P fimbriation among biochemically inactive strains of *Escherichia coli* of the group formerly called Alkalescens-Dispar

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Summary. Biochemically inactive, non-motile strains of *Escherichia coli* of the group formerly known as Alkalescens-Dispar (AD) and of known AD serogroups were analysed for biotype, resistotype, type-1 fimbriation, P fimbriation and haemolysin production. All strains of AD serogroups O1 and O2 examined were P-fimbriate and of closely related bio-resistotypes, suggesting that they may be representatives of two *E. coli* P-fimbriate clones, members of which have not infrequently been isolated from infected urine.

Introduction

Among uropathogenic strains of *Escherichia coli*, the property of P fimbriation correlates significantly with the ability to produce pyelonephritis (Källénus *et al.*, 1981; Väisänen-Rhen *et al.*, 1984). P fimbriae recognise specifically a minimal receptor structure α -D-Gal-(1-4)- β -D-Gal, a part of the glycosphingolipids related to the P blood-group antigens of human erythrocytes (Källénus *et al.*, 1980a). Although generally described as agglutinating human erythrocytes only (Källénus and Möllby, 1979), the majority of P-fimbriate *E. coli* strains, when tested at appropriate concentration, give mannose-resistant and eluting (MRE) haemagglutination of the erythrocytes of man, pig and sheep, i.e., their haemagglutination pattern is MPS (Old *et al.*, 1980; Old, 1985).

We have previously reported (Crichton *et al.*, 1981) that tests for the presence of MRE haemagglutinins (HAs) are helpful in differentiating biochemically inactive strains of *E. coli* of the Alkalescens-Dispar (AD) group from shigellae, which they mimic in many routine diagnostic characters. Thus, many AD bacteria (65%) produce MREHAs of the MPS or related haemagglutination patterns (Crichton *et al.*, 1981). In view of the suggestion that AD bacteria may be opportunist pathogens of the urinary tract (Kauffman, 1969), it is of interest that all strains of AD serogroups O1 and O2 are P fimbriate.

Materials and methods

Bacterial strains

Of the 42 AD strains of *E. coli* examined, 40 were described by Crichton *et al.* (1981); the other two strains were recent isolates from our own laboratory. Most (18 strains) belonged to the commonest AD serogroup O1 (Frantzen, 1950); six strains were either non-serotypable (NST) or autoagglutinating. The other strains belonged to seven AD serogroups: O2 (4 strains), O3 (4), O4 (4), O5 (3), O6 (1), O7 (1) and O8 (1). Of the 22 strains of serogroups O1 and O2, only two from the original collection of Kauffmann (1969) had not been examined to verify the absence of H antigen (Drs F. and I. Ørskov, personal communication).

Media

Nutrient Broth No. 2 and Nutrient Agar (CM3) were obtained from Oxoid. Phosphate-buffered broth (PBB) and phosphate-buffered agar (PBA) were, respectively, nutrient broth and nutrient agar with KH_2PO_4 0.35% w/v and Na_2HPO_4 0.65% w/v (pH 7.0).

Production and detection of haemagglutinins

For the production of type-1 fimbriae and mannose-sensitive (MS) HA, bacteria were grown serially in nutrient broth (10 ml) in cotton-wool stoppered test tubes incubated statically at 37°C for 48 h. A strain was designated as MSHA-negative if it gave a negative MSHA result in each of six serial broth cultures (Duguid *et al.*, 1979). For MREHA, strains were grown at 37°C either on PBA (20 ml/9-cm plate) for 24 h or in PBB (10 ml) for 48 h (Duguid *et al.*, 1979; Crichton, 1980).

Bacterial suspensions containing $c. (5-10) \times 10^{10}$ cfu/ml for MSHA tests and 1×10^{12} cfu/ml for MREHA tests were prepared as described previously (Duguid *et al.*, 1979; Old, 1985). Suspensions were mixed on a porcelain tile in the presence and absence of α -methyl-mannoside with the following erythrocyte species: fowl (F), guinea pig (G), horse (H), human, group O (M), ox (O), pig (P) and sheep (S). Tiles were rocked at room temperature ($c. 20^\circ\text{C}$) or at 4°C for 15 min. Cultures producing MSHA agglutinated, for example, guinea-pig erythrocytes at both low and high temperatures but only in the absence of α -methyl-mannoside; cultures producing MREHA agglutinated erythrocytes in the presence and absence of α -methyl-mannoside but did so better at lower than at higher temperatures and eluted from the erythrocytes when the temperature of the mixture was raised to $\leq 50^\circ\text{C}$ by gentle warming of the tile.

