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Functional Flexibility of the FimH Adhesin: Insights from a Random Mutant Library

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Type 1 fimbriae are surface organelles of *Escherichia coli* which mediate D-mannose-sensitive binding to different host surfaces. This binding is conferred by the minor fimbrial component FimH. Naturally occurring variants of the FimH protein have been selected in nature for their ability to recognize specific receptor targets. In particular, variants that bind strongly to terminally exposed monomannose residues have been associated with a pathogenicity-adaptive phenotype that enhances *E. coli* colonization of extraintestinal locations such as the urinary bladder. In this study we have used random mutagenesis to specifically identify nonselective mutations in the FimH adhesin which modify its binding phenotype. Isogenic *E. coli* clones expressing FimH variants were tested for their ability to bind yeast cells and model glycoproteins that contain oligosaccharide moieties rich in either terminal monomannose, oligomannose, or nonmannose residues. Both the monomannose- and the oligomannose-binding capacity of type 1 fimbriae could be altered by minor amino acid changes in the FimH protein. The monomannose-binding phenotype was particularly sensitive to changes, with extensive differences in binding being observed in comparison to wild-type FimH levels. Different structural alterations were able to cause similar functional changes in FimH, suggesting a high degree of flexibility to target recognition by this adhesin. Alteration of residue P49 of the mature FimH protein, which occurs within the recently elucidated carbohydrate-binding pocket of FimH, completely abolished its function. Amino acid changes that increased the binding capacity of FimH were located outside receptor-interacting residues, indicating that functional changes relevant to pathogenicity are likely to be due to conformational changes of the adhesin.

Bacterial adherence is normally critical for successful colonization of a specific host tissue. The best-characterized group of bacterial adhesins is constituted by fimbriae (11). Type 1, or mannose-sensitive, fimbriae are found on the majority of *Escherichia coli* strains and are widespread among other members of the *Enterobacteriaceae* (15). Interaction between type 1 fimbriae and receptor structures plays a key role in the colonization of various host tissues by *E. coli* (1, 36). Also, in certain strain backgrounds, type 1 fimbriae can be regarded as virulence factors. Indeed, we and others have previously shown that the expression of type 1 fimbriae in *E. coli* is linked to urinary tract pathogenesis (5, 22). In mouse models, immunizations with FimH and synthetic FimH peptides were shown to prevent urogenital mucosal infection by *E. coli* (19, 35).

A typical type 1 fimbriated bacterium has 200 to 500 peritrichously arranged fimbriae on its surface. A single type 1 fimbria is a 7-nm-wide, approximately 1-μm-long rod-shaped structure consisting of four different components that are added to the base of the growing organelle (21). The bulk of the structure is made up of about 1,000 copies of the major subunit protein, FimA, polymerized into a right-handed helical structure, but small quantities of the minor components, FimF, FimG, and FimH, are also present (12, 18). The receptor-recognizing element of type 1 fimbriae is the 30-kDa FimH protein (17). The FimH protein is located at the tip of the fimbria and is also interspersed along the fimbrial shaft (9, 17). The FimF and FimG components are probably required for integration of the FimH adhesin into the fimbriae (9, 12).

The components of the fimbrial organelle are encoded by the chromosomally located *fim* gene cluster (14). In addition to the structural components, this 9.5-kb DNA segment encodes the fimbrial biosynthesis machinery as well as regulatory elements (Fig. 1). The fimbrial organelle components, FimA, FimF, FimG, and FimH, are produced as precursors having an N-terminal signal sequence. This sequence is subsequently removed during export across the inner membrane. Thus, the FimH protein is produced as a precursor of 300 amino acids that is processed into a mature form of 279 amino acids (7, 12). Further export from the periplasm and across the outer membrane is dependent on a fimbria-specific export and assembly system constituted by the FimC and FimD proteins (8, 10, 13).

By virtue of the FimH adhesin, type 1 fimbriae mediate adhesion to a variety of mannosylated glycoproteins. The affinity of FimH variants toward mannose targets can vary due to changes in the primary structure of this protein. In about 80% of fecal *E. coli* isolates, the FimH adhesin is capable only of binding to trimannose receptors. In contrast, the FimH adhesins from approximately 70% of urinary tract isolates carry minor mutations (compared to the fecal isolates) which enhance their ability to recognize monomannose receptors (33). The mutant alleles confer a significantly higher tropism for the uroepithelium and dramatically enhance the ability of *E. coli* to colonize the mouse urinary tract (31). Some of the monomannose-binding *E. coli* strains are also capable of recognizing complex oligosaccharides with no terminally exposed mannose residues (34). Additionally, a recent study has revealed that FimH adhesins from meningitis-associated isolates of *E. coli* confer binding to collagens and that this specificity change is

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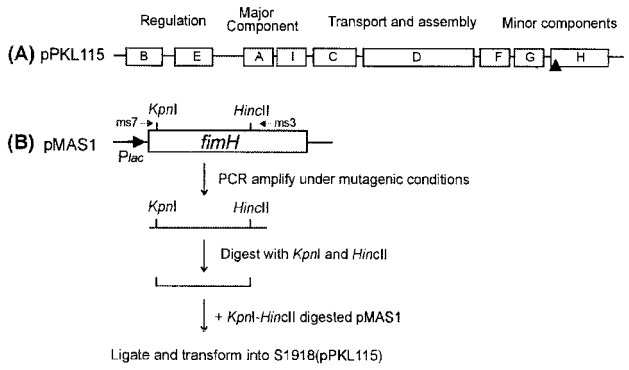

FIG. 1. Overview of the plasmids used to display FimH variants. Only relevant nonvector regions are shown. (A) Plasmid pPKL115 contains the entire *fim* gene cluster with a translational stop linker inserted into the *fimH* gene (triangle). (B) The *fimH* expression vector pMAS1 is shown along with the strategy employed to introduce random mutations into the receptor recognition domain of the *fimH* gene. Primers ms3 and ms7 are described in Materials and Methods.

due to a minor variation in the amino acid sequence of FimH (25).

