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Development and Testing of Invasion-Associated DNA Probes for Detection of *Shigella* spp. and Enteroinvasive *Escherichia coli*

MALABI VENKATESAN,* JERRY M. BUYSSE, ERIC VANDENDRIES, AND DENNIS J. KOPECKO

Department of Bacterial Immunology, Division of Communicable Diseases and Immunology, Walter Reed Army Institute of Research, Washington, D.C. 20307-5100

Received 29 June 1987/Accepted 29 October 1987

Genetic determinants of the invasive phenotype of *Shigella* spp. and enteroinvasive *Escherichia coli* (EIEC), two common agents of bacillary dysentery, are encoded on large (180- to 210 kilobase), nonconjugative plasmids. Several plasmid-encoded antigens have been implicated as important bacterial ligands that mediate the attachment and invasion of colonic epithelial cells by the bacteria. Selected invasion plasmid antigen (*ipa*) genes have recently been cloned from *Shigella flexneri* serotype 5 into the λ gt11 expression vector. Portions of three *ipa* genes (*ipaB*, *ipaC*, and *ipaD*) were tested as DNA probes for diagnostic detection of bacillary dysentery. Under stringent DNA hybridization conditions, all three DNA sequences hybridized to a single 4.6-kilobase *Hin*dIII fragment of the invasion plasmids of representative virulent *Shigella* spp. and EIEC strains. No hybridization was detected in isogenic, noninvasive *Shigella* mutants which had lost the invasion plasmid or had deleted the *ipa* gene region. Furthermore, these probes did not react with over 300 other enteric and nonenteric gram-negative bacteria tested, including *Salmonella*, *Yersinia*, *Edwardsiella*, *Campylobacter*, *Vibrio*, *Klebsiella*, *Aeromonas*, *Enterobacter*, *Rickettsia*, and *Citrobacter* spp. and various pathogenic *E. coli* strains. The use of unique invasion-essential gene segments as probes for the specific detection of invasive dysentery organisms should benefit both epidemiologic and diagnostic analyses of *Shigella* spp. and EIEC.

All four species of *Shigella* and selected enteroinvasive *Escherichia coli* (EIEC) strains are capable of causing bacillary dysentery, a major health problem throughout the developing world that contributes significantly to the loss of 4.6 million lives each year due to diarrheal diseases (5). Bacteriological identification of shigellae in stool or environmental specimens requires 48 to 72 h, and identification of EIEC entails the additional use of an expensive and time-consuming animal assay for virulence (18). Therefore, in conjunction with oral rehydration therapy, the development of methods for the rapid diagnosis of acute illness and for epidemiologic surveys of dysentery bacilli would benefit the management of this disease. This report describes the development and testing of DNA probes that may be useful for specific detection of shigellae and EIEC.

Bacillary dysentery is characterized by invasion of the superficial layers of the colonic epithelium by virulent bacteria after their ingestion by a primate host (9; D. J. Kopecko, M. Venkatesan, and J. M. Buysse, *in* M. Farthing and G. Keusch, ed., *Enteric Infection: Mechanisms, Manifestations and Management*, in press). Subsequently, intracellular multiplication of the bacteria and dissemination to adjacent epithelial cells occur, and the resulting dysentery is characterized by a localized inflammatory response, painful abdominal cramps, diarrhea, and a small-volume rectal discharge of blood and mucus. Genetic analysis of *Shigella* spp. has revealed that multiple and widely separated chromosomal loci encode determinants of virulence (5, 8). In addition, recent studies have shown that the initial invasion step is directed by gene products expressed by a large, ~180- to 210-kilobase (kb) nonconjugative invasion plasmid present in all virulent *Shigella* and EIEC strains (5, 8, 15, 16, 21). Loss of the invasion plasmid results in a loss of the invasive phenotype and in subsequent avirulence. Reintroduction of the *Shigella* invasion plasmid can restore invasiveness to plasmid-free EIEC and shigellae strains (5). Invasion plasmids from various *Shigella* spp. and EIEC isolates have been found to encode the syntheses of several outer membrane polypeptides that range in size from 12 to 140 kilodaltons (kDa) (5–7). At least five of these outer membrane polypeptides (i.e., of 140, 78, 57, 43, and 39 kDa) are important bacterial immunogens; convalescent antisera obtained from humans and monkeys subsequent to a *Shigella* infection contain significant titers of antibodies to these plasmid-encoded antigens (4, 6, 13).

