Controlling Biofilms Using Synthetic Biology Approaches

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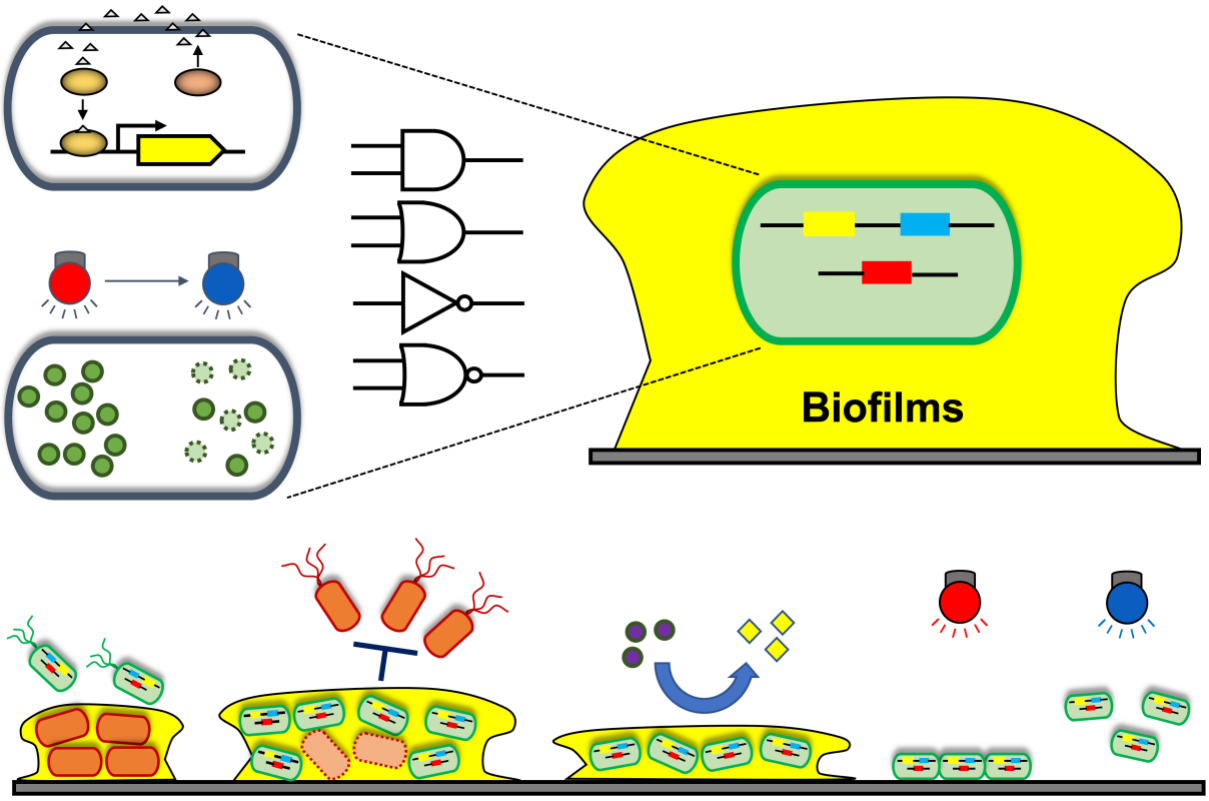
ABSTRACT

Bacterial biofilms are formed by the complex but ordered regulation of intra- or inter-cellular communication, environmentally responsive gene expression, and secretion of extracellular polymeric substances. Given the robust nature of biofilms due to the non-growing nature of biofilm bacteria and the physical barrier provided by the extracellular matrix, eradicating biofilms is a very difficult task to accomplish with conventional antibiotic or disinfectant treatments. Synthetic biology holds substantial promise for controlling biofilms by improving and expanding existing biological tools, introducing novel functions to the system, and re-conceptualizing gene regulation. This review summarizes synthetic biology approaches used to eradicate biofilms via protein engineering of biofilm-related enzymes, utilization of synthetic genetic circuits, and the development of functional living agents. Synthetic biology also enables beneficial applications of biofilms through the production of biomaterials and patterning biofilms with specific temporal and spatial structures. Advances in synthetic biology will add novel biofilm functionalities for future therapeutic, biomanufacturing, and environmental applications.

Keywords: biofilm control, synthetic biology, genetic circuit, protein engineering, quorum sensing, quorum quenching

Graphical Abstract



Synthetic biology can enable the eradication of harmful biofilms and the development of beneficial biofilms.

1. Introduction

Biofilms are sessile microbial aggregates resulted from cooperation and competition between microbes (Dang and Lovell, 2005; Elias and Banin, 2012; Nadell et al., 2016) within a self-produced matrix of extracellular polymeric substances (EPS) composed of polysaccharides, proteins, lipids and nucleic acids that enhance surface adherence and microbial aggregation (Costa et al., 2018; Flemming and Wingender, 2010). Typically, biofilm formation causes detrimental effects in various areas including industrial manufacturing (Xu et al., 2017), the environment (Beech and Sunner, 2004; Scheerer et al., 2009), food safety (Zhao et al., 2017), and health (Miquel et al., 2016). Many chronic infections are closely related to the biofilm state (Costerton et al., 1999; Lebeaux et al., 2014), and bacterial colonization of medical devices and implants such as catheters, contact lenses, mechanical cardiac valves, and dental implants can lead to device-related infections (Costerton et al., 2005; Stoodley et al., 2013). Biofilms formed on industrial production lines, heat exchangers, and working surfaces lead to corrosion and damage to machinery, as well as contamination of raw materials and products (Jia et al., 2019; Y. Li et al., 2018). In addition, biofilms formed in food processing facilities can contaminate food products (Brooks and Flint, 2008; Galié et al., 2018), contributing to foodborne outbreaks (Srey et al., 2013).

Biofilms serve to protect bacteria from antimicrobial agents by forming physical barriers composed of EPS that reduce the diffusion of toxic compounds and by slowing bacterial growth inside the biofilms, which mitigates the efficacy of antimicrobial agents (Mah and O'Toole, 2001). Although mechanical brushing and cleaning can effectively remove biofilms from accessible surfaces (Berger et al., 2018; González-Rivas et al., 2018), it is difficult or impossible to access biofilm-colonized surfaces in many cases. For example, biofilms on indwelling medical devices (Khatoon et al., 2018), industrial pipes (Liu et al., 2014), and food processing equipment (González-Rivas et al., 2018) are not easily accessible and require advanced physical, chemical, and biological methods for eradication. Advanced physical methods, such as pulsed electric (del Pozo et al., 2009; Khan et al., 2016), pulsed light (Garvey et al., 2015), magnetic (Geilich et al., 2017; H. Park et al., 2011), sonication (Baumann et al., 2009; Bjerkan et al., 2009), and cold plasma (Abramzon et al., 2006; Gilmore et al., 2018) approaches, have been used to remove or destroy surface biofilms. Chemical treatments, including the use of surfactants (Percival et al., 2017; Simões et al., 2005; Splendiani et al., 2006), disinfectants [*e.g.*, chlorine (Kim et al., 2008; Lee et al., 2011) and hydrogen peroxide (Lin et al., 2011; Lineback et al., 2018)], and antibiotics (Ciofu et al., 2017), have also been applied to control biofilms. Biological approaches for biofilm control (Roy et al., 2018) include interfering with signaling pathways via quorum sensing (QS) (*e.g.*, autoinducers) (Boles and Horswill, 2008; Brackman and Coenye, 2014; Hammer and Bassler, 2003; Herzberg et al., 2006; McNab et al., 2003) or secondary messenger molecules [*e.g.*, cyclic di-guanosine monophosphate (c-di-GMP)] (Arora et al., 2015; Barraud et al., 2015; Valentini and Filloux, 2016), inhibiting stringent