Furthermore, suspensions were tested with human erythrocytes of the rare $\bar{p}$ phenotype. Any strain that agglutinated human erythrocytes of P_1 or P_2 phenotypes but not those of the $\bar{p}$ phenotype in MRE fashion was designated P fimbriate (Källénus *et al.*, 1981; Väisänen *et al.*, 1981).

Hydatid-cyst fluid (HCF) from sheep or tissue-cultures infected with *Echinococcus granulosus* contains P_1 substance, a natural analogue of the glycoprotein receptor of P fimbriae (Parry *et al.*, 1984). Rocked-tile tests were performed as before, except that bacterial suspensions, tested neat and at dilutions of 1 in 5 and 1 in 10, were pre-incubated with α -methyl-mannoside and HCF for 30 min at 4°C before addition of erythrocytes (Parry *et al.*, 1984). The ability of HCF to inhibit MRE haemagglutination of human erythrocytes of P_1 or P_2 phenotype indicated the presence of P fimbriae (Parry *et al.*, 1984).

Haemolysin production

The ability of strains to form haemolysin was tested on plates of sheep-blood agar. They were examined for zones of clearing around isolated colonies after overnight incubation at 37°C .

Biotyping

AD strains of *E. coli* were biotyped by the two-tier scheme of Crichton and Old (1982). Strains were assigned to one of 16 primary biotypes on the basis of their reactions in tests with: raffinose (Raf), sorbose (Sor), ornithine (Orn) and dulcitol (Dul). Further discrimination was afforded by results in seven other secondary tests: rhamnose (Rha), lysine (Lys), aesculin (Aes), motility (Mot), type-1 fimbriation (Fim), 5-ketogluconate (Kgl) and prototrophy (Pro). Full details of the tests and their interpretation have been reported elsewhere (Crichton and Old, 1979, 1982 and 1985).

Resistotyping

Chemicals used in resistotyping tests were: A, sodium

arsenate; B, phenylmercuric nitrate; C, 4-4' diamidinodiphenylamine dihydrochloride; D, boric acid; E, acriflavine; F, 4-chlororesorcinol; G, cupric sulphate; and H, malachite green. Details of the method used, a modification of that originally developed by Elek and Higney (1970), have been published previously (Old *et al.*, 1980; Wilson *et al.*, 1981; Crichton and Old, 1983). The basis of the technique is the selective toxicity to strains of *E. coli* of these eight chemicals, each tested at a critical concentration in agar. On every occasion of testing, each chemical was tested at a narrow range of concentration around the optimum. The use of five reference strains on each plate with 20 test strains clearly showed the plate containing the concentration of chemical giving the expected growth pattern of the reference strains. From that plate, results for the test strains were read, thus ensuring their optimal type differentiation. Resistotypes were assigned as before (Wilson *et al.*, 1981): a strain of resistotype pattern AcDEF was resistant to chemicals A, D, E and F, partially resistant to chemical C and sensitive to chemicals B, G and H. When tested on the same plate, the resistotype pattern of different isolates of a strain is reproducible differing in one or, rarely, two minor characteristics (Elek and Higney, 1970; Old *et al.*, 1980). Thus, resistotypes acDEF, aCDEF and aCDeF would suggest strain identity.

Serological tests

Antisera were prepared in rabbits (Adegbola and Old, 1982) against authentic P-fimbriate *E. coli* strains ER2 and JR1 obtained from Dr G. Källénus. O, type-1 fimbrial and, for strain ER2, H antibodies were removed from each crude antiserum by appropriate absorptions to give pure P-fimbrial antisera which reacted to titres of 2560 and 5120, respectively, in tube-agglutination tests with homologous strains. All P-fimbriate AD strains of groups O1 and O2 were examined for agglutination in slide tests and several unselected strains were tested by tube agglutination. Type-1 fimbrial antiserum against *E. coli* strain A108 was available from another study (Crichton *et al.*, 1981).