All FimH variants and their corresponding adhesive profiles described hitherto have been characterized from fecal or clinical strains selected to play a specific role in nature, namely, interaction with mammalian host surfaces. Naturally occurring *fimH* mutants, adapted to enhance the colonization of either commensal intestinal or pathogenic extraintesintal niches, might therefore have a relatively tight group of receptor affinities that can be invoked only by highly specific structural changes. With a view to probing the binding potential of the FimH adhesin more extensively, we have created a random mutant library based on the *fimH* gene from *E. coli* K-12 strain PC31 and analyzed the functional impact of nonselective mutations.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The strains and plasmids used in this study are listed in Table 1. The *E. coli* K-12 strain HB101 (F′ *lacI kan*) (2) was used as an intermediate host during plasmid construction work. All subsequent phenotypic analyses were performed with the *E. coli* Δ*fim* strain S1918 (3). Cells were grown in Luria-Bertani (LB) broth (26) supplemented with the appropriate antibiotics.

DNA techniques. Plasmid DNA was isolated using the QIAprep spin plasmid kit (Qiagen). Restriction endonucleases were used as specified by the manufacturer (Biolabs or Pharmacia). The nucleotide sequences were determined on both DNA strands by the dideoxynucleotide chain termination method (27). Oligonucleotide primers were purchased from Gibco BRL. The primers ms7 (5′-TACCTGTTTGCTGTACTGCTGATGG) and ms3 (TTGCCGTTAATCC CAGACTCAC) were used.

Construction of the *fimH* mutant library. The 656-bp *Kpn*I-*Hinc*II fragment of the *fimH* gene was mutagenized by nucleotide misincorporation during suboptimal PCR conditions. Four reactions were performed with three of the four nucleotides present at 50 mM and the other present at 5 mM. Each reaction mixture also contained 7 mM $MgCl_2$ to increase the stability of noncomplementary base pairs and 0.5 mM $MnCl_2$ to reduce the template specificity of the polymerase. The error-prone PCR procedure was performed for 35 cycles with two primers (ms7 and ms3) that flank the *Kpn*I and *Hinc*II sites of the *fimH* gene. The amplification products were combined, digested with *Kpn*I and *Hinc*II, purified after agarose gel electrophoresis, and religated into similarly cut plasmid pMAS1 to construct a library of altered *fimH* genes. This ligation mix was used to transform S1918(pPKL115) cells. The transformation mixture was made up to 10 ml, grown to approximately 10 times the initial library diversity, and stored as aliquots at −80°C in 25% (vol/vol) glycerol.

Construction of defined *fimH* mutants. Gene changes encoding specific amino acid substitutions were introduced into the wild-type *fimH* sequence by exchange of restriction fragments from the mutant *fimH* genes. Unique restriction sites within the *fimH* gene permitted the exchange of these fragments using standard cloning procedures. Each construct was analyzed by restriction mapping and subsequent nucleotide sequencing. Plasmids containing these chimeric *fimH* genes were introduced into S1918(pPKL115) and tested in adhesion assays.

Agglutination of yeast cells. The capacity of bacteria to express a D-mannose-binding phenotype was assayed by their ability to agglutinate yeast (*Saccharomyces cerevisiae*) cells on glass slides. Aliquots of washed bacterial suspensions at an optical density at 550 nm (OD_{550}) of 0.5 and 1% yeast cells were mixed, and the time until agglutination occurred was measured. Furthermore, clones which did not cause any agglutination under these conditions were also tested at OD_{550} = 20 and/or low temperature but still did not react.

Yeast cell aggregation assay. Agglutination titers were determined by mixing a suspension of *E. coli* cells (serially diluted from OD_{530} = 0.4) with yeast cells (5 mg ml^{-1} in phosphate-buffered saline [PBS]). Aggregation was monitored visually, and the titer was recorded as the highest dilution giving a positive aggregation result. For inhibition experiments, bacteria (OD_{530} = 0.4) and yeast cells were mixed with serial dilutions of methyl-α-D-mannopyranoside solution and the results were recorded as the highest dilution able to inhibit aggregation.

Adhesion assays. Adhesion assays were performed essentially as previously described (30). In short, wells were coated with either yeast mannan, bovine RNase B (Sigma), or human plasma fibronectin (obtained as described in reference 34) at a concentration of 10 μg ml^{-1}, washed three times with PBS, and quenched with 0.2% bovine serum albumin (BSA) in PBS. Bacterial suspensions containing identical cell numbers (10^7 CFU/100 μl) were added to the wells and incubated at 37°C for 40 min. For determination of the mannose sensitivity of the binding, this incubation was performed in the presence of 1% methyl-α-D-mannopyranoside. The wells were washed with PBS, and the number of bound bacteria was determined by a growth assay as previously described (33).

Bacterial interaction with anti-type 1 fimbriae and anti-FimH antiserum was determined as follows. Polyclonal antiserum against purified type 1 fimbriae of *E. coli* K-12 or a naturally occurring FimH truncate (gift of S. Hultgren, Washington University, St. Louis, Mo.) was diluted 1:500 in 0.02 M sodium bicarbonate (Sigma) and used to coat microtiter plates as described previously (30). Binding of the bacteria to the immobilized antisera (in the presence of 2% methyl-α-D-mannopyranoside) was determined as described above.