responses [*e.g.*, alarmone (p)ppGpp] (Chávez de Paz et al., 2012; de la Fuente-Núñez et al., 2014), dispersing extracellular polymeric components by enzymatic disruption (Powell et al., 2018; Xavier et al., 2005), cleaving peptidoglycan [*e.g.*, transglycosylase (Stapleton et al., 2007) and endolysin (Shen et al., 2013)], and altering the membrane potential or permeabilization [*e.g.*, lantibiotics (Mathur et al., 2018) and polymyxins (Lima et al., 2019; S. C. Park et al., 2011)]. Furthermore, surface materials with anti-biofilm coatings (Cattò and Cappitelli, 2019) and smart antibacterial surfaces (X. Li et al., 2018) have been developed for anti-biofilm strategies. Various anti-biofilm compounds, including natural products [essential oils (Jafri et al., 2019) or fatty acids (Marques et al., 2015; Thibane et al., 2010)] and synthesized nanoparticles (Allaker, 2010; Mi et al., 2018), have been investigated for the inhibition or dispersion of biofilms.

Synthetic biology is the intersection of biology and engineering and has been harnessed to engineer commensal and probiotic bacteria as genetically programmable sensors and drug delivery devices (Bradley et al., 2016; Duan et al., 2015; Maxmen, 2017) and incorporate synthetic metabolic pathways to produce useful chemicals ranging from biofuels, foods, and pharmaceuticals in the form of microbial consortia (Carocho and Ferreira, 2013; Jia et al., 2016; Volke and Nikel, 2018). Tools employing synthetic biology approaches are also used to investigate the organization of biofilms, uncover the mechanisms of actions of anti-biofilm agents and design strategies to combat biofilms (Brenner and Arnold, 2011; Hong et al., 2012; Hwang et al., 2017). By understanding the formation of microbial consortia, we can design and engineer microbial ecosystems for biomedical, industrial and biotechnological purposes. Recent seminal reviews have summarized the synthetic biology tools used to engineer microbial communities (Bittihn et al., 2018; Jia et al., 2016; Kong et al., 2018). Here, we specifically focus on reviewing synthetic biology tools and strategies to eradicate and engineer biofilms.

2. Biofilms and Signaling Molecules

2.1 Biofilm development and persistence

Biofilms develop through the cellular processes of initial reversible and irreversible attachment, microcolony formation, and maturation. When biofilms become sufficiently mature, single planktonic cells are dispersed from the biofilms (Costerton et al., 1999) (**Fig. 1A**). Biofilm development involves the regulation of hundreds of biofilm-specific genes including those related to stress responses, QS, motility, cell-surface appendages, metabolism, and transport (Domka et al., 2007). Biofilm communities release diverse inter- and intra-cellular signaling molecules that directly affect the population and dynamic structure of biofilms (Giaouris et al., 2015; Karatan and Watnick, 2009). These bioactive compounds range from small signaling molecules known as autoinducers, D-amino acids, and metabolites, to higher-order proteins that mediate bacterial interactions (Karatan and Watnick, 2009; Kostakioti et al., 2013). As

a result, biofilms are quite robust and frequently require costly, repetitive physical and chemical treatment applications for removal. Biofilms are typically treated through the external addition of disinfectants or antimicrobials, unless physical debridement, such as mechanical brushing, is used (Berger et al., 2018; González-Rivas et al., 2018). However, external biocide addition shows very limited efficacy, mainly because of the mass transfer limitation in complex biofilms mixed with EPS, as well as the non-metabolizing nature of the cells inside biofilms (Anderson and O'Toole, 2008), which survive under high concentrations of antibiotics. Compared with planktonic counterparts, biofilms are 10- to 1,000-fold more resistant to various antimicrobials (Davies, 2003). Therefore, novel approaches are required to eradicate biofilm bacteria.

2.2 Regulation of biofilm formation via signaling molecules

Diverse signaling molecules are involved during bacterial biofilm formation (**Fig. 1B**). QS is a cell-cell communication process in bacteria mediated by the production and detection of extracellular chemicals known as autoinducers (Popat et al., 2015; Waters and Bassler, 2005). QS allows bacteria to coordinate their gene expression in a population-driven manner. Acyl-homoserine lactones (AHLs) are a major autoinducer signal mediating QS in Gram-negative bacteria (Papenfort and Bassler, 2016). The LuxI/LuxR system of *Vibrio fischeri* is known as a classical AHL QS system (Fuqua et al., 1994) (**Fig. 1C**). LuxI synthesizes the autoinducer *N*-(3-*oxo*-hexanoyl)-*L*-homoserine lactone (3*o*C6HSL), and LuxR forms a complex with 3*o*C6HSL, resulting in broad gene expression activation (Fuqua et al., 2001; Kumar and Rajput, 2018). In contrast, Gram-positive bacteria use modified oligopeptides as autoinducers, which are detected by membrane-bound two-component signaling proteins that transduce information via a series of phosphorylation events (Kleerebezem et al., 1997). The *agr* (accessory gene regulator) system of *Staphylcoccus aureus* is an example of a QS system in Gram-positive bacteria (Queck et al., 2008) (**Fig. 1D**). *agrD* encodes a propeptide possessing the autoinducing peptide (AIP) signal sequence (Zhang et al., 2002). The propeptide is processed by cleavage of the N-terminal signal peptide by *S. aureus* signal peptidase B (SpsB) and C-terminal tail by AgrB, and the mature AIP is then secreted into the extracellular environment (Kavanaugh et al., 2007). Sensor transmembrane histidine kinase AgrC and its cognate response regulator AgrA constitute a classical bacterial two-component signal transduction system. Once AIP binds to AgrC, AgrA is phosphorylated and subsequently binds to P2 and P3 promoter regions. This enables RNAII production that further triggers AIP synthesis, along with induction of RNAIII that regulates genes related to virulence, biofilm formation, and other processes (Koenig et al., 2004; Queck et al., 2008). Such QS signaling plays an important role in biofilm formation (Boles and Horswill, 2008). In *V. cholerae* and *S. aureus*, increased cell density inhibits biofilm formation (Boles and Horswill, 2008; Hammer and Bassler, 2003), while activation of QS circuits (two LuxI/R-type QS circuits, LasI/R and

