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Evaluation of DNA Colony Hybridization and Other Techniques for Detection of Virulence in *Yersinia* Species

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The virulence of yersiniae varies according to (i) species and biotype and (ii) possession of a 67- to 72-kilobase virulence plasmid. *Y. pestis*, *Y. pseudotuberculosis*, and biotypes 1B, 2, 3, 4, and 5 of *Y. enterocolitica* are inherently virulent but express full virulence only when in possession of a virulence plasmid. Other *Yersinia* species and biotypes 1A and 3B of *Y. enterocolitica* are seldom implicated in disease. In this study, we prepared DNA probes from eight nonoverlapping regions of the virulence plasmid of a strain of *Y. enterocolitica* and from the *inv* and *ail* chromosomal loci responsible for the invasive capacity of *Y. enterocolitica* and *Y. pseudotuberculosis*. The probes were used in colony hybridization experiments to investigate 156 yersiniae of various species and biotypes and of differing virulence. Probes prepared from the *inv* gene of *Y. pseudotuberculosis* hybridized with *Y. pseudotuberculosis* and *Y. pestis* only, whereas an analogous probe prepared from *Y. enterocolitica* hybridized with all species and biotypes of yersiniae (but not with other bacteria) regardless of virulence or potential virulence. Probes prepared from the *ail* region of *Y. enterocolitica* reacted almost exclusively with *Y. enterocolitica* strains of pathogenic biotypes. Probes prepared from the virulence plasmid of a serogroup O:8, biotype 1B isolate of *Y. enterocolitica* identified virulent yersiniae in all species with a high degree of sensitivity and specificity. These probes did not react with yersiniae of avirulent biotypes or species. Of the other assays of virulence evaluated (calcium dependence, binding of crystal violet, and pyrazinamidase activity), binding of crystal violet provided a simple means for identifying plasmid-bearing strains.

Yersinia enterocolitica and the closely related species *Y. frederiksenii*, *Y. intermedia*, and *Y. kristensenii* are widespread in nature. *Y. enterocolitica* is also a versatile enteric pathogen, causing a variety of disorders, including diarrhea, colitis, mesenteric adenitis, pharyngitis, hepatosplenic abscesses, and septicemia (10, 36). Infections with *Y. enterocolitica* are also noted for postinfectious immunologic sequelae, including arthritis, erythema nodosum, and glomerulonephritis (1, 10, 21). Not all strains of *Y. enterocolitica* are equally liable to cause disease, and because nonpathogens may readily contaminate food, water, or even clinical specimens, it is essential to be able to distinguish true pathogens from their similar, but comparatively benign, relatives.

Of the many serobiotypes of *Y. enterocolitica*, only a few regularly cause disease. Pathogenic strains appear to be restricted to strains of biotypes 1B, 2, 3, 4, and 5 (12). Thus, determination of the biotypes of isolates may provide useful information about their pathogenic potential. Recently, Kandolo and Wauters (19) reported that *Y. frederiksenii*, *Y. intermedia*, *Y. kristensenii*, and the nonpathogenic serobiotypes of *Y. enterocolitica* elaborate the enzyme pyrazinamidase, whereas strains belonging to pathogenic biotypes do not. This observation suggested that examining yersiniae for pyrazinamidase activity could form the basis of a useful screening test for virulence.

Pathogenic yersiniae are characterized by their ability to adhere to and invade cultured mammalian cells (25, 34, 37). Two genetic loci encoding invasive ability of yersiniae have recently been identified on the bacterial chromosome. These are the *inv* locus of *Y. enterocolitica* and *Y. pseudotuberculosis* and the *ail* (attachment invasion locus) region of *Y. enterocolitica* (18, 25). The relationships of these loci to each other and to the genetic determinants of serobiotype, including pyrazinamidase activity, are unknown.

