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Synthetic biology in biofilms: Tools, challenges, and opportunities

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Abstract

The field of synthetic biology seeks to program living cells to perform novel functions with applications ranging from environmental biosensing to smart cell-based therapeutics. Bacteria are an especially attractive chassis organism due to their rapid growth, ease of genetic manipulation, and ability to persist across many environmental niches. Despite significant progress in bacterial synthetic biology, programming bacteria to perform novel functions outside the well-controlled laboratory context remains challenging. In contrast to planktonic laboratory growth, bacteria in nature predominately reside in the context of densely packed communities known as biofilms. While biofilms have historically been considered environmental and biomedical hazards, their physiology and emergent behaviors could be leveraged for synthetic biology to engineer more capable and robust bacteria. Specifically, bacteria within biofilms participate in complex emergent behaviors such as collective organization, cell-to-cell signaling, and division of labor. Understanding and utilizing these properties can enable the effective deployment of engineered bacteria into natural target environments. Toward this goal, this review summarizes the current state of synthetic biology in biofilms by highlighting new molecular tools and remaining biological challenges. Looking to future opportunities, advancing synthetic biology in biofilms will enable the next generation of smart cell-based technologies for use in medicine, biomanufacturing, and environmental remediation.

KEYWORDS
bacteria, biofilms, synthetic biology

1 | INTRODUCTION

Bacteria are readily modifiable chassis organisms with diverse biochemical repositories of genes and proteins that could be leveraged for synthetic biology. However, much of bacterial synthetic biology remains focused on a handful of domesticated and planktonic bacterial species that have been optimized for the laboratory.[1] As a result, deploying these engineered bacteria into key target environments remains challenging since these cells experience heterogeneous conditions that result in non-optimal performance and an inability to persist in the environment.[2] Bacteria in natural environments predominately reside in the context of densely packed multicellular communities known as biofilms.[3] Biofilms account for nearly 80% of all bacteria on the planet, occupying environments that span from miles underneath the ocean floor to inside of the human gastrointestinal tract.[4] Bacteria within biofilms can undergo significant shifts in gene expression and participate in emergent social behaviors including division of labor and coordinated growth.[5-7] These processes enable collective organization and the formation of macroscopic structures that enable more efficient distribution of resources and mechanical resilience.[8,9] Furthermore, these bacteria facilitate population-level coordination though cell-to-cell signaling such quorum sensing and ion channel-mediated communication.[7,10,11]

Due to their prevalence in nature and innate emergent properties, biofilms present synthetic biology the attractive opportunity to deliver and operate engineered gene circuits in a range of desired target

environments, such as soil and the microbiome. More generally, collective organization has been a long-coveted goal for the field of synthetic biology and tapping into the native capabilities found in biofilms may enable the next generation of spatiotemporally controlled gene circuit designs. This review provides a brief overview of recent developments toward synthetic biology in biofilms, with focuses on molecular tools, biological challenges, and potential opportunities for engineered biofilms (Figure 1).

2 | TOOLS FOR SYNTHETIC BIOLOGY IN BIOFILMS

Inspired by the biology of natural microbial communities and biofilms, synthetic biology is shifting from engineering single cells and model species to engineering microbial consortia that may be composed of multiple species.[12-16] To realize this shift, the field will benefit from new tools that (a) expand the synthetic biology toolset toward non-model and undomesticated bacterial species; (b) harness optogenetics to control and coordinate densely packed native cellular populations; and (c) functionalize the biofilm extracellular matrix (ECM) to control the spatial and temporal arrangement of bacteria within the consortia. These tools will further enable the effective deployment of engineered bacteria into natural target environments by harnessing the unique physiology of biofilms.

