Engineered Stochastic Adhesion Between Microbes as a Protection Mechanism Against Environmental Stress

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Abstract

Introduction—Microbes aggregate when they display adhesive proteins on their outer membrane surfaces, which then form bridges between microbes. Aggregation protects the inner microbes from harsh environmental conditions such as high concentrations of antibiotics, high salt conditions, and fluctuations in pH. The protective effects of microbial aggregation make it an attractive target for improving the ability of probiotic strains to persist in the gut environment. However, it remains challenging to achieve synthetic microbial aggregation using natural adhesive proteins because these proteins frequently mediate microbial virulence.

Objectives—Construction of synthetic proteins that mediate aggregation between microbes to enhance the survival of cells delivered to stressful environments.

Methods—We construct synthetic adhesins by fusing adhesive protein domains to surface display peptides. The resulting aggregated populations of bacteria are characterized using immunofluorescence, microscopy, flow cytometry, and quantification of colony forming units.

Results—We assemble a series of synthetic adhesins, demonstrate their display on the outer membrane of *Escherichia coli*, and show that they mediate bacterial aggregation. Further engineering of the size and motif composition of the

adhesive domain shows that principles from natural adhesins can be applied to our synthetic adhesins. Finally, we show that aggregation allows *E. coli* cells to resist treatment with antimicrobial peptides and survive inside the gut of *Caenorhabditis elegans*.

Conclusions—Our results demonstrate that synthetic aggregation can allow bacteria to resist biocidal environmental conditions. Synthetic adhesins may be used to facilitate microbial colonization of previously inaccessible environmental niches, either in remote natural environments or inside living organisms.

Keywords—Synthetic biology, Adhesion, Adhesin.

INTRODUCTION

Intercellular adhesion leading to the aggregate formation is a widespread phenomenon across many microbial species and affects stress tolerance,²¹ resource distribution,⁴⁹ and communication³⁷ between cells. Indeed, aggregated bacteria have been shown to exhibit enhanced resistance to antibiotics^{4,10,15} and

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antimicrobial peptides.³⁶ Therefore, engineering synthetic adhesion modules may help to promote survival of beneficial microbes that are introduced into a new host environment. In nature, microbes use a variety of mechanisms to adhere to different surfaces, including membrane hydrophobicity, secretion of extracellular polysaccharides, and production of adhesive proteins.² We chose to focus our engineering efforts on adhesive proteins, also known as adhesins, for their high degree of customizability. Adhesins are known to mediate binding between microbes of one species, members of two species, and between a given species and abiotic surfaces.^{33,41,42,52} One class of these adhesins is known as the biofilm-associated protein (BAP). These adhesins tend to be large (~ 1000 kDa) membrane-associated proteins with tandem repeat regions that are required for at least one stage of biofilm formation.^{25,31} These proteins can compensate for a lack of biofilm matrix polysaccharide during aggregate formation.¹³ Intercellular aggregation has also been shown to be catalyzed by sugar-binding lectin proteins, which are displayed on the microbial surface and bind to sugar residues on the extracellular surface of other cells.^{17,42}

There is a growing body of work seeking to describe the potential of probiotic microbial strains to form intercellular aggregates. These studies have concluded that microbial adhesion and aggregation are important for colonization and persistence of probiotic strains inside the gastrointestinal tract.^{27,28,51,53} Although there have been many basic science studies on the adhesive properties of constituents of the microbiome, there has been comparatively small effort to engineer the adhesive properties of microbial strains.^{38,45} One reason for this disconnect might be that natural adhesins are frequently associated with increased virulence of their host strain,^{11,31,32} making them difficult to apply to an engineered probiotic context. Although natural adhesins may not be safe for engineered probiotic strains, we can take the fundamental principles of adhesion gleaned from studies on these proteins and apply them to the design of novel synthetic adhesins. Such principles include the use of hydrophobic protein interactions^{18,55} and the presence of repeated amino acid sequences^{17,55} in adhesin proteins. The discontinuity between the discovery and application of principles of intercellular adhesion represents an opportunity to build a novel biological toolkit to control adhesion and aggregation of microbes to survive in harsh environments such as the GI tract.

In this work, we create a genetically controlled adhesion toolbox for microbial systems. Instead of relying on natural adhesins, we construct synthetic biological adhesins using naturally adhesive protein domains from cellulosomes.⁷ Cellulosomes are extracellular enzymatic complexes involved in the degradation of cellulose that

are assembled by interactions between cohesin and dockerin domains.^{34,35} Cohesin and dockerin are excellent candidates for engineered adhesins because their structures and mechanisms of binding have been extensively characterized, and since these domains are highly specialized for extracellular assembly of protein complexes, it is unlikely they would mediate pathogenicity. These domains have been shown to use calcium-dependent interactions of F-hand motifs to enable tight binding and species-specific affinity between proteins containing cohesin and dockerin domains.^{26,46,48} Cohesin and dockerin also have the capacity to self-dimerize *via* hydrophobic protein interactions.^{1,47,50} In this work, we exploit the self-dimerization capability of cohesin and dockerin to engineer stochastic adhesion in a microbial population. Stochastic microbial adhesion is known to be important for biofilm formation in *Shewanella oneidensis*³⁰ and during colonization of the human oral microbiome.⁴³ By engineering stochastic adhesion between microbes, we aim to create a tool that is simultaneously useful as a probe to explore the principles of adhesion in microbial systems and as an engineered mechanism of protection for microbial cultures.

To achieve these goals, we first test adhesin expression and localization on the surface of *Escherichia coli*. Next, we express a series of adhesins and characterize their ability to mediate microbial aggregation when expressed in *E. coli*. Next, we show that synthetic adhesins can impart a protective effect on cells by examining the viability of planktonic vs. stochastically aggregated bacteria under antimicrobial peptide treatment. We also feed aggregated bacteria to *Caenorhabditis elegans* in order to demonstrate that the protective effect of synthetic adhesins can extend to aiding microbial survival in a simple gut model *in vivo*. Finally, we perform some preliminary experiments aimed at translating this adhesin system into *Saccharomyces cerevisiae*. This work demonstrates that microbial aggregation can be rationally designed using basic principles of adhesion and applied to protect microbes from harsh environmental conditions.

