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Penetration of human intestinal epithelial cells by *Salmonella*: Molecular cloning and expression of *Salmonella typhi* invasion determinants in *Escherichia coli*

(typhoid fever/*Salmonella typhimurium*/Tn*5* mutagenesis/*recA*/electron microscopy)

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ABSTRACT **Salmonella typhi, the causative agent of typhoid fever, must invade the human gastrointestinal tract and multiply within the host to cause disease. We have cloned from S. typhi Ty2 a chromosomal region that confers upon Escherichia coli HB101 the ability to invade cultured human intestinal epithelial cells. Three invasion-positive recombinant cosmids were isolated and restriction endonuclease analyses of the inserts showed a 33-kilobase region of identity. Transmission electron microscopy of epithelial cells invaded by S. typhi Ty2 or E. coli HB101 carrying an invasion cosmid showed intracellular bacteria contained within endocytic vacuoles. One of the invasion cosmids was mutagenized with transposon Tn5 to identify the cloned sequences that are required for the invasive phenotype. Seven of 92 independent Tn5 insertions within the common 33-kilobase region eliminated invasive ability and revealed at least four separate loci that are required for invasion. Penetration of epithelial cells by Ty2 and HB101 carrying the cloned invasion determinants was inhibited by cytochalasin B and D, indicating that epithelial cell endocytosis of S. typhi is a microfilament-dependent event. The invasion cosmids were found to carry the recA and srlC genes indicating that the cloned invasion determinants are located at about 58 minutes on the S. typhi chromosome. With a segment of the cloned S. typhi invasion region used as a probe, homologous sequences were isolated from Salmonella typhimurium. Two independent S. typhimurium recombinant cosmids containing the entire 33-kilobase common region identified in S. typhi were isolated, but these cosmids did not confer upon HB101 the ability to invade epithelial cells.**

Penetration of the epithelial mucosa is a key virulence mechanism and the first major step in the disease process of many pathogenic bacteria. Several genera of enteric bacteria, including *Salmonella, Shigella, Escherichia,* and *Yersinia* species, have been shown to invade human epithelial cells. *Salmonella typhi*, the causative agent of human typhoid fever, penetrates the intestinal mucosa and eventually spreads throughout the reticuloendothelial system.

Little is known about the genetic and biochemical bases of *S. typhi* pathogenesis. Most of this knowledge comes from studies of typhoid-infected humans and chimpanzees, research with *Salmonella typhimurium*, which causes a disease in mice apparently identical to human typhoid fever, and deduction from the results of studies on other bacterial enteric pathogens. *S. typhi* enters the body by ingestion of contaminated food or water and invades the ileal mucosal epithelium. Electron microscopic studies of *S. typhimurium* (1) and *Salmonella choleraesuis* (2) have shown a localized disruption of the microvilli during entry into epithelial cells. Ultrastructural analysis of HeLa cell invasion by *S. typhi* GIFU 10007 has shown bacterial penetration and multiplication within endocytic vacuoles (3). Current evidence indicates that after initial entry, *Salmonella* translocate through epithelial cells in endocytic vacuoles and are released at the basolateral surface of the epithelium (2).

Studies on the genetic and molecular bases of bacterial penetration of epithelial cells are beginning to provide insight into this key pathogenic mechanism. Invasion by *Yersinia* species is directed by the 3.2-kilobase (kb) *inv* locus (4, 5) that encodes invasin and confers upon *Escherichia coli* HB101 the ability to invade cultured epithelial cells (6). Additionally, *Yersinia enterocolitica* carries a second invasion system, the *ail* locus (5), which appears to occupy approximately 650 base pairs. Both the *inv* and *ail* loci are chromosomally located. In contrast to *Yersinia*, the invasive phenotype of all *Shigella* species and enteroinvasive *E. coli* (EIEC) strains is encoded on a large 180- to 210-kb plasmid. Invasion of epithelial cells by *Shigella flexneri* requires a 37-kb region of this plasmid DNA (7). Within this invasion region are several distinct loci, including the *ipa* genes which encode several immunogenic membrane-associated proteins that apparently trigger epithelial cell endocytosis (8). *S. typhimurium* contains a 90-kb plasmid that is associated with virulence but not with the ability to invade epithelial cells (9, 10). The chromosomal region associated with the invasive phenotype of *S. typhimurium* has not been identified, but transposon Tn*phoA* mutants of *S. typhimurium* that cannot invade epithelial cells have been isolated (11).

