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The *ail* Locus Is Found Uniquely in *Yersinia enterocolitica* Serotypes Commonly Associated with Disease

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***Yersinia enterocolitica* is a heterogeneous group of organisms with more than 50 serotypes and several biotypes. Only a few of these serotypes cause gastrointestinal disease in otherwise healthy hosts; these serotypes are the pathogenic serotypes. Although *Y. enterocolitica* requires a high-molecular-weight plasmid to cause disease, chromosome-encoded determinants are required for the full expression of virulence. The ability of *Yersinia* spp. to invade eucaryotic cells is thought to be a virulence factor, because nonpathogenic serotypes are noninvasive in animals and in tissue culture cell models. Current evidence indicates that invasion ability is chromosome encoded. We recently reported cloning two loci, *inv* and *ail*, from *Y. enterocolitica* O8 strain 8081c that allow *Escherichia coli* to invade tissue culture cells. We investigated the link between invasion in an in vitro tissue culture invasion (TCI) model and hybridization to probes derived from the two invasion loci, *inv* and *ail*. We examined 177 *Yersinia* strains. Strains of serotypes and species associated with disease were TCI^+, whereas strains of serotypes and species not associated with disease were TCI^-. Only TCI^+ strains had DNA homologous to probes derived from *ail*. All strains (TCI^+ and TCI^-) had DNA homologous to probes derived from *inv*, but there were certain restriction fragment-linked polymorphisms that were associated primarily with TCI^+ strains. These observations held true for strains epidemiologically associated with disease. Both the *inv* and *ail* loci were found to be clearly located on the chromosome. No other genera, including other invasive organisms, had DNA homologous to *inv* or *ail*. These data support the hypothesis that the *ail* locus encodes a *Y. enterocolitica* invasion factor that may be involved in pathogenesis.**

Yersinia enterocolitica is a facultative intracellular pathogen that causes a broad range of food- and waterborne gastrointestinal syndromes, ranging from mild diarrhea to mesenteric lymphadenitis (9, 12). Septicemia is a rare but serious complication of yersiniosis, occurring most often in immunocompromised patients (9, 12). *Y. enterocolitica*, like *Salmonella* spp. (50), traverses the intestinal epithelium and can be found in the underlying lymph tissues, where it proliferates (10, 51). *Y. enterocolitica* is a heterogeneous group of organisms with more than 50 serotypes and several biotypes (9, 14). There are two groups of serotypes pathogenic for humans: (i) O3, O9, and O5,27, which are the most common causes of human disease outside the United States (8, 13); and (ii) O8, O4,32, O13a,13b, O18, O20, and O21, which are isolated from cases of human disease in the United States and commonly designated American strains (9, 14). In addition, O1,2,3 is pathogenic for chinchillas, and O2,3 is pathogenic for hares (14). Other *Y. enterocolitica* serotypes can be isolated from healthy humans.

Y. enterocolitica and the other pathogenic *Yersinia* species, *Yersinia pestis* and *Yersinia pseudotuberculosis*, require a high-molecular-weight plasmid to cause disease in animal models (19, 41, 58); this plasmid is not observed in environmental isolates, nonpathogenic strains, or other *Yersinia* species (25, 46). Plasmid-associated phenotypes thought to play a role in pathogenesis include Ca^{2+} dependence for growth, mouse lethality, a positive Sereny test, temperature-inducible outer membrane proteins, cytotoxicity, serum resistance, V and W antigens, and autoagglutination (41). The contribution of each of these phenotypes to *Yersinia* pathogenicity has not been examined. Attempts have been made to correlate phenotypes associated with the virulence plasmid and pathogenesis (16, 19, 25, 43, 45, 46, 58). This approach may not be successful because the virulence plasmid is easily lost from the strain during laboratory cultivation. As a result, a strain that was fully virulent (that is, that had both plasmid and chromosomal markers) and the causative agent of disease in a patient may appear to be a nonpathogenic strain when tested in an animal model.

The plasmid is essential but is not alone sufficient for virulence; transfer of the virulence plasmid to a nonpathogenic *Y. enterocolitica* strain does not always confer full virulence (23, 24). This fact suggests that in addition to the plasmid, chromosome-encoded determinants are involved in the overall elaboration of the pathogenic or virulent phenotype. Bakour et al. (4) observed that although strains that lack the virulence plasmid rarely cause systemic disease in mice, they can often be isolated from the mesenteric lymph nodes. Similar results suggesting that the ability to penetrate the intestinal wall and establish an infection (although not disease) is not plasmid dependent have been obtained in gnotobiotic piglets (44).

Intracellular *Y. enterocolitica* can be found in tissues from both patients and infected animals (10, 51). In addition, after oral infection of animals, bacteria can be found in large numbers at extraintestinal sites such as the liver and spleen (4, 19, 21). The ability of *Yersinia* spp. to invade eucaryotic cells has been demonstrated in vitro by using a tissue culture model (15, 32, 52, 55). Serotypes that are usually virulent in animal models are able to invade tissue culture cells (32, 52). Several studies have also shown that clinical isolates, in

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TABLE 1. Correlation of hybridization to AilB and AilC probes with TCI phenotype

Probe phenotype	Hybridization[a]	No. of strains with given phenotype	
		TCI$^+$	TCI$^-$
AilC	+	85	1
	−	1	62
AilB	+	52	2
	−	7	61
	+w	27	0

[a] +, Hybridization; −, no hybridization; +w, weak hybridization, detectable only after several days of exposure to the film.

contrast to environmental and food isolates, can enter cultured animal cells (31, 32, 46). Invasion appears to be chromosome encoded. Isogenic strains with and without the virulence-associated plasmid are equally invasive in the tissue culture model (42, 56). A single gene, *inv* from *Y. pseudotuberculosis*, has been cloned (26). This locus allows *Escherichia coli* HB101 to invade HEp-2 tissue culture cells (26). Mutations in *inv* eliminate the ability of *Y. pseudotuberculosis* and *E. coli inv* to invade HEp-2 cells (26, 27). This gene encodes a polypeptide of 103 kilodaltons (27). We recently reported the cloning of two different loci, *inv* and *ail* from a serotype O8 *Y. enterocolitica* clinical isolate, which individually confer an invasive phenotype on noninvasive *E. coli* HB101 (38). The *inv* gene from *Y. enterocolitica* is homologous to the *inv* gene from *Y. pseudotuberculosis* (38); neither *inv* gene is homologous to *ail* (38).

Since not all strains of *Y. enterocolitica* can cause disease, we would like to know how pathogenic strains differ from nonpathogenic strains. Heeseman et al. (23, 24) showed that aside from the presence of the virulence plasmid, there are chromosomal differences between virulent and avirulent strains. Transferring the virulence plasmid to avirulent isolates confers some plasmid-associated phenotypes but does not result in full virulence (23, 24). Because avirulent strains are noninvasive, the invasion loci are prime candidates for this genetic difference.

We therefore investigated the link between invasion in an in vitro tissue culture invasion (TCI) assay and hybridization to probes derived from the two invasion loci, *inv* and *ail*. A total of 177 *Yersinia* strains were examined for TCI phenotype and hybridization to the probes. We found, as have others (31, 32, 46), that strains of *Y. enterocolitica* serotypes thought to be associated with disease are TCI$^+$, whereas strains of serotypes not thought to be associated with disease are TCI$^-$. Clear differences in hybridization to the probes were observed between TCI$^+$ and TCI$^-$ strains. Specifically, the *ailC* locus was uniquely found in TCI$^+$ strains.

