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PERSPECTIVE

Engineering microbial consortia for controllable outputs

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Much research has been invested into engineering microorganisms to perform desired biotransformations; nonetheless, these efforts frequently fall short of expected results due to the unforeseen effects of biofeedback regulation and functional incompatibility. In nature, metabolic function is compartmentalized into diverse organisms assembled into robust consortia, in which the division of labor is thought to lead to increased community efficiency and productivity. Here we consider whether and how consortia can be designed to perform bioprocesses of interest beyond the metabolic flexibility limitations of a single organism. Advances in post-genomic analysis of microbial consortia and application of high-resolution global measurements now offer the promise of systems-level understanding of how microbial consortia adapt to changes in environmental variables and inputs of carbon and energy. We argue that, when combined with appropriate modeling frameworks, systems-level knowledge can markedly improve our ability to predict the fate and functioning of consortia. Here we articulate our collective perspective on the current and future state of microbial community engineering and control while placing specific emphasis on ecological principles that promote control over community function and emergent properties.
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Diversity in the microbial world and its implications for biosystem design

Although ecologists have long regarded biological diversity as one of the main factors leading to gains in ecosystem stability and productivity, the mechanisms by which these gains arise have been the subject of a decades-long debate (Hooper *et al.*, 2005). We here consider ecosystem stability to be a composite of multiple stability-related properties, such as resilience, that together broadly refer to insensitivity to perturbation in ecosystem functioning (Song *et al.*, 2015). Historically, two major overarching explanations for gains in ecosystem stability have been proffered, although these mechanisms are not mutually exclusive. The first explanation, frequently termed the 'sampling hypothesis,' is that more diverse communities are statistically more likely to contain members with varying tolerance to environmental stressors, so that, as conditions change, organisms displaying a higher degree of fitness under given conditions fill the functional void left by intolerant members (Loreau *et al.*, 2001). In contrast, niche differentiation, and more specifically, functional complementarity, leads to gains in productivity- that is, 'overyielding'- through more efficient resource utilization and elevated resistance to environmental perturbation (Savage *et al.*, 2007). More recently, a view of communities as functionally degenerate networks has asserted that rewiring of individual member functions and interactions between members may buffer overall community function against environmental perturbation (Hastings, 2010; Shade *et al.*, 2012). Clearly, major strides are being made toward quantitative description of the effects of diversity upon microbial community higher-order properties, yet significant gaps remain in our understanding of mechanisms by which these properties emerge.

We argue that the paucity in mechanistic knowledge must not discourage biodesign efforts but rather leverage the available engineering strategies to test specific hypotheses. For example, if the sampling hypothesis is the most dominant explanation for biodiversity effects on productivity and stability, we should expect that organisms engineered for optimal properties and maintained under their optimal growth conditions in monoculture should outperform consortia. But if niche differentiation

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and/or networked buffering are causal contributors to community productivity or stability, we anticipate that multispecies consortia will outperform even the best-optimized species in monoculture. Our view is that recent technical advances now permit elucidation of the mechanisms driving diversity effects upon higher-order properties in microbial communities. We submit that research directed at uncovering such principles (Konopka *et al.*, 2014) will be useful for the engineering of 'synthetic' consortia to stably and efficiently perform desired functions, and even to the point of designing robust, self-regulating interactions between member species.

Gazing into the crystal ball: predicting the behavior of microbial consortia

Since the first published report of a sequenced genome in 1995 (Fleischmann *et al.*, 1995), there has been an explosion in microbial genomic data, which has greatly impacted the study of microbial populations and communities. Emergence of small subunit ribosomal RNA phylogeny-based approaches led to the first insights into the vastness of uncultivated microbial diversity. Since then, these studies have yielded to metagenomics as standard methods for studying the community functional and compositional dynamics. New tools begat new understandings that have led to new disciplines; in this case, the rising technological frontier of microbial community design and control. Although it is not our intent to provide an exhaustive treatment of new systems-level approaches pertinent to microbial community engineering, there are a few developments we deem most impactful upon the emergence of this discipline: advances in genome and transcriptome sequencing (Beliaev *et al.*, 2014; Shakya *et al.*, 2013), mass spectrometry-based metabolomic and proteomic approaches (Louie and Northen, 2014; Kurczy *et al.*, 2015), cell isolation and printing (Louie *et al.*, 2013), and a movement towards high-throughput cultivation (Knepper *et al.*, 2014; Long *et al.*, 2014).

