INFECTION AND IMMUNITY, Apr. 1990, p. 1059–1064
0019-9567/90/041059-06$02.00/0

Vol. 58, No. 4

Nonpathogenic Isolates of *Yersinia enterocolitica* Do Not Contain Functional *inv*-Homologous Sequences

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Received 1 August 1989/Accepted 15 December 1989

Previous studies have demonstrated a correlation between the ability of isolates of *Yersinia enterocolitica* to cause disease and to invade tissue culture cells in vitro. Two genes, *inv* and *ail*, isolated from a pathogenic strain of *Y. enterocolitica* have each been shown to confer this invasive phenotype upon *Escherichia coli*. Eighty pathogenic, invasive isolates studied by Miller et al. (Infect. Immun. 57:121–131, 1989) contained sequences homologous to both of these genes. Thirty-five nonpathogenic, noninvasive isolates similarly studied had no *ail* homology but carried *inv*-homologous sequences. We investigated *inv*-homologous sequences from four nonpathogenic isolates. Recombinant clones of these *inv*-homologous sequences did not confer the invasive phenotype upon *E. coli*. No RNA transcripts capable of encoding a full-length Inv protein were detected in the four noninvasive *Yersinia* strains. When the *inv* gene from a pathogenic isolate was introduced into two of these strains, the resulting transformants invaded tissue culture cells in vitro. The *inv* gene was transcribed in a pathogenic *Yersinia* isolate grown at 30°C but not at all in these cells grown at 37°C. The production of RNA transcripts homologous to *inv* in transformants was not regulated by temperature to the same degree as was seen for pathogenic isolates. We conclude that the *inv* gene in nonpathogenic strains of *Y. enterocolitica* is nonfunctional. *Y. enterocolitica* isolates epidemiologically linked to disease contain both a functional *inv* gene and a functional *ail* gene. Environmental isolates not associated with disease have a nonfunctional *inv* gene and no *ail* gene.

Yersinia enterocolitica is an enteric pathogen of humans which typically gains access to the host through contaminated foods (12, 23). The most common symptoms of infection with this organism are abdominal pain and diarrhea (5, 8). The infection can lead to mesenteric lymphadenitis and terminal ileitis (5, 8). Postinfectious complications, such as reactive arthritis and erythema nodosum (5, 8), are occasionally seen. These manifestations appear to be immunological in origin and are associated with particular serotypes of *Y. enterocolitica* and particular host haplotypes (1, 5). When bacteria present in contaminated food enter the small intestine, they are able to cross the intestinal epithelium and enter the underlying lymphatic tissue (24). Once there, the bacteria can establish infection within cells of the monocyte lineage (24). Some of the more severe consequences of infection by this organism seem to be a result of this colonization of local lymphatic tissue.

The ability to enter host cells appears to be an important first step in the infection process for this organism. Two chromosomal genes, *inv* and *ail*, have been associated with the ability of *Y. enterocolitica* to enter cultured human epithelial cells (16). When either gene is introduced into a laboratory *Escherichia coli* strain, it confers the invasive phenotype on this normally noninvasive organism. A gene homologous to the *inv* gene of *Y. enterocolitica* had previously been isolated from a related species, *Y. pseudotuberculosis*, and this gene also conferred the invasive phenotype on *E. coli* (9). In addition, when in vitro-generated mutations in *inv* were reintroduced into *Y. pseudotuberculosis*, these mutants became noninvasive (11). *Y. pseudotuberculosis inv* mutants also exhibit a delayed course of infection relative to that of the wild type when given orally to mice (21). All isolates of *Y. enterocolitica* epidemiologically linked to disease that have been studied have been shown to contain DNA sequences homologous to these two genes (17, 20). Environmental isolates of *Y. enterocolitica* not epidemiologically associated with disease and unable to invade cells in a tissue culture model have also been shown to have DNA homologous to the *inv* gene; they lack *ail*-homologous sequences (17, 20). The *inv* homology seen in these strains falls into a small number of groups based upon restriction fragment length polymorphisms (RFLPs) seen when chromosomal DNA from these strains is digested with the restriction endonuclease *Eco*RV (17). All of the pathogenic isolates contain *inv*-homologous sequences which fall into two RFLP groups, and all but a single nonpathogenic isolate have *inv*-homologous sequences that are in the other RFLP groups (17). Thus, there is an association between the *inv* homology RFLP pattern seen and pathogenicity. The fact that environmental isolates contain any sequences homologous to *inv* does, however, raise questions about the role of *inv* in the pathogenic process. To answer these questions, we have been studying the *inv*-homologous sequences from a number of non-disease-associated *Y. enterocolitica* isolates which do not invade tissue culture cells. We have isolated *inv*-homologous sequences from four noninvasive *Y. enterocolitica* strains and have shown that they do not promote *E. coli* invasion. *inv*-homologous RNA is not expressed in three of these strains and is only weakly expressed in the fourth. Finally, the presence of the wild-type *inv* gene in *trans* in two of these previously noninvasive isolates conferred the tissue culture-invasive phenotype. These experiments demonstrate that the *inv*-homologous sequences in nonpathogenic isolates are nonfunctional.

