes to attachment of $S$. Typhi to laminin (Fig. S2A, 1). This observation is supported by significantly reduced adhesion of the WT bacteria to laminin when it was preincubated with RAS or laminin was pre-

**Fig. 1.** $T2544$ is an outer membrane protein involved in host cell adhesion. (A) Bacterial adhesion to HT-29 cell monolayer detected by ELISA using polyclonal antiserum (PAS). Serial dilutions of bacteria were used for the experiment, and the result with $10^8$ bacteria is shown. (B) Confocal micrographs of GFP-expressing $S$. Typhi adhered to HT-29 cell monolayer. The lower panel shows superimposition of confocal and phase-contrast images. (C) Invasion assay studied by recovery of live intracellular bacteria. (D1) Recombinant $T2544$ detected by Coomassie Blue staining (Left) and monoclonal anti-His antibody (Right). (D2) Cellular fractions of Ty2 analyzed by Western blot probed with RAS. Ty2-comp expressed somewhat lesser amounts of $T2544$ compared with Ty2 (intensity 929.42 vs. 603.18 in the densitometry measurement using Quantity One, Biorad). TF, total cell fraction; MF, membrane fractions; CF, cytosolic fraction. (E) Adhesion of Ty2 or recombinant $T2544$ pre-incubated with PIS or RAS as detected by PAS (for bacteria) or RAS (for protein). (F) Adhesion of recombinant $T2544$; BSA was used as a control. All the above experiments were repeated three times, and data from a representative one are shown; bar diagrams show mean SD. *$P < 0.01$; NS, not significant.
treated with recombinant T2544. Laminin attachment of Ty2ΔpilS was comparable to that of Ty2 and also blocked by RAS, indicating that type IV plus of S. Typhi does not contribute to it (Fig. S2A, 2). Further studies revealed that Ty2 and LT2 were equally efficient in adhesion to laminin that was unaffected by PIS, whereas increasing concentration of RAS progressively inhibited binding of the former strain, but not the latter (Fig. S2A, 3). Conversely, adhesion of Ty2Δt2544 was markedly impaired whereas that of LT2ΔSTM0306 was comparable to that of WT bacteria and remained unaltered by RAS pretreatment (Fig. S3A). Together these results strongly suggest that T2544 directly binds to laminin and is responsible for adhesion of S. Typhi to it, and it remains redundant for adhesion of S. Typhimurium. Further studies showed a linear relation between the dose and the binding of T2544 to laminin at greater than a threshold concentration. However, purified BSA and an unrelated His-tag protein (FliC of S. Typhi) failed to show binding (Fig. 2B). In addition, T2544 did not bind to other ECM components, indicating specificity. Binding affinity was strong (K_a, 24.14 × 10^9 M; Fig. 2C), whereas a Kyte–Doolittle hydrophobicity plot showed two strong negative peaks at approximately 100 and beyond 200 aa from the N terminus of T2544, indicating probable hydrophilic regions in the protein that may be exposed outside (Fig. 2D). Subsequent computational analysis (22) suggested the predicted topology of T2544 as a nine-stranded barrel with two loop-like structures (aa 52–64 and 97–119) that may be involved in laminin binding (Fig. S3B).

**T2544 Contributes to S. Typhi Pathogenesis.** To investigate if the adhesion-impaired phenotype has reduced pathogenicity/virulence, we first determined the LD_{50} dose for Ty2 in Swiss Albino mice. To this end, iron-overloaded mice were challenged orally with different bacterial loads (10^5 to 10^{10}). Half were dead within the next 7 d. However, all animals that received similar doses of t2544- or pilS-mutant survived. As T2544 was found not to interfere with iron uptake and use by the bacteria, this result suggests less pathogenicity of Ty2Δt2544 as a result of impaired adhesion (Fig. 3A). Subsequent studies showed a 10-fold higher LD_{50} dose for Ty2Δt2544. In contrast, LD_{50} doses were the same for LT2 and LT2ΔSTM0306 strains. To investigate if reduced virulence of Ty2Δt2544 is a result of less number of bacteria reaching the systemic circulation, mice were fed with 10^6 each of the Ty2 or Ty2Δt2544 bacteria. Animals were killed on days 2, 4, and 6 and live bacteria were recovered from the blood and visceral organs. Five- to eightfold fewer live mutants were retrieved than the WT (P < 0.001), supporting the aforementioned hypothesis (Table S1). This issue was further addressed by analyzing the competitive index (CI) (23), where 10^6 each of Ty2 and Ty2Δt2544 were premixed before being fed to the mice and live organisms were subsequently recovered from the animals. The CI of the mutant was approximately 0.25 of the WT bacteria, indicating that systemic invasion by the Ty2Δt2544 is considerably less efficient (Fig. 3B). These results together suggest that T2544 contributes to pathogenicity and systemic infection of S. Typhi.

