Characterisation of a fimbrial, mannose-resistant and eluting haemagglutinin (MREHA) produced by strains of *Salmonella* of serotype Sendai

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Summary. Strains of *Salmonella* of serotype Sendai, producing a mannose-resistant and eluting haemagglutinin (MREHA) when cultured at 37°C but not at 18°C, were examined by electronmicroscopy after negative staining. Production of this MREHA, previously thought to be nonfimbrial, was correlated with the presence of thick fimbriae with an external diameter of 13.6 nm. These fimbriae were readily fragmented and, when purified, had an estimated M_r of 28 Kda. Production of fimbrial MREHA by Sendai strains was associated with the ability to adhere to a wide range of substrates and to form a fimbrial pellicle at the surface of liquid media incubated statically in air. The origin of this unusual Sendai fimbrial MREHA is unknown. Thin filamentous structures produced independently of fimbrial MREHA by Sendai strains were also described. Fimbrial MREHA was not produced by strains of the antigenically similar serotype Miami which, however, and unlike Sendai strains, formed mannose-sensitive haemagglutinin and type-1 fimbriae. The ability to differentiate strains of Miami and Sendai (serotype 1,9,12:a:1,5) by means of their fimbriae is noted.

Introduction

Most strains (80%) of *Salmonella* possess type-1 fimbriae (Duguid *et al.*, 1966; Duguid and Old, 1980; Duguid, 1985) which are polymers of fimbriin, a protein of 21 Kda with a high proportion (40%) of hydrophobic amino acids (Korhonen *et al.*, 1980). Type-1 fimbriae of salmonellae of different serotypes are antigenically diverse, share a major fimbrial antigen and carry a common adhesin which recognises mannosyl-containing residues (Duguid *et al.*, 1966; Duguid and Campbell, 1969; Old, 1972; Duguid, 1985). The adhesive properties of type-1 fimbriate salmonellae can be inhibited by D-mannose, its analogues and a few, highly specific oligosaccharides (Duguid *et al.*, 1966; Old, 1972; Firon *et al.*, 1983). Receptors for type-1 fimbriae are distributed in a wide range of cells including erythrocytes of many animal species, and detection of mannose-sensitive haemagglutinating activity (MSHA) in salmonella cultures affords good presumptive evidence of type-1 fimbriation (Duguid *et al.*, 1966; Old, 1985).

Other kinds of haemagglutinating activity not

inhibited by D-mannose have also been described in salmonellae. Some of these mannose-resistant haemagglutinating activities (MRHAs) are associated with fimbriae, others with non-fimbrial material (Duguid and Old, 1980; Old, 1985). Thus, rare strains of *Salmonella* of subspecies 1–3 possess thin fimbriae (of external diameter ≤ 4 nm) which render cultures inagglutinable in all available O antisera (Rohde *et al.*, 1975; Aleksić *et al.*, 1978). The thin fimbriae of subspecies-1 salmonellae are serologically related to the type-3 fimbriae of strains of *Enterobacter*, *Klebsiella* and *Yersinia* (Old and Adegbola, 1985) and, like them, are associated with a mannose-resistant and klebsiella-like haemagglutinin (MR/K-HA, see Duguid and Old, 1980) which agglutinates tannic acid-treated red-blood cells (Adegbola *et al.*, 1983). The thin fimbriae of salmonellae of subspecies 2 and 3 are also associated with a “tanned red-cell” haemagglutinin (Adegbola *et al.*, 1983; Old, 1985) but they are serologically unrelated to the type-3 fimbriae of other Enterobacteriaceae (Old and Adegbola, 1985).

Non-fimbrial MRHAs are produced by salmonellae of many different serotypes, mostly from subspecies 1 (Jones and Richardson, 1981; Tavenale *et al.*, 1983; Manning *et al.*, 1986; Old and

Tavendale, 1986; Halula and Stocker, 1987). It is generally agreed that many of these non-fimbrial MRHAs are diffusible materials not associated with the bacterial cells (Tavendale *et al.*, 1983; Halula and Stocker, 1987), but there is little agreement about their importance in the attachment of salmonellae to mammalian cells (Jones and Richardson, 1981; Tavendale *et al.*, 1983; Manning *et al.*, 1986; Old and Tavendale, 1986) possibly because different kinds of MRHA are involved.