RESULTS AND DISCUSSION

We first established the genetic constructs for achieving synthetic adhesion between bacteria. For the expression control of synthetic adhesion, we used the BL21DE3 *Escherichia coli* strain with a P_{Lac}-T7RNAP construct integrated into its genome, and the pET15b plasmid with a P_{T7/LacO1} promoter (Fig. 1a, “[Molecular Cloning: Bacterial Constructs](#)”). We chose the construct eCPX as a transmembrane scaffold for our adhesin because this protein was designed to present peptides on the surface of *E. coli* cells.⁴⁰ The functional

region of the adhesins was derived from cohesin and dockerin domains, as defined by the UniProt database.⁵ By presenting these domains on the cell surface, we expected that these adhesins could mediate intercellular binding and microbial aggregation by interacting with the extracellular domains of other adhesins (Fig. 1b). The functional domain and the scaffold were connected by stiff linkers with a high composition of proline and threonine to minimize the possibility of intra-protein interactions.¹⁹

Next, we validated the extracellular display of a synthetic adhesin using immunostaining (Fig. 1c). We made two constructs, one with eCPX fused directly to a 6xHis tag as a positive control (eCPX-6xHis), and the other with eCPX fused to Cel48s and a 6xHis tag (eCPX-Cel48s-Ct-6xHis) (Figs. 1d and 1e). Cel48s is a protein derived from *Clostridium thermocellum* that contains the dockerin domain. We chose to examine the localization of eCPX-Cel48s-Ct-6xHis because it was the largest protein (~ 100 kDa) used in this study, and larger proteins are more difficult to display on transmembrane protein scaffolds. We induced cells to express these constructs, then stained cells with 6x-His antibodies. We used an inverted fluorescent microscope to examine the localization of the fluorescently labeled secondary antibodies (“[Immunostain: Bacteria](#)”, “[Microscopy of Aggregates: Bacteria](#)”). We observed fluorescent signals in our samples expressing constructs tagged with 6x-His that we did not observe in the negative controls. Since we did not include a membrane permeabilization step, the antibodies cannot penetrate bacterial membranes, and we can conclude that the observed fluorescence on bacteria originated from eCPX-6xHis and eCPX-Cel48s-6xHis proteins that were displayed on the *E. coli* outer membrane. These results demonstrate that eCPX has the capacity to act as an effective scaffold for displaying a dockerin domain on the outer membrane of a bacterial cell. We note that these 6x-His-tagged constructs were intended only to confirm the extracellular display of cellulosome components. The 6x-His-tag inhibited the intercellular binding activity of adhesins and was omitted from all subsequent constructs.

After establishing the display of eCPX-6xHis and eCPX-Cel48s-6xHis, we set up the measurement protocols of synthetic adhesion between bacteria. We created two new synthetic adhesins with the cohesin and dockerin domains from *Acetivibrio cellulolyticus*^{22,54} (referred to as eCPX-Cohesin-Ac and eCPX-Dockerin-Ac). We controlled the expression of all the synthetic adhesins used in this study with IPTG (Isopropyl β -D-1-thiogalactopyranoside) (“[Adhesin Induction and Adhesion Protocol: Bacteria](#)”). This experimental set-up allowed us to correlate the presence of bacterial aggregates observed under the microscope with quan-

titative changes in the distribution of the particle sizes of these same bacterial populations. For our flow cytometry analysis, we used the forward scatter as a surrogate of aggregate size (FSC-A) based on previous work.⁴⁴ We analyzed differences in FSC-A in two ways. First, we directly overlaid the FSC-A intensity distributions for populations of cells with and without IPTG induction. Second, we performed a histogram subtraction (“[Flow Cytometry: Bacteria](#)”) that isolated the size distribution of particles found in the induced sample but not in the uninduced control.

We then investigated the capacity of these synthetic adhesins to mediate intercellular adhesion and bacterial aggregation. We found that our control cell lines, BL21DE3, and BL21DE3 expressing only eCPX experienced no visible aggregation under the microscope and a very small shift in the forward scatter upon induction with IPTG (Figs. 2a and 2b). Microscopy results from cells expressing eCPX-Cohesin-Ac and eCPX-Dockerin-Ac demonstrated small rod shapes characteristic of planktonic bacterial cells as well as irregularly shaped masses. The way that these irregularly shaped masses diffracted light suggested that they were composed of many smaller objects of a similar size to planktonic bacterial cells (Figs. 2c and 2d). Based on the complexity and ubiquity of these larger particles, we inferred that they were bacterial aggregates. In addition, the particle size distributions of induced vs. uninduced adhesins demonstrated a shift away from smaller particles and towards larger particles, with a unique population of large particles observed after a histogram subtraction (Figs. 2c and 2d). To ensure that the large particles were indeed composed of bacterial cells, we labeled cells expressing eCPX-Dockerin-Ac with Hoechst stain to test for the presence of DNA (“[Hoechst Staining: Bacteria](#)”). We found that Hoechst stain clearly indicated the presence of DNA in both planktonic and aggregated cells, and it showed that the aggregates are composed of clusters of single cells (Figs. 3a–3f). These results demonstrate that synthetic adhesins derived from cellulosome components are sufficient to catalyze the aggregation of a stochastically-selected subset of a microbial population. However, it is not clear whether the stochasticity in this process comes from variability in adhesin expression rates, display efficiency, or collision frequency, which may be further investigated in future work. We also note that aggregation was observed for a single bacterial population displaying either cohesin or dockerin, and that mixing of cohesin- and dockerin-bearing cells was not required to observe bacterial aggregate formation. This result suggested that cohesin-cohesin interactions and dockerin-dockerin interactions alone were sufficient to trigger aggregate formation.

