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Environmental Regulation of *Salmonella typhi* Invasion-Defective Mutants

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***Salmonella typhi* is the etiologic agent of human typhoid. During infection, *S. typhi* adheres to and invades epithelial and M cells that line the distal ileum. To survive in the human host, *S. typhi* must overcome numerous complex extracellular and intracellular environments. Since relatively little is known about *S. typhi* pathogenesis, studies were initiated to identify *S. typhi* genes involved in the early steps of interaction with the host and to evaluate the environmental regulation of these genes. In the present study, Tn*phoA* mutagenesis was used to study these early steps. We isolated 16 *Salmonella typhi* Tn*phoA* mutants that were defective for both adherence and invasion of the human small intestinal epithelial cell line Int407. Twelve of sixteen mutations were identified in genes homologous to the *S. typhimurium invG* and *prgH* genes, which are known to be involved in the type III secretion pathway of virulence proteins. Two additional insertions were identified in genes sharing homology with the *cpxA* and *damX* genes from *Escherichia coli* K-12, and two uncharacterized invasion-deficient mutants were nonmotile. Gene expression of Tn*phoA* fusions was examined in response to environmental stimuli. We found that the *cpxA*, *invG*, and *prgH* genes were induced when grown under conditions of high osmolarity (0.3 M NaCl). Expression of *invG* and *prgH* genes was optimal at pH 6.5 and strongly reduced at low pH (5.0). Transcription of both *invG* and *prgH* Tn*phoA* gene fusions was initiated during the late logarithmic growth phase and was induced under anaerobic conditions. Finally, we show that both *invG* and *prgH* genes appear to be regulated by DNA supercoiling, a mechanism influenced by environmental factors. These results are the first to demonstrate that in *S. typhi*, (i) the *prgH* and *cpxA* genes are osmoregulated, (ii) the *invG* gene is induced under low oxygen conditions, (iii) the *invG* gene is pH regulated and growth phase dependent, and (iv) the *prgH* gene appears to be regulated by DNA supercoiling. Since our experimental conditions were designed to mimic the in vivo environmental milieu, our results suggest that specific environmental conditions act as signals to induce the expression of *S. typhi* invasion genes.**

Typhoid fever is a human disease caused by the facultative, intracellular pathogen *Salmonella enterica* serovar typhi (*S. typhi*). Since 30 million cases occur annually, this disease is responsible for significant morbidity and mortality worldwide (20, 79). Indeed, this disease remains a major public health concern and continues to be a priority of the World Health Organization (20). According to the seminal studies of Hornick et al. (43, 44), after *S. typhi* is ingested in contaminated food and/or water, the organisms migrate to the distal ileum. Presumably, *S. typhi* then adheres to and invades the intestinal epithelium (56, 99), migrates to the underlying Peyer's patches, and disseminates throughout the reticuloendothelial system (43, 44). However, relatively little is known about the bacterium host interactions that occur at each of these steps in the pathogenesis of *S. typhi.*

S. typhimurium has been widely used as a model to study *S. typhi* pathogenesis since this organism causes a typhoid-like disease in mice, and no effective animal model currently exists for *S. typhi* (11, 76, 77, 83). Over the past 5 years, in studies using both in vivo and in vitro models, numerous *S. typhimurium* genes that are involved in the initial, essential step of bacterial-host interaction, the adherence to and invasion of intestinal epithelial cells, have been identified. These genes include *hilA* (6), *inv* (4, 13, 21, 32, 35, 38, 52, 53), *prg* (10), *spa* (41), *ssp* (*sip*) (54, 55), and *lpf* and *pef* (9).

In 1989, Elsinghorst et al. (22) identified the first *S. typhi* genes involved in invasion of intestinal epithelial cells. These investigators cloned from *S. typhi* a 33-kb chromosomal DNA fragment that conferred the ability to enter Int407 intestinal epithelial cells on the noninvasive *Escherichia coli* strain HB101. Since homologous sequences from *S. typhimurium* failed to confer invasiveness on this *E. coli* strain, these data suggested the existence of distinct invasion loci between *S. typhi* and *S. typhimurium*. However, Lee (58) suggested that the *S. typhi* invasion genes identified by Elsinghorst et al. (22) may not be involved in *Salmonella* invasion but instead may have indirectly altered the ability of *E. coli* to enter cells in vitro. More recently, DNA fragments containing the *S. typhimurium* invasion genes *invA*, *invE*, *invH*, and *orgA* have been identified on the *S. typhi* chromosome (4, 34, 38, 51). Miras et al. (71) have also identified two *S. typhi* genes, *iagA* and *iagB*, that appear to be involved in invasion of HeLa cells by *S. typhi*. The *iagA* gene was shown to be identical to the *S. typhimurium hilA* gene that encodes an activator of invasion gene expression (6). Finally, Hermant et al. (42) characterized additional invasion genes in *S. typhi*, *sipEBCDA*. These genes were shown to be homologous to the *S. typhimurium* genes *spaT*/*sicA* (54) and *sspBCDA*/*sipBCDA* (46, 54, 55) and to the *Shigella* genes *ipgC* and *ipaBCDA* (8, 88, 93, 94), which encode virulence factors.

Although the *S. typhi* invasion genes identified to date are homologous to the *S. typhimurium* genes, distinct invasion genes may remain unidentified, given that these two *Salmo-*

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nella species cause distinctly different diseases in humans; *S. typhi* causes the disseminated disease, typhoid fever, whereas *S. typhimurium* causes a relatively localized gastroenteritis. Moreover, recent data suggest that *S. typhi* and *S. typhimurium* differentially adhere to, invade, and stimulate intestinal epithelial cells to secrete soluble factors in vitro (95). Taken together, these findings imply that *S. typhi* may express distinct genes or may use different mechanisms of regulation for invasion of small intestinal epithelial cells than other *Salmonella* pathogens. One set of variables that could regulate invasion gene expression is environmental signals stimulated by the host. Thus, prior to entry of *S. typhi* into intestinal epithelial cells, the bacteria are exposed to a variety of host environmental conditions that may influence their functional capacity. Such environmental cues include temperature upshift after ingestion, acidity of the stomach, high osmolarity, and decreased oxygen tension within the intestine. Studies of *S. typhimurium* have shown that modulation of gene expression is required for bacteria to survive and replicate within these environments. For instance, concentrations of Ca^{2+} (74) and Mg^{2+} (36), nutrient availability (27), osmolarity (33), oxygen tension (24, 30, 52, 59, 89), pH (3, 10, 29), and other complex environments (reviewed in reference 66) have been shown to control virulence or virulence gene expression. However, little is known about environmental regulation of virulence genes in *S. typhi*. In this study, we report the identification and molecular characterization of 14 independent *S. typhi* invasion-defective Tn*phoA* mutants and their regulation by environmental stimuli.

(A preliminary account of this work has been presented elsewhere [57].)