RESULTS

Construction of a *fimH* mutant library. The FimH adhesin confers the ability to bind to various receptor targets by virtue of an NH_2-terminal receptor-binding domain (16, 19). To identify specific amino acids involved in receptor target recognition, we used the FimH expression vector pMAS1 (28). This vector contains the *fimH* gene from *E. coli* K-12 strain PC31 (14) in pUC19 under transcriptional control of the *lac* promoter. In addition, the plasmid contains unique *Kpn*I and *Hinc*II recognition sequences within the *fimH* gene, which flank the region encoding the proposed FimH receptor-binding domain (Fig. 1). Random mutagenesis was performed on the 656-bp *Kpn*I-*Hinc*II fragment of *fimH* using a modified PCR and *Taq* DNA polymerase. This polymerase possess an intrinsic error frequency under optimal conditions of approximately 10^{-4} to 10^{-5} error per bp (37). The error-prone PCR amplification products were digested with *Kpn*I and *Hinc*II and religated into similarly cut plasmid pMAS1 to reconstruct a library of altered *fimH* genes (Fig. 1). To permit expression of the corresponding FimH variants as functional constituents of type 1 fimbriae, the ligation mix was transformed into *E. coli* strain S1918 (Δ*fim*) containing an auxiliary plasmid, pPKL115, which encodes the entire *fim* gene cluster except *fimH*. Using this approach, we have specifically targeted our mutagenesis to the region encompassing amino acids 8 to 225 of the mature FimH protein.

Selection of clones with altered FimH phenotypes from the mutant library. To determine the phenotypic mutant frequency of the FimH library, 300 transformant colonies were randomly picked and screened for their ability to agglutinate yeast cells, the traditional assay for monitoring type 1 fimbria-mediated binding. Of these, 44% were negative and 56% positive. Fifteen clones from the negative group and 50 clones from the positive group were randomly picked for further characterization of their binding profiles and tested for their ability to bind model receptor-specific targets. As relevant target substrates, we used yeast mannan (Mn), bovine RNase B (RB), fibronectin (Fn), and BSA. Mn represents the model substratum for terminal monomannose-specific binding, RB

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant genotype	Reference or source
E. coli strains		
HB101	F′ *laqI*^{q}	2
KB53	*fimH* gene from wild-type strain CI#4	33
KB59	*fimH* gene from wild-type strain CI#3	33
KB91	*fimH* gene from wild-type strain F-18	33
KB96	*fimH* gene from wild-type strain MJ2-2	33
S1918	F′ *laqI*^{q} Δ*fimB-H*::*kan*	3
MS72	pMAS1 and pPKL115 in S1918	This study
MS205	pMAS63 and pPKL115 in S1918	This study
MS206	pMAS64 and pPKL115 in S1918	This study
MS208	pMAS66 and pPKL115 in S1918	This study
MS229	pMAS70 and pPKL115 in S1918	This study
MS233	pMAS72 and pPKL115 in S1918	This study
MS237	pMAS74 and pPKL115 in S1918	This study
MS239	pMAS75 and pPKL115 in S1918	This study
MS243	pMAS77 and pPKL115 in S1918	This study
MS272	pMAS88 and pPKL115 in S1918	This study
MS273	pMAS89 and pPKL115 in S1918	This study
MS276	pMAS92 and pPKL115 in S1918	This study
MS278	pMAS94 and pPKL115 in S1918	This study
MS279	pMAS95 and pPKL115 in S1918	This study
Plasmids		
pPKL4	Wild-type *fim* gene cluster	14
pPKL115	All *fim* genes except *fimH*	23
pMAS1	*fimH*	28
pMAS63	Modified *fimH* gene (L125F, F142Y)	This study
pMAS64	Modified *fimH* gene (G73E, L107F)	This study
pMAS66	Modified *fimH* gene (Y175F, A204E)	This study
pMAS70	Modified *fimH* gene (P490, P180Q)	This study
pMAS72	Modified *fimH* gene (A188E)	This study
pMAS74	Modified *fimH* gene (I52T, N152Y, G159V)	This study
pMAS75	Modified *fimH* gene (A25P, R92P, D141N)	This study
pMAS77	Modified *fimH* gene (L68F, S114R)	This study
pMAS88	Modified *fimH* gene (A18T, F43S, R132Q, F215Y)	This study
pMAS89	Modified *fimH* gene (R92H)	This study
pMAS92	Modified *fimH* gene (S17G, K101E, Y175H, S216P)	This study
pMAS94	Modified *fimH* gene (L183H, H201N)	This study
pMAS95	Modified *fimH* gene (H201Q)	This study
pPKL229	Modified *fimH* gene (A25P, R92P)	This study
pPKL230	Modified *fimH* gene (D141N)	This study
pPKL231	Modified *fimH* gene (A25P)	This study
pPKL232	Modified *fimH* gene (R92P, D141N)	This study
pTBK31	Modified *fimH* gene (G73E)	This study
pTBK32	Modified *fimH* gene (L107F)	This study
pTBK35	Modified *fimH* gene (P49Q)	This study
pTBK36	Modified *fimH* gene (P180Q)	This study
pTBK37	Modified *fimH* gene (L68F)	This study
pTBK38	Modified *fimH* gene (S114R)	This study

represents the model substratum for terminal oligomannose-specific binding, and Fn represents the model substratum for specific binding to oligosaccharides with no terminally exposed mannose residues; BSA served as a control substratum for monitoring of binding to protein substrates (30).

