Design, analysis and application of synthetic microbial consortia

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ABSTRACT

The rapid development of synthetic biology has conferred almost perfect modification on single cells, and provided methodological support for synthesizing microbial consortia, which have a much wider application potential than synthetic single cells. Co-cultivating multiple cell populations with rational strategies based on interacting relationships within natural microbial consortia provides theoretical as well as experimental support for the successful obtaining of synthetic microbial consortia, promoting it into extensive research on both industrial applications in plenty of areas and also better understanding of natural microbial consortia. According to their composition complexity, synthetic microbial consortia are summarized in three aspects in this review and are discussed in principles of design and construction, insights and methods for analysis, and applications in energy, healthcare, etc.

1. Introduction

With the rapid development of synthetic biology, designing and constructing synthetic microbial consortia has raised extensive attention, becoming one of the important frontiers for the second wave of synthetic biology,[1] but yet to be an important aspect of in-depth research.[2] As summarized by Ron Weiss and Cynthia Collins, there are three advantages of taking microbial consortia as the research object to engineer specific routes: (1) different strains are functionally divided to fulfill many complex tasks at the same time; (2) relationships between cells are dynamically balanced, leading to stronger adaptability and stability to the fluctuant environment; (3) elements and modules from different sources and with different functions can be built in different strains, reducing the metabolic load on single chassis as well as avoiding the cross-influence of different functions.[3,4]

There are mainly two ways for designing and constructing synthetic microbial consortia. The first one is to re-engineer naturally occurring microbial consortia, which is a top-down method.[5] That is, based on multiple omics analysis,[6–12] starting from the macroscopic microbial consortia, parsing the system principles, to explore the molecular mechanisms for the maintained systems. The other one is to design and construct artificial microbial consortia, which is a bottom-up method.[5] That is, based on the genetic elements, modules, circuits and metabolic pathways or networks,[13–16] with the rational guidance of engineering principles, to obtain microbial consortia with higher efficiency, stability and controllability. Considering the complexity and practicability of synthetic biology, currently the bottom-up method is the mostly used for constructing microbial consortia from simple to complicated. Moreover, about the synthetic systems, there are different statements on the concept: co-cultures,[17,18] mixed cultures,[19] microbial consortia,[4,20] and so on. Considering that the phrase "microbial consortia" indicates not only living together but also labor division, and covers all of conditions of their composition: by single, two, and multiple species,[2,6,21] we use "microbial consortia" in this review.

This review summarized current synthetic microbial consortia reported in literature from three aspects according to their composition complexity (composed of single species, two species or multiple species) and then discussed their design and construction strategies based on the interactions within microbial communities, their mechanism analysis methods, as well as their applications in many fields such as medicine and energy, etc.

2. Synthetic microbial consortia composed of single species

Research on synthetic microbial consortia composed of single species mainly focuses on pattern microbes, such as *Escherichia coli* and *Saccharomyces cerevisiae*, which have clear genetic backgrounds and mature molecular technologies. It mainly focuses on cell–cell communications and interaction analysis within the ecosystems.

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2.1. Design and construction of synthetic microbial consortia composed of single species

One of the basic cell–cell communications for constructing synthetic microbial consortia is quorum sensing (QS). The key of QS is some signaling molecules known as autoinducers, which diffuse from intracellular to extracellular. When reaching a certain threshold (usually in high cell concentration), they trigger or coordinate the expression of certain genes. Except communications within populations, one-way interactions between populations such as a pulse-generator system,[22] a pattern formation programming system,[23] and a sender–receiver communication network[24] are equally employed. Besides, there are two-way interactions. Brenner et al.[21] constructed a consortium with LasR/LasI and RhlR/RhlI QS systems in which gene-expression response if and only if both populations are present over a threshold cell densities (Fig. A1). These are all the basic interaction modes between microorganisms, revealing the molecular mechanisms, which provide the basis for the design of synthetic microbial consortia.

The most well studied form of QS is spatio-temporal, by which population density is coupled with some special module, with the help of a fluorescent protein, the consortia is periodically distributed.[25–29] For example,[30] the coupling of LasR/LasI as a density-sensing module, and coupling it with motility-control modules in *E. coli* could command population behavior: high cell density stopped the motility while low cell density drove the movement, according to the density difference, light and dark circular pattern were gradually formed (Fig. A2).

Recently, QS has been developed with more complicated population behavior and more novel control. Using the LasR/LasI and two dispersal proteins, a colonizer–disperser consortium is designed to control the biofilm breakdown, movement and formation.[31] Combining the LuxR/LuxI with a CcdA/CcdB toxin–antitoxin module, a typical Allee effect was constructed, which caused a tradeoff between population spread and survival.[32] Payne et al.[33] broke the traditional spatial cue for pattern formation which depended on morphogen gradient, using the morphogen served as a timing cue to trigger the formation and maintenance of the ring patterns.

