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# Organization of Genes Responsible for the Production of Mannose-Resistant Fimbriae of a Uropathogenic *Escherichia coli* Isolate

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A group of insertion mutants was used to define the genes of plasmid pDC5 required for the expression of mannose-resistant fimbriae. Minicell experiments identified four polypeptides (71,000, 45,000, 27,000, and 17,000 daltons) concerned with fimbrial production, the smallest of these being the fimbrial subunit. The approximate location of the structural genes encoding these polypeptides and one possible additional polypeptide not identified in minicell experiments has been established. Complementation experiments in vivo showed that these genes are arranged in more than one operon. The direction of transcription of the fimbrial genes was established by creating β-galactosidase fusions by using the mini-Mu d1681 kanamycin resistance transposon.

We have recently described the cloning of the genetic determinants encoding mannose-resistant (MR) fimbriae of a uropathogenic *Escherichia coli* strain (5). These fimbriae are thought to be virulence factors because they mediate attachment to uroepithelial cells and thereby facilitate colonization of the urinary tract by the bacteria (21). In vitro, these fimbriate bacteria can be differentiated from strains of *E. coli* producing type 1 or mannose-sensitive fimbriae by their ability to agglutinate human erythrocytes in the presence of the sugar D-mannose.

The genes encoding fimbrial biosynthesis and expression were cloned by cosmid cloning and in vitro packaging techniques (5). Following standard subcloning procedures, the smallest recombinant plasmid possessing a full complement of genetic information for the expression of fimbriae, pDC5, was found to comprise 12.3 kilobase pairs (kb). Chimeric plasmids which were smaller in size failed to transform a nonfimbriate strain of *E. coli* to hemagglutinating (HA) activity. Only those transformants possessing pDC5 exhibited HA activity and were observed to be fimbriate when examined by electron microscopy.

In the present study we used the transposable element Tn*5* to construct a number of insertion mutants which established the size of the genetic region necessary for fimbrial production. Also, deletion mutants were constructed and used to localize the genes on pDC5 involved in the expression of fimbriae. Evidence is presented that more than one operon is necessary for phenotypic expression and that these genetic elements can complement each other when carried on distinct DNA molecules.

## MATERIALS AND METHODS

**Bacterial strains, culture conditions, and plasmids.** *E. coli* HB101 (*hsdM hsdR recA*) was used in all transformation experiments involving recombinant plasmids. *E. coli* DS410 (8) was used as a source of minicells. Plasmid pDC5 was constructed as previously described (5). *E. coli* SC802 was derived from strain HB101 by transformation with pDC5.

Unless otherwise stated, all bacterial strains were grown in Luria broth or on Luria agar (14) for 18 to 24 h at 37°C. When necessary, media were supplemented with antibiotics at the following concentrations: ampicillin, 100 μg/ml; tetracycline, 20 μg/ml; kanamycin (Kn), 20 μg/ml; and chloramphenicol (Cm), 25 μg/ml (200 μg/ml for plasmid amplification).

**Isolation and analysis of plasmid DNA.** Plasmid DNA was isolated from transformed cells after sodium dodecyl sulfate (SDS) lysis and ethidium bromide-cesium chloride equilibrium density gradient centrifugation (10, 19). Rapid analysis of transformants for the presence of plasmid DNA was performed by the technique of Cameron et al. (3) as modified by Williams and coworkers (22).

Restriction endonuclease mapping of plasmid DNA was performed as previously described (5). Restriction enzymes were purchased from New England Biolabs and Bethesda Research Laboratories, Inc., and digestions were carried out according to the instructions of the manufacturer. Agarose electrophoretic analysis of restricted DNA has been described in detail elsewhere (5).

**Isolation of Tn*5* insertions.** The transposable Kn resistance-determining element Tn*5* (1) was inserted into plasmid pDC5 by using the phage λb211::Tn*5* cI857. *E. coli* SC802 was grown in tryptone broth (10