We report here the isolation of genetic determinants responsible for epithelial cell invasion by *S. typhi*. Approximately 30 kb of *S. typhi* chromosomal DNA is required for the invasive phenotype. These determinants allow normally noninvasive *E. coli* strains to invade epithelial cells. A comparable cloned region of *S. typhimurium* chromosomal DNA, isolated by its homology to the *S. typhi* invasion region, did not direct *E. coli* to invade epithelial cells. Although functionally similar to *Yersinia* and *Shigella* penetration mechanisms, the invasion system used by *S. typhi* appears to be genetically distinct from those described for other enteric pathogens.

MATERIALS AND METHODS

Tissue Culture Cells and Culture Conditions. Henle 407 (human embryonic intestine) epithelial cells were maintained in minimal essential medium containing 10% (vol/vol) fetal bovine serum and 2 mM L-glutamine (MEM) (GIBCO) at 37°C in a 5% CO_2/95% air atmosphere. Gentamicin (GIBCO) was added to the MEM at 100 μg/ml as indicated.

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Abbreviations: Amp, ampicillin; Kan, kanamycin; CFU, colony-forming unit(s).

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Where indicated, antibiotics were added to bacterial growth medium to a final concentration of (in μg/ml): ampicillin (Amp), 100; kanamycin (Kan), 40; streptomycin, 100.

Construction of *Salmonella* Cosmid Libraries. Left-hand and right-hand ends of the cosmid vector pHC79 (12) were constructed by digestion with either *Pst* I or *Sal* I and dephosphorylation with calf intestine alkaline phosphatase, followed by digestion with *Bam*HI. High molecular weight *S. typhi* Ty2 and *S. typhimurium* C5 chromosomal DNA was partially digested with *Sau*3A to give fragments with an average size of 35–45 kb, then dephosphorylated with alkaline phosphatase. This linear bacterial DNA (2 μg) was ligated with 4 μg of vector DNA (with equimolar concentrations of right- and left-hand ends) at a final concentration of 0.15 μg/μl, packaged *in vitro* (Packagene; Promega Biotec), and used to infect *E. coli* HB101 (*recA13 rpsL20 hsdS20*) (13). Recombinant cosmids from both libraries were screened for the ability to invade epithelial cells as described below. Recombinant cosmids from the *S. typhimurium* library were screened for DNA sequences that were homologous to an invasion-positive *S. typhi* cosmid, as described below.

Invasion Assay. Bacterial cells were grown to midexponential phase in L broth (14) at 37°C. Approximately 2×10^7 colony-forming units (CFU) was added to Henle 407 monolayers (6×10^5 cells in 35-mm tissue culture plates containing MEM), centrifuged at $600 \times g$ for 5 min, and then incubated at 37°C for 2 hr in a 5% CO_2/95% air atmosphere. After this invasion period, the plates were washed four times with Earles salts solution (GIBCO), and then incubated for an additional 2 hr in MEM containing gentamicin to kill extracellular bacteria. After this gentamicin-kill incubation, the plates were washed six times with Earles salts, and then internalized bacteria were released by lysis of the monolayer with 0.1% Triton X-100 in deionized water and quantitated by plate count.

In experiments determining the effect of cytochalasin B or D on invasion, 2 μM inhibitor was added to the monolayers prior to invasion and during the gentamicin-kill incubation. To measure bacterial intracellular growth, the length of the invasion period was reduced to 1 hr, and the total number of CFU surviving the gentamicin-kill incubation was determined at 1, 3, 6, and 10 hr. All wild-type strains, recombinant clones, and transposon mutagenized derivatives were assayed in duplicate at least four times; invasion data are average values from a single experiment and are representative of values obtained in other experiments.

