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Identification of amino acids in the Dr adhesin required for binding to decay-accelerating factor

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Summary

Members of the Dr family of adhesins of *Escherichia coli* recognize as a receptor the Dr^a blood-group antigen present on the complement regulatory and signalling molecule, decay-accelerating factor (DAF). One member of this family, the Dr haemagglutinin, also binds to a second receptor, type IV collagen. Structure/function information regarding these adhesins has been limited and domains directly involved in the interaction with DAF have not been determined. We devised a strategy to identify amino acids in the Dr haemagglutinin that are specifically involved in the interaction with DAF. The gene encoding the adhesive subunit, *draE,* was subjected to random mutagenesis and used to complement a strain defective for its expression. The resulting mutants were enriched and screened to obtain those that do not bind to DAF, but retain binding to type IV collagen. Individual amino acid changes at positions 10, 63, 65, 75, 77, 79 and 131 of the mature DraE sequence significantly reduced the ability of the DraE adhesin to bind DAF, but not collagen. Over half of the mutants obtained had substitutions within amino acids 63–81. Analysis of predicted structures of DraE suggest that these proximal residues may cluster to form a binding domain for DAF.

Introduction

Escherichia coli express various proteins on the surface of the bacterial cell that mediate attachment to mammalian receptors. The Dr family of adhesins of *Escherichia coli* is associated with urinary tract infections (UTI), in particular cystitis and pregnancy-associated pyelonephritis, and diarrhoeal disease (Arthur *et al*., 1989; Giron *et al*., 1991; Johnson, 1991; Stapleton *et al*., 1991; Jallat *et al*., 1993; Levine *et al*., 1993; Nowicki *et al*., 1994; Kaul *et al*., 1996). The Dr family includes fimbrial adhesins, such as the Dr haemagglutinin and F1845, and afimbrial adhesins, such as AFA-I, II, III and IV. Dr adhesins bind to the Dr^a blood-group antigen present on decay-accelerating factor (DAF or CD55) (Nowicki *et al*., 1988; 1990; 1993; Pham *et al*., 1995). DAF is a 70 kDa glycosylphosphatidylinositol (GPI)-anchored protein that is present on many mammalian cell types and has a role in complement regulation and cell signalling (Lublin and Atkinson, 1989; Nicholson-Weller, 1992; Nicholson-Weller and Wang, 1994). One member of the Dr family of adhesins, the Dr haemagglutinin, also binds to a second receptor, the 7S domain of type IV collagen (Westerlund *et al*., 1989; Carnoy and Moseley, 1997). Binding by the Dr haemagglutinin is inhibited in the presence of chloramphenicol, a property not shared by other Dr adhesins examined (Nowicki *et al*., 1988; Westerlund *et al*., 1989; Carnoy and Moseley, 1997).

The Dr adhesins share a high degree of similarity in the organization of their operons, as well as in their sequences, although there is greater divergence in the sequences of the genes that encode the major structural subunits. It has been shown that the major structural subunit of the Dr haemagglutinin, DraE, is the adhesive subunit for DAF and is responsible for the various binding phenotypes exhibited by the whole fimbriae (Swanson *et al*., 1991; Carnoy and Moseley, 1997; Van Loy *et al*., 2002). Initial studies showed that alterations in *draE* affect inhibition of binding by chloramphenicol (Swanson *et al*., 1991). It was later demonstrated using site-directed mutagenesis that changes in *draE* affect binding to DAF, type IV collagen and/or inhibition by chloramphenicol (Carnoy and Moseley, 1997). More recently, it was demonstrated directly that recombinant fusions of DraE mediate specific adherence to erythrocytes, CHO cells transfected with DAF and recombinant DAF (Van Loy *et al*., 2002).

Few studies have analysed the structure–function relationship in the Dr adhesive subunits and, furthermore, it has not been determined which regions within these subunits are important for binding to DAF. One study identified several residues within DraE that have a role in binding to type IV collagen and/or in inhibition by chloramphenicol (Carnoy and Moseley, 1997). The results indicated that DraE has a conformational binding domain for type IV collagen. However, this study did not identify amino acids that specifically affect the interaction between DraE and DAF.

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We investigated the Dr haemagglutinin, and its major structural subunit DraE, to identify which amino acids are specifically involved in binding to DAF. We subjected *draE* to random mutagenesis and developed an enrichment for mutants that do not bind to DAF but do retain binding to type IV collagen.

Results

Enrichment and screen for random mutants of DraE that do not bind DAF

We performed random mutagenesis of *draE* to identify amino acids in DraE involved in binding specifically to DAF. This adhesin provides an advantage because it binds to two receptors, DAF and type IV collagen, we therefore sought to obtain mutations that abolished binding to DAF but retained binding to type IV collagen. This would allow the determination of amino acids that are directly involved in binding or in maintaining an important local structure for binding specifically to DAF, but are unlikely to affect the overall conformation of the protein. Random mutations within *draE* were generated by a polymerase chain reaction (PCR) mutagenesis approach (Cadwell and Joyce, 1992; Muhlrad *et al*., 1992). *draE* was amplified under conditions promoting one to three base substitutions per 500 base pairs. The mutated *draE* amplicons were inserted into pUC-Cm and transformed into DH5α (pCC90-D54stop). This strain contains the *dra* operon with the genes necessary for assembly of the Dr fimbriae but has a premature stop codon in *draE*, resulting in the production of a non-functional truncated protein (Carnoy and Moseley, 1997).

The transformants were subjected to an enrichment procedure to obtain mutants in *draE* that do not bind DAF. This was accomplished by incubating pools of transformants with human erythrocytes and removing by centrifugation those mutants that bound to the erythrocytes. Bacteria remaining in the supernatant were incubated with fresh erythrocytes, and adherent bacteria again removed by centrifugation. After seven sequential incubations, the transformants that did not bind to erythrocytes and remained in the supernatant were plated for isolation. These transformants were then examined for the mannose resistant haemagglutination (MRHA) phenotype as a measure of the ability to bind and agglutinate erythrocytes expressing DAF. Approximately 85% of the selected transformants were MRHA$^-$. These transformants were then examined for the ability to bind to type IV collagen in a growth assay. Bacteria were added to microtitre plates coated with type IV collagen. Unbound bacteria were removed and culture medium was added to the wells followed by incubation at 37°C. Transformants that were positive for growth were further confirmed for binding to type IV collagen by the same assay. Approximately 3% of those transformants that were MRHA$^-$ bound to type IV collagen. Sequence analysis of *draE* was performed to identify mutations.

Using the enrichment and screen described above, 20 mutants that matched the desired criteria were obtained. Mutants resulting in one, two or three amino acid substitutions in the 141 amino acid sequence of DraE were obtained (Table 1). Each amino acid substitution resulted from a single base pair change. Some double mutants were obtained which had silent mutations that were distributed throughout the protein, indicating that the mutagenesis approach did not bias the changes to any particular region of the sequence. Multiple isolates were obtained for some of the mutations. These included identical isolates with the same sequence and isolates with synonymous mutations in other codons (Table 1).

Characterization of DAF-binding mutants

The 20 mutants isolated did not mediate MRHA but still bound to type IV collagen. Although the resulting substitutions were scattered throughout DraE, 15 of the mutants had substitutions that were clustered within residues 63–81. In particular, five mutants had substitutions at amino acid 79. We chose to further characterize the 13 mutants that contained single amino acid substitutions within DraE. These mutants were examined for DraE expression by immunoblot analysis of whole cell lysates. All of the mutants except F73S expressed DraE (Fig. 1B and data not shown). Although there was some variability in DraE expression as compared to wild-type Dr in the remaining mutants, only I75F showed consistently low expression. F73S was not analysed further.