RhlI/R) in *Pseudomonas aeruginosa* stimulates biofilm formation (Duan and Surette, 2007). Autoinducer-2 (AI-2) is a species-nonspecific autoinducer produced by both Gram-negative and Gram-positive bacteria (Schauder and Bassler, 2001). It is synthesized by *S*-ribosylhomocysteine lyase (LuxS), which converts *S*-ribosylhomocysteine to homocysteine and (*S*)-4,5-dihydroxy-2,3-pentanedione (DPD). DPD is then processed into AI-2 molecules (Xavier et al., 2007). AI-2 was studied in regulations of intra- and inter- species biofilms. In the environment of dental plaque, hundreds of bacterial species constitute mixed-species biofilms, and *Streptococcus gordonii* is a main colonizer among them as AI-2 production from *S. gordonii* on teeth induces the consecutive colonization by other bacteria such as *Porphyromonas gingivalis* (McNab et al., 2003). The addition of AI-2 leads to an increase in biofilm formation in *E. coli* (Herzberg et al., 2006). In addition, AI-2 from *Klebsiella pneumoniae* could promote its early biofilm formation (Balestrino et al., 2005).

Another common signaling molecule is c-di-GMP, a ubiquitous second messenger present in almost all bacteria. c-di-GMP is the central regulator of biofilm formation, as it mediates the switch between the motile and sessile forms of bacteria (Valentini and Filloux, 2016). c-di-GMP is synthesized from two guanosine-5'-triphosphate molecules by diguanylate cyclases (DGCs), and is degraded into 5'-phosphoguanylyl-(3'-5')-guanosine and guanosine monophosphate by phosphodiesterases (PDEs). Various microorganisms are reported to express multiple DGC and PDE enzymes (Hengge, 2009; Römling et al., 2013; Sondermann et al., 2012). This enzymatic redundancy might be beneficial to bacteria through each enzyme's specific activation and inactivation in response to different environmental conditions.

Indole is an intercellular signaling molecule produced from tryptophan by the enzyme tryptophanase TnaA (Lee and Lee, 2010). Indole has diverse roles including in spore formation, plasmid stability, drug resistance, biofilm formation, and virulence in indole-producing bacteria. The effect of indole on biofilm formation is controversial. Indole was initially reported to enhance biofilm formation in *E. coli* S17-1. However, indole inhibits biofilm formation in nine nonpathogenic *E. coli* as well as the pathogenic *E. coli* O157: H7 strain. Indole was recently reported to repress persister cells, which are metabolically dormant cell populations (J. H. Lee et al., 2016).

2.3 Regulation of biofilm dispersal via signaling molecules

Nitric oxide (NO) is a simple gas and a biological signaling molecule found to induce biofilm dispersal across a wide range of bacterial species (Arora et al., 2015; Barraud et al., 2015). Because of the broad-spectrum anti-biofilm effects of NO, NO-releasing materials and prodrugs have also been explored (Barraud et al., 2012; Hetrick et al., 2009). Increased understanding of the role of NO in biofilm formation through its regulation of intracellular c-di-GMP concentrations, QS, and cellular nitrogen

metabolism has helped reveal the action mechanism of known drugs and identify novel targets for drug development (Rinaldo et al., 2018). NO sensors such as H-NOX (heme-nitric oxide/oxygen binding) or NosP (nitric oxide sensing protein) affect biofilm formation by regulating c-di-GMP concentrations and QS (Hossain and Boon, 2017; Rinaldo et al., 2018). Understanding H-NOX and NosP mechanisms in bacteria could lead to better control of bacterial biofilms and biofilm-related infections (Williams et al., 2018).

Natural amino acids predominantly participating in protein synthesis are in the L-form, while D-amino acids are found in the cell walls of bacteria. Recently, D-amino acids have been demonstrated to act as regulatory signals for cell wall remodeling and biofilm disassembly (Cava et al., 2011; Kolodkin-Gal et al., 2010). In living organisms, D-amino acids are synthesized by the action of racemases that convert amino acids from L-form to D-form (Tanner, 2002). D-amino acids disperse biofilms by interfering with the anchoring of amyloid fibers that link biofilm cells together (Kolodkin-Gal et al., 2010; Oppenheimer-Shaanan et al., 2013) and prevent biofilm formation by altering the cell wall composition (Bucher et al., 2015). Furthermore, mixtures of D-amino acids have been shown to promote biocide treatments against biofilm communities in a water-cooling tower (Jia et al., 2017) and to reduce biofilms in dental unit waterlines (Ampornaramveth et al., 2018). Due to the distinctive mechanisms and biological roles of D-amino acids (Aliashkevich et al., 2018), the application of D-amino acids is an appealing anti-biofilm approach, either alone or in combination with established antimicrobials.

It should be noted that during biofilm formation, the synthesis and degradation of inter- and intra-cellular signaling molecules are regulated in response to key environmental factors such as temperature (Lee et al., 2008; Lee and Lee, 2010; Townsley and Yildiz, 2015), pH (Chopp et al., 2003; Lee and Lee, 2010), osmotic pressure (Hengge, 2008; Valverde and Haas, 2008), and nutrient conditions (Stanley and Lazazzera, 2004). Hence, signaling molecules are excellent candidates for controlling biofilm formation and eradication.

3. Synthetic Biology Approaches

With an enhanced understanding of biofilms (Flemming et al., 2016) and a growing synthetic biology toolkit (Bittihn et al., 2018; Brenner et al., 2008; Jia et al., 2016), the ability to control biofilms (Wood et al., 2011) continues to expand. An important strategy in controlling biofilms is based on the ability of molecules produced inside biofilms to bypass the mass transport barriers created by extracellular polymeric substances, thereby reaching concentrations sufficiently high to regulate target biofilms. This approach may address numerous biofilm-associated challenges in environmental, agricultural, industrial, and medical areas. The biofilm eradication strategies using protein engineering and synthetic biology are summarized below.

