Engineering Synthetic Biofilm-Forming Microbial Consortia

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"I've spent more time than many will believe [making microscopic observations], but I've done them with joy, and I've taken no notice of those who have said 'why take so much trouble and what good is it?"" — Antonie van Leeuwenhoek

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iv

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v

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Abstract

Most bacteria on earth live in heterogeneous surface-bound congregations called biofilms, and vast reaches of the earth are coated in these living films. In many cases, the microorganisms comprising this ubiquitous coating form complex, interactive communities called consortia. Microbial consortia are implicated in processes of great importance to humans, from environmental remediation and wastewater treatment to assistance in food digestion. Synthetic biologists are honing their ability to program the behavior of individual microbial populations, forcing the microbes to focus on specific applications, such as the production of drugs and fuels. Given that microbial consortia can perform even more complicated tasks and endure more changeable environments than monocultures can, they represent an important new frontier for synthetic biology. This thesis describes two engineered microbial consortia that live and perform their designed functions in biofilms. The biofilm consortium elucidated in Chapter 2 serves as a proof of concept for the development of the symbiotic biofilm consortium of Chapter 3. To provide a context for these two consortia, the first chapter highlights the salient features of microbial consortia that are of interest to synthetic biologists and reviews recent efforts to engineer synthetic microbial consortia, while the final chapter suggests challenges associated with and future directions for engineering microbial consortia.

Contents

Acknowledgements		iii
Abstrac	et	vii
Introduction		
1.1 1.2 1.3 1.4 1.5	Benefits and features of microbial consortia Mixed populations can perform complex tasks Mixed populations can be robust to changes in environment Communication organizes function in engineered consortia Synthetic consortia lend biological insight	1 2 4 4 4
Enginee	ered bidirectional communication mediates a consensus in a microbial	
biofilm	consortium	14
2.1 2.2 2.3 2.4 2.5	Abstract Introduction Results Discussion Methods	. 14 . 15 . 16 . 25 . 26
Structu	re and selection in an engineered symptotic biothin consol tuni	30
3.1 3.2	Abstract	30
3.3	Background: Evolution of communities	31
3.4	Background: Physical structure of biofilms	. 36
3.5	Design and construction of the symbiotic consortium	. 46
3.0	Function and stability of the biofilm consortium.	. 52
3./ 2.8	Aggregates in the transfer of emergent structure and growth advantage	. 30
3.0	Adaptation or polymorphism and the growth advantage	62
3 10	Third-generation biofilms reveal contributions of adaptation and aggregates	64
3 11	Discussion and conclusion	67
3.12	Methods	. 70

Conclu	sions	74
4.1	Challenges in engineering microbial consortia	
4.2	Synthetic consortia in healthcare	75
4.3	Summary	
Bibliog	raphy	78
Append	dix A	95
A.1	Plasmids encoding Circuits A and B	
A.2	Validation of model in liquid culture	
A.4	Biofilm experimental setup, imaging, and image processing	
A.5	Biofilm imaging equipment and settings	102
A.6	Three-dimensional image rendering	105
A.7	Image processing	105
Append	dix B	109
B.1	Details of construction of strains MGd- and MGfwc-	109
B.2	Stability of the engineered symbiotic biofilm	111
B.3	Metrics and COMSTAT image processing	112
B.4	Repeatability of growth advantage in untreated second-generation biof	film 113
B.5	Images of treated and untreated biofilms at the substrate	
B.6	Emergent structure does not reappear in the third-generation control bi	ofilm116
B.7	FACS was used to separate aggregates and YFP+ single cells	117

Chapter 1

Introduction

Portions of this chapter are published as [1].

1.1 Benefits and features of microbial consortia

Synthetic biology [2-6] has generated many examples of what microbes can do and what we can learn from them [7-12] when they are creatively engineered in the laboratory environment. From the synthesis of an anti-malarial drug [13] to the study of microbial genetic competency [14], engineered microbes have advanced technology while providing insight into the workings of the cell. Interest has recently emerged in engineering microbial consortia-multiple interacting microbial populations-because consortia can perform complicated functions that individual populations cannot and because consortia can be more robust to environmental fluctuations (Figure 1.1). These attractive traits rely on two organizing features. First, members of the consortium communicate with one another. Whether by trading metabolites or by exchanging dedicated molecular signals, each population or individual detects and responds to the presence of others in the consortium [15]. This communication enables the second important feature, which is the division of labor; the overall output of the consortium rests on a combination of tasks performed by constituent individuals or subpopulations. Let us briefly examine the complex functions that mixed populations perform, and the evidence for their robustness to environmental fluctuation. We will then explore how

engineers have employed communication and differentiation of function in designing synthetic consortia.



Figure 1.1 Processing of complex reactions by (A) a single population or (B) a microbial consortium. Generation of a product (P) might require multiple steps to convert the substrate (S), through the sequential synthesis of intermediates (X1 and X2). (A) A single population is responsible for synthesizing all the enzymes needed to carry out intermediate reactions and for balancing those reactions to optimize product yield. (B) Each population is dedicated to a single step. The reactions performed by each population can be coordinated by engineered cell–cell communication and balanced by engineering each population separately. A microbial consortium such as that illustrated in (B) has two potential advantages. First, limiting the number of exogenous elements in each population reduces the metabolic imbalance in the host cells. Such an imbalance often leads to growth retardation and suboptimal production. Second, the division of labor will simplify optimization of each reaction in the pathway by isolating the engineered circuit dedicated to each reaction.

1.2 Mixed populations can perform complex tasks

Mixed populations can perform functions that are difficult or even impossible for individual strains or species. Balancing two or more tasks so that they are efficiently completed within one organism can pose insuperable challenges in some situations. For example, it is difficult to engineer efficient, metabolically independent pathways within a single cell to enable it to consume the five- and six-carbon sugars produced by lignocellulose degradation; asynchrony in degradation, caused by glucose preference, lowers productivity [16]. These functions, however, can be separated into different, individually optimized populations. By compartmentalizing the molecular components of each pathway, transcriptional regulators and chemical intermediates in each can be modulated separately without regard for potential interactions. For example, two strains of *Escherichia coli* have been engineered so that one metabolizes only glucose and the other only xylose, and can be tuned so that they consume their substrates at similar rates. When grown in co-culture, the two strains ferment the sugars more efficiently than would any single engineered cell performing both functions [17].

Another important feature of microbial consortia is their ability to perform functions requiring multiple steps. Such tasks are possible when different steps are completed by dedicated cell types. For example, cellulolytic microbes make and excrete several different protein components (e.g., scaffolding proteins and enzymes) that assemble into an extracellular cellulosome that is capable of cellulose degradation. Various organisms in nature can secrete all of the necessary cellulase components, but these organisms are often difficult to manipulate genetically [18]. Attempts to engineer more genetically tractable organisms to secrete all of the cellulase components heterologously have not yet been successful. This might be because the heavy metabolic burden associated with expression of the cellulase-associated proteins inhibits cell growth, or because intracellular assembly of the cellulosomal complexes interferes with their excretion. However, two engineered strains of *Bacillus subtilis*—one secreting the scaffold and the other secreting either an endoglucanase or a xylanase that binds to the scaffold to become active—exhibit the predicted enzymatic activity in co-culture [19]. In each of these examples, a combination of populations was used to achieve a desired outcome that is currently difficult to engineer in a single population.

1.3 Mixed populations can be robust to changes in environment

Living in community is thought both to generate robustness to environmental fluctuations and to promote stability through time for the members of a consortium. Compared with monocultures, communities might be more capable of resisting invasion by other species [20]. Furthermore, they might be able to weather periods of nutrient limitation better because of the diversity of metabolic modes available to a mix of species combined with the ability to share metabolites within the community [21]. For example, when nutrients become limited, the most prevalent species in a community are not always the most metabolically active species. A minority population can become the most active population during nutrient limitation if it has a metabolic activity upon which survival of the entire consortium depends [22]. In fact, the consortium containing the minority species might have been retained by natural selection because the activity of the minority species caused it to withstand periods of nutrient limitation [22]. Diversity of species in a consortium does not guarantee survival [23, 24], but it might be that engineered consortia will perform most reliably in changeable environments when diverse metabolic modes are present among members [25].

1.4 Communication organizes function in engineered consortia

Communication among individuals or populations enables the division of labor that results in their ability to exhibit complex function. Communication in natural consortia can involve the exchange of dedicated signal molecules within or between single populations [15, 26]. Bacteria coordinate intra-population behaviors from biofilm formation [27-29] to virulence [30-32] with the exchange of acyl-homoserine lactone

(acyl-HSL) signaling molecules (in Gram-negative species) and small peptides (in Grampositive species) [26, 33, 34]. Inter-population communication between Gram-positive and Gram-negative species, through autoinducers 2 and 3, is less well characterized but might be implicated in enteropathogenic infections [35]. Microbes in consortia can also communicate by trading metabolites. For example, the member species of a consortium that degrades the herbicide diclofop methyl pass intermediate metabolites back and forth in the process of degrading the compound [36]. Additionally, species in a consortium can exert both positive and negative control over one another's activities by exchanging metabolic intermediates that either assist or compromise the growth of their neighbor [37].

Engineering cell–cell communication is a first step in constructing synthetic microbial consortia. To accomplish this, engineers have exploited components of bacterial quorum-sensing (QS). QS enables community-wide behaviors to be coordinated by the intercellular exchange of small molecules such as acyl-HSL signaling molecules [26]. Engineered acyl-HSL communication has been used in biological "circuits" that coordinate population-wide behaviors ranging from population-density-dependent fluorescence [38], cell suicide [39], and invasion of cancer cells [40] to pattern formation [41]. Chapter 2 of this thesis describes a mixed-population biofilm-based consortium that uses two-way engineered communication via acyl-HSLs to coordinate fluorescent gene expression [42]. The expression of fluorescent genes is possible if, and only if, both member populations are present at sufficiently high densities. This engineered "Microbial Consensus Consortium" (MCC) has a flexible output—in principle, any set of genes can be expressed when the populations co-localize and

accumulate—that invites the development of more complex consortium functions in biofilms.

Engineered communication with dedicated signal molecules can also be used to study the behavior of interacting populations or to mimic microbial interactions under controlled conditions. Balagadde et al. [43] constructed two populations of *E. coli* that, together, constitute a predator–prey ecosystem. As in the "consensus consortium" described above, the two populations communicate bi-directionally with acyl-HSL signals. Upon induction of the biological circuit that encodes the communication and the programmed cellular response, one population (the predator) dies out in the absence of the other (the prey). Communication between the two populations directs the prey to rescue the predator, but once the predator recovers to a sufficiently high density, it begins to kill the prey (Figure 1.2A). With appropriate parameters, including appropriate cellular growth rates for the two populations and the right concentrations of the inducing chemical isopropyl-b-D-thiogalactopyranoside (IPTG), the densities of the two populations begin to oscillate in a phase-shifted manner (Figure 1.2B).

In addition to programmed predation and rescue, the two populations in the synthetic predator–prey system also compete for nutrients in a co-culture. The relative contributions of predation and competition can be modulated by the induction level of the circuits that control the engineered behaviors. For instance, in the absence of circuit induction by IPTG, interactions between the two populations are dominated by competition for nutrients in the medium, where the predator drives out the prey owing to the growth advantage of the predator. Increasing the circuit induction level, however, activates the predator–prey dynamics and induces population oscillations, which allows

the two populations to co-exist despite their competition for nutrients. In other words, establishing predation dynamics enables greater biodiversity during long-term culturing. Also, when the dilution rate in the system is increased—increasing the rate at which any individual dies or leaves the environment—oscillations appear to have shorter periods until the predators die out. These results—the resource-based transition between competition and predation, and the out-competition of the predator at low population densities—might inform our understanding of other, more complex ecosystems.



Figure 1.2 A synthetic predator–prey ecosystem (A) consists of two engineered bacterial populations that control each other's survival through two different QS signals. Two QS modules, LuxI and LuxR from *Vibrio fischeri* and LasI and LasR from *Pseudomonas aeruginosa*, are used to enable two-way communication. When the prey density is low, the predator cells die, owing to constitutive expression of CcdB ("B"). In the prey cells, LuxI synthesizes a diffusible survival signal (3OC6HSL). At a sufficiently high prey density, 3OC6HSL accumulates in the culture and activates the transcriptional regulator LuxR in the predator cells leading to expression of an antidote CcdA ("A") to rescue the predator cells. In turn, LasI in the predator cells synthesizes a killing signal (3OC12HSL). The signal diffuses into the prey cells, where it can activate CcdB expression, effecting "predation." This system satisfies the broader definition of predation for a two-species ecosystem, in which one species (the prey) suffers from the growth of the second (the predator), and the latter benefits from the growth of the former. However, it differs from the canonical predator–prey system in two aspects. First, instead of acting as a food source, the prey provides an "antidote" to programmed cell death of the predator. Second, in a co-culture, the predator and the prey cells also compete for nutrients. (B) Typical oscillatory dynamics of the system with a period of ~180 h ([IPTG] = 5 mM, dilution rate = 0.1125 h_1). Figure adapted from Balagadde et al. [43].

Dedicated signals have also been used to implement communication between different kingdoms of organisms. Weber et al. [44] borrowed a mouse gene that converts ethanol into the volatile small molecule acetaldehyde. They installed this "sender" gene in Chinese hampster ovary (CHO) cells, human embryonic kidney (HEK) cells, E. coli, Saccharomyces cerevisiae, and Lepidium sativum (plant). All transformed cells were able to produce acetaldehyde from ethanol. CHO cells containing an Aspergillus *ridulans* hybrid promoter designed to detect the airborne acetaldehyde were engineered to respond to acetaldehyde by expressing a variety of genes. The researchers used this simple set of "sender" and "receiver" modules to engineer different intercellular interactions, including the following: commensalism, wherein one population benefits because of the association, while there is no effect upon the other; amensalism, wherein one population suffers, while there is no effect upon the other; mutualism, wherein both populations benefit from the interaction; parasitism, wherein the interaction is beneficial for one population and detrimental to the other; and parasitism leading to predation, in which antagonism between the populations causes oscillatory population densities (Figure 1.3).

In addition to the exchange of dedicated signal molecules, inter-population communication can also involve the exchange of chemicals involved in metabolism and growth [45]. An engineered consortium described by Shou et al. [46] provides insight into the exchange of metabolites in microbial consortia. Shou et al. [46] programmed two strains of *S. cerevisiae* to depend on one another for amino acid metabolism in a synthetic consortium they call CoSMO (cooperation that is synthetic and mutually

obligatory) [46]. One strain of *S. cerevisiae* is unable to make lysine but overproduces adenine, and the other cannot make adenine but overproduces lysine (Figure 1.4A).



Figure 1.3 Communication can occur between different kingdoms of organisms. Weber et al. [44] used a simple set of "sender" and "receiver" modules to engineer commensal (one population benefits because of the association, there is no effect upon the other), amensal (one population suffers, there is no effect upon the other), mutualistic (both populations benefit from the interaction), parasite (the interaction is beneficial for one population and detrimental to the other), and predatory (antagonism between the populations causes oscillatory densities) relationships between sending and receiving cells. In the engineered commensalism, amensalism and mutualism systems, E. coli cells growing in an open-air culture well with ethanol (EtOH) make volatile acetaldehyde (VA), which diffuses through the air to neighboring wells containing CHO cells. In each case, the VA activates a VA-sensitive promoter, p(VA), in CHO cells. In commensalism and mutualism, neomycin would kill the CHO cells if the VA did not activate production of the NeoR gene product, which rescues them from death. In the mutualistic case, ampicillin in the first culture well kills the E. coli, which would also eventually lead to CHO cell death due to lack of VA and therefore NeoR expression. However, the AmpR gene product, transferred periodically from the CHO cell culture well into the E. coli culture well, rescues the E. coli and thereby enables both populations to survive. In amensalism, VA produced by the E. coli induces apoptosis, through the production of the RipDD gene product, in neighboring CHO cells. Finally, in the cases of parasitism and predator-prey relationships, E. coli and CHO cells are cultured together. CHO cells express AmpR to rescue E. coli from ampicillin-mediated celldeath. However, because E. coli cells grow more quickly than CHO cells, they use more nutrients and begin to out-compete the CHO cells. When ampicillin is constantly re-supplied to the culture medium, however, E. coli cells require constant rescue by the CHO cells, resulting in predator-prey-type behavior. Figure adapted from Weber et al. [44].

The dynamics that emerge from the co-culture of these two auxotrophs reveal that, particularly if crucial metabolites are the mechanism of communication, the ability of one population to live in a consortium can depend on the rate at which the other dies. In this case, lysine and adenine are not released into the medium until the overproducing strain begins to die from lack of the amino acid that it cannot make itself. Despite this, both populations can survive in co-culture, and both grow once their partner begins to die (Figure 1.4B). This can serve as a guiding engineering principle; the onset of death might serve as an intrinsic delay mechanism for the onset of an engineered consortium function and as an alternative to QS-based coordination of population-wide function. Whereas engineered microbes that express QS genes can commence population-wide behavior gradually, as signal molecules accumulate in the growing community, CoSMO initiates a population-wide behavior only upon the advent of death within the community. Exploring how natural consortia exploit this mechanism might give us new insight into how we can use it to coordinate population-wide behaviors in synthetic consortia.

1.5 Synthetic consortia lend biological insight

Many questions remain regarding the evolution and stability of natural ecosystems. As Shou et al. [46] and Balagadde et al. [43] demonstrated, we can perturb microbial ecosystems by genetically engineering them to achieve different behaviors. Furthermore, we can control their growth environments. Given these abilities, we can explore the evolution of interacting species in ways that are impossible with larger organisms [47]. Such studies have already demonstrated that cheating strains, subpopulations that



Figure 1.4 Synthetic consortia communicate by exchanging chemicals involved in metabolism and growth. (A) Shou et al. [46] engineered two strains of auxotrophic yeast that depend upon one another for survival. One strain (strain 1) of *S. cerevisiae* is unable to make adenine but overproduces lysine, whereas the other (strain 2) cannot make lysine but overproduces adenine. (B) Lysine and adenine are not released into the co-culture medium until the overproducing strain begins to die from lack of the amino acid that it cannot make. Both populations can survive in co-culture, and both grow once their partner begins to die. Figure adapted from Shou et al. [46].

compete with the primary population by enjoying the benefits of a costly corporate behavior without contributing to it, arise within a population of cooperating bacteria more frequently when the individuals in the population are less related before the start of cheating [48]. Furthermore, Shou et al. [46] also demonstrated how the two populations in CoSMO adapt to co-habitation through time. Shou et al. mimicked population bottlenecks by repeatedly diluting and re-growing the co-culture. After ten cycles of dilution and re-growth, the engineered strains had adapted so that both populations were able to grow in co-culture when started from cell densities that were an order of magnitude smaller than was required before the cycles. Observing the dynamics and parsing the genetic mechanisms of co-adaptation will lend insight into the coevolution of species. Examples of co-evolution over longer periods of time can be studied as well. For example, the evolution and maintenance of microbial virulence factors might be directly correlated to competition or coordination between microbes in a given space. *Pseudomonas aeruginosa* binds, violates, and eats only the filamentous form of *Candida albicans*, which is the form of *C. albicans* that most commonly adheres to surfaces and therefore shares space with *P. aeruginosa* biofilms [49]. We can use engineered consortia to explore the evolution of cooperation and antagonism between populations in controlled environments to better understand these types of interactions.

