

Fimbrial Haemagglutinins in *Enterobacter* Species

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Fifty-two strains from seven species of *Enterobacter*, grown under a variety of conditions, were examined in rocked-tile tests for production of haemagglutinins and with the electron microscope for fimbriae. Thirteen non-haemagglutinating strains were non-fimbriate. Most (33) of the 39 haemagglutinating strains produced only one kind of haemagglutinin, either the mannose-sensitive haemagglutinin associated with type-1 fimbriae or, the mannose-resistant, klebsiella-like haemagglutinin associated with type-3 fimbriae. Multiply haemagglutinating strains were most common in *E. aerogenes*, in which species a third kind of haemagglutinin, also mannose-resistant, was found. The findings are discussed briefly in the light of the current taxonomy of *Enterobacter*.

INTRODUCTION

In contrast with well studied enterobacteria such as *Escherichia coli* and salmonellae, remarkably little is known of the fimbriae-mediated adhesive properties of bacteria from most other genera of Enterobacteriaceae (see Duguid & Old, 1980). Our recent studies have attempted to correct that deficiency (Adegbola & Old, 1982; Old & Adegbola, 1982).

Strains of *Klebsiella* and *Serratia* of the tribe Klebsielleae may produce one or more of up to three haemagglutinins (HAs), or may be HA⁻. The HAs detected were: (a) the mannose-sensitive HA (MS-HA), associated with type-1 fimbriae (Duguid, 1959, 1968; Adegbola & Old, 1982); (b) the mannose-resistant, 'tanned ox' HA (MR/K-HA), first described in *Klebsiella* and associated with type-3 fimbriae (Duguid, 1959, 1968; Duguid & Old, 1980; Adegbola & Old, 1982); (c) the mannose-resistant HAs active on untanned erythrocytes from different animal species, MR/P-HA, first described in *Proteus* (Duguid & Old, 1980). As judged by the distinct HA patterns given with erythrocytes of different animal species, there are diverse MR/P-HAs in different species and genera of Enterobacteriaceae; they have been grouped for convenience in the MR/P class (see Duguid & Old, 1980; Adegbola & Old, 1982). The MR/P-HAs in *Serratia* are fimbrial (Adegbola & Old, 1982); those in *Klebsiella* are non-fimbrial (D. C. Old & R. A. Adegbola, unpublished data).

Previous HA studies of *Enterobacter* have been confined to strains of *E. cloacae*, a species that produces only MS-HA and type-1 fimbriae (Constable, 1956; Duguid, 1959). Since then, however, the taxonomy of the genus *Enterobacter* has been modified considerably and at least seven species are now recognized (Brenner *et al.*, 1980; Farmer *et al.*, 1980; Izard *et al.*, 1980, 1981). In the present study, therefore, members of the genus *Enterobacter*, particularly representatives of more recently described species, were examined in order to extend our understanding of the fimbrial HAs in the tribe Klebsielleae.

Abbreviations: HA, haemagglutinin; HP, haemagglutinating power; MR/E-HA, mannose-resistant and eluting haemagglutinin; MR/K-HA, mannose-resistant 'tanned ox' haemagglutinin; MR/P-HA, mannose-resistant haemagglutinin active on untanned erythrocytes; MS-HA, mannose-sensitive haemagglutinin.

Table 1. *Enterobacter* strains examined

Species	No. of strains	Strain no. and source*
<i>E. aerogenes</i>	7	BR6; NCIB strains 10197, 10198 and 11460; NCTC strains 9735, 10006 and 10336
<i>E. agglomerans</i>	1	BR 8
<i>E. amnigenus</i>	8	CDC strains 1324-79, 238-80 and 2419-80; CUETM strains 78-77, 78-79, 78-88, 78-89 and 78-83
<i>E. cloacae</i>	16	BR6; JPD strains 035, 259, 438, 439, 793, 919, 923, 989 and 5936; NCTC strains 8137, 9711, 9529, 9785, 9842 and 10005
<i>E. gergoviae</i>	8	BR9; CDC strains 558-79, and 840-82; CUETM strains 79-101, 80-145, 80-146, 81-6 and 81-7
<i>E. intermedium</i>	8	CDC strains 1163-81, 1167-81 and 9011-82; CUETM strains 77-123, 77-136, 77-139, 77-140 and 77-147
<i>E. sakazakii</i>	4	JPD 889; NCTC strains 5920, 8155 and 9238

