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Peptide and Hydrophobin Interactions with Polymeric Substrates Screened by a Bacterial Surface Display Method

by Mark T Kozlowski, Randall A Hughes, Randi M Pullen, and Joshua A Orlicki

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14. ABSTRACT
Polymers are ubiquitous in the modern world, and yet their applications in some cases are limited by the difficulty of promoting adhesion to them. In this work, we use the techniques of synthetic biology and microbiology to explore the longstanding issue of controlling interactions at a polymer substrate. To probe these interactions, a cloned peptide library and a separate group of fungal proteins known as hydrophobins were prepared, expressed using the autotransporter surface-display system. We demonstrate that both peptides and hydrophobins can successfully be displayed on the surface of *Escherichia coli* using this system. We screen the peptide library against acrylic, polycarbonate, polystyrene, and polypropylene, and observe an enrichment of a surface-displaying population. Finally, using a fluorescent spot assay, we find that the hydrophobins Vmh2, Sc3, DEW, and NC2 appear to promote bacterial adhesion to acrylic, polystyrene, high-density polyethylene, and polyester.

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1. Introduction

Modern polymers have a wide range of surface energies, from polar materials such as polycarbonate, to highly nonpolar substrates such as polystyrene and polypropylene. The inert characteristics of many polymers makes adhesion or bonding to the substrates a longstanding limitation. This challenge is particularly pronounced in hydrophobic polymers, which require extensive pretreatment, priming, solvent plasticization, or high temperatures to adhere. While adhesion of more polar polymers is typically easier to achieve, these polymers are susceptible to water infiltration, which can lessen the strength of the adhesive contact in humid environments. New strategies to enable good adhesive bonding would allow for facile repair in the field and the creation of new types of composites for applications such as improved capacitors.

The large diversity of peptides in both sequence and functional groups makes them potentially useful to search for strong interactions with polymer substrates. Furthermore, the insights provided by peptide binding may eventually allow the design of more-effective synthetic adhesives. Screening the chemical compositional space afforded by peptides and the natural amino acids would be nearly impossible if undertaken in a serial fashion. Recently, the US Army Combat Capabilities Development Command Army Research Laboratory developed a peptide surface-display and high-throughput library screening system to find candidate peptides capable of adhering to polylactic acid[1] and gold nanoparticles.[2] The on-cell peptide screening has the advantages of easy recoverability, the ability to propagate and sequence the genetic code of the adhesive peptides, and possible further improvements of the peptides by directed evolution.

Aside from the display of peptide libraries, we were interested in exploring the possible adhesive properties of a class of fungal proteins known as hydrophobins, which are proteins produced by filamentous fungi such as *Trichoderma reesei*, *Schizophyllum commune*, and *Neurospora crassa*, among others. Hydrophobins are empirically classified into two groups based on their solubility. Class I hydrophobins are predicted to form aggregates that are soluble only in strong organic acids and have been seen to form self-assembled, insoluble rodlets with high β-sheet content similar to amyloid fibrils. Class II hydrophobins can be dissolved in aqueous-organic mixtures, dissociate more easily from surfaces, and appear to be globular and amphiphilic in nature.[3,4] Structurally, all hydrophobins are characterized by four disulfide bonds, though the other residues present are highly variable. Both classes form a four-stranded β-barrel core, but class I hydrophobins have a large disordered region, which the class II hydrophobins lack.[5]

Hydrophobins serve a wide range of functions, such as enabling the fungal hyphae to breach the air–water interface to release spores. Specific hydrophobins also facilitate fungal adhesion to hydrophobic surfaces, assist the penetration of hosts by certain pathogenic fungi, serve as a protective layer to prevent desiccation of spores and conidia, and are used as weapons against biological competitors.[6] These properties have made hydrophobins materials of interest to the adhesives community. Some hydrophobins have been explored as adhesives for paper products,[7] or as industrial surfactants[6] or immobilizing proteins and cells to hydrophobic surfaces.[5] Linder and coworkers demonstrated that the hydrophobin HfbI could be fused to endogluconase enzyme and used to adhere this enzyme to hydrophobic surfaces such as Teflon and silanized glass. In contrast, HfbII was able to adhere to such surfaces by itself, but not when employed as a fusion partner.[8] Recently, Sorrentino and coworkers demonstrated that a chimeric fusion protein consisting of the human antimicrobial peptide LL37 and the hydrophobin Vmh2 could adhere to a polystyrene surface, inhibit the formation of *Staphylococcus epidermis* biofilms, and have biocidal activity against an array of bacteria.[9] A similar effect was observed by Artini and coworkers, who were able to use Vmh2 to self-assemble monolayers on medically relevant materials and demonstrate that biofilm formation was inhibited by the layers of hydrophobin.[10]

In the present work, we demonstrate the display of a peptide library and a group of hydrophobins on the surface of *Escherichia coli*. The display uses the autotransporter display system derived from the EhaA adhesin protein of *E. coli*.[11,12] The advantages of a surface-display method as an initial screen, as opposed to testing lyophilized hydrophobin protein directly, are the surface-displayed hydrophobins can be more easily modified or mutated and this is a cheaper method of screening a large number of candidates. Further, purified protein is expensive and there is no guarantee that a given protein can be made in its functional form.

We further demonstrate this display and screening approach potentially lead to the adhesion of certain peptides to polymers of interest such as acrylic, polycarbonate, polystyrene, and polypropylene, and the surface display of hydrophobins Vmh2, DEW, Sc3, and NC2 appear to promote bacterial adhesion to acrylic, polystyrene, polyester, and high-density polyethylene (HDPE). We have therefore potentially identified positive noncovalent interactions for polymers of military interest, which should be further explored.

2. Materials and Methods

2.1 Materials

All gene blocks (Gblocks), primers, and deoxyribonucleic acid (DNA) duplexes were purchased from Integrated DNA Technologies (IDT; Coralville, Iowa). Restriction enzymes and DNA ligase were obtained from ThermoFisher Scientific (Waltham, Massachusetts) and New England Biolabs (NEB; Ipswich, Massachusetts). Lysogeny broth (LB) medium was purchased in powdered form from Fisher Scientific (Pittsburgh, Pennsylvania), dissolved in deionized (DI) water, and autoclaved according to manufacturer's instructions. LB agar, for use in plates, consisted of LB medium, supplemented with 2% bacteriological agar (SigmaAldrich, Milwaukee, Wisconsin). Molecular-biology-grade water and glycerol were purchased from Invitrogen (Waltham, Massachusetts).

Glycerol-yeast-tryptone (GYT) medium was made by mixing 100 mL of glycerol, 1.25 g of yeast extract, and 2.50 g of tryptone per liter of molecular-biology-grade water, which was then sterilized by autoclaving for 15 min at 121 °C. Super-optimal broth with catabolite repression (SOC) medium was made by first dissolving 20 g of tryptone, 5 g of yeast extract, and 0.584 g of sodium chloride (NaCl) per liter of distilled water, which was then autoclaved. Then 1M solutions of magnesium chloride ($MgCl_2$) and magnesium sulfate ($MgSO_4$) were made up and autoclaved separately. $MgCl_2$ and $MgSO_4$ were then added to the mixture of tryptone, yeast extract, and NaCl at a ratio of 100:1, such that the final concentrations of $MgCl_2$ and $MgSO_4$ in SOC were 10 mM.

2.2 Cloning Strategy, Library

An NNK site-saturation library duplex encoding 15 amino-acid-long peptides was purchased from IDT, with the library flanked by BsaI sites (i.e., BsaI site-$(NNK)_{15}$-BsaI site, where N = any DNA base and K = guanine or thymine). The NNK library codes for all 20 amino acids, yet each position has a roughly 3% chance of containing a stop codon, meaning that roughly 40% of all 15 amino-acid-long libraries will contain at least one stop codon. These stop codons result in a nonsense sequence, meaning that the effective size of the library is reduced by 40%. To reduce the frequency of these stop codons, the library is initially cloned into the plasmid pFES.2AB, which consists of an ampicillin-resistance cassette in a split intein system under a rhamnose-inducible promoter. If a stop codon is present between the BsaI sites in this plasmid, this should prevent full translation of the ampicillin-resistance cassette. Consequently, a plasmid that contains a stop codon will not confer ampicillin resistance and be removed by selection. To propagate

pFES.2AB2AB, kanamycin selection can be used, as this plasmid contains a constitutively expressed kanamycin-resistance cassette.

The duplex DNA could not be made long enough to contain both the BsaI restriction sites and the AarI restriction sites necessary for the two steps of inserting the library first into pFES.2AB and then inserting the library into a surface-display construct. For this reason, a polymerase chain reaction (PCR) was conducted using the duplex MTK-G003 and the primers pLibAmpF and pLibAmpR to add the BsaI sites. PCRs were conducted with an AccuPrime Pfx DNA polymerase mix kit (Invitrogen; Waltham, Massachusetts), according to manufacturer's instructions, starting with 50 ng of duplex DNA. Thermal cycling was conducted on a Bio-Rad T100 instrument, with the cycles arranged thusly: 2 min at 95 °C, followed by 35 cycles of 15 s at 95 °C, then 30 s at 50 °C, and then 30 s at 68 °C. After the 35 cycles were concluded, a final extension step was conducted for 5 min at 68 °C and then the sample was held at 4 °C. The resulting PCR is cleaned up using a Qiagen (Hilden, Germany) clean-and-concentrator kit following the manufacturer's instructions. This constituted the "NNK library".

Next, 300 ng of the NNK library was mixed with 150 ng of plasmid pFES.2AB in a PCR tube, along with 0.25 µL of BsaI, 0.25 µL of T7 DNA ligase, 1 µL of 10-mM adenosine triphosphate (ATP), 1 µL of enzyme Buffer G, and molecular-biology-grade water up to a volume of 10 µL (all from Invitrogen). This mixture is duplicated in eight different PCR tubes, which are assembled simultaneously in a Bio-Rad T100 PCR block (Bio-Rad Laboratories; Hercules, California) with the following heating cycles: 1 min at 37 °C and then 1 min at 16 °C, for 35 cycles. There was a final incubation at 37 °C for 1 h, followed by a denaturation step of 15 min at 85 °C, after which the temperature was lowered to 4 °C. After assembly, the library was cleaned up and concentrated using a Qiagen clean-and-concentrator kit following the manufacturer's instructions. In brief, in each 10-µL tube, 30 µL of DNA binding buffer was added. All eight fractions were then combined into a single tube and centrifuged through a clean-and-concentrator column at 16000 g's for 30 s. The column is then washed with 750 µL of wash buffer by spinning at 16000 g's for 30 s. The tube is then spun once, empty, at 16000 g's to remove any residual wash buffer, and the DNA is removed from the column by elution in 8 µL of molecular-biology-grade water. The concentration of DNA is then measured using a DeNovix DS-11+ spectrophotometer (DeNovix; Wilmington, Delaware) and kept on ice for transformation in electrically competent *E. coli* cells.

Electrically competent *E. coli* of strain DH10B was made in-house by the following method. Cells were grown overnight at 37 °C in LB medium. This overnight culture was used to inoculate 100 mL of 2xYT medium in a 250-mL baffled Erlenmeyer flask. The cells were allowed to grow to an optical density (OD) at 600 nm (OD600)

of 0.5. The cells were then placed on ice for 10 min and then centrifuged for 6 min at 5000 g's. The cell pellets were then resuspended in 100 mL of sterile 10% molecular-biology-grade glycerol in molecular-biology-grade water and centrifuged again. This wash process was repeated with 50 and 25 mL of 10% glycerol. Finally, the cells were resuspended in 2 mL of sterile GYT medium in molecular-biology-grade water. DNA was transformed into these cells immediately (*vide infra*).

Transformation of the library into DH10B cells was by electroporation in a Bio-Rad Micropulser electroporator. Approximately 1 μg of assembled DNA was added to 200 μL of cell suspension kept on ice. The 200 μL of cells were then placed in an electroporation cuvette (Bio-Rad) with a 0.2-cm gap. The cells were then shocked with a 2.5-kV pulse and the time constant was noted. After the pulse, 1 mL of sterile, warmed SOC medium supplemented with 0.04% L-rhamnose (SigmaAldrich) was immediately added to the cuvette and the cells were transferred to a sterile culture tube. The cells were allowed to incubate at 30 °C for 3 h, and then plated on a series of LB-agar plates supplemented with 50 mg/L of ampicillin and 0.04% L-rhamnose. An aliquot of the culture was also diluted 100- and 1000-fold, to be plated on separate agar plates, to determine approximately how many clones were obtained. Simultaneously, 10 pg of pUC19 plasmid (Invitrogen) was transformed into a separate aliquot of cells to test for electrical competence of the cells. The plates were incubated for 2 days at 30 °C, and colony counts were conducted on the 100- and 1000-fold diluted plates. Based on colony counts, the estimated size of the library was between 10^5 and 10^6 members. The transformations of pUC19 resulted in an observed transformation efficiency of approximately 10^9 colonies per microgram of DNA.

The plates were then scraped into 50 mL of LB medium supplemented with 50 mg/L of ampicillin. The cells were allowed to grow overnight at 30 °C and then miniprepped using a Qiagen miniprep kit according to manufacturer's instructions. The resulting plasmid prep was held at –20 °C until the library was to be removed from the plasmid and inserted into a surface-display construct. The resulting plasmid is known as pFES-Library.

