Review

Engineering microbial consortia: a new frontier in synthetic biology

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Microbial consortia are ubiquitous in nature and 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. Here, we review recent efforts to engineer synthetic microbial consortia, and we suggest future applications.

Benefits and features of microbial consortia

Synthetic biology [1–5] has generated many examples of what microbes can do and what we can learn from them [6–11] when they are creatively engineered in the laboratory environment. From the synthesis of an anti-malarial drug [12] to the study of microbial genetic competency [13], 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). 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 [14]. 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 sub-populations. Here, we briefly examine the complex functions that mixed populations perform, and the evidence for their robustness to environmental fluctuation. We then explore how engineers have employed communication and differentiation of function in designing synthetic consortia, and we comment on their future applications.

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 [15]. 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 [16].

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 [17]. 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 [18]. 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.

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0167-7799/$ – see front matter doi:10.1016/j.tibtech.2008.05.004 Available online 31 July 2008

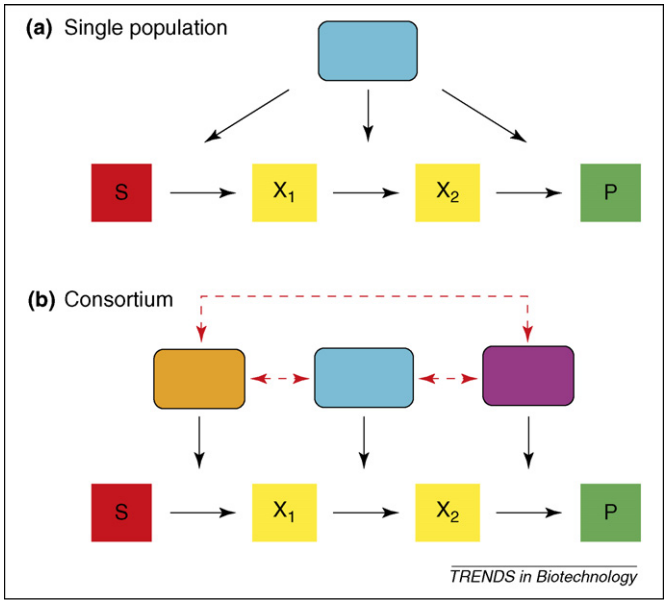

Figure 1. Processing of complex reactions by **(a)** a single population or **(b)** a microbial consortium. Generation of a product (P) might require multiple steps to convert the substrate (S), through the sequential synthesis of intermediates (X1 and X2). (a) A single population is responsible for 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.

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 [19]. 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 [20]. 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 [21]. 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 [21]. Diversity of species in a consortium does not guarantee survival [22,23], but it might be that engineered consortia will perform most reliably in changeable environments when diverse metabolic modes are present among members [24].

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 [14,25]. Bacteria coordinate intra-population behaviors from biofilm formation [26–28] to virulence [29–31] with the exchange of acyl-homoserine lactone (acyl-HSL) signaling molecules (in Gram-negative species) and small peptides (in Gram-positive species) [25,32,33]. 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 [34]. 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 [35]. 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 [36].

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 [25]. Engineered acyl-HSL communication has been used in biological 'circuits' that coordinate population-wide behaviors ranging from population-density-dependent fluorescence [37], cell suicide [38], and invasion of cancer cells [39] to pattern formation [40]. We recently described a mixed-population biofilm-based consortium that uses two-way engineered communication via acyl-HSLs to coordinate fluorescent gene expression [41]. The expression of fluorescent genes is possible if, and only if, both member populations are present at sufficiently high densities. This engineered 'consensus consortium' 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.* [42] 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 2a). With appropriate parameters, including appropriate cellular growth rates for the two populations and the right concentrations of the inducing chemical isopropyl-β-D-thiogalactopyranoside (IPTG), the densities of the two populations begin to oscillate in a phase-shifted manner (Figure 2b).

