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Cloning, mapping and expression of the genetic determinant that encodes for the K88ab antigen

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ABSTRACT

The K88 antigen, a plasmid-specified virulence factor of *E.coli* involved in porcine neonatal diarrhoea, is often found to be associated with the ability to metabolize raffinose (Raf). Plasmid pRI8801 (51 megadalton) was used to clone the determinants of K88 and Raf with the vector pBR322. K88 was found to be encoded by a 7.7 megadalton *Hin*dIII fragment. The expression was highly dependent on the orientation of the *Hin*dIII fragment within pBR322. By *in vitro* generation of deletions, the *Hin*dIII fragment was reduced in size to 4.3 megadalton. The expression of K88 by pRI8801 and the recombinant plasmids was studied using an enzyme-linked immunosorbent assay. Raf was found to be located on a 4.0 megadalton *Sal*I fragment. A physical map of pRI8801 was constructed. The K88 antigen and Raf genes are not closely linked but separated by a stretch of DNA of about 20 megadalton.

INTRODUCTION

Diarrhoeal disease in piglets is often caused by enteropathogenic strains of *Escherichia coli*. The diarrhoea is caused by the production of two types of enterotoxins, a heat-labile toxin (LT) and a heat-stable toxin (ST), both of which are plasmid-mediated (1). Frequently such strains have in addition to their normal surface antigens a particular antigen, that has been called K88 (2). This antigen has been shown to play an essential role in colonization of the small intestine of the piglet by its adhesive properties for epithelial cells of the gut (3, 4). The K88 antigen has been purified (5) and is composed of identical subunits that form filamentous surface appendages (6) also called pili. Serologically, at least three variants of the K88 antigen (K88ab, K88ac and K88ad) can be distinguished (7, 8). The K88 antigen is encoded by plasmids that can be transferred to other bacteria by conjugation (9). Smith and Parsell (10) found that the ability to utilize raffinose (Raf) as a carbon source is frequently co-transferred with the K88 plasmids. More recently, Shipley et al. (11) showed that the genetic determinants of K88 and Raf are usually located on the same plasmid.

After conjugal transfer to *E.coli* K12, two kinds of K88 plasmids were found; non-selftransmissable ones with a molecular weight of about 50 megadalton (Md) and occasionally plasmids of 90 Md in size that were self-transmissable. Bak et al. (12) reported the dissociation of a K88 plasmid into a large (40 Md) and a small (10 Md) component. It was suggested that the smaller component carried the K88 determinant, although no cells were explored carrying only this small plasmid.

Although the K88 antigen was first described in 1961, little is known about its structure, its biosynthesis, the organisation of the genes involved in expression of K88 and the regulation of its expression. Of particular interest is the very low production of K88 at temperatures of 18-20^{o}C (2). A similar temperature-dependent production has been found with other pilus-like colonization factors as K99 (13), 987P (14) and CFA (15). The study of such aspects would be greatly facilitated by the availability of a small plasmid carrying the K88 gene(s). The small plasmid described by Bak et al. (12) seemed promising in this respect. However, after introduction of this plasmid into an *E.coli* K12 strain by co-transformation, we could not detect expression of the K88 antigen nor raffinose metabolism (unpublished experiments). Therefore, we constructed small plasmids carrying the K88 and Raf determinants by *in vitro* recombination. In this study we report the molecular cloning of the K88 and Raf genetic determinants from plasmid pRI8801 using the vector pBR322 (16). Furthermore, the expression of the K88 antigen by the recombinant plasmids obtained has been studied by an enzyme-linked immunosorbent assay (ELISA). In addition a physical map of plasmid pRI8801 was constructed and the location of the genetic determinants for K88 production and raffinose utilization on pRI8801 was established.

MATERIALS AND METHODS

BACTERIAL STRAINS AND PLASMIDS

Strain G7 (1), a wild-type *E.coli*, is K88ab^{+}, Raf^{+} and produces heat-stable (ST) and heat-labile (LT) enterotoxin (W.H.Jansen, unpublished). *E.coli* K12, C600, *thr, leu, thi, sup E44, lac, ton A21* was used as host for the various plasmids studied. Strain 1801 (K88ab^{+}, Raf^{+}, LT^{-}, ST^{-}) is a transconjugant from the mating G7 x C600 and was obtained from Dr.P.A.M. Guinée and W.H.Jansen. The cloning vehicle used, pBR322 (16), was obtained from Dr.H.L.Heyneker.

CULTURE CONDITIONS

Unless otherwise stated bacteria were grown in Nutrient Broth (17), or on Nutrient Agar (17). Cells containing derivatives of pBR322 were cultured in the presence of ampicillin (100 µg/ml). Bacteria were screened for the ability to metabolize raffinose on MacConkey agar base (Difco) supplemented with 1% raffinose. Selection of Raf^+ transformants was done on M9 minimal medium (18) containing 1% raffinose.

