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Engineering biofilm formation and dispersal

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Anywhere water is in the liquid state, bacteria will exist as biofilms, which are complex communities of cells that are cemented together. Although frequently associated with disease and biofouling, biofilms are also important for engineering applications, such as bioremediation, biocatalysis and microbial fuel cells. Here, we review approaches to alter genetic circuits and cell signaling towards controlling biofilm formation, and emphasize utilizing these tools for engineering applications. Based on a better understanding of the genetic basis of biofilm formation, we find that biofilms might be controlled by manipulating extracellular signals, and that they might be dispersed using conserved intracellular signals and regulators. Biofilms could also be formed at specific locations where they might be engineered to make chemicals or treat human disease.

Genetic basis of biofilm formation and dispersal

Bacteria alternate between planktonic (free-swimming) and sessile states, with dense, multicellular communities called 'biofilms' being the more important state [1]. Nearly all cells make biofilms [1,2], which are formed in aquatic environments by the attachment of bacteria to submerged surfaces, to the air/liquid interface, and to each other. Biofilms attach via appendages, such as fimbriae and flagella [3], and microcolonies are formed by the production of microbial products including polysaccharides, proteins, lipids, and DNA [4]. Hundreds of genes are differentially controlled during biofilm development including stress-associated genes [5–8]; hence, these systems present an interesting challenge in terms of their control.

Biofilms are dynamic in that cells might detach from the biofilm matrix and disperse [9]. Active dispersal is initiated by the bacteria via a highly regulated process, whereas passive dispersal is mediated by external forces such as fluid shear and abrasion [9]. Changes in environmental conditions (e.g. nutrition level and oxygen depletion), whether favorable or unfavorable, can lead to biofilm dispersal [9], because it is beneficial to increase the biofilm at nearby locations when nutrients are plentiful and to colonize other locations when nutrients are scarce [3]. For example, *Pseudomonas aeruginosa* biofilms undergo dispersal in response to a sudden decrease [10] or increase [11] in nutrients. Furthermore, reproducible, periodic dispersal occurs in *Actinobacillus actinomycetemcomitans* [9],

P. aeruginosa [12] and *Serratia marcescens* [13]; hence, biofilm dispersal is important for the survival of the species because it allows the bacterial population to expand. For many pathogenic bacteria, biofilm dispersal also plays a crucial role in the transmission of bacteria from environmental reservoirs to human hosts, in the transmission of bacteria between hosts, and in the exacerbation and spread of infection within a single host [9].

Although complex and not fully understood (e.g. many bacteria possess redundant means to form biofilms), biofilm formation is an ordered process that is dependent on the response of the cell to environmental cues, which in turn regulates specific genes. Stages of biofilm formation include motility to the surface, attachment, formation of clusters, development of differentiated structures, and dispersal. In *P. aeruginosa,* these stages of biofilm formation depend in part on an ordered response of three two-component systems: BfiSR (biofilm initiation), BfmSR (biofilm maturation) and MifSR (microcolony formation) [14]. Two-component regulators usually sense an environmental signal via a membrane-bound histidine protein-kinase receptor, then communicate that change via phosphorylation of a response regulator. The three two-component regulators are sequentially phosphorylated. Inactivation of BfiSR causes biofilm formation to cease at the stage of irreversible attachment, whereas inactivation of BfmSR and MifSR stop biofilm maturation at later stages ($\sim$24 h and $\sim$72 h, respectively).

Biofilm formation and dispersal are ultimately genetic processes; therefore, they can be manipulated like other genetic systems using synthetic biology tools [15]. As an example, stress increases biofilm formation [16–18]. In Gram-negative bacteria, this occurs via the impact of stress on the concentration of the intracellular second messenger, 3,5-cyclic diguanylic acid (c-di-GMP) (Box 1). High concentrations of c-di-GMP lead to low motility and biofilm formation, whereas low concentrations of c-di-GMP lead to high motility and dispersal [9,19]. Several lines of evidence indicate that this control of c-di-GMP must be elegant and temporal; for most cells to form biofilms, they must interact with the surface, therefore, motility should be high [20]. Indeed, inactivation of diguanylate cyclases YeaI, YedQ and YfiN of *Escherichia coli*, which should reduce c-di-GMP levels, increases initial biofilm formation dramatically (T. Wood, unpublished). In addition, c-di-GMP levels are inversely related to extracellular DNA levels and are reduced by a tyrosine phosphatase [21]. Extracellular DNA is an important matrix component that

