Fimbriate Phase Variation and Purification of Type 1 Fimbriae from Salmonella kiambu A21

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Abstract: Salmonella kiambu, isolated from a diarrheal outpatient, was investigated the optimum fimbriae-producing process, fimbriate phase variation and characterization following purification of the fimbriae. By measuring hemagglutination against guinea pig and fowl erythrocytes and electron microscopic observation, it was demonstrated that strains tested had mannose sensitive (type 1 or common) fimbriae. The most optimum fimbriae-producing conditions are 48 hr static cultivation at 37℃ in modified TCG-broth. A simple procedure for purification was applied. After sheared from the cells by a blender, the fimbriae were concentrated by ammonium sulfate precipitation and purified by using urea treatment and sodium deoxycholate treatment, finally fimbriae were eluted in the void volume of a Sepharose CL-4B column. Purified fimbriae morphologically appeared as rigid, sylindrical or rod-like structures up to 1.5 μm in length and 5−7 nm diameter with an axial hole resembling other type 1 fimbriae of the Family *Enterobacteriaceae*, and failed to agglutinate guinea pig erythrocytes. The molecular weight of the purified fimbrial subunit was calculated as 20,834 dalton and the proportion of hydrophobic amino acid was 32.9% from amino acid analysis. By immunogold labeling, native fimbriae were labeled specifically with anti-fimbrial antiserum. Under cross-reactive analysis, fimbriated heterologous Salmonella serovars are agglutinated by anti-S. kiambu fimbrial antiserum. It is suggested that common fimbrial antigen exist among distinct serovars of Salmonella.

Key words: Salmonella, Bacterial fimbriae, Pili bacterial, Purification of fimbriae, Immuno gold labeling

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INTRODUCTION

Houwink and Iterson (1950) observed the numerous filamentous appendages other than flagella among *Escherichia, Proteus, Pseudomonas* and other bacterial species by electron microscopy. Duguid *et al.* (1955) proposed that these appendages be called "fimbriae" from the Latin word for threads, fibres or fringe, and Brinton (1959) had called them "pili" from the Latin, hair or fur. Subsequently, Duguid *et al.* (1966) found that many fimbriated Salmonella species agglutinated guinea pig or fowl erythrocytes, however, agglutination was inhibited in the presence of D-mannose. Then, they named this appendage mannose sensitive (MS) or type 1 fimbriae and additionally classified 6 types of fimbriae by hemagglutinating properties and one F fimbriae (F pilus). Type 1 fimbriae (or pili) were found to exist among *Escherichia, Klebsiella, Shigella* and *Serratia* species. It is believed that the attachment of pathogenic bacteria to mammalian mucosal surfaces is one of the essential steps in the pathogenic mechanism and a number of bacterial fimbriae have been known to mediate attachment as adhesions in the gastrointestinal or urinary tract. Type 1 fimbriae have been purified and their molecular weight and amino acid compositions have been described in *E. coli* (Salit and Gotschlich, 1977; Evans *et al.*, 1979; Korhonen *et al.*, 1980a), *Serratia marcescens* (Kohno *et al.*, 1984) and *Klebsiella pneumoniae* (Fader *et al.*, 1982). Concerning Salmonella species, the purification and characterization of fimbriae have been described for Salmonella typhimurium LT2 (Korhonen *et al.*, 1980b) and Salmonella enteritidis (Feutrier *et al.*, 1986). There have been very few observations on other serovars which cause diarrhea or gastroenteritis in humans. The present paper describes the fimbriae-producing process and, purification and chemical characterization of the fimbriae using the Salmonella kiambu strain A21, isolated from a diarrheal outpatient in a rural area of Kenya, East Africa, and the cross-reactivity of the fimbrial antigen among different serovars of Salmonella.

MATERIALS AND METHODS

Bacterial strains and media: The etiological and ecological studies of the bacterial diarrhea were carried out in a rural area of the Coast Province of Kenya from October 1981 to July 1982 (Utsunomiya *et al.*, 1982). Serovars of 35 isolates of Salmonella strains were studied and *Salmonella* choleraesuis subsp. *choleraesuis* serovar kiambu (Salmonella kiambu; according to the rule of International Enterobacteriaceae Subcommittee 1983, the discription of Salmonella-serovars were written by Roman letters) was the most predominant serovar (6 strains in 35 isolates; Utsunomiya, 1983). Isolates were inoculated on a semi-solid nutrient agar butt with several subcultures and maintained at room temperature.

Usually fimbriated bacteria agglutinate erythrocytes (Duguid *et al.*, 1966) and fimbriated strains of Salmonella species formed surface pellicles in broth culture in static conditions (Duguid *et al.*, 1966; Old *et al.*, 1968). In the first step, 6 strains of Salmonella kiambu (S. kiambu) were inoculated in liquid media to confirm the pellicle formation and hemagglutination using guinea pig and fowl erythrocytes. From these studies, one S. kiam-

bu strain, A21, was selected for the most part of this study.

TCG medium, developed by Ehara *et al.* (1986) was used with slight modification. The original formula was as follows; 1% bactotrypton, 0.2% yeast extract, 0.1% sodium L-glutamate mono, 0.02% thioproline (L-thiazolidine-4-carboxylic acid), 1 mM EGTA, 3% $NaHCO_3$, 0.5% NaCl, pH 8.4 with 1.5% agar. Modified TCG (mTCG) medium without EGTA and $NaHCO_3$ with the pH altered to 7.2 was used with and without 1.5% agar. Luria-broth (L-broth) pH 7.2 was used at the same time for comparison.

Fimbriate phase variation: Previously Duguid *et al.* (1966) described the optimum fimbriae-producing conditions for Salmonella strains. The test strain was inoculated on mTCG-agar and in mTCG-broth or L-broth, incubated at 37℃ with static or continuous shaking to reaffirm the optimum conditions of Duguid *et al.*

At 24, 48 and 72 hr after incubation, the agar cultures were suspended in physiological saline and the broth cultures were centrifuged at $1,500 \times g$ for 15 min. Each sedimented sample was placed on carbon-coated grids, negatively stained by 1% uranyl acetate, and about 200 cells were observed for their appendages using a JEM 100CX electron microscope at 10,000× magnification.