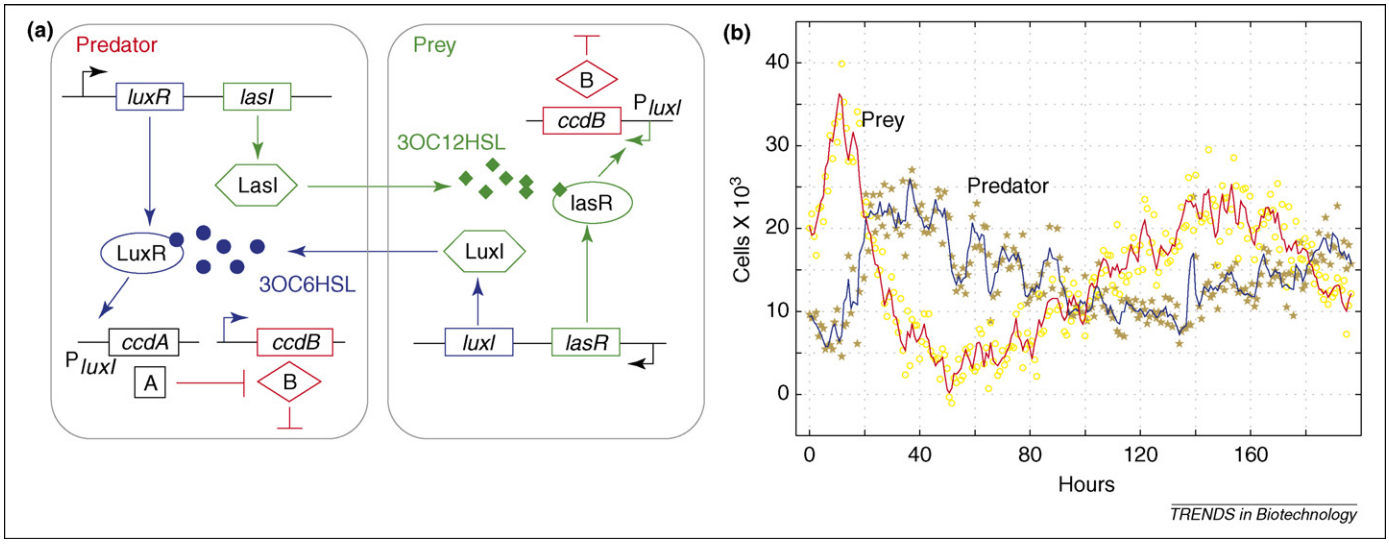

Figure 2. A synthetic predator–prey ecosystem **(a)** consists of two engineered bacterial populations that control each other's survival through two different QS signals. Two QS modules, LuxI and LuxR from *Vibrio fischeri* and LasI and LasR from *P. aeruginosa*, are used to enable two-way communication. 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 μM, dilution rate = 0.1125 h^{-1}). Figure adapted from Balagadde *et al.* [42].

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.

Dedicated signals have also been used to implement communication between different kingdoms of organisms. Weber *et al.* [43] 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 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 [44]. An engineered consortium described by Shou *et al.* [45] provides insight into the exchange of metabolites in microbial consortia. Shou *et al.* [45] 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) [45]. One strain of *S. cerevisiae* is unable to make lysine but overproduces adenine, and the other cannot make adenine but overproduces lysine (Figure 4a). 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 4b). This can serve as a guiding