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Box 1. c-di-GMP: a ubiquitous signal for controlling biofilm formation

c-di-GMP is an internal messenger that exists in almost all bacteria. It is the central regulator of biofilm formation because it mediates the switch between motile and sessile forms in Gram-negative bacteria [3]. High levels of c-di-GMP increase biofilm formation, whereas low levels reduce biofilm formation. c-di-GMP helps biofilms form by increasing exopolysaccharide production, cell size, and cell aggregation, but decreasing swimming motility and extracellular DNA production [63]. c-di-GMP also binds to the PilZ domains of proteins to regulate exopolysaccharide synthesis, twitching motility, and flagella activity [19]; for example, binding to the PilZ domain of YcgR of *E. coli* leads to decreased swimming motility via reduced flagella activity, which in turn increases biofilm formation. This molecule plays a significant role in biofilm dispersal events as well; decreases in c-di-GMP concentrations induce biofilm dispersal by decreasing exopolysaccharide production and increasing motility [9].

c-di-GMP is synthesized from two GTP molecules by diguanylate cyclase (DGC), and is degraded by phosphodiesterase (PDE) into 5′-phosphoguanylyl-(3′-5′)-guanosine and GMP [19]. There are 30 putative DGCs and PDEs in *E. coli* [63], although many of these proteins have not been studied. The duplication of enzymatic activity (synthesizing or degrading c-di-GMP) is unusual with respect to internal signals (e.g. intracellular cyclic AMP is synthesized by a single adenylate cyclase and degraded by a specific PDE [19]), but is a consistent feature for c-di-GMP in many genomes. One explanation is that DGCs and PDEs are individually activated and inactivated under different conditions, although the propagation of these environmental signals is not understood thoroughly. For example, although BdcA is not a PDE, it binds to c-di-GMP and primarily works toward biofilm dispersal. Consistently, its gene expression is the highest at stationary phase (8 h) [63]. Similarly, we hypothesize that for initial biofilm formation, c-di-GMP levels are low for surface attachment via high motility, and some specific DGCs might be inactivated (or their gene transcription is low), or some specific PDEs (or c-di-GMP-binding proteins) might be activated (or their gene transcription is high). During biofilm formation this gene regulation/protein modification that controls c-di-GMP levels is reversed. Hence, if we can understand the regulation of each DGC/PDE pair, we might be able to manipulate each stage of biofilm development under various growth conditions.

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is required for initial attachment [22] (when c-di-GMP should be low) and for biofilm maturation to develop a mushroom-shaped structure [23]. Hence, c-di-GMP levels should be low for enhanced motility and surface attachment, high for biofilm formation, and then low again for biofilm maturation and dispersal.

Biofilms and disease

Biofilms are important contributors to the state of human disease, considering 80% of bacterial chronic inflammatory and infectious diseases involve biofilms [24]. As the most common infection, biofilms of uropathogenic *E. coli* cause urinary tract infections that result in 8 million visits annually to physicians in the USA [25]. Enterohemorrhagic *E. coli* (EHEC) infections in the gastrointestinal tract are also important, and there are >76 million food-related infections annually in the USA that lead to 325 000 hospitalizations [26], 5000 deaths [26], and an economic cost of up to $US 1426 billion [27]. Furthermore, *P. aeruginosa* is an opportunistic pathogen that is responsible for many biofilm infections, including those associated with ventilator-associated pneumonia, urinary and peritoneal dialysis catheters, bacterial keratitis, otitis externa, burns, and lungs [28]. Another opportunistic pathogen, *Staphylococcus epidermidis*, frequently infects catheter materials; nearly 80% of the cells involved in biomaterial-associated infections are *S. epidermidis* [29].

Biofilms and engineering

The robust nature of biofilms (i.e. their ability to withstand chemical and physical stresses more than their planktonic counterparts [30]) makes them superior for many beneficial biotechnology applications. Their resiliency stems primarily from the physical barrier provided by the matrix to some chemicals [31] and predators, as well as from the physiological heterogeneity [32] caused by concentration gradients, mutation (e.g. enhanced genetic exchange [33]), and stochastic/environment-induced gene expression that leads to dormancy [34]. This sleeping state allows cells to withstand otherwise lethal environments because they are not metabolizing.