MATERIALS AND METHODS

Bacterial strains and tissue culture cells. Bacterial strains were maintained at −70°C in Luria broth (LB) medium (37) containing 25% (vol/vol) glycerol or on LB agar plates. *Yersinia* strains (listed in Tables 1 and 2) were tested for invasion in the TCI assay (TCI phenotype) and hybridization to the probes described below without prior knowledge of their serotype or source to ensure an unbiased evaluation. The *inv* and *ail* genes were cloned (37) from *Y. enterocolitica* O8 strain 8081c (41). *E. coli* HB101 (F$^-$ *hsdS20*[r_B^- m_B^-] *recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1*) (2) carrying the recombinant plasmid pRI203, pVM101, or pVM103 was maintained on LB agar plates containing 50 μg of ampicillin per ml. Human laryngeal epithelial (HEp-2) cells were maintained and prepared for the invasion assay as previously described (17). *Yersinia* strains were grown at 28°C with aeration for 12 to 18 h in LB for the invasion assays.

TABLE 2. TCI phenotype and hybridization results for *Y. enterocolitica* of known serotype

O-group serotype	No. of strains	TCI phenotype	Hybridization[a] to:		
			Inv-Ent	AilB	AilC
1,2,3	1	+	+ (I)	+w	+
13a,13b	3	+	+ (I)	+	+
13a,13b	6	+	+	+	+
18	1	+	+ (I)	+	+
20	3	+	+	+	+
	5	+	+ (I)	+	+
21	1	+	+	+	+
	2	+	+ (I)	+	+
3	16	+	+	+w	+
	6	+	+ (I-II)	+w	+
	4	+	+ (I)	+w	+
4	2	+	+	+	+
4,32	1	+	+ (I)	+	+
4,33	1	+	+	+	+
5,27	1	+	+ (I)	−	+
	3	+	+	−	+
	1	+	+	+w	+
	1	+	+	+	+
8	11	+	+	+	+
	4	+	+ (I)	+	+
	1	+	+ (I)	+	−
9	2	+	+ (I)	+w	+
	1	+	+ (I)	−	+
10	1	−	+ (IV)	−	−
13	1	−	+ (III)	−	−
20	1	−	+ (V)	−	−
22	1	−	+ (IV)	−	−
27	1	−	+ (II)	−	−
	3	−	+	−	−
28	1	−	+	−	−
2a,2b,3	1	−	+	−	−
3	1	−	+ (II)	−	−
34	1	−	+	−	−
	1	−	+ (I)	−	−
5	2	−	+	−	−
6	3	−	+	−	−
	1	−	+ (II)	−	−
6,30	4	−	+	−	−
	1	−	+ (II)	−	−
7,13	1	−	+ (II)	−	−
	3	−	+	−	−
7,19	1	−	+ (II)	−	−
7,8	3	−	+	−	−
9	2	−	+ (V)	−	−

[a] As defined in the footnote to Table 1. For the Inv-Ent probe, the type of hybridization is shown in parentheses. This could be determined only for those strains examined by Southern analysis.

Nucleic acid purification and probe preparation. Chromosomal DNA was isolated as previously described (36). Plasmid DNA was purified by a cleared-lysate method, followed by CsCl equilibrium density centrifugation as described elsewhere (34). DNA restriction enzymes were purchased from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.) and used according to the instructions of the manufacturer. DNA probes were prepared as follows. Plasmid DNA was digested with the appropriate restriction endonucleases, and the fragments were separated by electrophoresis

through a 0.7% agarose gel. The DNA fragments were purified from the agarose gel slices by using Geneclean (Bio101, La Jolla, Calif.). The purified fragments were then labeled with ^{32}P by nick translation for use as probes as previously described (35).

Southern hybridization analysis. Chromosomal DNA was digested to completion with *Eco*RV, and the fragments were separated by electrophoresis through a 0.7% agarose gel. The separated DNA fragments were transferred to nitrocellulose (Schleicher & Schuell, Inc., Keene, N.H.) as described elsewhere (47). Hybridizations were performed at either medium or low stringency. Medium-stringency conditions were as follows. The filter was prehybridized for 1 h at 37°C in 35% formamide–4× SSC (1× SSC is 0.15 NaCl plus 0.015 sodium citrate)–0.1% sodium dodecyl sulfate–1 mM EDTA–1× Denhardt solution. The prehybridization solution was then removed, and hybridization solution (same as prehybridization solution) containing 250 μg of calf thymus DNA per ml and the boiled probe was added to the filter. After hybridization for 12 to 18 h at 37°C, the filter was washed three times with 5× SSC–0.1% sodium dodecyl sulfate at 65°C for 15, 10, and 5 min. Then the filter was washed in 2× SSC at room temperature for 5 min, air dried, and exposed to XAR-5 film (Eastman Kodak Co., Rochester, N.Y.). Low-stringency conditions were the same as medium-stringency conditions except that 20% formamide was used in the prehybridization and hybridization solutions. The filters were reused after being washed in 0.25 M NaOH at room temperature for 6 to 10 h and rinsed briefly in 2× SSC. Probe results for a given strain were obtained by using a single filter that was reprobed several times. This procedure allowed direct comparison of fragments that hybridized to the various probes used.

Colony blots (34) were hybridized as described above. Individual colony blots were used for each probe rather than rehybridizing the same filter.

TCI assay. Either quantitative or qualitative TCI assays were performed. The quantitative assay was performed as follows. Bacteria (approximately 2×10^7) were added to each well of a 24-well microdilution plate that had been seeded with HEp-2 tissue culture cells the previous day as described elsewhere (17). The microdilution plates were centrifuged for 10 min at 162 × *g* and ambient temperature and then incubated in 5% CO_2 at 37°C. After 90 min, 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 monolayers were washed twice with phosphate-buffered saline to remove the gentamicin. The tissue culture cells were then lysed with 0.2 ml of 1% Triton X-100 to release intracellular bacteria. After 5 min, 0.8 ml of LB was added, bringing the final concentration of Triton X-100 to 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 % invasion = 100 × (number of bacteria resistant to gentamicin/number of bacteria added).

The qualitative assay was performed as described above. After being washed twice with phosphate-buffered saline to remove the gentamicin, the tissue culture cells were lysed to release intracellular bacteria by addition of 0.2 ml of 1% Triton X-100 to each well. After 5 min, 0.8 ml of LB was added. A 50-μl amount of this suspension was then spread on an LB agar plate. Results were scored as either TCI^+ or TCI^-. TCI^+ strains gave almost confluent growth on the LB plate after the assay, whereas TCI^- strains gave a few isolated colonies.

