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Identification of In Vivo-Induced Bacterial Protein Antigens during Human Infection with *Salmonella enterica* Serovar Typhi

Jason B. Harris,[1,2,3]* Andrea Baresch-Bernal,[1] Sean M. Rollins,[1] Ashfaqul Alam,[4] Regina C. LaRocque,[1,5] Margaret Bikowski,[1] Amanda F. Peppercorn,[1] Martin Handfield,[6] Jeffery D. Hillman,[7] Firdausi Qadri,[4] Stephen B. Calderwood,[1,5,8] Elizabeth Hohmann,[1,5] Robert F. Breiman,[4] W. Abdullah Brooks,[4] and Edward T. Ryan[1,5,9]

Division of Infectious Diseases, Massachusetts General Hospital, Boston, Massachusetts[1]; Department of Pediatrics, Massachusetts General Hospital, Boston, Massachusetts[2]; Department of Pediatrics, Harvard Medical School, Boston, Massachusetts[3]; ICDDR,B: Centre for Health and Population Research, Dhaka, Bangladesh[4]; Department of Medicine, Harvard Medical School, Boston, Massachusetts[5]; Department of Oral Biology, College of Dentistry, University of Florida, Gainesville, Florida[6]; Oragenics, Alachua, Florida[7]; Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts[8]; and Department of Immunology and Infectious Diseases, Harvard School of Public Health, Boston, Massachusetts[9]

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We applied an immunoscreening technique, in vivo-induced antigen technology (IVIAT), to identify immunogenic bacterial proteins expressed during human infection with *Salmonella enterica* serovar Typhi, the cause of typhoid fever. We were able to assign a functional classification to 25 of 35 proteins identified by IVIAT. Of these 25, the majority represent proteins with known or potential roles in the pathogenesis of *S. enterica*. These include proteins implicated in fimbrial structure and biogenesis, antimicrobial resistance, heavy metal transport, bacterial adhesion, and extracytoplasmic substrate trafficking as well as secreted hydrolases. The 10 remaining antigens represent proteins with unknown functions. Of the 35 identified antigens, four had no immunoreactivity when probed with control sera from individuals never exposed to serovar Typhi organisms; these four included PagC, TcfB, and two antigens of unknown function encoded by STY0860 and STY3683. PagC is a virulence factor known to be upregulated in vivo in *S. enterica* serovar Typhimurium infection of mice. TcfB is the major structural subunit of a fimbrial operon found in serovar Typhi with no homolog in serovar Typhimurium organisms. By examining differential immunoreactivities in acute- versus convalescent-phase human serum samples, we found specific anti-PagC and anti-TcfB immunoglobulin G responses in patients with serovar Typhi bacteremia. Serovar Typhi antigens identified by IVIAT warrant further evaluation for their contributions to pathogenesis, and they may have diagnostic, therapeutic, or preventive uses.

In humans, infection with *Salmonella enterica* serovar Typhi causes a spectrum of illness that includes diarrhea, a self-limited febrile illness, and most significantly, typhoid fever, a systemic infection characterized by persistent fever, lymphadenopathy, hepatosplenomegaly, encephalopathy, and intestinal hemorrhage and perforation (16). Worldwide, approximately 20 million cases of typhoid fever resulting in 200,000 deaths occur annually (2). The increasing incidence of disease caused by multidrug-resistant serovar Typhi organisms underscores the importance of developing novel approaches to the diagnosis, treatment, and prevention of typhoid fever (22).

Although many *S. enterica* serovars infect a broad range of animal hosts and cause gastroenteritis in humans, serovar Typhi is among the few serovars for which natural infection appears to be limited to human hosts. In humans, serovar Typhi organisms penetrate the gastrointestinal epithelial barrier and infect phagocytes within the lamina propria. However, unlike organisms from other broad-host-range serovars, serovar Typhi organisms are adapted for prolonged intracellular survival in human macrophages, allowing the bacteria to spread to reticuloendothelial organs, including the liver, spleen, and bone marrow (16).

Because serovar Typhi organisms are human specific, there is no optimal animal model of serovar Typhi infection, and presumed factors that contribute to the pathogenesis and immunology of typhoid fever are largely extrapolated from studies of *S. enterica* serovar Typhimurium infection of mice. Serovar Typhimurium is a broad-host-range pathogen that causes gastroenteritis in humans, but in mice it results in a systemic infection that resembles typhoid fever in humans. While the murine typhoid model has proven useful, there are limitations in its application to the study of human infection with serovar Typhi organisms. For example, serovar Typhimurium infection induces apoptosis in mouse macrophages in vitro and results in a highly virulent infection of genetically susceptible mice. Serovar Typhi infection causes less apoptosis in human macrophages, which may correlate with the prolonged survival of the organism in vivo (23). Furthermore, while serovars Typhi and Typhimurium share genetic homology in important pathogenicity elements, over 10% of the presumed open reading frames (ORFs) identified in sequenced genomes of serovar Typhi CT18 and serovar Typhimurium LT2 are unique with respect to each other (15). This divergence includes large clusters of ORFs found uniquely in serovar Typhi. These unique elements encode an array of proteins, including presumed fimbrial and

* Corresponding author. Mailing address: Division of Infectious Diseases, Massachusetts General Hospital, 55 Fruit Street, Boston, MA 02114. Phone: (617) 726-3812. Fax: (617) 726-7416. E-mail: jbharris@partners.org.

regulatory proteins, that may contribute to the host and disease specificity of the organism (24). In addition, serovar Typhi possesses a large number of pseudogenes compared to serovar Typhimurium, suggesting another possible mechanism for the host-restricted phenotype of serovar Typhi (15).