The λ gt11 expression vector has been used in this laboratory to clone invasion plasmid antigen (*ipa*) genes from a Tn*5*-tagged derivative (pWR110) of the *Shigella flexneri* serotype 5 (strain M90T-W) invasion plasmid (2). Polyclonal rabbit antisera specific for invasion plasmid antigens were used to select recombinant λ gt11 bacteriophage that synthesized the 57-, 43-, and 39-kDa antigens, the products of loci designated *ipaB*, *ipaC*, and *ipaD*, respectively. Insert DNA from these λ gt11 recombinants was subcloned into plasmid pUC8, and the purified fragments were used as radiolabeled probes for the detection of homologous sequences in enteric and nonenteric bacteria.

MATERIALS AND METHODS

Bacterial strains. Bacterial strains used in this study and their sources are listed in Table 1.

Media and culture conditions. Bacteria were routinely grown at 37°C in L broth or on plates of L broth agar. To examine the ability to bind Congo red dye, cells were grown on Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.) supplemented with Congo red and galactose at concentrations of 0.01 and 0.2%, respectively.

Isolation of invasion plasmid DNA. Large-scale isolation of plasmid DNA was carried out as previously described (2) and purified by two cycles of CsCl-ethidium bromide density gradient ultracentrifugation.

Restriction endonuclease digestion and gel electrophoresis.

* Corresponding author.

TABLE 1. Summary of in situ colony hybridization experiments

Bacterial species	Strain(s) tested	Source[a]	Response to probes[b]:		
			S12 *ipaB*	W22 *ipaC*	S10 *ipaD*
EIEC and invasive shigellae					
Shigella flexneri	M25-8 serotype 1b	WRAIR	+	+	+
	2457T serotype 2a	WRAIR	+	+	+
	J17B serotype 3	WRAIR	+	+	+
	M76-39 serotype 4	WRAIR	+	+	+
	M90T-W serotype 5	WRAIR	+	+	+
	2924-71 serotype 6	CDC	+	+	+
Shigella dysenteriae	11 Strains	CDC	+	+	+
Shigella boydii	8 Strains	CDC	+	+	+
Shigella sonnei	53G form I	WRAIR	+	+	+
	4 Strains	CDC	+	+	+
EIEC	4 Strains	WRAIR	+	+	+
Noninvasive shigellae					
Shigella flexneri	Serotype 1b	CDC	−	−	−
	Serotype 2b	CDC	−	−	−
	Serotype 3c	CDC	−	−	−
	Serotype 4b	CDC	−	−	−
	Serotype 5	CDC	−	−	−
	M90T-A_3 (serotype 5)	WRAIR	−	−	−
	Serotype 6	WRAIR	−	−	−
	10 Strains	B. Sachs	−	−	−
Shigella sonnei	53G form II	WRAIR	−	−	−
Other bacteria					
Salmonella typhi	WR4201 (ViaA$^+$ ViaB$^+$)	WRAIR	−	−	−
Salmonella typhi	6 Strains	CDC	−	−	−
Salmonella spp.	50 Serogroups	CDC	−	−	−
Edwardsiella hoshinae	1 Strain	CDC	−	−	−
Edwardsiella tarda biogroup 1	1 Strain	CDC	−	−	−
Citrobacter freundii	5 Strains	CDC	−	−	−
Citrobacter diversus	5 Strains	CDC	−	−	−
Campylobacter spp.	5 Strains	S. Kotarski	−	−	−
Enterobacter aerogenes	1 Strain	CDC	−	−	−
Enterobacter intermedium	1 Strain	CDC	−	−	−
Enterobacter cloacae	1 Strain	CDC	−	−	−
Klebsiella oxytoca	1 Strain	CDC	−	−	−
Vibrio parahemolyticus	2 Strains	J. Kaper	−	−	−
Vibrio cholerae	2 Strains	J. Kaper	−	−	−
Yersinia enterocolitica	100 Strains	M. A. Sodd	−	−	−
Aeromonas hydrophila	2 Strains	J. Kaper	−	−	−
Enterobacter taylorae	1 Strain	CDC	−	−	−
Enterobacter agglomerans	1 Strain	CDC	−	−	−
Escherichia coli K-12	C600	WRAIR	−	−	−
	HB101	WRAIR	−	−	−
	JM107	WRAIR	−	−	−
EHEC[c]	0157-H7 (6 strains)	CDC	−	−	−
EPEC	0111-H1	CDC	−	−	−
	0126-H27	CDC	−	−	−
	055-H6	CDC	−	−	−
	055-H$^-$	CDC	−	−	−
	0125-4115	CDC	−	−	−
ETEC LT[d]	7 Strains	CDC	−	−	−
ETEC ST[e]	3 Strains	CDC	−	−	−
ETEC LT/ST	2 Strains	CDC	−	−	−