Biofilms have been important for over a century for wastewater treatment [35] (Table 1) and for a recent extension of this process known as bioremediation. In bioremediation applications, biofilms are used for the removal of various environmental contaminants (e.g. substituted aromatics, heavy metals) [30] (Table 1) as well as many other processes, including rhizoremediation, where biofilms on the roots of plants degrade chlorinated ethenes [36]. Beneficial biofilms have also been proposed to decrease biocorrosion [37], as well as hold promise for other applications, such as biocatalysis [38], which includes the production of biofuels, specialty/bulk chemicals, biologics, and food additives (Table 1). For biofuels, such as ethanol from *Zymomonas mobilis*, greater productivity has been achieved in biofilms than by planktonic cells [38] (Table 1). Furthermore, biofilms are also important for bioelectrochemical systems, such as microbial fuel cells, where communities form on the surface of electrodes to produce power [39].

Early engineered biofilm systems: population control

To take advantage of the potential of biofilms, their formation must be controlled. The first engineered biofilm for any application was a consortium to reduce the biocorrosion of

Table 1. Representative applications of engineered biofilms

Application	Description	Refs.
Bioremediation	Removing hydrocarbons and heavy metals from the environment.	[30]
Wastewater treatment	Removing organic pollutants and ammonia from wastewater.	[35]
Biocorrosion control	Inhibiting the biocorrosion of steel, copper and aluminum.	[37,40–44]
Biofuels	Producing ethanol with *Z. mobilis*.	[38]
Disease treatment	Reducing EHEC and *Pseudomonas* infections with indole and 7-hydroxyindole.	[52,74]
BioMEMS	Spatially controlling the placement of bacterial 'nanofactories' in a device; synthesizing QS signals or QQ compounds to control cellular behavior.	[71]
Chemicals	Producing specialty and bulk chemicals in a biorefinery.	This review
Pharmaceutical testing	Testing drugs and pro-drugs.	This review

1018 mild steel and 304 stainless steel [40]. *Bacillus subtilis* has only one membrane; therefore, it has been engineered to secrete the human antimicrobial peptide bactenecin to inhibit the growth of the deleterious sulfate-reducing bacterium *Desulfovibrio vulgaris*. Growth of the corrosion-causing *D. vulgaris* was reduced by 36-fold in the biofilm, and corrosion was reduced by 12-fold [37]. This work has been corroborated by using natural bacterial strains, such as *Bacillus brevis* 18-3 and *Bacillus licheniformis*, which produce the antimicrobials gramicidin and γ-polyglutamate, respectively, to: inhibit the corrosion of stainless steel and mild steel by sulfate-reducing bacteria [41] and by iron-oxidizing bacteria [42]; to inhibit the corrosion of copper [43]; and to inhibit the corrosion of aluminum [44] (Table 1). These studies are important in that they have employed a consortium rather than a single species, which demonstrates that bacterial populations can be controlled in biofilms, and that cells can be engineered to achieve a biotechnological aim.

Using a synthetic circuit, an external stressor (UV light) has been applied to control the biofilm formation of a single *E. coli* strain (Figure 1a) [45]. UV light effectively damages DNA, which in turn activates the protease activity of RecA. RecA then cleaves the lambda *c*I repressor, which de-represses *traA*, the conjugation plasmid pilin gene that is under the control of the P_L promoter. Biofilm formation increases as a result of conjugation. This was one of the first studies to show that biofilm formation can be manipulated by an external stimulus, and that 'plug and play' genetic circuits can be developed.

Engineering quorum sensing circuits to control biofilm formation

Bacterial cells communicate via quorum sensing (QS) (Box 2): signals are secreted, their concentrations build extracellularly as a function of cell density, and then they are internalized or detected to coordinate gene expression via response regulators [46]. Building on this understanding, coordinated cell communication has been used to control fluorescence in biofilms between two *E. coli* populations via QS circuits [47]. The circuitry is flexible and should allow for future work with genes – other than those encoding fluorophores – to be manipulated in an analogous fashion [48].

The first synthetic circuit to control biofilm formation via QS utilized indole [49], which inhibits *E. coli* biofilm formation [49–53] in a QS fashion [52]. Based on these fundamental studies, a simple circuit has been built in which the extracellular concentration of indole in a consortium of *E. coli* and *Pseudomonas fluorescens* was altered by cloning toluene *o*-monooxygenase into the chromosome of the pseudomonad. Constitutive expression of the monooxygenase in the pseudomonad served to decrease the concentration of extracellular indole available to *E. coli* by converting it into insoluble isoindigo. The 22-fold reduction in the extracellular concentration of the biofilm inhibitor indole led to an 12-fold increase in the biofilm formation of *E. coli* (Figure 1b) [49], which demonstrated that the level of biofilm formation of a specific member of a consortium can be controlled by varying the concentration of a QS compound.