The study of organisms in cultured cells is another tool for modeling serovar Typhi infection and typhoid fever pathogenesis (4), but such models are limited to single stages of infection. Critical host-pathogen interactions as well as essential processes in the acquisition of immunity to serovar Typhi may occur prior to or after adaptation of the organism to survival within the *Salmonella*-containing macrophage vacuole.

To circumvent the limitations associated with animal and in vitro models of serovar Typhi infection and to identify serovar Typhi proteins that are immunogenic and expressed uniquely during human infection, we applied an immunologic screening technique termed in vivo-induced antigen technology (IVIAT) (5, 20). In summary, we screened a library of serovar Typhi proteins expressed in *Escherichia coli* to identify clones that were immunoreactive with convalescent-phase sera that had been adsorbed against in vitro-grown serovar Typhi and *E. coli* organisms. The adsorption process eliminates antibodies reactive with in vitro-expressed antigens and allows for the identification of clones that express protein antigens which are upregulated during human infection. Specifically, we hypothesized that by using IVIAT, we could identify proteins that play a role in the serovar-specific human-bacterium interactions unique to serovar Typhi infection of humans.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. We used genomic DNA from serovar Typhi CT18 (15) to construct a protein expression library in the host strain *Escherichia coli* BL21(DE3). The CT18 strain is a drug-resistant clinical isolate obtained in 1992 from a patient in Vietnam and was obtained through the Salmonella Genetic Stock Center (Calgary, Alberta, Canada). The construction of the library and generation of plasmids are described below. All strains were grown in vitro in Luria-Bertani (LB) medium (with kanamycin at 50 μg/ml for clones containing pET30 constructs) and maintained at −80°C in LB medium containing 15% glycerol.

Patient and control sera. We obtained paired acute- and convalescent-phase sera from 14 individuals presenting to the ICDDR,B: Centre for Health and Population Research's Kamalapur clinic in urban Bangladesh with febrile illness who had blood cultures positive for serovar Typhi (1). In addition, we used two sets of control sera in this study. One set of control sera included paired acute- and convalescent-phase samples from cholera patients from the same region of typhoid endemicity in Bangladesh (6). A second set of control sera was obtained from five North American volunteers with no history of serovar Typhi exposure (either travel to an endemic area or previous vaccination) (7). All human studies were approved by the institutional review board of the Massachusetts General Hospital, Boston, Mass., and the ethical and research review committees of the ICDDR, Dhaka, Bangladesh.

Adsorption of sera. We pooled convalescent-phase sera from eight patients with serovar Typhi bacteremia. We then allowed the pooled sera to adsorb extensively with in vitro-grown serovar Typhi CT18 organisms and with the expression host strain *E. coli* BL21(DE3), both of which were grown to late log phase in LB broth. Specifically, we concentrated serovar Typhi and *E. coli* cells 100-fold by centrifugation and resuspended them in phosphate-buffered saline with 0.05% sodium azide. We produced heat-denatured and nondenatured lysates of late-log-phase organisms grown in vitro by freeze-thawing the concentrated cell suspension and homogenizing it using 0.1-mm zirconia-silica beads in a Mini-Bead Beater tissue homogenizer (Biospec, Bartlesville, OK). The serum adsorption process consisted of serial stages using serovar Typhi and *E. coli* whole cells, followed by adsorption with nondenatured and heat-denatured whole-cell lysates immobilized on 0.5-μm polystyrene beads (Bangs Laboratories Inc., Fishers, IN). The resultant pooled serum sample retained no discernible reactivity in a whole-cell enzyme-linked immunosorbent assay (ELISA) format utilizing the CT18 strain (data not shown) and was aliquoted and stored at −80°C until use.

Construction of genomic and large plasmid expression libraries of serovar Typhi CT18. We purified genomic DNA from serovar Typhi CT18 by using Easy DNA (Invitrogen, Carlsbad, CA) and partially digested it with Sau3A under conditions optimized to yield fragments of 0.5 to 1.5 kb. We then gel purified the resulting fragments and extracted them using a QIAquick PCR purification kit (Qiagen, Valencia, CA). Serovar Typhi CT18 also harbors the large plasmids pHCM1 (218 kb) and pHCM2 (106 kb). These plasmids are not essential requirements for virulence; plasmid pHCM1 confers a drug resistance phenotype, while plasmid pHCM2 is phenotypically cryptic (15). Although neither plasmid is required to cause typhoid fever in humans, we included these in our screen since they may contribute to virulence and are often found in clinical isolates obtained from patients with typhoid fever. We purified plasmid DNA from serovar Typhi CT18 by using a QIAGEN large construct kit (Qiagen, Valencia, CA) to generate a heterogeneous pool of plasmid DNA, which was subjected to partial digestion as described above.

