Heterobinary Adhesins Based on the *Escherichia coli* FimH Fimbrial Protein

MARK A. SCHEMBRI AND PER KLEMM*
Department of Microbiology, Technical University of Denmark, DK-2800 Lyngby, Denmark

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The FimH adhesin of *Escherichia coli* type 1 fimbriae confers the ability to bind to α-mannosides by virtue of a receptor-binding domain located in its N-terminal region. This protein was engineered into a heterobifunctional adhesin by introducing a secondary binding site in the C-terminal region. The insertion of histidine clusters into this site resulted in coordinated bonding of various metal ions by recombinant cells expressing chimeric FimH proteins. In addition, libraries consisting of random peptide sequences inserted into the FimH display system and screened by a “panning” technique were used to identify specific sequences conferring the ability to adhere to Ni\(^{2+}\) and Cu\(^{2+}\). Recombinant cells expressing heterobifunctional FimH adhesins could adhere simultaneously to both metals and saccharides. Finally, combining the metal-binding modifications with alterations in the natural receptor-binding region demonstrated the ability to independently modulate the binding of FimH to two ligands simultaneously.

Expression systems for the display of heterologous protein segments facilitate the presentation of both defined and random peptide sequences at exposed regions of surface proteins of filamentous bacteriophage virions, bacteria, and yeasts (2, 4). We are particularly interested in the display of heterologous peptides in type 1 fimbriae. Such surface organelles are found on the majority of *Escherichia coli* strains and confer the ability to bind to specific surfaces. A single type 1 fimbria is a heteropolymer that is 7 nm wide and approximately 1 μm long. It consists of approximately 1,000 subunits of the major fimbrial protein, FimA, that are polymerized in a right-handed helical structure that also contains low levels of the minor components FimF, FimG, and FimH (9). The FimH protein has been shown to be the actual receptor-binding molecule which recognizes α-D-mannose-containing structures (10). Because of this, type 1 fimbriated bacteria readily agglutinate yeast cells (a rich source of mannann).

The FimH adhesin is located at the tip of each fimbria and also is interspersed along the fimbrial shaft (6, 10). The results of linker insertion mutagenesis (16) and analyses of naturally occurring variants (17–19) and hybrid proteins constructed by fusing FimH to FocH (8) and MalE (21) suggest that the FimH protein consists of two major domains, each constituting roughly one-half of the molecule; the N-terminal domain seems to contain the receptor-binding site, while the C-terminal domain seems to contain the recognition sequences for export and bioassembly.

In previous studies we investigated the ability of FimH to display heterologous peptides in connection with the development of vaccine systems. Various heterologous sequences, representing immune-relevant sectors of foreign proteins, were authentically displayed on the bacterial surface in FimH (12). These observations led us to believe that the FimH protein is an ideal candidate for display of random peptide sequences and for construction of designer adhesins (i.e., proteins manipulated to bind to targets of choice). Here we describe simultaneous heterobifunctional binding of recombinant cells expressing chimeric FimH proteins to metal and α-mannose targets.

MATERIALS AND METHODS

Strains and plasmids. *E. coli* strain S1918 (F− lacIq FimB-H:kan) (3) was used in this study. Strains were grown in Luria-Bertani medium supplemented with the appropriate antibiotics (14). The FimH expression vector pLPA30 is a pUC19 derivative containing the *fimH* gene downstream of the *lac* promoter and has a BglII linker inserted at position 225 (12). Plasmid pPKL115 is a pACYC184 derivative containing the whole *fim* gene cluster with a stop linker inserted into the *fimH* gene (12). Plasmid pMAS25 was made by inserting an 18-bp synthetic double-stranded DNA segment encoding six consecutive histidine residues and containing a BglII overhang at one end and a BamHI overhang at the other end into the BglII site of pLPA30. The double-stranded polynucleotide segment resulted from the annealing of two oligonucleotides, 5′-GATCATCACCATCACCATGACG and 5′-GATCATGGGATGATGATGATGAG. Plasmid pNSU36 was made by digesting pMAS25 with BglII and inserting a second polynucleotide DNA segment. Plasmid pMAS1 contained the *fimH* gene from *E. coli* strain PC31 (7) inserted into pUC19. Plasmid pMAS37 was made by performing overlapping PCR with a set of oligonucleotides which amplified the N-terminal half of *fimH* from *E. coli* strain CI#4 (19) and the C-terminal half of *fimH* from pNSU36 and then ligating the product into pUC19. Each PCR product was performed as previously described (12) with the Expand High Fidelity PCR system (Boehringer, Mannheim, Germany). DNA sequencing was carried out by the dideoxy chain termination technique (15) with a Sequenase version 2.0 kit (U.S. Biochemicals).