(This work was presented in part at the 89th Annual Meeting of the American Society for Microbiology [D. E. Pierson and S. Falkow, Abstr. Annu. Meet. Am. Soc. Microbiol. 1989, B263, p. 74].)

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TABLE 1. Strains used

Strain (serotype)	*inv* homology (RFLP group)[a]	*ail* homology[a]	Frequency of invasion (% ± SEM)[b] of:			Source (reference)
			HEp2	CHO	HEC-1B	
Y. enterocolitica						
8081c (O:8)	+ (I)	+	23.5 ± 3.2	40.0 ± 5.3	32.9 ± 6.6	Human septicemia (18)
Y312 (O:34)	+ (I)	−	0.028 ± 0.001	0.565 ± 0.015	0.200 ± 0	Food; W. Hill, FDA[c]
YF357	+ (II)	−	0.150 ± 0.014	0.865 ± 0.035	1.12 ± 0.18	Food; W. Hill, FDA
Y68	+ (III)	−	0.016 ± 0.002	0.360 ± 0.090	0.019 ± 0.001	Primate; W. Hill, FDA
MC7 (O:9)	+ (V)	−	0.269 ± 0.0375	0.970 ± 0.040	ND[d]	Human colitis; M. Cafferkey, Trinity College
E. coli						
DH5α	−	−	0.069 ± 0.008	0.022 ± 0.005	ND	Bethesda Research Laboratories
HB101	−	−	0.002 ± 0.0008	0.023 ± 0.003	0.004 ± 0.0005	6

[a] As determined by Miller et al. (17).
[b] Strains were assayed for the ability to invade tissue culture cell lines as described in Materials and Methods; values are the averages of triplicate samples from a single experiment on a single day.
[c] FDA, Food and Drug Administration.
[d] ND, Not determined.

MATERIALS AND METHODS

Bacterial strains and tissue culture lines. The bacterial strains used in these studies are described in Table 1. Strain 8081 has been defined as pathogenic by the Centers for Disease Control. Strains Y312, YF357, Y68, and MC7 have been defined as nonpathogenic by the Centers for Disease Control. Strains were maintained at −70°C in L broth (15) containing 50% glycerol. Bacteria were grown in L broth directly from these stock cultures. Hep2 and Hec1B (both from the American Type Culture Collection, Rockville, Md.) cells were grown in RPMI 1640 medium containing 5% fetal bovine serum. Chinese hamster ovary (CHO) (American Type Culture Collection) cells were grown in minimal essential medium with nonessential amino acids and 5% fetal bovine serum. Antibiotics were used in the following concentrations: ampicillin, 100 μg/ml, and chloramphenicol, 50 μg/ml.

Construction of probes. DNA from the *Y. enterocolitica inv* recombinant clone pVM101 (16) was digested with restriction endonucleases purchased from Bethesda Research Laboratories according to the directions of the manufacturer. DNA was subjected to electrophoresis on a 0.8% agarose gel (14). Fragments of interest were isolated by electroelution into DEAE paper (Schleicher & Schuell, Inc.). DNA was eluted from the paper onto 1 M sodium chloride–50 mM arginine, extracted with phenol, and precipitated with ethanol as described elsewhere (27). Fragments were labeled by nick translation (14).

DNA isolation and cloning of *inv* homologs. Gene banks of *Y. enterocolitica* Y312, YF357, Y68, and MC7 were constructed in the vector pMT11HC. This vector is a high-copy-number mutant (200 to 300 copies per cell) isolated from a deletion derivative of pBR322 from which the *Hae*II fragments corresponding to base pairs 238 to 2352 on the pBR322 sequence as defined by Maniatis et al. (14) have been removed and into which a polylinker has been inserted between the unique *Eco*RI and *Hin*dIII sites (Kevin Moore, unpublished results). Chromosomal DNA was isolated from these four strains by the method of Redfield and Campbell (19). DNA was partially digested with the restriction endonuclease *Sau*3A to generate fragments larger than 3 kilobase pairs (kbp). This partially digested DNA was fractionated by electrophoresis on a 0.8% agarose gel. Fragments ranging in size from 6 to 12 kbp were isolated by elution onto DEAE paper as described above. pMT11HC DNA was digested with *Bam*HI and treated with calf intestinal alkaline phosphatase (14). Vector and chromosomal DNAs were mixed in a molar ratio of 10:1 at a concentration of 200 μg/ml and ligated overnight at 14°C (14). Approximately 100 ng of this mixture was used to transform (14) *E. coli* HB101. Ampicillin-resistant transformants were screened for *inv*-homologous sequences by the method of Grunstein and Hogness (14) by using the *inv* probe B indicated in Fig. 1.