Using both genomic and large plasmid DNAs, we constructed expression libraries in pET30abc expression vectors (Novagen, San Diego, CA), allowing for cloning of the digested fragments in each of three possible open reading frames to generate fusion proteins under the control of an inducible T7 promoter. Vector DNA was prepared by digestion with BamHI and treated with calf and shrimp alkaline phosphatase.

We next ligated each of the three vectors separately with genomic and large plasmid DNA fragments to create six expression libraries. These libraries were electroporated into *E. coli* DH5α (Invitrogen, Carlsbad, CA) and spread on LB plates containing kanamycin. After overnight incubation, we collected colonies from plates by scraping and recovered plasmid DNA. We then electroporated the plasmid mixture into *E. coli* BL21(DE3), a protease-deficient strain optimized for expression of heterologous proteins. We then assessed the resulting libraries by PCR amplification of a random sample to determine the frequency and sizes of the insertions, and only libraries with >80% of inserts falling between 500 and 1,500 bp were used in subsequent screening assays.

Screening libraries for antigenic proteins expressed during serovar Typhi infection. In the primary screening of the expression libraries, we aliquoted small volumes of each library onto multiple plates containing kanamycin to achieve a colony density of approximately 500 colonies per plate. We incubated plates overnight and lifted the resultant colonies onto a nitrocellulose membrane that was then placed on an LB plate containing isopropyl-β-D-thiogalactopyranoside (1 mM) to induce the transcription of insert DNA. After 4 hours, we lysed colonies adhered to membranes by placing them on chloroform-soaked blotting paper. Membranes were then air dried and blocked with phosphate-buffered saline containing 5% nonfat milk. We then incubated the membranes overnight with adsorbed convalescent-phase pooled sera (described above) at a 1:5,000 dilution. We detected clones reacting with adsorbed sera using a peroxidase-conjugated goat anti-human immunoglobulin (Ig) antibody (MP Biomedicals/Cappel, Aurora, OH) at a 1:2,000 dilution and developed immunoblots using an enhanced chemiluminescence (ECL) kit (Amersham, Piscataway, NJ). Reactive clones were recovered from master plates and frozen as glycerol stocks.

We confirmed the immunoreactivities of clones selected in the primary screen by comparing them directly to the reactivity of a control strain, *E. coli* BL21(DE3) containing the pET30 vector with no insert, in a whole-colony immunoblot assay. We then purified plasmids from consistently reactive clones and sequenced the inserts by using pET30-specific upstream and downstream primers. For inserts that contained multiple ORFs, we performed a tertiary evaluation of the immunoreactivities of individual ORFs by cloning each of the entire predicted ORFs, generated by PCR amplification, into 5′ NdeI and 3′ NotI restriction enzyme sites in the pET30a vector and assaying the reactivity of each resultant clone compared to that of the control strain, *E. coli* BL21(DE3) containing the pET30 vector with no insert.

Prediction of function of antigens identified by IVIAT. The cellular localization of antigens identified by IVIAT was predicted using PSORTb, version 2.0.4 (http://www.psort.org). Functional classification was based, when available, on published studies of identified proteins in *S. enterica*. When no published functional studies for *S. enterica* were available, protein functional classification was based on predictive models using the Clusters of Orthologous Groups database (http://www.ncbi.nlm.nih.gov/COG/) and the Pfam database (http://www.sanger.ac.uk/Software/Pfam).

Screening antigens identified by IVIAT against sera from North American volunteers. To assess the degree of cross-reactivity of antigens identified by IVIAT, we also screened reactive clones for immunoreactivity against pooled sera from five North American volunteers with no prior history of exposure to serovar Typhi organisms or typhoid vaccines. Prior to screening, we allowed the pooled sera to adsorb with in vitro-grown *E. coli* BL21(DE3) (as described

above) to reduce background reactivity against the host strain used in the whole-colony immunoblot assay.

Evaluation of serum IgG responses to in vitro-transcribed and -translated PagC by Western blot analysis using paired acute- and convalescent-phase sera from patients with serovar Typhi bacteremia and from control patients. To confirm the significance of the serum immunoglobulin response to PagC in a whole-colony immunoblot assay, we applied an in vitro transcription-translation system using the *pagC* ORF cloned into the 5′ NdeI and 3′ NotI restriction enzyme cloning sites of pET30a to produce a T7 promoter-inducible template DNA, which was used in a commercial cell extract system (ActivePro; Ambion, Austin, TX) to yield a 20-kDa protein. Immune responses to PagC in paired acute- and convalescent-phase serum samples from 14 patients with serovar Typhi bacteremia and 7 control patients with cholera were then compared by Western blot analysis. For these studies, we used nonadsorbed serum from each patient at a 1:10,000 dilution. We detected immune responses with peroxidase-conjugated goat anti-human IgG (KPL, Gaithersburg, MD) at a dilution of 1:100,000, using an ECL kit (Amersham, Piscataway, NJ).