**T2544Induces Protective Immunity Against S. Typhi Infection.** RAS blocked adhesion of Ty2 and recombinant T2544 to host cells and ECM, suggesting induction of specific antibody response (Figs. 1 and 2). To further characterize T2544 antibodies, mice were injected s.c. with purified T2544 and titers of different antibody isotypes in the pooled preimmune and immune sera as well as sIgA titers in the intestinal lavage collected between 7 and 120 d after the first immunization were analyzed. Immunized mice showed an early increase of serum anti-T2544 IgM level, rapidly followed by increasing IgG1. Although the latter showed the greatest response, increased titers of all isotypes measured were found until 2 mo after immunization. However, serum IgG2a failed to show a response (Fig. 4A, 1). In addition, immunized, as opposed to unimmunized, mice had detectable levels of sIgA in stool, and when challenged with S. Typhi (10^8), the former shed significantly more number of bacteria (mean, 5.6 × 10^3 ± 6.20 cfu

**Fig. 2.** T2544 adhesion to laminin. (A1) ELISA showing bacterial adhesion to ECM-coated microtiter wells. (A2) ELISA showing adhesion of Ty2 or purified T2544 preincubated with or without PIS or RAS to laminin-coated microtiter wells. Ty2 was also added to laminin preincubated with recombinant T2544. (A3) Laminin adhesion of bacteria preincubated with serial dilutions of RAS. (B) Laminin-adhesion assay with recombinant His-tagged proteins (T2544 or FliC of S. Typhi) or BSA. (C) Scatchard plot showing association constant (K_a) of binding between T2544 and laminin. (D) Hydrophobicity plot of T2544 (window size = 9). All data are representative of three independent experiments; bar diagrams show mean SD. *P ≤ 0.01; NS, not significant.
but not Ty2

was seen with more than threefold recovery of live bacteria

collected 5 wk after the

Ty2 was incubated with complement and serial dilutions of RAS
correlate with protection against systemic infection. To this end,
investigated if anti-T2544 IgG may kill

independent experiments is shown here.

recovered from the blood and visceral organs. A representative of three
infected orally with 1:1 mixture of Ty2 and

challenged with bacteria. (Fig. 4)

plot of cumulative mortality after mice (Fig. 4, 2).

Fig. 4. Immunogenicity and protective efficacy of recombinant T2544. (A1) Titers of specific antibody isotypes in the pooled (n = 5) antisera and intestinal lavage of mice immunized with T2544. sigA, secretory IgA. (A2) In vitro bactericidal assay with RAS. (B1) Detection of anti-T2544 IgM. T2544 protein was probed with S. Typhi culture-positive (ST1, ST2, ST3 and ST4) and negative (both culture and PCR) (N51, N52 and N53) patients’ sera in a western blot experiment. (B2) Rising titres of T2544-specific IgG in the paired sera. A.S., acute phase sera (collected 3–7 d after the onset of fever); C.S., convalescent sera (collected ~2 mo from the onset of fever). (B3) In vitro bactericidal assay with patients’ sera adsorbed with Ty2ΔT2544. Plot shows mean (0.312, 0.748, 0.987,