MATERIALS AND METHODS

Bacterial strains, bacteriophage, plasmids, and growth conditions. *S. typhi* ISP1820 (clinical isolate, gift of David Hone, Medical Biotechnology Center, University of Maryland Biotechnology Institute, Baltimore, Md.) and its derivatives were routinely grown at 37°C in Luria-Bertani (LB) medium supplemented with 0.3 M NaCl unless otherwise specified. *E. coli* XL1-Blue MRF′ (Stratagene, Inc., La Jolla, Calif.) was used as the host strain for cloning experiments. Antibiotics were used at the following concentrations: kanamycin, 50 μg/ml; tetracycline, 12 μg/ml; ampicillin, 85 μg/ml; gentamicin, 100 μg/ml; and novobiocin, 2 to 10 μg/ml. 5-Bromo-4-chloro-3-indolyl phosphate (XP; Sigma) was used at a concentration of 40 μg/ml as the chromogenic substrate for detecting the alkaline phosphatase activity on LB agar plates. *p*-Nitrophenyl phosphate (Sigma) was used as the substrate for quantitative measurement of alkaline phosphatase activity. Plasmid pJM703.1 (gift from D. Hone), is a derivative of the suicide plasmid pGP704 that carries Tn*phoA* and confers both ampicillin and kanamycin resistance. Bacteriophage P22 HT*int* was propagated and used in transduction experiments as described previously (15).

Tn*phoA* mutagenesis. To eliminate the background activity of nonspecific periplasmic acid phosphatase encoded by the *phoN* gene, an *S. typhi* 1820 *phoN51*::Tn10d-Tet^r mutant was constructed. The *phoN51*::Tn10d-Tet^r mutation was introduced in *S. typhi* 1820 by P22 HT*int*-mediated transduction from *S. typhimurium* LT2 strain TT13216 (*nad*B499::MudJ *phoN51*::Tn10d-Tet) obtained from K. E. Sanderson (Salmonella Genetic Stock Center, Calgary, Canada). One transductant, DC27, that showed wild-type levels of cell-associated and invasion of Int407 cells was chosen for Tn*phoA* mutagenesis. Plasmid pJM703.1 was introduced in DC27 by electroporation, using a modification of the procedure described by O'Callaghan and Charbit (78). Briefly, the bacteria were grown in LB broth to an A_{600} of ≈0.6, chilled on ice, and washed three times with ice-cold distilled water. The bacterial pellet was suspended in 1/600 of the initial volume in ice-cold distilled water. Forty microliters of this suspension was used for the electroporation procedure. Tn*phoA* mutants were selected on LB agar plates supplemented with kanamycin, tetracycline, and the chromogenic substrate XP.

Invasion and adherence assays. Human small intestinal epithelial Int407 cells (Int407; ATCC CCL6; American Type Culture Collection, Rockville, Md.) were maintained and prepared for adherence and invasion assays as described elsewhere (96). The qualitative and quantitative invasion assays were conducted essentially as described by Tartera and Metcalf (91) and Weinstein et al. (96). Briefly, *S. typhi* strains were grown in a shaking water bath in LB medium supplemented with 0.3 M NaCl to mid/late logarithmic growth phase (A_{600} of ≈0.6). One milliliter of each culture was centrifuged and resuspended in an equal volume of saline. Twenty-five microliters of this suspension was added to 2.5×10^5 Int407 cells (multiplicity of infection of ~20). High osmolarity of the growth media has been shown to be optimal for *S. typhi* cell-associated and invasion of human small intestinal epithelial cells (91). The bacteria were centrifuged onto the monolayers to allow intimate contact. Bacteria and Int407 cells were incubated at 37°C in a 5% CO_2 atmosphere for 90 min and washed three times with Earle's balanced salt solution (EBSS; Gibco BRL). The monolayers were then incubated for 90 min in tissue culture medium containing 100 μg of gentamicin per ml to kill extracellular bacteria. Subsequently, the monolayers were washed three times with the EBSS and lysed with 0.2 ml of 1% Triton X-100 (Sigma) to release intracellular bacteria. Samples were vigorously mixed, and 0.8 ml of saline was added. In the qualitative invasion assays, 100 μl of this suspension were plated on LB agar plates. Under these conditions, the parental strain, DC27, gave confluent growth whereas invasion-defective mutants gave individual colonies. For quantitative invasion assays, the suspension was diluted and viable counts were quantified on LB plates. Percent invasion = (CFU released by 1% Triton X-100/CFU in the initial inoculum of bacteria added to each well) × 100.

For adherence assays, bacteria were allowed to attach for 90 min at 37°C in 5% CO_2 atmosphere and then washed six times with EBSS. The cell-associated bacteria were released by treatment of monolayers with 0.2 ml of 1% Triton X-100 in saline for 10 min, followed by addition of 0.8 ml of saline. CFU were quantified by plating appropriate dilutions on LB agar plates. Percent adherence = (CFU of cell-associated bacteria released by 1% Triton X-100/CFU in the initial inoculum of bacteria added to each well) × 100.

Phenotypic characterization of Tn*phoA* mutants. The presence of the capsule (Vi) and the lipopolysaccharide (LPS) O:9 antigen was determined by slide agglutination. Briefly, freshly grown, isolated bacterial colonies of *S. typhi* parental and mutant strains were suspended in either LPS O:9 *Salmonella* antiserum (Difco, Detroit, Mich.) or Vi antiserum (Difco), and agglutination was assessed. Motility of all bacterial strains was assessed by the ability of the bacteria to swarm on a semisolid (0.25%) LB agar plate. Tn*phoA* mutants were tested for nutritional requirements by their ability to grow on M9 minimal medium (86) supplemented with cysteine (20 μg/ml) and tryptophan (20 μg/ml).

Restriction enzyme, cloning, and Southern blot analysis. Restriction enzyme digestion was performed as recommended by the manufacturer (Gibco BRL). Plasmid DNA was purified by using Qiagen plasmid kits and QIAprep spin plasmid kits procedures as described by the manufacturer (Qiagen, Inc., Chatsworth, Calif.). *S. typhi* chromosomal DNA was purified by the procedure of Mekalanos (67), using proteinase K digestion for 18 h. DNA manipulations and Southern blot hybridization were carried out as described by Sambrook et al. (86). The oligonucleotide probes Km (5′-GTGGAGAGGCTATTCGGCTATG AC-3′) and *phoA* (5′-CAGAGCGGCAGTCTGATCAC-3′), complementary to the kanamycin and *phoA* genes, respectively, were labeled and detected by using the ECL 3′ oligolabeling system as described by the manufacturer (Amersham Life Science, Inc., Arlington Heights, Ill.). Chromosomal DNA from the Tn*phoA* invasion-defective mutants was digested to completion with *Sal*I or *Eco*RI, transferred onto a nylon membrane, and hybridized with the kanamycin or *phoA* oligonucleotide. *Eco*RI and *Sal*I cleave within the Tn*phoA* to generate fragments that yield bands representing the downstream and upstream sequences of the Tn*phoA* insertion, respectively.