Evaluation of serum IgG responses to purified TcfB by ELISA, using paired acute- and convalescent-phase sera from patients with serovar Typhi bacteremia and from control patients. To confirm the immunoreactivity of TcfB in the whole-colony immunoblot assay, we constructed an inducible TcfB fusion protein expressing an N-terminal hexahistidine residue coupled with the C-terminal 148 amino acids encoded by *tcfB*. The initial 43 amino acids of the full predicted peptide sequence were predicted to be a signal peptide, and we did not include them in the expressed fusion. We generated the encoding sequences on an NdeI-XhoI fragment from serovar Typhi CT18 genomic DNA, using the 5′ primer GGAGATATACATATGGTTCAGAAGGATATTACCGTC and the 3′ primer ATGCGGATCCTCGAGTTAGCCGGCAGTGACAGCCTGTG, and cloned this fragment into the pET15b (Novagen, San Diego, CA) expression vector. We induced the expression of TcfB at 37°C with 1 mM isopropyl-β-D-thiogalactopyranoside in *E. coli* BL21(DE3). We lysed TcfB-containing cells via sonication and purified the fusion protein by nickel-affinity chromatography under nondenaturing conditions to obtain a final 17.5-kDa product at a concentration of 100 μg/ml. We then diluted this product 1:100 in 50 mM carbonate buffer (pH 9.6) and allowed it to adsorb overnight at room temperature onto ELISA plates (Nunc Maxisorb; NalgeNunc, Rochester, NY), using 100 μl of sample per well. We applied acute- and convalescent-phase sera (1:100 dilution) from 14 patients with serovar Typhi bacteremia and 7 control patients with cholera, detected anti-TcfB IgG with horseradish peroxidase-conjugated rabbit anti-human IgG (Jackson Immunoresearch, West Grove, PA) at a 1:1,000 dilution, and measured peroxidase activity with the substrate 2,2′-azinobis(ethylbenzthiazolinesulfonic acid). The optical density at 405 nm was determined kinetically with plates read for 5 min at 19-second intervals, and the maximum slope for an optical density change was converted to ELISA units by comparison with pooled standard reference sera (obtained from healthy volunteers from Bangladesh), as described previously (10). We evaluated the statistical significance of the difference between acute- and convalescent-phase serum anti-TcfB IgG levels by using a nonparametric test (the Wilcoxon signed rank test) for comparing paired samples.

RESULTS

Identification of serovar Typhi antigens by IVIAT. In the primary screen of approximately 150,000 clones derived from the serovar Typhi protein expression library, we identified 352 immunoreactive clones. Of the initial isolates, 58 were robustly positive upon secondary screening. Sequencing of these inserts revealed 46 unique clones, some of which carried multiple potentially expressible ORFs, accounting for a total of 63 potentially inducible ORFs. Of these 63 potential IVIAT-derived antigens, 35 were confirmed by tertiary screening (cloning of the full ORF into a protein expression vector). The 35 putative in vivo-induced antigens are listed in Table 1 and represent <1% of the serovar Typhi genome. Of these 35 antigens, 10 were identified in multiple inserts. Four of the proteins identified by IVIAT were encoded on the drug resistance plasmid pHCM1, and one was encoded on the cryptic plasmid pHCM2. The remaining 30 antigens were encoded on the chromosome of serovar Typhi.

Functional classification of gene products identified by IVIAT. By evaluating previously published studies of *Salmonella* species and performing an analysis using predictive models, we were able to assign a functional classification to 25 of the 35 proteins identified by IVIAT. Of these 25, the majority represent proteins with a known, suspected, or possible role in the pathogenesis of *S. enterica* infection. These include proteins implicated in fimbrial structure and biogenesis, antimicrobial resistance, heavy metal transport, bacterium-host adhesion, and extracytoplasmic substrate trafficking, as well as several secreted hydrolases. The 10 remaining in vivo-induced antigens represent hypothetical or conserved hypothetical proteins. Two of the hypothetical proteins were predicted to localize to the outer membrane, and another five were predicted to localize outside the bacterial cytoplasm, suggesting a potential role in host-pathogen or pathogen-environment interactions.

Comparison of serovar Typhi genes identified by IVIAT to homologs in serovar Typhimurium. Of the 35 genes identified by IVIAT, 27 had ≥70% homology to ORFs found in the serovar Typhimurium LT2 genome. None of the five proteins encoded on pHCM1 or pHCM2 had a homolog in serovar Typhimurium LT2, but because these plasmids are not essential requirements for virulence (15), it is not likely that the five proteins are essential to the specificity of serovar Typhi-human interactions.

Of the chromosomally encoded serovar Typhi in vivo-induced antigens identified, all were present in both sequenced genomes of serovar Typhi (CT18 and Ty2), but three were not present in the genome of serovar Typhimurium LT2. These included STY1648 and STY3683, which are predicted to localize to the inner and outer membranes, respectively, and have no known function. The third in vivo-induced antigen with no known homolog in serovar Typhimurium was TcfB, which is predicted to be a major fimbrial structural protein. In contrast to most fimbrial operons found in serovar Typhi, the *tcf* operon is not widely distributed among other *S. enterica* serovars (24); however, TcfB shares approximately 30% homology at the amino acid level with CooA, the major structural subunit of the CS1 colonizing factor antigen of enterotoxigenic *E. coli*, as well as with the CblA major fimbrial subunit protein of the cable II pilus of cystic fibrosis-associated *Burkholderia cepacia*.

