

Identification of Regions on a 230-Kilobase Plasmid from Enteroinvasive *Escherichia coli* That Are Required for Entry into HEp-2 Cells

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Certain strains of *Escherichia coli* can cause an invasive diarrheal disease in humans which clinically resembles shigellosis. These strains share with *Shigella* species the ability to enter and replicate within colonic epithelial cells and the ability to bind Congo red dye in vitro when grown at 37°C. Like shigellae, they contain a large plasmid essential for virulence. A 230-kilobase (kb) plasmid from enteroinvasive *E. coli* was genetically marked with a transposon and mobilized into an *E. coli* K-12 background. This plasmid conferred upon *E. coli* K-12 the ability to enter and multiply within cultured epithelial cells, as well as the ability to bind Congo red. Expression of these phenotypes required growth at 37°C. Transposon mutagenesis was used to identify regions on the 230-kb plasmid required for virulence. All transposon insertions which resulted in loss of the ability to enter epithelial cells, as well as the ability to bind Congo red dye, were mapped to a single 25-kb *Bam*HI fragment. Subclones from this 25-kb region were tested for the ability to complement invasion in noninvasive derivatives. A subclone containing about 8 kb of the left end of the 25-kb *Bam*HI fragment was capable of complementing noninvasive mutants with Tn5 insertions in this region and restored to these noninvasive mutants the ability to enter epithelial cells.

Enteroinvasive *Escherichia coli* (EIEC) comprise a group of organisms known to cause a dysenteric enteritis in humans similar to that caused by *Shigella* species. Experiments with both chimpanzees and human volunteers conducted by Dupont et al. (5) demonstrated that EIEC have the capacity to invade bowel tissue, multiply intracellularly, and produce a disease indistinguishable from shigellosis. As in shigellosis, the infection is localized—organisms are not found beyond the lamina propria or in the bloodstream (6). In contrast to *Shigella* spp., however, a large inoculum of EIEC is required to produce disease. Whereas 200 *Shigella* organisms are capable of producing dysentery, over 10⁸ EIEC are required to produce disease (5). Thus, although it may be expected that the genetics of virulence in EIEC is similar to that of *Shigella* spp., one might expect to find differences as well.

EIEC share a number of biochemical characteristics with *Shigella* spp. They are lysine decarboxylase negative and usually nonmotile and often lack the ability to ferment lactose (21). Dysentery-producing isolates occur within a circumscribed group of serotypes, which includes O28a and c, O29, O42, O112a and c, O124, O136, O143, O144, and O152 (16, 17). There is recent evidence that other serotypes include EIEC as well (25). It is of interest that many of these serotypes are cross-reactive with *Shigella* serotypes (16).

At present, there exist no simple biochemical methods for identifying EIEC. Like *Shigella* spp., they have the ability to invade guinea pig conjunctiva (Sereny test) and to invade a variety of cultured epithelial cell lines. These diagnostic tests are beyond the capability of most clinical laboratories. Because of this, little is known about the epidemiology of

EIEC. More recently, DNA probes have been constructed which have shown diagnostic promise (2, 22).

Like *Shigella* species, virulent EIEC contain a large plasmid (200 to 250 kilobases [kb]) whose presence has been correlated with ability to produce disease (8). Phenotypes so far known to be plasmid encoded in *S. flexneri* include the ability to enter and multiply within epithelial cells (19) and the ability to bind Congo red dye (12, 20). This latter phenotype has proven useful as a marker for virulence in other *Shigella* species, as well as in EIEC. Both of these phenotypes, cell invasion and the ability to bind Congo red dye, require growth at 37°C for their expression (11).

Several lines of evidence suggest that the EIEC virulence plasmid is closely related to that of *Shigella* spp. Transfer of the virulence plasmid from *S. flexneri* to a plasmid-cured avirulent derivative of EIEC restores the ability of these bacteria to invade epithelial cells (18). Thus, the large plasmids in EIEC and *S. flexneri* appear to be functionally equivalent. Hale et al. have shown that the *S. flexneri* plasmid exhibits considerable DNA homology with large virulence plasmids in *S. sonnei* and EIEC and that both produce several plasmid-encoded proteins of similar sizes (7). Despite these similarities, restriction digests of the *Shigella*-EIEC group of virulence plasmids have shown few similarities (7). Questions of whether this restriction heterogeneity reflects differences within or outside of virulence genes or differences in gene order cannot be answered until further analysis of plasmid-mediated virulence determinants has been undertaken.

