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Distribution of the *invA*, *-B*, *-C*, and *-D* Genes of *Salmonella typhimurium* among Other *Salmonella* Serovars: *invA* Mutants of *Salmonella typhi* Are Deficient for Entry into Mammalian Cells

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Invasion of intestinal epithelial cells is an essential virulence factor of salmonellae. A group of genes, *invABC* and *invD*, that allow *Salmonella typhimurium* to penetrate cultured epithelial cells have previously been characterized (J. E. Galán and R. Curtiss III, Proc. Natl. Acad. Sci. USA 86:6383–6387, 1989). The distribution of these genes among *Salmonella* isolates belonging to 37 different species or serovars was investigated by Southern and colony blot hybridization analyses. Regions of high sequence similarity to the *invABC* genes were present in all *Salmonella* isolates examined, while regions of sequence similarity to the *invD* gene were present in all but one (*S. arizonae*) of the isolates tested, with little restriction fragment length polymorphism. Sequences similar to these genes were not detected in strains of *Escherichia coli*, *Yersinia* spp., or *Shigella* spp. *invA* mutants (unable to express the *invABC* genes) of several *Salmonella* species or serovars, including *S. typhi*, were constructed and examined for their ability to penetrate Henle-407 cells. All mutants were deficient for entry into cultured epithelial cells, indicating that the *invABC* genes were not only present in these strains but also functional.

A key pathogenic mechanism of salmonellae is their ability to invade the cells of the intestinal epithelium. Electron microscopic studies of *Salmonella*-infected laboratory animals (51) and cultured cells (13, 29) have shown that these organisms enter epithelial cells after transient disruption of their surface microvilli. Bacteria are later seen within endocytic vacuoles, in which, in some instances, they undergo replication. It is currently believed that *Salmonella* strains, unlike other invasive bacteria, such as *Shigella* spp. (3, 32, 46) or *Listeria* spp. (40, 52), do not leave the endocytic vesicle to gain access to the cytosol. Instead, it appears that *Salmonella* strains translocate through the epithelial cell in membrane-bound vesicles to later exit at the basolateral surface of the epithelium (13, 29, 51).

There are three different species of *Salmonella* (*Salmonella typhi*, *S. choleraesuis*, and *S. enteritidis*) and hundreds of serovars that infect a variety of different hosts (22). Some species and serovars are host adapted (e.g., *S. typhi* and *S. gallinarum*), while others can infect a variety of hosts (e.g., *S. typhimurium* and *S. enteritidis*). Although invasion of epithelial cells appears to be a common essential virulence factor of all salmonellae, it is not known whether all species and serovars interact with eukaryotic cells in a similar fashion. In fact, there is some evidence to suggest that differences may exist. Rough strains of *S. choleraesuis* and *S. typhi* are deficient in their ability to enter cultured mammalian cells (15, 41), while *S. typhimurium* rough strains are not (27, 28). In addition, Elsinghorst et al. (10) cloned a chromosomal region of *S. typhi* that conferred upon *Escherichia coli* HB101 the ability to enter Henle-407 cells. The same chromosomal region from *S. typhimurium* did not confer invasive properties upon *E. coli*, suggesting that the *S. typhi*-homologous genes are either defective or nonfunctional in *S. typhimurium* or are not expressed in *E. coli*.

We have recently cloned a group of genes (*invA*, *-B*, *-C*, and *-D*) that allow *S. typhimurium* to enter cultured epithelial cells (16). The *invA*, *-B*, and *-C* genes are arranged in the same transcriptional unit, while the *invD* gene is located downstream in a different transcriptional unit. Virulent strains of *S. typhimurium* carrying defined mutations in *invA* (and therefore unable to express *invA*, *-B*, and *-C*) had higher 50% lethal doses than their parent strains when administered orally to mice and were deficient in their ability to colonize Peyer's patches and the small intestinal wall. In contrast, *invA* mutants were fully virulent when administered intraperitoneally, suggesting that the *inv* genes are only needed for the display of virulence when *S. typhimurium* is administered by the natural route of entry (16). In addition, studies conducted with transcriptional and translational fusions of reporter genes to *invA* have established that the expression of the *inv* genes is regulated by changes in DNA supercoiling as a consequence of a variety of environmental signals, such as osmolarity, temperature, and oxygen tension (17). Conditions found in the gut are optimal for the expression of these genes.

Using Southern and colony blot hybridization analyses, we have now investigated the distribution of the *invABC* and *invD* genes among different *Salmonella* species and serovars as well as other enteric organisms. In addition, we have constructed Inv$^-$ mutants of a variety of *Salmonella* strains to investigate the function of these genes in different species and serovars. What follows is a report of our findings.