The MRHA produced by strains of *Salmonella* of serotype Sendai is readily distinguished from other salmonella MRHAs because it has the unusual property of "elution", i.e., adherent bacteria elute from the red cells as the temperature of the agglutinated mixture is increased (Duguid *et al.*, 1966). "Elution" is a property characteristic of the mannose-resistant and eluting (MRE) HAs found in *c.* 60% of strains of *Escherichia coli* (Duguid *et al.*, 1955, 1979) and, more rarely, with HAs of a few other species, e.g., *Haemophilus influenzae* (Scott and Old, 1981) and *Yersinia frederiksenii* (Old *et al.*, 1985). In this paper the characters of the Sendai MREHA, originally thought to be non-fimbrial (Duguid *et al.*, 1966), are described.

Materials and methods

Bacteria

Seven strains of *S. enterica* subsp. *enterica* (see Le Minor and Popoff, 1987) with the antigenic formula 1,9,12:a:1,5 were examined: three strains (NCTC 5772, 71.K and CNS 3666.51 [Hanoi]) were received as serotype Sendai and four strains (187.K, CNS 2.84, CNS 7.84 and CNS 1.85) as serotype Miami. Strain NCTC 5772 was obtained from the National Collection of Type Cultures, Colindale Avenue, London; the other six strains were kindly donated by Professor L. Le Minor, Service des Entérobactéries, Institut Pasteur, Paris, France.

Culture methods

Tryptone Soya Agar (CM 131) was from Oxoid. Otherwise, the media and methods of culture used were essentially as described before (Duguid *et al.*, 1979; Old *et al.*, 1987). Thus, in tests for the production of haemagglutinins, bacteria were grown serially for 48-h periods in static phosphate-buffered broth (pH 7.0, PBB) or for 24 h on phosphate-buffered agar (pH 7.0, PBA) at 18°C or 37°C. For the isolation of fimbriae from strains NCTC 5772 and 3666.51, bacteria were grown for 24 h at 37°C on PBA (500 ml) in glass-bottomed bioassay plates (25 cm², Mast Laboratories). Manipulation of Sendai cultures was performed in appropriate conditions of containment in a category 3 laboratory.

Haemagglutination tests

Bacteria were harvested and tested for their haemagglutination titres as before (Adegbola and Old, 1982). MREHA titres were expressed as the haemagglutinating power (HP) estimated in tests with the most sensitive species of red-blood cell and MSHA titres similarly in tests with guinea-pig cells (Old, 1985).

Improved salt-aggregation test

Different concentrations of ammonium sulphate (0.02–4.0 M) were prepared in 0.02 M sodium-phosphate buffer (pH 6.8) containing methylene blue. Equal volumes of bacterial suspension (*c.* 5×10^9 cells/ml) and solutions of ammonium sulphate at different concentrations were mixed with tooth picks on hydrophobic paper (Rozgonyi *et al.*, 1985). The concentration of ammonium sulphate at which bacterial aggregation occurred was scored immediately after mixing and confirmed next day by examination of the dried-up mixtures.

HEp2 cell-adhesion tests

HEp2 cells obtained from Flow Laboratories (Irvine, Scotland) were maintained according to the procedures detailed by Tavendale *et al.* (1983).

Bacterial cultures, grown in PBB or on PBA at 18°C or 37°C, were diluted in Eagle's Minimal Essential Medium (Gibco Europe Ltd, Paisley, Scotland) supplemented with fetal bovine serum 0.5% v/v, 20 mM glutamine and HEPES buffer (pH 7.3) (Gibco) 2% v/v, (1×10^9 bacteria/ml). Static overlay tests performed in the absence or presence of α -methyl-D-mannoside (α MM) 0.2% w/v were incubated for 1 h; washing and staining procedures followed the methods of Tavendale *et al.* (1983). The numbers of bacteria attached per epithelial cell and the percentages of epithelial cells showing adherent bacteria were estimated from counts of *c.* 200 cells.

