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Cloning and Expression of a Deoxyribonucleic Acid Fragment that Encodes for the Adhesive Antigen K99

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Deoxyribonucleic acid fragments of the naturally occurring conjugative K99 plasmid were cloned into vectors pBR322 and pBR325. The smallest deoxyribonucleic acid segment obtained that still expressed K99 was 4.5 megadaltons in size. With regard to the serological, adhesive, and morphological properties, no difference in the nature of the K99 antigen was observed between *Escherichia coli* strains carrying recombinant plasmids and those carrying pRI9901. Furthermore, the regulation of K99 expression from the recombinant plasmid deoxyribonucleic acid was similar to that from pRI9901. The low level of K99 expression in *E. coli* K-12 compared with natural K99-producing isolates seems to be host specific.

One of the prerequisites for the enteropathogenicity of certain *Escherichia coli* strains is ability to adhere to the mucosa of the small intestine. This adhesive property is promoted by the presence of filamentous appendages, usually called pili. Until now, five immunologically unrelated *E. coli* adhesions, K88, K99, 987P, colonization factor antigen I (CFAI), and CFAII, have been described (9, 15, 16, 18, 19, 21). The K99 antigen is found among enterotoxigenic strains of calves, lambs, and piglets (10, 21). Like other adhesins, the presence of K99 can be demonstrated by mannose-resistant hemagglutination of erythrocytes (25).

The production of K99 under various conditions has been the subject of several studies (8, 11, 12). K99 is not produced at growth temperatures below 30°C, and rich media act strongly inhibitory on the K99 production. Molecular cloning of deoxyribonucleic acid (DNA) can facilitate the study of the genetic organization, regulation, and biosynthesis of virulence factors (1, 7, 17, 22, 24). K99 is encoded by conjugative plasmids (21, 23) having sizes of about 50 megadaltons (Md) (22, 23). Because of the low genome complexity of such plasmids, molecular cloning is relatively easy to perform. In this report we describe the cloning of K99 encoding DNA from a naturally occurring K99 plasmid and the K99 expression of the recombinant plasmid obtained.

MATERIALS AND METHODS

Bacterial strains and plasmids. The K99-positive *E. coli* strains B41 (O101:K$^-$), WS10 (O8:K85), B117 (O8:K85) (reference 21), H416 [O101:K(A)?:NM], and H1242 (O101:K$^-$) (reference 8) were obtained from P. A. M. Guinée. *E. coli* K-12 C600 (F$^-$ λ^- *thr leu-6 thi-1 supE44 lacY1 tonA21*) was used as the recipient in transformation experiments. C600 Nal is a spontaneous nalidixic acid-resistant mutant of C600. M694 is a streptomycin-resistant K99$^+$ transconjugant of the mating B41 × C600 Nal. It carries a single plasmid species which was designated as pRI9901. M694 does not produce the heat-stable or heat-labile toxin (W. H. Jansen, unpublished data) and was used as the source of pRI9901 DNA. The cloning vehicles pBR322 and pBR325 (2, 3) were obtained from H. L. Heyneker.

Media. Unless otherwise stated, nutrient broth and nutrient agar (26) were used for growth of bacterial cells. For estimation of K99 production, cells were grown on Minca medium (11, 12) supplemented with vitamin B_1, 1 μg/ml, but not supplemented with IsoVitaleX (BBL Microbiology Systems, Cockeysville, Md.). Minimal medium contained: NH_4Cl, 2 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $FeSO_4 \cdot 7H_2O$, 5 mg; and glucose, 5 g; in 1 liter of 0.15 M phosphate buffer, pH 7.4. Bacteria carrying recombinant plasmids were grown on media supplemented with ampicillin (50 μg/ml), tetracycline (15 μg/ml), or chloramphenicol (40 μg/ml).

Isolation, characterization, and cloning of DNA. The procedures for the isolation of plasmid DNA, gel electrophoresis of cleaved DNA, and estimation of molecular weights have been described (17). The enzymes *Bam*HI, *Bgl*II, *Hin*dII, and *Hin*dIII were obtained from Boehringer Mannheim Corp., *Sal* *Xba*I, *Xho*I, *Pst*I, *Kpn*I, and *Sma*I were from Biolabs, *Hpa*I were from Bethesda Research Laboratories, and *Eco*RI and T_4 ligase were from Miles Laboratories, Inc., Elkhart, Ind. Digestion of DNA with the various restriction endonucleases was done according to the manufacterer's instructions. Ligation with T_4 ligase was carried out as described (17). Transformation was performed according to the method of Cohen et al. (6).