* CDC, Centres for Disease Control, Atlanta, Georgia, U.S.A.; CUETM, Collection Unité d'Ecotoxicologie Microbienne, Villeneuve-d'Ascq, France; NCIB, National Collection of Industrial Bacteria, Aberdeen, U.K.; NCTC, National Collection of Type Cultures, Central Public Health Laboratory, London, U.K.; BR, Mr Brian Reid, API Laboratory Products Ltd, Basingstoke, U.K.; JPD, Professor J. P. Duguid, Bacteriology Department, University Medical School, Dundee, U.K.

METHODS

Bacterial strains. The *Enterobacter* strains examined and their sources are shown in Table 1.

Media and culture methods. In general, the media and methods were as described previously (Adegbola & Old, 1982). Nutrient broth no. 2 and nutrient agar CM3 were Oxoid preparations. Phosphate-buffered agar (PBA) was nutrient agar with 0.36% KH_2PO_4 and 0.64% Na_2HPO_4 (pH 7.0).

Bacteria were grown statically in air in tubes of nutrient broth (10 ml) incubated as follows: (a) at 37 °C, subcultured six times at 3 d intervals; (b) at 30 °C, subcultured six times at 4 d intervals; (c) at 18–20 °C, subcultured four times at 1 week intervals. They were also grown on PBA at 37 °C for 24 h and at 18–20 °C for 48 h. Bacteria for HA testing were harvested as before (Adegbola & Old, 1982).

Erythrocytes and haemagglutination tests. The preparation of 3% (v/v) suspensions of erythrocytes of species of fowl (F), guinea-pig (G), horse (H), man, group O only (M), ox (O), pig (P) and sheep (S), and the tannic-acid treatment of ox erythrocytes were as before (Duguid, 1959; Adegbola & Old, 1982).

Haemagglutination tests by the rocked-tile method (Duguid *et al.*, 1955) were made with each of the seven species of untanned erythrocytes and tanned ox erythrocytes, at 4 °C and ambient temperature, and in the absence and presence of α -methyl-D-mannoside (Adegbola & Old, 1982). Suspensions of broth-grown bacteria (about 5×10^9 ml⁻¹) were tested for MS-HA, MR/K-HA and MR/P-HA activities and suspensions of agar-grown bacteria (about 10^{12} ml⁻¹) were tested for MR/E (mannose-resistant and eluting)-HA activity (see Duguid *et al.*, 1979). The criteria whereby the different HAs were characterized and the separate HA activities identified, when several were produced simultaneously by multiply haemagglutinating strains, have been fully described elsewhere (Duguid & Old, 1980; Adegbola & Old, 1982; Old & Adegbola, 1982).

Preparation of antisera. Fimbrial antisera were prepared in rabbits by the method of Adegbola & Old (1982) against type-1 fimbriate (MS-HA⁺) bacteria of *E. cloacae* strain 035 and '*Klebsiella aerogenes*' (i.e. the *Aerogenes* type of *K. pneumoniae sensu lato*) strain 55 (of capsular type K55); and against type-3 fimbriate (MR/K-HA⁺) bacteria of '*K. aerogenes*' strain 70/1 (of capsular type K70), the latter two strains from the collection of J. P. Duguid. Each fimbrial antiserum was made monospecific by a series of appropriate absorptions removing non-fimbrial antibodies (Adegbola & Old, 1982).

Electron microscopy. Portions of bacterial culture, sampled prior to testing for HA activities, were negatively stained for 1 min with 0.3% uranyl acetate (pH 4.6) as described by Adegbola & Old (1982). Grids were examined with a Jeol 100CX microscope and micrographs taken of different kinds of fimbriae observed on bacteria in different cultures.