Other adhesion tests

Preparation of, and adherence to, other mammalian cell types followed previously published methods: human buccal epithelial cells (Burke and Axon, 1987); mouse duodenal epithelial cells (Sato and Okinaga, 1987); and mouse enterocytes (Lindquist *et al.*, 1987).

Electron microscopy

PBB- or PBA-grown bacteria suspended in water were placed on clean dental-wax sheets (EMscope Laboratories Ltd, Ashford, Kent). A copper grid (3.05 mm diameter), coated with formvar and carbon, was placed on, and allowed to remain in contact with, the culture drop for *c.* 20 min. Bacteria were washed through 2 or 3 drops of glass-distilled water and stained for 1 min with uranyl acetate (pH 4.0) 0.3% w/v, ammonium molybdate (pH 7.0) 2% w/v or phosphotungstic acid (pH 6.8) 0.3% w/v.

After removal of excess stain, grids were dried and each side of the grid was exposed to ultraviolet light for 2 min before examination with a Jeol 100CX transmission electronmicroscope.

Isolation and purification of fimbriae

Bacteria from an overnight PBA culture grown on a bioassay plate (*vide supra*) were harvested in nutrient broth (10 ml) and the resultant dense suspension ($\geq 10^{12}$ bacteria/ml) was heated (60°C for 20 min) to detach fimbriae. Bacteria were deposited by centrifugation (3600g for 20 min) and the fimbriae in the supernate were concentrated either by centrifugation at 150 000g for 2 h or by precipitation with saturated ammonium sulphate 50% w/v overnight at 4°C. After dissolving the deposit in 50 mM Tris-HCl buffer (pH 8.0) or dialysing the precipitate against this buffer overnight at 4°C, the material was applied to an anion-exchange Mono Q column and chromatographed by a fast protein liquid chromatography (FPLC) system (Pharmacia Ltd). Material was eluted from the column with a linear gradient of 0–0.5 M NaCl and 1 M NaCl in 50 mM Tris-HCl buffer (pH 8.0). Column fractions were examined by electronmicroscopy and electrophoresis.

Polyacrylamide-gel electrophoresis (PAGE)

Column fractions and protein markers of known M_r (14.4–97.4 Kda) were analysed by sodium dodecyl sulphate (SDS)-discontinuous PAGE after the method of Laemmli (1970). Samples in disruption buffer (62.5 mM Tris-HCl, pH 6.8, containing SDS 2% w/v, glycerol 10% v/v, 2-mercaptoethanol 5% v/v, and a trace of bromophenol blue dye) were boiled for 2 min and applied to slabs of acrylamide 4% w/v in 125 mM Tris-HCl (pH 6.8) containing SDS 0.1% w/v over a resolving gel of acrylamide 15% w/v in 375 mM Tris-HCl (pH 8.8) containing SDS 0.1% w/v. Electrophoresis was carried out in the cold at 30 mA/gel in 25 mM Tris-HCl (pH 8.3) containing 192 mM glycine and SDS 0.1% w/v until the dye front reached the bottom of the gel. Gels were stained overnight in Coomassie Brilliant Blue R250 0.1% w/v in methanol:glacial acetic acid:water (50:10:40), destained in several changes of the same solvent and further washed in methanol:acetic acid:water (10:10:80). The stained gel was dried under vacuum.