While it seemed likely that cohesin and dockerin domains presented on the outside of *E. coli* were capable of mediating bacterial aggregation by dimerizing with adhesins on other cells, it was also possible that the production and exportation of these proteins to the cell membrane could lead to replication defects. Stress-induced replication defects are known to cause cytoplasmically-joined cells, which could appear as microbial aggregates if not carefully considered.¹⁶ Since previous work on natural adhesins had demonstrated that aggregates could be dissociated by proteinase K,²⁹ we treated aggregated bacteria expressing eCPX-Dockerin-Ac with proteinase K (“Proteinase K Assay: Bacteria”). If the bacterial aggregates were indeed composed of many individual bacteria connected by dimerized dockerin domains, we anticipated that proteinase K would degrade any extracellular proteins, dissociating aggregated bacteria into planktonic bacteria (Fig. 4a). We imaged and quantified the number of aggregates in samples with and without proteinase K, and found that the number of aggregates was significantly reduced when the cells were exposed to 0.1 $\mu\text{g/mL}$ of proteinase K for 1 h (Fig. 4b). The microscopy results also showed the aggregates were replaced by more planktonic cells (Figs. 4c and 4e). Treatment of planktonic cells did not appear to have any effect on the morphology of those cells (Figs. 4d and 4f). These results suggest that proteinase K treatment disrupts adhesion interactions between cells, releasing bacteria from aggregates to become planktonic again. This assay shows that extracellular protein interactions are necessary for bacterial aggregation catalyzed by the synthetic adhesins used in this study.

After our initial success with adhesins built using cohesin and dockerin domains from *A. cellulolyticus*, we engineered a series of adhesins using cellulosome components from *Clostridium thermocellum*^{26,48} (eCPX-Cohesin-Ct, eCPX-Dockerin-Ct, and eCPX-Cel48s-Ct) and *Ruminococcus flavefaciens*⁴⁶ (eCPX-Cohesin-Rf and eCPX-Dockerin-Rf). We found that eCPX-Cohesin-Rf and eCPX-Dockerin-Rf did not induce aggregate formation (Figs. 5a and 5b). The results of the adhesins from *Clostridium thermocellum* show that eCPX-Cohesin-Ct (Fig. 5c) successfully causing aggregation while eCPX-Dockerin-Ct (Fig. 5d) was inactive. To understand whether these results might be in part due to the small size of eCPX-Dockerin-Ct, we constructed eCPX-Cel48s-Ct from Cel48s, an 85 kDa native *Clostridium* protein that contains the dockerin domain (Fig. 5e). We found that eCPX-Cel48s-Ct was able to mediate aggregation in spite of its large size, suggesting that the distance of the adhesive domain from the cell surface plays an important role in the efficacy of an adhesin. In addition

to utilizing existing biological components, we applied our understanding of the importance of repeated sequences of amino acids in adhesins by constructing tandem versions of the *C. thermocellum* adhesins with duplicated extracellular domains (eCPX-2xCohesin-Ct and eCPX-2xDockerin-Ct) adjacent to one another (“Molecular Cloning: Bacterial Constructs”). We found that eCPX-2xCohesin-Ct (Fig. 5f) did not generate visible aggregation activity, while eCPX-2xDockerin-Ct (Fig. 5g) generated greatly increased aggregation capacity when compared to either adhesin with a single dockerin domain, eCPX-Dockerin-Ct, and eCPX-Cel48s-Ct. These results demonstrate that adhesin type, size, and the number of binding sites must be optimized to maximize the capacity of these proteins to mediate intercellular adhesion.

After characterizing the aggregation activity of microbial cultures expressing synthetic adhesins, we examined the resistance of bacterial cultures to biocidal treatments. We chose to test the efficacy of the eCPX-Dockerin-Ac construct even though it did not generate the highest percentage of bacterial aggregates in culture. We did this because it was unclear how duplicating the dockerin domain led to the high aggregation activity of the top performing adhesin, eCPX-2xDockerin-Ct. To understand the functional relevance of aggregation mediated by synthetic adhesins, we exposed aggregated and planktonic bacteria to different concentrations of the antimicrobial peptide LL37 (Fig. 6a, “Antimicrobial Peptide Assay: Bacteria”). LL37 is a small cationic peptide that attacks bacteria by forming pores in their membranes.²³ We chose an antimicrobial peptide as a stressor because these peptides are common in the gut environment, and many commensal organisms display resistance to these peptides.¹⁴ To perform our comparisons, we used unmodified, planktonic BL21DE3 cells and aggregated cells expressing eCPX-Dockerin-Ac. We found that at low doses of LL37, neither the planktonic cells or the stochastically aggregated cells were significantly affected (Figs. 6b and 6c). However, at high doses of LL37, the responses diverged, with planktonic cells experiencing significant mortality (Fig. 6b) and aggregated cells remaining viable (Fig. 6c). These tests establish the utility of synthetic adhesins as a defense mechanism that allows bacteria to persist in biocidal conditions.

To evaluate the capacity of our synthetic adhesins to improve the survival of microbes in the gut of a multicellular organism, we fed aggregated bacteria (expressing eCPX-Dockerin-Ac) and planktonic bacteria to *C. elegans*. Previous work has used *C. elegans* as a model system for bacterial persistence in the gut³⁹ and has shown that living bacteria in the gut can have a

FIGURE 1. Design of a genetic circuit that expresses synthetic adhesins. (a) A diagram of the genetic feedforward loop that controls the expression of each synthetic adhesin. IPTG activates the P_{Lac} promoter to turn on expression of T7 RNA polymerase (T7 RNAP). T7 RNAP and IPTG subsequently activate production of the synthetic adhesin from the $P_{T7/LacO1}$ promoter. The leaky transcriptional activity of T7 RNAP is reduced by LysS, which binds to and inactivates T7 RNAP. The P_{Lac} -T7RNAP module is located in the genome of the BL21DE3 cell line. The $P_{T7/LacO1}$ -adhesin module is located on the pET15b plasmid that is transformed into BL21DE3. LysS is constitutively expressed from the pLysS plasmid transformed into BL21DE3 (“[Molecular Cloning: Bacterial Constructs](#)”, “[Adhesin Induction and Adhesion Protocol: Bacteria](#)”). This genetic regulatory system is used to control the expression of all synthetic adhesins in this study. (b) An abstract representation of the cell–cell adhesion process mediated by the dimerization of adhesins displayed on the surface of *Escherichia coli*. (c) A depiction of the components necessary for the immunostaining process and their assembly onto cells presenting a dockerin-based adhesin with a six-histidine tag. (d, e) Immunostain for extracellularly-presented 6x-His-tagged proteins: negative controls (d) and experimental samples (e). (Panel i) Regulatory diagram and coding sequences of constructs used to confirm the display of synthetic adhesins on the outside of bacterial cells. (Panel ii) Brightfield image of stained bacteria cells. (Panel iii) Fluorescent overlay on brightfield image of samples expressing the corresponding protein (indicated by Panel i) (“[Immunostain: Bacteria](#)”). (Panel iv, e) 100 $\times$ view of antibody-stained samples to show the localization of fluorescence to the outer bacterial membrane. Pictures and graphs are representative of three biological replicates.