2.3 Cloning Strategy, Surface-Display System

The autotransporter system was cloned using a modification of the Modular Cloning (MoClo) toolkit developed by Lee and coworkers (this cloning strategy is more thoroughly explained in the Appendix).[13] The cloning strategy is illustrated in Fig. 1 and takes place in three steps. In brief, using the terminology of the MoClo strategy, the autotransporter surface display system is designed to enter into the

pYTK001 entry vector as a Type 3 part, with a slight modification to allow the insertion of the green fluorescent protein (GFP) dropout at the same time. Block 3, the promoter region, is designed as a Type 2 part, and Block 4, the terminator, is designed as a Type 4 part. All of these parts are placed in a Type 234 dropout plasmid, which is used for final insertion of the peptides or proteins of interest (Block 5), which can then be expressed.

More expansively, this strategy relies on cloning using the restriction enzymes BsmBI and BsaI, which are Type IIS enzymes. This means that the sticky end resulting from a BsmBI or BsaI digest is variable, and by judicious selection of sticky ends, it is possible to assemble a number of pieces of DNA in a desired order, a technique known as Golden Gate Assembly.

Referring to Fig. 1, Block 1 is obtained by PCR off of the pYTK001 plasmid, using primers MTK-p001F and MTK-p001R. This PCR adds the BsmBI and AarI restriction sites as noted on the diagram. Block 1 is intended to provide a GFP dropout construct to confirm eventual successful insertion of the library or hydrophobin construct. Block 2 is ordered from IDT with restriction sites already in place, as shown. PCRs were conducted with an AccuPrime Pfx DNA polymerase mix kit (Invitrogen), according to manufacturer's instructions. Block 3 is obtained by PCR from pDSJR, using primers araC-for and araC-rev. Block 4 is ordered as duplex DNA from IDT. Thermal cycling was conducted on a Bio-Rad T100 instrument, with the cycles arranged thusly: 2 min at 95 °C, followed by 35 cycles of 15 s at 95 °C, then 30 s at 50 °C, and then 30 s per kilobase at 68 °C. After the 35 cycles are concluded, a final extension step is conducted for 5 min at 68 °C and then the sample is held at 4 °C. The resulting PCR is cleaned up using a Qiagen clean-and-concentrator kit following the manufacturer's instructions.

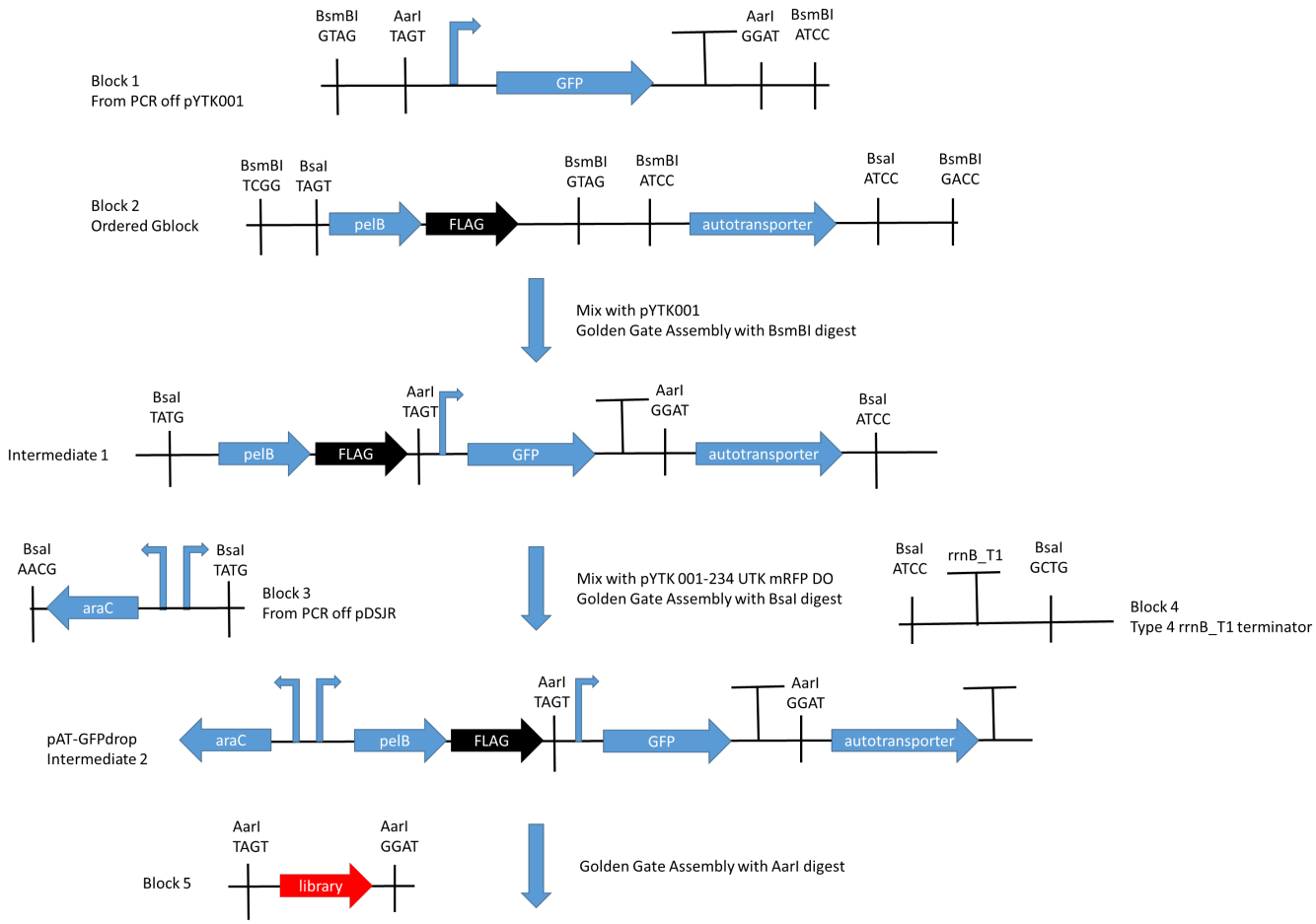

Fig. 1 Library and hydrophobin assembly strategy

Once all of the blocks are obtained, they are assembled in three steps. In the first assembly step, 150 ng each of blocks 1 and 2 are mixed with 100 ng of plasmid pYTK001 in a PCR tube, along with 0.25 µL of BsmBI, 0.25 µL of T7 DNA ligase, 1 µL of 10-mM ATP, 1 µL of enzyme buffer Tango, and molecular-biology-grade water up to a volume of 10 µL (all enzymes were obtained from Invitrogen). The tube was then thermally cycled for 1 min at 37 °C and 1 min at 16 °C, for 35 cycles. There was a final incubation at 37 °C for 30 min, followed by a denaturation step of 15 min at 85 °C, after which the temperature was lowered to 4 °C. Next, 1 µL of the assembly mix was transferred directly into 100 µL of electrocompetent cells, and electroporated in a 0.2-cm gap cuvette with a voltage of 2.5 kV. The cells are then immediately transferred into plain SOC medium, placed in a culture tube and allowed to recover for 1 h at 37 °C and 250 rpm. Then, 50 µL of cells are spotted and plated out onto LB-agar plates supplemented with 30-mg/L chloramphenicol. Next, 15–20 colonies were then selected by hand and allowed to grow overnight in LB medium supplemented with 30 mg/L of chloramphenicol. The overnight cultures were then miniprepped using a Qiagen miniprep kit according to the manufacturer's instructions. The miniprepped plasmids are sequence-verified using sequencing primers YTK-for, YTK-rev, and InsertSeqFor (see supporting information) by GENEWIZ (South Plainfield, New Jersey) and successful clones were separated for further use. This cloning intermediate is referred to as Intermediate 1.

Shown in Fig 1 are the steps for assembling the autotransporter constructs. Restriction sites are indicated with vertical lines and title. As BsmBI, BsaI, and AarI are Type IIS restriction enzymes, the sticky ends can be different at different recognition sites. Therefore, this figure also shows which sticky end is present at which site. In the first assembly step, Block 1 and Block 2 are assembled as inserts, with pYTK001 serving as the receiving vector. As the BsmBI recognition sites in pYTK001 are TCGG and GACC, the result of this assembly is Intermediate 1, carried on the pYTK001 backbone. In the second assembly step, Intermediate 1 is removed by PCR, then assembled with Block 3 and Block 4. Intermediate 1, Block 3, and Block 4 are the inserts, and pYTK001-234 UTK mRFP DO is the plasmid vector for assembly. The vector contains a constitutively expressing red fluorescent protein (RFP), so successful assembly is denoted by a loss of red fluorescence and a gain of green fluorescence. The assembly is done with a BsaI digest, and the pTYK 001-234 UTK mRFP DO plasmid has BsaI recognition sites of AACG and GCTG, leading to assembly of pAT-GFPdrop Intermediate 2 as shown.

Finally, to insert the peptide library or hydrophobin of interest, a digest of Intermediate 2 is done using AarI, with the recognition sites noted. This replaces

GFP with the library or hydrophobin, leading to the final constructs seen in Fig. 2A.

In Fig 2, Intermediate 1 is removed by PCR from its plasmid backbone by use of the primers InsertSeq-for and YTKseq-rev. In the second assembly step, this PCR product is then assembled with Block 3 (which was obtained by PCR from the plasmid pDSJR and contains the araBAD promoter) and Block 4 (ordered directly from IDT containing the rrnB_T1 terminator) using a BsaI digest, as described in the previous step. Intermediate 1, Block 3, and Block 4 are the inserts, and the vector used is the plasmid pYTK 001-234 UTK mRFP DO, which contains a constitutively expressing mRFP, which drops out upon successful insertion. The result of this second assembly is Intermediate 2, also called pAT-GFPdrop.

To transfer the library from pFES2-library, which enables it to be inserted into pAT-GFPdrop in the third and final assembly step, PCR was conducted using the primers LibOut-For and LibOut-rev. These primers are designed to add AarI restriction sites with appropriate sticky ends. Thermal cycling for the PCR was conducted with 2 min at 95 °C, followed by 35 cycles of 15 s at 95 °C, then 30 s at 50 °C, and then 30 s at 68 °C. After the 35 cycles are concluded, a final extension step is conducted for 5 min at 68 °C and then the sample is held at 4 °C. This PCR product is then cleaned up using a clean-and-concentrator kit, and inserted into pAT-GFPdrop in the third and final assembly step by Golden Gate Assembly using an AarI restriction enzyme. Golden Gate Assembly was performed by mixing 150 ng of library that had been removed by PCR, 100 ng of plasmid vector, 0.25 μL of AarI, 0.25 μL of T7 DNA ligase, 1 μL of 10-mM ATP, 1 μL of AarI enzyme buffer, and molecular-biology-grade water to a final volume of 10 μL in a PCR tube. This same mixture was made in eight identical PCR tubes. The tube was then thermally cycled for 1 min at 37 °C and 1 min at 16 °C, for 35 cycles. There was a final incubation at 37 °C for 15 min, followed by a denaturation step of 15 min at 85 °C. The resultant assembly was cleaned up and concentrated as previously described for inserting the library into pFES.2AB, and transformation was also conducted in a manner identical to pFES.2AB, without rhamnose supplementation in the SOC. The SOC recovery was plated in LB-agar plates supplemented with 30 mg/L of chloramphenicol, and 100- and 1000-fold dilutions were also made for the purposes of colony counting. The lawns on the chloramphenicol plates were then scraped into 500 mL of 2xYT medium supplemented with 30 mg/L of chloramphenicol and allowed to grow overnight at 37 °C. The OD600 was measured, then all cells were spun down at 5000 g's for 10 min. The cells were resuspended in LB medium supplemented with 15% glycerol, such that the concentration of cells in the frozen sample is approximately 1×10^{11} cells/mL, following a protocol established by Sarkes and coworkers.[14]

To insert the hydrophobins, a number of hydrophobin Gblocks were obtained from IDT, containing compatible AarI flanking sites for easy insertion (sequences of relevant hydrophobins and Gblocks are in the supporting information). The insertion of the hydrophobins into pAT-GFPdrop was conducted in a manner identical to the insertion of the library. Then, 0.5 µL of the resulting mixture was electroporated into electrocompetent cells and plated onto LB agar plates supplemented with chloramphenicol as previously described. Successful hydrophobin insertion was indicated by a colorless bacterial colony on the chloramphenicol plates, as the GFP construct should be replaced by the hydrophobin. A handful of colorless colonies were grown overnight at 37 °C in LB medium, miniprepped, and then sent to GENEWIZ for sequencing as previously described.

