Biofilm architecture: An emerging synthetic biology target

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1.1. Introduction

1.1.1. What are biofilms?

Biofilms are three-dimensional structures of various bacteria that adhere to biotic or abiotic surfaces. Generally, biofilms are founded by single cells or small groups of cells that then divide and differentiate into complex communities [1] with extracellular matrices [2,3] water channels [3] embedded extracellular proteins [4], extracellular lipids [5] and embedded extracellular nucleic acids [6]. Many biofilms also include humic [7,8] and uronic [8,9] acids. Biofilms contribute to bacterial fitness by increasing adherence to various surfaces, protection from predation, desiccation, immune attack, antibiotics, and protection from starvation via carbon storage [10]. Biofilms can contribute to pathogenesis and environmental survival of bacteria [11,12]. Biofilms also can have significantly different structural elements; while Pseudomonas aeruginosa biofilms classically have tall mushroom-like structures, and well-defined water-channels, other bacteria such as Francisella tularensis can have relatively flat and undifferentiated biofilms [13,14]. Being able to regulate the production of biofilm through exogenously added components such as small molecules or anti-biofilm peptides is a topic of significant research in the field [13,15].

Currently, extracellular proteins, carbohydrates and nucleic acids are considered the principal components of biofilm. When these central extracellular components are enzymatically degraded biofilms size can be reduced considerably [16–18]. The substances surrounding the cells in a biofilm are often referred to as extracellular polymeric substances (EPS). While EPS includes all of the extracellular lipids, carbohydrates, protein, and acids associated within the biofilm [19], the majority of current EPS research focuses on the carbohydrate components as they are believed to generally constitute a majority of biofilm biomass [5,20–22]. The actual percent biomass contribution is likely dependent upon the nature of the biofilm being studied. The identity of each sugar component, the mechanism of linkage and the order in which the sugars are joined are highly variable across different species/conditions. These carbohydrate chains contribute considerably to the incredible diversity of biofilms found throughout nature.

These complex biofilm structures are associated with disease states [23–25], biocorrosion [26] and biofouling [27,28]. They are also associated with food production [29] and the maintenance of human health [30]. On a more functional level, biofilms have very different properties than planktonic cells such as increased resistance to antibiotics [31], antiseptics [32], disinfectants [33], protists [34], phages [34], shear force [35], heat [36], desiccation [37] and UV [38,39] as well as additional properties. While not every studied biofilm has each of the above qualities relative to planktonic form, biofilm has nonetheless been established as a unique state. Recently, multiple transcriptomics studies highlighting the difference between biofilm and planktonic cultures have been performed [40–50]. These studies have largely enabled synthetic biologists to understand what exactly constitutes a biofilm and how each of these constituents might be artificially manipulated to optimize the use of biofilms as a production unit.
1.1.2. What is synthetic biology?

Synthetic Biology has been defined by a variety of scientists and engineers from diverse fields [51–54]. We have chosen to use the definition of “the design of life for useful purposes”. Those useful functions can generally be split into one of two categories: life designed in order to study life itself or life designed in order to produce a useful output. These are not necessarily exclusive categories as the latter generally involves pieces of the former in the design-build-test cycle. Important progress has been made in synthetic biology from the single-part standardization [55,56] and optimization [57,58], to the construction of modules/logic gates [59–62], to the engineering of whole systems [63–67]. On the applied science side of synthetic biology, whole systems are being developed to effectively produce specific outputs [68–71]. Since biofilms have additional properties compared to planktonic cells, synthetic biologists are interested in leveraging some of these properties to enhance preexisting biofilm production systems [72–75].

1.1.3. Why monoculture?

Following the traditional reductionist approach, bacterial organisms are generally studied in monoculture. This has the major advantage of reducing the complexity of systems. While these monoculture experiments may not reflect the real world, they do allow understanding of basic principles that can then be applied and modified in a more complex system. Aristotle aptly pointed out a flaw in this approach “The whole is greater than the sum of its parts”. Some of the more complex phenomena of interspecies interaction in a mixed culture will not exist at all in a monoculture system [76], just as some additional properties may be observed in a single type of organism grown as a biofilm as opposed to planktonic cells.

