INFECTION AND IMMUNITY, Jan. 1977, p. 272-279
Vol. 15, No. 1
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K99 Surface Antigen of *Escherichia coli*: Purification and Partial Characterization

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Received for publication 19 July 1976

K99, a presumed colonizing factor of enterotoxigenic *Escherichia coli* of calf origin, has been purified. K99 was removed from K99+ bacteria by salt extraction and subsequently purified by ammonium sulfate precipitation and column chromatography on diethylaminoethyl-Sephadex. The purified material was homogenous in size, having an $s_{20,w}$ of 13 to 15 *S*. It was composed of two subunits: a major component with a molecular weight of 22,500 and a minor component of 29,500. When observed in the electron microscope, K99 appeared to be rod-shaped, with a strong tendency for self-aggregation. At concentrations where aggregation was minimized, individual rods were observed with diameters of 8.4 nm and mean lengths of 130 nm. Based on the subunit structure, exterior location, and rod-like shape of K99, it is concluded that K99 is a pilus or pilus-like structure. Chemically, K99 is composed primarily of protein and has an isoelectric point of greater than 10. Purified K99 did not hemagglutinate guinea pig erythrocytes.

Colonization of the small intestine is a prerequisite for enterotoxigenic *Escherichia coli* to cause diarrheal disease. Smith and Linggood (16) reported on the identification of a transmissible, plasmid-mediated K antigen that was commonly found on calf and lamb enterotoxigenic *E. coli* strains and demonstrated it to be a colonization factor and an adhesive factor. This antigen has been designated K99 (15). The removal of the K99 plasmid and thus the K99 antigen from an enterotoxigenic calf strain resulted in an organism that no longer caused diarrhea. Reintroduction of the K99 plasmid into the same strain resulted in a virulent organism. Several more recent studies (9, 13, 14) have demonstrated the presence of K99 on a high percentage (76 to 95%) of enterotoxigenic and on a low percentage (0 to 14%) of nonenterotoxigenic *E. coli* strains isolated from calves.

These data are consistent with K99 being a colonization factor. The purification of K99 was undertaken as a prelude to determining the role that it plays in colonization of the calf small intestine. The results of K99 purification and partial characterization are presented here.

MATERIALS AND METHODS

E. coli strains and growth conditions. *E. coli* strain B41 (O101; K−,99:NM), which has been established as the K99 reference strain (15), was obtained from W. J. Sojka. *E. coli* strains 1474 (K99+) and 1475 (K99−) are isogenic K-12 strains that were obtained from C. L. Gyles. Bacteria were grown in 1-liter batches of Trypticase soy broth (BBL) in 2-liter Erlenmeyer flasks at 37°C with shaking (200 rpm) for 18 h.

Assay of K99. K99 was detected by a double-diffusion technique (Ouchterlony) in 1% agar (in 0.15 M sodium chloride). Wells were punched into the agar 4 to 5 mm apart and filled with 15 μl of either sample or antibody. Absorbed K99 antiserum was previously described (14) and was concentrated eightfold by precipitation with sodium sulfate. K99− antiserum was prepared from strain 1475 (K99−) by the method of Edwards and Ewing (5) and was also concentrated eightfold. K99 activity was quantitated by preparing serial twofold dilutions of the sample and determining the most dilute sample that would result in a precipitin arc when reacted with absorbed K99 antiserum after 24 h. The reciprocal of that dilution was used to designate K99 activity per 15 μl.

Chemicals. Reagents for polyacrylamide gel electrophoresis were obtained from Eastman Kodak. Diethylaminoethyl (DEAE)-Sephadex A-50, G-50, and G-100 were obtained from Pharmacia. Bio-Gel P60, A1.5, A5, and A15 were obtained from Bio-Rad. Carboxymethylcellulose, phosphocellulose, and molecular weight protein standards were purchased from Sigma Chemical Co.

Chemical assays. Protein was determined by the method of Lowry et al. (12) using bovine serum albumin as standard. Neutral sugars were determined by the indole test (1), using glucose as standard. Lipid was estimated by direct weight measurement after extraction with alcohol-ether (6). Hexosamines were determined in the amino acid analyzer.