3.1 Protein engineering of biofilm-controlling enzymes

Protein engineering is a potential strategy to enhance the activity of global regulator proteins related to biofilm formation (**Fig. 2A**). H-NS (histone-like nucleoid structuring protein) represses transcription by recognizing curved DNA sequences and was the first engineered regulator used to control biofilm formation without signaling molecules (Hong et al., 2010b). The variant H-NS K57N was found to reduce biofilm formation, showing an opposite function compared to the biofilm-promoting activity of wild-type H-NS (Hong et al., 2010b). Another global regulator Hha (high hemolysin activity) was engineered to promote biofilm dispersal, resulting in nearly complete biofilm dispersal (Hong et al., 2010a). Proteins with the ability to bind signaling molecules have been engineered for controlled biofilm formation and enhanced dispersal. *E. coli* does not produce AHLs because it lacks an AHL synthase, but it senses AHL signals through the AHL receptor SdiA, a homologue of LuxR (Dyszel et al., 2010). SdiA was engineered via random and site-directed mutagenesis to regulate biofilm formation in the presence of AHLs or indole (Lee et al., 2009). Like *E. coli*, the foodborne pathogen *Salmonella enterica* does not produce AHL signals but does contain the receptor SdiA for AHL, which regulates *S. enterica* adhesion as well as resistance to host immune responses (Bai and Rai, 2016). BdcA was identified as a c-di-GMP-binding protein and engineered to increase biofilm dispersal through a single amino acid replacement at E50Q (Ma et al., 2011a). In addition, BdcA of *E. coli* was found to control biofilm dispersal in *P. aeruginosa* and *Rhizobium meliloti* (Ma et al., 2011b). Therefore, protein engineering of global regulators or signaling molecule-binding proteins enables enhanced biofilm eradication or can be used to modulate the microbial activity of biofilm formation.

3.2 Synthetic biology for eradicating biofilms

3.2.1 Quorum sensing genetic circuits

Bacterial QS systems have been important components of a wide variety of engineered biological devices. Autoinducers are useful as input signals because they diffuse freely in liquid media and penetrate cells easily (Choudhary and Schmidt-Dannert, 2010). Because the engineered cells synthesize their own QS signals, they are able to self-monitor cell density and modulate their activities without oversight (Hong et al., 2012; Ryan and Dow, 2008). Synthetic QS circuit systems have great potential in that population-driven QS switches may be utilized to develop synthetic genetic networks for a variety of applications such as to engineer bidirectional communication, construct a predator-prey ecosystem, and create a synthetic symbiotic ecosystem (Wood et al., 2011). The LasI/R and RhlI/R pairs, the two best-characterized QS systems of *P. aeruginosa*, have been widely used for synthetic genetic circuits. LasI produces the autoinducer molecule, *N*-(3-*oxo*-dodecanoyl)-*L*-homoserine lactone (3*o*C12HSL), which is

sensed by LasR. Likewise, RhlI produces *N*-butyryl-*L*-homoserine lactone (C4HSL) that is sensed by RhlR (Pesci et al., 1997). For biofilm formation, the RhlI/R QS system was utilized to demonstrate important roles for self-organization and aggregation in a synthetic biofilm consortium. The LasI/R system in combination with the engineered biofilm-dispersal enzymes Hha and BdcA showed excellent biofilm displacement upon sensing QS signals (Hong et al., 2012). In this system, the second biofilm (disperser) is grown in the existing biofilm (colonizer), and QS signaling molecules are produced by LasI and accumulate inside the dual-species biofilm. The QS molecules form a complex with LasR, which triggers dispersal of the colonizer biofilm through increased c-di-GMP levels mediated by the BdcA variant (**Fig. 2B**). Then, the disperser biofilm can be disrupted by inducing the Hha variant with a chemical switch, resulting in cell death in the biofilm. The synthetic QS circuit was applied to prevent membrane biofouling and/or to degrade environmental pollutants (Wood et al., 2016). This beneficial biofilm was able to limit its own thickness on wastewater treatment membrane by secreting and sensing the signaling molecule controlling c-di-GMP levels mediated by the BdcA variant. In addition, the engineered biofilm also prevented biofilm formation by deleterious bacteria through NO generation and was able to degrade the environmental pollutant epichlorohydrin via epoxide hydrolase. Thus, the use of this beneficial biofilm enabled the development of a living biofouling-resistant membrane system. The QS circuit systems for controlling biofilms can provide insights into how beneficial biofilms can be developed to prevent or eradicate deleterious biofilms for various applications.

3.2.2 Quorum quenching enzymes

Finding ways to subvert microbes by interfering with their communication signals is important for combating antibiotic resistance and other biofilm-related situations (Marx, 2014). Quorum quenching (QQ) is the mechanism by which QS is inhibited or interrupted. One strategy here is to process, modify or degrade the signaling molecules that are required for cellular communication, thereby preventing the buildup of biofilms (Grandclément et al., 2016). The majority of QQ studies have focused on hydrolysis of N-acyl homoserine lactones using lactonases that break down lactone rings in AHLs along with acylases that cleave acyl groups (Oh and Lee, 2018) (**Fig. 2C**). Bacterial or enzymatic QQ has been applied for antifouling strategies in membrane bioreactors (MBRs) for wastewater treatment (Oh and Lee, 2018). For example, AHL-producing bacteria on the surface of membrane were decreased by recombinant *E. coli* producing lactonase AiiA from *Bacillus thuringiensis* (Oh et al., 2012) and AiiO from *Agrobacterium tumefaciens* (Oh et al., 2017). Production of EPS and expression of genes related to microbial attachment and agglomeration were found to be reduced with enzymatic QQ treatment (Kim *et al.*, 2013). *Rhodococcus erythropolis* W2 was used to degrade AHLs via both its oxido-reductase and AHL-acylase activities (Uroz et al., 2005). Because AI-2 signaling molecules are secreted by both Gram-

negative and Gram-positive bacteria, targeting AI-2 for QQ is another useful strategy. LsrK (*luxS*-regulated kinase) that phosphorylates AI-2 is considered to be a QQ enzyme, as purified LsrK with added ATP significantly decreased the AI-2 signaling of *S. typhimurium*, *E. coli,* and *V. harveyi* (Roy et al., 2010). Farnesol, a chemical compound secreted from *Candida albicans,* is effective in repressing AI-2 synthesis and mitigating biofouling in MBRs (K. Lee et al., 2016). Metagenomic approaches have been applied to find a system for modifying AI-2 (Weiland-Bräuer et al., 2016). An indigenous bacterium *Acinetobacter* sp. DKY-1 was found to inactivate AI-2 by secreting a hydrophilic AI-2 QQ compound with a molecular weight of less than 400 Da, but the mechanistic details remain to be determined (Lee et al., 2018). QQ enzymes or chemical compound production systems integrated into synthetic genetic circuits would enable elaborate control of biofilm prevention and eradication.