positive effect on the host,²⁴ corroborating the use of *C. elegans* as a platform for testing the survival of beneficial microbes. We introduced starved L1 worms to aggregated and planktonic bacterial cultures, let the worms feed, then collected them by centrifugation (“*C. elegans* Gut Survival Assay”). Next, we incubated the worms in M9 media containing gentamycin to kill bacteria outside the worm (Fig. 7a). After washing out the excess gentamycin and lysing the worms to release their gut contents, we quantified the colony forming units of each sample with *C. elegans* and compared their fold enrichment of bacteria as compared to ‘bacteria only’ controls that were never exposed to *C. elegans* (“*C. elegans* Gut Survival Assay”). We observed substantial variation between the colony forming units recovered from each biological replicate (see “[Conclusions](#)” for further discussion). Nonethe-

less, cells expressing synthetic adhesins were consistently enriched upon worm lysis compared to their ‘bacteria only’ controls. This suggests that a significant population of aggregated cells survived after ingestion while planktonic cells were killed (Fig. 7b). These results show that synthetic adhesins can improve the survival of bacteria in a gut environment and corroborate the utility of engineered microbial aggregation as a general defense strategy for bacteria.

CONCLUSIONS

We set out to develop a new biological toolkit to mediate microbial aggregation by designing synthetic adhesins and expressing them in engineered microbes. We developed several versions of these synthetic ad-

FIGURE 2. Optical microscopy and flow cytometry analysis of adhesin-based bacterial aggregation. (a, b) BL21DE3 cells or BL21DE3 cells expressing eCPX are primarily planktonic. (c, d) BL21DE3 cells expressing eCPX-Cohesin-Ac or eCPX-Dockerin-Ac show large clumps (indicated by red circles in panel ii) in cultures that appear to be composed of particles the size of planktonic bacteria. These clumps do not appear in the uninduced culture. There is a population of large particles unique to the induced cell culture, likely representing the large aggregated particles we see under the microscope. (a--d) (Panels i and ii) Microscope images of uninduced and induced cells (Panel iii) Overlay of particles sizes, as measured by forward scatter, for induced (grey) and uninduced (black) cellular populations. (Panel iv) Histogram subtraction between the induced and uninduced cells. Percentage in the upper right corner shows the proportion of events unique to the forward scatter distribution of the induced sample. (All panels) All pictures and graphs are representative of three biological replicates. Induction protocol ("[Adhesin Induction and Adhesion Protocol: Bacteria](#)"). Imaging protocol ("[Microscopy of Aggregates: Bacteria](#)"). Flow cytometry protocol ("[Flow Cytometry: Bacteria](#)").

FIGURE 3. DNA staining of microbial aggregates. (a) Brightfield image of unstained BL21DE3 cells expressing eCPX-Dockerin-Ac (“Adhesin Induction and Adhesion Protocol: Bacteria”). (b) DAPI filtered image of unstained BL21DE3 cells expressing eCPX-Dockerin-Ac (“Adhesin Induction and Adhesion Protocol: Bacteria”). Image shows that bacterial aggregates do not have autofluorescence on this fluorescent channel. (c) Overlay of brightfield and DAPI filtered image of BL21DE3 cells expressing eCPX-Dockerin-Ac (“Adhesin Induction and Adhesion Protocol: Bacteria”). (d) Brightfield image of Hoechst-stained BL21DE3 cells expressing eCPX-Dockerin-Ac (“Adhesin Induction and Adhesion Protocol: Bacteria”, “Hoechst Staining: Bacteria”). (e) DAPI filtered image of Hoechst-stained BL21DE3 cells expressing eCPX-Dockerin-Ac (“Adhesin Induction and Adhesion Protocol: Bacteria”, “Hoechst Staining: Bacteria”). Image shows that planktonic cells and aggregates exhibit DNA staining and that aggregates are composed of many small DNA-containing units, consistent with our conclusions. (f) Overlay of brightfield and DAPI filtered image of Hoechst-stained BL21DE3 cells expressing eCPX-Dockerin-Ac (“Adhesin Induction and Adhesion Protocol: Bacteria”, “Hoechst Staining: Bacteria”). Image shows that signal from Hoechst stain and brightfield images of planktonic and aggregated bacteria co-localize. (a–f) All pictures and graphs are representative of five biological replicates. Induction protocol (“Adhesin Induction and Adhesion Protocol: Bacteria”). Imaging protocol (“Hoechst Staining: Bacteria”).

hesins that produced microbial aggregates, suggesting a degree of modularity imparted by using distinct domains for anchoring the adhesin in the membrane, forming intercellular bonds, and linking the two functional domains. However, our adhesins were not uniformly successful. These discrepancies between our adhesins suggest that there are a number of factors to consider when designing a protein that mediates intercellular adhesion. The general success of cohesin- and dockerin-based adhesins show that the self-dimerization bonds due to hydrophobic interactions is largely sufficient to mediate cell–cell binding. We were also successful at applying another basic principle of intercellular adhesion by introducing repeated sequences into our dockerin-based adhesin from *C. thermocellum*. The increased adhesion between eCPX-

2xDockerin-Ct compared to the eCPX-Cel48S-Ct construct (which only has one copy of dockerin), shows that multiple copies of a functional unit can increase the adhesive capacity of these proteins. However, when we constructed eCPX-2xCohesin-Ct, it displayed negligible binding activity. It is possible that by putting two cohesin proteins adjacent to one another, we allow these domains to interact, inhibiting their interaction with adhesins on other cells and abolishing the aggregation activity we previously observed from the monovalent cohesion-based adhesin. This interaction could be prevented by incorporating shorter, stiffer linkers between the cohesin domains, but short linkers can prevent adjacent proteins domains from folding properly. Therefore, a future study that systematically varies the length and