5.07 cfu ± 11.5 cfu after 1 h; P < 0.001; Table S2), further suggesting protection. T2544 was found to be conserved in a large number of clinical S. Typhi and S. Paratyphi strains collected during 2003 to 2010 from different geographical locations in India. These strains reacted with RAS in a whole-cell ELISA, suggesting cell surface exposed T2544 epitopes (Fig. S4B, 1–3). Specificity of ELISA was suggested by restricted reaction of RAS to multiple subspecies of S. enterica, i.e., enterica (serovars Typhi, Paratyphi, Typhimurium), enteritidis, arizonae, and indicus, but not other enteropathogens, which do not express T2544 (Fig. S4D), and was confirmed by Western blotting (Fig. S4C). In vitro bactericidal activity of RAS against diverse S. enterica isolates suggested that T2544 antibodies may be involved in protection of humans against broad-range salmonellosis (Fig. S4E). To study immunogenicity of T2544 in humans, acute-phase sera of 17 S. Typhi culture-positive patients were analyzed. A majority of them (11 of 17; 65%) showed very high IgM response (detection level at dilutions as high as 1:512). In Western blot analysis, only S. Typhi-positive sera, but not the ones from other febrile patients, reacted with recombinant T2544 (clinical history provided in Dataset S1). This excludes cross-reactive antibodies in the clinical sera (Fig. 4B, 2). Paired typhoid sera showed a sharp increase of T2544-specific IgG titers from the acute phase to the convalescent sera (Fig. 4B, 2), whereas all sera, after being adsorbed with Ty2ΔT2544, showed complement-mediated lysis of Ty2 in vitro. This indicates bactericidal potential of T2544 antibodies that correlated with antibody titers present in the clinical sera (Fig. 4B, 3). In addition, adsorption of Ty2ΔT2544-adsorbed antisera with T2544 protein resulted in significant reduction of their bactericidal activities, further supporting the role of human T2544 antibodies in killing S. Typhi in vitro (Fig. 4B, 3). Finally, to study the protective immunity conferred by T2544, iron-overloaded mice were immunized with s.c. injection of recombinant
T2544 or Vi, followed by oral challenge with 5 × 10^9 bacteria. All immunized animals were healthy whereas 70% of the nonimmunized mice died within the next 7 d, suggesting strong protective immunity (Fig. 4C, 7). Similar protection was observed when mice were challenged with LT2, but not with LT2ΔSTM0306 (Fig. 5F). In separate experiments, passive immunization of mice with T2544 antiserum administered i.p. provided 100% protection against subsequent peritoneal challenge with a 10-fold higher dose of Ty2 compared with the dose that killed all unimmunized mice. Additionally, 50- and 100-fold higher doses resulted in 90% and 70% protection, respectively (Fig. 4C, 2). Together these results strongly suggest that T2544 is a potent immunogen and may confer protection against S. Typhi infection.

Discussion

In the postgenomic era, searches have been intensified to identify new virulence genes and immunogenic molecules of pathogenic microorganisms. A large volume of computer resources and tools has significantly helped these efforts (24). We have used computational approaches coupled with in vitro and in vivo experiments to identify hitherto undescribed pathogenicity-associated molecules of S. Typhi. A similar approach may be taken for high-throughput screening of virulence genes of other pathogens and may result in successful identification of new drug/vaccine targets. T2544 contributes to host adhesion of S. Typhi through binding to laminin with a strong affinity. Computational analysis (Fig. S3F) and a hydropophicity plot (Fig. 2D) suggested that at least one loop-like structure approximately 100 aa from the N terminus may be expressed on the outer membrane where it would greatly involve in laminin binding. The scanning mutation of the loop is currently under way in our laboratory to further characterize the binding residue(s). Laminin binding also explains T2544-mediated attachment to cell monolayers, as both IECs and THP-1 secrete laminin (25–27). However, we have not excluded binding of T2544 to the cell surface receptor (s). Redundancy of T2544 homologue to mediate cell adhesion of S. Typhimurium is explained by the fact that the latter bacterium expresses type 1 pili and additional molecules that help it to attach to both cells and ECM (10). We observed that T2544 contributes to pathogenicity and systemic infection of S. Typhi in a murine model. This is in agreement with the previous reports that suggested the requirement of ECM attachment for invasive pathogens (13) and our own finding that type IV pili of S. Typhi play no role in the bacterial adhesion to ECM. A 10-fold increase of LD50 dose for Ty2Δt2544 may not be the most dramatic of the effects, but is not unexpected considering that multiple adhesion molecules may take part in the pathogenesis and passive uptake of bacteria by the host cells is not significantly adhesion-dependent. Even a lesser difference of LD50 may be significant as was reported for fimbrial outer membrane usher IpfC of S. Typhimurium (28). Results of the lethality experiments correlate with five- to eightfold less tissue invasion and a comparable decrease in the cell and ECM attachment in vitro by Ty2Δt2544. In addition, the CI of the mutant bacteria was significantly less than that of the WT (P < 0.001) (29, 50). Considering the genomic location of t2544, to the best of our knowledge, this is the first report of functional characterization of PAI-VI and experimental evidence in favor of its role in Salmonella pathogenesis.