Chapter 3 of this thesis describes an engineered symbiotic biofilm consortium that can be used to explore adaptation of a consortium through time. Here, two otherwise nonviable populations of genetically engineered *E. coli* grow and form biofilms because they communicate. The first population cannot make lysine but can form biofilms, while the second population cannot form biofilms but can synthesize both lysine and an acyl-HSL that activates lysine production in the first population. Biofilms persist if and only if both populations are present and co-localized. The mature symbiotic biofilm consortium exhibits genetic and structural adaptations that enable it to colonize a fresh environment more quickly than the initial mixture of populations colonized the first environment. This consortium implements all of the features of consortia described above: communication between the two populations relies upon both dedicated signaling and metabolic processes, the consortium survives in a challenging environment (biofilm) because both populations are present, and from it we may decipher some of the mechanisms that underlie the persistence of natural symbiotic consortia. Overall, this thesis presents two novel engineered microbial consortia as platforms upon which a diverse array of other microbial consortia might be engineered. They are flexible in output and robust in performance. The microbial consensus consortium of Chapter 2 demonstrates that if complex combinatorial behaviors are engineered into two populations *E. coli*, the behaviors can persist when the populations are co-cultured in biofilms for long periods of time [42]. The symbiotic biofilm consortium of Chapter 3 capitalizes upon this knowledge and demonstrates that structural and genetic adaptations in two engineered collaborating populations contribute to enhanced biofilm formation when the populations are co-cultured for long periods of time and through multiple population bottlenecks [50]. Let us now explore these two consortia in more detail, before finally turning in Chapter 4 to examine the challenges associated with and potential future applications of engineering microbial consortia.

Chapter 2

Engineered bidirectional communication mediates a consensus in a microbial biofilm consortium

A version of this chapter is published as [42].

2.1 Abstract

Here we present an engineered microbial consortium in which the microbial members communicate with each other and exhibit a 'consensus' gene expression response. This serves as a proof of concept that engineered consortia can communicate via dedicated signaling molecules in a biofilm. Two co-localized populations of *Escherichia coli* converse bi-directionally by exchanging acyl-homoserine lactone (acyl-HSL) signals. The consortium generates the gene expression response if and only if both populations are present at sufficient cell densities. Because neither population can respond without the other's signal, this consensus function can be considered a logical AND gate in which the inputs are cell populations. The microbial consensus consortium (MCC) operates in diverse growth modes, including in a biofilm, where it sustains its MCC response for several days.

2.2 Introduction

Most bacteria live in heterogeneous surface-bound congregations called biofilms, and vast reaches of the earth are coated in these living films. In many cases, the microorganisms comprising this ubiquitous coating form complex, interactive communities called consortia [37, 51-54]. Despite their abundance, these microbial communities are poorly understood. Reflecting this relative ignorance of how bacteria behave in biofilms, efforts to program biofilm functions are still in their infancy. The ability to manipulate these films, however, would enable controlled studies of microbial ecosystem dynamics and micro-scale environmental manipulation. To begin to explore these possibilities, we have engineered *de novo* cellular circuits that control *Escherichia coli* behavior in a stable, robust mixed-population biofilm community. The populations communicate, come to a consensus, and respond to each other's presence with a flexible, combinatory gene expression output.

Engineered circuits have been used to control the behavior of single cells [38, 45, 55-59] and cell populations [38, 39, 41, 45, 60] in both time and space. Cell–cell communication is a prerequisite for coordination of cellular circuit dynamics on the population level. Engineered communication, via broadcasting and receiving small-molecule signals, can enable the programming of robust and predictable population dynamics [39]. One-way engineered cell–cell communication has been used to coordinate biofilm formation in a single population at a predictable cell density [38] and to engineer pattern formation in a mixed population [41, 60]. Here, we demonstrate the first engineered bi-directional cell–cell communication network that can coordinate gene

expression from a mixed population. We have characterized the spatial and temporal behavior of this communication network in liquid, agar, and biofilm growth systems.

2.3 Results

2.3.1 Microbial Consensus Consortium (MCC) design and implementation

The MCC signaling network was constructed in *E. coli* from components of the LasI/LasR and RhII/RhIR quorum sensing systems [61] found in *Pseudomonas aeruginosa*, an opportunistic pathogen that forms a biofilm in the lungs of cystic fibrosis patients (Figure 2.1). These two systems enable *P. aeruginosa* cells to sense their environment and population density and correspondingly regulate hundreds of genes [31, 62, 63]. LasI in Consensus Circuit A and RhII in Consensus Circuit B catalyze the synthesis of the small acyl-homoserine lactone (acyl-HSL) signaling molecules



Figure 2.1 The MCC. Two *E. coli* cell populations communicate by using *P. aeruginosa* quorum-sensing components to achieve a consensus response. In Circuit A, the LasI protein catalyzes synthesis of 3OC12HSL. 3OC12HSL diffuses into cells containing Circuit B, forms a complex with LasR, and activates the Las promoter. Similarly, RhII catalyzes production of C4HSL in Circuit B, which diffuses into Circuit A, forms a complex with RhIR, and activates the Rhl promoter. Expression of both Targets A and B constitutes the MCC response and can be regarded as implementing a logical AND gate operation (lower left) where the two cell populations are the inputs and target gene expression is the output. Diagrams of the plasmids encoding Circuits A and B can be found in Appendix A.1.

3-oxododecanoyl-HSL (3OC12HSL) and butanoyl-HSL (C4HSL). The LasR transcriptional regulator in Circuit B is activated by the 3OC12HSL signal emitted by Circuit A, whereas RhlR in Circuit A is activated by the C4HSL signal emitted by Circuit B. The acyl-HSL concentrations are low at low cell densities but rise as the densities of Circuit A and Circuit B cells increase. When the signal molecules accumulate at high enough concentrations to activate the R proteins, the active R proteins can up-regulate target gene expression. Thus, both Circuit A and Circuit B cells must be present and at sufficient density before generating a "consortium" response, in this case red and green fluorescence. The MCC signaling network implements a logical AND gate in which the two inputs are population levels of cells containing Circuit A and cells containing Circuit B, and the output is target gene expression by the two populations (Figure 2.1, lower-left corner).

Proper function of the MCC is defined by minimal target gene expression when the cells grow in isolation (neither can generate a response without a signal from the other) and high target gene expression when they are co-localized in sufficient densities to activate the R proteins. Preventing a single MCC member population from selfactivating in isolation requires minimal "crosstalk" interactions between the Rhl and Las signaling systems. This constraint means that the Rhl promoter p(rhl) must respond specifically to C4HSL, the primary Rhll product [64], and the Las promoter p(las) must respond specifically to 3OC12HSL, the primary LasI product [62, 65, 66]. However, initial experiments in which only the receiver elements of Circuits A and B were tested revealed minor crosstalk between these promoter–activator pairs; particularly, p(rhl)

responded to high levels of 3OC12HSL (Figure 2.2). Thus, engineering of the MCC began with modeling to investigate the effects of this crosstalk and how these effects



Figure 2.2 Receiver A and Receiver B (A) constructs and (B) dosage responses to 3OC12HSL and C4HSL. Engineering of the MCC began with the construction and characterization of these "receiver" circuits, designed to express a target gene in response to a specific acyl-HSL. The MCC requires that p(rhl) responds specifically to RhlR/C4HSL and that p(las) responds specifically to LasR/3OC12HSL. Therefore, we characterized the dosage responses of each to both C4HSL and 3OC12HSL. Median fluorescence values were converted to equivalent fluorescein molecule counts using SPHERO Rainbow Calibration Particles (Spherotech RCP-30-5A) that were measured during each session.

might be mitigated. The model was used to choose between circuit designs based on their ability to minimize the population densities required for activation when Circuit A cells and Circuit B cells are grown together (activation by consensus), while maximizing the population densities required for self-activation when Circuit A cells and Circuit B cells are grown in isolation (isolation activation). The model suggests that the MCC should be designed with positive feedback on the I proteins, as further described in the supplementary information to Brenner et al. [42]. The presence of the cognate signal, C4HSL, in cells containing Circuit A should be a prerequisite for expression of LasI and production of the signal 3OC12HSL; in this way, the crosstalk signal concentration is minimized in the absence of Circuit B. Likewise, 3OC12HSL should up-regulate expression of RhII in Circuit B, limiting the concentration of the crosstalk signal, C4HSL, in the absence of Circuit A. Modeling results illustrating target gene expression profiles in the presence of positive feedback are shown in Figure 2.3A. The construction of Circuit A and B therefore proceeded with *lasI* under control of p(rhl) in Circuit A and *rhII* under control of p(las) in Circuit B (Figure 2.1).

2.3.2 MCC validation in liquid culture

We confirmed these design choices by initial characterization of the MCC system in liquid culture. To eliminate behavioral differences arising from variations in fluorophore maturation time and toxicity between Circuits A and B, we used GFP as the target gene in both circuits (GFP replaced Ds-Red in Figure 2.1). Cells containing each circuit were grown in isolation. Single-cell fluorescence measured as a function of time demonstrated that isolated circuits are unable to produce a significant response (Figure 2.3B). Cells containing the two circuits were also grown in separate chambers that allowed passage of small molecules between the two populations through a 0.2 μ m membrane. When the two circuits were allowed to communicate with one another and grow to sufficient density, responses from both were > 100-fold greater than the responses of the circuits in isolation (Figure 2.3B). These results confirm our model-based design and verify that the response is specific and combinatorial: MCC components are distributed among different

cell populations, providing response control based on presence or absence of one of the cell populations from the mixture.



Figure 2.3 Initial characterization of the MCC. (A) Modeling results depicting AND gate activity of the Circuit A and B populations. Target genes are expressed at high levels only when both populations are present at adequate population densities. To optimize performance of the AND gate, it is necessary to maximize the population density required for one population to self-activate (isolation activation) while minimizing the population density required for activation of each circuit in the presence of the other (activation by consensus). A more formal analysis is included in Brenner et al. [42]. (B) Liquid phase characterization of the MCC confirms the modeling results in (A). Median single-cell fluorescence is depicted for each circuit as a function of the OD of cells containing Circuits A and B. When cells containing Circuits A and B are grown such that they can communicate with one another, fluorescence is > 100-fold higher than when they are grown in isolation. Circuit A cells grow more slowly than Circuit B cells in liquid phase, possibly because a higher metabolic cost is associated with production of 3OC12HSL (from LasI in Circuit A) than production of C4HSL (from RhII in Circuit B) or because high intracellular concentrations of 3OC12HSL may have toxic effects. However, both populations reach stationary phase within 20 h of growth in liquid culture (Inset). More information regarding characterization in liquid culture can be found in Appendix A.2.

2.3.3 MCC behavior requires co-localization

To explore the need for co-localization in preparation for biofilm experiments, we tested

MCC function in solid phase cultures. Circuit A cells were embedded in solid medium

and placed in physical contact with solid medium containing the same density of Circuit

B cells (Figure 2.4A). Function of both circuits was again indicated by green

fluorescence to enable quantitative comparison, and images of green fluorescence were

captured every 30 minutes. Image analysis (Figure 2.4B) revealed that fluorescence

emerges in the Circuit B cells closest to Circuit A within a few hours. The response of Circuit A cells as a whole is lower than that of Circuit B cells, likely because of the slower growth of Circuit A cells (Figure 2.3B, inset). Both populations reach maximal reporter gene expression within 20 hours of incubation in spatial proximity and maintain these fluorescence levels through at least 24 hours (Figure 2.4B). The level of fluorescence decreases with distance from the interface, reflecting the signal gradient. This illustrates the requirement that Circuits A and B grow to an adequate cell density in spatial proximity to one another to generate the consensus response.



Figure 2.4 The MCC response is achieved by spatial co-localization of cells containing Circuits A and B. (A) A gradient of fluorescence emerges from the interface between an agar slice with embedded Circuit A cells and another slice with embedded Circuit B cells. (B) Image analysis of the experiment in (A), depicting the log of fluorescence. The pixels immediately surrounding the interface between agar slices were not quantified and were replaced with a black strip, because fluorescence in the boundary region may not accurately represent target-gene expression. More information regarding characterization in solid phase can be found in Appendix A.3.

2.3.4 MCC function in biofilms

Biofilms enable spatially proximate, sheltered bacterial growth and provide for

development of predictable environmental niches in otherwise changeable macro-

environments [67]. The ability to engineer living films may enable unprecedented stand-

alone sensor design and environmental manipulation opportunities. To explore these

possibilities, we studied the behavior of MCC circuit-containing cells growing in

biofilms. First, thin conformal biofilms were imaged by Confocal Laser Scanning Microscope (CLSM) in order to determine whether Circuit A and B cells would respond to increasing concentrations of acyl-HSL with increasing levels of fluorescence in the same way that they do when grown in other culture methods. No biofilms used in this analysis were allowed to grow deeper than 10 µm, removing acyl-HSL diffusion through the biofilm as a variable. All cells expressed a constitutive cyan fluorescent protein (eCFP) to enable total cell counts [68], and both circuit responses were indicated by green fluorescence. Results revealed that Circuit A and Circuit B cells are individually able to initiate and maintain healthy monoculture biofilms for periods of up to two weeks. Consistent with the liquid phase results, both populations respond strongly to their cognate acyl-HSL, and Circuit B cells exhibit greater sensitivity to exogenous acyl-HSL than Circuit A (Figure 2.5).

Mixed-culture MCC biofilms were then monitored by CLSM. In contrast to the thin biofilm dosage experiments detailed above, in which acyl-HSL was provided exogenously and concentration was uniform throughout, here the medium served as a sink for endogenously produced signals. Therefore, the biofilms examined in the mixed-culture MCC analysis were allowed to grow deeper than the monoculture biofilms so that signal molecules from Circuits A and B could accumulate. These biofilms grew no deeper than 80 µm, a depth at which oxygen diffusion is not a variable in fluorophore expression [69]. Circuit A function was identified by green fluorescence and Circuit B function by red fluorescence.



Figure 2.5 Monoculture biofilms respond to higher concentrations of acyl-HSL with higher levels of GFP expression. (A) Circuit A fluorescess minimally when 0.1 μ M C4HSL is administered (blue), but fluorescence increases at 0.5 μ M (green) and 5.0 μ M (yellow) C4HSL, and saturates at 10 μ M (orange) and 25 μ M (red) C4HSL. (*Inset*) Mean fluorescence at each C4HSL concentration. (B) Circuit B fluorescess minimally in response to 0.001 μ M (purple) C12HSL, but fluorescence increases at 0.01 μ M (blue), 0.05 μ M (green), 0.1 μ M (yellow), and 0.5 μ M (orange) C12HSL, and saturates at 1.0 μ M (red) C12HSL. (Inset) Mean fluorescence at each C12HSL and saturates at 1.0 μ M (red) C12HSL. (Inset) Mean fluorescence at each C12HSL concentration. More information regarding image processing can be found in Appendices A.4–A.7.

As demonstrated in Figure 2.6A, Circuit A and B cells grow together and display MCC function in the mixed culture biofilm. Images of MCC biofilms taken between 24 and 120 hours after biofilm inoculation reveal that Circuits A and B grow in intimate contact within the biofilms. Cells containing Circuit A grow more slowly than Circuit B cells in liquid and solid cultures (Figure 2.3B, inset); fewer Circuit A cells are also present in the biofilm. Consistent with the liquid and solid phase results, fluorescence emerges in both strains within 24–36 hours of inoculation (Figure 2.6B). Steady MCC behavior, similar to that illustrated in Figure 2.6A, is observed for at least six days following inoculation, after which time biofilm depth generally exceeds the 80 µm experimental limit (Figure 2.6B). Neither circuit exhibits significant fluorescence when

grown separately in a similarly thick monoculture biofilm (Figure 2.6C, D). These results demonstrate sustained and specific consensus consortium behavior in an engineered biofilm.



Figure 2.6 The MCC functions for at least 6 days when grown in a biofilm. (A) Three-dimensional rendering of an MCC biofilm, 24 h after inoculation, shows that both Circuit A and B cells are present and fluorescing. Circuit A cells constitutively express enhanced yellow fluorescent protein (eYFP; shown in yellow) and express GFP when the circuit is "on." Circuit B cells constitutively express enhanced cyan fluorescent protein (eCFP; shown in cyan) and express ds-Red when the circuit is "on." Circuit A cells are a minority, possibly because of their slower growth. (B) Mean intensities for Circuit A and Circuit B cells remain significant for at least 120 h after inoculation. (C) Monoculture biofilms of Circuit A cells fluoresce minimally. (D) Monoculture biofilms of Circuit B cells fluoresce minimally. All gridlines are 20 µm apart. More information regarding imaging can be found in Appendices A.4–A.7.

2.4 Discussion

E. coli can be engineered to detect and respond to highly varied stimuli including temperature, light, pH, gas or liquid concentrations [38, 40, 41, 70]. The MCC's population-level AND gate enables a convenient and efficient integration of the function of multiple engineered cells that have each been specialized to sense and respond to particular conditions. The MCC might also be engineered into existing industrial strains, for example, to guarantee that in mixed culture batch reactors optimal population densities are reached prior to onset of multi-species enzymatic activity. We have demonstrated that *E. coli* growing in biofilms can be engineered like their planktonic counterparts. Communication among the cell populations in the MCC biofilm is essential, and it is noteworthy that some bacteria naturally depend upon quorum sensing to coordinate biofilm formation [29, 71], while others are known to disrupt their competitors' biofilms by intercepting these signals [72]. An engineered living film could comprise such natural systems and be tuned to interact with them in order to engineer its environment. For example, such an engineered biofilm might be used to better understand the interactions of, or to interrupt the normal processes of, a quorum-sensingdependent pathogenic biofilm.

In establishing the MCC, we have demonstrated the first step toward such an engineered living film. By segregating circuit components in different cells, we have introduced a new type of specificity in the response which eliminates the potential for "leakage" from any one population. The consensus response might be comprised of an enzyme and pro-drug pair, or two inactive fragments of a toxin. Leakage from either circuit in the absence of its partner or without adequate population density would be inert,

but a highly targeted therapeutic or destructive response would occur where and when the MCC becomes active. This type of multi-signal engineered living film could also be expanded to include many conversation partners, and to incorporate communication mechanisms other than quorum sensing. Potential applications of such multi-signal, synthetic multicellular systems include synthesis of materials [73] in response to integrated stimuli, or surveillance and early detection of environmental changes related to epidemiology or material degradation. As a medical technology, an engineered biofilm consortium might eliminate unwanted infection or even destroy harmful cells in the body [74]. In such applications, the engineered bacterial biofilm consortium would carry out its function over long periods of time, under a variety of conditions, with minimal human awareness or intervention.

2.5 Methods

2.5.1 Plasmids

The Circuit A plasmid pFNK-601 encodes lasI and gfp(LVA) under control of the *P*. *aeruginosa* rhlAB promoter (qsc119), as well as constitutive RhlR production from the p(lacIq) promoter. This plasmid was constructed from a PCR-amplified fragment of *P*. *aeruginosa* PAO-1 containing the lasI gene and from the Receiver A plasmid pFNK-202qsc119 [75], shown in Figure 2.2. The Circuit B plasmid pFNK-602 expresses rhlI and gfp(LVA) from the *P. aeruginosa* p(las) promoter p(rsal). This plasmid also encodes constitutive lasR from the p(lacIq) promoter. Plasmid pFNK-602 was constructed by inserting rhlI into parent plasmid pFNK-503-qscrsaL. Plasmid pFNK-503-qscrsaL is the Receiver B plasmid (Figure 2.2) and expresses lasR from p(lacIq) and gfp(LVA) from the *P. aeruginosa* p(rsal) promoter. Plasmid pFNK-602-red was constructed by replacing gfp(LVA) in pFNK-602 with dsred-exp from Clontech plasmid pDsRed-Exp. These plasmids are illustrated in Appendix A.1.