Nonetheless, the challenges of predictable systems-level understanding of complex microbiological systems, by and large, are not fully attributable to the lack of data or omics-level resolution. In fact, many microbial ecology questions do not require deeper sequence analysis and increased phylogenetic resolution, but rather studies that use the current technologies to explore the spatial scales, phenotypic diversity and temporality important to microbial communities (Prosser, 2012) that exert major influence upon the energy flux and nutrient cycling within even the simplest microbial consortia (Konopka *et al.*, 2014). Mechanistic understanding of interactive behaviors occurring within diverse, spatially organized communities is limited by methodological and theoretical issues related to predicting protein function from its sequence, inherent community dynamics, sampling at multiscale boundaries and our general inability to define a microbial 'species,' all of which hinder inter-community comparisons. Our perspective is aligned with Prosser (2012) in that improvements in our understanding of temporal and spatial dynamics will ultimately aid the ability to engineer microbial communities with outputs that can be predicted and, ultimately, controlled.

Furthermore, advancements of knowledge-based design and control of microbial consortia will increasingly rely on mathematical modeling, which can provide a better predictive understanding of microbial community dynamics and higher-order properties (Henson and Hanly, 2014). Any successful outcomes of *in silico* hypothesis testing, however, are contingent both upon our understanding of microbial metabolism and the accuracy of models, which describe community state in space and time as a function of metabolic adaptation of individual species as well as interactions among species. In this regard, the focus of microbial community modeling has bifurcated into two directions: first, population-based modeling for the prediction of the interspecies dynamics without detailed description of intracellular metabolism (Bouskill *et al.*, 2012) and, second, metabolic network modeling to analyze energy and material flows, and their partitioning in a community (Stolyar *et al.*, 2007; Taffs *et al.*, 2009). Integration of both approaches to understand the guiding role of interspecies interactions in governing community dynamics is becoming increasingly important (Song *et al.*, 2014).

The true asset of modeling, we believe, lies in its ability to predict community-level properties from individual variables through a rational description of nonlinearities arising from interactions between members. Accordingly, the prospect of *in silico* design of synthetic consortia will greatly benefit from expanding the knowledge of microbial metabolism and functional gene annotation, which, in turn, will facilitate the integration of heterologous data sets including omic, kinetic, and physiological data for improved prediction. This effort will lead to more accurate and realistic simulations of community functions that account for spatial heterogeneity (Zhuang *et al.*, 2012), single cell-level interactions (Lardon *et al.*, 2011) and/or population-controlling mechanisms (Klapper and Dockery, 2010). In sum, integrated, predictive modeling approaches will be required to describe the behavior of microbial communities and to discern the mechanisms by which higher-order properties arise. We also expect model accuracy to increase with our improved ability to test and validate model predictions through species-resolved measurements.

What are the known control points for microbial consortia?

Division of labor

It has been recognized for some time that engineered microbial consortia have the potential to be more productive and robust than monocultures

(Brenner *et al*., 2008). The question that remains, however, is how microbial communities can be designed and, more importantly, controlled. To date, successful examples of improvements by employing consortia rather than monocultures have not been based upon a mechanistic understanding of community function but have arisen either serendipitously or by adopting some of the most intuitive ecological principles. Chief among these is the division of labor concept based on rational assembly of functional specialists, which can be observed in cellular biology (Kirk, 2005), social economics (Becker and Murphy, 1994) and ecosystems (Shapiro, 1998). Division of labor among members mitigates constraints imposed by tradeoffs, whereby a population's pursuit of one objective is realized only at the cost of an alternative objective (Carlson and Taffs, 2010). Such inherent tradeoffs fundamentally prevent the existence of monoculture super-species that are simultaneously optimized for all niches (Law, 1979). Hence, when the first demonstrations of engineered division of labor in synthetic microbial consortia were reported (Shou *et al*., 2007), it was not surprising that carefully assembled specialists exhibited emergent properties, such as increased robustness and productivity, compared with their respective monoculture controls.

