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Better together: engineering and application of microbial symbioses

Stephanie G Hays[1,3], William G Patrick[2,3], Marika Ziesack[1,3], Neri Oxman[2] and Pamela A Silver[1]

Symbioses provide a way to surpass the limitations of individual microbes. Natural communities exemplify this in symbioses like lichens and biofilms that are robust to perturbations, an essential feature in fluctuating environments. Metabolic capabilities also expand in consortia enabling the division of labor across organisms as seen in photosynthetic and methanogenic communities. In engineered consortia, the external environment provides levers of control for microbes repurposed from nature or engineered to interact through synthetic biology. Consortia have successfully been applied to real-world problems including remediation and energy, however there are still fundamental questions to be answered. It is clear that continued study is necessary for the understanding and engineering of microbial systems that are more than the sum of their parts.

Addresses
[1] Department of Systems Biology, Harvard Medical School, 200 Longwood Avenue, WAB 563, Boston, MA 02115, USA
[2] MIT Media Lab, School of Architecture and Planning, Massachusetts Institute of Technology, 75 Amherst St.,Cambridge, MA 02139, USA

Corresponding author: Silver, Pamela A (pamela_silver@hms.harvard.edu, stephaniehays@fas.harvard.edu)
[3] First author.

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Introduction: the benefits of living together

Communities dominate the microbial world; coexisting organisms cannot help but interact. Interactions include touching, using dedicated signals, horizontal gene transfer, 'competitive or cooperative' scenarios where microbes compete for or provide resources, and alteration of environment to influence the growth of neighbors [1,2•,3–6]. Cultures that consist of multiple microbial species, by definition, contain an increased range of genes and metabolic capabilities in comparison to monocultures. This diversity allows for the emergence of communal properties such as robustness and division of labor. In order to engineer consortia, scientists take control of the environment and organisms through devices and synthetic biology. These tools enable the application of microbial communities to real-world problems where the natural attributes of consortia are valuable.