RNA isolation. Total bacterial RNA was isolated from cells growing logarithmically at 30 and 37°C by the hot phenol extraction method of von Gabain et al. (26). Briefly, cells were quickly chilled in an ice-water bath when they reached an A_{650} of 0.5. Cells were harvested by centrifugation at 3,000 × *g* for 10 min, and the pellet was suspended in ice-cold 0.3 M sucrose–0.01 M sodium acetate, pH 4.5. An equal volume of 2.5% sodium dodecyl sulfate–0.01 M sodium acetate, pH 4.5, was added, and this mixture was incubated at 65°C for 1.5 min. The mixture was then extracted three times with hot (65°C) phenol and then ethanol precipitated. The RNA pellet was suspended in 10 mM Tris–1 mM EDTA, pH 7.0, and treated with RNase-free DNase (Worthington Biochemicals) for 30 min at 20°C. This was then extracted with a 1:1 mixture of phenol and chloroform and ethanol precipitated. RNA was stored in 10 mM Tris–1 mM EDTA, pH 7.5, at −20°C.

Northern (RNA blot) analysis. A 15-μg portion of each RNA sample was denatured and separated on a 2.2 M formaldehyde–1% agarose gel (13) and blotted onto nitrocel-

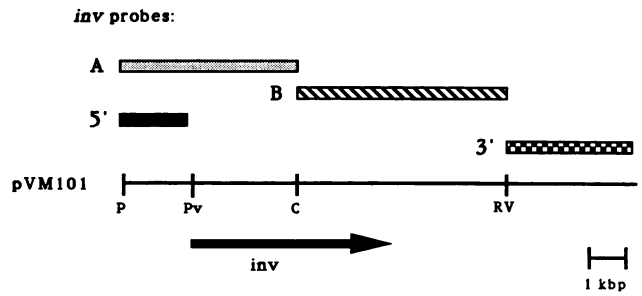

FIG. 1. *inv* probes used. Plasmid pVM101 was described by Miller and Falkow (16). The arrow indicates extent and direction of transcription of *inv* as determined from the *inv* gene sequence (V. Young, personal communication). Abbreviations: P, *Pst*I; Pv, *Pvu*I; C, *Cla*I; RV, *Eco*RV.

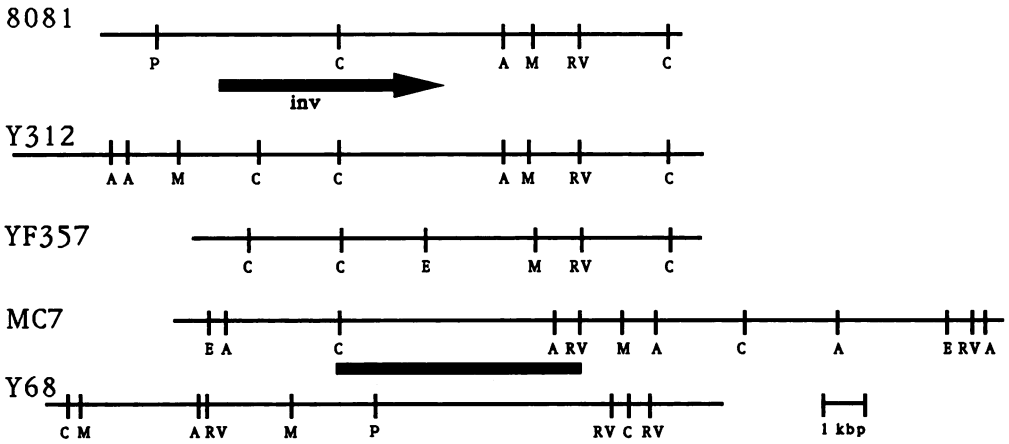

FIG. 2. Restriction maps of *inv*-homologous DNAs from the strains indicated. The arrow indicates extent and direction of the *inv* gene as derived from the *inv* gene sequence (V. Young, personal communication). The maps of Y312, YF357, and MC7 *inv* are aligned with that of 8081 relative to the common *Eco*RV site. The map of Y68 could not be aligned with these (see Results). The MC7 *inv* probe used for analysis of transcription in MC7 shown in Fig. 3C is indicated (▩). Abbreviations: E, *Eco*RI; P, *Pst*I; C, *Cla*I; A, *Ava*I; M, *Mlu*I; RV, *Eco*RV.

lulose (Schleicher & Schuell) by using standard procedures (13). For slot blot analysis, 10 μg of RNA was denatured by treatment with 7.4% formaldehyde in 1 M sodium chloride–100 mM sodium citrate at 56°C for 15 min and then spotted onto nitrocellulose filters (Bethesda Research Laboratories) (2). These filters were probed with nick-translated probes by using the hybridization and wash conditions described by Anderson and Young (2).

