Partial Purification and Characterization of Two Non K99 Mannose-resistant Haemagglutinins of *Escherichia coli* B41

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A K99⁻ variant of *Escherichia coli* B41 was produced by growing the parent strain in the presence of antiserum to *E. coli* K12K99. Two mannose-resistant and eluting (MRE) haemagglutinins with molecular weights greater than 20×10^6 were extracted from the cell surface of the variant. One was an anionic antigen, partially purified by ammonium sulphate and isoelectric point precipitation, which adhered to calf intestinal brush borders; it was a protein composed of subunits with mol. wt 34000. Electron microscopy showed that this material did not have a regular fimbrial appearance, but contained some fine fibrillar structures. A second MRE haemagglutinin which was also partially purified by ammonium sulphate precipitation, had a definite fimbrial structure, being a protein composed of two subunits of mol. wt 49500 and 48000. This antigen was probably responsible for the fimbrial appearance of the K99⁻ variant, but it was antigenically distinct from the anionic adhesin and did not adhere to calf intestinal brush borders.

INTRODUCTION

Adhesion to the surface of the small intestine, assisted by the fimbrial antigen K99 (Smith & Lingood, 1972), may be the first step in the colonization process by some enterotoxigenic *Escherichia coli* in calves, lambs and piglets (Moon *et al.*, 1977). Morris *et al.* (1980) described antibodies in antisera to all the K99 *E. coli* strains tested which reacted with a cationic antigen of *E. coli* B41; this antigen was K99. Antibodies in antisera to some K99 *E. coli* strains also reacted with an anionic antigen of *E. coli* B41. Antigen preparations that contained either, but not both antigens, caused MRE haemagglutination of horse and sheep erythrocytes. Only the preparation which contained the anionic antigen consistently inhibited adhesion of *E. coli* B41 to calf intestinal brush borders.

Chanter (1982) showed that a haemagglutinin of polypeptide subunits with mol. wt 19000 comprised the cationic antigen K99 and that the anionic antigen consisted of a haemagglutinin of polypeptide subunits with mol. wt 34000.

Further characterization of the anionic adhesin in this laboratory has been hampered by purification procedures (ammonium sulphate and isoelectric point precipitation, ion exchange and gel filtration chromatography, preparative electrophoresis, immunoelectrophoresis, and haemadsorption) that resulted in preparations contaminated by K99. Methods which overcame these difficulties have been developed and these have enabled demonstration of a third MRE haemagglutinin on the K99 reference strain *E. coli* B41.

Abbreviations: IE, Immunoelectrophoresis; IRA, immunoradiometric assay; MRE, mannose-resistant and eluting haemagglutinin; PBS, phosphate-buffered saline.

METHODS

Escherichia coli. *Escherichia coli* strains B41 (O101 : K99), B117 (O8 : K85, K99), S13 (O8 : K85, K99), a K12 and a K12 K99⁺ transconjugate of *E. coli* B41 were kindly supplied by Mr M. R. Burrows. Strains W1 (O149 : K91, K88ac), M2225 (O9 : K103, 987p) and K12 strains C600 and F711 were kindly supplied by Mr R. Sellwood. Unless otherwise stated, bacteria were grown at 37 °C for 18 h on modified TGX agar (Burrows *et al.*, 1976) containing 0.1% (w/v) Lab Lemco (Oxoid).

Antisera. Antisera to bacteria were prepared in rabbits (Sojka, 1965) and, where necessary, absorbed with homologous bacteria grown at 18 °C. Bacteria grown at 18 °C do not produce K99 or the anionic adhesin described by Morris *et al.* (1980). Antisera to the partially purified fractions of *E. coli* were prepared in rabbits using two subcutaneous inoculations, separated by 4 weeks, of 2 mg material in 2 ml Freund's incomplete adjuvant (Difco). Antisera were collected four weeks after the last inoculation.