To screen the *Salmonella* libraries for recombinant cosmids that were capable of invading Henle cells, aliquots of packaged cosmids sufficient to result in approximately 200 independent clones were used to transduce *E. coli* HB101 to Amp resistance. After infection, the cells were grown in L broth plus Amp to approximately 2×10^8 CFU/ml, and then assayed for invasion. Colonies recovered after the invasion assay were purified and retested for the invasive phenotype.

Screening of the *S. typhimurium* Cosmid Library by Colony Hybridization. The *S. typhimurium* library was screened by colony hybridization (15) to isolate cosmids that contained sequences homologous to the invasion-positive *S. typhi* cosmids. The largest *Pst* I fragment of pTY19 (see Fig. 3A) was purified by preparative agarose electrophoresis and electroelution, labeled with [^{32}P]dCTP by using a nick translation kit (New England Nuclear), and used as a probe in the screening [150 ng (1.3×10^7 dpm)]. The *S. typhimurium* library was replicated to nitrocellulose filters, lysed, and hybridized as described by Maniatis *et al.* (16). Colonies hybridizing to the probe were identified by autoradiography, and then purified.

Tn*5* Mutagenesis of pTY19. The F'_{ts114}*lac*::Tn*5* plasmid of *E. coli* strain WR6016 (17) was conjugally transferred to HB101(pTY19) in L broth at 32°C for 2 hr, then plated on L agar containing Amp, Kan, and streptomycin, and incubated at 42°C to select against the donor and maintenance of the F′ in the recipient. Antibiotic-resistant colonies were pooled and infected lytically with λgt4 (*c*I857) (18). The resulting lysate was used to transduce HB101 to Amp and Kan resistance. Mini preparations of cosmid DNA (19) from antibiotic-resistant transductants were used to transform (20) HB101 and *E. coli* strain EM3000 (*hsdR$_B$11 srlC300*::Tn*10*) (21) to Amp and Kan resistance. Complementation of the *srlC300*::Tn*10* mutation was determined by growth on minimal salts agar containing 0.5% sorbitol. Complementation of the *recA13* mutation was determined by sensitivity to UV irradiation. Insertion mutants were assayed for the invasive phenotype as described above. The position of each Tn*5* insertion was determined by digestion of the mutagenized cosmid DNA with *Eco*RI, *Hin*dIII, and *Pst* I.

Electron Microscopy. Invasion assays were performed as described above, except that 10^8 *S. typhi* Ty2, *E. coli* HB101, or HB101(pTY19) were added to 3×10^7 Henle cells in Lux Contur Paranox tissue culture plates (Miles), and the monolayers were not treated with gentamicin. The samples were then fixed, embedded, sectioned, and stained as described by Asafo-Adjei *et al.* (22). Infected monolayers were fixed in buffered 4% (vol/vol) formaldehyde/1% gluteraldehyde, postfixed in OsO_4, and stained with uranyl acetate. The samples were then dehydrated with ethanol, embedded in Poly/Bed 812 (Polysciences), sectioned, poststained with uranyl acetate and lead citrate, and examined on a Zeiss EM109 electron microscope.

RESULTS

Construction of an *S. typhi* Ty2 Cosmid Library and Isolation of Invasion Determinants. *S. typhi* Ty2 was used as the source of DNA for cloning experiments. This virulent strain, which lacks plasmid DNA (data not shown), is capable of invading Henle 407 epithelial cells (Table 1 and see Fig. 2). To clone the invasion determinants from Ty2, a chromosomal library was constructed in the cosmid vector pHC79. By using the described cloning protocol, reshuffling of chromosomal sequences was eliminated since only recombinant cosmids containing a single insert of the appropriate size were capable of being packaged. Each recombinant cosmid represents a contiguous segment of the *S. typhi* chromosome. This library was used to transduce the noninvasive *E. coli* HB101 to Amp resistance. Approximately 4500 independent recombinant cosmids in HB101 were screened for their ability to direct epithelial cell invasion. From this library, three separate recombinant cosmids that could invade Henle 407 cells were isolated; these were designated pTY2, pTY10, and pTY19. These clones directed HB101 to invade Henle cells with an efficiency of 4–7% of the parental *S. typhi* Ty2 strain (Table 1).