In synthetic biology it is well known that different chassis (organisms) are suited to different production needs. The most important factors to consider are going to be highly dependent upon the users end goal. For protein production, folding and secretion abilities might take precedence in design, whereas for secondary metabolite production, resistance to the produced product may be a primary concern. In addition to inherent biological limitations, factors such as ease of genomic manipulation and culturing may be considered as additional contributors to experimental costs.

1.1.4. Why not monoculture?

While multispecies biofilms are more complicated to create and control, these biofilms have the potential for novel properties unavailable in mono-species biofilms [38] and may have the potential to streamline the production/detector of various products [74]. Multi-species biofilms may be metabolically more efficient as they separate different aspects of a process into unique compartments [77,78]. Mixed biofilms may also be better able to survive environmental challenges [79–83] which may be necessary for synthetic biofilms. Complimentary metabolic waste products might further enhance biofilm maintenance/growth [84]. Different species also have different abilities to retain/ manufacture various chemical compounds such as iron containing magnetosomes [85] or various polyhydroxalkanoates [86] each of which may be useful in a production scenario. Alternatively, additional species allow modular tuning opportunities such as quorum sensing pathways only present in some of the members of the biofilm [87]. Another reason for using mixed species biofilms is the potential for inclusion of difficult-to-culture microorganisms [88–90]. A multispecies biofilm may also be useful to culture vulnerable cells such as L-form cells [91] which may possess desirable qualities for production/secretion [92–94], but lack the ability to outcompete wild type cells [94]. Multispecies biofilms may also have potential to include chassis that normally do not from sizeable biofilms [95]. The addition of novel chassis to synthetic biology is welcome as each new organism contains a set of novel parts ready to be manipulated. Finally, multispecies biofilms have the potential to form novel complex three-dimensional structures, which may affect the system in a number of ways. Herein we will discuss the altering of biofilm architecture by synthetic biologists as potential mechanism for optimizing the output of biofilm.

1.1.5. Genetic manipulation of biofilm architecture

The current manipulation of biofilm genetics is done by editing the genome of planktonic bacteria, and allowing these cells to form a biofilm. There are methods of blocking the transcription of certain genes in biofilms directly [96–99] or indirectly through the manipulation of quorum sensing [100–102]. These studies generally aim to eliminate or prevent biofilm completely. The ideal tool for genetic manipulation of transcription levels would allow for the fine tuning of a user specified gene. While there are a few promising new conjugation-based methods of multi-species biofilm genome editing [103,104], they have yet to mature into modular tools. For now, the best approach is editing the genome of individual planktonic species and letting those cells establish a biofilm.

To leverage a biofilms’ architecture to further enhance the efficiency of production, different aspects of biofilm architecture could be examined: the distribution quantity and relative function of the basic macro molecules, the distribution of cells across the biofilm as well as their spatial relationship to other species and the overall three-dimensional characteristics of the biofilm.

1.2. Macromolecules where and why

1.2.1. Protein

Embedded proteins are known to constitute a significant amount of biomass to biofilms [105]. These proteins are known to play roles in attachment of biofilm to both biotic and abiotic surfaces. New evidence points to the majority of proteins found in biofilm to not be secreted proteins or protein derived from lysed cells, but typically outer membrane proteins that may have been transported to the biofilm by outer membrane vesicles [106]. In contrast to embedded proteins, many of the secreted proteins bind specific biotic matrices such as fibronectin [107,108], chitin [109–111] or mannose [112,113] to mediate attachment. More general strategies for attachment involve the expression of pili [112,114,115], flagella [116] and the more recently uncovered fimbrae [117–121]. Both unique and generalist attachment strategies offer unique engineering opportunities. For example, a rhmnomose-inducible pili would yield a greater concentration of biofilm near the rhmnomose source. This serves two unique functions. First, this presents a relatively inexpensive way of structuring multispecies biofilms. Second, this serves as a potential fnd-the-leak type system, where biofilms preferentially form at higher concentrations of inducer identifying not just the presence but the sources of a contaminant. On the flip side, attachment can be directly selected against with the production of various biofilm disassembly agents, which can enhance motility and repress biofilm formation. These biofilm disassembly agents may be programmed to be species-specific and cell-surface attached [122] or they may be secreted [123].