2.5.2 Model and simulations

Continuous differential equations were used to model promoter activation by R-proteins, I-protein production and degradation (which is proportional to target protein expression at steady state), acyl-HSL synthesis and degradation, and saturation of acyl-HSL synthesis. The model is described in detail in [42].

2.5.3 Liquid phase data acquisition and analysis

To study the MCC response in liquid phase, starter cultures of *E. coli* JM2.300 cells (F⁻ *lacI22* λ^{-} e14⁻ *rpsL*135(StrR) *thi*-1) harboring either Circuit A or Circuit B plasmids were grown to OD < 0.3 in M9 medium (2 mM MgSO₄, 0.2% casamino acids, 0.5% glycerol, 300 µM thiamine) with 50 µgml⁻¹ kanamycin at 37°C in a shaking incubator. The cells were then washed and diluted to an OD of 0.02 in M9 medium supplemented with 50 µgml⁻¹ kanamycin. Holes were bored into the sides of two 50 ml Corning centrifuge tubes, and 20 ml of the Circuit A dilution was placed in one tube, 20 ml of the Circuit B dilution in the other. A Millipore Steriflip vacuum filtration unit was used to provide a 0.22 µM filter interface between two cultures for the consensus experiments. Both 50 ml tubes were affixed horizontally to the platform of a shaker at 37°C. Every hour, 1 ml samples were taken from each tube and replaced with 1 ml fresh M9 medium. Sample OD was measured using a Beckman Coulter DU 800 spectrophotometer, and
fluorescence measurements were taken on a Beckman Coulter Altra flow cytometer equipped with a 488 nm argon excitation laser and a 515–545 nm emission filter. Median fluorescence values were converted to equivalent fluorescein molecule counts using SPHERO Rainbow Calibration Particles (Spherotech RCP-30-5A) that were measured during each session. More information can be found in Appendix A.2.

2.5.4 Solid phase experiments

Two starter cultures of *E. coli* JM2.300, one harboring the Circuit A plasmid and one with the Circuit B plasmid, were grown to OD < 0.3 in M9 medium with 50 µgml⁻¹ kanamycin as described above. Cells from each culture were aliquotted into 6 ml of 37°C molten 1.5% low melt agarose (SeaPlaque) containing M9 and kanamycin 50 µgml⁻¹ to a final OD of 0.02. These solutions were poured into 60x15 mm Petri dishes (Falcon), and rectangular segments containing either Circuit A or Circuit B were excised from the solidified products. A Circuit A segment was placed end to end with a circuit B segment in a sterile WillCo glass-bottom dish. The plate was then incubated at 37°C, and images were taken every 30 minutes using a Zeiss Axiovert 200M microscope equipped with an AxioCam MR CCD camera. Images were captured with a 2.5x brightfield objective and a GFP filter with 470/40 excitation and 525/50 emission. Additional information is available in Appendix A.3.

2.5.5 Biofilm experiments

Starter cultures of *E. coli* JM2.300 harboring plasmid pMP4641 [68] and either the Circuit A or Circuit B plasmid were grown to saturation at 37°C in M9 biofilm medium,

(2 mM MgSO₄, 0.1% casamino acids, 0.4% glucose, 0.01% thymine, 100 µM CaCl₂), containing 50 µgml⁻¹ kanamycin and 20 µgml⁻¹ tetracycline. Starter cultures were then diluted to OD 0.2 in fresh M9 biofilm medium with 50 µgml⁻¹ kanamycin and 20 µgml⁻¹ tetracycline. Biofilms were grown in standard 1x4x40 mm flow chambers (Stovall Life Science, Inc.) with glass microscope coverslips. Monoculture biofilms were inoculated with 1 ml of the dilution of cells of the appropriate circuit, MCC biofilms were inoculated with a mixture of 500 µl of each. After inoculation, flow chambers were incubated for one hour without flow and then perfused at a low flow rate with M9 biofilm medium containing 50 µgml⁻¹ kanamycin and 20 µgml⁻¹ tetracycline. The flow chambers were incubated at 30°C. Images of the biofilms were captured at 24-hour intervals with a Zeiss 510 upright CLSM, controlled by Carl Zeiss AIM. A Zeiss Achroplan 40x/0.8 W objective was used to capture all images, images were captured with 512x512 pixel resolution, and all images used in quantitative comparisons were captured with identical pinhole and gain settings. eCFP excitation: 458 nm Argon laser, emission filter: BP 480-520 nm. GFP excitation: 488 nm Argon laser, emission filter: BP 500-530 nm. ds-Red excitation: 543 nm Helium-neon laser, emission filter: LP 560 nm. Images were processed for quantitative comparison with custom written Matlab-based tools. Threedimensional rendering was performed in Imaris 4.5.2. Biofilms prepared solely for threedimensional rendering incorporated a fourth fluorophore, eYFP on plasmid pMP4658 [68], excitation: 514 nm Argon laser, emission filter: LP 530 nm. More information regarding procedure, equipment settings, and processing can be found in Appendices A.4–A.7.

Chapter 3

Structure and selection in an engineered symbiotic biofilm consortium

Portions of this chapter are in submission [50].

3.1 Abstract

Microbial consortia constitute a majority of the earth's biomass, yet the evolutionary mechanisms by which they arise are debated. How do communities survive despite fitness differences, and therefore competition, between their constituents? Theory suggests that a community may adopt a spatial configuration, an "emergent structure," which gives the community a growth advantage over its members; natural selection can preserve a community if this structure and its growth advantage can be transferred to downstream environments. We present a synthetic symbiotic consortium in which two otherwise nonviable populations of genetically engineered *Escherichia coli* can complement each other, grow, and form biofilms. By exploring growth of the symbiotic biofilm through time, we discover emergent structure that can be transferred to downstream environments. When aggregates of the two populations are preserved through population bottlenecks, the emergent structure and a growth advantage are transferred to downstream environments, but when the aggregates are disrupted neither the emergent structure nor the full growth advantage is transferred. From such engineered consortia we may decipher some of the mechanisms that underlie the persistence of consortia in nature.

3.2 Introduction

The vast majority of living biomass consists of single-celled organisms, but the existence of higher organisms implies that natural selection can conserve interacting networks of cell populations despite competition between them [76, 77]. How nascent communities gain a growth advantage over their constituents is debated [47, 78-81], but highly complex cell–cell interactions [15, 29, 82-84], formation of multi-cellular structures [28, 85, 86], and the rise of genetic polymorphisms in spatially heterogeneous environments [87, 88] might contribute. Evaluating the role of emergent structure in evolution of natural communities poses a causality dilemma [77], yet de novo design of synthetic communities that exhibit emergent structure is difficult, so demonstrations of selection acting upon structure to preserve communities are few. It is known that competitors may coexist when cell-cell interactions occur over a small spatial scale between consistent neighbors through time, as they do in biofilm environments [89-92]. Additionally, emergent structure can arise when two populations that do not normally interact in nature are cultured together in a biofilm [93]. We set out to explore structure in a microbial community by engineering a synthetic symbiotic biofilm consortium.

3.3 Background: Evolution of communities

3.3.1 Kin selection

Theory suggests that multi-cellular entities can evolve from cooperating populations of single cells [94]. However, the mechanisms that provide for cooperating populations to survive in the face of cheats, individuals that take advantage of cooperation without assisting it, are not entirely clear. Therefore, to understand one path by which multi-

cellular organisms might have arisen, we must first understand the rise of cooperation in groups of individual cells. Kin-selection, or group-selection, theory proposes one mechanism by which cooperation can arise and become stable despite competition [95].

Kin- and group-selection were once considered separate entities but are now understood to be alternative statements of the same effect [96]. Kin-selection theory says that altruism, or gene expression that benefits a group at the expense of the individual, is more likely to arise in populations that exhibit less genetic diversity [85]. Thus, in a community where altruists are predominantly surrounded by other altruists, the outcome of a conflict between selection at the level of the individual (non-altruists are more fit, because they do not incur the cost of cooperation) and selection at the level of the group (individuals benefiting from cooperation are more fit) can be survival and dominance of the altruists. Stated another way, altruists must be the primary beneficiaries of the costly cooperative behavior in order for it to arise and become stable in a population. In one sense, then, kin-selection is selection at the level of the individual. However, the evolutionary outcome of the population cannot be predicted without knowledge of what is happening at a higher level of organization. That is, to predict the outcome we must examine not just fitness, but inclusive fitness, of the altruistic gene or genes [77]. Inclusive fitness takes into account the reproductive success of all individuals in a population possessing a given gene. The inclusive fitness of altruists depends upon how many of them are present, and/or upon how they are oriented in space with respect to one another.

3.3.2 Experimental demonstrations of community selection

Simple experimental demonstrations of how cooperation is maintained in laboratory populations exist. Many start with uniform populations of cooperators and observe the rise and impact of cheating subpopulations. For example, studies of *Pseudomonas fluorescens* reveal that cheats are more likely to take over through evolution when a population experiences large bottlenecks (many individuals from one community are propagated to the next) [97]. In contrast, when only a few individuals survive population bottlenecks, the survivors are more likely to be related and cooperation is thereby maintained. The same authors also show that cooperators can be selected when population bottlenecks occur at intermediate frequencies with respect to time (without regard to size), and that productivity in resulting populations is optimized [98, 99]. Importantly, spatial alignment between the bacteria was not preserved during bottlenecks in either study—cultures were homogenized during bottlenecks—so propagation was by truly random selection. As a result, the only factor determining survival of cooperation was the ratio of cooperators to cheats. In nature cells can stick together to form clusters, or aggregates, and these aggregates can be transferred between environments. It would be interesting to see how preserving aggregates through population bottlenecks might change the results of studies like these.

Pseudomonas aeruginosa is another common laboratory species that has been studied to examine the evolution of cooperation. For example, one challenge to kin selection arises under the condition where nutrients are limited. In this case, relatives living in close proximity become competitors (over a limited food supply) as well as cooperators, complicating the evolutionary outcome. One study examined this

phenomenon by varying both the relatedness of and the degree of competition in different cultures of *P. aeruginosa*. They showed that cooperation is advantageous in the face of global competition between subpopulations, despite the mitigating force of local competition within the subpopulations [100]. In another study, the authors watched the rise of quorum-sensing cheaters in populations of *P. aeruginosa*. The authors found that high relatedness allowed quorum-sensing variants to survive because cheaters were often localized in separate subpopulations. When populations began with lower relatedness, cheaters increased in frequency to a limit (the limit was determined by the fact that cheaters require the altruistic behavior of cooperators for optimal growth) [48]. In this latter set of experiments spatial structure was given no consideration, and in neither study did the authors examine whether the bacteria aggregated. Here again, it would be interesting to explore how spatial structure that arises and aligns the bacteria with one another impacts these studies' outcomes.

All of the examples discussed thus far employ populations of single species, but in nature microbes rarely exist as monocultures. As with most fields of biological study, theory precedes experimental validation, and theory suggests that in populations with multiple species, spatial self-structuring allows the formation of sub-communities that can be differentially adapted and between which natural selection differentiates [77, 101, 102]. One notable mixed-species community that has been examined experimentally is the symbiotic consortium of *Acinetobacter* and *Pseudomonas putida* [67, 93]. Initial studies of these two organisms co-cultured in biofilms demonstrated that *Acinetobacter* can metabolize benzyl alcohol to benzoate which leaks out of the cells of this population and can be metabolized by *P. putida*. An emergent structure arose in the mixed-species

biofilm after a few days; *P. putida* formed a mantle over *Acinetobacter* microcolonies. Next, some *Acinetobacter* microcolonies began to grow in the aerial regions of the *P. putida* structure, close to the bulk medium which was the source of benzyl alcohol [67]. This spatial arrangement optimized collaboration in benzyl alcohol degradation between the two species, allowing them to coexist in environments with lower benzyl alcohol concentrations than would normally support their coexistence [93]. By observing changes in colony morphology of *P. putida*, the emergent structure was found to be correlated to a change in colony phenotype, which was traced to a genetic polymorphism in the strain [93].

The simplicity of the *Acinetobacter* and *P. putida* experimental system enabled identification of causality: the spatial structure of the environment afforded the community an opportunity to organize spatially which, in turn, allowed the rise of mutations that were beneficial to the symbiosis. Although these authors identified emergent structure in the symbiotic consortium, and propagated individuals engaging in it to new environments, they did not propagate the actual structure to new environments. Given time, the individuals could re-establish the emergent structure, but it might be interesting to see how propagating pre-organized pieces of the structure to new environments would impact the fate of the consortium. Large pieces of physical structure are routinely conserved between biofilm environments by the propagation of aggregates, suggesting that biofilms might be a good place to look for evidence of community evolution.

3.4 Background: Physical structure of biofilms

3.4.1 Origins and evolution of biofilm structure

Within biofilms everything from species composition to cell density, and from gene expression to cell morphology, can vary by location and through time [69, 82, 103]. The physical structure of a biofilm is the result of a constant conversation between these celland population-dependent variables, and variables present in the physical environment such as temperature, pH, and dissolved oxygen concentration [104]. Biofilms may represent an important transition between uni- and multi-cellular life [76, 84, 94, 105, 106]. Evidence pointing to this includes the fact that bacteria commonly undergo gene transfer in biofilms [107-109]; the pool of available sequence space is larger than just the set of neutral or beneficial mutations available to the genome of the individual. Furthermore, bacteria exhibit direct metabolic interactions with one another even in laboratory biofilms, and proximity and community composition influence these types of interactions [110].

The formation of heterogeneous physical structure in biofilms hints that natural selection acts at the community level [77, 97]: the drive of individual cells to optimize their own access to nutrients while protecting themselves from environmental threats must play a role in determining biofilm structure [104], but some cells must sacrifice by colonizing the substrate, where access to nutrients is limited, in order to provide others with opportunity [105]. In other words, while it is detrimental to individual cells to become the basement layer in a biofilm, it is beneficial to the community to adhere and thereby to remain in an environment that supports life. The presence of surface colonizers cannot necessarily be predicted without knowledge of the higher level of

organization. The inclusive fitness conferred by surface-adhesion genes outweighs the costs associated with their expression.

Beyond the evolutionary origins of biofilms, thinking about evolution of biofilms and their structure requires us to think not only of the immediate environment in which the biofilm is found, but also of the global environment. The cells in a biofilm that are most likely to access nutrients are those that reach out furthest from the substrate into the environment, but these are also the most likely cells to be removed from the biofilm in the presence of flow. From the perspective of the immediate biofilm, the cost of being removed from the environment (essentially, death) outweighs the benefit of acquiring a small (not life-or-death) nutrient advantage. However, while the displaced cells "die" in the immediate environment, they can potentially be the first cells to colonize downstream environments. Therefore, their genes are propagated in a global sense and the inclusive fitness of the ability to leave a biofilm in search of new environments can be a net positive. Here is a potential example of Simpson's paradox: although the ability to "leave" a biofilm compromises the accumulation of "leavers" on a local scale, "leavers" will dominate on a global scale as long as they can colonize downstream environments as efficiently as "nonleavers" can [111, 112]. We will return to this theme later.

3.4.2 Development, composition, and propagation of biofilm structure

Most bacteria on earth can form biofilms, as can many other micro-organisms. Naturally occurring biofilms usually contain a mix of species, and environmental fluctuations can impact every species, or even each individual, differently [106, 113]. Mixed population biofilms can exhibit very different dynamics than their planktonic counterparts. For

example, neither planktonic growth rates nor monoculture biofilm growth rates accurately predict the growth rates exhibited by Burkholderia cepacia and Klebsiella oxytoca when both species are cultured together in a biofilm [114, 115]. Thus, it is virtually impossible to predict, *a priori*, what the structure of a particular biofilm will look like in a particular environment. Even if all controllable variables are known, stochastic fluctuations in the environment and in gene regulation can dramatically affect biofilm structure [104]. As a result of these uncertainties, most studies about biofilm structure are descriptive and not prescriptive, and many are qualitative [116]. Researchers most often use confocal laser scanning microscopy (CLSM) to study biofilms, but small pH and oxygen sensors can provide additional information about conditions within biofilms, fluorescent reporters can convey metabolic information [69], and staining cells and exopolysaccharides can provide endpoint information about biofilm composition [117]. Some efforts to quantify biofilm structure have been made, and the standard is a Matlab package called COMSTAT that calculates various metrics for CLSM images of biofilms including biomass and colony size [118].

There are two broad classes of biofilm structure. First, biofilms can be flat and monomorphic, although this is unusual in natural biofilms. In *Escherichia coli* the deletion of genes related to surface adhesion and cell–cell aggregation can cause flat structure under certain environmental conditions [117]. Deletion of metabolic genes that render a population very unhealthy, lack of cell surface appendages, or severe nutrient limitation, for example the absence of amino acids in the growth medium, can also lead to flat structure in *E. coli* biofilms (observations of present study). Providing citrate as the sole carbon source causes flat structure in usually robust and complex-structured *P*.

aeruginosa biofilms [119]. Additionally, in mixed-species microbial biofilms that are isolated from nature and cultured in the laboratory, severe nutrient limitation can cause flat structure [120].

Secondly and more commonly, biofilms can exhibit complex three-dimensional structure. Here, pillars (also referred to as mushroom clouds) of biomass that reach out from the substrate are surrounded by invaginations, tunnels, and caves through which liquid can flow or diffuse to deliver nutrients and remove waste products. Biofilms which form three-dimensional structure pass through five cyclical stages: initial adhesion to a surface, active growth, mature three-dimensional structure and/or formation of mushroom-shaped clouds, dispersal from the biofilm, and return to planktonic phase. Initial active growth in a biofilm is clonal, such that distinct clusters of whatever initially stuck to the surface will be observed growing from the point of initial adhesion with little exchange of biomass between these clusters [116]. Dispersal occurs with the greatest frequency after significant biomass has accumulated on the substrate. Then, single cells detach or are divided away from the biofilm to become planktonic, and large chunks— aggregates—of biofilm spontaneously detach and move downstream. We will return to a discussion of aggregates, but let us first examine observations of biofilm structure.

3.4.3 Development of structure in monoculture biofilms

Bacillus subtilis is a spore-forming bacterium that exhibits a coordinated structure, which is referred to as a colony biofilm, when colonies are grown on solid media. In one study *B. subtilis* colony biofilms were cultured on agar surfaces and the authors monitored expression of three different genes, whose expression indicates three separate

physiological states, with fluorescent reporters [121]. The study found that most cells were motile early in biofilm development, while midway through biofilm development most motile cells stopped producing motility genes and began to express a high amount of extracellular matrix. Cells expressing the most matrix were distributed in patches throughout the height and width of the biofilm and were theorized to provide the structure with integrity. Late in biofilm development some matrix-producing cells, particularly those in the aerial regions of the biofilm, began expressing spore-formation genes. Overall, this study demonstrated that a monoculture biofilm can exhibit complex differentiation through time and space because of coordinated gene expression [121].

P. aeruginosa is a common model organism for biofilm studies. One study demonstrates, much like the study of *B. subtilis* just described, that *P. aeruginosa* differentiates through space and time within biofilms based upon coordinated gene expression [122]. Using gene deletions and chemical treatments, the authors determined that expression of cell-surface appendages and chemotaxis-related genes, as well as quorum-sensing controlled release of DNA from cells, are required for formation of mature biofilm structures, including the caps on top of mushroom-shaped clouds [122]. Unlike the study of *B. subtilis*, the authors found that cells in the aerial regions (caps) on *P. aeruginosa* biofilms were more likely to contain motile cells, and that instead of progressing through every lineage, motile cells are present from the start of biofilm formation but swim up the mushroom stalk, via chemotaxis, to form the cap late in biofilm development [122]. These studies of *B. subtilis* and *P. aeruginosa*, taken together, demonstrate not only that differentiation occurs in monoculture biofilms, but also that no one species can be used to predict how another will act in a biofilm.