To determine DraE localization within the bacterial cell, surface expression of DraE was examined in the mutants. Bacteria were labelled with [^{3}H]-thymidine and incubated in microtitre wells coated with anti-Dr fimbriae antisera. The unbound bacteria were removed and the bound bacteria were quantified by scintillation counting. Wild-type Dr showed a high level of binding to anti-Dr fimbriae antibodies, whereas the D54stop vector control parent strain did not bind (Fig. 1A). All of the mutants bound to anti-Dr fimbriae antibodies, indicating that the mutant DraE proteins are expressed on the surface of the cell. Although there was some variability in the levels of binding, all of the mutants showed binding that was at least 50% of wild type. We conclude from these experiments that with the exception of F73S, mutations abolishing Dr fimbriae-mediated erythrocyte binding have not significantly affected levels of expression or surface localization of DraE.

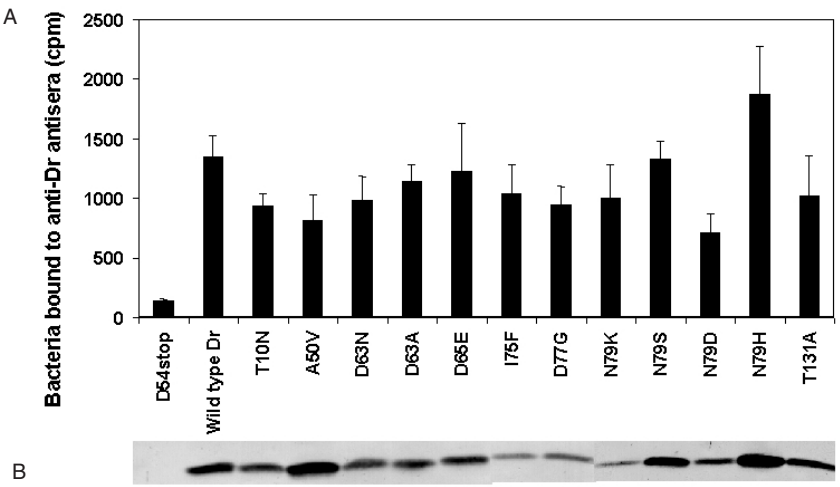

Fig. 1. DraE expression in DAF-binding mutants.
A. Surface expression of DraE mutant proteins. Bacteria were labelled with [^{3}H]-thymidine and incubated in microtitre wells coated with anti-Dr fimbriae antisera. Bound bacteria remaining in the wells were quantified by scintillation counting. Results shown are the average of three independent experiments with duplicate wells of each sample.
B. Immunoblot analysis of DraE expression in DAF-binding mutants. Whole cell lysates were separated by SDS–PAGE and transferred onto nitrocellulose. DraE was detected by immunoblot analysis using anti-Dr fimbriae antisera. D54stop, DH5α (pCC90-D54stop, pUC-Cm). Wild-type Dr, DH5α (pCC90-D54stop, pUC-R).

Type IV collagen binding phenotype of the DAF-binding mutants

The DAF-binding mutants identified retained binding to type IV collagen in the initial screen performed. We further investigated collagen binding of the mutants in a more quantitative assay. Bacteria were labelled with [^{3}H]-thymidine and incubated in microtitre wells coated with type IV collagen. Unbound bacteria were removed and bound bacteria were quantified by scintillation counting. D54stop showed no binding to type IV collagen, whereas wild-type Dr bound at a high level (Table 2 and Fig. 2). The mutants bound type IV collagen although at varying levels. Mutants A50V, D65E, N79S and N79H bound type IV collagen at a similar level as wild-type Dr. The remaining mutants demonstrated significant binding to type IV collagen albeit at a reduced level relative to wild-type Dr.

Wild-type Dr-mediated binding to DAF and collagen is inhibited by chloramphenicol. Previous studies have determined that mutations at various residues in DraE can result in binding that is not inhibited by chloramphenicol

Table 1. Sequence analysis of DAF-binding mutants.

Mutant	Codon change	Silent codon changes	Number of isolates	MRHA
D54stop				–
Wild type Dr				+
T10N	acc-aac		1	–
A50V	gtt-gtc	91, gat-gac; 25, gtt-gtc[a]	2	–
D63N	gac-aac		3	–
D63A	gac-gcc	13, acc-aca; 101, gtc-gtt	3	–
D65E	gat-gaa		1	–
F73S	ttc-tcc	41, gca-gcg	2	–
I75F	atc-ttc		1	–
D77G	gac-ggc		1	–
N79K	aat-aaa		1	–
N79S	aat-agt	30, gtg-gta 90, acg-aca; 115, ggg-ggt	4	–
N79D	aat-gat		1	–
N79H	aat-cat		2	–
T131A	aca-gca	81, gat-gac	2	–
T12S, T135S	acc-tcc, acc-tcc	79, aac-aat	1	–
R80G, D98V	agg-ggg, gac-gtc		2	–
T5P, T33I	acc-ccc, act-at		1	–
N79D, N124S	aat-gat, aac-agc	47, acc-act	1	–
F73L, N124Y	ttc-ctc, aac-tac		1	–
D81V, F102L	gat-gtt, ttc-tta		1	–
I75T, G108T, D119Y	atc-acc, ggg-cgg, gat-tat		1	–

a. Silent codon changes are designated by the codon number for the corresponding amino acid in the mature DraE sequence followed by the identified silent base pair change. Changes listed on separate lines indicate substitutions identified in different isolates.

Table 2. DAF-binding mutants and their properties.

Mutant	Binding to type IV collagen[a]	Adherence to[b]		
		J82 cells	T24 cells	CHO DAF$^+$
D54stop	–	–	–	–
Wild-type Dr	+++	+++	+ + +	+++
D63N	++	–	±	–
I75F	++	–	+	+
N79K	+	–	±	–
N79D	+	–	±	–
T10N	++	+	+	++
D63A	++	+	+	++
D65E	++	±	+	++
D77G	++	±	+	+
N79S	+++	±	++	++
N79H	+++	±	+	+
T131A	+	±	+	+
A50V	+++	+++	++	+++

a. +++, ++, +, ±, and – represent binding phenotypes relative to wild type from the results of the type IV collagen binding assay.
b. +++, ++, +, ±, and – represent binding phenotypes relative to wild type from the results of the percentage of cells with bound bacteria in the adherence assays.

(Carnoy and Moseley, 1997). Therefore, using the collagen binding assay, the DAF-binding mutants identified here were examined for inhibition of binding to type IV collagen by chloramphenicol. All of the mutants, except for A50V, exhibited binding to type IV collagen that was inhibited by the presence of chloramphenicol similar to wild-type Dr (data not shown). A50V therefore represents a unique mutation with decreased DAF binding and loss of inhibition of collagen binding by chloramphenicol.

Adherence of DAF-binding mutants to bladder epithelial cells expressing DAF

The enrichment for DAF-binding mutants was dependent upon the inability of bacterial cells to remain bound to erythrocytes subjected to centrifugation, and on the inability to agglutinate erythrocytes. These assays therefore demonstrated that the mutants did not interact normally with DAF on the surface of erythrocytes. To examine the effects of the mutations in a more relevant assay, the mutants were examined for adherence to two epithelial cell lines expressing DAF: T24 and J82 bladder epithelial cells. These cell lines were determined to express high and low levels of DAF on the surface, respectively, using fluorescence microscopy with antibodies to DAF (data not shown).