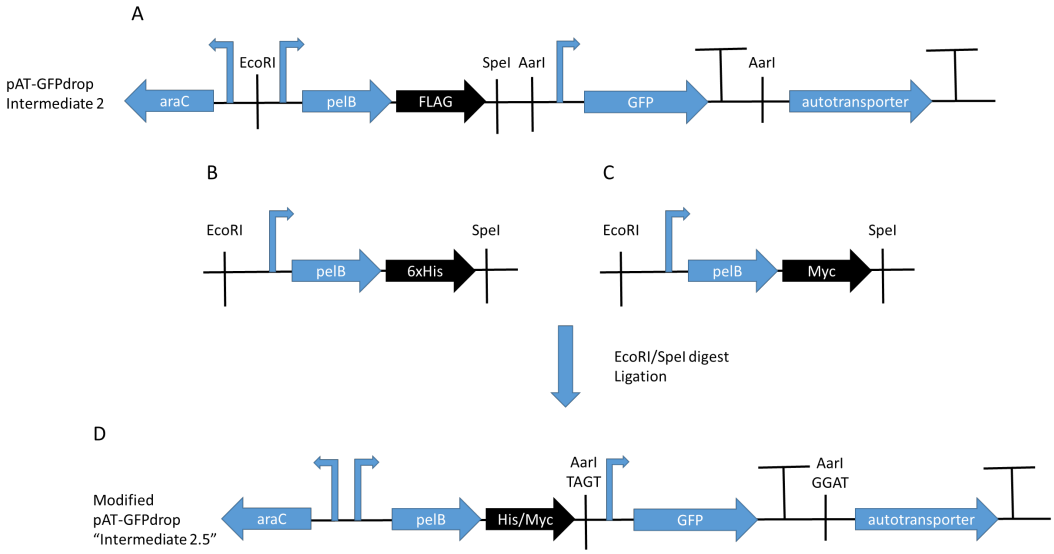

Fig. 2 Replacement of FLAG-tag with 6xHis and Myc tags

To replace the FLAG-tag in pAT-GFPdrop (panel A), we take advantage of conveniently located EcoRI and SpeI restriction sites, as shown. Gblocks MTK-G020 and MTK-G021 (panels B and C) contain the same araBAD promoter, ribosomal binding site, and pelB leader sequence as in Intermediate 2, but replace the FLAG epitope tag with 6xHis and Myc, respectively. A double-digest with EcoRI and SpeI, followed by ligation, results in the construct shown in panel D, where the FLAG-tag is replaced by either the His tag or the Myc tag. The GFP dropout can be removed by AarI digest, and handled the same way as pAT-GFPdrop in Fig. 1 to insert the library or a hydrophobin of interest.

2.4 Cloning Strategy, Replacement of FLAG-Tag by Myc and 6xHis Tags

During the course of experiments (*vide infra*; Section 3.1), it was found that autotransporter-containing constructs with the FLAG-tag did not appear to display. Furthermore, we did not have a known positive control for autotransporter-mediated display of a FLAG-tag. Difficulty displaying autotransporter constructs containing a FLAG-tag had been observed.[15] We therefore elected to switch to a 6xHistidine (6xHis) tag, for which a positive control was already available. We also cloned a construct employing a Myc tag in case this library was to be used to screen for metal-binding peptides, as the 6xHis tag is a known metal binder.[16]

Fortuitously, unique, conveniently located EcoRI and SpeI restriction sites were available upstream and downstream of the FLAG-tag. We therefore ordered EcoRI-HF, SpeI-HF, and calf intestinal phosphatase (CIP) from NEB, along with Gblocks MTK-G020 and MTK-G021 from IDT. The Gblocks contained the 6xHis and Myc tags, respectively, along with the appropriate araBAD promoter and pelB signal sequence that governs surface display. Next, 300 ng of plasmid pAT-GFPdrop Intermediate 2 were mixed with 0.5 µL of EcoRI-HF, 0.5 µL of SpeI-HF, 1 µL of NEB 10x CutSmart buffer, and molecular-biology-grade water to a total volume of 10 µL. Simultaneously, 150 ng of Gblocks MTK-G020 and MTK-G021 were mixed with EcoRI-HF, SpeI-HF, and CutSmart buffer. All three digests were then incubated at 37 °C for 1 h. At 1 h, 1 µL of CIP was added to the Intermediate 1 digest only and incubated for an additional hour at 37 °C. All of the digests were cleaned up with a Qiagen clean-and-concentrator kit according to manufacturer's instructions. Then, 50 ng of digested Intermediate 1 was mixed with 100 ng of digested MTK-G020 and MTK-G021, 0.5 µL of T7 ligase, 1 µL of ligase buffer, and molecular-biology-grade water up to 10-µL total volume. The ligations were then incubated for 1 h at 37 °C and 1 µL of each assembly was electroporated into electrocompetent DH10B as previously described. Then, 50 µL of recovery medium was plated onto LB agar plates supplemented with 30 mg/L of chloramphenicol and incubated overnight. A handful of colonies were selected from each plate, propagated in liquid LB-chloramphenicol, and sequence-verified by GENEWIZ.

To insert the library, or the hydrophobin constructs, Golden Gate Assembly with an AarI digest was conducted as previously described. The final surface display constructs are shown in Fig. 3.

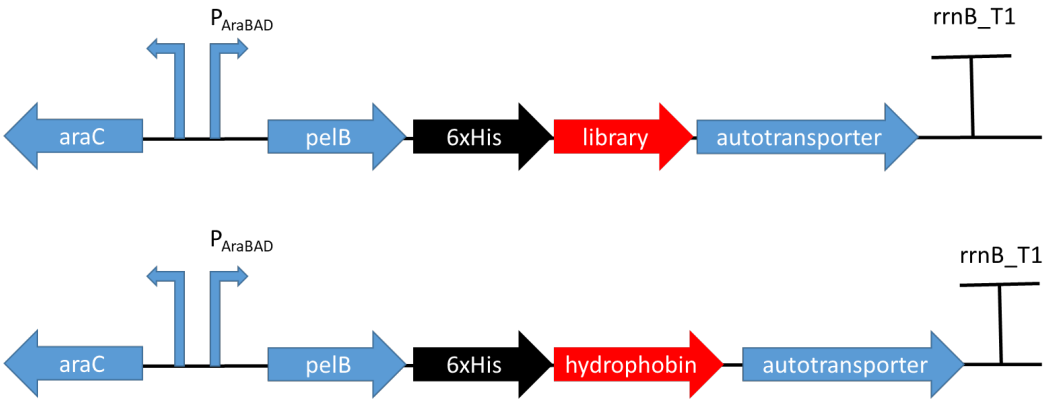

Fig. 3 Final surface-display constructs

Shown are the constructs that result after replacement of the FLAG-tag for a His tag and insertion of the library or appropriate hydrophobin. From left-to-right are the araC regulatory protein for the araBAD promoter system, the promoter for araC, and the promoter ParaBAD, which governs the expression of the construct of interest. The construct itself consists of the pelB secretion tag, which is essential for the display of the library or hydrophobin, the 6xHis epitope tag (HHHHHH), the library, and finally the autotransporter EhaA. The entire ensemble is terminated by the rrnB_T1 terminator.

2.5 Antibody Staining and Flow Cytometry to Verify Surface Display

To verify surface display, the final autotransporter constructs contained a 6xHis epitope tag immediately C-terminal to the pelB leader sequence. Single colonies were inoculated overnight at 37 °C in LB medium supplemented with 30 mg/L of chloramphenicol. The following morning, fresh cultures of LB medium supplemented with chloramphenicol were inoculated with overnight culture at a ratio of 100:1. The samples were allowed to incubate at 37 °C and 250 rpm until they reached an OD600 of approximately 0.5 (about 3 h). A 2-mL aliquot of culture was removed to serve as a control, and the remainder was induced to a final concentration of 0.1% sterile-filtered L-arabinose. Both cultures were incubated for a further 2 h post-induction. Then, 1 mL of each culture was centrifuged at 5000 g's for 5 m, and then washed once with 1 mL of cold phosphate-buffered saline (PBS; Sigma Aldrich) supplemented with 1% by weight of bovine serum albumin (BSA; Fisher Scientific). Of this solution, 50 µL of cells were mixed with 50 µL of PBS-1% BSA containing 40 µg/mL of anti-6xHis antibody conjugated to AlexaFluor 488 dye, making 20 µg/mL of the final antibody concentration used for staining (antibody was obtained from ThermoFisher Scientific). The cells were allowed to stain overnight at 4 °C. The cells were then washed twice with cold PBS.

The samples were then diluted 50-fold and run on a Sony SA3800 spectral cell analyzer (Sony Biotechnology; San Jose, California).

The positive control consisted of an autotransporter system previously reported by Kozlowski and coworkers.[17] The autotransporter was used to display SpyTag, SpyCatcher, and the leucine zippers SynZip17 and SynZip18, and stained in a matter identical to the one described previously. Successful expression of these proteins can be used to drive aggregation of cells displaying SpyTag with those displaying SpyCatcher, and the aggregation of cells expressing SynZip17 with those displaying SynZip18. Such aggregation was observed (data not shown), confirming that surface display had occurred.

2.6 Library Screening

To screen the libraries against polymers of interest, 1 mL of frozen stock was grown in a glass shaker flask at 37 °C at 250 rpm to an OD600 of 0.5 in 50 mL of LB medium supplemented with 30 mg/L of chloramphenicol. Library expression is then induced with a final concentration of 0.1% L-arabinose and allowed to proceed overnight. The following morning, two squares of material approximately 1 cm by 1 cm in size and made of acrylic, polycarbonate, polystyrene, or polypropylene are introduced to the culture. The materials are incubated for 15 min at 37 °C at 225 rpm. The medium is then removed and replaced with 50 mL of PBS supplemented with 1% (v/v) Triton X-100 (SigmaAldrich). The materials are then washed for 30 min at 37 °C at 225 rpm in the PBS-Triton X-100. The materials are removed and then placed in 2 5 mL of LB medium supplemented with 30 mg/L of chloramphenicol as well as 2% D-glucose (to repress the araBAD promoter). This new inoculum is grown overnight at 37 °C. The following day, the resulting culture is centrifuged for 6 min at 5000 g's and then resuspended in 2.5 mL of LB medium supplemented with 25% glycerol. The resulting pellets are frozen in liquid nitrogen and kept at –80 °C for future use.

To test for possible enrichment of polymer-binding peptides, 1 mL of frozen pellet was introduced into 50 mL of LB supplemented with 30 mg/L of chloramphenicol. The culture was allowed to grow at 37 °C and 225 rpm to an OD600 of 0.5, then induced with 0.1% L-arabinose. Expression was allowed to proceed for 2 h and then the samples were stained for flow cytometry analysis as previously described.

2.7 Contact-Angle Measurements of Substrates of Interest

The water contact angles of all materials were measured using a Ramé-Hart Model 290-F4 goniometer, running DROPimage Advanced software (Ramé-Hart; Succasunna, New Jersey). High-performance liquid chromatography (HPLC)-

grade water was used for all experiments (VWR International; Radnor, Pennsylvania). The mean and standard deviation of four spots are reported for both advancing and advanced (static) contact angles.

2.8 Spot Assay

The plasmids used to display the autotransporter were dual-transformed by electroporation with plasmid p15a-AmpR-TU2 (gift of Nathan Schwalm; Adelphi Laboratory Center, Maryland). This plasmid contains a GFP cassette under a constitutive promoter, an ampicillin-resistance cassette, and a p15a origin of replication. This causes the cells to fluoresce in the GFP channel, conveys ampicillin resistance, and the p15a origin is compatible with the ColE1 origin of the surface display plasmid.

To conduct the spot assays, overnight cultures of autotransporter-displayed hydrophobin constructs were inoculated at a ratio of 50:1 into LB medium supplemented with 100 mg/L of ampicillin and 30 mg/L of chloramphenicol. After 3 h, the cells were divided into two batches. One batch had sterile D-glucose added to a concentration of 2% by weight: this served as the control sample. The second batch was induced with 0.1% L-arabinose. The cells were then allowed to express overnight at 37 °C. The following morning, a sheet of polymer obtained from McMaster-Carr (Elmhurst, Illinois) was divided into circled spots (Fig. 4). Next, 100 µL of induced (experimental) or non-induced (negative control) culture was placed in the center of the appropriate marked spot and allowed to sit at room temperature for 1 h. Both control and experimental samples were run in triplicate. The spots were removed by pipetting, then were washed twice, by pipetting 100 µL of PBS onto the spot and removing the PBS, also by pipette. The polymer sheets were then imaged under a GFP channel on an Invitrogen iBright 1500 imager using an exposure time of 20 ms for all materials.

Fig. 4 Spot assay setup

Figure 4 illustrates the setup for spot assays. Locations on large slabs of polymers of interest, such as HDPE, polystyrene, and acrylic, are indicated by permanent marker. The 100 µL of bacterial cells are spotted in these circles and allowed to incubate for 1 h. The bacterial cells either are displaying a hydrophobin (experimental conditions) or contain the hydrophobin construct, but have the expression of this construct repressed (control conditions). In addition, the cells are made fluorescent through the constitutive expression of GFP located on a separate plasmid. After 1 h, the spots are removed and then 100 µL of PBS is used to wash each spot twice. The slabs are then fluorescently imaged on an iBright 1500 imager.

2.9 Attachments

Plasmid maps and Gblocks are available as SnapGene files attached to this report. An Appendix is also included, containing a glossary and description of the MoClo cloning system.