Results

Adhesins

Among the 42 AD strains, 28 (67%) produced MREHAs of one of three different haemagglutination patterns: 24 strains produced MREHA of pattern MPS; three strains produced MREHA of pattern GMPS (the additional reactivity with guinea-pig cells, though weak, was constant); and one strain produced a narrow-spectrum MREHA reacting with human erythrocytes only of the seven species tested (table I). Furthermore, the haemagglutination of erythrocytes of man (or pig or sheep or guinea pig) by strains producing MREHA

Table I. Distribution of MSHA, MREHA and P fimbriation among biochemically inactive AD strains of *E. coli*

AD O sero- group	Number of strains			MREHA pattern (number of strains)	HA of human erythrocytes of phenotype		Inhibition of HA by HCF (P fimbriation)
	tested	MSHA ⁺	MREHA ⁺		P ₁ ,P ₂	$\bar{p}$	
1	18	2	18	{ MPS (16) GMPS (2)	+	—	+
2	4	1	4	MPS (4)	+	—	+
3	4	2	0	...	...	...	...
4	4	0	0	...	...	...	...
5	3	0	1	M (1)	+	+	—
6	1	0	0	...	...	...	...
7	1	1	1	MPS (1)	+	—	+
8	1	0	0	...	...	...	...
NST	1	1	0	...	...	...	...
AA	5	0	4	{ MPS (3) GMPS (1)	+	—	+

NST = Non-serotypable; AA = autoagglutinating; HCF = hydatid-cyst fluid; ... = not applicable.

of patterns MPS or GMPS was inhibited by hydatid-cyst fluid (HCF) suggesting that these two haemagglutination patterns were associated with P fimbriae. The narrow-spectrum MREHA (pattern M) of the strain of serogroup O5 reacted with human erythrocytes only, including those of the $\bar{p}$ type; that activity was not inhibited by HCF and it was not, therefore, associated with P fimbriae. Indeed, that kind of MREHA (pattern 9 of Duguid *et al.*, 1979) is associated with either glycocalyx or fibrillar proteinaceous capsule (Williams *et al.*, 1984; Ørskov *et al.*, 1985). Our reporting of MREHA-type M, according to the terminology used by Old *et al.* (1980), should not be confused with the blood-group M-specific HA of glycoprotein A subsequently described by Väisänen *et al.* (1982).

Whereas all strains of serogroups O1 and O2 were P fimbriate, only one strain of serogroups O3–8 and NST was P fimbriate (table I). Again, four of five rough, autoagglutinable strains were P fimbriate (MREHAs of patterns MPS or GMPS). Thus, the association of P fimbriation with AD serogroups O1 and O2 was high.

The P-fimbriate AD strains reacted only to a moderate, or low, titre (≤ 640) in tube agglutination tests with P-fimbrial antisera against *E. coli* strains ER2 or JR1 and the agglutination reaction was weak (14 strains) or undetectable (13) in tests made on glass slides.

Only seven (17%) of the 42 AD strains formed MSHA in serial broth cultures and production of MSHA was as uncommon among P-fimbriate

strains of serogroups O1 and O2 as among other AD strains (table I). MSHA production was associated with type-1 fimbriae serologically related to those of *E. coli* strain A108.

Biotypes and resistotypes

All 18 strains of AD serogroup O1 belonged to primary biotype 11 (Raf[−]Sor⁺Orn[−]Dul⁺). Twelve strains were of full biotype 11bdef (indicating negative results in the secondary tests for Rha, Aes, Mot and Fim, symbols 'b', 'd', 'e' and 'f', respectively). Three Lys[−] strains (full biotype 11bcdef), two type-1 fimbriate strains (full biotype 11bde) and one strain that did not ferment 5-ketogluconate (full biotype 11bdefg) differed from the majority cluster in only one marker each (table II). In addition, their resistotypes (table II) fulfilled the criteria suggestive of strain identity: three (ADeFH, AcDeFH and acDeFH) differed from the majority resistotype AcDeFH in only one minor character and one (acDFH) in two minor characters.