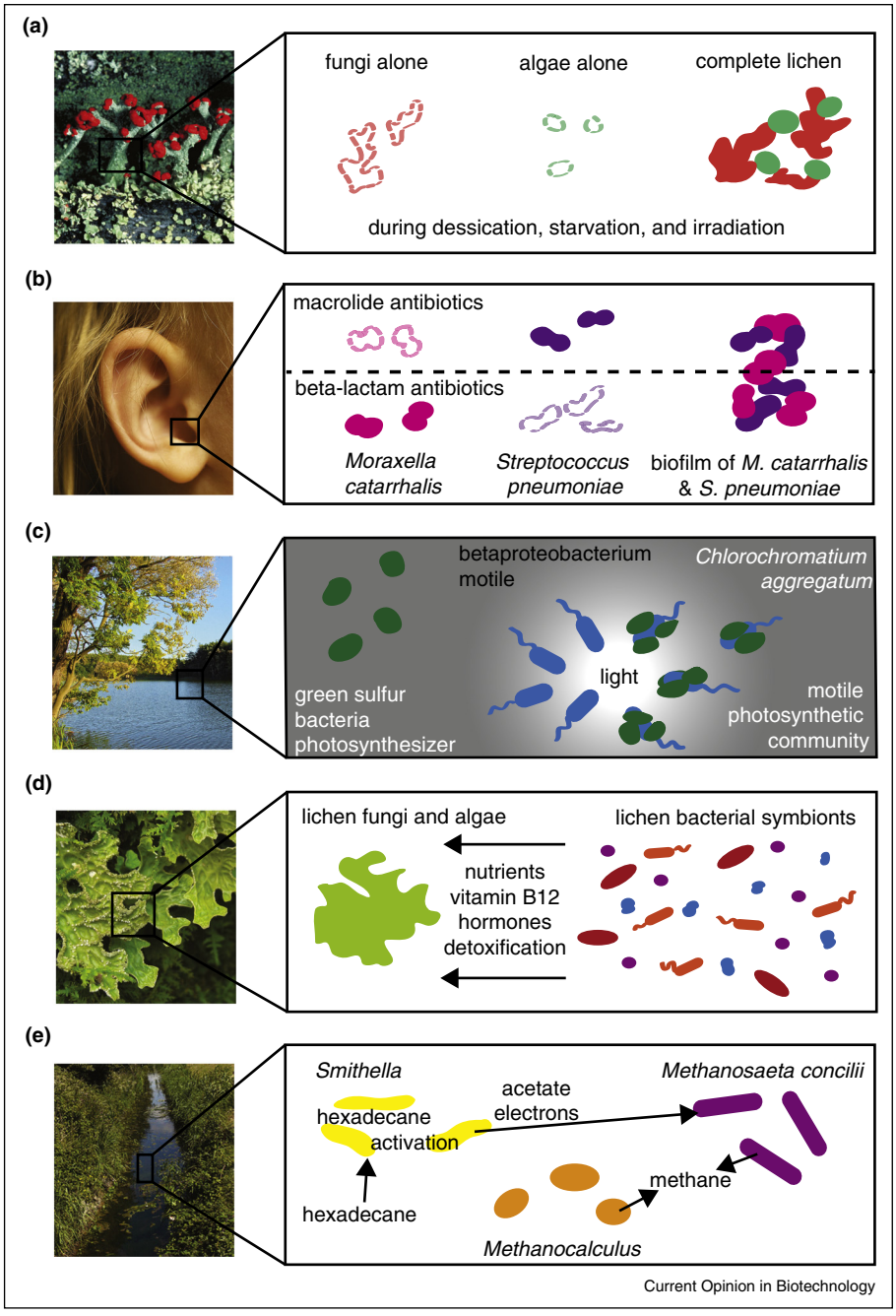
Robustness

Robustness — the ability to survive perturbation — is an emergent property of microbial communities and is necessary for survival in the wildly fluctuating world [5–7]. Such robustness is demonstrated in lichens and biofilms. Lichens are collections of bacterial, fungal, and algal symbionts that can survive desiccation, irradiation, and starvation better together than isolated organisms (Figure 1a) [8,9]. Biofilms, microbial communities that adhere to surfaces, exhibit similar community resilience [10,11]. Biofilms are a dominant form of bacterial growth and impact human health by coating our bodies and medical devices, contributing to biofouling, virulence, and pathogenesis. In a model of ear infections, Perez *et al.* shows biofilms of *Moraxella catarrhalis* and *Streptococcus pneumoniae* are more robust to antibiotic exposure than axenic films (Figure 1b). This results from *M. catarrhalis* protecting *S. pneumoniae* from β-lactam antibiotics and *S. pneumoniae* protecting *M. catarrhalis* from macrolide drugs [11]. This steadfastness of microbial communities may be one fundamental reason that biofilms form [12].

Division of labor

Consortia have access to expanded functional and metabolic capacities allowing for division of labor across organisms [5,6]. This is clearly seen in *Chlorochromatium aggregatum*, a motile photosynthetic community in which symbionts live on the surface of their partner, a central β-proteobacterium, as epibionts (Figure 1c) [13]. These green sulfur bacterial epibionts hitch a ride on the β-proteobacterium for chemotaxis and scotophobotaxis (movement away from the dark) [13]. The non-photosyntheic β-proteobacterium gains access to energy from the epibionts [14•]. Liu *et al.* determined that the β-proteobacterium depends largely on the photosynthetic epibionts for energy through metabolic coupling and, potentially, electron transfer [15•]. The β-proteobacterium has a reduced genome in comparison to free-living relatives but gains photosensitive motility despite its inability to do photosynthesis indicating increased evolutionary fitness when with its epibionts [15•].

Figure 1



Emergent properties in natural consortia. **(a)** The isolated fungal (red) and algal (green) symbionts of lichen, like the *Cladonia* species pictured [89], are less robust to harsh conditions than the complete lichen (red fungi and green algae together) [8,9]. **(b)** Biofilms that can cause inner ear infections [90] made up of *M. catarrhalis* or *S. pneumonia* are sensitive (dashed lines) to β-lactam and macrolide antibiotics, respectively, but can survive (solid coloration) both antibiotics when in coculture [11]. **(c)** Division of labor is accomplished in *C. aggregatum* with green sulfur bacteria photosynthesizing while the β-proteobacterium provides light-sensitive motility [91,13–15]. **(d)** Bacterial symbionts have been shown to contribute multiple processes the lichen *Lobaria pulmonaria* [92,18••]. **(e)** Potential roles of different organisms in methanogenic communities were determined by Embree *et al*. demonstrating division of labor within the community [93,19].