[a] CDC, Centers for Disease Control; WRAIR, Walter Reed Army Institute of Research.
[b] +, Hybridization to the probe; −, no hybridization.
[c] EHEC, Enterohemorrhagic *E. coli*.
[d] ETEC LT, Enterotoxigenic *E. coli* producing heat-labile toxin.
[e] ETEC ST, Enterotoxigenic *E. coli* producing heat-stable toxin.

Plasmid DNA was digested with *Hin*dIII restriction enzyme (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) as specified by the manufacturer. Whole and restriction enzyme-digested DNA was electrophoresed on agarose gels. The DNA restriction fragments were separated by electrophoresis in 0.8% agarose gels (made with 20 mM Tris acetate [pH 7.9]–20 mM sodium acetate–1 mM EDTA) for 5 h at 100 V and stained with 0.5 mg of ethidium bromide per ml.

DNA hybridizations. Southern blot analysis of plasmid DNA and colony blots of bacterial cultures were done under stringent DNA hybridization conditions. The DNA in the gels was depurinated with 0.25 N HCl for 15 min before denaturation and neutralization to ensure efficient transfer of the large plasmid DNA and its restriction fragments. Hybridization was carried out in 50% formamide–1× SSPE (1× SSPE is 0.18 M NaCl, 10 mM $NaPO_4$ [pH 7.7], and 1 mM EDTA)–5× Denhardt solution (1× Denhardt solution is 0.02% each of Ficoll [Pharmacia Fine Chemicals, Piscataway, N.J.], polyvinylpyrrolidone, and bovine serum albumin)–0.5% sodium dodecyl sulfate–200 μg of sonicated salmon sperm DNA per ml for 20 h at 37°C. After hybridization the filters were washed three times for 15 min each at room temperature in 2× SSPE–1% sodium dodecyl sulfate and then two times for 1 h each at 65°C in 0.1× SSPE–1% sodium dodecyl sulfate. Autoradiography was carried out at −70°C with XAR film and intensifying screens (Eastman Kodak Co., Rochester, N.Y.).

Preparation of filters for in situ colony hybridization. Cultures of various strains were streaked on LB plates, and single colonies were directly inoculated onto a nylon filter (colony plaque screen; New England Nuclear Corp., Boston, Mass.) placed on a grid. The inoculated filter was placed colony side up on another LB plate and incubated for 3 h at 37°C. The filters were then placed on 3MM filter paper (Whatman, Inc., Clifton, N.J.), soaked in 0.5 N NaOH–1.5 M NaCl, and steamed over a boiling-water bath (10). The filters, containing denatured DNA, were then immersed in 1.5 M NaCl–1 M Tris hydrochloride (pH 8.0) for 4 min, dried at room temperature, and hybridized with the appropriate DNA probes.

Preparation of ^{32}P-labeled DNA probes. Insert fragments encoding portions of the *ipaB*, *ipaC*, or *ipaD* gene were excised from pUC8 subclones (2), purified twice over agarose gels, and radiolabeled in vitro by nick translation with a kit from New England Nuclear Corp. ([α-^{32}P]dCTP [3,000 C/mmol]). Specific activity of the probes was 1×10^8 to 2×10^8 cpm/μg of DNA. For hybridization, 1×10^6 to 5×10^6 cpm per filter was used.