RESULTS

TCI phenotype. A total of 177 *Yersinia* strains (1 *Y. pestis*, 10 *Y. pseudotuberculosis*, 149 *Y. enterocolitica*, 4 *Y. frederiksenii* [54], 4 *Y. kristensenii* [6], 3 *Y. intermedia* [11], 2 *Y. aldovae* [5], and 4 *Y. rohdei* [1]) were examined for their TCI phenotype by using HEp-2 cells. These strains were isolated in a variety of geographic locales and over more than 10 years; some of the strains listed were isolated during outbreaks, and thus multiple isolates of essentially the same strain are represented. Twenty-six serotypes are represented (a complete listing of the strains used can be obtained by request from V. Miller). In the context of this study, serotypes commonly associated with disease, O3, O9, O5,27, O8, O4,32, O13a,13b, O18, O20, O21, O1,2,3, and O2,3, are designated pathogenic. Serotypes which are not ordinarily associated with clinical disease, or which cause disease only in immunocompromised individuals, are designated nonpathogenic. The same distinctions are made for the various *Yersinia* species. Clearly, the ability of a particular strain of a pathogenic serotype to cause clinical disease depends on the presence of the 70-kilobase (kb) virulence plasmid in addition to chromosomal determinants (23, 24). As mentioned above, this plasmid is readily lost during cultivation in the laboratory; therefore, phenotypes associated with the plasmid cannot always be used as an indicator of the pathogenicity of the strain at the time it was isolated. Consequently, the serotype of a particular strain was used in this study as a guide to its potential pathogenicity.

Forty strains were examined quantitatively and qualitatively. The invasion values for TCI^+ strains according to the quantitative assay ranged from 0.6 to 7.8%; values for TCI^- strains ranged from 0.014 to 0.023% (data not shown). In all cases, there was at least a 26-fold difference in values between the TCI^+ and TCI^- strains. Thereafter, the qualitative TCI assay was used. No strain gave ambiguous results; even strains that exhibited extensive cytotoxic activity were still clearly invasive. Serotypes that commonly cause disease (that is, pathogenic serotypes) were TCI^+, whereas serotypes not associated with disease were TCI^- (Tables 1 and 2). This result agrees with those of previous studies that have observed a good correlation between potential pathogenicity, as defined by serotype, and the ability to invade tissue culture cells (32, 46).

Probes. Four probes were used in our experiments. The Inv-Ent probe was a 3.6-kb *Cla*I fragment purified from pVM101 (38) (Fig. 1). This probe contained part of the *inv* gene from *Y. enterocolitica* in addition to adjacent sequences; it is known that DNA to the left of the *Eco*RV site (Fig. 1) is not required for *inv* activity (V. Miller, unpublished data). The Inv-Pstb probe was a 2.4-kb *Cla*I-*Xho*I fragment purified from pRI203 (27). This fragment contained only sequences internal to the *Y. pseudotuberculosis inv* gene. Although Southern blot analysis indicated that the *Y. enterocolitica* and *Y. pseudotuberculosis inv* genes were homologous, no similarity was observed in their restriction maps (38). Both of these *inv* genes promote a high level of invasion into a variety of tissue culture cell lines by *E. coli* HB101 (26, 38).

Two probes, AilB and AilC, from the *Y. enterocolitica ail* locus (38) were used. AilB was a 900-base-pair (bp) *Ava*I-

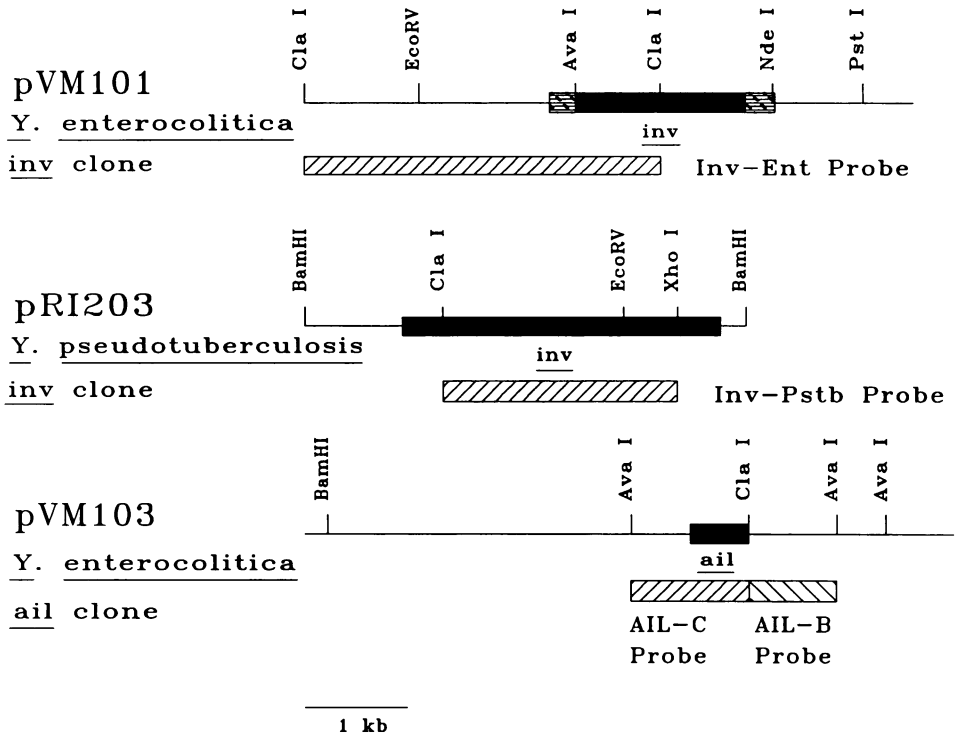

FIG. 1. DNA probes used. Plasmids pVM101 and pVM103 are described by Miller and Falkow (38); pRI203 is described by Isberg et al. (27). The heavy bar represents the coding regions of the *inv* and *ail* genes. The full extent of the *Y. enterocolitica inv* gene is not known but is contained within the *Eco*RV-*Pst*I fragment; the region shown by the heavy bar is required as determined by Tn*5* mutagenesis; the stippled bar indicates that the gene could extend beyond the heavy bar.

*Cla*I fragment from pVM103 (38). AilC was a 1.2-kb *Cla*I-*Ava*I fragment from pVM103. Genetic analysis with transposon Tn*5* insertions had indicated that DNA contained within the AilC probe was required for the Ail phenotype (38). No Tn*5* insertions eliminating the Ail phenotype have been isolated in AilB (38). Preliminary DNA sequence data suggest that only 6 bp of the Ail protein-coding sequence are located within AilB; the remainder of the Ail-coding sequence is contained within AilC (V. Miller, unpublished data). The *ail* gene promotes a high level of invasion of some tissue culture cell lines (CHO [Chinese hamster ovary]) and a low level of invasion of other cell lines (HEp-2 [human larynx epithelium]) by *E. coli* HB101 (38).

Location of *inv* and *ail*. The *inv* and *ail* genes were cloned from *Y. enterocolitica* O8 strain 8081c (38). Strain 8081c is a derivative of strain 8081 (42) that has been cured of the virulence plasmid. The 40-megadalton virulence plasmid is the only plasmid detected in 8081 (42). Strain 8081c lacks detectable plasmid DNA and plasmid-associated phenotypes (42), but it is possible that the plasmid has been integrated into the chromosome. To determine whether *inv* and *ail* are truly chromosomal genes rather than plasmid genes, we performed a Southern blot analysis of purified virulence plasmid DNA and chromosomal DNA from strain 8081c (Fig. 2). None of the probes hybridized to the plasmid DNA, which indicated that the *inv* and *ail* genes are not located on the virulence plasmid. The restriction endonuclease used to digest the DNA (*Eco*RV) cuts once within the Inv-Ent probe; therefore, we found two fragments from chromosomal DNA that hybridized to the Inv-Ent probe. The AilC probe hybridized to a single fragment from chromosomal DNA, but the AilB probe hybridized to several chromosomal DNA fragments. The restriction endonuclease used (*Eco*RV) does not cut within the AilB probe; therefore, a sequence within AilB is repeated within the chromosome. Although there are indications that the virulence plasmid may contain a repeated sequence (40), it appears to be distinct from the repeated sequence identified by the AilB probe. *inv* and *ail* are probably single-copy loci (Fig. 2) and, because probes derived from these genes hybridize to different fragments, are separated by at least 7 kb.

