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Construction and Characterization of Mutants Impaired in the Biosynthesis of the K88ab Antigen

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Plasmid pFM205 contains the genetic determinant for the K88ab antigen and is composed of a 4.3-megadalton DNA fragment derived from wild-type K88ab plasmid pRI8801 and cloning vehicle pBR322. The K88 DNA of pFM205 contains five genes, which code for polypeptides with apparent molecular weights of 17,000, 26,000 (the K88ab subunit), 27,000, 27,500, and 81,000. All five polypeptides were synthesized as precursors approximately 2,000 daltons larger than the mature polypeptides, indicating that they are transported across the cytoplasmic membrane by means of a signal sequence. A set of deletion derivatives of pFM205 was constructed, each containing a deletion in one of the five genes. In strains harboring derivatives of pFM205 containing a deletion in the gene for the 17,000- or 81,000-dalton polypeptide, the K88ab subunit was synthesized and transported to the outside of the cell. However, these strains did not adhere to brushborders or guinea pig erythrocytes, suggesting that the K88ab subunits were not assembled into normal fimbriae. Strains harboring plasmids containing a deletion in the gene for the 27,500-dalton polypeptide still adhered to brushborders and guinea pig erythrocytes, although very little K88ab antigen could be detected with an immunological assay. In strains harboring plasmids containing a deletion in the gene for the 27,000-dalton polypeptide, the K88ab subunit was synthesized but was probably subsequently degraded rapidly.

The K88 antigen is a plasmid-specified virulence factor which enables *Escherichia coli* strains to colonize the small intestines of piglets by means of specific adhesion to the epithelial cells of the gut (10). In an electron microscope the K88 antigen can be visualized as extracellular filamentous surface appendages (21), which are called fimbriae or pili. Several serologically distinct variants of the K88 antigen (K88ab, K88ac, and K88ad) have been described (9, 16); these variants have small differences in their amino acid compositions (7, 12). The K88 antigen is composed of identical proteinaceous subunits which have apparent molecular weights ranging from 23,500 to 26,000, depending on the variant analyzed (12).

Previously, we have described the cloning of the genetic determinant for the K88ab antigen (13) and the expression of the cloned DNA in minicells (14). It appeared that the cloned DNA, which was contained in recombinant plasmid pFM205, expressed at least six polypeptides in minicells. One of these polypeptides (molecular weight, 26,000) was the K88ab subunit. Through the construction of deletion derivatives of pFM205 the genes for these polypeptides were localized.

To study the process of fimbrial biosynthesis and to obtain information about the functions of the various gene products involved in this process, it was necessary to have a set of derivatives of pFM205, each containing a mutation in a different gene. In this paper we describe the construction of these plasmids and their properties. The results are discussed in relation to the possible functions of the polypeptides.

MATERIALS AND METHODS

Bacterial strains. *E. coli* K-12 (*thr thi lac-2 lacI recA171 supE*) was used as a host for the various plasmids studied. Minicells were isolated from *E. coli* strain DS410 (6).

Culture conditions. Unless otherwise stated, bacteria were grown in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) or on Trypticase soy agar. Cells containing derivatives of pBR322 (2) and pACYC184 (3) were cultured in the presence of ampicillin (500 μg/ml) and chloramphenicol (20 μg/ml), respectively.

Isolation of plasmid DNA. Plasmid DNA was purified from cleared lysates by cesium chloride-ethidium bromide density gradient centrifugation, as described previously (13). An alkaline extraction procedure (1) was used to analyze the plasmid contents of large numbers of transformants.

Enzyme reactions. Restriction endonucleases were obtained from New England Biolabs, Beverly, Mass.,

or Boehringer, Mannheim, Germany. Incubations were carried out according to the instructions of the manufacturers. The reaction conditions for S1 nuclease (Bethesda Research Laboratories, Cambridge, England) and alkaline phosphatase (Worthington Diagnostics, Freehold, N.J.) were as described by Rodriquez et al. (19) and Goodman and MacDonald (8), respectively. T4 DNA ligase was obtained from New England Biolabs. DNA fragments were ligated as described previously (13).

DNA gel electrophoresis. Electrophoresis of DNA was performed on horizontal or vertical slab gels in Tris-acetate buffer (40 mM Tris, 20 mM acetate, 2 mM EDTA, pH 7.7) or in Tris-borate buffer (90 mM Tris, 90 mM boric acid, 2.5 mM EDTA, pH 8.3). *Hin*dIII or *Hin*dIII-*Eco*RI fragments of λ (15) and *Hin*fI fragments of pBR322 (22) were used as molecular weight standards.

Construction of deletions with *Pst*I and S1 nuclease. An 80-μg amount of pFM205 was incubated with 20 U of *Pst*I in the presence of ethidium bromide (0.1 mg/ml) in a volume of 0.6 ml. After incubation for 2 h at 37°C, the reaction was stopped by adding excess EDTA. The reaction products were separated on a preparative horizontal gel (0.8% agarose in Tris-borate buffer), and linear full-length pFM205 molecules were isolated by electroelution. The single-stranded extensions of the linear molecules were removed with S1 nuclease, and the DNA was recircularized with T4 DNA ligase and was used to transform competent *E. coli* cells.