Extracts of bacteria. Material was removed from the surface of bacteria by homogenization of 10–20 g wet wt in a blender (MSE, Atomix) at half speed for 5 min in 100 ml volumes of 0.05 M-phosphate buffer, pH 7.2, containing 1 M-NaCl (Isaacson, 1977), followed by centrifugation at 20000 g at 4 °C for 60 min. Proteins were differentially precipitated from the supernatant buffer by stepwise increases in ammonium sulphate concentration. After each stepwise increase, precipitate was removed by centrifugation (20000 g at 4 °C for 10 min). Precipitates were then dialysed in 0.05 M-phosphate buffered isotonic saline (PBS), pH 7.0.

Antigen K99. K99 was prepared from *E. coli* K12 K99 by precipitation of an extract with 30% saturated ammonium sulphate and recovery of the precipitate by centrifugation and dialysis against PBS as described above.

Radioiodination of proteins. Partially purified proteins and antibodies to K99 were labelled with ¹²⁵I by the chloramine T method (Hunter, 1978).

Polyacrylamide gel electrophoresis (SDS-PAGE). Slab gels were run with the discontinuous buffer system of Laemmli (1970). Polypeptides were located after staining with Coomassie brilliant blue and, where appropriate, by autoradiography. Molecular weights (corrected to three significant figures) were calculated by reference to the mobility of bovine serum albumin, ovalbumin, lactate dehydrogenase and myoglobin, relative to that of bromothymol blue, and were derived from the mean figures for five gels.

Immuno-electrophoresis (IE). The method of Morris & Hussaini (1974) was used.

Immunodiffusion tests. The method of Ouchterlony & Nilsson (1978) was used. Gels containing radiolabelled antigen were washed in ten changes of PBS followed by one change of distilled water over 5 d. Where applicable, precipitin lines were cut out and boiled in an approximately equal volume of double strength sample buffer before layering onto gels for analysis by SDS-PAGE.

Column chromatography. Bacterial fractions (500–700 µg protein ml⁻¹) were applied in a sample volume of 1 ml to a Sepharose 4B CL column (15 × 1 cm; Pharmacia), equilibrated in PBS and eluted at a flow rate of 2.0 ml h⁻¹ at 4 °C. Fractions were collected every 20 min.

Chemical analysis. Protein concentrations were estimated spectrophotometrically by the method of Ehresmann *et al.* (1973). Carbohydrate concentrations were measured colorimetrically by the indole method for total carbohydrate (Ashwell, 1957), using sucrose as a reference.

Haemagglutination tests. The method of Burrows *et al.* (1976) was used. Tests were incubated at 4 °C for 2 h before reading. The test was modified (where applicable) for an assessment of mannose-sensitive haemagglutinins by excluding mannose from the buffer and by incubation of tests at 37 °C for 2 h. Bacterial extracts and suspensions were held at 100 °C for 30 min or treated with 0.5% (v/v) formaldehyde at 37 °C for 4 h prior to the test to assess temperature and chemical stability of the haemagglutinin. Resistance of haemagglutination to trypsin was examined as follows. 50 µl trypsin solution (10 mg ml⁻¹; Sigma, type III) was diluted in 450 µl of haemagglutinin preparation containing 200 µg of protein, and incubated at 37 °C for 1 h. To check the activity of the trypsin, samples were removed from the reaction mixture at intervals for analysis by SDS-PAGE. Trypsin was inactivated by incorporation of 2% (w/v) bovine serum albumin in buffer used in haemagglutination tests.

Haemagglutination inhibition tests. A three volume test was used (total volume, 60 µl) in which preparations were diluted to 2 haemagglutinin units in 3% (w/v) D-mannose. Antibody, serially diluted in a round-bottomed microtitre tray, and haemagglutinin were allowed to react for 30 min at 20 °C before washed erythrocytes were added. Tests were incubated at 4 °C for 2 h before reading.

Brush border adhesion tests. Brush borders were prepared from 2 lengths of small intestine, taken 1 m above the ileo-caecal valve from calves 1–2 d old. The adhesion test was performed as described by Burrows *et al.* (1976). Inhibition of adhesion to brush borders was carried out by the addition of 10 µl of isolated adhesin (200 µg ml⁻¹) to 100 µl of PBS which contained approximately 10⁶ brush borders and 10⁸ bacteria. Preparations were then incubated at 37 °C for 30 min. Tests for adhesion of bacterial fractions to brush borders were carried out by the addition of 10 µl of radiolabelled protein (200 µg ml⁻¹) to 90 µl of brush borders [10⁶ ml⁻¹ in PBS containing 1.5% (w/v) bovine serum albumin], which were incubated at 37 °C for 30 min. Brush borders were then washed twice in buffer and counted in a PRIAS PGD Autogamma (Packard Instruments). A negative control consisted of non-adhesive brush borders (brush-borders lacking in K99 receptors) from a calf more than 1 week old.