Hybridization of AilC to DNA from *Y. enterocolitica* strains. Strains from nonpathogenic serogroups of *Y. enterocolitica* were TCI^-. We supposed that they might be missing essential genes required for invasion or might contain the appropriate gene in a nonfunctional form. To investigate whether these possibilities pertained to *ail* or *inv*, we performed either Southern or colony blot analysis of 149 *Y. enterocolitica* strains (Table 1). The AilC probe did not hybridize to DNA from all strains (Table 1 and Fig. 3). If one compares hybridization to AilC with the TCI phenotype one finds that 85 of 86 TCI^+ strains were homologous to AilC whereas only 1 of 63 TCI^- strains was homologous to AilC. Thus, there was a better than 98% correlation between hybridization to AilC and the TCI phenotype. This result supports the hypothesis that the *ail* locus encodes a *Y. enterocolitica* invasion factor.

Hybridization of AilB to DNA from *Y. enterocolitica* strains. The AilB probe, like the AilC probe, did not usually hybridize to TCI^- strains (Table 1 and Fig. 3). However, the TCI^+ strains could be divided into two distinct groups: those that showed strong hybridization to AilB and those that showed weak or no hybridization to AilB. Strains that hybridized strongly to AilB always had multiple fragments that hybridized; the number and size of these fragments varied from strain to strain. Strains that hybridized only weakly to AilB

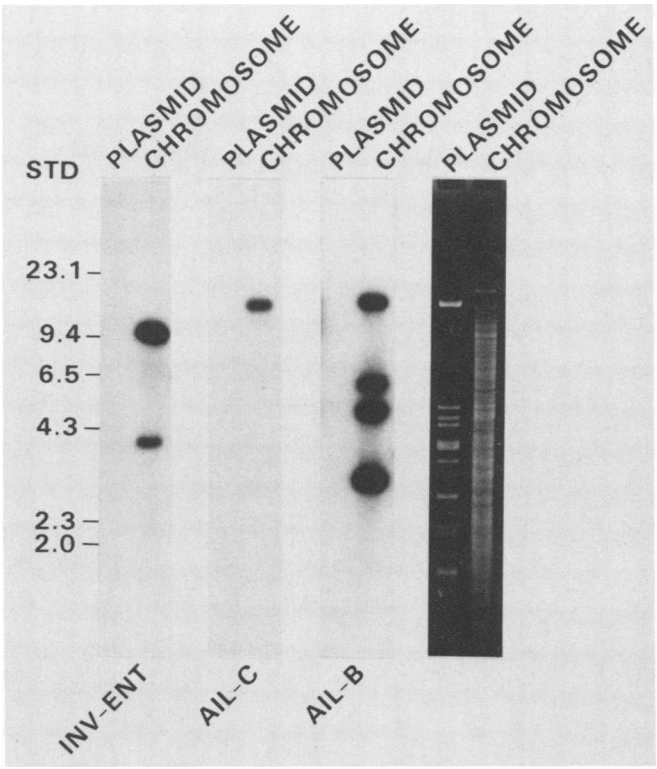

FIG. 2. Location of *inv* or *ail*. Chromosomal DNA was isolated from *Y. enterocolitica* 8081c. Plasmid DNA was isolated from *Y. enterocolitica* 8081. The DNA was digested with the restriction endonuclease *Eco*RV, and the fragments were separated by electrophoresis through a 0.7% agarose gel (rightmost panel). The DNA was then transferred to nitrocellulose and hybridized (medium stringency) to the Inv-Ent, AilC, and AilB probes as described in Materials and Methods. Each panel represents an autoradiograph of the same nitrocellulose filter probed separately with the probes listed. STD, Position and size (in kilobases) of λ DNA digested with *Hin*dIII.

always had only one fragment that hybridized. This fragment was usually identical to the fragment that hybridized to AilC. Preliminary sequencing results indicate that the Ail protein-coding region extends only 6 bp into AilB, although the *ail* gene itself may extend further. This suggests that the weak hybridization of AilB seen in these strains could have been due to a short sequence associated with *ail* but that these strains lack sequences present in AilB that are repeated in the chromosome of strains that hybridize strongly to this probe.

TCI^+ strains that hybridized strongly to AilB included serotypes O13a,13b, O18, O20, O21, O4, O4,32, O4,33, and O8. TCI^+ strains that hybridized weakly or not at all to AilB included serotypes O1,2,3, O3, O5,27, and O9. Interestingly, this AilB hybridization pattern reflected a clear division between the pathogenic American serotypes (that is, those normally isolated in North America) and other pathogenic serotypes isolated in Europe, Japan, southern Africa, and Canada. Although both groups of potentially pathogenic serotypes have the *ail* gene, the non-American strains appeared to lack a sequence located near *ail* that was found repeated in the chromosome of American strains.

Hybridization of Inv-Ent and Inv-Pstb to DNA from *Y. enterocolitica* strains. The Inv-Ent probe hybridized to DNA isolated from all strains (Table 2); nevertheless, there were differences between TCI^+ and TCI^- strains. We observed several distinct hybridization patterns, which were labeled types I, II, I-II, III, IV, and V. Type I strains had 9.5- and 4.0-kb fragments (Fig. 3, lanes 1 through 9), and type II had 9.7- and 3.8-kb fragments (lanes 10 through 12), that hybridized to the Inv-Ent probe. Of the 36 strains that were type I (including strain 8081c), 34 were TCI^+. Type I-II shared a 9.7-kb fragment with type II and a 4.0-kb fragment with type I (lanes 13); all six type I-II strains were TCI^+. The remaining four groups, II, III, IV, and V, were all TCI^-. Type V (not shown) shared a 9.5-kb fragment with type I and also had a 5.0-kb fragment that hybridized to Inv-Ent. Hybridization of Inv-Ent to type III was weak relative to the hybridization observed with other strains (lanes 14 and 15). The type III pattern appeared identical to the hybridization pattern observed for *Y. intermedia* (see below). Type IV is a catchall for strains with unique hybridization patterns. Overall, there was a correlation between strains that were TCI^+ and the type I or I-II hybridization pattern, whereas strains that were TCI^- exhibited the type II, III, IV, or V hybridization pattern.