Polyacrylamide gel electrophoresis. Sodium dodecyl sulfate-polyacrylamide gels (SDS-gels) were prepared by the method of Weber and Osborn (18)

and stained by the method of Fairbanks et al. (8). Molecular weight determination in SDS-gels has been previously described (18).

Isoelectric focusing. Isoelectric focusing was performed at 4°C in an LKB Ampholine column (model 8101). The column was prepared as described in the LKB manual and contained 1% carrier ampholytes (pH 3 to 10). K99 in distilled water was added to either the light or heavy sucrose solution before preparing the density gradient. Isoelectric focusing was carried out for 72 h at a constant voltage of 300 V.

Amino acid analysis. Purified K99 was dialyzed exhaustively against distilled water, lyophilized, and dissolved in 6 N hydrochloric acid. After being evacuated and sealed, individual samples were hydrolyzed at 110°C for 24, 48, and 72 h. After hydrolysis, the samples were taken to dryness and the amino acid composition was determined in a Beckman 121 automated amino acid analyzer.

Hemagglutination. Microhemagglutination of guinea pig erythocytes cells has been described (11).

Electron microscopy. K99 (1.5 mg/ml) was exhaustively dialyzed against distilled water. This material was then stained with potassium phosphotungstate as described elsewhere (R. E. Isaacson, B. Nagy, and H. W. Moon, submitted for publication).

RESULTS

K99 assay. An agar gel double-diffusion technique (Ouchterlony) was previously shown to be useful in the detection of K99 activity (9). The specificity of this precipitin reaction is demonstrated in Fig. 1 and 2. Materials in wells B through E of Fig. 1 (all containing K99) had a very slowly diffusing component that was precipitated by absorbed K99 antiserum, but not by antiserum prepared against the isogenic K99$^-$ parent. The materials in well A, which were prepared from strain 1475 (K99$^-$) did not have this slowly diffusing component when reacted with either serum. After extraction but before purification, materials from K99$^+$ or K99$^-$ strains contained a rapidly diffusing component that was precipitated by both sera. This component must therefore have been an antigen common to both K-12 strains and thus not K99. The slowly diffusing component obtained from 1474 (K99$^+$) was identical to the slowly diffusing component extracted from the K99 reference strain, B41 (Fig. 2). This is demonstrated by the lines of identity formed by the precipitin arcs of the two K99$^+$ strains. No spurring was observed. Guinée et al. (9) previously showed that this B41 component was K99. The double-diffusion technique therefore appeared to be a reliable and specific assay for K99 and was adapted to monitor K99 activity during purification.

Purification of K99. (i) Extraction of K99. Initial attempts to remove K99 from bacterial

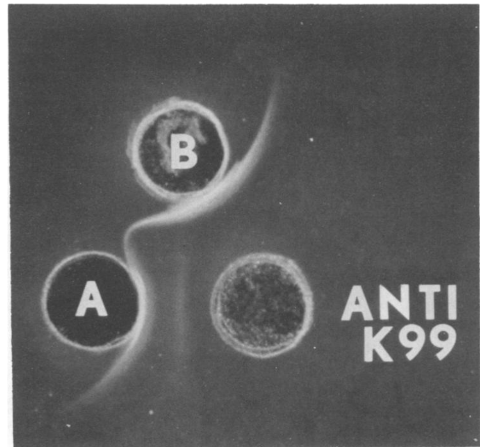

FIG. 2. *Comparison of K99 activity from B41 extract (A) and purified K99 from 1474 (K99$^+$) (B).*