The roles in microbial communities are not always well defined. 'Omics approaches enable the determination of organisms present in communities and help assign functions to those microbes [16,17]. Two recent examples include Grube *et al.*'s look at lichen and Embree *et al.*'s investigation of methanogenic communities (Figure 1d,e) [18••,19]. Both consortia are complex: they contain many species and can be hard to tease apart as many isolated

microbes are not readily culturable [20]. Grube and colleagues collected metagenomic and proteomic data for the lichen *Lobaria pulmonaria* and showed bacteria support lichen with increased nutrient access and resistance to external factors [18••]. Embree *et al.* used single-cell genomics and metatranscriptomics under different growth conditions to reveal the functions and interactions of *Smithella* species, *Methanosaeta consilii*, and other consortia members during methanogenic alkane degradation [19].

Not only are there advantages to dividing labor — there can be additive benefits from sharing labor with diverse partners. Ho and colleagues observed this in methanogenic communities composed of *Methylomonas methanica* and various heterotrophs seeing increased methane oxidation rates correlated with heterotrophic diversity [21•]. While this supports ecological theory and examples in higher organisms, experiments showing the importance of diversity have yielded varying results, highlighting the need for further study [22,23].

Engineering consortia: environment control and synthetic biology

Building a system in an effort to understand it is common throughout science [24]. While there are many approaches taken to engineer microbial communities, environmental control — control of physical space and chemical environment — is an important tool that engineers can leverage [25]. Building communities can also be accomplished from the bottom up with the tools of synthetic biology [24].

Environment control

Physical and chemical environments play critical roles in community function. Here we review several technologies that enable the exploration of various environmental parameters, such as the chemical concentration, timescale, volume and spatial distribution, and their effects on cocultures.

Microfluidics

Microfluidic devices provide the means to study microbial ecology and fabricate micron-scale environments with precisely tuned conditions (Figure 2a). Cocultures have been studied using microfluidics in several ways: generating gradients (Figure 2ai) [26–29], trapping cells to study interactions (Figure 2aii) [26,29], creating microhabitats to observe dynamics in particular relationships (e.g. predator–prey, resource-competition), constructing and dispersing biofilms [30], and high-throughput, droplet-based experiments (Figure 2aiii) [31]. While these devices have the advantage of being parallelizable [28,32], analysis is typically limited to optical measures of growth. Adapting microfluidic systems to other analytical techniques (e.g. flow cytometry, mass spectrometry, liquid chromatography) would enable high-throughput collection of richer data.

Membrane separation

Membranes used in coculture experiments can block the movement of cells while allowing small molecules (e.g. nutrients, gases, metabolites) to diffuse (Figure 2b). Membranes have been widely used to culture 'unculturable' microbial consortia *in situ* or in simulated soil and marine environments (Figure 2biii) [20,33–35]. Devices using this approach have isolated high-value microbes, such as methanothrophs from wetland and landfill environments [36] and, recently, over 10 000 soil microbes, one of which produces a novel antibiotic [37]. Clonal populations can be isolated from the environment by diluting environmental samples and inoculating membrane bound wells [35] or tubes [38], such that each chamber contains one cell. Many of these microbes may survive as individuals only because they can share molecules needed for growth between wells.

Membranes can also control species position within a coculture. *Kim et al.* used membranes to alter spatial distribution and research cell-to-cell communication influencing the success of syntrophic soil consortia (Figure 2bi). The distance between the three microbes was varied from 0 to 1.8 mm and cocultures stabilized only at intermediate distances (600–1200 μm) [39].

Spatial patterning

Spatial patterning (two and three-dimensional) has been used to explore how distribution affects microbial consortia (Figure 2c).