In a previous paper, we have described the construction of a K-12 derivative from a clinical EIEC strain. This derivative, HB101(pSF204), contains the virulence plasmid from EIEC and expresses the invasive phenotype of the parental strain (22). In this study, Tn5 mutagenesis and cloning techniques were used to identify regions on the virulence

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plasmid required for epithelial cell invasion, as well as for the Congo red-binding (CR⁺) phenotype.

MATERIALS AND METHODS

Media. HEP-2 cells were maintained in RPMI 1640 (Irvine Biochemical) supplemented with 5 mM glutamine (GIBCO Laboratories, Grand Island, N.Y.) and 5% fetal bovine serum (GIBCO). Bacteria were grown on Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.) plates, on minimal medium supplemented with thiamine (1 µg/ml) and 0.2% glucose, or in brain heart infusion broth. Antibiotics were added in the following concentrations: ampicillin, 100 µg/ml; kanamycin, 20 µg/ml; chloramphenicol, 20 µg/ml; tetracycline, 20 µg/ml. Plates for scoring Congo red dye-binding ability were made from Trypticase soy agar supplemented with 0.003% Congo red dye as previously described (12).

Construction of HB101 derivatives. A 230-kb plasmid from a clinical isolate of EIEC, *E. coli* 11 (O:124), was genetically marked with the ampicillin resistance transposon Tn801 by using plasmid pMR5 (13). This is a conjugative plasmid derivative of RP1 which is temperature sensitive for replication and carries a transposon for ampicillin resistance, as well as resistance to kanamycin and tetracycline. Plate matings between HB101(pMR5) and EIEC 11 were conducted at 30°C. Transconjugants were isolated on minimal medium containing appropriate antibiotics and purified. Purified colonies were grown at 37°C for 8 h and serially diluted 1:10 every hour. After this period, dilutions were plated on Trypticase soy agar plates containing ampicillin, and transposition events were identified by replica plating of ampicillin-resistant colonies to plates containing ampicillin and kanamycin. Isolates which were ampicillin resistant and kanamycin sensitive were examined by plasmid digestion and electrophoresis to confirm insertion of the ampicillin transposon into the virulence plasmid and to confirm the loss of pMR5. The virulence plasmid containing transposon Tn801 was then mobilized into *E. coli* K-12 HB101 by using *F'*lac ts:Tn10 (3). The mobilizing plasmid was lost at a high frequency when the strain was grown at 37°C in the absence of tetracycline. One derivative, HB101(pSF204), was used for further work. Kanamycin-resistant derivatives of HB101 (pSF204) were obtained by infection of HB101(pSF204) with lambda b221c1857::Tn5 Oam23 Pam80 (14). Kanamycin-resistant organisms were screened for the ability to infect HEP-2 cells. Plasmid digestions were undertaken to determine the locations of Tn5 insertions.

HEP-2 invasion assay. HEP-2 cells were cultured on glass cover slips (Bellco Glass, Inc., Vineland, N.J.) placed in the bottom of 1-dram (1 fluidram is 3.696 ml) glass shell vials (American Scientific Products, McGaw Park, Ill.) or directly on the bottom of glass vials as previously described (23). Infected monolayers were incubated for 2 h in 5% CO₂ at 37°C. The monolayers were washed three times with phosphate-buffered saline (pH 7.4), and fresh RPMI 1640 containing gentamicin (100 µg/ml) was added to kill extracellular bacteria. After 2 h, the monolayers were washed six times with phosphate-buffered saline and flooded with 1% Triton X-100 for 5 min to release intracellular bacteria. The lysed monolayers were plated, and the numbers of CFU per milliliter were determined. Cultures were made of supernatant from each well after 1 h of incubation in the presence of gentamicin to ensure that there was no survival of extracellular bacteria (data not shown). Experiments were run in triplicate and repeated at least five times per isolate.

Temperature dependence. Duplicate cultures of isolates to be tested were grown overnight at 30 and 37°C. On the following morning, tubes containing brain heart infusion broth were inoculated with these cultures and grown for an additional 2 h with shaking at 30 and 37°C. HEP-2 monolayers were infected for 2 h as described above, and incubation was continued for an additional 2 h in the presence of fresh RPMI 1640 containing gentamicin (100 µg/ml). Infected monolayers were washed, lysed, and plated to determine numbers of bacteria which had survived gentamicin killing. Experiments were done in triplicate and repeated at least five times per isolate.