Construction of the random library. The random library was constructed essentially as described by Brown (3). Briefly, a template oligonucleotide containing the sequence 5′-GGACGCAGATCT(VNN)AGATCTAGCACCAGT-3′ was chemically synthesized (N indicates an equimolar mixture of all four nucleotides, and V indicates an equimolar mixture of A, C, and G). A primer oligonucleotide, 5′-ACTGTCGTCGCTAGCT-3′, was hybridized to the template oligonucleotide and extended with the Klenow fragment of DNA polymerase I. The double-stranded oligonucleotide was extracted twice with phenol-chloroform and ethanol precipitated. Digestion with BglII released an internal 33-bp fragment which was purified by electrophoresis through a 12% polyacrylamide gel in TBE. The 33-bp fragment was excised and eluted from the gel with a buffer containing 10 mM Tris-HCl (pH 8.0), 2 mM EDTA, and 0.15 M NaCl. The eluate was filtered through a 0.22-μm-pore-size Qiagen filter, concentrated by ethanol precipitation, and redissolved in 10 mM Tris-HCl (pH 8.0)–1 mM EDTA–0.1 M NaCl. The redissolved 33-bp BglII fragment was ligated at various ratios to BglII-digested pLPA30. The ligation products were precipitated with ethanol and electrophoresed into S1918 (containing pPKL115).

The diversity of the library was calculated to be 4 × 10^14 individual clones based on extrapolation from numbers of transformants obtained in small-scale plateings. The volume of each transformation mixture was brought to 10 ml, and each eluate was filtered through a 0.22-μm-pore-size Qiagen filter, concentrated by ethanol precipitation, and redissolved in 10 mM Tris-HCl (pH 8.0)–1 mM EDTA–0.1 M NaCl. The redissolved 33-bp BglII fragment was ligated at various ratios to BglII-digested pLPA30. The ligation products were precipitated with ethanol and electrophoresed into S1918 (containing pPKL115).
Random screening of clones by PCR revealed a predominance of one to three
33-bp oligonucleotide inserts; sequencing of the inserts from randomly selected
clones revealed G+C contents ranging from 30 to 70%.

**Enrichment procedure.** Bacterial cells were bound to nickel ions by use of a
commercially available Ni²⁺-nitrilotriacetic acid (NTA) solid matrix (Qiagen).
The NTA ligand has four chelating sites which interact with each nickel ion. This
leaves two of the six ligand-binding sites in the coordination sphere of the Ni²⁺
ion to interact with the histidine tag. The enrichment procedure used to identify
Ni²⁺-bound clones from the random library was as follows. Mid-exponential-
phase cultures were diluted with M63 salts (11) containing 20 mM α-methyl-
mannopyranoside and 50% Percoll (Pharmacia). The α-methylmannopyranoside
was added to block the natural binding of the FimH adhesin, while the use of
Percoll resulted in the formation of a density gradient upon centrifugation.
This resulted in the formation of a distinct band by the Ni²⁺-NTA resin and specific
separation of any adherent bacteria from nonadherent bacteria. Under these
conditions, bacteria expressing wild-type FimH proteins as components of type 1
fimbriae did not coseparate with the Ni²⁺-NTA resin. The resin and bacteria
expressing the random library within FimH were mixed and allowed to adhere at
room temperature with gentle agitation. Centrifugation was then performed, and
the resin and any adhering bacteria were removed and plated onto L-agar
containing appropriate antibiotics. After overnight incubation colonies were
pooled from the surfaces of the plates, exponentially growing cultures were
established, and the enrichment procedure was repeated. Following each cycle
of enrichment aliquots of the populations were stored at −80°C. Plasmid DNA was
prepared from each aliquot and used in a PCR to monitor the size distribution
of the inserts in the population.