Unlike the Inv-Ent probe, the Inv-Pstb probe contained only the *inv* coding sequence. Therefore, fragments that hybridized to Inv-Pstb were those with sequences related to the *inv* gene. The Inv-Pstb probe hybridized to all *Y. enterocolitica* strains tested except for the type III strains (Fig. 3). In each case, the fragment that hybridized to Inv-Pstb was identical to one of the fragments recognized by Inv-Ent. Inv-Pstb hybridized to the 9.5-kb fragment of types I and V and to the 9.7-kb fragment of types II and I-II. This result indicated that the hybridization of Inv-Ent to these fragments probably represented homology in *inv* sequences. Hybridization of Inv-Ent to other fragments was probably due to homology to DNA sequences adjacent to *inv*. The results obtained by using the Inv-Pstb probe also suggested that type III strains did not have an *inv* gene and that the hybridization observed with the Inv-Ent probe was due to sequences adjacent to *inv*. Alternatively, but less likely, the type III strains may have diverged more from the *Y. pseudotuberculosis inv* gene than have other *Y. enterocolitica* strains. As mentioned above, the type III hybridization pattern was like that seen for *Y. intermedia* strains; therefore, it is possible that these strains have been incorrectly identified as to species.

Hybridization phenotypes of strains isolated during several outbreaks. As noted above, there was an extremely good correlation between the ability to invade tissue culture cells and hybridization to the Inv and Ail probes. Would a similar correlation be seen between *Y. enterocolitica* strains that clearly caused clinical disease and hybridization to the probes? To address this question, we examined strains previously characterized from outbreaks of yersiniosis. The strains examined included those isolated from patients and those isolated from asymptomatic individuals at the same time and in the same region. A summary of these results is shown in Table 3, and the Southern analyses are shown in Fig. 4. These data indicated that all *Y. enterocolitica* isolates that were strongly implicated as the cause of disease were TCI^+, whereas strains isolated from asymptomatic individuals (outbreaks 2 and 5, Table 3) were either TCI^- or TCI^+. Like other TCI^+ strains, these strains were type I with the Inv-Ent probe (Fig. 4, lanes 2, 3, and 5 through 7). A strain isolated from an asymptomatic family member (lanes 4) of the patient in outbreak 2 was TCI^- and was type II with the Inv-Ent probe. The strains isolated from patients were $AilC^+$ and $AilB^+$ (lanes 2, 3, and 5 through 7). The TCI^-

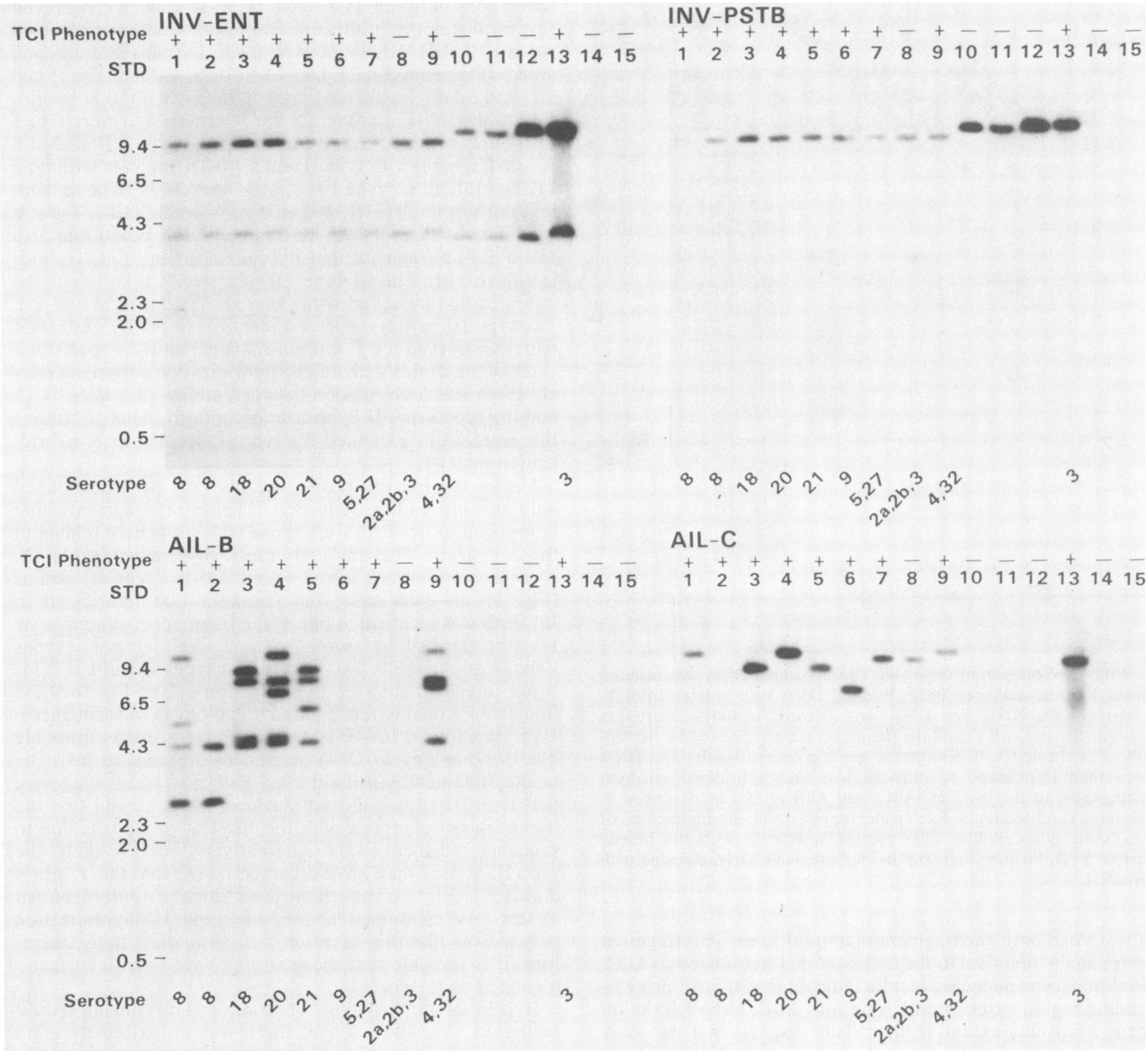

FIG. 3. Hybridization of DNA probes derived from *inv* and *ail* to chromosomal DNA purified from *Y. enterocolitica* strains. Southern analyses show examples of the different patterns of hybridization observed. Chromosomal DNA was purified from *Y. enterocolitica* strains of the serotypes listed (if known) and digested with the restriction endonuclease *Eco*RV, and the fragments were separated by electrophoresis through a 0.7% agarose gel. The DNA was then transferred to nitrocellulose and hybridized (medium stringency) to the Inv-Ent, Inv-Pstb, AilB, and AilC probes as described in Materials and Methods. Each panel represents an autoradiograph of the same nitrocellulose filter probed separately with the probe indicated. STD, Position and size (in kilobases) of λ DNA digested with *Hin*dIII. Lanes contain DNA isolated from the following strains: 1, 8081c (O8); 2, 641-83 (O8); 3, 655-83 (O18); 4, 657-83 (O20); 5, 658-83 (O21); 6, 642-83 (O9); 7, 637-83 (O5,27); 8, 632-83 (O2a,2b,3); 9, 634-83 (O4,32); 10, 661-83 (O27); 11, Y240; 12, Y219; 13, Y288 (O3); 14, Y68; 15, Y222.

strains were AilB$^-$ and AilC$^-$, as we had previously observed for other TCI$^-$ strains. These data show that nonpathogenic serotypes can also be isolated if extensive culturing is done during investigations of outbreaks.

