Identification of Novel Loci Affecting Entry of Salmonella enteritidis into Eukaryotic Cells

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There are an estimated 2 million cases of salmonellosis in the United States every year. Unlike the incidence of many infectious diseases, the incidence of salmonellosis in the United States and other developed countries has been rising steadily over the past 30 years, and the disease now accounts for 10 to 15% of all cases of acute gastroenteritis in the United States. The infecting organism is ingested and must traverse the intestinal epithelium to reach its preferred site for multiplication, the reticuloendothelial system. Despite several recent studies, the genetic basis of the invasion process is poorly understood. An emerging theme from these studies is that wild-type Salmonella organisms probably have several chromosomal loci that are required for the most efficient level of invasion. In this study, we have identified and characterized 13 TnphoA insertion mutants of Salmonella enteritidis CDC5 that exhibit altered invasion phenotypes. The mutants were identified by screening a bank of TnphoA insertions in S. enteritidis CDC5str for their invasion phenotype in three tissue culture cell lines (HEp-2, CHO, and MDCK). These 13 mutants were separated into six classes based on their invasive phenotypes in the tissue culture cell lines. Several mutants were defective for entry of some cell lines but not for others, while two mutants (SM6 and SM7) were defective for entry into all three tissue culture cell lines. This suggests that Salmonella spp. may express more than one invasion pathway. Southern analysis and chromosomal mapping indicated that many as nine chromosomal loci may contribute to the invasion phenotype. It is becoming clear that the invasive phenotype of Salmonella spp. is multifactorial and more complex than that of some other invasive members of the family Enterobacteriaceae.

Salmonella spp. are an important cause of disease worldwide and are responsible for an estimated 40,000 reported cases of gastroenteritis in the United States annually (5). Salmonellosis costs $50,000,000 per year in the United States because of lost work hours and medical expenses (5). In addition to gastroenteritis, Salmonella spp. cause enteric fever (Salmonella typhi) and septicemia (Salmonella cholerasuis). In contrast to the incidence of typhoid fever and many other infectious diseases in developed countries, the incidence of gastroenteritis due to Salmonella spp. has been steadily increasing for the past 30 years (5, 36). In all three diseases (enteric fever, septicemia, gastroenteritis), the infecting organism is food or water borne and must cross the intestinal mucosal barrier to reach its preferred site of multiplication, the reticuloendothelial system. Analysis of the mechanism Salmonella species use to cross this barrier has been the topic of several recent reports (7, 10, 11, 13), but the mechanism is still not well understood. What has become clear from these studies is that the genetic basis for epithelial cell entry by Salmonella spp. is complex and distinct from that of other invasive members of the family Enterobacteriaceae family: Yersinia spp., Shigella spp., and enteroinvasive Escherichia coli.

A single locus from Shigella spp. and individual genes from Yersinia spp. which are responsible for the invasive phenotype have been identified (18, 25, 29). It appears that the genes involved in epithelial cell entry by Shigella spp. are encoded on a 37-kb region of an essential 200-kb virulence plasmid (25). Salmonella and Yersinia spp. also have high-molecular-weight plasmids required for virulence, but curing the virulence plasmid from either Yersinia (34) or Salmonella (16) spp. does not affect invasion, suggesting that the genes required for invasion are located on the chromosomes of organisms in these genera. This has been clearly demonstrated in the case of Yersinia pseudotuberculosis and Yersinia enterocolitica, in which chromosomal genes (inv, p69, invC, and ail) have been cloned that individually confer an invasive phenotype on the noninvasive E. coli strain HB101 (18, 29).