Characterization of Invasion by *S. typhi* Ty2 and Recombinant *E. coli* HB101. To determine if the relatively low invasion efficiency of the recombinant HB101 strains was due

Table 1. Invasion of Henle 407 cells by *S. typhi* Ty2 and *E. coli* HB101 and HB101 carrying invasion clones

Strain	% recovery*	% relative invasion†
S. typhi Ty2	9.7	100
E. coli HB101	0.004	0.04
HB101 (pTY2)	0.36	3.7
HB101 (pTY10)	0.68	7.0
HB101 (pTY19)	0.69	7.1

*Percentage of bacteria added to Henle monolayers that resist treatment by gentamicin.
†Invasion relative to *S. typhi*, representing 100%.

to intracellular multiplication of Ty2 and a lack of such growth by recombinant HB101, intracellular growth was measured. Invasion assays were performed in which the gentamicin-kill incubation was 1, 3, 6, or 10 hr long. The bacterial recoveries from the various time points were compared to the recovery at the 1-hr time point. As gentamicin kills extracellular bacteria, an increase with time in the recovery of bacterial cells would reflect intracellular growth of the organism. *Shigella flexneri* is known to multiply within epithelial cells (23), and recovery of this organism increased consistently with time (Fig. 1). In contrast to this marked growth by *Shigella flexneri, S. typhi* recovery appeared to increase only slightly (Fig. 1). By 10 hr, the number of *S. typhi* recovered had doubled, but the rate at which this increase occurred appeared to be slowing. It is uncertain if this overall increase represents genuine intracellular multiplication by *S. typhi* or a division of those cells that were in the process of replication during invasion. These results cannot distinguish between bacterial turnover within the eukaryotic cell (i.e., concurrent multiplication and lysis) or static maintenance of bacterial numbers (i.e., no or limited growth without bacterial killing), either of which could be occurring with this strain. *E. coli* HB101 containing pTY19 did not appear to multiply intracellularly, as overall recovery had decreased by 10 hr.

Addition of 2 μM cytochalasin B to the MEM during the invasion and gentamicin-kill incubation periods inhibited recovery of *S. typhi* Ty2 and HB101 carrying the invasion cosmids by greater than 90% (Table 2). Similar results were obtained when cytochalasin D was used as the inhibitor. Cytochalasin B or D inhibition of invasion by *S. typhi* Ty2 and HB101 carrying the invasion cosmids indicates that this invasion system triggers a microfilament-dependent uptake by epithelial cells.

To verify that the invasion assay (i.e., protection from the bacteriocidal action of gentamicin) reflected actual bacterial penetration of Henle cells, invaded monolayers were examined by transmission electron microscopy. For *S. typhi* Ty2-infected cells, the bacteria were frequently seen associated with the eukaryotic cell membrane at the cell surface (Fig. 2*A*) and internalized within endocytic vacuoles (Fig. 2*B*). *E. coli* HB101 was not seen associated with or internalized within the epithelial cells. However, HB101 carrying the invasion cosmid pTY19 was observed internalized within endocytic vacuoles (Fig. 2*C*) but was rarely found associated with the eukaryotic cell membrane.

Chromosomal Location of the Invasion Region. Each of the *S. typhi* invasion cosmids was found to be capable of complementing the *recA13* mutation in HB101, thereby conferring resistance to UV irradiation on that strain. The *recA* gene of *E. coli* and *S. typhimurium* is closely linked to the *srl* gene locus, which is required for growth on D-sorbitol. To determine if the invasion cosmids carried this locus, they were transformed into strain EM3000, which contains the transposon insertion mutation *srlC300*::Tn*10*, and thus cannot grow on sorbitol minimal medium. EM3000 carrying any of the three invasion cosmids was capable of growth on sorbitol, indicating that each cosmid contained a functional *srlC* gene. The presence of the *recA* and *srlC* genes on the invasion cosmids indicates that the chromosomal location of the invasion genes is at approximately 58 minutes relative to the location of the *recA* and *srlC* genes in *E. coli* and *S. typhimurium*.

