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# Expression of *Salmonella typhimurium* Genes Required for Invasion Is Regulated by Changes in DNA Supercoiling

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**The ability to enter intestinal epithelial cells is an essential virulence factor of salmonellae. We have previously cloned a group of genes (*invA*, *B*, *C*, and *D*) that allow *S. typhimurium* to penetrate tissue culture cells (J. E. Galán and R. Curtiss III, Proc. Natl. Acad. Sci. USA 86:6383–6387, 1989). Transcriptional and translational *cat* and *phoA* fusions to *invA* (the proximal gene in the *invABC* operon) were constructed, and their expression was studied by measuring the levels of alkaline phosphatase or chloramphenicol acetyltransferase activity in mutants grown under different conditions. It was found that when strains containing the fusions were grown on media with high osmolarity, a condition known to increase DNA superhelicity, the level of *invA* transcription was approximately eightfold higher than that in strains grown on media with low osmolarity. The osmoinducibility of *invA* was independent of *ompR*, which controls the osmoinducibility of other genes. Strains grown in high-osmolarity media in the presence of subinhibitory concentrations of gyrase inhibitors (novobiocin or coumermycin $A_1$), which reduce the level of DNA supercoiling, showed reduced expression of *invA*. Nevertheless, *invA* was poorly expressed in *topA* mutants of *S. typhimurium*, which have increased DNA superhelicity. In all cases, the differential expression of the invasion genes was correlated with the ability of *S. typhimurium* to penetrate tissue culture cells. These results taken together indicate that expression of *S. typhimurium* invasion genes is affected by changes in DNA supercoiling and suggest that this may represent a way in which this organism regulates the expression of these genes.**

Invasion of the intestinal epithelial cells constitutes a crucial step in the pathogenesis of infections by many enteric bacteria (21). The use of cultured epithelial cells as an in vitro model to study bacterial invasion has begun to yield important information on the biochemical and genetic bases of cell penetration by a variety of bacteria (9, 15, 26, 27, 39, 44, 45, 57), but the molecular details of the bacterium-host cell interaction remain largely unknown.

There are three species of Salmonella (*S. typhi*, *S. choleraesuis*, and *S. enteritidis*) and hundreds of serovars that cause a variety of diseases in a large number of different hosts (25). The type of diseases caused by these organisms depends not only on the serovar or species of the infecting bacteria but also on the species of the infected host. The clinical and histopathological features of the disease may therefore vary greatly, from a localized gastroenteritis to a more invasive form like typhoid fever (25). In some cases salmonellae may cause little damage to the epithelial mucosa and simply gain access to deeper tissues, particularly those of the reticuloendothelial system, to establish a systemic infection. In other instances, they may establish a more localized infection of the intestine, with visible damage to the mucosa. One feature common to all salmonellae is that they penetrate the intestinal epithelial mucosa after oral ingestion (59). Because of the central importance of this process, a number of laboratories have begun to analyze the biochemical and genetic bases of *Salmonella* invasion of epithelial cells.

Many laboratories have isolated *Salmonella* mutants that are defective in their ability to penetrate cultured epithelial cells (11, 36, 46). Elsinghorst et al. (7) have recently reported the identification of a 33-kilobase region of *S. typhi* chromosomal DNA that conferred upon *Escherichia coli* HB101 the ability to penetrate tissue culture cells. We have recently cloned a group of genes (*invA*, *B*, *C*, and *D*) that allow *Salmonella typhimurium* to penetrate tissue culture cells (14). Infection studies with highly virulent *Salmonella* strains carrying defined mutations in these genes have established that the *inv* genes are involved in the establishment of infection of Peyer's patches and the small intestinal wall when the organisms are administered orally. Hybridization studies have determined that these genes are present in all *Salmonella* strains tested (including strains of *S. typhi* and *S. choleraesuis* and more than 40 serovars of *S. enteritidis*), although they are absent from other members of the family *Enterobacteriaceae* (unpublished data).