3. Results and Discussion

An illustration of the successful display of a protein of interest by the autotransporter is shown in Fig. 5. In brief, a 6xHis epitope tag is on the very N-terminus of the displayed protein. The protein of interest, or peptide library, are located C-terminal to the 6xHis tag. Finally, the C-terminal end of the protein is the autotransporter transmembrane protein, which anchors the epitope tag and protein of interest to the outside surface of the bacterial outer membrane. The 6xHis tag can be recognized by an Anti-His antibody, which is conjugated to the fluorescent

dye AlexaFluor 488. Therefore, if the protein of interest is present on the surface of the cell, staining with the anti-His antibody will cause that cell to be fluorescent. The number of fluorescent cells in a population can be determined using flow cytometry.

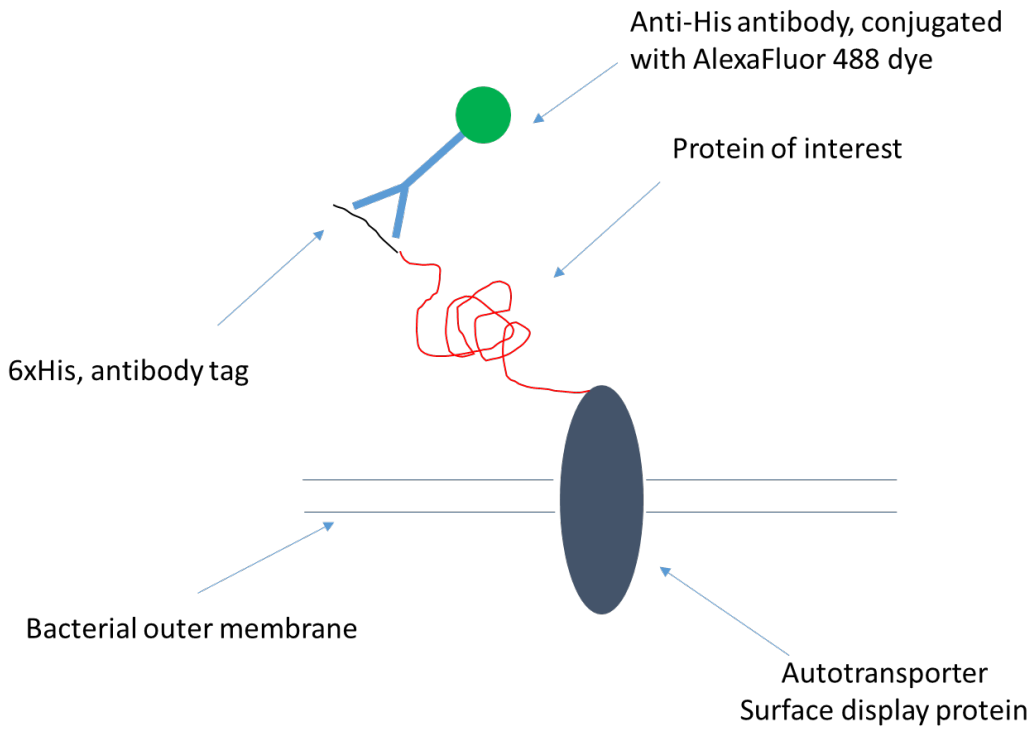

Fig. 5 Illustration of successful surface display, observable by flow cytometry

This is a schematic view of the successful display of a protein by the autotransporter system. The 6xHis antibody tag on the N-terminal end of the protein should be recognized by an Anti-His antibody, which is conjugated to a fluorescent dye. Therefore, a cell displaying the protein of interest should be fluorescent in the AlexaFluor 488 channel.

3.1 FLAG-Tagged Constructs Did Not Express Well

Initially, the autotransporter constructs were intended to have a FLAG epitope tag (sequence: DYKDDDDK), as the FLAG-tag is less active against metals than the 6xHis tag. Several hydrophobins were cloned into the autotransporter system under a FLAG-tag, along with a positive control consisting of a short FLAG peptide. When expression of these constructs were nominally induced, stained, and subjected to flow cytometry analysis, the resulting fluorescence observed in the population was no greater than the autofluorescence of control cells that did not contain any surface-display construct whatsoever (Fig. 6). As the putative positive

control also did not work, we could not be sure if the lack of display was caused by poor antibody, an error with the flow cytometer, or the inability of the cells to actually display a construct with a FLAG-tag. Based on these results, we elected to switch the FLAG-tag for a 6xHis tag as previously described, as we did have access to previously cloned positive controls with a 6xHis tag. All subsequent flow cytometry studies in this report were done with constructs containing the 6xHis tag, and staining is seen in these cases.

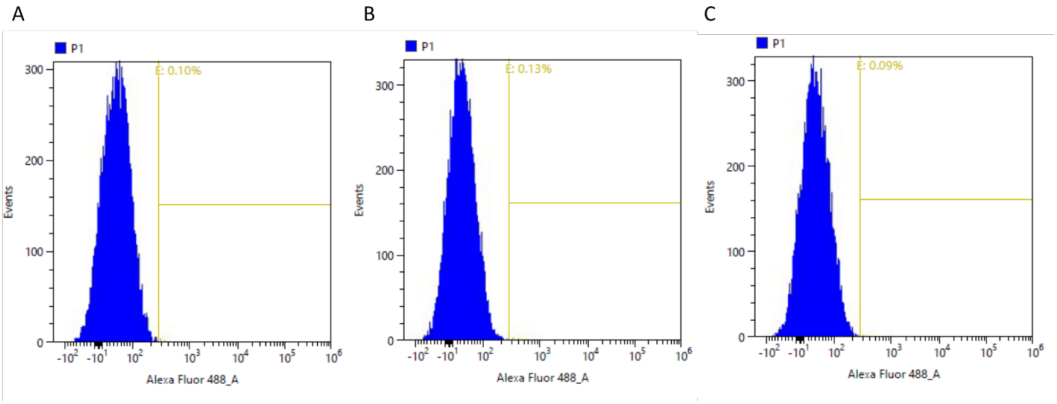

Fig. 6 Flow cytometry of select FLAG-tagged constructs

Flow cytometry indicates that constructs with a FLAG-tag appeared not to surface display. In all graphs in Fig. 6, the x-axis represents the fluorescent signal from AlexaFluor 488 in arbitrary units, and the y-axis represents a count of cells demonstrating a particular fluorescent signal. A vertical gate is drawn to exclude the entire population of cells in the negative control. Figure 6A is a negative control, showing ordinary DH10B cells, without any construct, that had anti-FLAG AlexaFluor 488 antibody added and then washed. Figure 6B shows an EAS hydrophobin autotransporter construct with FLAG-tag whose expression had been induced. Figure 6C shows the putative positive control, an autotransporter construct with FLAG-tag only, with expression induced. The populations of all three experiments show effectively the same level of fluorescence and leads us to conclude that surface display of FLAG-tagged constructs is not observed.

3.2 Using a 6xHis Tag, Surface Display of Library is Observed and Screening against Polymers Leads to Enrichment of Displaying Fraction

To see if any peptides in the displayed library actually bound to our materials of interest, we conducted flow cytometry at two points: first, the initial library and second, after one round of screening. The initial peptide library only had a small percentage of cells (~7%) that appeared to display a peptide, which could be due to

various reasons such as toxicity of certain peptides in the library, rare codons, stop codons, and so on. If none of the peptides displayed actually adhered to the screened materials, we would expect the bacteria cultivated after the first screen to have roughly the same fraction of cells displaying a peptide. However, if some peptides in the library did adhere to the tested materials of acrylic, polycarbonate, polystyrene, and polypropylene, we would expect the fraction of cells that display those peptides to be enriched after screening, and consequently, we would expect to see a higher percentage of cells undergoing surface display. In Fig. 7, after one round of screening, the population of displaying cells is larger than that of the initial library, and we therefore conclude that at least some peptides in the library promote adhesion to the materials of interest.

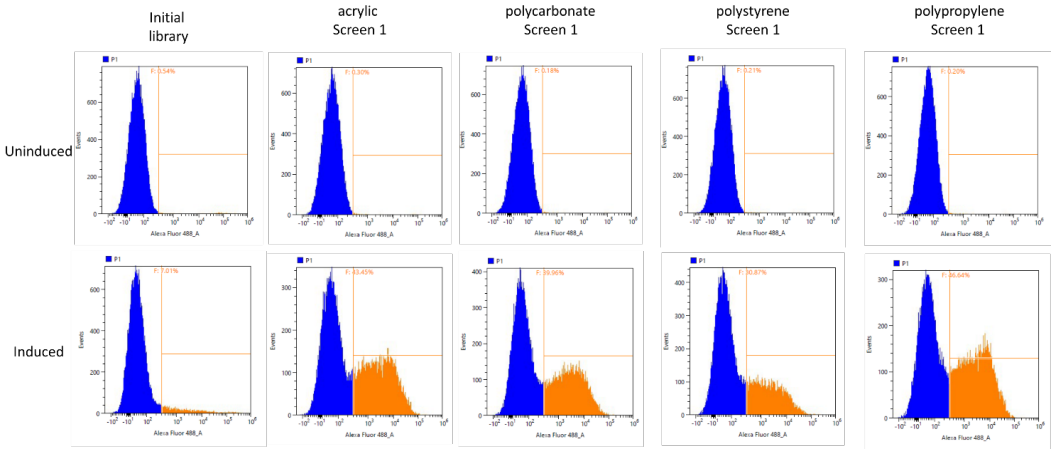

Fig. 7 Screening enriches fraction of displaying cells

The peptide library is cloned in to the autotransporter display system. The x-axes of these graphs show the fluorescence intensity of AlexaFluor 488 (the dye on the conjugated antibody), and the y-axes show the number of cells observed at a particular fluorescence intensity. The top row of graphs shows uninduced controls, and the bottom row of graphs shows induced experimental samples. A gate is drawn as shown to exclude all cells in uninduced samples. Cells with a fluorescence below the gate threshold are shown in blue, and those with a fluorescence above the gate threshold are shown in orange. In the initial library, only 7% of cells appear to display a peptide on their surface, as seen in the first column (“initial library”). However, after one round of screening, the number of cells that display a peptide upon induction increase, to 43% in the case of a screen against acrylic, 40% in the case of a screen against polycarbonate, 31% in the case of a screen against polystyrene, and 46% in the case of polypropylene. This enrichment in the displaying fraction suggests the screening favors the population of cells that actually do display in the initial library, and therefore that some of the displayed peptides in the initial library do in fact bind to these polymers of interest. Efforts at

sequencing this enriched fraction are ongoing, to determine which peptides may promote adhesion.

We note the diversity of the library, containing 10^5 members, is relatively small, and most libraries of this type aim for a diversity closer to 10^8 or 10^9. We are somewhat puzzled as to why our library diversity is small: the competence of the cells into which the library is transformed is approximately 10^9 colonies/μg when measured using a control pUC19 plasmid, and we are introducing approximately 1 μg of library DNA into the cells. The time constants of electroporation are low (3.5 ms) when the libraries are introduced, suggesting the presence of salts or other contaminants. Additional washes, or use of alternate cleanup-and-concentrator kits, may be required. Further troubleshooting is necessary to increase the diversity of the library.

3.3 Surface Display of Hydrophobins Is Strain-Dependent

The display of the library was conducted in *E. coli* strain DH10B, which had previously been used by Kozlowski and coworkers to display SpyTag, SpyCatcher, and leucine zippers.[17] As DH10B was used for cloning, it was convenient to attempt to display the constructs in the same strain, as that removed the necessity of an additional transformation step. However, we found this strain did not support surface expression of hydrophobins (Fig. 8). We then decided to attempt surface display in *E. coli* strain BL21, which is a protein-expression strain that is deficient in the proteases Lon and ompT.[18] The deficiency of ompT is particularly important, as this is a membrane protease that may digest membrane proteins such as autotransporter.[19] Therefore, hydrophobin constructs were electroporated into competent BL21 cells, and induced and stained as previously described. The result, as seen in Fig. 9, is that some fraction of the cells are observed to display hydrophobin on the surface. Encouraged by this result, we then proceeded to use BL21 for all subsequent surface-display experiments involving hydrophobins.