These initial studies beg the question of whether there are overarching engineering design principles that could predict how best to distribute metabolic labor across different microbial members to optimize a process objective. More specifically, how should metabolic processes be compartmentalized within different cells to optimize the performance of a desired metabolic transformation? Previous theoretical studies (Pfeiffer and Bonhoeffer, 2004) suggest that comprehending competition and conflict between metabolic processes could serve as a foundation for establishing such engineering design principles (Johnson *et al*., 2012). For example, consider a linear metabolic pathway where a primary substrate is consumed via an intermediate to an end product by two different enzymes, and that each of these different enzymes competes for the same finite pool of intracellular resources such as ATP or biomolecule precursors (Weisse *et al*., 2015) or for occupancy space within a cell (Beg *et al*., 2007). If the two competitive enzymes are contained within the same cell, then competition between the different enzymes could result in the accumulation of the intermediate within the cell. This would occur, for example, if the enzyme for the first metabolic transformation has preferential access to the finite supply of resources than the enzyme for the second metabolic transformation (Almeida *et al*., 1995). Conversely, if the two enzymes are segregated into different cells, then competition between the enzymes is eliminated, which in turn may reduce accumulation of the intermediate. This would occur, for example, if the enzyme for the first metabolic transformation no longer has preferential access to the finite supply of resources (Figure 1). The main outcome is that dividing metabolic labor should minimize the accumulation of intermediates, thus reducing the negative feedback effects that those intermediates might have on the cell and facilitating the consumption of the primary substrate. Indeed, the arguments above are analogous to those used for industrial assembly lines, where an important objective is to minimize the accumulation of manufacturing intermediates, thereby reducing any negative effects of accumulating those intermediates on the production of the final product.

A proven design principle that has emerged from the arguments above is that the division of metabolic labor among pre-assigned specialist cells enhances the substrate conversion efficiency, often leading to gains in biomass (Eiteman *et al*., 2008). This effect has special implications upon metabolite cross-feeding systems where the primary substrate is converted by one population to an inhibitory intermediate that can be concurrently consumed by a complementary specialist (Bernstein *et al*., 2012). If dividing metabolic labor reduces the accumulation