Members of different species form and populate three-dimensional biofilm structures dramatically differently in time and space, even when the overall biofilms exhibit similar three-dimensional architectures.

P. fluorescens is another well-characterized biofilm-forming strain that is commonly used in laboratory studies [87, 88, 97, 98]. When *P. fluorescens* is grown in spatially heterogeneous environments, such as stagnant liquid culture, genetic polymorphisms arise and different morphs, whose phenotypes are easily observed because of variations in colony morphology, populate different niches in the culture [87, 88]. After morphs arise, if the culture is sampled and samples are returned to a homogeneous environment (shaken liquid culture), the morphs revert to wild-type. As this study highlights, *P. fluorescens* is a model organism for adaptive radiation. Both *P. fluorescens* and *P. aeruginosa* are also used in laboratory studies of community evolution, as described in the previous background material, because both exhibit cooperative behavior that can be interrupted by the rise of cheats.

3.4.4 Aggregates in structure propagation and evolution: Simpson's Paradox Aggregates up to 500 μ m in diameter have been observed to detach from and move downstream in laboratory biofilms during detachment phases [123], but such large aggregates are unlikely to be found in our biofilm flow system given the dimensions of the flow chamber and tubing that we use. It is more likely that we see aggregates averaging between 50 and 60 μ m in diameter in our system [123]. Aggregates do not usually include the biomass from the substrate [123]. As a result, whatever cells initially colonize the substrate are likely to stay there, while detaching clusters will contain a

mixture of whichever cells inhabit layers of the biofilm above that basement layer [123]. This indicates that cells which grow away from the substrate faster will be more likely to detach in aggregates, and more aggregates will shed from biofilms that are able to accumulate more total biomass.

Once aggregates detach, they will either roll along the surface of the biofilm with potential to reattach to the same biofilm downstream, or leave the biofilm entirely to move downstream and potentially colonize a virgin surface [123]. Aggregate propagation may be a key mechanism by which a single type of biofilm structure takes over in a given environment. Aggregates can also convey mixed-population biofilms between environments. This second observation has important implications when examining the evolution of microbes in biofilms and perhaps of microbes in general. Because aggregates are pieces of biofilm within which cells may be pre-organized in an optimal physical structure, cells within them may work together more efficiently once they reach new environments than do naïve cells coming together for the first time. Thus, the inclusive fitness conferred by genes that cause mixed populations to form stable mixed-species structures may be higher than the inclusive fitness of genes that bolster the growth of individual cells. In other words, although theory and some experiments suggest that the primary benefit of three-dimensional structure formation is optimal growth and nutrient acquisition for individuals in a given environment [69, 124, 125], or protection for individuals from anti-microbial treatments [86, 126, 127], threedimensional structure may be equally important as a mechanism that promotes optimal downstream colonization by the whole community via aggregate detachment and downstream re-attachment.

This suggests a corollary. Hypothetically, since most bacteria on earth exist as biofilms, evolution of bacteria must be seen in light of the growth opportunities afforded by biofilms. If biofilm growth renders individuals less fit than aggregates to colonize downstream environments, the force of natural selection should yield individual bacteria that are optimized to interact with their community rather than individually most fit. Communities of bacteria can become symbiotic, even at the expense of individual fitness of community members, if symbiosis allows the community to dominate on a global level. Put another way, Simpson's Paradox predicts that individuals that are locally less fit can still be globally more fit, explaining the maintenance of apparently less-fit populations in local communities [111, 112]. One study using co-cultures of engineered cooperating and cheating strains of *E. coli* (which were grown without explicit physical structure in shaken liquid cultures) demonstrated Simpson's paradox. Even though cooperators grew more slowly, and therefore became a smaller fraction of their local populations in every instance, the populations that initially contained more cooperators grew better overall so that the global ratio of cooperators to cheats increased [111].

But does all of this speculation have any basis in reality? Do communities of symbiotic bacteria form structures that optimize cooperation? If so, can such structures be propagated to new environments by aggregates? If so, does having this pre-arranged structure confer any advantage to the community in its new environment? These are the questions we attempted to address in the present study.

We designed an engineered symbiotic ecosystem to explore its structure and function. It is plausible that adhesion and aggregation genes could be "lost" to some subpopulations in a stable biofilm community, if one subpopulation is primarily

responsible for cohesion of the overall biofilm and others come to depend upon the biofilm formation ability of the first. In our engineered system, one population has lost significant clusters of genes implicated in adhesion and aggregation so it depends upon the other population for biofilm formation. However, the biofilm-forming population is metabolically compromised and it forms only weak monomorphic biofilms alone. Its metabolic deficiency, and therefore its capacity to grow and form healthy biofilms, is compensated only in the presence of the biofilm-deficient population. We wanted to see, first, if this engineered symbiotic consortium would survive, how stable its function would be, and whether any discernable nonrandom three-dimensional structure would arise in the symbiotic biofilm. When we found that a particular three-dimensional structure did repeatedly emerge, we sought to determine whether it could be inherited by downstream communities. It could be inherited, and its presence was correlated with the presence of aggregates in the set of propagated cells. A growth advantage was also inherited by downstream communities that inherited these aggregates. Before turning to our results, let us put our study in perspective by examining what others have discovered about structure in microbial communities using engineering techniques.

3.4.5 Engineering approaches to studying microbial structure

The *Acinetobacter* and *P. putida* experimental system, described above, provides an entrée into a survey of how engineering techniques have been used to explore physical structure in microbial communities. That symbiotic system is natural in that the strains were not engineered, but it is synthetic in that these two strains do not necessarily coexist in nature. In another study of a natural-but-synthetic ecosystem, the authors used a

microfluidic device to simulate a spatially heterogeneous environment. Using it, they were able to culture a three-member community of soil bacteria [91]. Coexistence of multiple competing species can require persistent, self-organized, spatial arrangement [102] and this, in turn, can depend upon the natural environment in which the community originates. It is difficult to mimic complex natural environments in the laboratory, but microfluidic devices have the potential to come close. The three-member community cultured in this study, which is representative of similar communities found in nature, could not coexist in bulk medium, nor could any of the members grow alone. However, when the three populations were kept isolated but within several hundred microns from one another in the microfluidic device, and were allowed to communicate with one another through microfluidic channels, all three species survived and grew [91].

Several studies have examined physical structure in *E. coli* communities. A study employing microfluidic devices observed growth of *E. coli* in small, structured spaces that were perfused with nutrients [90]. The authors observed that the cells quickly selforganized to orient their long axes in parallel with the primary direction of nutrient diffusion, and they used computational models to confirm that the average shape and size of an *E. coli* cell is well adapted to colonizing small spaces while maximizing diffusion to the interior of the colony [90]. Yet another study also examined a community of *E. coli*, here with three different strains that compete in a canonical rock-paper-scissors ecosystem (one beats the second, who beats the third, who beats the first) [89]. The authors used simple plate-based culturing techniques to demonstrate that the three strains coexist when interactions are constrained to occur over only a local region, whereas the strains cannot coexist when cultured in the well-mixed environment of a shaken flask

[89]. A final study examined two competing polymorphic populations of *E. coli* in a microfluidic environment and discovered that isogenic aggregates of each population formed after co-culturing [92]. The formation of these aggregates was not dependent upon competition, but was a feature of the *E. coli* themselves. The authors suggest that selection is operating at multiple levels, including upon the physical structures present among bacteria in the microfluidic device, to promote fitness of both genotypes despite the competition between them [92].

Here, we present a synthetic symbiotic consortium in which two populations of *E*. *coli* depend upon one another for survival in biofilms. The first population cannot synthesize lysine but can form biofilms. The second population cannot form biofilms alone, but it can synthesize lysine and can activate lysine production in the first population. This consortium exhibits emergent structure, and we find that when aggregates of cells from initial biofilms are propagated to downstream biofilms, the emergent structure and a growth advantage are transferred also.

3.5 Design and construction of the symbiotic consortium

3.5.1 The biofilm-forming, but metabolically deficient, population

The symbiotic biofilm consortium consists of two engineered populations of *E. coli* MG1655, one of which is deficient in biofilm formation but otherwise healthy, while the other is metabolically compromised but capable of biofilm formation (Figure 3.1). To compromise metabolism in strain MG1655, we interrupted the biosynthetic pathway for lysine and diaminopimelate by deleting *dapD*, the gene encoding tetrahydrodipicolinate N-succinyltransferase, creating strain MGd- [128]. *DapD* was then replaced on an

engineered plasmid under control of the transcriptional regulator RhIR, which is activated by the small, freely diffusible acyl-homoserine lactone (acyl-HSL) butanoyl-homoserine lactone (C4HSL) [61]. This strain was marked by constitutive expression of eCFP, and will be called the blue population.



Figure 3.1 The synthetic symbiotic consortium. Two populations of *E. coli* coexist because they communicate. The blue population cannot synthesize diaminopimelate or lysine but can form biofilms. When grown as a biofilm in the absence of lysine or diaminopimelate, this population forms a scant biofilm that eventually dies. The yellow population cannot form biofilms alone, but it is otherwise healthy and it synthesizes a small molecule, C4HSL, that activates lysine production in the first population. Yellow cells cannot form biofilms unless they are bound within the biofilm formed by the blue population. Only when these two populations are grown together can they form viable biofilms that persist.

The engineered plasmid in the blue population was constructed from pFNK202 which encodes constitutive expression of *RhlR* [42]. Proper function of the symbiotic consortium requires that very little DapD be present in blue cells in the absence of the yellow population, while the presence of yellow cells should restore biological levels of DapD to blue cells. However, minor expression (promoter "leakage") of DapD allows the blue population to begin forming a sparse biofilm without C4HSL so that the symbiotic consortium can gain a foothold in the environment. We obtained an adequate

basal expression level of *dapD*, with wild-type levels of biofilm formation in the presence of saturating C4HSL, by placing *dapD* under control of the RhlR-activated promoter p(qsc119) with ribosome binding site (RBS) H, and attaching an LVA degradation tag to DapD. We tried various other permutations of this arrangement, including a variety of RBS strengths, and expressing *DapD* with and without the LVA tag, but this combination yielded optimal behavior.

3.5.2 The biofilm-deficient (but healthy) strain

To construct a biofilm deficient version of MG1655, we deleted three groups of genes that are implicated in biofilm formation. First, a primary determinant of both initial adhesion and three-dimensional structure formation in *E. coli* biofilms is the presence of the cell-surface appendage called curli. Curli can be seen in scanning and transmission electron microscope images as fibrous bundles protruding from and swirling around the cell wall of bacteria, and the curli of adjacent cells appear to intertwine. Curli are important for initial adhesion to abiotic surfaces [129] and also for cell-cell adhesion that leads to three-dimensional structure formation [117, 130]. In *E. coli*, curli are optimally expressed at 30°C under low nutrient and low osmolarity conditions [131, 132], which are similar to the conditions we use in our study. When *csgA* and *csgD* were deleted from *E. coli* in previous studies that used similar conditions to our study, a sparse monolayer was the best biofilm formed by the resultant strain [117].

Two operons, csgDEFG and csgAB, are responsible for the biosynthesis of curli monomers (CsgA) and their export. We deleted both operons entirely. One of the deleted genes, which is involved in regulation of curli expression, expresses a RhIR

homolog, CsgD [129]. It is not clear whether CsgD can either interact with C4HSL or bind the p(qsc119) promoter that we used in the engineered plasmid for the blue population, but its absence could potentially alter the effect of C4HSL upon the biofilmdeficient population relative to the blue population.

A secondary factor, which is involved in strong surface adhesion of *E. coli* biofilms but not as clearly involved in the formation of three-dimensional structure, is the presence of type I pili (also called fimbriae). These cell-surface appendages form catch bonds whose binding is characteristically tighter under higher stress [133, 134]. Genes responsible for fimbriae lie in the *fimA–fimH* locus, and the key gene whose product mediates catch bond formation is *fimH*. We used a mutant lacking the entire locus for the purposes of this study [133]. Many experimental studies use mannose-BSA to provide for catch-bond formation. Here, we used bovine ribonuclease B quenched with bovine serum albumin (BSA). In single mutants lacking the *fim* locus, we observed significantly less initial adhesion than in any other single mutant that we made.

A third factor, implicated in three-dimensional structure formation of *E. coli* biofilms but not in initial adhesion, is the presence of colanic acid (CA) [117]. CA is an excreted polysaccharide that surrounds cells in biofilms and creates space between them which presumably allows for diffusion of nutrients and wastes, for communication between cells, and for growth. CA is a constituent of the "slime" that is commonly mentioned in macroscopic observations of biofilms. It is not clear whether *E. coli* MG1655 produces CA when it is sessile [117], but CA is probably important in determining the initial three-dimensional structure of MG1655 biofilms while cells are actively growing and dividing. Both theory and experiments indicate that biofilms of

cells lacking the genes encoding CA are flatter and cells are more tightly packed [117, 135]. 19 genes in the *wca* locus are responsible for CA production and secretion. Genes encoding CA are most highly expressed at temperatures below 25°C and in minimal medium with an accessible carbon source [136]. These conditions are approximately those in our study so we deleted the entire locus *wcaL–wza*.

Many other gene products also influence the structure of *E. coli* biofilms. For example, when *E. coli* have the F plasmid that provides conjugative machinery, they exhibit better initial adhesion and also more prominent three-dimensional structure under conditions where medium is constantly refreshed but there is no clear pattern of flow [137]. The pilus structure expressed from the F plasmid seems to promote nonspecific cell–surface and cell–cell adhesion [137]. In contrast, under conditions more similar to those in our study, constant flow in a biofilm chamber, the F plasmid does not significantly enhance initial adhesion but it does contribute to the formation of three-dimensional structure [116]. The strain we used, MG1655, does not have the F plasmid or any conjugative machinery; this protects our two populations from exchanging the genetic material with which we programmed them.

Other genes that may contribute to biofilm formation in *E. coli* under some conditions include *flu*, which encodes a cell-surface autotransporter called antigen 43, and *flhD* and *flhC*, master regulators for the expression of flagellar genes. All three of these genes have been shown to be particularly important in biofilm development at 37°C [138, 139]. However, we made single, double, and triple mutants of the *fim* locus, *flu*, and *flhDC* and found that neither Δflu nor $\Delta flhDC$ significantly reduced biofilm formation under the conditions that we use when Δfim was present (data from observation). In fact, one or both of these deletions may actually enhance biofilm formation under the conditions we use (data from observation). The study which identified these genes as important did use biofilm flow chambers, but they did not coat the chambers with bovine ribonuclease B, they maintained the biofilm at 37°C rather than 30°C, they allowed a much longer time for initial adhesion (2 hours) than we do (5 minutes), and they flushed the chambers after incubation with a much higher flow rate (0.8 mL/min rather than 0.2 ml/min, which we use) [138]. This demonstrates how difficult it is to predict biofilm formation, and highlights the dramatic impact that environmental variables can have upon gene regulation and thereby upon biofilm formation.

Finally, strain MGfwc- was constructed from MG1655 lacking the curli locus ($\Delta csgC-csgG$), the type I fimbriae locus (Δfim), and the colonic acid locus ($\Delta wcaL-wza$). This population contained an engineered plasmid encoding strong constitutive expression of the C4HSL synthase, RhII. This engineered plasmid was constructed from pFNK102 [42]. Yellow cells must synthesize enough C4HSL to activate RhIR, and thereby to upregulate *dapD* expression, in the blue population early in the lifespan of the consortium so that the blue population does not die. The strong constitutive promoter J23100 combined with RBSII yielded enough C4HSL in the biofilm environment to activate the symbiotic function. The strong constitutive promoter p(lacIq), even coupled with RBSII, did not provide adequate C4HSL production to enable optimal function of the symbiotic consortium. This strain contained a YFP marker plasmid, and is called the yellow population. More information about the construction of both strains can be found in Methods and in Appendix B.1.

3.6 Function and stability of the biofilm consortium

3.6.1 Initial characterization of the function of the symbiotic consortium

To confirm that the symbiotic consortium functions as designed, we inoculated a 50/50 mixture of blue and yellow cells into biofilm flow cells alongside separate control monoculture biofilms of each population. No yellow biofilm was observed in the yellow control monoculture and the blue control monoculture formed a scant biofilm alone (Figure 3.2A). Only a small fraction of the yellow population remained in the mixed biofilm after 24 hours of growth, confirming the inability of yellow cells to form biofilms, but as the mixed biofilm matured it accumulated significantly more biomass than either control (Figure 3.2A). By 96 hours, the yellow population recovered to constitute half the total biomass, and that balance remained stable until after 120 hours (Figure 3.2B). This answered our first question: the engineered microbial consortium survived and grew. Next, we wondered how stable this coexistence and cooperation would be.

3.6.2 Stability of natural ecosystems

There are many theories about what makes natural ecosystems stable, but there are very few demonstrations of ecosystem stability that involve microbial ecosystems. One theory suggests that asymmetries in how dependent species are upon one another's presence, when coupled with differences in fitness, can lead to maintenance of species diversity in communities. This is true for some plant-animal mutualistic networks [140]. But a study



Figure 3.2 Initial characterization of the symbiotic consortium. (A) The symbiotic consortium functions as designed. The blue population control forms a biofilm which eventually dies, and the yellow population accumulates no biomass before 72 hours, and very little biomass over the lifespan of the experiment. When the yellow and blue populations are inoculated in a 50/50 mixture, significantly more biomass accumulates than when either population is inoculated alone. (B) The consortium functions when grown for long periods of time. At first the yellow population is a strict minority but by 96 hours it constitutes half the biomass of the consortium. Between 80 and 96 hours the yellow population shifts to accumulate primarily above the blue population. (Solid yellow areas, yellow biomass in consortium; solid blue areas, blue biomass in consortium; blue bars, blue control biomass; yellow bars, yellow control biomass plotted against right axis; yellow line, biomass median of yellow population; blue line, biomass median of blue population. All errors are standard deviations.)

by LaPara et al. challenges this theory [22]. Here, when a natural community taken from a waste-water treatment plant was subjected to decreasing nutrient concentrations, 16s rRNA and rDNA analyses revealed that while functions were conserved in the community, redundant populations were eliminated. Survival of this natural community occurred because of diversity, but also at the expense of diversity.

A second body of theory postulates that taxonomic diversity is the fundamental determinant of community stability [141]. Kiessling surveyed reef ecosystems and found that taxonomic diversity is related to ecological stability on evolutionary timescales [142]. However, studies on shorter times-scales do not agree with this theory. Rather, on shorter time-scales, a third body of theory claims that functional diversity within communities dictates invasibility and community stability. In other words, the more survival strategies that a community can try when it encounters stress, regardless of who tries them, the more likely it is that the community will survive. The study explicated

above by LaPara et al. supports this theory [22], but in one dissenting view Arenas et al. surveyed communities of algae and determined that specific species identities, rather than functional diversity, determined resistance to invasion [23]. The number of populations of algae considered by Arenas et al. was very small (< 4 species) so broad application is questionable.

Experimental evidence lags theory about ecosystem stability because naturally occurring symbiotic communities are difficult to culture in the laboratory. In a simple sense, our engineered symbiotic ecosystem exhibits behavior consistent with the third theory. That is, the presence of both engineered populations enables the entire community to survive in a biofilm and under nutrient stress, whereas neither population could do so alone.

3.6.3 Stability of biofilms in nature

There is much evidence that the biofilm mode of growth confers resistance to antimicrobial chemicals [126, 143]. Additionally, biofilm communities in nature are stable over periods of time on the order of years, even as the species balances fluctuate within that time in response to nutrient availability, temperature, and light (seasonal variance) [144-146]. Furthermore, evidence from geomicrobiology suggests that microbial communities may exist and perform their functions over evolutionary timescales [147]. Biofilm communities composed of species found in nature, but cultured in the laboratory, can be stable for periods of several weeks or months [36, 123] but there is little experimental work regarding the stability of engineered multi-species populations in biofilms.