**Binding assays.** Mid-exponential-phase cultures were washed, resuspended in
M63 salts and then mixed simultaneously with either Ni²⁺-NTA agarose beads
(Qiagen) and Saccharomyces cerevisiae cells or α-D-methylmannopyranoside aga-
rose beads (Sigma) and NiO. Samples were incubated at room temperature for
15 min with gentle agitation prior to examination by phase-contrast microscopy.
Where necessary, silver nitrate was used to block the natural FimH binding site.
α-D-methylmannopyranoside was used at a final concentration of 20 mM. The binding of cells to
the Ni²⁺-NTA resin was reversed by using an imidazole gradient (1 to 50 mM
(5)). Binding of cells to casein and yeast mannan was performed in microtiter
plates as previously described (18), except that bound cells were eluted without
prior incubation.

**Binding to metals.** Metal oxides (NiO, CuO, ZnO, and CdO) were purchased
from Aldrich. Particles of the appropriate size for microscopy were prepared by
directly centrifugation. Metal oxides were suspended in M63 salts before
bacteria were added. Samples were incubated at room temperature for 15 min
with gentle agitation and examined microscopically. An alternative procedure to
demonstrate metal-binding capacity was to measure the bioaccumulation of
either Ni²⁺ or Cd²⁺ by recombinant bacteria by atomic adsorption. Late-expo-
nential-phase cultures were washed in M63 salts and resuspended in the same
medium containing 20 μM NiCl₂ or CdCl₂. The cells were incubated for 30 min
to allow adsorption of the metal ions and washed twice in M63 salts. Samples
were prepared and analyzed with a Perkin-Elmer model 2100 atomic adsorption
spectrophotometer as previously described (13).

**RESULTS**

**Construction of a FimH-polyhistidine hybrid protein.** We previously
described two positions in the C-terminal domain of the FimH protein which can tolerate
the insertion of heterologous sequences (12). In this study, we used the FimH expression
vector pLP30, which contains the fimH gene with an in-frame BglII linker inserted at a position encoding amino acid residue 225 and placed under transcriptional control of the lac
promoter. In order to express chimeric FimH molecules as functional constituents of fimbriae, we also used an auxiliary plasmid (pPKL115) encoding the rest of the fim gene cluster (Fig. 1).

A synthetic DNA segment encoding six tandem histidine residues was constructed by annealing two complementary 24-bp oligonucleotides designed to create a final double-stranded DNA segment with a BglII overhang at one end and a BamHI overhang at the other end. This feature permitted the intro-
duction of one and two such segments into plasmid pLP30, resulting in plasmids pMAS25 and pNSU36, respectively. See que-
quen and inserted the insert orientation as inferred from the sequence of the reading frame in the chimeric fimH genes. Receptor
blots of the two chimeric FimH proteins with α-D-mannosylated bovine serum albumin indicated that these molecules were synthesized as full-length proteins. The presence of bio-
logically active chimeric FimH proteins on the surface of re-