Adhesion to host is the critical first step in microbial pathogenesis (10). By using a modified iron-overload mouse model (31), we have shown that T2544 contributes to S. Typhi pathogenesis. As the protein has no independent role in tissue invasion, impaired adhesion may be responsible for its reduced virulence. Given that S. Typhi fails to establish infection in mice as it is outperformed by the host for iron (32), an iron-overload mouse is more akin to the human host with respect to this infection. A recently developed humanized mouse model of S. Typhi failed to replicate enteric fever, but showed persistent infection (33). Moreover, the investigators used i.p. injection to infect the mice instead of the oral route, which is the natural portal of entry for S. Typhi. However, the humanized mice mounted immune response (IR) and it would be interesting to investigate T2544-induced IR in these mice, as well as the outcome of infection with Ty2Δt2544 in nonimmunized mice. Oral S. Typhimurium infection of mice mimics enteric fever and has been widely used as a model in which to study the human disease caused by S. Typhi (32). This model, however, is not suitable for our purpose to study the contribution of T2544 to the virulence/pathogenicity of S. Typhi, as the host immunity plays a critical role. Protein STM0306 of S. Typhi, the LPS protein of LT2, is not required for adhesion to the host. A mouse peritensis model of S. Typhi has been extensively used to investigate the protective efficacy of vaccine candidates (34). However, this does not replicate the progressive systemic infection we observe in humans and cannot be used as a model to study pathogenesis. Although i.p. administration along with excess of iron and iron chelator helps to establish a systemic disease (32), determination of LD50 may be difficult as a result of rapid induction of death by septic shock. Unlike the natural infection caused by S. Typhi, the i.p. route bypasses the gut IR, and adhesion molecules may be required exclusively during the initial attachment to the gut mucosa. This is underscored by the fact that an adhesion/invasion-impaired phenotype of S. Typhimurium may be attenuated in virulence after oral infection, but still able to cause systemic disease (35). Further, protective antibodies against adhesins may act by prevention of colonization of the mucosa by the pathogen (14). The oral route of administration of S. Typhi we have adopted here may be more appropriate to study the role of adhesins in pathogenesis and IR.

T2544 is strongly immunogenic. Increased titers of T2544-specific antibodies in mice persisted for at least 4 mo after s.c. immunization. The overall response in our experiments is comparable to that with a Vi-conjugate vaccine and substantially greater than that with Vi used alone (36). Given that IgG1 was the major antibody isotype induced in our studies, s.c. immunization of mice with recombinant long hairpin binders is recommended (37). The immune serum (i.e., RA5) reacted with a large number of clinical isolates of S. Typhi and Paratyphi recovered from different geographical locations in a whole-cell ELISA, suggesting cell-surface localization of T2544. At the same time, RA5 enhanced complement-mediated killing of Salmonella and uptake and clearance by macrophages in the in vitro experiments. T2544-specific antibodies were also present in the acute and convalescent sera of naturally infected humans and facilitated lysis of S. Typhi in vitro. Mice immunized with T2544 or passively immunized with anti-T2544 antiserum were protected against subsequent bacterial challenge. Together these findings suggest that circulating T2544 antibodies may be involved in the protection against systemic salmonellosis and a T2544-based vaccine may protect an endemic population against multiple S. Typhi and S. Paratyphi strains. Elevated intestinal and stool sIgA levels in the immunized mice with T2544 indicates locally induced protection. However, a mucosal (i.e., oral/intranasal) route of immunization may induce stronger sIgA response compared with s.c. injection (36, 38) and may be tried with T2544 in future studies. Published reports also suggest that serum IgG may transudate into the mucosal secretions after parenteral vaccination and may be sufficient to block colonization (14, 39). Cell-mediated IR (CMIR) is considered important for long-term protection against S. Typhi (38). Although we found low serum IgG2a response, a surrogate marker of CMIR in mice (37), similar observation was reported with the surface adhesin PsaA of pneumococci. However, PsaA significantly potentiated the IR against pneumococcal capsular polysaccharide (40). CMIR is modified by the route and schedule of immunization as well as the nature of the adjuvant agent used (41). Moreover, we did not examine other surrogate markers of CMIR in mice, such as IgG2b and IgG3; neither was CMIR against T2544 directly investigated. Finally, immunogenicity of a particular antigen may vary across mouse strains (42).