ISOLATION OF PLASMID DNA

pBR322 and the recombinant plasmids derived from it were isolated from cells grown in 250 ml of M9 minimal medium supplemented with 0.5% casamino acids (Difco). Chloramphenicol was added during the early exponential phase (E600 0.4) to a final concentration of 170 µg/ml (19). Purified plasmid DNA was obtained from cleared lysates which had been subjected to CsCl-ethidium bromide ultracentrifugation (20). Plasmid pRI8801 was isolated from 4 liter cultures. DNA was precipitated from the cleared lysate with polyethylene glycol 6000 (21) prior to CsCl-ethidium bromide ultracentrifugation. For screening of recombinant plasmid-containing transformants, the ultracentrifugation was omitted and cleared lysates were treated with RNase (100 µg/ml, heated for 10 min at 100°C to inactivate DNase) and subsequently with proteinase K (100 µg/ml), for one h at 37°C. The DNA solutions were extracted with an equal volume of phenol, followed by two extractions with equal volumes of chloroform. Finally, the solutions were dialyzed against 10 mM Tris-HCl, 10 mM NaCl, 1 mM EDTA, pH 8.0.

CLEAVAGE WITH RESTRICTION ENDONUCLEASES AND CLONING PROCEDURE[a)]

*Bam*HI was obtained from Boehringer Mannheim. *Eco*RI, *Hin*dIII, *Sal*I, *Pst*I, *Xho*I, *Xba*I and *Hae*II were obtained from New England Biolabs. *Hin*dIII, *Pst*I[c)] and *Hae*II digestions were performed in 6 mM Tris-HCl, 10 mM NaCl, 7 mM KCl, 7 mM $MgCl_2$, 6 mM dithiothreitol, pH 7.5. All other digestions were carried out according to the instructions of the manufacturer. T4 ligase was a gift from Dr.H.L.Heyneker. For cloning, DNA was digested to completion with a restriction endonuclease and extracted with phenol and chloroform as described above. After precipitation with ethanol, the DNA was dissolved in ligase buffer (20 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM dithiothreitol, pH 7.6). ATP and ligase were added to a final concentration of 0.5 mM and 20 unit/ml, respectively. The mixture was incubated for 16 h at 14°C. Prior to transformation, the solution was extracted with phenol and chloroform, precipitated with ethanol and dissolved in 10 mM Tris-HCl, 1 mM EDTA, pH 8. Transformation was performed according to the method of Cohen

et al. (22). Screening for tetracycline-sensitive colonies was done on plates containing 20 µg/ml tetracycline.

AGAROSE GEL ELECTROPHORESIS OF DNA

Electrophoresis of DNA was performed on horizontal slab gels (20 x 20 x 0.6 cm) containing 20 mM Tris, 20 mM sodium acetate, 2 mM EDTA, pH 7.7 and 0.7% or 1.4% agarose. After adding the DNA solution to the slots (0.8 x 0.5 x 0.1 cm) the bromophenol blue front was allowed to migrate 1 cm during 15 min at 100 mA. Subsequently, the slots were filled with agarose and electrophoresis was continued for 16 h at 40 mA. *Hin*dIII or *Hin*dIII/*Eco*RI fragments of λ (23) and *Hae*II fragments of ΦX174 (24) were used as molecular weight standards.

DETERMINATION OF THE MINIMAL INHIBITORY CONCENTRATION (MIC) OF TETRACYCLINE

Serial dilutions of overnight cultures were added to freshly prepared plates supplemented with tetracycline to final concentrations of 0.1, 0.2, 0.25, 0.5, 1.0, 2.0, 5.0 and 10.0 µg/ml, respectively. The plates were incubated overnight at 37°C. The MIC was taken as the concentration of tetracycline that resulted in a decrease of the efficiency of plating by at least 50%.

DETECTION OF THE K88 ANTIGEN

All sera were generously provided by Dr.P.A.M.Guinée and W.H.Jansen. Double diffusion tests (25) were performed using cell-free extracts from K88-producing strains prepared as described by Guinée et al. (26).