2.1 | Expansion of genetic tools toward non-model biofilm species

While domesticated bacterial strains can be used to prototype new synthetic designs in the lab, deploying these cells into nature remains a challenge as they experience heterogeneous environmental conditions that can impact cellular fitness.[2] To address this shortcoming, recent efforts have focused on expanding the synthetic biology toolbox toward new bacterial species beyond *Escherichia coli*. A foundational set of characterized promoters, ribozyme binding sites, and protein degradation tags was created for biofilm-forming *Bacillus subtilis*.[17] This was further expanded to include more inducible promoters and integration vectors for delivering DNA into the *B. subtilis* chromosome at specific sites such as the sacA and amyE loci.[18] The RSF1010 replicon was used to create a parts library that can be used to assemble broad-host-range plasmids in species of Proteobacteria that commonly colonize the bee gut.[19]

While the number of genetic parts for synthetic circuits is increasing, these parts continue to be created on a per-species basis. For engineering multispecies communities such as microbiomes in the soil or mammalian gut, there remains a need to broadly transform microbial systems in place using native microbial consortia. To address this need, bacterial conjugation has recently been leveraged to efficiently deliver DNA into undomesticated bacteria across a broad spectrum of species. During conjugation, a host cell attaches a pilus to a recipient allowing the direct cell-to-cell transfer of DNA and homologous recombination integrates this DNA into the recipient genome.[20] Engineered *E. coli* was able to deliver biosynthetic gene clusters into the chromosomes of bacteria species across multiple phyla, using conjugation to transfer DNA, a transposon system to integrate a landing pad into the recipient chromosome, and Lac-T7 expression system to tightly control expression of BGCs in the recipient.[21] The IncPα-family RP4 conjugation system enabled an *E. coli* donor strain to transfer a synthetic cassette into both Gram-negative and positive microbiota species in a mouse gastrointestinal tract.[22] Integrative and conjugative elements *B. subtilis* were engineered as to allow delivery synthetically designed DNA into the chromosomes of the recipient even under non-ideal conditions such as in the soil.[23]

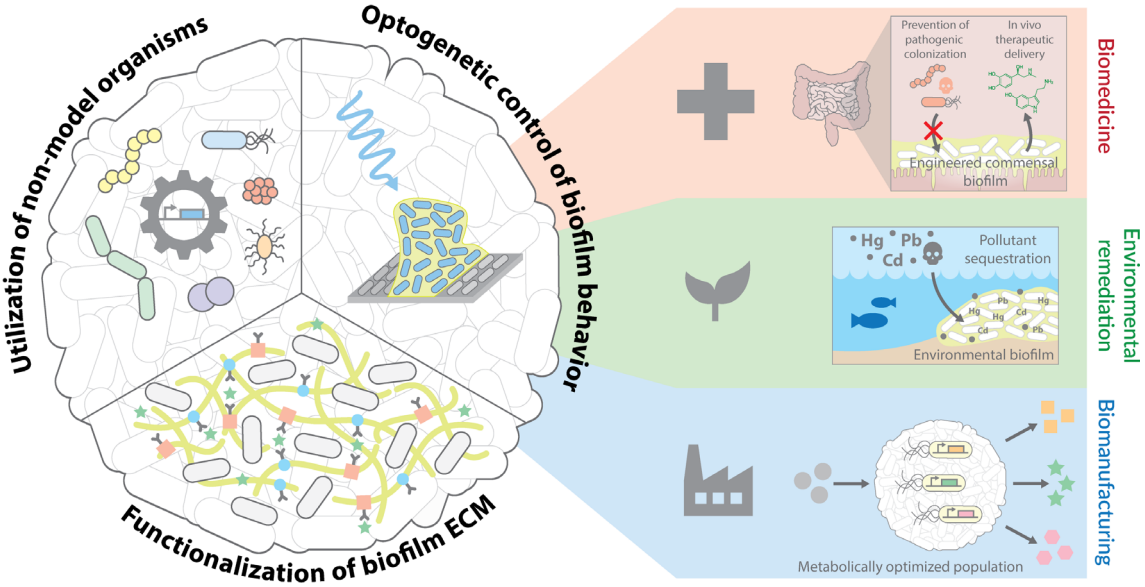

FIGURE 1 Recent tools and potential applications for synthetic biology in bacterial biofilms

The field of synthetic biology has begun to utilize non-model and undomesticated bacterial species though the creation of new genetic parts and broad range genetic transformation methods. Future challenges will include maintenance and containment of these engineered functions in their native contexts, such as soil and the microbiome.