Detection of K99 antigen. A specific K99 antise-

rum was obtained by vaccination of rabbits with K99 antigen, purified by preparative electrophoresis of an ultrasonic extract of *E. coli* strain B41 (kindly provided by P. A. M. Guinée; 11). Ultrasonic extracts of *E. coli* were prepared as described (11). The slide agglutination test and hemagglutination were carried out as described (10).

For the screening of large numbers of clones for the production of K99, a micro-enzyme-linked immunosorbent assay (ELISA) of whole cells was used as described previously for the detection of K88 (17), except that cells were grown in Minca medium. Quantitative estimation of K99 was done with ultrasonicated cell suspensions in microtiter plates (Cooke Engineering Co., Alexandria, Va.), using the ELISA. The trays were read visually, and the highest dilution of K99 antigen which still showed maximum coloring was taken as the titer (8, 17). Absorption of enzyme-labeled antibodies with ultrasonic extracts was done by 2-h incubation of a mixture of both at 37°C, followed by a 5-min centrifugation at 11,000 rpm in an Eppendorff centrifuge.

Brush border adhesion test. Brush borders were prepared from the small intestine of pigs. The procedure was essentially as described by Sellwood et al. (20). Isolated brush borders were washed and resuspended in phosphate-buffered saline to an optical density of 0.45 at 660 nm.

For adhesion tests, 0.1 ml of a brush border suspension was mixed with an equal volume of bacterial suspension in phosphate-buffered saline containing approximately 10^9 cells per ml. After 30 min of incubation with gentle mixing at 37°C, a drop of the mixture was examined by phase-contrast microscopy. Adhesion of *E. coli* K-12 derivatives was considered positive when an average of five or more cells adhered to one brush border.

Electron microscopy. In general, a two-step negative staining procedure was followed. Drops of a cell suspension were placed on glow-discharged "handle" grids coated with a carbon-evaporated Formvar film, and after 1 to 2 min the excess fluid was removed by a tip of filter paper, leaving behind a thin film of fluid. Settling of the bacterial cells on the grid during drying was observed by light microscopic observation. In this way, the concentration of cells in the grid squares could be judged and adjusted by dilution of the original cell suspension, and the degree of clumping of cells into large groups could be determined.

In a second step, cells were stained for 10 s with a solution containing either 1% phosphotungstic acid (pH adjusted to 5.2 or 7.0 with KOH) or ammonium molybdate (pH 5.1). Both staining solutions contained bacitracin (30 μg/ml) for spreading purposes. Drying was accomplished by removing the excess fluid after 10 s with filter paper under light microscopic control. Grids were examined in a Philips EM 200 or EM 201 electron microscope, operated at 80 kV. Electron micrographs were taken at magnifications of ×9,300 to 45,000.

Sometimes strains behaved differently in adherence to the grids. This hampered the judgment of relative frequencies in occurrence of cells with or without surface appendages. Therefore, it was sometimes necessary to find alternative procedures to prevent clumping of cells and to ensure optimal concentrations of cells on the grids. Consequently, one-step procedures (i.e., mixtures of bacterial suspensions with negative stains and bacitracin), one- or two-step floating procedures, or pseudo-replica techniques were occasionally included as well.

RESULTS

Cloning of the K99 determinant of pRI9901. Plasmid pRI9901 was used as the source of K99-encoding plasmid DNA. This plasmid originates from *E. coli* strain B41, and it was transferred to *E. coli* K-12 by conjugation. So et al. (22) reported that B41 carries a conjugative K99 plasmid which also encodes for streptomycin and tetracycline resistance. Our results differ from theirs in that the tetracycline resistance determinant of B41 was not cotransferred with the streptomycin resistance and the K99 determinants.

The restriction endonuclease *Hin*dIII cleaved pRI9901 in at least 13 fragments (Fig. 1, track B) with a total molecular weight of at least 57 × 10^6. These fragments were used to clone the K99 determinant into the unique *Hin*dIII site of the vector pBR322. Cloning of DNA into the *Hin*dIII site of pBR322 results in a loss of the vector-encoded tetracycline resistance expression, and such recombinants express only ampicillin resistance (3). A mixture of *Hin*dIII-cleaved pRI9901 DNA (3 μg) and pBR322 DNA (0.04 μg) was ligated and transferred to competent cells of *E. coli* K-12 C600. Forty-five ampicillin-resistant, tetracycline-sensitive clones were selected. One was streptomycin resistant and another one was K99 positive. The plasmid in the latter clone was designated pRI9902. *Hin*dIII cleavage of pRI9902 showed that a 11.1-Md fragment of pRI9901 had been inserted into vector pBR322 (Fig. 1, track C). A physical map of pRI9902 was constructed by analysis of digests and double digests of pRI9902 with the restriction endonucleases *Xba*I, *Xho*I, *Bam*HI, *Eco*RI, and *Pst*I. The result is shown in Fig. 2.