Purification of fimbriae: The test strain was pre-incubated in mTCG- or L-broth at 37℃ for 24 hr under static conditions. Ten milliliters each of culture was inoculated into 1,000 ml of fresh mTCG-broth in 5,000 ml Erlenmeyer flasks. Five flasks were incubated under optimum conditions. Purification procedures of Korhonen *et al.* (1980a) were used with the some modifications. Cultures were centrifuged at $9,000 \times g$ for 1 hr, unless otherwise stated, throughout purification, the temperature was at 4℃. The sediments were suspended in 5 times (w/v) of 10 mM potassium phosphate buffer pH 7.2 (PPB) containing 1 mM EGTA and 0.5 mM $MgCl_2$. The cell suspensions were blended by an Omnimixer (Sorvall) at position 3 for 5 min with cooling to shear the fimbriae from the cells and were centrifuged at $13,000 \times g$ for 30 min.

The supernatants were separated by decantation, sediments were resuspended with the above buffer, and the procedures were repeated 2 more times. Then crystalline ammonium sulfate was added to the convined supernatant to 50% saturation, and the solution was stirred for up to 1 hr. The salted out aggregates were obtained after centrifugation at $20,000 \times g$ for 30 min and were dissolved in a small amount (10 ml) of 10 mM PPB and dialyzed 2 days against 10 mM PPB with several changes of the buffer. After centrifugation at $25,000 \times g$ for 15 min, the supernatant was obtained. Crystalline urea was added to a final concentration of 6 M and the fimbriae-containing solution was kept at room temperature for 2 hr.

After dialyzing against 10 mM PPB overnight, sodium deoxycholate was added to the solution to the final concentration of 0.5% (w/v), and the solution was dialyzed against 10 mM PPB containing 0.5% sodium deoxycholate for 2 days. Following dialyses against 10 mM PPB for 2 days, the deoxycholate-insoluble outer membrane proteins were removed by centrifugation at $17,000 \times g$ for 15 min and the supernatant was concentrated to about 3 ml by ultrafiltration (PM 10 filter membrane, Amicon). The samples were applied

to a Sepharose CL-4B column (2.6 by 35 cm bed, Pharmacia Fine Chemicals) and were continuously eluted (flow rate 10 ml/hr, 2.5 ml each in collecting tubes) by 10 mM PPB.
Detection of fimbriae fraction: The protein contents of fractions were measured with UV light absorption at 280 nm (UV-visible recording spectrophotometer, UV-240, Graphicord, Shimadzu). The presence of fimbrial structures was determined using negatively stained samples by electron microscopy. The fimbriae-rich fractions were pooled (crude fimbriae) and a portion of sample was kept in a −80℃ deep freezer as an immunogen for rabbits and the remaining was processed to a second gel filtration. The fimbriae-rich fractions were pooled (pure fimbriae) and were acidified to pH values below 2 with 0.1 N HCl and heated at 100℃ for 5 min (McMichael and Ou, 1979). Following neutralization with 5 mM Tris-buffer, 0.5 ml were distributed into vials, then lyophilized for preservation.
SDS-polyacrylamide gel electrophoresis (SDS-PAGE): The sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the system of Laemmli (1970). The lyophilized samples were dissolved in distilled water, heated at 100℃ for 5 min with twice volume (v/v) of sampling buffer. The protein samples were applied to the 12.5% separating gel with a 4% stacking gel (1.5 mm thick) with a blended whole cell preparation (blended whole cell by Omnimixer) and were electrophoresed for 1 hr at 20 mA, followed by 5 hr at 40 mA constant current. For the calibration of the molecular weight, the low molecular weight marker kit (Pharmacia Fine Chemicals) was used. The gels were stained by Coomassie brilliant blue R250 and destained by a methanol-acetic acid solution.
Amino acid composition analysis: Amino acid analysis was carried out on the subunit band of the purified fimbriae extracted from SDS-PAGE. The electrophoretically collected protein was dialyzed against 0.2 M NaCl overnight and subsequently dialyzed against distilled water overnight at 4℃. The concentrated samples by lyophilization were hydrolyzed with 6 N HCl at 110℃ for 24 hr and analysed with a JEOL JLC-200A amino acid analyser at the Department of Biochemistry, Nagasaki University Scool of Medicine.
Preparation of anti-fimbrial antiserum: Antiserum was raised in adult New Zealand white rabbits by a subcutaneous injection of 0.5 ml of stocked antigen (crude fimbriae) mixed with Freund's complete adjuvant and 3 booster injections (1.0 ml of antigen with Freund's incomplete adjuvant) were carried out at 1 week intervals intramuscularly. Animals were bled 1 week after the last injection and the anti-fimbrial antiserum were separated.

To obtain the specific anti-fimbrial antiserum, antisera were absorbed with a homologous nonfimbriated live bacteria by the method of Logan and Trust (1983). Growth from one nutrient agar plate (thin layer and well dried plate; Duguid *et al.*, 1966) was suspended to 9 ml in saline, mixed with 1 ml of antiserum, and incubated at 4℃ overnight. Antibodies and antigens were separated by centrifugation at 1,500×*g* for 1 hr. The absorbed antisera were filtered by a 0.22 μm pore size filter (Millex-GS, Millipore). Absorbed and unabsorbed antiserum were preserved in a −80℃ deep freezer.
Agglutination tests: (1) Hemagglutination test: hemagglutination was examined using live bacteria incubated at 37℃ for 24 hr in broth. Guinea pig and fowl erythrocytes were

washed 3 times in saline and suspended to 3% (v/v) in saline alone and plus 1% D-mannose. One drop (0.05 ml) of culture (ca. 5×10^8 cfu/ml) and 1 drop of a 3% erythrocyte suspension were mixed on a white porcelain tile. Agglutination was read by the naked eye within a few minutes.

(2) Minimum hemagglutinating concentration (MHC): MHC, the smallest number of bacteria per milliliter which caused hemagglutination, was detected by the method of Leunk and Moon (1982). Fimbriated bacteria were incubated 48 hr in broth, and nonfimbiated bacteria were suspended in saline from an agar culture and centrifuged at $1,500\times g$ for 30 min, then the sediment was heavily suspended in saline. Live becteria were diluted by serial 2-fold dilutions with saline in U-bottomed microtiter plates. The same volume (0.025 ml) of a 3% guinea pig erythrocyte suspension was added to each well. Mixtures were incubated 2 hr at room temperature and agglutination was read by the naked eye. Bacterial suspension were assayed on agar plates for the viable cell counts.