For the two bladder epithelial cell lines examined, the percentage of cells with bound bacteria expressing mutant forms of DraE varied greatly compared with wild type (Table 2 and Fig. 3). Bacteria expressing wild-type

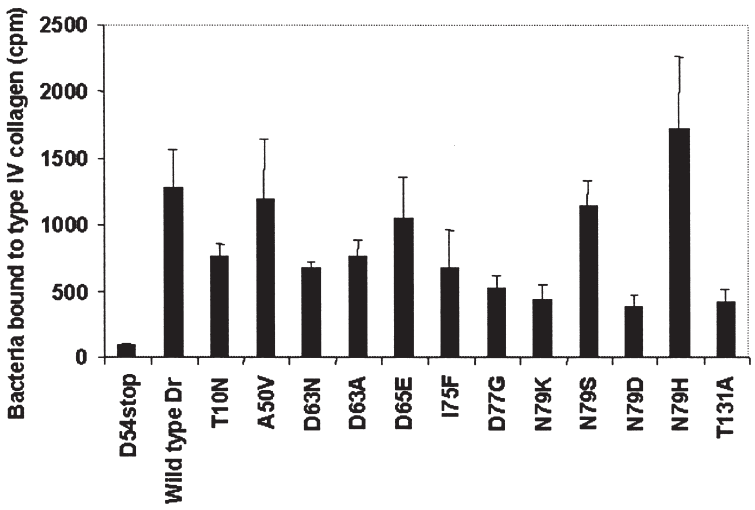

Fig. 2. Type IV collagen binding assay of DAF-binding mutants. Bacteria were labelled with [^{3}H]-thymidine and incubated in microtitre wells coated with 2 μg of type IV collagen. Bound bacteria remaining in the wells were quantified by scintillation counting. D54stop, DH5α (pCC90-D54stop, pUC-Cm). Wild-type Dr, DH5α (pCC90-D54stop, pUC-R). Results shown are the average of three independent experiments with duplicate wells of each sample.

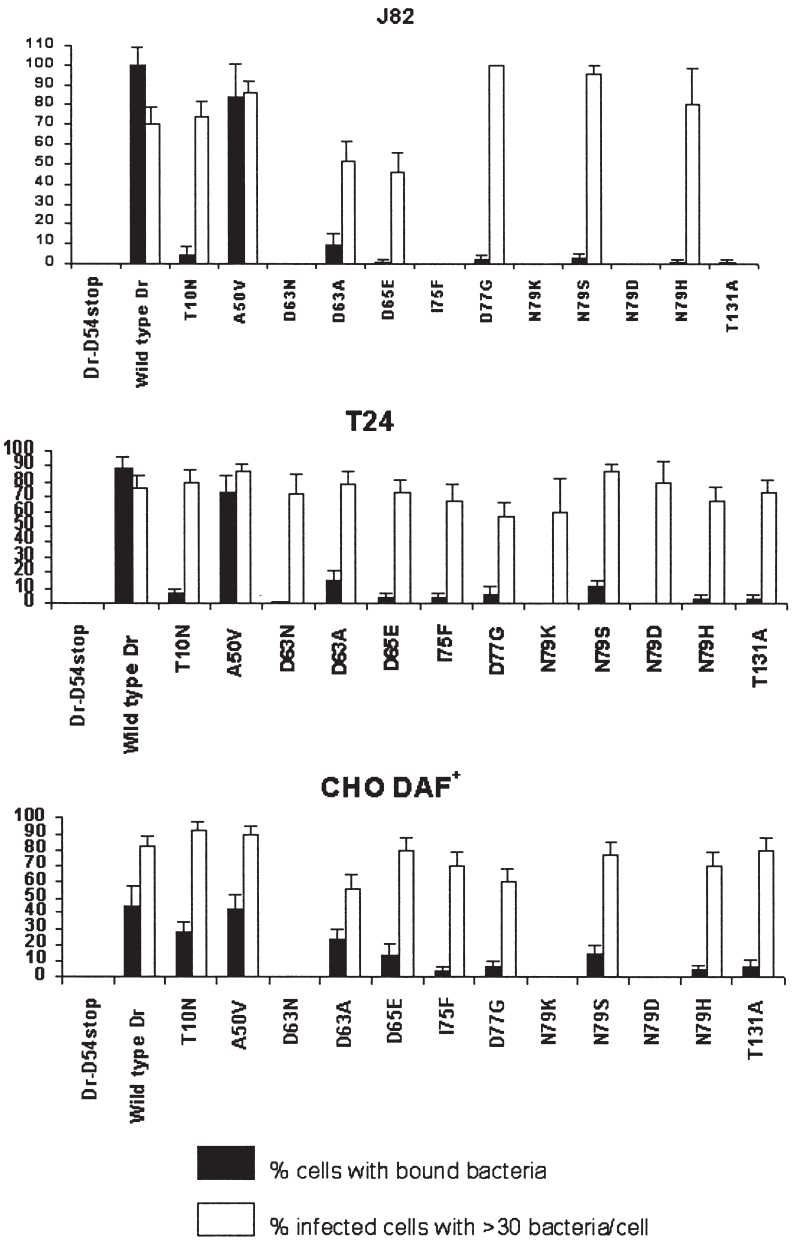

Fig. 3. Adherence assays of DAF-binding mutants. T24, J82, and CHO DAF$^+$ cells were grown to confluency on glass coverslips in 24-well tissue culture plates and 0.5 ml of bacteria at an OD_{600} of 0.6 were added to each well. Coverslips were fixed, stained, and examined by light microscopy. D54stop, DH5α (pCC90-D54stop, pUC-Cm). Wild-type Dr, DH5α (pCC90-D54stop, pUC-R). Black bars,% cells with bound bacteria. Results shown are the average of three independent experiments. Three fields were viewed from each experiment and an average of 90 cells were examined in each experiment. Error bars indicate standard deviation. White bars,% infected cells with >30 bacteria/cell. Adherent bacteria were counted on 30 cells including 10 cells from each of three independent experiments, except where 10 cells could not be found with adherent bacteria. In this case all cells with adherent bacteria were counted. Error bars indicate standard error of proportion.

Dr bound to 90% and 100% of T24 and J82 cells, respectively, whereas the D54stop parent strain did not bind to either cell line. Only mutant A50V bound to T24 and J82 cells at levels equivalent to wild-type Dr Mutants D63N, I75F, N79K and N79D did not bind to J82 cells but still bound to a low percentage of T24 cells. The remaining mutants bound to a small percentage of J82 and T24 cells ranging from 1% to 15%. To determine whether this also reflected a reduction in the number of adherent bacteria per cell, the percentage of infected cells with >30 adherent bacteria was quantified for each of the mutants. Interestingly, the number of adherent bacteria per infected cell was similar for wild-type Dr and the mutants (Fig. 3). The majority of cells with adherent bacteria had >30 bacteria for wild type and for each mutant, with the exception of mutant D65E on J82 cells. In this case, only 46% of cells with adherent bacteria had >30 bacteria. Additionally, mutant N79S had a higher proportion of cells with >30 bacteria compared with wild-type Dr. There was no correlation between the number of adherent bacteria per cell and levels of DAF expression by cells, as determined by staining with fluorescent antibody specific for DAF (data not shown).

These experiments revealed that while some of the mutants retained a degree of DAF binding capability, binding to epithelial cells was significantly reduced for all mutants except A50V. Furthermore, the results demonstrate that specific substitutions at amino acids 63, 75 and

79 in DraE severely reduce or abolish binding to T24 and J82 bladder epithelial cells, indicating that these residues are critical for binding.