Protein engineering to control biofilm formation

SdiA (suppress cell division inhibitors) detects *N*-acylhomoserine lactone (AHL) signals [54] – although *E. coli* makes no AHL of its own – and is required for sensing indole [49,53]. Hence, SdiA helps *E. coli* respond to the QS signals of other bacteria, as well as to its own QS signal, indole. Cementing the role of indole in *E. coli* biofilm formation and its relation to SdiA, protein engineering of SdiA has been performed via error-prone PCR [55]. Random mutagenesis of genes is a powerful approach to create proteins with novel functions; with this approach, knowing the structure of the protein is not necessary, because beneficial mutations are found everywhere – both near and far from active sites – and they are hard to find by rational approaches [56]. The key is to develop screening or, even better, selection methods to identify the beneficial mutations that lead to enhanced protein activity [57]. Using this approach, 4457 colonies have been screened for altered biofilm formation to identify an SdiA variant with amino acid replacements F7L, F59L, Y70C, M94K and K153X (Figure 2a); this variant had reduced biofilm formation by 20-fold relative to wild-type SdiA after 24 h owing to an increase in indole concentrations [55]. Another SdiA variant has been identified with four amino acid replacements (E31G, Y42F, R116H and L165Q), which has led to sevenfold higher biofilm formation in the presence of *N*-octanoyl-D,L-homoserine lactone and *N*-(3-oxododecatanoyl)-L-homoserine lactone produced by pseudomonads; this activity is opposite that of wild-type SdiA, which reduces biofilm formation in the presence of AHL signals [55]. Hence, protein engineering might be used to create tools to control biofilm formation. The regulator SdiA is not only converted into a more

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Box 2. Quorum sensing and quenching for controlling biofilms

QS is cell communication using extracellular signals that are secreted during specific growth stages. The external signal is then internalized or detected by surface proteins, which results in changes in the regulation of usually several genes. In effect, the cell is able to respond to changes in population density or changes in hydrodynamics that dictate signal concentrations. Typical signaling molecules include AHL in Gram-negative bacteria, and AI-2 in both Gram-negative and Gram-positive bacteria [46]. AHL signaling is usually species-specific, whereas AI-2 signaling is common to many bacteria, but is not universal. AI-2 increases biofilm formation by enhancing flagellar motility [72].