3.2.3 Bacteriophages

Bacteriophages can penetrate the inner layers of biofilms because phage depolymerases can degrade EPS components (Azeredo and Sutherland, 2008). Single-type phages (Curtin and Donlan, 2006; Pires et al., 2011) as well as multi-phage cocktails (Fu et al., 2010; Sillankorva et al., 2010) have been applied for biofilm destruction or inhibition. Bacteriophages have great potential for engineering as antimicrobial agents, vehicles for drug delivery and vaccines, and the assembly of new materials (Pires et al., 2016). Synthetic biology has been used to develop reinforced bacteriophages that can efficiently kill deleterious biofilm cells by introducing biofilm-degrading or -inhibiting enzymes or enhancing antibiotic penetration. For example, a T7 phage was engineered to produce the biofilm-degrading enzyme dispersin B (DspB) during phage infection (Lu and Collins, 2007). *dspB* from *Actinobacillus actinomycetemcomitans* was integrated into the phage genome under the T7 φ10 promoter, leading to *dspB* transcription by the T7 RNA polymerase upon phage infection of *E. coli* TG1 biofilms. Along with cell killing by the phages, DspB simultaneously attacked the biofilm matrix by hydrolyzing the biofilm-promoting adhesin β-1,6-N-acetyl-D-glucosamine of *E. coli*. The engineered enzymatic phage reduced the *E. coli* biofilm by 2 orders of magnitude compared to the wild-type non-enzymatic phage treatment. A bacteriophage was also designed to increase the antibiotic susceptibility of biofilm cells (Lu and Collins, 2009). The M13mp18 phage, a modified non-lytic filamentous M13 phage, was engineered to contain *csrA* that encodes a biofilm repressor CsrA with or without *ompF* that encodes a porin for quinolone penetration (**Fig. 2D**). Infection with the engineered phage enhanced the antibiotic ofloxacin's bactericidal effect, resulting in more effective killing of the biofilm as well as planktonic cells compared to unmodified phage treatment (Lu and Collins, 2009). In order to overcome the narrow substrate specificity of biofilm-degrading enzymes (*e.g.*, DspB), a QQ enzyme was integrated into bacteriophage that was more effective in

inhibiting mixed species biofilms by disrupting AHL signals (Pei and Lamas-Samanamud, 2014). Lactonase AiiA from *Bacillus sp.* cleaves the lactone rings of diverse AHLs (Wang et al., 2004). A T7 bacteriophage was engineered to express *aiiA* controlled by the T7 φ10 promoter, and this QQ phage treatment was effective in inhibiting *P. aeruginosa* and *E. coli* dual-species biofilm formation via both cell lysis and AHL degradation (Pei and Lamas-Samanamud, 2014). Genetically engineered phages will be further developed by integrating novel biofilm inhibitory functions.

3.2.4 Probiotics

Probiotics are beneficial microbes that enhance host immunity (Hill et al., 2014) and inhibit pathogens (Ohland and MacNaughton, 2010). Probiotic bacteria also have the ability to inhibit biofilm formation (Fang et al., 2018; Shao et al., 2019; Woo and Ahn, 2013). Due to their beneficial health effects, probiotics have been considered as an engineering host for human therapeutic application. *E. coli* Nissle 1917 strain (EcN) is one of the best characterized probiotics and has been used for the clinical treatment of intestinal disorders (Heselmans et al., 2005; Schultz, 2008; Sonnenborn and Schulze, 2009) and engineered for enhancing live biotherapeutics such as tumor detection (Ozdemir et al., 2018), hyperammonemia treatment (Kurtz et al., 2019), and as a drug delivery vehicle (Mckay et al., 2018). For biofilms, wild-type EcN has the ability to inhibit biofilm formation of pathogenic and non-pathogenic *E. coli* (Fang et al., 2018; Hancock et al., 2010) as well as the Gram-positive pathogens *Staphylococcus aureus* and *S. epidermidis* in co-cultures (Fang et al., 2018). EcN was engineered to sense, kill, and inhibit pathogenic biofilms for preventing *P. aeruginosa* gut infection in *Caenorhabditis elegans* and mouse models (Hwang et al., 2017) (**Fig. 2E**). The *alr* and *dadX* genes in the EcN genome were knocked out to enable the mutant EcN strain to become a D-alanine auxotroph, which stabilized retention of the plasmid expressing *alr*. The engineered EcN contained a synthetic genetic circuit. In response to the QS molecule 3*o*C12HSL from *P. aeruginosa*, the engineered EcN produced E7 lysis protein to open the host cell, S5 pyocin to kill *P. aeruginosa*, and DspB to degrade the biofilm matrix. The engineered EcN with anti-microbial and anti-biofilm enzymes disrupted the existing biofilm and prevented biofilm formation of *P. aeruginosa* (Hwang et al., 2017). Taken together, synthetic genetic circuits can enhance the prophylactic and therapeutic activities of probiotics against biofilm-forming pathogens.

3.3 Synthetic biology for engineering biofilms

Although the elimination of deleterious biofilm cells is crucial, biofilms may have beneficial potential if their pattern, thickness, composition, and metabolism can be controlled in a tunable, spatial, and temporal manner. Engineered biofilms can be applied for bioremediation (Brune and Bayer, 2012; Mangwani et al., 2016), wastewater treatment (Karadag et al., 2015; Lewandowski and Boltz, 2011),

biocorrosion control (Jia et al., 2019; Morikawa, 2006; Narenkumar et al., 2016; Zuo, 2007), biofuel production (Heimann, 2016; Hoh et al., 2016), specialty and bulk chemical biorefinery (Rosche et al., 2009; Wang et al., 2017), biomedical microelectromechanical systems (bioMEMS) devices (Fernandes et al., 2010), and pharmaceutical testing (Stewart, 2015). Synthetic genetic circuits and signaling can facilitate the design and development of such biofilm control systems.

3.3.1 Biofilm patterning

Biofilm formation requires complex gene regulation processes (Domka et al., 2007) that are difficult to manipulate when attempting to generate a desired structure or pattern. An optogenetic module was developed for microprinting biofilms (Huang et al., 2018; Ryu et al., 2017). Light-activated diguanylate (BphS) that synthesizes c-di-GMP under near-infrared light (Ryu and Gomelsky, 2014) and phosphodiesterase (BlrP1) that hydrolyzes c-di-GMP under blue light (Barends et al., 2009) were used to bidirectionally regulate c-di-GMP levels. Near-infrared light (632 nm) illumination increased the level of c-di-GMP, resulting in attachment of the cells to a cover glass surface, while blue light (434 nm) decreased the level of c-di-GMP to allow detachment. Dual-color illumination enabled biofilm patterning with a high spatial resolution (Huang et al., 2018) (**Fig. 3A**). Another biofilm patterning utilized the expression of membrane adhesion proteins in response to blue light (Jin and Riedel-Kruse, 2018). *E. coli* was engineered to contain a light-activated transcriptional promoter (pDawn) that optically controls the expression of an adhesin gene (Ag43). Upon blue light illumination, biofilm formation was increased and optically patterned with a 25 μm spatial resolution. Furthermore, a photoswitchable interaction between nMag and pMag proteins (Kawano et al., 2015) was also developed to control bacterial adhesion (Chen and Wegner, 2017) (**Fig. 3B**). pMag protein was produced on the surface of *E. coli* in the presence of blue light to allow the engineered strain to adhere to the immobilized nMag protein on the material surface. This adhesion was reversible. The binding was released in the dark, allowing tunable and biorthogonal control (Chen and Wegner, 2017). The ability to maintain biofilm levels at a desired thickness is important for bioremediation and bioproduction (Zhang and Poh, 2018). The CRISPRi/dCas9 system was applied to control the expression of the *wcaF* gene involved in the synthesis of colanic acid, a key EPS component in *E. coli* biofilm formation. Depending on the level of the guide RNA (gRNA) controlled by a chemical inducer, *wcaF* gene expression was regulated by gRNA-dCas9 binding to the chromosomal *wcaF* locus. Temporal induction resulted in different levels of biofilm thickness. When the circuit was combined with the blue light-mediated expression system, biofilm thickness could be controlled by switching the light. Furthermore, production of the antimicrobial peptide nisin was utilized to achieve robust and tunable spatial structures (Kong et al., 2017). The external nisin gradient resulted in no fluorescence or cell death at a low nisin concentration, fluorescence induction without killing the cells at