Transformation procedures. *E. coli* strains were transformed with plasmid DNA by the calcium chloride transformation protocol (14). *Y. enterocolitica* was transformed with plasmid DNA by the procedure of Balligand et al. (4).

Invasion assays. Invasion assays were performed as described by Miller and Falkow (16), with the following changes. Bacteria were incubated with the monolayer of tissue culture cells for 2 h at 37°C in a 5% CO_2 incubator, after which they were washed three times with phosphate-buffered saline. Fresh medium containing 100 μg of gentamicin per ml was added, and cells were incubated for 2 h at 37°C in a 5% CO_2 incubator, after which they were washed and lysed as described previously (16). Percent invasion was calculated as the number of bacteria surviving gentamicin treatment divided by the number of input bacteria.

RESULTS

Six *inv* RFLP categories have been defined by Miller et al. (17); these have been named types I, II, I-II, III, IV, and V. Type I isolates have two *inv*-homologous fragments of 9.5 and 4 kbp; type II strains have 9.7- and 3.8-kbp *inv*-homologous fragments; type I-II strains have a mixture of the type I and II patterns, with a 9.7-kbp fragment coming from the type II group and a 4-kbp fragment coming from the type I group; type III strains have two bands of 4.8 and 1.8 kbp that are only very weakly homologous to *inv* and may in fact represent homology to sequences adjacent to *inv* on the probe used; type V strains share the 9.5-kbp fragment with type I in addition to a 5-kbp *inv*-homologous fragment; and type IV strains are those that do not fall into any of the above-defined categories. All pathogenic strains, including the one used in this study, 8081c, fall into the type I and type I-II classes. The four nonpathogenic strains examined in these studies represent members of the type II, III, and V classes and the unique nonpathogenic type I isolate. The strain from which *inv* and *ail* were isolated (8081c), the four nonpathogenic isolates of *Y. enterocolitica*, and two *E. coli* control strains used in these studies are described in Table 1 with respect to the presence or absence of *inv*-homologous and *ail*-homologous sequences and the ability to invade three tissue culture cell lines. Of the seven strains described, only the strain capable of causing disease, 8081c, is able to invade the three tissue culture cell lines.

Isolation of *inv*-homologous sequences from noninvasive isolates. *inv*-homologous recombinant plasmids were isolated from *Sau*3A partial libraries of the four strains inserted into pMT11HC by using the *Cla*I-*Eco*RV probe (probe B) of *Y. enterocolitica inv* indicated in Fig. 1A as described in Materials and Methods. The restriction maps of these recombinant plasmids are shown in Fig. 2. The maps of the Y312, YF357, and MC7 inserts can be aligned with that of the *inv* gene from 8081c, as they share some common restriction sites. It would appear from this alignment that these three recombinant plasmids contain the full-length *inv* gene if it is indeed present in the parental strains. Additional evidence that these three inserts contain the full-length *inv* gene came from probing them with probes that flank the 5′ and 3′ ends of the gene as defined by the 8081c *inv* sequence (V. Young, personal communication). These probes are shown in Fig. 1. All three recombinant plasmids hybridized to both of these probes (data not shown), indicating that the full-length gene was present. It was not possible to align the map of the Y68 *inv* recombinant plasmid to that of 8081c *inv*, as there were no obvious similarities in the restriction maps of the two. The 5′ *inv* probe did not hybridize to either the Y68 *inv* clone or the Y68 chromosomal DNA (data not shown), suggesting that neither Y68 nor the *inv*-homologous sequences isolated from this strain contains a full-length *inv* gene.