Transformation. Transformation was performed by the method of Dagert and Ehrlich (4).

Isolation and labeling of minicells. Minicells were isolated and labeled as described elsewhere (14). Proteolytic processing in minicells was inhibited by adding 6 to 9% (vol/vol) ethanol (17).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography. Linear 12 to 20% sodium dodecyl sulfate–polyacrylamide gradient gels (length, 24 cm) were prepared and autoradiographed as described previously (14). These gels gave slightly better results than the 14 to 20% sodium dodecyl sulfate–polyacrylamide gradient gels used previously (14).

Immunological procedures. The amount of K88ab antigen produced was determined semi-quantitatively with an enzyme-linked immunosorbent assay (ELISA) (14).

Determination of the amount of K88ab antigen. Cells in the early exponential growth phase were harvested, washed once with 0.1 M phosphate buffer (pH 7.2), and suspended to an optical density at 660 nm of 1.0 in 0.1 M phosphate buffer (pH 7.2) containing 0.4% bovine serum albumin and 0.01% Tween 80. To prepare cell-free extracts, the cells were disrupted by ultrasonic treatment. After whole cells and large cell envelope fragments were removed, the cell-free extracts were used in the ELISA. To determine whether the K88ab antigen could be detected on the outside of the cell, the ultrasonic treatment was omitted and whole cells were used in the ELISA.

Adhesion to brushborders and agglutination of guinea pig erythrocytes. Brushborders from piglets were generously provided by N. Kortbeek and J. G. W. Bijlsma. Adhesion of *E. coli* strains to brushborders was performed in the presence of 0.5% D-mannose, as described by Wilson and Hohmann (24). Agglutination of guinea pig erythrocytes was performed as described by Guinée and Jansen (9).

RESULTS

Construction of plasmids. The principle of the method used to obtain deletion derivatives of pFM205 is illustrated in Fig. 1. Plasmid DNA was incubated with a restriction endonuclease that generated DNA fragments with single-stranded extensions. The incubation conditions were chosen so that most plasmid molecules were cleaved at only one restriction endonuclease recognition site. This was achieved by performing the incubation in the presence of ethidium bromide (18). Subsequently, the linear full-length plasmid molecules were isolated, treated with S1 nuclease, and recircularized with T4 DNA ligase. These manipulations resulted in a small deletion at a particular restriction endonuclease recognition site.

We used restriction endonuclease *Pst*I because it cleaves the K88 DNA eight times in an area containing four of the five K88ab genes (14), whereas it cleaves the vector DNA only once in the gene for β-lactamase (2). Since transformants were selected for ampicillin resistance, plasmids with a deletion in the β-lactamase gene were not isolated.

The *Pst*I cleavage patterns of pFM205 and the deletion mutants derived from it are shown in Fig. 2. We obtained five of the eight possible deletions in the K88 DNA. About 200 transformants were analyzed to get these results. Most transformants (85%) contained plasmids with large deletions resulting from the excision of one or more DNA fragments or from deletions generated in vivo due to transformation with linear DNA (23).

The order of the seven small *Pst*I fragments of pFM205 was not known in advance, but this order was deduced from the cleavage patterns of the deletion mutants. Each deletion resulted in the loss of a particular *Pst*I recognition site and, therefore, in the disappearance from the cleavage pattern of two DNA fragments which were located adjacent to each other in the plasmid. A new DNA fragment appeared with a molecular weight which was equal to the sum of the molecular weights of the two missing fragments, indicating that only a small deletion was introduced (Fig. 2). Deletion derivatives containing large deletions, which resulted from the excision of two or more *Pst*I fragments, also provided information about the order of the *Pst*I fragments. For example, one deletion mutant, designated pFM232 (Fig. 3), was missing the fourth and sixth *Pst*I fragments, implying that these fragments are located adjacent to each other in pFM205. Another deletion mutant, designated