A subtype of fimbrae are called curli [124]. As curli form en-masse and overlap they are known as amyloid fibers [125]. Amyloid fibers are commonly found in biofilms [126]. These projections from the cell have been used for a variety of disparate functions. Some interested in nanoscale engineering have been using the fibers as nucleation points for metal deposit [127], essentially building a functional nano-wire with the reasonably electro-stable curli at the core. While this is an achievement a bit removed from biofilm, the subsequent achievement of tunable curli length [128] is of immediate value in more environments beyond tightly controlled laboratory setups. Interestingly, some
bacterial species have the ability to elongate each other’s amyloid fibers [129] presenting yet another avenue of potential regulation. As amyloid fibers are known to provide structural stability to biofilms [130], these tunable amyloid fibers can be harnessed to dictate how difficult it is to remove an artificial biofilm via physical force. Others have discovered that amyloid fibers have the potential to bind and retain certain chemical compounds [131]. As amyloid fibers with engineered binding sites are developed [132], a greater number of substrates may be captured. There are also now a greater number of proteins with useful functions which may be displayed in biofilms, such as proteins that fend off grazing protists [133] or proteins that entice cellular aggregation [134]. Biofilm can also act as a reservoir which holds potential for inducible release. This has utility beyond general biofilm dispersal, as rebuilding a biofilm is resource-intensive so a minimal loss of biomass associated with product release is often desirable. As designer proteases [135–141] become more common and dependable it is conceivable that a net full of amyloid fibers might be sheared off by a user using specific protease and thus releasing concentrated cargo within. This type of approach may be useful in setups where collection is costly.

Often, but not always, cells in a biofilm will also form an S-layer. The S-layer is closer to the cell membrane than amyloid fibers. The S-layer is found in most archaea and some bacteria; it is a layer of protein adorning the cell beyond any cell wall that may be present [142]. S-layer engineering has contributed immensely to the efficiency of many production systems. These engineered proteinaceous alterations tend to act as scaffolds for specific enzymatic reactions. S-layers are also known to help shield bacteria from adverse environmental conditions [143] as well as influence attachment [144]. S-layers can also be utilized as a sort of sieve by the cell [142] preventing larger molecules from approaching the peptidoglycan layer. A potential problem with S-layer and amyloid fibers is the potential for them to get clogged with compounds [145]. This leads to three overlapping downstream problems: diffusion to and from the cell of various materials may be hampered, the concentrated compound may inhibit local cells’ ordinary function and the compound may be blocked from reaching a final (beneficial to the system) location.

1.2.2. Lipids

Outer membrane vesicles (OMVs), essentially lipid bilayers spheres that bleb off of cells, are one of the cellular products that may have altered formation/diffusion patterns in systems with extensive S-layer/amyloid fiber engineering. While these OMVs are known to be important contributors to biofilm protein and biomass overall, their role in contributing biofilm lipids is unclear. It is known that the cells which form biofilms alter their lipid membranes relative to planktonic cells [146]. A study pointed to the enrichment of lipids in cell membranes that provided more structure and less membrane fluidity [147]. While some studies have demonstrated that knocking out [148] or effectively reducing fatty acids [149] prevents full biofilm formation, these studies must be interpreted carefully as changes in lipid membranes inherently alter the viability of all cells. As lipidomics is a field largely in its infancy these early results prevent synthetic biologists from effectively editing the lipid content of biofilms beyond the simple increase in lipid production for harvesting.