The invasion frequencies of HB101 transformants carrying these recombinant plasmids into three different tissue culture cell lines are presented in Table 2. *E. coli* recombinants harboring the *inv* gene of strain YF357, Y68, or MC7 were no more invasive than the *E. coli* parent containing the cloning vector alone. *E. coli* recombinants harboring the Y312 *inv* gene appeared to be consistently slightly more invasive than strains bearing the vector alone, although the level of invasion was far less than that seen for recombinants harboring

TABLE 2. Invasion of tissue culture cell lines by *E. coli* carrying *Y. enterocolitica inv* recombinant plasmids

Plasmid (*inv* origin)	Frequency of invasion (% ± SEM)[a]		
	HEp2	CHO	HEC-1B
pINV (8081c)	1.45 ± 0.15	9.6 ± 0.4	13.5 ± 2.2
pINV (Y312)	0.031 ± 0.026	0.298 ± 0.21	0.181 ± 0.10
pINV (YF357)	0.006 ± 0.002	0.044 ± 0.024	0.066 ± 0.064
pINV (Y68)	0.004 ± 0.0004	0.034 ± 0.0015	0.004 ± 0.0003
pINV (MC7)	0.044 ± 0.012	0.023 ± 0.001	ND[b]
pBR322	0.003 ± 0.001	0.039 ± 0.017	0.004 ± 0.0005

[a] *E. coli* HB101 strains harboring the plasmids indicated were assayed for invasion of the three tissue culture cell lines and the percentage of intracellular bacteria was determined as described in Materials and Methods. Values given are averages of duplicate samples from a single experiment. These numbers are representative of similar experiments performed on different days.
[b] ND, Not determined.

the 8081c *inv* gene. This may suggest that the Y312 *inv* gene produces a defective Inv protein that is approximately 60 times less active than that produced by the 8081c *inv* gene.

Expression of *inv*-homologous sequences. RNAs were isolated from 8081c and the three noninvasive strains Y312, YF357, and Y68 growing at mid-log phase at 30°C. Equal amounts of RNA preparations were electrophoresed in a formaldehyde gel, transferred to nitrocellulose, and probed with the two *inv* probes, A and B, indicated in Fig. 1. The results are shown in Fig. 3. No RNA species homologous to the upstream portion of the *inv* gene (probe A) could be detected in the three noninvasive strains, although several RNA species of 3.2, 2.8, 1.5, and 1.2 kilobases (kb) could be detected in 8081c with this probe. In addition, the other RNA species seen in Y312 and YF357 homologous to the

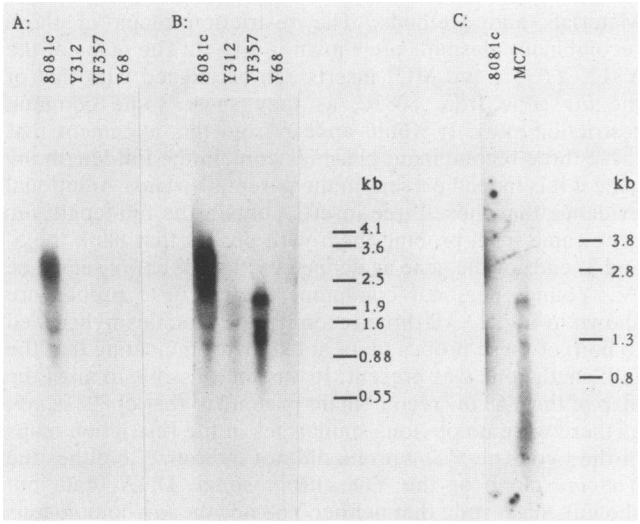

FIG. 3. Analysis of *inv*-homologous transcripts in noninvasive strains. RNA was isolated from cells growing exponentially at 30°C. A 15-μg portion of this preparation was subjected to electrophoresis on a formaldehyde-agarose gel, transferred to nitrocellulose, and hybridized with the probes indicated below as described in Materials and Methods. The lanes contained RNAs from the strains indicated. (A) Filter hybridized with *inv* probe A (Fig. 1). (B) Filter hybridized with *inv* probe B (Fig. 1). Molecular weights were determined by using *inv* DNA fragments treated in the same manner as RNA. (C) Filter hybridized with the 5.7-kbp *Cla*I-*Eco*RV fragment of MC7 *inv* (Fig. 2). Molecular weights were determined by using MC7 *inv* fragments treated in the same manner as RNA.

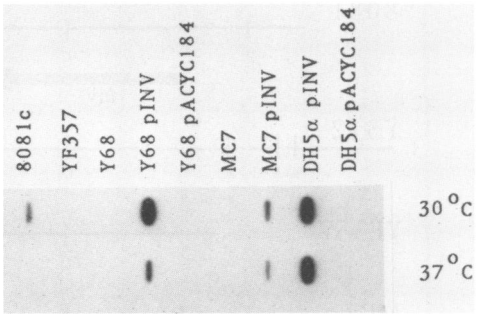

FIG. 4. Slot blot analysis of *inv*-homologous transcripts from noninvasive strains transformed with the 8081c *inv* gene. RNAs were isolated from the strains indicated growing exponentially at 30 or 37°C as indicated, denatured in the presence of formaldehyde and formamide, spotted onto filters, and hybridized as described in Materials and Methods. *inv* probe A (Fig. 1) was used to detect *inv*-homologous transcripts.

probe containing the 3′ end of the gene and downstream sequences (probe B) are not capable of encoding a full-length Inv protein, since at least 2.5 kb would be required, and the largest of these transcripts is 2 kb. These are most likely transcripts of genes downstream of *inv*. No *inv* RNA homology was seen in Y68 with probe B.