Results

Haemagglutinins

When cultured in PBB or on PBA at 37°C for 24 h, two of the Sendai strains (NCTC 5772 and 71.K) produced a haemagglutinin which in the presence of α MM agglutinated the red blood cells of fowl (F), guinea pig (G), man (M) and ox (O) (i.e., pattern FGMO) but not those of horse, pig

and sheep of the seven red cell species routinely used by us. This Sendai MREHA possessed the unusual property of "elution" whereby there was dispersion of the agglutinated red cell-bacteria mixture as the test was warmed from 4°C to 45°C; hence, it was a haemagglutinin of the mannose-resistant and eluting (MRE) kind. Furthermore, Sendai MREHA was not produced by either of these two strains when grown in PBB or on PBA at 18°C for 2 days. Sendai strain 3666.51 did not form this MREHA in either of the phosphate-buffered media even after eight serial 2-day subcultures at 18°C or 37°C. None of the three Sendai strains produced MSHA on serial subculture in nutrient broth or PBB at 18°C or 37°C.

When the four strains of serotype Miami were cultured similarly, they produced MSHA in serially-grown broth cultures at 18°C and 37°C but none formed MREHA in PBB or on PBA.

Adherence to epithelial cells

Bacteria from cultures of Sendai strain NCTC 5772 grown in PBB or on PBA at 37°C for 24 h formed moderate amounts of MREHA (titres of 835–875) (table I); when examined in the static overlay test in the absence of α MM, many (48–71%) of the HEp2 cells showed adherent bacteria (2–3 bacteria/epithelial cell). The levels of adherence by this strain were similar when tests were made in the presence of α MM (52–77% of the cells showed 3–4.2 bacteria/epithelial cell, table I). Thus, α MM did not inhibit the adherence of strain NCTC 5772, as would be expected for a strain that is type-1 non-fimbriate and does not form MSHA. Tests with the other two Sendai strains were made, therefore, only in the presence of α MM, i.e., only their MR adherence to HEp2 cells was assessed. MREHA⁺ cultures of strain 71.K showed the same overall pattern of adherence to HEp2 cells as had strain NCTC 5772, although its adherence was slightly less (0.7–0.9 bacteria/epithelial cell) than that of strain NCTC 5772 despite the fact that its MREHA titre was greater than that of strain NCTC 5772. Accurate titration of MREHA, however, is difficult (see Old, 1985).

Motility seemed to play no important role in the adherence of bacteria from MREHA⁺ cultures, because poorly motile bacteria from PBA cultures adhered as well as, if not better than, actively motile bacteria from PBB cultures. Neither strain NCTC 5772 nor strain 71.K was adherent after growth on PBA or in PBB at 18°C, i.e., conditions in which they were phenotypically MREHA[−] and their MREHA titres were 0 (table I).

Table I. MREHA and adhesion to HEp2 cells by broth- and agar-grown cultures of salmonellae of serotype Sendai

Strain no.	Phosphate-buffered medium (pH 7.0)	Temperature of incubation (°C)	MREHA* titre of culture	Percentage of HEp2 cells with adherent bacteria (and average number of attached bacteria/HEp2 cell) in static overlay tests	
				- α MM	+ α MM
NCTC	Agar	37	875	71 (2)	77 (3)
5772	Agar	18	0	2 (<0.1)	2 (<0.1)†
	Broth	37	835	48 (3)	52 (4.2)
	Broth	18	0	2 (<0.1)	2 (<0.1)
	Broth	37	4040	NT	43 (0.7)
71.K	Agar	18	0	NT	5 (<0.1)
	Broth	37	3005	NT	52 (0.9)
	Broth	18	0	NT	4 (<0.1)
	Broth	37	0	NT	3 (<0.1)
3666.51	Agar	18	0	NT	0
	Broth	37	0	NT	4 (<0.1)
	Broth	18	0	NT	0
	Broth	37	0	NT	0

* None of the Sendai strains was type-1 fimbriate; thus, the MSHA titres of their cultures were 0.

† <0.1 = 0–9 adherent bacteria/100 epithelial cells.

NT = not tested.

By contrast, bacteria from PBB- and PBA-grown cultures of strain 3666.51, which did not form MREHA at 18°C or 37°C, adhered poorly (table I). Indeed, bacteria from 37°C-grown cultures of this strain were as poorly adherent as the phenotypically MREHA⁻ bacteria of the two Sendai strains grown at 18°C (table I).

