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Evidence for Two Genetic Loci in *Yersinia enterocolitica* That Can Promote Invasion of Epithelial Cells

VIRGINIA L. MILLER[1]* AND STANLEY FALKOW[1,2]

Department of Medical Microbiology, Sherman Fairchild Science Building, Stanford University, Stanford, California 94305,[1] and Laboratory of Pathobiology, Rocky Mountain Laboratory, National Institute of Allergy and Infectious Diseases, Hamilton, Montana 59840[2]

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Virulent strains of *Yersinia enterocolitica* cause disease syndromes ranging from mild gastroenteritis to lymphadenitis and septicemia. The ability of these bacteria to invade intestinal epithelial cells to gain access to the reticuloendothelial system is thought to be an important aspect of their virulence. We report here on the cloning of two *Y. enterocolitica* chromosomal loci, *inv* and *ail*, each of which confers an invasive phenotype on *Escherichia coli* HB101. The *inv* locus allows a uniformly high level of invasion in several tissue culture lines and is homologous to the *inv* gene of *Yersinia pseudotuberculosis*. The second locus, *ail*, shows more host specificity than *inv* in that it allows invasion to a variable degree of some cell lines (e.g., HEp-2, HEC1B, and CHO cells) but allows no invasion of others (e.g., Madin-Darby canine kidney cells).

Many pathogenic bacteria are able to invade and survive within host cells. Invasion and intracellular survival are thought to be important aspects of the virulence of these bacteria, but the molecular basis of tissue invasion and intracellular replication remains to be determined. Among members of the family *Enterobacteriaceae*, yersiniae, salmonellae, shigellae, and enteroinvasive *Escherichia coli* have all been shown to invade human epithelial cells. Although these organisms cause similar diseases, they appear to interact with host cells in different ways. Salmonellae and yersiniae are thought to invade intestinal epithelial cells to gain access to the reticuloendothelial system, where they multiply and from which they may disseminate throughout the host (22, 25, 26). In contrast, shigellae and enteroinvasive *E. coli* usually remain in the colonic epithelial layer, where they invade and multiply locally (13, 23).

The genus *Yersinia* contains three species which are pathogenic for humans. *Yersinia pestis* is the causative agent of plague and does not cause gastrointestinal disease as do the other two species. *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* cause intestinal and extraintestinal syndromes of various severities, ranging from mild gastroenteritis to mesenteric lymphadenitis and septicemia (2). In addition, *Y. pseudotuberculosis* and *Y. enterocolitica* infections are associated with the subsequent development of ankylosing spondylitis and other reactive arthritis syndromes (24, 28). In all of the syndromes caused by these two organisms, tissue invasion is thought to be the first step of pathogenesis. The genes required for invasion are believed to be located on the *Yersinia* chromosome rather than on the virulence-associated plasmid, because strains lacking the plasmid remain invasive (8, 19, 20, 27). Recently, a single gene that facilitates invasion of human epithelial tissue culture cells (HEp-2) by normally noninvasive *E. coli* HB101 was cloned from the *Y. pseudotuberculosis* chromosome (10). This gene, designated *inv*, encodes a surface protein of about 108,000 daltons that promotes adherence to and invasion of tissue culture cells by these bacteria (10, 10a). Mutations in *inv* prevent invasion of tissue culture cells by *Y. pseudotuberculosis*, indicating that the *inv* gene product promotes entry by *Y. pseudotuberculosis* as well as by *E. coli* carrying the *inv* gene (10a).

Chromosomal DNA from *Y. enterocolitica* and *Y. pseudotuberculosis* is only 50% homologous (3). Thus, we reasoned that there might be a difference in the invasion mechanisms of these two species. Consequently, we have extended our genetic analysis of invasion to *Y. enterocolitica*. We show here that two *Y. enterocolitica* chromosomal loci, *inv* and *ail*, each confer an invasive phenotype on *E. coli* HB101. The *Y. enterocolitica inv* gene is homologous to *inv* from *Y. pseudotuberculosis*. The *ail* (for attachment-invasion locus) region is not homologous to either *inv* gene and confers a target tissue specificity on the bacterial host different from that conferred by the *inv* genes.