Pathogenic bacteria make use of a great diversity of genetic information to interact with the host. Expression of these genes are often not needed simultaneously, and therefore in many cases their expression is coordinately regulated (18, 38, 42). Recently, Finlay et al. (10) reported that exposure to epithelial cells induces de novo synthesis of *Salmonella* proteins that are required for invasion of tissue culture cells, and they suggested that this constitutes a mechanism of coordinate regulation of expression of invasion genes. We report here a different mechanism for regulation of expression of genes necessary for invasion of epithelial cells.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** The strains used in this study are listed in Table 1. Bacteria were grown in L broth (34) or in low- and high-osmolarity K medium as indicated. The composition of low-osmolarity K medium has been described (29), and when indicated, 0.3 M NaCl or 0.6 M sucrose was added to increase its osmolarity. The following antibiotics were used at the indicated concentrations

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TABLE 1. Bacterial strains

| Strain | Genotype | Description, reference, or source |
|---|---|---|
| *S. typhimurium* | | |
| SR11 | | |
| χ3181 | Wild type | SR11 (55) isolated from Peyer's patches of an infected mouse |
| χ3642 | *invA*::Tn*phoA* | 14 |
| χ3687 | *phoP12* | 13 |
| χ4108 | *phoP12 invA*::Tn*phoA* | P22 HT*int* (χ3643) → χ3687 |
| χ4109 | *phoP12* Y::Tn*phoA* | Random Tn*phoA* insertion from χ3689 transduced into χ3687 |
| χ4110 | *phoP12* Z::Tn*phoA* | Random Tn*phoA* insertion from χ3689 transduced into χ3687 |
| χ4111 | *phoP12 proU*::Tn*phoA* | P22 HT*int* (AD110) → χ3687 |
| χ4112 | *phoP12 invA*::Tn*phoA ompR1009* | P22 HT*int* (CFH1351) → χ4108 |
| χ4115 | *invA*::*cat* | $Ap^r$ $Cm^r$ transconjugant of χ3642 × SM10 λ *pir*(pSKCAT) |
| SL1344 | | |
| χ3339 | *rpsL hisG* | Mouse passage SL1344 24 |
| χ3643 | *rpsL hisG invA*::Tn*phoA* | 14 |
| χ3689 | *rpsl hisG phoP12* | 13 |
| LT2 | | |
| PM233 | *leu-500* Δ*topA24 ara-9* | 47 |
| PM596 | *leu-500 ara-9* | 47 |
| χ4113 | *leu-500* Δ*topA24 ara-9 invA*::Tn*phoA phoP12 purB*::Tn*10* | P22 HT*int* (χ3643) → PM233 |
| χ4114 | *leu-500 ara-9 invA*::Tn*phoA phoP12 purB*::Tn*10* | P22 HT*int* (χ3643) → PM596 |
| χ4282 | *leu-500* Δ*topA24 ara-9* Z::Tn*phoA phoP12 purB*::Tn*10* | Random Tn*phoA* insertion from χ3689 transduced into PM233 |
| AD110 | *proU*::Tn*phoA phoP purB*::Tn*10* | From E. Eisenstadt |
| CFH1351 | *ompR1009*::Tn*10* | 16 |
| *E. coli* | | |
| SM10 λ *pir* | *thi thr leu tonA lacY supE recA*::RP4-2Tc::Mu λ *pir* R6K | 31 |

(micrograms per milliliter): chloramphenicol, 25; kanamycin, 50; coumermycin $A_1$, 0.5 to 2 as indicated; novobiocin, 5 to 20 as indicated.

**DNA and genetic manipulations.** Chromosomal DNA isolation (14) and DNA hybridization (58) were performed as previously described. Bacteriophage P22 HT*int*-mediated transduction was performed as described elsewhere (3, 54). Conjugations were carried out by filter mating (61).

**Alkaline phosphatase and CAT assays.** Bacterial strains were grown under different conditions to an optical density at 600 nm ($OD_{600}$) of 0.5. Cells were washed twice with phosphate-buffered saline (PBS), suspended in 100 mM NaCl–10 mM Tris (pH 8) to 1/10 of the original volume, and disrupted by sonication. Cell debris were removed by centrifugation at 12,000 × *g* for 2 min. Alkaline phosphatase (PhoA) was assayed as described previously (20), and units were calculated by the following formula: units = $\{A_{405}/[\text{time (minutes)} \times \text{protein concentration (milligrams)}]\} \times 1{,}000$. Chloramphenicol acetyltransferase (CAT) was measured at room temperature by the colorimetric method of Shaw (56), and activity is expressed as nanograms of acetylated chloramphenicol per milligram of protein per minute.