MATERIALS AND METHODS

Bacterial strains, tissue culture cells, and growth conditions. The strains used in this study are listed in Tables 1 and 2. Bacteria were grown in L broth or on L agar plates (30).

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TABLE 1. *Salmonella* strains used in genetic manipulations

Strain	Species or serovar	Relevant genotype	Description, reference, or source
SL1344	*S. typhimurium*	Wild type	21
SB103	*S. typhimurium*	*invA*::*kan*	*invA* derivative of SL1344 (20)
Ty2	*S. typhi*	Wild type	Obtained from D. Hone (University of Maryland)
SB129	*S. typhi*	*invA*::*kan*	P22HT*int* (SB103) →Ty2
ISP2825	*S. typhi*	Wild type	Obtained from D. Hone (University of Maryland)
SB130	*S. typhi*	*invA*::*kan*	P22HT*int* (SB103) →ISP2825
7193	*S. enteritidis*	Wild type	Obtained from J. G. Morris (University of Maryland)
SB131	*S. enteritidis*	*invA*::*kan*	P22HT*int* (SB103) →7193
Stock	*S. gallinarum*	Wild type	Obtained from the Animal Disease Center (Ames, Iowa)
SB132	*S. gallinarum*	*invA*::*kan*	P22HT*int* (SB103) →Stock
Lane	*S. dublin*	Wild type	8
SB133	*S. dublin*	*invA*::*kan*	P22HT*int* (SB103) →Lane

When appropriate, 30 μg of kanamycin per ml was added to the growth media. Human intestinal Henle-407 cells were grown as described elsewhere (16).

Bacteriophage transductions. Bacteriophage P22HT*int*-mediated transductions were performed as indicated previously (47).

DNA isolation and probe preparation. Plasmid DNA was isolated by the method of Birnboim and Doly (4). Total-cell DNA was isolated as follows. Bacterial strains were grown overnight in L broth at 37°C in a rotating wheel. Cultures (5 ml) were washed twice in buffered saline containing 0.1% (wt/vol) gelatin and resuspended in 1 ml of cold lysing buffer (50 mM glucose–10 mM EDTA–25 mM Tris-HCl [pH 8.0]–1 mg of lysozyme [Sigma, St. Louis, Mo.] per ml). The suspensions were incubated for 10 min on ice, and EDTA (0.250 ml of a 0.5 M solution) and lauryl sarcosine (0.125 ml of a 10% [wt/vol] solution) were added. Samples were incubated for 20 min in a 55°C water bath. Cell lysates were extracted with phenol once, phenol-chloroform (1:1) twice, chloroform once, and ether twice. DNA samples were ethanol precipitated, resuspended in 0.5 ml of 10 mM Tris (pH 8.0)–1 mM EDTA, digested with RNase A (50 μg/ml) (Sigma) for 15 min at room temperature, and stored at −20°C until further use. Restriction endonuclease enzymes were purchased from Bethesda Research Laboratories (Gaithersburg, Md.) and International Biotechnologies, Inc. (New Haven, Conn.) and used in accordance with manufacturer instructions. DNA probes were prepared as follows. Plasmid DNA was digested with the appropriate enzymes, and DNA fragments were separated by electrophoresis on a 0.7% agarose gel. The DNA fragments of interest were isolated with Geneclean (Bio 101, La Jolla, Calif.), denatured by being heated at 90°C for 5 min, and labeled with [α-^{32}P]ATP (Amersham Corp., Arlington Heights, Ill.) by use of a random primer DNA labeling kit (Boehringer Mannheim, Indianapolis, Ind.).

Southern and colony blot hybridization analyses. Total-cell DNA samples were digested with *Eco*RI and *Pvu*II. DNA fragments were separated on a 0.7% agarose gel and transferred to nylon membranes (GeneScreen Plus; Dupont, Wilmington, Del.) by the method of Southern (49). In high-stringency hybridization experiments, membranes were prehybridized for 4 h at 37°C in 50% formamide–1% sodium dodecyl sulfate (SDS)–1 M sodium chloride–10% dextran sulfate. Hybridization was carried out at 37°C in the same solution containing 250 μg of denatured salmon sperm DNA per ml and boiled (10 min at 100°C) probe for 15 h. Membranes were washed two times for 5 min each time in 2× SSC (1× SSC is 150 mM sodium chloride–15 mM sodium citrate) at room temperature, two times for 30 min each time in 2× SSC–1% SDS at 65°C, and two times for 30 min each time in 0.1× SSC at room temperature. In low-stringency experiments, prehybridization and hybridization were carried out under similar conditions, except that 20% formamide was used and washes were performed at 55°C in buffer containing 0.1% SDS. Membranes were air dried and exposed to X-Omat AR film (Eastman Kodak Co., Rochester, N.Y.). Membranes were reused after being washed with 0.4 N sodium hydroxide at 42°C for 2 to 5 h and with 0.1× SSC–0.1% SDS–0.2 M Tris-HCl (pH 7.5) at 42°C for 2 h. Washed blots were exposed to X-Omat AR film to verify successful washing. Colony blots were prepared as described elsewhere (33) and hybridized as described above. These blots were also reused after being washed as described above. Stringency of hybridization was calculated with the assumption that there is a drop of 1°C in melting temperature (T_m) for every 1% base-pair mismatch (6) by use of the formula $T_m = 81.5 + 16.6 \log M + 0.41$(percent G+C content) − $(500/n)$, where n is the length of the probe in base pairs and M is the molarity. On the basis of sequencing data (18), our probe is 48% G+C; therefore high- and low-stringency experiments would allow 22 and 32% base pair mismatches, respectively.