For the semi-quantitative determination of the K88 antigen, a slightly different procedure was used for the preparation of cell-free extracts. Cells were grown in Nutrient Broth at 37°C or 19°C for 20 and 40 h, respectively. The cells were harvested, resuspended in 50 mM Tris-HCl, 140 mM NaCl, 0.01% Tween 80, pH 7.5 to a concentration of 10^9 cells/ml and disrupted by treatment with a sonifier (Branson Sonic Power Co, type B12) for 7 min at 100 Watt. Intact cells and large membrane fragments were removed by centrifugation (30 min, 30.000x g). The amount of K88 antigen in the supernatant was detected and estimated using an enzyme-linked immunosorbent assay (ELISA). IgG-enriched K88ab sera were prepared by treatment of antiserum with caprylic acid as described by Steinbuch and Audran (27). The labeling of IgG with horse radish peroxidase (Sigma type VI) was carried out basically according to the method of Nakane and Kawaoi (28). The following procedure was used for the semi-quantitative determination of the K88

antigen. Disposable polystyrene microtiter trays (Cooke, Microtiter) were coated with anti-K88 IgG, by filling each well with 0.20 ml of caprylated K88 antiserum (diluted in 140 mM NaCl to a final protein concentration of 4 µg/ml). After overnight incubation at room temperature, the wells were washed 3 times with a solution of 0.01% Tween 20 as described (29). In the coated wells, two-fold serial dilutions of K88-containing samples were made in BST (0.2% bovine serum albumine, 140 mM NaCl, 0.01% Tween 80) so that each well contained 0.10 ml of antigen solution. After incubation for 2 h at 37°C, the trays were washed as described above, 0.10 ml of conjugate (diluted 1:400 to 1:800 in BST) was added to each well and the trays were incubated again for 2 h at 37°C. After washing, 0.10 ml of freshly prepared substrate solution (0.08% 5-amino-5-hydroxy-benzoic acid, 0.05% H_2O_2, 2 mM EDTA, pH 6.0) was added to each well. The trays were incubated for 90 min at room temperature and read by eye. The highest dilution which still showed maximum coloring was taken as the titer. The sensitiviy of the test was determined using purified K88ab antigen (5) and amounted to about 0.1 ng of K88.

When large numbers of clones containing recombinant plasmids were screened for the production of the K88 antigen, overnight cultures were added to polystyrene cuvettes (LKB) coated with 1.2 ml of caprylated K88 antiserum or alternatively, colonies were suspended in BST in the cuvettes. The K88 antigen was detected as described above except that 1 ml of conjugate and 1 ml of substrate solution were used.

RESULTS

CLONING OF THE GENETIC DETERMINANTS THAT ENCODE FOR THE K88 ANTIGEN AND RAFFINOSE METABOLISM

To determine whether K88 and Raf were loacted on the same plasmid and to separate this plasmid from other plasmids that might have been co-transferred to the transconjugant strain 1801, plasmid DNA from this strain was used to transform competent cells of C600. Transformants were selected for their ability to metabolize raffinose. All of the 15 Raf^+ transformants tested produced the K88 antigen. Plasmid DNA from one of these strains was isolated and characterized. This strain contained a single plasmid species with a molecular weight of approximately 50 Md. This plasmid is designated as pRI8801 and was used to clone the genes encoding for the K88 antigen and raffinose metabolism. The restriction endonucleases *Hin*dIII and *Sal*I were

used for this purpose. Cloning with these enzymes leads to insertional inactivation of the tetracycline gene of pBR322 (16). pRI8801 has at least 14 *Hin*dIII and 4 *Sal*I cleavage sites (Fig. 1, lanes a and k). The cleavage pattern of pRI8801 as shown in Fig. 1, lane a resembles the *Hin*dIII cleavage pattern of plasmid pPS300, that has been published by Shipley et al. (11). Both plasmids, pPS300 and pRI8801, were derived originally from strain G7, which was first described in 1961 (2). These plasmids were characterized after being transferred independently by conjugation and transformation.

A mixture of pRI8801 and pBR322 was digested to completion with *Hin*dIII and subsequently treated with ligase (the total DNA concentration was 20 µg/ml; the molar ratio of pRI8801 to pBR322 was 5:1).The ligated mixture was used to transform competent *E.coli* cells. 121 out of the 257 ampicillin-resistant transformants tested were found to be tetracycline-sensitive. These were tested for K88 antigen production and six positive clones were detected. These six strains expressed the K88 antigen at different levels. The amount of K88 antigen produced by three strains could be easily detected with the ELISA (subsequently designated as the $K88^{+}$ phenotype), whereas expression of the K88 antigen by the three remaining strains was hardly detectable with the ELISA (subsequently designated as the $K88^{+/-}$ phenotype). The three $K88^{+}$