To further localize the K99 gene(s), deletions were introduced into pRI9902 by cleavage with *Sal*I and *Eco*RI, respectively, followed by ligation and transformation. Twenty transformants derived from the *Sal*I-treated pRI9902 DNA were tested, and all were K99 positive in the ELISA. The plasmid DNA in one of these, pRI9903-9, was characterized. As expected from the physical map, the small *Sal*I fragment (3.1 Md) of pRI9902 was deleted in pRI9903-9 (see Fig. 1, tracks E and F; Fig. 2). Plasmid pRI9903-9 was found to be extremely unstable. Growth in drug-free liquid medium of C600 carrying pRI9903-9 during 13 generations resulted in a

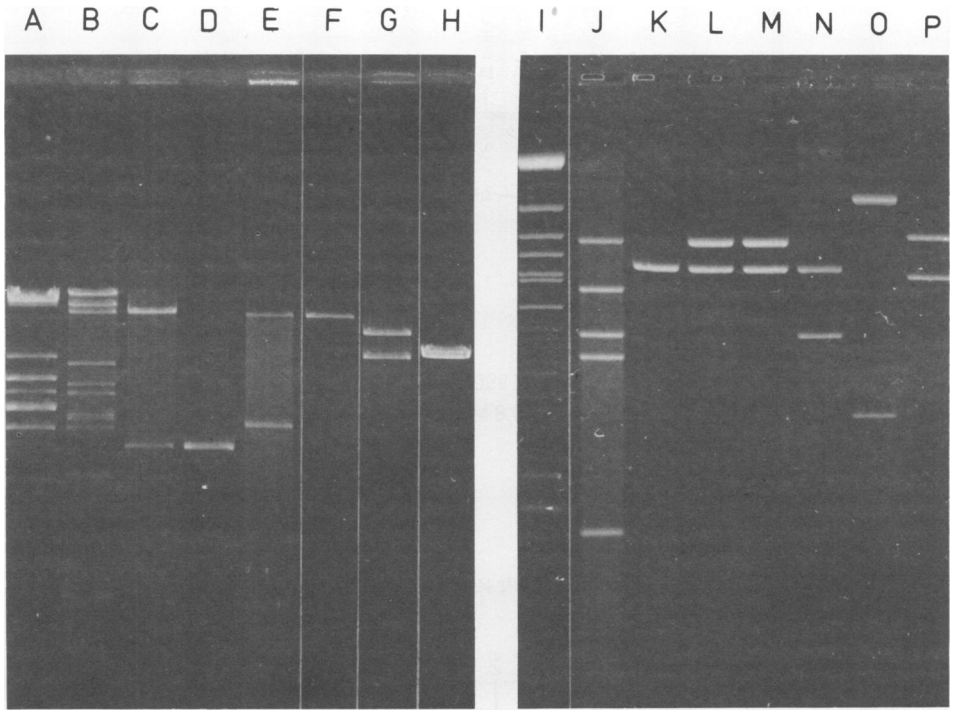


FIG. 1. *Agarose gel electrophoresis of restriction endonuclease-cleaved plasmids. (A) Mixture of λ/EcoRI and λ/HindIII; (B) pRI9901/HindIII; (C) pRI9902/HindIII; (D) pBR322/HindIII; (E) pRI9902/SalI; (F) pRI9903-9/SalI; (G) pRI9902/EcoRI; (H) pRI9904-1/EcoRI; (I) mixture of λ/EcoRI and λ/HindIII; (J) pRI9902/BamHI; (K) pBR325/BamHI; (L) pRI9906-1/BamHI; (M) pRI9906-13/BamHI; (N) pRI9906-16/BamHI; (O) pRI9906-1/EcoRI + XbaI; (P) pRI9912-1/EcoRI + XbaI.*

loss of the plasmid in 98% of the cells.

Twenty transformants derived from the *Eco*RI-treated pRI9902 DNA were tested, and all were K99 negative in the ELISA. One of these was analyzed. It carried a plasmid, pRI9904-1, in which the large *Eco*RI fragment of pRI9902 had been deleted (Fig. 1, tracks G and H; Fig. 2). These results indicate that the K99 gene(s) is located somewhere between coordinates 2.7 and 11.1 Md on the physical map of pRI9902.