**Tissue culture cell invasion assay.** Invasion of MDCK cells by *S. typhimurium* was performed in 24-well tissue culture plates as described previously (14), with some modifications. Infection was for 1 h in PBS with a multiplicity of infection of 10. After infection, monolayers were washed with PBS and incubated for 2 h with Eagle minimal essential medium containing gentamicin (100 μg/ml) to eliminate extracellular bacteria. Monolayers were then lysed with PBS containing 0.1% sodium deoxycholate to assess the total number of internalized bacteria.

**Isolation of random Tn*phoA* insertions into the *S. typhimurium* chromosome.** Plasmid pYA2207 is a temperature-sensitive plasmid derived from pSC304 (33) that carries a nonproductive insertion of Tn*5* $IS50_L$::*phoA* (Tn*phoA*) (37) (J. Galán, unpublished data). Secondary transpositions of Tn*phoA* from pYA2207 into the chromosome of *S. typhimurium* χ3689 (13) that yielded productive fusions to alkaline phosphatase were isolated as follows. Cells were grown in L (broth at 30°C to an $OD_{600}$ of 0.4, washed with 0.85% NaCl, and plated on a modified MOPS (morpholinepropanesulfonic acid) medium (48) in which EPPS [*N*-(2-hydroxyethyl)piperazine-*N*′-(3-propanesulfonic acid)] was substituted for MOPS and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) was substituted for $K_2HPO_4$ as the only source of phosphate. Plates were incubated at 30°C, and blue colonies were visible after 3 days. Blue colonies were then streaked for isolation on L plates containing BCIP, the plates were incubated at 37°C, and the process was repeated until only blue colonies (alkaline phosphate positive) were observed. Two random insertions in χ3689 termed Y::Tn*phoA* and Z::Tn*phoA* were transduced with P22 Ht*int* into χ3687 and PM233 for use as controls in all the experiments.

**Construction of *invA* transcriptional and translational fusions.** The construction of *S. typhimurium invA*::Tn*phoA* strain χ3642 has been described elsewhere (14). A *phoP12* mutation (30) from *S. typhimurium* AD154 (obtained from E. Eisenstadt, Harvard School of Public Health, Cambridge, Mass.) was introduced into this strain by moving a closely

TABLE 2. Effect of osmolarity on expression of *invA*[a]

| Strain | Relevant genotype | Growth condition(s) | PhoA units | CAT units |
|---|---|---|---|---|
| χ3687 | *inv*$^+$ | K | 12 | |
| | | K + NaCl (0.3 M) | 10 | |
| | | K + sucrose (0.6 M) | 14 | |
| χ4108 | *invA*::Tn*phoA* | K | 31 | |
| | | K + NaCl (0.3 M) | 242 | |
| | | K + sucrose (0.6 M) | 256 | |
| χ4109 | Y::Tn*phoA* | K | 140 | |
| | | K + NaCl (0.3 M) | 110 | |
| χ4110 | Z::Tn*phoA* | K | 420 | |
| | | K + NaCl (0.3 M) | 390 | |
| χ4111 | *proU*::Tn*phoA* | K | 90 | |
| | | K + NaCl (0.3 M) | 2,050 | |
| χ3181 | *inv*$^+$ | K | | 0 |
| | | K + NaCl (0.3 M) | | 0 |
| | | K + sucrose (0.6 M) | | 0 |
| χ4115 | *invA*::*cat* | K | | 8 |
| | | K + NaCl (0.3 M) | | 45 |
| | | K + sucrose | | 55 |

[a] Bacterial strains were grown as indicated, cells were disrupted by sonication, and PhoA or CAT activities were determined as described in Materials and Methods. Values are representative and are taken from one of three experiments with similar results.

