Development of activatable recognition proteins using the bacterial adhesin FimH

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Abstract

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Recognition molecules, such as antibodies, are widely useful in many applications within bioengineering, or life sciences in general. However, the high cost and variability in antibodies has led to the development of alternative protein scaffolds. While many such alternatives have high binding affinity, a range of specificities, and smaller sizes compared to antibodies, they do not have the advantage of regulated binding, a feature that could be hugely useful in controlled release applications, diagnostic tests, bioseparations, and more. Previously, no antibody or alternative scaffold had been used to develop regulated recognition molecules in an efficient and cost-effective way.

This work describes the use of the bacterial adhesin FimH as a protein scaffold for recognition molecules whose binding is regulated. FimH is a two-domain protein in which the binding pocket on one domain is allosterically regulated by the other domain, or parasterically regulated by triggers binding adjacent to the ligand in the pocket. This regulation is based on a major conformational change across the binding domain and binding pocket of FimH, which can switch from a loose to a tight conformation. We show here that this binding pocket contains many permissive positions which, when mutated, are tolerated by the protein and result in a new specificity towards both small molecules (i.e. nickel) and large molecules (i.e. Penta-His

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monoclonal antibody). Moreover, even after mutating the binding pocket, FimH not only still exists in both conformations, but can be induced to shift between both conformations. This results in the creation of new recognition molecules which are regulated parasterically using mannose, or allosterically using the wedging antibody mab21, when the FimH variant has an affinity that varies with conformation.

We then expand the capabilities of FimH as a scaffold by building a diversity library in which two of the binding pocket loops previously identified to carry permissive positions are mutated using NNK codons. The resulting library of 3.32×10^6 codons is characterized and then screened using a biomagnetic selection assay against the model target antibody Penta-His. Two high-affinity clones and one moderate-affinity clone are identified from the library with new specificity towards this antibody.

Overall, this work describes how a bacterial adhesin with conformation-dependent binding can be used to generate new recognition molecules which maintain the native protein's regulatory mechanisms. This has not been done previously with an allosteric scaffold, nor has FimH been utilized in this way before. Ultimately, this paper lays the groundwork for a new, useful tool for generating regulated recognition proteins that meet the ever-changing needs of life sciences research and applications.

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Chapter I: Introduction

Problem Statement

Recognition molecules are used widely in life sciences research and many biomedical applications. The gold standard for decades has been antibodies, the immunoglobulin molecules that can be readily plucked from the immune system to bind to an incredibly wide range of targets. This range of diversity, their high affinities, their bivalency, and their relative biocompatibility explain why antibodies remain so useful. However, antibodies are not perfect. Their large size, costly and time-consuming production, and batch-to-batch variability can limit their use, affordability, and reliability in many applications. Moreover, while antibodies bind to many targets, their capture or release of those targets cannot be naturally triggered. This unique ability of regulating binding of recognition molecules would be hugely useful in many life sciences applications, including diagnostics, bioseparations, and targeted imaging or drug delivery. While antibodies have been engineered with linkers or other additional domains to regulate binding, this is often difficult and not possible to be applied across a range of targets.

Recently, researchers have explored using alternatives to antibodies to address their binding needs. These are typically recognition molecules built upon other protein scaffolds that are smaller, produced quickly and easily via yeast or bacterial systems, and more amenable to engineering towards various applications. Several of these "antibody alternatives" have been commercialized, filling in gaps where antibodies may fail as reliable and affordable recognition molecules. However, these alternatives also lack regulated binding to their targets; they face similar challenges as antibodies when aiming to design a range of regulated recognition molecules. Thus, currently there does not exist a cost-efficient way to obtain regulated binding molecules to a range of targets.

Solution

Since scaffolds can serve as excellent binding molecules, it follows that the right scaffold could also generate regulated recognition molecules. Proteins already exist with conformational-dependent binding to their targets. However, to date, such proteins have not been used to generate *regulated* molecules to an array of targets. Therefore, it is worth exploring whether a protein with conformational-depending binding could serve as such a scaffold.

The bacterial adhesin FimH may address this need. FimH is a two-domain protein found on the tip of *Escherichia coli* fimbriae, or the long appendages responsible for bacterial adhesion. Naturally, the binding domain of FimH binds to the sugar mannose, thus enabling *E. coli* to attach to many cell surfaces. The second domain of FimH, the pilin domain, keeps the binding domain in a loose conformation that has a lower affinity for the target mannose. However, when some trigger such as force breaks the interactions between these two domains, a conformational change propagates across the whole binding domain to affect the binding pocket, which switches from a loose, low-affinity conformation to a tight conformation with high affinity for mannose (Le Trong, et al., 2010). Along with force, bulky molecules such as antibodies that bind within this interdomain region, distant from the binding domain, can also act as the trigger causing the conformational change (Tchesnokova, et al., 2008). In this way, FimH has conformational-dependent binding that is allosterically regulated. Recent evidence has shown that binding to mannose could also be regulated using parasteric inhibition, where the trigger is a molecule that acts adjacent to the ligand in the binding pocket (Kisiela, et al., 2015). Thus, FimH offers at least two mechanisms by which binding to its ligand can be regulated.

The FimH binding pocket consists of three loops which interact with the target mannose, presenting an ideal site for epitope display. By modifying these loops, FimH may serve as a scaffold for displaying epitopes that bind to a range of targets. Indeed, FimH has been explored as a scaffold for epitope display in the past, although with different strategies. Schembri, et al. used FimH to create chimeric structures with novel binding to heavy metals while retaining binding to mannose (Schembri & Klemm, 1998), while Sokurenko et al. used FimH display to gain a deeper understanding of the flexibility of the protein's specificity (Schembri, Sokurenko, & Klemm, 2000). These examples hint at the potential of FimH as a successful display scaffold. When combined with our more recent understanding of the conformational-dependent binding of FimH, FimH could be used to not only display binding epitopes, but to then regulate binding by those epitopes. To our knowledge, the use of a protein with conformational-dependent binding as a scaffold for generating regulated recognition molecules has never been reported. Moreover, parasteric inhibition has not yet been used as a mechanism for regulating binding in a display scaffold.

Hypothesis

The bacterial adhesin FimH can be used as a display scaffold in a way that retains its natural binding regulation. While some epitopes may require FimH to be locked in one conformation, others may be flexible enough to allow for conformational-dependent binding. We hypothesize that the loops of the binding site can be modified to bind to new targets while, in some cases, retaining the protein's ability to change conformation. Then, using either an allosteric trigger or a parasteric trigger, binding to the new target can be regulated. Using this information, a library of potentially regulated binding proteins can be created using FimH as the display scaffold. This library can then be screened for binders to new targets of interest.

Specific Aims

Aim 1: Demonstrate feasibility of FimH as a protein scaffold with conformation-dependent binding.

We used site-directed mutagenesis on each of the binding loops of FimH to generate FimH mutants with binding to an anti-6xHis antibody, showing that the specificity of FimH can be changed. By also measuring binding of these mutants to the anti-FimH monoclonal antibody mab824, which binds independent of the conformation of FimH and far from its binding pocket, we demonstrated that FimH tolerates changes to its binding loops, meaning that it still successfully expresses. After stabilizing these mutants in the loose or the tight conformation, we used a similar assay to show that FimH can still express in both of these conformations, further demonstrating the binding loops' tolerance of mutations. We then successfully used mannose to trigger conformational-dependent binding of one FimH mutant to its target Penta-His antibody, showing a 4-fold change in affinity via the parasteric mechanism. Lastly, we used mannose and mab21 to induce detachment of the target Penta-His antibody from another FimH mutant via parasteric and allosteric mechanisms, respectively. This demonstrated the flexibility of FimH as a scaffold for generating regulated proteins.

Aim 2: Develop a FimH-based library, optimized for capture of weak binders

We first selected and optimized an assay based on biomagnetic selection for identifying and enriching desired variants from a FimH library. Successful enrichment using multiple rounds of biomagnetic selection was shown using a mock FimH-based library. We then developed a new expression system for FimH using

a single plasmid system with an *araBAD* promoter, featuring tightly regulated, high FimH expression, demonstrated via flow cytometry. Next, we constructed a FimH library with either the CDR2 loop, the CDR3 loop, or both loops randomized, resulting in a library of 3.3×10^6 variants. Using flow cytometry with the appropriate monoclonal antibodies, we demonstrated that about 41% of the library expresses a functional FimH, with 92% expressing non-wild-type FimH mutants. Lastly, we used our optimized biomagnetic selection assay to identify two high-affinity binders and one moderate-affinity binder to the commercial monoclonal antibody Penta-His (Qiagen, Hilden, Germany).

Chapter II: Background

The World of Binding Molecules

Molecules that bind specifically to targets of interest are hugely useful in life sciences and bioengineering. Therapeutics, diagnostics, as well as basic and applied research all rely heavily on these binding molecules. Currently, antibodies dominate the field of binding molecules. One reason for this is historic; until about 20 years ago, the only source of diverse binding molecules which could be scanned for a desired specificity was the immune system (Binz, Amstutz, & Pluckthun, 2005). Yet even today, with the great range of protein engineering and directed evolution tools available, antibodies remain the most popular binding molecules. They have high specificities, with affinities often in the low nanomolar to picomolar range, surpassing most chemical drugs. Their immunoglobulin (IgG) structure provides bivalency, improving binding kinetics for many applications. When used in therapeutics, antibodies often have sufficiently long serum half-lives, and many can be made biocompatible by having fully human compositions.

However, antibodies still have many disadvantages. For many applications, the high molecular weight of antibodies poses a significant problem, limiting, for example, tissue penetration in drug delivery. Their unique structure of four polypeptide chains, with glycosylation and at least one structurally crucial disulfide bond, means that the production of antibodies must be done in eukaryotic expression systems, often mammalian cell lines (Gebauer & Skerra, 2009). This production is both laborious and costly, and has not improved significantly (Scolnik, 2009). Moreover, due to a lack of standardization and consistency in antibody production, batch-to-batch variability is often a problem (Buckingham, 2015). The antibody hybridoma can also become destabilized, preventing continued production of the same antibody. Lastly, many therapies involving antibodies require a large dose to be effective, which can result in potential immunogenicity. Given the high cost of antibody production, this also generally means that antibody-based therapies are very expensive.

To address the disadvantages of antibodies, smaller versions of these molecules have been engineered. These include Fab fragments, or monovalent antibody fragments produced via proteolysis, as well as single chain variable fragments, or ScFvs, which contain the variable heavy and light domains tethered together via a flexible polypeptide linker (Hudson & Sosuriau, 2003). Both fragments carry the advantages of being

smaller, and therefore having improved tissue penetration and better pharmacokinetics (Levin & Weiss, 2006). However, in making these antibodies smaller, stability is often sacrificed. These fragments still require the intradomain disulfide bond to remain stable, which does not form in reducing intracellular environments. Moreover, these fragments may aggregate, particularly if they are fused to additional domains for improved therapeutic efficacy. Thus, while antibodies are used widely, and while significant engineering is being done to modify and improve antibodies for use in various applications, they still carry significant limitations, chief among them the high production time and cost.

Non-Immunoglobulin Scaffolds

To address this issue, researchers have explored the use of alternative protein 'scaffolds', or polypeptide structures that are amenable to protein engineering purposes. Scaffolds are commonly used to display epitopes for binding new targets. Then, either these epitopes are isolated as useful binding peptides, or the whole scaffold is isolated as binding proteins.

The Ideal Protein Scaffold

Antibodies' high recognition function is partly due to its two-part structure, consisting of a structurally conserved core and a separate binding site that can be hypervariable in sequence and conformation. This is important for any protein scaffold—it must (as much as possible) structurally separate its conformation stability from its target recognition function and local shape (Skerra, 2000). This usually is possible when a structurally rigid core brings together several exposed loops which form the potential binding interface. However, as shown in many examples, the binding interface does not need to be limited to exposed loops. As long as the protein possesses 1) a well-defined hydrophobic core that provides significantly to the free energy of folding, and 2) a solvent-accessible active site or binding pocket that is spatially separated from the core, it may act as an excellent scaffold.

Beyond these structural traits, the ideal scaffold would also be smaller than the antibodies' 150 kDa. This makes it more useful for therapeutic applications, where smaller sizes are needed for improved pharmacokinetics. However, it must also be thermodynamically stable, an issue that often arises with very small protein scaffolds. Lastly, it must be possible (and relatively easy) to express the protein for protein purification.

The most ideal scaffold would improve on all the limitations of antibodies without compromising their high affinity and specificity range. However, the importance of each trait depends on the intended application.

Advantages of Non-Ig Scaffolds

Because these antibody alternatives are often smaller proteins that are expressed in yeast or bacterial systems, their production tends to be far less costly. Protein production via bacterial expression systems, for example, can be easily scaled up with less of an increase in cost, compared to scaling up antibody production via mammalian expression. It also takes far less time to produce the protein, on the order of a couple weeks compared to several months or longer. These savings in cost and time are huge for the life sciences industry and for academic life sciences research.

Antibody alternatives are also more amenable to engineering, meaning they can be engineered towards a particular application with more ease and success. Because they are produced via yeast or bacterial systems, there is less variability in batches, since successful production of the binding molecule does not rely on an animal's immune system (Hosse, Rothe, & Power, 2006).

Examples of Protein Scaffolds

Many different protein scaffolds have been explored. These include proteins that not only contain structures similar to immunoglobulins, but also structures with several domains, or tiny structures featuring one exposed loop, or even structures with a variable region in the secondary structure.

Table 1. Selected protein scaffolds and their structural properties

Scaffold name	Parent protein	Molar mass	Structure and randomization	Selection Method
Affibody	Staphylococcal protein A Z-domain	6 kDa	3-helix bundle; no disulfide bridges	Phage or ribosome display
Affilin	γ-B-crystallin	20 kDa	2 identical domains, active site in β-sheet	Phage or ribosome display
Anticalin	lipocalins	20 kDa	β-barrel with hypervariable loop region	Bacterial expression, phage display
DARPin	Ankyrin repeat proteins	10-19 kDa	3+ Ankyrin repeats (α-helices separated by loops)	Ribosome display
Knottin	Trefoil knot fold	3 kDa	Backbone twisted into knot, held by disulfide bonds	Yeast display
Kunitz domain	Kunits domains of protease inhibitors	6 kDa	Disulfide-rich, with α- and β- fold	Phage display
Monobodies/ Adnectins	10th type III domain of fibronectin	10 kDa	β-barrel with 3 exposed loops	Phage and yeast display

Table 1 lists just a few of these scaffolds and their key properties. Of these scaffolds, several have been widely studied and even commercialized. Two of these will be discussed a little further.

Affibody: Affibody molecules are based on the B-domain of the staphylococcal protein A. This domain consists of 58 amino acids folded into a three-helical bundle structure, making it one of the first non-β-sheet backbones used for a scaffold (Hosse, Rothe, & Power, 2006). The B-domain was altered for improved chemical stability, resulting in a new engineered variant called the Z-domain (Lofblom, et al., 2010). Additional advantages of this protein as a scaffold include its very small size, its high solubility, and relatively high thermal stability. Combinatorial libraries were made by randomizing 13 residues in two of the helices, residues which formed part of the original Fc-binding surface of the Z-domain and were solvent-accessible (Nord, Nilsson, Nilsson, Uhlen, & Nygren, 1995). Phage display was then used to display the protein library for biopanning against a number of targets, including gp120, insulin, and fibrinogen. Affibody molecules were identified with affinities in the micromolar to picomolar range, matching the high affinity of antibodies.

Anticalins: While many scaffolds can identify proteins or other high molecular weight targets, it has been difficult to find scaffolds capable of capturing low molecular weight ligands with high affinity (Gebauer & Skerra, 2009). The exceptions so far are scaffolds based on lipocalin proteins, called anticalins. These polypeptides contain 160-180 residues and can be found in a variety of organisms. What makes lipocalins particularly successful at capturing small molecules is the presence of a ligand-binding pocket formed from four hypervariable loops. These loops connect eight anti-parallel β-strands, forming a conical structure (Schlehuber & Skerra, 2005). This β-barrel is well-conserved among the many diverse lipocalin proteins, while the loop region shows significant differences in the residues, length, and conformation. As a result, there was already sufficient evidence in nature that the loop regions could tolerate combinatorial mutagenesis. In constructing anticalins, 16 residues across the four loops and adjoining portions of the β-barrel were randomized, using a variety of lipocalin proteins. Using phage display, then, the resulting anticalin library was screened against a large variety of targets, from small molecules like fluorescein (Beste, Schmidt, Stibora, & Skerra, 1999) to larger targets such as human hemoglobin (Vogt & Skerra, 2004). The conserved β-barrel structure and the existence of a hypervariable loop region make anticalins very similar to antibodies, and in the same way, successful as a binding molecule.

Both affibodies and anticalins have been commercialized, with some therapeutic variants under clinical development. Several other scaffolds have seen similar success as well. While these scaffold proteins are excellent as binding molecules, with various benefits, they all serve to only capture molecules. None can release molecules on cue, or otherwise control binding. This trait will be discussed next.

Regulation of Recognition Proteins

Binding molecules such as recognition proteins bind to their target when the thermodynamics and kinetics allow for binding to take place, and they unbind from their targets in a similar way. In most cases, if the protein and target are present in one system, binding and unbinding takes place without deliberate control by the observer. For example, one cannot control when an antibody binds its target, or easily force the antibody to release its target on cue, except by modifying the concentration of one or the other. Scientists have sought or identified many other ways to control binding and release of targets, because it adds a significant level of control to many applications and often improves them.

Applications for Regulated Binding

The ability to trigger binding and release of targets captured by proteins would be highly useful in many areas of biotechnology. The field of drug delivery, for example, requires controlled release of the drug payload and targeted delivery. Proteins or peptides are frequently used in designing drug carriers; so, it's important that the protein can be triggered to release the drug on cue. Similarly, imaging applications that rely on delivery of contrast agents would rely on regulated proteins as well.

Other applications include molecular sensors for diagnostics, food or environmental safety, or simply the study of a particular system. Regulated binding of the molecule of interest can improve sensitivity by essentially "pre-concentrating" the molecule. This would be done by first capturing molecules from the sample, removing the sample supernatant, and then releasing the molecules in a smaller volume, resulting in a higher concentration (Wang & Han, 2008). This sample preparation can improve sensitivity of existing tests.

Another application for regulated binding is bioseparations. Typically, samples are flowed through a column (with some sort of matrix) that captures the molecule of interest, while the rest of the sample elutes through. Then, the molecule of interest is eluted using a "trigger", which is typically a competing molecule that forces

the molecule of interest to release from the matrix. Other separation techniques take advantage of hydrophobic interactions or the ionic charge of the sample components. Still other cues that change the environment of the matrix to enable release of the molecule of interest would give more, and potentially cleaner, options for bioseparations than adding in a competing molecule.

Common Methods for Regulating Binding and their Flaws

In nature, there are many proteins or peptides that change conformation in response to external stimuli, a property which researchers have been quick to characterize and utilize into their own applications. This change is often in response to environmental cues such as temperature or a change in pH. Examples of this include the polymeric elastin-like polypeptide of the mammalian elastin protein, in which one residue can be changed to result in a sharp reversible hydrophobic-hydrophilic phase transition triggered by temperature, pH, or ionic strength (Chockalingam, Blenner, & Banta, 2007). Many others exist that change from a disordered polypeptide to a secondary structure, or switch secondary structures, or other similar structural changes. To generate proteins with regulated binding specificity, these "switchable" proteins or peptides found in nature must be applied to relevant binding proteins, such that ligand binding can be triggered by that switch. This can be difficult to do without changing the binding affinity or stability of the protein.

Most other examples of binding proteins with engineered regulation rely on a linker which must be cleaved to activate binding or release the bound molecule. 'Pro-ligands', for example, are protease-activated binding ligands in which substrate access to the active site is sterically blocked by an inhibitor that is attached to the ligand via a protease-cleavable linker (Thomas & Daugherty, 2009). One disadvantage here is that the extraneous tags are left attached to the final purified protein. More importantly, the linker strategy usually precludes binding reversibility, giving only one-way control.

One final method for ultimately forcing binding molecules to release their targets involves harsh conditions, such as highly acidic conditions or high temperatures that may even denature one or both molecules. While effective, it is obviously not ideal or suitable to most applications.

A Continued Need for Regulated Binding Proteins

Given the usefulness of regulating binding proteins and the flaws of most existing methods, it is clear that there continues to be a need for generating regulated proteins that bind to an array of targets, without

contaminating the final product. To this end, scaffolds for generating new binding proteins could be useful here.

The Ideal Scaffold – FimH

What are Fimbriae?

The gram-negative bacteria *Escherichia coli* adhere to surfaces using several different types of organelles called fimbriae (also pili). One of these are type 1 fimbriae, which bind specifically to mannosylated surfaces. These long arm-like appendages coat the cell at about 200-500 per cell (Schembri, Kjaergaard, Sokurenko, & Klemm, 2001). As shown in Figure 1, fimbriae consist of multiple protein subunits, each contributing to the structure or function of the pili.

Most of the fimbriae structure consists of about 1000 FimA subunits, which essentially determine the length of the fimbriae. These are polymerized into a right-handed helical structure. Small quantities of other subunits include the chaperone FimC and the usher FimD. These subunits help with the assembly of type 1 fimbriae via the chaperone/usher pathway in the periplasm of the cell (Munera, Hultgren, & Fernandez, 2007). Other minor components include FimF and FimG, as well as regulatory subunits FimB and FimE. These latter subunits regulate expression of fimbriae in a phase variable manner. *E. coli* shift periodically

Figure 1. Portion of type 1 fimbriae crystal structure (Le Trong, et al., 2010)

between a fimbriate and non-fimbriate state, often depending on whether the cells exist in suspension or in a biofilm (Klemm, 1986). All of the structural subunits connect via a β-strand donated by the previous subunit that stabilizes the Ig-like fold, in a process known as 'donor strand complementation'. This strand occupies the hydrophobic groove that would otherwise be left exposed.

The last subunit, FimH, is the first to be incorporated into the growing fimbrial filament. Unlike other subunits, this protein consists of two domains. The pilin domain interacts with the rest of the pili via a donated β-strand. The lectin domain is the final tip of the fimbriae and is the adhesive domain responsible for binding to mannose.