**Heterobifunctionality of the FimH adhesin.** To demonstrate that simultaneous heterobifunctional binding of the engineered FimH protein occurred, we presented the recombinants with targets for both the natural receptor site and the C-terminal polyhistidine insert at the same time (Fig. 2). We observed binding
in both directions (i.e., binding to the metal resin followed by the d-mannose target [yeast] or binding to d-mannose beads followed by NiO). Binding to the Ni²⁺-NTA resin was found to be dependent on the introduced polyhistidine clusters as a strain carrying the wild-type fimH gene did not adhere to the resin. In addition, binding to the Ni²⁺-NTA resin could be reversed by adding imidazole. The adherence of yeast cells to bacteria bound to the Ni²⁺-NTA resin could also be blocked by adding methyl-α-D-mannopyranoside (Fig. 2). Taken together,
these results demonstrate that two independent adhesive do-
main on the FimH protein can be used to bind cells to dif-
ferent target molecules simultaneously.

**Modification of the natural receptor-binding site of FimH.** The fimH gene used as a basis for manipulations was originally cloned from the E. coli K-12 strain PC31. The corresponding FimH molecule confers the ability to bind to α-D-mannosides but not to other targets, such as proteins. However, certain wild-type versions of FimH confer the ability to bind to protein targets and display higher affinity for α-D-mannosides due to
minor changes in the N-terminal receptor recognition domain (17–19). In order to demonstrate the ability to manipulate the natural binding site of the FimH adhesin, we exchanged this domain with that of the naturally occurring wild-type variant CI#4 (19). We used overlapping PCR to construct a hybrid fimH gene in which the first half originated from CI#4 and the second half originated from pNSU36. The FimH adhesin
from CI#4 has previously been shown to bind to protein targets, such as casein, and to possess enhanced affinity for mannan (19). The new hybrid FimH protein was shown to have the same binding phenotype for both casein and mannan (Fig. 3), while at the same time it also retained its ability to bind to Ni\textsuperscript{2+} ions. These results demonstrate that the natural binding domain in the N-terminal part of the FimH adhesin can be manipulated and that there is a change in receptor affinity. At the same time a heterologous insert in the C-terminal part of the same molecule confers the ability to bind to a secondary target (viz., nickel).

**Selection of Ni\textsuperscript{2+}-adhering bacteria from a random library.** Since we demonstrated that the FimH protein could be engineered to confer metal-binding properties on a recombinant cell, we were confident that the Ni\textsuperscript{2+}-NTA resin was a suitable target to evaluate the use of the fimbrial system for displaying random peptide sequences. A random library was constructed by inserting various numbers of synthetic double-stranded oligonucleotides into the BglII site at position 225 of the fimH gene. The double-stranded oligonucleotides consisted of nine random codons flanked by BglII restriction sites, encoding arginine and serine. This genetic structure allowed us to con-

![FIG. 2. Phase-contrast microscopy showing the heterobinary binding properties of cells expressing engineered FimH adhesins. S1918(pNSU36, pPKL115) was mixed with Ni\textsuperscript{2+}-NTA agarose beads and yeast cells in the absence (A) or presence (B) of 20 mM a-D-methylmannopyranoside. S1918(pMAS1, pPKL115) (C) and S1918 (pNSU36, pPKL115) (D) were mixed with a-D-methylmannopyranoside-coated agarose beads and Ni\textsuperscript{2+}.

![FIG. 3. Adhesion of cells expressing wild-type (pMAS1) (open bars) and hybrid (pMAS37) (solid bars) FimH proteins to casein and yeast mannan. Values are the mean numbers of bacteria bound per well + standard errors of the means (n = 4).](http://aem.asm.org/)

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struct libraries containing one or more double-stranded 33-bp oligonucleotides, a feature which greatly enhanced the complexity of the libraries. In addition, the distribution of the population through the enrichment procedure could be monitored by PCR amplification of the insert region with primers complementary to the vector sequence flanking the insertion site.