None of the clones that failed to agglutinate yeast cells in the initial screening were able to recognize any of the model substrates. Further studies revealed that all but one of these clones showed only marginal reaction with antiserum against type 1 fimbriae and purified FimH; i.e., they were similar to the *fimH*-null clone used as the negative control in these studies (data not shown). Indeed, sequence analysis of the corresponding *fimH* genes revealed that almost all of these clones had mutations in the *fimH* gene that would result in a truncated protein product. One of the nonagglutinating and nonbinding clones exhibited a relatively strong reaction with the anti-FimH serum and, possibly, expressed a nonfunctional FimH adhesin on the surface.

In contrast to the agglutination-negative clones, all of the 50 agglutination-positive clones were able to bind at a detectable level to at least one of the model substrates. These results confirm the reliability of yeast agglutination as an efficient screening assay for the identification of clones that express functional FimH. The ability to bind model substrates was assessed quantitatively to differentiate between clones with unchanged phenotype, i.e. the wild-type-like adhesion, and those with a variant FimH phenotype. The wild-type-like clones were considered to be the ones that exhibited less than 50% deviation from the substrate-binding level of the control strain expressing the original FimH adhesin from *E. coli* K-12 strain PC31 (i.e., strain S1918 containing pPKL115 and pMAS1). According to this criterion, 37 clones (74%) had an

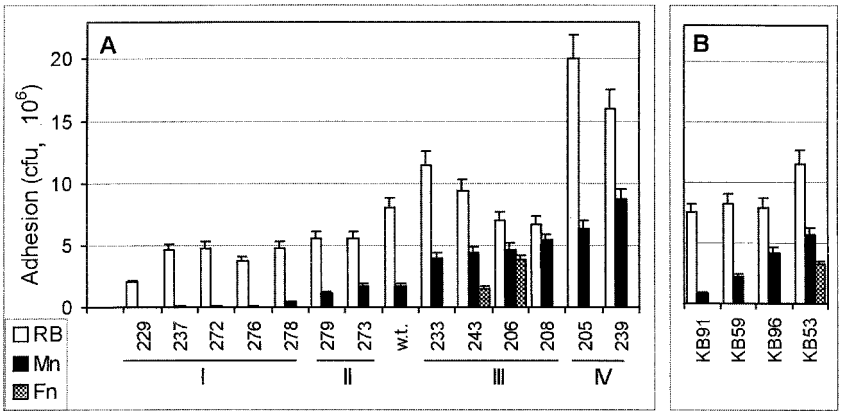

FIG. 2. Adhesion of *E. coli* expressing *fimH* variants selected from the mutant library (A) or naturally occurring *fimH* genes (B) to Mn, RB, and Fn. Clones are grouped (I to IV) according to their FimH monomannose-binding phenotype. The level of significance between each of the groups was as follows: groups I and II, $P < 0.0005$; groups II and III, $P < 0.001$; groups III and IV, $P < 0.02$. Additionally, a significant difference in RB binding was observed between groups III and IV ($P < 0.01$). Values are means and standard errors ($n = 3$).

unchanged binding phenotype while the remaining 13 (26%) clones demonstrated either a significantly lower or higher binding capability than the wild-type clone to at least one of the model substrates tested.

Since this study focused on FimH adhesive phenotypes and not on mutations which altered the fimbriation level, the variant clones were tested for their ability to react with a polyclonal anti-type 1 fimbria antiserum and anti-FimH antiserum (data not shown). Two of the clones demonstrated a significantly lower ability to react with the anti-type 1 fimbrial serum compared to the wild-type control and were not studied further. The remaining 11 clones exhibited a wild-type-like interaction with the fimbrial and FimH antisera, and we therefore concluded that their variant adhesive phenotype resulted primarily from alteration of the FimH receptor-binding domain. All of these and two additional clones, randomly picked from the wild-type-like receptor-binding group, were subjected to the in-depth functional characterization and structural analysis outlined below.

Detailed analysis of the functional variants of *fimH*. The receptor-binding profiles of the variant, wild-type, and wild-type-like clones are presented in Fig. 2A. Clones are listed based on the ability to bind Mn, because the monomannose-specific binding was previously shown to be the most variable property among naturally occurring FimH isotypes (30). Interestingly, all variant clones were able to bind the oligomannose-specific substrate, RB. Although the binding range differed 10-fold, from 2.0×10^6 to 20.0×10^6 CFU/well, the average difference from the wild-type level (8.0×10^6 CFU/well) was only 55.0% ± 11.4%. The same strains exhibited more than a 150-fold range of Mn binding (from 0.05×10^6 to 8.7×10^6 CFU/well), with the average difference from the wild-type level (1.7×10^6 CFU/well) being 161.4% ± 28.8% ($r < 0.015$). With regard to the other model substrates tested, only two clones, MS206 and MS243, were identified which, in contrast to the wild type, had acquired the ability to bind to Fn at detectable levels (Fig. 2A). No clones demonstrated an ability to bind the protein test substrate BSA (data not shown). The results suggest that the parental monomannose-binding phenotype, assayed by binding to Mn, was the most sensitive to randomly induced mutations, whereas the binding to the oligomannose target, RB, seemed to be a property of the FimH adhesin that is relatively resistant to structural alteration.