The Function of FimH

The FimH subunit of type 1 fimbriae has received significant attention because it is responsible for *E. coli* adhesion in many environments. Uropathogenic *E. coli*, in particular, utilize FimH to adhere to the urinary tract, making study of FimH important towards understanding the virulence factors of urinary tract infections and finding potential treatments or vaccines (Bouckaert, et al., 2005). As a result, both our group and many of our collaborators have studied this protein extensively and have come to understand a great deal about its function.

As mentioned, FimH adheres to mannosylated surfaces via its N-terminal lectin domain. Unique to this lectin domain, however, is its relationship with the anchoring pilin domain. This domain acts as an allosteric regulator of the lectin domain's binding to mannose. Under no flow or low flow conditions, FimH remains in an inactive state where its affinity towards mannose is low. Under moderate flow conditions, however, shear force breaks the interactions between the anchoring pilin domain and the lectin domain, such that FimH switches to an active state with high affinity for mannose. This unique behavior matches the catch bond model, in which the bond between FimH and its ligand mannose strengthens with force, up to a certain force threshold (Yakovenko, et al., 2008). It is because of this behavior that *E. coli* are able to adhere so well under moderate-flow conditions. The structural basis behind this allosteric behavior is discussed further.

The Structure of FimH

FimH is a 279-residue protein, of which 158 residues form the lectin domain. A 3-residue linker connects this domain to the rest of the FimH, or the pilin domain. The interactions between these two domains are the basis of the force-activated allosteric behavior of FimH.

As shown in Figure 2, the lectin domain of FimH is composed of a β sandwich fold, a structure commonly thought to be quite rigid (Le Trong, et al., 2010). It involves a large, twisted β-sheet and a β bulge, opposite of a split β-sheet. These form the hydrophobic core of the FimH lectin domain. To the top are three solvent-exposed loops forming the binding pocket of FimH, along with a fourth loop known as the 'clamp' loop. Towards the bottom, interacting with the pilin domain, is the swing loop, the linker loop, and an insertion loop that inserts into the pilin domain. These parts of FimH are named as such because of their movement when FimH undergoes a great conformational change.
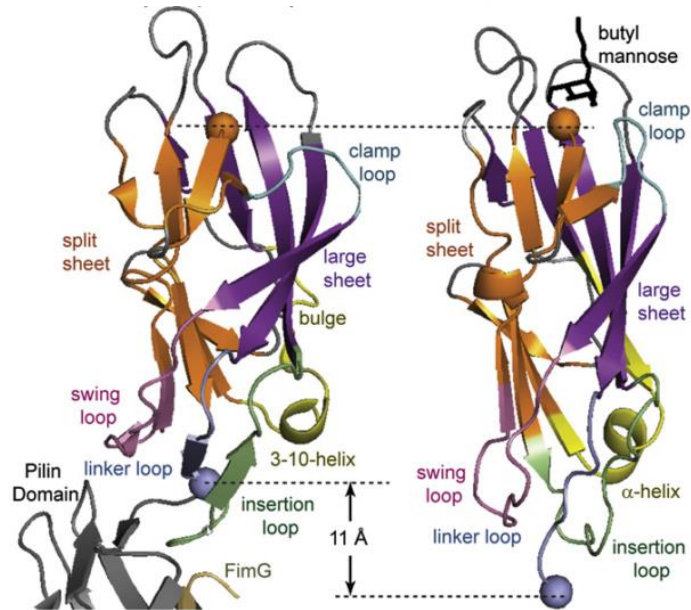
Figure 2. Conformation change in the FimH lectin domain from the "loose" conformation (left) to the "tight" conformation (right). (Le Trong, et al., 2010)

When triggered allosterically, such as by force, FimH undergoes a conformation change that propagates across the whole β-sheet. When switching from its loose conformation to the elongated, tight conformation, this β-sheet untwists, while the clamp loop at the top 'clamps' towards the binding pocket tightly. The swing loop, previously "swung" out in the compressed state, moves inward, while the linker loop extends outward. This latter change, combined with the rest of the conformational change across the β sandwich, is responsible for an 11 Å elongation in the structure, shown in Figure 2. FimH is the only example where an allosteric conformational change of this size propagates across a whole β-sheet (Rodriguez, et al., 2013). Another aspect of the structure of FimH that makes it interesting is its resemblance to the structure of immunoglobulins. Both have a hydrophobic core composed primarily of a β-sheet structure. Both also have a loop region which forms the binding site of the protein. Because of these similarities, the three loops of FimH that form the binding pocket are called CDR1, CDR2, and CDR3, since they are 'complementarity determining regions'.

FimH as a Regulated Binding Scaffold

For FimH to be a successful scaffold for building regulated binding molecules, it must be convenient to trigger binding and/or unbinding of targets. Naturally, FimH is triggered by force allosterically, which breaks the key interactions between the pilin domain and lectin domain, resulting in the conformational change to the active state (Fig. 3E). Analysis of this change found that any means of breaking the interactions between the domains, which keep FimH in the loose conformation (Fig. 3A), would result in a switch to the tight conformation (Fig. 3B). For example, the isolated lectin domain is in the tight conformation, because its interactions with the pilin domain have been completely broken.

Figure 3. Schematic of FimH states and triggers (Tchesnokova, et al., 2008). The pilin domain is the *lower shape*, the lectin domain is the *upper shape*; the black triangle is mannose, and the black rectangle is interdomain region.

Point Mutations

Other means of triggering this change can involve a specific mutation in or near the interdomain region that weakens the interactions (Fig. 3D). These mutations can sometimes be effective enough to sustain FimH in the tight conformation (Tchesnokova, et al., 2008). Examples of these include the point mutation A188D. Located in the pilin domain adjacent to the lectin domain, Ala[188] interacts with Val[118] of the lectin domain continuously when in the loose conformation, as has been demonstrated in MD simulations (Aprikian, et al., 2007). Mutation of this residue to Asp (D) resulted in a significant increase in FimH binding to mannose; further studies demonstrated that this increase was due to FimH switching to its tight conformation. While many other similar point mutations, such as Y186R and Y64R, have been identified (Aprikian, et al., 2007), A188D appears to have the largest affect, and has therefore been used as a key point mutation to stabilize FimH in the tight conformation as much as possible. Similarly, other point mutations stabilize the loose conformation of FimH to various degrees. One of these is R60P, a mutation of Arg[60] located in the β-bulge of FimH. This bulge is significant to FimH's allosteric pathway, and is thought to act as a 'release valve' for the strain caused by the more twisted conformation of the main β-sheet in the loose conformation (Rodriguez, et al., 2013). Therefore, mutating the Arg[60] to a proline destabilized the tight conformation by

preventing key movements, particularly since prolines are known to destabilize β-sheets. As a result, R60P is frequently used to stabilize FimH in the loose conformation. Finally, we must note that the stabilizing behavior of these point mutations applies to an otherwise wild-type FimH. It is possible that mutations to other parts of FimH may change the degree to which A188D or R60P keep FimH in one conformation.

The Wedging Effect: Antibodies

Antibodies can also be used to trigger the conformational change using a wedging effect, where binding to the interdomain region results in structural interference between the domains, causing a switch to the tight conformation (Fig. 3C). One such antibody is mab21, a mouse monoclonal antibody that was raised against the *E. coli* K12 FimH lectin domain. It was previously determined that mab21 can only recognize the tight conformation of FimH (Tchesnokova, et al., 2008). Its three-dimensional epitope is composed of positions 152-156 and 29 of the lectin domain, all of which lie in the interdomain region of FimH. Therefore, when FimH is in the loose conformation, the epitope is not accessible to mab21. However, FimH switches to the tight conformation when binding its ligand mannose; in that short time, mab21 can bind to its now exposed epitope. It was found that, upon binding to mab21, FimH remains in the tight conformation.

The Parasteric Mechanism

One final method for regulating FimH uses a relatively new concept of parasteric inhibition. Kisiela et al. discovered that an antibody raised against the loose conformation of FimH, called mab926, can bind to one of the binding pocket loops of FimH (CDR3 loop) and essentially "lock" FimH into the loose conformation (Kisiela, et al., 2015). Interestingly, this binding does not compete with the target mannose; it instead inhibits FimH in a non-competitive manner. To effectively bind mannose, FimH must normally shift to the tight conformation. Since mab926 binding prevents that shift, mannose binding is significantly reduced. Indeed, Kisiela et al. demonstrated that mab926 can induce detachment of *E. coli* biofilm, because mannose binding is reduced. This suggests that FimH binding to its target can be regulated via a trigger that acts adjacent to the target's epitope. Depending on the epitope, mab926 may be a parasteric trigger which induces FimH to shift to its loose conformation. Alternatively, since mannose binding shifts FimH to the tight conformation, one might hypothesize that it too can be used to parasterically regulated binding of FimH to its target.

These few methods show several ways for triggering binding that have been identified in the Thomas Lab or by our collaborators in Evgeni Sokurenko's group in UW Microbiology. However, other ways can be identified by engineering the interdomain region towards that end, such as by inserting a switchable peptide domain. While this is being explored by our group, that goal is beyond the scope of this thesis. What is relevant is that FimH can be triggered to shift between its tight and loose conformations using not just force, but a variety of triggers, some of which have already been identified, making FimH a useful scaffold for generating regulated binding molecules.

Understanding the Methodologies

Methods for Constructing a DNA Library

A DNA library is made typically to take advantage of directed evolution, or to access useful phenotypes otherwise not found in nature. A number of tools and methods are needed to develop a library of sufficient diversity, some of which are described here.

Gene Diversification: In an ideal world, a chosen sequence could be fully randomized, and the full sequence space could be explored and tested for desirable phenotypes. In reality, however, it is impossible to cover the full mutational space of most peptides and proteins, since it results in an enormous number of unique combinations that often exceeds the limits of most protein library creation methods. As a result, gene diversification strategies are often meant to obtain an optimal sample of the sequence space. This can be done using focused strategies that diversify specific regions of a protein, or random strategies that can diversify any residue of the protein. Methods for randomization include chemical mutagenesis, the use of mutator strains, error-prone PCR, homologous recombination such as DNA shuffling, and non-homologous recombination techniques (Packer & Liu, 2015). Chemical mutagenesis essentially involves randomly damaging the DNA with compounds such as ethyl methanesulfonate or deaminating compounds such as nitrous acid. While this has been used for many years, its use has been somewhat discontinued due to bias and concerns of damaging the chromosomal DNA. Error-prone PCR is a more commonly used method for randomly diversifying a gene. This takes advantage of low fidelity polymerases to generate DNA with changed bases, although these polymerases can still be 'too accurate'. Often Mn^{2+} and biased concentrations of dNTPs are added to further increase the error rate (Neylon, 2004).

Because we have structural knowledge of our protein FimH and have identified desired positions for randomization, our strategy will use focused diversification. For this, site-directed saturation mutagenesis is most commonly used. This involves the use of synthetic DNA oligonucleotides containing degenerate codons, or codons containing a mixed population of nucleotides at a given position. These codons can include N, which samples all four bases, K (samples G or T), S (samples G or C), or other options that sample two or three of the bases. Typically, NNK codons are used in order to reduce the frequency of stop codons, since the use of just G or T in the third position eliminates two of the three stop codon possibilities while still sampling all possible amino acids. Therefore the possibility of a stop codon is 1/32, or 3.125%, for each degenerate codon. Another option would be to use trinucleotide phosphoramidites in the synthesis of the diversified oligonucleotides (Neylon, 2004). This solves the problem of codon bias because each codon is synthesized one at a time. This can also eliminate stop codons. However, while effective, this method is not easy to prepare nor cheap, making it less desirable for library construction, where several iterations and several diversification strategies may be needed. Therefore, NNK randomization is the technique chosen here, due to its focused nature, its reduced stop codon frequency, and its lower cost.

Gibson Assembly: In order to assemble the diversified gene insert into the vector backbone, a number of assembly techniques can be used. Traditionally, restriction enzymes are used to cut an insert and a vector, such that sticky ends are produced for both fragments with the same restriction sites. As a result, they can be easily assembled using a ligase. If neither the vector nor insert have convenient restriction sites, as happens often with library construction, then blunt-end ligation is often used. Compared to sticky-end ligation, blunt-end ligation is less efficient, requiring longer reaction times since it depends on random collisions between blunt ends. In generating our vector, primers were designed such that inverse PCR led to overhanging bases. Therefore, overlapping extension techniques can be used instead, which are more specific too. These techniques were developed more recently and include commercial kits such as In-Fusion from Clontech® or sequence-independent and ligation-independent cloning (SLIC) (Li & Elledge, 2007).

One of these methods is known as Gibson Assembly. This can join products as large as 583 kb using reagents that are commercially available (Gibson D. G., et al., 2009), so that the Gibson mix can be made 'at home'. Briefly, an ideal ratio of insert to vector fragments are combined with relevant reagents in a one-step reaction that is carried out under isothermal conditions,
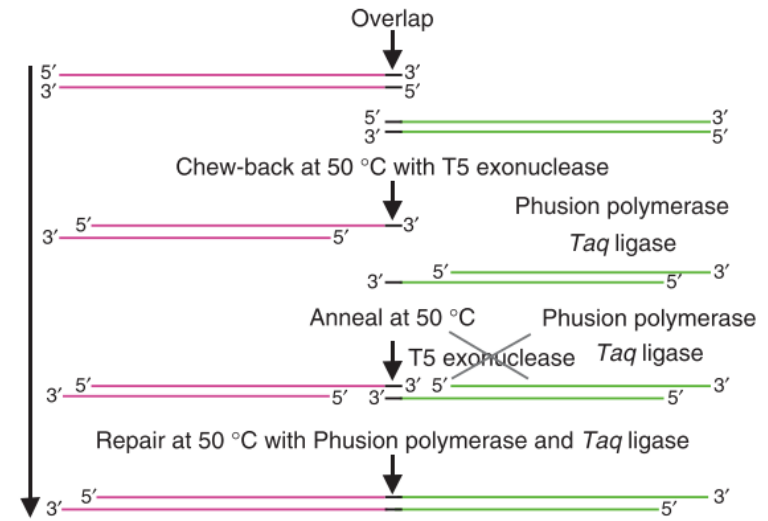
Figure 4. Gibson Assembly method (Gibson, et al., 2009)

or at a temperature of 50°C. The ratio varies depending on the size of the fragments, but typically falls around 3:1. During this reaction, a 5'-exonuclease chews back the double-stranded DNA segments from the 5' ends. The resulting single-stranded ends then anneal at 50°C. Next, a polymerase, typically the high-fidelity proofreading Phusion polymerase (New England BioLabs, Inc., Ipswich, MA), fills in the gaps of the annealed single stranded regions. Finally, a DNA ligase seals the remaining nicks in the DNA. This process is depicted in Figure 4. At this point, with the diversified gene inserted into the expression vector, the DNA library is ready for transformation.

Gibson assembly was chosen over other ligation methods due to the following reasons. First, ligation sites are not limited by the presence of restriction sites, enabling modification of and ligation around any region that interests us. Second, the one-step reaction uses reagents that any molecular biology lab would have, allowing us to make our own homemade Gibson mix at a significantly reduced cost. Third, multiple fragments can be ligated, rather than being limited to two fragments (insert and vector). Last, the reaction is significantly faster than traditional blunt-end cloning or even restriction enzyme-based cloning.

Methods for Displaying the Library

To screen a library for desired variants, the library must be displayed in a way that links the various genotypes to their respective phenotypes. The chosen method may depend on whether the proteins being

displayed require post-translational modifications, whether they can be expressed in a cell, the screening method chosen, or the phenotype being explored.

Phage display: The use of filamentous phages to display peptides or proteins is the oldest and most commonly applied tool for molecular display. Briefly, phage display uses bacteriophages to connect proteins with their genotypes. The DNA encoding the peptide or protein of interest is ligated into the phage coat protein of a bacteriophage such as the M13 filamentous phage. The resulting phage is then transduced into *E. coli* cells for replication, and the peptide or protein of interest is displayed on the phage outer coat (Smith, 1985). The resulting phage display library can then be screened for binding to various targets. While this method continues to be effective for many types of libraries, it does have some limitations. For example, phages can only display at most one to five copies of a polypeptide sequence fused to the phage coat, whereas other display methods can display hundreds to thousands of copies.

Yeast surface display: Display of proteins on cells gives the advantage of using flow cytometry for screening the library. A common system used is yeast display, which enables the display of mammalian proteins since the yeast cells' protein folding and secretory machinery is similar to that of mammalian cells. In yeast display, the protein of interest is fused to the Aga2p protein, resulting in surface localization of the protein fusion. The Aga2p protein mediates cell-cell adhesion during yeast cell mating. This means that the Aga2p-protein fusion in yeast display is displayed away from the cell for potential binding to targets (Boder & Wittrup, 1997).

Ribosomal and mRNA display: A relatively new form of molecular display uses cell-free protein engineering techniques to display proteins on ribosomes or mRNA *in vitro*. Briefly, ribosomal display accomplishes the link between genotype and phenotype during *in vitro* translation, where the complex of ribosome, mRNA, and the new folded polypeptide are stabilized. This complex is made possible by fusing a spacer sequence lacking a stop codon next to the gene of interest, preventing release factors from triggering disassembly of the ribosomal complex (Zahnd, Amstutz, & Pluckthun, 2007). The resulting complex displaying the folded polypeptide can then be screened. mRNA display works similarly by coupling the translated protein to its encoded mRNA through a puromycin analogue (Levin & Weiss, 2006). As cell-free systems, these avoid the growth and transformation steps of cell-based systems. As a result, the limitation of transformation efficiency is removed, resulting in libraries approaching 10^{13} or larger. While this

advantage is significant, these options are still novel and may have limits to the protein size and complexity that can be effectively displayed.

Bacterial surface display: Like yeast display, this method displays the protein of interest on the surface of cells as well, although in this case, the cells are bacterial. Several examples of bacterial display exist, in which the protein of interest is fused to some outer membrane protein, fimbrial protein, or other projected protein. The result is display on the order of hundreds or thousands of copies, as well as high transformation efficiency, ease of manipulation, and the ability to use flow cytometry to analyze the bacterial library (Levin & Weiss, 2006). One example of this involves fusion of 15-aa epitopes to the outer membrane protein OmpA, whose structure includes flexible extracellular loops (Bessette, Rice, & Daugherty, 2004). Another successful example uses the bacterial flagella protein FliC to display peptides, proving useful as a tool for mapping antibody epitopes (Lu, et al., 1995).

Methods of Screening the Library

Once a library has been developed and displayed, a method is needed for screening and selecting variants from the vast library of clones. Several methods are described here.

Screening of Spatially Separated Variants: Individual mutants can be physically separated in order to preserve the linkage between the genotype and the phenotype. With gene variants expressed via *E. coli*, for example, this may mean screening individual colonies on solid media or individual clones in multiwell liquid culture plates. This strategy is compatible with most assay techniques that enable testing of many phenotypes, including fluorescence, NMR, HPLC, gas chromatography, and more (Packer & Liu, 2015). This is ideal for testing various aspects of enzymatic activity as well. The distinct disadvantage of screening based on spatial separation is the limit on throughput, since, at most, 10^4 variants can be practically tested. It is significantly more time-consuming and infrastructure- and resource-intensive as well. For these reasons, these methods will not be explored in greater detail here.

High-throughput screening via flow cytometry: The greatest advantage of displaying libraries using cell-based systems is the ability to take advantage of flow cytometry and fluorescence-activated cell sorting (FACS). Flow cytometry analyzes the characteristics of particles in a fluidic sample as its passes through at least one laser. Hydrodynamic focusing is used to force cells to pass through in a 'single file' (generally) to enable accurate counting and characterizing of each particle, as shown in Figure 5. As each particle passes through the laser light source, light is scattered in a forward direction and to the side. Forward scatter is indicative of the size of the particle,
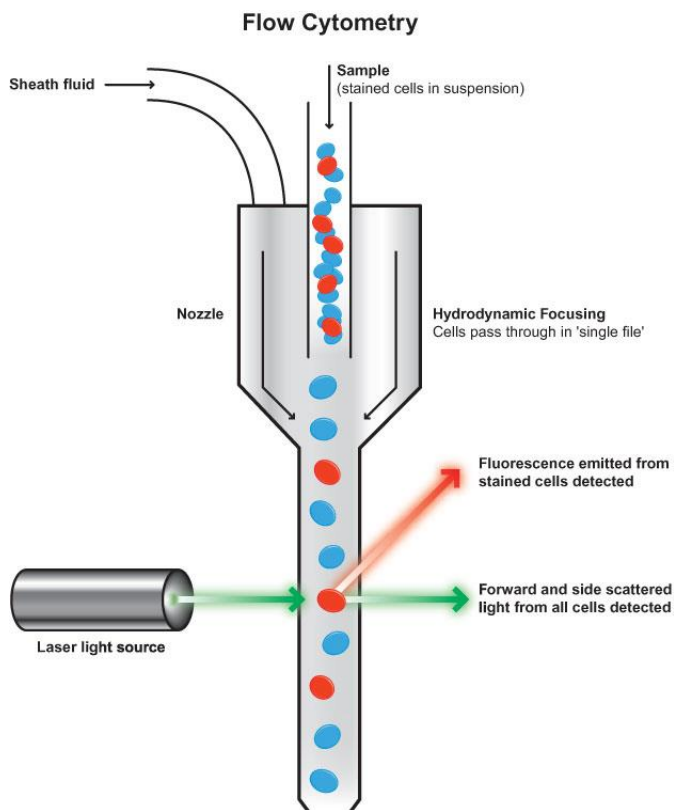
Figure 5. Schematic of flow cytometry mechanics (http://flowcytometry.med.ualberta.ca/)

whereas side scatter is generally indicative of the complexity or granularity of the particle. Other lasers of different wavelengths are also used to excite fluorophores attached to the particles of interest, allowing detection and quantification of specific populations. The combination of data (size, granularity, fluorescence, etc.) can give a breadth of information about the population being analyzed, such as potential aggregation and molecular binding due to fluorescence. As a result, compared to other screening methods, using flow cytometry for screening library clones allows for interrogating a bulk population, rather than individual clones, leading to more efficient and quantitative screening (Packer & Liu, 2015).

Additionally, FACS can be used to not just screen clones using flow cytometry, but also isolate desired clones. In this technique, the FACS machine uses electrostatic deflection of charged droplets to sort cells. Certain sorting settings control the formation of the drop and the criteria for charging the drop, allowing for either higher purity or higher yield (Davies, 2007).