In some examinations, bacteria were negatively stained, as above, after their interaction with type-1 or type-3 fimbrial antisera, following the procedure of Adegbola & Old (1982).

RESULTS

Haemagglutinins and fimbriae

The 19 haemagglutinating strains of *E. amnigenus*, *E. cloacae* and *E. sakazakii* produced MS-HA only (Table 2); its production was most marked with serial broth culture, a condition optimal for the selection of the MS-HA⁺ phase of other enterobacteria (Old & Duguid, 1970)

Table 2. *Haemagglutinins produced by Enterobacter species*

Species	No. of strains	No. of strains forming HAs* in the following combinations:				
		MS-HA only	MR/K-HA only	MS-HA and MR/K-HA	MS-HA, MR/K-HA and MR/P-HA	None
<i>E. amnigenus</i>	8	5	0	0	0	3
<i>E. cloacae</i>	16	12	0	0	0	4
<i>E. sakazakii</i>	4	2	0	0	0	2
<i>E. gergoviae</i>	8	0	7	0	0	1
<i>E. intermedium</i>	8	4	1	1	0	2
<i>E. aerogenes</i>	7	1	1	2	3	0
<i>E. agglomerans</i>	1	0	0	0	0	1

* For description of HAs, see Duguid & Old (1980).

and occurred at all three growth temperatures tested. Bacteria from MS-HA⁺ cultures bore several hundred thick, channelled fimbriae of external diameter 7–8 nm (Fig. 1a). The greater the haemagglutinating power (HP) (see Duguid, 1959) of the MS-HA⁺ culture, the greater the percentage of fimbriate bacteria. Few, if any, fimbriate bacteria were present in phenotypically MS-HA⁻ cultures of MS⁺ strains (e.g. those grown on agar or in early broth cultures of some series).

The seven haemagglutinating strains of *E. gergoviae* also produced only one kind of HA (Table 2), the MR/K-HA reacting with tanned, but not untanned, ox erythrocytes. The production of MR/K-HA was best in broth cultures and, like the MR/K-HA of *Klebsiella* and *Serratia*, was strongest in 30 °C-grown cultures though appreciable amounts were also detected in broth cultures after growth at 20 ° or 37 °C. The greater the HP of MR/K-HA⁺ cultures of MR/K⁺ strains, the greater the percentage of bacteria with thin, non-channelled fimbriae of external diameter 4–5 nm (Fig. 1b).

In the *E. intermedium* strains that produced MS-HA only (four strains) or MR/K-HA only (one strain), similar associations were noted between the presence of MS-HA and thick fimbriae, and that of MR/K-HA and thin fimbriae. Only one strain of the five species thus far discussed produced both MS-HA and MR/K-HA, and that, a strain of *E. intermedium*, formed both the thick and thin types of fimbriae.

All seven *E. aerogenes* strains were HA⁺ (Table 2); one each produced MS-HA or MR/K-HA only and their associated thick or thin fimbriae. The other five strains produced both MS-HA and MR/K-HA; three produced in addition a second MR-HA of the MR/P class (Duguid & Old, 1980) that agglutinated untanned guinea-pig and horse erythrocytes only of the seven species of erythrocytes tested. All five strains formed both thick and thin kinds of fimbriae. Whether fimbrial or non-fimbrial material is responsible for the observed MR/P-HA activity is not yet known.

The 13 HA⁻ strains of *E. amnigenus*, *E. agglomerans*, *E. cloacae*, *E. gergoviae*, *E. intermedium* and *E. sakazakii* (Table 2) were non-fimbriate.

Antigenic diversity

Immune electron microscopy studies showed that thick fimbriae of MS-HA⁺ bacteria of *E. amnigenus*, *E. cloacae* and *E. sakazakii* strains were coated by type-1 fimbrial antiserum of *E. cloacae* strain 035 but not by that of '*K. aerogenes*' strain 55. Those of *E. aerogenes* were coated by type-1 fimbrial antiserum of '*K. aerogenes*' (Fig. 1c) but not by that of *E. cloacae*; and, those of *E. intermedium* were not coated by either type-1 fimbrial antiserum (Table 3). Similar examinations showed that the thin fimbriae of MR/K-HA⁺ strains of *E. gergoviae* and most *E. aerogenes*, but not those of *E. intermedium*, were coated with type-3 fimbrial antiserum of '*K. aerogenes*' strain 70 (Fig. 1d).