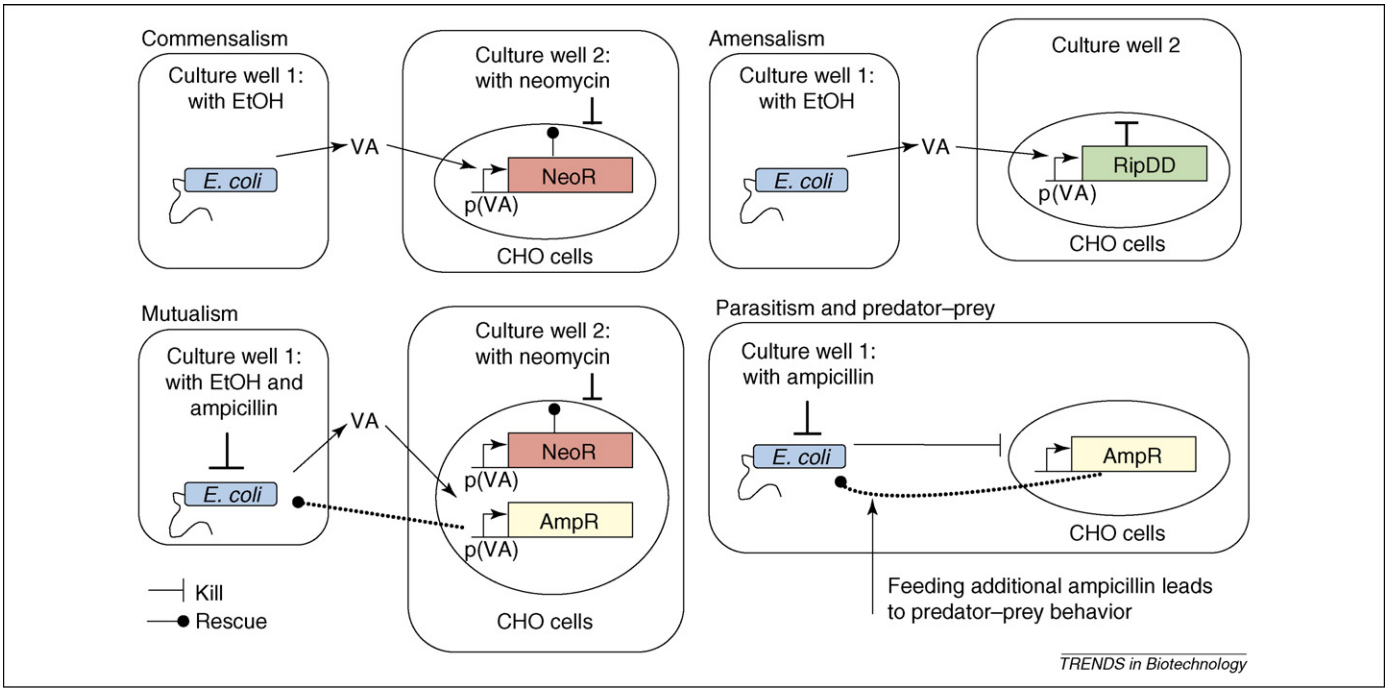

Figure 3. Communication can occur between different kingdoms of organisms. Weber *et al.* [43] used a simple set of 'sender' and 'receiver' modules to engineer commensal (one population benefits because of the association, there is no effect upon the other), amensal (one population suffers, there is 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 cell-death. 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.* [43].

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.

Synthetic consortia lend biological insight

Many questions remain regarding the evolution and stability of natural ecosystems. As Shou *et al.* [45] and Balagadde *et al.* [42] 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 [46]. Such studies have already demonstrated that cheating strains, sub-populations that 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 [47]. Furthermore, Shou *et al.* [45] 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 regrowth, 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 co-evolution 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 [48]. We can use engineered consortia to explore the evolution of cooperation and antagonism between populations in controlled environments to better understand the origins of these interactions.

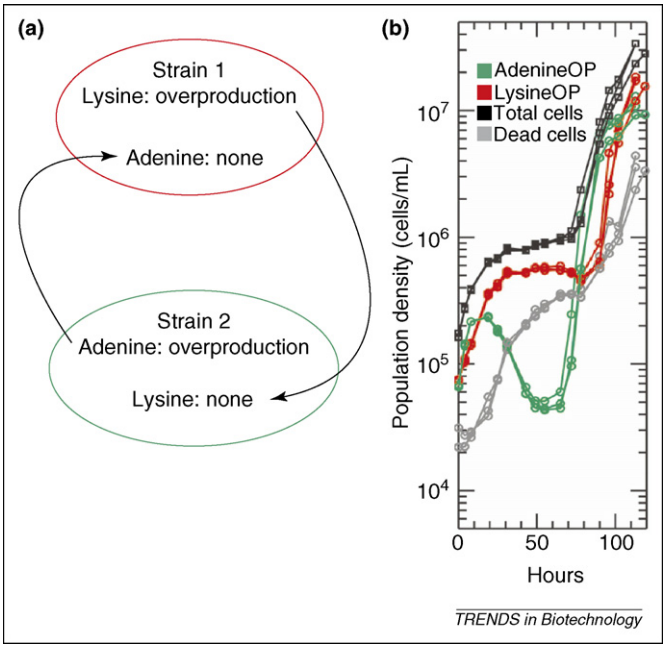

Figure 4. Synthetic consortia communicate by exchanging chemicals involved in metabolism and growth. **(a)** Shou *et al.* [45] engineered two strains of auxotrophic yeast that depend upon one another for survival. One strain (strain 1) of *S. cerevisiae* is unable to make adenine but overproduces lysine, 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.* [45].

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 medical technology. Engineers have developed bacteria that serve as drug-delivery devices [49–51] and gene-delivery vehicles [50,52,53], 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.* [42]) 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 [54,55]. A consortium of engineered commensal microbes might colonize and provide additional functionality, including detection warnings [56] 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.

Challenges in engineering microbial consortia

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 [57,58]. 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 [59]. 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 [17]. 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.

Conclusion

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 [60]. 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.

Acknowledgements

Our research on synthetic microbial consortia is funded by the National Institutes of Health grant NIH 1-R01-CA118486. Thanks to Kevin Boulware and Michael Dougherty for helpful conversations.

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