Combining the high-throughput screening capabilities of flow cytometry with the sorting capability of FACS leads to efficient and more quantitative library screening. However, there are several disadvantages of this

system. While FACS sorters are becoming more efficient as technology improves, they still cannot sort libraries larger than 10^8 within a reasonable amount of time and cost, since their typical sorting rates take hours to effectively sort through smaller libraries. More importantly, FACS can be quite stringent. Although the stringency level can be adjusted, conditions prioritizing yield may result in no effective enrichment, while conditions prioritizing purity are so stringent that weaker binders are not captured. Since we are aiming to capture regulated binding molecules that may vary between weak binding and strong binding, it is important that even weak binders be captured, particularly for the first pass at directed evolution of regulated binders. Lastly, to make capture of weaker clones at all possible, FACS would require high amounts of ligand, making it less desirable when screening against more valuable targets in limited quantity.

Selection assays using an immobilized target: Rather than inspecting each individual clone or even all the clones of a bulk population, desired clones can be selected first based on their activity of interest and physically separated from the rest of the library for later analysis. In this case, the activity of interest is binding specificity or affinity. So, an immobilized target can be used to capture protein library members with desired binding activity to that target (Packer & Liu, 2015), washing away any non-binding library members. This binding selection can occur using a plate-based assay, where the ligand of interest is first immobilized to individual wells of a microplate. Samples of the library (sampling the diversity by at least five-fold to ensure full representation) are then added to the ligand-coated wells, allowed to bind, then washed to remove non-binders. While this assay is a well-established one and less stringent than FACS, it can lead to high non-specific binding and therefore false positives.

Alternative assays immobilize the ligand on beads instead, such as magnetic beads. One example of this by Dane Wittrup's group used magnetic beads as an efficient and highly avid selection method for a yeast surface display library. Briefly, the multivalency of yeast display is coupled with the multivalency of the target displayed on magnetic microbeads by incubating both together in various tested ratios. Even weak binders can then be isolated, with significantly less antigen required to coat these beads. Following cell/bead incubation, a magnet is used to separate the binders from the non-binders. The bound population can then be analyzed using a variety of techniques. Repetition of this method over multiple rounds resulted in up to 30,000-fold enrichment (Ackerman, et al., 2009).

Characterization assays

Several assays are used to characterize the function and structure of *E. coli* clones containing various FimH variants. Flow cytometry is used for characterization purposes when analyzing the whole FimH library, as well as to optimize the chosen selection method and demonstrate enrichment success. Since the mechanism of flow cytometry and FACS has already been described, the details of these experiments will be discussed in later chapters. Two other assays will be described in some detail here, with specifics saved for their use in later chapters.

Crystal Violet assay: To analyze binding of FimH clones to various targets, a binding assay using crystal violet has been used frequently by our group and others in the field (i.e. Sokurenko group). Crystal violet is a triarylmethane dye more commonly used as a histological stain and for Gram staining bacteria. In this assay, the ligand of interest is immobilized to the bottom of microplate wells. *E. coli* cells carrying the FimH variant of interest are then allowed to bind to the ligand. After unbound cells are washed away, the remaining bound cells are heat-fixed to the well bottoms. This ensures that even binders with potentially quick off-rates are retained in the wells for measurement. The crystal violet dye is then added to bind to the lipopolysaccharides of these Gram-negative bacteria, resulting in a violet color indicative of the amount of cells. After unbound dye is washed off, ethanol is used to resuspend the remaining dye for an absorbance measurement. Because the time between cell-ligand binding and cell-fixation is minimal, this assay generates a signal for even low binders. The disadvantage is that high-affinity binders cannot be distinguished well, since they all present with a strong (saturated) signal.

TMB-based ELISA: A second assay used to analyze binding of FimH clones is an ELISA, or enzyme-linked immunosorbent assay. Briefly, *E. coli* cells carrying various FimH variants or isolated fimbriae are directly immobilized to wells of a microplate. Primary antibodies of interest are then added for binding, after which a secondary detection antibody is added. This secondary antibody is attached to the enzyme HRP, or horseradish peroxidase, which oxidizes the substrate TMB, or 3,3',5,5'-tetramethybenzidine. This is added last to the wells to give a blue color. As the reaction progresses, the color may darken for high amounts of HRP or even change to green and purple; as a result, a stop reagent such as sulfuric acid is added, turning the reaction yellow. Both colors can be measured via absorbance, although the former is best measured at various time intervals to ensure that measurements are within the linear range.

One major benefit of the TMB-based ELISA comes the use of the enzyme HRP. This contains mannose groups which bind to FimH in a monomannose manner. As a result, presence of FimH or successful binding by FimH to monomannose targets can be very quickly and directly tested by adding HRP to wells containing FimH, and then adding TMB for detection. Other benefits of this assay are its more quantitative nature and the lack of ligand avidity, allowing for a more quantitative analysis of binding affinity.

Limits of this assay include the need for a reporter enzyme. Targets (or the cells themselves) must be able to bind to an HRP-probe (or other reporter enzyme) in some way for later detection, which limits the scope of targets that can be used. Another disadvantage of this assay is the amount of time that passes between FimH-ligand incubation and absorbance measurement. An additional hour-long incubation with the secondary probe must take place (except for direct measurement of HRP binding to FimH), which may mean that binders with relatively quick off-rates are washed off before their presence is measured.

Chapter III: FimH as a Scaffold for Conformation-Dependent Molecular Recognition

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Publication

Abstract

Antibodies have been used widely as the gold standard for recognition molecules. However, because of their high cost, batch variability, or poor suitability to certain applications, researchers have turned to alternative protein scaffolds as recognition molecules. While smaller and better able to meet unique application needs, current scaffolds as well as antibodies both lack the ability to easily regulate binding or release to targets. This properly would be highly useful in developing more specific and sensitive binding agents for diagnostic, therapeutic, and bioanalytical applications. To address this need, we describe the use of the bacterial adhesin FimH as a scaffold for molecular recognition. Along with a convenient binding pocket for molecular recognition, FimH also has conformation-dependent binding to its native target, which we show can be retained even after changing the protein's specificity from the small molecule mannose to a large antibody. Moreover, we show that both the allosteric and parasteric mechanisms native to FimH can be used to regulate binding by the FimH mutant to its new target. The native target mannose acts as a parasteric trigger for the new target, while an anti-FimH antibody binding at an allosteric site acts as an allosteric trigger. This illustrates the first example of a scaffold with conformation-dependent binding being used to generate not only new recognition proteins, but regulated binding as well.

Background

Antibodies are widely useful recognition molecules because they can bind to many different proteins and small molecules. This range grows in part because antibodies can be so quickly generated against almost any desired target. Their high affinities, bivalency, and typically high biocompatibility make antibodies the gold standard in many life sciences applications. However, antibodies do have some limitations. For many applications, the high molecular weight of antibodies poses a significant problem, limiting, for example, tissue penetration in drug delivery. Their production method is both laborious and costly, and has not improved significantly or with confidence (Scolnik, 2009). Moreover, due to a lack of standardization in antibody production, batch-to-batch variability becomes a problem (Buckingham, 2015). The antibody

hybridoma can also become destabilized, preventing continued production of the same antibody. While smaller versions of antibodies, such as Fab fragments and single chain variable fragments, have been engineered to address some of these concerns, these often have stability issues and may aggregate. Thus, while antibodies are used widely, and while significant engineering is being done to improve them for some applications, they still carry significant limitations.

Recently, researchers have turned to protein 'scaffolds' as alternatives to antibodies, designed to display epitopes for binding new targets. Because these antibody alternatives are often smaller proteins that are expressed in yeast or bacterial systems, their production tends to be significantly less costly and easier to scale up. Moreover, it takes far less time to produce the protein, on the order of a couple weeks compared to several months or longer. There is less variability in batches, since successful production of the binding molecule does not rely on an animal's immune system (Hosse, Rothe, & Power, 2006). Antibody alternatives can also be engineered towards an application with more ease and success.

Many different protein scaffolds have been explored to date. Some of these scaffolds, such as lipocalins (Beste, Schmidt, Stibora, & Skerra, 1999), are structurally similar to immunoglobulins. Others may be tiny structures with one exposed loop, oligomeric proteins with several domains, or even scaffolds with a variable region in the secondary structure, such as Affibodies or DARPins (Skerra, 2007). Several of these scaffolds have been widely studied and even commercialized.

While these scaffold proteins are excellent for generating binding molecules, they can only capture molecules. None can release molecules on cue, or otherwise regulate binding to their own targets. The ability to trigger binding and release of targets would be highly useful in many areas of biotechnology, including targeted drug delivery, sample preparation for diagnostic tests, improved contrast for imaging, or even capture and downstream release of circulating tumor cells. To address this, researchers have meticulously engineered regulation into existing scaffolds by combining domains to create an allosteric protein, or by using cleavable linkers to temporarily block the binding site. This has seen varying degrees of success. In no previous case, though, has a scaffold with innate conformation-dependent binding been used for protein display with the aim of retaining the protein's ability to express in both conformations and potentially regulate binding. This would not only be a novel use of such a protein, but also a highly useful

tool for more easily generating regulated recognition molecules. This is what motivates our choice for our non-IgG scaffold: the bacterial protein called FimH.

FimH is the last subunit of the long organelles called fimbriae, or pili, protruding from *Escherichia coli* cells. This protein consists of two domains. The pilin domain interacts with the rest of the pili via a donated β-strand, while the lectin domain is the adhesive domain responsible for bacterial adhesion to mannosylated surfaces. The binding pocket of the lectin domain contains three
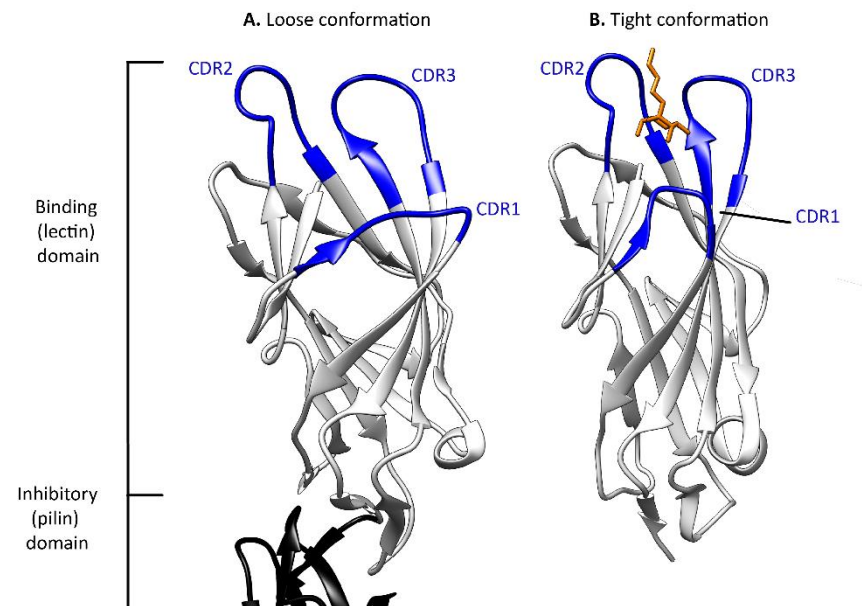
Figure 6. Structure of FimH and its conformational change. The CDR loops are in blue, the lectin domain in light gray, the pilin domain in black, and the ligand mannose in orange. A) Loose conformation of FimH, B) Tight conformation of FimH.

exposed loops which interact with mannose; these are called "CDR" loops for their similarity to antibodies' own complementarity determining regions. Unique to the FimH lectin domain is its relationship with the pilin domain, as shown in Figure 6. This pilin, or inhibitory, domain acts as an allosteric regulator of the lectin domain's binding to mannose. Under no flow or low flow conditions, the inhibitory domain keeps FimH in a "loose" conformation, where the binding pocket of FimH is more open, resulting in a low affinity towards its target mannose. Under moderate flow conditions, however, shear force breaks the interactions between the inhibitory pilin domain and the lectin domain. The resulting conformational change that propagates across the protein causes the binding pocket of FimH to tighten ("tight conformation"), resulting in a high affinity for mannose. This unique behavior matches the catch bond model, in which the bond between FimH and its ligand mannose strengthens with force, up to a certain force threshold (Yakovenko, et al., 2008). However, triggers other than force can also work in a similar way to induce conformational change. Bulky molecules such as antibodies that bind within this interdomain region can also act as an allosteric trigger by wedging between the two domains and breaking their interactions (Tchesnokova, et al., 2008).

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Additionally, FimH can be regulated using parasteric inhibition, where the trigger is a molecule that acts adjacent to the ligand in the binding pocket in a non-competitive way (Kisiela, et al., 2015). This was previously demonstrated by Kisiela et al, where an anti-FimH monoclonal antibody called mab926 bound to the CDR3 loop of the FimH binding pocket and locked FimH in the loose conformation. This did not compete with mannose binding directly, but non-competitively inhibited mannose binding and even induced detachment by forcing FimH to shift to the loose conformation. Similarly, mannose binding induces FimH to switch to the tight conformation, although this inducement is weaker than that of mab926. Therefore, it is possible that mannose could be used as a parasteric trigger for ligands binding adjacent to it in the binding pocket so long as it can induce detachment of that ligand.

Our understanding of both these mechanisms for conformation-dependent binding motivate us to consider FimH as a scaffold for displaying regulated proteins. While FimH has been used as a display scaffold previously (Schembri, Sokurenko, & Klemm, 2000), the binding regulation of the protein was not fully understood at the time. Initial studies included random mutagenesis using error-prone PCR of the whole *E. coli* FimH gene, which generated significant data on the flexibility of FimH specificity. However, it also resulted in far more truncated clones than useful clones. Later understanding of the two-domain nature of FimH led to an interest in displaying a random peptide library through a permissive position in the pilin domain of FimH, near the interdomain region. The goal was to maintain FimH's natural binding to mannose while adding binding to novel targets such as heavy metals or heavy metal oxides, resulting in chimeric structures (Schembri & Klemm, 1998). While this initial study was successful, later understanding of the importance of the interdomain region for mannose-binding and allostery limited the scope of the pilin-domain-expressed library; therefore, the potential of this library was not fully explored (Rice, Schohn, Bessette, Boulware, & Daugherty, 2006). Moreover, these fimbrial libraries could not fully take advantage of FimH's conformational-dependent binding behavior, since that knowledge, particularly the concept of parasteric inhibition, was not yet elucidated.

So, while significant for understanding various structural and functional aspects of FimH, the previous FimH scaffold work differ greatly from the current work. By deliberately mutating only regions of FimH known to contribute to its binding pocket specificity, we hypothesize that we can retain the conformation-dependent regulation of FimH. This enables the possibility of creating regulated proteins with new specificity.

Here, we show that FimH can serve as a scaffold for generating conformation-dependent recognition proteins. We demonstrate that all three CDR loops of the binding pocket of FimH carry permissive positions. This means that FimH still expresses as a functional protein, in both conformations, following modification of those residues in the binding pocket. Since we show the protein is not completely limited to one conformation, there is potential for conformation-dependent binding. We also show that changes to those permissive positions successfully result in a change to FimH specificity, yielding proteins with binding to new targets. While some of these new protein variants may bind their targets with equal affinity in both conformations, we identify two which retain conformation-dependent affinity for their new target, the anti-6xHis antibody Penta-His, using either parasteric or allosteric mechanisms.

Methods

Construction of pBAD-Fim Mutants—Strain pBAD-Fim was constructed using *Escherichia coli* cells from the MegaX DH10B™ T1^R Electrocomp™ cell line (Invitrogen™, Carlsbad, CA), a high-transforming derivative of the *fim* null *E. coli* K12 strain. The strain was transformed with the recombinant plasmid pBAD-Fim (10 kb), made from the pBAD/HisB vector (Invitrogen). This plasmid contains the *araBAD* promoter upstream of the full *fim* operon (excluding regulatory subunits *fimb* and *fime*), which was amplified using PCR from the plasmid pFim1a, a pCRii-TOPO derivative (provided by Dr. Veronika Tchesnokova from Evgeni Sokurenko's lab at the University of Washington, Seattle, WA). All leading peptide tags, including the His tag, were removed from the pBAD-Fim plasmid using inverse PCR to ensure proper expression of the *fim* genes. The *fim* operon was then inserted into the pBAD vector using Gibson Assembly (Gibson D. G., et al., 2009). Point mutations, substitutions, and insertions were introduced to the *fimh* gene in pBAD-Fim using the QuikChange II XL Site-Directed Mutagenesis kit (Agilent Technologies, Santa Clara, CA, USA). Primers were designed using the QuikChange Primer Design tool (Agilent Technologies, Santa Clara, CA, USA). The mutations were then verified by sequencing.

Anti-FimH antibodies—Mice anti-FimH monoclonal antibodies mab21 and mab824 were described previously (Tchesnokova, et al., 2008) (Kisiela D. I., et al., 2015). These purified antibodies were provided by Dr. Evgeni Sokurenko's lab at the University of Washington (Seattle, WA). Fab fragments of mab21 were generated using the Pierce™ Fab Preparation Kit (Thermo Fisher Scientific, Waltham, MA).

Pili purification—Fimbriae were purified from various *E. coli* pBAD-Fim strains as described previously (Tchesnokova, et al., 2008). Briefly, bacteria were grown overnight at 37°C in LB with 100 µg/mL ampicillin, then sub-cultured at a 1/100 dilution. After 3 hours of growth, 0.2% w/v arabinose was added to the culture to induce fimbrial expression. After overnight growth, cells were harvested, resuspended in a pili buffer (50 mM Tris-HCl, pH7.0, 150 mM NaCl buffer), then osterized four times. The cell debris was then spun down, and fimbriae were precipitated with 0.2 M $MgCl_2$ twice. The pili were then resuspended in HBS-EP buffer (10 mM Hepes, 150 mM NaCl, 3 mM EDTA, 0.01% Tween 20, pH 7.4). Protein concentration was measured using the Pierce™ BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA) after the fimbriae were heated for 5 min at 99°C in the presence of 0.1 M HCl.

Crystal Violet Assay—Bacterial adhesion to nickel was measured using Pierce™ nickel coated clear 96-well plates (Thermo Fisher Scientific, Waltham, MA). Briefly, plates were blocked with 0.2% bovine serum albumin (BSA) in phosphate-buffered saline (PBS). Meanwhile, *E.coli* pBAD-Fim strains were grown overnight at 37°C in LB with 100 µg/mL ampicillin, sub-cultured, then at log phase (3 hours later), incubated with 0.2% w/v arabinose and 2% methyl α-D-mannopyranoside (Sigma-Aldrich, St. Louis, MO). After 3-4 hours of *fim* expression induction, cells were harvested at 3200 rpm, washed using 1x PBS, and then resuspended in 0.2% BSA/PBS. Then, cell suspensions at OD_{600} of 4.0 was added to the blocked nickel plates and incubated at 37°C for 1 hour. After washing away unbound bacteria with PBS, bound bacteria were dried at 60°C for 10-15 minutes. Then, crystal violet dye was added to each well, incubated for 10 minutes at room temperature, and then rinsed with water 4 times. The remaining crystal violet stain was resuspended using 50% EtOH, so that bound bacteria could be visualized via absorbance measurement at 600 nm on a Biotek Synergy™ Neo2 Multi-Mode Microplate Reader.

ELISA—Microtiter plates were coated with either purified pili at 0.2 mg/mL in 0.02 M $NaHCO_3$ buffer, or with whole *E. coli* pBAD-Fim cells at an OD_{600} of 1.0 in 0.02 M $NaHCO_3$ buffer, for 1 hour at 37°C. The cells had been grown, harvested, induced for expression, and washed as described for the crystal violet assay. After washing wells with PBS to remove unbound pili or cells, all wells were quenched for 30 minutes with a blocking buffer, either 0.2% BSA/PBS or 0.5% casein (Sigma-Aldrich, St. Louis, MO). Then, primary antibody was added at concentrations ranging from 0.01 µg/mL to 20 µg/mL for the anti-6xHis antibody Penta-His (Qiagen, Hilden, Germany), or 5 µg/mL for mab824 or mab21. In some cases, 2% methyl α-D-

mannopyranoside or 10 µg/mL mab21 fab fragments was added during primary antibody incubation. All antibodies were diluted into the blocking buffer. After a 1 hour incubation at 37°C and 3 washes, goat anti-mouse IgG (H+L) Cross-Adsorbed secondary antibody, conjugated to HRP (Thermo Fisher Scientific, Waltham, MA) was added at 1:2000 dilution. Alternatively, when fab fragments were present, goat anti-mouse IgG Fc secondary antibody with HRP (Thermo Fisher Scientific, Waltham, MA) was added at 1:2000 dilution. Following washes, bound antibody was incubated with TMB, or 3,30,5,50-tetramethylbenzidine (KPL, Gaithersburg, MD). The resulting reaction was stopped after with 0.3 M sulfuric acid. Absorbance was then measured at 450 nm using a Biotek Synergy™ Neo2 Multi-Mode Microplate Reader.

A similar ELISA with mab21 as the primary antibody was performed, except mab21 was incubated with the pili for specific time intervals only before being washed, as described previously. (Yakovenko, Tchesnokova, Sokurenko, & Thomas, 2015). Another similar ELISA with Penta-His antibody (3.5 µg/mL) was performed in which, after primary antibody binding, a detachment period followed where either just buffer, mab21 fab fragments, or 2% methyl α-D-mannopyranoside was added to the pili. After detachment, pili were washed twice, and then incubated with the secondary antibody.