MATERIALS AND METHODS

Bacterial strains and tissue culture cells. *Y. enterocolitica* 8081c (19) and *E. coli* HB101 (1) were maintained at −70°C in Luria broth (LB) (17) medium containing 25% (vol/vol) glycerol or on LB agar plates. Antibiotics were used at the following concentrations: ampicillin, 50 μg/ml; chloramphenicol, 50 μg/ml; kanamycin, 40 μg/ml. Human laryngeal epithelium (HEp-2), Madin-Darby canine kidney (MDCK), and Chinese hamster ovary (CHO) cells were maintained and prepared for the invasion assay as previously described (5). Human endometrial (HEC-1B) tissue culture cells were maintained and prepared for invasion assays in the same manner as HEp-2 cells, with the exception that 10% fetal calf serum was used in the HEC-1B tissue culture medium. *Y. enterocolitica* 8081c and *E. coli* HB101 carrying recombinant plasmids with *Y. enterocolitica* invasion genes were grown at 28°C with aeration for 12 to 18 h in LB. HB101 carrying the type I pilus clone pSH2 was grown in LB containing chloramphenicol at 37°C without aeration (18). HB101 carrying the PAP pilus clone pPAP5 (15) was grown on tryptic soy agar plates containing ampicillin at 37°C. HB101 carrying the X-adhesion AFA-1 clone pIL14 (12) was grown on LB agar plates containing ampicillin at 37°C.

Nucleic acid preparation and analysis. High-molecular-weight chromosomal DNA was isolated as previously described (9). Plasmid DNA was purified by the alkaline lysis method (16). DNA restriction enzymes and bacteriophage

* Corresponding author.

T4 DNA ligase were purchased from Bethesda Research Laboratories, Inc. Calf alkaline phosphatase was purchased from Pharmacia, Inc. Restriction enzymes, ligase, and phosphatase were used according to the instructions of the manufacturers.

Invasion assay. Bacteria (2×10^7) were added to each well of a 24-well microdilution dish which had been seeded with tissue culture cells the previous day as previously described (5). The microdilution plates were centrifuged for 10 min at ambient temperature at $162 \times g$ and then incubated in a 5% CO_2 incubator at 37°C. After 3 h, the tissue culture medium was removed and the cells were washed three times with phosphate-buffered saline 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 phosphate-buffered saline 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%. The suspension was then diluted and plated on the appropriate bacteriological medium to determine viable counts. Viable counts of the initial bacterial culture were also determined. Results are expressed as follows: % invasion = 100 × (the number of bacteria resistant to gentamicin/the number of bacteria added).

The total number of cell-associated bacteria was determined in the same way as was the number of intracellular bacteria, with the following exception: after incubation of the bacteria with the monolayer for 3 h, the monolayer was washed five times with phosphate-buffered saline. The monolayer was then disrupted with 1% Triton X-100 as described above, and viable counts were determined.

Construction of a chromosomal DNA library of *Y. enterocolitica*. High-molecular-weight chromosomal DNA from *Y. enterocolitica* 8081c was partially digested with the restriction enzyme *Sau*3A as previously described (16). Fragments 7 to 10 kilobase pairs in size were isolated by sucrose gradient fractionation (16) and ligated into pBR322 that had been digested with *Bam*HI and treated with calf alkaline phosphatase. The resulting ligated DNA was used to transform competent *E. coli* HB101 cells (16). *E. coli* HB101 transformants carrying recombinant plasmids were selected on LB agar plates containing ampicillin.

Stained samples and electron microscopy. Samples of invasion assays were prepared and stained for electron microscopy as previously described (10).

Tn*5* mutagenesis. Strains bearing insertions of transposon Tn*5* into recombinant plasmid pVM102 were isolated as previously described (4).