linked Tn*10* by P22 transduction, yielding strain χ4108. This procedure eliminated acid phosphatase activity that interferes with the alkaline phosphatase assay (13). Conversion of *invA*::Tn*phoA* into a *cat* transcriptional fusion was carried out as described elsewhere (31). Briefly, *E. coli* SM10 λ *pir* carrying the plasmid pSKCAT was mated with the *invA*::Tn*phoA* *S. typhimurium* strain χ3642. Transconjugants were selected on minimal medium (48) containing ampicillin and kanamycin and scored for loss of alkaline phosphatase activity, an indication of the correct integration of pSKCAT. PhoA$^-$ transconjugants had pSKCAT integrated into the chromosome at the *invA* locus as indicated by Southern hybridization analysis (data not shown). One of these transconjugants was strain χ4115.

## RESULTS

**Effect of osmolarity on the expression of *invA*.** *S. typhimurium* χ4108 (carrying an *invA*::Tn*phoA* fusion) and χ4115 (carrying an *invA*::cat fusion) were grown in low-osmolarity K medium or in K medium containing either 0.3 M NaCl or 0.6 M sucrose. The steady-state expression of *invA* as measured by the levels of alkaline phosphatase or CAT activity was approximately eightfold higher when strains were grown in high-osmolarity medium (Table 2). No difference was observed in the expression of *invA* when either 0.3 M NaCl or 0.6 M sucrose was used as the osmolyte. In contrast, two randomly selected productive Tn*phoA* insertions in the same genetic background (strains χ4109 and χ4110) showed no difference in the levels of PhoA activity when grown in either high- or low-osmolarity medium (Table 2). An isogenic strain of *S. typhimurium* carrying a Tn*phoA* productive insertion in the osmoinducible gene *proU* (2, 40) (strain χ4111) showed a more than 20-fold higher level of alkaline phosphatase when organisms were grown in high-osmolarity medium (Table 2).

**Effect of *ompR* on expression of *invA*.** The *ompB* loci of *E. coli* and *S. typhimurium* are each composed of two genes, *ompR* and *envZ*, which osmoregulate the expression of the outer membrane porin genes *ompF* and *ompC* (22). In order to determine whether *invA* osmoregulation was also dependent on *ompB*, we analyzed the effect of *ompR* on the expression of *invA* by constructing an *invA*::Tn*phoA* *ompR* isogenic mutant. The *ompR1009*::Tn*10* mutation was moved from *S. typhimurium* CFH1351 (16) into strain χ4108 by P22 HT*int* transduction, yielding strain χ4112. This strain was tested for retention of the Tn*phoA* insertion and *ompR* phenotype. The latter was tested by determining the altered expression of *ompC* and *ompF* by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of outer membrane preparations of mutants after growth in low- and high-osmolarity media. The presence of *ompR* did not affect the expression of *invA*::Tn*phoA* under either osmotic condition (PhoA units in K medium, 37; PhoA units in K medium plus 0.3 M NaCl, 310 [representative values from one of three experiments with similar results]); therefore, the osmoinducibility of *invA* is most likely independent of the *ompB*-mediated regulation.

**Effect of gyrase inhibitors on *invA* expression.** *proU* is an osmoinducible locus of *E. coli* and *S. typhimurium* that encodes a glycine betaine transport system that is important in the protection of these organisms from osmotic stress (2, 40). It has been established that the osmoinducibility of *proU* is determined by changes in the degree of DNA supercoiling (23). An increase in DNA superhelicity as a consequence of high osmotic conditions causes the induction of *proU*. The negative superhelicity of chromosomal DNA in procaryotes is in a dynamic state and changes in response to several extracellular stimuli (1, 4, 17, 32, 50, 62). The degree of supercoiling is determined for the most part by the coordinate action of two enzymes, gyrase and topoisomerase I (6, 52). Gyrase introduces negative supercoils in an energy-dependent manner, while topoisomerase I relaxes the DNA in an energy-independent way. We used inhibitors of DNA gyrase to determine if, like induction of *proU*, induction of *invA* expression requires an increase in DNA supercoiling. Inhibitors of DNA gyrase relax DNA (6) and therefore might be expected to inhibit expression of *invA*. *S. typhimurium* strains carrying *invA*::Tn*phoA* (strain χ4108) and *invA*::*cat* (strain χ4115) fusions were grown in high-osmolarity K medium containing increasing amounts of an inhibitor of the B subunit of DNA gyrase, coumermicin $A_1$ or novobiocin. The drug concentrations used in these experiments did not significantly inhibit cell growth. Coumermicin $A_1$ and novobiocin reduced the steady-state levels of expression of *invA* as measured by the levels of PhoA and CAT (Table 3). The inhibitory effect of novobiocin was dose dependent, although the effect of coumermicin $A_1$ was not dose dependent at the drug levels tested. As previously reported (22), expression of *proU* was also reduced by the gyrase inhibitors. In contrast to these two genes, two randomly selected isogenic Tn*phoA* insertions were either not affected (Z::Tn*phoA*) or stimulated (Y::Tn*phoA*) by the inhibitors (Table 3).