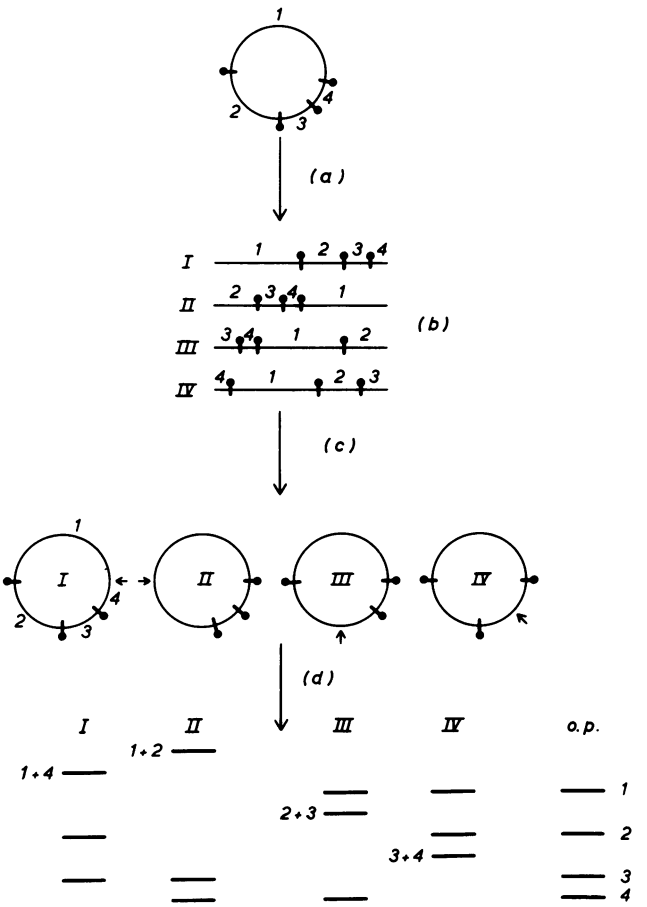

FIG. 1. Principle of the method used to construct deletion derivatives. The plasmid depicted was chosen arbitrarily and serves only to illustrate the method. (a) Plasmid DNA was digested in the presence of ethidium bromide with a restriction enzyme which generated single-stranded extensions. (b) The reaction products were separated on a preparative gel, and linear full-length molecules were extracted from the gel. The four possible permuted molecules are shown. (c) The single-stranded extensions were removed with S1 nuclease, and the DNA was circularized with T4 DNA ligase and used to transform competent cells. (d) Plasmid DNA was isolated from the transformants and cleaved with the restriction enzyme. From the cleavage patterns the order of the DNA fragments and the location of the deletion could be deduced. The cleavage patterns of the four possible deletion derivatives and of the original plasmid (o.p.) are shown. The DNA fragments generated by the restriction enzyme are indicated by the numbers 1 to 4. ↟, Cleavage sites of the restriction enzyme. The deleted cleavage sites are indicated by arrows.

pFM229 (Fig. 3), was missing the third, seventh, and ninth *Pst*I fragments, so these must also be located adjacent to each other. Combined, these results allowed us to derive a physical map of pFM205 for *Pst*I and to determine the locations of the deletions (Fig. 3).

The gene for the 27,500-dalton polypeptide does not contain a *Pst*I recognition site (14). Therefore, another procedure was used to introduce a deletion into this gene. The gene for the 27,500-dalton polypeptide and a large part of the gene for the 26,000-dalton polypeptide are located on a 1.1-megadalton *Eco*RI fragment (14). Since the complete nucleotide sequence of the gene for the 26,000-dalton polypeptide has been determined (7a), we were able to look for a restriction endonuclease which cleaved the 1.1-megadalton *Eco*RI fragment frequently but not in the region encoding the 26,000-dalton polypeptide. Restriction endonuclease *Hae*III fulfilled these requirements. The construction of pFM259, which contains a deletion in the gene for the 27,500-dalton polypeptide, is shown in Fig. 4.

Plasmid pFM261 was used for complementation studies. This plasmid was constructed by

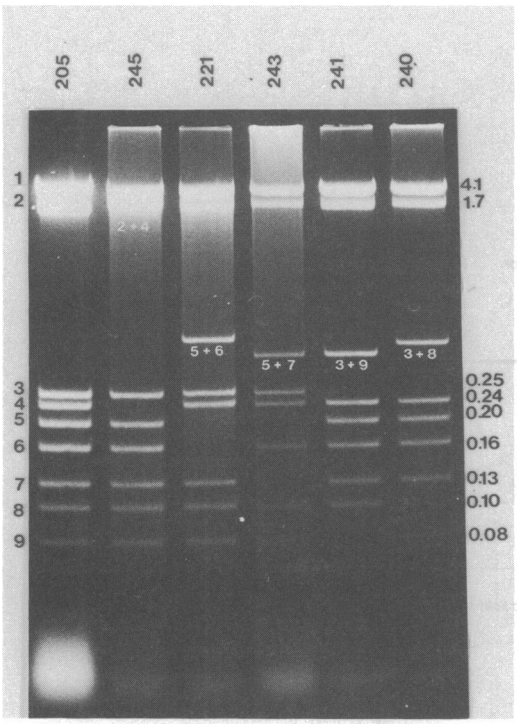

FIG. 2. Cleavage patterns of pFM205 and its deletion derivatives. A 2.5% agarose gel was used. The numbers on the left indicate the nine *Pst*I DNA fragments of pFM205. The compositions of the new *Pst*I DNA fragments of the deletion derivatives are indicated. The numbers on the right indicate the molecular weights of the *Pst*I DNA fragments ($\times 10^6$).

inserting a 4.3-megadalton *Sau*3AI fragment derived from pFM245 (Fig. 3) into the *Bam*HI site of pACYC184 (3). The *Sau*3AI fragment derived from pFM245 contains a deletion in the gene for the K88ab subunit. In pTM261, this fragment is located in the tetracycline operon of pACYC184 in the correct orientation relative to the tetracycline promotor, and possibly is transcribed from it. In minicells pFM261 expressed all of the K88 polypeptides except the K88ab subunit. Since pACYC184 and the deletion derivatives of pFM205 are compatible, pFM261 could be used to determine whether mutations in genes other than the gene for the K88ab subunit could be complemented in *trans*.