Results

The CDR loops in the FimH binding pocket carry permissive positions. To test whether the CDR loops carry positions that can be mutated without destabilizing the protein, a hexa-histidine tag was substituted into each loop. In the CDR1 loop ([10]AIPIGGG[16]), positions I11 through G16 were replaced with six histidine residues. The same was done to positions D47 through T53 in the CDR2 loop ([46]NDYPETITD[54]) and to positions N135 through D140 in the CDR3 loop ([133]QTNNYNSDDF[142]). Each of these mutations were made to the *fimh* gene in the plasmid pBAD-Fim, which contains the full *fim* operon downstream of the *araBAD* promoter. The resulting mutants were called CDR1-6xH, CDR2-6xH, and CDR3-6xH, respectively. Further mutations were added to stabilize either the loose conformation or the tight conformation. Mutations A188D (located on the pilin domain in the interdomain region) and Y64R (located on the β-sheet of the lectin domain), for example, both stabilize the tight conformation of FimH (Aprikian, et al., 2007). While A188D alone causes an otherwise wild-type FimH to strongly favor the tight conformation, it may not be enough if FimH also has mutations in the binding pocket that favor the loose conformation. Therefore, for CDR2-6xH

and CDR3-6xH, Y64R was also added to further stabilize the tight conformation, resulting in the mutants CDR2-6xH-AY and CDR3-6xH-AY. Only A188D was needed for the CDR1-6xH mutant, resulting in CDR1-6xH-A. On the other hand, since the mutation R60P strongly stabilizes the loose conformation of FimH (Rodriguez, et al., 2013), it was used to keep FimH in all three mutants in the loose conformation (resulting in, for example, CDR2-6xH-R). The final plasmids were then expressed in *E. coli* MegaX DH10B™ cells, a *fim* null strain. Successful expression of a properly-folded FimH was then measured via binding to the anti-FimH monoclonal antibody mab824 in an ELISA. This antibody has been described to bind equally well to either conformation of FimH (see appendix A), and its epitope does not include any of the interdomain region residues or the binding pocket CDR loops (Kisiela D. I., et al., 2015). Therefore, for our applications, it serves as an appropriate indicator for proper FimH expression.
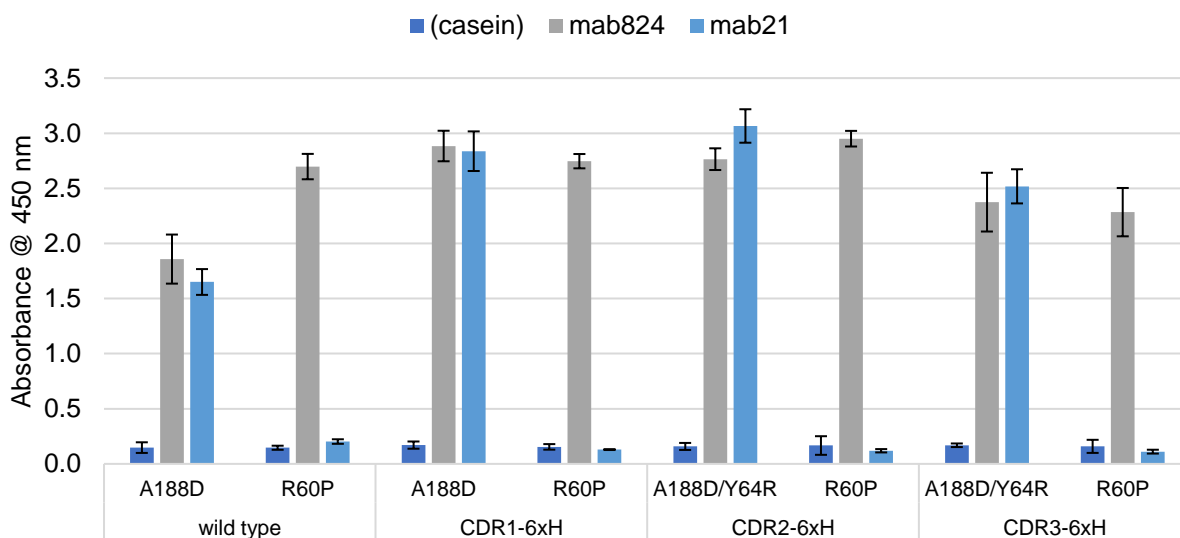
Figure 7. Expression of FimH mutants as measured by binding to mab824. Binding to mab824 (gray bars) indicated that FimH expressed as a properly-folded protein even with the hexa-histidine tag substitution to any CDR loop. Mutants with the hexa-histidine tag and stabilized in either the loose (R60P) or tight conformations (A188D or A188D/Y64R) were also incubated with mab21 (light blue bars) to confirm their conformation. The lack of binding to the blocking buffer casein (dark blue bars) showed a lack of nonspecific binding. Error bars represent the standard deviation of the sample (n=3).

Figure 7 depicts all mutants binding to mab824, showing that FimH expressed even with mutations to most of the residues of each CDR loop. Binding to mab21 was also measured to confirm the stabilized conformation of each mutant. Mab21 is known to bind to the interdomain region of FimH when FimH is in the tight conformation. It cannot bind to FimH in the loose conformation (Tchesnokova, et al., 2008). Thus,

in Figure 7, the lack of binding to mab21 for all mutants with the loose conformation stabilized by R60P, and the strong binding to mab21 for all mutants with the tight conformation stabilized by A188D or A188D/Y64R, confirms their expected conformation. Knowing this, since all mutants bound to mab824 in both conformations, it can be concluded that FimH is capable of expressing in both conformations even with mutations to its CDR loops.

Changes to the FimH CDR loops result in a change in specificity. To test whether mutations to the CDR loops result in a change in FimH specificity, the previously designed mutants CDR1-6xH, CDR2-6xH, and CDR3-6xH were tested for binding to nickel, which is known to bind to histidine residues. A bacterial adhesion assay was performed, in which crystal violet was used to stain cells expressing FimH mutants that were bound to nickel-coated wells in a microtiter plate. Binding to just the blocking buffer BSA was measured in a standard microtiter plate without nickel. A similar assay had been used previously to measure biofilm formation to immobilized mannosylated proteins (Kisiela D. I., et al., 2015). The results of this assay, shown in Figure 8, depict binding by all 6xHis mutants to nickel. This shows the change in FimH specificity caused by the histidine mutations.
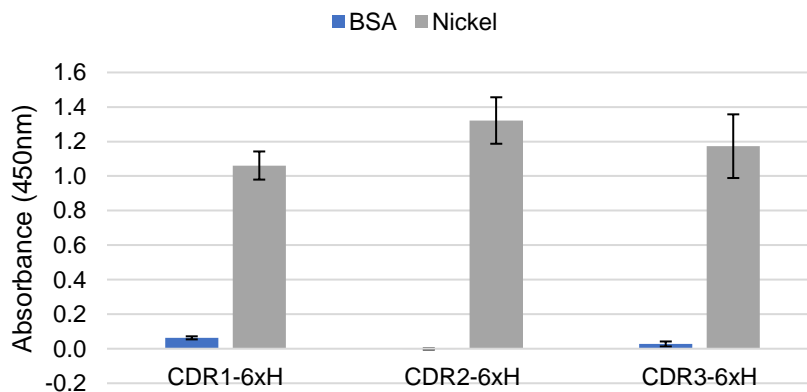
Figure 8. Bacterial adhesion assay showing binding by 6xHis mutants to nickel. Cells expressing the 6xHis mutants were added to nickel-coated plates for binding to nickel (gray bars) or to uncoated plates to measure nonspecific binding to the blocker BSA (blue bars). Bound cells were quantified via absorbance measurement of crystal violet stain. Error bars represent standard deviation of sample (n=3).
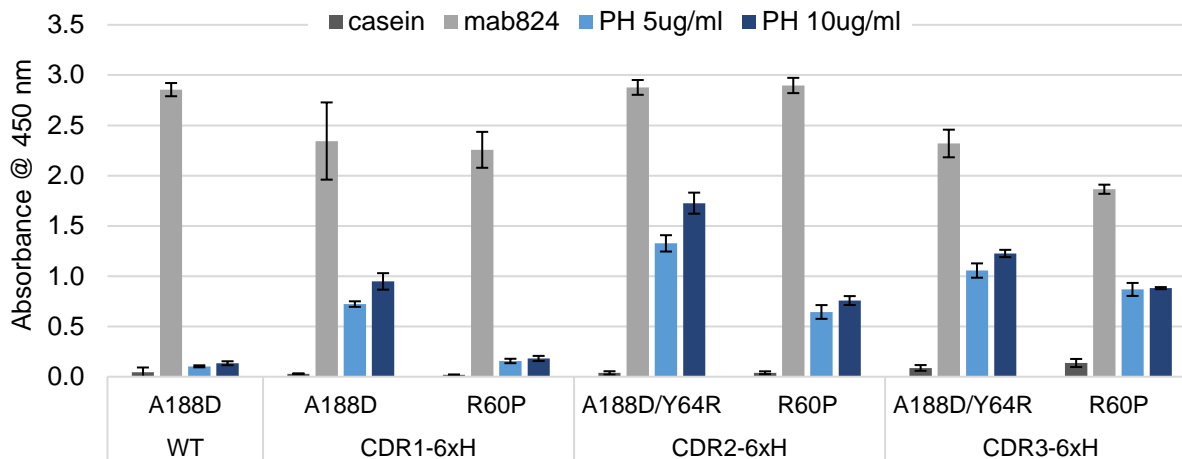
Figure 9. ELISA of 6xHis mutants' binding to Penta-His (PH) antibody. Whole cells expressing the 6xHis mutants were immobilized and incubated with the negative control casein buffer (dark gray bars), the positive control mab824 (gray bars), and Penta-His antibody at 5 µg/mL (light blue bars) or 10 µg/mL (dark blue bars). Bound antibody was detected using an HRP-conjugated goat-anti-mouse secondary antibody and the substrate TMB. Error bars represent standard deviation of sample (n=3).

In addition to binding to nickel, the mutants CDR1-6xH, CDR2-6xH, and CDR3-6xH were also tested for binding to the target anti-6xHis antibody Penta-his (Qiagen). This antibody has an affinity for the hexa-histidine tag that typically ranges from 1 nM to 50 nM when the tag is added to a protein terminus, though this can change when the tag is added to the middle of a protein. An ELISA was performed to measure binding of these mutants to the target antibody Penta-His. Whole *E. coli* cells expressing fimbriae with the 6xHis mutations were immobilized to a 96-well microtiter plate. These mutants also included point mutations to stabilize either the loose or tight conformations, as described previously. Immobilized cells were incubated for 1 hour with the Penta-His antibody at two concentrations: 5 µg/mL and 10 µg/mL. Binding to mab824 was used as the positive control. The results in Figure 9 show binding to Penta-His by the 6xHis mutants only, and not the wild-type. Therefore, this also illustrates that the changes to the CDR loops resulted in a change in FimH specificity towards a new target. Interestingly, for CDR1-6xH and CDR2-6xH, binding to Penta-His appeared to be conformation dependent. Since whole cells were used in this assay, the concentration of fimbriae was not controlled; therefore, we could not conclude from this assay alone that binding was conformation dependent, since the variation in binding to Penta-His may be due to a difference in FimH expression rather than a difference in affinity. Alternative methods were needed, which are described next.

The CDR1-6xH mutant has conformation-dependent affinity for the Penta-His antibody. To test whether the CDR1-6xH and CDR2-6xH mutants truly had conformation-dependent affinity for the target antibody Penta-His, the fimbriae (pili) of these mutant strains were isolated. This allowed for better control of the fimbrial concentration in the binding assays. CDR1-6xH and CDR2-6xH mutants without other point mutations (such as R60P or A188D) were used to allow for any possible conformational change. Following purification of the pili, an ELISA was performed using immobilized pili. Binding to Penta-His antibody was measured at a range of concentrations, from 0.01 µg/mL to 20 µg/mL (0.06 nM to 129.03 nM), with or without the addition of free mannose (2% methyl α-D-mannopyranoside). Mannose was added to further stabilize the tight conformation, since previous experiments suggested that both mutants retained some affinity for mannose when in the tight conformation (data not shown). To determine whether mannose induced a shift from the loose to the tight conformation, binding to mab21 was also measured with or without mannose. Since the concentration of FimH in the wells was significantly less than the expected K_d of Penta-His for the hexa-histidine tag in these FimH mutants (1 – 50 nM), we were able to use the Penta-His binding measurements from this ELISA to fit a binding curve model to the data.

Figure 10. A. Conformation of CDR1-6xH with or without mannose, as indicated by binding to mab21. **B.** Binding curve of FimH mutant CDR1-6xH to ligand Penta-his antibody. The affinity of CDR1-6xH for Penta-His was 21.77±2.73 nM in the presence of mannose (blue squares) and 93.35±9.27 nM without mannose (purple circles). Error bars represent the standard deviation (n=3). Binding curves were fitted using GraphPad Prism 7 software.

As shown in Figure 10A, CDR1-6xH appears to shift to a tight conformation when mannose is added, indicated by the binding to mab21. This is similar to what is seen with wild-type FimH. The binding curve in Figure 10B shows that CDR1-6xH has an affinity for the Penta-His antibody of 93.35 ± 9.27 nM when no

mannose is present, or when it is in the loose conformation (as shown by the lack of mab21 binding in Figure 10A). When mannose is added, apparently inducing the mutant to switch to the tight conformation, the affinity of CDR1-6xH for Penta-His antibody increases more than 4-fold, shown as a lower k_d of 21.77 ± 2.73 nM. This indicates that mannose triggers a change in affinity for CDR1-6xH.

A similar test with CDR2-6xH found that addition of mannose did induce a shift to the tight conformation, as shown in Figure 11A. However, the binding curves in Figure 11B indicate that there is no change in affinity when mannose is added. While this may suggest that CDR2-6xH has the same affinity for Penta-His regardless of its conformation, there is one caveat. It is possible that mannose alone cannot induce the shift to the tight conformation in this case. Figure 11A shows binding to mab21 when mannose is added; it is possible that both mannose and mab21 are needed to shift CDR1-6xH to the tight conformation. This is explored next.
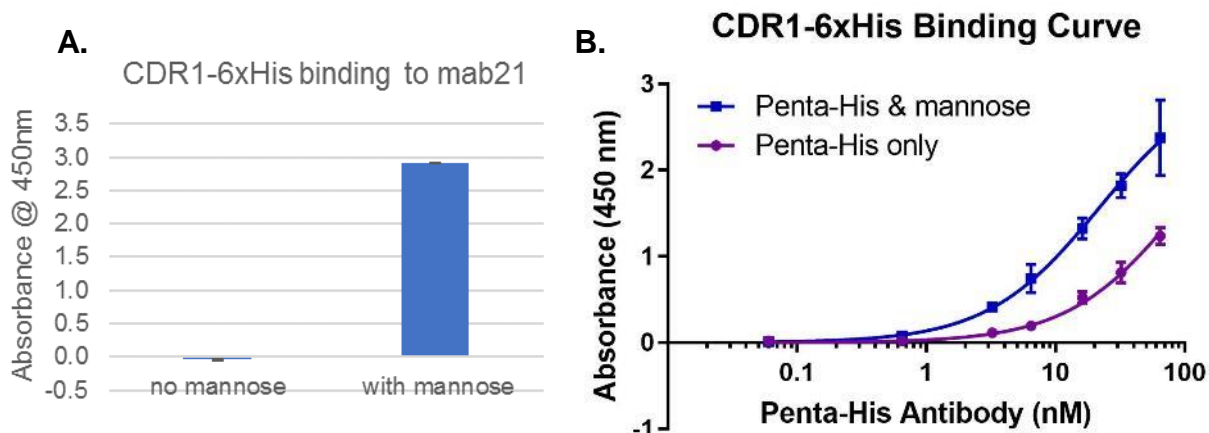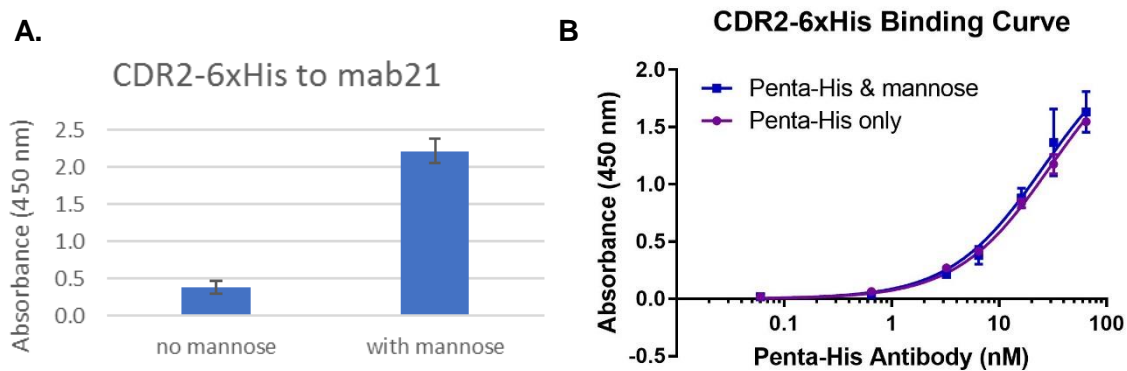

Figure 11. A. Conformation of CDR2-6xH with or without mannose, as indicated by binding to mab21. **B.** Binding curve of FimH mutant CDR2-6xH to ligand Penta-his antibody. The affinity of CDR2-6xH for Penta-His in the presence of mannose (blue squares) or without mannose (purple circles) is the same, 27.34 ± 2.98 nM. Error bars represent the standard deviation (n=3). Binding curves were fitted using GraphPad Prism 7 software.

The CDR2-6xH mutant has conformation-dependent affinity for the Penta-His antibody that is regulated by mannose or mab21. To test the extent to which the CDR2-6xH mutants existed in their expected conformations, a kinetics-based ELISA was performed in which the attachment of mab21 was measured at set time intervals. Pili from CDR2-6xH mutants with the A188D/Y64R mutations (CDR2-6xH-AY), no additional point mutations (CDR2-6xH-K), or the FimH mutant FocH were compared to wild-type FimH pili with the A188D point mutation. FocH is a mutant in which 16 amino acids of the FimH pilin domain are

mutated to match the corresponding sequence in the bacterial adhesin FocH. This FocH mutant is known to have the same affinity for mannose as the isolated lectin domain (Yakovenko, Tchesnokova, Sokurenko, & Thomas, 2015). Data in Figure 12 shows that CDR2-6xH-AY binds quickly to mab21, and therefore spends significant time in the tight conformation. It binds even more quickly than FocH, which appears to saturate, while A188D reaches about 50% binding. Meanwhile, mutant CDR2-6xH-K is evidently in the loose conformation, shown by the very slow or lack of increase in mab21 binding even after 30 minutes of incubation. All mab21 binding data was normalized based on their mab824 binding to adjust for any varying expression levels. This was only needed for A188D, which had a lower level of expression; the other three mutants had the same levels of expression.

Binding to Mab21 over time
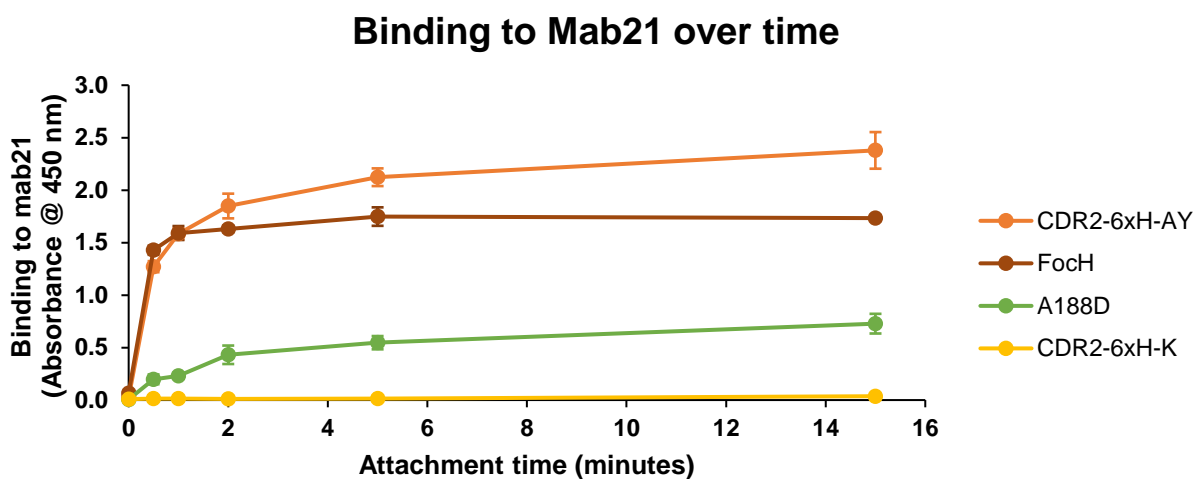
Figure 12. Time course of mab21 binding to FimH CDR2-6xHis mutants (orange or yellow), the wild-type FimH with A188D mutation (green), or the FimH mutant FocH (red). Error bars represent the standard deviation (n=3).

Preliminary data using a crystal violet assay suggested that CDR2-6xH-AY binding to Penta-His may be impacted by mab21 and/or by mannose (see appendix B). Since this data also shows that adding mannose further stabilizes this mutant's tight conformation (indicated by the higher binding to mab21 when mannose is added), this suggested that the conformation of CDR2-6xH-AY could be further stabilized in the tight state, or that it may still exist in the loose conformation when, for example, binding to Penta-His. In other words, while it bound tightly to mab21, CDR2-6xH-AY could still possibly switch to the loose conformation if needed. This was tested by running an ELISA in which binding to Penta-His was measured using CDR2-

6xH-K (loose conformation), CDR2-6xH-AY (tight conformation, but potentially switchable), and CDR2-6xH-AY plus mab21 ("locked" in the tight conformation via the antibody). Figure 13A first demonstrates that both mutants have equal expression levels, which justifies the use of a binding curve model fit in which the maximal binding (b_{max}) parameter is constrained to be the same across all three data sets. Using this model, Figure 13B shows that CDR2-6xH in its loose conformation (CDR2-6xH-K) has an affinity that is 7-fold higher than when it is forced into its tight conformation using mab21 (CDR2-6xH-AY + m21). This data also illustrates that CDR2-6xH-AY without mab21 serves as more of a "moderate" binder, relative to when it is

A. ELISA: Expression Levels

BSA mab824

B.
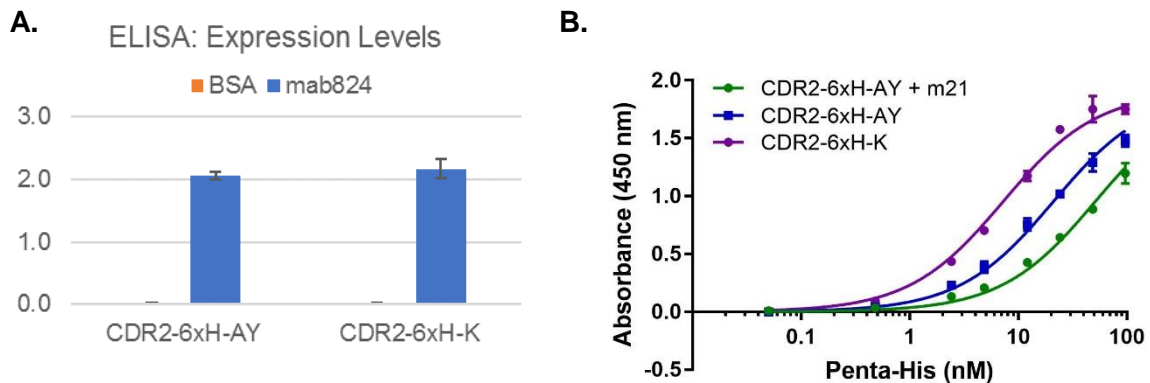
Figure 13. **A.** Equal expression levels of each mutant, as measured by mab824 (blue), with blocking buffer (orange) as the negative control. **B.** Binding curve of FimH mutant CDR2-6xH to ligand Penta-his antibody. The affinity of CDR2-6xH-K for Penta-His (purple circles) is 7.25 ± 0.41 nM. The affinity of CDR2-6xH-AY without mab21 (blue squares) is 21.06 ± 1.07 nM, while addition of mab21 (green circles) further lowered the affinity to 51.19 ± 2.45 nM. Error bars represent the standard deviation (n=3). Binding curves were fitted using GraphPad Prism 7 software.

fixed in either conformation. This also implies that the CDR2-6xH-AY mutant is not truly "locked" in the tight conformation on its own.