Evaluation of immunoreactivities of serovar Typhi antigens identified by IVIAT to sera from North American volunteers. Many serovar Typhi antigens are conserved across *Salmonella enterica* species as well as across the *Enterobacteriaceae* in general. To identify a subset of serovar Typhi antigens identified by IVIAT whose immunoreactivities are most specific to sera from individuals with serovar Typhi infection, we tested each IVIAT-derived clone for reactivity to sera obtained from North American volunteers with no history of exposure to serovar Typhi or previous immunization against typhoid. We identified four clones expressing serovar Typhi gene products identified by IVIAT with no detectable degree of reactivity with North American volunteer sera compared to control colonies without an insert. The immunoreactivities of the clones expressing these four IVIAT-derived antigens with the pooled sera from serovar Typhi bacteremic patients compared to their reactivities with sera from North American volunteer control subjects are shown in Fig. 1.

TABLE 1. Thirty-five proteins identified by IVIAT in *S. enterica* serovar Typhi

Functional category	ORF	Gene product—description, function (reference)[a]	Predicted cellular location	Location of ORF	Homolog in serovar Typhimurim LT2
Pilus structure, regulation, and biogenesis	pHCM1.99	TrhW—plasmid transfer protein, fimbrial regulation	Extracellular	pHCM1	No
	STY0346	TcfB—fimbrial subunit, fimbrial major structural subunit	Extracellular	Chromosome	No
	STY0370	StbD—fimbrial protein, fimbrial regulation and bacterial adhesion (25)	Extracellular	Chromosome	Yes
	STY1176	CsgG—assembly and transport component in curli, fimbrial regulation (21)	Outer membrane	Chromosome	Yes
	STY1177	CsgF—assembly and transport component in curli, fimbrial biogenesis (21)	Unknown	Chromosome	Yes
	STY1178	CsgE—assembly and transport component in curli, fimbrial biogenesis (21)	Unknown	Chromosome	Yes
Antimicrobial resistance	STY0520	AcrA—acriflavin resistance protein, antibiotic and bile resistance (17)	Periplasm	Chromosome	Yes
	STY1373	PspC—phage shock protein C, stress response and antimicrobial resistance (3)	Inner membrane	Chromosome	Yes
	STY1374	PspD—phage shock protein D, stress response and antimicrobial resistance (3)	Inner membrane	Chromosome	Yes
	STY1375	PspE—phage shock protein E, stress response and antimicrobial resistance (3)	Periplasm	Chromosome	Yes
Other pathogenesis functions	STY0405	Putative autotransporter, virulence factor, putative integrin binding adhesin	Outer membrane	Chromosome	Yes
	STY1878	PagC—outer membrane invasion protein, macrophage survival (12, 19)	Outer membrane	Chromosome	Yes
	STY2755	ShdA—fibronectin binding adhesin (11)[b]	Outer membrane	Chromosome	Yes
Heavy metal transport resistance	pHCM1.153	MerP—mercuric transport protein, heavy metal transport and resistance	Periplasm	pHCM1	No
	STY0909	Conserved hypothetical protein, heavy metal transport and resistance	Unknown	Chromosome	Yes
Other transport and binding functions	STY1304	Periplasmic oligopeptide-binding protein, peptide transport	Periplasm	Chromosome	Yes
	STY2852	Putative lipoprotein, large substrate and ligand binding	Outer membrane	Chromosome	Yes
	STY3796	Putative ABC transporter, carbohydrate transport	Periplasm	Chromosome	Yes
	STY4194	YhjE—metabolite transport protein, small-molecule transport	Inner membrane	Chromosome	Yes
Extracellular hydrolase functions	STY1255	Putative exported protein, cell wall catabolism	Extracellular	Chromosome	Yes
	STY1522	Putative secreted protein, conjugated bile acid hydrolase	Extracellular	Chromosome	Yes
	STY2659	Putative outer membrane protein, probable hydrolase	Outer membrane	Chromosome	Yes
Metabolic and regulatory functions	STY0482	CyoD—ubiquinol oxidase subunit B, energy metabolism	Cytoplasm	Chromosome	Yes
	STY1816	TopB—DNA topoisomerase III, DNA uncoiling	Cytoplasm	Chromosome	Yes
	STY3756	YijO—AraC family transcriptional regulator, transcriptional regulation	Cytoplasm	Chromosome	Yes
Hypothetical/conserved hypothetical proteins of unknown function	pHCM1.176	Hypothetical protein	Unknown	pHCM1	No
	pHMC1.257	Putative periplasmic protein	Periplasm	pHCM1	No
	pHCM2.0028	Putative lipoprotein	Outer membrane	pHCM2	No
	STY0860	Conserved hypothetical protein	Extracytoplasmic	Chromosome	Yes
	STY1648	Hypothetical protein	Inner membrane	Chromosome	No
	STY2156	Hypothetical protein	Inner membrane	Chromosome	Yes
	STY2195	Conserved hypothetical protein	Cytoplasm	Chromosome	Yes
	STY2761	Putative outer membrane protein	Outer membrane	Chromosome	Yes
	STY3683	Putative membrane protein	Outer membrane	Chromosome	No
	STY3684	Hypothetical protein	Unknown	Chromosome	Yes

[a] Results are based on published data for *S. enterica* serovars, as indicated, or were predicted using the Cluster of Orthologous Groups and Pfam databases.
[b] Listed as a pseudogene in primary annotation. The sequence contains a frameshift mutation at the C terminus of the sequence leading to termination 5′ of the autotransporter domain, although the hypothetical protein contains the conserved pertactin-like domain.