Characterization of the deletion mutants. The effects of the deletions on the expression of the K88 genes were studied in minicells. The K88ab DNA contained in pFM205 expresses six polypeptides in minicells; these polypeptides have apparent molecular weights of 17,000, 26,000, 27,000, 27,500, 30,000, and 81,000 (Fig. 5). Previously (14), we described the locations of the genes for the 26,000-, 27,500-, and 81,000-dalton polypeptides. The genes for the 17,000-, 27,000-, and 30,000-dalton polypeptides were found to be located between the genes for the 26,000- and 81,000-dalton polypeptides, but the order of these genes could not be determined at the time. The deletion mutants described in this paper enabled us to locate the genes for the 17,000- and 27,000-dalton polypeptides. Moreover, it appeared that the 30,000-dalton polypeptide was a precursor of the 27,000-dalton polypeptide (see below).

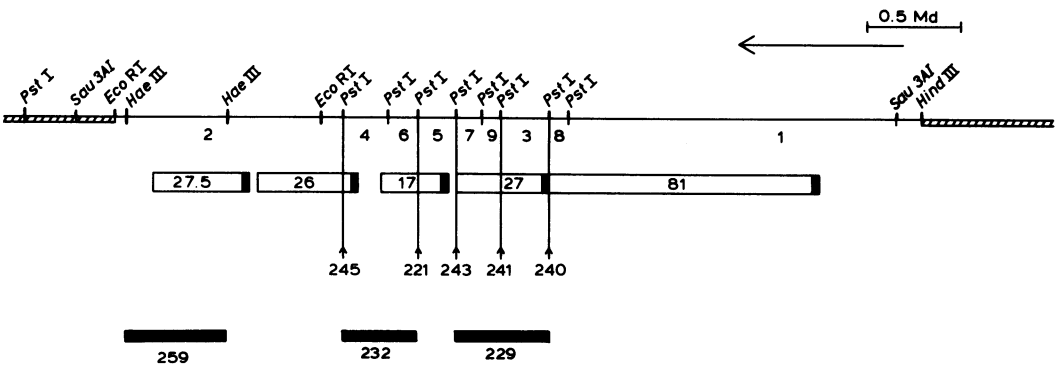

FIG. 3. Physical and genetic map of the K88ab DNA contained in pFM205. The arrow indicates the direction of transcription. pBR322 DNA is indicated by the cross-hatched areas. The numbers below the physical map indicate the nine DNA fragments generated by *Pst*I. The locations of the K88 genes are indicted by the bars. The lengths of the bars correspond to the lengths of DNA required to code for the polypeptides, assuming that the average molecular weights of an amino acid and a base pair are 110 and 640, respectively. The numbers in the bars indicate the molecular weights of the mature polypeptides ($\times 10^3$). The solid parts of the bars indicate the parts of the genes encoding the signal peptides. The deletions at the various *Pst*I sites are indicated by arrows. The numbers below the arrows refer to the plasmids containing the deletions. The deletions in pFM259, pFM232, and pFM229 are indicated by the thick black lines. The small *Hae*III fragment retained in pFM259 has been omitted. Md, Megadalton.