RESULTS

Cloning invasion genes from *Y. enterocolitica*. We used *Y. enterocolitica* 8081c (serotype O8), which had been isolated from a patient with septicemia, as our prototype strain (19). Strain 8081c lacks the 47-megadalton virulence-associated plasmid, as well as any other plasmid, yet is still able to invade HEp-2 cells as efficiently as is the plasmid-containing strain (19). This suggests that, as in *Y. pseudotuberculosis* (10), the invasion determinants are chromosomally encoded. Consequently, to clone the genes from *Y. enterocolitica* 8081c which were involved in invasion, we first constructed a library of chromosomal DNA from strain 8081c in the plasmid vector pBR322. This library was used to transform the normally nonadherent and noninvasive *E. coli* HB101. Adhesive and invasive clones were selected by pooling the resulting transformants and infecting a monolayer of HEp-2 tissue culture cells as described in Materials and Methods. After 3 h of incubation, the monolayer was washed 15 times with phosphate-buffered saline and then lysed with 1% Triton X-100 to release any intracellular or adherent bacteria. The suspension was then spread on LB plates containing ampicillin. Gentamicin was not used during this enrichment procedure because we felt that invasion may be a two-step process involving at least two loci, one for attachment and a second for invasion. If this were the case, the attachment factor would be a prerequisite for identifying a recombinant strain carrying the invasion gene.

Clones from the enriched population were then tested individually in the invasion assay. Approximately 50% of the clones that survived the enrichment were able to invade HEp-2 tissue culture cells to some degree (data not shown).

E. coli HB101 transformants expressing an invasion phenotype fell into two classes (Table 1). The first class, represented by clones 6, 8, and 9, demonstrated a high level of invasion similar to that of wild-type *Y. enterocolitica* 8081c and 800- to 1,400-fold higher than the background level seen with *E. coli* HB101. The second class, represented by clones 3 and 7, demonstrated a relatively low level of invasion, which was nevertheless 40- to 100-fold higher than that of HB101.

Characterization of *Y. enterocolitica* invasion clones. Isolation and characterization of plasmid DNA from clones 6, 8, and 9 indicated that they all contain the same fragment of *Y. enterocolitica* DNA. The recombinant plasmid from clone 6 was called pVM101 (Fig. 1A). Southern hybridization analysis indicated that plasmid pVM101 shares homology with the *inv* gene from *Y. pseudotuberculosis* (data not shown) and thus is a clone of the *Y. enterocolitica inv* locus. The homology shared with *Y. pseudotuberculosis inv* is not observed at the restriction map level. Further characterization of this clone will be presented elsewhere.

Isolation and characterization of plasmid DNA from clones 3 and 7 indicated that they have overlapping inserts of *Y. enterocolitica* DNA (Fig. 1A). These two plasmids were renamed pVM103 and pVM102, respectively. Plasmids pVM102 and pVM103, while they share homology with each other, are not homologous to pVM101, as demonstrated by both Southern hybridization and restriction map analysis (Fig. 1A; data not shown), and thus probably represent a new locus involved in invasion.

A high proportion of *E. coli* HB101 recombinant cells carrying either pVM102 or pVM103 adhered to the HEp-2 cell monolayers (data not shown). This raised the possibility that the low level of invasion observed with the quantitative invasion assay reflected a small proportion of adherent bacteria that were protected from the gentamicin treatment. To determine whether bacteria were actually being internalized, we performed electron microscopy on samples of HEp-2 cells infected with HB101(pVM102) (Fig. 2A, B, and C). We frequently found bacteria in very close association with eucaryotic cell membranes (Fig. 2A and B). We also observed a small number of intracellular bacteria (Fig. 2C), suggesting that pVM102 confers a phenotype of adhesion and low-level invasion of HEp-2 tissue culture cells on *E. coli* HB101. Electron-dense eucaryotic structures resembling coated pits (7) were frequently found in association with attached bacteria, regardless of whether the bacteria were *Y. enterocolitica* 8081c or *E. coli* HB101 carrying either of the recombinant plasmids, pVM101 or pVM102 (Fig. 2A,

TABLE 1. Relative invasion of *Y. enterocolitica* clones into HEp-2 cells[a]