3.6.4 Stability of the engineered symbiotic biofilm consortium

We observed that the symbiotic biofilm ecosystem was stable, both populations continued to co-exist, for up to 288 hours after inoculation (the experiment was terminated at that point). During this time, the biofilm exhibited oscillations in total biomass characteristic of biofilm growth and detachment/sloughing phases (see section on biofilm structure for a description of these phases), and the timing of these phases was remarkably repeatable in independent biofilms that were grown months apart [analysis of variance, ANOVA, $F_{0.05}(3,28) = 0.47 < F_{crit} = 2.947$, P = 0.70] (see also Appendix B.2). In no case did either population die off during the length of the co-culture experiment.

Additionally, the blue and yellow populations function as designed—the yellow population does not recover wild-type biofilm forming ability and the blue population does not grow without exogenous C4HSL—for at least 288 hours in the biofilm, and during this time the engineered plasmids also remain unchanged (data from sequencing and observation). This was somewhat unexpected; we thought an engineered biological system, particularly under the selective pressures of biofilm growth in minimal medium, would mutate to escape engineered control much more quickly. This result begs for further experimentation to determine whether engineered consortia are more stable than engineered monocultures [1, 148]. However, it has been shown that although engineered control can be lost in *E. coli* within 70 hours in batch culture, control is retained over a period of 200 hours when the same monoculture is grown in a micro-chemostat [149]. It is not entirely surprising that the biofilm environment might afford an equal opportunity. This answered our second question: the engineered symbiotic consortium was stable over at least a period of 12 days.

3.7 Physical structure in the engineered symbiotic consortium

3.7.1 Initial observations of structure

We explored the physical structure of the mixed biofilm by calculating the biomass median for each population at each time-point (Figure 3.2B). The biomass median is an indicator of the location of individual populations with respect to the substrate in mixed biofilms; 50% of the biomass of a given population is located between the substrate and the biomass median of that population. If a population has a larger biomass median, its biomass is primarily localized away from the substrate, whereas a population with a smaller biomass median grows close to the substrate. The minimum biomass median (1 µm) indicates that most cells in the population are attached to the substrate (see also Appendix B.3). Between 80 and 96 hours of growth a significant shift occurred in the symbiotic consortium; although there was still more blue biomass than yellow, the yellow population moved from below or within the blue population to a position significantly further from the substrate than the blue population (Figure 3.2B). Observations corroborate this distinct phenotypic change—clumps of yellow biomass form a mantle over the blue biomass (Figure 3.3).

We identified this change in the symbiotic biofilm consortium as emergent structure, reminiscent of the structure that arose between *Acinetobacter* and *P. putida* in studies described above. It is interesting to note that our results stand in contrast to results generated in one theoretical paper [135]. There, the authors used computational modeling to predict three-dimensional structure in a mixed biofilm containing two populations: extra-cellular matrix producers (like the blue population in our study), and



Figure 3.3 Projections showing emergent structure in a 38 μ m-thick symbiotic biofilm. (A) After 96 hours of growth, the consortium exhibits emergent structure. The blue population grows primarily near the substrate, as shown in this projection that is taken at the level of the substrate. (B) The yellow population forms clouds on top of the blue population, as shown in this projection taken at 1/3 the total height of the biofilm, above most of the blue biomass.

nonproducers (like the yellow population in our study). In the outcome of that model, the producers formed bulbous structures very similar to the ones we observe to be formed by our nonproducers (our yellow population) on top of a flat, nonproducer biofilm. This is exactly the opposite of the emergent structure that we observe. A direct comparison between our experiments and their model cannot be made because of the additional mutations which we introduced into strain MGfwc-; however, it is still an interesting juxtaposition. Overall, this result answered our third question: a discernable, nonrandom, repeatable structure emerged in the co-culture biofilm of the symbiotic consortium. Did the structure appear in downstream environments more quickly than it initially emerged in this first generation?

3.7.2 Exploring the transfer of the emergent structure

In biofilms, initial growth is clonal, but cells later detach from the biofilm and move downstream to populate new environments [116]. Under these conditions, a given clone or community can be selected if it grows away from the substrate most quickly, detaches to move downstream first, adheres, and grows best in the downstream environment. We wondered whether the emergent structure observed in the symbiotic biofilm could be transferred to downstream environments, and whether it was related to a growth advantage there. We simulated a population bottleneck by propagating two samples into fresh flow cells: one from a 48-hour-old consortium, before the emergent structure appears, and one from an 80-hour-old consortium exhibiting emergent structure. In both cases, the number of cells transferred was the same.

When the 48-hour-old consortium was propagated, the second-generation biofilm accumulated less biomass than the blue monoculture control (from Figure 3.2A). Presumably, early propagation transfers an immature community in which the blue population is sessile or dying as it awaits recovery of the yellow population. However, when the 80-hour-old consortium was propagated, biomass medians of the second generation revealed that the emergent structure was transferred to the second generation: within 48 hours the yellow biomass was found significantly further from the substrate than the blue biomass (Figure 3.4A). This answered part of our question: once established, the emergent structure could re-establish much more quickly in a downstream environment. But would the downstream biofilm have any advantage over a biofilm that began with naïve cells?

3.7.3 The second generation has an advantage

We observed that the second generation biofilm exhibited a growth advantage over the first generation. It formed a biofilm far more quickly: after 48 hours of growth, second generation consortia accumulated an average of 15 times, and as much as 30 times, the biomass accumulated by first-generation consortia in their first 48 hours [ANOVA $F_{0.05}(3,4) = 0.358 < F_{crit} = 6.591$, P = 0.78, see also Appendix B.4]. Not only did the second generation accumulate biomass more quickly, but it also accumulated more total biomass. After 48 hours of growth, the average total biomass of the second generation was double the average of the highest ever recorded first-generation biomasses (Figure 3.4A, see also Appendix B.4). Overall, when the symbiotic biofilm consortium is mature, it exhibits emergent structure which can be transferred to downstream environments. Additionally, the downstream biofilm consortium exhibits a growth advantage, consisting of faster and greater total accumulation of biomass, over the initial consortium. These results raise two further questions: first, how is the emergent structure transferred? Second, why is there a growth advantage in the second generation?

3.8 Aggregates in the transfer of emergent structure and growth advantage

3.8.1 Aggregates and emergent structure

Evidence suggests that aggregates can detach from mature biofilms and seed downstream communities [123]. To evaluate the role of aggregates in the transfer of the emergent structure between generations, we performed the propagation experiment described above but treated the first generation to break up multi-cellular structures before inoculating it



Figure 3.4 Biomass comparisons between generations. (A) Both second-generation consortia accumulate biomass more quickly than the first generation. The untreated second-generation accumulates biomass more quickly and accrues more total biomass than either the first- or treated second-generations. The biomass medians (plotted against the right axis) of the untreated second-generation indicate that the emergent structure is present. (Grev bars, first-generation biomass; green bars, untreated second-generation biomass; pink bars, treated second-generation biomass; blue line, biomass median of untreated blue population; yellow line, biomass median of untreated yellow population.) (B) The treated and untreated consortia start with the same amount of total biomass, but the treated biofilm has only 10% vellow biomass whereas the yellow population constitutes 50% of the starting biomass of the untreated second-generation biofilm. Yellow biomass comprises significantly more than 50% of the second- and third-generation biofilms after 24 hours (vellow biomass percentages plotted against right axis). Total biomass accumulation for the treated second- and the untreated third- (propagated from the treated second-) generations was equivalent after 48 hours, whereas total biomass accumulation for the untreated secondgeneration was double that total. (Pink bars, treated second-generation biomass; pink line, percentage vellow in treated second-generation; green bars, untreated second-generation biomass; green line, percentage yellow in untreated second-generation; orange bars, third-generation biomass; orange line, percentage yellow in third-generation.) (C) The biomass medians of the treated second-generation populations diverge less than those corresponding to the first-generation (see Figure 3.2B) and untreated second-generation populations (shown here). The biomass medians of the third-generation populations parallel one another and stay low, indicating that the emergent structure is not present. (Solid yellow line, biomass median of treated second-generation yellow population; solid blue line, biomass median of treated second-generation blue population: long-dashed vellow line, biomass median of untreated secondgeneration yellow population; long-dashed blue line, biomass median of untreated second-generation blue population; short-dashed vellow line, biomass median of third-generation yellow population; short-dashed blue line, biomass median of third-generation blue population.) (D) The YFP+ fraction was able to form a biofilm, but the aggregate fraction biofilm formed most quickly and accumulated the most total biomass. Biomass medians from the aggregate biofilm reveal that the emergent structure is present from the start, and that both populations grow away from the substrate together. (Yellow bars, biomass of YFP+ fraction biofilm; red bars, biomass of aggregate fraction biofilm; yellow line, biomass median of aggregate fraction yellow population; blue line, biomass median of aggregate fraction blue population. All errors in this figure are standard deviations.)

into the second generation (an equal number of cells was inoculated into the first, and treated and untreated second-generation biofilms). We observed that the biomass medians of the blue and yellow populations diverged less in the treated second generation than they did in the first and the untreated second generations (Figures 3.2B, 3.4C). This suggested that the treated second generation did not exhibit the same emergent structure as the first and untreated second generations, but it did not provide conclusive evidence that aggregates convey this structure.

Upon closer examination, we noticed that although the treated and untreated consortia were made from the same inoculum (first-generation effluent), and thus contained the same 50/50 composition of yellow and blue biomass, the treated biofilm had only 10% yellow biomass after 12 hours of growth whereas the yellow population constituted 50% of the 12 hour biomass of the untreated second-generation biofilm (Figure 3.4B). This result indicated that treatment in some way prevented yellow cells from sticking in the second-generation biofilm. We first examined the possibility that treatment damaged the cells. Such damage should have been done to cell-surface structures of both populations and would compromise initial adhesion. However, after only 12 hours of growth, the treated and untreated second-generation biofilms contained essentially equal amounts of total biomass (although, as mentioned above, the fraction of yellow biomass was dramatically different). This suggested that initial adhesion was not compromised in the treated case. Images captured at the substrate after 12 hours of growth in the untreated case show clumps, perhaps aggregates, of blue and yellow cells co-localized on the substrate (see Appendix B.5). In contrast, images of the substrate in

the treated case show clumps of blue cells unassociated with widely dispersed single yellow cells and small yellow clusters (see Appendix B.5).

Given all of these observations combined, we hypothesized that more of the yellow population remains in the untreated biofilm primarily because the yellow cells are aggregated with blue cells. Put another way, sticking to the blue population could help more members of the yellow population to stick and stay in the second-generation biofilm. Further, if the aggregates are pre-organized pieces of the emergent structure, the emergent structure might arise more quickly (as we observe that it does) when the inoculum contains aggregates and it might not emerge at all if the aggregates are disrupted.

3.8.2 Aggregates and the growth advantage

We wondered whether the presence of aggregates in the inoculum conferred a growth advantage to the untreated second generation. While the treated second-generation consortium still grew more quickly than the first generation, it accumulated only half the total biomass of the untreated second-generation consortium in the same amount of time and its maximum total biomass was closer to that of the first generation (Figure 3.4A). This suggested that the presence of aggregates in the inoculum was correlated with at least a portion of the growth advantage—the greater total accumulation of biomass.

3.9 Adaptation or polymorphism and the growth advantage

The observation that some yellow cells were stuck to the substrate after 12 hours of growth in both the treated and untreated second generations is important (see Appendix

B.5). In the untreated second generation, adhesion of yellow cells could be explained if the yellow cells are stuck to the blue cells and the blue cells bring the yellow cells into contact with the substrate. But we observed some single yellow cells and small clusters sticking to the substrate in the treated second generation. We did not observe yellow cells to adhere at all in the first-generation control. This hints at the possibility that an adaptation—perhaps a polymorphism like those described in above sections about *P. fluorescens* biofilms, or a change in gene expression as has been observed in subpopulations of *P. aeruginosa* and *B. subtilis* biofilms—has arisen that makes the yellow population more sticky.

A stickier yellow population could have three effects on the biofilm consortium. First, it could contribute to the emergent structure; as yellow cells become more adhesive, perhaps they form the balls of yellow cells that we observed sticking to one another and to the blue cells in the emergent structure (Figure 3.3). Second, sticky yellow cells might associate more tightly and/or more permanently with blue cells, helping the symbiosis along. Third, sticky yellow cells, if given the opportunity, might disrupt coexistence of the consortium since, being sticky, they no longer need blue cells. We wanted to learn whether the yellow cells had indeed become more adhesive, and to parse the relative contributions made to emergent structure and to the growth advantage by the propagation of aggregates and by the theoretical "sticky adaptation" in the yellow population. Thus, we used cell sorting to separate the yellow cells from the aggregates in the effluent from the treated second generation, and started three third-generation biofilms: a separate biofilm from each sorted fraction, and one control biofilm comprised of untreated effluent from the treated second-generation biofilm.
3.10 Third-generation biofilms reveal contributions of adaptation and aggregates

3.10.1 The control third-generation biofilm

After 12 hours of growth, the third-generation biofilm consortium control, started from untreated effluent of the treated second-generation biofilm, contained an even higher percentage of yellow biomass than both of the second-generation consortia. Curiously, this third generation initially accumulated biomass faster than any of its precursors (Figure 3.4B). We reasoned that biomass might accumulate faster if yellow cells adapt to grow faster (note that "growth" is a product of cell division whereas "biomass accumulation" results from a combination of cell division and adhesion). If faster growth is the primary adaptation, the yellow biomass should increase at the same rate simultaneously in the treated second generation and in the third generation (which was propagated directly from it) biofilms, because the vellow cells in these two biofilms are clones and nothing happens in the transfer between the generations that should change the rate of yellow cell growth. However, the rate of increase of yellow biomass in the third-generation biofilm was at least seven times greater than that in the treated second-generation biofilm, leading us to conclude that a change in growth rate was not the primary adaptation. Since the yellow cells are metabolically healthy, they grow faster than the blue cells. If they can stick to the substrate and in the biofilm better, yellow biomass, and therefore total biomass, should accumulate much more rapidly, particularly if they are able to colonize more of the substrate in the third generation than in the second. We speculated that the primary adaptation in the yellow cells enabled them to stick more effectively. Importantly, the third generation consortium never accumulated

more biomass than its predecessor, the treated second-generation biofilm, which accumulated only half the total of the untreated second-generation consortium. Additionally, the emergent structure definitely did not reappear in this third-generation control biofilm (Figure 3.4C and Appendix B.6).

From all of these observations, we concluded that at the third generation the yellow cells no longer absolutely require the blue population, so the blue population can be sifted out by population bottlenecks (thus, we see progressively higher proportions of yellow biomass in successive generations). However, this change might be globally maladaptive for two reasons. First, it appears that the consortium cannot accumulate the optimal total biomass without the blue population which is conveyed in aggregates. Second, even though the yellows can stick, they still do not colonize the substrate as effectively as blue cells. From a global evolutionary perspective, the biofilm formed by an inoculum containing aggregates might still be better than the biofilm formed by adapted yellow cells alone. We explored this with biofilms started from separate, sorted fractions of the same effluent that started this control biofilm.

3.10.2 Probing adaptation by cell sorting

We sought to explore biofilm formation by the adapted yellow population and by aggregates separately by using FACS to sort the effluent from the treated secondgeneration consortium. We gathered two fractions (see Appendix B.7). The first fraction contained single yellow cells (YFP+ fraction) while the second contained aggregates of unknown, assorted size and composition (aggregate fraction). We inoculated these two fractions into separate biofilms. Both fractions were able to form biofilms, although the initial biomass accumulation of both was slower than in the control third-generation biofilm (Figure 3.4B, D). Here, it is possible that the cells were stressed during cell sorting, leading to slower biomass accumulation. Alternatively, the high speed of biomass accumulation found in the control third generation might be a product of both fractions coexisting, rather than purely a function of the yellow adaptation. This conclusion is consistent with our observation that the adapted yellow cells can stick, but do not colonize the substrate as effectively as blue cells (see also Appendix B.5).

The YFP+ fraction biofilm accumulated biomass more quickly and accrued more total biomass than the first-generation vellow population control (Figures 3.2A, 3.4D). We concluded from this that the yellow population did, indeed, undergo some form of adaptation. To test whether the enhanced ability of the yellow population to form biofilms was a reversible change, we passed a subset of cells from the YFP+ fraction through growth on solid and in liquid media before inoculating into fresh flow cells. The growth of this population mirrored the behavior of the first-generation yellow population control—it formed no biofilm within 72 hours—suggesting that a reversible adaptation (perhaps a genetic polymorphism, or a regulatory change, or both) was responsible for the improved ability of the yellow population to form biofilms. Because the biofilm formed by the YFP+ fraction initially accumulated biomass more slowly, and accumulated less maximum total biomass, than the aggregate fraction biofilm, the adaptation of the yellow population alone cannot confer the growth advantage that we observed in the untreated second-generation biofilm (Figure 3.4A, D). Furthermore, we observed flat structure in the YFP+ fraction biofilm, indicating that it was unable to form healthy three-dimensional structure.

The biofilm formed by the aggregate fraction accumulated biomass more quickly, and also accumulated more total biomass, than the YFP+ fraction biofilm (Figure 3.4D). Total biomass accumulation of the aggregate fraction biofilm was twice that of the YFP+ fraction biofilm (Figure 3.4D). Together, all of these results suggest that the presence of aggregates is necessary for the speed of biomass accumulation and is sufficient to optimize total biomass accumulation. Additionally, the aggregated fraction biofilm starts with the emergent structure—the yellow population resides above the blue population from the start of the lifespan of the biofilm—which appears to enable both the blue and the yellow populations to recover and grow away from the substrate together (Figure 3.4D). Noting that the emergent structure did not re-emerge in the control thirdgeneration biofilm, this indicates that inoculating only the aggregates recovers structure that is otherwise lost in the presence of the full effluent from the treated biofilm. These results demonstrate that the aggregates are at least correlated with the presence of the emergent structure.

3.11 Discussion and conclusion

We constructed a synthetic symbiotic consortium from two populations of engineered *E. coli* that functions stably over long periods of time and through multiple population bottlenecks. After the consortium grew for 80 hours, we observed emergent structure which could be transferred to downstream environments and was correlated with a growth advantage (more total and faster biomass accumulation). Only when aggregates of the two populations were preserved through population bottlenecks were the emergent structure and greater total accumulation of biomass found in downstream biofilms. The

secondary component of the growth advantage, faster biomass accumulation, is the combined effect of an adaptive change in the yellow population, which enabled cells of the yellow population to adhere better, and the presence of aggregates of both populations.

Overall, these results suggest that aggregates are the primary conveyors of the emergent structure and the growth advantage of the consortium; the adaptive change in the yellow population, which hastens biofilm formation, also threatens to take over and dismantle the consortium if the aggregates are disrupted during population bottlenecks. If the yellow population takes over, the resulting uniform population is less well adapted than the consortium, as measured by the speed and amount of biomass accumulation.

The aggregates may preserve emergent structure by being readily assembled pieces of that structure, but how they convey a growth advantage is an open question. Aggregates may colonize a fresh substrate in a manner that enables more biomass to accumulate, or they may provide proximity between the two populations to enhance cooperation and growth. Either way, we see that three-dimensional structure is indeed an important mechanism that promotes optimal downstream colonization by the whole community, via aggregate detachment and downstream re-attachment. The spatial organization of the community into aggregates provides for both populations to survive, and to be inherited together so that the community functions better in a new environment (as measured by speed and amount of biomass accumulation) than either population can alone. As best we can tell, the aggregates are composed of 1/3 blue and 2/3 yellow biomass. It would be interesting to explore whether there is an optimal composition by artificially constructing and inoculating aggregates of varying composition.