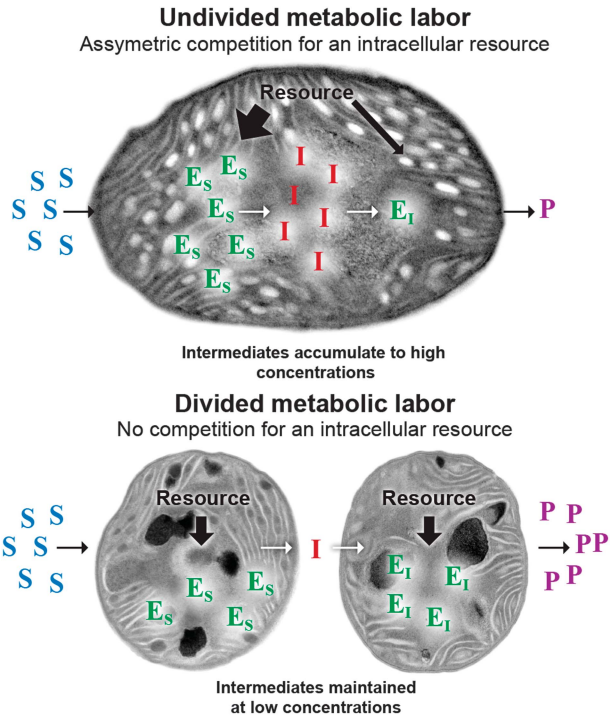

Figure 1 Example of how competition and conflict between different metabolic processes could promote the division of metabolic labor. In this hypothetical pathway, an enzyme (ES) transforms a substrate (S) into an intermediate (I). A second enzyme (EI) then transforms the intermediate into a product (P). These two enzymes may compete for the same intracellular resource, such as cellular space, co-factors for enzyme activity or building blocks for biosynthesis. If both enzymes are contained within the same cell and competition is asymmetric (for example, the enzyme for the first step has preference over the enzyme for the second step), then this would lead to the accumulation of the intermediate. If each enzyme is contained within different cells, then competition is lost, thus preventing one enzyme from outcompeting the other and reducing the accumulation of the intermediate.

of toxic intermediates, enhanced mechanistic knowledge about inhibition may soon guide design of new communities with optimal reaction compartmentalization assigned to distinct members. There is some empirical evidence to support this expectation as different steps of pollutant biotransformation pathways, which produce inhibitory intermediates, are often compartmentalized within different cells across the population (de Souza *et al*., 1998; Moller *et al*., 1998; Pelz *et al*., 1999). However, there is only limited evidence to date that dividing metabolic labor enables or promotes the consumption of inhibitory compounds. Such a generalization will require cross-system comparisons where the performance of communities, in which the metabolic labor is differentially compartmentalized, can be compared.

Spatial and temporal organization

In nature, incorporation of incompatible members or processes is fostered through the development of spatial and/or temporal segregation. For example, aerobic and anaerobic microbial populations may be co-cultured within a single system containing spatially defined oxic/anoxic habitats collocated within micrometer proximities; this can arise spontaneously (Field *et al*., 1995) or by design (Kim *et al*., 2008). Controlling spatial partitioning of populations in artificial habitats (for example, microfluidic and biofilm reactors) is also a promising technique for rationally engineering system robustness by simultaneously mitigating competition (or other antagonisms) and promoting beneficial interactions (Brenner and Arnold, 2011). It has been shown that in a spatially structured environment, strongly cooperating species will intermix, whereas two species engaging in competition or commensalism will spatially segregate resulting in a laminated consortium (Figure 2a; Estrela and Brown, 2013; Momeni *et al*., 2013; Muller *et al*., 2014). Another example of manipulating community spatial organization through interaction strength is based on a co-culture of *Sphingobium chlorophenolicum* and *Ralstonia metallidurans* engineered to perform environmental remediation (Kim *et al*., 2011). *S. chlorophenolicum* can degrade the highly toxic environmental pollutant pentachlorophenol (PCP) but is sensitive to Hg^{2+}, which is often present with PCP in contaminated sites. Microfluidic laminar flow techniques have been used to spatially position the PCP degrader *S. chlorophenolicum* in a core layer protected by an outer shell of *R. metallidurans*, which is a reducer of Hg^{2+} (Figure 2b). This spatially structured community can simultaneously remove PCP and Hg^{2+}; a capability not possessed by a well-mixed consortium containing the same members.

Physiological or metabolic incompatibilities among community members can also be resolved via temporal separation, which involves sequential activities or operation in distinct phases (Bond *et al*., 1995). Rational design and operational control based on this concept may draw inspiration from nature. A well-known and ecologically critical example is diel separation of nitrogen fixation and energy acquisition via oxygenic photosynthesis in diazotrophic cyanobacteria and their associated communities (Bebout *et al*., 1987). However, to our knowledge, synthetic consortia have yet to be designed specifically around a temporal separation concept, although temporal mechanisms have been specifically designed in quorum-sensing-based synthetic biology framework (Garcia-Ojalvo *et al*., 2004). New design principles based on concepts such as analog memory (Farzadfard and Lu, 2014), time-lag and temporal shifts are likely to be realized in future generations of synthetic microbial consortia platforms.