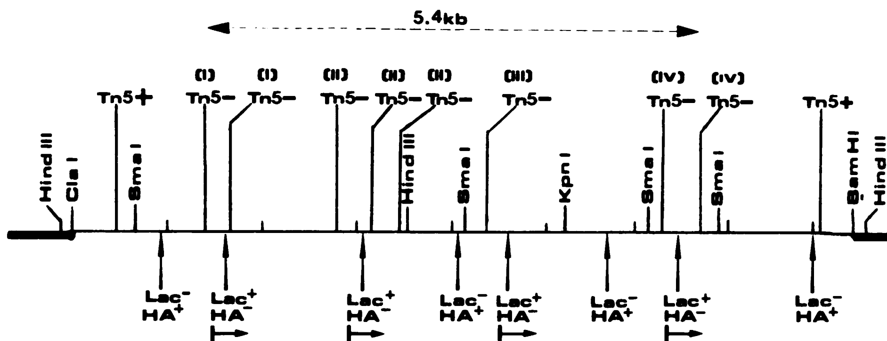


FIG. 1. Location of Tn*5* and mini-Mu insertions into pDC5. The symbols + or − immediately following Tn*5* indicate the ability of each insertion mutant to cause MR HA of human erythrocytes. The dotted line represents the length of DNA between the two most distant Tn*5*−ve insertions. The solid vertical arrows below the plasmid indicate representative insertion sites of mini-Mu, and solid horizontal arrows represent the direction of transcription at these points. Lactose-positive or -negative phenotypes are indicated as Lac$^+$ or Lac$^-$, respectively.

ml) containing 0.2% maltose to a cell density of approximately $5 \times 10^8$ bacteria per ml; after centrifugation, the cells were resuspended in 2 ml of 0.01 M magnesium sulfate. Bacteria were infected with λ:Tn*5* phage at a multiplicity of infection slightly greater than 1 by mixing aliquots (0.1 ml) of phage and bacteria, followed by incubation of the mixtures at 30°C for 1 h. The infected bacteria were plated on Luria agar containing Kn and Cm, and incubated for 18 to 24 h at 30°C; Kn$^r$ Cm$^r$ bacteria were harvested from the plates. Plasmid DNA was prepared and used to transform the nonfimbriate *E. coli* HB101 strain. The Kn$^r$ Cm$^r$ transformants were tested for HA activity, and the insertion site of Tn*5* into HA$^+$ and HA$^-$ derivatives of pDC5 was determined by restriction enzyme digestion.

**Insertion of mini-Mu transposon.** Mini-Mu transposon 1681 was constructed from the Mu-lac bacteriophage (4) by M. Casadaban and co-workers. The method of inserting the mini-Mu transposon into a plasmid has been described in detail elsewhere (9). Briefly, after transformation of *E. coli* pOI1681 (*araD araB*::Mucts Δ*lac recA strA*), which contained mini-Mu (Mu d1681; Kn$^r$), by pDC5, the strain was heat induced, and a phage lysate was prepared. This lysate was used to infect the recipient strain, *E. coli* MH 3497 (Rec$^+$ Mucts), and infected bacteria were selected after plating on Luria agar containing Kn and Cm. To ensure selection of those bacteria in which the mini-Mu had inserted into pDC5, plasmid DNA was prepared from these cultures and used to transform a *recA lac* deletion strain, *E. coli* GS162 (*pheA thi ara recA* Δ*lac*) (kindly provided by G. V. Stauffer). Transformants were plated on lactose-MacConkey agar to test for their ability to ferment lactose, and plasmid-containing strains were analyzed for MR HA activity after growth on antibiotic-containing Luria agar. The direction of insertion and position of the mini-Mu transposon in pDC5 was determined by restriction endonuclease analysis.

**Preparation and labeling of minicells.** After transformation with the appropriate plasmid, an overnight broth culture (10 ml) of *E. coli* DS410 was used to inoculate a 1.5-liter volume of Luria broth. After incubation for 24 h at 37°C with shaking, minicells were isolated by centrifugation through 10 to 30% (wt/vol) linear sucrose gradients at 4°C (5,000 rpm; 20 min). The minicells were suspended in M9 medium (14) supplemented with 20% glycerol to a final concentration of approximately $2 \times 10^{10}$ minicells per ml (optical density of 2 at 600 nm). Samples were distributed in 1-ml aliquots and stored at −70°C. Plasmid-encoded polypeptides were labeled with [$^{35}$S]methionine or [$^{35}$S]cysteine (Amersham Corp.) at a final activity of 50 μCi/ml. Radiolabeling of minicells (0.1 ml) was performed at 37°C for 10 min in M9 medium plus 0.2% glucose and D-cycloserine (20 μg/ml). Any label not incorporated into minicells was removed by centrifugation.

**Electrophoresis and autoradiography.** SDS-polyacrylamide gels were prepared by the method of Laemmli (13). Slab gels (15% acrylamide) electrophoresed for 6 h at 300 V were fixed, stained, and prepared for autoradiography by the technique of Bonner and Laskey (2).