In nature, it is likely that mixed populations attain spatial structure together that is conducive to their collaboration. The stable spatial structure afforded by biofilms provides for prolonged interactions between neighbors and for the development of emergent structure in communities [105, 106]. Selection then acts not just upon individual populations, but upon whole communities, and structures that enable communities to colonize downstream environments better than their constituent populations may be conserved through evolution. This engineered symbiotic consortium allowed us to uncover and study such interactions precisely, demonstrating the utility of engineered synthetic consortia to a wide range of scientific fields.

3.12 Methods

3.12.1 Strains and plasmids

Strains MGd- and MGfwc- were constructed by recombination with the lambda red recombinase plasmid pKD46, as outlined in [150]. More information about strain construction can be found in Appendix B.1. Plasmids were constructed as outlined in the text and in [42].

3.12.2 Growth conditions

Throughout all experiments, cultures and biofilms were grown at 30°C in M9-AADO medium containing 50 µgml⁻¹ kanamycin and 20 µgml⁻¹ tetracycline to maintain the engineered and the marker plasmids, respectively [68].

M9-AADO (per litre): 200 mL 5xM9, 100 mL 10x Amino Acid Dropout Solution without Lysine, 2 mM MgSO₄, 0.5% glycerol, 0.01% thymine.

5x M9 (per litre): 18g anhydrous Na₂HPO₄, 15g KH₂PO₄, 5g NH₄Cl, 2.5g NaCl.

10x Amino Acid Dropout Solution without Lysine (per litre): 300 mg L-Isoleucine;
1500 mg L-Valine; 200 mg L-Adenine hemisulfate salt; 200 mg L-Arginine HCl; 200 mg
L-Histidine HCl monohydrate; 1000mg L-Leucine; 200 mg L-Methionine; 500mg LPhenylalanine; 2000 mg L-Threonine; 200 mg L-Tryptophan; 300 mg L-Tyrosine; 200
mg L-Uracil.

3.12.3 Biofilm preparation and inoculation

The biofilm flow apparatus was described previously in Chapter 2 and Appendix A.4 [42] with two exceptions. First, here inoculation was performed into the Tygon tubing via inoculation ports installed into three-way connectors one inch upstream of each flow lane (connectors, Cole Parmer and inoculation ports). Second, two inches of tubing upstream of flow lanes, including the inoculation port, was removed within 48 hours of inoculation to prevent upstream biofilm formation from affecting results within the flow cells. To begin first-generation biofilms, separate overnight cultures of blue and yellow populations were shaken in M9-AADO medium with antibiotics, as described above, to saturation. Cultures of the blue population were supplemented with 10 µM C4HSL (Sigma, O9945). Cultures were centrifuged at 4000 RPM for 8 minutes, cells were resuspended in 1mL 0.9% NaCl solution containing the same antibiotics, then diluted into 0.9% NaCl solution with the antibiotics to an OD_{600} of 0.07, which corresponds to approximately 4×10^7 cellsmL⁻¹. 1 mL of a 50/50 mixture of blue and yellow cells was inoculated into each flow lane for experimental replicates. Control lanes contained a 50/50 mixture of blue or yellow cells and 0.9% NaCl solution.

To begin untreated second- and third-generation biofilms, effluents from three separate replicates (in separate lanes) of the generation to be propagated were mixed, OD_{600} was adjusted to 0.07 as necessary, and 1 mL was inoculated into each fresh flow lane. To begin treated second-generation biofilms, effluents were taken as above, but prior to adjusting the OD_{600} the effluent was vortexed at top speed for 5 minutes and then

passed through a 40 μ m cell strainer (BD Falcon, #352340). All inoculates were plated in parallel with inoculation to confirm cell counts.

Prior to inoculation each 1x4x40 mm lane of each flow chamber (Stovall Life Sciences, ACFL0001) was incubated for at least 90 minutes at 37°C with 200 μ L of a solution of 10 mg/mL bovine ribonuclease B (Sigma, R7884) suspended in 0.02 M bicarbonate buffer. Each lane was then quenched with 200 μ L of 0.2% Bovine Serum Albumin (Sigma, A4503). Flow of M9-AADO with antibiotics through the flow chambers was initiated for five minutes prior to inoculation. After inoculation, flow chambers were incubated glass-coverslip-down for 4 minutes, and then flow was reinstated for 4 minutes prior to returning the flow chambers to the upright position. The flow rate of medium through each lane was approximately 230 μ Lmin⁻¹ and flow cells were incubated at 30°C ± 2°C throughout the length of each experiment. Medium reservoirs were replaced every 12 hours to ensure freshness of the antibiotics.

3.12.4 Imaging

Images of the biofilms were captured with a Zeiss 510 upright confocal laser scanning microscope (CLSM), controlled by Carl Zeiss AIM. A Zeiss Achroplan 40x/0.8 W objective was used to capture all images, images were captured with 512x512 pixel resolution, and all image stacks were captured with identical pinhole and gain settings. eCFP excitation: 458 nm Argon laser, emission filter: BP 480–520 nm. eYFP excitation: 514 nm Argon laser, emission filter: LP 530 nm.

3.12.5 Metrics

Measurements were calculated using the COMSTAT biofilm image processing package in Matlab. At least three biological replicates were grown at a time for each condition, and every condition was repeated on at least two different days. Averages were taken of COMSTAT results from at least three randomly selected images, taken at a variety of locations within the flow lane. More information about quantitative processing in COMSTAT, and changes made to COMSTAT to incorporate calculations of the biomass median can be found in Appendix B.3.

Chapter 4

Conclusions

Portions of this chapter are published as [1].

4.1 Challenges in engineering microbial consortia

There are several examples of synthetic microbial consortia in the literature [43, 44, 46], and in this thesis we have presented two engineered synthetic consortia that grow and function in biofilms [42]. However, there are significant challenges associated with engineering microbial consortia, and these will require attention as engineers consider their potential applications. Although many of the challenges are shared with those faced when engineering single microbial populations, some are particular to controlling the behavior of multiple, interacting populations. First, natural microbial communities can maintain homeostasis; members generally do not out-compete one another and do not exhaust the resources in their environments [151, 152]. However, it is difficult to design either long-term homeostasis or long-term extinction into a synthetic consortium, because long-term behavior, and even the long-term genetic composition of an engineered organism, is unpredictable. Thus, engineered consortia should be designed for contexts

in which members of the consortium can be re-introduced or eliminated as needed, and in which their behavior can be monitored over time. A second challenge is that, at least in nature, gene transfer between microbes is common [109]. As a result, engineered consortia should function despite horizontal gene transfer, or even exploit it. A third challenge will be to develop methods for incorporating stable changes into the genomes of microbes that are not currently commonly engineered. Horizontal gene transfer is limited when engineers make stable changes to the chromosome. In addition, organisms currently recalcitrant to genetic modification methods often perform very useful functions that are difficult to engineer into other organisms. For example, species of Clostridia (e.g., *Clostridium thermocellum*, for which there are no established genetic cloning protocols, and *Clostridium acetobutylicum*, the protocols for which are difficult and proprietary) live in consortia with other microbes and naturally secrete powerful cellulases [18]. A fourth major challenge inherent in engineering consortia is fine-tuning the performance of multiple populations. Techniques such as directed evolution that can optimize the behavior of a single population must be extended for application to multiple populations and varying environments. High-throughput screening methods and inexpensive gene-chip assay procedures will be extremely useful for the efficient construction and evaluation of synthetic consortia.

4.2 Synthetic consortia in healthcare

Microbial consortia can carry out more complex functions, and they might be more robust to changes in their environments than are individual populations. These two traits make microbial consortia attractive as platforms for a variety of technologies, if the

engineering challenges such as those listed above can be met. The field of medical technology stands to benefit greatly from the ability to engineer communities of microbes. Engineers have developed bacteria that serve as drug-delivery devices [153-155] and gene-delivery vehicles [154, 156, 157], but these technologies suffer a lack of precision in targeting and release. The greater complexity of function available, coupled with longevity and stability through environmental change, might make consortia a better starting-point for microbial drug-delivery and gene-delivery technologies. For example, a healthcare technology requiring the delivery of two therapeutic components in succession with a defined time-offset could potentially employ an oscillatory system (e.g., the predator-prey ecosystem of Balagadde et al. [43]) as a platform. Such an application would require much greater understanding of both the dynamics of mixed populations and how to control them in a robust fashion. Researchers have also introduced genetically engineered commensal bacteria into mammals as sentry cells. Such efforts have successfully prevented colonization by problematic organisms at epithelial barriers in the reproductive and digestive tracts of the mammals [158, 159]. A consortium of engineered commensal microbes might colonize and provide additional functionality, including detection warnings [160] or protection against multiple infectious agents, over longer periods of time. Similar strategies might also be considered to detect and prevent pathogenic colonization of wounds and the lungs.

4.3 Summary

Because members of microbial consortia communicate and differentiate, consortia can perform more complex tasks and can survive in more changeable environments than can

uniform populations. Simple engineered consortia might be described through mathematical models more easily than natural systems are, and they can be used to develop and validate models of more complex systems [161]. Furthermore, their behavior can be controlled by externally introduced signals (e.g., circuits can be induced by small molecules such as IPTG). To date, engineers have successfully constructed microbial consortia by implementing cell-cell communication and differentiation of function in traditional, laboratory microbes. To fully exploit the potential of engineered consortia, we must learn to stably engineer organisms that are currently recalcitrant to genetic manipulation. Furthermore, when engineering new technologies, we should prioritize safety by beginning with innocuous or commensal organisms. As a result of engineered communication and differentiation of function, engineered consortia do exhibit complex functions that can be difficult to engineer into single populations. If they are to be used in future technologies, engineered consortia will need to be tested and optimized for their ability to persist and withstand environmental fluctuations. In addition to "pushing the envelope" of synthetic biology, with promising health, environmental, and industrial applications, engineered microbial consortia are potentially powerful and versatile tools for studying microbial interactions and evolution.

Bibliography

- [1] Brenner, K., et al. (2008) Engineering microbial consortia: a new frontier in synthetic biology. *Trends Biotechnol* 26, 483–489.
- [2] Arkin, A.P., and Fletcher, D.A. (2006) Fast, cheap and somewhat in control. *Genome Biol* 7, 114.
- [3] Drubin, D.A., et al. (2007) Designing biological systems. Genes Dev 21, 242–254.
- [4] Endy, D. (2005) Foundations for engineering biology. *Nature* 438, 449–453.
- [5] Yeh, B.J., and Lim, W.A. (2007) Synthetic biology: lessons from the history of synthetic organic chemistry. *Nat Chem Biol* 3, 521–525.
- [6] Di Ventura, B., et al. (2006) From *in vivo* to *in silico* biology and back. *Nature* 443, 527–533.
- [7] Hasty, J., et al. (2002) Engineered gene circuits. Nature 420, 224-230.
- [8] Keasling, J.D. (2008) Synthetic biology for synthetic chemistry. *ACS Chem Biol* 3, 64–76.
- [9] McDaniel, R., and Weiss, R. (2005) Advances in synthetic biology: on the path from prototypes to applications. *Curr Opin Biotechnol* 16, 476–483.

[10] Voigt, C.A. (2006) Genetic parts to program bacteria. *Curr Opin Biotechnol* 17, 548–557.

[11] Marguet, P., et al. (2007) Biology by design: reduction and synthesis of cellular components and behaviour. *J R Soc Interface* 4, 607–623.

[12] Sprinzak, D., and Elowitz, M.B. (2005) Reconstruction of genetic circuits. *Nature* 438, 443–448.

[13] Ro, D.K., et al. (2006) Production of the antimalarial drug precursor artemisinic acid in engineered yeast. *Nature* 440, 940–943.

[14] Suel, G.M., et al. (2006) An excitable gene regulatory circuit induces transient cellular differentiation. *Nature* 440, 545–550.

[15] Keller, L., and Surette, M.G. (2006) Communication in bacteria: an ecological and evolutionary perspective. *Nat Rev Microbiol* 4, 249–258.

[16] Zaldivar, J., et al. (2001) Fuel ethanol production from lignocellulose: a challenge for metabolic engineering and process integration. *Appl Microbiol Biotechnol* 56, 17–34.

[17] Eiteman, M.A., et al. (2008) A co-fermentation strategy to consume sugar mixtures effectively. *J Biol Eng* 2, 3.

[18] Lynd, L.R., et al. (2002) Microbial cellulose utilization: fundamentals and biotechnology. *Microbiol Mol Biol Rev* 66, 506–577.

[19] Arai, T., et al. (2007) Synthesis of *Clostridium cellulovorans* minicellulosomes by intercellular complementation. *Proc Natl Acad Sci U S A* 104, 1456–1460.

[20] Burmolle, M., et al. (2006) Enhanced biofilm formation and increased resistance to antimicrobial agents and bacterial invasion are caused by synergistic interactions in multispecies biofilms. *Appl Environ Microbiol* 72, 3916–3923.

[21] Fay, P. (1992) Oxygen relations of nitrogen fixation in cyanobacteria. *Microbiol Rev* 56, 340–373.

[22] LaPara, T.M., et al. (2002) Functional and structural adaptations of bacterial communities growing on particulate substrates under stringent nutrient limitation. *Microb Ecol* 44, 317–326.

[23] Arenas, F., et al. (2006) The invasibility of marine algal assemblages: role of functional diversity and identity. *Ecology* 87, 2851–2861.

[24] Briones, A., and Raskin, L. (2003) Diversity and dynamics of microbial communities in engineered environments and their implications for process stability. *Curr Opin Biotechnol* 14, 270–276.

[25] Kato, S., et al. (2008) Network relationships of bacteria in a stable mixed culture.*Microb Ecol* 56, 403–411.

[26] Fuqua, C., et al. (2001) Regulation of gene expression by cell-to-cellcommunication: acyl-homoserine lactone quorum sensing. *Annu Rev Genet* 35, 439–468.

[27] Davies, D.G., et al. (1998) The involvement of cell-to-cell signals in the development of a bacterial biofilm. *Science* 280, 295–298.

[28] Greenberg, E.P. (2003) Bacterial communication and group behavior. *J Clin Invest* 112, 1288–1290. [29] Parsek, M.R., and Greenberg, E.P. (2005) Sociomicrobiology: the connections between quorum sensing and biofilms. *Trends Microbiol* 13, 27–33.

[30] Clarke, M.B., and Sperandio, V. (2005) Events at the host-microbial interface of the gastrointestinal tract III. Cell-to-cell signaling among microbial flora, host, and pathogens: there is a whole lot of talking going on. *Am J Physiol Gastrointest Liver Physiol* 288, G1105–1109.

[31] De Kievit, T.R., et al. (2001) Quorum-sensing genes in *Pseudomonas aeruginosa* biofilms: their role and expression patterns. *Appl Environ Microbiol* 67, 1865–1873.

[32] Passador, L., et al. (1993) Expression of *Pseudomonas aeruginosa* virulence genes requires cell-to-cell communication. *Science* 260, 1127–1130.

[33] Kleerebezem, M., and Quadri, L.E. (2001) Peptide pheromone-dependent regulation of antimicrobial peptide production in Gram-positive bacteria: a case of multicellular behavior. *Peptides* 22, 1579–1596.

[34] Lyon, G.J., and Novick, R.P. (2004) Peptide signaling in *Staphylococcus aureus* and other Gram-positive bacteria. *Peptides* 25, 1389–1403.

[35] Kaper, J.B., and Sperandio, V. (2005) Bacterial cell-to-cell signaling in the gastrointestinal tract. *Infect Immun* 73, 3197–3209.

[36] Wolfaardt, G.M., et al. (1994) Multicellular organization in a degradative biofilm community. *Appl Environ Microbiol* 60, 434–446.

[37] Kato, S., et al. (2005) Stable coexistence of five bacterial strains as a cellulosedegrading community. *Appl Environ Microbiol* 71, 7099–7106. [38] Kobayashi, H., et al. (2004) Programmable cells: interfacing natural and engineered gene networks. *Proc Natl Acad Sci U S A* 101, 8414–8419.

[39] You, L., et al. (2004) Programmed population control by cell-cell communication and regulated killing. *Nature* 428, 868–871.

[40] Anderson, J.C., et al. (2006) Environmentally controlled invasion of cancer cells by engineered bacteria. *J Mol Biol* 355, 619–627.

[41] Basu, S., et al. (2005) A synthetic multicellular system for programmed pattern formation. *Nature* 434, 1130–1134.

[42] Brenner, K., et al. (2007) Engineered bidirectional communication mediates a consensus in a microbial biofilm consortium. *Proc Natl Acad Sci U S A* 104, 17300–17304.

[43] Balagadde, F.K., et al. (2008) A synthetic *Escherichia coli* predator-prey ecosystem.*Mol Sys Biol* 4, 187.

[44] Weber, W., et al. (2007) Synthetic ecosystems based on airborne inter- and intrakingdom communication. *Proc Natl Acad Sci U S A* 104, 10435–10440.

[45] Bulter, T., et al. (2004) Design of artificial cell-cell communication using gene and metabolic networks. *Proc Natl Acad Sci US A* 101, 2299–2304.

[46] Shou, W., et al. (2007) Synthetic cooperation in engineered yeast populations. *Proc Natl Acad Sci U S A* 104, 1877–1882.

[47] West, S.A., et al. (2006) Social evolution theory for microorganisms. *Nat Rev Microbiol* 4, 597–607.

[48] Diggle, S.P., et al. (2007) Cooperation and conflict in quorum-sensing bacterial populations. *Nature* 450, 411–414.

[49] Hogan, D.A., and Kolter, R. (2002) *Pseudomonas-Candida* interactions: an ecological role for virulence factors. *Science* 296, 2229–2232.

[50] Brenner, K., and Arnold, F.H. (2009) Structure and selection in an engineered symbiotic biofilm consortium. *In submission*.

[51] Hooper, L.V., et al. (2002) How host-microbial interactions shape the nutrient environment of the mammalian intestine. *Annu Rev Nutr* 22, 283–307.

[52] Kleessen, B., and Blaut, M. (2005) Modulation of gut mucosal biofilms. *Br J Nutr*93 Suppl 1, S35–40.

[53] Macfarlane, S., et al. (2005) Colonization of mucin by human intestinal bacteria and establishment of biofilm communities in a two-stage continuous culture system. *Appl Environ Microbiol* 71, 7483–7492.

[54] Mishra, S., et al. (2001) In situ bioremediation potential of an oily sludge-degrading bacterial consortium. *Curr Microbiol* 43, 328–335.

[55] Becskei, A., and Serrano, L. (2000) Engineering stability in gene networks by autoregulation. *Nature* 405, 590–593.

[56] Becskei, A., et al. (2001) Positive feedback in eukaryotic gene networks: cell differentiation by graded to binary response conversion. *Embo J* 20, 2528–2535.

[57] Kramer, B.P., et al. (2004) An engineered epigenetic transgene switch in mammalian cells. *Nat Biotechnol* 22, 867–870.

[58] Elowitz, M.B., and Leibler, S. (2000) A synthetic oscillatory network of transcriptional regulators. *Nature* 403, 335–338.

[59] Atkinson, M.R., et al. (2003) Development of genetic circuitry exhibiting toggle switch or oscillatory behavior in *Escherichia coli*. *Cell* 113, 597–607.

[60] Basu, S., et al. (2004) Spatiotemporal control of gene expression with pulsegenerating networks. *Proc Natl Acad Sci U S A* 101, 6355–6360.