2.2 | Optogenetic control over bacterial biofilm behavior

Most synthetic biology designs utilize small molecule inducers which require sufficient concentration in the environment as well as homogenous diffusion throughout the cellular community. These requirements can be difficult to achieve in conditions of nonideal environmental mixing and dense cellular growth, such as found in biofilms. As a result, the field is moving to leverage optogenetics, where exposure to specific wavelengths of light trigger gene expression on demand in a defined manner. Such an approach would allow control of engineered cells even in complex natural environments where it is not practical to achieve high and uniform inducer concentrations. Considering these challenges, optogenetic approaches may provide dynamic control of spatiotemporal induction that could not be achieved with a small molecule inducer alone.

With precise spatiotemporal light exposure across multiple wavelengths, biofilm cells can be patterned to aggregate in specific patterns with micron precision. Photoreceptors and their associated transcriptional regulators from plants and cyanobacteria have been leveraged to create light-responsive elements that regulate expression of biofilm matrix components and subsequently biofilm structure. Blue light was used to activate the transcriptional promoter pDawn and adhesion gene Ag43 expression, enabling lithography of *E. coli* biofilms.[24] Blue light exposure was also shown to persistently and robustly change the membrane potential dynamics in a *B. subtilis* biofilm, with the effect remaining for hours after the initial stimulus suggesting a form of cellular memory.[25] Near-infrared (NIR) was used to control target gene expression in *E. coli* via NIR-responsive photoreceptor BphP1 and its interacting transcriptional repressor PpsR2.[26] Multiple light-responsive elements have also been integrated together in a single strain. A dual-sensing optogenetic module was installed into *Pseudomonas aeruginosa* to sense both NIR and blue light, regulate intracellular levels of c-di-GMP and pattern biofilm formation based on exposure to each light type.[27] The expression of the chromophore phycocyanobilin (PCB) and PCB-enabled red/green light photoswitchable two-component system allowed for multimodal transcriptional regulation in a *B. subtilis* biofilm.[28] Expression of the *E. coli* matrix protein CsgA fused with various peptide tags were transcriptionally activated via multiple different wavelengths of visible light, allowing for tunable control over *E. coli* matrix production and composition.[29]

Optogenetics has enabled precise spatiotemporal control over bacterial gene expression and biofilm formation, with a growing list of available wavelengths and responsive cellular machinery. Future development will need to address delivery of light to engineered biofilms in natural target locations as well as advancing switching and multiplexing kinetics of light-activated transcriptional regulation.

2.3 | Functionalization of biofilms into engineered living materials

During biofilm formation, individual motile bacteria adhere to a surface and begin to secrete exopolysaccharides, DNA, and proteins to form an ECM that serves as a biofilm scaffold.[8,30,31] In particular, matrix proteins exist play a critical role, providing both macroscopic structure and distinct material properties that dictate cellular organization. Recent efforts are leveraging these proteins to transform biofilms into engineered living materials that can self-organize, regenerate, and interface with inorganic materials.

Biofilm matrix proteins secreted by the cell can self-assemble into long structures that form the basis for the biofilm ECM. These fibrils can be genetically modified to include different functional tags that imbue different material properties. The *E. coli* biofilm amyloid protein CsgA was genetically modified with various peptide domains to create custom fusion proteins that could be produced by a host and self-assemble into ECM.[32] CsgA protein expression across multiple length scales has also been engineered to be driven by inducible gene circuits and quorum sensing, and later used to interface *E. coli* biofilms with inorganic materials such as quantum dots and gold nanoparticles.[33] Further tunable control over CsgA allowed for *E. coli* biofilms to serve as 3D pattered scaffolds for gold nanoparticles, creating resettable living pressure sensors.[34] The *B. subtilis* biofilm matrix amyloid protein TasA was functionalized with the adhesive mussel protein Mefp5 to transform *B. subtilis* biofilms into living and regenerating glues.[35] This strategy has been further expanded to genetically modify TasA with many different proteins and peptide domains, resulting in biofilms with tunable viscoelasticity and hydrogel properties that can be 3D printed into robust and self-healing materials.[36]

Functionalization of biofilm matrix components has begun to transform biofilms into novel living materials with tunable physiochemical properties. Looking to the future, more work will be needed to fully characterize ECM properties over time in a natural target environment, as well as developing increased control over ECM monomer assembly.