Caution should be exercised in extrapolating results from animal studies to humans. A FimH-based vaccine, although found to be highly protective in mice and nonhuman primates, failed in humans (39). We found rapid seroconversion against T2544 in all immunized animals, healthy whereas 70% of nonimmunized mice. Moreover, we did not examine other surrogate markers of CMIR as well as the nature of the adjuvant agent used (41). Furthermore, a mucosal (i.e., oral/intranasal) route of immunization may induce stronger sIgA response compared with s.c. injection (36, 38) and may be tried with T2544 in future studies. Published reports also suggest that serum IgG may transudate into the mucosal secretions after parenteral vaccination and may be sufficient to block colonization (14, 39). Cell-mediated IR (CMIR) is considered important for long-term protection against S. Typhi (38). Although we found low serum IgG2a response, a surrogate marker of CMIR in mice (37), similar observation was reported with the surface adhesin PsaA of pneumococci. However, PsaA significantly potentiated the IR against pneumococcal capsular polysaccharide (40). CMIR is modified by the route and schedule of immunization as well as the nature of the adjuvant agent used (41). Moreover, we did not examine other surrogate markers of CMIR in mice, such as IgG2b and IgG3; neither was CMIR against T2544 directly investigated. Finally, immunogenicity of a particular antigen may vary across mouse strains (42).
titers were collected from children younger than 5 y of age. This raises the hope that a T2544-based vaccine may benefit very young children, in whom the currently licensed vaccines are less effective (6).

Several Vi–protein conjugates have shown better IR than Vi alone in animals and the results of field trials with a Vi-RePA conjugate have been highly encouraging (6, 7). However, the latter is yet to receive approval for clinical use, probably because of the lack of precedence for EPA use (6). As Vi-negative strains are also capable of systemic invasion, these strains may be preferentially selected in the population if Vi is used for mass vaccination. This may be avoided if T2544, an intrinsic protein of Salmonella, is added to Vi or used as a Vi-conjugate vaccine.

Bacterial adhesin-based therapy has advantages and drawbacks. Because these agents do not act by killing or arresting growth of the pathogen, as antibiotics do, the propagation and spread of resistant strains are much less likely to occur (39). The major drawback of such therapy is the redundancy in their function because most pathogens may express more than one adhesin during the infectious process (10). However, a vaccine-based therapy may still be effective for invasive pathogens, as we found for LT2 in infection in mice, because the antibodies may enhance complement-mediated lysis of circulating bacteria, despite being unable to prevent adhesion. Alternatively, immune serum may contain functional antibodies that prevent adhesion whereas opsonizing antibodies facilitate macrophage and complement-mediated killing. A recent study reported that complement-mediated killing of nontyphoidal Salmonella by HIV-noninfected serum is mediated specifically by antibodies against outer membrane proteins (44). Antigenic variability of protein adhesins may also compromise the efficacy of the vaccine (39). However, a high degree of antigenic conservation is an attribute of many adhesins, which make them ideal vaccine candidates (14). This is also likely for T2544, as the S. Typhi genome shows only limited variations (45).

Materials and Methods

An iron-overload mouse model was generated as previously described (31, 32). Swiss Albino mice were intraperitoneally injected with FeSO₄ (0.32 mg/g) and desferrioxamine (0.025 mg/g; Novartis) 4 h before the bacterial challenge. Log-phase cultures of bacteria resuspended in PBS solution were fed orally with neutralization of gastric acidity with sodium bicarbonate (0.5 mL). In case of lethal infections, mice were monitored for the next 7 d for the occurrence of death. For sublethal doses, blood and visceral organs (liver and spleen) were collected every 2 d after the infection. In both cases, viable bacteria recovered from mouse tissues were grown in Luria agar (LA) plates. A detailed description of all other materials and methods is provided in SI Materials and Methods.

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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-Δ2544 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, Δ2544, t2769, tasC, and pISS genes of Ty2 and STM1036 gene of LT2 were PCR-amplified from E. coli DH41 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. Ty2Δ2544 strain was complemented with Δt2544 by transforming competent bacteria with pQE60-Δt2544 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’Ferrell 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 (10^9 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 Ty2Δ2544 (10^3 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<sup>2+</sup>-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<sup>2+</sup>-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<sub>d</sub> 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<sub>d</sub> value was calculated by using GraphPad Prism software.