Infecting strain	% Invasion
Y. enterocolitica 8081c	26.8 ± 3.6
E. coli HB101	0.0075 ± 0.0035
HB101(pBR322-8081c) (clone 3) (= pVM103)	0.82 ± 0.24
HB101(pBR322-8081c) (clone 7) (= pVM102)	0.37 ± 0.19
HB101(pBR322-8081c) (clone 6) (= pVM101)	6.2 ± 2.1
HB101(pBR322-8081c) (clone 8)	10.8 ± 0.1
HB101(pBR322-8081c) (clone 9)	6.9 ± 0.32

[a] Strains were used to infect a monolayer of HEp-2 tissue culture cells as described in Materials and Methods. Values are the averages of duplicate samples, with the ranges indicated, and reflect similar results from several experiments.

B, and D). Whether or not these structures play a role in microbial attachment or entry or both is not known at this time. Strain 8081c, unlike HB101 carrying either pVM101 or pVM102, is seen almost exclusively intracellularly after 3 h of infection (Fig. 2D). This observation would suggest either that there are other gene products directly involved in invasion or that the gene products encoded by pVM101 and pVM102 do not function as effectively in an *E. coli* background. Electron microscopy of negatively stained cultures of HB101 pVM102 and pVM101 do not indicate the presence of any piluslike structures on the surface of these bacterial cells (data not shown).

We tested the ability of *E. coli* HB101 carrying several cloned virulence-associated adhesins to invade HEp-2 cells (Table 2) to determine whether adherence alone promoted a low level of invasion. HB101 harboring recombinant plasmids encoding type 1 pili (18), PAP pili (15), or X-adhesin AFA-1 (12) was cultured in such a way as to maximize expression of the adhesin. While all three of these adhesins promoted adherence of *E. coli* HB101 to HEp-2 cells, as determined by examination of Giemsa-stained cover slips and by quantitative assay (data not shown), the presence of type 1 pili, PAP pili, or X-adhesin did not confer an invasive phenotype on *E. coli* HB101 (Table 2), nor were coated pits seen associated with the adherent bacteria. This result