3 | CHALLENGES FOR SYNTHETIC BIOLOGY IN BIOFILMS

Compared to their domesticated laboratory counterparts, the genetic and biochemical profiles of cells in the biofilm state often present conditions that are not amenable to current genetic circuit designs. Ongoing challenges that must be considered for engineering biofilms include extracting microscopic and macroscopic measurements among millions of biofilm cells and contending with bacterial cell fate changes that occur during biofilm community development.

3.1 | Measurements of densely packed communities

Biofilms are densely packed communities that contain millions of bacterial cells. The challenge remains to extract high-quality single cell measurements amidst the noise of heterogeneous biofilm cells. Special care must be taken in amplifying desired readouts and understanding the cellular dynamics and heterogeneity of the biofilm population.

Recent methods have begun to address these challenges though use of microfluidics, microscopy, and high-throughput sequencing. Microfluidics have been used to overcome the noisiness arising from dense cellular through constricting biofilm growth to only a few cell layers thick. Using such devices, it has been observed that synthetic microbial consortia can coordinate across great length scales using quorum sensing genetic positive feedback loops.[37] Undomesticated *B. subtilis* biofilms were shown to spatiotemporally oscillate in growth and membrane potential in a microfluidic chamber.[10] Microfluidics have also enabled the study of diffusion-mediated interactions between spatially separate microbial communities.[38] To dissect biofilms with established 3D structure, light-sheet microscopy has been able to dissect *Vibrio cholerae* biofilms and track migration of individual cells within the developing community.[39] Finally, for determining bulk species composition in a community, high throughput sequencing has enabled quantification of relative abundance of microbiota in mucosal and luminal layers of the murine gut.[40] While these methods have provided critical biofilm-related measurements, live and in situ monitoring of biofilms in nonconstrained natural conditions would benefit from further development.

3.2 | Control over bacterial cell fate

The majority of synthetic biology work is performed in bacterial cells during the exponential growth phase in order to take advantage of rapid cell replication and protein turnover. However, outside of the laboratory, most bacteria in nature do not appear to exist in this growth phase, instead transitioning to a variety of cell fates including stationary phase, cell death, and biofilm formation. These cellular fates and the cellular decisions that influence them are intertwined with metabolism and transcriptional networks, resulting in many genes being influenced by cell fate. When engineering biofilms, the cellular commitment to form a biofilm can be convoluted by other cell fate pathways, such as sporulation, dispersal, and localized cell death. This issue is further compounded by the lack of molecular and genetic tools that are designed to work in nonexponential growth phases.

Even within biofilms, not all cells behave similarly in terms of matrix production and motility. In *V. cholerae* biofilms, cells that grew at the biofilm front were transported from a founder population in a fountain-like pattern, whereas the remaining biofilm population near the substrate surface remained relatively immobile.[39] In *B. subtilis* biofilms, motile and sessile cells experience different transcriptional regulation of time spent in a lifestyle, suggesting that cells do not have to fully commit to biofilm formation.[41] Some species, including biofilm-forming *B. subtilis* also possess the propensity to form endospores in lieu of biofilms, resulting in a completely different transcriptomic profile.[42] Additionally, the current set of synthetic biology tools to engineer cells in stationary phase remain in their infancy. While some stationary phase promoters have been discovered and characterized, their numbers remain low and only have seen use in recombinant protein production where cells do not form biofilms.[43] Furthermore, the biological mechanisms occurring during stationary phase are still being elucidated as nongrowing bacteria have been shown to display a low but surprisingly constant protein production rate.[44] Cellular memory of stationary phase can also lead to a heterogeneous population, with the creation of persister cells.[45]