Production and isolation of K99⁻ variants to *E. coli* B41. Ethidium bromide (BDH) was sterilized by filtration and added to Oxoid nutrient broth no. 2 at 100 µg ml⁻¹. Broth cultures inoculated with *E. coli* B41 to give an initial concentration of 100 c.f.u. ml⁻¹ were incubated statically at 37 °C for 18 h. After being subcultured in small numbers until the bacteria had passed through approximately 60 generations, broths were diluted and spread onto agar plates. Plates were incubated at 37 °C for 18 h before colonies were tested for K99 production by slide agglutination, immunoradiometric assay, SDS-PAGE and IE. Alternatively, *E. coli* B41 was serially subcultured at 43 °C for 72 h, diluted and spread onto agar plates which were incubated at 37 °C for 18 h before colonies were tested for K99 production.

Selection and isolation of K99⁻ variants of *E. coli* B41. *Escherichia coli* B41 (10⁹ organisms) were added to 4 ml of rabbit antiserum raised against *E. coli* K12 K99 and 1 ml of rabbit serum which had been stored at -70 °C and served as a source of complement. The bacteria were incubated at 37 °C for 24 h. Serial dilutions were spread on plates and incubated at 37 °C for 18 h; colonies were examined for K99 production.

Immunoradiometric assay (IRA) for K99. Anti-K99 antibodies were purified by an immunosorbent method. A K99 preparation (5 mg) was further purified by adsorption to washed erythrocytes at 4 °C followed by three washes in PBS at 4 °C and by elution and separation from erythrocytes at 37 °C. The yield was 3 mg and according to SDS-PAGE the preparation contained one polypeptide with mol. wt of 19000. Purified K99 was attached to Sepharose 4B CL (Pharmacia) by the CN-Br method of Fuchs & Sela (1978). The immunosorbent was mixed with 2 ml of antiserum to *E. coli* B41 for 15 min at 20 °C and washed five times in PBS. K99 antibody was eluted by resuspension of the immunosorbent in 2 ml of 0.1 M-glycine/HCl buffer pH 2.0 for 15 min. The eluate was removed and the pH adjusted to 7.4 with 1 M-Tris/HCl buffer.

Antibody to K99 (50 µg) was labelled with 0.5 mCi (18.5 MBq) of ¹²⁵I, centrifuged at 10000 g for 15 min and diluted to 10000 c.p.m. (10 µl)⁻¹ in IRA buffer [1.5% (w/v) bovine serum albumin, 10% (v/v) foetal calf serum in PBS]. Suspensions (100 µl) of approximately 10⁹ c.f.u. ml⁻¹ were mixed with 10 µl of ¹²⁵I-labelled anti-K99 antibody, incubated at 4 °C for 30 min, 37 °C for 30 min and centrifuged at 10000 g for 2 min. The supernatant was removed and the pellet washed twice by resuspension in IRA buffer and centrifugation at 10000 g for 2 min. The radioactivity of both centrifuge tubes and pellets was counted.

Electron microscopy. Fractions of bacteria (200 µg protein ml⁻¹) in PBS were dropped onto Formvar/carbon-coated grids. After 30 s, excess fluid was removed with filter paper. The grids were then stained with 1% (w/v) sodium phosphotungstate (pH 6.8).

Bacteria, on Formvar/carbon-coated grids, were fixed with 1% (w/v) aqueous osmium tetroxide, washed with distilled water and coated with gold/palladium at an angle of 45° in an Edwards coating unit (Bradley, 1965). All specimens were examined in a Philips EM 300 at instrumental magnifications of 20000-55000 with an acceleration voltage of 80 kV.