The thin fimbriae of MR/K-HA⁺ were not coated with type-1 fimbrial antisera and the thick fimbriae of MS-HA⁺ bacteria were not coated by type-3 fimbrial antisera.

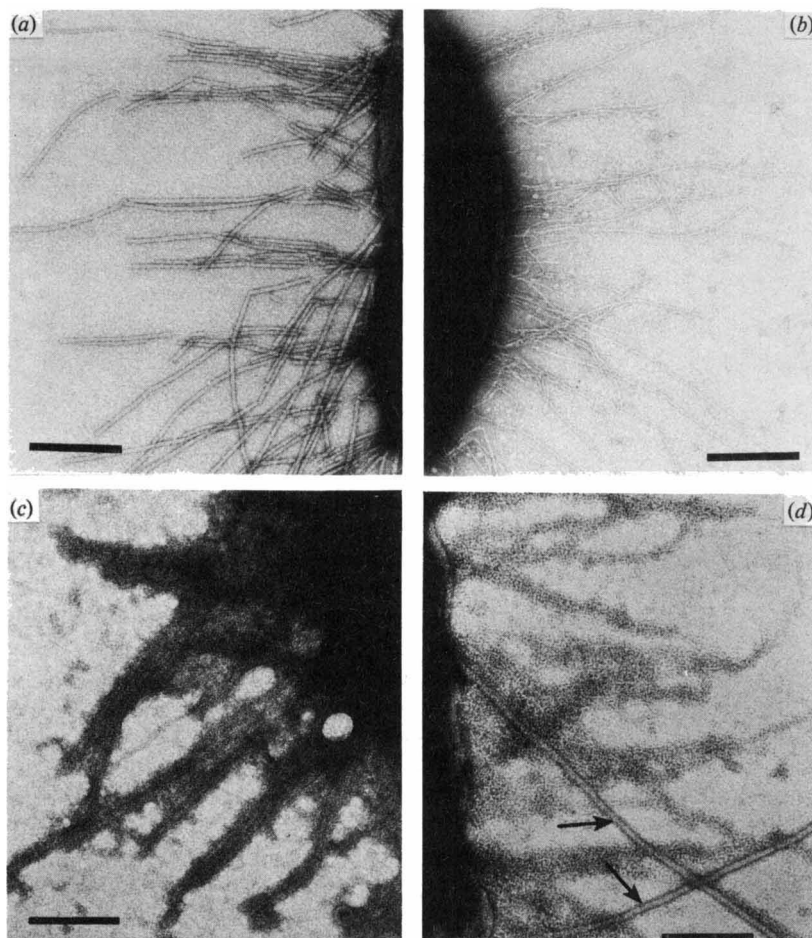


Fig. 1. Uncoated and coated fimbriae of *Enterobacter*. (a) Thick, channelled fimbriae of *E. amnigenus* CUETM 78-89 from MS-HA⁺ broth culture grown at 30 °C. (b) Thin, non-channelled fimbriae of *E. gergoviae* BR9 from an MR/K-HA⁺ broth culture grown at 30 °C. (c) Thick fimbriae of *E. aerogenes* NCTC 10006 (coated, +++) from an MS-HA⁺ broth culture after treatment with type-1 fimbrial (MS-HA) antiserum of '*K. aerogenes*' 55. (d) Thin fimbriae of *E. gergoviae* CDC 840-82 (coated, ++) and flagella (arrowed, no coating) from an MR/K-HA⁺ broth culture after treatment with type-3 fimbrial (MR/K-HA) antiserum of '*K. aerogenes*' 70. All the bar markers represent 200 nm.