In addition to belonging to selected serobiotypes and carrying specific chromosomal genes, virulent *Y. enterocolitica* (and virulent strains of *Y. pestis* and *Y. pseudotuberculosis*) also invariably carry a 67- to 72-kilobase (kb) virulence plasmid (12, 28–30, 33). Bacteria bearing this plasmid exhibit a distinctive phenotype characterized by dependence on calcium ions for growth at 37°C, expression of novel outer membrane proteins, increased hydrophobicity, increased affinity for crystal violet and Congo red, a tendency to agglutinate in media containing mammalian serum, and enhanced resistance to the bactericidal effects of normal human serum (6, 12, 28, 31). Possession of these properties has been used to infer virulence of strains isolated from food or clinical specimens, but many isolates give anomalous or contradictory results in these assays (20, 27, 31, 38). Accordingly, the reliability of these indirect assays of virulence is in doubt.

DNA from the virulence plasmids of yersiniae of various serogroups and even of different species shows considerable nucleotide homology. Hill et al. (17) and Gemski et al. (14) have shown that probes derived from plasmid DNA from one strain hybridize with plasmid DNA from other strains and thus can be used to detect virulent strains in a wide range of serogroups. In this study, we evaluated a series of radiolabeled DNA probes prepared from the virulence plasmid of a serogroup O:8 *Y. enterocolitica* isolate and from the cloned *inv* and *ail* regions of the *Yersinia* chromosome. These probes were examined for their ability to hybridize with colonies of virulent and avirulent yersiniae of various

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TABLE 1. Characteristics of *Yersinia* species used in this study

Species and biotype	No. of strains	No. of virulent strains[a]	Serogroup(s)
Y. enterocolitica[b]			
1A	42	0	O:5; O:6,30; O:6,31; O:7,8; O:7,13; O:9b; O:10; O:14; O:16; O:22; O:36; O:41,42; O:41,43
1B[c]	18	6	O:8; O:13a,13b; O:18,13b; O:20; O:21
2[c]	5	4	O:5,27; O:9
3[c]	5	4	O:1,2,3
3B	1	0	O:16,58
4[c]	27	19	O:1,3; O:3
5[c]	2	1	O:2; O:2,3
Y. frederiksenii	13	0	O:16,29; O:35; O:38; O:39; O:44; O:44,45
Y. intermedia	17	0	O:4,33; O:17; O:37; O:40; O:48; O:49; O:52,53; O:52,54; O:55,57
Y. kristensenii	7	0	O:11; O:12,25; O:12,26; O:28,50; O:46; O:50; O:52
Y. pestis[c]	6	3	
Y. pseudotuberculosis[c]	13	11	Types I, II, and III

[a] Determined by inoculating strains into mice pretreated with iron dextran and desferrioxamine B (32).
[b] Biotype determined by the schemes of Wauters and Bercovier et al. (4, 12).
[c] Biotypes or species known to include virulent strains.

species, biotypes, and serogroups. The results of these assays were compared with those of other in vitro and animal tests of virulence.

MATERIALS AND METHODS

Bacteria. The reference strain for all virulence assays and the source of plasmid DNA from which a number of radiolabeled probes were prepared was *Y. enterocolitica* A2635, a serogroup O:8 strain originally isolated during an outbreak of yersiniosis in which contaminated chocolate milk was the likely vehicle of infection (7). This well-characterized strain expresses all the traits associated with virulence, including lethality for mice, a positive guinea pig keratoconjunctivitis (Serény) test, calcium dependence, serum resistance, and increased affinity for crystal violet and Congo red (20, 32). The plasmidless derivative of A2635, A2635c, is negative in all these assays. Both strains demonstrate no pyrazinamidase activity. *Escherichia coli* HB101 (F$^-$ *hsdS recA ara proA galK lacY rpsL xyl mtl supE*) was used as a recipient of hybrid plasmids constructed during the cloning experiments (see below) (3).

Test bacteria comprised 156 *Yersinia* isolates, including 100 strains of *Y. enterocolitica* (57 of which were virulent biotypes), 13 of *Y. frederiksenii*, 17 of *Y. intermedia*, 7 of *Y. kristensenii*, 6 of *Y. pestis*, and 13 of *Y. pseudotuberculosis*. The characteristics of these bacteria are given in Table 1. The test bacteria were chosen from our culture collection to include representatives of almost all known serotypes and biotypes of *Y. enterocolitica* and related organisms, isolated from a variety of sources in widespread geographic areas.