**Immune precipitation.** Minicell extracts were prepared after sonication and centifugation, and 100 μl of extract was mixed with an equal volume of 0.05 M Tris (pH 7.5)–2 M potassium chloride–2% Triton X-100. Rabbit antiserum (50 μl) raised against purified fimbrial antigen (6) was added, and the reaction mixture was placed at 4°C for 18 h. Immune complexes were precipitated by goat anti-rabbit immunoglobulin G (50 μl) after overnight incubation at 4°C and subsequently recovered by centrifugation. The pellet was washed once with 0.05 M Tris (pH 7.5)–1.2 M potassium chloride–1.2% Triton X-100 and once with 0.05 M Tris–0.1 M sodium chloride. The immune precipitate was dissolved in SDS electrophoresis sample buffer and run on SDS-polyacrylamide gels as described above.

## RESULTS

**Identification of the DNA segment of pDC5 encoding fimbriae.** To define the DNA region necessary for fimbrial expression, a number of plasmids were isolated which contained the

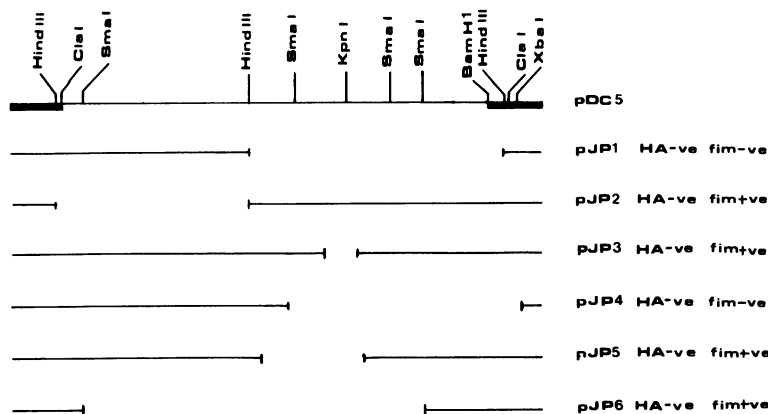


FIG. 2. Map of recombinant plasmid pDC5 and its deletion derivatives. The thick black line represents pACYC184 DNA sequences. The fragments of DNA remaining in the deletion derivatives are shown as solid lines. All deletions were HA negative (HA−ve); pJP2, pJP3, and pJP5 encoded the 17,000-dalton subunit (fim+ve).

transposable element Tn*5*. Figure 1 shows the location of 10 independent Tn*5* insertions into pDC5 as determined by endonuclease mapping. Eight of these insertions resulted in the loss of MR HA activity by bacteria possessing these plasmids, whereas two insertions had no effect on HA activity. The length of DNA between the two most distant insertions eliminating HA activity was calculated to be 5.4 kb (Fig. 1).

To determine whether different $HA^-$ insertion mutants could complement each other to restore HA activity, the nonfimbriate HB101 strain was transformed with two plasmids, each possessing Tn*5* inserted at a different site. The two plasmids also differed with respect to the cloning vector used (pACYC184 and pBR322), so that double transformants could be selected by plating on appropriate antibiotic agar. As shown in Fig. 1, pDC5 consists of at least four complementation groups. The Tn*5* insertion mutants were tested in all possible pairwise combinations, and the HA activity of the double transformants was consistent with the sites of Tn*5* insertions shown in Fig. 1. Each group is characterized by the fact that its members do not complement each other to restore HA activity, whereas members of different groups may act, in *trans*, to produce hemagglutinating transformants.

**Direction of transcription of the *fim* cistrons.** Insertion of the transposable element mini-Mu carrying a promotorless *lacZ* gene and the Kn resistance determinant was used to detect the direction of transcription of the genes responsible for fimbrial expression. Insertion of the mini-Mu in one orientation downstream from a promoter in the cloned DNA results in the synthesis of a functional β-galactosidase which can be detected by plating on lactose-MacConkey agar. However, insertion of the mini-Mu in the opposite orientation would be expected to produce phenotypically $Lac^-$ clones. A transposition event leading to insertion of the mini-Mu into a gene required for fimbrial expression leads to loss of HA activity.

A total of 45 mini-Mu insertions into pDC5 were mapped by restriction endonuclease analysis. Of these, 34 were $HA^-$. Location of the 11 $HA^+$ isolates within pDC5 suggested that the genetic elements required for the expression of MR HA activity consist of more than one operon. Thus, 6 $HA^+$ mini-Mu insertions were found between the sites of insertion of $HA^-$ mutants. The location of $HA^-$ isolates was consistent with the sites of the complementation groups determined by analysis of Tn*5* insertion mutants (Fig. 1).