Table 3. *Antibody coating of fimbriae of Enterobacter species*

Species	Coating of fimbriae as observed in immune electron microscopy tests with:		
	Type-1 fimbriate, MS-HA ⁺ strains and type-1 (MS-HA) antiserum from:		Type-3 fimbriate, MR/K-HA ⁺ strains and type-3 (MR/K-HA) antiserum from:
	<i>E. cloacae</i> strain 035	' <i>K. aerogenes</i> ' strain 55	' <i>K. aerogenes</i> ' strain 70
<i>E. amnigenus</i>	+++	0	-
<i>E. cloacae</i>	+++	0	-
<i>E. sakazakii</i>	+++	0	-
<i>E. aerogenes</i>	0	+++	+++
<i>E. intermedium</i>	0	0	0
<i>E. gergoviae</i>	-	-	+++

0, No coating; +++ strong coating; -, type-1 fimbriae (and MS-HA) not formed by *E. gergoviae* and type-3 fimbriae (and MR/K-HA) not formed by *E. amnigenus*, *E. cloacae* and *E. sakazakii*.

DISCUSSION

Most (75%) of the *Enterobacter* species were HA⁺, but generally produced only one kind of HA. Thus, the correlation of fimbriae with HAs was less difficult than it had been with the multiply haemagglutinating strains of *Serratia* species (Adegbola & Old, 1982) and '*K. aerogenes*' (D. C. Old & R. A. Adegbola, unpublished data). In *Enterobacter*, therefore, as in other enterobacteria (Duguid & Old, 1980) thick, channelled fimbriae and thin non-channelled fimbriae (types 1 and 3, respectively, of Duguid *et al.*, 1966) were associated with, respectively, MS-HA and MR/K-HA.

Both phenotypic and genetic studies have shown that *E. amnigenus*, *E. cloacae* and *E. sakazakii* are closely related species (Farmer *et al.*, 1980; Izard *et al.*, 1978, 1981). It was not surprising, therefore, to find that HA⁺ strains of these species were of a uniform HA type (MS-HA only) and had type-1 fimbriae antigenically distinct from those of other *Enterobacter* species.

Enterobacter gergoviae strains, a homogeneous group both biochemically and genetically (Brenner *et al.*, 1980), also show an essentially homogeneous HA type. Though *E. gergoviae* is most similar biochemically to *E. aerogenes*, the two species are distinguishable by a few biochemical characters (Brenner *et al.*, 1980), by distinct esterase patterns (Goulet, 1980) and, from this study, by the inability of *E. gergoviae* to form type-1 fimbriae and MS-HA (Table 2).

Both phenotypic and genetic studies have shown the clustering of *E. aerogenes* and '*K. aerogenes*' (Bascomb *et al.*, 1971; Johnson *et al.*, 1975; Sakazaki *et al.*, 1976) and further phenotypic similarities have been demonstrated in their esterase patterns (Goulet, 1980). In species of *Enterobacter* and *Klebsiella*, the production of multiple HAs was not a common property. Nevertheless, most strains of *E. aerogenes* and '*K. aerogenes*' were multiply haemagglutinating producing MS-, MR/K- and MR/P-HAs, and their type-1 and type-3 fimbriae were antigenically similar.

The relationship of the recently described species of *E. intermedium* to other enterobacters is not yet clear and DNA-DNA hybridization studies have indicated that it is as closely related to *K. pneumoniae* as to *E. cloacae* (Izard *et al.*, 1981). It was of some interest, therefore to find that both type-1 and type-3 fimbriae of *E. intermedium* were antigenically distinct from those of other *Enterobacter* species.

Enterobacter species are frequently isolated from diverse clinical specimens, particularly in nosocomial infections. Adhesive properties may be important in the establishment or maintenance of these infections. However, before the ecological role of their adhesive properties can be decided, it is essential that the HAs (adhesins) produced *in vitro* be fully and carefully characterized. This preliminary study has considerably increased our understanding of the fimbrial HAs beyond the limited information available from previous studies (Constable, 1956; Duguid, 1959; Nowotarska & Mulczyk, 1977). Further work in progress is directed particularly to elucidating the antigenic relationships of the type-1 and type-3 fimbriae in *Enterobacter* and the nature of the MR/P-HA of *E. aerogenes*.

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