Given that CDR2-6xH has conformation-dependent affinity in which the tight conformation has a lower affinity for the ligand, we next tested whether bound Penta-His could be detached using either mab21 (10 µg/mL mab21 fab fragments) or mannose (2% methyl α-D-mannopyranoside) as the trigger, since both molecules cause FimH to shift to the tight conformation. For this test, CDR2-6xH-AY was used instead of CDR2-6xH-K, since the former required just mab21 or just mannose to shift to the tight conformation, while the latter would require both molecules to shift. This was done using an ELISA in which, after Penta-His binding, the CDR2-6xH-AY pili were exposed to either just buffer, or buffer mixed with the trigger, for 60

minutes. After that time, the solution was removed, the pili were washed twice, and the secondary antibody was added for detection.
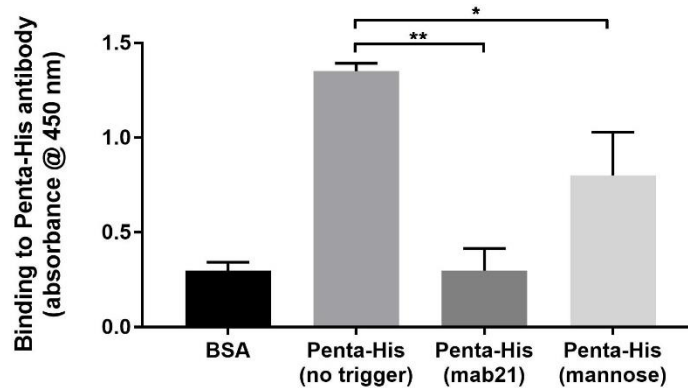
Figure 14. Detachment of Penta-His Antibody from CDR2-6xH-AY. The ELISA compares target antibody that remains after one hour without any trigger added, to antibody that remains after induced detachment by mab21 or mannose. Statistical significance was determined using two-tailed Welch's t-test (GraphPad Prism 7 software), where ** indicates $p \leq 0.005$, and * indicates $p \leq 0.05$. Error bars represent the standard deviation (n=3).

Results show that, after one hour, mab21 and mannose can each induce detachment of Penta-His antibody from CDR2-6xH-AY pili by 78% ± 8.6% and 41% ± 16%, respectively. This detachment is found as a percentage of the Penta-His antibody that remained after one hour in buffer alone, therefore accounting for any loss due to its own dissociation rate. This demonstrates that the CDR2-6xH-AY FimH mutant not only has conformation-dependent binding, but also regulatable binding which can be triggered using the same molecules which trigger conformational change for the wild-type FimH.

Discussion

Here, we demonstrate for the first time that the bacterial protein FimH can be used as a conformation-dependent binding scaffold for displaying new, regulated recognition proteins. While FimH has been used as a scaffold previously, none of the previous works takes advantage of the allosteric or parasteric regulatory mechanisms of FimH. These mechanisms are our primary motivation for pursuing FimH as a scaffold, since they enable us to generate binding molecules with regulated binding for use in a wide range of life sciences applications.

Beyond its regulatory mechanisms, FimH possesses many characteristics of an ideal protein scaffold. These include 1) a well-defined hydrophobic core, 2) a solvent-accessible binding pocket that is spatially separated from the core, 3) a smaller size of about 34 kDa, and 4) a soluble binding domain. The binding pocket, composed of three "CDR" loops as shown in Figure 6, is ideal for modification and protein display, in a way mimicking the immunoglobulin structure. Moreover, E. coli cells already conveniently display FimH at 300-500 (or more) molecules per cell, resulting in an avidity advantage. Because FimH is expressed on the tips of fimbriae, it is expressed far from the cell, thereby avoiding possible interference by lipopolysaccharides or any other structures near the cell. Lastly, unlike many protein scaffolds, FimH can express disulfide bonds, since they form in the periplasmic space as the fimbrial subunits are secreted.

We first show that the CDR loops of FimH are permissive positions, meaning that they can be mutated to display new recognition epitopes without destabilizing FimH entirely. Moreover, because we are using a scaffold with conformation-dependent binding, our definition of 'permissive positions' expands to describe those which, after mutations in the binding pocket, still allow FimH to exist in both its loose and tight conformations. These mutations may still cause FimH to favor one conformation over another; however, one or two point mutations may encourage the mutant to switch to its otherwise less favored state. Even the wild-type FimH prefers the loose conformation, and requires either force, point mutations, or other disruptions to its interdomain region to shift the protein to its tight conformation. So, we consider positions to be permissive so long as, following mutations to those positions, FimH is not totally destabilized after being made to switch to its less favored conformation.

To demonstrate permissiveness, we used the hexa-histidine tag (6xHis) as a model epitope, because its target is known, is easy to obtain, and fits well into ELISAs and other simple binding assays. This practice has been used previously as a convenient method for testing permissive sites before investing in random mutagenesis (Hoffman, et al., 2010). Insertions into the FimH binding loop CDR2, specifically at position I52, had already been shown to be tolerated (Bhomkar, Materi, Semenchenko, & Wishart, 2010). Therefore, our work focused on substitutions of the hexa-histidine tag into each of the loops, rather than insertions, since the former is usually less likely to be tolerated by the protein, and since substitutions allow us to query most of the residues of the CDR loops as permissive positions. Moreover, substitutions into the protein are more likely to retain interactions with a larger portion of the binding pocket, and therefore take advantage

of the protein's conformational change. Using binding to mab824 as a proxy for proper FimH expression, we demonstrated that FimH tolerated the substitution of its CDR loops with the hexa-histidine tag, and could express stably in both conformations. Therefore, each of the CDR loops were shown to carry permissive positions. Future work may include testing other epitope tags, or conducting a more thorough investigation of each individual residue. While histidines were tolerated, other residue mutations may not be tolerated at all. It would be advantageous to know which residues are deleterious to the stability of FimH overall.

It should be noted here that, although binding to mab21 was used to confirm that the mutants were able to express in the tight conformation, it does not necessarily mean that that mutant is completely locked in that conformation. Mab21 binds whenever FimH shifts to the tight conformation. Wild-type FimH switches at a slow rate, whereas FimH mutants with R60P almost do not switch at all and instead remain in the loose conformation. Conversely, mutants with A188D or A188D/Y64R point mutations shift frequently to the tight conformation, and therefore mab21 can bind and effectively keep it in the tight conformation. This detail is important because it must be emphasized that even mutants with two point mutations that favor the tight conformation can still exist in the loose conformation and even preferentially bind ligands in that conformation.

The 6xHis mutants designed here were also shown to have new specificity—that is, mutations to the binding pocket successfully resulted in binding by FimH to targets other than mannose. Most display scaffolds are able to successfully bind only to large molecules such as proteins; binding by protein display scaffolds to small molecules is notoriously difficult. In fact, one of the few non-immunoglobulin scaffolds to bind small molecules is the anticalin, based on the lipocalin fold (Gebauer & Skerra, 2009). This scaffold and FimH share the key trait of a deep binding pocket capable of capturing small molecules. Since FimH's natural target is the small molecule mannose, we expected that FimH would be an ideal scaffold for a range of targets that included both large proteins and small molecules. To that end, our data showed binding by these FimH mutants to both a small molecule (nickel) and to a large protein (Penta-His monoclonal antibody).

To truly call FimH a scaffold for regulated binding, it had to be shown that FimH was capable of displaying conformation-dependent affinity towards a new target. The mutant CDR1-6xH demonstrated this via a more than 4-fold increase in affinity for the antibody Penta-His when mannose was present, compared to the

loose conformation with no mannose. The addition of mannose to CDR1-6xH also induced its binding to mab21, and therefore its switch to the tight conformation. There are two possible explanations for this modest increase in affinity. Mannose also binds to the binding pocket, with most of its epitope occupying residues in the CDR2 and CDR3 loops. Therefore, it's possible that its binding to the pocket helps form additional interactions with Penta-His, therefore improving the FimH mutant's affinity for the antibody. The alternative explanation is that mannose binding induces CDR1-6xH to shift to the tight conformation, which subsequently has a higher affinity for Penta-His. This can be explored by working with CDR1-6xH mutants that have been "locked" in one conformation or the other.

More interestingly, the mutant CDR2-6xH also demonstrated conformation-dependent affinity via a 7-fold *decrease* in affinity for the antibody Penta-His when binding in its tight conformation (which was maintained with the help of mab21), compared to the loose conformation. Further data showing the induced detachment of Penta-His antibody from CDR2-6xH-AY when mab21 or mannose was added shows that neither behavior is due to competitive inhibition, but instead due to allosteric and parasteric mechanisms, respectively. The antibody mab21 binds far from the binding pocket, in the interdomain region, and is already known to be an allosteric regulator for the wild-type FimH because it essentially wedges between the two domains and causes FimH to shift to the tight conformation (Tchesnokova, et al., 2008). Therefore, its ability to induce conformational shift and therefore detachment of Penta-His antibody from CDR2-6xH-AY by 78% is reminiscent of FimH's own allosteric regulation. Mannose, on the other hand, must bind adjacent to Penta-His. While some of its epitope has been mutated to histidine residues that now bind Penta-His, the rest of its epitope remains available for binding to mannose. Competitive inhibition cannot explain this since detachment of Penta-His caused by the presence of mannose is significantly greater than detachment of Penta-His due to its own dissociation rate; therefore, mannose must be able to bind even before Penta-His has dissociated. This therefore describes a parasteric mechanism for inhibition.

It must be emphasized again that, while CDR2-6xH-AY was shown to bind quickly to mab21, we also showed that its binding to mab21 could be improved with the addition of mannose, and that the addition of mab21 resulted in a lower affinity to the target Penta-His antibody. This information suggests that CDR2-6xH-AY exists in perhaps an "intermediary" state, in which its interdomain region captures mab21 quickly, but its binding pocket may not be fully in the tight conformation. However, when mab21 or mannose binds,

the binding pocket may switch quickly to the tight conformation. In other words, despite the addition of point mutations A188D and Y64R, the mutant is still switchable, and not locked in the tight conformation. Understanding the complexity in the conformational states of FimH is beyond the scope of this paper; however, it is worth exploring, and may shed light on the many ways in which FimH can be used as a regulated protein.

While the affinity changes described have been small, if one were interested in a larger affinity change, additional mutations, such as through affinity maturation, could be made to further increase or decrease affinity in one conformation as needed. Ultimately, however, this example shows that both allosteric and parasteric regulatory mechanisms of FimH can be retained even after modifying the binding pocket and changing the protein's specificity. This has not been done previously with FimH or any other allosteric protein.

Conclusion

Taken together, our findings show that our initial hypothesis is correct: by mutating only the binding loops of FimH to successfully generate new specificity, we can potentially retain the regulatory capabilities of FimH. Because this has not been done previously with any protein scaffold, there is significant potential for using FimH to generate regulated binding molecules to any target of interest. Now that the permissive positions of FimH have been identified, those positions can be randomly or rationally mutated to generate binders to a wide range of targets. Members of our group have already begun work on developing and screening a library of such FimH variants. Some of these new binders may not present with conformation-dependent affinity, but for those that do, there is room to explore the mechanism of that regulation. Thus, FimH as an allosteric or parasteric scaffold opens many future possibilities towards generating regulated binding molecules.

Chapter IV: Developing and Screening a Library of Activatable Recognition Proteins

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Publication

This is a working manuscript and will be submitted for publication.

Abstract

Recognition molecules based on scaffolds other than antibodies are crucial not only to protein design, but also for developing improved binding reagents for diagnostic, therapeutic, and bioanalytical applications. The use of a scaffold with conformation-dependent affinity enables the development of activatable recognition proteins, which can be triggered to bind or release targets. Previously, we have shown that the bacterial adhesin FimH can be used as a scaffold to generate such activatable proteins. Here, we describe the generation of a library built by modifying two of the binding pocket loops of FimH. The resulting library of 3.32×10^6 variants was then screened using biomagnetic selection, a method shown here to enrich FimH variants with affinities as low as 1.2 μM from a mock library. Successful screening yielded two high-affinity binders and one moderate-affinity binder to the target anti-His6 antibody. This is the first example of the use of an allosteric protein as a scaffold for library display.

Background

Recognition molecules are essential for life sciences research and biomedical applications. Proteins, specifically antibodies, have been the gold standard for decades due to their wide range of specificities and high affinities. More recently, alternative scaffolds are being explored to develop recognition proteins with the ideal characteristics for a given application. These may include a smaller size, greater thermostability, high solubility, and a lack of post-translational modifications or glycosylation (Diem, et al., 2014). Along with greater amenability towards engineering for unique applications, alternative scaffolds generate recognition proteins far more quickly and at a lower cost due to their development via yeast or bacterial expression systems.

One of the characteristics sought from recognition molecules is the ability to regulate their binding of targets or their release of targets. This is often necessary in drug delivery or imaging applications, where the drug payload or contrast agent needs to be released in a controlled and targeted manner. Diagnostic tests also benefit from this, since "capture-and-release" recognition proteins can be used to improve assay sensitivity

and specificity. In short, regulation of recognition molecules is hugely useful in many life sciences research and applications. Current techniques for regulating recognition molecules rely on the addition of linkers or switchable peptides or polymers that are triggered by environmental cues (Thomas & Daugherty, 2009) (Ostermeier, 2005). While some successful switchable proteins exist, these are all built on a case-by-case basis for specific targets and often have one-way control. No method exists yet for generating regulated proteins to any target of interest, where the regulation can trigger binding or release of the target.

To this end, our group has explored the use of the bacterial adhesin FimH as a scaffold for generating regulated proteins. This two-domain protein, which exists at the tip of *Escherichia coli* type I pilus, exhibits conformational-dependent binding to its natural target, mannose. This is caused by the interactions between the binding domain and the pilin domain, which keep FimH in a loose conformation with low affinity for mannose. If force or a wedging antibody breaks those interactions, FimH switches to a tight conformation with high affinity for mannose. This large conformational change propagates from the interdomain region up through to the binding pocket of the binding domain, which loosens or tightens. This pocket contains three loops, called complementarity determining regions (CDR loops), that present an ideal site for modification. In our previous work, we demonstrated that these loops are permissive sites that tolerate mutagenesis, and that their modification results in a change in FimH specificity. We also demonstrated that the conformational change experienced by FimH can be maintained following CDR loop mutagenesis, such that the new recognition protein created also has regulated binding. In short, FimH seems to be the ideal scaffold for more conveniently generating molecules with regulated binding.

While our previous work focused on directed mutagenesis to evaluate the feasibility of FimH as a scaffold, truly generating unique, regulated recognition molecules would require having a full ensemble of mutants for evaluation. To access the rich diversity available to a scaffold, researchers typically generate protein libraries that enable display of many of the possible mutants. While phage display libraries are most common for displaying varying peptides, cell surface display of libraries via yeast or bacteria are useful for easily linking genotype to phenotype via the cell, presenting hundreds to thousands of copies per cell, and enabling use of flow cytometry and cell sorting for quantitative screening of the libraries (Levin & Weiss, 2006). Thus, to access the diversity available to a FimH scaffold, one could generate a library of FimH mutants. This has been done previously in two ways. Schembri, Sokurenko, & Klemm randomized the

whole FimH protein using error-prone PCR, resulting in a random mutant library which greatly increased our understanding of the functional flexibility of FimH and its structure (Schembri, Sokurenko, & Klemm, 2000). Schembri and Klemm also generated chimeric FimH mutants in which a permissive position in the pilin domain was mutated to generate binders to heavy metals, while maintaining the binding domain's specificity for mannose (Schembri & Klemm, 1998) (Kjærgaard, 2001). While these initial studies were highly useful, later understanding of the importance of the interdomain region for mannose-binding and binding regulation limited the scope of these libraries; therefore, the potential of either library was not fully explored (Rice, Schohn, Bessette, Boulware, & Daugherty, 2006). Moreover, these fimbrial libraries could not truly take advantage of FimH's conformational-dependent binding behavior, since that knowledge, particularly the concept of parasteric inhibition, was not yet elucidated.

In this aim, we use the scaffold FimH to generate a library of recognition molecules, to be used as a tool for identifying regulated binding proteins. We demonstrate here the development of such a library with high, regulated expression, as well as an optimized method for screening this library for a range of binders. Finally, our work uses this screening method to probe our library and successfully identify several binders to a target antibody, Penta-His. To our knowledge, this illustrates the first example of a library based on a scaffold with conformational-dependent binding, in which that regulation can be retained.

Methods

Strains, Plasmids, and Mutagenesis—Strain pBAD-Fim was constructed using *Escherichia coli* cells from the MegaX DH10B™ T1^R Electrocomp™ cell line (Invitrogen™, Carlsbad, CA), a high-transforming derivative of the *fim* null *E. coli* K12 strain. The strain was transformed with the recombinant plasmid pBAD-Fim (10 kb), made from the pBAD/HisB vector (Invitrogen). This plasmid contains the *araBAD* promoter upstream of the full *fim* operon (excluding regulatory subunits *fimb* and *fime*), which was amplified using PCR from the plasmid pFim1a, a pCRii-TOPO derivative (provided by Dr. Veronika Tchesnokova from Evgeni Sokurenko's lab at the University of Washington, Seattle, WA). All leading peptide tags, including the His tag, were removed from the pBAD-Fim plasmid using inverse PCR to ensure proper expression of the *fim* genes. The *fim* operon was then inserted into the pBAD vector using Gibson Assembly (Gibson D. G., et al., 2009). Point mutations A188D or R60P were introduced to the *fimh* gene in pBAD-Fim using the QuikChange II XL Site-Directed Mutagenesis kit (Agilent Technologies, Santa Clara, CA, USA). Primers

were designed using the QuikChange Primer Design tool (Agilent Technologies, Santa Clara, CA, USA). The mutations were then verified by sequencing. Strain pBAD-Fim with point mutations in FimH are named as: pBAD-Fim/*mut*, for example, pBAD-Fim/A188D.

Anti-FimH antibodies—Mouse anti-FimH monoclonal antibodies mab21, mab824, mab926, and mab475 were described previously (Tchesnokova, et al., 2008) (Kisiela D. I., et al., 2015). These purified antibodies were provided by Dr. Evgeni Sokurenko's lab at the University of Washington (Seattle, WA).

Testing FimH Expression via Flow Cytometry— E.coli pBAD-Fim strains were grown overnight at 37°C in LB with 100 μg/mL ampicillin, and sub-cultured into two vials. Once at log phase (3 hours later), 0.2% w/v arabinose and 2% methyl α-D-mannopyranoside (Sigma-Aldrich, St. Louis, MO) were added to one vial. After 3-4 hours of *fim* expression induction, cells were harvested at 3200 rpm, washed using PBS, and then resuspended in 0.2% bovine serum albumin in PBS as a blocking buffer. 4×10^8 cells were then incubated with the primary mouse monoclonal antibody mab926 (1/200 dilution) for 1 hour at 4°C while rotating. The cells were then spun down at 6000 rpm for 3 minutes. After removing the supernatant, cells were washed once, and then mixed with a 1/1000 dilution of the secondary antibody goat-anti-mouse IgG conjugated with Alexa-Fluor® 488 (Thermo Fisher Scientific, Waltham, MA). In one case, cells were only incubated the secondary antibody, and not the primary. Following one spin and one wash, cells were diluted 1/10 into PBS and run for flow cytometry on a BD FACSCanto II system (BD Biosciences, Franklin Lakes, NJ) at the lowest flow rate. For each sample, 100,000 events were collected, and then analyzed using FlowJo software (FlowJo LLC, Ashland, Oregon).