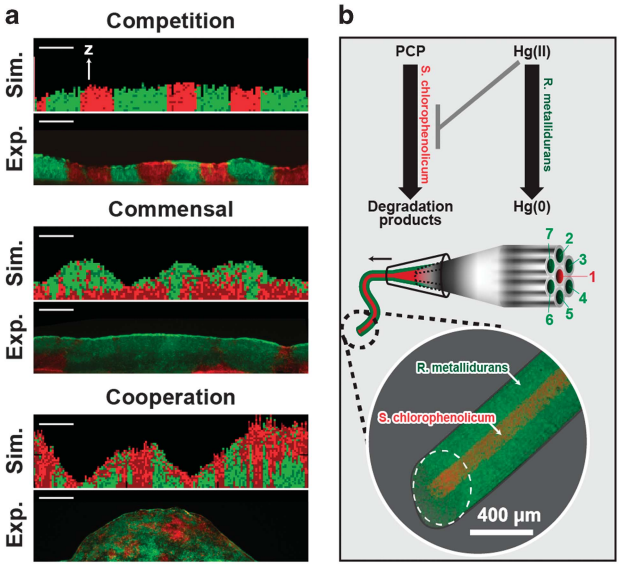

Figure 2 (**a**) Interactions and community behaviors can be manipulated by modifying the abiotic environment (Momeni *et al*., 2013). Vertical cross sections of communities of two non-mating *S. cerevisiae* strains, one requiring lysine and releasing adenine (red) and one requiring adenine and releasing lysine (green). The two strains engaged in competition (top) if both adenine and lysine are exogenously provided by the agarose medium, commensalism if, for example, lysine but not adenine is supplied (middle), and cooperation if neither lysine nor adenine is supplied (bottom). Different interactions lead to different spatial organization in experiments and simulations (scale bar, 100 µm). (**b**) Community behaviors can be manipulated by modifying the spatial organization of cells (Kim *et al*., 2011). A sufficiently thick 'shell' of *R. metallidurans* can be deposited around a 'core fiber' of *S. chlorophenolicum*, using microfluidics. *R. metallidurans* protects *S. chlorophenolicum* by reducing the toxic Hg(II) to Hg (0), and *S. chlorophenolicum* can in turn detoxify PCP.

Cumulative behavior as a function of interactions and environmental context

The collective action of individual physiologies and resulting interactions at various scales of time and space yield systems-level community behavior that is more than sum of all parts (Levin, 1992). Interactions between members can encompass physical contact, chemical signaling and metabolic exchange. For example, in a community comprised of two species cooperating through metabolite

exchange, exogenous addition of one or both metabolites will change their relationship from cooperation to competition or commensalism, respectively, with the potential to markedly change community behaviors. In commensalism and cooperation, but not in competition, disparate species ratios can potentially converge to a steady-state value yielding non-linear response which is basis for the emergence of higher-order properties (Momeni *et al.*, 2013). Further examples of modifying community properties through the modulation of individual behaviors have been demonstrated both in clonal populations (Hu *et al.*, 2010; Moon *et al.*, 2011) and synthetic multispecies communities design based on metabolic cross-feeding (Harcombe, 2010) or obligate syntrophy (Zhou *et al.*, 2015).

What's next in microbial community engineering?

As generalizable biological principles governing the functioning of microbial communities continue to emerge, they will expand the foundation of biological systems design. In our view, one of the primary objectives of microbial community engineering is enhanced control over composition and behavior. Ideally, advances in this field will bring to bear ability to control safety, productivity and stability of both natural and synthetic microbial ecosystems. The two fundamental approaches for managing the structure and the function of consortia may include extrinsic and intrinsic mechanisms. Although extrinsic methodologies have already been established and successfully demonstrated through environmental control and introduction of selective pressure, or substrate availability (Shou *et al.*, 2007; Bayer *et al.*, 2009; Minty *et al.*, 2013; Mee *et al.*, 2014), intrinsic control through programmable regulatory circuits is a relatively new concept that has been largely applied to isogenic populations of microorganisms (Elowitz and Leibler, 2000; Gardner *et al.*, 2000). Recent demonstrations have proven that population-level behaviors can be engineered into synthetic systems that through quorum-sensing and metabolite-sensing intrinsic devices (Brenner *et al.*, 2007; Marchand and Collins, 2013; Chen *et al.*, 2015). With further development of intrinsic devices that render cells capable of decision-making, the ability to control cellular behavior through logic-based operations will provide means for tighter control and a higher level of communication between members of the consortia (Brophy and Voigt, 2014; Church *et al.*, 2014). We envision new design concepts to take advantage of consortial modularity (Figure 3) and create new opportunities for metabolic engineering by offering robust, multicellular engineering platforms that can be stabilized by metabolic coupling and forced interdependency. In fact, the state of the science is ready to conceive designs in which output(s) can be controlled and customized simply by interchanging specialist members or 'modules'. This 'plug-and-play' concept will be particularly effective for implementation of automated, intrinsic control processes designed after established feedback motifs constituting of sensor, controller and actuator system components.