TABLE 3. Effect of gyrase inhibitors on expression of *invA*[a]

| Strain | Relevant genotype | Coumermycin (μg/ml) | PhoA (U) | Novobiocin (μg/ml) | PhoA (U) |
|---|---|---|---|---|---|
| χ4108 | *invA*::Tn*phoA* | | 258 | | 258 |
| | | 0.5 | 38 | 5 | 193 |
| | | 1.0 | 35 | 10 | 116 |
| | | 2.0 | 43 | 20 | 60 |
| χ4111 | *proU*::Tn*phoA* | | 2,168 | | 2,168 |
| | | 0.5 | 1,228 | 5 | 1,759 |
| | | 1.0 | 977 | 10 | 1,650 |
| | | 2.0 | 759 | 20 | 849 |
| χ4109 | Y::Tn*phoA* | | 96 | | 96 |
| | | 0.5 | 150 | 5 | 137 |
| | | 1.0 | 214 | 10 | 183 |
| | | 2.0 | 110 | 20 | 135 |
| χ4110 | Z::Tn*phoA* | | 386 | ND[b] | |
| | | 0.5 | 342 | ND | |
| | | 1.0 | 313 | ND | |
| | | 2.0 | 420 | ND | |

[a] See Table 2, footnote *a*. Equivalent results were obtained in similar experiments conducted with *invA*::*cat* fusions.
[b] ND, Not done.

**Effect of *topA* on the expression of *invA*.** To further explore the effects of DNA supercoiling on the expression of *inv* genes, we examined the effect of a *topA* mutation on *invA* expression. The lack of topoisomerase I activity in *topA* mutants brings about an increase in the overall level of negative superhelicity of the DNA (6, 52, 53). We chose to examine the effect of the well-characterized *topA* mutation Δ*topA24* (formerly *supX24*) (35, 47). We introduced the *invA*::Tn*phoA* fusion into the chromosome of the *S. typhimurium* Δ*topA24* strain PM233 and into its wild-type parent strain PM596 (35, 47) by P22 HT*int* transduction, yielding strains χ4113 and χ4114, respectively. The correct position of *invA*::Tn*phoA* in the transductants was determined by Southern hybridization (data not shown), and the *topA* phenotype was confirmed by examining phage P22 plaque morphology (49) and the *leu* phenotype (51). *invA* was poorly expressed in the *topA S. typhimurium* strain χ4113 (Table 4). In contrast, expression of a random Tn*phoA* fusion, Z::Tn*phoA*, in the same genetic background (i.e., χ4282) was unaltered. These results were unexpected since *topA* mutants exhibit a higher level of DNA supercoiling than their wild-type parents (51) and therefore *invA* should be expressed under low osmotic conditions. Nevertheless, these results were similar to those reported by Higgins et al. (23) for the expression of *proU* in a Δ*topA* background. It is possible, as suggested by Higgins et al., that Δ*topA* mutants oversupercoil DNA to a degree beyond the optimal window of supercoiling at which *invA* transcription can efficiently proceed.