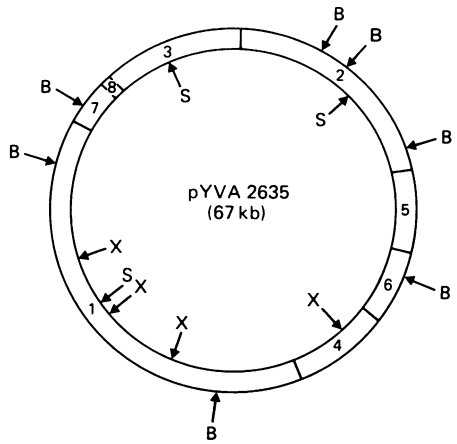

FIG. 1. *Bam*HI restriction map of the virulence plasmid of *Y. enterocolitica* A2635, showing the cleavage sites for *Bgl*II (B), *Sal*I (S), and *Xba*I (X). The sizes of *Bam*HI restriction fragments 1 to 8 are 26, 14, 8.5, 6, 5, 4.2, 2.8, and 0.5 kb, respectively.

Of the *Yersinia* species tested, 74 were obtained from clinical specimens from humans, 29 were from animals, and 42 were of food or environmental origin. Included among these was a reference collection of 47 strains incorporating all known serogroups of *Y. enterocolitica* and related bacteria, kindly provided by G. Wauters, Université Catholique de Louvain, Brussels, Belgium. We also tested 11 derivatives of virulent yersiniae (6 of *Y. enterocolitica*, 3 of *Y. pestis*, and 2 of *Y. pseudotuberculosis*) from which the virulence plasmid had been cured by sequential passage on agar containing magnesium oxalate at 37°C (11, 16).

DNA probes were also evaluated on a collection of 56 bacteria, mostly enterobacteria other than yersiniae, isolated from clinical material. These bacteria included *E. coli* (19 isolates, representing the major diarrheagenic categories: enteropathogenic, enterotoxigenic, enterohemorrhagic, enteroinvasive, and enteroadherent-aggregative), *Salmonella typhi* (2 isolates), nontyphoid salmonellae (13 isolates), *Shigella* species (11 isolates), *Enterobacter* species (3 isolates), and 2 isolates each of *Klebsiella* species, *Plesiomonas shigelloides*, *Pseudomonas* species, and *Vibrio cholerae*. All bacteria were maintained in Luria broth (24) containing 30% (vol/vol) glycerol at −70°C or on brain heart infusion agar (Difco Laboratories, Detroit, Mich.) at 4°C.

Plasmids. pYVA2635 is the 67-kb virulence plasmid of strain A2635 (Fig. 1). pBR322 and pBR325 are cloning vectors that carry determinants for resistance to ampicillin and tetracycline (8, 9). pRK404 is a 10.6-kb broad-host-range plasmid, derived from pRK290, that encodes resistance to tetracycline and includes a unique *Bam*HI target within a *lacZ* gene (13).

pRI203 is a derivative of pBR325 that contains a 4.5-kb insert, encompassing the *inv* locus from a *Y. pseudotuberculosis* strain (18). This locus encodes the ability of *Y. pseudotuberculosis* to invade cultured epithelial cells. A homologous locus from a serogroup O:8 strain of *Y. enterocolitica*, 8081c, has been cloned into the vector plasmid pBR322 to give the recombinant plasmid pVM101 (25). pVM103 is another derivative of pBR322 and contains the cloned *ail* region from the chromosome of 8081c (Fig. 2). This region, which shares no homology with the *inv* locus, confers a phenotype of adhesion and low-level invasion on *E. coli* HB101 (25). The properties of these plasmids and

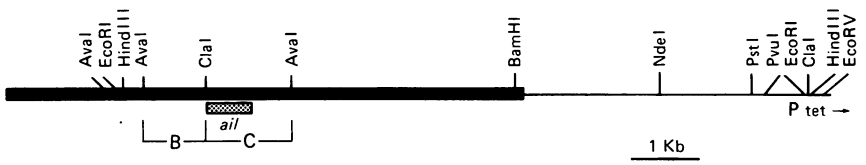

FIG. 2. Restriction map of pVM103 showing the *ail* region and the location of fragments B and C used to prepare the Ail-B and Ail-C probes, respectively. The thick line represents DNA cloned from the chromosome of *Y. enterocolitica* 8081c; the thin line represents the cloning vector pBR322. Modified from a previous study of Miller and Falkow (25).

others constructed during the course of this study are given in Table 2.