Library Construction—A FimH library was constructed using site-directed saturation mutagenesis. Briefly, all 9 codons of loop CDR2 (from N46 to D54) were replaced with NNK degenerate codons, with a 45 bp overhang on the 5' end and a 48 bp overhang on the 3' end. This 120 bp oligo was purchased as an Ultramer® from Integrated DNA Technologies (IDT, San Diego, CA). Similarly, all 10 codons of loop CDR3 (from Q133 to F142) were replaced with NNK degenerate codons, with a 46 bp overhang on the 5' end and a 44 bp overhang on the 3' end; the resulting 120 bp was also purchased as an Ultramer®. These single-stranded oligos were made double-stranded by first annealing to an 18-bp complementary primer, and then using the DNA Polymerase I, Large (Klenow) Fragment (New England BioLabs, Inc., Ipswich, MA) to fill in the remaining 5' overhang to form a blunt end. Meanwhile, inverse PCR was used to remove the CDR2

loop or the CDR3 loop from the pBAD-Fim plasmid containing the *fimh* gene. Primers pointing outward from the CDR2 loop, with their inward ends complementary to the overhang region of the randomized loop oligo, were purchased from IDT (5'–GATTTGCGTCGAAAGATCCAC–3' and 5'–CAACGAGGCTCGGCTTAT–3'). Similar primers were purchased for removing the CDR3 loop (5'–AATGAGCCAGCTTTAATCGCC–3' and 5'–ACGCCAATAATGATGTGGTGG–3'). The template used, pBAD-Fim, was modified with the A188D mutation prior to inverse PCR. Alternatively, the CDR2 library DNA was used as the template for CDR3 randomization to generate a library with both loops randomized (called CDR2+3). Following PCR, any remaining template was digested using DpnI. The final vector backbone was then isolated via gel extraction from a 0.7% agarose gel in TBE buffer using the QIAquick Gel Extraction kit (Qiagen), and then cleaned and concentrated using the Zymo DNA Clean & Concentrator kit (Zymo Research, Irvine, CA). To assemble the library DNA, the CDR2 and CDR3 double-stranded oligos with randomized loop regions were each ligated into the pBAD-Fim/A188D backbone vector using a homemade Gibson Assembly mix at a 3:1 insert-to-vector molar ratio (Gibson, et al., 2009). 8 reactions of 20 μL containing 50 ng of vector were run per library. This mix contained concentrated ISO buffer (1 mM dNTPs, 50mM DTT, 25% w/v PEG-8000, 500mM Tris-HCl pH 7.5, and 50mM $MgCL_2$), T5 exonuclease (Epicentre, San Diego, CA), Taq DNA ligase (New England BioLabs, Ipswich, MA), and Phusion DNA polymerase (New England BioLabs, Ipswich, MA). The resulting 20 μL reaction was incubated for 1 hour at 50°C, then cleaned using the Zymo Research Clean & Concentrator kit and concentrated into 6 μL. Finally, each Gibson reaction DNA (CDR2, CDR3, or CDR2+3) was transformed into *E. coli* MegaX DH10B cells via electroporation. Cells were made electrocompetent by harvesting in the log phase (OD_{600} 0.4-0.45), chilling the cells, and then quickly washing the cells with cold ddH2O using centrifugation. Using several centrifugation and wash steps, the initial 400 mL of cells were concentrated into 400 μL for immediate use. For electroporation, 95 μL of cells were gently mixed with each Gibson reaction DNA in a pre-chilled 0.1 cm electroporation cuvette (Bio-Rad, Hercules, CA); this was repeated for 8 Gibson Assembly reactions' worth of library DNA. Cuvettes were then loaded into a Bio-Rad MicroPulser™ Electroporator, and the cells were zapped using the Ec.1 program (1.8 kV). Cells were immediately resuspended into a flask of the pre-warmed LB media for recovery at 37°C. Following recovery and before cell doubling began, a dilution of cells was plated to quantify the library diversity, while the remainder was grown overnight with ampicillin for library DNA amplification.

Library Characterization—Characterization of the three libraries was done using flow cytometry. Briefly, LB media with ampicillin was inoculated with 10-fold sampling of library diversity for each library. Following overnight growth, cells were sub-cultured, grown to log phase, and then induced for fimbrial expression with 0.2% w/v arabinose and 2% methyl α-D-mannopyranoside for 3-4 hours. Cells were then harvested, washed, and prepared for flow cytometry as described above. Samples of 4×10^8 cells were incubated with 10 µg/mL of either mab475, mab21, or mab824 as the primary antibodies. During mab824 incubation, cells were also mixed with 2% methyl α-D-mannopyranoside to reduce aggregation. After secondary antibody incubation with goat-anti-mouse IgG conjugated with Alexa-Fluor® 488 (1/1000 dilution) and washes, cells were diluted into FACS tubes with PBS, and then run on a BD FACSCanto II system (BD Biosciences, Franklin Lakes, NJ) at the lowest flow rate. For each sample, 100,000 events were collected, and then analyzed using FlowJo software (FlowJo LLC, Ashland, Oregon).

Biomagnetic Selection: Mock Enrichment—The selection assay for the library and its use in mock enrichment is described here. First, magnetic Dynabeads M-280 Streptavidin (2.8 µm diameter) were washed to remove preservatives using PBS. This was done by placing the tube of beads in a magnetic strip and removing the supernatant. Washed beads were then incubated with biotinylated mannose (α-D-Man-*sp*-biotin, Glycotech, Gaithersburg, MD) at 600 pmol per 100 µL beads. Meanwhile, *E. coli* cells expressing each FimH variant were grown in LB media with ampicillin and chloramphenicol, harvested, and washed as described for flow cytometry experiments, with the blocking buffer alternated between 0.2% bovine serum albumin in PBS and 0.5% casein (Sigma-Aldrich, St. Louis, MO) in PBS for each round of mock enrichment. The FimH variants tested were FimH/A188D-fluor (fluorescent FimH mutant with 1.2 µM affinity for mannosylated groups on bovine serum albumin, or mannose-BSA) or FimH/R60P (a non-fluorescent FimH mutant with millimolar or lower affinity for mannose). Note that these were expressed in an older low-expression system, in which the *fimh* gene was on a low-copy plasmid while the remaining *fim* operon was on a high-copy plasmid. In this system, FimH/R60P acted essentially as a non-binder. For mock enrichment, a mock library mixture of both variants was made with either 0.0001% or 0.001% FimH/A188D-fluor in a background of FimH/R60P, for a total of 1.66×10^7 cells. These "mock libraries" were mixed with 1×10^7 bare streptavidin beads in a tube for 30 minutes with rotation at room temperature as a depletion step to reduce nonspecific binders (to mimic depletion needed during actual library screening). After incubation, the

magnetic strip was used to isolate all beads, and the supernatant was carried on for a second round of depletion. After this depletion, the supernatant was combined with 1×10^7 of either the previously ligand-coated beads for specific interactions or bare beads to measure nonspecific interactions. These incubated for 1 hour with gentle rotation at 4°C. Beads were then collected by magnet, and the supernatant was saved for analysis. Bound cells were then washed twice, and the supernatant of each wash was combined with the first supernatant to form the unbound population. The remaining beads were saved as the bound population, and resuspended in 0.5 mL of LB media with ampicillin and chloramphenicol for overnight growth and enrichment. Fractions of the bound and unbound populations were then diluted into FACS tubes containing PBS and analyzed via flow cytometry. Samples were run for flow cytometry on a BD FACSCanto II system (BD Biosciences, Franklin Lakes, NJ) for 20 seconds at the lowest flow rate, and the data was analyzed using FlowJo software (FlowJo LLC, Ashland, Oregon). During analysis, beads and cells were distinguished from one another using forward scatter vs. side scatter, and fluorescence was used to distinguish between the A188D FimH variant and the non-fluorescent, non-binding mutant FimH/R60P. Gates were constructed to isolate the bound or unbound population of each cell type, thus obtaining quantitative results.

Library Screening and Enrichment—Screening of the actual library used the above biomagnetic selection assay with some changes. Dynabeads Protein G (Thermo Fisher Scientific, Waltham, MA) were used as the magnetic beads given our target choice of an antibody (10 µg antibody for 50 µL protein G beads). This target was the commercial monoclonal antibody Penta-His (Qiagen, Hilden, Germany).[1] Meanwhile, *E. coli* cells expressing each of the three FimH libraries (with CDR2, CDR3, or CDR2+3 randomized) were grown, induced for fimbrial expression, harvested, and washed as described above. At this point, a 5x sampling of each library's diversity was combined into a tube, resulting in a total of 1.66×10^7 cells per sample. These were then incubated with 1×10^7 bare protein G beads for 30 minutes with rotation at room temperature as a depletion step to reduce nonspecific binders. Samples included 2% methyl α-D-mannopyranoside to block any mannose-related interactions by wild-type variants. After incubation, the magnetic strip was used to isolate all beads, and the supernatant was carried on for depletion again (except for the first round of library

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[1] For other targets tested, including materials information and results, see Appendix C and D.

screening, which had just one round of depletion). After depletion, enrichment was conducted by incubating the supernatant with 1×10^7 of the previously ligand-coated beads for 1 hour with gentle rotation at 4°C. Beads were then collected by magnet, and the supernatant was removed. For the first round of enrichment, the beads were not washed. In later rounds, beads were washed once. These bead-bound cells were then resuspended in 0.5 mL of LB media with ampicillin. A portion (0.5 μL and 0.05 μL) was plated onto LB plates with ampicillin, while the rest was grown overnight in 5 mL of LB media and ampicillin. For each successive round, the blocking buffer used alternated between 0.2% BSA/PBS and 0.5% casein/PBS. After each round, the next morning, the plated colonies were counted and recorded, and 1 mL of the overnight stock was saved in 15% glycerol. Following successful library enrichment, at least 8 clones from the final round of enrichment were grown overnight, and their DNA was extracted using the QIAprep Spin Miniprep kit (Qiagen, Hilden, Germany) and sent for sequencing to Genewiz (Seattle, WA).

Clonal Analysis via ELISA—The binding capability of each isolated clone was measured using an ELISA. Briefly, microtiter plates were coated with whole *E. coli* cells expressing each isolated variant at an OD_{600} of 1.0 in 0.02 M $NaHCO_3$ buffer, for 1 hour at 37°C. The cells had been grown, induced for expression, harvested, and washed as described for the flow cytometry studies above. After washing wells with PBS, all wells were quenched for 30 minutes with 0.2% BSA/PBS. Then, the target antibody Penta-His (Qiagen, Hilden, Germany) was added to cells at 2 μg/mL, or at concentrations varying from 0.01 μg/mL to 20 μg/mL; the anti-FimH monoclonal antibodies mab21 and mab926 were also added to some cells (1/100 dilution). After a 1 hour incubation at 37°C and 3 washes, goat anti-mouse IgG (H+L) cross-adsorbed secondary antibody, conjugated to HRP (Thermo Fisher Scientific, Waltham, MA) was added at 1:2000 dilution. Following washes, bound antibody was incubated with TMB, or 3,30,5,50-tetramethylbenzidine (KPL, Gaithersburg, MD). The resulting reaction was stopped after 1-2 minutes with 0.3 M sulfuric acid. Absorbance was then measured at 450 nm using a Biotek Synergy™ Neo2 Multi-Mode Microplate Reader.

Results

Design of a FimH Library with High, Regulated Expression. To design a library of recognition molecules using FimH as a scaffold, it was first necessary to develop an expression system for FimH with high, regulated expression. It was demonstrated previously that the use of the arabinose inducible promoter from the *araBAD* operon provided the sufficiently tight regulation needed for library expression (Bessette, Rice,

& Daugherty, 2004). For this reason, we developed an expression system using the commercial plasmid pBAD/HisB from Thermo Fisher, which has a high copy number and a multiple cloning site downstream of the *araBAD* promoter. The *fim* operon, excluding regulatory subunits *fimB* and *fimE*, was cloned directly downstream of the promoter. This plasmid was transformed into the *fim*-null strain MegaX DH10B. Expression of fimbriae using this strain, called pBAD-Fim, was measured using flow cytometry. Cells were sub-cultured and grown to log-phase, at which point one group of cells was induced with 0.2% w/v arabinose while another continued to grow without arabinose. Previous experiments involving pBAD-Fim following arabinose induction showed cellular aggregation that appeared to be directly correlated with that strain's FimH affinity for mannose; for this reason, 2% methyl α-D-mannopyranoside (or aMMP) was added to successfully eliminate aggregation following arabinose induction.[2] Expression of fimbriae was marked by binding to the anti-FimH monoclonal antibody mab926, previously identified to bind at a high affinity to wild-type FimH (Kisiela, et al., 2015). The tight regulation and high expression level is shown in Figure 15, where the ON peak (FimH expression turned on by arabinose induction) is a distinct, high peak with a mean

fluorescence intensity that is two orders of magnitude higher than that of the OFF peak (FimH expression not induced).

Using this expression system, a FimH library was constructed in which either the CDR2 loop, the CDR3 loop, or both loops were randomized using NNK degenerate codons. These loop regions were previously identified as permissive
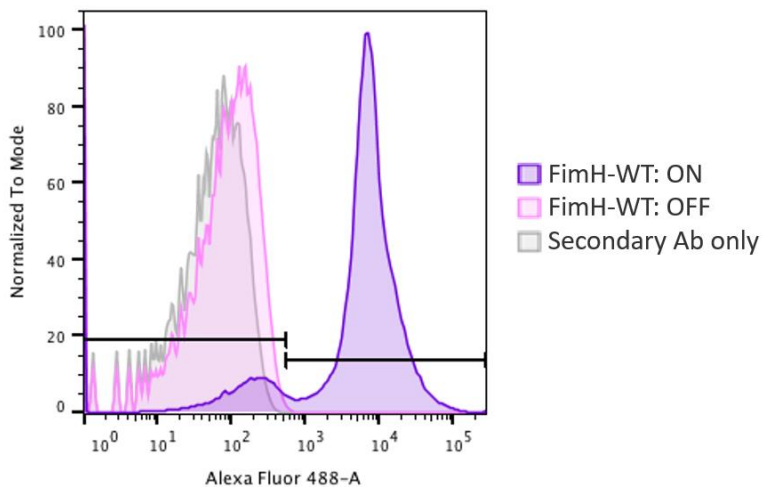
Figure 15. Expression levels of strain pBAD-Fim (with wild-type FimH gene) with arabinose (ON, in purple) or without arabinose induction (OFF, in pink). The negative control (gray) are cells incubated with the secondary antibody only.

regions that, upon mutation, resulted in a change in FimH specificity as well as potentially retained binding regulation. NNK codons were chosen to reduce the frequency of stop codons to a theoretical rate of 3.1%

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[2] Data regarding arabinose induction optimization and addressing aggregation can be found in appendix E.

for each degenerate codon (compared to NNN codons). This results in a predicted rate of truncated FimH library variants of 28% for the CDR2 library, 31% for the CDR3 library, or 59% for the CDR2+3 library. For the backbone vector, pBAD-Fim/A188D was used rather than the wild-type FimH in order to improve the stability of the tight conformation. Preliminary results had suggested that most mutations to the CDR loops caused FimH to favor the loose conformation. Therefore, we further stabilized the tight conformation of FimH with A188D such that, when screening the library, binders could be found to either the loose or tight conformation, rather than to just one conformation. Oligo inserts for each loop region, with NNK codons and sufficient overhang regions, were assembled into the pBAD-Fim/A188D backbone vector using Gibson Assembly, an isothermal blunt-ended ligation method with relatively high efficiency. The resulting library DNA was then electroporated into the previously described MegaX DH10B strain, using an optimized and scaled up method with higher transformation efficiency.[3] Table 2 shows the size of each of the libraries, found by counting colonies plated from the recovered electroporated cells. These libraries are a sampling of the sequence space available to the randomized 9-mer (CDR2) or 10-mer (CDR3) regions, but provide a sufficient starting point for understanding the utility of a FimH library based on the CDR loops.

Table 2. Size and characteristics of the FimH libraries.

Library (randomized loop)	Library size (CFU)	Mab824 binding*	Mab21 binding*	Mab475 binding*
CDR2	2.0×10^6	50%	40%	9%
CDR3	8.5×10^5	38%	10%	7%
CDR2+3	4.7×10^5	6%	3%	3%

* Percentages given for antibody binding have been normalized to antibody binding levels for the library scaffold, pBAD-Fim/A188D, which serves as a positive control for all three antibodies.

Each of the libraries was also characterized using flow cytometry. The anti-FimH antibody mab824 was used as a proxy for FimH expression, since it binds to FimH away from the binding pocket and the interdomain region, and it binds regardless of the conformation of FimH (see data in appendix A) (Kisiela, et al., 2015). The anti-FimH antibody mab21 was used as a proxy for FimH in the tight conformation, since it only binds to FimH in the tight conformation. Lastly, anti-FimH antibody mab475 was used as a proxy for wild-type FimH, since it binds to residues in loops CDR2 and CDR3, and therefore would only bind FimH if

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[3] Transformation optimization data for this library can be found in appendix F.

neither of those loops were mutated. These characteristics are also shown in Table 2. When all three libraries are combined, as is done during library screening, the total FimH library contains 41% clones which properly express FimH (typical to many scaffold libraries), 27% clones in the tight conformation, and 8% wild-type clones.

Biomagnetic Selection for Enrichment of Variants from a Mock FimH Library. To screen the library and identify variants with the desired binding specificity, a method was needed which would quickly screen the full library with minimal target and stringency. Biomagnetic selection was chosen for its quick selection and isolation of desired variants and its high avidity. Briefly, magnetic microbeads (2.8 μm diameter) were coated with the desired target antigen, after which *E. coli* cells expressing the FimH library were mixed with the beads for binding. Any cells bound to the beads were then pulled aside using a magnetic strip, so that the supernatant could be removed. Bound cells were then grown overnight, during which cells doubled and "grew off" of the beads, eliminating the need for elution from the binding surface. To demonstrate the suitability of this screening method for our FimH library, enrichment using biomagnetic selection was tested using a mock library.[4] FimH/A188D, which has an affinity of about 1.2 μM for mannose groups on mannosylated bovine serum albumin, (Aprikian, et al., 2007), was spiked into a background of FimH/R60P, a FimH mutant locked into the loose conformation. In our high expressing system with the pBAD-Fim plasmid, pBAD-FimH/R60P can still bind to mannose-coated microbeads, even with likely millimolar affinity, as is shown in appendix G. However, in the lower expression system used for this assay, FimH/R60P was essentially a non-binder, and thus representative of non-binding clones from the FimH library. FimH/A188D represented the minimal desired affinity for a new binder from the library. More than three rounds of biomagnetic selection were run; however, only three were needed to reach complete enrichment of the FimH/A188D mutant from the non-binding FimH/R60P variants. The initial amounts of FimH/A188D were either 10, 100, or 1000 cells out of 10^7 total cells in the mock library, or 0.0001%, 0.001%, and 0.01%, thus representing likely rarity fractions of a desired binder. Enrichment of each of these mock libraries is shown in Figure 16, showing up to 950,000-fold enrichment. While this is a different expression system,

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[4] Studies showing assay specificity, the optimization of bead/cell incubation time, and the effect of various blocking agents are shown in appendix H.

success seen with a less optimal expression system is indicative of success with the improved, high expression system.
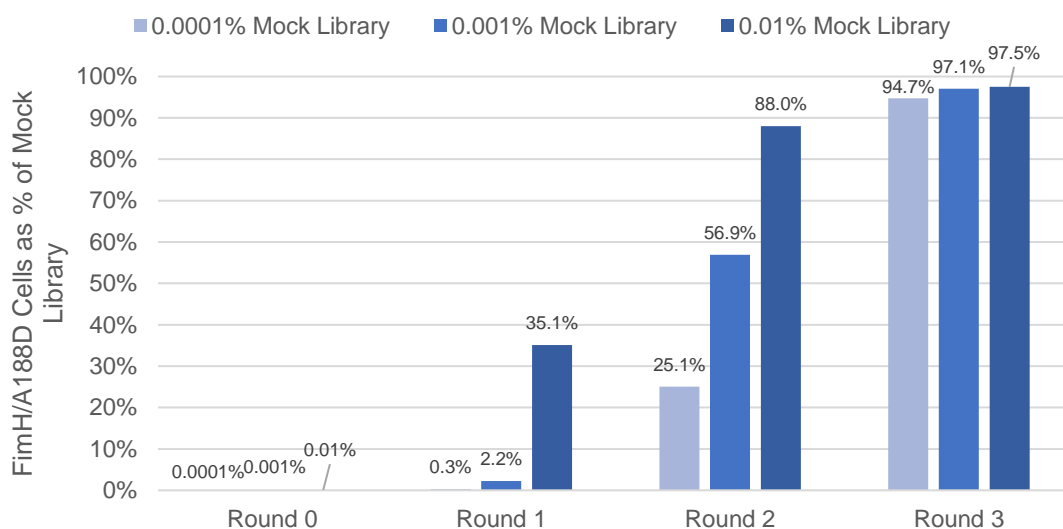

Figure 16. Enrichment of FimH/A188D from a background of non-binding FimH variants. This study was conducted using a FimH expression system with lower and variable expression.

Screening of the FimH Library against the antibody target Penta-His. To determine whether the FimH library could be useful for producing new recognition proteins, the library was screened against a model target. This target was the commercial monoclonal antibody Penta-His, from Qiagen, chosen for its easy availability for later binding characterization. The combined FimH library (CDR2, CDR3, and CDR2+3) of 3.32×10^6 variants was screened against Penta-His over the course of four rounds of biomagnetic selection. The number of cells captured in each round by the magnetic microbeads increased from a background level of about 100,000 in round 1 (with less stringent conditions), to over 4 million by round 4 (with more stringent conditions). From this final enriched population, 8 clones were sequenced. These sequences and their frequencies based on that sampling are shown in Table 3. From the 8 clones sequenced, four unique clones were identified, three of which are shown to be enriched based on their repeated occurrence even from a very small sampling of the enriched population.

Table 3. Potential Penta-His antibody binding FimH clones identified from the FimH library

FimH Clone	CDR2 sequence*	CDR3 sequence*	Frequency (out of 8 clones)
PH2	*(N D Y P E T I T D)*	L D S H A Y R L G V	3
PH3	A E G R W W V S D	*(Q T N N Y N S D D F)*	2
PH4	P H A R R T Q G A	*(Q T N N Y N S D D F)*	2
PH5	T F H S R R N Y F	*(Q T N N Y N S D D F)*	1

* Italicized sequences in parentheses are wild-type sequences.