Congo red dye-binding ability. Isolates to be tested were spread on Trypticase soy agar plates containing 0.003% Congo red dye and grown for 24 to 30 h at 37°C. Congo red-positive (CR⁺) isolates appeared orange-red after this period of time, whereas Congo red-negative (CR⁻) isolates had a pale orange tint.

Isolation of noninvasive mutants by Tn5 mutagenesis. HB101, containing virulence plasmid pSF204 from EIEC 11, was infected with a lambda derivative, b221 c11857::Tn5 Oam8 Pam29 (14). Mutations induced by transposition of Tn5 to the 230-kb virulence plasmid pSF204 were isolated in the following manner. Dilutions of HB101(pSF204) infected with lambda b221 c11857::Tn5 were plated on Trypticase soy agar containing 20 µg of kanamycin per ml and 0.003% Congo red dye. All CR⁻ colonies and several CR⁺ colonies were purified, screened for the ability to invade HEP-2 cells, and examined for plasmid content. Insertions into the virulence plasmid were also identified by mobilization of pSF204::Tn5 from HB101 into a noninvasive *S. flexneri* strain (25-8A) lacking the *S. flexneri* virulence plasmid (19). Transconjugants were screened for the invasiveness and Congo red dye-binding phenotypes and analyzed for plasmid content.

Plasmid isolation and electrophoresis. Plasmid DNA was prepared by the method of Birnboim and Doly (1) and purified through CsCl-ethidium bromide density gradient centrifugation. DNA was electrophoresed in horizontal 0.6% agarose gels in Tris-acetate buffer (9).

Restriction endonuclease digestion. DNA was digested by restriction endonucleases in a final volume of 20 µl under appropriate conditions (9). Each reaction mixture contained 1 to 2 µg of plasmid DNA and 1 µl of enzyme (New England BioLabs, Inc., Beverly, Mass.).

Cloning and transformation. Insert DNA for cloning was isolated from low-melting-point agarose (SeaPlaque; FMC Corp., Marine Colloids Div., Rockland, Maine) and purified by phenol extraction and ethanol precipitation. Plasmid vectors were digested to completion with *Cla*I. Vector ends were dephosphorylated, and cloning reactions were performed as described by Maniatis et al. (9). Ligated clones were transformed into cells made competent by CaCl₂ treatment (4). Clones were selected by using appropriate antibiotics and analyzed via restriction endonuclease digestion and electrophoresis.

Preparation of DNA probes and hybridization. A Tn5 probe was isolated from pEYDGE1 (26) and nick translated with ³²P-labeled deoxyribonucleotides (New England Nuclear Corp., Boston, Mass.) as previously described (9). DNA to be probed was digested with appropriate enzymes, Southern blotted (24), and hybridized with probe DNA under stringent conditions (50% formamide, 5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]). Tn5 insertions were mapped by using cloned DNA fragments from pSF204 as probes.

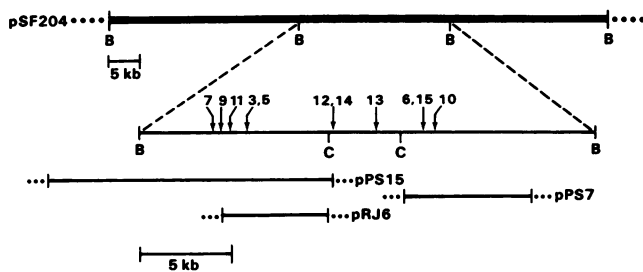


FIG. 1. Partial *Bam*HI map of pSF204, showing Tn5 insertions in virulence-associated regions. B, *Bam*HI; C, *Cla*I. Numbered arrows identify specific Tn5 insertions shown in Table 1. pPS15, pRJ6, and pPS7 are cloned fragments of pSF204 DNA which cover some virulence-associated regions.

Deletion mutants. DNA from pPS15, a subclone of pSF204, was digested with *Bam*HI and *Pst*I, phenol extracted, ethanol precipitated, religated, and transformed into *E. coli* K-12 HB101. Deletion mutants were isolated, purified, and assayed for the abilities to bind Congo red and complement Tn5 insertions in noninvasive mutants.