4 | OPPORTUNITIES FOR ENGINEERED BIOFILMS

Bacterial biofilms currently provide benefits for wastewater treatment and microbial fuel cells due to their ability to adhere, densely pack, and persistence in the environment.[46,47] With improved understanding of biofilm biology and creation of new synthetic biology tools, biofilms are poised to advance synthetic biology efforts in medicine, manufacturing, and environmental remediation (Table 1).

4.1 | Cell-based medicine

Within the gastrointestinal microbiota, probiotic and biofilm-forming species have been highlighted in recent studies as critical for healthy gut symbiosis. Engineering biofilms in this context could lead to the creation of new cell-based therapies where engineered bacteria could provide extended diagnostics and therapeutic delivery. Commensal *E. coli* Nissle (EcN) biofilms were engineered to outcompete pathogenic species, such as enterohemorrhagic *E. coli*, *Staphylococcus aureus*, and *Staphylococcus epidermis* through expression and secretion of the protease DegP.[48] Synthetic biosensing modules have created for EcN, allowing engineered strains to colonize the mouse gut, detect the inflammation marker tetrathionate, and genetically record inflammation exposure over months in vivo[49]. The EcN biofilm matrix itself has been employed as a modality to retain engineered cells in the mammalian gut. EcN curli fibrils were fused to the trefoil family of human cytokines and when delivered to a mouse gut, allowed for engineered EcN biofilms to entrain themselves in the mucosal later and influence epithelial cell behavior.[50] Biofilm ECM was also used to coat probiotic *B. subtilis* cells, improving their gut mucoadhesion and bioavailability when delivered to both mouse and swine guts.[51] Other native biofilm-forming species could also serve as powerful tools, as many already interact and influence their host through secreted neuroactive molecules. *Providencia* bacteria living in the gut of *C. elegans* worms were found to modulate host sensory decision via tyramine production.[52] With special care taken to understand and control immunogenicity and behavior, engineered biofilms could act as sentinel organisms in the mammalian gut and even deliver therapeutic payloads.

TABLE 1 Potential applications for synthetic biology in biofilms

Engineered biofilm applications	Description	Current challenges	Enabling synthetic biology tools
Microbiome diagnostics	Biofilms as sentinel organisms in the mammalian gut to sense disease and pathogens	Quantifying readout from biofilm sensor, multispecies cooperation	Broad-spectrum species genetic transformation,[21,22] expansion of standardized genetic parts,[17-19] biofilm ECM functionalization[32-36]
Microbiome therapeutics	Engineered biofilms regulate host microbiome through therapeutic production	Long-term retention of engineered biofilm in gut, stationary phase gene circuit performance	biofilm ECM functionalization,[32-36] expansion of standardized genetic parts,[17-19] engineered production of therapeutic and signaling biomolecules[48,50,51]
Biomanufacturing	Metabolic burden split across multiple cell populations within a biofilm for increased efficiency	Control over intraspecies cell fate, control over interspecies biofilm population distribution	Light-responsive optogenetic biofilm gene circuits,[24-29] broad-spectrum species genetic transformation[21,22]
Novel biomaterials	Biofilm ECM with engineered biochemical properties to enable novel biomaterials	Stationary phase gene circuit performance, scale of material production	Light-responsive optogenetic biofilm gene circuits,[24-29] 3D bioprinting[29,34,36]
Environmental remediation	Removal of heavy metals and hazardous compounds, stored safely in the biofilm matrix	Biosensing of pollutants, long-term control of engineered biofilm	Biofilm ECM functionalization,[32-36] biofilm morphology control, novel biosensing gene circuits[52-54]
Biofouling prevention	Seeding surfaces with engineered biofilms to prevent attachment of microbial species	Long-term control of engineered biofilm, multispecies cooperation	Light-responsive optogenetic biofilm gene circuits,[24-29] biofilm ECM functionalization[32-36]