377 medium nisin level, and cell death without fluorescence at a high level of nisin, which created band-pass
378 patterns. Mixed nisin producer and responder species generated dynamic spatial structures consistent with
379 the computational model (Kong et al., 2017).
380
381 **3.3.2 Biomaterial production**
382 Biofilms can be developed as a biological platform for producing self-assembling functional materials
383 (Nguyen et al., 2014) (**Fig. 3C**). Biofilm-Integrated Nanofiber Display (BIND) was developed to produce
384 engineered amyloid protein CsgA, a major component of the curli fibrils of *E. coli* biofilms. The
385 engineered CsgA containing functional peptide domains was self-assembled upon secretion and
386 facilitated nanoparticle biotemplating, substrate adhesion, and site-specific protein immobilization on the
387 BIND system (Botyanszki et al., 2015; Nguyen et al., 2014). The same amyloid protein was applied to
388 create environmentally switchable conductive biofilms by using an inducible synthetic riboregulator
389 circuit and interfacing the self-assembled curli fibrils with inorganic materials such as gold nanoparticles
390 to introduce an electro-conductive property (Chen et al., 2014). 3D printing of bacteria was used to create
391 biofilm-based functional materials for bioremediation and biomedical applications (Schaffner et al., 2017).
392 Patterned biofilms were demonstrated by engineered curli production on the 3D-printed *E. coli*
393 (Schmieden et al., 2018). Synthetic biology will guide the engineering of self-assembled polymer
394 production and direct the assembly of patterned biofilms (Majerle et al., 2019).
395
396 **4. Perspective**
397 Intra- or inter-species phenomena occur in mixed-species biofilms, which exhibit dynamic
398 interactions among bacteria (Giaouris et al., 2015). The cooperative interactions between biofilm bacterial
399 species are achieved through cell-cell communication, metabolic cooperation, or spatial organization
400 (Elias and Banin, 2012). However, there are also competitive interactions regarding nutrient uptake,
401 occupation of spatial resources, or with the production of anti-biofilm agents (Giaouris et al., 2015).
402 Synthetic biology approaches can help understand and engineer such cooperative and competitive
403 behaviors among different bacterial species in biofilms. Studies on the beneficial characteristics of
404 probiotic bacteria in inhibiting deleterious biofilms are growing (Fang et al., 2018; Hager et al., 2019;
405 Wasfi et al., 2018). Ribosomally-synthesized antimicrobial proteins such as pyocins (Oluyombo et al.,
406 2019; Smith et al., 2011) or colicins (Brown et al., 2012; Jin et al., 2019, 2018; Rendueles et al., 2014)
407 that exhibit target-specific bacterial killing could be used with probiotics to eradicate harmful biofilms
408 without affecting the overall beneficial or commensal microbial consortia.
409 Biofilms with higher productivity and tolerance to toxic inhibitors can serve as microbial cell
410 factories (Berlanga and Guerrero, 2016) for producing chemicals such as ethanol (Todhanakasem et al.,

411 2014), acetone, butanol (Förberg and Häggström, 1985), and succinyl acid (Urbance et al., 2004).
412 Coordinating synthetic biofilm communities is becoming more important in industrial biochemical
413 production (Berlanga and Guerrero, 2016). The morphology and spatial organization of catalytic biofilms
414 must be programmed along with engineering of their metabolic pathways for biochemical production
415 (Volke and Nikel, 2018). A 3D printing approach combined with synthetic genetic controls will enhance
416 the design and assembly of synthetic biofilm catalysts.

417 In addition to bacterial biofilms, fungal biofilms on implanted devices and on epithelial and
418 endothelial surfaces can cause recurrent infections with increased drug resistance (Desai et al., 2014;
419 Kernien et al., 2018). *Candida*, *Aspergillus*, and *Cryptococcus* are the most prominent clinically relevant
420 fungi involved in the resilience of fungal biofilms to host immunity (Kernien et al., 2018). Antimicrobial
421 peptides naturally found in living organisms can effectively treat fungal biofilms without eliciting an
422 immune response. For example, histatin-5 (Hst-5) from human saliva is an antifungal peptide that can
423 inhibit the growth of *Candida albicans* (Baev et al., 2002) but has limited antifungal activity due to its
424 rapid degradation at the site of action (Moffa et al., 2015a). Recently, liposome encapsulation has enabled
425 the prolonged delivery of Hst-5 (Zambom et al., 2019), and the design of proteolysis-resistant peptides
426 has been shown to stabilize Hst-5, resulting in enhanced antifungal activity (Ikonomova et al., 2019,
427 2018), which may be applied for the control of fungal biofilms (Moffa et al., 2015b). In contrast to
428 harmful fungal biofilms, some fungal biofilms are beneficial. For example, the formation of fungal–
429 bacterial biofilms on the plant root promotes plant growth by supplying essential nutrients and providing
430 plant growth-promoting substances (Gentili and Jumpponen, 2006; Herath et al., 2015). Such symbiotic
431 relationships between plants and microbes, including fungi and bacteria (Goh et al., 2013; Hassani et al.,
432 2018), have resulted the development of biofilmed biofertilizers, presenting a viable alternative for
433 chemical fertilizers in agriculture (Zakeel and Safeena, 2019). Despite the need to control fungal biofilms
434 in medical, industrial, and agricultural applications, synthetic biology techniques for fungal cells are still
435 in the early developmental stages (Hennig et al., 2015). Fungal QS (Albuquerque and Casadevall, 2012)
436 and pheromone communication (Hennig et al., 2015) may be attractive targets for modulating fungal
437 biofilms.