1.2.3. Nucleic acids

Extracellular DNA (eDNA) is DNA that is found in the biofilm matrix (EPS), outside of the cellular membrane. The tools and methods to isolate, manipulate, interpre and build nucleic acids are much farther along than their lipid counterparts. Originally, it was thought that the nucleic acids associated with biofilms were just remnants of lysed cells [150], but it became clear that in addition to lysis [151], many of these molecules were also released within OMVs [152–156]. The degree to which the outer membrane vesicle release of DNA and the quorum sensing regulated release of DNA (via lysis or OMV) are related is not yet clear [157–159]. OMVs are also known to be responsible for the release of many intracellular proteins. This highlights the ability for protein and/or eDNA release to be potentially tuned at a high level by regulating outer membrane vesicle release [160]. This may be useful as a method to lower signal-to-noise ratios in regards to extracellular molecule monitoring in biofilms. If this tunable circuit of outer membrane vesicle release is restricted to a single species this also allows for opportunities for ratioed protein contribution adjustment to biofilm.

eDNA has recently been shown to be important for the formation of biofilm [6,155,161,162]. This may be via a combination of enhanced attachment [161] and added structural stability [162]. It appears that eDNA is mostly randomly fragmented genomic DNA [163]; no studies have yet to investigate non-B form DNA structures (Z, G-quad, etc.) in relation to biofilm stability or attachment. These future studies might provide additional modular ways to use eDNA in a biofilm shaping manner, especially when combined with the idea that DNA fragment length (something that is easily artificially controlled) may be used to tune attachment. An experiment with Listeria monocytogenes showed that shorter pieces of DNA prevented attachment and that if added prior to long pieces of DNA, these short pieces could prevent subsequent long DNA-assisted biofilm attachment [161]. In mixed species biofilms, the release of eDNA from biofilm is more complex, as different organism have different propensities for releasing eDNA [163,164] and those propensities may be changed based on the other species in the biofilm [163,165]. The same study showed that both eDNA and peptidoglycan were codependent for attachment, further illustrating the opportunities for cooperation and involvement of eDNA in biofilm formation. Studies have shown that the lattice or the mechanism of cross-linking of eDNA contributes to the strength and resilience of the resulting biofilm [166].

eDNA presents unique control opportunities for biofilm attachment and structural stability. Its release might be modified in a species specific manner via specific protein mechanisms [156] or phage-based lysis [162] or quorum-sensing based OMV release [158] or antibiotic-based OMV release [152] that may be applied to alter mechanical biofilms properties. eDNA can also play additional roles in biofilm beyond basic establishment and maintenance.

eDNA can be taken up by living cells via a process known as competence. Sometimes this can just be a useful background mechanism for cells to survive during stationary phase using the DNA of previously lysed cells as a carbon source in order to survive longer [167]. Competence is also known to contribute to the effective transfer of genes and plasmids between bacteria [168–170]. This has been demonstrated to be a method of antibiotic resistance marker transfer. A distinct mechanism of antibiotic resistance caused by eDNA has also been uncovered in which neighboring cells detect eDNA with two component systems and upregulate various genes in response [171]. eDNA can also lead to antibiotic resistance via a structural mechanism. As DNA (as well as most bacterial membranes) has a negative charge, a layer of eDNA outside of biofilm cells can help shield biofilm cells from amnoglycosides and cationic antimicrobial peptides which are positively charged [172]. Releasing eDNA in a temporal manner might be ideal for generating a biofilm which can be made more resistant to positively charged molecule attack upon demand. This may be ideal if the user does not know exactly which cationic antimicrobials the biofilm will be exposed to. In synthetic biology, even if your machine (biofilm) gets destroyed and the integrated circuit chip gets broken in half, that circuit is dead. Barring elaborate unprecedented artificial surgical reconstruction, that physical circuit chip will never contribute to a circuit again. In synthetic biology, even if your machine (biofilm) gets destroyed there is a reasonable chance parts of its code (eDNA) might be integrated into a whole new system. This is an interesting
opportunity that has yet to be explored.

Much as eDNA can help physically stabilize biofilm, exopolysaccharides are also known to have this effect from both antibiotic based destabilization [173] and immune system based attack [174–176]. Constitutive antibiotic resistance engineered into biofilm may also be useful as a mechanism to thwart competing biofilms from getting established. An often overlooked facet of biofilm is that some cells that facultatively replicate intracellularly might form biofilm in an effort to be more efficiently recognized and phagocytosed [177]. Thus, it may be important to form a larger biofilm if the end goal is to insert your organism into another.