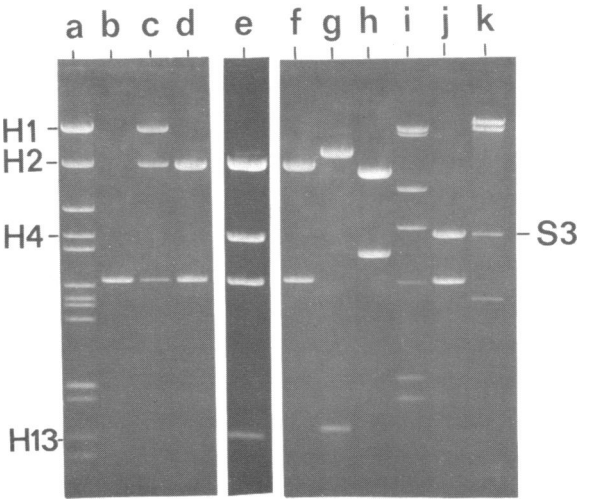

Fig. 1 - Agarose gel (0.7%) electrophoresis of restriction endonuclease-generated fragments of pRI8801 and recombinant plasmids. a, *Hin*dIII/pRI8801; b, *Hin*dIII/pBR322; c, *Hin*dIII/pFM65; d, *Hin*dIII/pFM38; e, *Hin*dIII/pFM12; f, *Hin*dIII/pFM200; g, *Bam*HI/pFM38; h, *Bam*HI/pFM200; i, *Hin*dIII/g (17.7, 14.9, 6.1, 4.1, 2.7, 1.4 and 1.1 Md); j, *Sal*I/pFM100; k, *Sal*I/pRI8801.

strains contained the recombinant plasmids designated as pFM32, pFM65 and pFM79 in which the cloned DNA was found to be composed of the two largest *Hin*dIII fragments of pRI8801 (Fig. 1, lane c). These fragments, H1 and H2, were 15 Md and 7.7 Md in size. After construction of the physical maps (data not shown), it became apparent that these three plasmids were identical with respect to the orientation and order of the cloned *Hin*dIII fragments in pBR322. The mutual orientation of the H1 and H2 fragments in these recombinant plasmids differed from that in the parental plasmid pRI8801 (see below). Two of the $K88^{+/-}$ strains contained the recombinant plasmids designated as pFM29 and pFM38, and were composed of the H2 fragment and pBR322 (Fig.1, lane d). The orientation of the H2 fragment within pBR322 was identical in these two plasmids. This could be determined by comparing the *Bam*HI-generated fragments (see below). The third $K88^{+/-}$ strain contained a third type of recombinant plasmid, pFM12, (Fig. 1, lane e) in which the cloned DNA was composed of the 3 fragments H2, H4 (3.9 Md) and H13 (0.94 Md). The H2 fragment had the same orientation as in pFM29 and pFM38 and was flanked by the fragments H4 and H13 (data not shown). All the recombinant plasmids that expressed K88 had only one particular fragment, H2, in common. We conclude that this fragment contains the K88 determinant. The location of the K88 determinant on a *Hin*dIII fragment of similar size has also been suggested by Shipley et al. (11). The orientation of the fragment H2 in the plasmids that expressed K88 at a low level was opposite to the orientation in the plasmids expressing K88 at a high level. Therefore, it seemed that the orientation of the H2 fragment within pBR322 determined the level of expression of the K88 antigen. To test this hypothesis, pFM38 was digested to completion with *Hin*dIII and ligated. After transformation 23 tetracycline-sensitive colonies were screened for K88 antigen production. Fourteen showed the $K88^{+/-}$ and the nine $K88^{+}$ phenotype. Plasmid DNA was isolated from one $K88^{+}$ transformant and the *Bam*HI-generated fragments of this plasmid, pFM200, were compared to those of pFM38 (Fig. 1, lanes g and h). The orientation of H2 in pFM200 and pFM38 was found to be opposite. Digestion of pFM200 with *Hin*dIII (Fig. 1, lane f), followed by ligation, transformation and screening of 11 tetracycline-sensitive colonies for K88 production again revealed the two phenotypes (7 colonies $K88^{+}$, 4 colonies $K88^{+/-}$).

To determine whether the Raf locus of pRI8801 could also be cloned with *Hin*dIII, transformants were plated on MacConkey medium supplemented with raffinose and ampicillin. No Raf^{+} colonies were found among 400 tetracycline-sensitive transformants screened.

The cloning procedure was repeated with the restriction endonuclease *Sal*I. In none of the 18 tetracycline-sensitive colonies examined, the K88 antigen was detected. However, one of these transformants did reveal the Raf^+ phenotype on MacConkey plates. This strain contained a recombinant plasmid, pFM100, composed of the S3 fragment (4.0 Md) of pRI8801 and pBR322. (Fig. 1 lanes j and k).