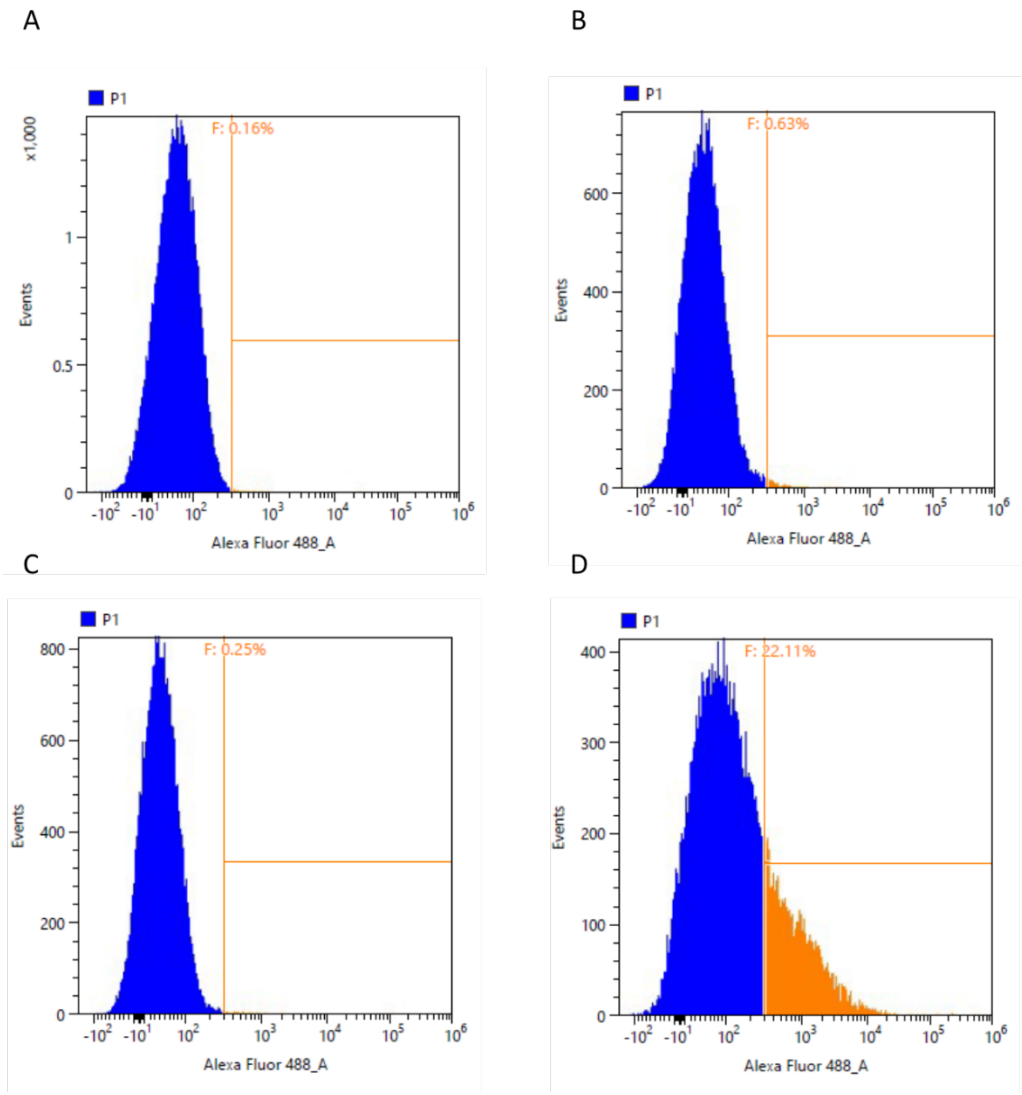

Fig. 8 Hydrophobin expression did not proceed in DH10B, but appears to work in BL21

In Fig. 8A, hydrophobin HfbII is present in *E. coli* DH10B and not induced. In Fig. 8C, expression of HfbII is induced by the addition of 0.1% L-arabinose. However, no difference in surface fluorescence can be observed between the uninduced and induced samples, implying that surface display of the construct is not observed. Other hydrophobins attempted showed a similar lack of expression in DH10B (data not shown). The same construct was then electroporated into *E. coli* strain BL21. The uninduced HfbII construct is shown in Fig. 8B. After induction with 0.1% L-arabinose, a population of fluorescent cells can be observed (Fig. 9D), implying that HfbII does express on a population of BL21 cells: approximately 22% of induced BL21 cells show the HfbII hydrophobin on the surface. Also of note, BL21 cells that are not induced have a small (0.6%) population of cells that appear to show HfbII on the surface, suggesting that

expression of this construct is somewhat leaky. These images are broadly representative of what was observed with hydrophobins other than HfbII, namely, non-expression in DH10B and some degree of expression in BL21.

3.4 Vmh2 Appears to Promote Bacterial Adhesion to Polystyrene, as Do Sc3 and DEW

An important positive control in our spot assays was to see if the work of Sorrentino and coworkers could be duplicated.[9] In particular, we wanted to observe whether the display of hydrophobin Vmh2 could induce bacterial adhesion to polystyrene. Two different versions of Vmh2 appear in the UniProt database, the first version, Vmh2-1 being the one used by Sorrentino and coworkers (UniProt: Q8WZI2_PLEOS)[9] and the second, Vmh2-2, being from the work of Peñas and coworkers (UniProt: Q8WZI1_PLEOS).[20] Both versions were spot-tested on a polystyrene surface, as shown in Fig. 9. Spots of cells that had hydrophobin expression induced are still fluorescent after washing. However, the corresponding spots of control samples, consisting of cells whose hydrophobin expression was catabolically repressed, are much less fluorescent. Therefore, presence of the hydrophobin on the cell surface appears to be correlated to cellular adhesion to a polystyrene surface, and it appears to be the case that surface display of the appropriate hydrophobin can promote adhesion of bacterial cells to a tested substrate.

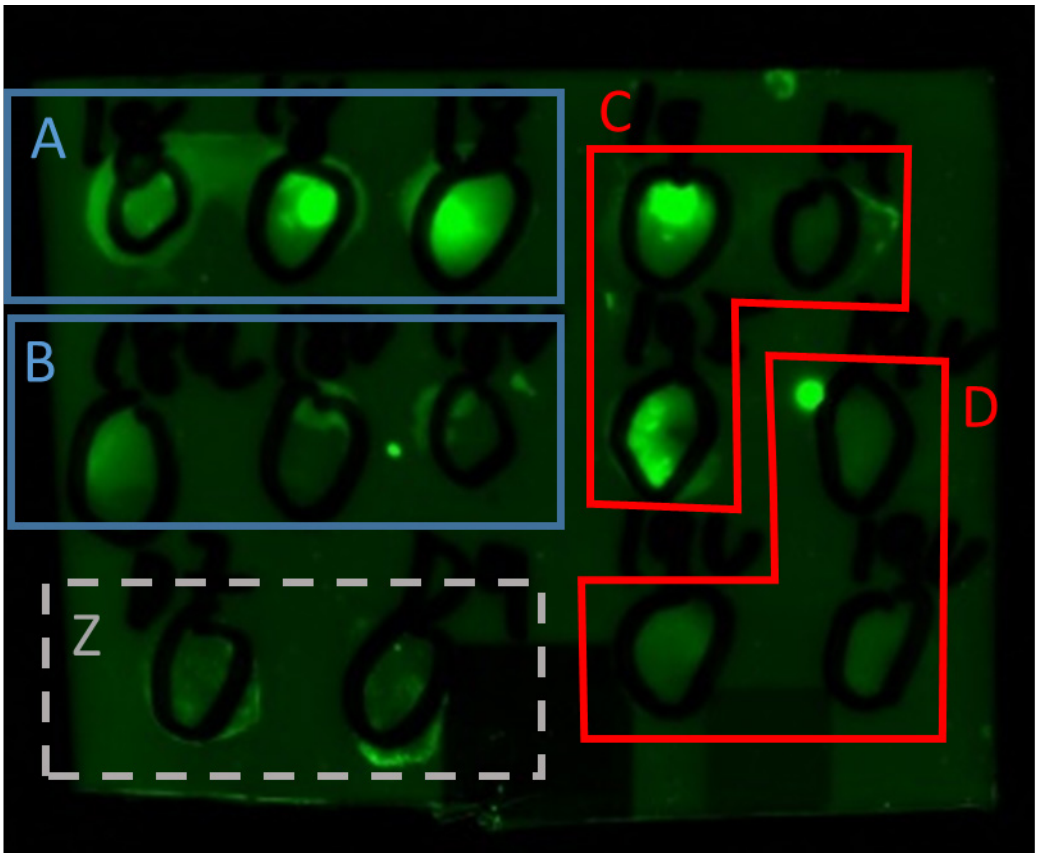

Fig. 9 Spot assay of Vmh2 on polystyrene. Fluorescent cells containing the plasmid for expression of Vmh2-1 are in locations A and B, with A being where expression was induced and B being the control uninduced/catabolically repressed sample. Similarly, cells containing the Vmh2-2 construct are in locations C and D, with C being the induced sample in C and D being the uninduced sample. The cells sat on the polystyrene surface for 1 h and then each spot was washed twice with PBS. Note the bright spots in locations A and C, and the correspondingly much dimmer spots in locations B and D, suggesting the fluorescent cells expressing Vmh2-1 and Vmh2-2 are more adhesive to the polymer than the corresponding controls, which are more easily washed off by PBS. Note also the gray dashed-line box Z, which shows spots where two peptides not known to bind to polymers were surface displayed. Exposure time was 250 ms.

Further tests were run with other hydrophobins on polystyrene, as seen in Fig. 10. Notably, the hydrophobins Sc3 and DEW appear to have promoted bacterial adhesion to the polystyrene, as bright spots are left behind in the experimental samples after washing, whereas the corresponding controls do not show such bright spots. In the NC2 hydrophobin samples; however, there is not much difference between the experimental and control spots. Also of note, the hydrophobin HfbII did not appear to promote adhesion and, curiously, in this experiment, Vmh2-2 did not appear to adhere very well either, as while some fluorescence can be observed, it is very dim in contrast to the results in Fig. 9. We note that Fig. 9 was taken with a slightly longer exposure time, as can be seen by the higher background

fluorescence of the material itself. These results suggest that Sc3 and DEW may actually be better adhesive partners for polystyrene than Vmh2-1, though this would require further experimentation and better quantitation.

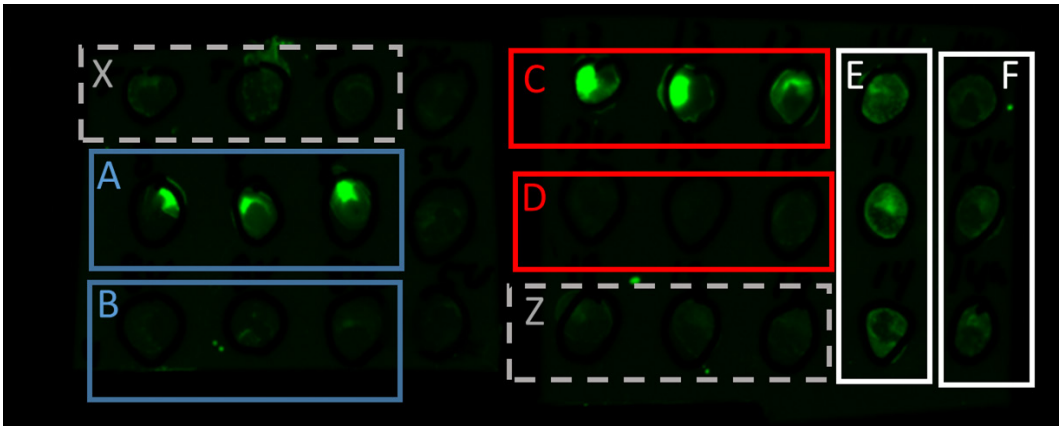

Fig. 10 Spot assay of various hydrophobins on polystyrene

The hydrophobin Sc3 was induced and spotted in box A, whereas the corresponding uninduced/catabolically repressed sample is seen in box B. The hydrophobin DEW is induced in box C and uninduced in box D. As can be seen, the spot with induced cells gives a fluorescent signal, whereas the corresponding control does not. The hydrophobin NC2, when induced, was placed in box E and the corresponding uninduced sample is placed in box F. No particular difference between induced and uninduced sample can be seen here, and in fact, the washing may have been insufficient as the fluorescence from the control is still visible. The two dashed boxes correspond to induced samples of HfbII (top-left, labeled box X) and Vmh2-2 (bottom-center, labeled box Z). Surprisingly, the Vmh2-1 induced sample appears to have a very dim fluorescent spot. The exposure time for this image was 20 ms, shorter than that of Fig. 9, which may explain some of this discrepancy and would suggest that Sc3 and DEW better promote cell binding to polystyrene.

3.5 Hydrophobins Sc3, DEW, and NC2 Appear to Promote Bacterial Adhesion to HDPE and Polyester

For spot assays, two different types of HDPE were used, one obtained from McMaster-Carr and one scrap of material that was available in the Polymers Branch. In both cases, following washing, a fluorescent spot can be observed in a spot where Sc3, DEW, and NC2 hydrophobin display was induced, but not in the corresponding control spot where the sample was not induced. Therefore, the hydrophobins Sc3, DEW, and NC2 appear to be plausible candidates for promoting bacterial adhesion to HDPE (Figs. 11 and 12).