FIGURE 4. Dissociation of bacterial aggregates by proteinase K treatment. (a) The cartoon demonstrates the experimental setup using proteinase K to disperse adhesion-based bacterial aggregates. (b) Comparison of the number of aggregates found in samples expressing eCPX-Dockerin-Ac after treatment with proteinase K to untreated samples. * indicates that differences between samples are significant below a p-value less than 0.05. Significance was determined by a one-tailed *t* test with an unequal variance that returned a p-value of 0.013. (c) Control sample demonstrating normal aggregate formation by expression of eCPX-Dockerin-Ac. Red circles highlight aggregates. (d) Control sample demonstrating normal planktonic state of BL21DE3 cells. (e) An experimental sample of cells expressing eCPX-Dockerin-Ac treated with 0.1 $\mu\text{L/mL}$ proteinase K demonstrating dissociation of aggregates found in the untreated sample (b). (f) Control sample demonstrating the normal planktonic state of BL21DE3 cells treated with 0.1 $\mu\text{L/mL}$ proteinase K. (c–f) Each picture is representative of at least 3 biological replicates. Induction protocol (“[Adhesin Induction and Adhesion Protocol: Bacteria](#)”). Proteinase K protocol (“[Proteinase K Assay: Bacteria](#)”). Imaging protocol (“[Microscopy of Aggregates: Bacteria](#)”).

properties of linkers between adhesive protein domains might reveal rules for reducing interactions between two adjacent domains and promoting their binding to protein domains on other cell surfaces.

We also highlighted the ability of stochastically aggregated bacterial cultures to resist biocidal compounds. This aspect of the project is significant because of the existing body of work connecting the survival of natural probiotic strains to their capacity to survive exposure to biocidal compounds.²⁷ While microbial aggregation has primarily been considered in a natural context, our work applies protein engineering to make stochastic aggregation and stress resistance an engineered property of microbial systems. Future work could use the synthetic adhesins developed in this study as defensive tools for engineered probiotic strains to persist in the harsh environment of the GI tract.

We also showed that stochastically aggregated cultures (mediated by eCPX-Dockerin-Ac) consistently survived better in the gut of *C. elegans* than planktonic cells did. While the increased survival of synthetically aggregated microbes has implications for the application of synthetic microbial adhesion to probiotic strains, the variation between the biological replicates for this data was substantial. Although the source of this variation is not clear, we noted that some *C. elegans* cultures showed more motility than others when added to the bacteria, which could have affected the feeding rate. This variation in motility of the worms may have been caused by variability in the amount of time the worms remained on the starved plates for synchronization (“*C. elegans* Gut Survival Assay”). Alternatively, variation in the amount of time the worms spent in M9 before being introduced to the

FIGURE 5. Engineering of adhesins using alternative biological components and duplicated protein domains. Induced vs. uninduced *E. coli* populations containing the coding sequence for eCPX-Cohesin-Rf (a), eCPX-Dockerin-Rf (b), eCPX-Cohesin-Ct (c), eCPX-Dockerin-Ct (d), eCPX-Cel48s-Ct (e), eCPX-2xCohesin-Ct (f), or eCPX-2xDockerin-Ct (g). The overlays are almost identical in (a), (b), (d), and (f), suggesting that this adhesin does not mediate bacterial aggregation. The overlays show that the induced population of cells is shifted towards higher forward scatter values in (c), (e), and (g), suggesting the formation of bacterial aggregates. (g) In cell expressing eCPX-2xDockerin-Ct, there is a bigger population of large particles unique to the induced population than in cells expressing eCPX-Dockerin-Ct or eCPX-Cel48s-Ct. Since eCPX-Dockerin-Ct and eCPX-Cel48s-Ct only contain a single dockerin domain each, these results suggest that introducing a repeated dockerin sequence improved the efficacy of this synthetic adhesin to mediate bacterial aggregate formation. (a–g) (Panel i) Flow cytometry overlay for forward scatter of induced (grey) vs. uninduced (black) *E. coli* populations containing the coding sequence for one of a series of synthetic adhesins. (Panel ii) Histogram subtraction of the forward scatter of the induced bacterial population compared to the uninduced bacterial population. The percentage in the upper right corner shows the proportion of events unique to the forward scatter distribution of the induced sample. (All panels) All graphs are representative of three biological replicates. Induction protocol (“[Adhesin Induction and Adhesion Protocol: Bacteria](#)”). Flow cytometry protocol (“[Flow Cytometry: Bacteria](#)”).

bacterial cultures (“*C. elegans* Gut Survival Assay”) may have affected their activity. Even with the high degree of variability observed in the *C. elegans* feeding assays, the difference in enrichment between the planktonic and stochastically aggregated populations is significant, with the stochastically aggregated cultures showing substantially higher amounts of bacteria released from the lysed worms (Fig. 7b). Taken together, the results of the antimicrobial peptide assay and the *C. elegans* feeding assay suggest that synthetic adhesins are a useful defense strategy that improves the survival and efficacy of engineered probiotics. Further

work must be done, however, to ensure that these synthetic adhesins do not impart virulence on their host strain.

In addition to engineering our adhesins in *E. coli*, we wanted to explore the possibility of applying the same principles of adhesin construction to *Saccharomyces cerevisiae*. We fused cohesin and dockerin from *R. flavefaciens* to the surface display peptide Aga2⁸ and expressed these adhesins under the control of GAL1p in *Saccharomyces* cells (“[Molecular Cloning: Yeast Constructs](#)”). When we examined cells expressing these adhesins under the microscope (“[Ad-](#)