438 Signaling molecules exhibit some drawbacks in the control of biofilms and thus require further
439 improvement. As mentioned above, QS molecules have been widely utilized in synthetic biology
440 (Choudhary and Schmidt-Dannert, 2010; Hong et al., 2012; Ryan and Dow, 2008), as signals produced in
441 the host cell can bind to receptors of the target cell, resulting in population-driven responses (Popat et al.,
442 2015; Waters and Bassler, 2005). However, QS signal production and detection are strain-specific
443 (Hawver et al., 2016); therefore, it is difficult to apply QS circuits to target non-model strains or species
444 that have different QS systems or that lack QS signal recognition, which commonly arise in real-world

situations. In contrast, c-di-GMP is a nearly ubiquitous bacterial signal (Hengge, 2009; Römling et al., 2013; Sondermann et al., 2012) that regulates biofilm formation, but it acts intracellularly (Valentini and Filloux, 2016). This lack of signal diffusion to other cells limits the development of a c-di-GMP genetic circuit and the corresponding control strategy to the host cells. Nitric oxide (NO) signaling in nitrogen metabolism is involved in c-di-GMP metabolism (Rinaldo et al., 2018), and NO production can be triggered by the external addition of chemicals (Barraud et al., 2012; Hetrick et al., 2009) to modulate c-di-GMP production in a broad range of bacteria. Therefore, combining ubiquitous c-di-GMP regulation and strain-specific QS systems will enable the development of a broad spectrum of synthetic genetic circuits for the control of complex biofilms. Additionally, bioactive phytochemicals found in natural products, such as green tea leaves (Qais et al., 2019) and medicinal plant extracts (Shukla and Bhathena, 2016), that exhibit broad-spectrum QS and biofilm inhibition may be integrated in the development of biofilm-controlling genetic circuits. Furthermore, models of the effects of signaling molecules in biofilm communities (Abisado et al., 2018; Emerenini et al., 2015; Frederick et al., 2011) can aid in the design and validation of synthetic biological circuits for effective biofilm control.

5. Conclusion

Control of biofilms, including their eradication and utilization, has been hampered due to insufficient knowledge of biofilm development and the limitations of biological toolkits. Recent investigations of biofilm physiology and synthetic biology advancements can facilitate fine control of biofilms, resulting in the efficient eradication of deleterious biofilms without the use of antibiotics and beneficial utilization of engineered biofilms. However, such synthetic biology approaches for controlling biofilms remain in the early stages. Rather than a single gene or signaling molecule, multiple factors contribute simultaneously or in series at the different stages of biofilm development. Hence, multi-stage and multi-target strategies may be required to achieve the desired level of biofilm control, which will be enabled by mimicking native biofilm formation and dispersal processes. Growing sets of synthetic biology tools as well as continued investigations into biofilm regulation will provide insights for biofilm-controlling strategies and their application in medical, food-processing, agricultural, industrial, and environmental fields.

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FIGURE LEGENDS

Figure 1. Biofilm formation and signaling. A) Biofilm developmental stages: **1**) attachment, **2**) cell-to-cell adhesion, **3**) proliferation, **4**) maturation, and **5**) dispersal. **B**) Signaling molecules involved in biofilm formation: acylhomoserine lactone (AHL), autoinducing peptide (AIP), autoinducer-2 (AI-2), cyclic di-guanosine monophosphate (c-di-GMP), indole, and nitric oxide (NO). **C**) Gram-negative quorum sensing (QS). In *V. fischeri*, LuxI synthesizes 3*o*C6HSL (AHL). LuxR forms a complex with AHL, and the complex activates target gene expression. **D**) Gram-positive QS. In *S. aureus*, AgrD is processed to form AIP. Upon sensing AIP, AgrC phosphorylates AgrA, which in turn induces RNAIII production. RNAIII activates or inhibits target gene expression.

Figure 2. Biofilm cell killing and eradication. A) Protein engineering via random or site-directed mutagenesis to induce biofilm dispersal. **B**) Synthetic QS genetic circuit to enable biofilm displacement. LasI in the green cell produces AHL, and the LasR/AHL complex in the red cell induces biofilm dispersal [biofilm images from (Hong et al., 2012)]. **C**) Quorum quenching to disrupt AHL. Lactonase hydrolyzes lactone rings, and acylase cleaves acyl groups, which inhibits biofilms. **D**) Engineered bacteriophage for biofilm cell killing via enhanced antibiotic penetration along with biofilm inhibition via induction of the biofilm-inhibiting enzyme CsrA. **E**) Engineered probiotic strain to sense and kill pathogen biofilms. Colin E7 lysin (E7) disrupts the probiotic host cells, pyocin S5 (S5) kills *P. aeruginosa* in biofilms, and dispersin B (DspB) degrades the biofilm matrix.

Figure 3. Biofilm utilization for patterning and biomaterial production. A) Optogenetic biofilm patterning using light-switchable c-di-GMP regulation. BphS activated by near-infrared light synthesizes c-di-GMP, while BlrP1 activated by blue light degrades c-di-GMP, resulting in biofilm formation and dispersal, respectively [biofilm images from (Huang et al., 2018)]. **B**) pMag on the microbial surface and nMag on the material surface form heterodimers with blue light. **C**) Engineered microbe produces the self-assembled curli nanofiber CsgA with a variable peptide domain, which confers new functions.

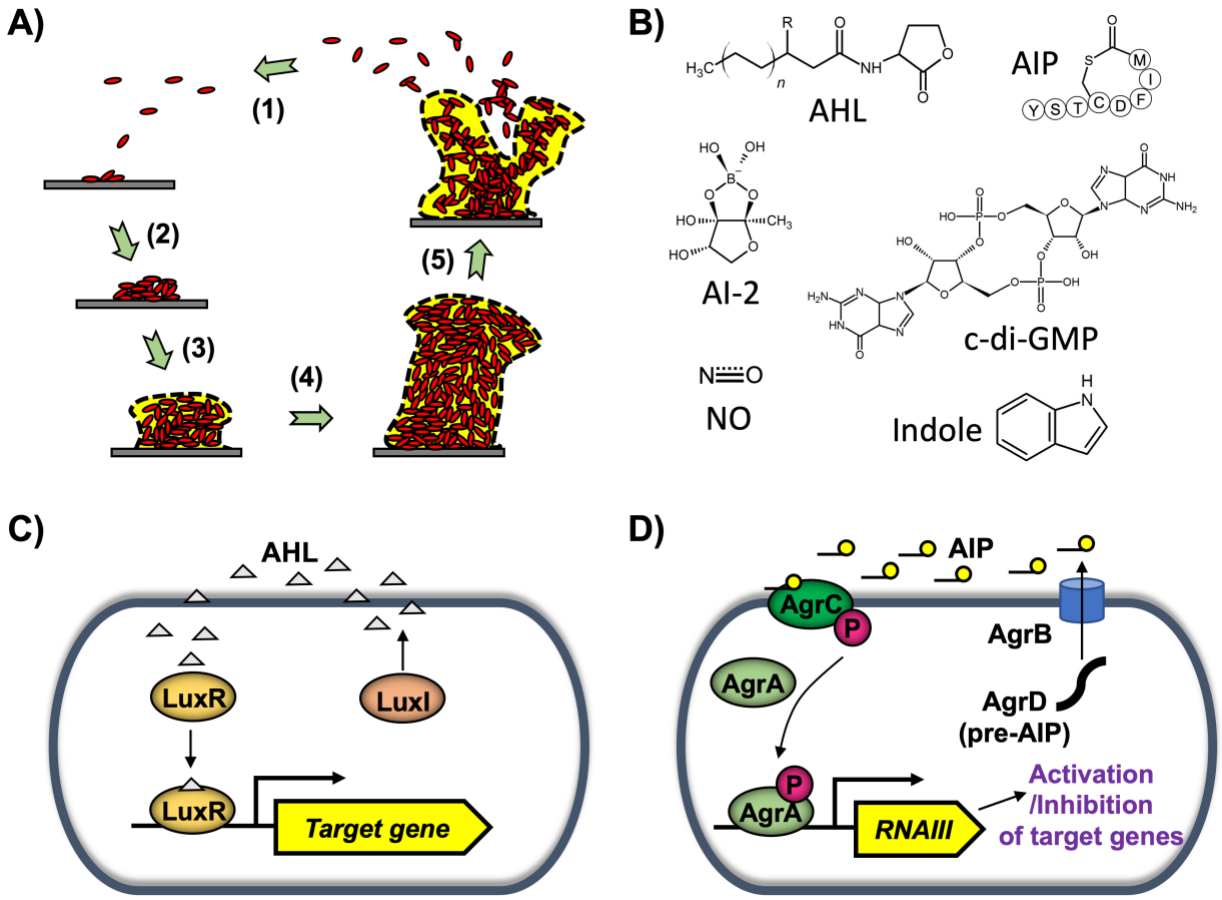
A)
(1)
(2)
(3)
(4)
(5)
B)
AHL
AIP
AI-2
c-di-GMP
NO
Indole
C)
AHL
LuxR
LuxI
LuxR
Target gene
D)
AIP
AgrC
P
AgrB
AgrA
AgrD
(pre-AIP)
P
AgrA
RNAIII
Activation
/Inhibition
of target genes