Of the 45 isolates, 27 were phenotypically $Lac^+$. The orientation of mini-Mu in these strains indicated that the genes are transcribed in the direction shown in Fig. 1. $Lac^-$ $HA^-$ plasmids consistently had mini-Mu inserted in the opposite orientation to $Lac^+$ $HA^-$ ones. All $HA^+$ mini-Mu insertions were $Lac^-$ regardless of orientation, indicating that the mini-Mu had inserted into a segment of DNA the transcription of which is not necessary for expression of the hemagglutinin.

**Construction of deletion mutants of pDC5.** Deletion mutants were constructed after digestion of pDC5 with either specific restriction endonucleases or the exonuclease *Bal*31. Plasmids pJP1 and pJP2 (Fig. 2) were produced after digestion of pDC5 with *Hin*dIII and subsequent subcloning of the 3.5- and 5.1-kb fragments into the *Hin*dIII site of pACYC184. Similarly, pJP6 was constructed after digestion of pDC5 by the restriction enzyme *Sma*I, followed by religation with T4 DNA ligase. This procedure removed a

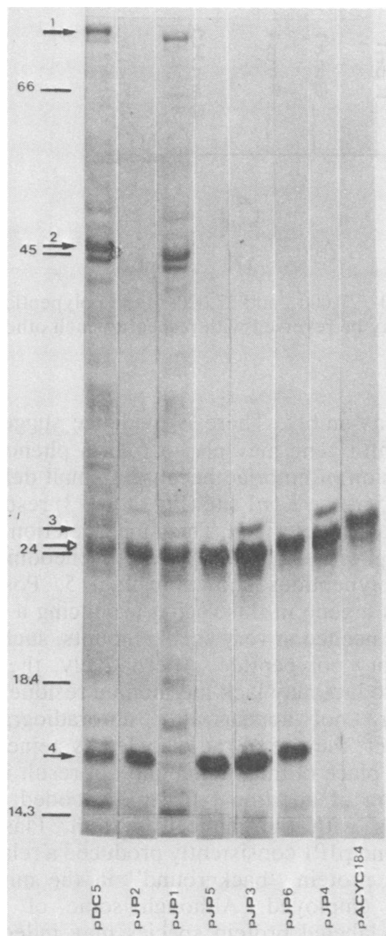


FIG. 3. Plasmid-encoded polypeptides synthesized in minicells by pDC5 and its deletion derivatives. The plasmids used are indicated below the lanes, and the numbers on the left represent the molecular weights ($\times 10^3$) of standards. The four polypeptides involved in MR fimbrial expression are indicated by the solid arrows, and the pACYC184-encoded gene product is indicated by an open arrow.

6-kb DNA fragment containing the two *Sma*I sites of pDC5 (Fig. 2).

The remaining deletions were made by digestion of pDC5 at the single *Kpn*I site, incubation with limiting concentrations of *Bal*31 for various times, and religation. The sizes of the deletions were calculated after restriction endonuclease analysis. For example, the sizes of the deletions of pJP3, pJP4, and pJP5 (Fig. 2) were approximately 0.6, 4.3, and 2.1 kb, respectively. Transformation of *E. coli* HB101 with the deletion mutants described above resulted in the production of $Cm^r$ $HA^-$ isolates.

**Polypeptides involved in the expression of MR fimbriae.** The minicell-producing strain *E. coli* DS410 was used to determine the effects of the various deletions on expression of the MR fimbrial genes. Minicells containing the parental plasmid pDC5 express detectable levels of at least four polypeptides apparently involved in the synthesis and expression of MR fimbriae (Fig. 3). The molecular weights of these polypeptides were estimated to be 71,000, 45,000, 27,000, and 17,000. The 17,000-dalton polypeptide was not produced by minicells harboring pJP1 or pJP4, but was expressed by the remaining plasmids. This would suggest that the 17,000-dalton polypeptide is encoded on the 1.3-kb *Sma*I-*Hin*dIII fragment of pDC5 (see Fig. 5). The 71,000- and 45,000-dalton polypeptides were produced by pJP1 but not by pJP2, indicating that these two polypeptides are encoded by genes located on the 3.5-kb *Hin*dIII fragment of pDC5. Interestingly, the 71,000- and 45,000-dalton polypeptides could not be detected in minicell extracts containing pJP3, pJP4, or pJP5 (Fig. 3), although intact genes encoding these two polypeptides would appear to be present on these plasmids. Therefore, it is possible that the expression of these two genes may be regulated by a gene located near the *Kpn*I site of pDC5.