For the cloning of DNA fragments containing a portion of the Tn*phoA* sequence, chromosomal DNA from invasion-defective mutants was digested to completion with *Eco*RI or *Sal*I, cloned into Bluescript plasmids (Stratagene, Inc.), and then transformed into *E. coli* XL1-Blue MRF′. Recombinants were selected for kanamycin resistance encoded by the Tn*phoA* transposon. The presence of the kanamycin resistance gene was confirmed by Southern hybridization analysis with the labeled oligonucleotide Km probe as described above.

DNA sequence analysis. The nucleotide sequence of the chromosome-Tn*phoA* junction was carried out on double-stranded plasmid DNA templates, using the dideoxy-chain termination method of Sanger et al. (87) and Sequenase (U.S. Biochemical, Cleveland, Ohio) as instructed by the manufacturer. The *phoA* reverse primer (5′-CAGAGCGGCAGTCTGATCAC-3′) and IS*50*R primer (5′-TAGGAGGTCACATGGAAGTCAGAT-3′), complementary to the *phoA* and IS*50*R sequences, respectively, were used to generate nucleotide sequences from upstream and downstream regions of the transposon insertion. Nucleotide and deduced amino acid sequences data were analyzed with the Genetics Computer Group software package, version 8.0 (16), and the BLAST program at the server of the National Center for Biotechnology Information at the National Library of Medicine (5).

Environmental regulation. To determine the osmoregulation of selected Tn*phoA* gene fusions, each mutant was grown to mid-logarithmic phase (A_{600} of ≈0.6) in 10.0 ml of LB with 0.3 or 0.06 M NaCl containing tetracycline and kanamycin. Cells were washed twice with phosphate-buffered saline and resuspended in 1 ml of 100 mM NaCl–10 mM Tris (pH 8.0). Bacterial cells were disrupted by sonication in a model 550 sonic dismembrator (Fisher Scientific, Pittsburgh, Pa.), and cell debris was removed by centrifugation at 12,000 rpm for 5 min. Protein concentrations were determined by using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, Calif.). Samples containing 50 μg of protein were assayed to measure the alkaline phosphatase activity. Specific activity units were calculated and expressed as described by Galán and Curtiss (33), using the following formula: units = {optical density at 420 nm [OD_{420}]/[time (minutes) × protein (milligrams)]} × 1,000.

To analyze the effect of the pH on the expression of the Tn*phoA* gene fusions, strains were grown to mid-logarithmic phase (A_{600} of ≈0.6) in LB buffered with 0.1 M MES (2-[*N*-morpholino]ethanesulfonic acid) (69) at pH 5.0 or 6.5, supplemented with the appropriate antibiotics from overnight cultures grown under the same conditions. Alkaline phosphatase activity was measured per milligram of protein as described above for the osmoregulation analysis.

For analysis of the effect of oxygen tension on the expression of the Tn*phoA* gene fusions, *S. typhi* strains were grown aerobically in 500-ml Erlenmeyer flasks with 15 ml of LB medium containing either 0.06 or 0.3 M NaCl. Bacteria were incubated at 37°C with strong agitation in a rotary shaker until the cultures reached an A_{600} of 0.6. Anaerobiosis was achieved by growing the bacteria in Brewer GasPak jars under an atmosphere of 4 to 10% CO_2 generated by the GasPak Plus system (Becton Dickenson, Cockeysville, Md.). The cultures were incubated at 37°C with agitation, and samples processed after the A_{600} of each culture in LB medium supplemented with either 0.06 or 0.3 M NaCl was about 0.3. Alkaline phosphatase activity was measured per milligram of protein as described above for the osmoregulation analysis.

To assay growth phase-related expression of Tn*phoA* gene fusions, bacterial strains were grown in LB with 0.3 M NaCl containing tetracycline and kanamycin. Three 1.0-ml samples were removed every 30 min, and the A_{600} was measured. In these experiments, the cells were treated with 0.1% $CHCl_3$ and 0.1% sodium dodecyl sulfate and directly assayed for alkaline phosphatase activity, expressed in Miller units (69). Units were calculated by the following formula: units = $\{[OD_{420} - (1.75 \times OD_{550})]/[\text{time (minutes)} \times \text{volume} \times OD_{600}]\} \times 1{,}000$. For strains containing either an *invG* or *prgH* Tn*phoA* insertion, the alkaline phosphatase activity was also assayed per milligram of protein as described above.

TABLE 1. Adherence, invasion, and characterization of *S. typhi* invasion-defective Tn*phoA* mutants

Strain	% Adherence[a]	% Invasion[b]	Locus[c]/amino acid position of Tn*phoA* insertion
HB101	6.5	0.003	NA[d]
DC27	66.7	90.9	NA
A1[e]	61.6	80.0	NA
K14	1.4	0.010	*invG*/53
H5	0.4	0.016	*prgH*/184
I25	1.9	2.1	*cpxA*/89
L5	12.9	14.9	*damX*/222

[a] Percentage of cell-associated bacteria after 90 min of incubation with respect to the initial inoculum. Values are representative of two independent experiments.

[b] Percentage of bacteria resistant to gentamicin treatment after 90 min with respect to the initial inoculum. Values are representative of two similar experiments.

[c] Deduced amino acids from partial DNA sequences were determined and used to search the GenBank database. The *invG* and *prgH* genes are from *S. typhimurium*; the *cpxA* and *damX* genes are from *E. coli* K-12.

[d] NA, not applicable.

[e] Mutant A1 represents a randomly selected Tn*phoA* insertion with wild-type levels of adherence and invasion and serves as a positive control.

RESULTS

Isolation of Tn*phoA* mutants that are defective in invasion of Int407 small intestinal epithelial cells. To identify *S. typhi* genes involved in the invasion of human small intestinal epithelial cells, *S. typhi* DC27 was mutagenized with the Tn*phoA* transposon. DC27 is a derivative of wild-type *S. typhi* ISP1820 from which the periplasmic nonspecific acid phosphatase was eliminated by the introduction of the *phoN51*::Tn*10* mutation. The suicide plasmid pJM703.1, containing Tn*phoA*, was electroporated into DC27, and PhoA$^+$ fusions were identified on LB agar plates containing tetracycline, kanamycin, and the chromogenic substrate XP. From 15 independent electroporations, yielding approximately 27,000 Tn*phoA* mutants, 600 blue colonies expressing the alkaline phosphatase were purified and frozen for further analysis; 437 PhoA$^+$ cells were tested qualitatively for the ability to invade the small intestinal epithelial Int407 cell line when grown in LB with 0.3 M NaCl. Seventeen noninvasive mutants, which showed decreased invasiveness compared to the wild-type strain DC27, were identified. Examples of each mutant type are shown in Table 1. All of the mutants and the wild-type strain DC27 had similar growth characteristics on M9 supplemented minimal medium and growth rates in LB medium. All mutants expressed both the LPS O:9 and Vi antigens, and two mutants, A19 and H1021, were nonmotile (data not shown). Since mutations affecting chemotaxis or motility have been reported to confer a noninvasive phenotype on *S. typhi* (63), A19 and H1021 were not characterized further at this time.