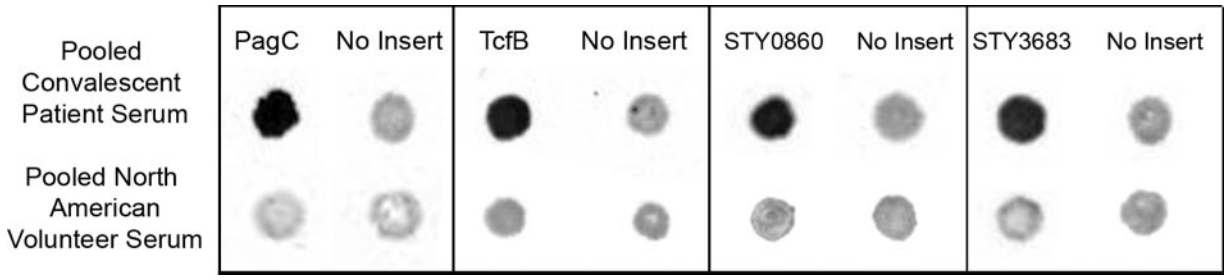

FIG. 1. Evaluation of the specificity of immunoreactivity of three fimbria-related antigens and PagC. We compared the immunoreactivity to each antigen identified by IVIAT in a whole-colony blot assay using pooled sera from patients with serovar Typhi bacteremia to the reactivity of pooled sera from adult North American volunteers. Four of the IVIAT-identified antigens (PagC, TcfB, STY0860, and STY3683) had no detectable immunoreactivity with the North American volunteer sera compared to control colonies with no insert. Each antigen was screened in triplicate with both patient and control sera, and a single representative immunoblot is shown.

Given the specificity of their immunoreactivity, and hence their potential to be serovar Typhi-specific in vivo-induced antigens, we were particularly interested in the four serovar Typhi antigens identified by IVIAT that exhibited no detectable reactivity with sera from North American volunteers. These included two antigens of unknown function encoded by the STY0860 and STY3683 genes. STY0860 is a conserved hypothetical protein with a predicted extracytoplasmic location. STY0860 has a 100% identical homolog in serovar Typhimurium LT2. STY3683 is a putative outer membrane protein with no known homolog in serovar Typhimurium, although orthologs have been found in *E. coli* CFT073 and *Shigella boydii* Sb227. As shown in Fig. 1, among the antigens with a predicted functional classification, only PagC, a known virulence factor required for macrophage survival, and TcfB, a major fimbrial subunit described above, showed no detectable immunoreactivity with sera from North American volunteers.

Evaluation of immune responses to PagC and PagC homologs. Among the serovar Typhi antigens identified by IVIAT, PagC was one of the most immunoreactive proteins. We thus compared humoral immune responses to PagC in individual paired sera from 14 patients with fever and confirmed serovar Typhi bacteremia (including samples from the 8 patients used to generate the pooled sample). We also measured humoral immune responses to PagC using sera from seven control individuals with confirmed *Vibrio cholerae* infection who presented to the same care facility in Bangladesh as did the typhoid patients. Although our initial attempts to purify a full-length PagC fusion protein by affinity chromatography were unsuccessful, we utilized an in vitro transcription and translation system to generate the full-length PagC protein and found that 11 of 14 patients with symptomatic serovar Typhi bacteremia had a detectable increase in circulating anti-PagC IgG, as detected by Western blot analysis, while none of the