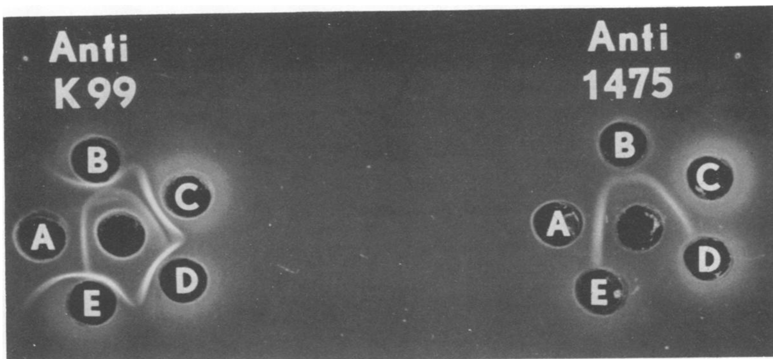

FIG. 1. *Assay of K99 from 1474 (K99$^+$) at various stages of purity and 1475 (K99$^-$) using absorbed K99 antiserum and antiserum prepared against 1475 (K99$^-$). Well A contains 1475 (K99$^-$) extract, B contains 1474 (K99$^+$) extract, C contains K99 after ammonium sulfate precipitation, D contains K99 after DEAE-Sephadex, and E contains K99 after carboxymethylcellulose.*

cells made use of a short, high-speed homogenization: 2 min at a setting of 10 in a Sorvall Omnimixer. After homogenization, bacteria and large debris were removed by centrifugation. K99 activity could be detected in the resultant supernatant. However, a two- to fourfold increase in K99 yield could be achieved by using the following salt extraction. Cells from 12 liters of 18- to 24-h-old cultures were harvested by centrifugation (17,000 × g for 10 min) and resuspended in 200 to 300 ml of 0.05 M sodium phosphate buffer, pH 7.2 (PB), containing 1.0 M sodium chloride. K99 was extracted at 4°C by homogenization in a Sorvall Omnimixer (setting of 4) for 30 min. Bacteria were removed by centrifugation at 17,000 × g for 10 min at 4°C and reextracted. The two cleared supernatants were pooled and stored at −20°C for further use. When the extracted bacteria were assayed for K99 activity by slide agglutination, residual activity was detected. Additional extraction of the bacteria did not substantially increase the amount of K99 liberated, and therefore two extractions were adapted for the purification schema. The amounts of K99 and protein extracted and the specific activity of the K99 are shown in Table 1. Figure 3 shows an SDS-gel of the extracted material.

(ii) Ammonium sulfate precipitation. All subsequent steps were performed at 4°C. Ammonium sulfate (10.6 g/100 ml) was slowly added to the pooled extract with constant stirring. After 30 min, the insoluble material was removed by centrifugation (20,000 × g for 10 min) and discarded. Ammonium sulfate (11.3 g/100 ml) was again added to the supernatant. After another 30 min of stirring, the insoluble material was collected by centrifugation, resuspended in PB, and dialyzed overnight against PB. The ammonium sulfate precipitation resulted in a recovery of 44% of the total extracted K99 activity and a 3.2-fold increase in specific activity (Table 1). SDS-gels of this material (Fig. 3) looked similar to the pattern observed from the extract, except that there was a substantial increase in the amount of material in

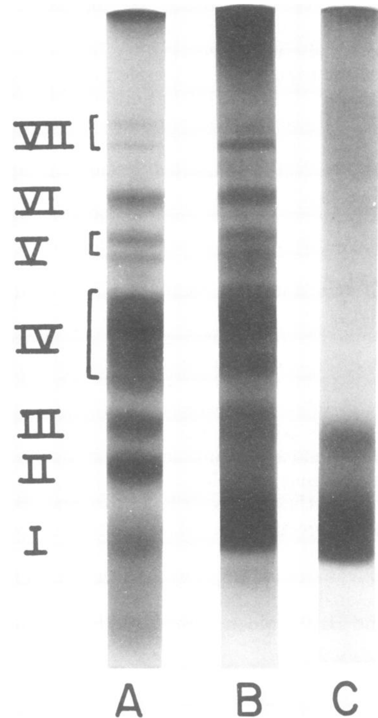

FIG. 3. *SDS-gels of 1474 (K99+) extract (A), ammonium sulfate precipitate (B), and DEAE-Sephadex-purified K99 (C). Bands or band clumps are numbered with Roman numerals.*

band I with respect to the other bands, and band II was absent. Assay for K99 activity (Fig. 1) showed the presence of the slowly diffusing K99 component and the rapidly diffusing non-K99 component.