FIGURE 6. Cell viability assay for planktonic vs. stochastically aggregated cells after antimicrobial peptide treatment. (a) Abstract representation of differential killing of planktonic vs. stochastically aggregated bacterial cells. Outer members of bacterial aggregates may protect inner members of bacterial aggregates from lysis by LL37, allowing greater survival for the stochastically aggregated culture as a whole. (b) Fold enrichment of viable cells between untreated planktonic cells (represented by dashed line) and planktonic cells treated with 12.5 or 25 $\mu\text{g/mL}$ LL37 (represented by bars). A *t*-test between the untreated and 12.5 $\mu\text{g/mL}$ LL37 treated populations shows a *p*-value of 0.139. A *t*-test between the untreated and 25 $\mu\text{g/mL}$ LL37 treated populations shows a *p*-value of 0.0025. This graph shows that 25 $\mu\text{g/mL}$ LL37 is capable of significantly reducing the viability of a planktonic microbial population. (c) Fold enrichment of viable cells between untreated stochastically cells (represented by dashed line) and stochastically aggregated cells treated with 12.5 or 25 $\mu\text{g/mL}$ LL37 (represented by bars). A *t*-test between the untreated and 12.5 $\mu\text{g/mL}$ LL37 treated populations shows a *p*-value of 0.611. A *t*-test between the untreated and 25 $\mu\text{g/mL}$ LL37 treated populations shows a *p*-value of 0.204. This graph shows that 25 $\mu\text{g/mL}$ LL37 is not capable of significantly reducing the viability of a stochastically aggregated microbial population. (b, c) Planktonic cells are represented by the untransformed BL21DE3 cell line, and aggregated cells are represented by cells expressing eCPX-Dockerin-Ac. Stars represent the degree of significance from two-tailed *t*-tests with unequal variance: ***p*-value < 0.01, NS represents 'Not Significant'. Each bar represents data from two biological replicates with three technical replicates apiece. Induction protocol ("[Adhesin Induction and Adhesion Protocol: Bacteria](#)"). Antimicrobial peptide treatment and CFU protocol ("[Antimicrobial Peptide Assay: Bacteria](#)").

FIGURE 7. Survival of planktonic vs. stochastically aggregated cells in the *C. elegans* gut. (a) Depiction of the analysis of bacterial survival in the gut of *C. elegans*. (Left Panel) Experimental sample with worms that have fed on bacteria. (Right Panel) Control sample with bacteria, but no worms. (Both Panels) After allowing time for *C. elegans* feeding, external bacteria (colored black) were killed with gentamycin treatment, then the worms were subjected to grinding by a sterile pestle, which released any bacteria in the worm gut (labeled grey). The number of viable cells in both samples were measured by counting the colony forming units, and compared by dividing the number of bacteria in the samples with worms by their corresponding "cells only" control. This metric demonstrates the fold enrichment in the number of bacteria coming from the *C. elegans* gut. (b) Fold enrichment of viable cells between planktonic and stochastically aggregated cultures fed to *C. elegans*. Planktonic cells are represented by the untransformed BL21DE3 cell line, and aggregated cells are represented by cells expressing eCPX-Dockerin-Ac. Bars represent the median of each data set. A *t*-test between the two populations returns a *p*-value 0.0249. Star represents degree of significance from a two-tailed *t*-test with unequal variance: **p*-value < 0.05. Both sets of data were gathered from three biological replicates with three technical replicates apiece. Biological replicates are indicated by color, light grey—replicate 1, dark grey—replicate 2, black—replicate 3. Induction protocol ("[Adhesin Induction and Adhesion Protocol: Bacteria](#)"). Worm feeding and CFU protocol ("[C. elegans Gut Survival Assay](#)").

hesin Induction and Adhesion Protocol: Yeast", "[Microscopy of Aggregates: Yeast](#)"), we found that they formed large clumps of cells not observed in the

control cells only displaying the Aga2 cell presentation peptide (Fig. S1). To confirm that these results indeed represent true microbial aggregation, future work

would have to confirm the expression of the adhesin on the yeast cell wall, construct additional adhesins to establish the ubiquity of this approach, and back up the microscopy results with another particle size assay, such as flow cytometry.

In conclusion, we have created a novel biological toolkit that allows for stochastic microbial aggregation, and we have demonstrated how these tools may be applied as a defensive strategy for improving the survival of probiotics. Future work could focus on improving the performance of these synthetic adhesins by exploiting other basic principles of microbial adhesion or could apply these synthetic adhesins to improving the survival and performance of existing probiotic strains.

METHODS

Molecular Cloning: Bacterial Constructs

The coding sequence for eCPX was a gift from the Daugherty lab, and the coding sequences for the cohesin and dockerin modules and their linkers were provided by the Nash lab. The eCPX sequence, all cohesin sequences, and all dockerin sequences were amplified by PCR with Q5 polymerase (NEB), gel extracted (NEB), and quantified using a Nanodrop (Thermo Fisher). All adhesins were assembled into the pET15b-mOrange backbone that had been digested with NcoI and BamHI with buffer 3.1 (all NEB) for 37 °C in a static incubator overnight. The resulting backbone had mOrange excised, was purified by gel extraction, and was combined with an eCPX insert, a single or dual cohesin or dockerin domain, then assembled *via* the Gibson Assembly Master Mix (NEB) for 1 h at 50 °C following the DNA molar ratios suggested by the company's protocol. Successful transformations were assayed by digestion with NcoI, BamHI, and buffer 3.1 and confirmed by Sanger sequencing.

Media Preparation

All cells were grown in liquid M9 media (Amresco) unless otherwise noted. Media was prepared by autoclaving the salts dissolved in water for 30 min, allowing the solution to cool overnight, then adding 20% glucose, 1 M CaCl₂ and 1 M MgSO₄ following product recommendations. Media was also supplemented with 0.1% casamino acids. The resulting mixture was filtered through a 0.2 µm filter and used the day of the experiment. All experiments were done using sterile technique unless otherwise noted.

Adhesin Induction and Adhesion Protocol: Bacteria

On the first day of the experiment, each sample was grown overnight in M9 media ("[Media preparation](#)"). BL21DE3 cells were grown without antibiotics, cells expressing synthetic adhesins were grown in 50 µg/mL carbenicillin to ensure retention of the pET15b backbone and 33 µg/mL chloramphenicol to ensure retention of the pLysS plasmid. Each sample was diluted in fresh M9 media to 0.5 OD600. Samples were then left on ice for 5 h, then diluted 1:100 into 3 mL cultures with fresh M9 media and the appropriate antibiotics. Culture tubes were put on an angled shaking incubator at 200 rpm and 37 °C for one and a half hours. After this, all samples were pulled out of the incubator, and the samples intended for induction were treated with 1 mM IPTG. All samples were returned to the incubator and allowed to continue incubating for 17 h at 200 rpm and 37 °C.

