A new method for the extraction and purification of K99 pili from enterotoxigenic *Escherichia coli* and their characterization

K. ALTMANN,* N. A. PYLIOTIS† and T. K. S. MUKKUR‡§

*Department of Veterinary Pathology, University of Sydney, Glebe, N.S.W. 2037, †Prince Henry's Hospital, Melbourne, Vic. 3004, and ‡C.S.I.R.O. Division of Animal Health, McMaster Laboratory, Glebe, N.S.W. 2037, Australia

(Received 28 September 1981/Accepted 26 October 1981)

It was found that K99 pili from enterotoxigenic *Escherichia coli* (of bovine origin) could be extracted by treatment with 3 M-KSCN solution. The K99 pili were purified by preparative isoelectric focusing to apparent homogeneity as judged by the presence of a single band on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis; the molecular weight of this component was calculated to be 12600 ± 300 . This indicated that the K99 pili were composed of a single subunit. On analytical ultracentrifugation, a single boundary with an $s_{20,w}$ of 12.2S at a concentration of 0.42 mg/ml was observed. The average length of purified pili at zero concentration was approx. 160 nm and the diameter was 7.4 ± 0.6 nm. Amino acid analysis of the purified K99 pili revealed that sulphur-containing amino acids, cysteine and methionine, were absent. Aromatic amino acids, phenylalanine and tyrosine, previously reported to be absent [Isaacson (1977) *Infect. Immun.* **15**, 272–279], constituted 7.14% of the total amino acid residues present. On immunoelectrophoresis, purified K99 pili migrated towards the cathode and caused mannose-resistant haemagglutination of horse, but not of sheep or guinea-pig, red blood cells. Pili from enterotoxigenic *E. coli* of porcine and human origin and from another bacterial species, namely *Fusiformis nodosus*, could also be extracted by the treatment of respective micro-organisms with 3 M-KSCN.

Enterotoxigenic *Escherichia coli* cause an acute cholera-like diarrhoea in both animals and humans. The disease process initially involves the colonization of the mucosal surface of the small intestine, followed by the elaboration of a heat-labile enterotoxin and/or heat-stable enterotoxin. Intestinal colonization by enterotoxigenic *E. coli* is generally mediated by a specific surface-associated pilus or fimbrial antigen that endows the bacteria with the capacity to adhere to epithelial-cell surface (Smith & Linggood, 1972). Such pili, which are plasmid-mediated, are the K88 antigens of swine-specific (Jones & Rutter, 1974), the K99 antigen of calf- and sheep-specific (Ørskov *et al.*, 1975) and the colonization-factor antigens of human-associated enterotoxigenic *E. coli* (Evans *et al.*, 1975, 1977; Evans & Evans, 1978). K88, K99 and colonization-factor antigens are antigenically unrelated to the type 1 pili generally associated with *E. coli* and are dis-

tinguishable from the latter by their mannose-resistant haemagglutinating activity (Burrows *et al.*, 1976; Ørskov *et al.*, 1977; Evans *et al.*, 1979).

K99 has been reported to be associated with a majority of enterotoxigenic *E. coli* isolated from cases of diarrhoeal disease in neonatal calves and lambs (Guinée *et al.*, 1976; Moon *et al.*, 1976; Myers & Guinée, 1976; Sivaswamy & Gyles, 1976).

K99 was previously reported to have been purified and composed primarily of protein subunits of 22500 and 29500 daltons with a pI in excess of 10 (Isaacson, 1977). Such purified pili did not haemagglutinate guinea-pig erythrocytes. On the other hand, Morris *et al.* (1977, 1978) reported that K99 pili isolated in their laboratory haemagglutinated guinea-pig erythrocytes and had a pI of 4.2. Isaacson (1978) later reported that if K99 pili were prepared by acid precipitation, they caused guinea-pig erythrocytes to agglutinate, presumably because of the presence of a second antigen with a pI of 4.2. However, if the K99 pili were prepared by ion-exchange chromatography on DEAE-Sephadex, guinea-pig erythrocytes were not agglutinated. More

Abbreviations used: SDS, sodium dodecyl sulphate; RNAase, ribonuclease.

§ To whom requests for reprints should be addressed.

recently, Morris *et al.* (1980) presented evidence suggesting the existence of two types of adhesive antigens, namely cationic and anionic haemagglutinins, in their K99-pili preparations.