(3) Hamagglutination by purified fimbriae: 0.05 ml each of purified fimbriae obtained from the 1st and 2nd gel filtrations was mixed with same volume of 3% guinea pig erythrocytes on a white porcelain tile. Agglutination was read within a few minutes.

(4) Quantitative agglutination of antiserum: 0.5 ml of 2-fold serial dilutions of antiserum were added to test tubes and the same volume of fimbriated live bacterial cultures which had incubated 48 hr in broth were mixed and incubated for 2 hr at 37℃. The titer was read as the highest dilution of the serum giving visible floccular agglutination. Five other strains of S. kiambu were examined for homogeneity and cross-agglutination tests were performed using as the agglutinogens *Salmonella choleraesuis* subsp. *choleraesuis* serovar senftenberg, braenderup, zanzibar, weltevreden, typhimurium (S. senftenberg, S. braenderup, S. zanzibar, S. weltevreden and S. typhimurium) which are the heterologous serovars isolated from Kenya (Utsunomiya, 1983).

Immunogold labeling: Immunogold labeling was performed according to the method of Faulk and Taylor (1971). Ten microliters of a washed suspension of fimbriated cells (incubated 48 hr) was placed on carbon-coated grids and allowed to fix under a light bulb for 5 min. The excess liquid was removed using filter paper and the grids were immediately placed face down onto a drop (15 μl) of 10-fold-diluted absorbed-antiserum for 15 min. The excess liquid was removed and the grids were washed 3 times using distilled water. The grids were then placed on a drop (15 μl) of goat anti-rabbit IgG-gold colloidal particles (diameter 15 nm, E-Y Labs., Inc., USA) for 15 min. After washed 3 times using distilled water, the grids were negatively stained 30 sec with 1% uranyl acetate and observed by electron microscopy.

RESULTS

Pellicle formation and hemagglutination: Six strains of S. kiambu were inoculated into L-broth and incubated at 37℃ under static conditions. After 24 hr incubation, all of the strains formed surface pellicles. Subsequently, hemagglutination was tested using guinea

pig and fowl erythrocyte suspensions with the same bacterial culture. All the strains agglutinated both kinds of erythrocytes but agglutination was inhibited by the presence of 1% D-mannose (Table 1). Additionally, electron microscopy confirmed the presence of fimbriae. From these results, it was determined that all the strains have mannose sensitive or type 1 fimbriae and A21 was randomly selected as the representative strain.

Growth condition and fimbriate phase variation: To determine the optimum fimbriae-producing conditions, strain A21 was cultured in different media under different conditions and the numbers of fimbriated and flagellated cells were counted under electron microscopy. The percentage was calculated from about 200 cells in each stage (Table 2). A cell

Table 1. Pellicle formation and hemagglutination

Strain No.	Pellicle formation in broth*	Agglutination with erythrocytes of	
		guinea pig	fowl
A 12	+	MS**	MS
A 16	+	MS	MS
A 18	+	MS	MS
A 21	+	MS	MS
A 25	+	MS	MS
A 33	+	MS	MS

*: Bacteria were incubated at 37℃, 24 hr in L-broth.

**: Mannose sensitive agglutination.

Table 2. Fimbriate phase variation in mTCG-broth and on mTCG-agar at different conditions incubated at 37℃

Culture conditions	Numbers and percentages of cell have			Total No. of cells examined
	Flagella only	Flagella & Fimbriae	Fimbriae only	
0 hr*	189 (94.5)	8 (4.0)	3 (1.5)	200
24 hr agar	218 (97.7)	5 (2.2)	0	223
broth/shaking	150 (63.2)	47 (19.8)	40 (16.8)	237
broth/static	83 (38.6)	72 (33.4)	60 (27.9)	215
48 hr agar	209 (94.5)	12 (5.4)	0	221
broth/shaking	168 (77.7)	15 (6.9)	33 (15.2)	216
broth/static	3 (1.3)	29 (12.9)	192 (85.7)	224
72 hr agar	182 (83.8)	26 (11.9)	9 (4.1)	217
broth/shaking	172 (83.9)	12 (5.8)	21 (10.2)	205
broth/static	48 (23.5)	41 (20.0)	115 (56.3)	204

*: Suspended in saline from the overnight culture on nutrient agar.

The number of the cells were examined by electron microscope at 10,000× magnification, and the numbers in parentheses are percentage.

suspension in saline from overnight culture on nutrient agar showed 5.5% fimbriated (only fimbriated plus both fimbriated and flagellated) cells. This suspension was inoculated onto agar and in broth media. In mTCG-agar cultures, the percentage of fimbriated cells was 2.2%, 5.4% and 16% at 24 hr, 48 hr, 72 hr respectively. In shaking broth cultures, fimbriated cells increased to 36.6% at 24 hr, after which the percentage decreased.

In contrast, in static mTCG-broth cultures, fimbriated cells increased to 61.3% at 24 hr and 98.6% at 48 hr which was the maximum level, then decreased to 76.3% at 72 hr. In addition, in static L-broth cultures, the maximum level was 88.0% at 48 hr (data not shown).

According to the above results, a 48 hr mTCG-broth static culture represented the optimum condition and was used for the following experiments. Observations on this phase variation by electron microscopy are presented in Figs. 1−3.

Purification of fimbriae: The yield of 20−22 g (wet weight) of cells was obtained from 5 liters of mTCG-broth culture. Fimbriae were purified by ammonium sulfate precipitation, urea treatment, sodium deoxycholate treatment and Sepharose CL-4B gel filtration in the 10 mM PPB. The fimbriae were eluted in the void volume of the column (Fig. 4A). These fimbriae-rich fractions were combined and a portion of this was used for the immunogen (crude fimbrial protein).

Since this combined fraction was contaminated with very small amounts of cellular debris or outer membranes, the remaining was treated again with 6 M urea. After dialyzing against same buffer, gel filtration was repeated to obtain the purified fimbriae. Following the second gel filtration, the fimbriae were eluted again in the void volume of the column (Fig. 4B) and no contaminants were observed by electron microscopy.