Immunostain: Bacteria

After induction of synthetic-adhesin expression ("[Adhesin Induction and Adhesion Protocol: Bacteria](#)"), bacteria were gently spun down in a sterile 1.5 mL tube at 5000×g for 20 min and resuspended in 4% PFA, and allowed to mix for 20 min at room temperature on a Variable Orbital Shaker (GeneMate). Samples were spun down at 5000×g and resuspended in PBS three times to remove excess PFA, and left spinning on an end-over-end agitator at 4 °C for 2 days. After 2 days, cells were diluted 1:10 into 0.5 mL tubes for a total volume of 0.5 mL, spun down at 5000×g for 20 min, then resuspended in blocking solution (3% BSA in PBS) allowed to block on an end-over-end agitator for 20 min at 4 °C. After this, 1 µL of His-tag antibody (Mouse–Thermo Fisher) was put in each tube and incubated overnight on an end-over-end agitator at 4 °C. The next day, cells were spun down 5000×g for 20 min three times and resuspended in PBS to wash out excess primary antibody, before being spun and resuspended in blocking solution. Samples were allowed to block for 1 h on an end-over-end agitator at 4 °C before 0.5 µL of secondary antibody tagged with Alexafluor 488 (Goat-antimouse Thermo Fisher) was added to the solution. Tubes were covered with foil and incubated overnight on an end-over-end agitator at 4 °C. The next day, each sample was spun down 5000×g for 20 min three times and washed in PBS to get rid of excess secondary antibody. Negative control samples expressing surface displayed peptides without 6xHis tags were also included in these experiments but did not demonstrate the same signal observed in the stains of samples expressing surface-

displayed peptides with 6xHis tags. Samples were prepared for imaging by drawing squares on a glass slide using a hydrophobic barrier pen (VWR), pipetting 1 μ L of each sample into the square, then sandwiching the sample with a coverslip and imaging the slide on an inverted Nikon Eclipse Ti microscope with a 40 $\times$ and 100 $\times$ objective.

Microscopy of Aggregates: Bacteria

Following an induction protocol (“[Adhesin Induction and Adhesion Protocol: Bacteria](#)”), samples were prepared for imaging by drawing squares on a glass slide using a hydrophobic barrier pen (VWR), pipetting 1 μ L of each sample into the square, then sandwiching the samples with a coverslip. Samples were imaged using the brightfield of an inverted Nikon Eclipse Ti microscope with a 40 $\times$ objective. 4 $\times$ 4 grids of images were recorded for each sample.

Flow Cytometry: Bacteria

After adhesin expression was induced (“[Adhesin Induction and Adhesion Protocol: Bacteria](#)”), cells were diluted 1:10 into fresh M9 media and analyzed using an Attune Nxt (Life Tech) flow cytometer. Ten thousand events were collected for each sample, and data from each sample was analyzed by histogram subtraction using FCS Express version 4. (De Novo Software, Los Angeles, CA). Percentage of positive events unique to the FSC-A distributions of induced cells were calculated by dividing the number of unique positive events as determined by the histogram subtraction by the total event count of the induced cell population.

Hoechst Staining: Bacteria

After adhesin expression was induced (“[Adhesin Induction and Adhesion Protocol: Bacteria](#)”), 200 μ L of culture was pipetted into a clear, round bottomed, 96 well plate. Next, 2 μ L of 10 mg/mL Hoechst stain (Sigma-Aldrich) was added to the well, the plate was protected from light, and incubated at 37 $^{\circ}$ C with no shaking for 2 and a half hours. Negative controls without stain added to the solution were included in the experiment to demonstrate that bacterial autofluorescence was not responsible for the observed signal. Samples were imaged using the DAPI filter an inverted Nikon Eclipse Ti microscope with a 40 $\times$ objective (“[Microscopy of Aggregates: Bacteria](#)”).

Proteinase K Assay: Bacteria

After expression was induced (“[Adhesin Induction and Adhesion Protocol: Bacteria](#)”), 200 μ L of culture was pipetted into a 96-well plate with the indicated concentration of Proteinase K (Thermo Fisher). The 96-well plate was covered with a plastic lid and put in a shaking incubator for 1 h at 200 rpm and 37 $^{\circ}$ C. Samples were prepared for imaging following “[Microscopy of Aggregates: Bacteria](#)”. Number of aggregates was compared by hand counting images in Image J.

Antimicrobial Peptide Assay: Bacteria

After expression was induced (“[Adhesin Induction and Adhesion Protocol: Bacteria](#)”), 200 μ L of culture was pipetted into a 96-well plate with the indicated concentration of the antimicrobial peptide LL37 (AnaSpec). The 96-well plate was covered with a plastic lid and put in a shaking incubator for 6 h at 200 rpm and 37 $^{\circ}$ C. Each sample was added to a 96 well plate and serially diluted between 10¹ and 10⁶ in fresh M9 media by transferring 20 μ L of cells between wells loaded with 180 μ L of M9 media. 10 μ L of all serial dilutions of each sample was transferred to a 24-well plate filled with LB agar (Amresco) autoclaved and poured 1 day before the experiment. The liquid sample was evenly spread over each well by tilting the plate, each plate was placed agar side down in a static 37 $^{\circ}$ C incubator. Bacteria on each plate were allowed to grow for 17 h. The number of colony forming units was calculated by counting the number of colonies in each well. Wells were chosen to represent the total concentration of each sample by looking at colony definition and having a total colony count between 20 and 40 when possible. Each sample was pipetted in triplicate to account for technical error. Significance was determined by using a two-sample equal variance, two-tailed *t* test implemented in Microsoft Excel.

C. elegans Gut Survival Assay

C. elegans were maintained at 20 $^{\circ}$ C on MYOB plates with a lawn of OP50 *E. Coli*.^{9,12}

One week ahead of the experiment, chunks of agar from a plate with *C. elegans* starved at the L1 stage of development were transferred to fresh plates with a lawn of *E. coli* OP50. These worms were allowed to feed for approximately 1 week at 20 $^{\circ}$ C until they ran out of bacteria and starved; starved plates were filled with primarily L1 worms, along with a few dauer worms (estimated to be less than 5%).

To obtain synchronized populations, worms were allowed to reproduce for 1 week at 20 °C until the bacteria was consumed, which causes L1 stage worms to arrest.^{3,6} Because the presence of older larval and adult worms varied, plates were left for 1–4 days until those worms died and the starved plates contained primarily synchronized L1 worms, along with a few dauer worms (estimated to be less than 5%).