Serial selection and enrichment of the random library was performed with the Ni\(^{2+}\)-NTA resin. PCR monitoring of the insert population revealed a distinct change in the size distribution after four cycles of selection and enrichment. In a control experiment, 10 cycles consisting of growth of the population, washing in M63 salts in the absence of Ni\(^{2+}\)-NTA resin, and regrowth did not alter the size distribution of the insert sequences. Of 50 randomly selected colonies from the fourth enrichment, 11 were shown to bind to the Ni\(^{2+}\)-NTA resin and were examined further. The FimH-containing plasmids were isolated from each strain, and the insert region was sequenced. Ten different insert sequences were identified (Fig. 1). Interestingly, the insert sequence encoded by plasmid pMAS38 was identified in 2 of the 11 plasmids. This sequence contained a unique ScaI restriction site which could be used to monitor the prevalence of the insert in the fifth enrichment. Eight of 12 clones identified as binding to Ni\(^{2+}\) from the fifth enrichment contained this unique restriction site, indicating that this insert was the dominant sequence enriched throughout the selection procedure. The remaining four inserts were also examined and contained sequences which differed from those identified in the previous enrichment (Fig. 1). All of the insert sequences contained histidine residues, providing further evidence that this amino acid plays a role in the binding of proteins to Ni\(^{2+}\).

**Binding of selected clones to metal oxides.** The 14 different plasmids identified from the random library which conferred affinity to Ni\(^{2+}\) were purified and retransformed into S1918 (pPKL115). The new recombinant clones had the same binding phenotype as the original isolates, indicating that the binding phenotype was indeed plasmid encoded. Although these clones were originally selected in M63 salts containing 20 mM α-methylmannopyranoside and 50% Percoll, they also had the same binding phenotype in M63 salts alone, indicating that these reagents had no effect on the stability of the metal-binding capacity. The binding of these clones to the Ni\(^{2+}\)-NTA resin could be inhibited by adding imidazole, as previously observed with the clones harboring one and two histidine clusters. The agglutination titers of these cells were similar to those of a control strain expressing wild-type FimH, indicating that

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**FIG. 4.** Phase-contrast microscopy showing the adherence of S1918 cells containing plasmids expressing various chimeric fimH genes to metal oxides. The plasmids used were pLPA30 (wild-type fimH), pMAS25 (containing one polyhistidine insert), and pNSU36 (containing two polyhistidine inserts), as well as pMAS38 and pMAS42 (random clones). Cells were incubated in M63 salts medium alone or in M63 salts medium containing NiO, CuO, or CdO.
the presence of the inserts did not influence the natural binding domain of FimH or significantly alter the number of fimbriae on the surfaces of the cells.

To investigate whether the plasmids isolated conferred the ability to recognize other metals, transformants of S1918 (pPKL115) harboring these plasmids were examined in binding assays with NiO, CuO, and CdO by phase-contrast microscopy. All of the clones formed aggregates when they were mixed with either NiO or CuO but not when they were mixed with CdO. Figure 4 shows the binding of clones harboring plasmids pMAS38 and pMAS42. Recombinant clones harboring pMAS25 and pNSU36 (containing one and two histidine clusters, respectively) formed aggregates with all three metal oxides. The different sizes of the cell-metal aggregates indicated that there were differences in the avidities of the various clones for each of the metals. In a separate assay to monitor avidity for metal ions, atomic adsorption spectroscopy was used to measure the amounts of Ni\textsuperscript{2+} or Cd\textsuperscript{2+} associated with clones harboring either pMAS25, pNSU36, or pMAS38. The amount of metal associated with these cells was significantly different from the amount of metal associated with cells expressing wild-type FimH-containing fimbriae (Fig. 5).

**DISCUSSION**

In this study we developed heterobifunctional adhesins based on the FimH component of *E. coli* type 1 fimbriae. This novel technology is based on the following rationale. Insertion of heterologous sequences in the C-terminal part of FimH does not disturb the natural receptor-binding domain located in the other end of the FimH protein, which mediates targeting to D-mannosides. Consequently, random peptide libraries can be displayed in one sector of the molecule and can be selected based on affinity to a given target. At the same time the natural binding domain remains intact and, in the case of wild-type affinity, confers the ability to bind to D-mannosides. This permits immobilization of bacteria on a matrix containing one type of receptor, while at the same time the secondary binding site is available for interaction with its cognate target.

