Purification and Characterization of Fimbriae from Salmonella adelaide and Salmonella agona

Masahiko EHARA, Mikako ISHIBASHI and Yoshio ICHINOSE

Department of Bacteriology, Institute of Tropical Medicine, Nagasaki University, 12-4 Sakamoto-machi, Nagasaki 852, Japan

Sueli A. FERNANDES

Department of Bacteriology, Institute of Adolfo Lutz, Avenida Dr. Arnaldo 355, Cerqueira Cesar, Sao Paulo, SP, CEP 01246, Brazil

Abstract: Fimbriae from S. adelaide and S. agona were purified and characterized. These fimbriae were 5 to 7 nm in diameter. The molecular weight of fimbrial subunits was 21,000 Da as estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and this molecular weight was similar to that of S. typhimurium fimbriae (Mr. 22,000) (Korhonen *et al.*, 1980). Hydrophobic amino acids of the fimbriae comprised 32% of the total amino acids. Whole cells agglutinated chicken erythrocytes but purified fimbriae did not have haemagglutination activity. Antibodies raised against the native fimbriae recognized only native fimbriae but not fimbrial subunits. Both fimbriae shared common antigenic determinants exposed on both sides of the fiber.

Key words: Fimbriae, Salmonella, Immunoelectron microscopy

INTRODUCTION

Fimbriae are proteinaceous, filamentous bacterial cell surface structures that are composed of identical subunits. Many kinds of fimbriae have been described for *Escherichia coli*, *Salmonella* and the other bacteria. These types of fimbriae have been broadly divided into 7 types (Duguid *et al.*, 1966; Clegg and Gerlach, 1987). The common fimbriae of *E. coli* are very stable protein. These fimbriae, called type 1 fimbriae or type 1 pili, are firmly attached to the bacterium (Mcmichael and Jonathan, 1979). Most Salmonella species are known to have type 1 fimbriae and their synthesis has been shown to be chromosomally coded in *S. typhimurium* (Duguid *et al.*, 1976; Sanderson and Hartman, 1978). The physiological function of type 1 fimbriae of *Salmonella* is to adhere to enterocytes (Lindquist *et al.*, 1987). The subunit of *S. typhimurium* type 1 fimbriae has higher molecular weight and different amino acid composition compared to those of type 1 fimbriae of

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E. coli and no serological cross-reactivity (Korhonen *et al.*, 1980). On the other hand, type 1 fimbrial molecule of *Salmonella enteritidis* is not markedly different from other type 1 fimbrillin (Josiane *et al.*, 1986). Other serotypes of *Salmonella* have also been reported to produce mannose sensitive fimbriae, but there is no detailed biochemical information concerning *Salmonella* fimbriae. Salmonella agona and Salmonella adelaide are commonly associated with diarrhea due to *Salmonella* infection in humans. In this paper, we describe the purification and characterization of the fimbriae of S. adelaide and S. agona.

Materials and Methods

Becterial strains: Two strains of *Salmonella* were chosen from stock strains in our laboratory. A26 strain (S. adelaide) was isolated in Kenya in 1981 and KS132 strain (S. agona) was isolated in Japan in 1985. Both strains were isolated from diarrhea patients.

Purification of fimbriae: Selected strains were subcultured in L-broth to fimbriate phase. The fimbriate cells were used as inocula (seed). Ten ml of the seed was added to 6 Erlenmeyer flasks for 5 liter containing one liter medium and further grown at 37℃ for 48 h. Cells were harvested by centrifugation at 9,000x*g* for 1 h at 4℃. Whole cells were suspended at the concentration of 0.5 g (wet weight)/ml in 0.1 M potassium phosphate buffer, pH 7.0 (PPB) containing 0.5 mM $MgCl_2$ and 1 mM EGTA, then homogenized in a cooled Sorvall Omnimixer at setting 5 for 5 min. The homogenate was centrifuged at 16,000x*g* for 1 h. The resultant supernate was pooled at 4℃ and the pellet was resuspended with fresh PPB and the homogenization was repeated 3 times. Each supernatant was combined (shear fraction). Solid ammonium sulfate was added to the shear fraction to a final concentration of 50% and stirred for 30 min at 4℃. The salted out proteins were pelleted by centrifugation and resuspended in a small volume of 20 mM PPB following dialysis against the same buffer. The dialysate was treated with 6 M urea at room temperature overnight to solubilize flagella and dialyzed against 20 mM PPB at 4℃ overnight. After clarification by centrifugation, deoxycholate was added to the supernatant to a final concentration of 0.5% (w/v) and dialyzed against 20 mM PPB containing 0.5% deoxycholate at 4℃ for 48 h. Hydrophobic membrane vesicles were removed by low speed centrifugation. The supernatant was then dialyzed against 20 mM PPB again for 24 h. After centrifugation, the supernatant was applied to a Sepharose CL−4B column and eluted with 10 mM PPB.