Several recent studies have sought to define the genetic basis of epithelial cell entry by Salmonella spp. Finlay et al. (11) identified mutants of S. cholerasuis that were unable to transcytose polarized MDCK cells; all of these mutants were defective for cell entry to some degree, entering at levels between 1 and 57% of wild-type levels. Elsinghorst et al. (7) isolated cosmids carrying S. typhi chromosomal DNA that could confer an invasive phenotype for Henle 407 cells on E. coli. The level of invasion exhibited by E. coli carrying S. typhi invasion genes was 4 to 7% that of wild-type S. typhi. The homologous region from Salmonella typhimurium cloned into E. coli did not confer the ability to invade Henle 407 cells. Using a slightly different approach, Galan and Curtiss (13) identified a cosmid carrying S. typhimurium chromosomal DNA that could complement the invasion defect of a noninvasive S. typhimurium laboratory strain. However, when transferred to E. coli, this cosmid did not confer an invasive phenotype. The relationship between this clone and that identified by Elsinghorst et al. (7) is unknown, but comparison of restriction maps and gene locations suggests that they are not the same.

Together, these results suggest that wild-type Salmonella spp. probably have several chromosomal loci that are required to fully express the invasive phenotype. To begin to

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were cultured in medium to bacteriological medium containing 25% glycerol or on LB agar plates at 4°C. Antibiotics were used at the following concentrations: streptomycin (Str), 100 μg/ml; ampicillin (Ap), 100 μg/ml; kanamycin (Kn), 40 μg/ml; gentamicin, 100 μg/ml. Strains containing plasmids were routinely grown in LB supplemented with the appropriate antibiotics. A spontaneous streptomycin-resistant mutant of *S. enteritidis* CDC5 was obtained by spreading the bacterial cells collected from a 10-ml overnight culture grown in LB on an LB agar plate containing streptomycin. Nine Str+ colonies, all of which had the same invasion phenotype and growth rate as the parent strain, were observed. One of these mutants was selected for further studies and designated CDC5str. The mutagenized CDC5str strain was incubated at 37°C in an atmosphere containing 5% CO₂. After 3 h, the tissue culture medium was removed and the monolayers were washed six times with PBS. The tissue culture cells were then lysed, and viable counts were determined as described above.

Isolation and qualitative (for screening) invasion assays were performed. *S. enteritidis* strains were grown at 28°C with aeration for 12 to 18 h in LB or in LB containing the appropriate antibiotic. The bacteria (ca. 2.0 × 10⁸) were added to the inoculated monolayer. The microtiter plates were then incubated at 37°C in an atmosphere containing 5% CO₂. After 3 h, the tissue culture medium was removed and the cells were washed three times with phosphate-buffered saline (PBS) to remove nonadherent bacteria. Fresh tissue culture medium containing 100 μg of gentamicin per ml was then added, and the plates were reincubated as described above. After 90 min, the medium was removed and the cells were washed twice with PBS to remove the gentamicin. The tissue culture cells were then lysed to release intracellular bacteria by adding 0.2 ml of 1% Triton X-100 to each well. After 5 min, 0.8 ml of LB was added; the final concentration of Triton X-100 was 0.2%. For the qualitative assay, 100 μl of this suspension was plated on the appropriate bacteriological medium. The wild-type strain CDC5 under these conditions gave nearly confluent growth, whereas invasion-defective mutants gave individual colonies. For the quantitative assay, the suspension was diluted and plated on the appropriate bacteriological medium to determine viable counts. Quantitative invasion assay results are calculated as follows: percent invasion = 100 × (number of bacteria resistant to gentamicin/total number of bacteria added).

Attachment of bacteria was assessed by determining the total number of cell-associated bacteria. *S. enteritidis* strains were cultured at 28°C with aeration for 12 to 18 h in LB. Approximately 2 × 10⁷ bacteria were added to the indicated monolayer. The microtiter plates were incubated at 37°C in an atmosphere containing 5% CO₂. After 3 h, the tissue culture medium was removed and the monolayers were washed six times with PBS. The tissue culture cells were then lysed, and viable counts were determined as described above.
incubated with a second antibody (Bethesda Research Laboratories) conjugated to AP. Antibody binding was visualized by incubating the filter with XP and p-nitroblue tetrazolium chloride as described previously (3). In some experiments, the cells were separated into membrane and soluble fractions, before separation on an acrylamide gel, by a modification of the Schnaitman method (37) as described by Pepe and Miller (32); when alterations in membrane protein profile were screened for, the proteins were visualized by staining with Coomassie blue R-250.