QS is integral to biofilm formation; therefore, it is possible to interfere or quench QS (QQ) to control biofilm formation, which would be beneficial for biofouling applications. Unlike antimicrobials, QQ compounds (also known as anti-virulence compounds) are utilized to disrupt cell communication without affecting growth; these compounds hold much promise because there should be less Darwinian selection pressure for resistance to them [77]. By disrupting communication, pathogens become more susceptible to the host immune system and build less-effective biofilms. Biofilms of pathogens are notoriously difficult to eradicate with antibiotics. To this end, brominated furanones have been utilized to reduce biofilm formation in *E. coli* via QQ of both AHL- and AI-2-mediated signaling [78]. Also, nitric oxide disperses biofilm formation by disrupting c-di-GMP internal signaling [79], and D-amino acids trigger biofilm dispersal by causing the release of amyloid fibers that link cells together in the biofilm [80].
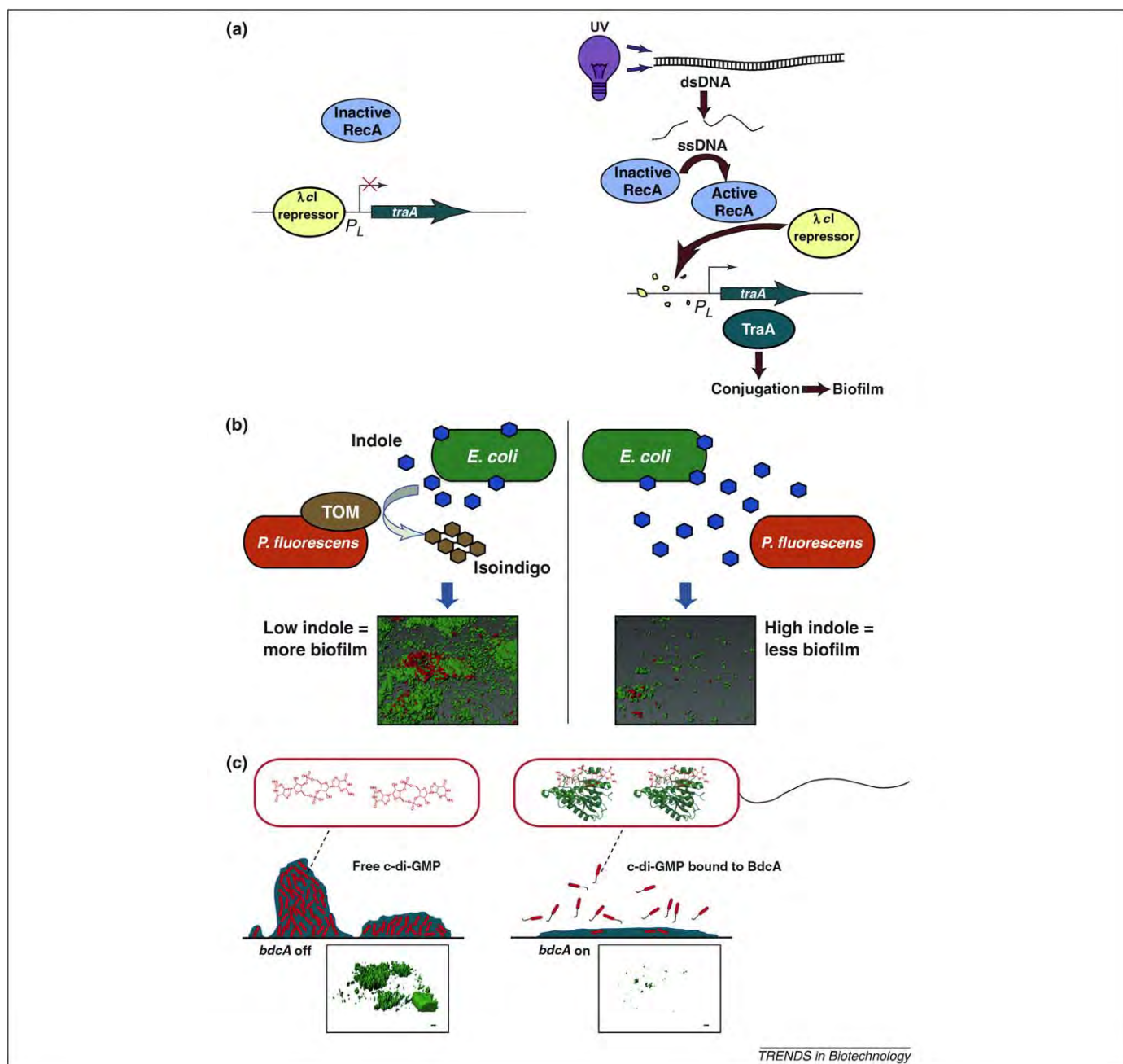
Figure 1. Engineered genetic circuits to control biofilm formation. **(a)** Genetic circuit to control *E. coli* biofilm formation using an environmental signal (e.g. UV light) [45]. Phage λ *cI* repressor silences *traA* transcription by binding its promoter. Application of UV light converts dsDNA into ssDNA, which activates the RecA protease. Active RecA degrades λ *cI*, which induces *traA*. *traA* expression increases biofilm formation by enhancing conjugation. **(b)** Genetic circuit to control dual-species biofilm formation using QS [49]. Toluene *o*-monooxygenase (TOM) is produced by cloning *tomA0A1A2A3A4A5* of *Burkholderia cepacia* G4 into *P. fluorescens* using a constitutive promoter, which converts indole into insoluble isoindigo. Once indole is removed by the pseudomonad, indole no longer inhibits *E. coli* biofilm formation. Representative confocal consortial biofilm images are shown in which *P. fluorescens* is tagged with a red fluorescent protein and *E. coli* is tagged with a green fluorescent protein. **(c)** Genetic circuit to control *E. coli* biofilm dispersal using a c-di-GMP-binding protein [63]. Engineered BdcA variant binds c-di-GMP, which decreases the concentration of free c-di-GMP. Lower c-di-GMP concentrations lead to increased motility and reduced adhesin production, which results in biofilm dispersal. Representative confocal biofilm images are shown in which *E. coli* is tagged with a green fluorescent protein.

sensitive inhibitor of biofilm formation via its own signal indole, but also its activity is reversed via its conversion into a stimulator of biofilm formation in the presence of signals that are produced by other bacteria.