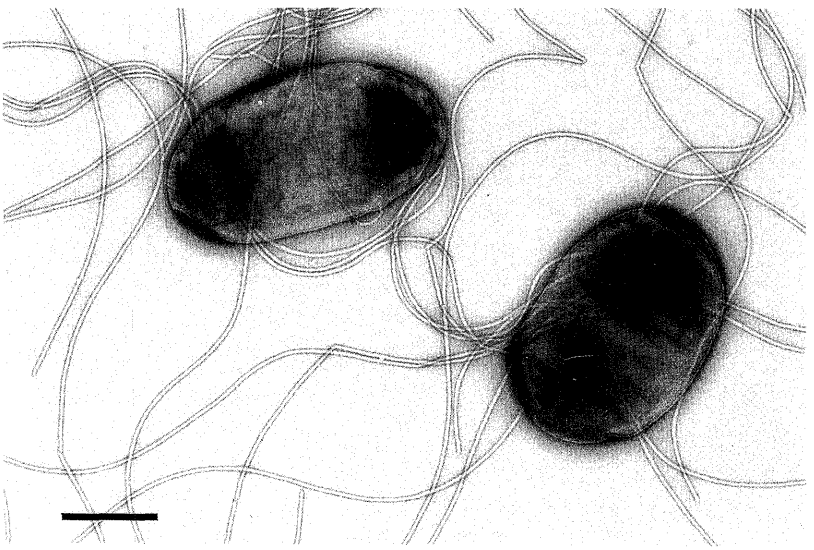

Fig. 1. Electron micrograph of S. kiambu, suspended in saline from the nutrient agar culture for 24 hr. Cells have only flagella. Bar, 500 nm.

Structure of fimbriae: An electron micrograph of negatively stained purified fimbriae (fraction from 2nd gel filtration) is presented in Fig. 5. Fimbriae appeared as rigid, cylindrical or rod-like structures up to 1.5 μm in length and 5−7 nm diameter with an axial hole penetrated by the uranyl acetate.

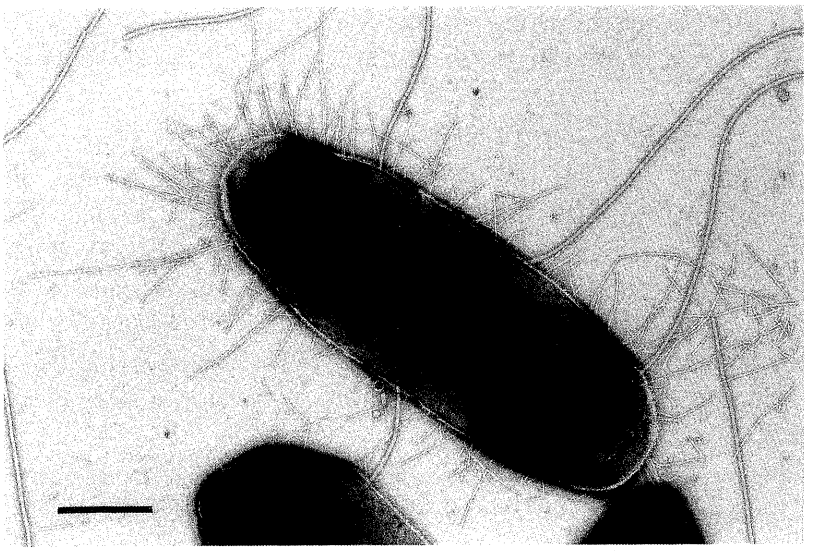

Fig. 2. Cells having both flagella and fimbriae from static mTCG-broth culture for 24 hr. Bar, 500 nm.

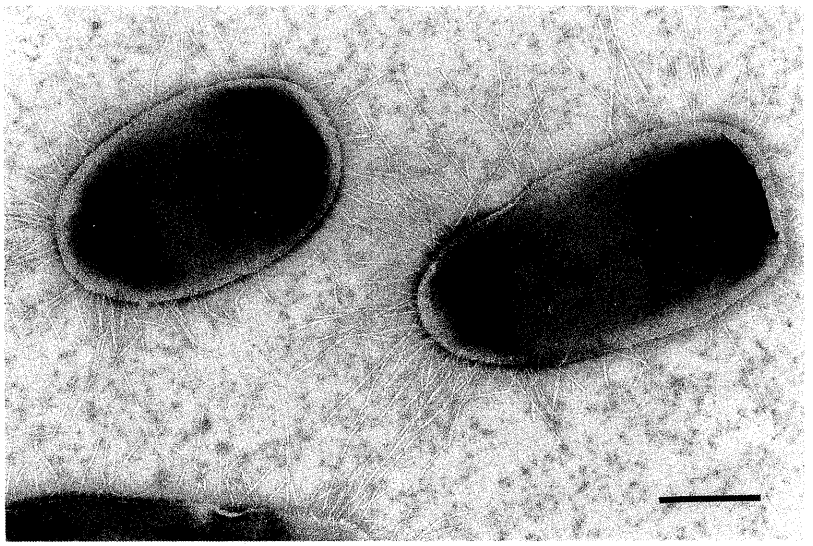

Fig. 3. Cells having only fimbriae from static mTCG-broth culture for 48 hr. Bar, 500 nm.

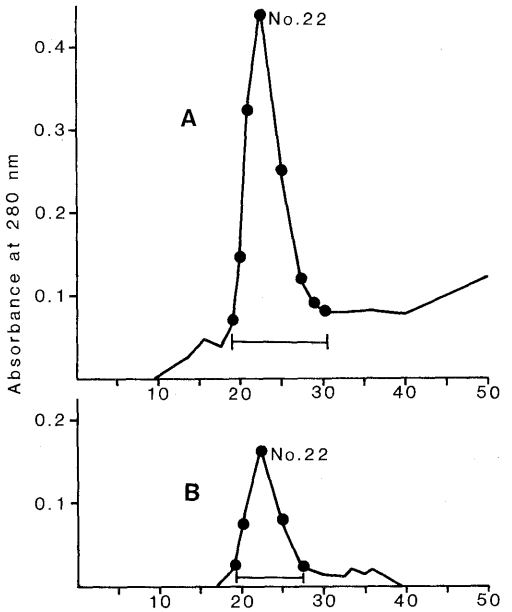

Fig. 4. Gel filtration profiles of A21 fimbrial preparation on Sepharose CL-4B column eluted in 10 mM PPB at 4℃. Fimbriae were eluted in the void volume (A). The bar (fraction number 19 to 30) represents fimbriae-rich fractions. The combined fraction was purified by 2nd gel filtration (details were shown in the text). Following the 2nd gel filtration, the fimbriae were eluted again in the void volume of the column (B). The bar (fractions 20 to 27) represents pure fimbriae fractions.