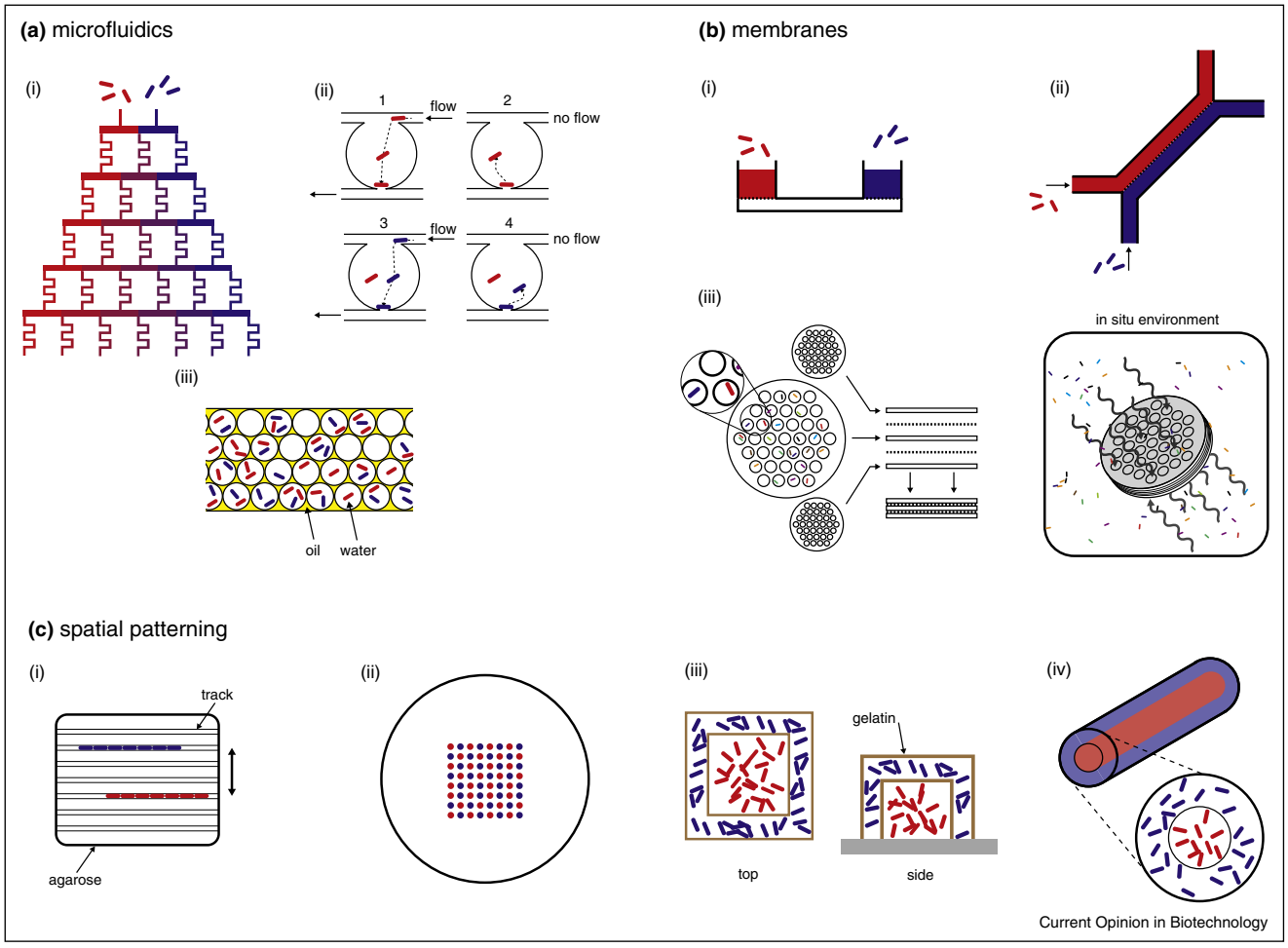
Molding: Moffitt *et al.* molded tracks to embed a single cell into solid media (Figure 2ci), allowing the authors to optically measure growth of microbial consortia for 30–40 generations and examine how the vertical spacing of *E. coli* auxotrophs in separate linear tracks alters growth [40].

Inkjet printing: Inkjet printers can pattern picoliter-scale droplets of microbes, mammalian cells, and relevant biomolecules at high resolution ($\leq$25 μm, Figure 2cii) [41]. Drachuk *et al.* recently demonstrated inkjet printing environmental biosensors. Two strains of *E. coli*, each modified to respond to an environmental chemical, were printed on arrayed silk ionmers, which improved their functionality for up to 3 months [42••].

3D printing: Multi-photon polymerization can be used to 3D print microscale geometries around bacteria (Figure 2ciii). Connell *et al.* used this method to create spatially separated communities of *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The bacteria demonstrated greater resistance to β-lactam antibiotics when separated by the 3D printed gelation walls than when living together in a mixed environment [43•].

Fiber structure: Kim *et al.* used a coaxial extruder to create calcium alginate fibers embedded with *Sphinogobium*

Figure 2



Engineering environment. **(a)** Microfluidics are used to create specific environments, miniaturize and parallelize coculture chambers. These systems include (i) cell density and chemical gradient generators [26,30], (ii) cell traps designed to capture single cells of two different species [26], (iii) and droplet generators that encapsulate single cells of multiple species [94]. **(b)** Membranes can separate species. They include (i) transwell membranes that spatially separate cells [39] and (ii) membranes in flow devices [32]. Membranes can be used to culture 'unculturable' species *in situ* by separating single cells from samples within a device that is then submerged [33,34,36]. **(c)** Techniques are used to pattern cells in 2 and 3D, including (i) molding single-cell wide tracks in agarose [40], (ii) inkjet printing cells [41], (iii) 3D printing microenvironments using multiphoton 3D printing of gelatin [43•], and (iv) creating coaxial calcium-alginate fibers with one species in the core and another in the exterior [44].