Engineering a global regulator to control biofilm formation

The first engineered regulator to control biofilm formation was the histone-like nucleoid structuring protein (H-NS) of *E. coli* [58]. H-NS is a global regulator that represses transcription by recognizing curved DNA sequences [59,60] and is widely conserved in Gram-negative bacteria [61]. H-NS is very abundant, with more than 20 000 copies per cell [60], and consists of three domains: N-terminal oligomerization, C-terminal DNA-binding, and a flexible linker [59]. Using error-prone PCR, 2104 mutants have been screened for altered biofilm formation, and H-NS variant K57N has been identified as a mutant that reduces biofilm formation 10-fold compared to wild-type H-NS [58]. Hence, the activity is reversed: wild-type H-NS increases
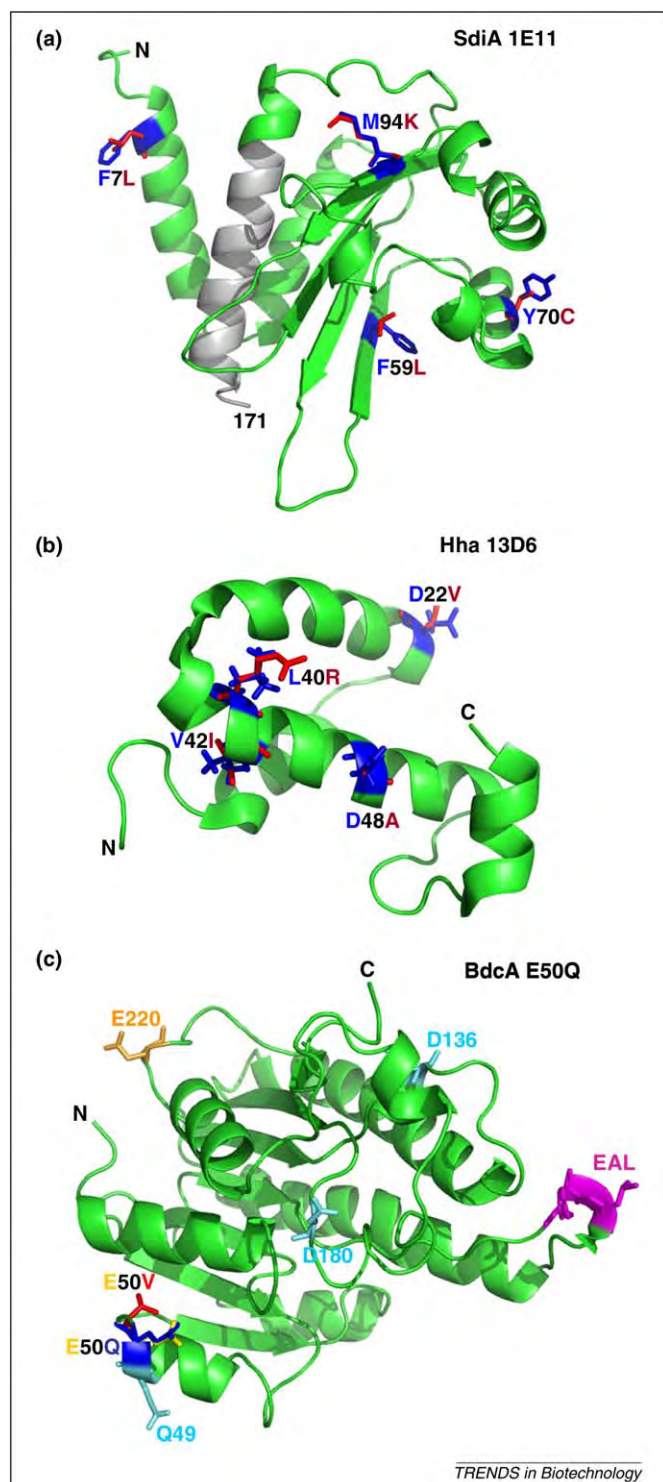
Figure 2. (a) Protein structure of evolved SdiA to decrease biofilm formation [55]. Truncated region (residues 153–171) is shown as a grey ribbon, and substituted residues of SdiA 1E11 (F7L, F59L, Y70C and M94K) are shown in red, whereas the original residues are shown in blue. (b) Protein structure of evolved Hha to disperse biofilms [62]. Substituted residues of Hha 13D6 (D22V, L40R, V42I, and D48A) are shown in red, whereas the original residues are shown in blue. (c) Protein structure of evolved BdcA to disperse biofilms [63]. The E50 residue is indicated in yellow. The replaced valine in the BdcA E50V variant is indicated in red. The replaced glutamine in the BdcA E50Q variant is indicated in blue. The typical c-di-GMP PDE contains EALXR for coordinating Mg^{2+}, Q/R/D/D for c-di-GMP binding, and T/E for catalysis. Here the possible EAL-domain residues are indicated: EAL in pink as part of the EALXR motif; Q49/D136/D180 in turquoise as part of the Q/R/D/D motif; and E220 in orange as part of the T/E motif. The amino and carboxy termini are marked as N and C, respectively.