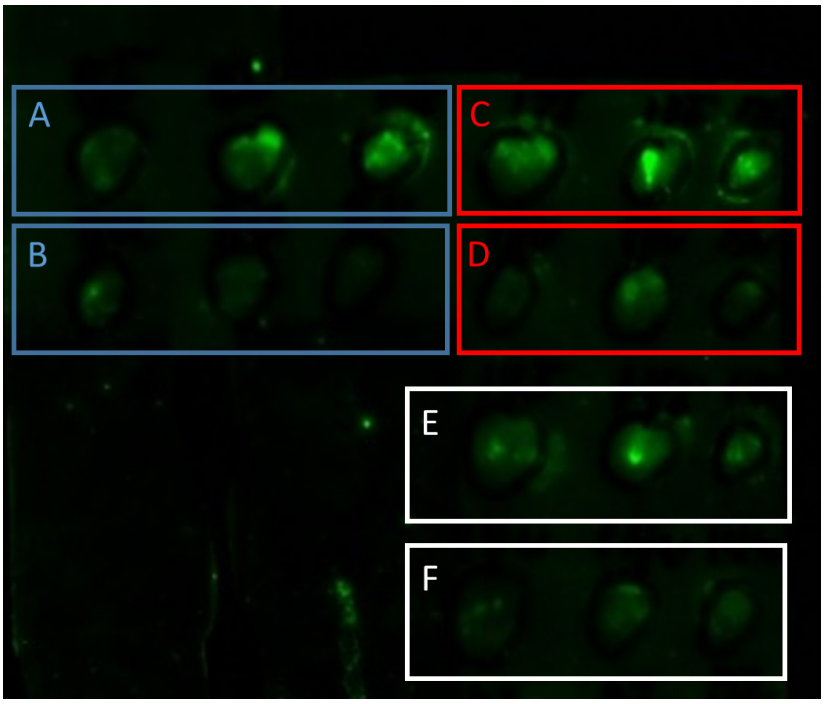

Fig. 11 Spot assay on HDPE from the Polymers Branch

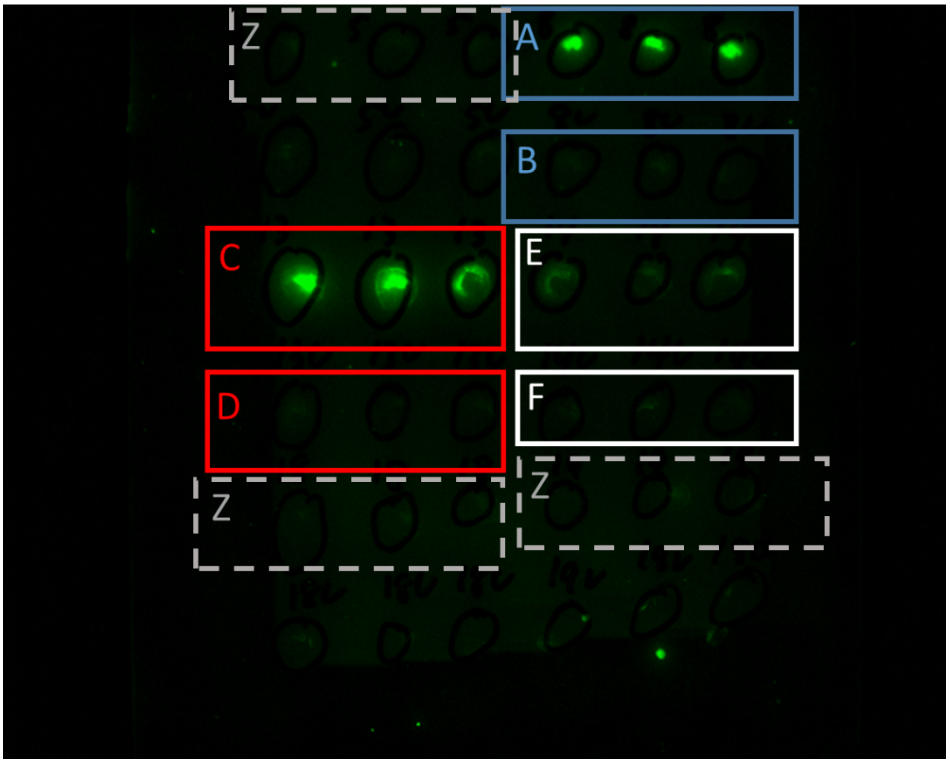

Fig. 12 Spot assay on HDPE from McMaster-Carr

On the piece of HDPE from the Polymers Branch, we spotted cells containing the surface-display construct for Sc3 (blue boxes, A and B), DEW (red boxes, C and D), and NC2 (white boxes, E and F). Each spot was done in triplicate. Expression of the construct was induced for the top row of spots (boxes A, C, and E) and catabolically repressed for the bottom row of spots (boxes B, D, and F). Boxes A, C, and E are therefore experimental samples, and boxes B, D, and F are their corresponding controls. After washing, we noted that bright spots appear to persist in the induced Sc3, and they do not persist when this expression is repressed (box B). Similarly, expression of DEW appears to lead to spots remaining (box C), whereas repression of DEW causes the spots to be removed by washing (box D). There is not a particularly large contrast between induced and repressed NC2 (boxes E and F). Based on these results, we believe Sc3 and DEW may be promoting bacterial adhesion to HDPE.

On the other piece of HDPE, we spotted cells containing the surface-display construct for Sc3 (blue boxes, A and B), DEW (red boxes, C and D), and NC2 (white boxes, E and F). Expression of the construct was induced for the top row of spots (boxes A, C, and E) and catabolically repressed for the bottom row of spots (boxes B, D, and F). Boxes A, C, and E are therefore experimental samples, and boxes B, D, and F are their corresponding controls. After washing, we noted that bright spots appear to persist in the induced Sc3, and they do not persist when this expression is repressed (box B). Similarly, expression of DEW appears to lead to spots remaining (box C), whereas repression of DEW causes the spots to be removed by washing (box D). There is some difference in the brightness of spots where NC2 was induced (box E) compared to where NC2 expression was catabolically repressed (box F), meaning this may be a plausible candidate hydrophobin worth further exploration. The dashed boxes in Fig. 12 (labeled boxes Z) indicate locations where cells were placed while expressing HfbII, Vmh2-1, and Vmh2-2, and no fluorescent signal can be seen, suggesting that expressing any hydrophobin construct is not sufficient to enhance bacterial adhesion (i.e., it is not the expression of the autotransporter protein causing the observed differences elsewhere).

Similar results are observed on polyester obtained from McMaster-Carr, as shown in Fig. 13, where spots composed of cells expressing Sc3, DEW, and NC2 are more fluorescent than their corresponding controls, though the effect of NC2 expression is the weakest of the three. Also notable is that three other surface-displaying hydrophobins do not appear to promote adhesion to this polymer. This is important to note as high levels of surface-protein expression can lead cells to become nonspecifically sticky, regardless of the protein displayed. This nonspecific stickiness does not appear to be a major factor in these experiments. Once again,

Sc3 and DEW appear to be good candidates for promoting binding to this polymer. NC2, however, does not appear to be a good candidate.

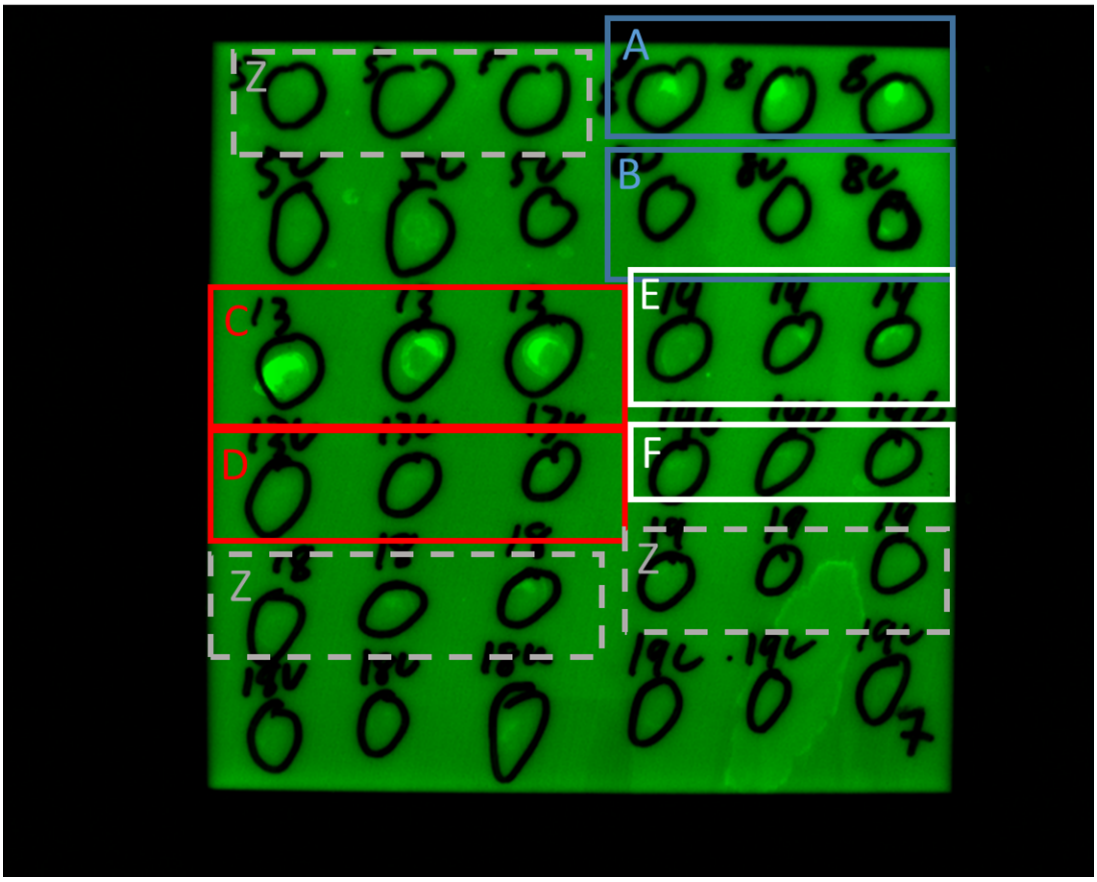

Fig. 13 Spot assay on polyester

Cells expressing Sc3 are shown in box A, whereas the corresponding uninduced control is shown in box B. Cells expressing DEW are shown in box C, whereas the corresponding uninduced control is shown in box D. Cells expressing NC2 are shown in box E, whereas the corresponding uninduced control is shown in box F. Box E shows very faint spots, suggesting that the binding activity of NC2 is weaker than that of Sc3 and DEW. The gray dashed boxes correspond to cells that are expressing HfbII, Vmh2-1, and Vmh2-2: note that fluorescence is either very faint, or not observed, in these gray dashed-line boxes (boxes labeled Z), suggesting that not all hydrophobins tested are capable of binding to polyester and expression of the autotransporter construct does not necessarily lead to bacterial adhesion. Note, also, the considerable fluorescence of the polyester itself and the fluorescent "splash" near the spot labeled 19U. This splash corresponded to a region of the polymer that, when spotted, appeared to be wetting, in contrast to the rest of the sample.

3.6 Hydrophobins and Acrylic: Possible Limitations or Need for Modification of Spot-Assay Approach for More-Hydrophilic Surfaces

The least-hydrophobic polymer we tested was acrylic, with a static contact angle of 73.2° (Table 1). We hypothesized that hydrophobins would have the lowest activity for acrylic because the proposed mechanism for hydrophobin binding is the interaction of a hydrophobic patch on the protein with a hydrophobic surface, specifically with a hydrophilic portion of the protein facing the surrounding solvent.[5] As acrylic was the least-hydrophobic surface measured, we expected this type of assembly to be weakest on such a surface, and consequently, expected to see a weak interaction between the hydrophobins and acrylic. To our surprise, DEW appears to enhance binding of the bacterial cells to acrylic fairly strongly, whereas the evidence of Sc3 and NC2 binding is more ambiguous. However, in contrast to polystyrene, HDPE, and polyester substrates, control spots throughout the panel appear to be dimly fluorescent, suggesting that the wash conditions used did not fully remove cells that did not display a surface construct, and therefore, more stringent wash conditions are likely required for acrylic.

Shown in Table 1 are the means and standard deviations of four different spots for advancing contact angle and advanced contact angle. All measurements are reported in degrees. Polyester was the most hydrophobic polymer studied, and acrylic was the least.

Table 1 Contact angles of tested polymers

Polymer	Advancing mean	Advancing SD	Advancing mean	Advancing SD
Acrylic	74.2	2.2	73.2	2.2
Polystyrene	105.1	3.3	101.8	1.5
Polyester	118.2	5.6	114.8	4.3
HDPE	97.4	4.5	90.8	3.7

In Fig. 14, cells expressing Sc3 are shown in box A, whereas the corresponding uninduced control is shown in box B. Cells expressing DEW are shown in box C, whereas the corresponding uninduced control is shown in box D. Cells expressing NC2 are shown in box E, whereas the corresponding uninduced control is shown in box F. The gray dashed boxes (boxes Z) correspond to cells that are expressing HfbII, Vmh2-1, and Vmh2-2 (top row of the box) or the corresponding controls (bottom row of the box). While the evidence for DEW promoting adhesion to acrylic is compelling, the other spots are very faint. Furthermore, the spots of control samples also show a faint fluorescence, suggesting that the wash conditions

used were not stringent enough to remove cells that were not displaying anything on the surface.

We hypothesize that harsher conditions are needed for more hydrophilic surfaces, and additional evidence for this hypothesis can be found in the image presented for polyester (Fig. 13). In this figure, there is a large, dimly fluorescent "splash" that can be observed near the spots labeled 19 and 19U. When spot 19U (Vmh2-2, uninduced) was placed in this location, we observed surface wetting, in contrast to the high-contact-angle spots that were observed elsewhere. Despite doing four washes with PBS of this spread-out spot, we were not able to entirely eliminate the splash that can be observed in the fluorescent channel.