Preparation of antiserum against native fimbriae: Rabbits were injected subcutaneously at 2 sites in the back and intramuscularly at 2 sites in the thigh 4 times at 2 week intervals with 0.5 ml of fimbrial antigen mixed with an equal volume of Freund's complete adjuvant. From the second injection, Freund's incomplete adjuvant was substituted for the complete adjuvant. They were bled 1 week after the last injection. The IgG fraction of the antisera was prepared by DEAE-Sephadex A-50 column chromatography and was used for immunoblotting and immunoelectron microscopy after absorption with non-fimbriate *Salmonella* at 4℃ overnight.

Titration of antiserum (tube method): The cell suspensions used in the agglutination tests consisted of 2×10^8 c. f. u./ml.The fimbriate phase of S. adelaide and S. agona were prepared in L-broth cultured for 48 h and were confirmed as the fimbriate phase by electron microscopy. Non-fimbriate phase cells were obtained by cultivating *Salmonella* on BTB agar plate at 18℃ for 24 h. O and H antibodies were removed from the fimbrial antiserum by absorption with live non-fimbriate phase bacteria. Fimbriate phase cell-suspension and non-fimbriate phase cell suspensions were mixed with an equal volume of serially diluted antisera against the respective fimbriae (0.5 ml of cell suspension and 0.5 ml of the diluted antisera). A control tube contained normal saline in place of the diluted antisera. The mixtures were incubated at 37℃ for 1 h in a water bath. The titers was defined as the reciprocal of the highest dilution of the antisera in which cell agglutination was clearly visible.

SDS-polyacrylamide gel electrophoresis: Fimbrial preparations were acidified to pH 1.8 with 0.1 N HCl and boiled for 3 min following neutralization with Tris-buffer. The solubilized samples of fimbriae were heated at 100℃ for 5 min in sample buffer (0.05 M Tris-HCl, pH 6.8) containing 2.5% (w/v) sodium dodecyl sulfate (SDS), 10%(v/v) glycerol, 5% (v/v) β-mercaptoethanol and a trace of bromophenol blue. Polyacrylamide gel electrophoresis (PAGE) was performed in 1.5mm thick slab gels according to the system of Laemmli (1970). Samples were applied to the polyacrylamide slab gel (12.5%) using a 4% stacking gel and were electrophoresed for 7 h at 20 mA constant current per slab. The protein bands were stained with Coomassie Brilliant Blue R250. For molecular weight calibration, the low molecular weight marker kit (Pharmacia) was used.

Western blotting: Immunoblotting was performed according to the techniques of Towbin, *et al.* (1979) and Burnett (1981). Purified fimbriae were subjected to SDS-PAGE in duplicate on different gels, one of which was stained with Coomassie blue and the other electroblotted onto a nitrocellulose membrane using a Bio-Rad electroblotting apparatus. Horseradish-peroxidase conjugated goat anti-rabbit IgG was from Cappel Laboratories Inc., USA, and 4-chloro-1-naphthol used in the development of the colour reaction was from Bio-Rad Laboratories.

Haemagglutination test: Techniques for the quantification of haemagglutination activity (HA) was adapted from Jones and Freter (1976). Serial two-fold dilution of fimbrial preparations were prepared in U-bottomed microtiter plates (25μl). An equal volume of 2.5% chicken red blood cells with and without 1% D-mannose were added to each well. The plates were tapped to mix the well contents and the erythrocytes were allowed to settle at room temperature for 1 h. The titer was defined as the reciprocal of the highest dilution in which HA was clearly visible.