**Effect of osmolarity and Δ*topA* on the ability of *S. typhimurium* to penetrate tissue culture cells.** Highly virulent strains of *S. typhimurium* were grown in low-osmolarity K medium or K medium containing 0.3 M NaCl to increase osmolarity and then tested for their ability to penetrate MDCK cells as indicated in Materials and Methods. Strains grown in low-osmolarity medium were significantly impaired in their ability to penetrate tissue culture cells (Table 5). However, the presence of Δ*topA* significantly impaired the ability of PM233 to invade MDCK cells, a result in accord with its inability to express *invA*.

To determine if the induction of *invA* after the shift from low- to high-osmolarity medium correlated with an increase in the invasiveness of *S. typhimurium*, the following experiment was conducted. *S. typhimurium* χ4108 (carrying an *invA*::Tn*phoA* fusion in its chromosome) was grown in low-osmolarity K medium to an $OD_{600}$ of 0.4. NaCl was then added to a final concentration of 0.3 M, and levels of alkaline phosphatase were measured in samples taken at different times after the addition of NaCl. Cultures were maintained at an $OD_{600}$ of 0.3 to 0.6 throughout the experiment. In addition, wild-type *S. typhimurium* χ3181 was grown under similar conditions and samples were taken at different times after the addition of NaCl to test for the ability to penetrate tissue culture cells. The kinetics of induction of the expression of *invA* after the addition of 0.3 M NaCl closely correlated with an increase in the ability of a wild-type

TABLE 4. Effect of *topA* on expression of *invA*[a]

| Strain | Relevant genotype | Growth condition(s) | PhoA units |
|---|---|---|---|
| χ4113 | *invA*::Tn*phoA* Δ*topA* | K | 20 |
| | | K + NaCl (0.3 M) | 23 |
| χ4114 | *invA*::Tn*phoA* | K | 35 |
| | | K + NaCl (0.3 M) | 270 |
| χ4282 | Z::Tn*phoA* Δ*topA* | K | 405 |
| | | K + NaCl (0.3 M) | 415 |

[a] See Table 2, footnote *a*.

TABLE 5. Effect of Δ*topA* and osmolarity on the ability of *S. typhimurium* to penetrate tissue culture cells[a]

| Strain | Relevant genotype | Growth condition(s) | % Invasion |
|---|---|---|---|
| χ3181 | Wild type | K | 0.14 ± 0.02 |
| | | K + NaCl (0.3 M) | 4.05 ± 0.59 |
| χ3339 | Wild type | K | 0.08 ± 0.01 |
| | | K + NaCl (0.3 M) | 4.2 ± 0.15 |
| PM233 | Δ*topA* | L broth[b] | 0.001 ± 0.0005 |
| PM596 | *topA*$^+$ | L broth | 1.5 ± 0.24 |

[a] Strains were grown under the condition indicated to an $OD_{600}$ of 0.5, and the ability to enter MDCK cells was assayed as described in Materials and Methods. Invasion values are expressed as the percentage of the initial inoculum of bacteria that was insensitive to gentamicin due to cell invasion. The values represent the averages ± standard deviations for three samples.
[b] The composition of L broth has been described (34), and *inv* genes are fully expressed when bacteria are grown in this medium.