Tissue culture cell invasion assay. Invasion by *Salmonella* strains of cultured Henle-407 cells was carried out in 24-well tissue culture plates as described elsewhere (16). For qualitative screening of the invasiveness of a large number of *Salmonella* strains, a variation of this assay was performed essentially as described by Miller et al. (37). In brief, after gentamicin treatment, tissue culture cells were washed twice with Hanks balanced salt solution and lysed with 0.5 ml of the same solution containing 0.1% sodium deoxycholate. After 5 min, 1.5 ml of buffered saline containing 0.1% (wt/vol) gelatin was added to each well and 100 μl of the suspension was plated. Invasive strains gave almost confluent growth on the plates after overnight incubation, while noninvasive strains gave few isolated colonies.

RESULTS

Tissue culture cell invasion by *Salmonella* strains. A total of 91 *Salmonella* strains (Table 2) were tested for their ability to invade tissue culture cells. All strains were clinical isolates from humans and a variety of other animal species. These isolates represent the three *Salmonella* species (*S. typhi*, *S. choleraesuis*, and *S. enteritidis*) and a large number of

TABLE 2. Bacterial strains tested for hybridization to the *invABC* and *invD* probes

Organism	O group or serotype	No. of isolates tested	Origin, description, or reference
Salmonella species or serovars			
S. typhimurium	B	20	Human, bovine, equine, porcine, avian
S. enteritidis	D1	15	Human, avian
S. dublin	D	4	Human, bovine
S. typhi	D1	3	Human
S. choleraesuis	C1	3	Porcine
S. gallinarum	D	3	Avian
S. pullorum	D1	2	Avian
S. arizonae	61	2	Avian
S. anatum	E1	3	Human
S. infantis	C1	3	Human
S. hadar	C2	2	Human
S. heidelberg	B	2	Human
S. london	E1	2	Human
S. panama	D1	2	Human
S. bovismorbificans	C2	2	Human
S. manhattan	C2	2	Human
S. agona	B	1	Human
S. albany	C3	1	Human
S. branderup	C1	1	Human
S. brandenburg	C1	1	Human
S. bredeney	B	1	Human
S. cerro sieburg	K	1	Human
S. derby	B	1	Human
S. glostrup	C2	1	Human
S. give	E1	1	Human
S. montevideo	C1	1	Human
S. nienstedten	C4	1	Human
S. othmarschen	C1	1	Human
S. schwarzengrund	B	1	Human
S. thompson	C1	1	Human
S. vejle	E1	1	Human
S. virchow	C1	1	Human
S. java	B	1	Human
S. duisburg	E1	1	Human
S. tennessee	C1	1	Human
S. ohio	C1	1	Human
S. newport	C2	1	Human
E. coli strains[a]			
χ289			Wild-type K-12
EIEC1	O28ac:H−		Enteroinvasive
EIEC5	O28ac:H−		Enteroinvasive
EIEC10	O29:H−		Enteroinvasive
EIEC15	O29:−		Enteroinvasive
EIEC19	O112ac:H−		Enteroinvasive
EIEC22	O124:H−		Enteroinvasive
EIEC32	O136:H−		Enteroinvasive
EIEC36	O136:H−		Enteroinvasive
EIEC42	O143:H−		Enteroinvasive
EIEC50	O144:H−		Enteroinvasive
EIEC55	O152:H−		Enteroinvasive
EIEC65	O164:H−		Enteroinvasive
EIEC74	O167:H−		Enteroinvasive
E2348/69	O127:K63:H6		Enteropathogenic
E851/71	O142		Enteropathogenic
E2831/70	O142		Enteropathogenic
Other bacterial strains			
Shigella flexneri 2a BS185			34
Yersinia pseudotuberculosis YPIII			5
Yersinia enterocolitica 8081			43

[a] All but χ289 were obtained from J. Kaper (Center for Vaccine Development, University of Maryland).