Subcloning of pRI9902 into vector pBR325. The K99 determinant of pRI9902 was subcloned with *Bam*HI, using vector pBR325. Insertion of foreign DNA in the unique *Bam*HI site of this vector results in the inactivation of the vector-encoded tetracycline resistance, whereas resistance to ampicillin and chloramphenicol remains intact (2). A mixture of pRI9902 and pBR325 (molar ratio, 1:3) was cleaved with *Bam*HI, ligated, and used for transformation of *E. coli* K-12 C600. Sixteen tetracycline-sensitive and ampicillin- and chloramphenicol resistant transformants were analyzed. The plasmids in these transformants were designated pRI9906-1 through pRI9906-16. Three of them, pRI9906-1, pRI9906-10, and pRI9906-11, reacted strongly in the ELISA, whereas two clones, carrying pRI9906-13 and pRI9906-16, respectively, reacted only weakly in the ELISA. The remaining 11 clones did not produce any detectable amount of K99.

Plasmid pRI9906-1 was composed of the 4.5-Md *Bam*HI pRI9902 fragment and vector pBR325 (Fig. 1, tracks J through L). pRI9906-1 was cleaved at unique sites by the enzymes *Bgl*II, *Kpn*I, and *Sma*I. *Hpa*I cleaved the K99 fragment in pRI9906-1 at four sites, and *Hin*dII cleaved this fragment at five different sites. The cleavage sites of these enzymes on pRI9906-1 were determined by analyses of fragment patterns resulting from various double digestions. The physical map of pRI9906-1 is depicted in Fig. 3. Plasmids pRI9906-10, pRI9906-11, and pRI9906-13 were identical to pRI9906-1 with respect to the size of the inserted *Bam*HI fragment and also with respect to the orientation of this fragment, as was demonstrated by double digestions with *Eco*RI plus *Xba*I (Fig. 1, tracks L through O).

The low K99-expressing plasmid pRI9906-16 carried the 2.5-Md *Bam*HI fragment of pRI9902.

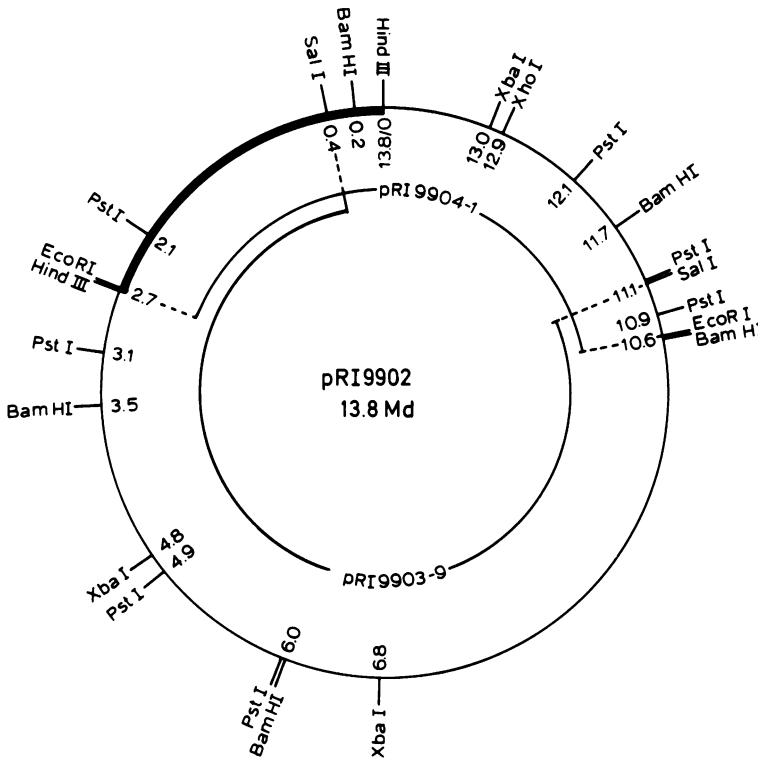


FIG. 2. *Physical map of the K99⁺ recombinant plasmid pRI9902. The coordinates are expressed in megadaltons. The heavy line represents the pBR322 vector DNA. The DNA contained in the EcoRI-generated deletion mutant pRI9904-1 and in the Sal-generated deletion mutant pRI9903-9 is depicted in the inner circles.*

To learn whether or not the expression of K99 depends on the orientation of the K99 fragment in the vector, pRI9906-1 was digested with *Bam*HI and ligated, and six transformants were analyzed. In three of them the K99 fragment was found to be reversed in orientation, as was shown by double digestion with *Eco*RI plus *Xba*I. A double digest of one of these, pRI9912-1, is shown in Fig. 1, track O. No difference in the level of K99 production was found between cells carrying pRI9906-1 and those carrying pRI9912-1 (see Table 1).