Our perspective is that these expansions will continue to move away from the traditional monocultures

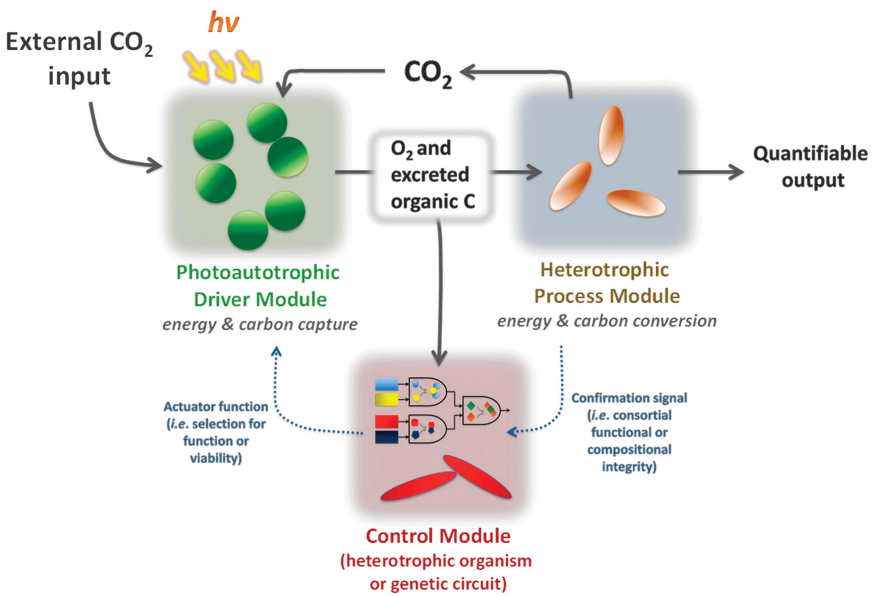

Figure 3 Conceptual design of a self-assembling autonomous, synthetic microbial consortium built through photoautotroph–heterotroph interactive partnership. Genetically tractable photoautotrophic organisms, such as cyanobacteria, can sustain and drive an engineered consortium through metabolic exchange reactions (black arrows), which include photosynthetic production of O_2 and organic C; these serve as respiration substrates for the heterotrophic module(s) to generate CO_2. This creates opportunities for metabolic engineering using interdependent modules, whose functional outputs and interactions can be programed for additional control via synthetic regulatory circuitry (blue arrows).

which rely heavily upon cultivation of domesticated and often highly engineered strains, and move more towards multi-organism designs. Traditional monoculture microbial technology is limited by poor resistance and resilience to fluctuating environmental conditions and competition from indigenous microorganisms; designed microbial consortia may greatly increase robustness against such insults, especially in open systems (for example, raceway ponds). Reconciliation of fundamental ecology with new biodesign framework is poised to overcome these barriers, drawing inspiration from the modular design of natural systems, which is a common paradigm in synthetic biology and standard for genetic recombination, device synthesis and protein engineering (Purnick and Weiss, 2009). The promise that this field has to offer is great—not only because transformative biotechnologies will help overcome the energy, health and environmental problems of the future but also because the process of learning to design and control ecological phenomenon has and will undoubtedly continue to yield new insights on the fundamentals of life.

Conflict of Interest

The authors declare no conflict of interest.

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