THE CONSTRUCTION OF PHYSICAL MAPS

Physical maps of the recombinant plasmids pFM38, pFM200 and pFM100 were inferred from single and double digestions with *Bam*HI, *Eco*RI, *Hin*dIII, *Sal*I, *Xba*I and *Xho*I. The interpretation of the cleavage patterns was facilitated by the availability of a detailed map of pBR322 (16). The physical maps of pFM38 and pFM200 are shown in Fig. 2. The H2 fragment contained within pFM38 and pFM200, has one asymmetric *Bam*HI site, two *Eco*RI sites, but no *Sal*I, *Xba*I or *Xho*I sites. The asymmetric *Bam*HI site was used to determine the orientation of H2 in pFM38 and pFM200, which was found to be opposite in these plasmids.

The physical map of pFM100 is shown in Fig. 3. Of the restriction endonucleases tested, only *Xba*I did not cleave the S3 fragment.

A physical map of pRI8801 was also constructed and the positions of the

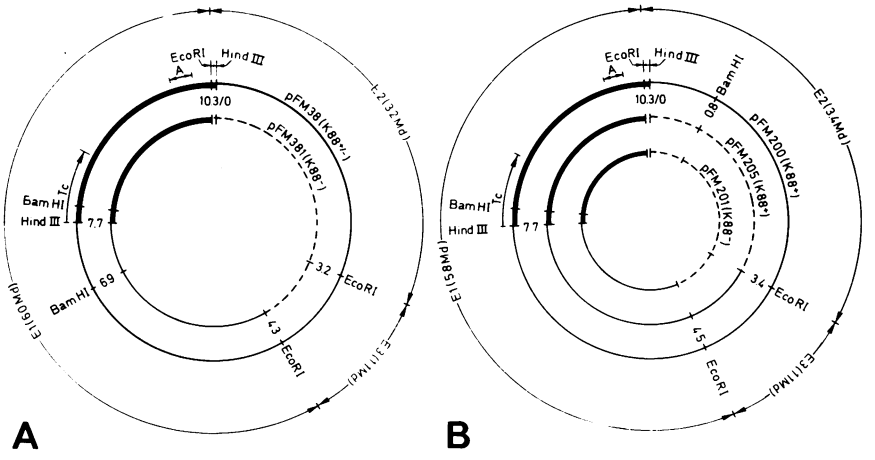

Fig. 2 - Physical maps of pFM38 (a) and pFM200 (b) and the plasmids derived from them by generating deletions. The outermost segments depict the *Eco*RI-generated fragments. The dotted areas refer to the segments which have been deleted and the heavy lines represent pBR322. The tetracycline (Tc) and ampicillin (A) resistance genes have been depicted (16). The co-ordinates are in Md.

cleavage sites of *Bam*HI, *Sal*I, *Xba*I and *Xho*I are shown in Fig. 4. The molecular weight of pRI8801 (51.4 Md) was deduced from the sum of the *Eco*RI (51.6 Md) and the *Hin*dIII (51.2 Md) generated fragments, respectively. Mapping of the restriction endonuclease sites of pRI8801 was facilitated by the availability of physical maps of the various recombinant plasmids in which about 50% of the pRI8801 genome was cloned (see also Fig. 1). The positions of the cloned fragments H1,H2 and S3 are included in Fig. 4.

GENERATION OF DELETIONS IN pFM38 AND pFM200

To obtain insight into the location of the K88 determinant within the H2 fragment, deletions were generated in the plasmids pFM38 and pFM200. The H2 fragment contains two *Eco*RI sites and pBR322 has an additional site (16). The opposite orientation of H2 in pFM38 and pFM200 allows the removal of different parts of H2 with *Eco*RI (Fig. 2a and 2b). Digestion of pFM38 or pFM200 with *Eco*RI generates three fragments (Fig. 5, lanes a and c). The smallest *Eco*RI fragment of pFM38, designated as 38-E3, is identical to that of pFM200 (i.e. 200-E3).

pFM38 was digested to completion with *Eco*RI and ligated (DNA concentration 20 µg/ml). After transformation and selection for ampicillin resis-

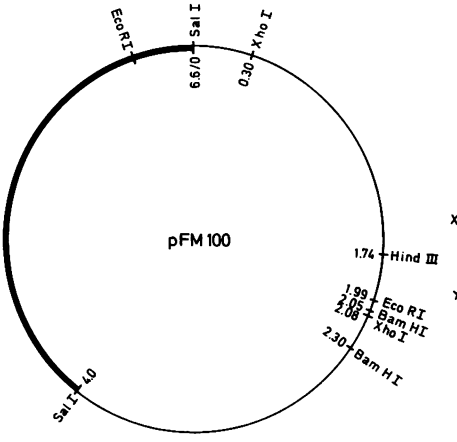

Fig. 3 - A physical map of pFM100 which contains the Raf determinant. The heavy line represents pBR322 and the co-ordinates are in Md.