Because of fairly recent optimistic reports regarding the feasibility of using pili preparations as possible vaccines for the control of scours (diarrhoea) in calves, lambs and piglets (Isaacson *et al.*, 1977; Acres *et al.*, 1978; Sojka *et al.*, 1978), it was considered important to develop an efficient method for the isolation and purification of K99 pili to facilitate the evaluation of purified pili as a possible vaccine for the control of neonatal-calf scours, and to study the physicochemical properties of purified K99 pili with the ultimate aim of achieving maximum immunopotential via antigenic modification, with or without the assistance of appropriate adjuvants. In the present paper, data are provided on: (i) the comparative evaluation of methods developed in this laboratory for the isolation of purified K99 pili, and (ii) some of the physicochemical properties of thus-purified K99 pili.

Materials and methods

Bacteria

Two strains of enterotoxigenic *E. coli* were used in the present investigation. One, C1443, was kindly provided by Dr. J. Craven of the Veterinary Research Laboratory, 'Attwood', Westmeadows, Vic., Australia, and had been isolated from an outbreak of calf scours. Its serotype was 020: KX106:K99⁺, H⁻ [I. Links (Regional Veterinary Laboratory, Wagga Wagga, N.S.W., Australia), personal communication]. The organism produced both heat-stable and heat-labile enterotoxins. The other *E. coli* strain used was the K99 reference strain (B41) and was obtained through the courtesy of Dr. H. W. Smith.

Growth conditions

For the extraction of K99, *E. coli* was grown at 37°C in a batch fermenter (New Brunswick) containing 10 or 18 litres of Minca medium (Guinée *et al.*, 1976), unless otherwise indicated. The bacterial culture was aerated at the rate of 3 litres/min.

When micro-organisms were to be used for absorption purposes for the production of specific K99 antiserum, they were grown at 18°C in stationary flasks. Under these conditions, the *E. coli* strain (C1443) was found to be devoid of pili as judged by transmission electron microscopy of negatively stained preparations, thus confirming previous observations (Ørskov *et al.*, 1975), although occasionally cells possessing a long, single pilus (resembling a sex pilus) were encountered. Whereas the cells grown as such caused neither mannose-sensitive nor

mannose-resistant haemagglutination, K99⁺ cells caused a mannose-resistant haemagglutination of horse red blood cells.

Extraction procedures

(i) *Ultrasonication*. Details of the procedure used have been described previously (Clements & Finkelstein, 1979).

(ii) *Homogenization*. The conditions used were essentially those described by Isaacson (1977).

(iii) *Heating of broth cultures of E. coli*. This method of extraction of K99 pili has been described previously (Morris *et al.*, 1977).

(iv) *Extraction with KSCN*. As revealed by the kinetics of the release of K99 activity as a function of total protein released, enterotoxigenic *E. coli* were extracted with a final concentration of 3 M-KSCN, pH 7.1, for 1 h at 22°C, followed by centrifugation at 12350g in a Sorvall centrifuge (RC-2B) for 30 min in order to sediment the extracted cells, which were discarded. The supernatant (referred to hereafter as the 'crude KSCN extract') was dialysed exhaustively against phosphate-buffered saline (Morris *et al.*, 1977) and stored frozen at -20°C.

Assay of K99 activity

K99 activity was detected by the following methods.

(i) *Ouchterlony double-diffusion technique in 0.85% agarose*. Wells punched in the agar (5 mm apart) were filled with 20 µl of either sample or specific K99 antiserum, which was prepared in rabbits as described below. K99 activity was quantified by determination of the most dilute sample that resulted in the development of a precipitin arc when made to react with specific K99 antiserum for up to 96 h at 4°C. The reciprocal of that dilution was designated as the K99 activity/20 µl.

(ii) *Haemagglutination of horse red blood cells*. Haemagglutination of horse red blood cells was carried out as described by Morris *et al.* (1980) in round-bottomed microtitre plates. Serial 2-fold dilutions of samples (25 µl) to be assayed for K99 activity were made in phosphate-buffered saline. K99 activity was quantified by determining the last dilution of the sample that resulted in haemagglutination. The reciprocal of that dilution was used to designate the K99 activity/25 µl.

Haemagglutination was also carried out with guinea-pig and sheep red blood cells, but only with purified K99 preparations.

Preparation of K99-specific antisera

Antisera directed against O antigens (0:20), polysaccharide capsule (KX 106) and K99 pili (OK antisera) were prepared essentially as described by Sojka (1965) (hereafter referred to as 'unabsorbed C1443 OK antisera') absorbed repeatedly with live

cells grown at 18°C until no agglutination of the latter was encountered. Such antisera agglutinated the Minca-grown *E. coli* strain C1443 (K99⁺) to a titre of 8–16. However, no agglutination was observed with (a) enterotoxigenic *E. coli* (C1443) grown at 18°C or (b) enterotoxigenic *E. coli* (C1443) grown in trypticase soy broth at 37°C, heated for 1 h at 100°C, with the agglutination reaction being carried out at 56°C.