chlorophenolicum, a pentachlorophenol (PCP) degrader, in a core layer surrounded by *Ralstonia metallidurans*, a mercury ion reducer (Figure 2civ). In liquid coculture, *S. chlorophenolicum* suffers from mercury exposure, but in the structured fiber, *R. metallidurans* protects *S. chlorophenolicum*. The resulting coculture degrades both mercury and pentachlorophenol [44].

Synthetic biology

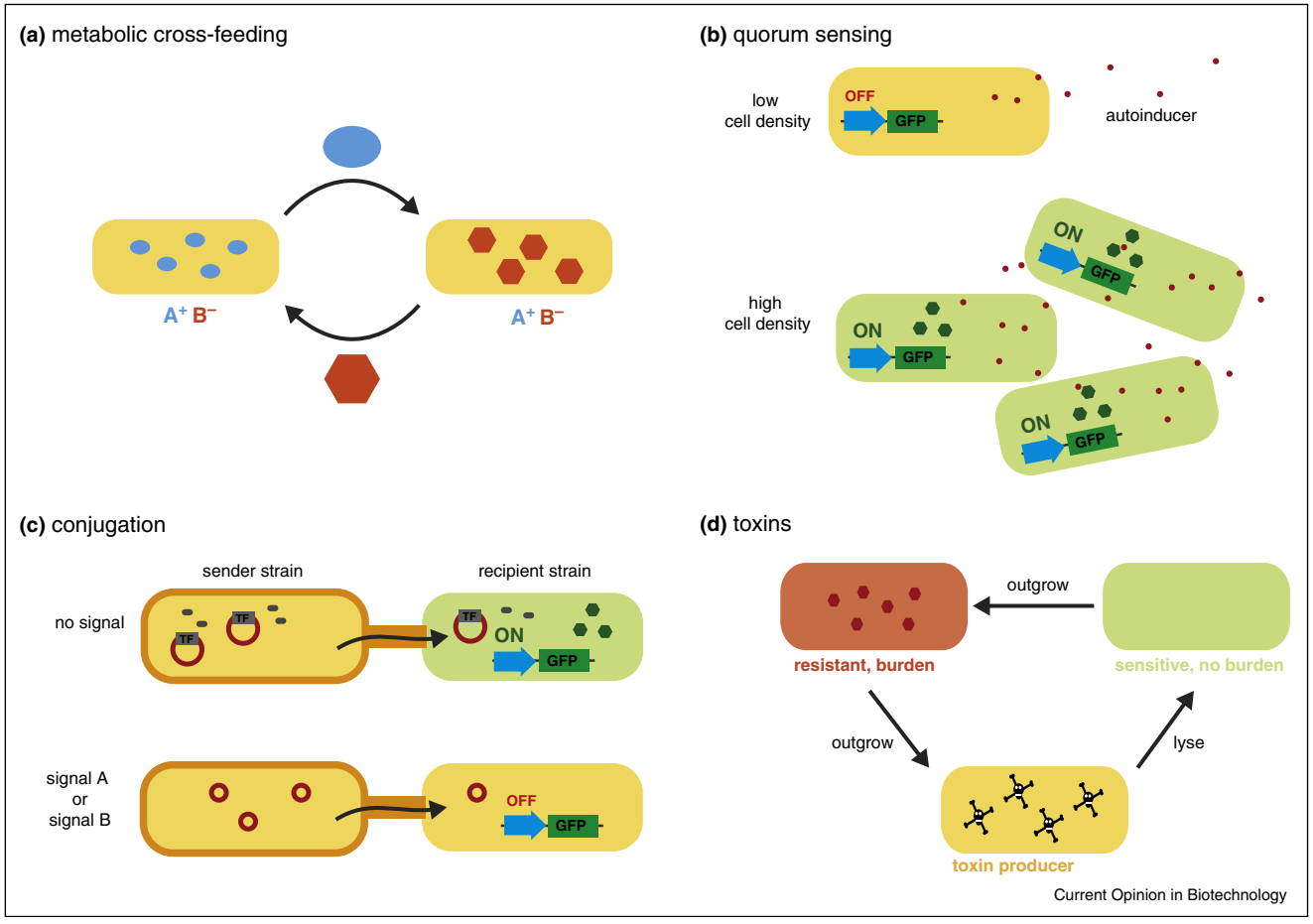
Synthetic biologists strive to rationally engineer new functions into biological systems [24]. While many synthetic transcriptional, translational, and post-translational components and devices have been built in single cells, engineering entire populations is a new frontier [5]. Here we review work engineering bacterial communication and the application of these devices to generate complex synthetic population behaviors.