An ELISA was run to characterize the clones' binding to the antibody Penta-His, as well as their conformation and level of specificity. Cells expressing each FimH mutant were immobilized to the assay plate such that their fimbriae were available for binding to the target. The results (Figure 17) indicated clones had varying affinities for Penta-His, ranging from higher affinities by PH3 and PH4, to moderate affinity for Penta-His by PH5, to little to no affinity by PH2. Binding to mab21 indicates that mutants PH3 and PH4 exist primarily in the tight conformation. Since both these antibodies were mouse monoclonal antibodies, binding to mab926 was also measured to check that the specificity was not simply for any mouse monoclonal antibody. Thus, the lack of binding to mab926 by all the mutants demonstrates that binding to Penta-His is specific to that target.
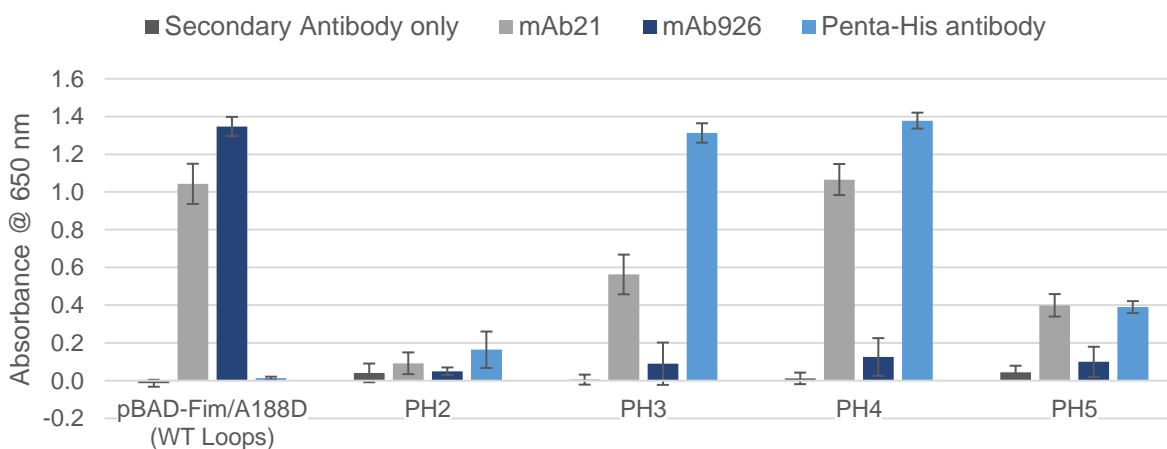
Figure 17. ELISA characterization of binding by isolated FimH library clones. Whole cells expressing either the FimH library template (pBAD-Fim/A188D) or the FimH mutants were immobilized and incubated with the secondary antibody only (dark gray bars), the tight-conformation binding anti-FimH mab21 (light gray bars), the wild-type loop binding anti-FimH mab926 (dark blue bars), and Penta-His antibody at 2 µg/mL (light blue bars). Bound antibody was detected using an HRP-conjugated goat-anti-mouse secondary antibody and the substrate TMB. Error bars represent standard deviation of sample (n=3).

A further ELISA was done to determine the binding affinity of clones PH3 and PH4, which seemed to have the highest binding to the Penta-His antibody. Clones were incubated with a range of concentrations of Penta-His antibody. The binding curves (Figure 18) show that both shared a similar affinity of 79 ± 18 nM.

Binding Curve of Penta-His Binders from FimH Library

Figure 18. Binding curves of Penta-His binding clones identified from the FimH library. The curves fit to both mutants' binding data corresponded to an affinity of 79 ± 18 nM. Error bars represent the standard deviation (n=3). Binding curves were fitted using GraphPad Prism 7 software.
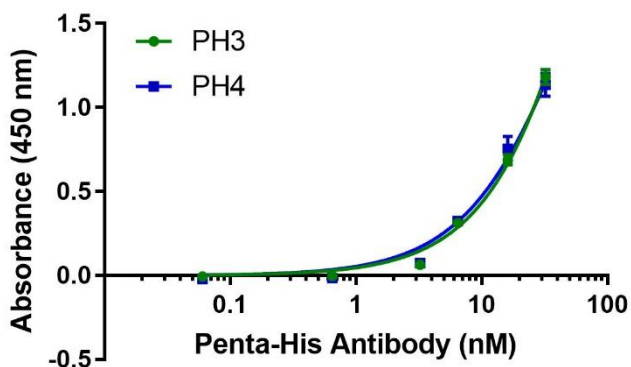
Discussion

The goal of this paper was to demonstrate the utility of a FimH library in which the binding pocket was modified in a deliberate way that did not preclude regulation of binding. Since previous work already established the permissive positions that would allow this, this work focused on the design and screening of the library. This differs from previous FimH libraries precisely because it builds from the previous knowledge of FimH as a regulated scaffold; instead of generating isolated epitopes or peptides with binding to new targets, whole proteins are generated which can take advantage of the full FimH binding pocket, as well as the interdomain interactions which regulate binding specificity, since those interactions will be retained. Previous FimH libraries lacked that knowledge, and thus could not take advantage of it at that time. Moreover, this work is unique in describing the development of a library based on a regulated scaffold, which to our knowledge has not been done to date.

The design of the library relied first on a tight, regulated FimH expression system. Expression of proteins in libraries requires tight regulation, since constitutive, high expression can be toxic to the host, leading to undesired bias against certain library variants (Daugherty, Olsen, Iverson, & Georgiou, 1999). Moreover, once induced, expression should be consistent among individual library variants to reduce unintended biases. Finally, high expression was desired for our fimbrial library to increase the avidity effect and improve capture of weaker affinities. This was important since the FimH library is a naïve library unbiased towards

any specific target or epitope; thus, interactions with a new target were more likely to be initially weak, or with faster dissociation rates.

Our combined FimH library, consisting of either loop CDR2 randomized, loop CDR3 randomized, or both loops randomized (CDR2+3), had 3.32×10^6 variants. In comparison to other bacterial libraries, this is somewhat smaller, and samples a smaller possible sequence space given that up to 19 residues have been randomized. However, even at that size, the library could be successfully screened to generate a binder to the target antibody Penta-His. It is interesting to note that none of the identified Penta-His binders have a repetitive histidine sequence, like the highly charged and basic hexa-histidine tag which is the known epitope for the Penta-His antibody. Instead, the identified Penta-His binders with nanomolar affinity range from being positively charged and basic (PH4 sequence of PHARRTQGA) to slightly negatively charged and acidic (PH3 sequence of AEGRWWVSD). This range in the epitope hydrophilicity and its difference from the hexa-histidine tag demonstrates that the library can generate entirely different binders to the target, even at a size of 10^6 variants. For screening other targets, the library size can be improved in the future to increase the probability of identifying strong binders to a broader range of targets, including small molecules.

Beyond size, several other aspects of the FimH library were characterized using flow cytometry. Roughly 41% of the clones properly express FimH, which is typical to many scaffold libraries. While in this work, the truncated or otherwise non-expressing clones were left in the library, future work may involve sorting out any non-expressing clones using fluorescence-activated cell sorting (FACS), thus increasing the probability of isolating a desired binding variant. Characterization also revealed that 8% of the total library were wild-type clones. While this is slightly higher than expected, their impact during library screening was reduced by including free mannose in the biomagnetic selection process to effectively deplete any mannose binders. If needed, these too can be depleted from the library using FACS and the same antibodies.

The use of biomagnetic selection as a screening method, particularly for *de novo* binders from a protein library, has been described previously for a yeast-displayed library (Ackerman, et al., 2009) and a bacterial-displayed library (Bessette, Rice, & Daugherty, 2004). However, the method has not previously been applied to a fimbrial library, nor has it been applied to a scaffold with conformation-dependent binding. The work here has shown that such a method is successful for screening a fimbrial library based on a scaffold

with conformation-dependent binding. The flexibility of this method was hugely important in its choice. While FACS is the preferred method for many cell-based libraries, it would have been far too stringent for our library due to the lack of multivalency and the significant amount of time between ligand-cell interactions, removal of excess ligand, secondary reagent incubation, and isolation of the bound variants. During that time, binders with quick dissociation rates would be missed. In biomagnetic selection, however, those variants are isolated almost immediately after removal of excess ligand. Moreover, stringency can be added easily as needed by increasing the number of depletion steps, increasing the number of wash steps after cell capture, or reducing the amount of ligand-coated beads interacting with the cells. While this method captures weaker affinity variants, FACS can be used later for affinity maturation when finer discrimination is needed.

The library described here is a first-pass effort at showing the utility of a library with fully-randomized binding loops. With our improved understanding of the impact of randomization on the conformational stability of FimH, the same library can be re-designed in various ways to improve the expression rate or increase the percentage of likely binders. This can involve excluding certain codons, avoiding mutations of certain residues in the loops, or simply sorting out non-expressing variants in order to improve screening probabilities.

Conclusion

Taken together, our findings indicate that a FimH-based library, in which the binding pocket is carefully randomized to maintain potential regulated binding, would be able to successfully generate new recognition molecules against targets of interest. When combined with our previous knowledge of the regulation of FimH variants with new specificities, we have presented a complete tool for generating regulated proteins to any target of interest. The effort involved in screening such a library is far less than the effort needed to individually engineer a recognition molecule to be regulated, or engineer a regulated protein to have the desired binding affinity. Thus, such a tool could be used to produce regulated binders more conveniently for a wide range of life sciences applications.

Chapter V: Conclusions

This work describes a new tool for more easily developing recognition molecules with regulated binding. While protein engineering methods continue to advance and provide researchers with ways to engineer regulation into existing recognition proteins, the scaffold and resulting library described here present a much simpler and elegant way to generate regulated binding to many different targets. Along with developing the first library based on a scaffold with regulated binding, this work also generated significant insights into the coupling between binding regulation and the binding pocket residues. For example, we have learned that the pocket is far more likely to prefer the loose conformation after mutation than the tight conformation. We have also continued probing the parasteric mechanism of inhibition that was first identified with FimH. This knowledge not only assists us in the future when improving the FimH library, but also can provide insight to other researchers working to understand the complex binding behaviors of native FimH, or those working to develop strategies to inhibit FimH binding in order to prevent adhesion by uropathogenic *E. coli*.

While we have shown the strong potential of FimH as a scaffold for generating regulated binding proteins, the limited success of our library screenings limited the scope of the work. The full potential would have involved identifying binders to disease-relevant targets, and thus showing how a FimH-derived regulated protein could be used in an actual application. This can still be done, but would require significant improvements to the FimH library. This may include rebuilding the library using a two-plasmid expression system that still retains the same high, regulated expression, but places FimH on its own, much smaller plasmid. This would enable more efficient transformations, resulting in larger libraries. It would also simplify modifications to the *fimh* gene, since it would be on a smaller plasmid. Lastly, oddities such as cellular aggregation after inducing fimbrial expression may be solved by this segregation. Another improvement to the library would involve more selectively randomizing residues. For example, certain codons could be avoided during mutagenesis, or certain residues could be left as wild-type to improve the chances of successful FimH expression. These decisions could be informed by the immense amount of data that can be gathered after deep sequencing the current FimH library. Lastly, all of the work done here has treated FimH as a two-domain protein that is part of the larger fimbrial structure. Recent work by other researchers have identified ways in which the two-domain protein itself can be isolated; or, if one is only interested in

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molecular recognition, the lectin domain itself can be isolated. This presents opportunities for exploring how FimH-derived recognition molecules could actually be used in an application.

In summary, there is still more to learn about how to effectively use FimH to better generate regulated recognition proteins. However, the work described in this thesis has laid the groundwork for this new and exciting tool, and it is only the beginning of our understanding of how a little bacterial protein can potentially exceed antibodies as the next gold standard for life sciences applications.

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A.
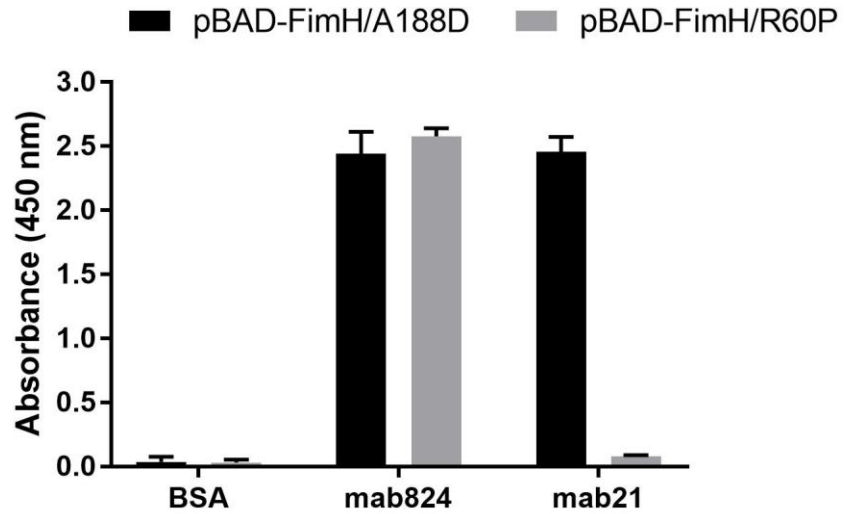
ELISA: Binding of FimH variants in tight or loose conformations to mab824. This data demonstrates that mab824 can bind to FimH in either conformation, even when stabilized in just the loose or just the tight conformation. The conformation of each variant is confirmed by the binding or lack of binding to mab21, which only binds FimH in its tight conformation. It remains possible that mab824 can induce FimH to switch to the loose conformation, even when it has the A188D point mutation. Therefore, the exact mechanisms and kinetics of mab824 binding to FimH have yet to be probed. However, based on this data, we are confident that mab824 can be used as a proxy for FimH expression with most of our FimH variants.

B.
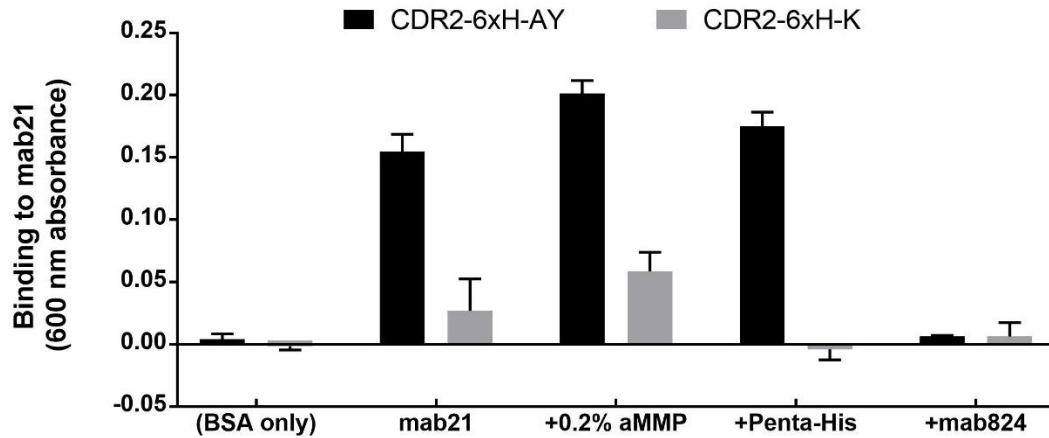
Crystal Violet Assay: Binding to mab21 by CDR2-6xHis mutants under various conditions. This binding assay was run similarly to the crystal violet assay described in the Methods section of Chapter III. The key difference was the immobilization of mab21 to the plate surface, and the inclusion of other molecules with the cells. For example, 0.2% aMMP (methyl α-D-mannopyranoside), 2 µg/mL Penta-His antibody, or 2 µg/mL mab824 was mixed with cells (OD_{600} of 2.0) prior to their incubation with mab21. This graph suggests that CDR2-6xH-AY may not be "locked" in the tight conformation, since the presence of mab824 results in a lack of mab21 binding. While the mechanism behind this is not known yet, this implies that CDR2-6xH-AY could still exist in either conformation, even if it prefers the tight conformation when mab21 is present. This is further backed by the fact that the addition of even just 0.2% aMMP (1/10th the usual concentration for inducing conformational shift) can increase CDR2-6xH-AY binding to mab21. Plus, binding to mab21 even when Penta-His is present could mean that either Penta-His is induced to detach by mab21, or that it can still bind mab21 while bound to Penta-His. Given the data found in Chapter III, the former is more likely. Lastly, CDR2-6xH-K is definitely in the loose conformation, and Penta-His does not induce its shift to the tight conformation, although 0.2% aMMP does increase mab21 slightly.
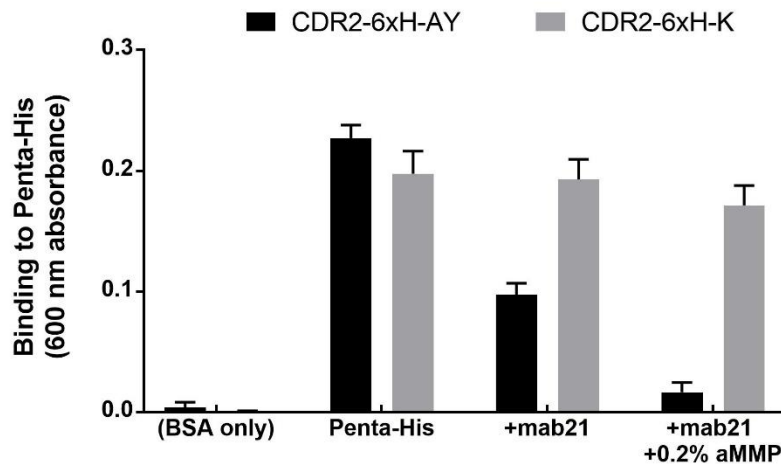
Crystal Violet Assay: Binding to Penta-His by CDR2-6xHis mutants under various conditions. This binding assay was run as described in the above caption, except 2 µg/mL Penta-His antibody was immobilized instead. This data suggests that when CDR2-6xH-AY cells have pre-incubated for just minutes with mab21 or mab21 + aMMP, Penta-His binding can be inhibited, likely because CDR2-6xH-AY has been locked in the tight conformation by those molecules. CDR2-6xH-K is also modestly affected when both mab21 and aMMP are present.

C.

Data below shows attempts at screening the small molecules cortisol and digoxigenin. These were provided as biotinylated molecules by Jiayi Dou from David Baker's lab at the University of Washington.

Total cells bound	DIG	Cortisol	Mannose (pBAD-Fim/A188D cells)
Round 1	347,000	212,000	2,200,000
Round 2	338,000	298,000	N/A
Round 3	278,000	469,000	2,000,000
Round 4	460,000	769,000+	N/A
Round 5	2,000,000	2,200,000	N/A

Cells captured during each round of screening of FimH library by cortisol and digoxigenin (DIG). Biomagnetic selection was performed at each round using magnetic microbeads coated with either of these small molecules. Captured cells were quantified by plating a dilution of the capture population. This data suggested successful enrichment of cortisol or DIG binders. Binding of pBAD-FimH/A188D cells by mannose was used as a positive control to benchmark specific binding. *This data was collected by Bill Koski.*

8 clones from each enriched library were sequenced. A number of these clones had truncated sequences or lost the majority of the *fim* operon. Clones with non-wild-type and successful sequencing are shown here.

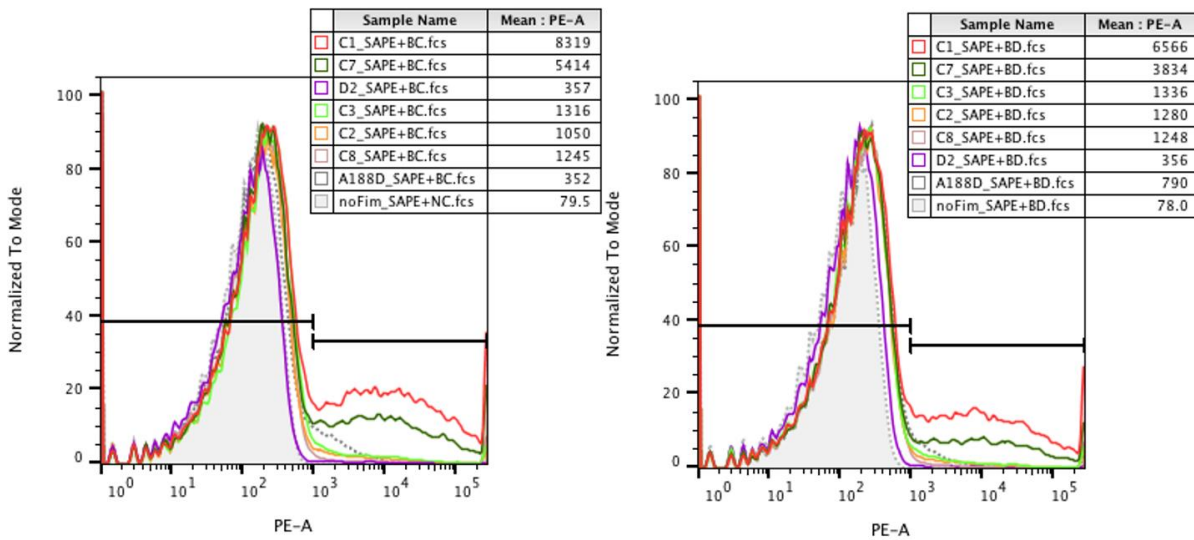| FimH Clone	CDR2 sequence*	CDR3 sequence*	Frequency (out of 8 clones)
C1	G R Y G R N G K R	*(Q T N N Y N S D D F)*	1
C2/C3	S K Y R Q S V R Q	*(Q T N N Y N S D D F)*	2
C5	S P S C C H T T L	*(Q T N N Y N S D D F)*	1
C7	R L R L L R A P A	*(Q T N N Y N S D D F)*	1
C8	R R M S S D S A D	*(Q T N N Y N S D D F)*	1
D1	G Y L P G S R H I	*(Q T N N Y N S D D F)*	1
D2	*(N D Y P E T I T D)*	S T L T I W V I H S	1

Sequences of potential cortisol (C*) or DIG (D*) binding FimH clones identified from the FimH library. Italicized sequences in parentheses are wild-type. One clone was shown to be enriched based on its frequency (2 found within 8 clones sequenced).

Characterizing binding of these clones to their small molecule targets was difficult; the usual plate-based assays did not work due to either steric hindrance by larger proteins during small molecule immobilization, or due to the total dissociation of binders during secondary antibody incubation. For this reason, biomagnetic selection was used again to probe binding by individual clones. We correlated high amounts of cell capture with specific binding by those FimH clones. Moreover, all clones were tested for binding to both small molecules, since it has been found that clones enriched towards one target often have an affinity for the other.