The K99 antisera were further judged to be specific by the following additional criteria.

(i) When immunoelectrophoresis of the crude KSCN extract was carried out with K99 antiserum, only a single arc migrating towards the cathode was observed (Guinée *et al.*, 1976).

(ii) Haemagglutination of horse red blood cells by the crude KSCN extract containing cell-free K99 antigen was inhibited (Morris *et al.*, 1977).

Assay for heat-labile enterotoxin activity

The heat-labile enterotoxin activity was assayed by using y-1 adrenal tumour cells (Donta *et al.*, 1974).

Preparative isoelectric focusing

Preparative isoelectric focusing was carried out with Sephadex IEF with 2% Pharmalyte, pH 3–10 (Pharmacia Fine Chemicals, Sydney, N.S.W., Australia). The power limit was set at 2000 V, with the current limit being set at 8 W (LKB power supply 2103) and the total isoelectric-focusing time was approx. 24 h. A portion (30 ml) of the sample was dialysed against phosphate-buffered saline, pH 7.4 (0.05 M-K₂HPO₄/0.15 M-NaCl) overnight and diluted to 100 ml by addition of 6% D-Mannose in deionized water before mixing with Sephadex IEF. After isoelectric focusing, the Sephadex IEF was sliced into 30 equal segments and eluted with 5 ml of phosphate-buffered saline. Removal of Pharmalyte was accomplished by the addition of (NH₄)₂SO₄ to 50% final saturation to precipitate proteins. The latter were removed by ultracentrifugation at 105 000 g for 60 min and reconstituted in 1.0 ml of phosphate-buffered saline.

Analytical polyacrylamide-gel electrophoresis

Polyacrylamide-gel electrophoresis in SDS was carried out in 10% gels as described by Weber & Osborn (1969). Electrophoresis was carried out at 5 mA/gel for approx. 4 h. Staining of the gels was accomplished by using 0.25% Coomassie Blue in methanol/acetic acid/water (5:1:5, by vol.). Destaining was achieved with 7.5% acetic acid containing 5% methanol. SDS/polyacrylamide-gel electrophoresis was used for the determination of the molecular weight of purified K99 as well as to analyse: (a) fractions with K99 activity at various

steps of purification; (b) immunoprecipitates obtained by allowing specific K99 antiserum to react with crude K99 prepared from enterotoxigenic *E. coli* (C1443) by other methods (Isaacson, 1977; Morris *et al.*, 1977); (c) the crude K99 pili prepared by KSCN extraction of K99 reference strain (B41).

Chemical analysis

Protein concentration was determined as described by Bradford (1976) using the Bio-Rad protein kit. For the detection of DNA, the method used was that of Ceriotti (1952). The method used for the detection of RNA was based on a standard curve depicting change in A_{260} of yeast RNA caused by hydrolysis with RNAase and has been described previously (Mukkur & Pyliotis, 1981). The lipopolysaccharide content was determined by using 2-oxo-3-deoxyoctonate as a marker as described by Weissbach & Hurwitz (1959) with the modification that the hydrolysis was carried out at 100°C for 20 min, and the concentrations of periodic acid and sodium arsenite were those recommended by Osborn (1963). Total carbohydrate was determined by using the anthrone reagent as described by Mokrasch (1954); glucose was used as a standard.

Immunoelectrophoresis

Immunoelectrophoresis was carried out by the method of Scheidegger (1955) with 0.85% agarose C dissolved in barbitone/acetate buffer, pH 8.6 (Oxoid). The antisera used for the development of immunoelectrophoretic patterns (4°C) included specific K99, unabsorbed C1443 OK and unabsorbed KSCN-extract antisera. These were used as indicated in the Results and discussion section.

Electron microscopy

Whereas the preparations of cells (untreated and KSCN-extracted) suspended in saline were stained in 0.5% sodium phosphotungstate, pH 6.0, those of the extracts were stained in 2% sodium phosphotungstate, pH 7.0, with standard negative-staining procedures. The preparations were then examined in a Philips EM301 electron microscope.

Determination of sedimentation coefficient

The $s_{20,w}$ of purified K99 pili was determined by analytical ultracentrifugation in a Beckman analytical ultracentrifuge equipped with a multiplex scanner. Centrifugation was carried out at 37 020 rev./min in a titanium rotor (AnG-Ti) at 20°C. The concentration of purified K99 pili employed was 0.42 mg/ml.