(iii) DEAE-Sephadex column chromatography. The sample from the ammonium sulfate precipitation step was next applied to a DEAE-Sephadex A-50 column (2.5 by 40 cm) that was equilibrated with PB and developed with 300 ml of PB. A 400-ml 0 to 1.0 M NaCl gradient (in PB) was then applied to the column. Fractions were assayed for absorbance at 280 nm (A_{280}) and for K99 activity (Fig. 4). All recovered K99 activity was located at the approximate location of the column void volume (fractions 14 to 42). No K99 activity was detected in the material eluting with the NaCl gradient. Material pooled from fractions 14 to 42 contained 26% of the original K99 activity (Table 1) and showed an additional 2.45-fold increase in specific activity. SDS-gels (Fig. 3) showed that material from the DEAE-Sephadex column contained only bands I and III. Assay of K99 activity (Fig. 1) showed that the DEAE-Sephadex step removed the rapidly diffusing material and that

TABLE 1. *Purification of K99*[a]

Step	Protein (mg)	K99 (U)	Sp act (U/mg of protein)	Yield[b] (%)
Crude extract	3,887	207,000	53	100
Ammonium sulfate	526	92,000	174	44
DEAE-Sephadex ...	125	53,360	427	26

[a] Data are based on purification from 72 liters of broth grown 1474 (K99+).

[b] Based on the amount extracted from whole cells.

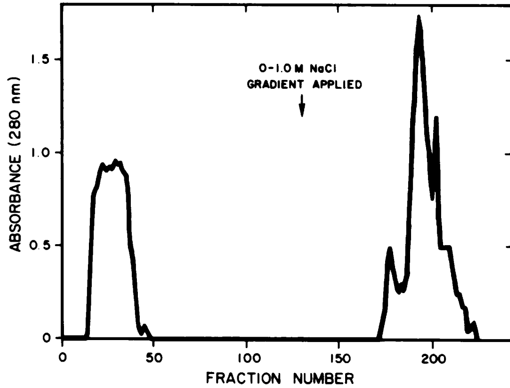

FIG. 4. *DEAE-Sephadex chromatography profile of ammonium sulfate precipitate from 1474 (K99$^+$). Conditions are described in Results.*

only the slowly diffusing K99 component was present.

(iv) Cation-exchange columns. Aliquots from the pooled DEAE-Sephadex step were applied to columns of carboxymethylcellulose or phosphocellulose, both equilibrated and eluted with PB. With each column, a single peak containing K99 activity was obtained. No increase in specific activity was detected, and SDS-gels demonstrated the presence of bands I and III. The K99 obtained from these steps behaved identically to the DEAE-Sephadex-derived K99 in the double-diffusion assay (Fig. 1).

(v) Gel filtration chromatography. Columns of Sephadex G-50 and G-100 and of Bio-Gel P60, A1.5, and A5, and A15 in PB were used in an attempt to separate bands I and III from the DEAE-Sephadex-purified K99. In each case, both bands (I and III) co-migrated in the column void volume and could not be separated, indicating that K99 is very large (Bio-Gel A15 excludes particles of 15 × 10^6 daltons or larger). The cation-exchange and gel filtration columns were therefore not included in the purification schema since they did not result in any increase in purification.

SDS-gel bands I and III were not present in materials extracted from 1475 (K99$^-$), which lacked the K99 plasmid (Fig. 5). Therefore, the presence of these two components was conferred on the strain carrying the K99 plasmid only. Also, treatment of absorbed K99 antiserum with purified K99 resulted in the loss of the ability of the antiserum to agglutinate the two K99$^+$ strains. These data along with the data demonstrating the specificity of K99 precipitation in the double-diffusion assay are convincing evidence that the purified material was indeed K99. All subsequent reference to purified K99 will refer to the material obtained from the DEAE-Sephadex column chromatography step.

Isoelectric focusing. In an attempt to separate bands I and III and to characterize these components, purified K99 was subjected to isoelectric focusing in a pH 3 to 10 gradient. Isoelectric focusing resulted in a single A_{280}-absorbing peak with a corresponding pI of 10.1 (Fig. 6). Assay of K99 activity also resulted in a single peak located at the same place as the A_{280}-absorbing material. SDS-gels of this material showed the presence of both bands I and III.