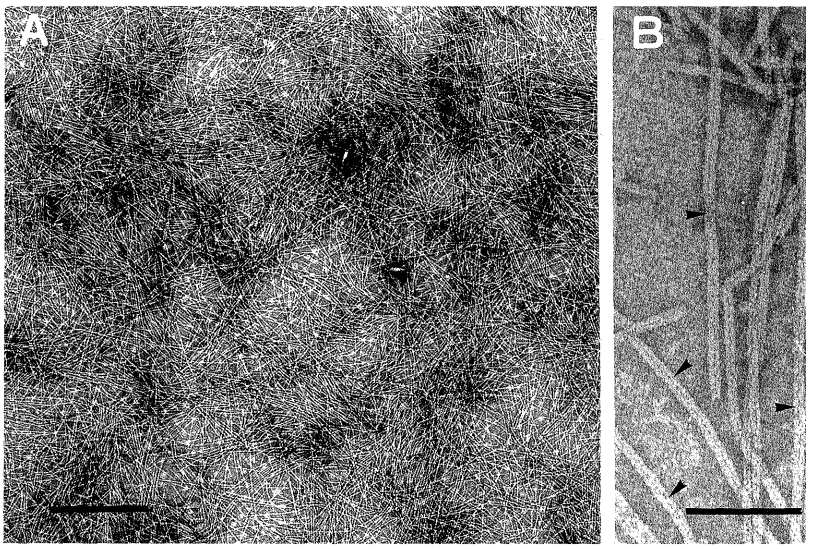

Fig. 5. Electron micrographs of purified fimbriae obtained from the 2nd gel filtration. Low magnification (A), Bar, 50 nm, and high magnification (B), Bar, 100 nm. Arrows show axial holes.

Molecular weight and amino acid composition of purified fimbriae: The subunit of purified fimbriae had an apparent molecular weight of 21,000 dalton by SDS-PAGE (Fig. 6) and the amino acid composition of the purified fimbrial protein is shown in Table 3. The proportion of hydrophobic amino acids (Pro, Ala, Val, Met, Ile, Leu and Phe) was 32.9% and the molecular weight calculated from the amino acid composition was 20,834 dalton.

Quantification of antibodies: Antibody titers in rabbit serum before and after immunization with crude fimbriae were detected using live A21 cells. The anti-A21 fimbrial antibody titer was significantly raised to $\times 32,768$ (2^{15}) from below 2^1 before immunization. After absorption with nonfimbriated live A21 cells, the agglutination titer of absorbed-antiserum which was diluted to 10-fold in the process, was $\times 2,048$ (2^{11}) as presented in Table 4. At the same time, a cross-reaction were tested using 5 heterologous serovars of Salmonella strains. S. senftenberg and S. braenderup showed similar agglutinating titers, and S. zanzibar, S. typhimurium and S. weltevreden showed a slightly lower agglutinating titers (Table 4). The absorbed antiserum was named as specific anti-fimbrial antiserum.

Immunogold labeling: Immunogold labeled specimens were examined by electron microscopy (Fig. 7). Fimbriae (fim) were labeled with anti-fimbrial antiserum, whereas longer appendages such as flagella (fla) were not labeled with anti-fimbrial antiserum.

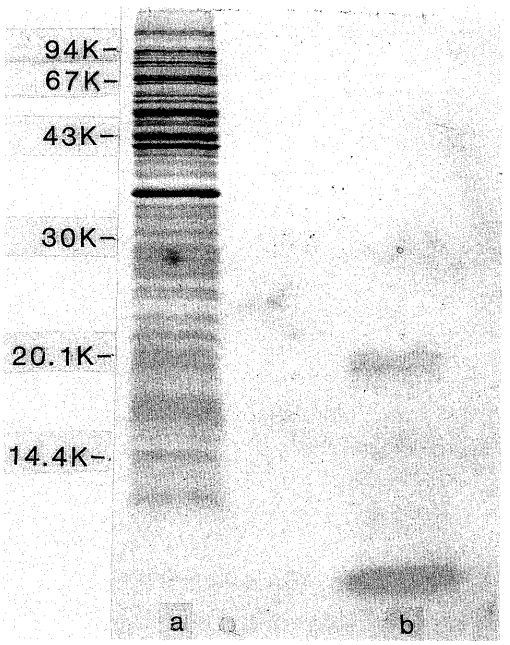

Fig. 6. SDS-PAGE profile in 12.5% separation polyacrylamide gel, blended whole cell preparation (lane a) and purified fimbriae (lane b). The molecular weight markers were phosphyrylase (94K), bovine serum albumin (67K), ovalbumin (43K), carbonic anhydrase (30K), soybean trypsin inhibitor (20.1K) and α-lactoalbumin (14.4K). The gel was stained with Coomassie brilliant blue R250. The migrated subunit indicated an apparent molecular weight of 21,000 dalton.

Table 3. Amino acid composition of type 1 fimbriae purified from S. kiambu and other Salmonella serovars and *E. coli*

Amino acids	No. of residues per molecule			
	S. kiambu	S. typhimurium*	S. enteritidis**	*E. coli* ***
Asp	15	22	13	18
Thr	8	25	17	20
Ser	16	23	11	9
Glu	20	19	14	16
Pro	8	11	8	2
Gly	33	23	22	21
Ala	19	34	21	34
Val	9	16	13	14
Met	1	tr	0	0
Ile	6	7	5	5
Leu	10	12	4	14
Tyr	4	12	4	0
Phe	5	9	7	8
His	5	3	1	2
Lys	13	9	4	4
Arg	4	4	2	2
Total no. of residues	176	221	145	173
Molecular weight	20,834	22,100	14,400	17,099
Hydrophobic residues (%)	32.9	40.5	40.0	44.5

*: Data from Korhonen *et al.* (1980).
**: Data from Feutrier *et al.* (1986).
***: Data from Salit and Gotschlich (1979).