**LPS analysis.** Lipopolysaccharide (LPS) was purified by the method of Darveau and Portnoy as described by Martinez (24) from *S. enteritidis* CDC5str and invasion-defective mutants grown aerobically for 12 to 18 h in LB at 37°C.

**Mapping invasion-defective loci.** To determine the chromosomal location of the *TnphoA* insertions in invasion-defective mutants, the chromosome-*TnphoA* junction was cloned from each mutant and used as a probe against a set of mapping phages carrying defined portions of the *S. typhimu-
rium* chromosome. To clone the chromosome-*TnphoA* junction, chromosomal DNA was purified from each mutant and digested with either BamHI or SalI, as these restriction endonuclease enzymes each have a single site within *TnphoA* just 3' of the Kan' gene. The chromosomal DNA fragments were then ligated either to pUC19 (Amp') or pACYC184 (Cam') digested with the same enzyme. Recombinant plasmids carrying the chromosome-*TnphoA* junction were identified by transforming *E. coli* DH5a and selecting for the resistance marker of the vector and for kanamycin resistance. Because probes derived from *TnphoA* alone do not hybridize to *Salmonella* chromosomal DNA, these chromosome-*TnphoA* clones were used directly to probe the set of mapping phages.

Y anderian et al. (43) constructed a set of mapped, locked-in Mud-P22 prophages along the *S. typhimurium* chromosome. Each of these prophages is capable of packaging and segregating several minutes of chromosomal DNA adjacent to the site of prophage insertion after induction of the prophages with mitomycin C. DNA was purified from the phage particles, and dot blot filters were prepared with 0.5 μg of DNA from each phage. The filters were hybridized under medium stringency (30) with probes derived from chromosomal-*TnphoA* junctions of each of the invasion-defective mutants.

**RESULTS**

**Isolation of invasion-defective mutants of *S. enteritidis.*** We and others have been unable to directly clone and express in noninvasive *E. coli* genes encoding the invasion phenotype from *Salmonella* strains responsible for gastroenteritis (7, 13). The reasons for this are unclear, but the data suggest that unlike the situation in *Y. pseudotuberculosis* (17) and *Y. enterocolitica* (29), at least two chromosomal loci acting in concert are required to convert a noninvasive *E. coli* to an invasive organism. Therefore, the genetic basis for epithelial cell invasion by *S. enteritidis* was studied by identifying mutations in *S. enteritidis* that affect this phenotype. The number of insertion mutants to be screened was narrowed by making the assumption that at least some components of the invasion apparatus would be secreted proteins. To this end, random *TnphoA* insertion mutants of *S. enteritidis* were screened for changes in the invasion phenotype in the tissue culture invasion (TCI) assay. The transposon insertions into the chromosome of *S. enteritidis* CDC5str were generated by conjugation of a suicide plasmid carrying *TnphoA*. Insertion mutants expressing AP activity were examined in the TCI assay with HEp-2 and CHO cells. In this way, from approximately 300 AP* insertion mutants screened, 13 invasion-defective mutants were identified.

**Invasion phenotype of invasion-defective mutants.** Thirteen putative invasion mutants identified by the qualitative TCI assay screen were subsequently examined quantitatively for their TCI phenotypes in HEp-2, CHO, and MDCK cells (Table 1). As a result of these analyses, the 13 mutants fell into six phenotypic classes. All the mutants except SM3 and SM5 were derived from independent matings. Class I mutants (SM1, SM2, and SM8) had a severe defect for invasion of HEp-2 cells but only a slight to moderate defect for invasion of MDCK or CHO cells. Class II mutants (SM3, SM4, and SM5) had the phenotype opposite that of class I mutants; they were moderately to severely affected in invasion of CHO cells and MDCK cells but had only a moderate defect for HEp-2 cells. The class III mutant (SM6) was of particular interest because it was noninvasive in all cell types tested. The class IV mutant (SM7), like SM6, was also severely affected for entry into all cell types but was about 10-fold more invasive than SM6. The class V mutant (SM9) had a severe defect for entry into HEp-2 and MDCK cells but only a slight to moderate defect for entry into CHO cells. Class VI mutants (SM10 to SM13) had a slight but reproducible defect in invasion relative to wild type regardless of which cell line was used for the assay.