All four strains of serogroup O2 belonged to primary biotype 16 (Raf[−]Sor[−]Orn[−]Dul[−]); two strains were of full biotype 16bdefg (indicating negative results in secondary tests for Rha, Aes, Mot, Fim and Kgl); two Lys[−] strains were similar in their secondary biotype characters (negative in Rha, Lys, Aes, Mot and Kgl) except that one was type-1 fimbriate (biotype 16bcdeg) and the other was type-1 non-fimbriate (biotype 16bcdefg) (table II). These four strains were of closely related

Table II. Biotype and resistotype profiles of P-fimbriate AD strains of *E. coli*

AD O sero- group	Primary biotype*	Full biotype* (number of strains)	Resistotype†
1	11 (18)	{ 11bdef (12) 11bcdef (3) 11bde (2) 11bdefg (1)	{ AcDeFH (13) acDFH (1) ADeFH (1) AcdeFH (1) acDeFH (2)
2	16 (4)	{ 16bdefg (2) 16bcdeg (1) 16bdefg (1)	{ ADEF (3) AdEF (1)
7	7 (1)	7bde (1)	AcDEF (1)
AA	11 (4)	{ 11bdef (3) 11bcdef (1)	{ AcdeFH (1) AcDeFH (2) acDeFH (1)

AA = autoagglutinating.

* See Crichton and Old (1982 and 1985).

† See Crichton and Old (1983).

resistotypes (ADEF and AdEF) each differing from the majority pattern of O1 strains in both a minor (c) and a major (H) character.

Of the other five P-fimbriate strains, one strain of serogroup O7 was of biotype 7bde and resistotype AcDEF; the other four (rough) strains were of full biotype 11bdef and resistotype AcDeFH, or similar types (table II).

None of the P-fimbriate AD strains was haemolytic.

Discussion

Biochemically inactive strains of *E. coli*, formerly called AD, merit no special taxonomic position nowadays (Farmer *et al.*, 1985), being distributed instead among appropriately specified *E. coli* O serogroups (Kauffmann, 1969; Edwards and Ewing, 1972). For example, strains of AD serogroup O1 contain the same O antigens as *E. coli* O1 (Frantzen, 1950). The 18 strains of AD serogroup O1 examined by us were anaerogenic, non-haemolytic, non-motile (and H⁻), P fimbriate and generally type-1 non-fimbriate. Furthermore, all belonged to primary biotype 11 and to closely related full biotypes. Yet, among 599 biochemically active strains of *E. coli* previously described, many of them of urinary origin (Crichton and Old, 1982), the unusual combination of secondary biotype characters (bcdef) was never found among strains of primary biotype 11 which itself was uncommon

(Crichton and Old, 1982). The observed stability of the biotype-resistotype profiles of the AD strains of serogroup O1 was all the more remarkable because they had been isolated from different countries in North America and Scandinavia and from the UK over more than 60 years and it seems likely that they belong to a clone with a long history. Furthermore, the finding that four other P-fimbriate, rough AD strains were also of full biotype 11bdef and of resistotypes similar to AcDeFH suggested that they too might be related to the serotypable strains of serogroup O1.

Among a total of 55 strains of *E. coli* of serotype O1:K1 isolated in Europe and USA, two clones were identified that had a distinct outer-membrane-protein (omp) pattern and biotype and were thought to be of independent origin (Achtman *et al.*, 1983; Ørskov and Ørskov, 1983). Väisänen-Rhen *et al.* (1984) by definition of full serotype, omp pattern, adhesins and haemolysin production recognised seven major pyelonephritogenic clones of *E. coli* and showed similarly that one particular omp pattern was predominant among strains of serotype O1:K1:H7 but that another omp pattern was associated with O1 strains of faecal origin. Whereas the former (consisting of two subclones different in type-1 fimbriation) were P fimbriate, the latter were generally P non-fimbriate (Väisänen-Rhen *et al.*, 1984).