RESULTS

*Isolation and characterization of a K99⁻ variant of *E. coli* B41*

Attempts to induce K99⁻ variants of *E. coli* B41 by growth either in the presence of ethidium bromide or at high temperatures failed.

96-97% of colonies of *E. coli* B41 which had been incubated with antiserum to *E. coli* K12 K99 were entire, 2 mm in diameter and agglutinated by antisera to *E. coli* K12 K99 and to *E. coli* B41 that were absorbed with organisms grown at 18 °C; this indicated the presence of K99. The remaining colonies were 4 mm in diameter, crenated and were agglutinated by absorbed antiserum to *E. coli* B41. They were not agglutinated by absorbed antiserum to *E. coli* K12 K99. This pattern of agglutination remained constant after six subcultures at 37 °C and indicated that the variants did not possess K99 but produced another antigen at 37 °C, but not at 18 °C.

One K99⁻ variant was chosen for further characterization. The IRA revealed that suspensions of the variant grown at 37 °C containing > 10⁹ organisms ml⁻¹ did not contain K99. The IRA was regularly capable of detecting K99 in suspensions of 10⁶ *E. coli* B41 ml⁻¹, but did not react with K99⁺ *E. coli* grown at 18 °C.

SDS-PAGE of cell-free extracts, concentrated by precipitation in 36% saturated ammonium sulphate from the variant grown at 37 °C, revealed the absence of the 19000 mol. wt polypeptide subunit of K99. The 34000 mol. wt polypeptide subunit of the anionic adhesin was of comparable intensity to that of the parent strain (Fig. 1).

IE performed with cell-free extracts from the K99⁻ variant and absorbed antiserum to *E. coli* B41 produced one anionic precipitin line. The cationic line produced in this test by cell-free extracts from K99⁺ *E. coli* B41 was absent.

Fig. 1

Fig. 2

Fig. 1. SDS-PAGE of cell extracts from *E. coli* B41 (lane 2) and the K99⁻ *E. coli* (lane 3). Lane 1 contains the molecular weight standards. This result has been reproduced five times.

Fig. 2. SDS-PAGE of a cell extract from *E. coli* B41 (lane 2), 30% ammonium sulphate fraction of a cell extract from the K99⁻ *E. coli* B41 (lane 3) and a subsequent 45% ammonium sulphate fraction (lane 4). Lane 1 contains the molecular weight standards. This result has been reproduced four times.

The variant when grown at 37 °C adhered to brush borders (>10–12 bacteria per brush border). By contrast, the variant when grown at 18 °C did not adhere to brush borders but did adhere to other refractile material in brush border preparations. The wild-type *E. coli* B41 reacted in brush border tests in the same way.

Partial purification and characterization of the anionic adhesin

A cell extract from the K99⁻ variant of *E. coli* B41 made 30% saturated with ammonium sulphate developed a precipitate which was soluble in PBS and contained approximately 50% protein and 50% carbohydrate. SDS-PAGE revealed a single polypeptide of mol. wt 34000 (Fig. 2). The carbohydrate component was reduced to 4% without loss of haemagglutinating activity by four serial precipitations at pH 4.2 (Stirm *et al.*, 1967). This material produced a single anionic line when tested by IE against *E. coli* B41 antiserum. Antiserum to this preparation agglutinated K99⁺ *E. coli* B41 organisms grown at either 18 or 37 °C. When absorbed with K99⁻ organisms grown at 18 °C, this antiserum agglutinated only K99⁻ *E. coli* grown at 37 °C. These findings suggested that the partially purified polypeptide was contaminated with small amounts of O-antigen.

A solution of the anionic adhesin, which contained 30 µg protein ml⁻¹, caused agglutination of sheep and horse erythrocytes to titres of 1/256 and 1/64, respectively. Treatment with trypsin or formaldehyde did not affect the haemagglutination titre but the titre was reduced to less than

20% by prior treatment of the adhesin at 100 °C for 30 min. In contrast, the K99 haemagglutination titre was reduced by all three treatments.