***Hpa*I-generated deletions of pRI9906-1.** Deletion mutants of pRI9906-1 were obtained by partial digestion of pRI9906-1 with *Hpa*I, ligation, and transformation to C600. Plasmid DNA was isolated from 31 clones, and 5 of them carried a plasmid that was smaller than pRI9906-1. All five deletion mutants were K99 negative in the ELISA. Among these mutants three different deletions were found. One mutant, pRI9910-16, lacked the *Hpa*I fragments C (0.87 Md) and D (0.35 Md), pRI9910-12 and pRI9910-23 were deleted in the *Hpa*I fragments B (1.03 Md) and C (0.87 Md), and plasmids pRI9910-7 and pRI9910-25 lacked the three HpaI fragments B, C, and D. The deletions obtained are depicted in Fig. 3.

Expression of K99 in the various recombinant plasmids. The K99 expression level of the various recombinant plasmids was determined by the ELISA of ultrasonicated cells. The results are shown in Table 1.

The level of K99 production in *E. coli* K-12 C600 of the K99⁺ recombinant plasmids was generally 16 to 32 times higher than that of the parental plasmid pRI9901. The low K99 expression level of pRI9903-9 is probably related to the extreme instability of this plasmid. Even after growth in the presence of ampicillin, the majority of the cells were found to have lost the plasmid. As mentioned before, cells carrying pRI9906-13 produced very little K99. However, no structural difference between pRI9906-13 and pRI9906-1 was observed: double digestion of pRI9906-13 with *Xba*I plus *Bam*HI and *Eco*RI plus *Bam*HI resulted in fragmentation patterns identical to double digests of pRI9906-1.

Cells carrying pRI9906-16 were also slightly positive in the ELISA, although this plasmid

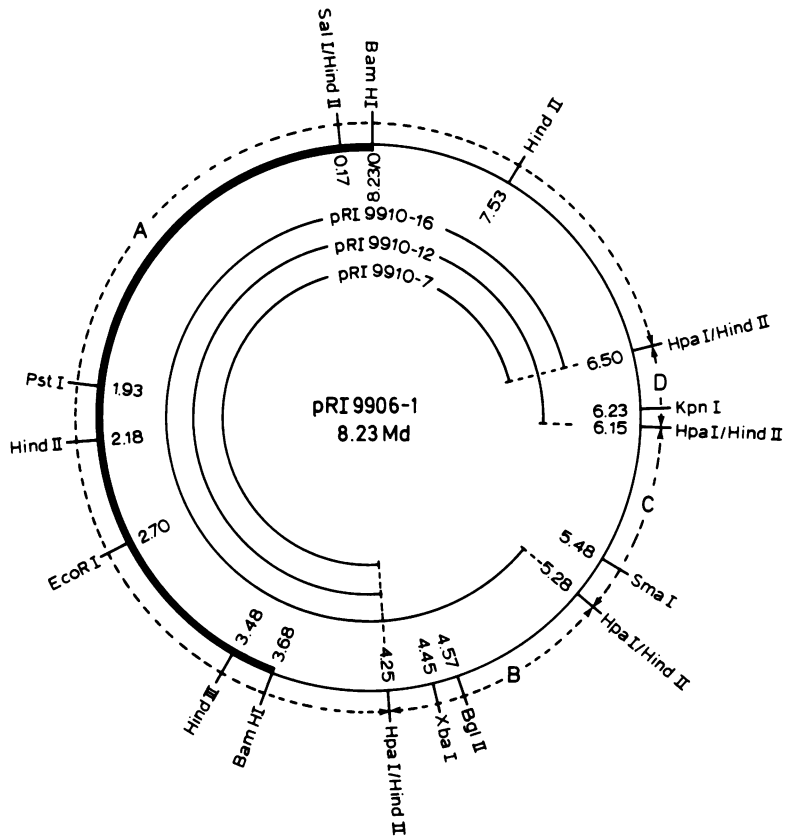


FIG. 3. *Physical map of the K99⁺ recombinant plasmid pRI9906-1. The coordinates are expressed in megadaltons. The heavy line represents the pBR325 vector DNA. The HpaI fragments of pRI9906-1 are designated A, B, C, and D in order of their size. The heavy line represents the vector DNA of pBR325. Three representative HpaI-generated deletion mutants, pRI9910-7, pRI9910-12, and pRI9910-16, are depicted in the inner circles.*