Amino acid analysis

Purified K99 pili were dialysed exhaustively against water, freeze-dried and dissolved in 6 M-HCl. After hydrolysis of the sample at 110°C for 24 h

under vacuum, the amino acid composition was determined in an automated amino acid analyser (JLC-6AH). Tryptophan was determined spectroscopically (Edelhoch, 1967). The results were calculated as mol of amino acid residues recovered/mol of purified K99.

Results and discussion

Different methods were compared for their efficiency of extraction of K99 pili from an enterotoxigenic *E. coli* strain of bovine origin. The methods evaluated included homogenization (Isaacson, 1977), heating of an enterotoxigenic *E. coli* broth culture at 60°C for 30 min (Morris *et al.*, 1977), ultrasonication [employed by Clements & Finkelstein (1979) for the extraction of heat-labile toxin from enterotoxigenic *E. coli*], and extraction with a chaotropic agent, namely KSCN. The latter had been successfully employed previously to extract protective antigens of *Pasteurella multocida*, which were protein in nature (Mukkur, 1979; Mukkur & Pylotis, 1981). The assay systems used for monitoring the presence of K99 pili were: (a) transmission electron microscopy of negatively stained preparations; (b) haemagglutination of horse red blood cells (Morris *et al.*, 1980); and (c) precipitation reactions in gel using specific K99 antisera (Isaacson, 1977). When the enterotoxigenic *E. coli* extracted by various methods were negatively stained and examined by transmission electron microscopy for the efficiency of removal of K99 pili from the bacterial surface, it was discovered that whereas the cells extracted with KSCN for $\frac{1}{2}$ –1 h were completely devoid of pili (Plate 1), the surface of those prepared either by homogenization (Isaacson, 1977) or heating (Morris *et al.*, 1977) was still associated with significant numbers of pili. Also, the percentage yield of K99 activity per mg of protein was the greatest when the cells were extracted with KSCN, the lowest yield being obtained by ultrasonication (Table 1). It was decided therefore to

devise procedures for the purification of K99 pili extracted with KSCN in order to characterize their physicochemical and biological properties.

At first the kinetics of the release of protein and K99 activity from enterotoxigenic *E. coli* (C1443) by the KSCN extraction procedure, as judged by haemagglutination of horse red blood cells, was determined. Fig. 1 shows that the total protein released was essentially the same after $\frac{1}{2}$ h and 1 h of KSCN extraction. If the extraction was allowed to proceed for any further length of time, the total amount of protein released from the bacterial cells was increased, although the haemagglutinating activity actually decreased, possibly due to the release of as-yet-unidentified haemagglutination inhibitors. In this regard it would be interesting to examine the possible correlation, if any, between the release of carbohydrates, lipopolysaccharide, DNA and RNA with the increased release of protein after 1 h. All available K99 activity had apparently been

Fig. 1. Kinetics of the release of total K99 activity (●) and proteins (○) as a function of time employed for the extraction of enterotoxigenic *E. coli* (C1443) with 3 M-KSCN

The K99 activity was measured by haemagglutination of horse red blood cells.

Table 1. Comparative efficiency of various methods in affecting the release of K99 activity from enterotoxigenic *Escherichia coli*

Abbreviations used: H.A., haemagglutination units; P.U., precipitation units; N.D., not detectable.

Method of extraction	Total protein (mg)	Total H.U.*	H.U. per mg of protein	Total P.U.*	P.U. per mg of protein	Reference
(i) KSCN	35.84	71 680	2000	10 400	290	The present paper
(ii) Ultrasonication	426.2	15 360	360	8000	18.7	The present paper
(iii) Homogenization	100.6	64 000	636	5760	57	(Isaacson, 1977)
(iv) Heating broth culture at 60°C	1860	240 000	129	N.D.	N.D.	(Morris <i>et al.</i> , 1977)

* Based on activity released from either 10 litres of Minca-grown *E. coli* (5×10^9 bacteria/ml) (i–iii) or 6 litres of broth (TSB)-grown *E. coli* (1×10^{10} bacteria/ml).

EXPLANATION OF PLATE 1

Electron micrographs of negatively stained preparations

(a) Untreated enterotoxigenic *E. coli* (C1443) (magnification $\times 35\,000$); (b) enterotoxigenic *E. coli* (C1443) after extraction with 3M-KSCN ($\times 35\,000$); (c) crude KSCN extract rich in K99 pili ($\times 136\,000$); (d) purified K99 pili ($\times 136\,000$). P denotes pili and the bars equal $1\,\mu\text{m}$ (a, b) and $0.25\,\mu\text{m}$ (c, d).

extracted within the first $\frac{1}{2}$ h. The amount of protein released was much greater (ranging from 3- to 12-fold) when other methods were used for the preparation of pili (Table 1).