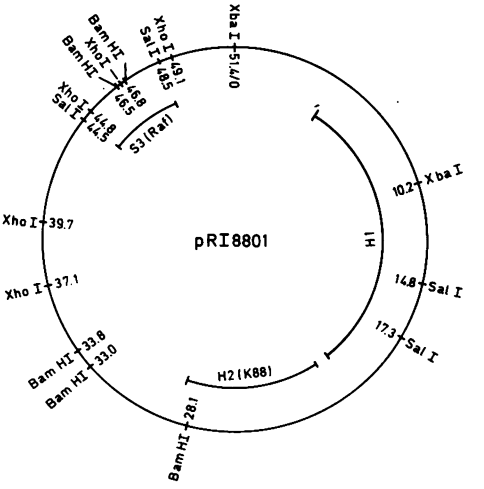

Fig. 4 - A physical map of the K88 plasmid pRI8801. The cloned fragments H1, H2 and S3 have been depicted. H2 contains the K88 and S3 the Raf genetic determinant. The co-ordinates are in Md.

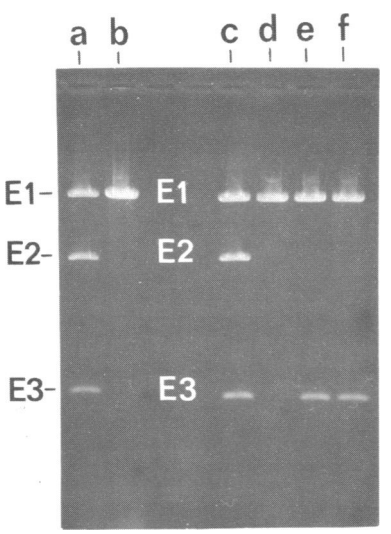

Fig. 5 - *Eco*RI restriction endonuclease cleavage pattern of pFM38, pFM200 and plasmids derived from them by generating deletions. The digested DNA was subjected to electrophoresis on a 0.7% agarose gel. a, pFM38; b, pFM381; c, pFM200; d, pFM201; e, pFM205; f, pFM204.

tance, 60 colonies were screened for K88 antigen expression. None were found to be K88-positive. Plasmid DNA from four transformants was analyzed and in all cases the *Eco*RI-generated fragments 38-E2 and 38-E3 were deleted. One of these plasmids, designated as pFM381, is shown in Fig. 2a and Fig. 5, lane b.

A similar procedure was carried out with pFM200. Twenty four of the 240 ampicillin-resistant colonies tested produced the K88 antigen. The plasmid DNA of 17 K88-positive strains was analyzed. Sixteen of these contained identical plasmids in which the 200-E2 *Eco*RI generated fragment was found to be deleted. A representative of these plasmids, pFM205, is shown in Fig. 2b. The cleavage pattern with *Eco*RI is depicted in Fig. 5, lane e. The remaining K88-positive clone carried a recombinant plasmid that was identical to pFM200, except that the 200-E2 fragment was inverted (data not shown). Four K88-negative strains were examined and three of them contained plasmid DNA in which both the 200-E2 and 200-E3 fragments were removed. A representative of these plasmids, pFM201, is shown in Fig. 2b and Fig. 5, lane d. The other K88-negative clone analyzed harbored a plasmid, pFM204, that was indistinguishable from pFM205 with respect to its molecular weight and cleavage pattern with *Eco*RI (Fig. 5, lane f). The observed difference in expression of K88 by pFM204 and pFM205 might be caused by an opposite orientation of the 200-E3 fragment in these two plasmids. To test this possibility, a double digestion with *Hae*II and *Pst*I was carried out. As can be seen in Fig. 6, lanes e and f, the resulting cleavage patterns of pFM204

and pFM205 are identical except for four bands. The four non-identical bands contain the *Eco*RI sites, as could be demonstrated by a triple digestion with *Hae*II, *Pst*I and *Eco*RI (Fig. 6, lanes b and c), and thus form the junctions between the 200-E1 and 200-E3 fragments. These results indicate an opposite orientation of the 200-E3 fragment in pFM204 and pFM205. Furthermore, the orientation of the 200-E3 fragment in pFM200 and pFM205 is identical, because only one of the two pFM205 bands containing an *Eco*RI site is not present in the *Hae*II-*Pst*I cleavage pattern of pFM200 (see Fig. 6, lane d).

EXPRESSION OF THE K88 ANTIGEN BY THE RECOMBINANT PLASMIDS

To determine whether there was any difference between the K88ab antigen encoded by pRI8801 and pFM205, double diffusions with K88ab and K88b sera were performed. Cell-free extracts of C600 (pRI8801) and C600 (pFM205) were tested against these sera. Only one line of identity without spurs (30) was observed. Furthermore, after absorption of the K88ab serum with C600 (pFM205) (0.5×10^{11} cells/ml of serum) no remaining K88ab antibodies were detectable. By these criteria, the K88 antigen expressed by the recombinant plasmid did not differ structurally from that expressed by the parental K88 plasmid.