Adherence of DAF-binding mutants to CHO cells expressing DAF

The mosaic nature of the binding of some of the mutants to epithelial cells described above was unexpected. If the mutations had simply resulted in a reduced affinity of the fimbrial adhesins for DAF, one might expect to find a similar number of cells with bound bacteria but a reduced number of bound bacteria per cell, or a correlation between the number of bound bacteria and DAF levels. The mosaic binding exhibited by these mutants therefore raises the possibility that the mutations have altered the binding specificity of Dr fimbriae. To examine the specificity of binding of the mutants, Chinese hamster ovary (CHO) cells expressing DAF (CHO DAF$^+$) were used (Lublin and Coyne, 1991). These cells were determined to express variable levels of DAF on the surface as described above (data not shown). As a control, CHO cells transfected with vector alone were also analysed (CHO DAF$^-$). Adherence assays with these two cell lines demonstrated that the mutants that retained binding to T24 and J82 bladder epithelial cells bound to CHO DAF$^+$ cells but not to CHO DAF$^-$ cells, as has been shown previously for wild-type Dr (Nowicki *et al*., 1993) (Table 2 and Fig. 3; data not shown). As with the adherence assays using T24 and J82 cells, some of the mutants bound to a lower percentage of the cells while exhibiting a similar number of bacteria bound per cell as wild-type Dr. However, these mutants bound a higher percentage of the cells than with the other two cell lines, ranging between 4% and 30%. Additionally, mutations at amino acids 63 and 79 abolished binding to CHO DAF$^+$ cells further emphasizing the critical role of these residues for binding to DAF on these cells.

The mutants were additionally examined for specificity of binding by analysing the ability to adhere to CHO DAF$^+$ cells in the presence of anti-DAF antibodies specific for SCR3. This cell line was used due to the higher percentage of cells with bound bacteria, allowing the effect of the antibodies to be more discernible. Binding by wild-type Dr and by the mutants that retained adherence was abolished by treatment with the anti-DAF antibodies (data not shown) as has been shown previously for the Dr haemagglutinin (Nowicki *et al*., 1990; Van Loy *et al*., 2002). These results demonstrate that the mutants are binding to DAF and not another surface exposed molecule.

Analysis of DAF and type IV collagen binding by Dr-D63N, Dr-N79S and Dr-N79H fimbriae

Comparison of the binding phenotypes of the mutants shown in Table 2 indicates that amino acids 63, 75 and 79 have a particularly important role in binding to DAF. Substitutions at these residues abolish cell adherence while the ability to bind type IV collagen is retained. However, some of the phenotypes observed in the previous assays may have resulted from differences in DraE surface expression by the mutants. To examine the direct effects of the mutations in DraE upon its binding properties, fimbriae were removed from the surface of the bacterial cells. Preliminary experiments with crude fimbrial preparations were performed to analyse the binding of the mutant fimbriae to a DAF fusion protein containing SCRs 2, 3 and 4 (DAF234) (Powell *et al*., 1997) and to type IV collagen in an enzyme immunoassay. According to the DAF234 binding phenotypes, the mutants can be divided into three groups (Table 3). D63N, I75F and N79K showed essentially no binding to DAF234. N79D, N79H and T131A showed low binding to DAF234. T10N, A50V, D63A, D65E, D77G and N79S showed wild-type or moderately reduced binding to DAF234. All of the mutant fimbriae bound to type IV collagen at wild type or moderately reduced levels.

Three mutants representative of the phenotypes observed, D63N, N79S and N79H, were selected for a more detailed analysis of their DAF and type IV collagen binding properties. Fimbriae were isolated from the mutants by shearing and separation on a CL-4B Sepharose column. Wild-type Dr fimbriae were isolated

Table 3. DAF234 and type IV collagen binding of crude fimbrial preparations of the DAF-binding mutants.

Mutant	Binding to DAF234 (mean ± SD)[a]	Binding to type IV collagen (mean ± SD)[b]
D54stop	0.9 ± 0.6	0.4 ± 0.6
Wild-type Dr	100.0 ± 4.8	100.0 ± 3.6
D63N	0.3 ± 0.2	66.3 ± 10.6
I75F	0.6 ± 0.2	78.7 ± 8.4
N79K	0 ± 0.7	77.3 ± 7.5
N79D	3.3 ± 1.5	87.4 ± 3.3
N79H	2.9 ± 0.4	93.4 ± 1.3
T131A	28.5 ± 3.0	77.3 ± 6.9
T10N	54.1 ± 18.2	73.4 ± 5.0
A50V	93.0 ± 8.5	39.9 ± 1.1
D63A	71.6 ± 23.9	89.4 ± 2.3
D65E	58.8 ± 4.8	98.4 ± 5.3
D77G	61.2 ± 6.8	63.0 ± 3.0
N79S	61.8 ± 3.3	100.5 ± 2.8

a. The values correspond to percentage of wild-type Dr binding to DAF234 in an enzyme immunoassay. The assays were performed in triplicate. The values represent the average of triplicate wells from a representative assay with standard deviations.
b. The values correspond to percentage of wild-type Dr binding to type IV collagen in an enzyme immunoassay. The assays were performed in triplicate. The values represent the average of triplicate wells from a representative assay with standard deviations.
SD, standard deviation.

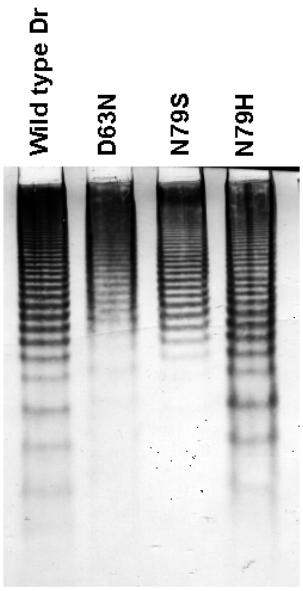

Fig. 4. Immunoblot analysis of fimbrial preparations. Equivalent concentrations of each fimbrial preparation were separated by native gel electrophoresis and blotted onto nitrocellulose. DraE was detected by immunoblot analysis using anti-Dr fimbriae antisera. Wild-type Dr, DH5α (pCC90-D54stop, pUC-R).

for comparison. The use of purified fimbriae allowed a more precise quantitative binding analysis of the wild-type and mutant fimbriae which could not be assessed using the crude fimbrial preparations, since the crude preparations contained additional contaminating proteins which prevented determination of the concentration of DraE.

The isolated fimbriae were examined by native gel electrophoresis to compare the structures formed by each of the mutants to that of wild-type Dr. The mutant fimbriae were similar to the wild-type Dr fimbriae, and were visualized as high molecular weight multimers forming distinct bands of decreasing size (Fig. 4). This shows that these mutations in DraE have not affected the ability of the subunits to polymerize into high molecular weight structures. However, wild-type Dr and N79H fimbriae formed several lower molecular weight multimers that were not observed in D63N and N79S.

The fimbriae were compared for their ability to bind DAF234 and type IV collagen in an enzyme immunoassay. DAF234 or type IV collagen was bound to the wells of microtitre plates and different concentrations of fimbrial proteins were added and detected with anti-Dr fimbriae antisera. As shown in Fig. 5A, fimbriae from the mutants differed in their affinity for DAF. Wild-type Dr fimbriae bound DAF at high levels, D63N fimbriae did not bind to DAF, N79S fimbriae bound to DAF at an intermediate level, and N79H fimbriae bound DAF at a very low level. In contrast, all the mutant fimbriae bound to type IV collagen with an affinity similar to wild type (Fig. 5B). A control preparation made in the same manner from the D54stop parent strain did not bind DAF234 nor type IV collagen (data not shown). These results demonstrate that differences in cell binding phenotypes by bacteria expressing mutant fimbriae are due to differences in interactions between DraE and the receptor, rather than differences in surface expression of the fimbriae.