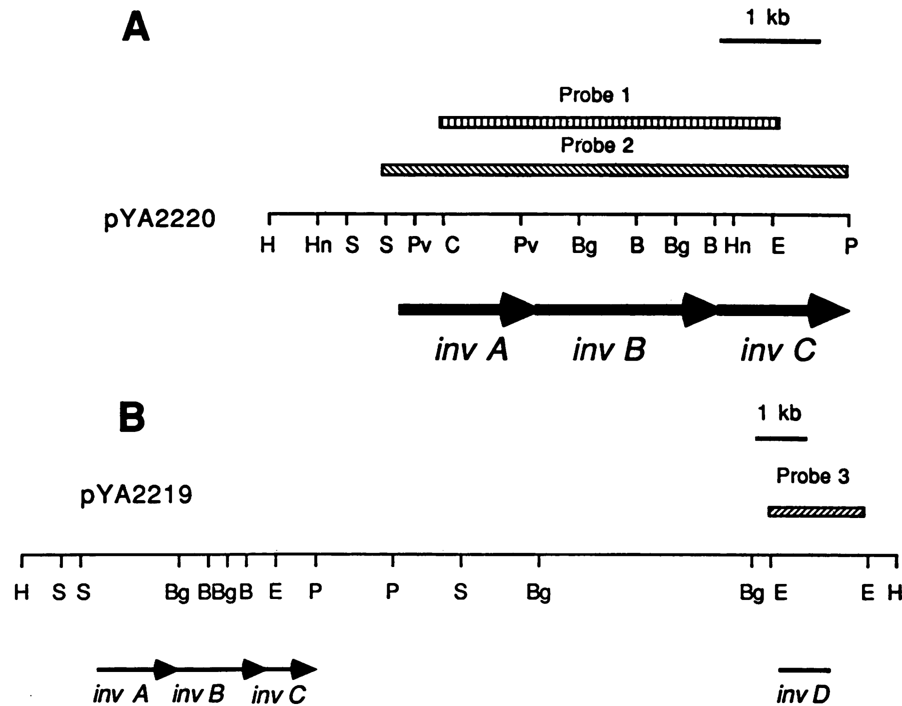

FIG. 1. DNA probes used. Probes were derived from pYA2220 (A) and pYA2219 (B). Both plasmids have been previously described (16). The positions of the *invABC* genes and the direction of transcription are indicated by horizontal arrows. The position of the *invD* gene is indicated by the heavy line underneath pYA2219. The precise location of *invD* is not known, but *invD* is contained within the 2.4-kb *Eco*RI fragment of pYA2219; the region shown by the heavy line was determined by Tn*phoA* mutagenesis (16). B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; Hn, *Hin*cII; P, *Pst*I; Pv, *Pvu*II; S, *Sal*I.

serovars belonging to a variety of O-antigenic types. With the qualitative test described in Materials and Methods, all *Salmonella* strains tested, with the exception of strains of *S. arizonae*, were unambiguously shown to be invasive. Thirty strains were tested quantitatively, and the invasion values ranged from 1 to 30% (data not shown). These values represent the percentage of the initial inoculum that was insensitive to 2 h of gentamicin treatment because of invasion of tissue culture cells.

Conservation of the *invABC* operon in *Salmonella* species and serovars. The *invABC* genes were originally cloned from *S. typhimurium* SR-11 (16). To determine whether these genes were present in other *Salmonella* species and serovars, we performed colony blot hybridization analysis of 91 *Salmonella* strains (Table 2). The probe used was a 3.4-kb *Cla*I-*Eco*RI fragment of pYA2220 (probe 1; Fig. 1) that contains most of the *invABC* operon, with no flanking sequences, as determined by preliminary sequence analysis (18). All *Salmonella* strains tested hybridized to the probe under high-stringency conditions (see Materials and Methods). No qualitative difference between the intensities of the signals of the positive control strain (*S. typhimurium* SR-11) and the other *Salmonella* strains was detected.

To test the degree of restriction fragment length polymorphisms of the *invABC* genes, we carried out Southern blot hybridization analysis of a number of *Salmonella* strains. Total-cell DNA samples were digested with *Eco*RI and *Pvu*II and hybridized to a 4.5-kb *Sal*I-*Pst*I fragment of pYA2220 (probe 2; Fig. 1) that contains the entire *invABC* operon. This combination of probe and restriction enzymes allowed us to obtain information on polymorphisms of the *invABC* genes themselves and their flanking sequences, since *Eco*RI and *Pvu*II generate internal fragments and probe 2 can hybridize to DNA fragments that span beyond the *invABC* operon. Figure 2 shows the results of the analysis of representative strains. The 2.6-kb *Eco*RI-*Pvu*II and 1.1-kb *Pvu*II-*Pvu*II fragments internal to the *invABC* operon were conserved in all strains tested, except for *S. arizonae* strains, which showed a single high-molecular-weight hybridizing fragment. Some polymorphisms were observed in the restriction fragment containing sequences flanking the *invABC* genes.