To determine the number of the Tn*phoA* insertions in each mutant and to obtain an estimate of the number of different chromosomal loci involved in the invasion-defective phenotype observed, the Tn*phoA* mutants were analyzed by Southern blot hybridization (data not shown). Chromosomal DNA was digested with *Eco*RI and *Sal*I and probed with labeled oligonucleotides generated from the kanamycin or *phoA* sequences present on Tn*phoA*. No signal was observed with the parental strain DC27 chromosomal DNA. Sixteen of the mutants had a single insertion, while one mutant, H1026, contained a double Tn*phoA* insertion and therefore was not characterized further. At least four different band patterns were readily identified. Results obtained from the *Eco*RI digestions hybridized with the *phoA* probe and the *Sal*I digestions hybridized with the Km probe allowed the identification of four distinct classes of mutants (data not shown).

The percentages of both adherence and invasion of small intestinal epithelial cells were determined for the 14 mutants. Examples of the mutant classes are shown in Table 1. One randomly selected Tn*phoA* insertion, mutant A1, showed wild-type levels of adherence and invasion and was used as a positive control. As expected, *E. coli* HB101 modestly adhered to but did not invade Int407 cells. The majority of the mutants were 5,000- to 10,000-fold less invasive than the parental strain DC27. In contrast, Tn*phoA* mutants I25 and L5 were only 43- and 6-fold, respectively, less invasive than DC27. Interestingly, none of the mutants was able to adhere to the Int407 monolayers as well as the wild type did, which suggests that the defects in invasion could be due to the inability of the mutants to attach to the Int407 monolayers. Decreases in cell-associated bacteria ranged from 35- to 166-fold compared to the parental strain DC27, except for the cell association of mutant L5, which was decreased approximately 5-fold. A similar cell association-defective phenotype was observed with *S. typhi* SB130 (*invA*::Km) and H553 (*invE*::Km), which suggests that adherence and invasion may be tightly linked in *S. typhi* (95).

Identification and characterization of *S. typhi* Tn*phoA* gene insertions. To characterize the 14 Tn*phoA* invasion-defective mutants, *Eco*RI and *Sal*I chromosomal DNA fragments containing a portion of the Tn*phoA* insertion were cloned into the pBluescript plasmid. Recombinant plasmids were identified by restriction enzyme analysis, and the presence of the kanamycin gene in the inserts was confirmed by Southern hybridization analysis using a Km oligonucleotide as a probe (data not shown). Nucleotide sequences were determined from the junction of the Tn*phoA* insertion by using the IS*50*R or *phoA* reverse primers which generated sequences from the downstream or upstream flanking region of the insertion, respectively. The DNA sequence data were compiled and analyzed with the Genetics Computer Group software program (16). Deduced amino acid sequences were used to search the GenBank database sequences. Six independent mutations, A25, F106, L106, K14, N16, and O1012, were identified in a gene sharing homology with the *invG* invasion gene of *S. typhimurium* (53). One example is shown in Table 1. Ginocchio et al. (39) demonstrated that the *invG* gene product is required

for the formation of bacterial organelles expressed at the surface of *S. typhimurium* and is involved in entry into cultured epithelial cells. Kaniga et al. (53) showed that InvG is homologous to the PulD family of protein translocases and suggested that the gene encoding this protein could be involved in the secretion apparatus or assist in the translocation of target proteins through the outer membrane. It has been shown that the *invG* gene product is involved in the secretion of InvJ, which is required for *S. typhimurium* invasion (13). InvG shares homology with the *Shigella* sp. MxiD (1), *Yersinia enterocolitica* YscC (68), *E. coli* SepD (48), *Pseudomonas solanacearum* HrpA (40), *Pseudomonas syringae* HrpH (45), and *Xanthomonas campestris* HrpA1 (97). The InvG, MxiD, YscC, SepD, and HrpA proteins are all involved in a type III secretion pathway of virulence factors (reviewed in reference 31).

The deduced amino acid sequences from K14, L106, and O1012 Tn*phoA* insertions ranged between 82 and 85% identity over the 44 to 60 amino acids analyzed compared to *S. typhimurium* InvG. These data suggest that the InvG amino-terminal domain is highly conserved between *S. typhi* and *S. typhimurium*. In contrast, the amino acid sequence deduced from mutants N16 and F106, in which the Tn*phoA* insertions were located in the carboxyl-terminal region of InvG, showed homology of only 63% and 68% amino acid identity. These data suggest that the carboxyl-terminal domain may be less conserved between *S. typhi* and *S. typhimurium.*

Six additional independent Tn*phoA* mutations, G3, H5, I17, M9, and M29, were identified in a gene that shares extensive homology to the *S. typhimurium* PhoP-repressed gene, *prgH*, a part of the *prgHIJK* operon (10, 80). Table 1 shows one example of this mutant class. Pegues et al. (80) have suggested that products of some genes encoded by the *prgHIJK* operon are necessary for synthesis or secretion of Ssp (Sip) proteins required for bacterial entry into epithelial cells. PrgH is homologous to MxiG from *Shigella flexneri*, a protein that is involved in the secretion of Ipa proteins (2). Since no Tn*phoA* insertions were obtained in the amino-terminal domain of PrgH, we speculate that the amino-terminal domain of PrgH is located in the cytoplasm. This notion is supported by the conjecture that the amino terminus of MxiG is also located in the cytoplasm (2). The deduced amino acid sequences obtained from the regions downstream of the Tn*phoA* insertions of mutant G3 showed 83% identity over 47 amino acids analyzed with the *S. typhimurium prgI* located downstream from the *prgH* gene (80). This observation suggests that the *prgHIJK* operon may be organized in the same manner for both *S. typhi* and *S. typhimurium.*

The insertion in mutant I25 was shown to be in a gene sharing homology to the *cpxA* gene from *E. coli* K-12. The deduced amino acid sequence of mutant I25 revealed 91% identity over 105 amino acids with CpxA (17). The *cpxA* gene encodes a sensor kinase that is a part of an operon also encoding the transcriptional regulator, *cpxR* (17, 85); CpxA and CpxR belong to a family of two-component regulatory systems. Danese et al. (14) suggested that the activated CpxA/CpxR signal transduction pathway is capable of relieving certain envelope-associate stresses and may play a role in directing protein trafficking functions within the cell envelope by inducing the production of multiple extracytoplasmic factors such as the DegP (HtrA) protease.

Finally, the mutant L5 has an insertion in a gene sharing homology with the *damX* (urf-74.3) gene from *E. coli* K-12 (51). Although the function of the *damX* gene is unknown, it has been suggested that this gene may be involved in cell cycle regulation because overexpression induced cell filamentation (65). The *damX* gene is a part of a superoperon containing genes of unrelated function such as aromatic amino acid biosynthesis (*aroK*, *aroB*, and *trpS*), cell cycle regulation (*damX*), DNA adenine methyltransferase (*dam*), and carbohydrate metabolism (*gph* and *rpe*) (65).