RESULTS

Homology of *ipa* gene sequences in *Shigella* spp. and EIEC. Representative virulent strains of EIEC, *S. sonnei*, *S. dysenteriae*, and *S. bodyii* and all six serotypes of *S. flexneri* were isolated initially as red colonies on Congo red dye agar medium. Loss of invasiveness in *Shigella* spp. has been correlated with the loss of the ability of cells to bind Congo red dye (3, 17; Kopecko et al., in press). Subsequently, these strains were tested in the HeLa cell invasion assay and guinea pig keratoconjunctivitis test for invasiveness (11, 18). *S. flexneri* serotype 5 (strain M90T-A_3), which appeared as a white colony on Congo red plates (Pcr$^-$) did not penetrate HeLa cells and was negative by the Sereny test. Western blot (immunoblot) analysis, with antisera against *S. flexneri* 2a, of 20 invasive, virulent *Shigella* and EIEC strains showed reaction with a common set of protein bands corresponding to the products of *ipaI* (140 kDa), *ipaA* (78 kDa), *ipaB* (57 kDa), *ipaC* (43 kDa), and *ipaD* (39 kDa) (data not shown). Avirulent noninvasive *S. flexneri* did not synthesize these proteins.

The detection of common antigens among these strains indicated the presence of homologous DNA sequences encoding the antigens. Previously, invasion plasmid DNA from representative *Shigella* and EIEC strains had been compared by restriction enzyme analyses and DNA hybridization (16). Although the plasmids share very few restriction endonuclease fragments in common with one another, DNA hybridization studies indicated that all *Shigella* and EIEC invasion plasmids were at least 80% homologous. We isolated plasmid DNA from representative virulent strains of *S. sonnei*, *S. flexneri*, *S. boydii*, *S. dysenteriae*, and EIEC and from the noninvasive strain M90T-A_3 and, with agarose gel electrophoresis, separated the large invasion plasmid DNA from the smaller (2- to 4-kb) plasmid DNAs normally present in these strains (Fig. 1A). Considering that large plasmids between ~180 and 210 kb are difficult to isolate without physical shearing, different structural forms of the same plasmid were sometimes seen as multiple, high-molecular-sized bands; this interpretation was confirmed by the hybridization data discussed below. A considerable amount of invasion plasmid DNA did not enter the gel, suggesting concatenation or aggregation of sheared and nicked large plasmid DNA (Fig. 1A). Also, the area on the gel corresponding to linear fragments of average size (>23 kb) was shown to be a mixture of degraded chromosomal DNA, as well as fragmented large plasmid DNA (see below).

Nick-translated DNA probes derived from the *ipaC* (a 1,750-base-pair *Eco*RI fragment originating in λ gt11 recombinant W22; Fig. 2), *ipaB* (a 375-base-pair *Eco*RI fragment originating in λ gt11 recombinant S12; Fig. 2), and *ipaD* (an 870-base-pair *Eco*RI fragment originating in λ gt11 recombinant S10; Fig. 2) genes (2) were found by Southern blot analysis to hybridize only the large plasmid DNA, which they did in all of the invasive strains tested (Fig. 1B). None of the probes hybridized to the large plasmid molecule isolated from M90T-A_3, an avirulent strain of M90T-W, whose mobility was slightly greater than that of the corresponding plasmid in the invasive M90T-W strain (Fig. 1B).

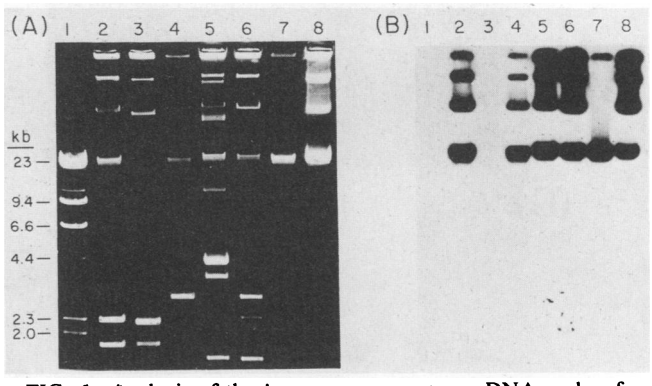

FIG. 1. Analysis of the *ipa* gene sequences as DNA probes for *Shigella* and EIEC strains. (A) Plasmid DNAs were isolated from *S. flexneri* serotype 5 (strain M90T-W) (lane 2), *S. flexneri* serotype 5 (strain M90T-A_3) (lane 3), *S. sonnei* 53GI (lane 4), *S. sonnei*PA2325 (lane 5), *S. boydii* CG1159 (lane 6), *S. dysenteriae* serotype 1 (strain CG1314) (lane 7), and EIEC M41-63T (lane 8); electrophoresed on 0.8% agarose gels; and stained with ethidium bromide. Lane 1 contained *Hin*dIII-digested λ DNAs as molecular size markers. (B) Plasmid DNA from the gel in panel A was bound to nitrocellulose and hybridized to nick-translated W22 insert DNA.