Stringent hybridization conditions were used for this Northern analysis, as we knew that these conditions would allow detection of DNA homology with these probes and these strains (data not shown). As these hybridization conditions are too stringent to detect MC7 *inv* homology in Southern analysis, we repeated the experiment for MC7 RNA by using a probe derived from the MC7 *inv* clone that hybridized to the 8081c *inv* gene under less stringent conditions. By Northern analysis (Fig. 3C), RNA homologous to this probe was detected; however, these transcripts (of 1.9 and 1.3 kb) were insufficient in length to encode a full-length Inv protein, as had been seen with Y312 and YF357 *inv*-homologous RNA. Thus, the *inv* genes in these nonpathogenic strains are not expressed, possibly explaining the noninvasive phenotype of these strains.

The effect of wild-type DNA in *trans*. The *inv* gene from 8081c was introduced on the plasmid pACYC184 into two of the noninvasive strains of *Y. enterocolitica*, Y68 and MC7. This plasmid remains extrachromosomal, and the copy number appears to be approximately equal to that seen in *E. coli* (data not shown), that is, approximately 20 molecules per cell (7). RNA was isolated from the resulting transformants and their parental strains from mid-log-phase cells growing at 30 or 37°C. These were spotted onto nitrocellulose and probed with the *inv* probe A shown in Fig. 1. The results of this are shown in Fig. 4. *inv*-homologous RNA was produced by the two *Y. enterocolitica* strains and the *E. coli* strains containing the *inv* recombinant plasmid but not by their parental strains lacking this recombinant plasmid. In contrast to what was observed for 8081c, for which there was no detectable *inv* transcription in cells grown at 37°C, the expression in these transformants was not strictly regulated by temperature to the same extent. *inv*-homologous transcripts were seen in cells of the three strains containing the *inv* plasmid grown at both 30 and 37°C. Although there seemed to be some temperature regulation in Y68 and MC7 harboring the wild-type *inv* recombinant plasmid, the level of expression was high enough to render any quantification

TABLE 3. Invasion of tissue culture cell lines by noninvasive bacteria with and without the 8081c *inv* clone

Strain	Frequency of invasion (% ± SEM)[a]	
	HEp2	CHO
8081c	3.38 ± 0.86	9.75 ± 0.75
Y68	0.004 ± 0.002	0.095 ± 0.015
Y68(pINV)	1.24 ± 0.53	3.47 ± 1.1
Y68(pACYC184)	0.001 ± 0.0002	0.029 ± 0.002
MC7	0.004 ± 0.002	0.215 ± 0.005
MC7(pINV)	6.1 ± 2.8	18.9 ± 9.7
DH5α(pINV)	0.560 ± 0.420	1.2 ± 0
DH5α(pACYC184)	0.002 ± 0.001	0.43 ± 0.29

[a] Strains were assayed for the ability to invade tissue culture cell lines as described in Materials and Methods. The percentages of intracellular bacteria given are averages of duplicate samples from a single experiment.

difficult. Certainly there was some transcription at 37°C in these strains, which was never seen in 8081c.

These transformants were tested for their ability to invade HEp2 and CHO cells in culture (Table 3). These previously noninvasive strains were now able to invade these two cell lines as well as 8081c could. The expression of the wild-type *inv* sequences introduced into the two nonpathogenic strains Y68 and MC7 was sufficient to confer the invasive phenotype to these two strains.

DISCUSSION

Tissue culture invasion has been used as one model to determine the pathogenic potential of a microorganism. *Y. enterocolitica* appears to require invasion as an early step in the pathogenic process (12, 22, 25). For that reason, the identification of factors that promote this process has been used as one approach to study some of the virulence factors of this microorganism. To that end, Miller and Falkow (16) identified two such putative chromosomal virulence factors, Inv and Ail, in *Y. enterocolitica*. However, the discovery that nonpathogenic environmental isolates of *Y. enterocolitica* have DNA sequences homologous to one of these genes, the *inv* gene, has raised some doubts about the relevance of this gene in the pathogenic process. In contrast, the observation that only pathogenic isolates have *ail* homology is consistent with the role of this gene in virulence.