TABLE 2. Effect of osmolarity on the expression of invasion-defective Tn*phoA* gene fusions

Strain[a]	Locus/amino acid position	Mean PhoA activity (Miller units)[b] ± SEM		Ratio[c]
		LB–0.3 M NaCl	LB–0.06 M NaCl	
χ3642[d]	*invA*	129 ± 2.3	34 ± 1.1	3.8
A1	*Z*::Tn*phoA*	190 ± 53	329 ± 52	0.6
K14	*invG*/53	216 ± 18	17 ± 7	12.7
H5	*prgH*/184	1278 ± 422	110 ± 5	11.6
I25	*cpxA*/89	166 ± 11	38 ± 11	4.4
L5	*damX*/222	156 ± 6	192 ± 15	0.8

[a] Strains were grown to mid-logarithmic phase (OD_{600} of ≈0.6) in LB supplemented with 0.3 or 0.06 M NaCl and with kanamycin and tetracycline. Cell extracts were prepared by sonication, and the amount (milligrams) of protein was determined by the Bio-Rad assay.

[b] Alkaline phosphatase activity was measured as described in Materials and Methods. The data represent means of duplicate samples from three independent experiments.

[c] Ratio of PhoA activity LB–0.3 M NaCl/LB–0.06 M NaCl.

[d] *invA*::Tn*phoA* mutant of *S. typhimurium.*

Environmental signals that regulate *S. typhi cpxA*, *damX*, *invG*, and *prgH* gene expression. (i) Effect of medium osmolarity. Osmolarity has been identified as an environmental signal that controls virulence gene expression in several organisms, including the expression of the invasion genes *invA*, *invF*, *prgH*, *prgK*, *orgA*, *sspA*, and *sspC* of *S. typhimurium* (7, 33) and the *tcp* pili of *Vibrio cholerae* (70). In *S. typhi*, osmolarity and growth phase overlap in the regulation of adherence to and invasion of human intestinal epithelial cells (91). However, no *S. typhi* invasion genes have been shown to be regulated by these environmental signals. To evaluate the osmoregulation of the *cpxA*, *damX*, *invG*, and *prgH* genes, each of the 14 Tn*phoA* gene fusions was analyzed. Whole-cell extracts of mid-logarithmic-phase cultures grown in LB medium with either 0.06 NaCl or 0.3 M NaCl were assayed for alkaline phosphatase (PhoA) activity. The results are shown in Table 2. As previously reported, expression of *invA*::Tn*phoA* gene fusion from *S. typhimurium* χ3642 was osmoregulated (33). The random Tn*phoA* insertion mutant, strain A1, showed no significant difference in the level of PhoA activity when grown in either high- or low-osmolarity medium. In the *invG*::Tn*phoA* and *prgH*::Tn*phoA* gene fusions, the phosphatase activity was induced in high-osmolarity LB medium. The *invG* gene expression was induced 5- to 12-fold, whereas *prgH* gene expression was induced 9- to 20-fold in high-osmolarity medium. In addition, the phosphatase activity in the *cpxA*::Tn*phoA* gene fusion was induced fourfold in high-osmolarity medium. Conversely, no induction or repression of the PhoA activity was observed with the *damX*::Tn*phoA* gene fusion under the same conditions. These results indicate that *invG*, *prgH*, and to a lesser extent *cpxA* are osmoregulated genes that may be involved in the entry of *S. typhi* into intestinal epithelial cells.

(ii) Effect of gyrase inhibitor on *cpxA*, *invG*, and *prgH* gene expression. Previous studies have shown that DNA supercoiling can modulate gene expression in response to high osmolarity, anaerobiosis, and growth phase (19, 73). Galán and Curtiss (33) reported that the osmoinduction of *invA* was independent of the *ompB* (*ompR*/*envZ*) regulon but affected by the degree of DNA superhelicity. The level of DNA supercoil-

TABLE 3. Effect of novobiocin on expression of *cpxA*, *invG*, and *prgH*

Strain[a]	Locus	Novobiocin concn (μg/ml)	Mean PhoA activity (Miller units)[b] ± SEM
A1	Z::Tn*phoA*	0	124 ± 10
		2	160 ± 5
		5	38 ± 6
		10	109 ± 6
K14	*invG*	0	376 ± 47
		2	42 ± 0.2
		5	39 ± 1
		10	46 ± 3
H5	*prgH*	0	928 ± 367
		2	53 ± 4
		5	27 ± 1
		10	29 ± 1
I25	*cpxA*	0	127 ± 2
		2	177 ± 6
		5	169 ± 7
		10	119 ± 4

[a] Strains were grown to mid-logarithmic phase (OD_{600} of ≈0.6) in LB–0.3 M NaCl with or without various concentrations of novobiocin. Cell extracts were prepared by sonication, and the amount (milligrams) of protein was determined by the Bio-Rad assay.

[b] Alkaline phosphatase activity was measured as described in Materials and Methods. The data represent means from two independent experiments.

TABLE 4. Effect of pH on the expression of invasion-defective Tn*phoA* gene fusions

Bacterial strain[a]	Locus/amino acid position	Mean PhoA activity (Miller units)[b] ± SEM		Ratio[c]
		LB, pH 5.0	LB, pH 6.5	
A1	*Z*::Tn*phoA*	662 ± 229	187 ± 34	0.3
K14	*invG*/53	22 ± 11	497 ± 98	22.5
H5	*prgH*/184	171 ± 70	1,027 ± 145	6.0
I25	*cpxA*/89	165 ± 23	128 ± 4	0.8
L5	*damX*/222	120 ± 39	125 ± 10	1.0

[a] Strains were grown to mid-logarithmic phase (OD_{600} of ≈0.6) in LB buffered with 0.1 M MES at pH 5.0 or 6.5 with kanamycin and tetracycline. Cell extracts were prepared by sonication, and the amount (milligrams) of protein was determined by the Bio-Rad assay.

[b] Alkaline phosphatase activity was measured as described in Materials and Methods. The data represent means of duplicate samples from four independent experiments.

[c] Ratio of PhoA activity LB at pH 6.5/LB at pH 5.0.

ing in vivo can be perturbed by using novobiocin, which specifically inhibits the β subunit of DNA gyrase and results in relaxation of intracellular DNA (37). To determine whether the *cpxA*, *invG*, and *prgH* genes were affected by DNA supercoiling, the effects of novobiocin were examined. Tn*phoA* mutants A1, K14, H5, and I25 were grown overnight in LB medium with 0.3 M NaCl and increasing amounts of novobiocin and then subcultured into fresh medium of the same characteristics. In contrast to *S. typhimurium*, 10 to 20 μg of novobiocin per ml significantly inhibited the growth of *S. typhi* (data not shown). More importantly, when the *S. typhi* mutants were grown in LB supplemented with 2 μg of novobiocin per ml, expression of *invG*::Tn*phoA* and *prgH*::Tn*phoA* fusion products was inhibited 9- and 17-fold, respectively (Table 3). In contrast, the PhoA expression from the *cpxA*::Tn*phoA* or the random *Z*::Tn*phoA* insertion fusion was not affected by changes in DNA supercoiling. Collectively, these data suggest that transcription from chromosomal *invG* and *prgH* promoters is sensitive to the degree of DNA supercoiling in response to different environmental stimuli such as osmolarity.