Building communication

Interaction is key to building consortia. Recent approaches in engineering microbial communication have yielded sophisticated ways to control population density [45,46], gene expression within one [47–49] or multiple species [50] as well as biofilm formation [30,51].

Communication within cocultures can be controlled through metabolite crossfeeding (Figure 3a) [1,2,5,6]. Much work has been done using auxotrophs [52–54, 55•,56] and mixed species systems [57,58]. Wintermute *et al.* performed a comprehensive analysis of pairwise

Figure 3



Engineering microbial communication. **(a)** Metabolic crossfeeding. One strain of *E. coli* overproduces metabolite A (blue oval) but cannot produce metabolite B (red hexagon). The second strain cannot produce metabolite A but overproduces B. Neither strain can survive in isolation but they can survive as part of a community [49,50]. **(b)** Quorum sensing [1,2,5,6]. Cells produce a diffusible molecule (autoinducer, red dot) at a constant rate. At low cell density, the concentration of autoinducer remains low and the cell maintains an 'OFF' state. At high cell densities, local autoinducer concentration increases and a responsive promoter (blue arrow) is switched ON resulting in expression changes (green hexagon) [54]. **(c)** Conjugation. The sender strain contains a plasmid (red circle) encoding a transcription factor (TF, grey oval). Upon conjugation into the recipient TF induces GFP expression. In the presence of signal A or B TF is recombined out of the plasmid. Then when the plasmid is conjugated it no longer induces transcription [56]. **(d)** Toxins. The consortium consists of three strains: strain 1 (yellow) produces a toxin, strain 2 (red) is resistant to that toxin but overexpresses a certain molecule that constitutes a metabolic burden and strain 3 (green) is sensitive to the toxin but does not contain a burden [57]. This system is called a 'Rock-Paper-Scissors' scenario and can be used to study ecological phenomena.

E. coli auxotroph interactions identifying a subset of highly cooperative partners [54]. Mee *et al.* extended this idea by systematically testing interactions between auxotrophs in multi-member *E. coli* communities. Costly amino acids (Met, Lys, Ile, Arg and aromatics) promoted strong cooperative interactions and complex cocultures of 14 auxotrophs revealed a number of key dominant strains and syntrophic interactions [55•].

Quorum sensing (QS), a density-dependent form of communication, also serves as a means to program population interactions (Figure 3b) [1,2,5,6]. Youk and Lim showed that a simple secrete-and-sense QS motif could generate a wide range of 'social behaviors'. Secretion rate and receptor abundance were systematically altered while additional logic functions (e.g. feedback loops) were introduced. Single cell behaviors ranged from communication with neighbors to communication with ones self and intermediates thereof [59]. This is just one example of the flexibility of QS which can, subsequently, be engineered into more complex systems spanning different kingdoms [60].

Recently Goni-Moreno and colleagues engineered interactions via conjugation. Conjugation is a method of communication that can transfer more information, in the form of DNA, and be more specific than either metabolic crossfeeding or QS. Using conjugation, Goni-Moreno

et al. constructed a multicellular system of several strains acting analogously to computational networks for the biocomputation of logic functions (Figure 3c) [61]. They first built a consortium that fluoresces only in the absence of two signaling molecules, a computation known as a NOR function. This concept was then successfully expanded to a three-strain system [61].

Another way to engineer communication is to exploit toxin production. Weber and colleagues used toxin production to construct a synthetic microbial consortium to study species dispersal [73]. The model three-strain *E. coli* consortium consisted of: (i) a strain capable of toxic colicin production, (ii) a colicin-sensitive strain, and (iii) a resistant strain (Figure 3d). Strain growth rates were tuned via genetic engineering and the system was modeled computationally. Consortia exhibited robust proliferation with balanced growth rates when low numbers of toxin producers were included at inoculation or toxin range was short [62•]. A major challenge for ecological research is to understand how competing species survive in rapidly changing environments and here a synthetic consortium elucidated basic principles that may help to explain more complex ecosystems [62•,63,64].