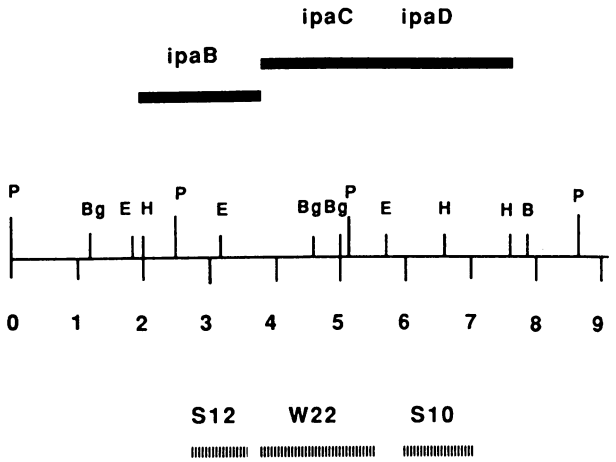

FIG. 2. Restriction enzyme map of the *ipa* region. The boundaries of the DNA fragments used as probes are shown below the map. E, *Eco*RI; Bg, *Bgl*II; H, *Hin*dIII; B, *Bam*HI; P, *Pst*I. Scale at bottom is in kilobases.

Invasion plasmid DNA from each of the virulent strains was digested with *Hin*dIII, transferred to nitrocellulose, and probed with the same *ipa* gene segments as described above (Fig. 3A through D). A single 4.6-kb band was found to hybridize under high-stringency hybridization conditions in all of the virulent strains tested (Fig. 3B through D). No hybridization was seen with *Hin*dIII-digested plasmid DNA from strain M90T-A_3, although the restriction enzyme digestion pattern was generally similar to that of the parental strain M90T-W (Fig. 3A).

In situ colony blot hybridization. The above studies indicated that these sequences might constitute excellent diagnostic probes for the detection of invasive bacteria causing dysentery. To further test this possibility, the probes were used in colony blot hybridization assays against a variety of strains which included *Campylobacter*, *Edwardsiella*, *Citrobacter*, *Yersinia*, *Vibrio*, *Aeromonas*, *Klebsiella*, and *Enterobacter* species; enterohemorrhagic, enterotoxigenic, and enteropathogenic *E. coli*; as well as isolates representing the more than 50 *Salmonella* serogroups. The *ipa* gene probes defined and used in this study were highly specific for invasive *Shigella* and EIEC strains and did not react with more than 300 other nondysenteric, pathogenic gram-negative bacteria (Table 1). The *ipa* gene probes also did not react with DNA from *Rickettsia conori* and *Rickettsia tsutsugamushi* in a Southern blot analysis.

Spontaneous Pcr^- colonies of all six serotypes of *S. flexneri*, which were noninvasive in HeLa cell and Sereny assays and did not express the *ipa* gene products as determined by Western blot analysis (data not shown), did not hybridize the *ipa* gene probes (Table 1). A number of