Conservation of *invD* in *Salmonella* strains. The *invD* locus was originally identified by transposon insertional mutagenesis of pYA2219, a plasmid that contains DNA from *S. typhimurium* SR-11 and was able to complement an invasion-deficient strain of *S. typhimurium* (16). Transposon insertions in the *invD* locus diminished, but did not abolish, the complementing ability of pYA2219 and also eliminated the expression of a 30-kDa polypeptide in an in vitro transcription-translation system (16). To test for the presence of *invD*-like sequences in other *Salmonella* strains, we performed high-stringency colony blot hybridization analysis. The probe used was a 2.4-kb *Eco*RI fragment of pYA2219 that contains the *invD* locus (probe 3; Fig. 1). The precise boundaries of this gene have not yet been determined, but it is expected that probe 3 has sequences that flank *invD*, since some transposon insertions in pYA2219 that mapped near one end of the 2.4-kb *Eco*RI fragment did not affect the complementing ability of this plasmid and therefore are assumed to be outside *invD*. In addition, probe 3 contains more DNA than would be needed to encode a 30-kDa polypeptide, the product of *invD*. All *Salmonella* strains tested (Table 2) hybridized to the probe, although the

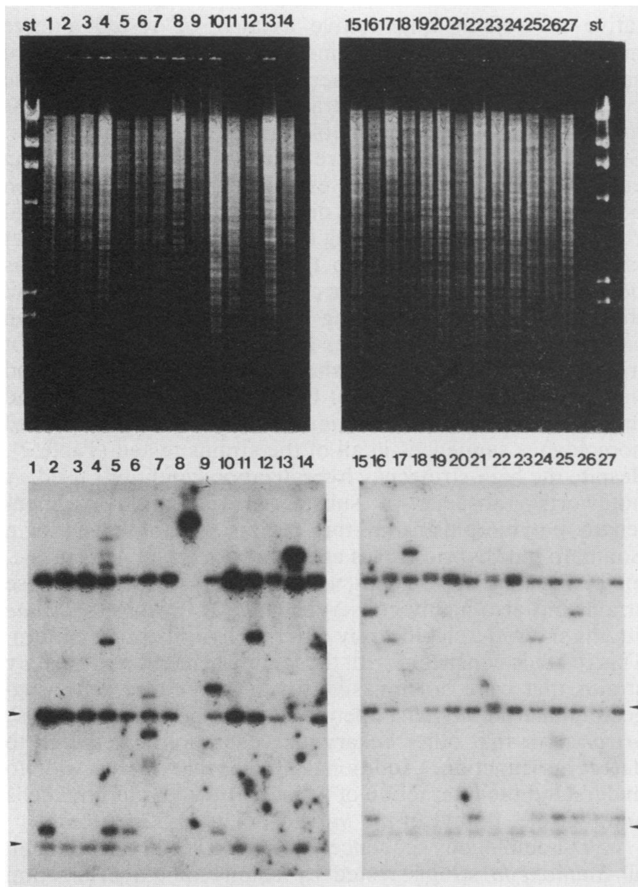

FIG. 2. Hybridization of the *invABC* DNA probe to total-cell DNA from *Salmonella* strains. Total-cell DNA was isolated from the different strains and digested with *Eco*RI and *Pvu*II, and fragments were separated by electrophoresis through a 0.7% agarose gel and transferred to nylon membranes as described in Materials and Methods. Membranes were hybridized to probe 2 (Fig. 1), which contains the *invABC* locus. Top panels show the agarose gels, and bottom panels show the Southern blots. Arrowheads to the left and right of the bottom panels denote the positions of the internal *invABC* fragments. Lanes contain DNA isolated from the following *Salmonella* strains: 1, *S. typhimurium* (SR-11); 2, *S. dublin*; 3, *S. enteritidis*; 4, *S. choleraesuis*; 5, *S. typhi*; 6, *S. pullorum*; 7, *S. gallinarum*; 8, *S. arizonae*; 9, *S. heidelberg*; 10, *S. manhattan*; 11, *S. newport*; 12, *S. ohio*; 13, *S. tennessee*; 14, *S. duisburg*; 15, *S. typhimurium*; 16, *S. london*; 17, *S. java*; 18, *S. bovismorbificans*; 19, *S. infantis*; 20, *S. hadar*; 21, *S. othmarschen*; 22, *S. virchow*; 23, *S. vejle*; 24, *S. thompson*; 25, *S. schwarzengrund*; 26, *S. panama*; and 27, *S. typhimurium* (SR-11). st, λ *Hin*dIII digest size standards.