Applying consortia

There are many complex tasks that consortia are well suited to address. In this review we emphasize applications in bioremediation and bioenergy. However, important work with microbial communities has also been done in wastewater treatment, nitrogen fixation in soil, and other fields.

In bioremediation, which repurposes or engineers organisms to clean up pollutants, division of labor is crucial because complex toxic compounds often require several steps for degradation and cultures must be robust to whatever compounds are present making microbial communities an ideal solution [65–71]. Petroleum can be degraded naturally by consortia that produce biosurfactants which make hydrocarbons accessible through emulsification [69]. Mnif *et al.* successfully mimicked this concept synthetically by pairing a three-microbe degradation consortium with surfactant producing strains of *Bacillus subtilis* and *Acinetobacter radioresistens*. Diesel biodegradation was more successful in cocultures with surfactant producers than in cultures of degraders alone (Figure 4a) [72].

Movement towards a greener economy will be aided by consortia, notably in the areas of renewable feedstocks, bioproduction, and biofuels. For example, consider the growth of oleaginous microalgae for the production of biofuels. Do Nascimento *et al.* showed that culturing the microalgae *Ankistrodesmus*, with *Rhizobium* increased growth as well as lipid content in cocultures in comparison to axenic cultures [73]. Similar benefits from communities have been seen in efforts to use cellulosic feedstocks for energy, which is a costly metabolically but is effectively achieved in consortia [74,75]. Cocultures have been engineered to take the system one step further by going from cellulose to product [76–78]. Minty and colleagues accomplished this by pairing a fungus that breaks down cellulose with engineered *E. coli* that make isobutanol (Figure 4b) [79••]. This is an excellent example of marrying natural and synthetic systems as the fungus, *Trichoderma reesei*, can degrade feedstocks without modification while the *E. coli* was engineered and optimized for isobutanol production [79••].

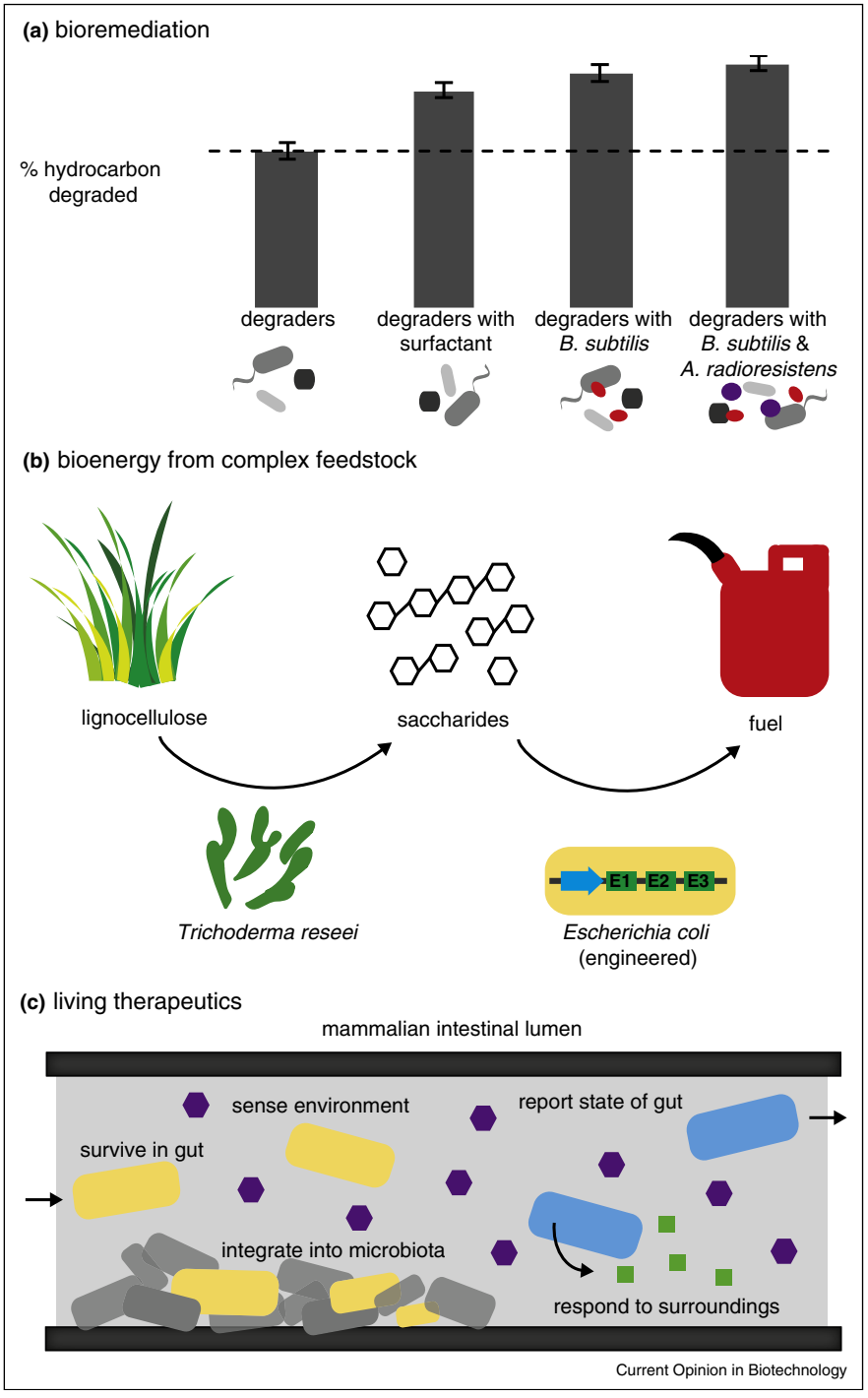
Microbial fuel cells (MFCs) are another bioenergy application for microbial consortia. These systems can treat wastewater and simultaneously generate energy. In MFCs, microbes degrade complex compounds in wastewater into substrates for 'exoelectrogens' [81]. These electrochemically active bacteria transfer electrons onto an anode via direct contact, secreted mediators or nanowires [81]. In order to gain better insight into interactions in MFC consortia, Bourdakos *et al.* constructed a synthetic consortium consisting of *E. coli* and *Geobacter sulfurreducens*. They found that *E. coli* scavenge oxygen that is toxic to *G. sulfurreducens* enabling MFC operation in aerobic conditions. However, production of succinate by *E. coli* reduced the efficiency of the fuel cell. It would be desirable to include a species that generates less succinate in this synthetic system [82••].