**Attachment phenotype of invasion-defective mutants.** To determine whether the observed defect in invasion was due to an inability to adhere to the monolayers, the invasion-defective mutants were assayed for attachment to tissue culture cells by measuring total cell-associated bacteria (Table 1); this value included both attached and intracellular bacteria. Interestingly, the LPS mutant, SM13 (see below), is considerably more adherent than wild type for MDCK cells yet has only 10% of the wild-type invasion capability. This confirms previous observations (29) that adherence is

<table>
<thead>
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<th>Mutant</th>
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<th>HEp-2</th>
<th>CHO</th>
<th>MDCK</th>
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<tr>
<td></td>
<td></td>
<td>TCI</td>
<td>TCA</td>
<td>TCI</td>
</tr>
<tr>
<td>SM1</td>
<td>I</td>
<td>1.6</td>
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<td>I</td>
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<td>SM5</td>
<td>II</td>
<td>10.5</td>
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<td>1.7</td>
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<td>V</td>
<td>0.5</td>
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<td>SM13</td>
<td>VI</td>
<td>14.9</td>
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* The amounts of TCI and total cell-associated (TCA) bacteria were determined as described in Materials and Methods. The results are expressed as a percentage of wild-type level and represent the average of at least three separate assays, each performed in duplicate.
not sufficient for entry to occur. In general, the number of cell-associated bacteria was slightly less for the mutants than for the wild-type strain and was probably due in part to fewer intracellular bacteria. In some cases, notably with SM9, attachment was essentially at wild-type levels for all three tissue culture cell lines. The mutants in classes I to V, therefore, appear to have more-pronounced defects in the mechanism(s) of cellular invasion than in cellular attachment. In most cases, the class VI mutants had defects in attachment similar in magnitude to their defects in invasion. Together, these data indicate that with the exception of the class VI mutants, it is unlikely that the alteration in total cell-associated bacteria could alone account for the change in the invasion phenotype.

Characterization of invasion-defective mutants. To determine whether the invasion-defective phenotype of the mutants was a result of secondary effects, the following properties were investigated: growth rate, motility, and cell surface composition. All 13 invasion-defective mutants had a growth rate in LB medium similar to that of the wild-type parental strain CDC5str (data not shown). In addition, the invasion-defective mutants were as motile as the parent strain in soft-agar plates (0.4% agar) (data not shown). LPS was purified from CDC5str and the mutants, separated on a 15% SDS-polyacrylamide gel, and visualized by staining with silver (Fig. 1). Twelve of the 13 mutants had an LPS profile similar to that of CDC5str both in the amount synthesized and in the extent of O-side chain polymerization. Only mutant strain SM13 failed to synthesize O side chains, and only a minor invasion defect was observed for this mutant. It therefore appears that LPS is not a major contributor to the invasion phenotype of S. enteritidis.

Comparison of the outer membrane protein profiles of invasion mutants with that of wild type showed the absence of certain proteins in some of the mutants (Fig. 2). All the class II mutants (SM3, SM4, and SM5) were missing a protein of ~18 kDa. The class III mutant, SM6, was missing a 52-kDa protein in addition to this 18-kDa protein. Loss of lipoprotein as identified by Western analysis with an antibody to lipoprotein (data not shown) was evident only in class VI mutant SM10. For these mutants, it is likely that the TphA::Tn insertion is in either the gene or the operon encoding the missing protein(s) or is in a gene that affects synthesis or localization of the missing protein(s).