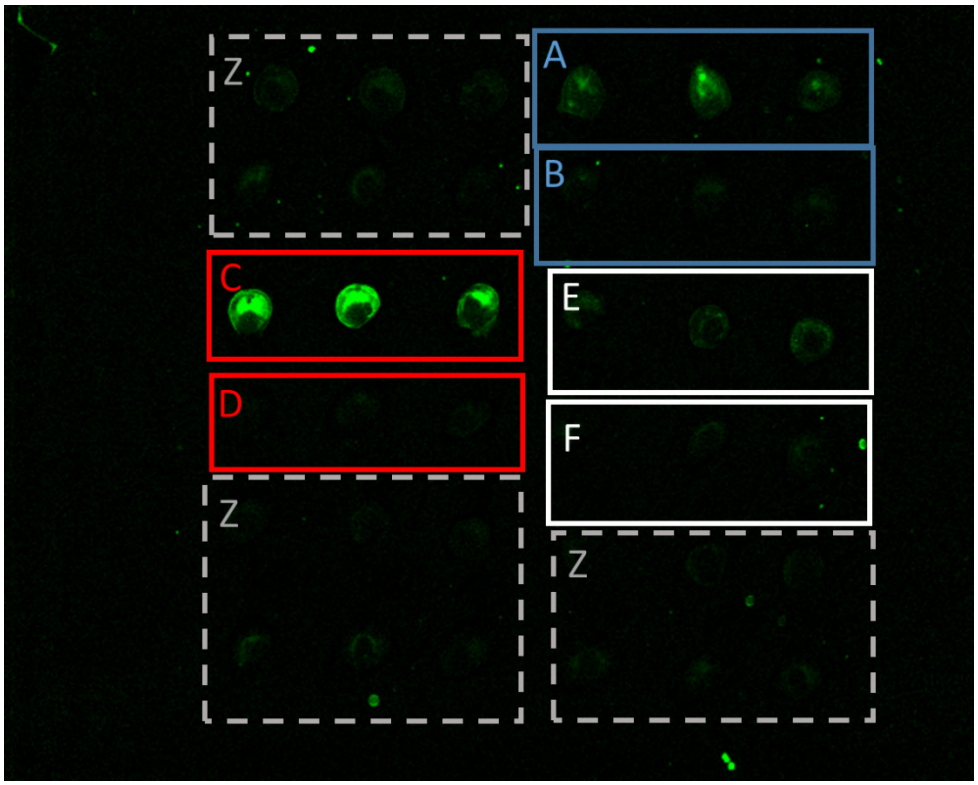

Fig. 14 Spot tests against acrylic may need additional washing

3.7 Comparing Surface Display of HfbII, Sc3, DEW, and NC2

It may be argued that the adhesive hydrophobins are simply the ones that were most expressed on the cell surface. Here, we discuss Fig. 15, which is a comparison of the flow cytometry results for HfbII (which did not bind to our substrates of interest), and Sc3, DEW, and NC2, which did. Note it is actually Sc3 that shows the least evidence of surface display, with hardly any cells exceeding the gate drawn in the figure. There is a very small shift in the peak of the fluorescence, however, that suggests some surface display did in fact take place. HfbII shows the next-

smallest population of expressing cells, at approximately 22%, and DEW and NC2 both have approximately 40% of cells that appear to be expressing. Based on the shape of the population, however, it appears to be the case that NC2 expressed better, as the curve of the induced sample has more events closer to 10^4 arbitrary units than does DEW.

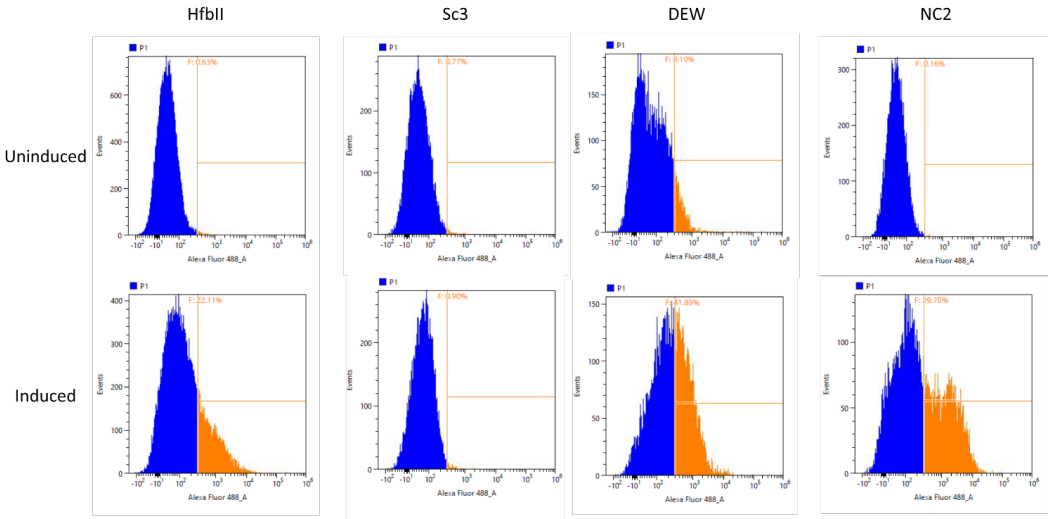

Fig. 15 Comparing surface display of HfbII, Sc3, DEW, and NC2

It may be the case that HfbII simply expresses too poorly to be seen adhering to the polymer substrates of interest. However, it should be noted that in the spot assays presented earlier, DEW appeared to facilitate better binding to the polymer surface than NC2, despite NC2 having an equal or better level of surface display.

The flow cytometry results for uninduced and induced autotransporter-hydrophobin constructs are as-marked previously. Based on using the same gating for all samples, in HfbII, approximately 22% of cells show evidence of surface display; in Sc3, 1% of cells show some evidence of surface display; in DEW, 41% of cells show evidence of surface display and there is additional evidence of leaky expression; and in NC2, approximately 40% of cells show evidence of surface display.

Hydrophobin Sc3 is somewhat puzzling. Figure 16 would suggest that no surface expression is observed. However, there is a slight upward shift in the fluorescence of the experimental sample, and the average cell in the induced sample is also more fluorescent, suggesting that surface expression of this hydrophobin does occur, but is very slight.

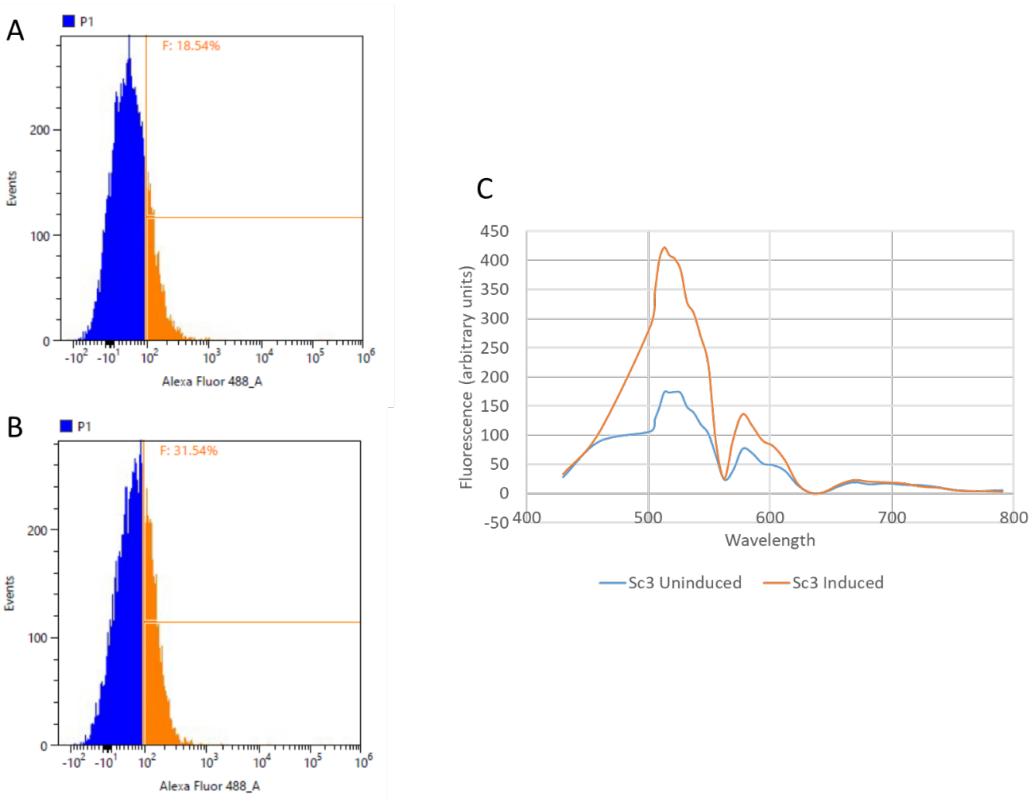

Fig. 16 Evidence for surface display of Sc3

If the gates from the flow cytometry are drawn arbitrarily at 10^2, we find that the percentage of cells that exceed this arbitrary gating is higher when Sc3 expression is induced (panel B) as opposed to uninduced (panel A). Note also the peak has shifted slightly to the right (higher fluorescence). When doing an overlay of overall fluorescence (panel C), we find that the induced sample does have higher average fluorescence than the uninduced sample, in the wavelengths corresponding to AlexaFluor 488. We therefore conclude that some Sc3 is being expressed on the surface of the cells, but that such expression is weak.

Despite relatively low levels of surface expression, Sc3 promoted good adhesion in the spot assays, suggesting that this may be a strong candidate for further research. However, the surface display of this hydrophobin should be optimized, or perhaps studied using a different surface-display system.

3.8 Limitations of Study and Future Improvements

While these initial results appear promising, we urge caution in interpretation of these spot-assay results. First, we note the washing conditions used were not especially rigorous, as the PBS was not supplemented with detergent, and the PBS was only pipetted onto and off of the spots rather than being extensively agitated.

While these wash conditions appear to be sufficient to remove the control spots in materials other than acrylic, the mildness of the conditions means that hydrophobin adhesion may not be especially strong. Indeed, when a large amount of water is poured over the samples, no fluorescent spots are observed (data not shown).

These studies also do not allow us to draw strong conclusions on the relative performance of the hydrophobins, as the surface display of these hydrophobin constructs has not been optimized and a considerable proportion of the induced cells do not appear to display the construct. Spot assays to date have not been normalized for the number of cells displaying the hydrophobin of interest (i.e., there may simply be more potentially adhesive cells present in Sc3 or DEW experimental samples than in Vmh2 samples). However, while the method shown here is not quantitative, it does show that certain hydrophobins appear to be good candidates for further study for promoting adhesion of bacterial cells to hydrophobic polymers.

One major limitation of the spot-assay method is that a number of the polymer materials are fluorescent at the wavelengths being observed. In particular, a nylon slab obtained from McMaster-Carr was too fluorescent to distinguish the presence of bacterial cells from the background fluorescence (data not shown). As can be seen in Fig. 13, polyester also shows considerable fluorescence. This will make quantification difficult and also imposes a limit of detection as setting too long an exposure time on the camera results in a massive fluorescence signal from the material. The substrates used in this study were all off-the-shelf, commercially available samples used without modification and, so far, no attempt has been made to quantify or modify surface roughness. Surface roughness no doubt plays a role in the ability of the bacteria to adhere, and this variable may be a fruitful topic of further study.

These polymer coupons should be observed under a confocal fluorescent microscope with laser excitation to see if a layer of cells can be seen on the polymer surface and provide a semi-quantitative measure of adhesion. Such an attempt was made, but the working distance on the inverted confocal microscope used was too long and the sample blocks are too large to be mounted on the microscope stage. The samples must be cut down to an appropriate size, roughly that of a 96-well plate, before they can be mounted on a microscope stage. This approach is not high-throughput, however, and is probably most useful once promising candidates have been identified through the sorts of spot tests that have been run to date.

Finally, even after normalization of the number of hydrophobin-expressing cells, these results are not quantitative and must be made so if directed evolution or other methods of protein engineering are to be used to improve adhesive performance. We may wish to take advantage of microfluidic methods, where the flow rate of a

wash solution can be controlled and, consequently, so can the shear stresses on an individual adhered cell. Being able to remain adhered at higher shear stresses may give a semi-quantitative measure of the performance of the individual hydrophobin.

Future studies might include examination of a wider range of hydrophobins in addition to the six shown here and a more systematic study as to whether Class I or Class II hydrophobins are better suited to potentially serving as adhesives. We may also wish to examine materials with a wider range of contact angles to determine the minimum contact angle at which adhesion can be observed. Structural studies of each hydrophobin may give insight as to how it adheres to a surface, and an alanine scan of the hydrophobin may help determine what structural elements are critical for adhesion to occur.

4. Conclusion

In this study, we successfully cloned a peptide library into the autotransporter surface-display system, as well as a family of fungal-derived hydrophobins. We demonstrated that the library, and the hydrophobins, can be surface-displayed in *E. coli*. We further demonstrated that screening the peptide library against acrylic, polycarbonate, polystyrene, and polypropylene leads to enrichment of a peptide-displaying fraction, which we interpret to mean that the library contains peptides that bound to these polymers of interest. Finally, we showed that displaying the hydrophobins Vmh2, Sc3, DEW, and NC2 enhances the adhesion of bacterial cells to polymers such as polystyrene, HDPE, polyester, and acrylic. In so doing, we have identified proteins that may be promising for future use as biologically inspired polymer adhesives.