(iii) Effect of pH. Acid adaptation is likely to be an important variable in *Salmonella* pathogenicity, since pH has been identified as a regulator of virulence gene expression in both human and plant pathogens. These genes include the *invF*, *pagC*, *prgH*, *prgK*, *orgA*, *sspA*, and *sspC* genes of *S. typhimurium* (3, 7, 10), the *inv* gene of *Y. enterocolitica* (81), the *hrp* genes from *P. syringae* pv. phaseolicola (84), and the *vir* gene of *Agrobacterium tumefaciens* (98). To determine whether *S. typhi* invasion genes are regulated by pH, expression of the Tn*phoA* gene fusions was analyzed at pH 5.0 and 6.5. Bacterial strains were grown to mid-logarithmic phase in LB medium buffered with 0.1 M MES at pH 5.0 and 6.5 from overnight cultures grown under the same conditions. Cell extracts were prepared, and alkaline phosphatase activity was assessed. The growth curves of the mutants in the different conditions tested were similar (data not shown). As shown in Table 4, the *invG*::Tn*phoA* and *prgH*::Tn*phoA* gene fusion products were highly reduced at pH 5.0. In contrast, expression of neither *cpxA*::Tn*phoA* nor *damX*::Tn*phoA* gene fusion products was altered by pH. These results indicate that *invG* and *prgH* genes are repressed at low pH. A low-pH environment is encountered in the stomach by the pathogens during passage to the intestine or intracellularly within endosomes after invasion of intestinal epithelial cells or macrophages (90).

(iv) Effect of oxygen tension. Another variable that *S. typhi* may encounter when entering a host is the reduced oxygen tension of the distal ileum (90). Independent studies have shown that reduced oxygen tension during growth of bacteria enhanced attachment and invasiveness of *S. cholerasuis* and *S. typhimurium* in vitro, which suggested that invasion genes may be induced under anaerobic conditions (24, 30, 52, 59, 60, 89). For instance, the *invF*, *prgH*, *prgK*, *orgA*, *sspA*, and *sspC* genes, which are involved in the virulence of *S. typhimurium*, have been shown to be induced under anaerobic conditions (7, 52). However, Behlau and Miller (10) showed that the *S. typhimurium prgH* invasion locus was repressed under anaerobic conditions. To determine the effect of oxygen tension on *cpxA*, *damX*, *invG*, and *prgH* gene expression in *S. typhi*, Tn*phoA* mutants A1, I25, K14, H5, and L5 were grown anaerobically and cell extracts were assayed for alkaline phosphatase activity (Table 5). In comparison to growth in media on a rotary shaker in air, anaerobiosis decreased the *S. typhi* growth rate (data not shown). Nonetheless, as shown in Table 5, low oxygen tension had a strong effect on the expression of the *invG* and *prgH* genes. The alkaline phosphatase activity of the *invG* Tn*phoA* mutant was 14-fold higher under anaerobic conditions and was enhanced similarly in both high- and low-osmolarity media. The *prgH* gene was also strongly regulated under low-oxygen conditions when bacteria were grown in LB medium supplemented with 0.06 M NaCl (low osmolarity). In the cultures, the alkaline phosphatase activity of the gene fusion was 29-fold higher when bacteria were grown under anaerobic conditions compared to the aerobic cultures. In contrast, the osmoinduction of *cpxA* was not affected by oxygen tension, while *invG* and *prgH* genes were strongly affected. The activity of the *cpxA*, *damX* (data not shown), and control *Z*::Tn*phoA* fusions was not affected by oxygen tension in either high- or low-osmolarity conditions. These results suggest that osmolarity and oxygen tension appear to be the major environmental signals controlling the expression of *invG* and *prgH* genes.

(v) Effect of growth phase. Previous studies from our laboratory demonstrated that high osmolarity and growth phase

TABLE 5. Effect of oxygen tension on the expression of invasion-defective Tn*phoA* gene fusions

Strain, Tn*phoA* fusion	Medium	Mean PhoA activity (Miller units)[a] ± SEM		Ratio[d]
		Aerobic[b]	Anaerobic[c]	
A1, *Z*::Tn*phoA*	LB–0.06 M NaCl	182 ± 72	133 ± 25	0.7
	LB–0.3 M NaCl	127 ± 29	194 ± 21	1.5
I25, *cpxA*	LB–0.06 M NaCl	21 ± 2	19 ± 2	0.9
	LB–0.3 M NaCl	64 ± 1	80 ± 20	1.3
K14, *invG*	LB–0.06 M NaCl	10 ± 3	105 ± 39	10.5
	LB–0.3 M NaCl	28 ± 6	377 ± 78	13.5
H5, *prgH*	LB–0.06 M NaCl	11 ± 2	314 ± 79	28.6
	LB–0.3 M NaCl	96 ± 1	370 ± 42	3.8

[a] Alkaline phosphatase activity was measured as described in Materials and Methods. The data represent means of duplicate samples from two independent experiments.

[b] Strains were grown with aeration and agitation to mid-logarithmic phase (OD_{600} of ≈0.6) in LB–0.06 M NaCl or LB–0.3 M NaCl containing kanamycin and tetracycline. Cell extracts were prepared by sonication, and the amount (milligrams) of protein determined by the Bio-Rad assay.

[c] Strains were grown under an anaerobic atmosphere of 4 to 10% CO_2 with agitation to mid-logarithmic phase (OD_{600} of ≈0.3) in LB–0.06 M NaCl or LB–0.3 M NaCl with kanamycin and tetracycline. Cell extracts were prepared by sonication, and the amount (milligrams) of protein was determined by the Bio-Rad assay.