two *S. arizonae* strains tested showed a weaker signal than did the positive control strain (*S. typhimurium* SR-11). These data suggested that the *invD* gene may be present in most (if not all) *Salmonella* strains tested, but since the probe contained flanking sequences, these results were not conclusive. We tested by Southern blot hybridization analysis the conservation among *Salmonella* strains of the 2.4-kb *Eco*RI fragment that contains the *invD* region, a better indicator of the distribution of the *invD* locus. We used the same blots as those used in the *invABC* analysis (see Material and Methods), since there are no *Pvu*II sites within the 2.4-kb *Eco*RI fragment that contains the *invD* region. The results of this analysis are shown in Fig. 3. The 2.4-kb fragment was present in most of the strains tested, although several strains

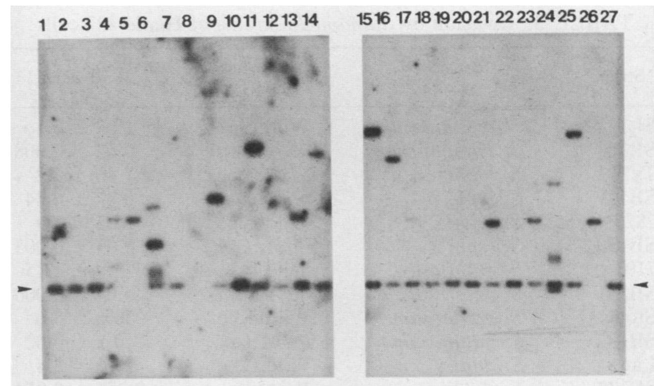

FIG. 3. Hybridization of the *invD* DNA probe to total-cell DNA from *Salmonella* strains. Blots described in the legend to Fig. 2 were washed to strip the probe as described in Materials and Methods and reprobed with probe 3 (Fig. 1), which contains the *invD* locus. Arrowheads to the left and right denote the position of the 2.4-kb fragment that contains the *invD* locus. Samples are the same as those described in the legend to Fig. 2.

showed additional hybridizing fragments. The latter may indicate the existence of more than one copy of this gene in some strains, although a probe with *invD*-internal sequences would be necessary to confirm this hypothesis. Three strains (isolates of *S. choleraesuis*, *S. typhi*, and *S. panama*) showed a different pattern of hybridization. An isolate of *S. arizonae* that had shown a weak signal in the colony blot hybridization analysis showed a weak high-molecular-weight hybridizing fragment (barely visible in Fig. 3) only upon prolonged exposure. These results strongly suggest that the *invD* locus is widely distributed among most *Salmonella* species and serovars.

Construction of *invA* mutants of different *Salmonella* species and serovars. Having established the presence of *invABC*-homologous sequences in all *Salmonella* strains analyzed, we were interested in testing whether these genes were functional. Therefore, *invA* mutants (unable to express the *invABC* genes) of *S. typhi*, *S. gallinarum*, *S. dublin*, and *S. enteritidis* were constructed by transducing these strains to kanamycin resistance with a P22HT*int* lysate prepared on strain SB103. SB103 is an *S. typhimurium* strain that carries an insertion of a kanamycin resistance gene cassette in the *Cla*I site of *invA* (20). The correct position of *invA*::*kan* in the transductants was verified by Southern blot hybridization analysis (data not shown). *invA* mutants were tested for their ability to penetrate cultured Henle-407 cells. The results of these experiments are shown in Table 3. *invA* mutants of *S. typhi* (SB129 and SB130), *S. enteritidis* (SB131), *S. gallinarum* (SB132), and *S. dublin* (SB133) were significantly impaired in their ability to penetrate cultured epithelial cells. These data indicate that, in these strains, the *invABC* genes are not only present but also functional.

Presence of the *inv* genes in other invasive enteric bacteria. Several enteric bacteria other than *Salmonella* strains have been shown to invade cultured epithelial cells (38, 48). We therefore were interested in testing the possibility that DNA sequences similar to *invABC* or *invD* are present in these invasive bacteria. We tested several strains of bacteria, such as *Yersinia* spp., *Shigella* spp., and enteroinvasive and enteropathogenic *E. coli*, that have been shown to invade cultured epithelial cells. The 3.4-kb *Cla*I-*Eco*RI fragment of pYA2220 (probe 1; Fig. 1) that contains most of the *invABC* operon with no flanking sequences was used to detect