Plasmid purification. Plasmid DNA was extracted from *Y. enterocolitica* A2635 by the alkali lysis method and purified by ultracentrifugation in a cesium chloride gradient (22). A restriction map of the plasmid (Fig. 1) was obtained by incubating purified plasmid DNA with the restriction endonucleases *Bam*HI, *Bgl*II, *Sal*I, and *Xba*I (Bethesda Research Laboratories, Gaithersburg, Md.), alone or in pairs, under conditions specified by the manufacturer. Fragments thus obtained were separated by electrophoresis in a 0.7% (wt/vol) agarose gel at 70 V for 4 h and sized by comparing their rates of migration with that of a *Hin*dIII digest of DNA from bacteriophage lambda.

Because the virulence plasmid of *Y. enterocolitica* A2635 is present at a low copy number and is subject to a high rate of spontaneous curing, we experienced some difficulty in obtaining sufficient quantities of pYVA2635 DNA from which to prepare DNA probes. We therefore cloned the eight largest *Bam*HI fragments of this plasmid into a multicopy vector plasmid in *E. coli*. This was achieved by cleaving pYVA2635 and pBR325 with *Bam*HI, ligating the mixture with T4 ligase, and using it to transform *E. coli* HB101. Transformants selected on agar containing 30 mg of chloramphenicol per liter were replica plated on tetracycline-containing agar to permit identification of plasmid-bearing strains that contained an insert in the unique *Bam*HI site in the tetracycline resistance gene of pBR325. Plasmids purified from these bacteria were digested with *Bam*HI; their inserts were identified by comparing their rates of migration with that of pYVA2635 reacted with the same enzyme, and subsequently by Southern blotting (see below). *Bam*HI fragments of pYVA2635 amplified in this way were eluted from agarose gels and ligated into the *Bam*HI cloning site within the *lacZ* gene of pRK404 (13; M. D. Miliotis et al., manuscript in preparation). The hybrid plasmids obtained from these experiments served as the source of DNA for probes derived from the virulence plasmid (Table 2).

TABLE 2. Characteristics of plasmids used in this study

Plasmid	Description	Reference
pBR322	4.3-kb cloning vector (Ap^r Tc^r)	9
pBR325	6-kb cloning vector (Ap Cm^r Tc)	8
pRK404	10.6-kb cloning vector (Tc *lacZ*)	13
pYVA2635	67-kb virulence plasmid of *Y. enterocolitica* A2635	This study
pCVD777	Clone of 26-kb *Bam*HI fragment of pYVA2635 in pRK404	This study
pCVD787	Clone of 14-kb *Bam*HI fragment of pYVA2635 in pRK404	This study
pCVD771	Clone of 8.5-kb *Bam*HI fragment of pYVA2635 in pRK404	This study
pCVD775	Clone of 6-kb *Bam*HI fragment of pYVA2635 in pRK404	This study
pCVD795	Clone of 5-kb *Bam*HI fragment of pYVA2635 in pRK404	This study
pCVD794	Clone of 4.2-kb *Bam*HI fragment of pYVA2635 in pRK404	This study
pCVD788	Clone of 2.8-kb *Bam*HI fragment of pYVA2635 in pRK404	This study
pCVD797	Clone of 0.5-kb *Bam*HI fragment of pYVA2635 in pRK404	This study
pRI203	Cloned *inv* locus of *Y. pseudotuberculosis* in pBR325	18
pVM101	Cloned *inv* locus of *Y. enterocolitica* in pBR322	25
pVM103	Cloned *ail* locus of *Y. enterocolitica* in pBR322	25

[a] Ap, Tc, and Cm, Resistance to ampicillin, tetracycline, and chloramphenicol, respectively.