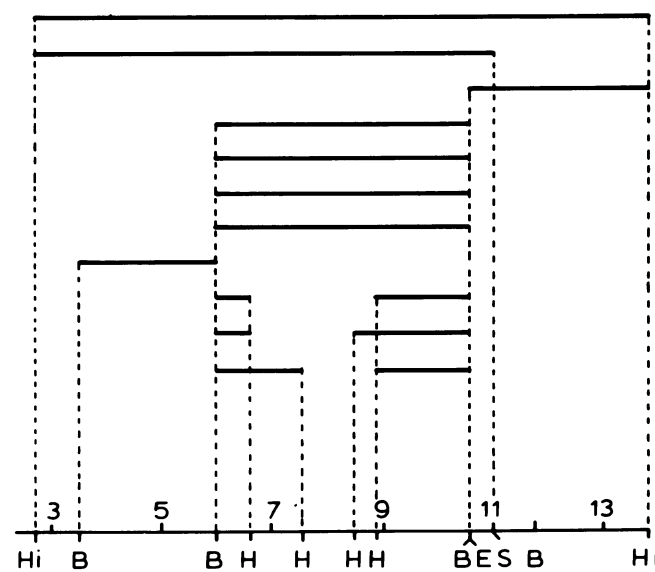
does not share common K99 sequences with the recombinant plasmid pRI9906-1. To determine whether or not pRI9906-16 encoded for antigen(s) related to K99, ELISAs were performed in which the peroxidase-labeled antibodies were preabsorbed with ultrasonic extracts of cells carrying pRI9906-1. Antisera that were absorbed with ultrasonic extracts of C600 (pRI9906-1) did not result in a negative ELISA of C600 (pRI9906-16) and vice versa. As expected, preabsorption with the homologous antigen, however, resulted in a completely negative test. We conclude that the K99 antiserum used contains not only antibodies against K99, but also antibodies against an unrelated antigen that is encoded by the 2.5-Md *Bam*HI fragment of pRI9902.

No significant differences in K99 expression were observed after growth of cells with recombinant plasmids in minimal or Minca medium under aerobic or anaerobic conditions. However, the K99 production was greatly reduced by growth in nutrient broth or at 30°C in Minca medium. de Graaf et al. (8) showed that the expression by K99 plasmids in *E. coli* K-12 transconjugants is generally at least one order of magnitude lower than in the natural isolates. Consistently, C600 (pRI9901) produces about 16 times less K99 than the original donor strain B41 (Table 1). We attempted to transfer pRI9906-1 by transformation to various natural *E. coli* isolates; however, this was only successful with the recipient H1242. This strain is a K99⁺ strain, which produces about 16 times more K99 than strain B41. Introduction of pRI9906-1 into H1242 results in a two- to fourfold increase in K99 production (Table 1). This suggests that pRI9906-1 is well expressed in H1242 and that host factors are responsible for the relatively low K99 production in *E. coli* K-12.

The level of K99 expression as measured by the ELISA correlated well with the agglutination, hemagglutination with horse erythro-

TABLE 1. *K99 expression of various recombinant plasmids and comparison to the presence of various K99 DNA regions of pRI9902*

Strain	K99 production	Agglutination	Hemagglutination	Adhesion to brush border	Presence of K99 filaments[a]	Segment(s) of pRI9902 present[b]
C600	$<2^0$	-	$<2^0$		-	
C600 (pRI9901)	2^4	+	2^3	-	-	
" (pRI9902)	2^9	+	2^3	+	+	
" (pRI9903-9)	2^2	+/-	$<2^0$	-	-	
" (pRI9904-1)	$<2^0$	-	$<2^0$	-	nd	
" (pRI9906-1)	2^8	+	2^4	+	+	
" (pRI9906-10)	2^8	+	2^3	+	nd	
" (pRI9906-11)	2^8	+	2^4	+	nd	
" (pRI9906-13)	2^0	-	$<2^0$	-	-	
" (pRI9906-16)	$<2^0$ [c]	-	$<2^0$	-	-	
" (pRI9910-7)	$<2^0$	-	$<2^0$	-	nd	
" (pRI9910-12)	$<2^0$	-	$<2^0$	-	nd	
" (pRI9910-16)	$<2^0$	-	$<2^0$	-	nd	
" (pRI9912-1)	2^8	+	2^4	nd	nd	
B41	2^8	+	2^3	+	+	
H1242	2^{12}	+	2^3	+	nd	
H1242 (pRI9906-1)	2^{14}	+	2^3	nd	nd	



nd= not done

a) As detected by electron microscopy.

b) The coordinates (in Md) of pRI9902 are represented (see figure 2). Only the relevant Hind III (Hi), Bam HI (B), Hpa I (H), Sal I (S) and Eco RI (E) sites are depicted.

c) The K99 production was just above the detection level.

cytes, and adhesion to brush borders from piglets (Table 1). Furthermore, spontaneous clumping of K99-producing cells was observed after 24 h of incubation in liquid Minca medium. Double diffusion tests of ultrasonic preparations of cells carrying K99$^+$ recombinant plasmids and an ultrasonic extract of B41 showed one single common precipitation line, without spurs. Therefore, the K99 antigen encoded by the recombinant plasmids does not seem to differ from the K99 produced by the natural isolate B41.