When KSCN was previously used to extract protective antigens from *P. multocida* type A (Mukkur, 1979; Mukkur & Pylotis, 1981), it was discovered that such extraction resulted not only in the release of proteins, carbohydrates, lipopolysaccharides but also DNA and RNA. Therefore we decided to determine if, in addition to the protein, other macromolecules were also present in the crude KSCN extract. All the above-mentioned macromolecules were detectable in the crude KSCN extract prepared from enterotoxigenic *E. coli* (C1443). These components were not quantified except where indicated. Since recently it was reported (Clements & Finkelstein, 1979) that the best source for the isolation of heat-labile enterotoxin was the whole-cell lysate, it was considered important to determine its presence in the KSCN extract. By using the y-1 adrenal-tumour-cell assay (Donta *et al.*, 1974), the heat-labile enterotoxin activity ranging from 1/4 to 1/16 was detected.

The initial method attempted for the purification of K99 pili from the KSCN extract of enterotoxigenic *E. coli* (C1443) was that reported by Isaacson (1977), which involved precipitation of K99 pili with $(\text{NH}_4)_2\text{SO}_4$ to a final saturation of 14%, discarding the precipitate, followed by a second precipitation of the supernatant with $(\text{NH}_4)_2\text{SO}_4$ to a final saturation of 31%. However, when using this procedure we discovered that approx. 40% of the K99 activity was non-precipitable, as judged by haemagglutination of horse red blood cells and by the Ouchterlony gel-diffusion test using specific K99 antisera. However, using $(\text{NH}_4)_2\text{SO}_4$ to a final saturation of 50%, approx. 90% of the available K99 activity was precipitated. In this regard, it is noteworthy that Wevers *et al.* (1980) recently reported precipitation of pili associated with enterotoxigenic *E. coli* of human origin (O:18ac) with only 10% final saturation of $(\text{NH}_4)_2\text{SO}_4$. This step of 'salting out' resulted in 1.7-fold purification as judged by the increase in

specific activity per mg of protein (Table 2). The lipopolysaccharide, DNA, RNA and the heat-labile enterotoxin were still detectable in the enriched K99-pili preparations, however. In order to remove the heat-labile enterotoxin activity, the latter were now subjected to batchwise absorption with Bio-Gel A5m equilibrated with TEAN buffer (Clements & Finkelstein, 1979) until no such activity was detected at a final concentration of 150 μg of protein/ml. After this step, the pili-containing preparation was subjected to preparative isoelectric focusing using Pharmalyte (pH range 3–10). A typical isoelectric-focusing profile is shown (Fig. 2). After removal of the Pharmalyte and concentration of the fractions, they were analysed by the gel-diffusion test using specific K99 antisera, and by immunoelectrophoresis using unabsorbed C1443 OK antisera. Specific K99 activity was found to be localized in the first seven fractions, the greatest activity being associated with fraction number 2. On immunoelectrophoresis, a single cationic arc was discernible on development either with unabsorbed C1443 OK or rabbit anti-(crude KSCN extract) antisera. However, on SDS/polyacrylamide-gel electrophoresis, whereas fractions 1–3 showed a single band, the molecular weight of which corresponded to $12\,600 \pm 300$ (Figs. 3 and 4), fractions 4–7 showed a minimum of five additional bands with mol.wts. greater than 39 800 (result not shown).

On immunoelectrophoresis of the crude KSCN extract with unabsorbed C1443 OK antisera, one faint and two dense arcs were observed. Whereas one of the dense arcs was cationic, the second was anionic. The faint arc was localized in line with the well (Fig. 5). The anionic component was found to be distributed in an immunoelectrophoretically pure state in fractions 8–29. Fraction 30 was found to contain both the anionic as well as the third component represented by the faint arc. The presence of the latter component in fraction 30 was surprising in view of its position in the immunoelectrophoretic profile; the reason for this behaviour is not clear at present.

Anionic antigen titration was accomplished by immunoelectrophoresis of serially diluted fractions 8–30 with unabsorbed C1443 OK antiserum. A

Table 2. *Purification of K99 from enterotoxigenic E. coli*

Step	Total protein (mg)	Total P.U.*	Specific activity (P.U./mg)†	Purification (fold)	Yield (%)
Crude KSCN extract	54.4	22 000	404	—	100
$(\text{NH}_4)_2\text{SO}_4$ precipitation	30.0	20 000	667	1.7	91
Bio-Gel A5m absorption	15.0	16 000	1067	2.6	73
Isoelectric focusing (pure pili)	2.9	11 200	3862	9.6	51

* P.U., precipitin units.