Cloning of the K88 determinant might also influence the level of its expression. This was determined with the semi-quantitative ELISA. The

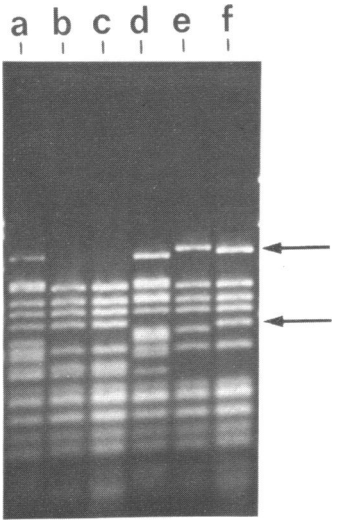

Fig. 6 - The cleavage patterns of pFM200, pFM204 and pFM205. The arrows indicate the non-identical bands of pFM204 and pFM205. A 1.4% agarose gel was used. a, *Hae*II-*Pst*I-*Eco*RI/pFM200; b, *Hae*II-*Pst*I-*Eco*RI/pFM205;c. *Hae*II-*Pst*I-*Eco*RI/pFM204; d, *Hae*II-*Pst*I/pFM200; e, *Hae*II-*Pst*I/pFM205. f, *Hae*II-*Pst*I/pFM204. The largest fragment in lane a is a partially digested fragment.

results as shown in Table 1 indicate that, at 37°C, strains harboring the recombinant plasmids pFM200 or pFM205 produce four times as much of the K88 antigen as strains harboring the parental plasmid pRI8801. The dependency of the expression of K88 on the orientation of the H2 fragment in pBR322, earlier described, is clearly demonstrated in Table 1. In one orientation (as in pFM200) at least 250 times more K88 antigen is produced than in the other orientation (as in pFM38).

The expression of K88 was also studied at a temperature of 19°C. The results (Table 1) showed that the expression by the recombinant plasmids pFM200 and pFM205 was 32-fold less at 19°C than at 37°C. Expression by pRI8801 at this low temperature was at least 64-fold less. The low production measured at 19°C was not due to detachment of K88 from the cells and release into the medium during culturing, because the titer of K88 in the supernatants of all cultures tested was less than 2. This low titer was also observed at 37°C.

EXPRESSION OF TETRACYCLINE RESISTANCE BY THE RECOMBINANT PLASMIDS

Insertion of foreign DNA into the *Hin*dIII site of pBR322 does not always result in complete loss of the expression of tetracycline resistance. This is because the *Hin*dIII site is not located within the structural genes, but in the promotor region of the tetracycline resistance determinant (31). Therefore we determined the minimal inhibitory concentration of tetracycline for strains harboring the various K88 recombinant plasmids. In contrast to plasmids pFM38 and pFM381, some expression resistance was observed by the

Table 1 Production of the K88 antigen by strains carrying the parental and the various recombinant plasmids

Strain	K88 antigen production[a]	
	37°C	19°C
C600 (pRI8801)	64	<2
C600 (pFM200)	256	8
C600 (pFM205)	256	8
C600 (pFM38)	<2	<2
C600	<2	<2

[a] The K88 production was measured semi-quantitatively and is expressed as the titer of K88 as described in Materials and Methods

Table 2 Tetracycline resistance level of strains carrying different recombinant plasmids

Strain	MIC[a] (µg/ml)
C600	1.0
C600 (pFM38)	1.0
C600 (pFM381)	1.0
C600 (pFM200)	5.0
C600 (pFM201)	5.0
C600 (pFM204)	5.0
C600 (pFM205)	5.0
C600 (pBR322)	>20

[a]MIC: Minimal inhibitory concentration

plasmid pFM200 and its derivatives (Table 2).

DISCUSSION

Molecular cloning revealed that the genetic determinant of the K88 antigen is located within the 7.7 Md *Hin*dIII fragment, H2, of plasmid pRI8801. The expression of the K88 antigen by the recombinant plasmids was highly dependent on the orientation of H2 in the cloning vehicle pBR322. In the one orientation, as in pFM200, at least 250 times more K88 is produced than in the other orientation, as in pFM38 (Table 1). This phenomenon remains rather mysterious. Such a large difference in expression is difficult to explain by differences in copy numbers. Moreover, no significant differences in plasmid yield between strains containing the plasmids pFM38 and pFM200 were observed. Orientation-dependent expression might by attributed to the presence of promotors on the cloning vehicle as has been described by Timmis et al. (32). In our case it is possible that the cloned K88 locus is dependent on a pBR322 promotor for its expression. Alternatively, a pBR322 promotor might interfere with the expression of the cloned K88 determinant. It is unlikely that the tetracycline promotor is involved here, because it is split by the insertion of DNA into the *Hin*dIII site (31). Moreover, removal of a part of the tetracycline promotor area by deletion of the small *Eco*RI-*Hin*dIII fragment of pBR322, as in pFM205, does not influence the expression of K88 (Table 1).