[61] Pesci, E.C., and Iglewski, B.H. (1997) The chain of command in *Pseudomonas* quorum sensing. *Trends Microbiol* 5, 132–134; discussion 134–135.

[62] Pearson, J.P., et al. (1994) Structure of the autoinducer required for expression of *Pseudomonas aeruginosa* virulence genes. *Proc Natl Acad Sci U S A* 91, 197–201.

[63] Pesci, E.C., et al. (1997) Regulation of las and rhl quorum sensing in *Pseudomonas aeruginosa*. *J Bacteriol* 179, 3127–3132.

[64] Winson, M.K., et al. (1995) Multiple N-acyl-L-homoserine lactone signal molecules regulate production of virulence determinants and secondary metabolites in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A* 92, 9427–9431.

[65] Gould, T.A., et al. (2006) Specificity of acyl-homoserine lactone synthases examined by mass spectrometry. *J Bacteriol* 188, 773–783.

[66] Passador, L., et al. (1996) Functional analysis of the *Pseudomonas aeruginosa* autoinducer PAI. *J Bacteriol* 178, 5995–6000.

[67] Christensen, B.B., et al. (2002) Metabolic commensalism and competition in a twospecies microbial consortium. *Appl Environ Microbiol* 68, 2495–2502. [68] Bloemberg, G.V., et al. (2000) Simultaneous imaging of *Pseudomonas fluorescens* WCS365 populations expressing three different autofluorescent proteins in the rhizosphere: new perspectives for studying microbial communities. *Mol Plant Microbe Interact* 13, 1170–1176.

[69] Sternberg, C., et al. (1999) Distribution of bacterial growth activity in flow-chamber biofilms. *Appl Environ Microbiol* 65, 4108–4117.

[70] Levskaya, A., et al. (2005) Synthetic biology: engineering *Escherichia coli* to see light. *Nature* 438, 441–442.

[71] Eberl, L. (1999) N-acyl homoserinelactone-mediated gene regulation in gramnegative bacteria. *Syst Appl Microbiol* 22, 493–506.

[72] Houdt, R., et al. (2006) N-acyl-l-homoserine lactone signal interception by *Escherichia coli*. *FEMS Microbiol Lett* 256, 83–89.

[73] Klaus, T., et al. (1999) Silver-based crystalline nanoparticles, microbially fabricated. *Proc Natl Acad Sci U S A* 96, 13611–13614.

[74] Swartz, J.R. (2001) Advances in *Escherichia coli* production of therapeutic proteins. *Curr Opin Biotechnol* 12, 195–201.

[75] Karig, D., and Weiss, R. (2005) Signal-amplifying genetic circuit enables in vivo observation of weak promoter activation in the Rhl quorum sensing system. *Biotechnol Bioeng* 89, 709–718.

[76] Shapiro, J.A. (1998) Thinking about bacterial populations as multicellular organisms. *Annu Rev Microbiol* 52, 81–104.

[77] Johnson, C.R., and Boerlijst, M.C. (2002) Selection at the level of the community: the importance of spatial structure. *Trends Ecol Evol* 17, 83–90.

[78] West, S.A., et al. (2002) Conflict and cooperation—cooperation and competition between relatives. *Science* 296, 72–75.

[79] Lion, S., and van Baalen, M. (2008) Self-structuring in spatial evolutionary ecology.*Ecol Lett* 11, 277–295.

[80] Millstein, R.L. (2006) Natural selection as a population-level causal process. *British J Phil Sci* 57, 627–653.

[81] Urban, M.C., and Skelly, D.K. (2006) Evolving metacommunities: toward an evolutionary perspective on metacommunities. *Ecology* 87, 1616–1626.

[82] Crespi, B.J. (2001) The evolution of social behavior in microorganisms. *Trends Ecol Evol* 16, 178–183.

[83] West, S.A., et al. (2007) The social lives of microbes. *Annu Rev Ecol Evol Systematics* 38, 53–77.

[84] Ben Jacob, E., et al. (2004) Bacterial linguistic communication and social intelligence. *Trends Microbiol* 12, 366–372.

[85] Wingreen, N.S., and Levin, S.A. (2006) Cooperation among microorganisms. *PLoS Biol* 4, e299.

[86] Fux, C.A., et al. (2005) Survival strategies of infectious biofilms. *Trends Microbiol* 13, 34–40.

[87] Rainey, P.B., et al. (2000) The emergence and maintenance of diversity: insights from experimental bacterial populations. *Trends Ecol Evol* 15, 243–247.

[88] Rainey, P.B., and Travisano, M. (1998) Adaptive radiation in a heterogeneous environment. *Nature* 394, 69–72.

[89] Kerr, B., et al. (2002) Local dispersal promotes biodiversity in a real-life game of rock-paper-scissors. *Nature* 418, 171–174.

[90] Cho, H., et al. (2007) Self-organization in high-density bacterial colonies: efficient crowd control. *PLoS Biol* 5, e302.

[91] Kim, H.J., et al. (2008) Defined spatial structure stabilizes a synthetic multispecies bacterial community. *Proc Natl Acad Sci US A* 105, 18188–18193.

[92] Keymer, J.E., et al. (2008) Computation of mutual fitness by competing bacteria. *Proc Natl Acad Sci U S A* 105, 20269–20273.

[93] Hansen, S.K., et al. (2007) Evolution of species interactions in a biofilm community. *Nature* 445, 533–536.

[94] Bonner, J.T. (2000) *First Signals: The evolution of multicellular development*.Princeton University Press.

[95] Maynard Smith, J. (1964) Group selection and kin selection. *Nature* 201, 1145–1147.

[96] Keller, L., ed. (1999) Levels of selection in evolution. Princeton University Press.

[97] Brockhurst, M.A. (2007) Population bottlenecks promote cooperation in bacterial biofilms. *PLoS ONE* 2, e634.

[98] Brockhurst, M.A., et al. (2007) Cooperation peaks at intermediate disturbance. *Curr Biol* 17, 761–765.

[99] Buckling, A., et al. (2000) Disturbance and diversity in experimental microcosms. *Nature* 408, 961–964.

[100] Griffin, A.S., et al. (2004) Cooperation and competition in pathogenic bacteria. *Nature* 430, 1024–1027.

[101] Johnson, C.R., and Seinen, I. (2002) Selection for restraint in competitive ability in spatial competition systems. *Proc Biol Sci* 269, 655–663.

[102] Hassell, M.P., et al. (1994) Species coexistence and self-organizing spatial dynamics. *Nature* 370, 290–292.

[103] Stoodley, P., et al. (2002) Biofilms as complex differentiated communities. *Annu Rev Microbiol* 56, 187–209.

[104] Tolker-Nielsen, T., and Molin, S. (2000) Spatial organization of microbial biofilm communities. *Microb Ecol* 40, 75–84.

[105] Costerton, J.W., et al. (1994) Biofilms, the customized microniche. *J Bacteriol* 176, 2137–2142.

[106] O'Toole, G., et al. (2000) Biofilm formation as microbial development. *Annu Rev Microbiol* 54, 49–79.

[107] Christensen, B.B., et al. (1998) *In situ* detection of gene transfer in a model biofilm engaged in degradation of benzyl alcohol. *APMIS Suppl* 84, 25–28.

[108] Christensen, B.B., et al. (1998) Establishment of new genetic traits in a microbial biofilm community. *Appl Environ Microbiol* 64, 2247–2255.

[109] Davison, J. (1999) Genetic exchange between bacteria in the environment. *Plasmid* 42, 73–91.

[110] Moller, S., et al. (1998) In situ gene expression in mixed-culture biofilms:
evidence of metabolic interactions between community members. *Appl Environ Microbiol* 64, 721–732.

[111] Chuang, J.S., et al. (2009) Simpson's paradox in a synthetic microbial system. *Science* 323, 272–275.

[112] Wagner, C.H. (1982) Simpson's paradox in real life. Am Statistician 36, 46–48.

[113] Kolter, R., and Greenberg, E.P. (2006) Microbial sciences: the superficial life of microbes. *Nature* 441, 300–302.

[114] Komlos, J., et al. (2005) Interaction of *Klebsiella oxytoca* and *Burkholderia cepacia* in dual-species batch cultures and biofilms as a function of growth rate and substrate concentration. *Microb Ecol* 49, 114–125.

[115] Komlos, J., et al. (2006) Effect of substrate concentration on dual-species biofilm population densities of *Klebsiella oxytoca* and *Burkholderia cepacia* in porous media. *Biotechnol Bioeng* 93, 434–442.

[116] Reisner, A., et al. (2003) Development and maturation of *Escherichia coli* K-12 biofilms. *Mol Microbiol* 48, 933–946.

[117] Prigent-Combaret, C., et al. (2000) Developmental pathway for biofilm formation in curli-producing *Escherichia coli* strains: role of flagella, curli and colanic acid. *Environ Microbiol* 2, 450–464.

[118] Heydorn, A., et al. (2000) Quantification of biofilm structures by the novel computer program COMSTAT. *Microbiology* 146(10), 2395–2407.

[119] Klausen, M., et al. (2003) Biofilm formation by *Pseudomonas aeruginosa* wild type, flagella and type IV pili mutants. *Mol Microbiol* 48, 1511–1524.

[120] Stoodley, P.I., et al. (1999) Influence of hydrodynamics and nutrients on biofilm structure. *J Appl Microbiol* 85, 198–288.

[121] Vlamakis, H., et al. (2008) Control of cell fate by the formation of an architecturally complex bacterial community. *Genes Dev* 22, 945–953.

[122] Barken, K.B., et al. (2008) Roles of type IV pili, flagellum-mediated motility and extracellular DNA in the formation of mature multicellular structures in *Pseudomonas aeruginosa* biofilms. *Environ Microbiol* 10, 2331–2343.

[123] Stoodley, P., et al. (2001) Growth and detachment of cell clusters from mature mixed-species biofilms. *Appl Environ Microbiol* 67, 5608–5613.

[124] Malik, A., and Kakii, K. (2008) Novel coaggregating microbial consortium: testing strength for field applications. *Bioresour Technol* 99, 4627–4634.

[125] Rani, S.A., et al. (2007) Spatial patterns of DNA replication, protein synthesis, and oxygen concentration within bacterial biofilms reveal diverse physiological states. *J Bacteriol* 189, 4223–4233.

[126] Hoffman, L.R., et al. (2005) Aminoglycoside antibiotics induce bacterial biofilm formation. *Nature* 436, 1171–1175.

[127] Roberts, M.E., and Stewart, P.S. (2005) Modelling protection from antimicrobial agents in biofilms through the formation of persister cells. *Microbiology* 151, 75–80.

[128] Richaud, C., et al. (1984) Regulation of expression and nucleotide sequence of the *Escherichia coli dapD* gene. *J Biol Chem* 259, 14824–14828.

[129] Prigent-Combaret, C., et al. (2001) Complex regulatory network controls initial adhesion and biofilm formation in *Escherichia coli* via regulation of the *csgD* gene. *J Bacteriol* 183, 7213–7223.

[130] Vidal, O., et al. (1998) Isolation of an *Escherichia coli* K-12 mutant strain able to form biofilms on inert surfaces: involvement of a new *ompR* allele that increases curli expression. *J Bacteriol* 180, 2442–2449.

[131] Olsen, A., et al. (1989) Fibronectin binding mediated by a novel class of surface organelles on *Escherichia coli*. *Nature* 338, 652–655.

[132] Olsen, A., et al. (1993) The RpoS sigma factor relieves H-NS-mediated transcriptional repression of CsgA, the subunit gene of fibronectin-binding curli in *Escherichia coli. Mol Microbiol* 7, 523–536.

[133] Blomfield, I.C., et al. (1991) Type 1 fimbriae mutants of *Escherichia coli* K12: characterization of recognized afimbriate strains and construction of new fim deletion mutants. *Mol Microbiol* 5, 1439–1445.

[134] Thomas, W., et al. (2006) Catch-bond model derived from allostery explains forceactivated bacterial adhesion. *Biophys J* 90, 753–764. [135] Xavier, J.B., and Foster, K.R. (2007) Cooperation and conflict in microbial biofilms. *Proc Natl Acad Sci U S A* 104, 876–881.

[136] Markovitz, A. (1977) Genetics and regulation of bacterial capsular polysaccharide synthesis and radiation sensitivity. In *Surface carbohydrates of the prokaryotic cell* (Sutherland, I.W., ed.), 415–462, Academic Press.

[137] Ghigo, J.M. (2001) Natural conjugative plasmids induce bacterial biofilm development. *Nature* 412, 442–445.

[138] Schembri, M.A., et al. (2003) Global gene expression in *Escherichia coli* biofilms.*Mol Microbiol* 48, 253–267.

[139] Van Houdt, R., and Michiels, C.W. (2005) Role of bacterial cell surface structures in *Escherichia coli* biofilm formation. *Res Microbiol* 156, 626–633.

[140] Bascompte, J., et al. (2006) Asymmetric coevolutionary networks facilitate biodiversity maintenance. *Science* 312, 431–433.

[141] Loreau, M., et al. (2001) Biodiversity and ecosystem functioning: current knowledge and future challenges. *Science* 294, 804–808.

[142] Kiessling, W. (2005) Long-term relationships between ecological stability and biodiversity in Phanerozoic reefs. *Nature* 433, 410–413.

[143] Al-Bakri, A.G., et al. (2005) Influence of gentamicin and tobramycin on binary biofilm formation by co-cultures of *Burkholderia cepacia* and *Pseudomonas aeruginosa*. *J Basic Microbiol* 45, 392–396.

[144] Moss, J.A., et al. (2006) Stability and change in estuarine biofilm bacterial community diversity. *Appl Environ Microbiol* 72, 5679–5688.

[145] Lyautey, E., et al. (2005) Bacterial community succession in natural river biofilm assemblages. *Microb Ecol* 50, 589–601.

[146] Canstein, H.F., et al. (2001) Long-term stability of mercury-reducing microbial biofilm communities analyzed by 16S-23S rDNA interspacer region polymorphism. *Microb Ecol* 42, 624–634.

[147] Newman, D.K., and Banfield, J.F. (2002) Geomicrobiology: how molecular-scale interactions underpin biogeochemical systems. *Science* 296, 1071–1077.

[148] Turner, P.E., et al. (1996) Tests of ecological mechanisms promoting the stable coexistence of two bacterial genotypes. *Ecology* 77, 2119–2129.

[149] Balagadde, F.K., et al. (2005) Long-term monitoring of bacteria undergoing programmed population control in a microchemostat. *Science* 309, 137–140.

[150] Datsenko, K.A., and Wanner, B.L. (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci U S A* 97, 6640–6645.

[151] Kuramitsu, H.K., et al. (2007) Interspecies interactions within oral microbial communities. *Microbiol Mol Biol Rev* 71, 653–670.

[152] Marsh, P.D. (2006) Dental plaque as a biofilm and a microbial community implications for health and disease. *BMC Oral Health* 6 Suppl 1, S14.

[153] Bermudes, D., et al. (2002) Live bacteria as anticancer agents and tumor-selective protein delivery vectors. *Curr Opin Drug Discov Devel* 5, 194–199.

[154] Lin, J., et al. (2004) Bacteria in the treatment of cancer. *Curr Opin Mol Ther* 6, 629–639.

[155] Steidler, L. (2004) Live genetically modified bacteria as drug delivery tools: at the doorstep of a new pharmacology? *Expert Opin Biol Ther* 4, 439–441.

[156] Liu, S.C., et al. (2002) Anticancer efficacy of systemically delivered anaerobic bacteria as gene therapy vectors targeting tumor hypoxia/necrosis. *Gene Ther* 9, 291–296.

[157] Ryan, R.M., et al. (2006) Use of bacteria in anti-cancer therapies. *Bioessays* 28, 84–94.

[158] Beninati, C., et al. (2000) Therapy of mucosal candidiasis by expression of an antiidiotype in human commensal bacteria. *Nat Biotechnol* 18, 1060–1064.

[159] Rao, S., et al. (2005) Toward a live microbial microbicide for HIV: commensal bacteria secreting an HIV fusion inhibitor peptide. *Proc Natl Acad Sci U S A* 102, 11993–11998.

[160] Burlage, R.S., and Kuo, C.T. (1994) Living biosensors for the management and manipulation of microbial consortia. *Annu Rev Microbiol* 48, 291–309.

[161] Kambam, P.K., et al. (2008) Design and mathematical modelling of a synthetic symbiotic ecosystem. *IET Syst Biol* 2, 33–38.

[162] Cherepanov, P.P., and Wackernagel, W. (1995) Gene disruption in *Escherichia coli*: TcR and KmR cassettes with the option of Flp-catalyzed excision of the antibiotic-resistance determinant. *Gene* 158, 9–14.

Appendix A

Supplementary information for Chapter 2: Engineered bidirectional communication mediates a consensus in a microbial biofilm consortium

A.1 Plasmids encoding Circuits A and B





A.2 Validation of model in liquid culture

We validated the model-informed design choices by testing cells containing Circuits A and B in liquid culture. Cells containing each circuit were grown both in isolation and in communication with one another. As per details provided in the materials and methods section of the paper, isolated circuits were unable to produce a significant response, but when cells containing the two circuits were grown to sufficient density in separate chambers that allowed passage of small molecules between the two populations, responses from both were over 50-fold greater than the responses of the circuits in isolation (Figure 2.3 and A2).



Figure A2 Fluorescence of cells containing Circuits A and B rises with time when the two are grown in communication with one another. Neither cell population fluoresces significantly in the absence of the other population.

A.3 Solid phase imaging equipment and settings

Axiovision 4.5 software was used to capture mosaic images every 30 minutes on a Zeiss Axiovert 200M microscope equipped with an AxioCam MR CCD camera. Images were captured using a 2.5x objective and a GFP filter with 470/40 excitation and 525/50 emission, and the exposure time for all mosaic tiles was 50 ms. Image acquisition bit depth was 12, and each mosaic tile was stored as a 16-bit grayscale image. Each pixel represents an area of 9.8039 µm by 9.8039 µm. The number of tiles per mosaic and pixels per tile for the experiment in Figures 2.4A, B were as follows: 8x5 tile mosaic with 282x188 pixels in each tile. Tiles overlapped 10% to form the full mosaic. Control experiments were performed in which two rectangular agarose slices containing Circuit A were placed in contact with one another and two slices containing Circuit B cells were placed in contact with one another. In both of these control experiments, no gradient formed at the interface between the adjacent agarose slices.

Matlab was used to perform background correction and image normalization. Background correction was implemented by first selecting a set of tiles within the agarose regions of the mosaic from the initial image (time = 0 hours). A single, representative "background" tile was created from the median intensities of this set of tiles. This background tile was then subtracted from all tiles over all times. A 51-pixel moving average filter was used to further remove the effects of bias within each tile. A one-dimensional spatial representation for each time point was then created by taking the mean of each column of pixels.

A.4 Biofilm experimental setup, imaging, and image processing

A.4.1 Equipment specifications

An image of the biofilm flow apparatus can be found in Figure A3. The interior of the biofilm flow apparatus was kept sterile during the duration of each experiment. Biofilms were grown in M9 biofilm medium which was not recycled, and which was maintained at room temperature. Freshly prepared medium with appropriate antibiotics (50 µgml⁻¹ kanamycin and 20 µgml⁻¹ tetracycline) was placed in the sterile reservoirs every 24 hours. Medium was pumped from the reservoirs by a Watson-Marlowe peristaltic pump (205U) with 16-channel capacity. Oxygen-permeable Tygon tubing (ABW00002) carried medium from the reservoirs, through bubble traps which reduced pulsatile action in the flow (Biosurface Technologies, BSTFC34), and through a custom-made heat strip which prevented bacteria from swimming upstream to the medium reservoirs. Medium then entered the flow chambers (Stovall Life Sciences, Inc., ACCFL0001) and finally exited the flow apparatus into sterile effluent reservoirs. The flow chambers and tubing and medium approaching them were maintained at 30°C in a small custom-built incubated chamber (not shown).