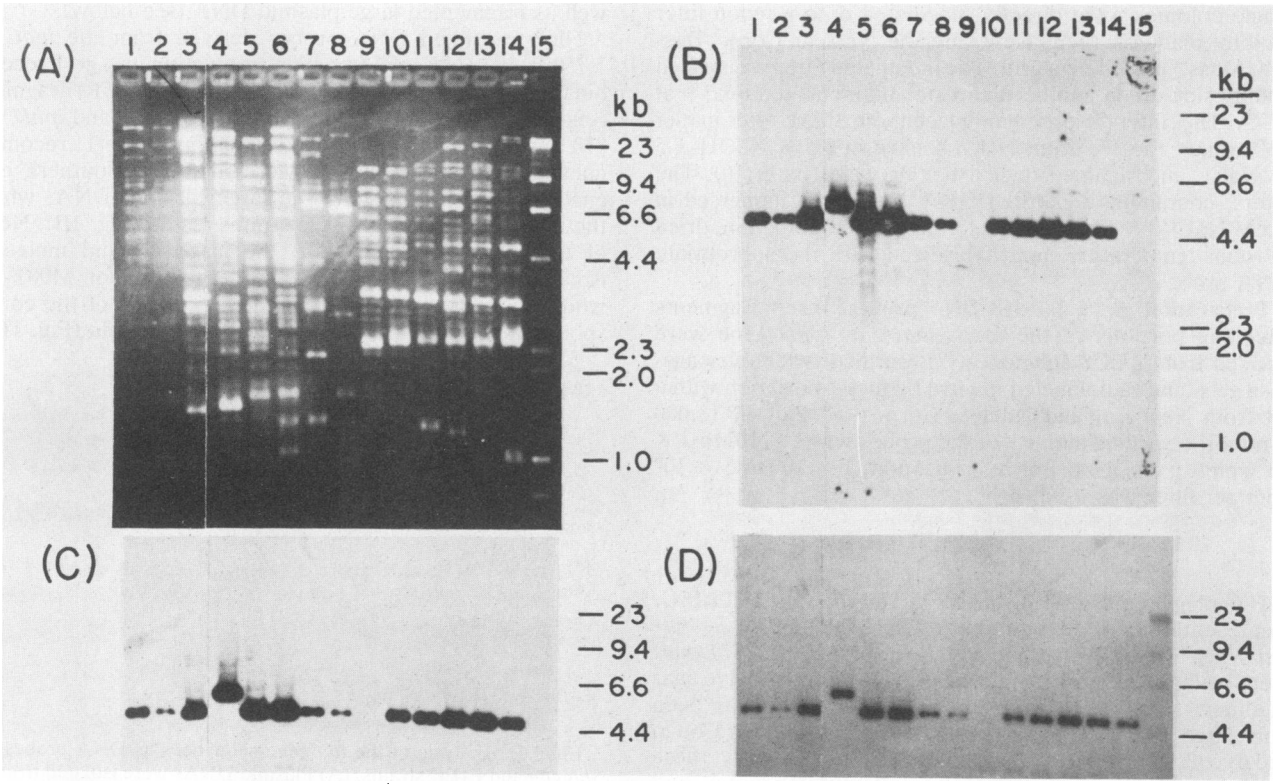

FIG. 3. Analysis of *ipa* genes with restriction enzyme-digested plasmid DNA. (A) Plasmid DNA from EIEC M41-63T (lane 1), *S. dysenteriae* serotype (strain JVA/70) (lane 2), *S. dysenteriae* serotype 1 (strain 1617) (lane 3), *S. dysenteriae* CG1314 (lane 4), *S. boydii* CG1159 (lane 5), *S. sonnei* PA2325 (lane 6), *S. sonnei* 53GI (lane 7), *S. flexneri* serotype 6 (strain CDC) (lane 8), *S. flexneri* serotype 5 (strain M90T-A_3) (lane 9), *S. flexneri* serotype 5 (strain M90T-W) (lane 10), *S. flexneri* serotype 4 (strain M76-39) (lane 11), *S. flexneri* serotype 3 (strain J17) (lane 12), *S. flexneri* serotype 2 (strain 2457T) (lane 13), and *S. flexneri* serotype 1 (strain M25-8) (lane 14) were digested with *Hin*dIII, electrophoresed on agarose gels, and stained with ethidium bromide. Lane 15 contained *Hin*dIII-digested λ DNA and *Hin*cII-digested ϕXRF DNA as molecular size markers. Plasmid DNA from the gel in panel A was bound to nitrocellulose and hybridized with W22 (B), S10 (C), and S12 (D). Numbers on the side of the figures indicate sizes of DNA size markers.

Shigella strains obtained as stock cultures also did not hybridize the probes (Table 1). Subsequent testing of these strains in the HeLa cell invasion assays showed them to be noninvasive.