Several clones were examined by electron microscopy for the presence of pili. K99 pili were found on all K99$^+$ strains tested, except on C600 (pRI9901) and C600 (pRI9903-9) (Table 1). Such pili were not found when cells were incubated at 18°C. In contrast to the rigid, straight type I pili, also present on the C600 derivatives, the K99 pili were visible as flexible, curved, filamentous structures. The K99 pili were much thinner than type I pili. Occasionally, these pili had a zigzag configuration (Fig. 4A). Therefore, it was difficult to assign a realistic cross-diameter to the K99 pilus. We estimate it to be in the range of 2 to 4 nm. No morphological difference was found between the K99 pili of strain B41 and strains carrying recombinant plasmids (Fig. 4A and B). Electron microscopy of the K99$^+$ natural isolates WS10, B117, and H416 showed the presence of thin pili that were morphologically identical to those of B41.

DISCUSSION

The 4.6-Md *Bam*HI fragment from pRI9901 was the smallest DNA segment obtained in this study that still encoded for K99. Such a region has the capacity to encode for polypeptides with a total molecular weight of about 200,000. This is amply sufficient to accommodate the structural genes of the two polypeptide subunits of 22,500 and 29,500 daltons, which are presumed to be the unassembled subunits of the K99 pilus (13, 14). The smallest *Hpa*I-generated deletion of pRI9906-1 of 1.22 Md resulted in a loss of K99 production. This suggests that this region contains at least a part of the structural or regulatory K99 gene(s).

Inversion of the 4.5-Md *Bam*HI fragment in vector pBR325 did not influence the level of K99 production. This suggests that K99 expression on the cloned fragment is independent of transcription signals of the vector. Cells carrying K99 recombinant plasmids produced 16 to 32 times more K99 than did *E. coli* K-12 carrying the parental plasmid pRI9901. The molecular weight of pRI9901 was found to be at least 57 $\times$ 10^6, and plasmids of this size are usually present to the extent of one to two copies per bacterial cell. The cloning vehicles used, pBR322 and pBR325, are multicopy plasmids (2, 3), and therefore the high level of K99 expression in cells containing recombinant plasmids can be explained by a gene dose effect.

Based on the morphological, immunological, and adhesive properties, the nature of the K99 antigen expressed by recombinant plasmids was identical to K99 encoded by the wild-type plasmid pRI9901 in *E. coli* K-12 and B41. Also, the regulation of K99 expression of recombinant plasmid DNA was similar to that of natural K99$^+$ isolates (8).

The electron micrographs of K99$^+$ bacteria showed the presence of very thin (2 to 4 nm), flexible surface appendages which occasionally showed a zigzag configuration. This morphology of the K99 pilus differs from that observed by Isaacson (13) on purified K99 pili isolated from a B41 $\times$ *E. coli* K-12 transconjugant. The diameter of these purified pili was estimated to be between 7.0 and 9.8 nm, with an average diameter of 8.4 nm. The electron microscopic techniques used were basically the same as ours, and we also included strain B41 in our analysis. Perhaps the K99 pili are morphologically modified as a consequence of the purification procedure. In other reports on the electron microscopy of K99, shadow casting was used to visualize the pili (5, 8). However, by this procedure the fine structure of the thin K99 pili is obscured in contrast to the negative staining used by us and by Isaacson.

One of the recombinants obtained, pRI9906-13, produces minimal amounts of K99, although the plasmid carries the same K99 segment as pRI9906-1. Also, cleavage with the multiply cutting enzymes *Hin*dII, *Alu*I, *Sau*96I, and *Hpa*II did not reveal any fragmentation difference between pRI9906-13 and pRI9906-1 (unpublished data). Presently we cannot rule out the possibility that pRI9906-13 carries a very small *Bam*HI fragment derived from pRI9901, in addition to the 11.1-Md pRI9901 fragment. Such a fragment might exert a polar effect on the K99 expression. It is tempting to speculate on the possibility that pRI9906-13 carries the intact K99 gene(s) and that the cells are in a phase of nonpiliation. The phenomenon of metastable phase variation has been described previously for type I, 987P, CFAI, and CFAII pili (4), but not for K88 and K99. The subcloning of pRI9902 might have resulted in the cloning of the K99$^-$ phase variant pRI9906-13, which does not frequently seem to revert to the K99$^+$ phase, perhaps because regions of pRI9902 bordering the 4.5-Md *Bam*HI fragment are involved in the phase variation and these are missing in pRI9906-13.