Based on the receptor-binding phenotypes depicted in Fig. 2A, the variant clones could be divided into four groups in comparison to the wild-type phenotype. Group I clones are distinguished by reduced binding characteristics compared to the wild-type-like group (group II). Two other groups (III and IV) exhibited significantly stronger binding to Mn than did the wild-type control. However, while the group III clones showed wild-type-like binding to RB, the two clones comprising group IV exhibited significantly enhanced binding to both Mn and RB in comparison to the wild-type control. In light of the binding pattern exhibited by the clinical isolates (Fig. 2B), the group III clones fall within the receptor-binding spectrum toward Mn and RB observed in such strains selected in nature. Furthermore, the binding patterns of the mutant strains of groups I and III provide additional evidence for the notion that monomannose-specific binding is a variable property of the FimH adhesin while oligomannose binding is a conserved trait. This would also suggest a certain level of structural and/or functional autonomy between the two characteristics. The remaining group IV clones demonstrated binding phenotypes with significantly increased affinity toward both Mn and RB relative to the wild type. These types of alterations in FimH receptor specificity are different from those seen in naturally occurring FimH isotypes and possibly represent adhesive phenotypes that are selected against in nature.

Agglutination and inhibition. The classical assay for monitoring type 1 fimbria-mediated adhesion to eucaryotic cells is agglutination of erythrocytes or yeast cells. Yeast cell agglutination is the most highly conserved binding property among natural *E. coli* isolates and was used in this study to evaluate the variation in receptor binding exhibited by the *fimH* mutant clones examined in this study. The ability to bind yeast was expressed as the highest dilution of bacterial suspension that could confer agglutination. Bacterial adhesion to eucaryotic cells under natural conditions is a function of the ability of the adhesin to interact with the cognate receptor on the cell surface but also depends on the sensitivity of the adhesin to soluble inhibitory compounds that could bathe the cellular target. Accordingly, we also determined the sensitivity of the various clones to yeast cell agglutination in the presence of a soluble inhibitor, methyl-α-D-mannopyranoside.

When the clones from the mutant library were tested for their ability to agglutinate yeast cells, it became apparent that the clones with wild-type-like receptor-binding profiles (i.e., clones from the wild-type-like group II and "flanking" clones

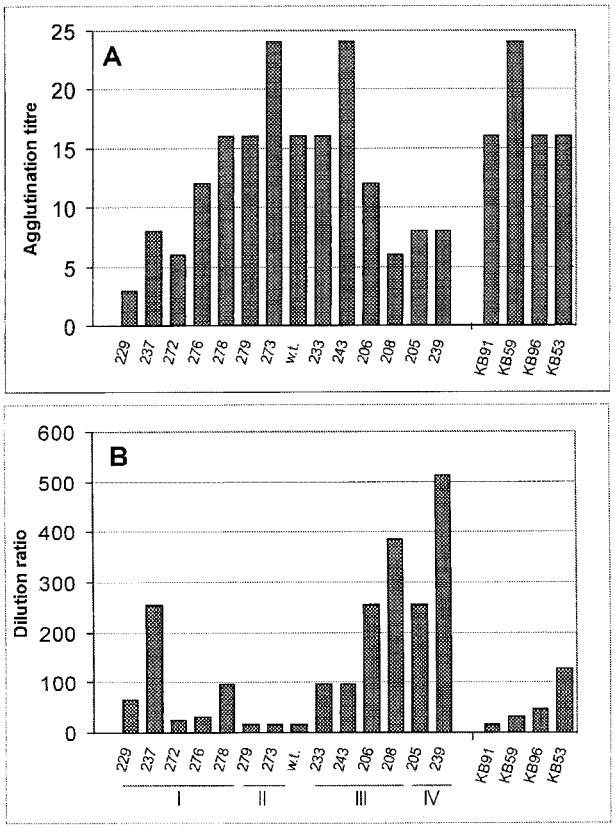

FIG. 3. (A) Agglutination titer of *E. coli* expressing *fimH* variants selected from the mutant library. The titer is a measure of the highest dilution of bacterial cells (from OD_{540} = 0.4) still able to give a positive aggregation result. (B) Agglutination of *E. coli* expressing *fimH* variants selected from the mutant library in the presence of the soluble inhibitor methyl-α-D-mannopyranoside. The dilution ratio is a measure of the highest dilution of methyl-α-D-mannopyranoside (from the starting concentration of 1%) still able to inhibit aggregation. Values are means and standard errors (n = 3).

from groups I and III) had relatively high agglutination titers and ability to resist inhibition. However, this ability generally dropped as the clones became more unlike the wild-type clones. Notably, the "outer" group I and III clones, as well as the two group IV clones, possessed a significantly lower capacity to agglutinate yeast cells and/or to resist specific inhibition (Fig. 3). These clones also differed most from the wild type in Mn binding and RB binding, having significantly lower and higher titers, respectively, than the wild type. Taken together, the data indicate that mutants with adhesion profiles that are significantly altered in monomannose and (especially) oligomannose binding tend to have an inferior ability to adhere to eucaryotic cells. Interestingly, increased sensitivity to soluble inhibitors has been reported for natural variants that bind very strongly to monomannose-like receptors, although it was not accompanied by the decreased ability of the isolates to agglutinate yeast cells (Fig. 3B).

Characterization of the amino acid substitutions introduced by random mutagenesis. The nucleotide sequences of the *fimH* genes of the 11 variant and 2 wild-type-like clones were determined; the corresponding amino acid sequences are presented in Fig. 4. Overall, the PCR mutagenesis of the 656-bp region of *fimH* resulted in one to four (an average of two) nonsynonomus mutations per clone. The changes were randomly distributed along the target sector, and the observed amino acid changes were of diverse nature. It should be noted that the nucleotide substitution rate gave an average of 3.5 substitutions per clone; however, we have reported only the nucleotide changes which alter the amino acid sequence of the protein. Taken together, these data suggest that the PCR mutagenesis technique we used was an adequate method for the introduction of a limited number of random structural alterations in the FimH primary sequence.