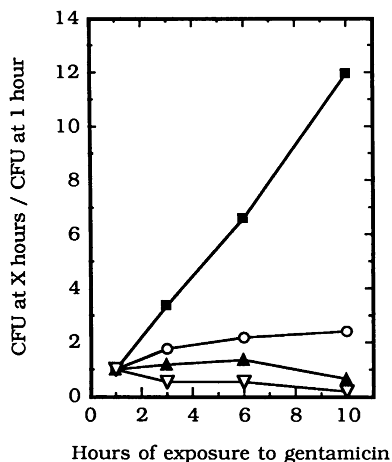

FIG. 1. Bacterial multiplication within Henle cells. ■, *Shigella flexneri* 2457T; ○, *S. typhi* Ty2; ▽, *E. coli* HB101; ▲, *E. coli* HB101 (pTY19).

Table 2. Invasion inhibition by cytochalasin B

Strain	Total recovery, no.*		% inhibition
	+ CB†	− CB	
S. typhi Ty2	1.3×10^5	1.8×10^6	92.7
HB101 (pTY2)	3.1×10^3	6.7×10^4	95.3
HB101 (pTY10)	7.4×10^3	1.2×10^5	93.8
HB101 (pTY19)	4.4×10^3	1.3×10^5	96.6

*Total recovery is the total bacterial count after lysis of the epithelial cell layer with Triton X-100.

†Invasion assay protocol performed, except 2 μM cytochalasin B was added to the MEM in the invasion period and the gentamicin-kill incubations.

Physical Mapping of the Invasion Cosmids. Restriction maps of the invasion cosmids were generated by single and multiple restriction endonuclease digestions (Fig. 3*A*). These maps, as well as DNA hybridization analyses (data not shown), indicated that the inserts of the three cosmids shared a sequence of approximately 33 kb. To define the specific region(s) within this 33-kb sequence required for the invasive phenotype, pTY19 was mutagenized with transposon Tn*5*. Each independent pTY19::Tn*5* derivative was analyzed for its ability to direct Henle cell invasion and to complement *recA* and *srlC* mutations. Insertion mutants that eliminated invasion fell into one of at least four regions in pTY19 (Fig. 3*A*). We refer to these four genetic regions as *invA, -B, -C,* and *-D*. Additionally, several Tn*5* insertion mutants were isolated that decreased, but did not eliminate, the capacity of pTY19 to direct the invasion of Henle cells. These ± mutants invaded at approximately 20% of the pTY19 efficiency and were located in three of the four regions identified by the fully noninvasive Tn*5* mutants (Fig. 3*A*). This ± phenotype may indicate that these mutants are effected in the expression of cloned structural genes or may encode truncated structural proteins that retain limited function.

Each of the *inv* loci were located within the 33-kb area common to the three invasion clones and were separated from the other *inv* loci by insertion mutants that did not alter the invasive phenotype of pTY19. Additionally, transposon insertions which eliminated *recA* or *srlC* complementing activity were isolated, thus allowing for the mapping of these genes on the invasion clones. Based upon the positions of flanking Tn*5* insertions that did not affect invasion, the maximum sizes of the four *inv* regions are: *A*, 2.6 kb; *B*, 2.1 kb; *C*, 1.5 kb; *D*, 1.6 kb.