Identification of the AP fusion proteins. Western analysis using antibody directed against AP was performed to confirm that AP fusion proteins were being synthesized and to distinguish mutants within a class if possible (Fig. 3). This
polyclonal serum had weak cross-reactivity to parental strain CDC53tr. Fusion proteins ranging in size from >110 to 49 kDa could be identified in all mutants except SM13. The amount of AP produced by SM13 may be below the level of detection with this antibody; consistent with this is the observation that this mutant is very pale blue on plates containing X-P. In addition to a larger product, most mutants had a protein similar in size to AP that was recognized by the antibody; it is quite common to see AP size breakdown products from AP fusion proteins (23). Expression of the invasion genes from *Yersinia* spp. is regulated by temperature (20, 33, 42), so the effect of growth temperature on expression of the fusion proteins was tested. The same fusion products were produced at similar levels regardless of whether the cells were grown at 37 or 28°C (data not shown). Class I mutants, SM1, SM2, and SM8, had the same invasion phenotype, and the locations of the *Tphoa* insertion in these three strains could not be distinguished by Southern analysis with the *Tphoa* probe (see below), yet the hybrid protein produced by SM1 was a different size from those observed from SM2 and SM8. These data together suggest that these mutants may have insertions in neighboring genes or at different places within the same gene, with the insertion in SM1 being promoter proximal.

**Southern hybridization analysis and mapping of invasion-defective mutants.** To determine the number of *Tphoa* insertions in each mutant and to obtain an estimate of the number of chromosomal loci contributing to the invasion phenotype, Southern hybridization analysis of the mutants was performed by using a probe containing sequences internal to *Tphoa* (Fig. 4). Parental strain CDC53tr had no sequences recognized by this probe, indicating that the hybridization observed in the mutants was due to *Tphoa* sequences only. This probe was used to carry out Southern analysis of chromosomal DNA isolated from the mutants digested with three different restriction enzymes (Fig. 4; data not shown). Two of the restriction endonucleases, EcoRV and SacI, do not cut within *Tphoa*, while HpaI cuts 186 bp upstream of the 3' end of *Tphoa*. Mutants SM3 and SM6 appeared to have two insertions of *Tphoa*. The insertions in SM6 were subsequently separated by phage P22 transduction; the invasion phenotype was associated with only one of the two *Tphoa* insertions. The class I mutants (SM1, SM2, and SM8) appeared to have *Tphoa* inserted on the same fragment regardless of which restriction enzyme was used, suggesting that the insertions in these mutants are in the same locus. Likewise, the three class II mutants (SM3, SM4, and SM5) also had *Tphoa* inserted in the same fragment, but this fragment was distinct from that observed for the class I mutants. Southern hybridization analysis of all the other *Tphoa* insertion mutants revealed unique combinations of fragments when results from the three restriction endonucleases were compared.

To more clearly define the number of invasion loci identified by the *Tphoa* invasion-defective mutants, the approximate chromosomal location of *Tphoa* was determined for each mutant. The chromosome-*Tphoa* junction was cloned from SM1, SM2, SM5 to SM9, and SM11 to SM13. These clones were then used as probes against a set of mapping phages constructed and described by Youderian et al. (43) (Table 2). The class I mutants mapped to the 57- to 60-min region of the chromosome. A previously characterized invasion locus, *inv* (13), also maps to this region. Therefore, Southern hybridization analysis was performed using probes derived from the *inv* locus (data not shown). Only class I mutants (SM1, SM2, and SM8) appeared to have *Tphoa* insertions in this area. The nucleotide sequences of the chromosome-*Tphoa* junctions of SM1, SM2, and SM8 were determined (data not shown). Comparison of these sequences with the *inv* locus indicated that these three mutants have *Tphoa* insertions into *invH* (1). The class II mutants, represented by SM5, mapped to the 25- to 28.5-min region of the chromosome. Each of the remaining six mutants mapped to different regions of the chromosome. Although we were unable to map the insertion in SM10, we believe, on the basis of its invasion phenotype, its loss of lipoprotein, and Southern hybridization analysis, that the *Tphoa* insertion in this strain is distinct from the other mutations. Thus, it appears that *S. enteritidis* has at least nine chromosomal loci that influence the invasion phenotype. Some of these loci may actually have several genes contributing to invasiveness, as has been demonstrated for the *invA* locus (13).