Evidence from animal studies by Roqvist et al. (21) does suggest a role for *inv* in pathogenesis, as *Y. pseudotuberculosis inv* mutants show a delayed rate of infection compared with the wild type. However, in spite of this delay, infection does occur, indicating that there are other factors that can substitute for Inv.

We have presented evidence here that, although nonpathogenic isolates of *Y. enterocolitica* have *inv* sequence homology, these *inv* sequences are nonfunctional. First, these strains are not invasive in vitro, supporting the idea that in vitro invasiveness can be correlated with pathogenicity. While *E. coli* strains carrying the functional *inv* gene recombinant plasmid are invasive, the *inv*-homologous sequences from nonpathogenic isolates have little or no effect upon the ability of the *E. coli* strain harboring them to invade. In all four *Y. enterocolitica* isolates studied, no expression of these genes could be detected by Northern analysis. Finally, we have introduced the functional *inv* gene from a pathogenic strain into two of these nonpathogenic strains and shown that they then express *inv*-homologous RNA. These strains are apparently able to place the Inv protein on their surface in a functional configuration, as they exhibit the capacity to invade tissue culture cells in vitro. Therefore, the presence of *inv* homology in nonpathogenic strains does not rule out a role for *inv* in the infection process.

It is not clear why these bacteria retain this *inv* homology if the gene is not expressed. One possible explanation is that they have recently expanded into a new niche in which the expression of this gene would be deleterious to the organism. This retention (or acquisition) of nonexpressed virulence factor genes is not without precedent. The *ptx* operon, which encodes the virulence factor pertussis toxin in *B. pertussis*, is found in a nonexpressed form in two closely related species, *B. parapertussis* and *B. bronchiseptica* (3). As with *inv* in *Y. enterocolitica*, it is not known why these unexpressed virulence factor genes have not been lost from these organisms.

There remains the possibility that the *inv*-homologous sequences in the noninvasive strains are expressed under conditions that we have not examined in our experiments. Although this is a possibility that we cannot rule out, the fact that the wild-type *inv* gene could confer the invasive phenotype to these strains under the conditions we examined suggests that whatever is required by these organisms to express a wild-type *inv* gene is present.

We have also shown, as has been previously demonstrated for *Y. pseudotuberculosis inv* (10), that *Y. enterocolitica inv* expression is regulated by temperature. Furthermore, this temperature regulation has been shown, by slot blot analysis of RNA isolated from cells grown at different temperatures, to be at the level of transcription. At 30°C, *inv* RNA is expressed well, while at 37°C there is no detectable *inv* transcription. The *inv* gene in *E. coli* is not subject to this temperature regulation, as approximately equal amounts of *inv* RNA are detected at these two temperatures. In the noninvasive strains with the wild-type *inv* gene on a plasmid, there is some suggestion of temperature regulation; however, the extent of this regulation does not appear to be as great as that seen in the strain from which the *inv* gene was originally derived. The wild-type *inv* gene is in multiple copies in these strains, which may act to titrate out a factor with a role in temperature regulation (a factor which is presumably absent in *E. coli*). Alternatively, transcription of *inv* may be occurring from both its own promoter, which is temperature regulated, and from a plasmid promoter, which is not. This would lead to higher overall levels of *inv* transcripts, making it difficult to detect any effects of temperature. We are currently extending our investigation of the regulation of the *inv* gene by temperature.

ACKNOWLEDGMENTS

We thank D. Relman and J. St. Geme for critical reading of the manuscript. We thank V. Young for unpublished results.

This work was supported by Public Health Service grant AI 26195-02 from the National Institutes of Health. D. E. Pierson was supported by American Cancer Society fellowship no. PF-2963.

LITERATURE CITED

1. **Aho, K., M. Leirisalo-Repo, and H. Repo.** 1985. Reactive arthritis. Clin. Rheum. Dis. **11**:25–40.
2. **Anderson, M. L. M., and B. D. Young.** 1985. Quantitative filter hybridization, p. 73–111. *In* B. D. Hames and S. J. Higgins (ed.), Nucleic acid hybridisation, a practical approach. IRL Press, Ltd., Oxford.
3. **Arico, B., and R. Rappuoli.** 1987. *Bordetella parapertussis* and *Bordetella bronchiseptica* contain transcriptionally silent pertussis toxin genes. J. Bacteriol. **169**:2847–2853.
4. **Balligand, G., Y. Larouche, and G. Cornelis.** 1985. Genetic analysis of virulence plasmid from a serogroup 9 *Yersinia*

enterocolitica strain: role of outer membrane protein P1 in resistance to human serum and autoagglutination. Infect. Immun. **48**:782–786.