biofilm formation, whereas H-NS K57N reduces its formation (but does not alter dispersal). H-NS K57N reduces biofilm formation through its interaction with the nucleoid-associated proteins Cnu and StpA, which leads to enhanced excision of one out of nine resident cryptic prophages in *E. coli* – the defective prophage Rac (wild-type H-NS represses excision). Excision of the prophage results in cell lysis through the production of a host killing toxin HokD [58] of the cryptic prophage Qin. A global regulatory system might therefore be evolved through a single amino acid change in the N-terminal oligomerization domain of a protein to control biofilm formation, prophage excision, and programmed cell death. Such tools might be important for helping to remove biofilms.

Engineering regulators to control biofilm dispersal

Arguably one of the loftiest goals related to biofilm formation is to be able to remove existing biofilms, either to treat disease or to control biofilm formation for engineering applications. Recently, two approaches have been attempted, using internal circuits to effect biofilm dispersal: one involves the global regulator Hha (high hemolysin activity) [62] and the other relies on a novel c-di-GMP-binding protein, BdcA (biofilm dispersal via c-di-GMP; formerly YjgI), that titrates c-di-GMP, thereby causing dispersal [63].

Hha regulates 162 loci via its partner, H-NS [64], and has been linked to biofilms. *hha* is induced 30-fold in *E. coli* biofilms [7], and Hha decreases initial biofilm formation by repressing the transcription of both rare codon tRNAs and fimbrial genes [65]. Hha is also toxic and leads to cell lysis and biofilm dispersal as a result of the activation of prophage lytic genes [66] and the induction of the protease ClpXP [65]. Hence, Hha is a global transcriptional regulator in biofilm formation, therefore, it is reasoned that it can be engineered to control biofilm dispersal [62]. Using random mutagenesis of *hha* and a direct screen for biofilm dispersal, Hha variant 13D6 (D22V, L40R, V42I and D48A) has been obtained (Figure 2b). Hha 13D6 causes nearly complete (96%) biofilm dispersal in flow cells by increasing programmed cell death [62]. Importantly, it induces dispersal without affecting initial biofilm formation.

Unlike Hha 13D6, BdcA has been engineered to cause biofilm dispersal by decreasing the concentration of second messenger c-di-GMP [63] (Figure 1c). Once c-di-GMP levels are reduced, genetic cascades are influenced, thus leading to phenotypic changes such as increased motility and decreased adhesin formation (e.g. curli or cellulose) [19]. As a c-di-GMP-binding protein, BdcA exhibits pleiotropic effects, such as reduction in exopolysaccharide, cell length and aggregation, and increases motility and extracellular DNA [63]. BdcA was discovered during a biofilm screen of uncharacterized genes related to the protein TqsA that exports the bacterial signal AI-2 [67]; the *bdcA* mutant was found to be deficient in biofilm dispersal [63]. Hence, it was reasoned that BdcA positively regulates biofilm dispersal. ^{31}P-NMR spectroscopy has been used to show that BdcA binds to c-di-GMP, but does not act as a phosphodiesterase [63]. To increase biofilm dispersal, protein engineering has been used to evolve BdcA for greater c-di-GMP binding [63]. A novel screen of 6000 mutants has been developed that relies on the increase in swimming motility that

occurs as c-di-GMP levels are decreased [63]. The best BdcA variant has the single amino acid change E50V (Figure 2c), which causes fourfold greater biomass dispersal in flow cells than the wild-type BdcA. Saturation mutagenesis has been used to increase biofilm dispersal further by testing all 20 possible amino acid substitutions at this position [68], because a single mutation randomly placed in codons generates, on average, only 5.6 out of 19 possible substitutions, and multiple mutations in a single codon are rare [69]. Using this approach, BdcA E50Q was identified (Figure 2c), which causes near-complete removal of biofilms in flow cells (18-fold better than wild-type BdcA), without affecting initial biofilm formation. Hence, a second genetic switch has been created based on a novel c-di-GMP-binding protein that might be used to remove biofilms after they are formed. c-di-GMP is used by nearly all bacteria, therefore, this approach might be useful in many strains.