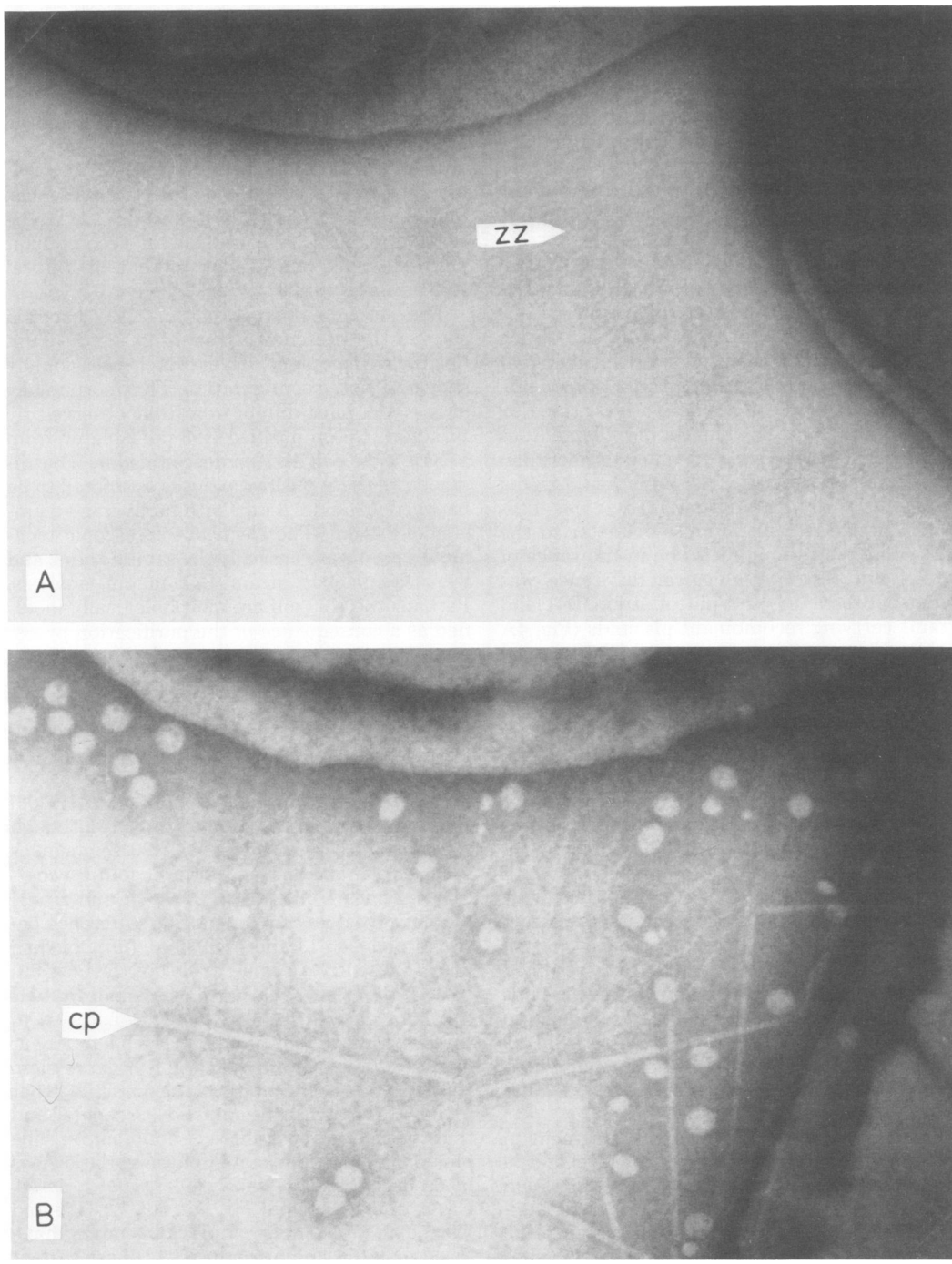


FIG. 4. *Electron micrographs of K99 pili on cells of strain (A) B41 and (B) C600 (pRI9906-1). Note that the K99 pili are visible as flexible filaments occasionally showing a zigzag (zz) structure; (cp) common pili. (A) Negative staining with ammonium molybdate; ×200,000. (B) Negative staining with phosphotungstic acid; ×200,000.*

Strains carrying recombinant plasmids such as pRI9906-1 could be useful for production of K99-specific antisera. Vaccination with such strains will not result in the generation of antibodies against antigens that are not related to K99 and which are encoded by other regions of naturally occurring K99 plasmids. In this study we accidently detected such antibodies in K99 antiserum. This antigen was encoded by the recombinant plasmid pRI9906-16, which carried a DNA segment that maps close to the K99 gene(s) in pRI9901.

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