The smallest number of changes was, not unexpectedly, found in the wild-type-like group II, with the two clones MS279 and MS273 having single substitutions. In contrast to the wild-type-like clones, the *fimH* genes of all the variant clones had accrued nonsynonymous substitutions, varying from one (MS233, group III) to four (MS272 and MS276, group I). Within the groups, the mutations were distributed along the FimH region without a notable phenotype-specific clustering. Therefore, the substitution pattern observed in the mutagenized FimH resembles the one defined previously for the functional variations in FimH that occur under natural selection; i.e., mutations are limited in number and spread throughout the protein.

In general, the substitutions observed in the variant clones were of a diverse nature. The structural changes within FimH in the MS229 and MS239 clones, which constitute the phenotypically most altered FimH variants, involved alteration or introduction of proline residues. Proline often defines turning regions within the peptide chain, and therefore such mutations would be expected to cause dramatic changes in conformation and function.

Amino acid changes that confer shifts in receptor specificity. The majority of FimH variants identified in this study with altered phenotypes were found to contain multiple amino acid changes. To specifically define the functional impact of some of these individual substitutions, we took advantage of unique restriction sites to exchange segments with the wild-type *fimH* gene.

The group III clones are particularly interesting since their phenotype resembles those of naturally occurring FimH variants found in uropathogenic *E. coli*. Therefore, detailed analyses of these clones could provide information on the mechanism of natural selection of the FimH adhesin. Compared to the parental FimH, the mutant FimH expressed by the group III clone MS206 is characterized by a threefold increase in the ability to bind Mn (the monomannose-specific substrate) and in the acquired ability to bind human plasma Fn (the non-mannose-specific substrate). Two mutations were defined in the FimH-MS206 protein, G73E and L107F. When these changes were introduced separately into the parental FimH background, the G73E substitution was identified as the functionally critical one (Fig. 5). Interestingly, a G73E mutation has been identified as the functional change in a naturally occurring FimH variant that has a similar adhesive profile to MS206 (clone KB53, Fig. 2B) (34).

FimH from another group III clone, MS243, has two changes, L68F and S114R. When these mutations were introduced separately into the parental background, none of them were individually capable of altering the wild-type phenotype (Fig. 5). Therefore, both are required to increase the FimH capability to bind monomannose receptor targets. Such a phenomenon has not previously been seen among naturally occurring FimH variants, where single mutations have been pinpointed to account for phenotypic changes.

The group I clone MS229 expresses a FimH variant with a decreased ability to recognize any of the tested receptor substrates, a phenotype not found among clinical isolates. We hypothesized that the mutations introduced into this FimH

w.t.	229	237	272	276	278	279	273	233	243	206	208	205	239	KB91	KB59	KB96	KB53
S17				G													
A18			T														
A25													P				
V27*														A	A	A	A
F43			S														
P49	Q																
I52		T															
T57																	
G66*																D	
L68									F								
N70*														S		S	S
G73										E							E
S78*														N		N	N
R92							H						P				
K101				E													
L107										F							
S114									R								
A118*															V		
L125												F					
R132			Q														
D141													N				
F142												Y					
N152		Y															
G159		V															
V163*																A	
Y175				H							F						
P180	Q																
L183					H												
A188								E									
H201					N	Q											D
A204											E						
A213																	
F215			Y														
S216				P													
group	I					II		III				IV		clinical isolates			

FIG. 4. Summary of mutations resulting in altered FimH phenotypes from the *fimH* mutant library and selected wild-type isolates. Asterisks indicate specific mutations identified only in clinical isolates.

variant may therefore interfere with critical functional regions of the adhesin. To investigate this, we introduced the mutations separately in the FimH-K12 background. The P49Q substitution was found to be responsible for the abolition of FimH function, while the P180Q mutation appeared to be functionally neutral. The P49 amino acid residue therefore appears to be critical for receptor recognition by FimH.

We also examined the effect of the individual mutations in the group IV clone MS239. This clone contains three amino acid changes (A25P, R92P, and D141N) and possesses a phenotype of increased affinity to both Mn and RB. Dissection of this clone to determine the effect of the individual changes revealed that this phenotype was directly associated with the A25P mutation (Fig. 5).

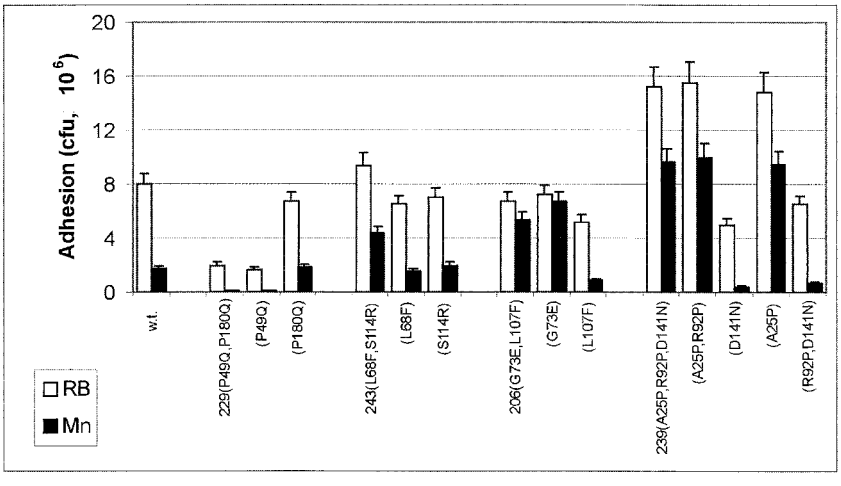

FIG. 5. Adhesion of *E. coli* expressing *fimH* genes with specific individual mutations to yeast Mn and RB. Chimeric *fimH* genes were constructed using unique restriction sites to exchange fragments between mutant and wild-type genes. Values are means and standard errors ($n = 3$).