Engineering bacteriophages to control biofilm dispersal

Bacteriophages have also been engineered to control bacterial biofilm dispersal. Dispersin B from *A. actinomycetemcomitans*, an enzyme which degrades the biofilm adhesion β-1,6-*N*-acetyl-D-glucosamine, has been produced in T7 phage, which resulted in nearly 100% dispersal of an *E. coli* biofilm [70]. *dspB* was placed under control of the T7φ10 promoter so that it was transcribed during infection. This demonstrates that combining a polysaccharide-degrading enzyme with normal phage lytic mechanisms is more effective than using an unmodified phage. Different bacteria utilize different biofilm extracellular polymeric building blocks (some cells utilize more than one building block), and phages are usually very strain-specific, therefore, several enzymes and several types of phage are probably required to disperse multi-species biofilms. However, the approach holds promise for removing one strain specifically in a multi-species biofilm.

Targeting gene expression and patterning in biofilms

Targeting gene expression within biofilms is desirable for certain biotechnological applications, such as tumor-specific anticancer therapies and quorum quenching (QQ) measures (Box 2). One successful approach in targeting gene expression has been to anchor cells at specific locations where chitosan has been previously deposited in a biological microelectromechanical systems (bioMEMS) device, to create bacterial 'nanofactories' that are targeted to a specific location, assemble, synthesize the QS signal AI-2, and detect AI-2 [71]. Pfs (*S*-adenosylhomocysteine nucleosidase) and LuxS (*S*-ribosylhomocysteinase) convert *S*-adenosylhomocysteine to AI-2; therefore, a (His)$_6$-protein G-LuxS–Pfs-(Tyr)$_5$ fusion protein has been used to target cells specifically to areas where chitosan has been deposited via the Tyr tag. Protein G helps to assemble *E. coli* cells via an antibody against *E. coli* that binds to the fusion protein, and GFP has been used to detect the AI-2 signal. Hence, spatially selective synthesis, capture and manipulation of a QS signal that is known to increase biofilm formation [72] can be achieved within a bioMEMS device (Table 1). This opens doors to future studies that involve the impact of AI-2 on biofilm formation, as well as for the synthesis of QQ compounds at specific locations; that is,

within biofilms, to thwart pathogens using truly novel therapies.

Another approach to direct biofilm development to specific regions includes the creation of regions that resist biofilm formation. Using self-assembled monolayers on gold film that have been terminated with D-mannitol, biofilm formation has been controlled for *E. coli*, *P. aeruginosa*, and *Candida albicans* for up to 26 days [73]. This achievement allows gaps at the micrometer scale to be formed between biofilms without a physical barrier, such that gene expression can be studied as a function position relative to the gap. Also, bacterium–surface and bacterium–host interactions might be explored.

Future prospects

To engineer is to control. Therefore, an overarching goal is to discern the complete genetic basis of biofilm formation and dispersal so that this information can be used to control these communities of associated cells for biotechnological applications (Table 1). The studies highlighted in this review demonstrate that once individual biofilm regulatory circuits are understood, biofilm formation and dispersal can be manipulated.

Biofilms can be formed in a spatially selective manner; cells can be engineered to secrete compounds and QS signals to affect the behavior of neighboring cells in a consortium; and biofilms can be dispersed. Further refinements in this nascent field include the need to demonstrate that biofilms can be generated and removed as desired (i.e. a reversible process), which will facilitate the re-use of platforms. In addition, it should be demonstrated that beneficial cells can be engineered to displace or integrate into an existing biofilm. This might allow treatment of disorders in the complex gastrointestinal tract, and allow one to prevent colonization by pathogens, such as EHEC and *P. aeruginosa*, by secreting compounds known to inhibit these strains [52,74] (Table 1). The introduction of beneficial biofilms will also facilitate engineering applications, such as the reduction of biocorrosion.

Along with spatiotemporal control of biofilm formation and dispersal, gene expression might be controlled as a function of depth at a single position. This could perhaps be achieved by using oxygen-responsive promoters, because oxygen levels will drop to zero at the base of the biofilm in an aerobic environment. This depth-controlled expression can be monitored with new techniques, such as direct RNA sequencing [75] of cells taken from specific positions using laser capture microdissection [76]. As a result, biofilm synthetic capabilities might be optimized as a function of depth at each position. Synthetic biology and protein engineering approaches can be utilized to make these depth-controlled circuits. The ability to harness biofilms in a spatiotemporal manner via engineered cellular communication could allow the creation of sophisticated reactor systems. Such reactor systems could lead to formation of bio-refineries on large and small scales in which plant-derived feedstocks are converted into a wide array of products, thereby making simultaneous use of the growth compounds and metabolites produced from complex feedstocks.

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