Predicted structure analysis of DraE

Of the residues we identified as being important for the interaction with DAF, residues 63, 65, 75, 77 and 79 constitute two groups of proximal amino acids, suggesting that they may comprise two loops that form a binding domain for DAF. In particular, the binding assays indicate that amino acids 63 and 79 are critical for binding to DAF. Because of the difficulty of obtaining soluble, monomer adhesins for crystallography studies (Van Loy *et al*., 2002), the structure of DraE has not been determined. This has impeded a further analysis of the effect the mutations in DraE have upon the structure and the interaction with its receptors. Therefore, to gain a better understanding of how the residues identified are involved in binding to DAF, programs in the RAMP software suite were used to create three-dimensional predicted structures of DraE (Samudrala and Moult, 1998a,b; R. Samudrala, unpublished). This method uses the conditional probabilities of pairwise atom–atom distances from proteins with known structures and a hybrid Monte Carlo/genetic algorithm minimization protocol to predict the native folds of proteins with unknown structures. Methods implemented by the RAMP software suite were used to make 11 blind predictions for targets that had no detectable sequence relationships at the fourth Critical Assessment of Structure Prediction experiment (Moult *et al*., 2001). The methods produced nine predictions with accuracies ranging from 4.0 to 6.0 Å root mean square deviation (RMSD) for 60–100 C_α atoms. The DraE structure with the highest probability is shown in Fig. 6. DraE is predicted to form a globular structure. Residues Asp-63, Asp-65, Ile-75, Asp-77 and Asn-79 are indicated in dark grey. These amino acids are predicted to cluster together on one face of the globular molecule to form two proximal, surface-exposed loops. All nine structures examined predicted a similar structure for these amino acids.

Analysis of the effects the mutations would have upon the predicted structure of DraE was performed. Individual amino acid changes for all of the mutants were made in two of the predicted structures and conditional probability scores were calculated. For Asn-79, the structures with amino acid changes N79K and N79D had scores similar to the wild-type DraE structures, indicating that these mutations have little or no effect on the overall structure

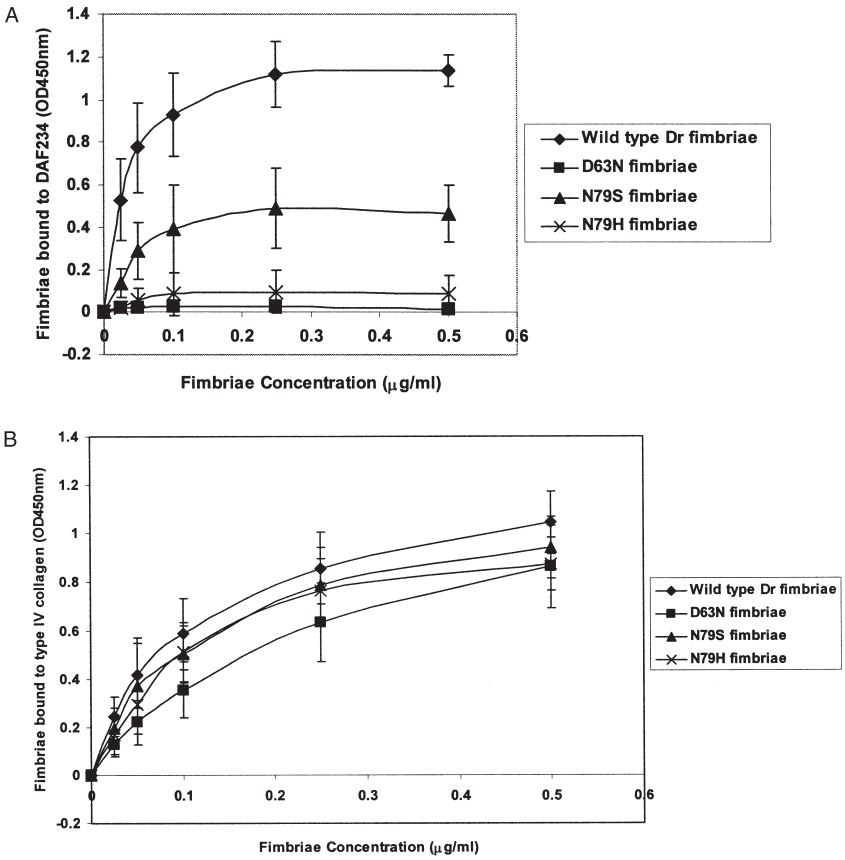

Fig. 5. DAF and type IV collagen binding assays of fimbrial preparations from wild-type Dr, D63N, N79S, and N79H. Fimbriae were purified by shearing and separation on a CL-4B Sepharose column. Varying concentrations of fimbriae were incubated in microtitre wells coated with 1.5 μg of DAF234 or with 2 μg of type IV collagen. Bound Dr fimbriae were detected using anti-Dr fimbriae antisera.
A. DAF234 binding assay of fimbrial preparations.
B. Type IV collagen binding assay of fimbrial preparations.
Results shown are the average of three independent experiments with duplicate wells for each sample.

of the protein. This is consistent with the possibility that changes at this residue may directly affect the interaction with DAF rather than disrupt the local structure of the protein. Amino acid changes T10N, D65E, D77G and T131A had slightly lower scores than wild-type DraE, suggesting that they also have little effect on the overall structure of DraE. The remaining mutations were predicted to have an effect on the local or global structure of DraE, particularly F73S and I75F, which had the lowest scores compared with wild-type DraE. This is consistent with the

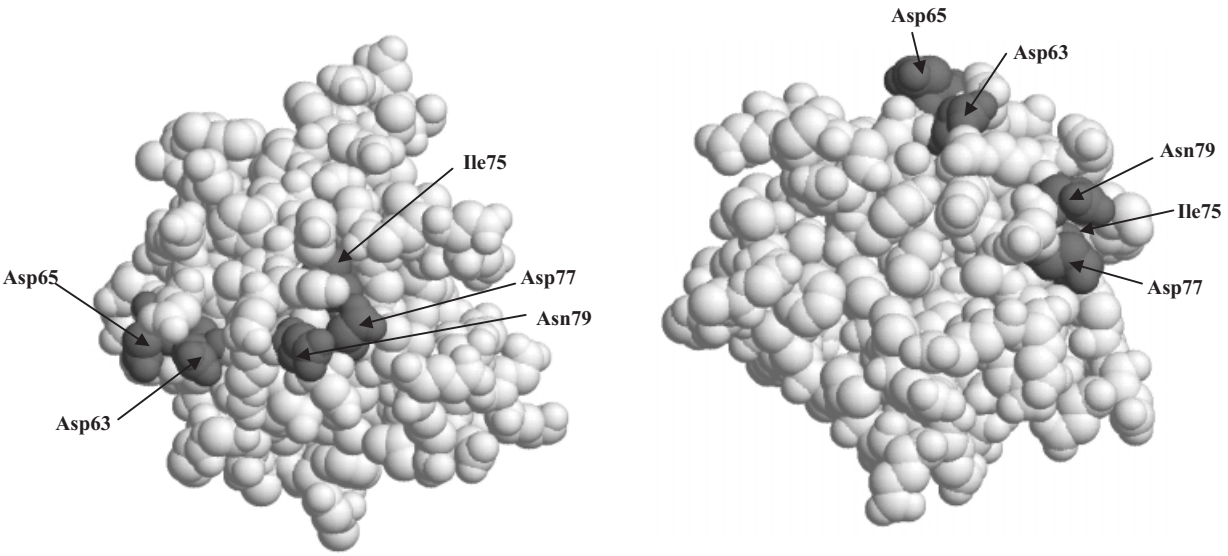

Fig. 6. Predicted structure of DraE. Two views are shown of the structure with the highest probability. Residues identified to be involved in binding to DAF are indicated in dark grey.

significantly reduced DraE expression in the immunoblot analysis of these two mutants.