The heterobifunctionality of engineered FimH adhesins was demonstrated by using two different binding targets. Cells expressing these proteins as fimbrial components were able to adhere to both metal and D-mannose targets simultaneously. Insertion of polyhistidine clusters into FimH resulted in recombinant cells that were able to form aggregates with NiO, CuO, and CdO, as well as ZnO (data not shown). It has been reported previously that histidine residues inserted into the LamB protein conferred the ability to bind to Ni\textsuperscript{2+} and Cd\textsuperscript{2+} on recombinant cells (20). Our results are consistent with these findings and support the observation that increased binding avidity occurs when there are tandem inserts. However, the attractions of the present display system are the availability of two functionally distinct binding domains in the same protein and the ability to manipulate these domains independently.

The random peptide display technology allows large populations of diverse peptides to be screened for specific binding affinity to an immobilized target. A number of peptide sequences conferring the ability to coordinate Ni\textsuperscript{2+} ions were selected from the random library constructed in the FimH fimbrial protein. A common feature found in all of the sequences was the presence of at least one histidine residue. Histidine has previously been shown to participate in the coordination of various metals (Ni\textsuperscript{2+}, Cu\textsuperscript{2+}, Zn\textsuperscript{2+}) by proteins (1). The insert present in the most enriched clone (pMAS38) is interesting since it contains only one histidine residue but is rich in proline residues. Taking into account the VNN coding scheme which we used in the genetic structure of our library, we also observed enrichment for arginine residues. Interestingly, both proline and arginine provide a nitrogen-located electron lone pair which perhaps could be involved in chelating metal ions. No similarities to other nickel-binding sequences in the database were identified. It is apparent that this display system provides a plethora of structural solutions to metal binding. Analysis of all of the clones indicated that in some cases only one histidine residue in the insert sequence is required to confer the ability to bind to metal ions on recombinant cells expressing the chimeric FimH proteins. It is possible that the folding of the chimeric FimH proteins is such that naturally occurring histidines in the protein (of which there are two) also participate in the construction of the Ni\textsuperscript{2+}-binding site. Alternatively, the fimbrial structure may be such that more than one FimH molecule is able to participate in metal ion chelation. This may be particularly significant in light of the observations of Jones et al. (6), who observed tip fibrillar complexes at the distal ends of type 1 pilus rods, suggesting that multiple copies of FimH are present. The correct interpretation of these observations awaits resolution of the FimH three-dimensional structure by X-ray crystallography.

The N-terminal part of the FimH protein contains a natural receptor-binding domain which recognizes D-mannoside-containing structures. Molecular exchange techniques were used to demonstrate that this domain can be manipulated to confer the ability to bind to protein targets. We envisage the possibility that an interesting range of heterobinary adhesins can be constructed by combining the two types of technology (i.e., an
altered natural binding site in the N-terminal end of this molecule combined with the ability to display peptides with binding affinity at the C terminus).

There are obvious advantages inherent in using the FimH display system for the construction of heterobinary adhesins. Two functionally distinct binding domains can be engineered into one (binary) adhesin and presented on the bacterial surface, which permits easy clone selection by selective binding and enrichment procedures. In addition, immobilization of the resulting cells by one adhesive domain could facilitate the use of the cells in detection systems for metals or perhaps directly as biosorption agents for the removal of toxic or precious metals from the environment. It is also possible to purify fimbriae by blending if the chimeric proteins are used without bacteria. Furthermore, there is also the potential to use the major fimbrial structural protein, FimA, to present binding sequences in high-valency display formats. Such technology may have a role in the construction of ordered nanostructures.

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