DISCUSSION

Most of the type 1-fimbriated *E. coli* strains of human fecal origin primarily recognize oligomannose-like receptors. In contrast, FimH variants that enable *E. coli* to bind strongly to monomannose-like receptors provide an adaptive advantage for the bacterial colonization of the urinary tract. Indeed, the monomannose-specific phenotype is dominant among uropathogenic *E. coli* isolates while the oligomannose phenotype is most common among fecal *E. coli* (31). A number of studies have attempted to define the integral parts of the FimH adhesin that contribute to receptor recognition. In-frame linker insertion mutagenesis of *fimH* in positions corresponding to amino acids 56 and 136 in the mature protein completely abolished the ability of FimH to bind to D-mannose receptors (29). Minor structural variations occurring naturally in the FimH adhesin that can lead to physiologically important changes in the pattern of receptor recognition have also been found primarily within the N-terminal half of the FimH protein (25, 34). Two studies involving fusions of sectors of FimH with either MalE or FocH showed that a segment encompassing amino acid residues 3 to 158 constituted a core region for receptor recognition, with additional information residing in the region from residues 159 to 201 (16, 35).

On this background, we created a mutant library by introduction of mutations in the first two-thirds of the *fimH* gene from the *E. coli* K-12 strain PC31 (whose sequence is identical to that of the reference strain MG1655). Random mutagenesis was performed on the region covering bp 85 to 740 (codons 8 to 225) of the 900-bp *fimH* gene by PCR mutagenesis, the same sector previously identified to encode the receptor recognition part of the adhesin. To construct a library of altered *fimH* genes, the PCR amplification products were exchanged with the wild-type gene copy in plasmid pMAS1 and introduced into *E. coli* strain S1918 (Δ*fim*) containing an auxiliary plasmid, pPKL115, which carries the entire *fim* gene cluster except *fimH*. This permitted expression of the corresponding FimH variants as functional constituents of type 1 fimbriae and therefore allowed specific phenotypic analysis of the mutants. Using this approach, we were able to analyze a wide spectrum of FimH variants without alteration of the other fimbrial structural components, namely, FimA, FimF, and FimG.

The PCR mutagenesis resulted in 44% of the mutants being nonfunctional, primarily due to the introduction of premature stop codons in the *fimH* gene. The bacterial clones containing truncated FimH proteins were unable to agglutinate yeast cells or bind to any of the model receptor substrates. These results concur with those of previous studies indicating that without the C-terminal region, which is critical for the interaction of FimH with the molecular chaperone FimC, the FimH protein is highly unstable and is not incorporated into the fimbrial organelle (9). These mutants were also characterized by a severe decrease in the number of fimbriae visualized at the cell surface, an observation consistent with the notion that FimH is involved in the initiation of fimbrial organelle synthesis.

The majority (56%) of the clones were capable of causing agglutination of yeast cells to various degrees. Fifty bacterial clones selected randomly from this group were all able to bind at least one of the model receptor substrates. Eleven of these clones (22%) exhibited a FimH-specific receptor-binding phenotype that differed significantly from that of the parental K-12 FimH. Detailed analyses of the functionally altered mutants revealed that the capacity of the FimH adhesin to recognize monomannose-like receptor substrates is highly prone to changes induced by the random mutagenesis while the oligomannose recognition phenotype is a much more stable functional property. This phenomenon corresponds to the pattern observed in naturally selected FimH variants, which are characterized by up to 15-fold variation in their ability to bind monomannose but by only minor deviations in their oligomannose-binding capacity (30).

Four (36%) of the functionally modified mutants (group III) closely resembled the natural pathogenicity-adaptive phenotype of FimH. In comparison to the original wild-type K-12 FimH, these clones were able to bind monomannose at a dramatically higher level, while their oligomannose-binding capability remained unchanged. In addition, two of these mutants exhibited binding to human plasma Fn, a phenotype highly specific to uropathogenic but not fecal *E. coli* isolates (32). Therefore, the naturally occurring pathogenicity-adaptive phenotype of the FimH adhesin could be induced at a relatively high rate under nonselective conditions. The G73E substitution, which was found to be responsible for the strong monomannose and Fn binding of the mutant clone MS206, has also previously been identified in the uropathogenic strain KB53 (33). However, the KB53 variant of *fimH* differs from the K-12 variant in four other positions, V27A, N70S, S78N, and H201D. Therefore, the G73E mutation imparts the same functional effect on two structurally different FimH alleles, suggesting that the same types of pathogenicity-adaptive mutations might provide the selective functional changes in various clonal variants of FimH across the *E. coli* species. The random mutagenesis has also provided strong evidence that the pathogenicity-adaptive phenotype of the FimH adhesin can be induced via a cumulative effect of two functionally neutral substitutions. Indeed, the L68F and S114R replacements do not affect the K-12 FimH phenotype as separate changes, but their simultaneous presence in the mutant clone MS243 dramatically increases the monomannose-binding capability. This would suggest that certain allelic variants of FimH that bear one of the neutral replacements would be more primed than other FimH alleles to evolve into the pathogenicity-adaptive variant.