Isolation of *S. typhimurium* C5 Sequences Homologous to the *S. typhi* Invasion Region. *S. typhimurium* C5 is capable of invading Henle cells and was used as the source of DNA for cloning experiments. This pathogenic strain contains a virulence plasmid, but this plasmid is not associated with the ability to invade epithelial cells (9, 10). With the protocols described for *S. typhi*, a C5 cosmid library was constructed and then screened for the ability to direct HB101 to invade epithelial cells. Although approximately 6500 independent

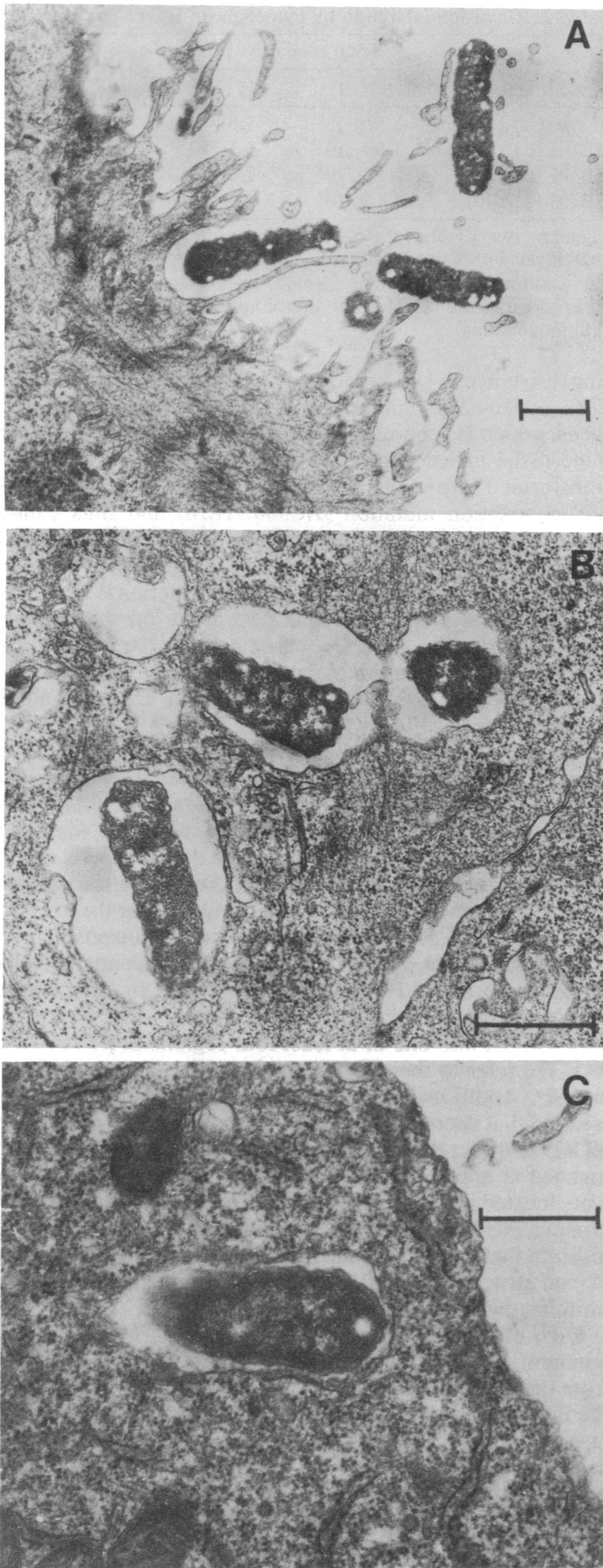

FIG. 2. Transmission electron micrographs of Henle 407 intestinal epithelial cell monolayers infected with *S. typhi* Ty2 or *E. coli* HB101(pTY19). (*A*) Association of *S. typhi* Ty2 with the eukaryotic membrane at the cell surface. (*B*) *S. typhi* Ty2 cells located within endocytic vacuoles. (*C*) *E. coli* HB101 (pTY19) located within an endocytic vacuole. (Bars = 1.0 μm.)

recombinant cosmids in HB101 were screened, no invasion-positive clones were found.

The C5 library was then examined for recombinants that contained sequences homologous to the *S. typhi* invasion region. From approximately 1200 independent cosmids, three cosmids carrying *S. typhi* homologous sequences were isolated and were designated pTM2, pTM4, and pTM8. pTM2 and pTM8 were found to be capable of complementing *recA* and *srlC* mutations in *E. coli* and were studied further. Restriction maps of pTM2 and pTM8 were generated by single and multiple restriction endonuclease digestions (Fig. 3*B*). The restriction patterns observed in the *S. typhimurium* cosmids were very similar to those of the *S. typhi* invasion cosmids and suggested that the inserts of pTM2 and pTM8 contained the entire 33-kb sequence common to the invasive *S. typhi* cosmids (Fig. 3). Although the restriction data indicated that all the *S. typhi inv* loci should be present in pTM2 and pTM8, both of these cosmids were unable to direct the invasion of epithelial cells by HB101.