Preparation of DNA probes. Restriction fragments used to prepare DNA probes were as follows: the eight largest *Bam*HI fragments of pYVA2635 (26, 14, 8.5, 6, 5, 4.2, 2.8, and 0.5 kb; Fig. 1), a 4.5-kb *Bam*HI fragment from pRI203 encompassing the *inv* locus of *Y. pseudotuberculosis*, a 3.7-kb *Cla*I fragment of pVM101 incorporating part of the *inv* locus of *Y. enterocolitica* 8081c and adjacent sequences, and two fragments derived from pVM103 (a 1.2-kb *Ava*I-*Cla*I fragment incorporating the *ail* region of 8081c [the Ail-C probe] and an adjacent 0.9-kb *Ava*I-*Cla*I fragment [the Ail-B probe] [Fig. 2]). DNA fragments intended for use as probes were separated from unrequired plasmid DNA by electrophoresis and labeled with ^{32}P by nick translation (Bresa Ltd., Adelaide, South Australia, Australia) (2).

Hybridization experiments. For colony hybridization, bacteria were cultivated on nylon membranes (Hybond-N; Amersham International, Buckinghamshire, England) on brain heart infusion agar at 30°C for 24 h. The membranes were processed to extract bacterial DNA and fix it to the filters as recommended by the manufacturer. To reduce nonspecific binding of probe DNA, membranes were further treated by immersion in 0.05 M Tris hydrochloride (pH 7.5) containing 2 g of proteinase K per liter and 0.5% Triton X-100 for 15 min at room temperature. Hybridization with radiolabeled probe DNA was carried out at moderately high stringency (incubation at 65°C and inclusion of a final wash in low-salt buffer at 65°C) for the plasmid-derived probes and at very high stringency (incubation at 78°C and a final wash in low-salt buffer at 78°C) for the Inv and Ail probes (23). Bound label was visualized by autoradiography, using X-Omat RP X-ray film (Eastman Kodak Co., Rochester, N.Y.).

For Southern hybridization, DNA was transferred to Zeta-Probe blotting membranes (Bio-Rad Laboratories, Richmond, Calif.). Hybridization and autoradiography were carried out as described above.

Determination of bacterial virulence in vivo and virulence-associated properties in vitro. Bacterial virulence was determined in mice pretreated with iron dextran and desferriox-

TABLE 3. Hybridization (at high stringency) of bacteria with probes derived from virulence determinants cloned from the chromosomes of *Y. enterocolitica* and *Y. pseudotuberculosis*

Species	No. of strains examined	No. of strains belonging to virulent biotype	No. of strains that hybridized with probes derived from locus:			
			Y. pseudotuberculosis inv	*Y. enterocolitica*		
				inv	*ailB*	*ailC*
Y. enterocolitica	100	57	0	100	18[a]	53[b]
Y. frederiksenii	13	0	0	13	0	0
Y. intermedia	17	0	0	17	0	1
Y. kristensenii	7	0	0	7	0	0
Y. pestis	6	6	6	6	0	0
Y. pseudotuberculosis	13	13	13	13	0	0
Nonyersiniae	56		0	0	0	0

[a] All were biotype 1B strains.
[b] Fifty-two strains belonged to virulent biotypes.

amine B (32). Assays for calcium dependence, binding of crystal violet, and pyrazinamidase activity were performed in accordance with published methods (6, 16, 19). For all assays, test bacteria were coded so that results could be read independently without knowledge of the outcome of other assays. The sensitivity and specificity of the various assays were calculated by published formulae (15).

RESULTS

Of the 156 *Yersinia* strains, 48 (31%) were virulent as judged by their capacity to kill mice given iron dextran and desferrioxamine B. All these strains belonged to species (*Y. enterocolitica*, *Y. pestis*, and *Y. pseudotuberculosis*) and biotypes (*Y. enterocolitica* biotypes 1B, 2, 3, 4, and 5) known to harbor virulent strains. Evidently, these bacteria possess a full complement of chromosomal and plasmid-borne genes required for virulence. All virulent strains carried plasmids of approximately 67 to 72 kb (data not shown). Furthermore, no strain that lacked a plasmid in this size range, nor any of the 11 virulent strains cured of their plasmids, was virulent for mice.

Virulence assays associated with chromosomal determinants. (i) DNA probes. Under the high-stringency conditions used, the Inv probe cloned from *Y. pseudotuberculosis* hybridized only with colonies of *Y. pseudotuberculosis* and *Y. pestis*. All 19 isolates of these species reacted with this probe, including the five isolates from which the virulence plasmid had been cured.