TABLE 3. Invasion by *Salmonella* strains of Henle-407 cells

Strain	Species or serovar	Relevant genotype	% Invasion[a]
SL1344	*S. typhimurium*	Wild type	19.2 ± 6.1
SB103	*S. typhimurium*	*invA::kan*	0.02 ± 0.008
Ty2	*S. typhi*	Wild type	7.46 ± 5.2
SB129	*S. typhi*	*invA::kan*	0.07 ± 0.04
2825	*S. typhi*	Wild type	25.2 ± 1.9
SB130	*S. typhi*	*invA::kan*	0.03 ± 0.003
7193	*S. enteritidis*	Wild type	12.9 ± 2.15
SB131	*S. enteritidis*	*invA::kan*	0.04 ± 0.006
Stock	*S. gallinarum*	Wild type	30.6 ± 5.6
SB132	*S. gallinarum*	*invA::kan*	0.1 ± 0.02
Lane	*S. dublin*	Wild type	27.6 ± 2.4
SB133	*S. dublin*	*invA::kan*	0.258 ± 0.054
77-85	*S. arizonae*	Wild type	0.515 ± 0.01
875-84	*S. arizonae*	Wild type	0.07 ± 0.01

[a] Invasion is expressed as the percentage of the initial inoculum of bacteria that was insensitive to gentamicin because of cell invasion. The values represent the averages ± standard deviations for three samples. Similar results were obtained in several repetitions of this experiment.

invABC-like sequences, and the 2.4-kb *Eco*RI fragment of pYA2219 that contains the *invD* locus (probe 3; Fig. 1) was used to detect *invD*-like sequences. Colony blot hybridization analysis was performed under low-stringency hybridization conditions (see Materials and Methods). Under these conditions, neither of the probes hybridized to any of the strains tested, indicating that *inv*-like sequences were not detected in these organisms.

DISCUSSION

A number of enteric bacteria have developed the ability to penetrate nonphagocytic cells. This ability constitutes a very important pathogenic property. In some cases, reaching intracellular compartments may constitute a powerful mechanism of evasion of host defenses, while in others, it may simply be a step on the way to a more favorable niche. Although there are some common features in the mechanisms by which these organisms become intracellular, it appears that different bacterial species have evolved their own specific mechanisms (9, 12, 34, 36–39). *Shigella* strains, for example, enter epithelial cells in membrane-bound vesicles but shortly thereafter gain access to the cytosol (3, 40, 46). *Yersinia* and *Salmonella* strains, on the other hand, remain inside endocytic vesicles throughout their intracellular stage (12, 38, 48). It is also becoming more apparent that some bacteria may have developed more than one mechanism to accomplish this task (23, 36).

A number of laboratories are currently studying the molecular basis of how these organisms invade (9, 10, 14, 24, 25, 36, 52) and survive (11, 35) within host cells. We have initiated studies to understand the molecular mechanisms by which one of these invasive pathogens, salmonellae, enters cultured mammalian cells. We have identified four genes of *S. typhimurium*, *invABC* and *invD*, that allow this organism to penetrate cultured epithelial cells (16). We have also demonstrated that the *invABC* operon is involved in the establishment of infection of Peyer's patches and the intestinal wall when *S. typhimurium* is administered orally to BALB/c mice. Since invasion of the intestinal mucosa is believed to be a common feature of all pathogenic salmonellae, we were interested in investigating the distribution of these genes among other *Salmonella* serovars. Using qualitative as well as quantitative assays, we tested a large number of *Salmonella* strains belonging to a variety of species and serovars for their ability to penetrate tissue culture cells (Table 2). All strains, with the exception of *S. arizonae*, were fully capable of penetrating Henle-407 cells. Since all of these strains were clinical isolates, this finding confirms earlier reports that established a good correlation between the ability to cause disease and cultured epithelial cell invasion (19). This finding is also in agreement with that of Barrow and Lovell, who tested 59 *Salmonella* strains belonging to 19 different species and serovars and found them all capable of invading cultured cells (2). We then examined all of these isolates by colony and Southern blot hybridization analyses for the presence of *invABC*- and *invD*-homologous sequences. Using a probe that contained internal sequences of the *invABC* operon, we detected homologous sequences in all of the strains tested (Table 2), despite the high-stringency hybridization conditions used. A noteworthy absence of substantial restriction fragment length polymorphisms in this region was observed in a Southern blot hybridization analysis with an *invABC* probe, indicating that these genes were not only present in these strains but also highly conserved (Fig. 2). Only *S. arizonae* strains showed a significantly different hybridization pattern. This result is consistent with the fact that these were the only strains that were not invasive for tissue culture cells, suggesting that the *invABC* operon may not be functional in *S. arizonae* or that other eukaryotic cells may be required to detect its function. Additional studies are under way to understand the interaction of *S. arizonae* with cultured cells and the role, if any, of the *invABC* locus in *S. arizonae*.

invD-homologous sequences were also readily detected in all *Salmonella* strains tested by colony blot hybridization analysis, although *S. arizonae* strains hybridized more weakly to the *invD* probe. In fact, *invD*-homologous sequences were barely detectable in Southern blots of *S. arizonae* strains, suggesting that the *invD* gene may not be as conserved in *S. arizonae* or that it may be absent (Fig. 3). The *invD* probe used in this study is expected to have flanking sequences; therefore, conclusions should be drawn with caution. Nevertheless, the presence of the 2.4-kb *Eco*RI fragment in Southern blots of most of the strains tested strongly suggests that *invD*-like sequences are present in the vast majority of these organisms (Fig. 3).