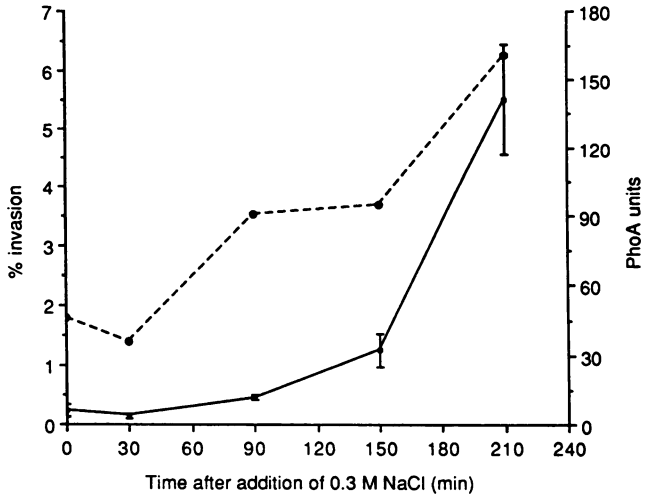


FIG. 1. Kinetics of induction of *invA* after addition of 0.3 M NaCl to low-osmolarity medium. *S. typhimurium* χ4108 was grown in low-osmolarity K medium to an $OD_{600}$ of 0.4. Levels of alkaline phosphatase were measured as described in Materials and Methods in samples taken at different times after the addition of NaCl to a final concentration of 0.3 M. Cultures were maintained at an $OD_{600}$ of 0.3 to 0.6 throughout the experiment. Equivalent results were obtained with *invA*::*cat* fusions. In addition, the wild-type *S. typhimurium* strain χ3181 was grown under similar conditions, and samples were taken at different times after the addition of NaCl and tested for the ability to penetrate tissue culture cells as described in Materials and Methods. ——, PhoA units; -----, percentage of the inoculum of *S. typhimurium* χ3181 that survived the treatment with gentamicin due to cell invasion.

virulent *S. typhimurium* strain to invade cultured epithelial cells after a shift from low- to high-osmolarity medium (Fig. 1).

## DISCUSSION

A crucial virulence attribute of all *Salmonella* species is their ability to penetrate epithelial cells. The complexity of this event is suggested by the large number of genes involved, as indicated by the relatively large number of mutations that affect the ability of *Salmonella* species to penetrate tissue culture cells (11, 14; J. E. Galán and R. Curtiss III, unpublished data). These data may also indicate that perhaps *Salmonella* species can use different pathways of invasion, which seems likely considering both the central importance of this event in their life cycles and the great variety of hosts that *Salmonella* species are able to infect by similar routes of entry.

Coordinate regulation of gene expression is an emerging theme in bacterial pathogenesis, and several global regulatory networks that operate under different stimuli are beginning to be characterized (18, 42). In *Salmonella* species, for example, it is now clear that *phoP* is a global regulator that governs the ability of these organisms to survive inside macrophages (8, 13, 43). Finlay et al. (10) have recently reported that certain *Salmonella* genes required for attachment and invasion are induced upon contact with cultured epithelial cells.

In order to study the regulation of a group of invasion genes that we have recently cloned and characterized (14), we constructed translational and transcriptional *phoA* and *cat* fusions to one of these *inv* genes (*invA*). We chose to construct *invA* fusions because *invA* is the proximal gene in the *invABC* operon and insertions in this gene eliminate the expression of *invB* and *invC*. We then examined the effect of several environmental stimuli on the level of expression of *invA* by measuring the levels of alkaline phosphatase or CAT activity in mutants grown under different conditions. We found that when strains containing these fusions (i.e., χ4108 and χ4115) were grown in media with high osmolarity achieved by the addition of 0.3 M NaCl or 0.6 M sucrose, the level of *invA* transcription was approximately eightfold higher than that of strains grown in media with low osmolarity. In contrast, strains with two randomly chosen Tn*phoA* fusions (i.e., χ4109 and χ4110) obtained from a *S. typhimurium* insertion library did not exhibit this effect (Table 2).

*ompC* and *ompF* are two well-characterized osmoinducible genes of *S. typhimurium* and *E. coli* that encode two outer membrane porins, and their expression is coordinately regulated by the *ompB* locus, composed of the *ompR* and *envZ* genes (22). *ompR* mutants of *S. typhimurium* have been reported to be avirulent, while *ompF* and *ompC* mutants retained virulence (5). These data suggest the possibility that other virulence genes are regulated by *ompR*. Since *Salmonella inv* genes are also osmoinducible, we tested the possibility that *invA* may also be regulated by *ompR* by constructing *invA ompR* isogenic mutants of *S. typhimurium* SR11. The presence of an *ompR* mutation did not significantly affect the expression of *invA*, indicating that osmoinduction of *invA* is independent of the *ompB* regulatory network.