4.2 | Biomanufacturing

Synthetic biology could enable the development of more efficient methods to synthesize biochemical compounds of interest. One common challenge with expressing multiple enzymes in a pathway is to minimize toxicity and metabolic burden on the host. Biofilms could potentially avoid this issue altogether through division of labor within its population. Such behavior occurs naturally during production of ECM components in *B. subtilis* biofilms, where cells cooperate to produce complementary products and contribute to the public goods pool of matrix.[6] Additionally, the biofilm ECM can be genetically modified to display specific affinities and crosslinking to transform the biofilm itself into a regenerating biomaterial with multimodal properties. Both *E. coli* and *B. subtilis* amyloid fibers where genetically modified to express proteins or peptide domains that allowed for the biofilm matrix to act as a renewable and robust biomaterial.[32,35,36] Additionally, these strategies can be combined in tandem with 3D printing to rapidly and precisely print biofilms into a desired shape. Utilizing division of labor in engineered biofilms will enable the next generation of biotechnologies for manufacturing sophisticated biochemical products and renewable biomaterials.

4.3 | Environmental remediation

The ability to persist in natural environments makes biofilms an ideal platform for deploying engineered bacteria to directly mitigate and treat pollution. Bacterial biofilms already enjoy wide use in wastewater treatment where biofilms break down organic pollutants in controlled ponds. In more natural settings such as waterways and soil, synthetic biology could expand the role of biofilms as platforms for on-site remediation and upstream sequestration of pollutants. Indeed, recent studies have established that biofilms are able to sequester pollutants from their environment. Rare earth elements can be captured by *E. coli* biofilms expressing genetically modified CgsA matrix protein.[53] Heavy metals such as mercury can also be sequestered by biofilms, as *E. coli* biofilms have been engineered to produce CsgA in the presence of mercury, which can immobilize mercury compounds in the fibrils.[54] Toxic halogenated compounds can also be degraded by biofilms, as *Pseudomonas putida* biofilms were engineered to express haloalkane dehalogenases, and this catalytic activity was further enhanced with tunable control over biofilm formation.[55] Biofouling on osmotic membranes have also been mitigated with use of programmable biofilms, as quorum-quenching *E. coli* biofilms were seeded into membrane materials and optogenetically controlled to prevent formation of biofilms from other species.[56]

5 | CONCLUSIONS AND FUTURE PERSPECTIVES FOR SYNTHETIC BIOLOGY IN BIOFILMS

Developing biofilms as next generation synthetic biology chasses holds great promise, yet important challenges remain to realize this vision. Current tools have only begun to address non-model biofilm-forming bacterial species and their complex social behaviors. Spatial heterogeneity and temporal dynamics associated with cell state and species composition in biofilms remain poorly understood.

Furthermore, the environmental persistence of biofilms raises some concern about biocontainment, as biofilms have been associated with chronic infections and biofouling. Despite these challenges, the opportunity remains to co-opt the complex social behaviors of biofilms (e.g., cell-to-cell signaling, division of labor, and matrix production) for medicine, biomanufacturing, and environmental remediation. Additionally, basic scientific study of these processes could provide inspiration for more sophisticated synthetic gene circuits beyond the biofilm context. In addition to intercellular coordination, the physical robustness and environmental persistence of biofilms could enable new living materials and robust deployment of engineered bacteria into target environments. These advances may also prove valuable beyond synthetic biology, impacting fields spanning materials science, ecology, and medicine. Overall, engineering individual bacteria has been instrumental to the advancement of synthetic biology thus far and the field is now poised to leverage bacterial biofilms for next generation synthetic biology applications.

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CONFLICT OF INTEREST

Both authors declare no conflict of interests.

AUTHOR CONTRIBUTIONS

Peter Tran: Conceptualization; writing-original draft; writing-review and editing. **Arthur Prindle**: Conceptualization; supervision; writing-review and editing.

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DATA AVAILABILITY STATEMENT

Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

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