In contrast, the Inv probe derived from the chromosome of *Y. enterocolitica* 8081c hybridized with strains of all *Yersinia* species regardless of virulence or potential virulence. In some instances, notably with *Y. pestis* and *Y. pseudotuberculosis* types I and II, the binding of probe DNA was relatively weak, as evidenced by a reaction of low intensity in the autoradiographs.

The Ail-B probe, which also was cloned from the chromosome of *Y. enterocolitica* 8081c, hybridized with all 18 *Y. enterocolitica* strains of biotype 1B irrespective of serogroup or plasmid profile. No other strains were recognized by this probe.

The Ail-C probe, derived from a region of the chromosome adjacent to Ail-B (Fig. 2), also recognized all biotype 1B isolates but was less specific than Ail-B insofar as it reacted with 1 (serogroup O:22) of 42 biotype 1A strains, 4 of 5 biotype 2 strains, all 5 biotype 3 strains, 23 of 27 biotype 4 strains, and both biotype 5 strains, as well as with 1 (serogroup O:37) of 17 isolates of *Y. intermedia*. Here too, binding of the probe was unrelated to plasmid carriage. Of the *Y. enterocolitica* strains not recognized by Ail-C, only one, a serogroup O:3 biotype 4 strain, was plasmid bearing and virulent for mice.

No strain of *Y. frederiksenii*, *Y. kristensenii*, *Y. pestis*, or *Y. pseudotuberculosis* was recognized by either Ail probe. Moreover, none of the Inv or Ail probes recognized any bacteria other than yersiniae. The results of these assays are given in Table 3.

(ii) Pyrazinamidase activity. Pyrazinamidase was not detected in 66 of 76 (87%) yersiniae potentially lethal to mice (i.e., *Y. pestis*, *Y. pseudotuberculosis*, and virulent biotypes of *Y. enterocolitica*). Enzyme activity was present, however, in the following potentially virulent strains: 8 *Y. enterocolitica* strains (4 of 5 biotype 3, 2 of 25 biotype 4, and both biotype 5) and two *Y. pseudotuberculosis* strains. One *Y. enterocolitica* strain of biotype 1A was negative in this assay, a result expected with potentially virulent strains only.

Virulence assays associated with plasmid carriage. (i) Plasmid-derived probes. The results of the colony hybridization experiments performed with the plasmid-derived probes are given in Table 4. Probe positivity correlated closely with virulence. False-positive reactions were observed with two strains only. One was a serogroup O:3 strain that reacted

TABLE 4. Results of in vitro assays of virulence of *Yersinia* species related to presence of virulence plasmid

Species	No. of strains examined	No. of virulent strains[a]	No. of strains positive in the following assays:									
			Calcium dependence	Crystal violet binding	Hybridization with probe derived from plasmid fragment[b]:							
					1	2	3	4	5	6	7	8
Y. enterocolitica	100	34	36 (5)[c]	37 (4)	32 (0)	35 (1)	34 (0)	34 (0)	34 (0)	34 (0)	36 (2)	34 (1)
Y. frederiksenii	13	0	0	0	0	0	0	0	0	0	0	0
Y. intermedia	17	0	1 (1)	2 (2)	0	0	0	0	0	0	0	0
Y. kristensenii	7	0	0	0	0	0	0	0	0	0	0	0
Y. pestis	6	3	3 (0)	3 (0)	3 (0)	3 (0)	3 (0)	3 (0)	3 (0)	3 (0)	3 (0)	3 (0)
Y. pseudotuberculosis	13	11	10 (0)	11 (0)	11 (0)	11 (0)	11 (0)	11 (0)	11 (0)	11 (0)	11 (0)	11 (0)
Nonyersiniae	56	0	NT[d]	NT	0	0	0	0	0	0	0	0

[a] Virulence was determined in mice treated with iron dextran and desferrioxamine B (32).
[b] Refer to Fig. 1 and Table 2 for explanation.
[c] Number of false-positive results is given in parentheses.
[d] NT, Not tested.