This point of view offers interesting potential to the engineer. Now by using biofilm as a sort of entry vector (which is modifiable in a myriad of ways), organisms that may be phagocytosed present a new opportunity to edit other chassis that may be more resistant to genetic engineering. This may provide an interesting backdoor to genetically engineering unculturable protists [178,179] as a prerequisite to genetic engineering is traditionally the ability to culture the recipient organism.

1.2.4. Carbohydrates

Along with antibacterial agents, various exopolysaccharide agents can also trap micronutrients [180,181], which is essential for biofilms grown under flow conditions. Extracellular carbohydrates in EPS are also known for their ability to enhance attachment and therefore enhance biofilm formation [182–184]. Optimizing nutrient intake in biofilms grown in flow systems is a balancing act as ideally the biofilm will capture what it was designed to capture yet will not inhibit the flow of fluid (which more exopolysaccharide will contribute to).

Many complex polysaccharides are produced within biofilms in the EPS. Exopolysaccharide production in biofilms can be manipulated at the transcriptional level with repressors [185], at the post transcriptional level via withheld precursors which can therefore not be operated on by downstream enzymes [186] or via degradation of previously secreted sugars [16]. Manipulation of these processes can be utilized for synthesis of desired molecules such as the production of Glucosamine from Chitin [187]. Many of the carbohydrate-control-loci (in addition to expression of proteins and of eDNA) are influenced by intercellular small-molecule messages called quorum signals [188], meaning that quorum signals often regulate sugar production.

1.3. Interspecies biofilm relationships

Quorum signals are chemical species that get produced by prokaryotes and some eukaryotes when the population has amassed to a certain density. These signals are generally sensed by two component systems, where a membrane bound receptor binds the extracellular signal. This receptor then interacts with a response regulator which alters transcriptional patterns. While quorum signals could potentially regulate any transcriptional levels, the majority of established research has focused on the effects of quorum sensing on antibiotic resistance, virulence, and biofilm formation [189].

Quorum sensing logic gates provide fantastic opportunities for modular tunable control. Building artificial quorum sensing systems as a means for regulating biofilm continues to be the most prevalent way of genetically manipulating artificial biofilms. For a full review on artificial quorum systems see Hennig et al. [190]. The utility of quorum sensing systems is largely based on two facets, modularity and tunability. Quorum systems are ubiquitous in nature. Often distantly related organisms can communicate through the same molecule. While the actual production of certain molecules may prove difficult in certain chassis, the expression of a selected two-component system is usually a much easier feat. The great variety of quorum signals even within the same families of organisms can communicate through the same molecule. While many complex polysaccharides are produced within biofilms in the EPS. Exopolysaccharide production in biofilms can be manipulated at the transcriptional level with repressors [185], at the post transcriptional level via withheld precursors which can therefore not be operated on by downstream enzymes [186] or via degradation of previously secreted sugars [16]. Manipulation of these processes can be utilized for synthesis of desired molecules such as the production of Glucosamine from Chitin [187]. Many of the carbohydrate-control-loci (in addition to expression of proteins and of eDNA) are influenced by intercellular small-molecule messages called quorum signals [188], meaning that quorum signals often regulate sugar production.

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1.4. Biofilm shape

There is a non-trivial relationship between cell shape and biofilm organization. While coccolid cells have the advantage of a high surface area to volume ratio allowing them to exchange more easily than bacilli the cocci are generally at a disadvantage when it comes to attachment area. Cell shape in isolation is not a powerful enough factor to completely dictate attachment rate in nature. Under artificial conditions though, cell shape can absolutely be applied to altering biofilm arrangement. This was cleverly demonstrated by Smith et al. [205]. They made single amino acid changes to MreB (cell partitioning protein) in Escherichia coli resulting in substrains that replicated at nearly identical rates. They then observed these substrains when cultured together seeing a clear gradient from bacillus at the basal surface towards more coccus like cells near the top of the colony. This relationship is depicted in Fig. 1. This study highlights a unique single protein tunable structure that has profound effect on how cells relate structurally to one another. This is a potentially useful way to optimize metabolic consortia or to shield vulnerable population from external forces. For example, one might engineer coccos cells normally found on the periphery of the biofilm to be more bacillus like which would likely sequester them closer to the basal surface of the biofilm which would partially shield these cells from changing environmental conditions such as flow rate. Alternatively cell shape may be altered not to change cells local positioning but to alter the vertical height of a biofilm [206]. A useful review by Caccamo and Brun [207] highlights other potential proteins that might be manipulated to alter cell shape and therefore arrangement in biofilm.