Anionic adhesin inhibited adhesion of the K99⁻ *E. coli* B41 to brush borders by more than 90%, but did not inhibit adhesion of K99⁺ *E. coli* B41 or *E. coli* K12 K99 to brush borders. Radiolabelled anionic adhesin reacted with adhesive brush borders with a binding ratio (defined as count/background count) of three, but did not react with non-adhesive brush borders. In contrast, radiolabelled K99 reacted with adhesive brush borders with a binding ratio of less than two, which was not considered significant. Unlabelled K99 was unable to prevent the adhesion of *E. coli* B41, K99⁻ *E. coli* B41 or *E. coli* K12 K99 to brush borders.

Anionic adhesin eluted from a Sepharose 4B CL column in the void volume, which indicated that the 34000 mol. wt subunits were assembled into structures of mol. wt greater than 20×10^6 . Electron micrographs of negatively stained preparations of anionic adhesin revealed both long parallel-sided processes, approximately 3 nm in diameter (Fig. 3b) and structures with irregular morphology which predominated (Fig. 3a).

Partial purification and characterization of a non-K99 non-anionic adhesin MRE haemagglutinin

The extract of the K99⁻ variant of *E. coli* B41 from which the anionic adhesin had been removed by 30% saturated ammonium sulphate was made 45% saturated with respect to ammonium sulphate and stirred at 4 °C for 2 h. The precipitate was redissolved and dialysed in PBS and, following centrifugation (20000 g, 10 min), contained 95% protein and 5% carbohydrate. SDS-PAGE revealed two closely spaced polypeptides of 49500 and 48000 mol. wt (Fig. 2). A solution of 35 µg protein ml⁻¹ caused agglutination of sheep erythrocytes to a titre of 1/1024, but did not agglutinate horse or guinea pig erythrocytes. Sheep haemagglutinin eluted within 30 min at 37 °C. This haemagglutinin was resistant to prior treatment by heating to 100 °C for 30 min and to the action of formaldehyde and trypsin. SDS-PAGE of trypsin-treated haemagglutinin revealed that the trypsin autodigested after 1 h, whilst the strength of the stained 49500 and 48000 mol. wt polypeptides remained constant as did the sheep haemagglutination titre. Partially purified haemagglutinin was unable to cause haemagglutination of sheep or horse erythrocytes at 37 °C in the absence of D-mannose.

Antiserum to this preparation inhibited haemagglutination at a titre of 1/64, whereas the same antiserum did not inhibit haemagglutination by the anionic adhesin. Antiserum to the anionic adhesin inhibited agglutination of sheep erythrocytes caused by both the 30% and 45% saturated ammonium sulphate fractions at titres of 1/128 and 1/128, respectively. However, normal rabbit serum was equally inhibitory to haemagglutination by the 45% ammonium sulphate fraction. Thus the interaction between antiserum to anionic adhesion and the 45% ammonium sulphate fraction was probably not due to specific antibody. This was supported by the fact that antiserum to the anionic adhesin gave no line of precipitin in immunodiffusion tests with the 45% saturated ammonium sulphate fraction.

Since absorption of OK-antiserum with bacteria grown at 18 °C did not affect the production of an anionic line in IE, the 45% ammonium sulphate fraction is presumably antigenically different from the anionic adhesin.

SDS-PAGE of extracts of K99⁻ *E. coli* B41 grown at 18 °C revealed the 49500 and 48000 mol. wt polypeptides, but not the 34000 mol. wt anionic adhesin. The mixture containing 49500 and 48000 mol. wt polypeptides was unable to prevent adhesion of the K99⁻ *E. coli* B41, *E. coli* B41 and *E. coli* K12 K99 to brush borders. Likewise, neither K99⁻ *E. coli* B41 grown at 18 °C nor radiolabelled 49500 and 48000 mol. wt polypeptides adhered to brush borders.

In preparations of the 45% ammonium sulphate fraction applied to a Sepharose 4B column, both haemagglutinin activity and the 49500 and 48000 mol. wt polypeptides eluted together within the void volume. This indicated that either or both of the 49500 and 48000 mol. wt polypeptides could be aggregated in the haemagglutinin having a mol. wt of greater than 20×10^6 . Electron micrographs of negatively stained haemagglutinin revealed a mass of long parallel-sided processes of approximately 10 nm in diameter (Fig. 3c), which tended to aggregate in a regular fashion.