The pattern of hybridization of AilB to DNA from random *Y. enterocolitica* isolates varied from strain to strain. However, strains isolated from the same outbreak exhibited identical hybridization patterns to the AilB probe (Fig. 4, lanes 2, 3, and 5 through 7). This probe may therefore serve as a useful marker for identifying the source and following a particular epidemic.

Presence of *inv* and *ail* sequences among the yersiniae. Three species of the genus *Yersinia* are generally recognized as primary pathogens for humans and animals: *Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica* (14). Several other *Yersinia* species have been defined, but these are generally considered to be either nonpathogenic or opportunistic pathogens. Are the differences between pathogenic and nonpathogenic *Y. enterocolitica* serotypes observed by using the *inv* and *ail* probes also true for the genus *Yersinia* as a whole? DNA isolated from all *Yersinia* species tested exhibited homology to the Inv-Ent probe (Fig. 5), but only

TABLE 3. TCI phenotype and hybridization results for *Y. enterocolitica* associated with outbreaks[a]

Strain	Serotype	TCI phenotype	Hybridization[b] to:			Source or comment
			Inv-Ent	AilB	AilC	
Outbreak 1						
9341-78	3	+	+	+w	+	Sick chinchilla
Outbreak 2						
9286-78	20	+	+ (I)	+	+	Patient
9287-78	20	+	+ (I)	+	+	Dog feces; dog died
9291-78	6	−	+ (II)	−	−	Cousin, asymptomatic
9292-78	UND[c]	−	+	−	−	Aunt, asymptomatic
9293-78	UND	−	+	−	−	Grandfather, asymptomatic
9294-78	UND	−	+	−	−	Cousin, asymptomatic
Outbreak 3						
Y137	13	+	+ (I)	+	+	Patient
Y135		+	+ (I)	+	+	Patient
Y164		+	+ (I)	+	+	Patient
Y165		+	+ (I)	+	+	Patient
Outbreak 4						
Y124		+	+ (I)	+	+	Patient
Y121	8	+	+ (I)	+	+	Patient
Outbreak 5						
JCFA2650	8	+	+	+	+	Patient
JCFA4182	6	−	+	−	−	Not associated
JCFA4186	4	+	+	+	+	Not associated

[a] Outbreaks: 1, California (3); 2, Kentucky, 1974 (3); 3, Tennessee, Mississippi, and Arkansas, 1982 (49); 4, Oregon (48); 5, see references 3 and 7.
[b] As defined in the footnote to Table 1. For the Inv-Ent probe, the type of hybridization is shown in parentheses.
[c] UND, O subgroup undetermined.

the pathogenic species showed strong homology to the *inv* gene-specific Inv-Pstb probe. The DNA fragments hybridizing to Inv-Pstb and Inv-Ent for *Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica* were the same (data not shown), which suggested that these fragments encode the *inv* gene. In contrast, *Y. aldovae*, *Y. intermedia*, *Y. frederiksenii*, *Y. kristensenii*, and *Y. rohdei* showed only very weak hybridization to Inv-Pstb, and the fragments hybridizing to Inv-Pst were not the same as those hybridizing to Inv-Ent, which suggested that the hybridization to Inv-Ent may have been due to sequences present in the probe adjacent to *inv* rather than to *inv* itself.

The AilB and AilC probes both hybridized to DNA isolated from *Y. pestis* and *Y. pseudotuberculosis* as well as to pathogenic *Y. enterocolitica* serotypes. The fragments that hybridized to these two probes differed, however, which indicated either that the AilB and AilC sequences are separated on the chromosome of these species (unlike the case with *Y. enterocolitica*, in which AilB and AilC are usually located on the same *Eco*RV fragment) or that an *Eco*RV site separates these sequences. The AilB probe hybridized strongly to only one DNA fragment in *Y. pestis* and *Y. pseudotuberculosis* YPIII, but after longer exposure several additional weak hybridization signals could be observed for *Y. pestis*.

DNA isolated from 25 different species (*Salmonella typhimurium*, *Salmonella choleraesuis*, enteroinvasive *E. coli*, enteropathogenic *E. coli*, *Enterobacter agglomerans*, *Enterobacter cloacae*, *Citrobacter freundii*, *Klebsiella pneumoniae*, *Haemophilus influenzae*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Campylobacter jejuni*, *Francisella tularensis*, *Morganella morganii*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Serratia marcescens*, *Aeromonas hydrophila*, *Acinetobacter* sp., *Chlamydia trachomatis*, *Vibrio parahaemolyticus*, *Pasteurella multocida*, *Bordetella pertussis*, *Bordetella parapertussis*, *Bordetella bronchiseptica*, and *Bordetella avium*) in addition to the *Yersinia* spp. was also examined for hybridization to these probes under low-stringency conditions (data not shown). No hybridization to the Inv-Ent probe was observed, which suggested that this probe contained *Yersinia*-specific sequences. No hybridization to the AilC probe was observed either, which suggested that this probe contained DNA sequences specific to pathogenic yersiniae. Only DNA isolated from a strain of *K. pneumoniae* hybridized to AilB; four hybridizing fragments were observed upon digestion with the restriction endonuclease *Eco*RV, which suggested that the sequence is repeated in *K. pneumonia* as it is in *Y. enterocolitica* (data not shown).

Hybridization of *inv* and *ail* to *Y. pseudotuberculosis*. As noted above, all four probes used in this study hybridized to DNA isolated from *Y. pseudotuberculosis* YPIII. To determine whether this is generally true, we examined nine other *Y. pseudotuberculosis* strains isolated from both humans and animals (generously provided by Tom Quan, Division of Vector-Borne Viral Diseases, Centers for Disease Control, Atlanta, Ga.). All of these strains were TCI^+ and hybridized to all probes (data not shown). Two different hybridization patterns were observed with the Inv-Pstb probe, but there was no obvious correlation between serotype or source and hybridization pattern. In addition to the fragment that hybridized to AilB in all strains, a few strains also showed four other weakly hybridizing fragments; these fragments were the same for all strains that exhibited the phenotype. Although only a few *Y. pseudotuberculosis* strains were examined, it appeared that the species *Y. pseudotuberculosis* was more homogeneous with regard to the *inv* and *ail* loci than was *Y. enterocolitica*.