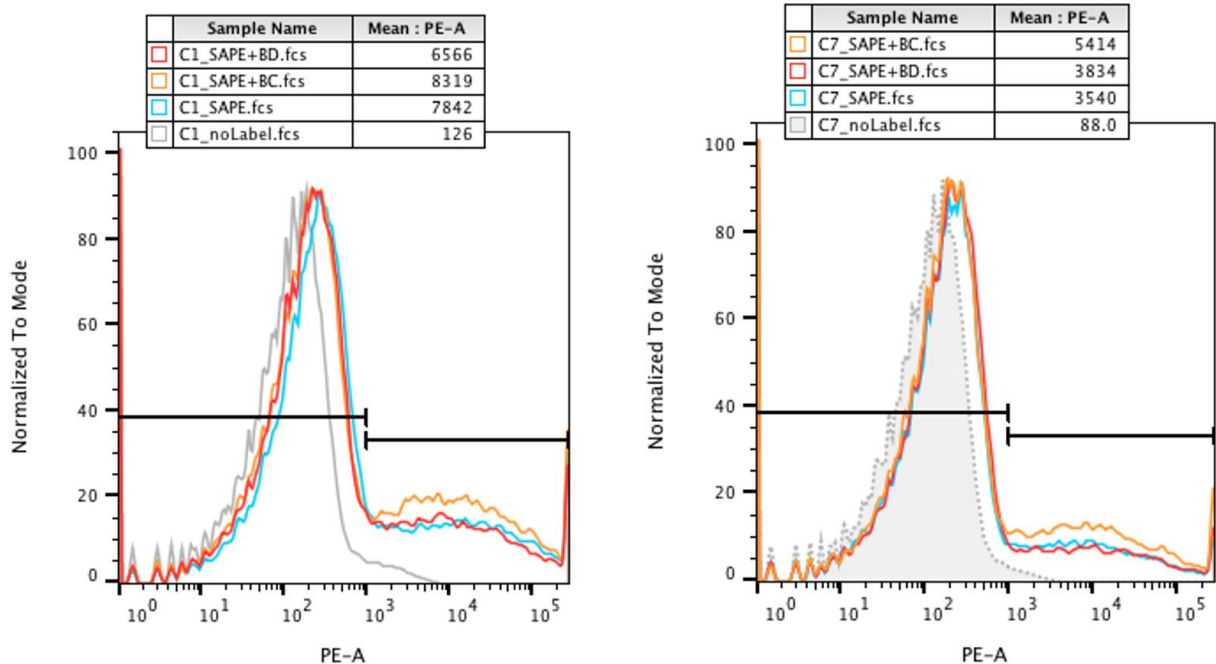| Clone	DIG	Cortisol
C1	66,000	139,000
C2	23,000	111,000
C3	14,000	115,000
C5	28,000	48,000
C7	474,000	194,000
C8	111,000	227,000
D1	33,000	11,000
D2	39,000	292,000

Cells captured during biomagnetic selection assay probing each FimH clone. Clones C2 and C3 share the same sequence; the similar numbers of captured cells by both clones suggests that the assay gave consistent results. Clones C1 and C2/3 had more specific binding to cortisol than to DIG, while C7 appeared to have high binding to both DIG and cortisol. Clone D2 had far more specific binding to cortisol than to DIG. Clones C5 and D1 appeared to be non-binders.

In an attempt to better quantify this binding, flow cytometry was run to measure capture of cortisol and DIG by these clones. Briefly, 5×10^6 cells were incubated with 3 µM of either biotin-cortisol or biotin-DIG mixed with 0.75 µM streptavidin-phycoerythrin (SAPE, Vector Labs). Some cells were mixed with just SAPE. These incubated for 1-2 hours in the dark with rotation, although the reaction volume was just 50 µL. Cells were spun down for 3 minutes to remove excess ligand, and washed once with 50 µL PBS. Cells were then immediately run on a BD FACSCanto II system (BD Biosciences) at the lowest flow rate, and 100,000 events were collected.

Sample Name	Mean : PE-A
C1_SAPE+BC.fcs	8319
C7_SAPE+BC.fcs	5414
D2_SAPE+BC.fcs	357
C3_SAPE+BC.fcs	1316
C2_SAPE+BC.fcs	1050
C8_SAPE+BC.fcs	1245
A188D_SAPE+BC.fcs	352
noFim_SAPE+NC.fcs	79.5

Sample Name	Mean : PE-A
C1_SAPE+BD.fcs	6566
C7_SAPE+BD.fcs	3834
C3_SAPE+BD.fcs	1336
C2_SAPE+BD.fcs	1280
C8_SAPE+BD.fcs	1248
D2_SAPE+BD.fcs	356
A188D_SAPE+BD.fcs	790
noFim_SAPE+BD.fcs	78.0

Flow cytometry histogram showing binding of clones to either biotinylated-DIG (left) or biotinylated cortisol (right). The gray peak illustrated the negative control. Clones C1 (red) and C7 (dark green) show the highest amount of binding; however, binding to both cortisol and DIG suggests a lack of specificity.

The next figure shows histograms of just clones C1 and C7.

Sample Name	Mean : PE-A
C1_SAPE+BD.fcs	6566
C1_SAPE+BC.fcs	8319
C1_SAPE.fcs	7842
C1_noLabel.fcs	126

Sample Name	Mean : PE-A
C7_SAPE+BC.fcs	5414
C7_SAPE+BD.fcs	3834
C7_SAPE.fcs	3540
C7_noLabel.fcs	88.0

Flow cytometry histogram showing binding of C1 (left) and C7 (right) to biotinylated cortisol (BC), biotinylated DIG (BD), or the secondary reagent streptavidin-phycoerythrin. The gray peak illustrated the negative control. While these clones had the highest binding to DIG and cortisol, they also appear to have high binding to SAPE. This suggests that these are nonspecific binders.

Because the enriched binders turned out to be nonspecific binders, we are not confident that the library screening yielded success. It is possible instead that nonspecific interactions were encouraged somehow in the assay.

D.

Data below shows attempts at screening the large protein hemagglutinin from the H1N1 influenza virus, as well as glycoprotein 1B (GP1B), found on the surface of platelets.

Total cells bound	Hemagglutinin	GP1B
Round 1	48,000	131,000
Round 2	31,000	137,000
Round 3	113,000	69,000
Round 4	291,000	469,000
Round 5	1,507,000	270,000
Round 6	507,000*	N/A

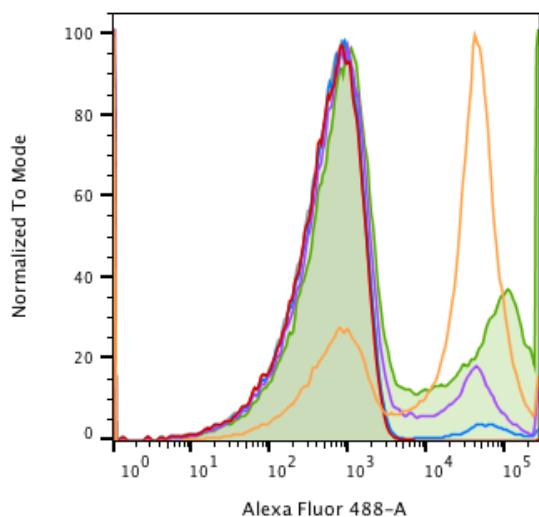**Cells captured during each round of screening of FimH library by hemagglutinin or GP1B.** Biomagnetic selection was performed at each round using magnetic microbeads coated with either of these large molecules. Captured cells were quantified by plating a dilution of the capture population. All cell/bead incubations were done in the presence of free mannose to avoid enrichment of the wild-type FimH by binding to mannosylated groups on these proteins. This data suggests a lack of enrichment by either target.

While hemagglutinin seemed to suggest success, during round 6, binding was also measured to the bare streptavidin-coated beads and to mannose-coated beads. This enriched population bound far more to mannose (1,700,000) or to the bare breads (2,600,000) than to hemagglutinin. Therefore, we concluded that enrichment was not successful.

E.
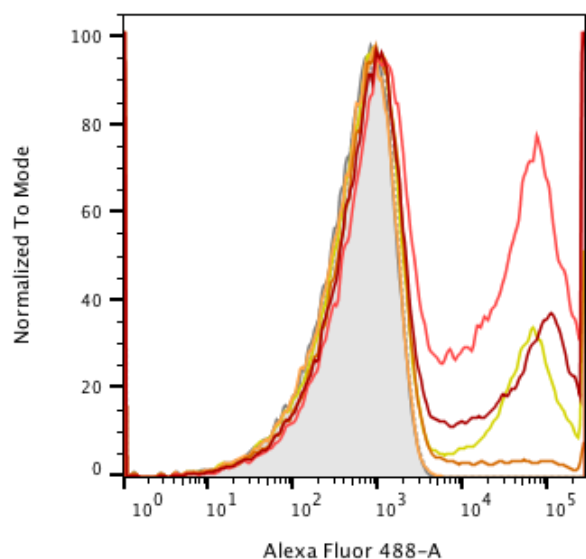
	Sample Name
☐	pBAD-Fim 02_mab926_05082015180332.fcs
☐	pBAD-Fim 002_mab926.fcs
☐	pBAD-Fim 0002_mab926_05082015181338.fcs
☐	pBAD-Fim 00002_mab926.fcs
☐	pBAD-Fim 000002_mab926.fcs
☐	pBAD-Fim 0_mab926.fcs
☐	pBAD-Fim 0_2nd only.fcs

Flow cytometry histogram of pBAD-Fim induced by varying concentrations of arabinose. Concentrations of arabinose ranged from 0.2% to 0.00002%. The anti-FimH monoclonal antibody mab926 served as a proxy for fimbrial expression since it is known to bind to FimH in either conformation. The highest concentration (0.2%) was chosen to maximize fimbrial expression.
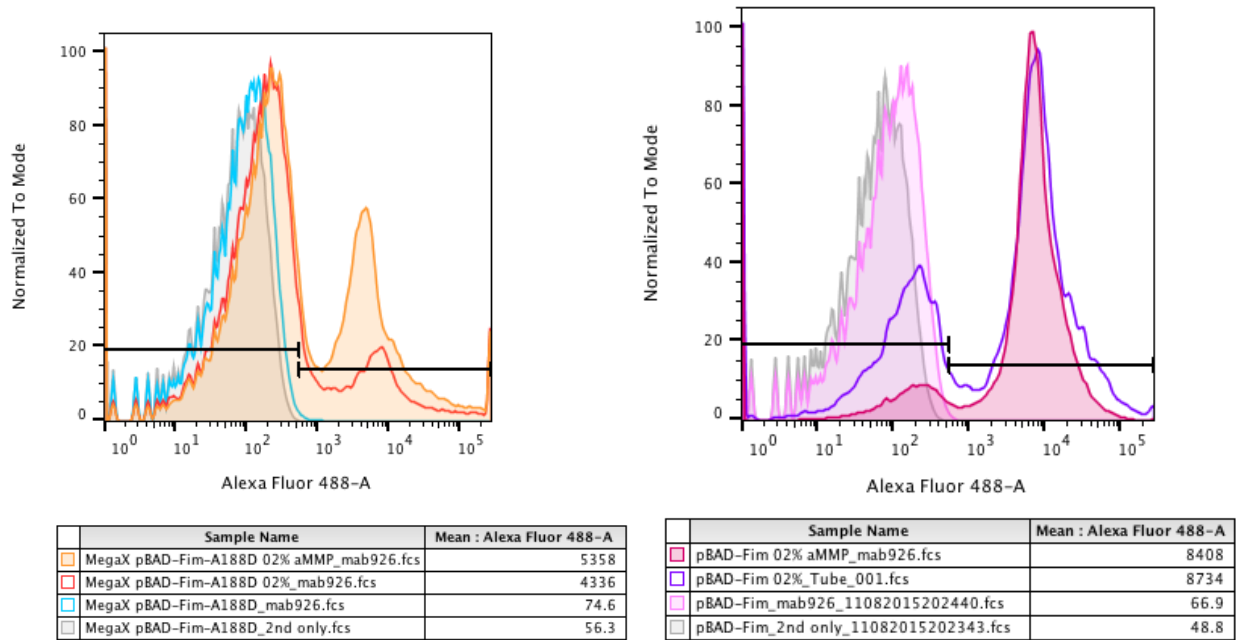
It should be noted that expression at 0.2% appeared to be lower than at 0.02%. Further analysis showed that cells at the higher concentration had begun to aggregate, which led to a lower concentration of events. This in turn led to fewer events in the positive peak, a clump of cells still appears as just one event.

	Sample Name	Mean : Alexa Fluor 488–A
☐	MegaX pBAD-Fim-R60P 02%_mab926.fcs	31214
☐	MegaX pBAD-Fim-R60P_mab926.fcs	585
☐	MegaX pBAD-Fim-A188D 02%_mab926_05082015181205.fcs	6028
☐	MegaX pBAD-Fim-A188D_mab926.fcs	2469
☐	pBAD-Fim 02_mab926_05082015180332.fcs	30518
☐	pBAD-Fim 0_mab926.fcs	460

Flow cytometry histogram of pBAD-Fim strains with and without arabinose induction.
Orange – pBAD-Fim/R60P, ON
Red – pBAD-Fim/R60P, OFF
Purple – pBAD-Fim/A188D, ON
Blue – pBAD-Fim/A188D, OFF
Green – pBAD-Fim, ON
Gray – pBAD-Fim, OFF
This graph shows an interesting correlation between the FimH variant and fimbrial expression. The three variants have varying affinity for mannose, from 298 µM for R60P, to moderate affinity for pBAD-Fim (wild-type), to 1.2 µM for A188D. Expression decreases with increased affinity; at the same time, aggregation also increased. In fact, the A188D cells were so aggregated that its concentration was very low, leading to artificially low counts in the positive peak. This suggests that the aggregation seen is somehow related to mannose binding.

To determine if mannose binding was the cause of aggregation, 2% methyl α-D-mannopyranoside was added to cells during arabinose incubation.

	Sample Name	Mean : Alexa Fluor 488–A
☐	MegaX pBAD-Fim-A188D 02% aMMP_mab926.fcs	5358
☐	MegaX pBAD-Fim-A188D 02%_mab926.fcs	4336
☐	MegaX pBAD-Fim-A188D_mab926.fcs	74.6
☐	MegaX pBAD-Fim-A188D_2nd only.fcs	56.3

	Sample Name	Mean : Alexa Fluor 488–A
☐	pBAD-Fim 02% aMMP_mab926.fcs	8408
☐	pBAD-Fim 02%_Tube_001.fcs	8734
☐	pBAD-Fim_mab926_11082015202440.fcs	66.9
☐	pBAD-Fim_2nd only_11082015202343.fcs	48.8

Flow cytometry histograms of FimH expression for pBAD-Fim/A188D (left) or pBAD-Fim (wild-type, right) with or without mannose during arabinose induction. Both graphs show that the addition of mannose causes a reduction in the OFF peak or an increase in the ON peak. pBAD-Fim shows the greatest improvement in expression by the simple addition of mannose. pBAD-Fim/A188D still has a lower expression peak. This shows that mannose-caused aggregation is the reason for the lower ON expression peak, rather than actual lower expression. During the study, cells still appeared clumped together, although the clumps were easier to break apart when mannose had been added during incubation.

F.

The greatest bottleneck to the size of a cell-displayed library is transformation of the library DNA to the expression system. To maximize the library size, it is important to optimize transformation conditions. Our method of transformation was electroporation, which is known to have far higher efficiency than chemical heat-shock treatment.

For our first optimization test, we experimented with increasing the volume of the cell/DNA mixture that is added to the electroporation cuvette. While manufacturers of the cuvette recommend no more than 50 µL in these cuvettes, we tested the maximum volume of 95 µL. Following electroporation and recovery, the final cell population was plated to obtain an estimate of the library size.

Volume	Plating volume	CFU	CFU/µg of DNA
50 µL	5 µL	500+	8.70E+07
	0.5 µL	62	1.08E+08
95 µL	5 µL	700+	1.22E+08
	0.5 µL	88	1.53E+08

Effect of cuvette volume on electroporation efficiency. In both cases, the same total volume of DNA was electroporated; however, the cell/DNA mixture was aliquoted among cuvettes in either 50 µL volumes or 95 µL volumes. This data suggests that using a higher volume in the electroporation cuvettes results in a higher number of colonies per µg/DNA.

It must be noted that this electroporation was done using a single plasmid of about 4 kb, rather than the pBAD-Fim library plasmid which is 10 kb, and thus less efficient. However, this optimization should also improve transformation of the larger plasmid.

G.
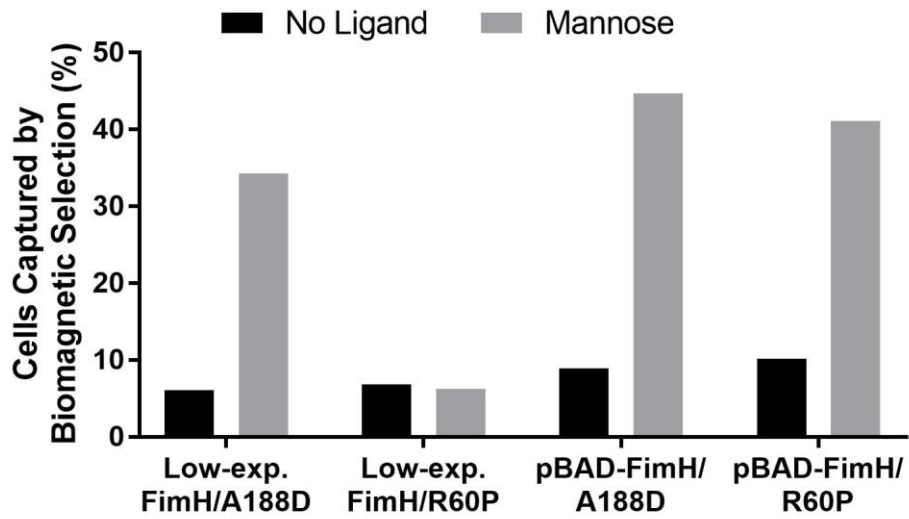
Biomagnetic Selection: Comparison of previous plasmid expression system to the new pBAD-Fim expression system. While binding to bare beads ("no ligand", black bars) is slightly higher in the new pBAD-Fim system, there is still higher specific binding to mannose-coated beads (gray bars) by the pBAD-Fim system. Most importantly, the high-expression system resulted in capture of the low-affinity variant FimH/R60P at a level similar to the high-affinity variant FimH/A188D.

H.

Many biomagnetic selection parameters were optimized by Ackerman, et al. when in use with the yeast display system. We optimized several of these for use with our system.
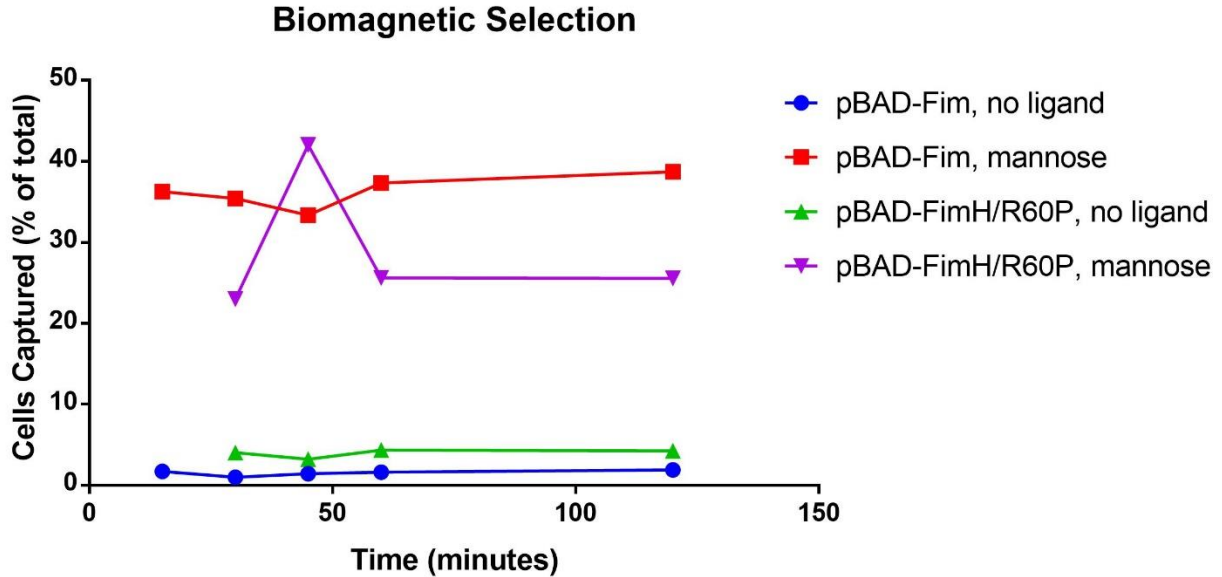
Cells captured by biomagnetic selection after varying incubation times. This data suggests that 30-60 minutes is sufficient for capture of cells. The consistently low levels of capture by cells when beads lack any ligand is indicative of the specificity of this assay.
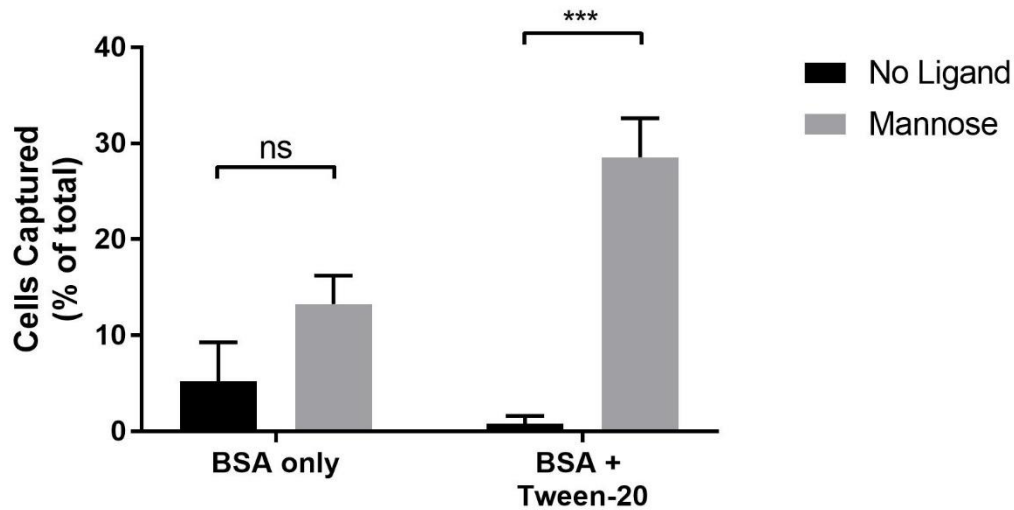
pBAD-Fim/A188D cells captured by biomagnetic selection with different blocking agents. 0.2% BSA/PBS is used in both cases. However, on the right, 0.05% Tween-20 was also added. This data shows that adding Tween-20 improves assay specificity by reducing non-specific binding and even increasing specific binding. While this works with mannose-binding, it remains to be tested if Tween-20 has an impact on small hydrophobic molecules. Statistical significance was determined using a t-test (GraphPad Prism 7 software), where *** indicates $p \leq 0.0005$, using data from n=3 experiments.