† Data are based on purification from 18 litres of Minca-grown *E. coli* (5×10^9 bacteria/ml).

Fig. 2. Isoelectric-focusing profile of $(\text{NH}_4)_2\text{SO}_4$ precipitate of crude KSCN extract of enterotoxigenic *E. coli* (C1443), obtained by using Pharmalyte, pH 3–10

Δ , A_{280} ; $\bullet$, $\log_2$ (precipitin titre) obtained by Ouchterlony gel-diffusion test using specific K99 antisera for development; $\circ$, $\log_2$ (precipitin titre) of the anionic component obtained immunoelectrophoretically with unabsorbed enterotoxigenic *E. coli* (C1443) antisera for development; $\square$, pH gradient.

bimodal pattern of activity represented by peaks II and III (Fig. 2) was revealed. However, the Ouchterlony gel-diffusion test on peaks II and III revealed lines of complete identity on development with unabsorbed C1443 OK antiserum (result not shown).

The yield of K99 pili was 51% on the basis of specific K99 activity/mg of protein (Table 2). This yield was significantly greater than that reported by Isaacson (1977). The total purification achieved was 9.6-fold.

The introduction of gel filtration on Bio-Gel A-5m as an additional purification step after Bio-Gel A-5m absorption resulted neither in any increased resolution nor in the purification of K99. In fact, an approx. 4-fold decrease in the final yield of K99 pili was the result (results not shown). Whether the losses incurred were due to non-covalent interaction between the galactose residues of Bio-Gel A-5m or to non-specific absorption remains to be determined.

Although the Pharmalyte used in the isoelectric focusing of K99-pili-rich preparations was in the pH range 3–10, a gradient shift was always recorded. That this shift did not result from the presence of mannose or the buffer system used in the isoelectric-focusing run was revealed when a control run performed under the same conditions showed a similar gradient shift. Regardless of differences in the pH gradient, maximum K99 activity always was confined to the fraction with a pH between 8 and 9 (Fig. 2).

The cationic characteristic of purified K99 pili was consistent with a fairly recent report by Morris *et al.* (1980), who stated that the pili belonging to group 0:20 were cationic. This was supported by the finding that the intact enterotoxigenic *E. coli*

(C1443) and purified pili caused a strong mannose-resistant haemagglutination of horse, but not of sheep or guinea-pig, red blood cells.

The $s_{20,w}$ of purified pili was calculated to be 12.2S at a concentration of 0.42 mg/ml, the latter value being in good agreement with that reported by Isaacson (1977). Electron microscopy of purified pili revealed that they were aggregated not only in a parallel arrangement but also in a linear fashion as judged by an increase in the average pilus length as a function of increased protein concentration (Fig. 6). Whereas the diameter of an individual pilus was measured to be 7.4 ± 0.6 nm, the length at zero concentration was estimated to be approx. 160 nm (Fig. 6). These data are in good agreement with those reported by Isaacson (1977).

No lipopolysaccharide, DNA, RNA or heat-labile enterotoxin activity were detectable in the purified K99-pili preparations. However, the amount of total carbohydrates was estimated at $51 \mu\text{g}/\text{mg}$ of protein.

On Ouchterlony gel diffusion, the purified K99 pili showed a reaction of complete identity with a component in the KSCN extract of the K99 reference strain (B41), on development both with specific K99 as well as with the unabsorbed C1443 OK antisera (results not shown). This presumably represented the presence of the cationic K99 pili (Morris *et al.*, 1980) in the KSCN extract of strain B41.

Isaacson (1977) reported K99 pili to comprise two subunits: a major component with a mol.wt. of 22 500 and a minor component with a mol.wt. of 29 500. Because of the discrepancy, it was considered important to ascertain that the lower molecular weight of the K99 subunit obtained in this

Fig. 3. SDS/polyacrylamide-gel electrophoresis of (a) the crude KSCN extract (100 µg), (b) $(\text{NH}_4)_2\text{SO}_4$ precipitate of the crude KSCN extract (100 µg) and (c) purified K99 pili (100 µg).