Discussion

Previous studies of the Dr family of adhesive subunits have not indicated which amino acids are important for binding to the DAF receptor. We chose to use a random mutagenesis approach to find amino acids in DraE, the adhesive subunit of the Dr haemagglutinin, that are involved in binding to DAF. The Dr haemagglutinin provides a model adhesin for this procedure because it binds to two receptors, DAF and type IV collagen. Therefore, we sought to obtain mutants that have changes that affect binding to DAF but do not affect binding to type IV collagen, suggesting that the overall structure of the adhesin is not altered. In this study, we identified 20 mutants that showed altered binding to DAF and had one, two or three amino acid changes within DraE.

The mutations identified are distributed throughout the DraE sequence, which suggests that there is a conformational binding domain for DAF as has been described for type IV collagen (Carnoy and Moseley, 1997). Over half of the mutants obtained had changes in amino acids 63–81, suggesting that this region may be of particular importance for binding to DAF. All of the mutants containing a single amino acid change, except F73S, showed expression of DraE, indicating that the observed phenotypes were not due to instability of the protein. Furthermore, the surface expression assay demonstrated that the mutant DraE proteins were localized to the surface of the bacterial cells. Although this assay provides a qualitative measure of DraE surface expression, it does not provide a quantitative measure of the surface levels of DraE. Therefore, this assay cannot be used to determine if the mutants are forming fimbrial structures of equivalent size compared with those of wild-type Dr.

The collagen binding assay showed that the DAF-binding mutants bound to type IV collagen but at varying levels. This variation in binding may be due to variation in surface expression of DraE as the isolated fimbrial preparations that were normalized for DraE showed binding similar to wild-type Dr (Table 3 and Fig. 5B). It is also possible that the fimbrial structures of these mutants are not stable and can break off from the cell resulting in reduced bacterial adherence. The binding assays performed with the fimbrial preparations are therefore more sensitive than the whole cell binding assays and may allow a better understanding of the effects the mutations have upon binding to type IV collagen.

Only one of the mutants obtained, A50V, showed binding that was not inhibited by the presence of chloramphenicol. Previously, another residue in this vicinity, Asp-54, has been shown to be involved in inhibition by chloramphenicol (Carnoy and Moseley, 1997). The mechanism of chloramphenicol inhibition remains to be elucidated. A50V was also the only mutant obtained that could not mediate MRHA but exhibited wild-type levels of binding in the other functional assays examined. This mutation may have lowered the affinity for DAF, which would result in an MRHA$^-$ phenotype, but still retain cell adherence due to the different nature of these assays. This mutation may also have resulted in fragile fimbriae that break more easily and cannot sustain MRHA due to sheer stress. The results with this mutant suggests that Ala-50 may not have a direct role within the binding site for DAF.

The adherence studies with cell lines expressing DAF showed that although none of the mutants mediated MRHA, some bound to cells expressing DAF. These differences may be due to the properties of each assay. Those mutants that are MRHA$^-$ but adhere to tissue culture cells may have less stable fimbrial structures that cannot mediate agglutination of cells expressing DAF. The MRHA$^-$ phenotype may also be due to a lower affinity for DAF. It may also be associated with the complex pattern of adherence that resulted in mosaic binding that was observed for some of the mutants. This phenomenon was not due to binding to another molecule on the surface of the cells because binding was inhibited by an anti-DAF antibody specific for SCR3. Furthermore, bacteria that bound to CHO DAF$^+$ cells did not bind to CHO DAF$^-$ cells (data not shown). This mosaic binding does not appear to correlate with DAF expression levels since cells with bound bacteria did not consistently show higher DAF expression as examined by fluorescence microscopy (data not shown). It is possible that the mutants are binding to alternate forms of DAF that are only expressed by a small subpopulation of human bladder cells, but a larger proportion of CHO DAF$^+$ cells. One variant of surface-associated DAF, termed DAF-2 has been described. This variant has been shown to involve covalent dimerization of DAF. Additionally, it has been found that DAF-2 is expressed in CHO DAF$^+$ cells (Nickells *et al.*, 1994). This possible role of DAF-2 in the mosaicism observed remains to be explored.

The fimbriae of three representative mutants, D63N, N79S and N79H, were isolated and examined quantitatively for their binding to DAF and type IV collagen. The native gel analysis of the fimbriae indicated that although all of the mutant fimbriae assemble into high molecular weight multimers similar to wild-type Dr fimbriae, D63N and N79S fimbriae did not form as many low molecular weight multimers. It is possible that these mutations may have increased the affinity of the DraE subunits for each other, resulting in greater stability of the higher molecular weight structures. The DAF binding assay demonstrated that equal concentrations of fimbriae showed different

levels of binding. The collagen binding assay demonstrated that the three mutants bound to type IV collagen at a similar level as wild-type Dr. Therefore, although the binding domain for DAF has been altered, the binding domain for type IV collagen has not been affected. This also confirms that DAF and type IV collagen binding are separable phenotypes.

The functional studies identified two regions in DraE that include residues 63, 65, 75, 77 and 79 that are important for binding to DAF. Analysis of predicted structures of DraE suggests that these residues cluster to one face of the protein, implying that these residues may form part of a binding pocket for DAF constituted by two proximal surface exposed loops. The RMSD accuracies obtained from the RAMP predictions indicate that the general topologies of the target proteins are predicted accurately. Similar results have been observed in internal tests in which we compared predicted structures to known experimental results, where we are able to predict 70–80% of the proteins to a topological level (for three test sets consisting of >30 proteins) (Samudrala, 2002a,b; R. Samudrala and M. Levitt, unpublished). We therefore expect that there is a 70–80% probability that the general topology of DraE is accurately predicted. Additionally, the mutagenesis results indicate that amino acids 63 and 79 are critical for binding to DAF, which would support this prediction. Residues that have been previously implicated in type IV collagen binding (Carnoy and Moseley, 1997) do not show the clustering that was observed with the DAF-binding residues (data not shown). This is of interest because in contrast to Dr fimbriae, purified DraE adhesive subunits do not bind to type IV collagen (Van Loy *et al*., 2002). This observation is consistent with the hypothesis that the type IV collagen binding site is comprised of multiple fimbrial subunits as opposed to the DAF binding site that is contained within a single DraE subunit.

Taken together, the results of this study suggest that amino acids 10, 63, 65, 75, 77, 79 and 131 have important roles in binding to the DAF receptor. In particular, residues 63–81 may be critical for this function. This provides the first characterization of a DAF binding region in a Dr family adhesin. These mutants together with previously described mutants that retain DAF binding but have lost collagen binding may be valuable in determining the relative importance of each receptor, DAF and type IV collagen, during establishment of experimental infections in the urinary tract by *E. coli* expressing Dr fimbriae. Additionally, these results can aid in determining the residues in other Dr family members that are necessary for binding to DAF.

Experimental procedures

Strains and plasmids

Bacterial strains were grown in Luria–Bertani (LB) or Super Broth (SB) medium at 37°C (Maniatis *et al*., 1982). Derivatives of pUC-Cm were grown in the presence of 25 μg ml^{-1} chloramphenicol. Derivatives of pCC90-D54stop were grown in 100 μg ml^{-1} ampicillin. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) was used at a concentration of 80 μg ml^{-1}. *E. coli* DH5α (Life Technologies, Rockville, MD) was host for the plasmids. Plasmid DNA was prepared using the Easyprep method (Berghammer and Auer, 1993). Enzymes were purchased from New England Biolabs (Beverly, MA) and used as recommended by the manufacturer.