Ultracentrifugation. The sedimentation coefficient of K99 was determined by two methods. The first involved the cosedimentation of purified K99 with 16*S* and 23*S* ribosomal ribonucleic acid through a neutral 5 to 20% sucrose density gradient. An $s_{20,w}$ of 15.2 was calcu-

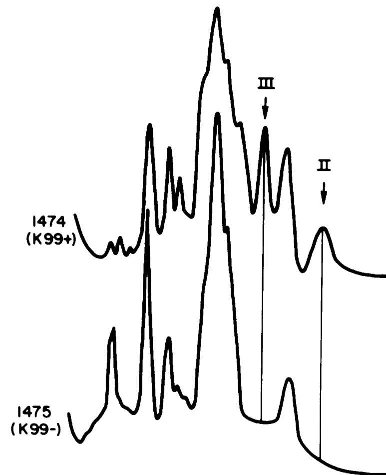

FIG. 5. *Scan at 550 nm of Coomassie blue-stained SDS-gels of extract from 1474 (K99$^+$) and 1475 (K99$^-$). Scan was performed using a Gilford recording spectrophotometer.*

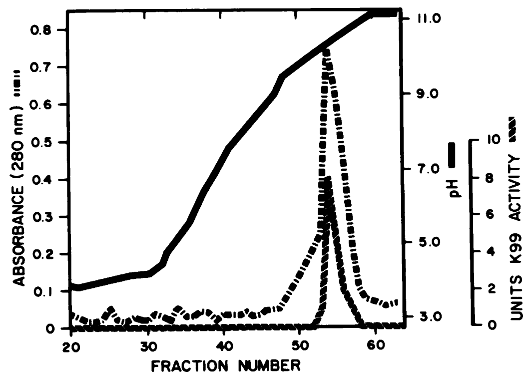

FIG. 6. *Isoelectric focusing of purified K99. Conditions are described in Materials and Methods.*

lated. K99 sedimented as a single symmetrical peak containing all of the measurable K99 activity. Fractions from various peak locations were analyzed in SDS-gels and were shown to contain bands I and III. In the second method, the sedimentation coefficient of purified K99 (in 0.05 M PB) was determined by sedimentation at 56,000 rpm at 20°C in a 2°, 12-mm Kel-F aluminum cell in a Spinco analytical ultracentrifuge. An $s_{20,w}$ of 12.96*S* was calculated. In the analytical ultracentrifuge, a single, sharp, symmetrical peak was observed (Fig. 7).

Molecular weight determination. The method of Weber and Osborn (18), which uses SDS-gels, was used to determine the molecular weights of the two K99 components (Fig. 8); band I had a molecular weight of 22,500, and band III had a molecular weight of 29,500. Incubation and electrophoresis of K99 in 2% SDS-gels yielded the same molecular weights. Also, omission of 2-mercaptoethanol from the incubation mixture did not affect the band patterns or molecular weights. The molecular weights obtained from SDS-gels were considerably lower than would be expected for a particle of 13 to 15*S*. This discrepancy probably indicates that native K99 is composed of subunits. The SDS-gels were scanned at 550 nm in a Gilford recording spectrophotometer, and the results indicate that there was approximately five times more material in band I than in band III. Assuming that each component stains identically with Coomassie brilliant blue, there were five band I subunits for every one band III subunit.