The FimH mutant clone MS229 from group I and the two group IV clones demonstrate the most profound functional alterations of FimH since they affect its highly conserved oligomannose-binding property. Such types of functional alterations have not been previously observed among commensal or pathogenic isolates of *E. coli*. It is possible that the altered oligomannose-binding phenotype of the FimH protein is physiologically detrimental to *E. coli* in its natural environment. Indeed, the most distinctive clones from group I (MS229) and group IV (MS239) demonstrate a significantly decreased ability to bind to model target cells (yeast) and to overcome interference with adhesion by soluble inhibitors. It is possible, however, that such mutants do occur and are selectively advantageous under some yet unidentified natural conditions. The group I mutant clone MS229 contains the amino acid replacement P49Q that is responsible for abolishing the oligomannose-binding capability of the FimH adhesin, while the group IV clone MS239 demonstrates an increased oligomannose-binding capability due to the A25P substitution in its FimH. Interestingly, the P49Q substitution occurs in the YPETITD54 amino acid stretch that is homologous to the YPNTD16 region of the mannose-specific jack bean lectin, concanavalin A (ConA). Based on studies that have resolved the three-dimensional structure of the ConA lectin cocrystallized with trimannoside compounds, the YPNTD16 region of ConA is built by the residues that form a pocket capable of accommodating a complex oligomannose receptor structure (20). This would suggest that the P49 residue of FimH is part of its oligomannose-combining site. Further evidence that the P49 residue could be within the FimH receptor-binding site

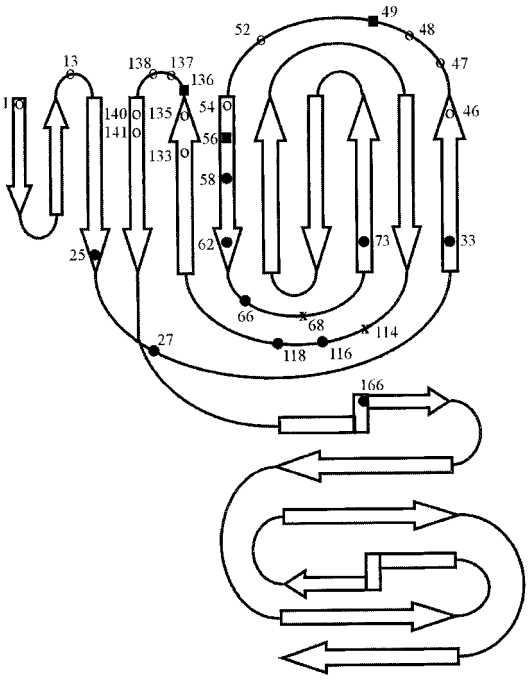

FIG. 6. β-sheet topology diagram of the lectin (top) and pilin (bottom) domains of FimH (4), indicating structural changes that alter FimH function. Although the length of the sheets and loops does not reflect the actual size, the relative position of the labeled residues is indicated accurately. The data include functional amino acid changes identified in this study, along with changes previously observed in wild-type clones (25, 33) and FimH linker mutagenesis studies (29). Residues are indicated as substitutions causing enhanced monomannose binding (solid circles), substitutions destroying monomannose binding (solid squares), and individually neutral substitutions that act in concert to enhance monomannose binding (crosses). The C-HEGA-interacting residues in the FimH carbohydrate-binding pocket are also indicated (open circles).

was provided by the X-ray structure of the FimH adhesin (in complex with the molecular chaperone, FimC) that was reported during the preparation of this paper (4). According to the X-ray study, the P49 residue is located within a pocket of the FimH protein that binds to a molecule of cyclohexylbutanoyl-*N*-hydroxyethyl-D-glucamide (C-HEGA). C-HEGA is not a known inhibitor of FimH-mediated mannose binding but was used as the cocrystallizing compound to obtain FimH-FimC crystals of the necessary quality. The defined carbohydrate-binding pocket was shown to accommodate only the glucamide moiety of C-HEGA, a relatively small molecule. Therefore, it is difficult to speculate how FimH interacts with larger receptor compounds like trimannose or with other known oligosaccharide inhibitors that exhibit 10- to 30-fold-higher binding affinity to FimH than does monomannose (6).

According to the reported structure of the FimH-FimC complex, the FimH protein is folded into two domains, an NH_2-terminal lectin domain (residues 1 to 156) and a COOH-terminal pilin domain (residues 160 to 279). An important pattern emerges from the analysis of the distribution of functional amino acid substitutions that have been identified in this and previous studies (Fig. 6). The decreased binding capability of FimH (i.e., group I clones) is caused by mutations that occur either within or near the carbohydrate-interacting residues that form the FimH-binding pocket at the tip of the jelly-roll-shaped lectin domain. Conversely, the changes resulting in an increased monomannose-binding capability of FimH (i.e., group III and IV clones) do not interact directly with the mannose receptor site. Instead, these mutations occur in the "bottom" part of the lectin domain. Such a mirror-image distribution of the monomannose-binding enhancing substitutions is highly nonrandom ($P < 0.0001$) and could not be recognized without knowledge of the FimH crystal structure. Therefore, the enhanced monomannose binding of the mutants is most probably conformational, possibly due to alteration of the conformational stability of the protein loops that carry the receptor-interacting residues. This type of phenomenon has also been observed in serine proteases, where substitution of such rigidity-providing residues has been shown to result not in the abolition but in the broadening of substrate specificity (24). This specificity-broadening functional pattern is characteristic of the monomannose- and fibronectin-binding FimH variants.

It is very likely that a thorough understanding of the structural and functional basis of the natural adaptability of the *E. coli* FimH adhesin will require cocrystallization of different FimH variants with various types of receptor molecules and, possibly, with other associated proteins within the fimbrial organelle structure. In this context, the FimH variants identified in this study constitute a well-defined group of variants based on the K-12 FimH allele. Studies on the adaptability of the *E. coli* FimH protein may serve as a model paradigm for the analysis of other bacterial adhesins or, basically, of any microbial traits that can be functionally modified by naturally occurring mutations to result in enhanced virulence.

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