DISCUSSION

Genetic determinants directing the invasion of epithelial cells were cloned from *S. typhi* Ty2 and expressed in *E. coli* HB101. Approximately 4500 independent recombinant cosmids were screened for the invasive phenotype, and three invasive cosmid clones were isolated. These three cosmids share a 33-kb region, suggesting that a large segment of DNA is required for the invasive phenotype. Tn*5* mutagenesis confirmed the need for a large sequence by identifying at least four separate loci, termed *invA*, *-B*, *-C*, and *-D*, with a maximum distance of 30 kb between the two most distal regions, *invA* and *invD*. Because the entire 33-kb sequence common to the three invasive clones was not mutagenized with Tn*5*, there is the possibility that other yet-to-be-identified loci are involved in this invasive phenotype. The number of invasion-specific genes contained within the cloned *S. typhi* invasion loci is unknown. Analysis of recombinant HB101 by PAGE and immunoblotting with human typhoid convalescent serum has not yet consistently revealed the synthesis of immunogenic peptides.

Relative to *S. typhi*, the lower recovery of the invasive recombinant HB101 strains from invasion assays does not appear to reflect intracellular growth of Ty2 and a lack of such growth of recombinant HB101. The absent or slight intracellular growth of Ty2 in Henle cells appears to contrast with the intracellular growth of *S. typhi* GIFU 10007 in HeLa cells, as reported by Yokoyama *et al.* (3). The intracellular growth of strain GIFU was measured for 24 hr, whereas Ty2 growth was measured for 10 hr. Extended time points with Ty2 may reveal a greater degree of multiplication, but any increase in Ty2 recovery due to intracellular growth during a standard invasion assay (i.e., 2-hr invasion and gentamicin-kill incubations) would not account for the large observed difference in CFU recovery between Ty2 and recombinant HB101. Therefore, the lower recovery of HB101 carrying an invasion cosmid appears to be due to a lower efficiency of invasion relative to the parent *S. typhi* strain.

The reason for the low efficiency of invasion by HB101 carrying the invasion cosmids is unknown. A similar situation was encountered in the cloning of a minimum DNA sequence necessary for invasion of epithelial cells by *Shigella flexneri* (7). In *Shigella flexneri*, a regulatory locus unlinked to the cloned invasion determinants is required for maximum invasion efficiency (24). Unlinked sequences may also be required for maximum invasion by the *S. typhi* invasion cosmids. Alternatively, the lowered invasion frequency may be due to a lack of epithelial cell attachment factors in HB101, decreased transcription or translation of *S. typhi* sequences in *E. coli*, or the presence of more than one invasion system in *S. typhi* Ty2.