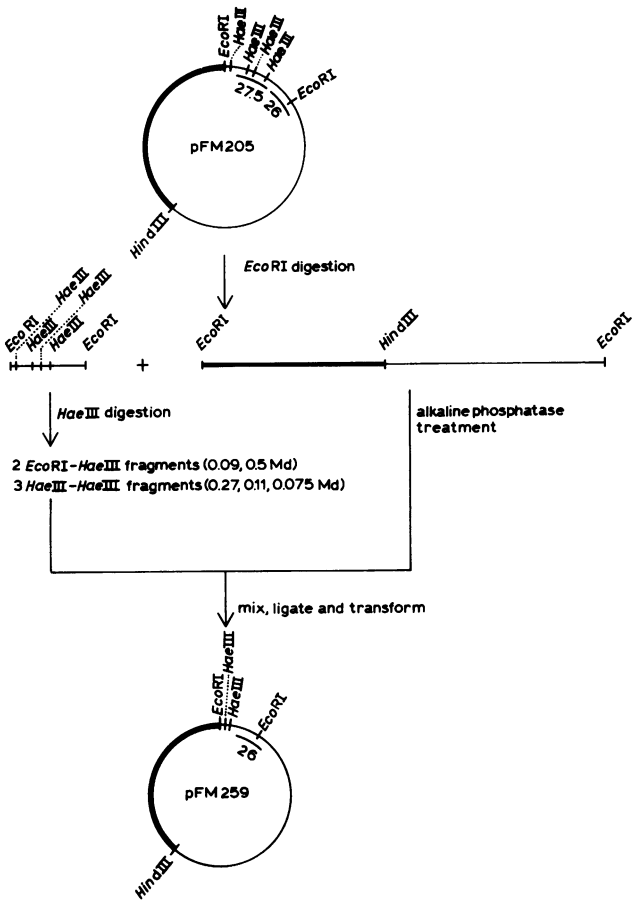

FIG. 4. Construction of pFM259. Plasmid pFM205 was cleaved with *Eco*RI, and the two resulting DNA fragments were isolated. The large fragment was treated with alkaline phosphatase to prevent it from being recircularized by T4 DNA ligase. The small fragment was cleaved with *Hae*III. Subsequently, the DNA fragments were mixed, ligated, and used to transform competent cells. A plasmid, designated pFM259, which contained the large *Eco*RI-*Hae*III fragment in the same position as pFM205 (i.e., it harbored an intact K88ab subunit gene), was isolated and used for further study. In addition to the two *Eco*RI-*Hae*III fragments, pFM259 also contained one *Hae*III-*Hae*III fragment (0.075 megadalton [Md]). The heavy line indicates pBR322 DNA. The locations of the genes for the 26,000- and 27,500-dalton polypeptides are shown. Only the relevant restriction enzyme recognition sites are shown. The order of the *Hae*III-*Hae*III fragments is tentative.

Minicells harboring pFM240 did not synthesize the 81,000-dalton polypeptide (Fig. 5). Instead, a new, slightly smaller polypeptide was observed. This indicated that the *Pst*I site deleted in pFM240 is located in the C-terminal end of the gene for the 81,000-dalton polypeptide.

The deletion in pFM245 resulted in the disappearance of the 26,000-dalton polypeptide and in the appearance of a new small polypeptide fragment (Fig. 5). Apparently, the *Pst*I site deleted in pFM245 is located in the gene for the 26,000-dalton polypeptide. DNA sequence data have confirmed the presence of a *Pst*I site in the N-terminal end of the K88ab subunit gene (7a).

Plasmid pFM259 contains a large deletion in the gene for the 27,500-dalton polypeptide (Fig. 3). Previously (14), we referred to this gene product as the 29,000-dalton polypeptide because it was detected after inhibition of proteolytic processing, when it appeared as a polypeptide with an apparent molecular weight of 29,000. At the time, we could not determine whether the 29,000-dalton polypeptide was a precursor because its processed form was obscured by the 27,000-dalton polypeptide. By improving the resolution of our gel system, it became possible to resolve the 27,000-dalton band into two bands with apparent molecular

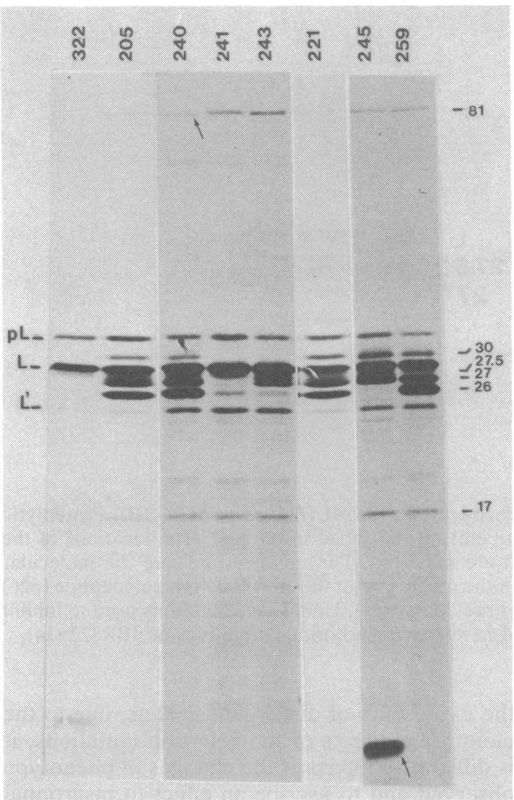

FIG. 5. Autoradiograph of polypeptides synthesized in minicells by pBR322 and by pFM205 and its deletion derivatives. The numbers on the right indicate the molecular weights of the K88 polypeptides ($\times 10^3$). The plasmids used are indicated above the lanes. The arrows indicate polypeptide fragments. The locations of the pBR322-encoded β-lactamase (L), its precursor (pL), and a β-lactamase fragment (L') are indicated on the left.

weights of 27,000 and 27,500. The 27,500-dalton band was absent in minicells containing pFM259 (Fig. 5). Furthermore, pFM259 did not synthesize the 29,000-dalton polypeptide when proteolytic processing was inhibited (data not shown). Therefore, we concluded that the 29,000-dalton polypeptide is a precursor of the 27,500-dalton polypeptide.

Plasmid pFM221 did not express the 17,000-dalton polypeptide (Fig. 5), indicating that the *PstI* site deleted in pFM221 is located in the gene for this polypeptide. The gene encoding the 17,000-dalton polypeptide extends into the fourth *PstI* fragment of pFM205 (Fig. 3), because plasmids missing only this DNA fragment did not express the 17,000-dalton polypeptide (data not shown). Furthermore, this gene is located to the left of the *PstI* site deleted in pFM243 (Fig. 3) because the 17,000-dalton polypeptide is still expressed by pFM243 (Fig. 5). On the basis of these data, the position of the gene for the 17,000-dalton polypeptide was derived (Fig. 3).