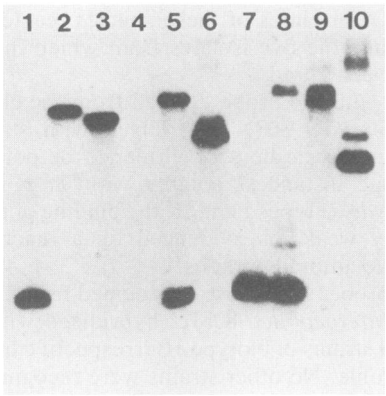

FIG. 3. Southern blot analysis of virulence plasmids of *Yersinia* species. DNA was extracted from overnight cultures of bacteria by the alkali lysis method (22), digested with *Bam*HI, separated by electrophoresis on a 0.7% (wt/vol) agarose gel at 70 V for 4 h, and transferred to nitrocellulose paper. ^{32}P-labeled probe DNA was prepared from the 2.8-kb *Bam*HI fragment (fragment 7) of pYVA2635. Lane 1, *Y. enterocolitica* A2635 (serogroup O:8); lane 2, *Y. enterocolitica* serogroup O:1,2,3; lane 3, *Y. enterocolitica* serogroup O:3; lane 4, *Y. enterocolitica* serogroup O:3 cured of the virulence plasmid; lane 5, *Y. enterocolitica* 8081 (serogroup O:8, see reference 30); lane 6, *Y. enterocolitica* serogroup O:9; lane 7, *Y. enterocolitica* serogroup O:13a,13b; lane 8, *Y. enterocolitica* serogroup O:20; lane 9, *Y. enterocolitica* serogroup O:5,27; lane 10, *Y. pestis*. Note that despite differences in the restriction patterns of these plasmids, much of the DNA sequence in fragment 7 is conserved. Similar results were obtained with probes prepared from other regions of the plasmid (data not shown).

with probes derived from fragments 2, 3, 7, and 8 but was avirulent. This strain carried a 60-kb plasmid from which a spontaneous deletion of a region analogous to *Bam*HI fragments 4, 5, and 6 of pYVA2635 had evidently taken place (data not shown). The fragment 7 probe also reacted with a second serogroup O:3 strain which was negative with all the other plasmid-derived probes. The reaction of these probes with DNA from virulent *Y. enterocolitica* strains in various serogroups and with *Y. pestis* was confirmed by Southern blotting (Fig. 3).

False-negative reactions with plasmid-derived probes were uncommon but were observed with fragment 1: two strains (one serogroup O:3 and one O:5,27) were lethal for mice but were probe negative. The O:3 strain was also negative with the probe derived from fragment 8. None of the plasmid-derived probes reacted with *Y. frederiksenii*, *Y. intermedia*, or *Y. kristensenii* or with *Y. enterocolitica* of biotypes 1A or 3B. These probes also did not recognize any bacteria other than yersiniae.

(ii) Binding of crystal violet. The crystal violet-binding assay was approximately as sensitive as, but somewhat less specific than, that with the plasmid-derived probes (Table 4). False-positive reactions were obtained with two isolates of *Y. intermedia* and four isolates of *Y. enterocolitica* (including both strains that gave false-positive reactions with the fragment 7 probe). Only one false-negative result was observed. This was with the O:5,27 isolate that failed to hybridize with the fragment 1 probe.

(iii) Calcium dependence. Calcium dependence was the least sensitive and specific of the assays of plasmid carriage. False-positive reactions occurred with five isolates of *Y. enterocolitica* and one isolate of *Y. intermedia*. False-negative results were obtained with three isolates of *Y. enterocolitica* and one isolate of *Y. pseudotuberculosis*.

DISCUSSION

In this study we confirmed the usefulness of probes derived from the virulence plasmid of *Y. enterocolitica* in detecting virulent strains of this species and virulent *Y. pestis* and *Y. pseudotuberculosis*. Despite the reported low degree of homology between some regions of the virulence plasmids of *Y. enterocolitica* and *Y. pseudotuberculosis* (30), probes derived from every region of the *Y. enterocolitica* plasmid hybridized with all virulent *Y. pseudotuberculosis* and *Y. pestis* isolates examined. This indicates that substantial regions of the virulence plasmids are highly conserved and is consistent with the observation that these plasmids confer similar phenotypes on each of the three pathogenic species that carry them (12, 28).