DISCUSSION

All four *Shigella* species, representing 32 different serotypes, and certain *E. coli* strains (EIEC), falling into at least 9 serotypes, are potential pathovars of the dysenteric syndrome. The identification and cloning of homologous gene sequences among these organisms, whose protein products contribute to the invasive phenotype, represent the first steps toward the development of rapid and specific diagnostic probes for bacillary dysentery. The immunological cross-reactivity of *ipa* antigens in all *Shigella* spp. and EIEC reflects a common mechanism of entry into epithelial cells. In as much as the Sereny and the HeLa cell invasion assays measure this invasive phenotype, a simple and direct assay to detect the genetic components of invasion could eliminate the need for these expensive and time-consuming laboratory models.

Although the pattern of restriction endonuclease fragments obtained when various invasion plasmids were digested with *Hin*dIII demonstrated a number of differences, very little restriction fragment length polymorphism was detected when the *ipa* gene probes were hybridized to Southern blots of the *Hin*dIII-digested plasmid DNA, reflecting the conserved invasion-essential DNA sequences present in these related organisms. The absence of the *ipa* gene region in M90T-A_3 is interesting and confirms our interpretation that a specific deletion of the *ipa* loci has occurred in the strain and may account for the resulting noninvasiveness and avirulence. It also correlates well with the observed propensity of *S. flexneri* invasion plasmids to generate specific deletions with defined endpoints that result in a Pcr^- noninvasive phenotype (3, 17; Kopecko et al., in press). Spontaneous mutants of virulent *S. flexneri* that are Pcr^-, noninvasive, and negative by the Sereny test did not hybridize with the *ipa* gene probes in colony blots (Table 1). Lack of hybridization in these strains could be due to either the loss of the whole invasion plasmid or a specific deletion of the *ipaB*, *ipaC*, and *ipaD* gene cluster, as has been observed with the avirulent strain M90T-A_3. Since noninvasive shigellae and EIEC mutants occur spontaneously (often at frequencies as high as 1 in 100), it is tempting to speculate that a sequence highly susceptible to deletion borders these *ipa* gene loci and frequently causes their removal.

Although other *Shigella* invasion plasmid DNA segments have been cloned (11, 15, 21), there have been only four studies in which these segments have been tested as diagnostic probes (1, 19, 20, 22). Boileau et al. (1) have reported on the use of an invasion-associated 17-kb *Eco*RI fragment, obtained from the *S. flexneri* serotype 5 invasion plasmid, as a DNA probe. This 17-kb sequence, which has also been tested on clinical samples (14, 19), is derived from a region of the large plasmid separated from the *ipa* gene cluster and was contained in a cosmid clone that could complement the HeLa cell invasion phenotype (11). Small and Falkow (20) reported the results of a preliminary study (testing 34 strains) on the use of a functionally uncharacterized 2.5-kb *Hin*dIII fragment derived from an EIEC invasion plasmid. Wood et al. (22) recently compared the use of these two probes with the Sereny test in the detection of invasive organisms among clinical isolates. In that study, all 20 *Shigella* and 19 EIEC strains that were Sereny positive hybridized both probes; none of 9 non-EIEC strains reacted with either probe. However, both probes inappropriately reacted with 3 of 23 (13%) Sereny-negative (i.e., noninvasive) *Shigella* and EIEC strains. Thus, although these plasmid-specific probes are useful in identifying *Shigella* and EIEC strains, it is apparent from this limited study and other reports that the probes are not invasion specific and will react with certain avirulent *Shigella* and EIEC strains as well (14, 19, 22). Aside from the large size of the 17-kb *Eco*RI fragment probe, which limits hybridization reaction specificity, this fragment has been found to contain at least one copy of IS*1*, a common component of enterobacterial chromosomes and plasmids (2, 12). These facts appear to limit the usefulness of this probe.

DNA probes, such as the *ipaB*, *ipaC*, and *ipaD* gene fragments, that encode the synthesis of known invasion peptides would better satisfy the requirement for specificity. In fact, of 14 noninvasive EIEC isolates, all of which inappropriately hybridized the 17-kb *Eco*RI probe, only 1 has been observed to hybridize the *ipaC* probe (David N. Taylor, personal communication). Thus, although further testing is necessary, these *ipa* gene probes appear to be very specific indicators of invasive dysentery bacilli. Future studies will be aimed at sequencing the *ipaB*, *ipaC*, and *ipaD* gene regions, constructing short synthetic oligonucleotide probes from these genes that can be used in nonradioactive colorimetric DNA hybridization assays, and developing methods for the direct analysis of dysentery bacilli in stool and environmental samples.

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