The gene for the 27,000-dalton polypeptide maps around the *Hin*dIII site of pDC5. The finding that a protein necessary for fimbrial expression is encoded in this region of the plasmid is consistent with the fact that the 3.5- and 5.1-kb *Hin*dIII fragments do not complement each other to produce $HA^+$ transformants.

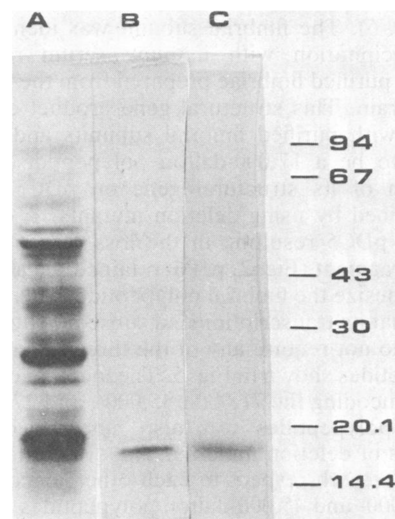


FIG. 4. Identification of the fimbrial subunit encoded by pDC5. Lane A is an autoradiograph of pDC5-encoded polypeptides, and lane B shows the 17,000-dalton plasmid-encoded fimbrial subunit after immune precipitation. Lane C is a Coomassie blue-stained SDS gel of purified fimbrial subunits isolated from the wild-type strain *E. coli* IA2.

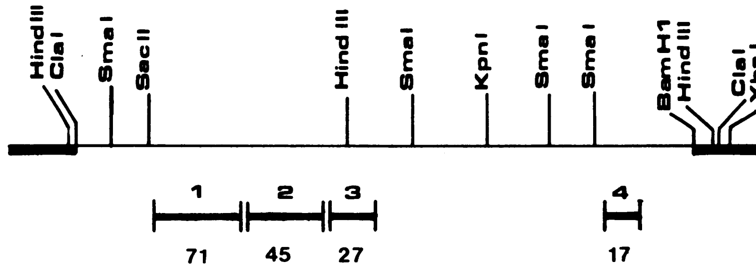


FIG. 5. The location of the genes encoding the 71,000-, 45,000-, 27,000-, and 17,000-dalton polypeptides on the recombinant plasmid pDC5. The location of genes 1 and 2 may be reversed with respect to each other (see text).

**Identification of the fimbrial gene product.** The fimbrial subunit was identified by precipitation with immune serum raised against the purified fimbrial antigen (Fig. 4). Minicells harboring pDC5 were lysed, and fimbrial antigen-antibody complexes were precipitated by goat anti-rabbit immunoglobulin G. The 17,000-dalton fimbrial subunit which reacts with fimbrial antiserum shows the same electrophoretic mobility as purified antigen prepared from fimbriae of the wild-type, uropathogenic strain.

## DISCUSSION

These experiments describe the position and organization of genetic elements of the plasmid pDC5 involved in fimbrial expression. This plasmid was found to encode at least four polypeptides necessary for the production of MR fimbriae (Fig. 5). The fimbrial subunit was identified by precipitation with immune serum raised against purified fimbriae prepared from the wild-type strain. This structural gene product comigrates with purified fimbrial subunits and was found to be a 17,000-dalton polypeptide. The location of its structural gene on pDC5 was ascertained by using deletion mutants. A deletion of pDC5 resulting in the loss of a 6.7-kb *Sma*I fragment (Fig. 2, pJP6) retained the ability to synthesize the fimbrial polypeptide. This indicates that its transcription and subsequent translation do not require any of the three remaining polypeptides shown in Fig. 5. The location of the genes encoding the 71,000-, 45,000-, and 27,000-dalton polypeptides was also determined by analysis of deletion mutants. The orientation of the genes with respect to each other, encoding the 71,000- and 45,000-dalton polypeptides (Fig. 5), is speculative at present. We are now constructing deletion mutants of plasmid pJP1 to more accurately map the sites of these genes. As yet the function of these gene products in fimbrial biosynthesis and expression is unknown, but they may play a role in polymerization, secretion, and/or anchorage of the fimbrial subunits during assembly. There is evidence suggesting that a fifth gene may play a role in phenotypic expression of fimbriae because a small deletion of pDC5 at the *Kpn*I site (Fig. 2, pJP3) results in a loss of HA activity. This small deletion does not map within the DNA regions encoding the four polypeptides shown in Fig. 5. Possibly there is a gene in this region producing a polypeptide needed in very small amounts, such as a regulatory polypeptide. Alternatively, the final gene product may lack methionine residues and therefore not appear after autoradiography. However, the use of radiolabeled cysteine residues in place of methionine did not result in the detection of additional plasmid-encoded polypeptides with the minicell system. Plasmids pDC5 and pJP1 consistently produced a relatively high protein "background" in the minicell system employed. Although some of these weakly labeled protein species may indeed be plasmid encoded, we have only considered those polypeptides which can be mapped by using the parental plasmid and the deletion mutants. The reason for this high background when these two plasmids are used is unknown and may possibly reflect a difficulty in separating minicells from parental cells due to surface properties of the minicells when transformed by these two plasmids.