Amino acid composition: Amino acid analysis was carried out on the purified fimbrial subunit eluted from a preparative SDS-PAGE. The electrophoretically purified fimbrial subunit was first dialyzed against 0.2 M NaCl to remove the glycine in the electrophoresis buffer before dialysis against distilled water. The samples were hydrolyzed with 6 N HCl at 110℃ for 24 h in evacuated sealed tubes. The hydrolysates were analyzed with a JEOL

JLC-200A amino acid analyzer.

Electron microscopy: Labeling of bacteria with immunogold was carried out essentially as described by Faulk and Taylor (1971). Formvar-coated copper grids with air-dried Salmonella cells were reacted with anti-native fimbriae antisera (400-fold diluted with normal saline) for 15 min and washed with 3 serial drops of distilled water and reacted for 15 min with a drop of 15 nm-colloidal gold labeled anti-rabbit IgG goat serum (E. Y. LABS, INC. SANMAEO, CA 94401, USA). The specimen was then stained with 4% uranyl acetate for 30 sec and examined with a JEM 100CX electron microscope operated at 80 kV.

RESULTS

Purification of fimbriae: Fimbriae rich fractions eluted at the void volume (Fig. 1) were combined and used for further analysis under electron microscopy and by SDS-PAGE. The molecular weight of the structural subunit of S. agona fimbriae was 21,000 Da as shown in Fig. 2. The fimbrial subunit of S. adelaide also had the same molecular weight (data not shown). Electron micrographic examination of the fimbriae purified from

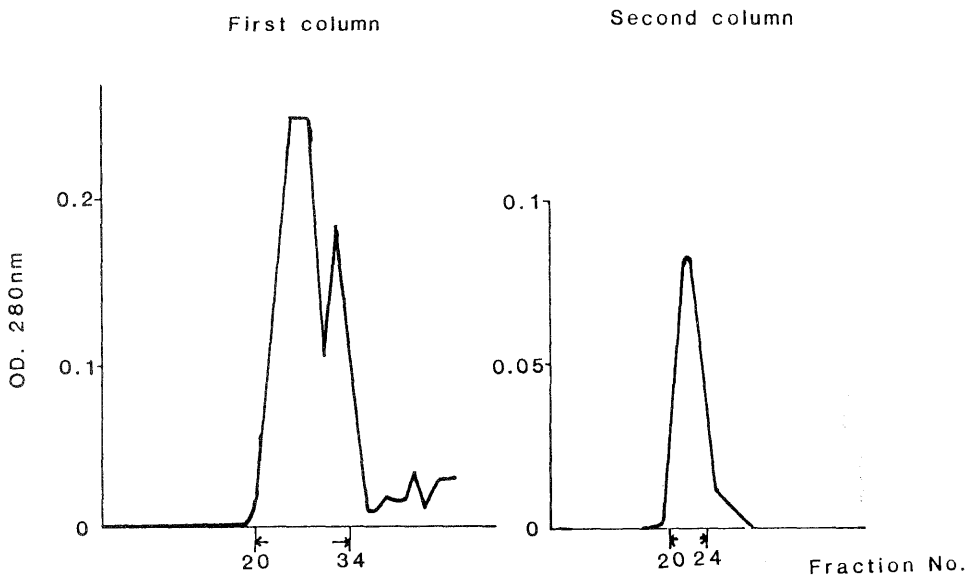


Fig. 1. Sepharose CL-4B gel filtration of Salmonella agona fimbriae (column size; 50 by 2.5cm). Samples (4 ml) were applied and eluted at 8.8 ml/h with 20 mM PPB; 2-ml fractions were collected and checked for purity by SDS-PAGE and EM. Fractions No. 20 to 34 of the first column were combined and treated again with 6 M urea following dialysis in the same buffer. The dialysate was concentrated with an Amicon PM 10 membrane and rechromatographed. Fractions No. 20 to 24 of the second column were used as the sample foor immunization, SDS-PAGE (fig. 2) and EM (fig. 3). The same results were obtained from the sample of Salmonella adelaide fimbriae.

S. adelaide and S. agona showed the presence of an axial hole and chanelled fimbriae of external diameter 5 to 7 nm (Fig. 3) typical of type 1 fimbriae.

Western blot: Antisera raised against native fimbriae did not recognize their structural subunits in a western blot (Data not shown).