The presence of homologous sequences does not necessarily mean that these genes are functional in all of these different species and serovars. Pierson and Falkow, for example, recently showed that *Yersinia enterocolitica inv*-homologous sequences from nonpathogenic strains of this organism were not functional (42). We therefore wanted to investigate whether the *inv* genes of *S. typhimurium*, in particular, *invABC*, were functional in different *Salmonella* species and serovars. We were particularly interested in knowing the function of the *invABC* genes in *S. typhi*. *S. typhimurium* infections in mice have been historically used as a model for the study of typhoid fever in humans because of the similarities of these diseases. Nevertheless, it has been suggested that the cell invasion mechanisms of *S. typhi* and *S. typhimurium* are probably different. It was shown previously, for example, that a region of the *S. typhi* chromosome conferred upon a noninvasive strain of *E. coli* the ability to enter epithelial cells (10). A homologous region of the *S. typhimurium* chromosome was not able to render invasive the same strain of *E. coli*. In addition, rough strains of *S. typhi* were deficient in their ability to enter epithelial cells (41), while rough mutants of *S. typhimurium* were

invasion proficient (27, 28). We constructed *invA* mutants of *S. typhi* (unable to express the *invABC* genes) and tested them for their ability to enter cultured epithelial cells. *invA* mutants of *S. typhi* were significantly impeded in their ability to enter cultured Henle-407 cells (Table 2). Our results clearly indicate that the *invABC* operon is functional in *S. typhi* and plays a critical role in the ability of this organism to penetrate epithelial cells. Therefore, despite some differences in the ways that *S. typhi* and *S. typhimurium* interact with epithelial cells, the *invABC*-mediated pathway of invasion is conserved and operational in both. In fact, the *invABC* operon is functional in all strains so far tested. The inactivation of these genes rendered several *Salmonella* species and serovars belonging to a variety of O-antigenic groups invasion defective (Table 3).

A surprisingly large number of different genetic loci that affect the ability of *Salmonella* strains to enter cultured cells have been identified by a number of laboratories (7, 10, 13, 15, 16, 31, 41). These may reflect the complexity of this event and perhaps the existence of multiple pathways of cell penetration. On the other hand, many of these studies have not separately assayed attachment and invasion, nor have they tested the possibility that invasion occurs but it is soon followed by bacterial death. Therefore, it is possible to have an invasion pathway with multiple types of genetic blocks that can result in the same apparent Inv^- phenotype.

A number of laboratories have identified several genes from other enteric bacteria, such as *E. coli*, *Yersinia* spp., and *Shigella* spp., that allow these organisms to enter tissue culture cells. Examples of these are the *inv* and *ail* genes of *Yersinia* spp. (24, 36), the *eae* gene of enteropathogenic *E. coli* (26), and the *ipa* genes of *Shigella* spp. (34). Interestingly, relationships among invasion genes from different species are beginning to emerge. The nucleotide sequence of the recently identified *eae* gene of enteropathogenic *E. coli* showed striking similarity to that of the *inv* gene of *Yersinia* spp. (26). The *ail* gene of *Yersinia* spp. was recently shown to have similarities to *pagC* (44), a *phoP*-regulated *Salmonella* gene involved in the ability of *S. typhimurium* to survive inside macrophages, to *lom* (1), a bacteriophage lambda-encoded gene of unknown function, and to *ompX* (50), an *Enterobacter cloacae* outer membrane protein-encoding gene also present in a number of enteric bacteria. Therefore, we investigated the presence of *invABC*- and *invD*-like sequences by colony blot hybridization analysis in a number of invasive enteric bacteria (Table 2). No DNA sequences sharing homology to *invABC* or *invD* were detected in these organisms. In fact, we have extended our analysis to other organisms by using polymerase chain reaction technology with primers derived from the *invA* sequence and have been unable to detect sequences sharing homology to this gene in a variety of organisms (45). The use of these sequences as a diagnostic probe is currently being evaluated in our laboratories.

In conclusion, we have shown that regions of high sequence similarity to the *invABC* and *invD* genes of *S. typhimurium* are present in all *Salmonella* species and serovars tested, although they are absent from other invasive enteric bacteria. We have also presented evidence that indicates that the *invABC* operon is functional in *S. typhi* and other *Salmonella* species and serovars, indicating that the *invABC*-mediated invasion of epithelial cells is highly conserved. Studies to unravel the function of this operon will therefore be relevant to most *Salmonella* species and serovars.

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