Higgins et al. (23) have recently reported that the regulation of expression of the osmoinducible gene *proU* is determined by changes in DNA supercoiling in response to different environmental stimuli. These investigators demonstrated that the growth of cells at high osmolarity leads to an increase in the mean negative superhelix density of chromosomal DNA and this causes the induction of *proU* expression. The level of supercoiling of the DNA in bacteria is mostly maintained by the coordinate action of two enzymes: DNA gyrase (encoded by the *gyrA* and *gyrB* genes), which introduces negative supercoils in an energy-dependent manner, and DNA topoisomerase I (encoded by the *topA* gene), which removes negative supercoils in an energy-independent fashion (6, 52). We tested the influence of the degree of DNA supercoiling on the expression of *invA* by measuring the level of expression of *invA* in cells grown at high osmolarity in the presence of subinhibitory concentrations of coumermycin $A_1$ or novobiocin. These antibiotics inhibit the B subunit of DNA gyrase and therefore cause a reduction in the negative supercoiling of the DNA (6). Increasing amounts of coumermycin $A_1$ or novobiocin decreased the level of expression of *invA*, as well as of *proU* (Table 3). In contrast, these antibiotics increased or did not affect the expression of two random Tn*phoA* fusions in *S. typhimurium* SR11. These results indicate that increased relaxation of the DNA causes a decrease in the expression of *invA*. In fact, the expression of *invA* was also lower (twofold) when cells were grown at 28°C (data not shown), a condition that is also known to cause a reduction in the linking number of DNA (16). The sensitivity of *invA* expression to DNA supercoiling was further confirmed by the fact that this gene was poorly expressed in a Δ*topA* mutant of *S. typhimurium* regardless of the osmotic conditions (Table 4). It is likely that the DNA in this mutant is supercoiled beyond the optimal level for the expression of *invA*. Nevertheless, we were not successful in reversing the effect of the Δ*topA* mutation by using DNA gyrase inhibitors. This is not a surprising finding since fine modulation of DNA supercoiling

with gyrase inhibitors is complex. Under certain conditions (i.e., low concentrations) gyrase inhibitors can also increase supercoiling by stimulating *gyrA* transcription (12).

We were interested in determining if our findings on the differential expression of *invA* under different conditions translated into a biological effect. In fact, mouse-virulent *S. typhimurium* strains χ3181 and χ3339 grown under low osmotic conditions were significantly impaired in their ability to penetrate tissue culture cells, compared with their growth under high osmotic conditions (Table 5). A *topA* mutant of the partially virulent *S. typhimurium* LT-2 strain (PM233) was also impeded in its ability to invade MDCK cells (Table 5). In addition, the kinetics of induction of the expression of *invA* after the addition of 0.3 M NaCl closely correlated with an increase in the ability of a wild-type virulent *S. typhimurium* strain to invade tissue culture cells after a shift from low-osmolarity to high-osmolarity medium (Fig. 1). These data taken together further suggest that DNA supercoiling has an effect on the expression of genes involved in *S. typhimurium* invasion.

There is cumulative evidence that demonstrates that promoter function can be affected both in vivo and in vitro by the degree of DNA supercoiling (41, 52, 60). In one study Jovanovich and Lebowitz (28) examined the effect of coumermycin $A_1$ on the expression of 67 random Mu d1-8 $Lac^+$ operon fusions to random *S. typhimurium* promoters. They found that this drug increased the expression of 70% of the fusions, decreased the expression of 16%, and did not affect 13%. These studies suggest that most of the promoters in *S. typhimurium* are affected in vivo by changes in the negative superhelicity of the DNA. It has been shown that a number of environmental stimuli such as osmolarity (19, 23), temperature (17), oxygen levels (4, 32, 62), and starvation (1, 4) affect DNA supercoiling. It therefore seems probable that pathogenic organisms make use of this system to regulate the expression of virulence genes in response to a diversity of stimuli. This regulatory network may operate by directly influencing the expression of a given virulence gene or, alternatively, by affecting the expression of regulatory genes that control the expression of additional virulence genes. The regulation of the *inv* genes of *S. typhimurium* by changes in the degree of DNA supercoiling may be an example of the end effect of such a regulatory network. At present it is not known if the *inv* promoters themselves are sensitive to supercoiling or if an as yet unidentified gene whose promoter is sensitive to changes in DNA superhelicity modulates their expression. It is noteworthy that conditions of temperature and osmolarity found in the intestinal tract are optimal for the expression of *S. typhimurium* genes that are uniquely necessary in that environment for display of virulence (14).

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