In order to understand and control the effects of different inputs leading to varying outputs in biofilm systems, it would be useful to have computational models of biofilm formation and organization. Recent studies with non-typeable Haemophilus influenzae have revealed difference between in vitro biofilm architecture versus in vivo biofilm architecture, with clusters being 1/10 the size in vivo. This was attributed to the removal of planktonic cells from the biofilm through the host response, leading to a similarly organized but structurally different biofilm [208]. An in silico model of this biofilm formation was then generated, which will allow the study and isolation of different parts of the biofilm production process, to potentially identify the parts of the system responsible for the differences between the morphologies of in vitro compared to in vivo biofilms. This is the first report that we have identified of a computational model that can integrate various inputs and predict the biofilm output.

Surface area analysis can also be applied to apical surfaces. The apical surface area has potential engineerable factors including surface roughness and fractal dimensionality. Surface roughness is essentially a measure of the distance from a predefined smooth surface (having consistent curvature). This measure can be contrasted with fractal dimensionality, a metric of the degree to which an answer changes when the scale at which the object is measured is varied. At first glance fractal dimensionality may appear to be merely a measure of surface roughness something akin to fluorescence within a mixed species biofilm.

standard deviation. In actuality, fractal dimensionality is a combination of the deviation from average surface and a measure of how regularly deviations occur over distances. The relationship between surface roughness and fractal dimensionality can be seen in Fig. 2. While applying fractal dimensionality to biofilm height and length is certainly useful, comparing that fractality to other dimensions such as height and width provides more advanced measurements for understanding architecture in response to environmental stimuli especially that of flow [209]. As biofilms are not rectangular prisms, but complex non-euclidian shapes there are a theoretically infinite amount of semi overlapping two-dimensional dimension comparisons a user could study. The limit to which these relationships are useful is likely defined by the regularity of flow with a pipe model being a simple model with fluid flow coming from one direction and a lattice of interconnected tubes being a complicated model with more complex dynamics. These deeper understandings of biofilm shape are necessary to understand to establish ideal biomass to productivity ratios. The idea is the more input your biofilm/factory needs to provide output, the less efficient a factory it is. This mechanical relationship is further complicated by the fact that biofilms can be extremely ephemeral in nature. So one must also ensure that the biofilm will both stay where it needs to be (attachment) and not get destroyed regularly (resisting shear force).

Surface roughness and fractal dimensionality are important as they influence both the kinetics of chemical reactions and fluid dynamics. This can be more broadly thought of with the truism shape dictates function. As fractal dimensionality and surface roughness are properties of shapes they are part of defining this function. This was aptly demonstrated by Dewey who was studying the kinetics of proton exchange within lysozymes. Dewey showed using tritium that as reaction space got smaller relative to the unit that causes the change (in this case proton movement) the reaction sped up [210]. Beyond speeding up or slowing down the rate of reaction, optimizing the fractal dimensionality may also be useful for obtaining a more consistent rate of reaction which may be desirable if user input is required at discrete steps during biofilm establishment or maintenance. A more elaborate discussion of fractal dimensionality and how that affects the rates of chemical reactions in many different biological contexts may be found in a review by Gabriele Angelo Losa [211]. When looking at multispecies biofilms exchanging different kinds of chemical species, as opposed to subcellular structures and singular chemical species, these relationships
Table 1: Current biofilm engineering targets.