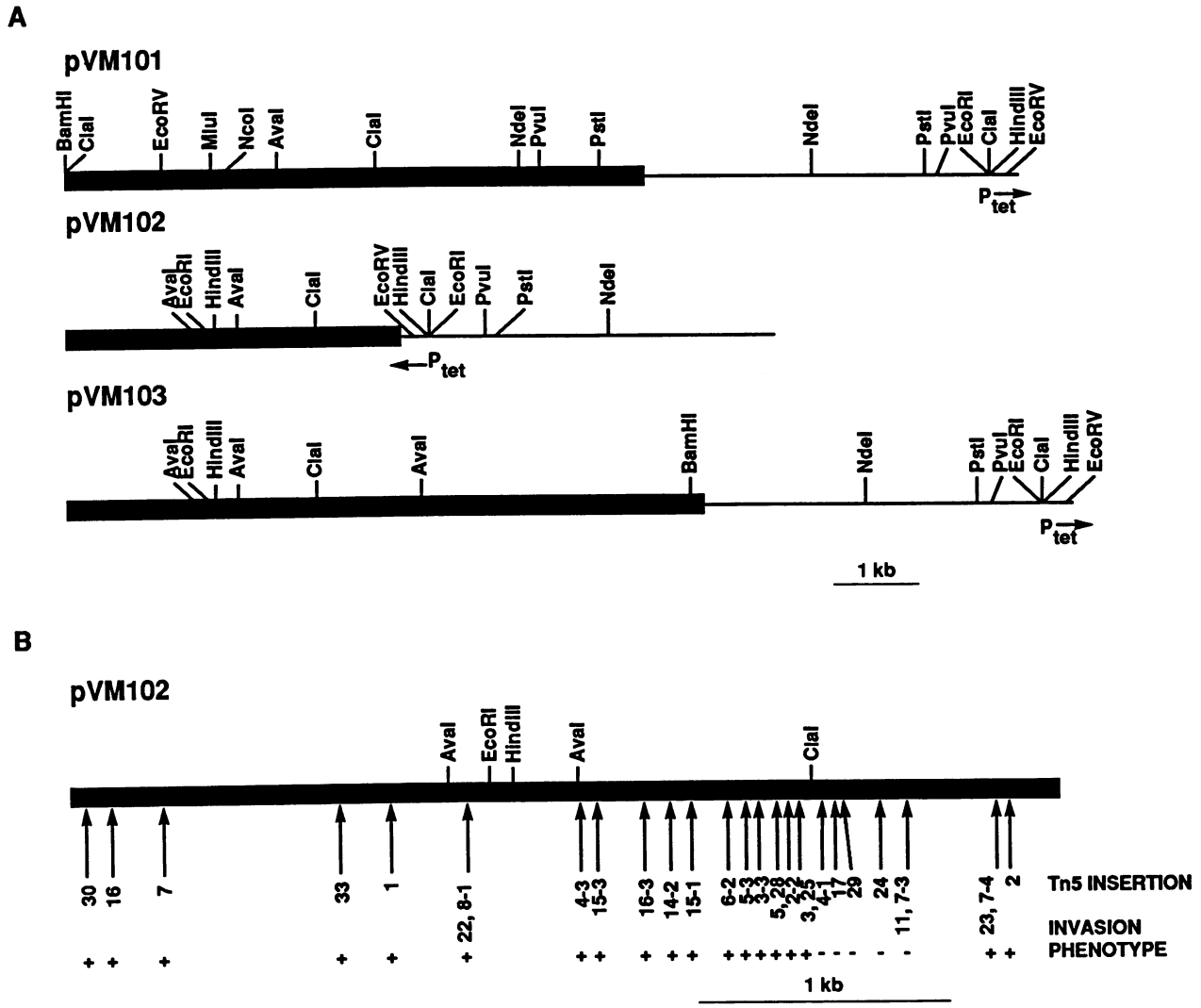

FIG. 1. Restriction map of recombinant plasmids that confer an invasive phenotype on *E. coli* HB101. Symbols: ▬, insert DNA; ——, cloning vector pBR322. (A) Restriction maps of the three recombinant plasmids. (B) Expanded view of the insert DNA of pVM102 with the location and phenotype of Tn*5* insertions shown below. P_{tet}, Tetracycline promoter; kb, kilobase.