Table 4. Bacterial agglutination with homologous and heterologous strains against absorbed anti-A21 fimbrial antiserum*

Agglutinogen**	Agglutination titer 2^n
S. kiambu	
A 21	11^a ($\times 2,048$)
A 12	11
A 16	11
A 18	10
A 25	10
A 33	10
S. braenderup	11
S. senftenberg	11
S. zanzibar	10
S. typhimurium	9
S. weltevreden	9

*: Immunizing and absorption methods were described in the text.
**: Live bacteria ($1.5-2.0\times10^9$ cfu/ml), incubated at 37℃ for 48 hr.
a: Before absorption, agglutination titer was 2^{15}.
Antigenic structures are as follows: S. kiambu (4,12:z:1,5), S. braenderup (6,7:e,h:e,n,z_{15}), S. senftenberg (1,3,19:g,s,t:−), S. zanzibar (3,10:k:1,5), S. typhimurium (1,4,5,12:i:1,2) and S. weltevreden (3,10:r:z_6).

MHC and agglutination test of purified fimbriae: The results of the agglutination reaction are shown in Table 5. In fimbriate phase cells, the MHC was 4.68×10^8 cells/ml and repeated test showed 3.82×10^8 cells/ml. In contrast, the nonfimbriate phase cells failed to cause hemagglutination at a concentration of 1.0×10^{10} cells/ml. Additionally, crude fimbriae (fraction from 1st gel filtration) and purified fimbriae (fraction from 2nd gel filtration) failed to show hemagglutination.

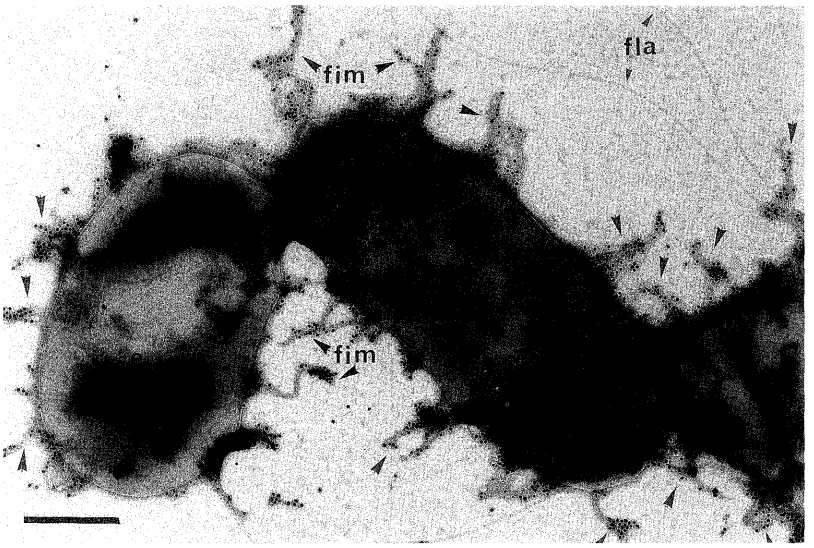

Fig. 7. Electron micrograph of fimbriated S. kiambu A21 labeled by immunogold. Antiserum labeled native fimbriae (fim, large arrows) and not labeled flagella (fla, small arrows). Bar, 500 nm.

Table 5. Minimum hemagglutinating concentration (MHC) and hemagglutination of purified fimbriae for guinea pig erythrocytes

Bacteria/Fimbriae	MHC or agglutination of purified fimbriae
Fimbriate phase cell	1 st exp. 4.68×10^8 2 nd exp. 3.82×10^8
Nonfimbriate phase cell	Negative*
Purified fimbriae	Negative**

*: Bacteria failed to cause hemagglutination at doses of 1.0×10^{10} cells/ml.
**: Neither fimbrial fractions from 1st nor 2nd gel filtration agglutinated erythrocytes.
Fimbriate phase: incubated for 48 hr in broth.
Nonfimbriate phase: suspension in saline from 24 hr incubation on nutrient agar plates.

DISCUSSIONS

Duguid *et al.* (1966) established the optimum fimbriae-producing conditions, such as nutrient broth, 37℃ for 48 hr, aerobic and static culture for Salmonella species. Many conditions have been reported: nutrient broth, 37℃ at 48 hr static culture for *Shigella flexneri* (Gillies and Duguid, 1958); minimal medium containing glucose, 41℃ at 36-48 hr culture with gyratory shaking for *E. coli* (Salit and Gotschlich, 1977); L-broth, 37℃ at 48 hr static culture for *E. coli* (Korhonen *et al.*, 1980a), S. typhimurium (Korhonen, 1980; Korhonen *et al.*, 1980b) and S. enteritidis (Feutrier *et al.*, 1986); tryptosoy broth or brain heart infusion broth, 37℃ at 48 hr static culture for *Klebsiella pneumoniae* (Fader *et al.*, 1982) and CFA-broth, 37℃ overnight culture with continuous shaking for *Serratia marcescens* (Kohno *et al.*, 1984) and so on. In this study, mTCG medium was used instead of the ordinary liquid media. After 48 hr of static cultivation at 37℃ in mTCG-broth, the population of the fimbriated cells reached to the highest 98.6%, comparing with shaking broth cultures and also agar cultures, and with 88% of 48 hr static culture in L-broth (data not shown). The maximum level of 98.6% resembled those of 90% in Salmonellae (Duguid *et al.*, 1966) and 97% of *S. marcescens* (Kohno *et al.*, 1984). The percentage of fimbriated cells was reduced to 76.3% at 72 hr from 98.6% at 48 hr. These results support the optimum conditions of Duguid *et al.* (1966).

From the results in Figs. 1−3 and Table 2, the fimbriate phase variations can be explained as follows. In the starting step, bacterial appendages are almost flagella (Fig. 1, Table 2, 0 hr). After incubation in broth, the cells start to produce fimbriae (the second step; Fig. 2, Table 2, 24 hr). In the third step, bacterial appendages are almost fimbriae (Fig. 3, Table 2, 48 hr). Thereafter, in the forth step, the population of the fimbriated cells were declined to 76.3% (Table 2, 72 hr). The further step, the percentage of the fimbriated cells and the numbers of fimbriae per cell decreased and the length of fimbriae became short at 96 hr comparing with 72 hr cultivation in this study (data not shown).

TCG medium was originally developed for pili-production of *Vibrio cholerae* (Ehara *et al.*, 1986). The present study proposes similar results in the production of fimbriae in Salmonellae although the efficiency of the TCG medium remains to be elucidated.