Other adhesive properties

When the MREHA⁺ strains NCTC 5772 and 71.K were grown statically in air in tubes of PBB (10 ml) at 37°C, MREHA activity was first detected after 12 h; a pellicle of growth at the broth-air interface first appeared between 18 and 24 h, and was observed in all static PBB cultures incubated for ≥ 24 h. The MREHA⁻ strain 3666.51 grown similarly did not form a pellicle. Measurement of the amount of growth (as estimated by turbidity values at 540 nm) of cultures of the MREHA⁻ and MREHA⁺ strains showed that the amounts of growth of MREHA⁺ strains incubated for 24–72 h were consistently 1.65–2.35 times greater than those of the MREHA⁻ strain grown similarly (data not shown). No such growth advantage for the MREHA⁺ strains was observed in PBB cultures grown at 37°C for <24 h (i.e., before pellicle formation appeared) or when the strains were cultured in PBB at 18°C.

Both MREHA⁺ strains also bound to human buccal epithelial cells (100% of cells, 33 bacteria/

cell), mouse duodenal epithelial cells (95% of cells, five bacteria/cell) and mouse colonocytes (94% of cells, three bacteria/cell) in the presence of α MM. The MREHA⁻ strain, tested similarly, showed negligible adherence to these same mammalian cell types (data not shown). Yet both the MREHA⁺ and MREHA⁻ strains were equally hydrophobic, aggregating in 0.02 M ammonium sulphate. Thus, hydrophobicity alone did not account for adherence to epithelial cells.

Fimbriae

The approximate percentages of fimbriate bacterial cells detected in cultures of the three Sendai strains after growth in phosphate-buffered media at 18°C or 37°C are shown in table II. Three different kinds of fimbrial (or fibrillar) structures were noted. First, thick fimbriae with an external diameter of 13.6 nm were present on a high percentage of bacteria in MREHA⁺ cultures of strains NCTC 5772 and 71.K. When these thick fimbriae were attached to the bacterial cells, they appeared long and straight (fig. 1a), but they fragmented readily even in the course of the gentle in-situ washings with water required for electron-microscopical examination. Thus, most preparations of MREHA⁺ cultures contained large amounts of thick fimbrial fragments detached from the bacterial cells (fig. 1b). The observation that thick fimbriae were more readily observed on

Table II. Fimbriation of salmonellae of serotype Sendai grown in phosphate-buffered medium

Strain no.	MREHA production	Phosphate-buffered medium	Growth temperature (°C)	Percentage of fimbriate bacteria	Kinds of fimbriae* present
NCTC 5772	+	Agar	37	63	Thick and thin
		Agar	18	0	None
		Broth	37	55	Thick and thin
		Broth	18	0	None
71.K	+	Agar	37	65	Thick and thin
		Agar	18	0	None
		Broth	37	61	Thick and thin
		Broth	18	0	None
3666.51	—	Agar	37	17	Thin
		Agar	18	0	None
		Broth	37	16	Thin
		Broth	18	0	None

* Fimbriae and fibrillar material described are shown in figs. 1–4.

bacteria of Sendai cultures grown in static liquid PBB rather than on solid PBA may have been associated with that lability. Furthermore, the finding that thick fimbriae were readily seen in preparations stained with neutral stains such as ammonium molybdate or phosphotungstate, but not after staining with uranyl acetate (pH 4.0), suggests that the thick fimbriae may also be acid-labile.

In MREHA⁺ Sendai cultures, there was a tendency for the cells to aggregate, e.g., as in the formation of fimbrial pellicles (*vide supra*). Examination of these cultures indicated that the clumping of bacteria was mediated by tangled masses of thick fimbriae which, unlike the straight form shown in fig. 1a, had a distinct curly appearance reminiscent of flagellar structures (fig. 2a). However, the flagella

of Sendai strains, like those of other salmonellae (MacNab, 1987), have an external diameter of c. 19 nm, i.e., about 50% thicker than the Sendai thick fimbriae. Furthermore, immuno-electronmicroscopy tests with flagellar antiserum of *Ha* specificity (Wellcome Reagents Ltd, Beckenham) showed that Sendai flagella were stained strongly with flagellar antibody whereas the thick curly fimbriae remained uncoated (fig. 2b). Thick fimbriae, straight or curly, were not detected on bacteria from cultures of the MREHA[−] strain 3666.51 nor from phenotypically MREHA[−] cultures of the other two Sendai strains grown at 18°C.