Conclusions

Most natural microbes exist in consortia that provide robustness and broad metabolic capacities and these traits are attractive for applications in energy, environment, and potentially healthcare. For example, microbial communities could be living therapeutics with health-promoting functions. Microbes have been engineered to sense and kill pathogens [83–85] however integration of engineered bacteria into the gut microbiota, a complex microbial community, is key [86•,87,88]. To this end, Kotula and colleagues engineered bacteria that survive in the gut to detect signals that generate memory assessable in fecal samples [86•]. Next steps for microbial consortia in healthcare could include combining *in vitro* sensing, killing, and nutrition augmentation systems with *in vivo* integration strategies to generate living therapeutics (Figure 4c) that can compete with natural microbiota, disperse pathogens, and foster healthy human microbiomes.

Advances in both environment engineering and synthetic biology have enabled engineering of 'simple' microbial consortia. The challenges faced constructing these rather basic consortia reveal the vast complexity of microbial interactions. We believe that to realize the full potential of consortia-based biotechnological applications 'omics scale data paired with computational models are needed

Figure 4



Applied microbial consortia. **(a)** Bioremediation of petroleum contaminants by degrader consortia (grey) show that the addition of surfactant makes hydrocarbons more available to degradation and cocultures with *B. subtilis* (red) and *A. radioresistans* (purple) producing biosurfactants enable better contaminant degradation than the degrader strains alone [72]. **(b)** Bioenergy from complex feedstocks. The fungi *Trichoderma reseei* (green) secretes cellulase which degrades weed lignocellulose into accessible saccharides (black hexagons). An engineered *Escherichia coli* strain (yellow) converts these sugars into fuel compounds [79••]. **(c)** Living therapeutics. An engineered bacteria (yellow) containing a trigger and a memory device were fed to mice and reported an event in the gut with a colorimetric output (blue) [86]. Moving forward to engineer living therapeutics, it is important to note that engineered cells (yellow) must (i) survive in the gut (ii) integrate into the complex gut microbiota (grey), (iii) sense their environment (purple hexagons), (iv) respond to the environment (e.g. changing gene expression (blue coloration), providing something helpful (green squares), killing pathogens), and (v) report the state of the gut after excretion [3,86].

to inform the selection of organisms and genetic parts and understand these systems. Much progress has been made in these fields and each is worthy of a devoted review. In future work, we hope the scientific community will engineer understandable, controllable consortia of higher complexity with increased robustness and longevity. For that goal further understanding of natural consortia and advances in both device and genetic engineering will be vital.

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- •• of special interest

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