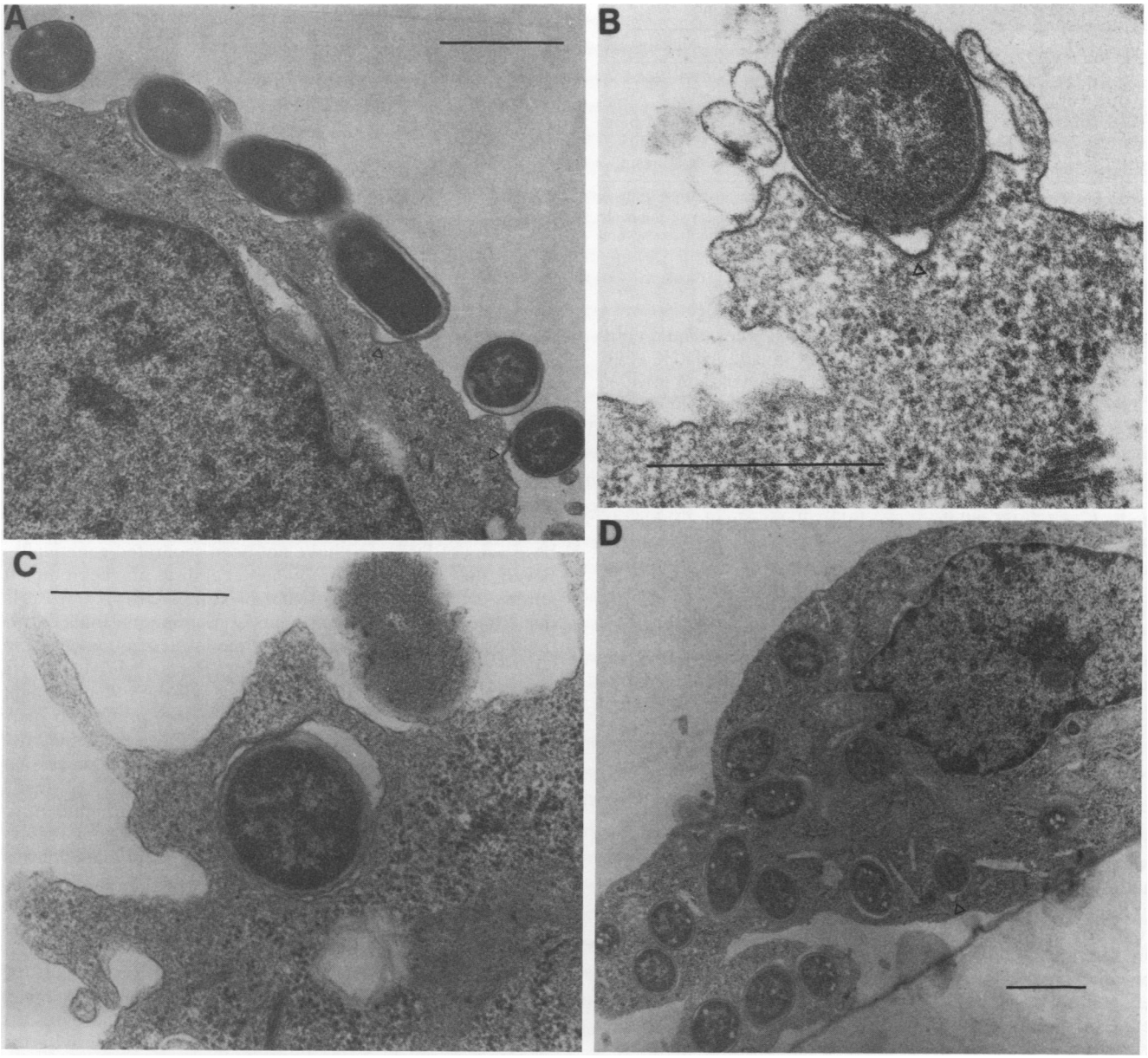

FIG. 2. Electron micrographs of invasion assays with HEp-2 cells. (A, B, C) HEp-2 cells infected with HB101(pVM102) (magnifications: A, ×11,000; B, ×34,000; C, ×27,000). (D) HEp-2 cells infected with 8081c (magnification, ×8,200). Some of the coated pitlike structures are indicated (△). The bar in each panel represents approximately 1 μm.

indicates that the invasive phenotype of HB101(pVM102) is a specific property of this recombinant strain and not a manifestation of simple adhesion.

Localization of the invasion locus of pVM102. Insertions of transposon Tn*5* into pVM102 DNA were isolated and characterized to localize the gene(s) involved in the invasion promoted by this plasmid (Fig. 1B). Independent Tn*5* insertions were mapped, and their invasive phenotypes were determined. Six insertions that eliminated invasion were clustered to the right of the unique *Cla*I site. Insertion mutants defective for invasion were found only in this region, suggesting that there is only one region on this plasmid that is responsible for both adherence and invasion. There are only approximately 650 base pairs between the Inv^+ insertions flanking the Inv^- insertions, suggesting that this region contains a very small gene(s). We have designated this region *ail* for attachment-invasion locus.

Invasion of other tissue culture cell lines. We were curious about the role that the *ail* gene(s) plays in *Y. enterocolitica*. One possibility is that it defines target cell types other than those defined by *inv*, thus giving *Y. enterocolitica* a different range of target host cells than might result from the presence of *inv* alone. To test this hypothesis, we examined invasion by HB101 carrying either of the cloned *Y. enterocolitica* invasion loci, *inv* or *ail*, in several cell lines (Table 3). All cell lines were invaded by *Y. enterocolitica* 8081c, although to various degrees. Plasmid pVM101 promoted only a low level of invasion of MDCK cells by HB101. Plasmid pVM101 caused HB101 to invade all tested cell lines, except the MDCK cells, at a relatively high level. HB101(pVM102)