It is also improbable that the promotor of the ampicillin resistance gene (Amp) is implicated, because the *Hin*dIII site, into which the H2 fragment was inserted, is located upstream relative to the Amp operon (H.L.Heyneker, personal communication). Therefore, the most likely location for a promotor responsible for the orientation-dependent expression observed, would be the area between the Amp gene and the *Eco*RI site of pBR322, which originates from the transposon TnA (32). To affect the expression of the cloned H2 fragment, transcription from this promotor should be clockwise in Fig. 2.

Recently, Widera et al. (34) reported that replacement of the small *Eco*RI-*Hin*dIII pBR322 fragment by foreign DNA resulted in plasmids that expressed tetracycline-resistance at low levels in some instances. This was attributed to the presence of a promotor on the inserted DNA, which allowed transcription of the tetracycline operon. Several of the recombinant plasmids described in this study also expressed tetracycline resistance at a low level (Table 2). If the low level of expression of tetracycline resistance by plasmid pFM200 and its derivatives is caused by read-through from a promotor on the cloned H2 fragment, then another promotor should be involved in K88 expression, because the low expression of tetracycline resistance was not abolished in the $K88^-$ deletion mutant plasmid, pFM201. Alternatively, the low level of tetracycline resistance might be due to the presence of the pBR322 promotor we presupposed above, to explain the orientation-dependent expression of K88.

By introducing deletions we were able to locate the K88 determinant within the H2 fragment more precisely. The smallest plasmid, pFM205, that still expressed the K88 antigen contained a 4.3 Md fragment derived from pRI8801. Such a fragment can code for proteins with a combined molecular weight of 200.000 dalton and it is much larger that the minimal length needed to encode for the protein subunit of the K88 antigen which has an apparent molecular weight of 23.500 d (5). Further reduction of the size of the cloned DNA fragment by removal of a 1.1 Md *Eco*RI fragment resulted in inactivation of the K88 gene(s). The 1.1 Md fragment itself does not appear to contain all the information necessary to express the K88 antigen, because recombinant plasmids containing only this fragment do not express the K88 antigen (unpublished experiments). Furthermore, inversion of this fragment, as in pFM204, also caused loss of K88 expression.

Cloning of the K88 determinant resulted in a four-fold higher production of K88 at 37^oC. This increased production might be a reflection of the difference in copy numbers between pRI8801 and the recombinant plasmids. The

results obtained with the ELISA (Table 1) show that the recombinant plasmids pFM200 and pFM205 produce about 30 times more K88 antigen at 37°C than at 19°C. The production of K88 by the parental plasmid at 19°C is very low and could only be detected when concentrated cell-free extracts were used.

The Raf determinant was contained within the 4.0 Md *Sal*I fragment, S3, of pRI8801. According to Schmid et al. (35), four proteins are involved in raffinose metabolism, an α-galactosidase, a permease, an invertase and a regulatory protein. We do not know if all genes coding for these proteins, are contained within fragment S3, however the α-galactosidase and the permease are probably essential for the Raf phenotype. The inability to clone the Raf determinant with *Hin*dIII might be attributed to the presence of a *Hin*dIII cleavage site in an area essential for Raf expression. In this connection it should be noted that the S3 fragment contains a centrally-located *Hin*dIII site, in addition to a centrally-located *Eco*RI and *Xho*I site. This makes the Raf determinant potentially useful for insertional inactivation. Screening of large numbers of colonies for the presence of recombinant DNA would be simplified by the use of such a metabolic marker, because Raf^- colonies can be distinguished from Raf^+ colonies on MacConkey medium, eliminating the necessity of replica-plating.

A physical map of pRI8801 was constructed and the location of the K88 and Raf determinants on this map was established (Fig. 4). The reason why K88 and Raf are often found to be associated is not known, but it is apparent from Fig. 4 that they do not map close together on pRI8801. Although it has been shown that K88 plasmids endow certain *E.coli* strains with the capacity to colonize the small intestine and thus play an essential role in neonatal diarrhoea in piglets (3, 36), it is not clear whether the production of the K88 antigen alone is sufficient for this, or if other genes located on the K88 plasmids are also involved. Experiments with recombinant plasmids which contain defined parts of K88 plasmids might aid in determining whether other genes are also involved in colonization.

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