5. **Bottone, E. J.** 1977. *Yersinia enterocolitica*: a panoramic view of a charismatic microorganism. Crit. Rev. Microbiol. **5**:211–241.
6. **Boyer, H. B., and D. Roulland-Dussoix.** 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J. Mol. Biol. **41**:459–472.
7. **Chang, A. C. Y., and S. N. Cohen.** 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. J. Bacteriol. **134**: 1141–1156.
8. **Cornelius, G., Y. Larouche, G. Balligand, M.-P. Sory, and G. Wauters.** 1987. *Yersinia enterocolitica*, a primary model for bacterial invasiveness. Rev. Infect. Dis. **9**:64–87.
9. **Isberg, R. R., and S. Falkow.** 1985. A single genetic locus encoded by *Yersinia pseudotuberculosis* permits invasion of cultured animal cells by *Escherichia coli* K-12. Nature (London) **317**:262–264.
10. **Isberg, R. R., A. Swain, and S. Falkow.** 1988. Analysis of expression and thermoregulation of the *Yersinia pseudotuberculosis inv* gene with hybrid proteins. Infect. Immun. **56**: 2133–2138.
11. **Isberg, R. R., D. L. Voorhis, and S. Falkow.** 1987. Identification of invasin: a protein that allows enteric bacteria to penetrate cultured mammalian cells. Cell **50**:769–778.
12. **Lee, W. H., P. P. McGrath, P. H. Carter, and E. L. Eide.** 1977. The ability of some *Yersinia enterocolitica* strains to invade HeLa cells. Can. J. Microbiol. **23**:1714–1722.
13. **Lehrach, H., D. Diamond, J. M. Wozney, and H. Boedtker.** 1977. RNA molecular weight determinations by gel electrophoresis under denaturing conditions, a critical reexamination. Biochemistry **16**:4743–4751.
14. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
15. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
16. **Miller, V. L., and S. Falkow.** 1988. Evidence for two genetic loci in *Yersinia enterocolitica* that can promote invasion of epithelial cells. Infect. Immun. **56**:1242–1248.
17. **Miller, V. L., J. J. Farmer III, W. E. Hill, and S. Falkow.** 1989. The *ail* locus is found uniquely in *Yersinia enterocolitica* serotypes commonly associated with disease. Infect. Immun. **57**: 121–131.
18. **Portnoy, D. A., S. L. Moseley, and S. Falkow.** 1981. Characterization of plasmids and plasmid-associated determinants of *Yersinia enterocolitica* pathogenesis. Infect. Immun. **31**:775–782.
19. **Redfield, R. J., and A. M. Campbell.** 1984. Origin of cryptic lambda prophages in *Escherichia coli* K-12. Cold Spring Harbor Symp. Quant. Biol. **49**:199–206.
20. **Robins-Browne, R. M., M. D. Miliotis, S. Cianciosi, V. L. Miller, S. Falkow, and J. G. Morris, Jr.** 1989. Comparison of DNA colony hybridization and other techniques for the detection of virulence in *Yersinia* species. J. Clin. Microbiol. **27**:644–650.
21. **Rosqvist, R., M. Skurnik, and H. Wolf-Watz.** 1988. Increased virulence of *Yersinia pseudotuberculosis* by two independent mutations. Nature (London) **334**:522–525.
22. **Schiemann, D. A., and J. A. Devenish.** 1982. Relationship of HeLa cell infectivity to biochemical, serological, and virulence characteristics of *Yersinia enterocolitica*. Infect. Immun. **35**: 497–506.
23. **Shayegeani, M., I. DeForge, D. M. McGlynn, and T. Root.** 1981. Characteristics of *Yersinia enterocolitica* and related species isolated from human, animal, and environmental sources. J. Clin. Microbiol. **14**:304–312.
24. **Une, T.** 1977. Studies on the pathogenicity of *Yersinia enterocolitica*. I. Experimental infection in rabbits. Microbiol. Immunol. **7**:349–363.
25. **Une, T.** 1977. Studies on the pathogenicity of *Yersinia enterocolitica*. II. Interaction with cultured cells *in vitro*. Microbiol. Immunol. **7**:365–377.
26. **von Gabain, A., J. G. Belasco, J. L. Schottel, A. C. Y. C. Chang, and S. N. Cohen.** 1983. Decay of mRNA in *Escherichia coli*: investigation of the fate of specific segments of transcripts. Proc. Natl. Acad. Sci. USA **80**:653–657.
27. **Winberg, G., and M.-L. Hammarskjold.** 1980. Isolation of DNA from agarose gels using DEAE-paper. Application to restriction site mapping of adenovirus type 16 DNA. Nucleic Acids Res. **8**:253–264.