Overall, assays with probes prepared from *Bam*HI fragments 4, 5, and 6, which coincide with the most highly conserved region of the plasmid responsible for calcium dependence (30; Miliotis et al., in preparation), were the most sensitive and specific of all the assays investigated (Table 4). The comparatively low sensitivity of the probe prepared from the largest (26-kb) *Bam*HI fragment of the plasmid may have been related to the size of the fragment, as large pieces of DNA are not always suitable for probe preparation by nick translation (2).

Under the high-stringency conditions used for colony hybridization in this study, the Inv probe derived from the chromosome of *Y. pseudotuberculosis* detected only strains of the same species and the genetically indistinguishable species, *Y. pestis* (5). Surprisingly, the analogous Inv probe cloned from the chromosome of *Y. enterocolitica* was far less specific, hybridizing with all *Yersinia* strains examined, regardless of species, virulence, or potential virulence. The probable explanation for this apparent discrepancy is that the Inv probe of *Y. enterocolitica* includes DNA from regions adjacent to the *inv* locus, whereas the analogous probe from *Y. pseudotuberculosis* is wholly contained within the *inv* region (25, 26). The *Y. enterocolitica* Inv probe does appear to be genus specific, however, as it did not react with bacteria in several other genera.

The Ail-B probe cloned from the chromosome of *Y. enterocolitica* was extremely sensitive and specific, reacting only with *Y. enterocolitica* strains of biotype 1B, the so-called American biotype (12, 35). This indicates the presence of a conserved region of the chromosome in these serologically heterogeneous bacteria and suggests that they may have evolved from a common ancestor. In contrast, the Ail-C probe, from a portion of the chromosome adjacent to Ail-B, detected potentially virulent *Y. enterocolitica* of all biotypes and serogroups with a sensitivity of 91%, a specificity of 99%, and positive and negative predictive values of 98 and 94%, respectively.

The results of the hybridization experiments with probes derived from the bacterial chromosome are in broad agreement with those recently reported by Miller et al. (26). In addition to the findings reported here, however, Miller et al. observed hybridization of the Inv probe of *Y. pseudotuberculosis* to *Y. enterocolitica* and of the Ail-B and -C probes to *Y. pestis* and *Y. pseudotuberculosis*. This apparent conflict is explained by the conditions of reduced stringency used by these workers for their hybridization studies.

Although the assays for calcium dependence, binding of crystal violet, and pyrazinamidase activity have been evaluated previously (6, 19, 27, 31), they had not heretofore been compared with each other or with mouse virulence. Of these assays, binding of crystal violet provided a reasonably

accurate yet simple means of identifying plasmid-bearing yersiniae, although false-positive reactions with clearly avirulent strains did occur. Interpretation of calcium dependence is difficult, even with experience. This assay is not recommended, particularly because it selects for avirulent variants, the growth of which is favored by the conditions of the test (11). The assay for pyrazinamidase activity may also be difficult to interpret, particularly with *Y. enterocolitica* strains of biotypes 3 and 5. Although clearly a useful addition to the biotyping scheme, the pyrazinamidase assay is not a substitute for full biotyping.

In summary, this study showed that DNA probes prepared from the virulence plasmid of *Y. enterocolitica*, in particular from the region associated with calcium dependence, provide a highly sensitive and specific means for testing the virulence of yersiniae of all species. Such probes may be used to detect pathogenic yersiniae in laboratory specimens. Probes derived from the bacterial chromosome, on the other hand, could be used to identify members of the genus *Yersinia* (the Inv probe of *Y. enterocolitica*), the species *Y. pestis* and *Y. pseudotuberculosis* (the Inv probe of *Y. pseudotuberculosis*), and pathogenic biotypes of *Y. enterocolitica* (Ail probes of *Y. enterocolitica*). Because these probes hybridize with plasmidless strains, however, they cannot be used to determine virulence per se.

This study also revealed new information about the relationship of the plasmid to virulence, first by demonstrating that no bacterium of an avirulent species or biotype carried DNA that hybridized with probes prepared from the virulence plasmid (although a number of these bacteria harbor plasmids in the 50- to 70-kb range [31, 38]) and second by uncovering two isolates that apparently lacked a substantial region of the plasmid, yet retained virulence. Further studies with these organisms are in progress in order to learn more about the genetic regulation of virulence in yersiniae.

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