TABLE 2. Effect of adhesion on invasion[a]

E. coli strain	% Invasion	Adhesin type
HB101(pBR322)	0.015 ± 0	—[b]
HB101(pVM102)	0.770 ± 0.03	—
HB101(pSH2)	0.067 ± 0.001	Type 1 pilus
HB101(pPAP5)	0.020 ± 0	PAP pilus
HB101(pIL14)	0.007 ± 0.001	X-adhesin

[a] Strains were used to infect a monolayer of HEp-2 tissue culture cells as described in Materials and Methods. Values are the averages of duplicate samples, with the ranges indicated, and reflect similar results from several experiments.
[b] —, Not known.

invaded the tested cell lines to different degrees. The recombinant strain HB101(pVM102) did not invade MDCK cells, invaded HEp-2 and HEC1B cells at a low level, and invaded CHO cells at a high level. This suggests that cell lines may vary in their capacity to phagocytize bound bacteria and that this variability can be specific to the invasion factor expressed by the bacteria.

The total number of cell-associated bacteria, both attached and intracellular, was also determined (Table 4). Although HB101(pVM102) invaded HEp-2 cells at only a low level, it bound to HEp-2 cells as well as if not better than it did to CHO cells and to a slightly higher degree to both these cell lines than did HB101(pVM101) (Table 4). Indeed, there is no correlation between number of bacteria bound and number of bacteria internalized, suggesting that the interaction of these bacteria with tissue culture cells that leads to internalization involves more than just attachment to the cell surface.

DISCUSSION

As with many other bacterial pathogens, *Y. enterocolitica* carries a high-molecular-weight plasmid (40 to 48 megadaltons) that is required for virulence in animal models (6, 19) and, presumably, in humans. However, there are several lines of evidence to suggest that chromosomal genes are also required for virulence. Isberg et al. have clearly demonstrated that an invasion gene of *Y. pseudotuberculosis* is chromosomally located and that this gene is required for efficient invasion in vitro (10, 10a). In addition, Heesemann et al. (8) have found that several phenotypes which correlate with virulence, such as Congo red binding and HEp-2 cell invasion, are not plasmid mediated. Other reports have also indicated that epithelial-cell invasion by *Y. enterocolitica* is at least in part chromosomally encoded (19, 20, 27). In keeping with these observations, we were able to clone chromosomal DNA from *Y. enterocolitica* 8081c into *E. coli* HB101 and thereby confer an invasion phenotype on the latter. We found that two chromosomal loci can independently enable HB101 to invade tissue culture cells. To the degree that bacterial invasion of tissue culture cells in vitro correlates with invasion in vivo (14, 26), these cloned genes are probably involved in epithelial-cell invasion by *Y. enterocolitica* in its natural hosts.

One of the cloned invasion loci, designated *inv*, allows a uniformly high level of invasion in several tissue culture lines and is homologous to the *inv* gene of *Y. pseudotuberculosis*. The second invasion locus of *Y. enterocolitica* is *ail*. Bacteria carrying *ail* exhibit several invasion phenotypes, depending on which cell line is infected. *ail* promotes a high level of invasion of CHO cells and a low to moderate level of invasion of other cell lines (HEp-2 and HEC1B) but allows no invasion of MDCK cells. The recombinant plasmid pVM102 strongly promotes adherence of *E. coli* HB101 to many cell lines, including those cell lines for which it does not promote efficient invasion (e.g., HEp-2). It is remarkable that although *E. coli*(pVM102) adheres as well to HEp-2 cells as to CHO cells, more intracellular bacteria are found in CHO cells. Clearly these two tissue culture cell lines interact differently with the bound bacteria. The phenotypes of attachment and invasion which are associated with the presence of pVM102 appear to be encoded by fewer than 650 base pairs of DNA and have not been separated genetically. Given the difference in invasive ability of recombinant strains carrying *inv* versus that of strains carrying *ail*, it is debatable whether transformants containing plasmids carrying the *ail* region would have been identified had gentamicin been used in the initial enrichment. Because HB101 *inv* is more invasive than HB101 *ail*, clones of the *ail* gene might have been missed, since they would be outnumbered (perhaps by as much as 10 to 1) by clones of the *inv* gene.