Fig. 3. Electron micrographs of negatively stained anionic adhesin found in 30% saturated ammonium sulphate precipitates of cell-free extracts from a K99⁻ *E. coli* B41 showing (a) irregular structures and (b) fimbriae-like structures; (c) heat resistant fimbrial haemagglutinin found in 45% saturated ammonium sulphate precipitates, see Methods; (d, e) electron micrographs of shadow cast K99⁻ *E. coli* B41 and *E. coli* K12 grown at 18 °C. This result has been reproduced twice. The bar markers represent 100 nm (a, b, c) and 200 nm (d, e).

Assessment of heat resistant haemagglutinin production by different E. coli

All K99⁺ and K12 *E. coli* produced a mannose- and heat-resistant sheep haemagglutinin when grown at 18 and 37 °C. The 987p (M2225) and K88 (W1) strains did not.

Electron micrographs of *E. coli* B41, K99⁻ B41, K12 K99, K12 and K12 C600 grown at 18 °C showed the presence of fimbriae (Fig. 3), whereas electron micrographs of W1 and M2225 cultured at 18 °C did not reveal fimbriae. SDS-PAGE of surface proteins from B41, K12, K99, K12, S13 and B117 revealed a couplet of polypeptides with molecular weights identical to those in the haemagglutinin preparation.

The heat- and mannose-resistant haemagglutinins of *E. coli* K12 and K99⁻ *E. coli* B41 were antigenically similar, since antiserum to the haemagglutinin preparation caused strong slide agglutination of K99⁻ *E. coli* B41 and *E. coli* K12. Absorption of the antiserum with either *E. coli* K12 or *E. coli* B41 removed the ability to slide agglutinate K99⁻ *E. coli* B41 and the ability to form a precipitin line in immunodiffusion tests with partially purified haemagglutinin. SDS-PAGE analyses by autoradiography of the precipitin line between radiolabelled haemagglutinin and wells containing antiserum to whole live *E. coli* B41 revealed the 49 500 and 48 000 mol. wt polypeptides.

Escherichia coli strains K12 and K99⁻ B41 did not produce a heat- and mannose-resistant sheep haemagglutinin when grown on 5% (v/v) bovine blood agar at 18 °C or 37 °C. The K99⁻ *E. coli* B41, however, did produce a heat-sensitive, mannose-resistant sheep and horse erythrocyte haemagglutinin on this medium when grown at 37 °C. However, electron micrographs revealed that less than 10% of bacteria produced fimbriae similar in appearance to those produced at 18 °C on TGX medium. SDS-PAGE analysis of the 45% saturated ammonium sulphate fraction from the K99⁻ *E. coli* B41 grown on 5% bovine blood agar at 37 °C for 18 h revealed traces of 34 000, 48 000 and 49 500 mol. wt polypeptides. Haemagglutination tests with this fraction showed the presence of both a heat-sensitive horse haemagglutinin and a heat-resistant sheep haemagglutinin. Thus, the fractionation procedure presumably concentrated the heat-resistant haemagglutinin, which was otherwise undetectable.

DISCUSSION

Chanter (1982) purified the anionic adhesin by IE, but this yielded a preparation contaminated by agar and antibody and was suitable only for subunit mol. wt determinations. Other purification procedures consistently resulted in preparations contaminated with K99. The production of a K99⁻ variant of *E. coli* B41 seemed critical to the characterization of the anionic adhesin both *in vitro* and *in vivo*. *E. coli* B41 is a complement-sensitive strain (N. Chanter & P. W. Jones, unpublished results) and it was thought that killing might be enhanced by the presence of specific antibody thus facilitating isolation of K99⁻ variants. This was confirmed when 2–4% of *E. coli* B41 incubated in antiserum to *E. coli* K12 K99 lost the ability to produce K99, but retained the ability to produce the anionic adhesin. *Escherichia coli* K12 K99 produced K99 but not the anionic adhesin. Anionic adhesin could be purified from the variant to give a 98% pure preparation composed of the 34 000 mol. wt polypeptide. This preparation behaved in IE, inhibition of brush border adhesion tests, brush border adhesion tests and haemagglutination tests as previously described by Morris *et al.* (1980). Polymerization of the 34 000 mol. wt subunits, resulted in an aggregate with mol. wt in excess of 20×10^6 . Attempts to visualize fimbriae in preparations of the aggregated anionic adhesin were unsatisfactory. However, occasionally fimbriae-like structures were seen which were finer than those seen in K99 preparations and in preparations containing the heat-resistant sheep haemagglutinin. The anionic adhesin may be a fragile fimbrial structure which readily breaks down into the irregular structures which predominated in negatively stained preparations. It seems, however, that a fimbrial structure is not required for haemagglutination and brush border adhesion.