Chemical analysis. Less than 7 μg of neutral sugar and 66 μg of lipid were detected per mg of K99 protein. The relative amino acid composition of K99 is given in Table 2. Calculation of the number of residues of each amino acid was based on a molecule of approximately 22,500 daltons. At this stage of purity, two proteins were present, and therefore the results of amino acid analysis should be interpreted as such. K99 was very low in aromatic amino

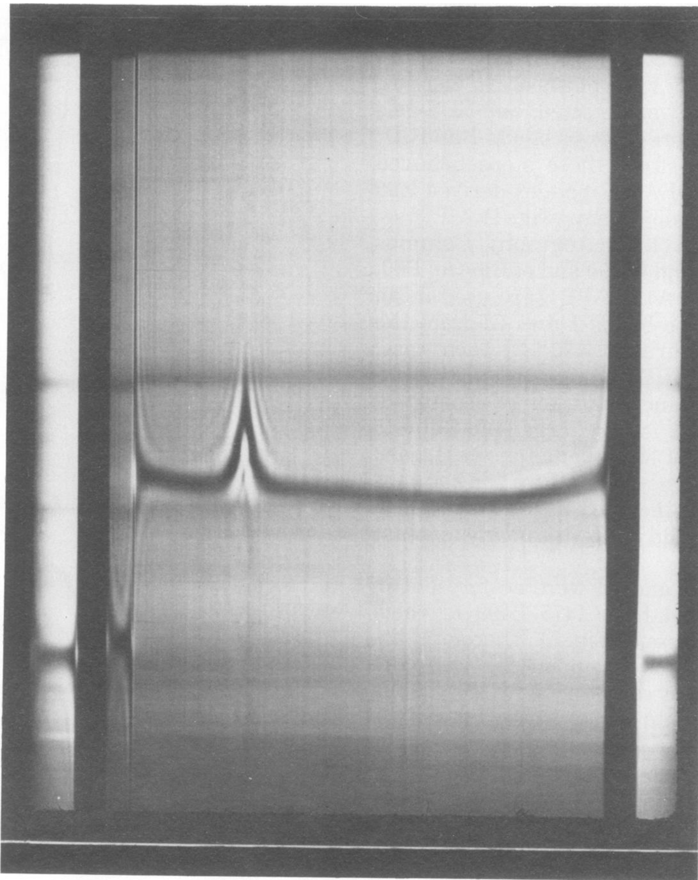

FIG. 7. *Schlieren pattern obtained for purified K99 in the analytical ultracentrifuge. Conditions for centrifugation are described in Results. Concentration of K99 was 1.5 mg/ml.*

acids, with three tryptophan residues and no tyrosine or phenylalanine residues. Three residues of an unusual amino acid, hydroxylysine, were found. Eight percent of the amino acids were basic (lysine, hydroxylysine, and arginine). Based on the experimentally measured pI, it is assumed that all or most aspartic and glutamic acid residues were present as asparagine and glutamine before hydrolysis. No hexosamines were detected.

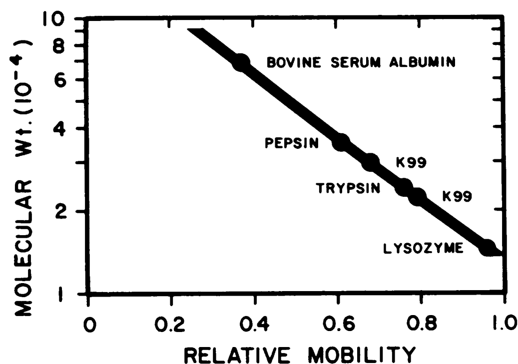

FIG. 8. *Determination of K99 molecular weights in SDS-gels. Conditions for electrophoresis are described in Materials and Methods. The molecular weights of marker proteins are: bovine serum albumin, 68,000; pepsin, 35,000; trypsin, 24,000; lysozyme, 14,600. The relative distance of migration is expressed as the distance of migration with respect to the bromphenol blue tracking dye.*

TABLE 2. *Amino acid composition of K99*

Amino acid	Calculated[a]	Assumed
Cysteine[b]	2.0	2
Aspartic	25.5	25
Methionine[c]	3.3	3
Threonine[d]	20.2	20
Serine[d]	11.8	12
Proline	2.6	3
Glutamic	7.2	7
Glycine	15.2	15
Alanine	46.4	46
Valine	13.6	14
Isoleucine	13.1	13
Leucine	7.5	7
Lysine	9.4	9
Histidine	3.4	3
Arginine	4.2	4
Hydroxylysine	2.8	3
Tryptophan[e]	2.9	3

[a] Based on 2.0 cysteine. Numbers are average values for 24-, 48-, and 72-h hydrolysates except where noted.