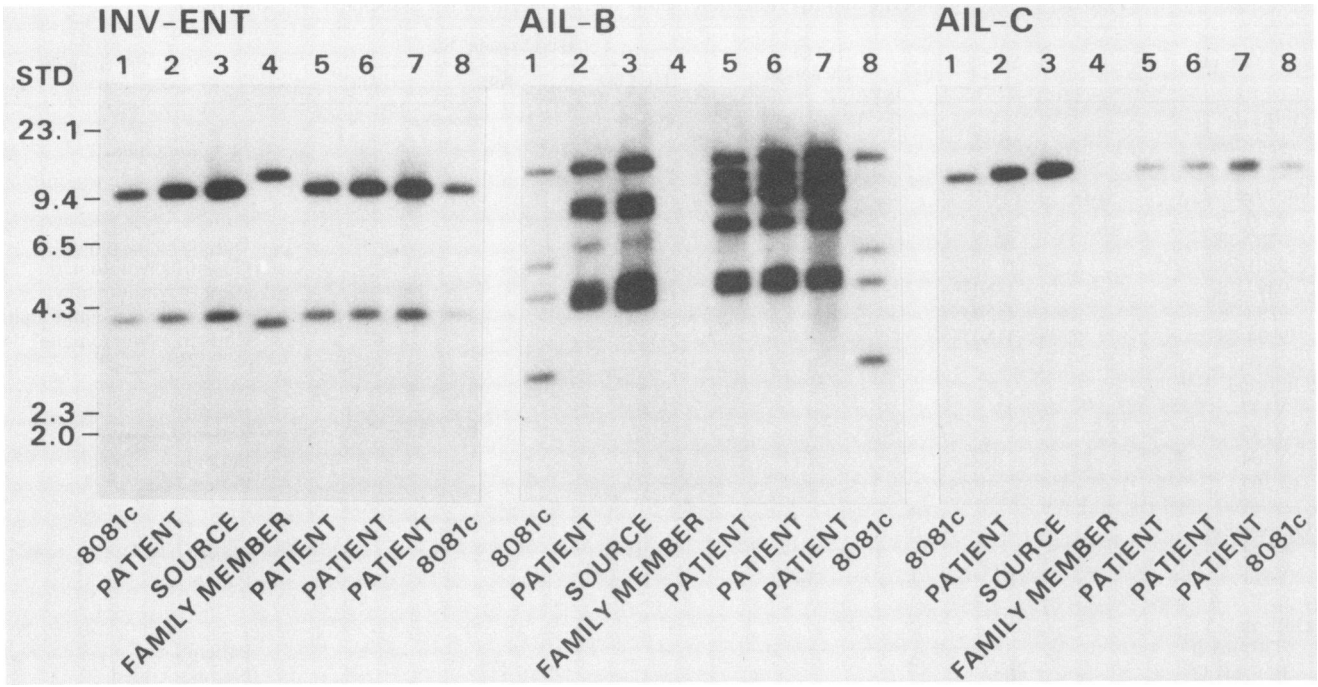

FIG. 4. Hybridization phenotypes of *Y. enterocolitica* strains isolated during outbreaks. Chromosomal DNA was purified from the strains listed below and treated as described in the legend to Fig. 3. STD, Position and size (in kilobases) of λ DNA digested in *Hin*dIII. Lanes contain DNA isolated from the following strains: 1, 8081c (O8); 2, 9286-78 (O20); 3, 9287-78 (O20); 4, 9291-78 (O6); 5, Y137 (O13a,13b); 6, Y135 (O13a,13b); 7, Y165 (O13a,13b); 8, 8081c (O8). Lanes 2, 3, and 4 contain DNA isolated from strains from outbreak 2. Lanes 5 through 7 contain DNA isolated from strains from outbreak 3.

DISCUSSION

Several distinct patterns emerge after examination of a large number and variety of *Y. enterocolitica* strains for ability to invade tissue culture cells (TCI phenotype) and for hybridization of chromosomal DNA to probes derived from the *inv* and *ail* loci. Strains of serotypes that were associated with disease were TCI^+, whereas strains of serotypes not normally associated with disease were TCI^-. It appears, therefore, that tissue culture invasiveness is a good indicator of potentially pathogenic serotypes. This conclusion, which has been proposed in several other studies (31, 32, 46, 52), is strengthened by our observation that strains that were strongly implicated as the cause of outbreaks of gastrointestinal disease were exclusively TCI^+ and that strains isolated from healthy individuals at the same time and in the same locale as strains associated with disease were often TCI^-. The ability to invade tissue culture cells is a good in vitro assay for identifying potentially pathogenic strains. Unlike plasmid-associated phenotypes, TCI is stably maintained even after prolonged storage or cultivation.

We recently reported the cloning of two different invasion genes, *inv* and *ail*, from a *Y. enterocolitica* serotype O8 strain (38). These genes confer on *E. coli* HB101 the capacity to enter cultured human cells (38). Definitive proof that one or both of these genes are required by *Y. enterocolitica* for invasion in vivo remains to be obtained, but several lines of evidence suggest that this is the case. First, mutations in the *inv* gene of *Y. pseudotuberculosis* render the bacteria TCI^- for HEp-2 cells (27). Second, strains of *Y. enterocolitica* and *Y. pseudotuberculosis* that are TCI^+ are invasive in animal models (51–53). Third, the results presented above demonstrate clear differences between both the *inv* and *ail* loci of potentially pathogenic strains and nonpathogenic strains of the genus *Yersinia*.

Perhaps the most striking result is that TCI^- *Y. enterocolitica* strains entirely lack any sequences homologous to the *ail* gene. In contrast, 85 of 86 TCI^+ strains contain sequences homologous to the *ail* locus. By using probes derived from DNA around *ail*, we determined that this region of DNA unique to potentially pathogenic strains is at least 6 kb (V. Miller, unpublished data); we have yet to find a probe from the cloned *ail* locus that hybridizes to TCI^- strains. This result suggests that *ail*, or a gene near *ail*, is an essential virulence factor. This hypothesis is further supported by the observation that the other two *Yersinia* species generally recognized as pathogenic, *Y. pestis* and *Y. pseudotuberculosis*, have sequences homologous to *ail* whereas nonpathogenic *Yersinia* species do not. It has frequently been observed in other genera that nonpathogenic strains or species lack sequences homologous to recognized virulence factors. Some examples are the cholera toxin genes of *Vibrio cholerae* (39), the diphtheria toxin gene of *Corynebacterium diphtheriae* (18, 20), the hemolysin gene of *E. coli* (29, 33), and the immunoglobulin A protease gene of the neisseriae (30). Virulence genes such as those listed above may be inherited as a block as part of either a phage, a plasmid, a transposon, or a structure that shows remnants of a transposonlike (13) structure. Could the *ail* locus of *Y. enterocolitica* be another example of this?

TCI^- *Y. enterocolitica* strains do not exhibit homology to the AilB probe. Single fragments of DNA isolated from TCI^+ strains of serotypes commonly isolated in Europe,