| Species                  | Molecular Target                      | Mechanism                                                                 | Goal                                                                 | Reference |
|--------------------------|---------------------------------------|---------------------------------------------------------------------------|----------------------------------------------------------------------|-----------|
| *Pseudomonas aeruginosa* | PqsE/PqsC (Quorum Sensing)            | Elimination of anaerobic repression leading to enhanced intracellular metabolism | Enhanced Electric Production                                        | [223]     |
|                          |                                       | this used some products of the primary strain to grow enhancing total growth |                                                                        |           |
|                          |                                       | Raising the level of cyclic-di-GMP which lead to a thicker biofilm           |                                                                        |           |
| *Escherichia coli*       | PtG/PtsM/Glk/Gcd (Carbon utilization) | Raising the levels of cyclic di-GMP which allowed large biofilms to speed the biodegradation of haloalkanes | Enhanced Biomass/Haloalkane degradation | [225]     |
|                          |                                       | Raising the levels of cyclic di-GMP which allowed large biofilms to speed the biodegradation of haloalkanes | Enhanced Biomass/Haloalkane degradation | [225]     |
| *Pseudomonas putida*     | PrgR (regulator of extracellular polyglutamate, caprolactam) | Increasing extracellular glutamate which lead to a thicker biofilm           | Enhanced Biomass/Enhanced Attachment/Altered Surface Charge/styrene-oxide | [226]     |
|                          |                                       | Upregulating the amount of surfactin lead to enhanced attachment and then larger biofilms to produce styrene-oxide |                                                                        |           |
| *Escherichia coli*       | OmpR (regulator of curli production)  | Raising the levels of cyclic di-GMP which allowed large biofilms to speed the biodegradation of haloalkanes | Enhanced Biomass/Enhanced Attachment/Altered Surface Charge/styrene-oxide | [226]     |
|                          |                                       | Upregulating the amount of surfactin lead to enhanced attachment and then larger biofilms to produce styrene-oxide |                                                                        |           |
| *Pseudomonas taiwanensis*| BslA like protein (s-CaM phospholipases) | Raising the levels of cyclic di-GMP which allowed large biofilms to speed the biodegradation of haloalkanes | Enhanced Biomass/Enhanced Attachment/Altered Surface Charge/styrene-oxide | [226]     |
|                          |                                       | Upregulating the amount of surfactin lead to enhanced attachment and then larger biofilms to produce styrene-oxide |                                                                        |           |

As fractal dimensionality and surface roughness are already standard metrics for biofilms [221] it seems likely that these will be a greater focus in the future of biofilm optimization projects. Engineering surface roughness and fractal dimensionality may be accomplished via a variety of mechanisms depending on the starting composition of biofilm. Initial colonizers may be employed to change the underlying architecture which may influence apical surfaces on cells, or the periphery of the biofilm may be engineered, either constitutively or in response to a gradient, to produce more or less of a desired protein, carbohydrate etc. While biofilm surfaces are composed of a great many things (discussed above) the relative contribution of different types of molecules is not necessarily equal. A group studying *Bacillus* showed that at one point on the biofilm cycle surface roughness was largely attributable to a single protein BslA [222]. This highlights the feasibility of surface roughness engineering in biofilms. Alternatively, an external agent may be added which may bind the biofilm and essentially alter its surface topography.

The great challenge is altering roughness or fractal dimensionality in an orthogonal manner. Generally surface roughness and fractal dimensionality are thought of as consequences as other more central
1.5. Conclusion

Biofilms are the new factories of tomorrow. As more and more design teams seek to harness biofilm abilities, a greater need for system efficiency is demanded. While metabolic engineering-type approaches are absolutely essential in directing metabolic flux, the underlying biofilm architecture should not be ignored. Each of the components of biofilm and the shape of the biofilm itself significantly affects the efficiency of any system. Efforts by biologists to optimize biofilms are summarized in Table 1 whereas future potential targets are summarized in Table 2. While not all modulations are possible in all biofilms, it is important to appreciate the plasticity of each chassis or group of chassis to best optimize performance to a certain task.

Declaration of competing interest

None.

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