Two other kinds of fimbrial (or fibrillar) structures were observed, each of which was much thinner than the thick 13.6 nm fimbriae. First, thin fibrillae of c. 3 nm external diameter were present

Fig. 1. Ammonium molybdate-stained preparations of MREHA⁺ Sendai strain NCTC 5772 showing thick fimbriae (diameter 13.6 nm): (a) attached to the bacterial cell; (b) fragmented and detached from the bacterial cell. Bar = 100 nm.

Fig. 2. Ammonium molybdate-stained preparations of MREHA⁺ Sendai strains showing: (a) thick fimbriae of curly appearance on Sendai strain 71.K; (b) antibody-coated flagella and uncoated thick fimbriae of strain NCTC 5772. Bar = 100 nm.

on bacteria of all three Sendai strains grown at 37°C, and were more readily seen on bacteria grown in PBB than on PBA (fig. 3). A second kind of fibrillar material with an external diameter of *c.* 2 nm was found on all three Sendai strains grown at 37°C; it was detected equally well on bacteria from PBB- and PBA-grown cultures (fig. 4). Although we estimated that *c.* 16–17% of the bacteria from 37°C-grown cultures of Sendai strain 3666.51 possessed one or other, or both, of the thin kinds of fibrillae, we experienced considerable difficulty in seeing these structures and it may be that we have underestimated the actual percentages of bacteria bearing fibrillae. We made no attempt to score the numbers of individual bacteria possessing each of the different kinds of fibrillae and fimbriae.

The OD₂₈₀ profile of the material eluted from the Mono Q column, after loading with supernate from a heated and centrifuged suspension of Sendai

strain NCTC 5772, is shown in fig. 5. Three major peaks were present at fractions 54, 78 and 95. Samples of fractions 72–80, eluting at 0.4 M NaCl, were shown by electronmicroscopy to contain thick fimbriae (fig. 6). Furthermore, these thick fimbriae, when detached, gave the same pattern of MRE haemagglutination as the original suspension of MREHA⁺ bacteria, suggesting that MREHA was mediated by thick fimbriae. Strain 71.K behaved similarly. When fractions 72–80 from Sendai strain NCTC 5772 (or strain 71.K) were pooled and analysed by SDS-PAGE, the subunit M_r of the thick fimbriae was estimated as being *c.* 28 Kda (fig. 7). Samples of fractions 54 and 95 from strain NCTC 5772, eluting at 0.25 M and 1 M NaCl, respectively, were found by electronmicroscopy to contain fibrillar material. Fig. 8a shows fibrillae from fraction 95; sometimes, however, as with material from fraction 54, the filaments were stacked together in sheets (fig. 8b).

Fig. 3. Ammonium molybdate-stained preparation showing thin fibrillae (diameter *c.* 3 nm) on Sendai strain NCTC 5772. Bar = 100 nm.

Fig. 4. Phosphotungstic acid-stained preparation showing thin fibrillae (diameter *c.* 2 nm) (arrows) on Sendai strain 71.K. Bar = 100 nm.

Fig. 5. OD₂₈₀ profile of material eluted with salt from the Mono Q column loaded with fimbriae-rich material from MREHA⁺ Sendai strain NCTC 5772.

Fig. 6. Ammonium molybdate-stained preparation of material from column fractions containing thick fimbriae from MREHA⁺ Sendai strain NCTC 5772. Bar = 100 nm.