[b] Determined as cysteic acid after oxidation by performic acid (10).

[c] Determined as methionine sulfone after oxidation by performic acid (10).

[d] Values obtained by extrapolation to zero time.

[e] Determined spectrophotometrically (4).

Hemagglutination. Strains 1474 (K99$^+$) and 1475 (K99$^-$), when grown in Trypticase soy broth and concentrated to 10^{10} cells/ml were able to hemagglutinate guinea pig erythrocytes in a D-mannose-resistant manner. The ammonium sulfate-precipitated 1474 (K99$^+$) material also retained this ability. However, purified K99 no longer hemagglutinated guinea pig erythrocytes. The hemagglutination activity was recovered from the DEAE-Sephadex column in the material eluted after application of the salt gradient, but contained no K99 activity. The *E. coli* K-12 strains used in this study did possess somatic pili in addition to K99 (unpublished data). Therefore, it is possible that the hemagglutination that was observed was a result of the somatic pili.

Electron microscopy. Purified K99 was negatively stained and examined in an electron microscope (Fig. 9). Numerous rod-like structures were observed. At high protein concentrations (1.5 mg/ml), the rods had a strong tendency to aggregate and form long, thick, somewhat irregular filamentous structures (Fig. 9A). When the K99 concentration was diluted several-fold, aggregates were no longer observed and instead short rod structures were seen (Fig. 9B). These shorter structures had an average diameter of 8.4 nm (range = 7.0 to 9.8 nm) and a length of 130 nm (range = 84 to 183 nm). It is probable that the large, filamentous aggregates seen at high protein concentrations were composed of the shorter rod structures.

DISCUSSION

K99 has been purified and has been shown to be composed primarily of protein. Less than 0.6% is carbohydrate, and 6.6% is lipid. Data obtained from ultracentrifugation, gel filtration, double-diffusion precipitation, and isoelectric focusing indicate that K99 has been purified to homogeneity. However, under strong denaturing conditions (treatment with SDS), K99 can be dissociated into two protein components or subunits (SDS-gel bands I and III) having molecular weights of 22,500 and 29,500, respectively. In the native state, it is assumed that the two subunits are aggregated in an orderly manner to form a long filamentous structure. This interpretation is confirmed by observation of purified K99 in the electron microscope. The data from double diffusion, ultracentrifugation, and gel filtration indicating a very large size for native K99 are also consistent with this interpretation. It is also possible that K99 is composed of a single subunit and that the other component is a K99