RESULTS

Tn5 mutagenesis. Using Tn5 transposon mutagenesis, we obtained 1.3×10^4 colonies of kanamycin-resistant HB101 (pSF204) derivatives. Since pSF204 was too large to transform, we used the Congo red-binding phenotype to identify insertions in plasmid DNA. Fourteen CR⁻ colonies were identified and purified. When assayed for the ability to enter HEp-2 cells, all 14 of these CR⁻ mutants had lost this ability. Analysis of plasmid DNAs from these derivatives digested with *Bam*HI showed that Tn5 had been transposed onto pSF204. Five CR⁺ isolates were also assayed for the ability to enter HEp-2 cells, and all five had retained this ability. We also examined the invasiveness and Congo red-binding phenotypes of 19 insertional mutants isolated by mobilization of pSF204::Tn5 into *S. flexneri* M25-8A (19), a noninvasive strain lacking the virulence plasmid. Successful mobilization of the kanamycin marker was interpreted as evidence that Tn5 insertions had occurred in the plasmid. Investigation of these 19 isolates revealed that 15 were CR⁻ and noninvasive and 4 were CR⁺ and invasive. These results demonstrated that pSF204 expressed the Congo red dye-binding and invasiveness phenotypes in both an EIEC and an *S. flexneri* background and that in each case there was a correlation between the presence of the plasmid and the abilities to invade epithelial cells and bind Congo red.

Mapping virulence regions in pSF204. Plasmid analysis of 14 CR⁻, noninvasive Tn5 mutants (in HB101) revealed that in three cases deletion or inversion of pSF204 DNA had occurred along with transposition. These mutants were not analyzed further. Probing the remaining 11 insertional mutants with Tn5, we found eight independent insertions. These mapped to a single 25-kb *Bam*HI fragment flanked by a 31-kb *Bam*HI fragment to the left and a 26-kb *Bam*HI fragment to the right (Fig. 1). With DNA cloned from the left end of the 25-kb *Bam*HI fragment as a probe, Tn5 insertions were mapped along this fragment. The locations of these insertions are shown in Fig. 1. It can be seen that one group of Tn5 insertions is clustered to the left end of the fragment, separated by about 10 kb from insertions in the right half of the fragment. Insertions in the right portion of the 25-kb fragment are less tightly clustered than those in the left.

TABLE 1. Complementation of Tn5 insertions with cloned fragments

Plasmids	Ability of subclones from pSF204 to invade HEp-2 cells		
	pPS15	pRJ6	pPS7
pSF204::Tn5-3	+	+	-
pSF204::Tn5-5	+	+	-
pSF204::Tn5-6	-	-	-
pSF204::Tn5-7	+	-	-
pSF204::Tn5-9	+	+	-
pSF204::Tn5-10	-	-	-
pSF204::Tn5-11	+	+	-
pSF204::Tn5-12	-	-	-
pSF204::Tn5-13	-	-	-
pSF204::Tn5-14	-	-	-
pSF204::Tn5-15	-	-	-

Cloning and complementation of the invasiveness phenotype. In an attempt to define virulence regions covered by the Tn5 insertions further, we made subclones from the right and left regions of the 25-kb *Bam*HI fragment. One clone, pPS15, contained a 15-kb *Cla*I fragment which covered 8 kb from the left end of the 25-kb *Bam*HI fragment and extended 7 kb into the adjacent 31-kb *Bam*HI fragment. We also derived several classes of deletion mutants by digesting and religating pPS15 with *Pst*I and *Bam*HI. One of these, pRJ6, contained a 6-kb fragment internal to the left end of the 25-kb *Bam*HI fragment. A *Cla*I fragment containing 7 kb from the right end of the 25-kb fragment was cloned into pBR325 (pPS7). The relationships of these cloned fragments to the Tn5 insertions in the 25-kb virulence-associated *Bam*HI fragment are shown in Fig. 1.

Deletion derivatives of pPS15, as well as pPS15, were transformed into 11 derivatives of HB101(pSF204) containing single Tn5 insertions. The resulting strains were assayed for the ability to complement the invasiveness phenotype. A clone encompassing 7 kb from the right end of the 25-kb *Bam*HI fragment, pPS7, was also assayed for ability to complement insertions in the 25-kb *Bam*HI fragment. Results of these experiments are shown in Table 1. When transformed into noninvasive mutants containing insertions within the left half of the 25-kb *Bam*HI fragment, pPS15 was able to restore to noninvasive mutants the ability to enter and replicate within epithelial cells (Table 2). Expression of this invasiveness phenotype, as well as expression of the Congo red-binding phenotype, required growth at 37°C (Table 2). A deletion derivative, pRJ6, complemented all but one insertion in the left half of the 25-kb plasmid (Table 1). Only the most leftward insertion was not complemented. Although complementation *in trans* of invasion was complete, complementation of the Congo red-binding phenotype was only partially restored, as indicated by a less intense reddish color found in complemented isolates as compared with the parental strain. We were unable to complement any insertions in the 25-kb *Bam*HI fragment with pPS7 for either invasiveness or Congo red binding.