The day before the experiment, *E. coli* BL21DE3 cells containing the indicated adhesin on the pET15b backbone and the pLysS plasmid were induced with IPTG for 17 h (“Adhesin Induction and Adhesion Protocol: Bacteria”). Cells were left on the bench at room temperature for 3 h after induction while reagents were prepared for the experiment. While cells were at room temperature, four 60 mm plates of *C. elegans* were washed in fresh M9 media and collected by centrifuging them in a Heraeus Pico 17 Centrifuge (Thermo Fisher) at 200×g for 1 min. The supernatant was discarded, the plates were each washed again, and combined again the same tube, spun down at 200×g for 1 min. All but 100 µL of media was removed from the tube, and the worms were quantified by serially diluting them, then counting the number of worms in an adequately dilute sample. For the first biological replicate, worm quantification was done from a parallel aliquot after mixing worms and bacteria. For both subsequent biological replicates, worm quantification was done before mixing worms and bacteria, to ensure that a similar number of worms was added as for the first replicate.

Bacterial cells were gently resuspended, and two samples of each biological replicate were added to a 24-well plate. One full biological replicate included two samples of BL21DE3 with no IPTG treatment, and two samples of cells expressing eCPX-Doc(Ac) with 1 mM IPTG. For one well of BL21DE3 and one well of eCPX-Doc(Ac) per biological replicate, 20 µL of 300 worms/µL were added. The other wells, containing only BL21DE3 and only eCPX-Doc(Ac) respectively, were treated exactly the same as the wells with worms and bacteria together for all subsequent steps, constituting the ‘bacteria only’ controls for both groups. The 24-well plate was then left to gently agitate for 2 h on a Variable Orbital Shaker (GeneMate) at a speed that allowed the sample to remain mixed, but was not high enough to spin all the worms into the center of each well.

After 2 h of feeding, all samples were collected by centrifuging them at 200×g for 1 min in a sterile 1.5 mL tube and washing them in 1 mL fresh M9 media with 25 mM levamisole and 100 µg/mL gentamicin. Levamisole serves to induce pharyngeal paralysis and prevents the worms from continued feeding, expelling their stomach contents, or ingesting

gentamicin. Samples were each washed two times, reducing the total volume down to 100 µL in order to preserve the loose worm pellet. After the second wash, the samples were allowed to incubate at room temperature for 3 h on an end-over-end agitator to kill the free-living bacteria.

After 3 h, the samples were collected by 200×g for 1 min and washing them four times in 1 mL fresh M9 media to remove levamisole and gentamycin, reducing the total volume down to 100 µL in order to preserve the loose worm pellet.

Next, each sample was washed with 1 × PBS containing 1% Triton-X and reduced down to 100 µL. All samples were lysed by grinding an autoclaved pestle against the base of the tube for 3 s, ten times before being spun down at 8000×g. This process was repeated three times.

80 µL of each lysed sample was added to a 96 well plate and serially diluted between 10¹ and 10⁵ fold in fresh M9 media. 10 µL of all serial dilutions of each sample was transferred to a 24-well plate filled with LB agar (Amresco) autoclaved and poured 1 day before the experiment. The liquid sample was evenly spread over each well by tilting the plate, each plate was left to dry under a flame with the lid cracked for 5 min before being placed agar side up in a static 37 °C incubator. Bacteria on each plate were allowed to grow for 17 h before being counted by hand the next day. Wells were chosen to represent the total concentration of each sample by looking at colony definition and having a total colony count between 20 and 40 when possible. Each sample was pipetted in triplicate to account for technical error. Significance was determined by using a two-sample, unequal variance, two-tailed *t*-test implemented on Microsoft Excel.

Molecular Cloning: Yeast Constructs

The genes coding for cohesin III and XMod-dockerin III of *R. flavefaciens* were amplified by using two primers carrying the *KpnI* and *XhoI* restriction sites. After restriction digestion, the two DNA fragments were ligated to the recipient pYD1 plasmid digested with the same restriction enzymes generating the plasmids pYD1_CohIII and pYD1_Xmod-DocIII. Successful cloning was also confirmed by Sanger sequencing.

Adhesin Induction and Adhesion Protocol-Yeast

Both yeast adhesin plasmids were transformed into separate *Saccharomyces cerevisiae* EBY100 lines following a typical lithium acetate transformation procedure²⁰ and selecting the positive colonies on SD agar 2% (w/v) glucose plates lacking tryptophan. Resulting colonies were cultivated in liquid SD-TRP liquid

medium with 2% glucose for 24 h at 30 °C with continuous shaking at 200 rpm. Protein expression and display were then induced by transferring the culture to a fresh liquid medium lacking tryptophan containing 0.2% (w/v) glucose and 1.8% (w/v) galactose, and shaking for 24 h at 30°C.

Microscopy of Aggregates: Yeast

Once the display of the constructs was confirmed, the formation of cellular aggregates was verified through optical microscopy using an Olympus Inverted Microscope IX81 by spotting 10 μ l of the culture on a glass slide under the microscope. All the analyses were carried out two independent times and without any additional washing of the sample in order to not disturb eventual cell aggregates with pipetting and centrifugation.

ELECTRONIC SUPPLEMENTARY MATERIAL

The online version of this article (<https://doi.org/10.1007/s12195-018-0552-9>) contains supplementary material, which is available to authorized users.

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AUTHOR CONTRIBUTIONS

DL and CT designed the study and wrote the manuscript. DL performed all wet lab experiments involving bacteria, RV performed all wet lab experiments involving yeast. CV assisted with Hoescht staining and proteinase K experiments. MN and RV provided cohesin and dockerin components as well as advice for adhesin design. LR helped design *C. elegans* experiments and analyzed the results. MN, RV, LR all helped edit the manuscript.

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DATA AVAILABILITY

The datasets generated during and/or analyzed during the current study are available from the corresponding author upon request.

CONFLICT OF INTERESTS

All authors, including D. Lewis, R. Vanella, C. Vo, L. Rose, M. Nash, and C. Tan, declare that they have no conflict of interest.

ETHICAL APPROVAL

This article does not contain any studies with human participants or animals performed by any of the authors.

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