Haemagglutination (HA) activity of fimbriae: Whole cells of the fimbriate S. adelaide and S. agona possessed HA activity and these HAs were inhibited with D-mannose. However, as reported by Clegg and Gerald (1987), fimbriae purified from both strains possessed no HA activity. This result also indicates that isolated fimbriae of S. adelaide and S. agona are not involved in the agglutination of chicken erythrocytes (Table 1).

Titration of antisera: Antisera raised against purified fimbriae of S. adelaide and S. agona were absored with non-fimbriate cells. The resultant antisera were highly specific to fimbriae and shared cross-reactivity with each other as shown in Table 2.

Amino acid composition: The amino acid analysis of the fimbriae molecule is shown in Table 3. Based on these compositions, the culculated molecular weight of S. adelaide fimbrillin was 20,705 Da and that of S. agona was 20,265. The fimbrillin of S. adelaide had a relative hydrophobicity of 31.4% and that of S. agona fimbrillin was 32%

Immunoelectron microscopy: Both *Salmonella* strains, grown in Luria broth at 37℃ for 48 h, were processed for immunogold labelling and electron microscopy. The electron micrographs revealed that the antibodies were specific for native fimbriae of both strains independent of serotype (Fig. 4).

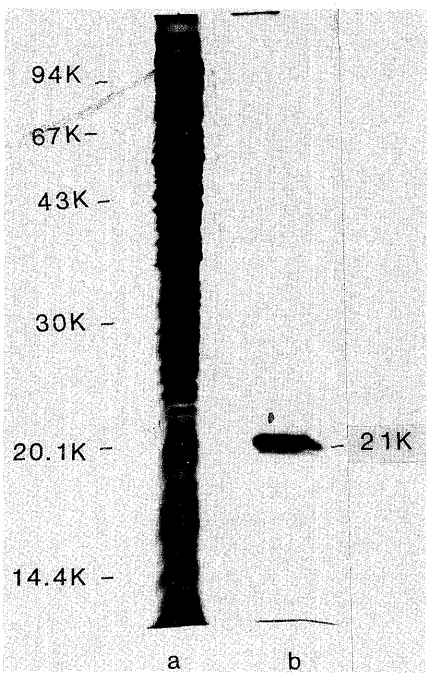
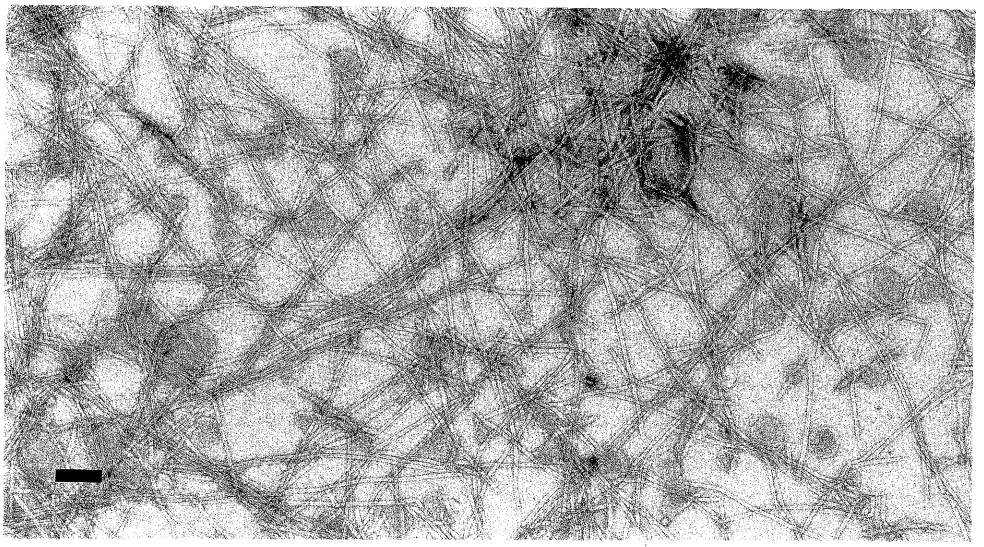


Fig. 2. SDS-PAGE analysis of the fimbrial preparation purified from Salmonella agona.
a) Whole cell b) purified fimbriae