DISCUSSION

The ability of certain bacterial pathogens to enter and survive within host epithelial cells has been the focus of much current research. Work from several laboratories indicates that several plasmid-mediated determinants appear to be necessary for expression of the invasiveness pheno-

TABLE 2. Complementation of a noninvasive derivative of HB101(pSF204) for invasiveness, intracellular multiplication, and ability to bind Congo red dye^a

Strain	Bacteria (CFU/ml) grown at 30°C surviving gentamicin addition at:		Congo red binding	Bacteria (CFU/ml) grown at 37°C surviving gentamicin addition at:		Congo red binding
	90 min	210 min		90 min	210 min	
HB101	ND	$(2.0 \pm 1.2) \times 10^3$	—	ND	$(1.0 \pm 0.0) \times 10^3$	—
HB101(pSF204)	$(1.0 \pm 0.1) \times 10^3$	$(2.0 \pm 0.8) \times 10^3$	—	$(5.3 \pm 1.6) \times 10^4$	$(7.0 \pm 0.0) \times 10^5$	+
HB101(pSF204::Tn5-3)	$(2.6 \pm 1.2) \times 10^3$	$(3.0 \pm 0.1) \times 10^3$	—	$(1.0 \pm 0.4) \times 10^3$	$(4.0 \pm 0.8) \times 10^3$	—
HB101(pSF204::Tn3, pPS15)	$(1.0 \pm 0.0) \times 10^3$	$(2.3 \pm 0.4) \times 10^3$	—	$(7.3 \pm 1.8) \times 10^4$	$(1.0 \pm 0.0) \times 10^6$	+/-
HB101(pPS15)	$(1.0 \pm 0.0) \times 10^3$	$(2.0 \pm 0.4) \times 10^3$	—	$(1.0 \pm 0.0) \times 10^3$	$(1.0 \pm 0.7) \times 10^3$	—

^a Plasmid designations are as follows: pSF204, virulence plasmid from a virulent isolate of EIEC (23); pSF204::Tn5-3, noninvasive derivative of pSF204; pPS15, subclone containing virulence determinants derived from pSF204. The values represent the mean plus or minus the standard deviation ($n = 3$). The data are from one of five experiments. ND, Not determined.

type in *Shigella* spp. (10, 15, 20). We present evidence in this paper that the genetics of the invasiveness phenotype in EIEC is similarly complex. Initial mapping of plasmid regions required for epithelial cell invasion suggests that two or perhaps three distinct regions within a 25-kb *Bam*HI fragment are required for epithelial cell invasion. We suspect that other areas may be involved as well, since cosmid clones containing the 25-kb *Bam*HI fragment are not invasive.

Complementation data provide evidence that at least one soluble gene product required for invasion can complement noninvasive Tn5 mutants. Although pPS15 complemented the invasiveness phenotype completely, the Congo red-binding phenotype was only partially restored. In *S. flexneri*, it appears that more than one Congo red-binding determinant is found on the virulence plasmid (15). This may be true in EIEC as well. We were disappointed that pPS7 failed to complement insertions in the right half of the 25-kb *Bam*HI fragment and are in the process of cloning larger fragments from that portion of the plasmid to determine whether the failure to get complementation was because we had interrupted a gene during cloning or because insertions in this area cannot be complemented in *trans*.

The invasiveness phenotype in EIEC, as in *Shigella* spp., involves both the ability to enter the host cell and the ability to escape from an endocytic vacuole and gain access to the cytoplasm, where rapid multiplication takes place. It is reasonable to hypothesize that the ability to enter a host cell and the ability to escape from the endocytic vacuole are encoded by separate loci. We have no evidence to support this, however. In fact, we were unable to obtain a mutant which retained the ability to enter a host cell and yet was incapable of intracellular multiplication. It is possible that EIEC enter the host cell and gain access to the cytoplasm by the same mechanism.

How *Shigella* spp. and EIEC gain entrance to nonprofessional phagocytes such as epithelial cells is of great interest. One possibility is that the bacteria carry on the surface a biochemical structure which is recognized by a specific eucaryotic cell receptor and that recognition of this structure sets in motion a series of events leading to endocytosis. Compared with *Shigella* spp., EIEC are less efficient in their invasion of epithelial cells. This may be due to differences in outer membrane proteins.

Further mapping of virulence-associated regions and identification of the gene products of these regions are necessary before basic questions regarding the bacterium-host cell interactions involved in pathogenesis can be answered.

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