Insertion of transposable elements into pDC5 further supports the evidence that MR fimbrial expression is due to several operons. Nonhemagglutinating, independent insertion mutants were found to complement each other when acting in *trans* and thus restore HA activity. Clearly diffusable gene products from distinct transcription units can cooperate for phenotypic expression of fimbriae. Evidence suggests that most of these genes are transcribed in the same direction (Fig. 1). However, no mini-Mu insertions were obtained in the region of pDC5 corresponding to the fimbrial gene; therefore, the direction of transcription of this gene is unknown.

Recently the genetic determinants encoding

the K88ab (15, 16) and K88ac (7, 20) fimbrial antigens have been described. The expression of these fimbriae was determined to be due to the interaction of at least four (K88ac) or six (K88ab) gene products. The K88 antigens are found on porcine strains of enterotoxigenic *E. coli* (12). It is a plasmid-encoded antigen (18), in contrast to the MR hemagglutinins of human uropathogenic *E. coli*, which appear to be chromosomally determined (5, 11). However, in both cases the genetic organization of the relevant determinants appears to involve a number of distinct genes which are necessary for the expression of functional antigen. Further analysis of plasmid pDC5 and its derivatives will be necessary to determine other similarities with known fimbrial genetic elements. For example, we propose to use the minicell system to search for precursor polypeptides as well as to quantitate the amount of fimbrial subunits produced by the various deletion mutants.

Initial evidence suggests that not all the fimbrial genes are coordinately expressed from a single promoter. For example, the fimbrial subunit is synthesized in minicells harboring the extensive deletion plasmid pJP6, whereas the 71,000- and 45,000-dalton polypeptides are encoded by pJP1. These results would indicate that the 17,000-dalton fimbrial subunit gene has its own promoter which is distinct from that required for expression of the two larger polypeptides. Alternatively, the gene encoding the fimbrial subunit may be expressed by a promoter located on the vector DNA. However, the presence of both Tn*5* and mini-Mu insertion mutants, which retain HA activity and yet map between the 17,000-dalton subunit gene and vector DNA, would indicate that transcription of this gene is not under the control of a promoter on the vector. DNA sequence analysis of this region should provide a better resolution of the transcription initiation site.

The two largest plasmid-encoded polypeptides detected by SDS-polyacrylamide analysis do not appear to be synthesized constitutively by pDC5. Although the 71,000- and 45,000-dalton polypeptides are encoded by the 3.1-kb *Hin*dIII fragment of pDC5, relatively small deletions some distance from this fragment dramatically decrease the expression of these gene products. This is evident in minicell experiments with plasmid pJP3, which retains the coding region of the large polypeptides but has a 600-base deletion at a site distant from this region. Therefore, expression of the 71,000- and 45,000-dalton polypeptides may be controlled by a gene product synthesized by a distinct operon.

Recently, Normark et al. (17) reported the genetic organization of an MR adhesin derived from a uropathogenic *E. coli*. A comparison of restriction endonuclease sites of pDC5 with their recombinant plasmid indicates a large degree of sharing of these sites. However, significant differences in the number of locations of *Hin*dIII, *Hpa*I, and *Pst*I restriction endonuclease sites exist between the two recombinant molecules. Also, the approximate location of the genes encoding the fimbrial subunit would appear to be significantly different on the two plasmids. As reported previously, these two fimbrial preparations are not serologically cross-reactive (5), and therefore the structural genes may be significantly different. A comparison of the nucleotide sequences of these two genetic regions would prove useful in determining the degree of homology of the two genes encoding MR fimbriae.

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