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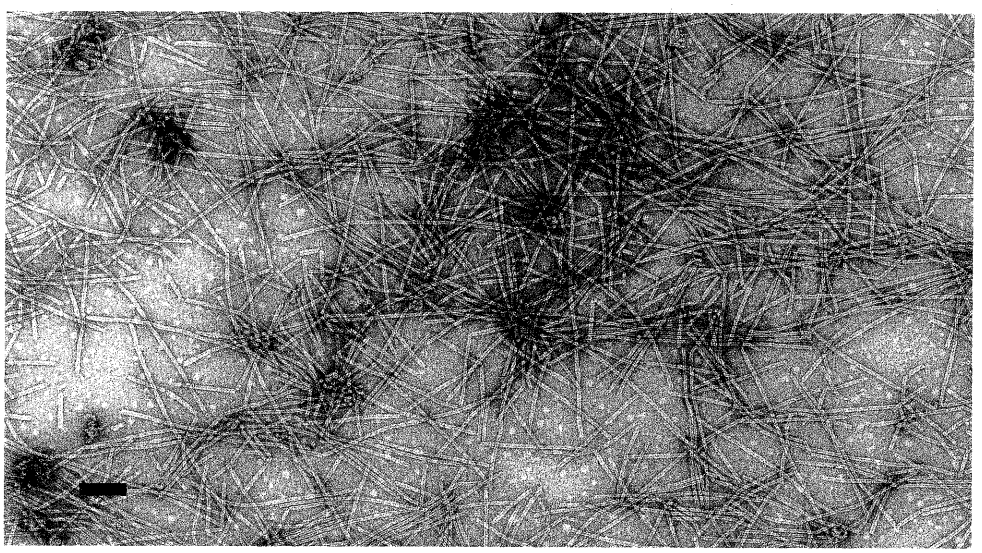

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Fig. 3. Electron micrographs of purified fimbriae.
(a) purified fimbriae from S. adelaide
(b) purified fimbriae from S. agona
Bars indicate 100 nm.

Table 1 HA titer of Salmonella agona and Salmonella adelaide to chicken erythrocytes

	whole cell[a]	shear fraction	purified fimbriae
S. adelaide	128	0	0 (500 μg/ml)
S. agona	64	0	0 (250 μg/ml)

[a] Whole cells of both strains showed D-mannose sensitive haemagglutination.

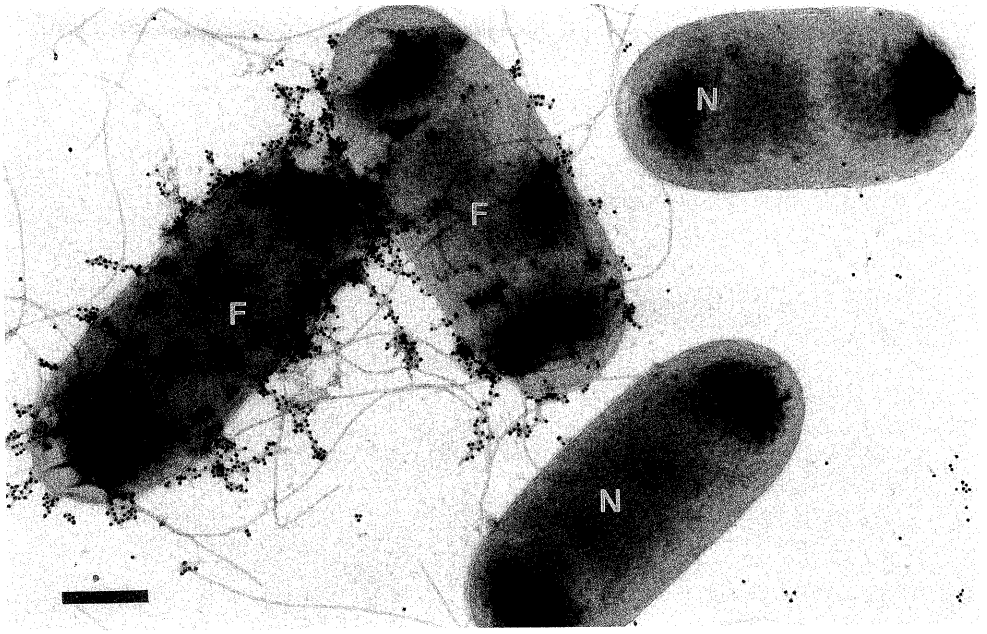
Table 2 Titration of the antisera raised against native fimbriae