Many of our AD strains of serogroup O1 possessed K1 antigen though that information was not available for all of them. It is important, therefore, to establish whether these AD strains belong to previously described clones of serotype O1:K1. AD strains of serogroup O1, though similar to the non-motile strains of omp-pattern 5 of Achtman *et al.* (1983) in not requiring nicotinamide for growth, differed from them in three important biotype characters (indole, dulcitol, rhamnose). The AD strains of serotype O1 also differed from the generally motile strains of the pyelonephritogenic clone of serotype O1:K1:H7 in four biotype characters (ornithine, nicotinamide requirement, rhamnose, salicin) of the nine characters described by Achtman *et al.* (1983). Thus, although we did not perform omp analysis, the overall properties of AD strains of serogroup O1 seem to distinguish them from the two O1 clones of Achtman *et al.* (1983) and Väisänen-Rhen *et al.* (1984).

AD serogroup O2 is closely related, but not identical, to *E. coli* O group 25 (Frantzen, 1950) among which P-fimbriate clones have not been described (Ørskov *et al.*, 1982; Väisänen-Rhen *et al.*, 1984). Thus, the P-fimbriate strains of serogroup O2 examined here, isolated in different

countries over more than 35 years, may represent yet another P-fimbriate clone, members of which are non-motile (and H⁻), anaerogenic, non-haemolytic and belong to closely related biotypes and resistotypes. AD serogroup O2 is also related, though poorly, to other *E. coli* O serogroups including O16, 19 and 68 (Frantzen, 1950; Kauffmann, 1969; Edwards and Ewing, 1972). Any relationship, therefore, between P-fimbriate strains of AD serogroup O2 and some of these latter types, though unlikely, should be investigated.

It was of particular interest that the AD strains identified as P fimbriate in our tests with $\bar{p}$ erythrocytes and HCF reacted only weakly, or not at all, in slide-agglutination tests with pure P-fimbrial antisera. These findings illustrate yet again the antigenic heterogeneity existing among P fimbriae and extend the results of Rhen *et al.* (1983) who, by ELISA testing, demonstrated major differences in cross-reactivity among P fimbriae of different pyelonephritogenic isolates of *E. coli*. The minor differences shown here among AD strains of serogroups O1 and O2 with absorbed P-fimbrial antisera may reflect quantitative variation in P-antigen production.

The consistent, albeit weak, agglutination of guinea-pig erythrocytes shown by only three of the P-fimbriate AD strains has also been noted for a few other P-fimbriate strains of *E. coli* of urinary origin (Crichton, 1980; Källenius *et al.*, 1980*a* and *b*). The inhibition of that (guinea-pig) MREHA reaction by HCF showed its association with P fimbriation and extends the findings of Parry *et al.* (1984) who similarly demonstrated the P-specific haemagglutination of sheep, pigeon and chicken erythrocytes.

In this study, the clinical information available to us was in many cases insufficient to establish

whether the AD strains had been associated with pyelonephritis or even with urinary-tract infection. Furthermore, when in the past AD bacteria were considered as distinct entities, it was interest in their biochemical and serological relationships to shigellae and their possible role in gastroenteritis that attracted attention (Frantzen, 1950; Edwards and Ewing, 1972). Nevertheless, several early studies provide enough evidence to show that Alkalescens bacteria were responsible for a not inconsiderable number of infections of the urinary tract and often for pyelonephritis (Bamforth, 1934; Neter and Heide, 1940; Weil, 1943).

The need to identify clones of *E. coli* in human infection is important for pinpointing their likely virulence determinants. It also draws attention to previously overlooked and yet distinctive groups, such as the two P-fimbriate clones of *E. coli*, of wide geographic distribution and historic lineage that are here described. Though typing of *E. coli* by observations of many different phenotypic characters is sufficient to indicate in the short term probable clonal relationships, it is insufficient to demonstrate clonal disseminations that have taken place over long periods, especially when there has been divergence in their phenotypic markers. To answer the question as to which types of strains isolated from different sources today might many years ago have descended from a common ancestral bacterium, i.e., are members of a clone, evidence of genetic homology is required but for the P-fimbriate strains of *E. coli* this has yet to be demonstrated.

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