Minicells harboring pFM241 did not synthesize the 27,000- and 30,000-dalton polypeptides (Fig. 5). Previously (14), we assigned two separate genes to these polypeptides. These genes should be located between the genes for the 17,000- and 81,000-dalton polypeptides (Fig. 3), because only deletions in this area influence the production of the 27,000- and 30,000-dalton polypeptides. However, the DNA contained in this area does not have the coding capacity for two unrelated polypeptides with these molecular weights. Therefore, it seems likely that the 30,000-dalton polypeptide is a precursor of the 27,000-dalton polypeptide. This would also explain why the small deletion in pFM241 influences the production of both polypeptides.

The deletion in pFM241 also affected the 26,000- and 27,500-dalton polypeptides. The 27,500-dalton polypeptide could not be detected at all in minicells containing pFM241, whereas a faint band with an apparent molecular weight of 26,000 could be detected only after prolonged exposure of the autoradiograph (Fig. 5). When processing was inhibited, the precursors of both polypeptides were observed (Fig. 6).

In minicells harboring pFM243 the 30,000-dalton polypeptide could not be detected, whereas the 27,000-dalton polypeptide was synthesized (Fig. 5). On the basis of its location, we expected the deletion in pFM243 to be located in the C-terminal end of the gene for the 27,000-dalton polypeptide. When proteolytic processing was inhibited, the 27,000-dalton band was diminished, whereas the 30,000-dalton band reappeared (Fig. 6), again indicating that the 30,000-dalton polypeptide is a precursor of the 27,000-dalton polypeptide. As observed for minicells containing pFM241, the deletion in pFM243 also affected the 26,000-dalton polypeptide. A faint band with an apparent molecular weight of 26,000 could be detected only after prolonged exposure of the autoradiograph (Fig. 5). When proteolytic processing was inhibited, the precursor of the 26,000-dalton polypeptide was not detected (Fig. 6).

The results of the minicell experiments are summarized in Table 1.

To determine the influence of the deletions on the level of K88ab production, the amounts of K88ab antigen were determined in cell-free extracts and in the culture supernatant with an ELISA. To ascertain whether the K88ab antigen was transported to the outside of the cell, whole cells were also tested in the ELISA (Table 1). Three deletion derivatives of pFM205 still produced the K88ab antigen in detectable amounts

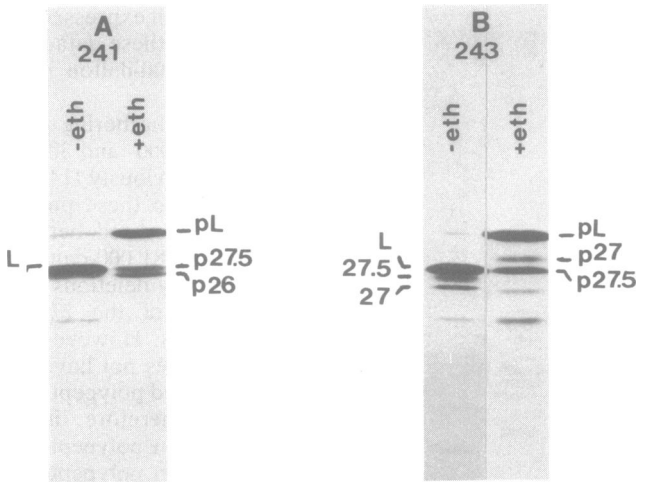

FIG. 6. Accumulation of precursors of polypeptides encoded by pFM241 (A) and pFM243 (B). Proteolytic processing was inhibited in minicells by adding ethanol (+eth), as described in the text. The locations of the pBR322-encoded β-lactamase (L) and its precursor (pL) are indicated. The numbers indicate the molecular weights of the mature polypeptides ($\times 10^3$). The locations of the precursors of the 26,000-dalton polypeptide (p26) and the 27,500-dalton polypeptide (p27.5) were established previously (14). Under the conditions used to inhibit processing, only the precursor of β-lactamase is observed in minicells harboring derivatives of pBR322 (14).

(i.e., pFM240, pFM221, and pFM259). None of the deletion mutants released significant amounts of K88ab antigen into the culture medium. Whole cells containing pFM240, pFM221, or pFM259 were K88ab$^+$ in the ELISA, indicating that the K88ab antigen was present on the outside of the cell.

We also investigated the ability of strains harboring the various plasmids to adhere to brushborders and to agglutinate guinea pig erythrocytes (Table 1). Only strains harboring pFM205 and pFM259 were able to adhere to brushborders or to agglutinate guinea pig erythrocytes.

To ascertain whether the observed effects were due to a diffusable function and not, for example, to polar effects, we determined whether the mutations could be complemented in *trans*. To do this, the various plasmids were introduced into an *E. coli* strain containing pFM261 (Table 1). Only the mutations in pFM243 and pFM259 could not be complemented.