The major difference in the OD₂₈₀ profile of material from the MREHA⁻ strain 3666.51, treated and fractionated similarly, was the absence of the major peak corresponding to that of the thick fimbriae (data not shown). Because strain 3666.51 did not produce thick fimbriae, we tried to characterise the thin fimbrial type(s) from that strain. We have been unable, however, to obtain by SDS-PAGE analysis bands whereby the M_r of the thin fimbrial type(s) could be assessed.

Discussion

Considerable advances in the techniques of electronmicroscopy prompted us to seek an explanation of the "non-fimbrial" MREHAs of *Salmonella* (Duguid *et al.*, 1966) and we have recently established that Salinatis MREHA, agglutinating the red cells of fowl, guinea pig, mouse and ox

Fig. 7. Coomassie Blue-stained SDS-polyacrylamide gel of thick fimbriae of MREHA⁺ Sendai strain NCTC 5772 (lane B). The M_r values (Kda) of protein markers (lane A) are indicated.

(Duguid *et al.*, 1966), is associated with thin fimbriae which have an external diameter of 3.6 nm and a subunit M_r of 19 Kda (Yakubu *et al.*, 1989). In the present study, by contrast, we have shown that Sendai MREHA, agglutinating the red cells of fowl, guinea-pig, man, monkey, ox and rabbit (Duguid *et al.*, 1966), is associated with thick fimbriae which have an external diameter of 13.6 nm and a subunit M_r of 28 Kda. Furthermore, thick fimbriae in Sendai are extremely fragile, readily fragmented and the associated Sendai MREHA, unlike that of Salinatis (Yakubu *et al.*, 1989), is destroyed by formaldehyde (Duguid *et al.*, 1966). Formaldehyde sensitivity and mechanical fragility were the likely reasons why the thick Sendai fimbriae were not visualised in earlier studies in which formaldehyde-killed cultures were washed twice by centrifugation in distilled water before examination by electronmicroscopy; again, any short stumps of thick fimbriae remaining after these aggressive preparative techniques, would

Fig. 8. Ammonium molybdate-stained preparations of fractionated materials from Sendai strains showing material of *c.* 2-nm diameter (a) and *c.* 3-nm diameter (b), with the latter forming sheets. Bar = 100 nm.

have been difficult to detect by the relatively insensitive shadowing techniques then used (Duguid *et al.*, 1966).

It was interesting that the unusual property of MREHA production by salmonellae should be found in two such infrequently occurring salmonellae as *Salinatis* and *Sendai* and that their MREHAs should be so different. These fimbrial MREHAs can be readily distinguished by several criteria from other salmonella MRHAs including both the widely distributed, diffusible, non-fimbrial MRHAs (Tavendale *et al.*, 1983; Halula and Stocker, 1987) and the less common type-3 fimbrial MRHAs (Adegbola *et al.*, 1983). Although a relationship between *Salinatis* and *Sendai* MREHAs and some of the many MREHAs found in human strains of *E. coli* might seem likely, none of the 10 important classes of fimbrial and "non-fimbrial" MREHAs of *E. coli* described by Duguid *et al.* (1979) had a spectrum of haemagglutinating activity like those of salmonellae.

In their excellent electronmicroscopical study of the colonisation factors present on bovine, enterotoxigenic strains of *E. coli*, Duchet-Suchaux *et al.* (1988) reported that the structure of F41 colonisation factor was complex. Thus, although individual F41 filaments were thin and had a diameter of *c.* 3 nm, they were more generally present as thick, coil-like structures consisting of two or more filaments tightly wound on themselves to form dense spirals; again, large amounts of thick surface structures lay free in the medium. F41, therefore, had an appearance not unlike that of the thick fimbriae of *Sendai* (see figs. 1–4, Duchet-Suchaux *et al.*, 1988) and their subunit M_s (29.5 Kda for F41, de Graaf and Roorda, 1982) were also similar. But F41 coils were much thicker (17–20 nm) than

Sendai fimbriae and their associated MRHAs were different (Morris *et al.*, 1982; Duchet-Suchaux *et al.*, 1988). Furthermore, the finding that all three *Sendai* strains formed thin filaments, whereas thick fimbriae were formed by MREHA⁺ strains only, led us to conclude that *Sendai* thick fimbriae were neither related to F41 nor formed similarly.