5. References

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Appendix. Supporting Information

Table A-1 Sequences of hydrophobins used in this work

Hydrophobin	Species of origin	Class	Protein sequence	UniProt accession number
HfbII	*Trichoderma reesei*	II	AVCPTGLFSNPLCCATNVLD LIGVDCKTPTIAVDTGAIFQ AHCASKGSKPLCCVAPVADQ ALLCQKAIGTF	P79073
Sc3	*Schizophyllum commune*	I	MFARLPVVFLYAFVAFGALV AALPGGHPGTCTTGSLSCCN QVQSASSSPVTALLGLLGIV LSDLNVLVGISCSPLTVIGV GGSGCSAQTVCCENTQFNGLINIGCT PINIL	P16933
DEW	*Aspergillus nidulans*	I	LPASAAKNAKLATSAAFAKQ AEGTTCNVGSIACCNSPAET NNDSLLSGLLGAGLLNGLSG NTGSACAKASLIDQLGLLAL VDHTEEGPVCKNIVACCPEG TTNCVAVDNAGAGTKAE	P52750
NC2	*Neurospora crassa*	II	APAAMERQVPYTPCSGLYGT AQCCATDVLGVADLDCANPP ATLANATHFESTCAAIGQRA RCCVLPILGQDILCQTPAGL	Q7S3P5
Vmh2 version 1	*Pleurotus ostreatus*	I	IPRTDTPSCSTGSLQCCSSV QKATDPLASLLIGLLGIVLG PLDLLVGVTCSPITVIGVGG TSCTQQTVCCTGNSFNGLIA IGCSPINISL	Q8WZI2
Vmh2 version 2	*Pleurotus ostreatus*	I	IPRTDTPSCSTGSLQCCSSV QKASDPLVGIIVALLGIVLG PLDLNVGLTCSPITVIGVGG TSCTQQTVCCTGNNFNGLIV AGCSPINIGL	Q8WZI1

Table A-2 Primers used in this work

Primer	Sequence	Purpose
pQE for	Agcggataacaatttcacacag	Amplification of hydrophobin Gblocks
pQE rev	Ttctgaggtcattactggatc	Amplification of hydrophobin Gblocks
LibraryOut-for	GGTGATGGATTGTCTGACAGG	Remove library from pFES.2AB
LibraryOut-rev	GTTTCACGAGTACCGATATTGTCC	Remove library from pFES.2AB
MTK-p1001F	CGCGTCTCTGTAGTTTTTGCAGGTGGGAAAGTGAAACGTGATTTC	Remove GFP from pYTK001 for use as a dropout, and add BsmBI restriction site
MTK-p1001R	GCGTCTCAGGATCCAAAAGCAGGTGTAAACGCAGAAAGGCCCACC	Remove GFP from pYTK001 for use as a dropout, and add BsmBI restriction site
pLibAmpF	GATACGGTCTCCAGGTACACCTGCGTACTAG	Initial amplification of library, adding BsaI sites
pLibAmpR	ATCTGGTCTCGAAGCCACCTGCGAACATC	Initial amplification of library, adding BsaI sites
araC-for	GCATCGTCTCATCGGTCTCAAACGtgctactccgtcaagccgtc	Remove araC and ParaBAD from pDSJR
araC-rev	ATGCCGTCTCAGGTCTCACATAacctcaaaggtaccgagctcg	Remove araC and ParaBAD from pDSJR
InsertSeq-for	GCTCTTCTCGCTAACCAAACC	Sequencing constructs in pYTK001 and dropout derivative
YTKseq-for	GCGTTATCCCCTGATTCTGTG	Sequencing constructs in pYTK001 and dropout derivative
YTKseq-rev	CGCTTGGACTCCTGTTGATAG	Sequencing constructs in pYTK001 and dropout derivative

Table A-3 Gene blocks (Gblocks) used in this work

Gblock	Sequence	Purpose
MTK-G002	gcatcgtctcatcggtctcatatgATGAAATACCTATTGCCTACGGCAGCC GCTGGATTGTTATTACTCGCGGCCCAGCCGGCCATGGCGGAC TACAAAGACGATGATGATAAAACTAGTgGTAGtGAGACGaggct cagataCGTCTCtATCCGGTTCTGGCTCTGGTTCCGGTAGCGGTA GCGGTAGCGGCTCTGGTAGCGGTTCCATCGACAATTCAGCCG CAATTAGTATGGCAAATCCACGTCCACCAACACCGCGGacacct acaccgggtccggaccttaatgttgataacgatctgcgtccagaggcgggttcatatattgcgaatctt gcggcggcaaatacaatgttcacaactcgcttgcatgagcgcctgggtaatacttactataccgatat ggtaactggcgagcagaaacagacgacgatgtggatgcgccatgaaggaggccataacaaatgg cgcgatgggtccggtcaattaaaaacccagtctaatcgttacgtgcttcaattaggtggtgatgttgctc aatggtcccagaatgggtctgatcgttggcacgtcggcgtgatggcgggttatgggaacagcgata gtaaaactatttctagtcgtacagggtaccgtgcaaaagcttcagtcaatggatactccacaggtctgt acgctacttggtatgctgatgatgaatctcgcaacggagcatatctggattcatgggcccaatacagtt ggttcgacaatacggttaagggcgatgatttacaaagcgagtcttataaatcaaaaggattcactgcat cccttgaagccggttacaaacataaactggccgagttcaatggcagccaaggaactcgcaacgaat ggtatgtgcagccgcaagctcaggtgacttggatgggggtgaaagcggataagcatcgtgaatcaa atggtacattggtacattctaacggcgacggtaatgtccaaacccgcttgggtgttaagacctggctta agtcccatcataagatggatgacggtaaatcccgtgagtttcagccctttgtcgaggttaactggctgc ataacagcaaagactttagtacttcgatggatggcgtttctgttacccaagatggggctcgtaatattgc ggagattaaaacaggggtggaagggcaacttaatgctaacctgaacgtctggggtaatgttggtgttc aagtagcggaccgcggttataacgacacttctgctatggtcggaatcaagtggcagttctgacGAT CCTGAGACCTGAGACGGCAT	Block 2 of figure 1. Autotransporter, with FLAG tag, and space for insertion of GFP dropout.
MTK-G003	CTCCAGGTACACCTGCGTACTAGTNNKNNKNNKNNKNNKNN KNNKNNKNNKNNKNNKNNKNNKNNKNNKGGATGTTCGCAG GTGGCTTCG	Library
MTK-G005	agcggataacaatttcacacagGCATCGTCTCATCCACCTGCgtacTAGTgct gtatgtccaactggtctgttctctaatccgctgtgttgcgcgactaacgtcctggatctgattggcgtgg actgcaaaacgcctactatcgctgttgacactggtgctatcttccaggctcactgcgcgagcaaaggt tccaaaccgctgtgctgcgttgcaccagttgcagaccaggccctgctgtgccagaaagcaatcggc accttGGATgttcGCAGGTGGGATCCTGACCTGAGACGGCATgatcc agtaatgacctcagaa	HfbII insert
MTK-G008	agcggataacaatttcacacagGCATCGTCTCATCCACCTGCgtacTAGTatgt ttgcgcgtctgcctgttgtctttctgtacgcgtttgtagcgttcggtgcgctggttgctgcgctgccggg cggccacccgggcacttgtaccacgggttctctgagctgctgtaaccaggtgcagagcgcgagca gctccccggtaacggctctgctgggcctgctgggtatcgttctgtccgacctgaatgttctggtgggc atcagctgctctccgctgaccgttatcggtgtgggtggctccggttgctctgctcagactgtttgctgcg aaaacacccagttcaacggcctgatcaacatcggctgtaccccgatcaacattctgGGATgttcG CAGGTGGGATCCTGACCTGAGACGGCATgatccagtaatgacctcagaa	Sc3 insert
MTK-G013	agcggataacaatttcacacagGCATCGTCTCATCCACCTGCgtacTAGTctg cctgcttctgctgctaaaaacgctaaactggccacctctgcagcttttgcgaagcaggcggaaggtac tacgtgcaacgttggctctattgcgtgctgtaactctcctgccgagaccaacaacgatagcctgctga gcggtctgctgggtgctggtctgctgaacggcctgagcggtaatacgggtagcgcttgtgccaaag ccagcctgattgatcagctgggtctgctggccctggttgatcataccgaagagggtccagtttgtaaa aacattgttgcttgttgcccggaaggtaccacgaattgcgtagctgttgataacgctggtgcgggtacc aaagcagaaGGATgttcGCAGGTGGGATCCTGACCTGAGACGGCAT gatccagtaatgacctcagaa	DEW insert
MTK-G014	agcggataacaatttcacacagGCATCGTCTCATCCACCTGCgtacTAGTgca ccggcagccatggagcgtcaagttccatatactccttgttctggcctgtacggtactgcacagtgctgt gcgacggatgttctgggtgtggcggatctggattgcgctaacccgccggctactctggcaaacgcc actcacttcgaatctacctgcgcagcaattggccagcgtgcacgctgttgcgtactgccaatcctggg tcaggacattctgtgccaaacgccggcgggtctgGGATgttcGCAGGTGGGATCCT GACCTGAGACGGCATgatccagtaatgacctcagaa	NC2 insert

Table A-3 Gblocks used in this work (continued)

Gblock	Sequence	Purpose
MTK-G018	agcggataacaatttcacacagGCATCGTCTCATCCACCTGCgtacTAGTattc cgcgtactgataccccgtcttgttctactggttccctgcagtgctgttcctccgtacagaaggctactga tccgctggcttctctgctgatcggcctgctgggcattgttctgggtccgctggacctgctggttggtgtc acctgtagcccaattactgtaattggcgttggtggcacttcctgcactcagcaaactgtatgctgtaccg gcaacagctttaacggtctgatcgctatcggttgtagcccaatcaacatctctctgGGATgttcGC AGGTGGGATCCTGACCTGAGACGGCATgatccagtaatgacctcagaa	Vmh2-version 1 insert
MTK-G019	agcggataacaatttcacacagGCATCGTCTCATCCACCTGCgtacTAGTattc cacgtaccgacacccctttcctgttctaccggttctctgcaatgctgtagcagcgtgcagaaagcgtct gaccctctggtgggcattatcgtggcgctgctgggtatcgttctgggtccgctggatctgaacgttggt ctgacgtgcagcccgatcaccgttattggtgtaggtggtacctcctgtactcagcagaccgtttgctgt acgggtaacaactttaatggcctgatcgtggctggctgctctcctattaacatcggcctgGGATgtt cGCAGGTGGGATCCTGACCTGAGACGGCATgatccagtaatgacctcag aa	Vmh2-version 2 insert
MTK-G020	agcggataacaatttcacacagGAATTCGAGCTCGGTACCTTTGAGGTTA TGATGAAATACCTATTGCCTACGGCAGCCGCTGGATTGTTAT TACTCGCGGCCCAGCCGGCCATGGCGcatcaccaccatcaccatACTA GTgatccagtaatgacctcagaa	6xHis tag, to replace FLAG tag.
MTK-G021	agcggataacaatttcacacagGAATTCGAGCTCGGTACCTTTGAGGTTA TGATGAAATACCTATTGCCTACGGCAGCCGCTGGATTGTTAT TACTCGCGGCCCAGCCGGCCATGGCGgaacaaaaattgatttccgaggaag atcttACTAGTgatccagtaatgacctcagaa	Myc tag, to replace FLAG tag
Type4Term	gcatcgtctcatcggtctcaatcctaactcgagCACATCAGCCAGTGGAAGCCG AAGGTCCCGAACCGCGAGGACAAATACAAGAAGTAAagcggat aacaatttcacacagATAAAACGAAAGGCTCAGTCGAAAGACTGGGC CTTTCGTTTTATgatccagtaatgacctcagaaGCTGTGAGACCTGAGAC GGCAT	Block 4 of figure 1. rrnB_T1 terminator cloned as a Type 4 part

List of Symbols, Abbreviations, and Acronyms

ARL	Army Research Laboratory
ATP	adenosine triphosphate
BSA	bovine serum albumin
CIP	calf intestinal phosphatase
DEVCOM	US Army Combat Capabilities Development Command
DI	deionized
Gblocks	gene blocks
GFP	green-fluorescent protein
GYT	glycerol-yeast-tryptone
HDPE	high-density polyethylene
HPLC	high-performance liquid chromatography
IDT	Integrated DNA Technologies
LB	lysogeny broth
$MgCl_2$	magnesium chloride
$MgSO_4$	magnesium sulfate
MoClo	Modular Cloning
NaCl	sodium chloride
NEB	New England Biolabs
OD	optical density
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
SOC	super-optimal broth with catabolite repression

1 (PDF)	DEFENSE TECHNICAL INFORMATION CTR DTIC OCA
1 (PDF)	DEVCOM ARL FCDD RLD DCI TECH LIB
4 (PDF)	DEVCOM ARL FCDD RLW MG M T KOZLOWSKI J A ORLICKI FCDD RLH BA R A HUGHES R M PULLEN