DISCUSSION

To obtain information about the functions of the gene products involved in the production of the K88ab antigen, we constructed a set of plasmids, each containing a mutation in a different K88 gene. One way to obtain mutations in genes located on a cloned DNA fragment is by means of transposons. However, insertions of transposons in an operon exert polar effects on the expression of distal genes. Thus, due to the pleiotropic effects of the insertion mutations, it is difficult to interpret the changes in phenotype observed and to ascribe an effect to insertional inactivation of a particular gene. Furthermore, analyses of insertion mutants in minicells are often complicated by the presence of polypeptides encoded by the transposon.

To avoid these drawbacks, we used a method in which it is possible to obtain a set of deletion mutants with small deletions located at different recognition sites of a particular restriction endonuclease. By chosing the right restriction endonuclease it is possible to predetermine whether the average distance between two deletions will be large or small. Detailed knowledge of the positions of the recognition sites of the restriction endonuclease used is not a prerequisite because a map of the recognition sites and the locations of the deletions can be deduced from an analysis of the deletion mutants (Fig. 1).

We used restriction endonuclease *Pst*I, which generated single-stranded extensions that were removed by S1 nuclease. As Fig. 3 and Table 1 show, plasmids with deletions in all four genes containing *Pst*I sites were obtained. An analysis of the deletion mutants in minicells allowed us to complement and adjust some of our previous observations (14).

It appeared that a significant fraction of one gene product, the 27,000-dalton polypeptide, was present in a precursor form (30,000 dalton). Even after a pulse of 60 s followed by a chase with excess unlabeled amino acids, the 30,000-

TABLE 1. Properties of the K88ab deletion mutants

Plasmid	Polypeptides detected in minicells[a]					K88ab production in:[b]				Adhesion[c]		Agglutination[c]	
	27,500 daltons	26,000 daltons	17,000 daltons	27,000 daltons	81,000 daltons	Culture supernatant	Whole cells	Cell-free extract					
								−pFM261	+pFM261[d]	−pFM261	+pFM261[d]	−pFM261	+pFM261[d]
pFM205	+	+	+	+	+	2^4	2^9	2^{13}	2^{13}	++	++	2^8	2^9
pFM240	+	+	+	+	D	$<2^1$	2^3	2^5	2^{13}	−	++	$<2^1$	2^8
pFM241	−	±	+	D	+	$<2^1$	$<2^1$	$<2^1$	2^{13}	−	++	$<2^1$	2^7
pFM243	+	±	+	+	+	$<2^1$	$<2^1$	$<2^1$	$<2^1$	−	−	$<2^1$	$<2^1$
pFM221	+	+	D	+	+	$<2^1$	2^8	2^8	2^{13}	−	++	$<2^1$	2^8
pFM245	+	D	+	+	+	$<2^1$	$<2^1$	$<2^1$	$<2^1$	−	−	$<2^1$	$<2^1$
pFM259	D	+	+	+	+	$<2^1$	2^3	2^4	2^4	+	+	2^3	2^4

[a] +, Polypeptide present in minicells; −, polypeptide absent in minicells; ±, faint bands observed after prolonged exposure; D, polypeptide absent in minnicells due to a deletion in the gene for the polypeptide.

[b] K88ab antigen production was determined by using an ELISA and is expressed as the highest dilution that was still positive in the test.

[c] Adhesion to brushborders and agglutination of guinea pig erythrocytes were determined as described in the text.

[d] Strains contained pFM261 in addition to the plasmids indicated.

dalton band remained prominent in minicells harboring pFM205 (data not shown). A fraction of this gene product probably accumulates in a form that cannot be processed by minicells.

Because of better resolution of our gel system, a new polypeptide (27,500 daltons) was detected, which appeared to be the processed form of the 29,000-dalton polypeptide described previously (14). It was determined previously that the 17,000-, 26,000-, and 81,000-dalton polypeptides are synthesized as precursors (14); therefore, it appears that all five gene products expressed by the K88 DNA of pFM205 are processed. This indicates that these gene products are transported through the cytoplasmic membrane by means of a signal sequence (5). Additional evidence for this was obtained by localization studies, which revealed that the 81,000-dalton polypeptide is located in the outer membrane, whereas the 17,000-, 27,000-, and 27,500-dalton polypeptides are located in the periplasmic space (23a). The presence of a signal sequence in the precursor of the K88ab subunit has been confirmed by DNA sequence data (7a).

From the characterization of the deletion mutants, it became apparent that the 17,000-, 26,000-, 27,000-, and 81,000-dalton polypeptides and probably also the 27,500-dalton polypeptide are involved in the biosynthesis of K88ab fimbriae.