Several lines of evidence presented here indicate that invasion by HB101 carrying the recombinant plasmids pVM101 or pVM102 involves more than just attachment to the tissue culture cell surface. First, HB101 carrying recombinant plasmids which encode previously described adhesins (12, 15, 18) is not invasive. Second, pVM101 and pVM102 both promote a similar level of attachment of HB101 to HEp-2 and CHO cells, yet the degree of invasion differs considerably between HB101 cells carrying these two plasmids. Third, HB101(pVM102) binds at least as well to HEp-2 cells as to CHO cells, yet more than 10 times as many bacteria are internalized by the CHO cells. These results suggest that the interaction of HB101 carrying *inv* with the eucaryotic cell differs from the interaction of HB101 carrying *ail* with the eucaryotic cell and, in addition, that cell lines differ in their abilities to respond to the bound bacteria.

It appears from the results presented here that *Y. enterocolitica* synthesizes at least two factors that can mediate attachment and invasion of tissue culture cells. The products of the *inv* and *ail* genes are involved either directly or

TABLE 3. Invasion of tissue culture cell lines by *Y. enterocolitica* and *Y. enterocolitica* clones[a]

Infecting strain	% Invasion of:			
	HEp-2	MDCK	CHO	HEC1B
8081c	26.8 ± 3.6	0.93 ± 0.07	20.4 ± 5.9	124.3 ± 5.0
HB101(pBR322)	0.0075 ± 0.0035[b]	<0.0001	0.08 ± 0.01	0.005 ± 0.001
HB101(pVM101)	6.2 ± 2.1	0.015 ± 0.0015	15.2 ± 1.3	19.1 ± 2.4
HB101(pVM102)	0.37 ± 0.19	<0.0001	12.7 ± 0.2	5.5 ± 1.2

[a] Strains were used to infect monolayers of the tissue culture cell lines as described in Materials and Methods. Values are the averages of duplicate samples, with the ranges indicated, and reflect similar results from several experiments.
[b] From infection of HEp-2 with HB101. Results were similar for HB101 and HB101(pBR322).

TABLE 4. Percent cell-associated bacteria in tissue culture cell lines[a]

Infecting strain	% Bacteria associated with:			
	HEp-2	MDCK	CHO	HEC1B
8081c	67.0 ± 4.0	15.0 ± 1.7	51.0 ± 5.0	104.0 ± 53
HB101(pBR322)	11.0 ± 1.7	0.34 ± 0.07	3.6 ± 0.7	7.4 ± 2.3
HB101(pVM101)	34.0 ± 2.0	4.4 ± 0.05	26.0 ± 3.0	58.0 ± 5.2
HB101(pVM102)	43.0 ± 11.0	4.2 ± 0.2	37.0 ± 4.0	10.0 ± 1.6

[a] Strains were used to infect monolayers of the tissue culture cell lines as described in Materials and Methods. Values are the averages of duplicate samples, with the ranges indicated, and reflect similar results from several experiments.

indirectly in invasion. Neither *inv* nor *ail* promotes invasion to the degree seen with wild-type *Y. enterocolitica* 8081c. This may be due simply to differences in strain background between *E. coli* HB101 and *Y. enterocolitica* 8081c, though it is also possible that *Y. enterocolitica* possesses more than two factors that can promote invasion. It is already known that, in addition to these two chromosomally encoded attachment and invasion factors, *Y. enterocolitica* possesses an attachment factor that is plasmid encoded and is thought to be protein 1, which is responsible for autoagglutination as well as adhesion (11, 21, 29). Why *Y. enterocolitica* should express several different factors involved in adhesion and invasion is unclear. It is possible that they are expressed at different times during infection or that each promotes interactions of the bacterium with different cell types. A more detailed study of these genes and their products should lead to a better understanding of the interaction of this invasive bacterial pathogen with its host.

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