Antisera	Antigens			
	S. agona		S. adelaide	
anti-	fimbriate	non-fimbriate	fimbriate	non-fimbriate
S. adelaide fimbriae	1600	0	6400	0
S. agona fimbriae	6400	0	3200	0

Table 3 Amino acid composition of fimbrillin purified from Salmonella agona and Salmonella adelaide

Amino acid	Residues per molecule[a]	
	S. agona	S. adelaide
Aspartic acid	15	15
Threonine	9	8
Serine	18	16
Glutamic acid	20	21
Proline	8	8
Glycine	39	35
Alanine	16	15
Valine	8	8
Methionine	1	1
Isoleucine	7	7
Leucine	11	11
Tyrosine	2	4
Phenylalanine	5	5
Histidine	4	5
Lysine	8	9
Arginine	4	6

[a]Numbers of residues are given as the nearest whole number. Calculations are based on the molecular weight 21 kDa determined by SDS-PAGE.



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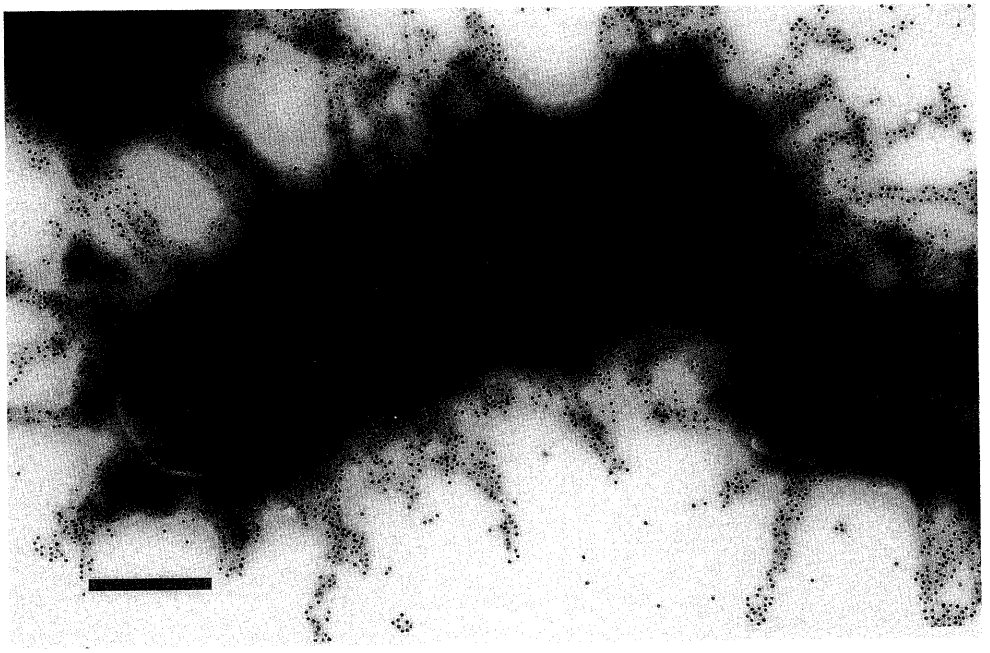