PCR mutagenesis of draE

draE was subjected to PCR mutagenesis based on previous methods (Cadwell and Joyce, 1992; Muhlrad *et al*., 1992). Briefly, standard PCR reactions were carried out using reaction buffer (0.166 M $(NH_4)_2SO_4$, 0.67 M Tris-Cl, pH 8.8, 0.067 M EDTA, pH 8.0), 0.17 mg ml^{-1} bovine serum albumin, 10% dimethyl sulphoxide, 10 mM β-mercaptoethanol, 0.2 mM $MnCl_2$, 3 mM $MgCl_2$, Taq DNA polymerase, 0.2 mM dGTP, 0.2 mM dATP, 1 mM dTTP and 1 mM dCTP. The pCC90 vector containing the *draB–E* genes was used as a template at a final concentration of 0.5 ng (Carnoy and Moseley, 1997) with the following primers for the *draE* gene: 5ranmut, 5′-CCCGCCCGCCGATTCGGGGTAAGACAGC-3′ and 3ranmut, 5′-CCCGCCCGCCCTGCAGTCATTTTGCCCAGT AACC-3′. The final concentration of each primer was 1.25 μM. Following the PCR reaction, the product was quantified and digested with *Pst*I and *Eco*RI and inserted into pUC-Cm. The resulting plasmids (pR-X, containing X amino acid change) were transformed into *E. coli* DH5α (pCC90-D54stop) (Carnoy and Moseley, 1997). This strain contains the other necessary genes from the *dra* operon for fimbrial expression with a premature stop codon at codon 54 within *draE*; no full-length DraE can be detected in this strain. The resulting transformants were selected on LB medium containing X-gal, ampicillin and chloramphenicol.

A wild-type copy of the *draE* gene was PCR amplified using pCC90 as a template with the primers 5ranmut and 3ranmut. After amplification, the PCR product was digested with *Pst*I and *Eco*RI and inserted into pUC-Cm. The resulting plasmid (pUC-R) was transformed and selected as described above. The construct was confirmed by sequencing using Big Dye Terminator method and ABI sequencing (PE Applied Biosystems, Foster City, CA). A vector control strain was also generated by transformation of pUC-Cm into *E. coli* DH5α (pCC90-D54stop).

Erythrocyte suspension preparation and MRHA assays

The human erythrocyte suspension was prepared from blood group O whole human blood donated by a healthy volunteer. The whole blood samples were diluted to 2% or 8% erythrocyte suspensions in phosphate-buffered saline (PBS). Erythrocytes were washed twice in PBS and then resuspended in PBS containing 2% D-mannose. The suspensions were stored at 4°C for up to two weeks. Mannose resistant haemagglutination (MRHA) assays were performed by resuspending bacterial strains in PBS to an OD_{600} of 0.6 and mixing with an equal volume of 2% erythrocyte suspension.

Enrichment for DAF-binding mutants

Transformants containing the mutated *draE* genes were resuspended in PBS to an OD_{600} of 0.15. Two millilitres of the bacterial suspension were mixed with 2 ml of an 8% human erythrocyte suspension followed by rocking for 30 min. The erythrocytes were then pelleted at 120 *g* for 5 min and the supernatant was added to 2 ml of freshly pelleted erythrocytes from an 8% suspension so as not to increase the volume of the suspension. These steps were repeated successively a total of seven times. The final supernatant was then collected and plated on LB medium containing ampicillin, chloramphenicol and X-gal. White colonies were selected and examined for erythrocyte binding by MRHA. Those transformants that were $MRHA^-$ were analysed for type IV collagen binding.

Type IV collagen binding growth assay and sequencing of mutants

Those transformants that were $MRHA^-$ were examined for the ability to bind type IV collagen as described previously (Sokurenko and Hasty, 1995). Type IV collagen (from human placenta, Sigma-Aldrich, St Louis, MO) was used to coat 96-well microtitre plates at a concentration of 20 μg ml^{-1} in PBS at 4°C overnight. The wells were blocked with PBS containing 1% BSA for 1 h at 37°C. Then, 100 μl of an overnight culture of each transformant were added to individual wells and allowed to bind for 45 min at 37°C. The unbound bacteria were removed with PBS and 150 μl of Super Broth were added to each well. The plates were incubated with shaking at 37°C for 4 h. The resulting growth was then measured at OD_{600}. Those transformants that exhibited growth were further confirmed for collagen binding using the same assay and were retained for analysis.

The transformants that were $MRHA^-$ but were positive for type IV collagen binding were subjected to sequence analysis to identify the resulting base pair changes within *draE*. Sequencing was performed with Big Dye Terminator Reagent by ABI Sequencing (PE Applied Biosystems, Foster City, CA).

Immunoblot analysis for DraE expression

Bacterial strains were resuspended in PBS to an OD_{600} of 1.0 and 20 μl aliquots were mixed with sample buffer containing DTT and boiled for 5 min. The samples were loaded onto a 15% polyacrylamide gel and separated by SDS–PAGE. The proteins were transferred onto nitrocellulose and the membrane was blocked in PBS containing 0.1% Tween 20 and 5% non-fat milk for 1.5 h. The membrane was washed and anti-Dr fimbriae rabbit antisera (Bilge *et al*., 1989; Carnoy and Moseley, 1997) was added at a 1:500 dilution for 1 h. A goat anti-rabbit alkaline phosphatase (AP) conjugated antibody (Pierce, Rockford, IL) was then added at a 1:5000 dilution for 1 h. The blot was developed using nitro blue tetrazolium/5-bromo-4-chloro-3-indolylphosphate (NBT/BCIP) in AP assay buffer (0.1 M Tris pH 9.5, 5 mM $MgCl_2$, 100 mM NaCl). All washes were performed with PBS containing 0.1% Tween 20. All antibodies were diluted in PBS containing 2% BSA and 0.1% Tween 20.

Surface expression assay

Anti-Dr fimbriae antibodies were bound to the wells of a 96-well microtitre plate at a 1:500 dilution of specific antisera in PBS at 4°C overnight. The wells were blocked with PBS containing 1% BSA for 1 h at 37°C. The different bacterial strains were grown in SB supplemented with 10 μCi ml^{-1} of [^{3}H]-thymidine overnight at 37°C. The cells were then pelleted and resuspended in PBS and adjusted to a final OD_{600} of 2.0. 50 μl of each of the bacterial strain suspensions were added to the wells of the microtitre plate together with 50 μl of PBS containing 0.2% BSA and allowed to bind for 1 h at 37°C. Unbound bacteria were removed by washing with PBS and the wells were dried at 60°C. The wells were then placed in scintillation fluid and quantified by scintillation counting. Efficiency of labelling of the bacterial cells with [^{3}H]-thymidine was the same for all of the bacterial strains used.

Radioactive collagen binding assay

This assay was performed in the same manner as the surface expression assay with the following modifications. Plates were coated with type IV collagen at a concentration of 20 μg ml^{-1} in PBS. Bacterial strains were adjusted to a final OD_{600} of 1.4 before addition to the coated plates.