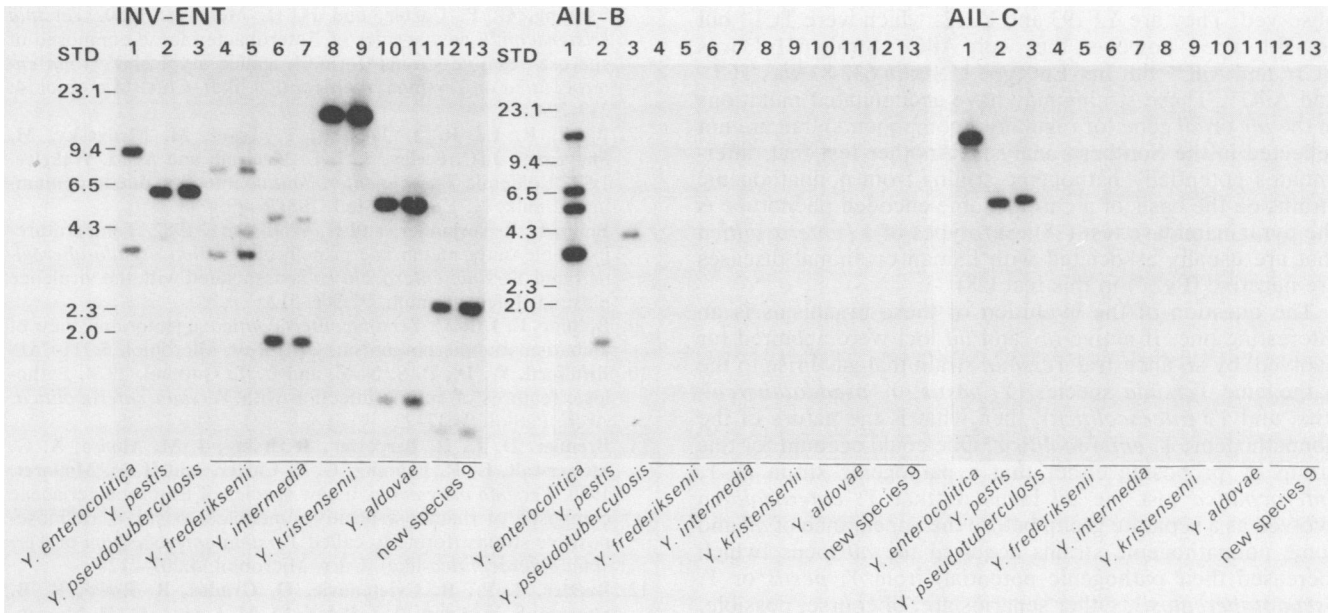

FIG. 5. Presence of *inv* and *ail* sequences among the yersiniae. Chromosomal DNA was purified from the strains listed below and treated as described in the legend to Fig. 3. STD, Position and size (in kilobases) of λ DNA digested with *Hin*dIII. Lanes contain DNA isolated from the following strains: 1, 8081c; 2, EV76-6; 3, YPIIIc; 4, Y225; 5, Y226; 6, Y227; 7, Y228; 8, Y232; 9, Y233; 10, 669-83; 11, 670-83; 12, 3022-85; 13, 3435-85. New species 9 (lanes 13) has been renamed *Y. rohdei*.

Japan, and Canada hybridize only weakly to this probe. These results suggest either that only a very short sequence present on the probe is found in these strains or that there has been extensive divergence of this sequence in these strains. There is strong hybridization to multiple DNA fragments of AilB by DNA isolated from the American serotypes, O13a,13b, O18, O20, O21, O4, O4,32, O4,33, and O8 (the *ail* locus was cloned from an American O8 strain). Phenotypic differences have previously been observed between American and other pathogenic *Y. enterocolitica* strains (9, 14). The American strains are considered to be more virulent by several criteria: they cause extraintestinal infections more frequently than do the non-American strains, some can cause keratoconjunctivitis in a guinea pig Sereny test, and some are lethal in several small rodent models (9, 14). Our data illustrate differences at the genetic level between these two sets of serotypes.

The pathogenic American strains not only have homology to AilB but also have multiple copies on the chromosome of a sequence present in AilB. The AilB probe is only 900 bp, and Southern analysis indicates that the sequence that is repeated elsewhere on the chromosome is entirely contained within AilB (V. Miller, unpublished data). Therefore, the repeated sequence is less than 900 bp, a size similar to those of described insertion sequences (13). It is not known whether this sequence is an insertion sequence, but consistent with this possibility is the observation that both the number of repeats and their locations on the chromosome vary from strain to strain. Although there is considerable variability among strains, strains isolated from the same outbreak have the same pattern of hybridization to AilB. This probe may therefore be useful in epidemiological studies for identifying the source cases of an outbreak and for distinguishing epidemiologically related clinical isolates.

Pathogenic and nonpathogenic serotypes of *Y. enterocolitica* also exhibit differences at their *inv* loci. All strains, both TCI^+ and TCI^-, have DNA homology to the Inv-Ent probe, which contains both *inv* and adjacent sequences. The Inv-Pstb probe, which contains only *inv* sequences, also hybridizes to DNA isolated from most of these strains. Therefore, we conclude that *inv*-related sequences are ubiquitous among *Y. enterocolitica* strains. These *inv*-related sequences may or may not represent functional genes.

Although both virulent and avirulent strains have the *inv* locus, distinct differences were observed between these groups on the basis of the sizes of the fragments that hybridized to the Inv-Ent probe. Potentially pathogenic serotypes were always either type I or type I-II in hybridization to the probe. Of particular interest are the six strains that exhibited the type I-II pattern. All six were serotype O3 (but not all O3 strains were type I-II); two strains were implicated as the cause of postinfectious arthritis, and one strain was implicated in a case of Graves disease (57). No other strains in our collection were known to be associated with these postinfectious complications of yersiniosis. It has long been appreciated that serotype O3 is associated with these syndromes, which raises the suggestion that there are "arthritogenic" strains (9). However, no clear evidence other than association with serotype O3 has been found for an arthritogenic strain. Our sample is far too small to allow us to draw any conclusions, but the type I-II hybridization pattern to Inv-Ent may serve as a marker for such strains.

The probes derived from the *inv* and *ail* loci may be useful in clinical laboratories to identify pathogenic *Yersinia* isolates because, as we have shown, there are clear differences between potentially pathogenic serotypes and nonpathogenic serotypes in response to the probes. This is true not only for *Y. enterocolitica* but for the genus *Yersinia* as a whole. The Inv-Ent probe is specific for the genus *Yersinia*, whereas the AilC probe is specific for the pathogenic members of the genus *Yersinia*. These genes are chromosomally located; therefore, they may be a better predictor of previously pathogenic serotypes than are probes derived from the virulence plasmid. Of 177 strains examined, only four ex-

ceptions to the patterns we have described above were observed. They are YF193 and Y312, which were TCI^- but Inv-Ent type I; however, they were $AilC^-$. Strain 641-83 was TCI^+ and $AilC^-$ but Inv-Ent type I. Strain 632-83 was TCI^- and $AilC^+$. These strains may have accumulated mutations in the *inv* or *ail* gene (or regulatory components) that are not reflected in the Southern analysis. Another test that differentiates potentially pathogenic strains from nonpathogenic strains on the basis of a chromosome-encoded phenotype is the pyrazinamidase test (28). Serotypes of *Y. enterocolitica* that are usually associated with human or animal diseases are negative (Pyz^-) in this test (28).

The question of the evolution of these organisms is an interesting one. If active *inv* and *ail* loci were acquired (or evolved) by an ancestral *Yersinia* strain that gave rise to the pathogenic *Yersinia* species (*Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica*), then what is the nature of the nonpathogenic *Y. enterocolitica*? One could account for this group by proposing either that a pathogenic strain of *Y. enterocolitica* lost the *ail* locus or that *Y. enterocolitica* evolved as a separate group before the appearance of *ail* and some nonpathogenic strains acquired the *ail* locus (which increased their pathogenic potential) from *Y. pestis* or *Y. pseudotuberculosis*. Other senarios are, of course, possible. Complicating the issue is that not one but two loci separated by at least 15 kb are involved, since the *inv* locus also exhibits differences between these two groups. The observation that Ail^+ is always associated with Inv I or Inv I-II whereas Ail^- is always associated with Inv II, Inv III, Inv IV, or Inv V suggests that the pathogenic and nonpathogenic strains represent two distinct clonal groups. This type of clonality has been observed for *E. coli* (22), which, like *Y. enterocolitica*, is a heterogeneous species (9).

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