Figure 1.

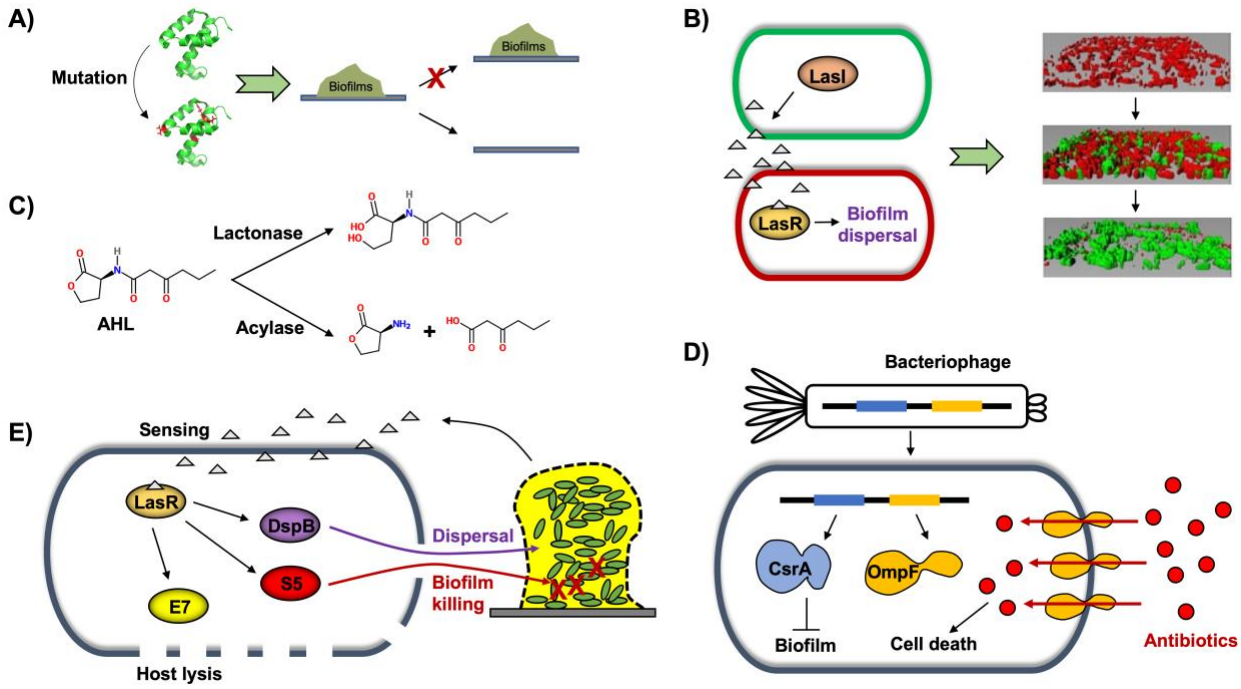
A)
Mutation
Biofilms
Biofilms
C)
Lactonase
AHL
Acylase
B)
LasI
LasR
Biofilm dispersal
D)
Bacteriophage
CsrA
OmpF
Biofilm
Cell death
Antibiotics
E)
Sensing
LasR
DspB
S5
E7
Dispersal
Biofilm killing
Host lysis

Figure 2.

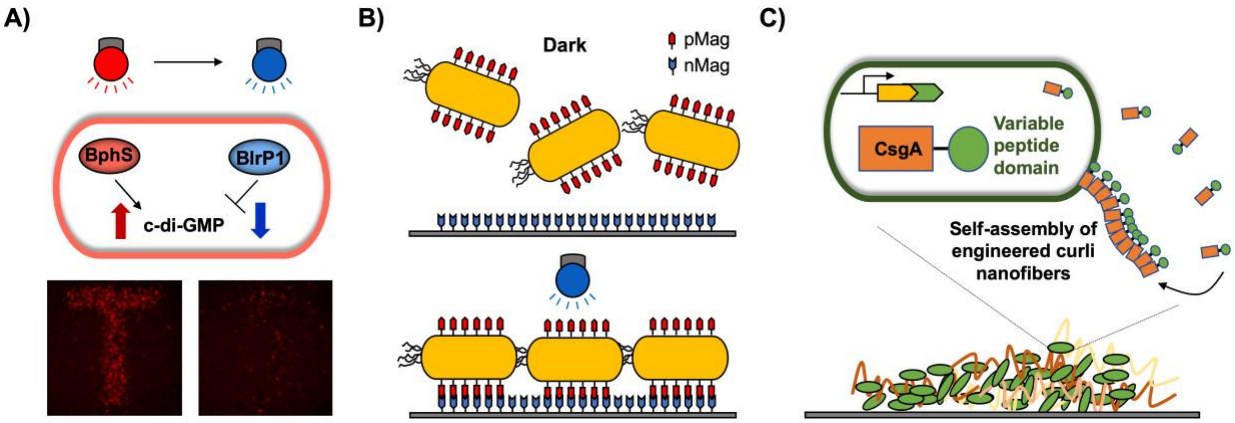
A)
BphS
BlrP1
c-di-GMP
B)
Dark
pMag
nMag
C)
CsgA
Variable peptide domain
Self-assembly of engineered curli nanofibers

Figure 3.