[d] Ratio of PhoA activity, anaerobic/aerobic.

conditions overlap for optimal induction of adherence of *S. typhi* to intestinal epithelial cells (91). These studies also showed that optimal adherence occurred during the mid- to late log growth phase. Lee and Falkow (59) have also shown that the capacity of *S. cholerasuis* to enter mammalian cells was a function of the bacterial growth state. Moreover, Behlau and Miller (10) demonstrated that expression of all *S. typhimurium prg* loci was maximal in late logarithmic growth phase. Collectively, these data suggested that *S. typhi* invasion genes may be also temporally regulated by the growth phase. To test whether expression of *S. typhi cpxA*, *damX*, *invG*, and *prgH* genes is growth phase dependent, the PhoA activity of the Tn*phoA* gene fusions was analyzed at different phases of growth. Bacterial strains were grown in optimal conditions using LB medium with 0.3 M NaCl; samples were removed periodically and assayed for alkaline phosphatase activity. The results presented in Fig. 1 show that the expression of *prgH* and *invG* genes was induced in late logarithmic phase or early stationary phase, indicating that expression of both genes was growth phase dependent. Similar results were obtained when PhoA activity was assayed per milligram of protein (data not shown). In addition, the expression of the *invG* and *prgH* genes was reduced approximately 50% in late stationary growth phase (data not shown). In contrast, the *cpxA* and *damX* genes were not regulated by growth phase. The timing of *invG* and *prgH* gene expression correlates with the timing of maximal expression of all *S. typhimurium prg* loci and secretion of the Ssp (Sip) proteins (10, 80).

DISCUSSION

To persist in the human host, *S. typhi*, as well as other facultative intracellular pathogens, must encounter and survive numerous complex extracellular (stomach, intestine, lymphatic system, and bloodstream) and intracellular (phagocytic and nonphagocytic cells) environments. Previous studies have suggested that some of these environments modulate the expression of virulence factors (reviewed in reference 66). However, relatively little is known about the effects of these environmental signals on the expression of *S. typhi* virulence genes. Because no physiologically relevant animal model exists for *S. typhi*, investigators have analyzed the response to *S. typhimurium*, which causes a typhoid-like disease in mice, in an attempt to understand the molecular mechanisms of *S. typhi* pathogenesis. In this report, we have identified and characterized 14 *S. typhi* Tn*phoA* mutants that are involved in the initial interactions of *S. typhi* with the host and analyzed gene expression in response to environmental stimuli. The results demonstrate that 12 of 16 mutations were in genes that shared homologies with *S. typhimurium* genes known to be involved in the type III secretion pathway of virulence proteins. Six independent mutations in the invasion gene, *invG*, and six in the PhoP repressed gene, *prgH*, were found. Two additional insertions were identified in genes sharing homology with the *cpxA* and *damX* genes of *E. coli* K-12. Finally, two invasion-deficient mutants (A19 and H1021) were found to be nonmotile.

Initially, our Tn*phoA* mutants were screened only for defects in invasion of intestinal epithelial cells. However, our results demonstrated that Tn*phoA* insertions in *invG*, *prgH*, *cpxA*, and *damX* genes also rendered *S. typhi* defective in adherence to, as well as invasion of, the small intestinal epithelial cell line Int407. The possibility that the Tn*phoA* insertion has exerted a polar effect on other adherence/invasion genes located downstream cannot be excluded at this time. Nonetheless, this linkage between adherence and invasion has also been observed in *S. typhi* mutants SB130 (*invA*::Km) and H553 (*invE*::Km) (95). However, in *S. typhimurium*, the results are different. *S. typhimurium invA* and *invE*, as well as *invC*, *invF*, *invG*, *invI*, *invJ*, and *prgH*, invasion mutants are able to adhere to intestinal epithelial cell monolayers at levels similar to wild-type levels (10, 13, 21, 35, 38, 53). These observations suggest that adherence and invasion mechanisms of the two organisms may be distinct. Differences between *S. typhimurium* and *S. choleraesuis* have also been reported. Tn*phoA* mutants of *S. choleraesuis* that were unable to pass through (transcytose) polarized MDCK epithelial monolayers were also unable to adhere to or invade MDCK cells (28). An *S. choleraesuis invH* mutant was shown to be deficient in both attachment and invasion of cultured epithelial cells (4). Taken together, our results and those of Altmeyer et al. (4) suggest that host-adapted pathogen adherence/invasion genes have acquired or modified their function for optimal adherence and invasion of intestinal epithelial cells. It is also possible that adherence and invasion phenotypes are mediated by the same protein(s) in *S. typhi* as previously shown for the *inv* gene of *Yersinia* spp. (47). Mutations in the *inv* genes of *Y. enterocolitica* and *Y. pseudotuberculosis* altered both the ability to enter and to adhere to eukaryotic cells (47). Alternatively, the adherence/invasion defect phenotype that we observed in *S. typhi* could be caused by the nonspecific toxic effect exhibited by an active $PhoA^+$ fusion that could block the export pathway of other proteins needed for invasion. In this case, it is possible that Tn*phoA* mutations in the *cpxA* and *damX* genes indirectly affect the adherence/invasion mechanism of *S. typhi.*

In *E. coli*, the CpxA/CpxR signal transduction pathway regulates the expression of the DegP (HtrA) protease under certain envelope stresses (14). Thus, the function of the CpxA/CpxR regulon in bacterial pathogenesis may be related to the role of the DegP (HtrA) protease. In *S. typhimurium*, mutations in the *htrA* gene were shown to be avirulent or highly attenuated when given orally to mice (12, 25, 49). Interestingly, Everest et al. (25) showed that expression of the *htrA* gene was

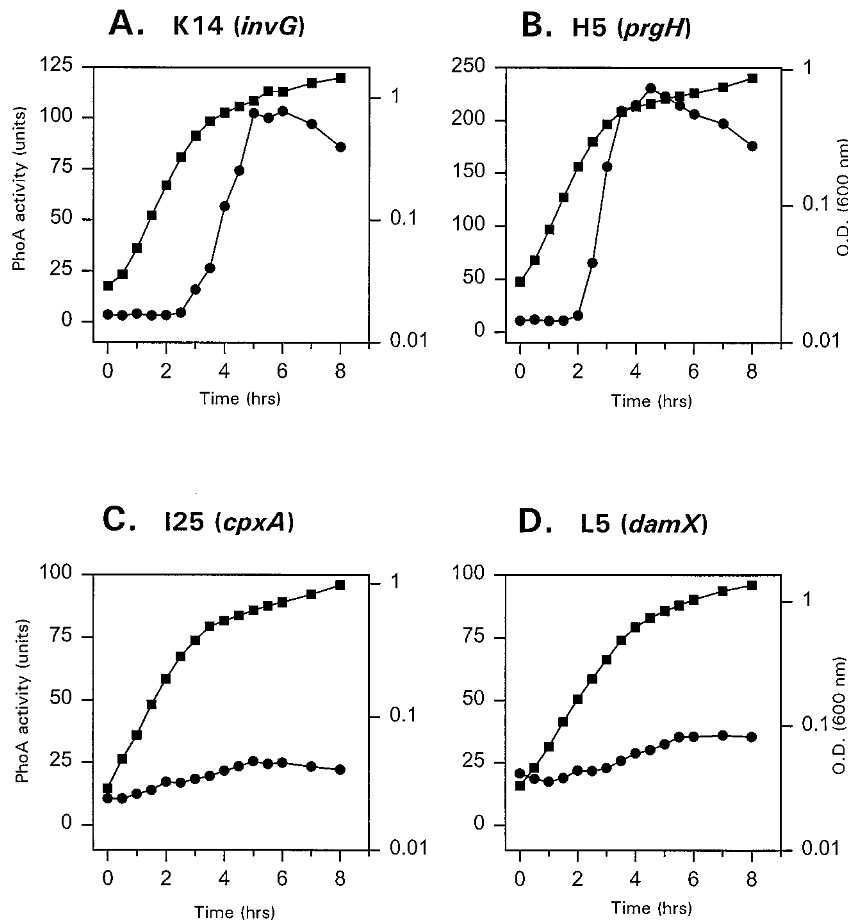

FIG. 1. Temporal expression of *invG*, *prgH*, *cpxA*, and *damX*::Tn*phoA* gene fusions. Strains were grown in LB–0.3 M NaCl containing kanamycin and tetracycline. Samples were removed at 30-min intervals, and cell density (■) and PhoA activity (●) were measured. Alkaline phosphatase activity is expressed in Miller units as described in Materials and Methods. Data are representative of two independent experiments done in duplicate.