**Discussion**

Both in *vivo* (40) and in *vitro* (15, 21, 38) studies indicate that *Salmonella* spp. are capable of invading and passing through eukaryotic cells (10). Recently, genetic and molecular analyses of this process were initiated. Finlay et al. (11) isolated several mutants of *S. choleraesuis* defective in transcytosis of polarized MDCK cells. All of these mutants were down 2- to 50-fold for invasion of MDCK cells compared with wild type. These mutants also had a defect in adherence that roughly paralleled the invasion defect of individual mutants. Almost half of these mutants had alterations in their LPS. Why we obtained so few LPS mutants compared with Finlay et al. (11) is not clear. This could reflect differences between *S. enteritidis* and *S. choleraesuis* or differences in the initial screening protocol (i.e., we used the invasion phenotype, whereas Finlay et al. [11] used the transcytosis phenotype).

Using an alternate approach, Elsinghorst et al. (7) and Galan and Curtiss (13) directly cloned invasion loci by screening for clones that conferred the invasion phenotype on *E. coli* or complemented a *S. typhimurium* invasion mutant, respectively. The clone obtained by Elsinghorst et al. (7) from *S. typhi* allowed *E. coli* HB101 to invade Henle

![FIG. 4. Southern hybridization analysis of *S. enteritidis* CDC5 and the invasion mutants. Chromosomal DNA was purified from *S. enteritidis* CDC5 and the invasion mutants as described in Materials and Methods and then digested with restriction endonuclease *EcoRV*. DNA fragments were separated by electrophoresis in a 0.7% agarose gel, transferred to nitrocellulose, and probed with a fragment derived from *Tphoa* (see Materials and Methods for details). Shown on the left are the positions of the fragments derived from bacteriophage λ DNA digested with HindIII in kilobases.](image-url)
407 cells at 4 to 7% of the efficiency of the parental S. typhi strain. This result suggested either that the invasion genes (or their products) were not fully active in E. coli or that S. typhi has additional invasion factors. Four regions of this clone were required for the invasion phenotype. Interestingly, the corresponding region from S. typhimurium cloned into E. coli did not promote invasion of Henle 407 cells. Galan and Curtiss (13) identified a recombinant clone that complemented the invasion defect of a S. typhimurium laboratory strain. However, this clone did not confer the invasion phenotype on E. coli. Insertions of TnphoA into three genes carried on this clone, invA, invB, and invD, resulted in failure to fully restore the invasion phenotype of the S. typhimurium mutant. invA and invB form an operon with invC; invD, while carried on the same cosmID, is not part of the invABC operon. This invasion locus was recently shown by Southern hybridization analysis to be present in other Salmonella spp., including S. enteritidis (14), and to contain additional genes, invEFGH (12). An invA mutation was recombinated onto the chromosome of a virulent S. typhimurium strain, and the dose resulting in death of 50% of mice infected orally with the invA mutant was found to be 50-fold higher than that of the wild-type strain (13). However, the invA mutant was still able to infect and kill mice. This result also suggested that additional entry mechanisms may exist in Salmonella spp.

The phenotypes of the 13 invasion-defective mutants of S. enteritidis CDC5str described here provided additional evidence that more than one entry pathway exists. By screening our mutants in several cell lines (Hep-2, CHO, and MDCK), mutants defective for entry of some cell lines but not others were identified. For example, the class I mutants SM1, SM2, and SM8 were 50- to 100-fold less invasive than wild type for Hep-2 cells but were nearly as invasive as wild type for MDCK cells. In contrast, the class V mutant SM9 was more than 200-fold less invasive than wild type for both Hep-2 and MDCK cells but retained 25% of wild-type activity for CHO cells. The class III and IV mutants SM6 and SM7 are particularly interesting because they were noninvasive for all three cell types and thus may be affected in steps common to all entry pathways or may have mutations in regulatory genes.