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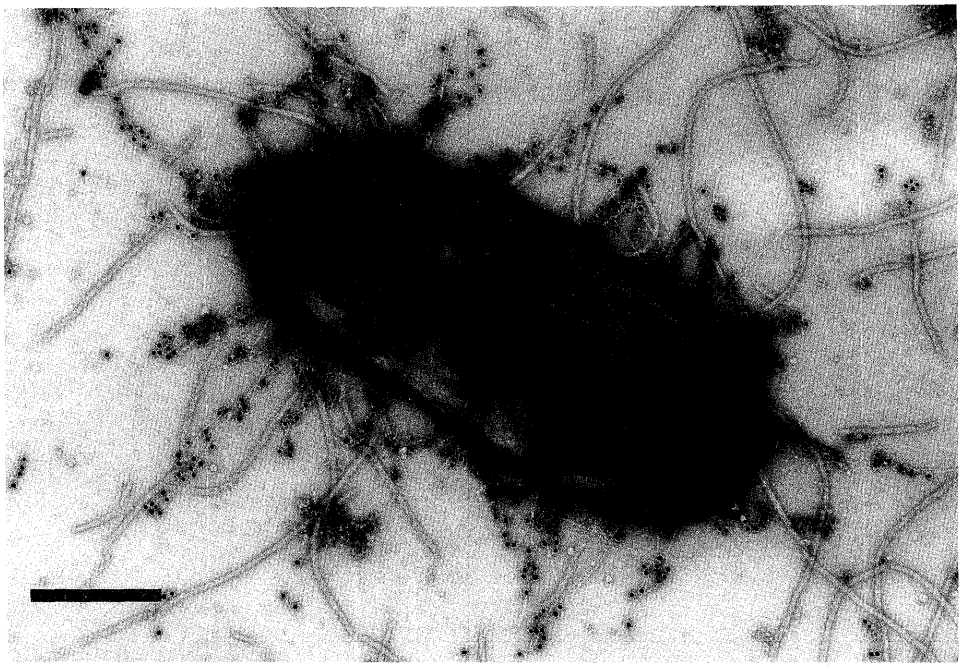

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Fig. 4. Electron micrographs of immunogold-labeled Salmonella.
(a) S. adelaide reacted with anti-S. adelaide fimbriae antiserum. Note that only fimbriate cells (F) contain immunogold. Non-fimbriate cells are indicated as "N".
(b) S. agona reacted with anti-S. agona fimbriae antiserum.
(c) S. agona reacted with anti-S. adelaide fimbriae antiserum.
Bars indicate 500 nm.

DISCUSSION

Fimbriae of S. adelaide and S. agona were purified by ammonium sulfate precipitation and 2 cycles of gel filtration. This convenient procedure provided high yields of fimbriae of high purity. We showed that S. adelaide and S. agona had fimbriae with a diameter of 5 to 7 nm composed of protein subunit with a molecular weight of 21,000 Da as estimated by SDS-PAGE. Fimbriae of *S. typhimurium* LT2 have molecular weight similar to those of S. adelaide and S. agona (Waalen *et al.*, 1983). But the molecular weight of *S. enteritidis* fimbriae (Josiane *et al.* 1986) was lower than that of S. adelaide and S. agona. Fimbrial subunits were not detected by the antisera directed against native fimbriae in immunoblot, however, using the same antisera, native fimbriae were immunodecorated. This fact revealed that antigenic determinants of the fimbriae require tertiary structure and are destroyed when dissociated into monomer. Fimbriae of S. agona reacted with anti-S. adelaide fimbriae antibody and *vice versa*. This cross-reactivity among

type 1 fimbriae of Salmonella strains was also confirmed by immunoelectron microscopy (Adegbola and Old, 1987). These fimbriae had similar morphological properties of *E. coli* type 1 fimbriae under electron microscopy. Both Salmonella strains had HA activity to chicken erythrocytes, however shear fraction and purified fimbriae showed no HA activity. This fact may suggest that fimbriae of both strains are necessary to bind LPS to exhibit HA activity. The amino acid composition of S. adelaide and S. agona fimbrillin were similar to each other and different from other type 1 fimbrial subunits due to the lower content of hydrophobic amino acids (30%). The detailed function of type 1 fimbriae of Salmonella still remains unknown.

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S. adelaide と S. agona の線毛の精製，及びその性状

江原雅彦，石橋美雅子，一瀬休生（長崎大学熱帯医学研究所病原細菌学部門）
Sueli A. FERNANDES（アドルフ　ルッツ研究所）

D-mannose sensitive な HA 活性をもつ S. adelaide と S. agona の2株を教室保存株から選定し，それぞれの線毛を精製した．精製に関しては Korhonen, T. K らの方法に準じて行った．線毛の幅は約 5－7 nm でaxial hole を有し，subunit の分子量は 21,000 Da で，アミノ酸分析の結果，疎水性アミノ酸の含量は約32%であった．赤血球凝集試験では Whole cell (fimbriate phase) では高い凝集能を示したが，精製した線毛は全く凝集能を示さなかった．また，この精製した線毛を用いて家兎を免疫し，得られた抗体は線毛の subunit は認識せず，native fimbriae のみを認識した．このことは線毛の抗原決定基には蛋白質の三次構造が何らかの役割を担っていることを示唆した．

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