It is believed that the fimbriate phase variation or conversion occurs by some kind of inherited factor and not environmental factors. The fimbrial synthesis in S. typhimurium has been shown to be chromosomally coded (Sanderson and Hartman, 1978). The genes encoding type 1 fimbriae of *Enterobacteriaceae* are cloned in *E. coli.* (Clegg *et al.*, 1985) and the organization of genes encoding type 1 fimbriae are determined (Clegg *et al.*, 1987). While, Novotny and Fives-Taylor (1974) described that F pili of *E. coli* disappeared in the presence of NaCN and pili had retracted into the cell, new F pili were produced at a normal rate 3 min after NaCN was removed. Lowe *et al.* (1987) analyzed the location of assembly and elongation of *E. coli* type 1 fimbriae. They removed the fimbriae from the cells by blending and found that the cells regenerate their new fimbriae for approximately 25 min. However, mutual relationships between the fimbrial syntheses and organelles

phase variation (flagellate phase and fimbriate phase) *in vitro* are still unknown.

Although the classification of bacterial fimbriae are not yet established, Duguid *et al.* (1966) described fimbrial types according to hemagglutination and the numbers or length of fimbriae. Evans *et al.* (1979) presented the fimbrial types by hemagglutination patterns. Type 1 fimbriae (or common fimbriae) were characterized as mannose sensitive and were found among the Family *Enterobacteriaceae*. Under electron microscopic examination, the morphology of typical type 1 fimbriae were rigid, cylindrical or rod-like up to 1.5 μm in length and 5−7 nm diameter and the fimbriae of this type were found among *E. coli* (Salit and Gotschlich, 1977), S. typhimurium (Korhonen *et al.*, 1980b), *K. pneumoniae* (Fader *et al.*, 1982), S. enteritidis (Feutrier *et al.*, 1986) and so on. With an exception, an uncommon type 1 fimbriae of *Serratia* (Kohno *et al.*, 1984) showed flexible fine filamentous structures in 3 nm diameter. In this study, S. kiambu exhibited mannose sensitive fimbriae (Table 1) and displayed a similar morphology to typical type 1 fimbriae with an axial hole penetrated by uranyl acetate (Fig. 5B) so that these fimbriae are corresponded to type 1.

Purified fimbrial subunit has been "pilin" by Brinton or "fimbriin" by Old (cited in Old and Payne, 1971). The molecular weight of the purified S. kiambu fimbrial subunit is about 21,000 dalton in SDS-PAGE (Fig. 6) and the calculated molecular weight from the amino acid composition analysis is 20,834 dalton (Table 3). In Table 3, the molecular weight and amino acid composition reported for fimbriae from 2 other serovars of Salmonella and *E. coli* are also shown for comparison. The molecular weight of S. kiambu fimbrial subunit is slightly lower than that of S. typhimurium but distinctly higher than that of S. enteritidis and *E. coli*. Regarding the composition of hydrophobic amino acids, similar percentages are reported among 2 Salmonella spp. and *E. coli* with little high value. Whereas the composition of hydrophobic amino acids of S. kiambu is markedly low showing 32.9%. Among the 3 species of Salmonellae, the ratio of threonine and valine (both are hydrophobic amino acids) are low but the numbers of residues of glycine (hydrophilic) and lysine (basophilic) are high in S. kiambu.

Table 6 summarizes molecular weights and hydrophobicities of fimbriae from other bacteria. S. kiambu fimbriae are similar to levels of molecular weight for *K. pneumoniae* type 1, *Bordetella pertussis* type 2 and type 6, and similar to levels of hydrophobicities for *K. pneumoniae* type 3. But it seems very difficult to conclude the relationships between the molecular weight, the hydrophobicity and the type of fimbriae.

Fimbriated bacteria (native fimbriae) agglutinate guinea pig and fowl erythrocytes and the MHC levels using guinea pig erythrocytes are 3.82 and 4.68×10^8 cells/ml. These levels are lower than the 8.6×10^7 in S. typhimurium (Leunk and Moon, 1982) and 2.4×10^6 in *E. coli* (Salit and Gotschlich, 1977). Hemagglutination by purified type 1 fimbriae are confirmed in *E. coli* (Salit and Gotschlich, 1977), S. typhimurium (Korhonen *et al.*, 1980b; Leunk and Moon, 1982) and *S. marcescens* (Kohno *et al.*, 1984). In contrast to that, purified fimbriae failed to agglutinate guinea pig erythrocytes in this study.

Antisera raised in adult rabbits immunized by crude fimbriae show 2^{15} agglutination titer. After absorption by homologous nonfimbriated cells (consisting only of O and H an-

Table 6. The molecular weight and calculated relative hydrophobicity from purified fimbriae in another species of bacteria listed in Table 3

Bacteria and HA-type* or another expression	Molecular weight	Hydrophobicity (%)	References
E. coli CFA/I	23,800	42.9	Evans *et al.* (1979): Infect. Immun., 25, 738−740
E. coli MR**	17,767	43.0	Korhonen *et al.* (1980): Infect. Immun., 27, 569−575
E. coli Type 1*	17,000	40.7	Esdat (1981): J. Bact., 148, 308−314
E. coli Type 1*	18,000	44.8	O'Hanley *et al.* (1983): J. Exp. Med., 158, 1713−1719
E. coli	15,700	43.6	Klemm (1984): Eur. J. Biochem., 143, 395−399
Ser. marcescens Type 1*	22,000	42.0	Kohno *et al.* (1984): Infect. Immun., 46, 295−300
Kleb. pneumoniae Type 1*	21,500	39.7	Fader *et al.* (1982): J. Biol. Chem., 257, 3301−3305
Kleb. pneumoniae Type 3*	24,000	33.6	Korhonen *et al.* (1983): J. Bact., 155, 860−865
Prot. mirabilis Type 2*	17,600	37.5	Wray *et al.* (1986): Infect. Immun., 54, 43−49
Pseu. aeruginosa Type K	17,800	43.0	Paranchych *et al.* (1978): J. Bact., 134, 1179−1180
Haemo. influenzae	25,000	36.4	Guerian *et al.* (1985): J. Exp. Med., 161, 145−151
Neiss. gonorrhoeae	17,500	41.1	Schoolnik *et al.* (1984): J. Exp. Med., 159, 1351−1370
Neiss. gonorrhoeae	16,580	35.4	Olafson *et al.* (1985): Infect. Immun., 48, 336−342
Bact. nodosus	18,400 19,300	no data no data	Every (1979): J. Gen. Microbiol., 115, 309−316
Bact. nodosus	17,000	no data	Mattick *et al.* (1984): J. Bact., 160, 740−747
Bact. gingiralis	43,000	40.5	Yoshimura *et al.* (1984): J. Bact., 949−957
Bord. pertussis Type 2*	21,933	35.8	Zhang *et al.* (1985): Infect. Immun., 48, 422−427
Bord. pertussis Type 2*	22,000	35.4	Cowel *et al.* (1987): Infect. Immun., 55, 916−922
Bord. pertussis Type 6*	21,500	34.9	

*: Type 1,2,3 and 6 were according to the classification of Duguid *et al.* (1966).