Tissue culture

All cell lines were cultured in a 37°C incubator with 5% CO_2 and split every 2–3 days. Chinese hamster ovary cells with DAF (CHO/DAF/A9, CHO DAF^+) and without DAF (CHO/SFFV/8G*, CHO DAF^-) were a generous gift from Dr Douglas Lublin at Washington University. CHO cells were cultured in Ham's F12 medium supplemented with 10% fetal bovine serum (FBS), 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 25 μg ml^{-1} G418 (Geneticin, Life Technologies, Rockville, MD). T24 bladder epithelial cells (HTB-4) were obtained from ATCC (Manassas, VA) and were cultured in McCoy's 5A medium supplemented with 10% FBS, penicillin and streptomycin. J82 bladder epithelial cells (HTB-1) were a generous gift from Dr Robert Vessella at the University of Washington and were cultured in minimum essential medium (MEM) supplemented with 10% FBS, penicillin and streptomycin. All cell lines were passaged using 0.25% trypsin containing EDTA.

Adherence assays

CHO DAF^+, CHO DAF^-, T24 and J82 cells were split into 24-well plates with glass coverslips and grown to confluency in the appropriate culture medium. Before the assay, cells were washed twice with Hanks' balanced saline solution (HBSS) and incubated with fresh medium without antibiotics and without FBS for 1 h. The different bacterial strains were grown overnight on LB medium and harvested and resuspended in PBS to an OD_{600} of 0.6. The bacterial cells were pelleted at 2700 *g* for 5 min and resuspended in the corresponding tissue culture medium for each cell line. Then 0.5 ml of each bacterial strain were added to each well. The plates were incubated on ice for 1 h to allow bacterial cells to bind. The wells

were then washed five times with HBSS and stained using the Diff-Quik stain set as recommended by the manufacturer (Dade Behring, Neward, DE). The coverslips were air dried, mounted onto glass slides and viewed by light microscopy. Three fields of cells were counted from each coverslip (average of 90 cells total). The experiment was repeated in triplicate. Anti-SCR3 antibody IC6 (Wako Bioproducts, Richmond, VA) was used for inhibition of adherence at a 1:50 dilution and was added to the tissue culture cells for 1 h on ice prior to addition of bacterial cells.

DAF234 expression and purification

DAF containing short consensus repeats (SCRs) 2, 3 and 4 and an oligohistidine tag at the C-terminal end (DAF234) was purified from *Pichia pastoris*-DAF234, generously provided by Dr Susan Lea (Oxford University, Oxford, UK). DAF234 was expressed in *Pichia pastoris* and purified from the supernatant of induced cultures by nickel chromatography as described previously (Powell *et al*., 1997). Briefly, *P. pastoris* was grown on yeast extract peptone dextrose (YPD) medium supplemented with 4 mg ml^{-1} G418 at 30°C and used to inoculate buffered minimal glycerol (BMG) medium. After overnight culture the cells were pelleted by centrifugation at 3000 *g* for 20 min. The pellet was then resuspended in buffered minimal methanol (BMM) medium to an OD_{600} of 1.0. The cells were incubated with shaking at 30°C for 72 h with the addition of methanol to a final volume of 0.5% of the total culture every 24 h for induction. The culture was then adjusted to a final concentration of 20 mM imidazole, pH 8.0 and centrifuged at 3000 *g* for 30 min. The supernatant was collected and DAF234 was purified by affinity chromatography using HisBind resin matrix with immobilized Ni^{2+} (Novagen, Madison, WI). The column was first equilibrated with binding buffer (20 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 8.0) and then the supernatant was added over the column. The column was washed with binding buffer and DAF234 was eluted using 300 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 8.0. The protein was aliquoted and stored at –80°C.

Crude fimbrial preparation binding analysis

Bacterial strains were grown overnight on LB agar and resuspended in PBS. The suspensions were then vortexed for 30 min to remove fimbriae from the surface of the cells. The bacterial cells were removed by centrifugation at 14 000 *g* and the levels of DraE in the supernatants were determined by immunoblot analysis as described above. The levels of DraE were normalized and the supernatants were analysed for binding to DAF234 and type IV collagen in an enzyme immunoassay (EIA) as described below for the purified Dr fimbriae with the exception that 50 µl of the crude supernatants were added to the coated plates together with 50 µl of PBS containing 0.1% Tween 20.

Purification of wild-type and mutant Dr fimbriae

Bacterial strains were grown in SB overnight. The cultures were centrifuged at 5000 *g* for 15 min and the pellet resuspended in 10 mM Tris-HCl, pH 7.0. The bacterial suspension was subjected to mechanical shearing in a blender (Hamilton Beach) to remove the fimbriae and the cells were pelleted by centrifugation at 12 000 *g* for 20 min. The fimbriae were then precipitated from the supernatant with 40% ammonium sulphate and centrifuged at 12 000 *g* for 20 min. The pellet was resuspended in 50 mM phosphate, 50 mM NaCl, 2 M urea, pH 7.4 buffer and dialysed for 48 h in the same buffer. The fimbriae were then purified by gel-filtration chromatography using a sepharose CL-4B column (Amersham Pharmacia Biotech, Piscataway, NJ) in the same buffer. Eluted fractions containing fimbriae were identified by SDS–PAGE and were pooled and dialysed extensively against PBS. Fimbrial samples were stored at 4°C. A control preparation was made in the same manner from the D54stop parent strain and the same fractions that contained fimbriae in the other samples were collected for the negative control and dialysed extensively against PBS. Type 1 fimbriae were found in the fimbrial samples at a very low concentration. However, these did not bind to DAF nor type IV collagen and they did not cross-react with the anti-Dr fimbriae antisera.

The fimbrial samples were examined by non-denaturing gel electrophoresis. Fimbriae were separated on an 8% non-denaturing polyacrylamide gel, blotted onto nitrocellulose and examined by immunoblot analysis as described above.

DAF and type IV collagen binding EIAs of purified fimbriae

DAF234 was diluted in PBS to a concentration of 15 or 30 µg ml^{-1} and 100 µl were added to each well of a 96-well microtitre plate and incubated at 4°C overnight. These concentrations were determined to provide maximal and consistent coating of the well surfaces. The wells were then washed with PBS and blocked with PBS containing 0.1% Tween 20 for 2 h at 37°C. The blocking solution was removed and 100 µl of different concentrations of the fimbrial preparations were added in PBS containing 0.05% Tween 20 at varying concentrations and incubated for 1 h at 37°C. Following various washes with PBS, the bound proteins were detected with anti-Dr fimbriae antisera at a 1:500 dilution for 1 h at 37°C. This dilution of anti-Dr fimbriae antisera was determined to bind equally to wild-type fimbriae and the mutant fimbrial samples examined. The bound anti-Dr antibodies were detected with goat anti-rabbit horseradish peroxidase (HRP) conjugated antibodies at a 1:5000 dilution for 30 min at 37°C. All antibodies were diluted in PBS containing 0.05% Tween 20. The bound antibodies were quantified using TMB Peroxidase substrate as directed by the manufacturer (Bio-Rad, Hercules, CA). A PBS control was used to subtract background binding values due to the antibodies. The type IV collagen binding EIA was performed in the same manner with the exception that 20 µg ml^{-1} of type IV collagen was used to coat the wells of microtitre plates.

DraE structure prediction

Structure prediction was performed using selected programs in the RAMP software suite (http://compbio.washington.edu/ramp/), mcgen_semfold_ss, potential, and scgen_mutate,

which have performed well in the CASP blind prediction experiments (Samudrala and Moult, 1998a,b; R. Samudrala and M. Levitt, unpublished data). Eleven high probability structures were selected using a primary scoring function as described (Samudrala and Moult, 1998a). The resulting structures were analysed visually using RasMol 2.7.1 software (Sayle and Berstein, 1999). The mutations identified were created in the parent structure and the conditional probability scores of protein conformation were compared to that of the parent structure to identify those mutations that have an effect upon the overall structure (Lee and Levitt, 1991).

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