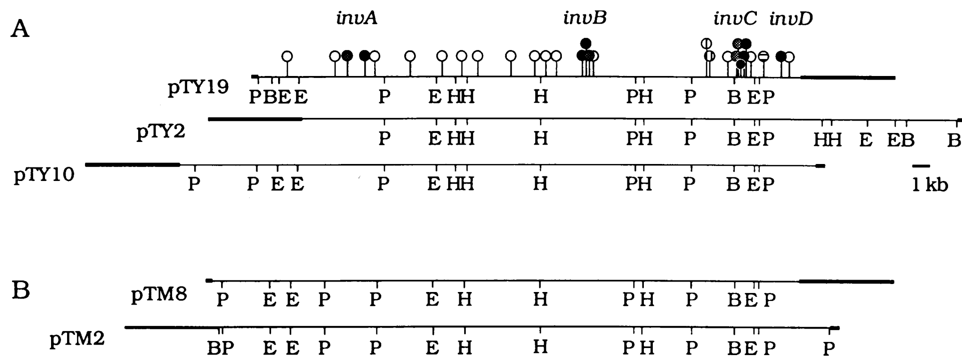

FIG. 3. Restriction maps of *Salmonella* recombinant cosmids. The thin lines represent insert DNA, and the thick lines represent DNA of the vector, pHC79, cut at its *Eco*RI site. Restriction sites in the vector are not shown. B, *Bam*HI; E, *Eco*RI; P, *Pst* I; H, *Hin*dIII. (*A*) *S. typhi* cosmids that confer epithelial-cell invasiveness on *E. coli* HB101 and the location of transposon insertions within cosmid pTY19. The insert sizes of the cosmids are, in kb: pTY19, 36.1; pTY2, 44.4; pTY10, 42.5. Tn*5* insertion mutants are represented by circles above the map of pTY19. The phenotype of mutants is indicated as follows: ○, invasive; ●, noninvasive; ⊛, reduced invasiveness; ⊘, sorbitol negative (*srlC*$^-$); ⊖, UV-irradiation sensitive (*recA*$^-$). Invasion-negative insertions defined four *inv* loci, the location of which is shown above the map of pTY19. (*B*) *S. typhimurium* cosmids that are homologous to the invasive *S. typhi* cosmids but do not direct the invasion of epithelial cells. The insert sizes of the cosmids are, in kb: pTM2, 40.9; pTM8, 39.1.

Attempts to directly clone functional *S. typhimurium* invasion determinants by screening the cosmid library for recombinants that would allow HB101 to invade epithelial cells were unsuccessful. With a fragment of the cloned *S. typhi* invasion region used as a probe, cosmids carrying homologous *S. typhimurium* sequences were isolated. Similarities in the restriction patterns between the Ty2 and C5 cosmid clones suggested that the *inv* loci of *S. typhi* should be present in the *S. typhimurium* cosmids. However, the *S. typhimurium* cosmids were unable to direct the invasion of epithelial cells. The inability of the *S. typhimurium* cosmids to express the invasive phenotype may be related to restriction site variation observed in the region identified as the *invA* locus of *S. typhi* (Fig. 3). Further experimentation will be required to determine if any *inv* loci analogous to those identified in *S. typhi* are responsible for invasion by *S. typhimurium*.

The invasion system of *S. typhi* presents a unique mechanism employed by an enteric bacterium to gain entry into epithelial cells. The large segment of chromosomal DNA required for the *S. typhi* invasion system is in sharp contrast to the relatively small DNA region required for invasion by *Yersinia* species. The *inv* locus of *Yersinia pseudotuberculosis* occupies 3.2 kb and encodes a single protein (6), whereas the *ail* locus of *Yersinia enterocolitica* occupies 650 base pairs (5). Similar to our findings for *S. typhi*, the invasive phenotype of *Shigella flexneri* serotype 5 requires a large (37 kb) segment of DNA from its 180- to 210-kb virulence plasmid (7). Contained within this segment are several separate invasion-associated loci, including the adjacent *ipaA*, *-B*, *-C*, and *-D* genes (8). However, *ipaB*, *-C*, and *-D* have been shown to lack homology with *Salmonella* species, including *S. typhi* (25). Additionally, the *ipa* gene products are highly antigenic, even when expressed in *E. coli* (8). These considerations indicate that *Yersinia*, *Shigella*, and *Salmonella* species use analogous but genetically different systems to invade epithelial cells.

It is interesting to consider that in three closely related genera of enteric pathogens, at least three separate systems capable of epithelial cell penetration have been developed. Indeed, within a single genus such as *Yersinia*, several independent invasion systems exist and may function during the disease process. The invasion system we describe in *S. typhi* may be one of several such mechanisms in *Salmonella* species. Further analysis of this system will aid in determining whether other routes of eukaryotic cell penetration are functioning in *S. typhi* and provide specific biochemical details concerning this virulence mechanism. This information may be useful in the development of a vaccine that is more effective than those currently available for protection against typhoid fever.

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