significantly increased when the *Salmonella* entered eukaryotic cells. The HtrA protease has also been shown to be involved in the virulence of *Y. enterocolitica* and *Brucella abortus* (23, 62). Recently, Nakayama and Watanabe (72) reported that *cpxA* could be involved in a pH-dependent regulation of the expression of *Shigella sonnei virF* gene, which positively regulates *ipaBCD* gene expression. In contrast, our results shown that the expression of the *S. typhi cpxA* gene is pH independent but osmoregulated, indicating that *Shigella* and *Salmonella* might sense different environmental signals to control the expression of virulence factors. Taken together, these data suggest that the CpxA/CpxR regulon may play a role in *S. typhi* virulence as well as other pathogens, by modulating the expression of the HtrA protease.

Recently, Hermant et al. (42) identified 15 noninvasive Tn*phoA* mutants from *S. typhi* Ty2 that were unable to enter HeLa cells. Two mapped within the *iagAB* locus, and six others mapped in the *sipEBCDA* locus. It is interesting that none of our 16 Tn*phoA* insertions were in the *sipEBCDA* locus but were found primarily within the *invG* and *prgH* genes. It is possible that the conditions used for our invasion assay favored the identification of some osmoregulated invasion genes such as the *invG* and *prgH* instead of the *sip* genes. In our standard invasion assay, the bacteria are grown in LB with 0.3 M NaCl, the optimal growth conditions for *S. typhi* adherence to and invasion of intestinal epithelial cells (91). Our results suggest that a more substantial decrease in invasion is observed when a subset of osmoregulated invasion mutants are compared to the wild type under conditions of high osmolarity.

Our studies demonstrate that osmolarity is one of the major environmental signals controlling the expression of *S. typhi* virulence genes. It is interesting that osmolarity not only controls the expression of *S. typhi invG* and *prgH* invasion genes but also appears to regulate the level of Vi capsule antigen synthesis (82). This notion is consistent with our published and preliminary data that show optimal adherence occurs at high osmolarity when the capsule is the smallest (91, 92, 95). Our results also show that low oxygen tension is an important factor that regulates the expression of genes involved in the invasive phenotype of *S. typhi* and are in agreement with studies conducted with *S. typhimurium* (7, 52). However, our results are the first to show that anaerobiosis affects the expression of the *invG* gene under both high- and low-osmolarity conditions. The *prgH* gene is also induced under low oxygen tension, but the effect is stronger under low-osmolarity conditions. It is possible that the effects of osmolarity, pH, and anaerobiosis are exerted on the posttranslational modification of the gene products. However, since other investigators have reported direct regulation on related genes (7, 33), we have assumed that the effects observed in our studies are at the transcriptional level. Discrepancies between our results and data from

other investigators could be explained by differences in the growth conditions of the bacterial cultures used.

Expression of the *S. typhi invG* and *prgH* genes was also shown to be growth phase dependent. Transcription of *invG* and *prgH* genes was initiated in the late log/early stationary phase (Fig. 1). This observation suggests that these two genes may be regulated by the RNA polymerase subunit σ^S (RpoS) that is required for expression of stationary phase as well as osmotically regulated genes in *E. coli* (reviewed in reference 64). In *S. typhimurium*, the *rpoS* gene mediates the expression of the *spv* plasmid virulence genes during bacterial starvation (26, 75). Interestingly, the *S. typhi invG* and *prgH* genes were repressed under starvation conditions (data not shown). In addition, it has been shown that σ^S is required for a sustained acid tolerance response in virulent *S. typhimurium* (61). A sustained acid tolerance response also provided cross-protection to a variety of other environmental stresses such as heat, H_2O_2, and osmolarity (61). Taken together, our findings suggest that *S. typhi invG* and *prgH* invasion genes can be regulated by the sigma factor σ^S (RpoS) in response to environmental signals such as osmolarity, pH, and growth phase.

Gene expression can be modulated by changes in DNA supercoiling in response to high osmolarity, anaerobiosis, and growth phase (19, 73). Increases in DNA superhelicity can either activate expression of some genes or repress others (18). *Salmonella* invasiveness is affected by environmental conditions that influence the degree of DNA supercoiling (33, 59). For instance, the *S. typhimurium invA* gene has been shown to be induced by DNA supercoiling (33). Our findings support the view that changes in DNA supercoiling may be a regulatory mechanism for the control of virulence gene expression (18). Expression of *S. typhi invG* and *prgH* genes was significantly decreased in the presence of novobiocin, an inhibitor of DNA gyrase (Table 3). The mechanism of gene regulation by DNA topology, in response to environmental signals, is still unknown. However, such a mechanism may require a signal transduction system to sense changes in the environment for controlling gene expression. Studies have shown that in *S. typhimurium*, *invF* and *prgH* genes were activated by HilA or by a mutation that overexpressed the *hilA* gene (6, 10). Bajaj et al. (6) showed that the *hilA* gene encodes a transcriptional regulator. Recently, Bajaj et al. (7) reported that *S. typhimurium invF*, *prgH*, *prgK*, *orgA*, *sspA*, and *sspC* genes were coordinately regulated by oxygen, osmolarity, pH, PhoP/Q, and HilA. The expression of the *hilA* gene was shown to be activated by *sirA*, a newly identify phosphorylated response regulator gene (50). A homologous *hilA* gene has been identified in *S. typhi* (71), which suggests that *S. typhi invG* and *prgH* gene can also be regulated by the regulatory cascade SirA-HilA. Consequently, the *sirA* and *hilA* genes may be excellent candidates to control invasion gene expression by DNA supercoiling in response to environmental conditions.

In summary, we have attempted to establish in vitro culture conditions that reflect the environmental milieu present in the intestinal lumen of the human host. We have analyzed the effects of these conditions on expression of genes involved in invasion of intestinal epithelial cells by *S. typhi*. Our studies suggest that specific environmental conditions act as signals to induce the expression of *S. typhi* invasion genes. These studies further our understanding of the initial steps in the pathogenesis of *S. typhi* and the interactions of this pathogen with intestinal epithelial cells.

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