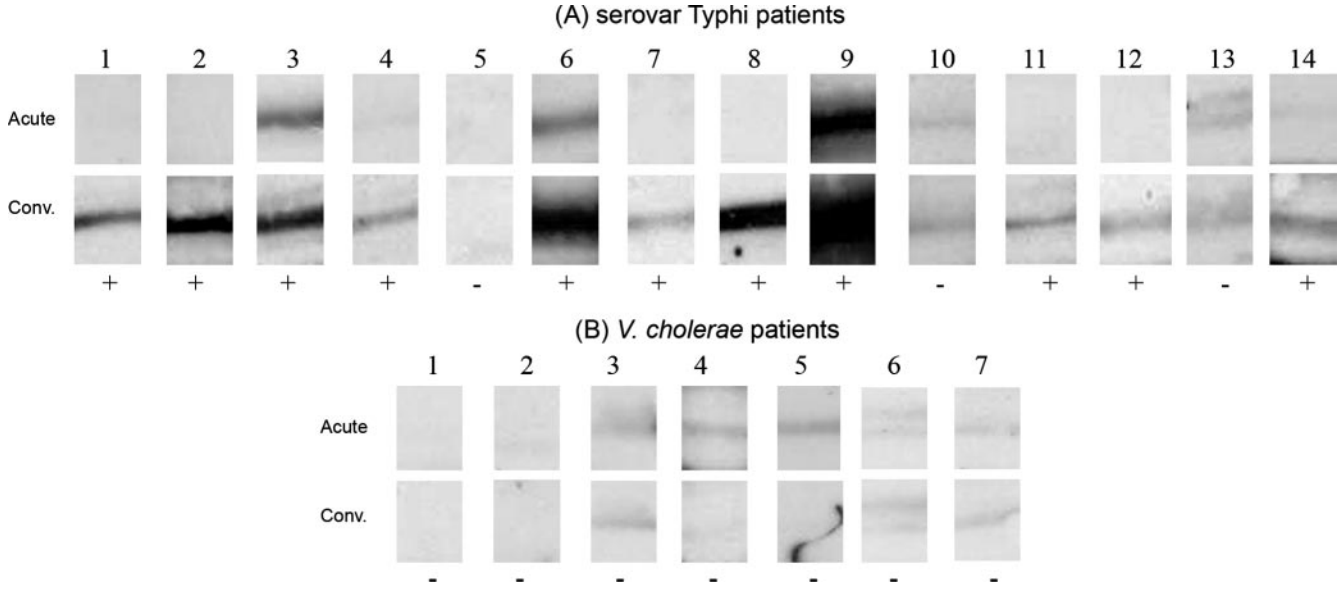

FIG. 2. Responses to PagC in paired serum samples obtained from patients infected with *S. enterica* serovar Typhi or *V. cholerae* (controls). PagC was produced by a cell-free in vitro transcription-translation system, and individual patient immune responses were visualized by Western blotting. (A) Eleven of 14 patients infected with serovar Typhi had a visible increase in anti-PagC IgG antibodies during convalescence (conv.). +, visually significant increase in reactivity between paired acute- and convalescent-phase samples by Western blotting; −, no response. (B) None of the patients infected with *V. cholerae* had a visible increase in PagC reactivity with convalescent-phase serum. None of the North American volunteers had visible reactivity against PagC in this assay (data not shown).

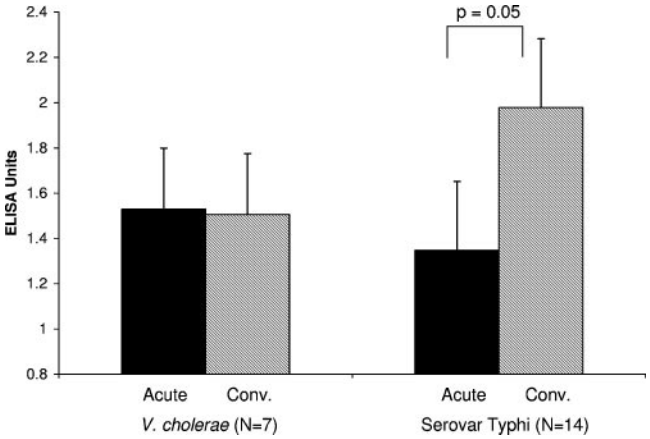

FIG. 3. Responses to TcfB in paired serum samples obtained from patients infected with *S. enterica* serovar Typhi or *V. cholerae* (controls). Sera were collected at the time of presentation to a health care facility (acute phase) and again 2 to 3 weeks later (convalescent phase [conv.]). Columns represent geometric means, and error bars represent the standard errors of the means.

controls had an apparent increase in their anti-PagC antibodies (Fig. 2). Notably, some Bangladeshi patients and controls residing in an area of serovar Typhi endemicity did have detectable anti-PagC IgG in acute-phase serum samples, possibly representing prior infections or low levels of cross-reactive antibodies. However, we did not observe reactivity to PagC when we used sera from North American volunteers who were never exposed to serovar Typhi organisms or vaccines (data not shown).

To assess the possibility that immunoreactivity to PagC was based on cross-reactive immune responses to homologous proteins within the serovar Typhi genome, we also cloned and expressed the three serovar Typhi ORFs with >30% predicted similarity at the amino acid level to PagC, including STY0380, STY0872 (*ompX*), and STY3179. Although OmpX did show a low level of immunoreactivity, the response was nonspecific, and the protein was equally reactive with control sera from North American volunteers. Clones expressing STY0380 and STY3179 did not react with patient or control sera (data not shown).

Evaluation of immune responses to TcfB. To further evaluate the anti-TcfB responses in individuals with documented serovar Typhi infections, we purified a His-TcfB fusion protein and compared anti-TcfB humoral immune responses in 14 serovar Typhi-infected patients and 7 control patients with cholera. During convalescence, in contrast to the case during the acute stage of infection, anti-TcfB IgG responses increased in individuals with serovar Typhi bacteremia but not in individuals with cholera ($P = 0.05$) (Fig. 3).

DISCUSSION

Applying IVIAT to *S. enterica* serovar Typhi, we identified 35 immunogenic bacterial proteins which are expressed uniquely in vivo and are reactive with convalescent-phase sera from humans with serovar Typhi bacteremia. The validity of this screening process is supported by our identification of PagC as an antigen identified by IVIAT. In the mouse serovar Typhimurium model, PagC has previously been identified as an in vivo-induced virulence protein, and PagC has been shown to be coordinately regulated by the PhoP/PhoQ regulon (12), a two-component regulatory system that controls the expression of a large number of genes required for intracellular *S. enterica* adaptation and survival, with functions that include the modification of outer membrane components, resistance to antimicrobial peptides and bile acids, and growth at low Mg^{2+} concentrations and under low-pH conditions (14). Although PagC is not constitutively expressed during log-phase growth in vitro (19) and its expression is repressed in the presence of bile acids (18), the transcription of *pagC* is highly upregulated during intracellular growth of serovar Typhimurium organisms in macrophages, and PagC is required for bacterial survival within macrophages and virulence in the murine serovar Typhimurium typhoid model (12). It is thus likely that PagC is specifically expressed during serovar Typhi infection of humans and is required for virulence, although a definitive mechanism for its function in macrophage survival and virulence in humans has not yet been established. While fusion proteins under transcriptional control of the *pagC* promoter in serovar Typhimurium vector organisms have previously been shown to generate immunity against heterologous antigens in mice (reflecting upregulation of PagC expression in vivo) (8), PagC itself has not previously been shown to be immunogenic in mice or humans (13).