The letter D denotes the dye marker; the arrow points to the K99 subunit.

investigation was not a consequence of the method used for the extraction of K99 pili. Therefore, crude extracts containing K99 pili prepared by the method of Isaacson (1977) and Morris *et al.* (1977) and purified K99 pili (the present investigation) were subjected to immunoprecipitation using specific K99 antiserum. A 0.5 ml portion of various crude preparations was mixed with 0.5 ml of specific K99 antisera and incubated at 4°C for 48 h. The precipitates were washed once with ice-cold saline (0.9% NaCl), dissolved in 1% SDS, and subjected to SDS/polyacrylamide-gel electrophoresis. In all cases, the molecular weight of the precipitated K99 pili was found to be 12600 (result not shown). Therefore it is probable that the K99 pili purified by Isaacson (1977) represented either a different type of

Fig. 4. Determination of the molecular weight of purified K99 pili by SDS/polyacrylamide-gel electrophoresis. The molecular weights of the marker proteins are: bovine serum albumin, 68 000; ovalbumin, 45 000; aldolase subunit, 40 000; pepsin, 35 000; subtilisin, 27 500; chymotrypsin, 25 000; trypsin, 23 800; ribonuclease A, 13 700; cytochrome c (horse heart), 12 100.

Fig. 5. Immuno-electrophoretic patterns of the crude KSCN extract of enterotoxigenic *E. coli* (C1443) and purified K99 pili.

The antisera used for development were (a) specific K99 antiserum and (b) unabsorbed enterotoxigenic *E. coli* (C1443) antiserum. Wells 1 and 4 contained purified K99 pili, wells 2 and 3 contained the crude KSCN extract.

pili or partially purified pili. In this regard it is noteworthy that heterogeneity of molecular weights in the case of colonization factor antigens I and II was recently reported (Wevers *et al.*, 1980).

The general similarity observed between the amino acid composition of K99 pili reported previously by Isaacson (1977) and that determined in this laboratory (Table 3) was in the order in which

Fig. 6. Determination of the length of K99 pili as a function of increasing protein concentration

Table 3. Amino acid composition of purified K99 isolated from enterotoxigenic *Escherichia coli* (C1443)

Amino acid	Content (mol of amino acid residue/ mol of K99 pili)
Lysine	6
Histidine	2
Arginine	3
Aspartic acid	13
Threonine	10
Serine	12
Glutamic acid	9
Proline	4
Glycine	16
Alanine	10
Cysteine	Nil
Valine	5
Methionine	Nil
Isoleucine	5
Leucine	7
Tyrosine	3
Phenylalanine	5
Tryptophan	2

the percentages of various groups of amino acid residues were present, viz., aliphatic > dicarboxylic and hydroxy amino acids > basic amino acids. However, significant differences observed were that whereas hydroxylysine, cysteine and methionine were absent, tyrosine and phenylalanine were present in our purified K99 pili preparations. Since a high proportion of acidic amino acid residues is uncharacteristic of proteins with cationic isoelectric points, it was assumed that most of the aspartic and glutamic acid residues were present as asparagine and glutamine before acid hydrolysis. The molecular weight of purified K99 was calculated to be 13687 from its amino acid composition, which appeared to be in reasonable agreement with that obtained by SDS/polyacrylamide-gel electrophoresis ($12600 \pm$

300). Whether more than one type of K99 pilus exists in Nature will require isolation and immunochemical characterization of K99 pili from additional enterotoxigenic *E. coli* strains.

Because of the ease with which K99 pili could be extracted with KSCN, we decided to determine whether pili from enterotoxigenic *E. coli* of porcine (e.g. K88) and human (e.g., colonization-factor antigens) origin could also be extracted using the same procedure. Electron microscopy of one KSCN-treated enterotoxigenic *E. coli* of porcine (P155) and human origin (H10407) revealed that these pili were extractable. Similarly, pili of *Fusiformis nodus* (the bacterium causing foot-rot in sheep) was also found to be extractable with KSCN. Therefore it appears feasible that this method of extraction of pili may be applicable to other pilated micro-organisms as well, e.g., *Moraxella bovis*, *Neisseria gonorrhoea* and some *Salmonella* species.

This project was supported by a grant from the Australian Dairying Research Committee made to T. K. S. M. The excellent technical assistance of Mrs. D. Stewart is gratefully acknowledged. Also acknowledged is the part-time assistance of Ms. H. Thomas. We are thankful to Mr. Berto Reibero, Department of Veterinary Medicine, University of Sydney, for donating pilated cultures of *Fusiformis nodus*. Finally, we thank Mr. L. Higginbottom for performing the amino acid analysis and Dr. Alex Reisner (C.S.I.R.O. Cellular and Molecular Biology Unit, North Ryde, N.S.W., Australia) for permitting us to use their Beckman analytical ultracentrifuge.