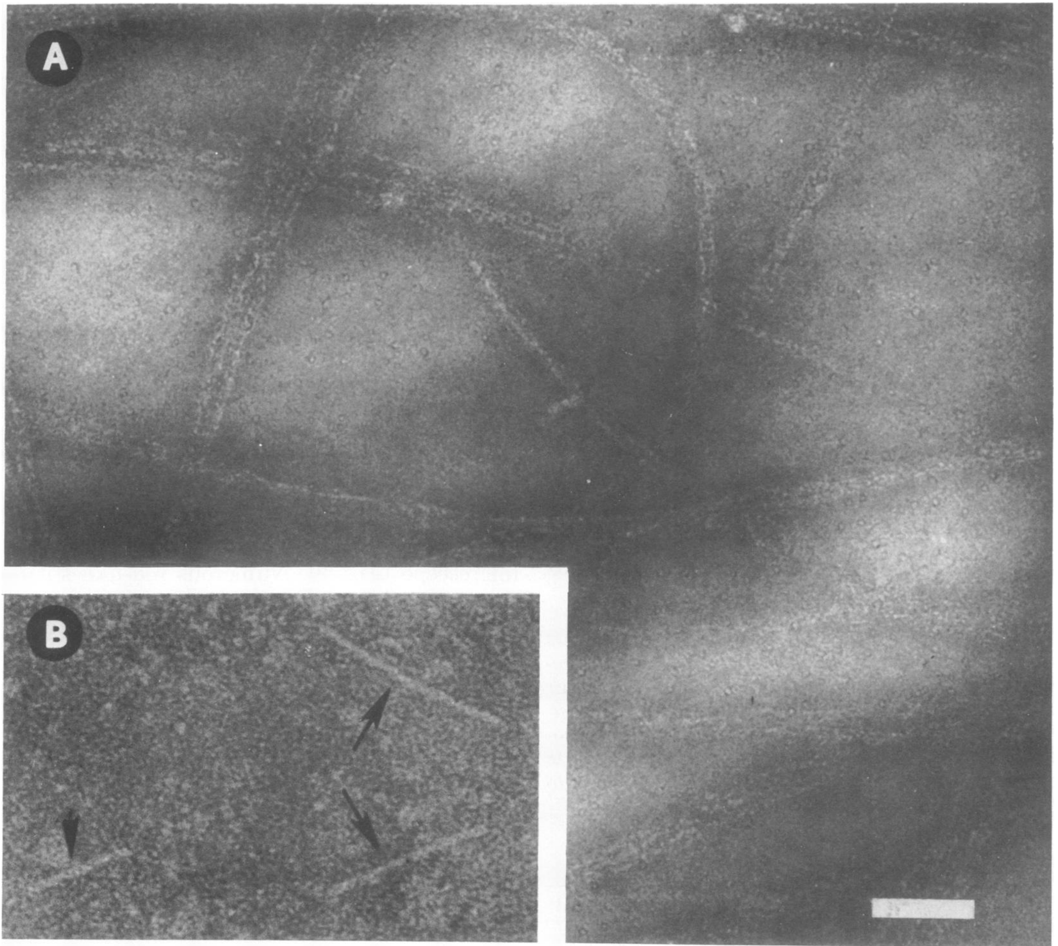

FIG. 9. *Electron microscopy of purified K99 negatively stained as described in Materials and Methods with 1% potassium phosphotungstate. (A) 1.5 mg/ml; (B) 150 μg/ml. Magnification, ×140,500. Bar equals 100 nm.*

plasmid-specific protein that copurifies with K99.

The native filamentous structure of K99, its subunit structure, and its physical location on the bacterial cell envelope are consistent with it being a pilus or pilus-like structure. Pili are elongated or filamentous protein containing structures that reside on the outside of the bacterial cell envelope (2). Like K99, pili are aggregates of smaller subunits, called pilin. The postulated role of K99 is as a colonizing factor. In this regard, several other pili or pilus-like structures have been shown to be involved in the colonization of the small intestine by enterotoxigenic *E. coli* (7, 17; Isaacson et al., submitted for publication). By analogy, it can be hypothesized that K99 plays a similar role.

Isoelectric focusing showed that K99 has a pI of greater than 10, and therefore at the physiological pH of the small intestine it has a positive charge. The positive charge makes K99 particularly suitable as an adhesive structure involved in colonizing the small intestine. Besides the possibility of K99 attaching to specific intestinal epithelial cell receptors, it would also be expected to stick avidly in a nonspecific manner to the acidic mucopolysaccharides found in intestinal mucus. One can envision a two-step process for $K99^+$ *E. coli* strains to colonize calf small intestines. The first step is based on a charge attraction to intestinal mucus. Once the bacteria are immobilized, a second, highly specific interaction between K99 and an epithelial cell receptor occurs, further anchoring the bacteria in place. The second step would be required, since it would be expected that, with time and increase in intestinal secretion due to the induced diarrhea, a substantial amount of

mucus would be washed out of the host. Therefore, a secondary attachment would be necessary.

The role that K99 plays in colonization is at present uncertain. With the availability of pure K99, it will now be possible to study interactions of K99 with intestinal epithelium. Such experiments will be useful in elucidating the role played by K99 in the colonization of the calf small intestine.

ACKNOWLEDGMENTS

I wish to thank R. A. Schneider and Gary Witmer for excellent technical assistance. I also thank Robert Patterson and A. Baetz for assisting in analytic ultracentrifugational analysis and amino acid analysis, respectifully.

This work was supported by the Agricultural Research Service and the U.S. Army Medical Research and Development Command (grant no. DADM 17-75-C-5014 to H. W. Moon).

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