The presence of type-1 fimbriae in salmonellae is associated not only with mannose-sensitive adhesive properties but also with the ability to form a type-1 fimbrial pellicle at the surface of liquid media incubated statically in air (Old and Duguid, 1970, 1971); pellicle formation by type-1 fimbriate salmonellae is also mannose-sensitive (Old *et al.*, 1968). It was interesting, therefore, to find that type-1 non-fimbriate *Sendai* strains also formed fimbrial pellicles in liquid media but by a process that was mannose insensitive and restricted to MREHA⁺ strains of *Sendai*. Although pellicle formation by type-1 fimbriate salmonellae and MREHA⁺ fimbriate *Sendai* is associated *in vitro* with the attainment of growth yields greater than those achieved by non-fimbriate or MREHA⁻ cultures, it is not clear what, if any, function this property fulfils *in vivo*. The demonstration in this study and elsewhere (Duguid *et al.*, 1966) that adherence by *Sendai* strains to a wide range of substrates is MREHA-dependent suggests that the thick fimbriae may contribute to the overall adhesiveness of *Sendai* strains; however, the finding that this fimbrial MREHA is not produced by all naturally occurring *Sendai* strains makes it unlikely that it is an essential virulence determinant.

Most of the salmonellae recovered from human infections belong to subspecies 1 (Le Minor, 1988) and to prototrophic, ubiquitous serotypes which inhabit a wide range of hosts; in man, they usually

cause a mild gastroenteritis. A few salmonellae, however, belong to serotypes—such as Paratyphi A and Typhi—which are auxotrophic and host-adapted to man in whom they cause systemic disease. Among salmonellae of group O9, two serotypes of subspecies I have the antigenic formula 1,9,12:a:1,5, namely, Sendai and Miami (Le Minor and Popoff, 1988). Sendai, first isolated from cases of enteric fever in Japan (Aoki and Sakai, 1925), is another, albeit less-well known, example of a serotype adapted to man and its biochemical properties are remarkably similar to those of Paratyphi A (1,2,12:a:[1,5]) (Ewing, 1985); there are, however, few reports of its isolation in Japan, or elsewhere, in recent years (Professor L. Le Minor, personal communication). Miami, by contrast, is a prototrophic, ubiquitous serotype causing a mild gastroenteritis in man; it too is infrequently recovered, e.g., only 13 isolations of Miami among 67 767 strains of salmonellae in France in 1980–83 (Le Minor *et al.*, 1985). Because strains of Sendai and Miami can be differentiated by biochemical properties only, the question has been raised as to whether both serotype names should be retained in the Kauffmann-White scheme (Le Minor and Popoff, 1988). However, this study has established that Miami strains, like most other salmonellae, produce type-1 fimbriae but never MREHA-associated fimbriae whereas Sendai strains, even if they produce MREHA, are never type-1 fimbriate.

Thus, these two serotypes can be readily differentiated, albeit by fimbrial antigens which are not customarily considered when designating the antigenic formulae of *Salmonella* serotypes. Accordingly, the proposal to remove serotype Miami from the Kauffmann-White scheme because it is a biotype variant of Sendai would seem premature.

In the course of identifying the MREHA of Sendai strains as fimbrial, additional problems have been raised by this study. Thus, all three strains of Sendai produced thin filamentous structures—perhaps even two kinds—which, though not associated with adherence to epithelial cells, may have contributed to the high hydrophobicity of these strains. Despite many attempts, we were unsuccessful in detecting bands on gels of the column-purified fibrillar structures seen by electronmicroscopy. We can only assume that, because of the highly hydrophobic nature of the molecule, significant amounts were lost during the procedures of purification, dialysis and PAGE analysis. Although we know that other salmonellae host-adapted to man or other animals do not form MREHA (Duguid *et al.*, 1966), we have no information about how widely distributed these filamentous structures are in host-adapted or ubiquitous serotypes of *Salmonella*.

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