References

- Acres, S. D., Isaacson, R. E., Khachatourians, G., Babiuk, L. & Kapitany, R. A. (1978) in *Proceedings of the Second International Symposium on Neonatal Diarrhoea* (Acres, S. D., ed.), pp. 433–455, Vido, Saskatoon
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- Burrows, M. R., Sellwood, R. & Gibbons, R. A. (1976) *J. Gen. Microbiol.* **96**, 269–275
- Cerioti, G. (1952) *J. Biol. Chem.* **198**, 297–303
- Clements, J. D. & Finkelstein, R. A. (1979) *Infect. Immun.* **24**, 760–769
- Donta, S. T., Moon, H. W. & Whipp, S. C. (1974) *Science* **183**, 334–336
- Edelhoc, H. (1967) *Biochemistry* **6**, 1948–1954
- Evans, D. G. & Evans, D. J., Jr. (1978) *Infect. Immun.* **21**, 638–647
- Evans, D. G., Silver, R. P., Evans, D. J., Jr., Chase, D. G. & Gorbach, S. L. (1975) *Infect. Immun.* **12**, 656–667
- Evans, D. G., Evans, D. J., Jr. & DuPont, H. L. (1977) *J. Infect. Dis.* **136**, S118–S123
- Evans, D. J., Jr., Evans, D. G. & DuPont, H. L. (1979) *Infect. Immun.* **23**, 336–346
- Guinée, P. A. M., Jansen, W. H. & Agterberg, C. M. (1976) *Infect. Immun.* **13**, 1369–1377
- Isaacson, R. E. (1977) *Infect. Immun.* **15**, 272–279
- Isaacson, R. E. (1978) *Infect. Immun.* **22**, 555–559

- Isaacson, R. E., Morgan, R. L., Moon, H. W. & Brinton, C. C. (1977) in *Proceedings of the 13th Joint U.S.-Japan Conference on Cholera, Atlanta, GA*, pp. 285-293, National Institutes of Health, Bethesda
- Jones, G. W. & Rutter, J. M. (1974) *J. Gen. Microbiol.* **84**, 135-144
- Mokrasch, L. C. (1954) *J. Biol. Chem.* **208**, 55-59
- Moon, H. W., Whipp, S. C. & Skartvedt, S. M. (1976) *Am. J. Vet. Res.* **37**, 1025-1029
- Morris, J. A., Stevens, A. E. & Sojka, W. J. (1977) *J. Gen. Microbiol.* **99**, 353-357
- Morris, J. A., Stevens, A. E. & Sojka, W. J. (1978) *Infect. Immun.* **19**, 1097-1098
- Morris, J. A., Thorns, C. J. & Sojka, W. J. (1980) *J. Gen. Microbiol.* **118**, 107-113
- Mukkur, T. K. S. (1979) *J. Gen. Microbiol.* **113**, 37-43
- Mukkur, T. K. S. & Pyliotis, N. A. (1981) *J. Comp. Pathol.* **91**, in the press
- Myers, L. L. & Guinée, P. A. M. (1976) *Infect. Immun.* **13**, 1117-1119
- Ørskov, I., Ørskov, F., Smith, H. W. & Sojka, W. J. (1975) *Acta. Pathol. Microbiol. Scand. Sect. B* **83**, 31-36
- Ørskov, I., Ørskov, F., Jann, B. & Jann, K. (1977) *Bacteriol. Rev.* **41**, 667-710
- Osborn, M. J. (1963) *Proc. Natl. Acad. Sci. U.S.A.* **50**, 499-506
- Scheidegger, J. J. (1955) *Int. Arch. Allergy Appl. Immunol.* **7**, 103-109
- Sivaswamy, G. & Gyles, C. L. (1976) *Can. J. Comp. Med.* **40**, 247-251
- Smith, H. W. & Linggood, M. A. (1972) *J. Med. Microbiol.* **5**, 243-250
- Sojka, W. J. (1965) *Escherichia coli in Domestic Animals and Poultry*, Review Series no. 7, Commonwealth Bureau of Animal Health, Weybridge, Surrey, U.K.
- Sojka, W. J., Wray, C. & Morris, J. A. (1978) *J. Med. Microbiol.* **11**, 493-499
- Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406-4412
- Weissbach, A. & Hurwitz, J. (1959) *J. Biol. Chem.* **234**, 705-709
- Wevers, P., Picken, R., Schmidt, G., Jann, B., Jann, K., Golecki, J. R. & Kist, M. (1980) *Infect. Immun.* **29**, 685-691