Our identification of PagC by IVIAT, the differential anti-PagC reactivity in convalescent- versus acute-phase sera from 11 of 14 patients with confirmed serovar Typhi bacteremia, the absence of comparable seroreactivity in individuals with cholera, and the absence of seroreactivity in North American volunteers with no prior exposure to serovar Typhi organisms or vaccines suggest that immune responses to PagC may be specific to patients with at least *S. enterica* (if not more specifically serovar Typhi) infection. The potential diagnostic significance of anti-PagC humoral immune responses, as well as the roles of humoral and cellular anti-PagC responses in adaptive immunity to serovar Typhi infection, requires further evaluation.

Of the other potential virulence factors identified by IVIAT, the largest functional category included six fimbria-related proteins, including proteins derived from three separate fimbrial operons, i.e., *tcf*, *stb*, and *agf*. These results are consistent with findings from the murine serovar Typhimurium typhoid model, in which only one fimbrial antigen, FimA, is detectable in organisms grown in vitro but infected mice develop humoral immune responses to 11 fimbrial antigens that are detectable by Western blotting, suggesting a significant upregulation of these fimbrial antigens in vivo (9). In concordance with these data, we identified several *S. enterica* fimbrial antigens by using IVIAT, including proteins involved in the biogenesis of aggregative fimbriae (or curli) and two chaperone-usher class pili encoded by the *stbABCDE* and *tcfABCD* operons (24). This is relevant given the significance of fimbrial antigens both in the pathogenesis of bacterial infections and in host innate and adaptive immune responses to bacterial pathogens.

Our identification of the fimbrial antigen TcfB by IVIAT is of particular interest. Unlike the majority of the 12 fimbrial operons predicted or described for the sequenced isolates of serovar Typhi organisms, the *tcf* operon is not found in serovar Typhimurium, nor is this operon widely distributed among

other *S. enterica* serovars. Interestingly, the *tcf* operon is found in serovar Paratyphi A organisms, which, like serovar Typhi organisms, are restricted to human hosts and are another major etiologic agent of enteric fever (24). Based on this genomic evidence alone, *tcf* has been theorized to play a role in the host specificity of serovar Typhi. TcfB represents the major structural antigen of this fimbrial operon, and as with PagC, we found no appreciable immunoreactivity to TcfB in a serovar Typhi-naive population. We also found that there was a significant increase in circulating anti-TcfB IgG antibodies in patients presenting with serovar Typhi bacteremia, which were absent in control patients from the same area of serovar Typhi endemicity. Our identification of the main structural subunit encoded by the *tcf* operon as an in vivo-induced antigen provides support for the hypothesis that this protein plays a role in the host specificity of serovar Typhi. However, because TcfB is not found in serovar Typhimurium, additional experiments will be required to evaluate the role of Tcf in natural infection and pathogenesis.

In addition to TcfB, we also identified two other serovar Typhi antigens (STY1648 and STY3683) by IVIAT that may play a role in the host-restricted phenotype of serovar Typhi, based on their absence from the serovar Typhimurium genome. Of these, the putative membrane protein STY3683 is of particular interest for future evaluation since, like TcfB, no cross-reactive antibodies to this antigen were found in a serovar Typhi-naive population.

Although IVIAT has been used to identify proteins specifically expressed in vivo during infection with a number of organisms (20), IVIAT has particular utility when in vitro and animal models present only limited representations of human infection and when genomic and proteomic analyses are limited by a small number of recoverable organisms during the infectious process, such as during human infection with serovar Typhi organisms. As with any technology, IVIAT does have potential limitations, including (i) the identification of exclusively in vivo-expressed protein antigens, with the exclusion of potentially important in vitro-expressed and nonprotein antigens; (ii) cross-reactivity of homologous antigens; (iii) the identification of targets of humoral but not cellular immunity; and (iv) the fact that while IVIAT identifies genes that are upregulated in vivo and are antigenic, assessing the potential roles of these antigens in pathogenesis and protective immunity requires additional study (20). IVIAT does, however, identify a subset of antigens that warrant additional evaluation for their roles in pathogenesis and immunity and their use in clinical applications.

In summary, applying IVIAT to *S. enterica* serovar Typhi, we identified 35 immunogenic bacterial proteins which are expressed uniquely in vivo and are reactive with convalescent-phase sera from humans with serovar Typhi bacteremia. A subset of these antigens are present in serovar Typhi organisms and are not present in serovar Typhimurium (STY1648, STY3683, and TcfB), and another subset does not have any reactivity with sera from North American volunteers who were never exposed to serovar Typhi organisms or vaccines (STY0860, STY3683, PagC, and TcfB). Only TcfB and STY3683 are both unique to serovar Typhi organisms and are not cross-reactive with naïve sera, and as such, we believe that these antigens warrant focused evaluation of possible contributions to the host specificity of systemic serovar Typhi infection.

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