In Vitro Bactericidal Assay. Sera collected from typhoid and non-typhoid patients were preincubated with Ty2Δ2544 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 × 10⁵ 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 10⁶ 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 matter was collected from 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 × 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 10⁷, 10⁶, 5 × 10⁵, and 10⁴ 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 ≤ 0.01.


**Bioinformatic Prediction of Novel Virulence Factors of S. typhi**

- S: typhi genome (4000+ genes)
- Genomic island prediction (Design Island)
- >90% genes
- PAIDE search
- 338 potential virulence genes (known:98 + unknown:240)
- BLAST search against non-redundant database
- Functional annotation of unknown S. typhi genes (on the basis of >95% identity, e-value<0.0001, gap=0%)
- Adh/Inv | Secretion | Transport | Effector | Inner/mem | Others
- 15 16 20 23 7 11 6 7 5 20

**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. 1A. (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. 1E. All data represent one of three independent experiments; bar diagrams show mean ± SD. *P ≤ 0.01; NS, not significant.
Fig. S3.  (A) Dose-dependent inhibition by RAS of bacterial adhesion to laminin as described in Fig. 2A, 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. 4A, 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. 4C, 1. Mice were immunized with recombinant T2544 and subsequently challenged with LT2 or LT2ΔSTM0306. All data represent one of three independent experiments; bar diagrams show mean ± SD.
Table S1. Systemic invasion of mice

<table>
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<tr>
<th>Organ/DPI</th>
<th>Mean difference in bacterial count log10 values</th>
<th>t Statistic</th>
<th>Significance</th>
<th>95% CI of difference</th>
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<td>Blood</td>
<td></td>
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<td>&lt;0.001</td>
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<td>2</td>
<td>0.59</td>
<td>7.54</td>
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<td>0.41−0.77</td>
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<tr>
<td>4</td>
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<td>17.14</td>
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<td>0.59−0.78</td>
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<td>0.69</td>
<td>11.88</td>
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<td>0.56−0.83</td>
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<td>Liver</td>
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<td></td>
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<tr>
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<td>0.52</td>
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<td>6</td>
<td>0.43</td>
<td>11.25</td>
<td>&lt;0.001</td>
<td>0.34−0.52</td>
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<td>Spleen</td>
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<td>6</td>
<td>0.78</td>
<td>23.90</td>
<td>&lt;0.001</td>
<td>0.70−0.86</td>
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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

<table>
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<tr>
<th>Opsonized with</th>
<th>Mean ± SD, CFU × 10^4/well</th>
<th>0 min</th>
<th>60 min</th>
<th>90 min</th>
<th>120 min</th>
<th>t Statistic</th>
<th>Significance</th>
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<tr>
<td>T2544 antisera</td>
<td></td>
<td>25.9 ± 5.7</td>
<td>23.4 ± 5.07</td>
<td>42.3 ± 7.8</td>
<td>82.8 ± 11.6</td>
<td>1.60</td>
<td>&lt;0.001</td>
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<tr>
<td>Preimmune sera</td>
<td></td>
<td>20.5 ± 1.2</td>
<td>93.4 ± 11.5</td>
<td>109 ± 11.2</td>
<td>110 ± 11</td>
<td>9.52</td>
<td>&lt;0.001</td>
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</table>

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

<table>
<thead>
<tr>
<th>Strain name</th>
<th>Phenotype</th>
<th>Genotype</th>
<th>Source</th>
<th>Reference</th>
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<td>—</td>
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<td>Commercial</td>
<td>ATCC 700931</td>
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<td>S. Typhi</td>
<td>O9, 12, (Vi) Hd</td>
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<td>Clinical</td>
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<td>S. Paratyphi A</td>
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<td>S. Typhimurium</td>
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<td>—</td>
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<td>Clinical</td>
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<tr>
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<tr>
<td>ETEC (1)</td>
<td>O6:H16</td>
<td>LT+</td>
<td>Clinical</td>
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<td>ETEC (2)</td>
<td>OUT</td>
<td>LT+</td>
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<td>ETEC (3)</td>
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<td>V. cholerae O139 Bengal (SG24)</td>
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<td>Ctx(+), tcp(+)</td>
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<td>S. Typhi Ty2Δt2544</td>
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<td>t2544Δ, PilSΔ</td>
<td>Laboratory</td>
<td>Present study</td>
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OUT, O-antitypable antigen.

Dataset S1.