**: Mannose resistant agglutination for human erythrocytes.

tigens), the agglutination titer was 2^{11}, therefore a reduced titer by absorption was very low because antiserum was previously diluted to 10-fold by cell suspension. Anti-fimbrial antibody reacted with only fimbriae and recognized neither flagella (H) antigen nor somatic (O) antigens under immunogold methods, hence, it is suggested that fimbrial antigens are distinguished from H and O antigens. Another 5 strains of S. kiambu show almost similar bacterial agglutination titers (Table 4). These results indicate the species specificity. At the same time, agglutinations to another 5 heterologous Salmonella serovars were tested and the results obtained from Table 4 showed similar or slightly lower agglutination titers. It can be said that there is a common antigenicity among fimbriae of Salmonella strains belonging different serovars by the agglutination test. Previously, Old and Payne (1970) showed cross reactions between type 1 and type 2 fimbriae of S. paratyphi B by bacterial agglutination. In contrast, Feutrier *et al.* (1986) obtained no cross-reactions between specific anti-S. enteritidis fimbrial antibody and 5 heterologous Salmonella serovars using immunodot-blot analysis. On the contrary, Adegbola and Old (1987) described that the 4 distinct serovars of Salmonella were strongly coated by the type 1 fimbrial antiserum against S. paratyphi B under immuno electoron microscopy. Hence, further study is needed to observe the cross-reactivity using immunologold methods between anti-S. kiambu fimbrial antibody and heterologous serovars.

With regard to the attachment or adherence to some kind of cell surface, the functional analysis of type 1 fimbriae was performed with mouse livers (Leunk and Moon, 1982), Henle cells (Mintz *et al.*, 1983) and HEp2 cells (Tavendale *et al.*, 1983) using S. typhimurium. Additionally, at least 10 kinds of fimbriae-like structures *i. e.* colonization factor antigen (CFA) are recognized among enterotoxigenic *E. coli* (ETEC) (references not shown) which relate to adherence properties to host cells. Under experimental infection, Duguid *et al.* (1976) demonstrated that fimbiated S. typhimurium was more virurent in oral challenges than nonfimbriated ones. Jones *et al.* (1982) proposed 60-megadalton plasmids influenced adhesive, invasive properties in S. typhimurium.

In the mechanism of Salmonella-mediated diarrhea, Giannella *et al.* (1975) found increased levels of cyclic AMP like cholera toxin in the intestinal epithelium in the rabbit ileal loop test. Later, Molina and Peterson (1980) reported that several serovars of Salmonella released the cholera-like toxin into the medium and this toxin caused elongation of Chinese hamster ovary cells.

However, the relationships between fimbrial synthesis, functions of fimbrial attachment to eukaryotic cells and pathogenesis (including some kinds of toxin) still remains unknown.

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Salmonella kiambu の線毛保有相の変異と線毛の精製，諸性状および抗原性に関する研究

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ケニアにおいて，下痢患者から分離したサルモネラのうち，最も優位な血清型であった S. kiambu 株を用いて線毛保有相の変異と，線毛の精製，精製線毛の諸性状および線毛の抗原性に関する検討を行った．異なる培地と培養条件のもとで経時的に線毛と鞭毛の保有状況を電顕で観察したところ，変法 TCG 液体培地，48時間の静置培養で線毛保有細胞が最高の98.6%となり，鞭毛保有細胞から線毛保有細胞への変異が認められた，この至適条件は Duguid ら (1966) の説を再確認するものであったが，従来の L ブイヨンよりも今回の変法 TCG ブイヨンが優れ，また各時間での線毛保有率と電顕的形態の観察は従来報告のなかったものである．この線毛はモルモット，ニワトリ赤血球に対しマンノース感受性の凝集能を示した．形態学的には中心に酢酸ウラニールで染色される中空 (axial hole) を有する，長さが 1.5 μm，幅 5−7 nm の管状構造で，他の腸内細菌で認められてきた1型線毛，あるいは普通線毛と一致するものであった．変法 TCG ブイヨンで培養した菌を，ホモジナイザーにかけて線毛を菌体から分離した後，硫安塩析，尿素およびデオキシコール酸ナトリウム処理，アミコン濃縮，ゲル濾過により線毛の精製を行った．SDS-PAGE での精製線毛サブユニットの分子量は，21,000 ダルトンとなり，アミノ酸組成分析の結果からは 20,834 ダルトンとなった．疎水性アミノ酸の比率は32.9%であり，これらの数値について既に報告されているサルモネラ，あるいは他の菌についての成績との比較を行った．部分精製した線毛を家兎に免疫したところ，高い線毛凝集力価を示す抗血清が得られた．さらに無線毛菌での吸収を行ったところ，抗体価の低下はわずかで，線毛特異抗血清が得られた．吸収後の抗血清と，gold をラベルした二次抗体を用いた間接標識抗体法を行うと，培養菌の線毛は認識されたが，鞭毛は認識されなかった．これによっても，吸収抗血清の線毛特異性が証明された．この特異的抗血清に対する5株の S. kiambu と，血清型の異なる5株のサルモネラの生菌凝集試験により，すべてに交差反応が見られた．これはサルモネラの抗原構造において，線毛抗原は菌体 (O) 抗原，鞭毛 (H) 抗原とは異なり，各血清型の菌に共通するものであることを示唆するものであるが，標識抗体法を用いた交差反応の立証による共通性については、さらに検討の予定である．

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