Southern hybridization analysis and chromosomal mapping showed that at least nine chromosomal loci affect the invasion phenotype of S. enteritidis; their chromosomal map locations and additional phenotypes are summarized in Table 2. The class I mutants (SM1, SM2, and SM8) mapped to 57.0 to 60.0 min on the S. typhimurium chromosome. The inv locus described by Galan and Curtiss also maps to this region (12, 13). Comparison of the chromosomal sequence upstream of the TnphoA insertions in SM1, SM2, and SM8 with the sequence of the inv locus indicated that all three class I mutants have TnphoA insertions into invH (1). The class II mutant SM5 mapped to 25 to 28.5 min, a region that contains pagC, a gene previously implicated in macrophage survival (28). These mutants are missing an outer membrane protein of 18 kDa which is the size of PagC, suggesting that class II mutants have a TnphoA insertion in pagC (35). The class VI mutants all have TnphoA insertions at sites distinct from each other and from the sites in other classes of invasion mutants. In general, these mutants had a relatively minor invasion defect which was paralleled by a similar defect in attachment. While it is possible that these mutations directly affect invasion, an alternative explanation is that the invasion defect observed in this class of mutants is an indirect effect of the mutation. Consistent with this interpretation is the observation that two of these mutants, SM10 and SM13, were missing components of the cell

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Class</th>
<th>Strong hybridization</th>
<th>Moderate hybridization</th>
<th>Map position (min)</th>
<th>Locus or additional phenotype</th>
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<tbody>
<tr>
<td>SM1</td>
<td>I</td>
<td>cysHIJ1574::MudP</td>
<td>proU1888::MudP</td>
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<td>invH</td>
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<tr>
<td>SM2</td>
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<td>cysHIJ1574::MudP</td>
<td>proU1888::MudP</td>
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<td>SM8</td>
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<td>cysHIJ1574::MudP</td>
<td>proU1888::MudP</td>
<td>57.0-60.0</td>
<td>invH</td>
</tr>
</tbody>
</table>

a Chromosomal locations of invasion mutants were determined from hybridization of excision-defective phage DNA (Materials and Methods; 43) to probes made from the TnphoA junction of each mutant. ND, not determined; NMH, no moderate hybridization observed for this mutant.
surface and outer membrane (lipoprotein and LPS, respectively) that are likely to perturb the interaction and presentation of other outer membrane and cell surface components.

The remaining three mutants (SM6, SM7, and SM9) are missing a gene that promotes in their capacities to enter eukaryotic cells and thus probably have mutations in genes whose products directly influence this phenotype. The TphoA insertions in these mutants map to different locations on the chromosome and do not fall into the previously identified inv locus of Salmonella spp. We have designated these three new invasion loci sinA (Salmonella invasion), sinB, and sinC for the mutations present in SM6, SM7, and SM9, respectively. Not all of the loci identified here necessarily encode products that directly interact with the eukaryotic cell, as has been demonstrated for invasin of Y. pseudotuberculosis (18, 19). Rather, the products of these genes may play a role in secretion, localization, or assembly of an “invasion factor” or may possibly play a role in regulating expression of an invasion factor. The mutant SM6 is a good candidate for a regulatory mutant because several proteins are missing from its membrane protein profile.

It is becoming clear that the invasion phenotype of Salmonella spp. is multifactorial, involving several unlinked chromosomal genes. This is in striking contrast to the invasion phenotype of another invasive member of the Enterobacteriaceae, Yersinia spp. (17, 29). The early evidence appears to suggest that the invasive capacities of Salmonella and Yersinia spp. evolved independently. Although Yersinia spp. also appear to have several entry mechanisms, single genes (either inv, invA, invB, or invC) can confer an invasive phenotype on E. coli. Homologs of invA and invC of Yersinia spp. have not been identified in Salmonella strain that causes gastroenteritis. The reasons for this are not clear, but they could include problems with expression of the cloned invasion gene in E. coli or a need for the products of unlinked genes to work together. Cloning and further analysis of the invasion genes and their products should shed light on the molecular basis of entry into eukaryotic cells by Salmonella spp.

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