Although strains harboring pFM240, which contains a deletion in the gene for the 81,000-dalton polypeptide, did produce K88ab subunits, they did not adhere to brushborders or agglutinate guinea pig erythrocytes (Table 1). However, whole cells containing pFM240 reacted with antibodies directed against the K88ab antigen (Table 1), indicating that the fimbrial subunits are transported to the outside of the cell. Apparently, these fimbrial subunits are not assembled into normal fimbriae in the absence of the 81,000-dalton polypeptide. The absence of normal fimbriae was also suggested by the observation that the K88ab antigen produced by these strains differed from the antigen produced by pFM205 in that it was more thermosensitive and was not released from the cells at 65°C (14). The 81,000-dalton polypeptide might be involved in the assembly of the fimbrial subunits or anchorage of the fimbriae or both.

Plasmid pFM241 contains a deletion in the gene for the 27,000-dalton polypeptide (Fig. 3). In strains harboring pFM241, the K88ab subunit could not be detected with the ELISA, and these strains did not adhere to brushborders or agglutinate guinea pig erythrocytes. In minicells very low amounts of the K88ab subunit were observed, whereas the 27,500-dalton polypeptide was not detected at all. These observations cannot be explained by polar effects, because

the gene for the 17,000-dalton polypeptide, which is also located downstream from the deletion, was expressed normally. Moreover, the production of K88ab antigen reached normal levels when an intact gene for the 27,000-dalton polypeptide was present in *trans* (Table 1). When proteolytic processing was inhibited in minicells containing pFM241, the precursors of the K88ab subunit and the 27,500-dalton polypeptide were observed (Fig. 6), indicating that both genes were expressed. Possibly, the two processed gene products are susceptible to proteolysis in the absence of the 27,000-dalton polypeptide and are degraded rapidly. The 27,000-dalton polypeptide is located in the periplasmic space, and we presume that this polypeptide interacts at some stage of fimbrial biosynthesis with the K88ab subunit and the 27,500-dalton polypeptide. Preliminary experiments have indicated that the 27,000-dalton polypeptide forms large complexes with the fimbrial subunit in the periplasmic space.

The deletion in pFM243 is probably located in the C-terminal end of the gene for the 27,000-dalton polypeptide. This deletion did not result in a detectable change in the mobility of the 27,000-dalton polypeptide. However, lower amounts of the 27,000-dalton polypeptide were detected in minicells containing pFM243. This might explain why its precursor (the 30,000-dalton polypeptide) was not observed. In minicells containing pFM243 the K88ab subunit was present in very low amounts. When proteolytic processing was inhibited, the precursor of the K88ab subunit was not detected (Fig. 6). The low amounts of the K88ab subunit cannot be explained by polar effects, because the gene for the 17,000-dalton polypeptide, which is also located downstream from the deletion, was expressed normally. Since the mutation in pFM243 could not be complemented, it seems likely that the K88ab subunit is present at low levels due to a second mutation in or near the gene for the subunit.

Plasmid pFM221 contains a deletion in the gene for the 17,000-dalton polypeptide. As observed for cells harboring pFM240, cells containing pFM221 did synthesize the K88ab subunit and transport it to the outside of the cell, but they did not adhere to brushborders and agglutinate guinea pig erythrocytes, suggesting that the K88ab subunits were not assembled into normal fimbriae. Possibly, the K88ab subunit has to be modified by the 17,000-dalton polypeptide, which is located in the periplasmic space, in order to be assembled into normal fimbriae.

Strains harboring pFM259, which contains a deletion in the gene for the 27,500-dalton polypeptide, still adhered to brushborders and agglutinated guinea pig erythrocytes (Table 1). Very low amounts of K88ab antigen were detected in cell-free extracts of these strains. The low values obtained in the ELISA could not be enhanced by complementation. This indicates that the observed effects were not caused by the absence of the 27,500-dalton polypeptide, but by the deletion itself. Possibly, the deletion affects the stability of the mRNA. However, it seems likely that the 27,500-dalton polypeptide is involved in the biosynthesis of the K88ab fimbriae, because deletions in the gene for the 27,000-dalton polypeptide affect both the K88ab subunit and the 27,500-dalton polypeptide (Table 1).

Thus, the biosynthesis of the K88ab fimbriae is a complex process involving at least four and possibly five polypeptides. The K88ab subunit is probably transported across the cytoplasmic membrane by means of its signal sequence during translation. In the periplasmic space it interacts with the 17,000- and 27,000-dalton polypeptides and possibly also with the 27,500-dalton polypeptide. These polypeptides might be involved in modification of the subunit and in the assembly of the subunits into fimbriae. The presence of the 81,000-dalton polypeptide in the outer membrane is necessary for the subunits to be assembled into normal fimbriae. Possibly, the fimbriae are anchored to the outer membrane by means of this polypeptide.

Shipley et al. (20) have cloned the K88ac genetic determinant and identified six K88 polypeptides in minicells. By analyzing Tn*5* insertion mutants of the cloned K88ac determinant, Kehoe et al. (11) derived a genetic map very similar to the one derived for K88ab. It is interesting to speculate whether the serological differences found in the K88ab, K88ad, and K88ac antigens only reflect differences in the nucleotide sequence of the fimbrial subunit gene. Possibly, the serological differences are also caused by divergence of other genes (for example, the genes involved in the modification of the fimbrial subunit).

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