Awad-Masalmeh *et al.* (1982) isolated a non-fimbrial mannose-resistant haemagglutinin from a K88⁻ K99⁻ 987⁻ enterotoxigenic *E. coli* which assisted intestinal colonization by attachment to intestinal cells. This adhesin was antigenic and was not produced at 18 °C. These properties

are similar to those of the anionic adhesin from *E. coli* B41. The porcine adhesin, however, had a subunit molecular weight described as only slightly greater than that of K99. The pattern of resistance of the anionic adhesin to the action of trypsin and formaldehyde was quite different from that of K99. The sensitivity of K99 to these treatments may indicate a generally labile nature which might explain its apparent inactivity, following purification, in brush border adhesion tests when compared with the anionic adhesin (Morris *et al.*, 1980). The anionic adhesin and the heat stable haemagglutinin were unable, however, to prevent the adhesion of *E. coli* B41 and *E. coli* K12 K99 to brush borders. Under these conditions, adhesion of whole bacteria caused by anionic adhesin or heat-stable haemagglutinin would presumably be blocked, so that the observed adhesion of bacteria must have been caused by bacteria-bound K99. K99 preparations contained fimbriae (unpublished result). The stability of the fimbrial structure of K99, therefore, did not ensure biological activity.

To the author's knowledge, the presence of haemagglutinating fimbriae on *E. coli*, resistant to 100 °C for 30 min and composed of two polypeptide subunits of different size has not been described previously. These structures were unlikely to be flagella since they had little or no amplitude. In addition, flagella of *E. coli* are 13 nm in diameter (De Pamphilis & Adler, 1971) and those of the *Enterobacteriaceae* are generally composed of a single polypeptide subunit of mol. wt 40000 (Iino, 1969). Heat-resistant haemagglutinin was purified from bacteria grown on solid media and electron microscopy of bacteria grown under these conditions showed an absence of flagellate cells. Eshdat *et al.* (1981) indicated that type 1 fimbriae could not be disaggregated by denaturing conditions commonly employed in SDS-PAGE so that the possibility that the long parallel-sided structures seen in preparations of heat-resistant haemagglutinin were type 1 fimbriae could not be excluded. However, partially purified haemagglutinin did not contain any mannose-sensitive haemagglutinating activity at 37 °C. Also the heat-resistant sheep haemagglutinin does not seem to be related to the major outer membrane proteins of *E. coli* (Hofstra & Dankert, 1979; Paakkanen *et al.*, 1979).

The heat resistance of this haemagglutinin is exceptional for a protein. It is possible that its structure is stabilized by cystine disulphide bridges. The exclusion of 2-mercaptoethanol from the sample buffer for SDS-PAGE did not reduce the number of bands, an effect observed with other fimbriae which contain intrasubunit cystine disulphide bridges (Isaacson, 1981; Chanter, 1982). However, inter-polypeptide disulphide bridges are less likely when the distance between cysteine residues in the primary structure of polypeptides is small (Martin, 1964).

The subunit molecular weights of the heat-resistant sheep haemagglutinin are unusually large compared to nearly all of the previously described fimbriae. However, Lugtenberg *et al.* (1976) in a study of the outer membrane proteins of *E. coli* K12 identified a 50000 mol. wt band in SDS-PAGE gels as pilin subunits of fimbriae found on some, but not all, *E. coli* K12 strains.

The heat-resistant sheep haemagglutinin did not seem to be involved in adherence to calf intestinal brush borders. It is possible that this haemagglutinin binds a receptor in the mucosal layers that would have been removed during brush border preparation. Alternatively, this component may possess another as yet unidentified role in virulence.

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