Inoculation of bacteria into the flow chambers was performed with sterile 1cc syringes directly into the Tygon tubing, approximately 3 cm before the flow chambers. Flow chambers were left to incubate coverslip-down for an hour without flow, and then were incubated coverslip-down for an additional 24 hours with flow. They were then incubated coverslip-up with flow for the remainder of experiments and all imaging.



Figure A3 Photograph of the flow apparatus. The entire interior was kept sterile, and flow chambers were maintained at 30°C in an incubator (not shown).

A.4.2 Monoculture dosage experiments

Circuit A monoculture dosage experiments were initiated as described above. To enable identification of all bacteria in the biofilm, an eCFP expression plasmid, pMP4641 [68], provided a constitutive marker in all Circuit A cells. This plasmid was chosen for its demonstrated retention in *E. coli* cells, even in the absence of antibiotic pressure. Tetracycline was administered with the biofilm medium to maintain the plasmid. However, its degradation properties in this medium are uncharacterized. Thus, retention in the absence of antibiotic was an attractive feature.
After the first 48 total hours of Circuit A incubation, sterile M9 biofilm medium containing the appropriate antibiotics and concentrations of C4HSL (Sigma) was placed into the medium reservoirs. The Circuit A biofilm was then incubated for 18 hours with medium containing acyl-HSL prior to dosage response imaging. This induction time was chosen based upon the determined time-points of maximal expression in the solid-phase MCC studies. Dosage response imaging therefore took place after 66 total hours of Circuit A biofilm growth. At this timepoint and with a constant flowrate of 125 µlmin⁻¹ (speed setting 1.5 on the Watson-Marlowe peristaltic pump), Circuit A biofilms were robust monolayers which provided reproducible imaging data.

Circuit B monoculture dosage experiments were also initiated as described above. Again, to enable identification of all bacteria in the biofilm, the eCFP expression plasmid pMP4641 provided a constitutive marker in all Circuit A cells. Circuit B biofilms grow and thicken more quickly than Circuit A biofilms, so Circuit B biofilms were incubated for a total of 24 hours prior to induction with C12HSL (Sigma). The Circuit B biofilm was induced with M9 biofilm medium containing appropriate antibiotics and concentrations of C12HSL for an additional 18 hours prior to imaging. Hence, after 42 total hours of growth, dosage response imaging took place for Circuit B biofilms. At this timepoint and with a constant flowrate of 125 μ lmin⁻¹ (speed setting 1.5 on the Watson-Marlowe peristaltic pump), Circuit B formed conformal monolayer biofilms which coated the substrate to provide reproducible imaging data.

A.4.3 MCC experiments

In MCC biofilms used for quantitative imaging (Figure 2.5), separate cultures of Circuit A and Circuit B cells, all containing the eCFP plasmid pMP4641, were first grown to saturation and then diluted to OD 0.2. These were mixed in a 50/50 ratio immediately prior to inoculation of the MCC biofilms. Biofilms were incubated after inoculation, coverslip-down without flow, for one hour. Flow was then resumed at a flow rate of 20 μ lmin⁻¹, (speed setting 0.5 on the Watson-Marlowe peristaltic pump), and flow chambers were left coverslip-down for a total of 24 hours. For the remainder of the experiment and for all imaging, flow chambers were left coverslip-up. Images were taken a total of 24, 48, 72, 96, and 120 hours past inoculation.

For the four-color images of the MCC biofilm (Figure 2.6), cells containing the Circuit A plasmid also contained plasmid pMP4658, which is identical to plasmid pMP4641 but which constitutively expresses eYFP in place of eCFP [68]. Cells containing the Circuit B plasmid also contained pMP4641. The images generated by these biofilms were not used for quantitative analysis because (1) the two different fluorophores may interact differently with the cellular environments, and (2) differentiation between the potentially overlapping spectra of these four fluorophores with certainty is difficult. These biofilms were otherwise prepared and analyzed by a method identical to that described above for quantitative MCC biofilms.

A.5 Biofilm imaging equipment and settings

A.5.1 Microscope settings

Images taken for quantitative analysis were all taken with identical settings. All images taken for a given dosage analysis were taken from the same experiment, at the same time and on the same day. In addition, all biofilms described in this paper were grown and imaged at the same time of day, and the microscope and flow apparatus were maintained in the same room with the same lighting and temperature. Controlling these variables enabled reliable, reproducible growth of the biofilms.

A.5.2 Settings for all imaging

Microscope:	Zeiss 510 upright CLSM
Control software:	Carl Zeiss AIM
Objective:	Zeiss Achroplan 40x/0.8 W
Pixel resolution:	512x512
Data depth:	12 bit
Scan speed:	5–12.8 µs pixel time
Averaging:	2 for all single images, 1 for stacked images

A.5.3 Settings specific to dosage experiments

Channel 1: green	Pinhole setting: 250 (2.39 Airy)
Excitation: 488 nm Argon laser, 11%	Gain: 800
Dichroic: 488/543	Amplifier Offset: -0.048
Secondary Beamsplitter: NFT 545 nm	Amplifier Gain: 1
Emission filter: BP 500–530 nm	

Channel 2: cyan	Pinhole setting: 250 (2.46 Airy)
Excitation: 458 nm Argon laser, 76%	Gain: 1000
Dichroic: 458/514	Amplifier Offset: 0.1
Emission filter: BP 480–520 nm	Amplifier Gain: 1

A.5.4 Settings specific to MCC experiments

Channel 1: green	Pinhole setting: 250 (2.39 Airy)
Excitation: 488 nm Argon laser, 11%	Gain: 875
Dichroic: 488/543 nm	Amplifier Offset: -0.043
Secondary Beamsplitter: NFT 545 nm	Amplifier Gain: 1
Emission filter: BP 500-530 nm	
Channel 2: cyan	Pinhole setting: 250 (2.46 Airy)
Excitation: 458 nm Argon laser, 80%	Gain: 1000
Dichroic: 458/514 nm	Amplifier Offset: -0.04
Emission filter: BP 480-520 nm	Amplifier Gain: 1
Channel 3: red	
Excitation: 543 nm HeNe laser, 80%	Pinhole setting: 250 (2.12 Airy)
Dichroic: 488/543 nm	Gain: 1000
Secondary Beamsplitter: NFT 545 nm	Amplifier Offset: 0.1
Emission filter: BP 480-520 nm	Amplifier Gain: 1

103

A.5.5 Settings specific to 4-color MCC imaging

Channel 1: green	Pinhole setting: 104 (0.99 Airy)
Excitation: 488 nm Argon laser, 11%	Gain: 860
Dichroic: 488/543 nm	Amplifier Offset: -0.038
Secondary Beamsplitter: NFT 545 nm	Amplifier Gain: 1
Emission filter: BP 500-530 nm	
Channel 2: cyan	
Excitation: 458 nm Argon laser, 80%	Pinhole setting: 102 (1.00 Airy)
Dichroic: 458/514 nm	Gain: 1000
Emission filter: BP 480–520 nm	Amplifier Offset: -0.04
	Amplifier Gain: 1
Channel 3: red	
Excitation: 543 nm HeNe laser, 80%	Pinhole setting: 117 (0.99 Airy)
Dichroic: 488/543 nm	Gain: 1000
	Amplifier Offset: -0.0
Secondary Beamsplitter: NFT 545 nm	Amplifier Gain: 1
Emission Filter: LP 650 nm	
Channel 4: yellow	
Excitation: 514 nm Argon laser, 80%	Gain: 1000
Dichroic: 458/514 nm	Amplifier Offset: -0.05
Emission filter: LP 530 nm	Amplifier Gain: 1
Pinhole setting: 115 (1.00 Airy)	

A.6 Three-dimensional image rendering

The stacked images were captured with settings listed above, at 1 μ m spacing. The entire field was captured for each channel at each depth, prior to moving to a new depth. LSM files from the stacks were imported directly into Imaris 4.5.2. Throughout the depth, cyan was used to mask the red channel (red pixels without cyan were set to 0) and yellow was used to mask the green channel (green pixels without yellow were set to 0). All channels were then rendered in Imaris as isoforms (lower threshold cutoffs of 100, Gaussian filter diameter of 1.584 μ m). Colors were generated by a default full-range linear look-up table.

A.7 Image processing

Step 1—The input

The input is always a set of 512x512 pixel RGB TIFF-chunky images exported from LSM files. For each image, RGB colors correspond to detector channels on the microscope ("R" is emission from dsRed, "G" is emission from GFPlva, "B" is emission from eCFP). All images for a given dosage experiment were taken on a single day, 18 hours after induction with acyl-HSL. All images for a given day in the MCC experiments were taken at the same time on that day. Each biofilm grew in one "lane" of a flow chamber and at least two lanes were used for each acyl-HSL concentration (dosages, Figure 2.5) or for each day (MCC experiments, Figure 2.6). Images were directly imported into Matlab.

Step 2—Obtaining information from the eCFP image

Maxima were first extracted from the "B" layer of the RGB image (eCFP emission). The image containing only these maxima was then adjusted to fill the entire spectrum, and the regional maxima were extracted from it. The image containing only these regional maxima was essentially a digital matrix of pixels which were "1" if a "significantly cyan" pixel is present, and "0" if not. A significantly cyan pixel could be assumed to be associated with a cell in the biofilm, because all cells in the biofilm constitutively express eCFP. The total number of cell-associated pixels in the histogram was counted and assigned to the variable TotalCyan.

Step 3—Using eCFP information to threshold the GFP image

The "G" layer of the TIFF image reports GFPlva expression, or circuit-function-related green fluorescence. From the raw histogram for the "G" image, the top TotalCyan pixels were chosen for inclusion in a new "green" histogram. When DsRed-exp was also present (MCC experiments), the "R" layer of the TIFF image reported DsRed-exp expression. The top TotalCyan pixels were chosen from it for inclusion in a new "red" histogram. Pixels were chosen from the top intensity bin first, then the next intensity bin, and so on, until TotalCyan pixels were incorporated into the new histogram. The new histograms therefore included only cell-related green or red pixels.

Step 4—Generating comparable histograms from all images

All intensity bins in the cell-related fluorescence histograms ("green" and "red") were divided by TotalCyan such that they represented a percentage of total pixels in the image,

106

rather than a raw total. This enabled quantitative comparison of various images, even when they did not contain the same number of cell-related pixels. We called these the percentage histograms.

Step 5—Intensity weighting

The percentage histograms were retained, (only "green" histograms for dosages, and both "green" and "red" for MCC experiments) but also used to generate weighted histograms. Each element (bin) of the percentage histogram was multiplied by the intensity it represented (1 to 256), yielding a weighted histogram for mean calculations.

Step 6—Averaging over a single concentration or lane

Percentage histograms for images taken of lanes that are induced with the same acyl-HSL concentration (dosages) or on the same day (MCC experiments) were averaged by intensity bin. This resulted in an average intensity histogram for each acyl-HSL concentration or day. The "green" averaged percentage histograms reporting dosage experiment results were displayed in Figure 2.5.

Step 7—Mean intensity calculations

The weighted histograms for images taken of lanes that were induced with the same acyl-HSL concentration (dosages) or on the same day (MCC experiments) were averaged by intensity bin. Then, for each concentration or day, the mean of this averaged-weighted histogram was taken. This calculation yielded a mean intensity for each dosage or day, for each fluorophore present. The mean intensity for each color for each day was then plotted, in Figure 2.5, insets (dosages), and Figure 2.6B (MCC experiments).

Appendix B

Supplementary information for Chapter 3: Structure and selection in an engineered symbiotic biofilm consortium

B.1 Details of construction of strains MGd- and MGfwc-

Strains MGd- and MGfwc- were constructed by recombination with the lambda red

recombinase plasmid pKD46, as outlined in [150]. The chromosomal inserts to replace

dapD, csgG-csgC, and wza-wcaL were all constructed by PCR with template plasmid

pKD4.

The primers used were:

dapD-P1-fwd: 5'-ATGCAGCAGTTACAGAACATTATTGAAACCGCTTTTGAACGCCGGTGTAGGC TGGAGCTGCTTC and dapD-P2-rev: 5'-TTAGTCGATGGTACGCAGCAGTTCGTTAATGCCGACTTTGCCGCATATGAATA TCCTCCTTA; csgG-P1-fwd: 5'-TCAGGATTCCGGTGGAACCGACATATGGCGGTATTTCACCAGAATGTCATGT GTAGGCTGGAGCTGCTTC and csgC-P2-rev: 5'-TTAAGACTTTTCTGAAGAGGGCGGCCATTGTTGTGATAAATGAAGTGACTGC ATATGAATATCCTCCTTA;

wza-P1-fwd: 5'-ATGATGAAATCCAAAATGAAATTGATGCCATTATTGGTGTCAGTAACCTTGTG TAGGCTGGAGCTGCTTC and wcaL-P2-rev: 5'-CTATAAAGCCTGCAGCAAGCTGGCGAGTTCTCGATTGATCACCTGCTGGTCAT ATGAATATCCTCCTTA

Recombinant clones were selected with 50 µgml⁻¹ kanamycin, cured at 42°C, and tested with colony PCR reactions using internal primers to confirm the presence of the kanamycin resistance gene and absence of the target genes. Plasmid pCP20, containing the Flp recombinase, was transformed into cells containing successful kanamycin inserts to remove the inserts [162]. Finally, clones were again cured at 42°C to remove pCP20, and the same colony PCR reactions with internal primers were repeated to confirm the deletions of target genes and of the kanamycin resistance gene insert.

B.2 Stability of the engineered symbiotic biofilm

The blue and yellow populations coexisted for periods of up to 288 days in the biofilm environment (experiments were terminated after that, and imaging data was not collected past 178 hours). Biofilm sloughing and growth phases occurred at repeatable times from experiment to experiment, even when experiments were conducted months apart (Figure B1). Repeatability over the first 120 hour time period—the length of time for which we obtained the most independent replicates—was confirmed with analysis of variance (ANOVA), F0.05(3,28) = 0.47 < Fcrit = 2.947, P = 0.70. This demonstrates that the biofilm environment we created provides repeatable results over at least a period of 120 hours, which is longer than necessary to validate all quantitative comparisons that we make between generations. However, some weeks all biofilms accumulated more total biomass than other weeks (see November versus December in Figure B1) which was probably due to changes in incubation temperature, which was difficult to precisely control. The relationships between relevant biofilms were repeatable from week to week even if total biomass accumulation was not equivalent.



Figure B1 Mean total biomass in μ m, with respect to hours past inoculation, for independent symbiotic biofilms cultured months apart. These data reveal that biomass accumulation, including timing of growth and dispersal phases, of the symbiotic ecosystem is very repeatable between independent biological replicates (P = 0.7).

111

B.3 Metrics and COMSTAT image processing

Image stacks were pre-processed with the COMSTAT script LOOK, the background cutoff threshold was set at 15 for all images, and any images still containing background auto-fluorescence of the substrate were deleted from the stack (thus, any errors in the measured values will be too low, rather than too high). Options chosen for processing in COMSTAT were 1 (biomass calculation) and 24 (no connected volume filtration, because yellow and blue populations were intermingled in the biofilm but were captured in parallel image stacks, so not all clusters captured in each single-channel stack were connected to the substrate). We also edited the code for option 1—the biomass calculation—to generate the biomass median as well as biomass totals for each layer in the stack (allowing us to confirm total biomass and biomass median calculations). The COMSTAT code that we used to generate the biomass totals and medians is below.

```
function [y,bmp,hs]=biomass_func(bb,xyarea,voxel)
count=0; hs=0; layersum=0; bmpcount=0; halfsum=0;
for side=1:size(bb,3)
   loc1=bb(:,:,side)>0;
   count=count+sum(sum(loc1));
end
y=count*voxel/(xyarea*size(bb,1)*size(bb,2));
halfcount=count/2;
for side=1:size(bb,3)
   loc1=bb(:,:,side)>0;
   layersum=sum(sum(loc1));
   bmp(side)=(layersum/count)*100;
end
for side=1:size(bb,3)
   loc1=bb(:,:,side)>0;
   halfsum=halfsum+sum(sum(loc1));
   if halfsum>=halfcount
       hs=side;
       break;
   end
end
```

B.4 Repeatability of growth advantage in untreated second-generation biofilm



Figure B2 The untreated second-generation biofilms (Untreated G2) always exhibited a growth advantage over the first-generation biofilms (G1). The second-generation consortia formed biofilms far more quickly: on average, after 48 hours of growth, second-generation consortia accumulated 15 times the biomass accumulated by first-generation consortia in their first 48 hours (ANOVA $F_{0.05}(3,4) = 0.358 < F_{crit} = 6.591$, P = 0.78). In one example (JanI) the untreated second generation accumulated 30 times the biomass of the first generation.

Not only did the second-generation biofilms accumulate biomass more quickly than the first generation, as seen in Figure B2, but second-generation biofilms also accumulated more total biomass. We first averaged the highest ever recorded total biomasses from four separate first-generation biofilms (regardless of the time point). We then averaged total biomass after 48 hours of second-generation growth from the same four separate trials. By comparing these two averages we found that, on average, the second generation accumulated two times more biomass than the first generation ever accumulated in its lifespan.



B.5 Images of treated and untreated biofilms at the substrate

Figure B3 (A) and (B) Blue and yellow biomass is co-localized at the substrate in untreated 12-hour second-generation (G2) biofilms. (C) and (D) Single and very small clusters of yellow cells are distributed across the substrate in 12-hour treated G2 biofilms, but blues are still found in clusters. (E) and (F) In the untreated G2 case, blue and yellow continue to be co-localized and to grow well at 24 hours. (G) and (H) In the treated G2 case, blues appear less healthy and yellows are distributed across the substrate in small clusters rather than being co-localized with blues after 24 hours.



Figure B4 Note that this is a different layout of images from Figure B3. All images are taken after 72 hours of growth. (A)–(D) are untreated biofilms, (E)–(H) are treated biofilms. (A) and (B), (C) and (D) are paired blue and yellow images at the substrate from untreated second-generation (G2) biofilms. These show that blue and yellow are healthy, co-localized, and significant biomass has accumulated. (E) and (F), (G) and (H) are paired blue and yellow images at the substrate from treated G2 biofilms. These show that blue biomass is no longer healthy or has died, while yellow biomass is forming a scant biofilm. Overall, we can see that there is a significant difference in substrate colonization between the treated and untreated second-generation biofilms.

B.6 Emergent structure does not reappear in the third-generation control biofilm



Figure B5 A projection taken at the substrate of the control third-generation biofilm, which was started from untreated effluent of the treated second-generation biofilm. It shows that emergent structure is no longer present in the third-generation biofilm. Although there are regions of co-localization of blue and yellow, the blue biomass and yellow biomass are both primarily localized near the substrate.



B.7 FACS was used to separate aggregates and YFP+ single cells

Figure B6 Representative data from FACS (this was repeated twice, this data is from one of the cases). The YFP+ fraction is conservatively gated to contain only single cells (based upon fluorescent and no-stain controls). The YFP+ fraction fluoresces in the yellow but not in the blue range, with a subpopulation that appears to lack fluorescence entirely (this could be single blue cells that are dead—they do not appear to adhere). The aggregate fraction contains groups of cells of unidentified shape and composition. It appears that most of these contain cells that fluoresce in the yellow range (although the smattering of dots below the primary group suggests the possibility of groups of just blue cells), and that most of the aggregates also exhibit fluorescence in the blue range (thus, most aggregates probably do contain at least some blue and some yellow biomass).