Of course, QS is not the only principle for designing the communities. Based on nutrition complementarity, microbes can form symbiosis relationship, such as consortium composed of isoleucine auxotroph and leucine auxotroph, strains not only acquired what they lacked, but also over-supplied amino acids required for partner
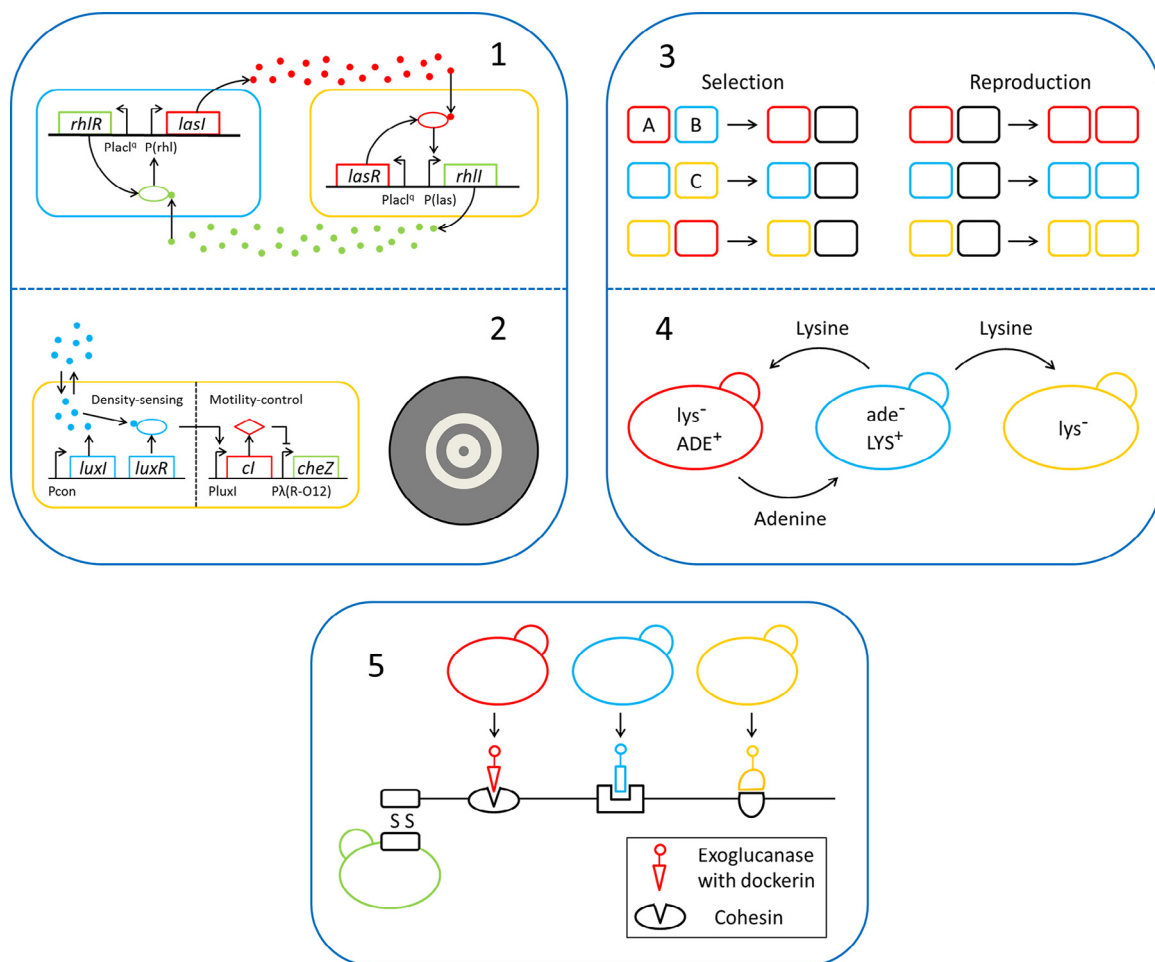

Fig. A. Design, analysis and application of synthetic microbial consortia composed of single species. (1) A bidirectional QS system. (2) A spatio-temporal system coupled QS and density-sensing module. (3) Local reactions of designed rock–paper–scissors relationship. (4) Interactions of a cooperator–cheater system. (5) A minicellulosome yeast consortium. Part 1 is adapted by permission from *Proceedings of the National Academy of Sciences*[21] ©. Part 2 is adapted by permission from *Science*[30] ©. Part 3 is adapted by permission from Macmillan Publishers Ltd: *Nature*[43] ©. Part 4 is adapted by permission from *Proceedings of the National Academy of Sciences*[45] ©. Part 5 is adapted by permission from *Microbial Cell Factories*[49] ©.

strains.[34] On the basis of a stable system, further regulating the expression of genes encoded with amino acids could tune the metabolite exchanges, which changed the growth rate and ratio of the two strains in a wide range.[35]

Due to the relatively low complexity of single-species microbial consortia, it is more suitable for further mechanisms study, with the design and construction principle getting more and more systematic. However, single-species microbial consortia are limited to *E. coli* and *S. cerevisiae*. Besides, the universality of existing principles in other species still needs to be examined.

2.2. Analysis of synthetic microbial consortia composed of single species

Built ecosystems, as the mimic of natural ecosystems, demonstrated diverse relationships, such as mutualism, commensalism, parasitism or predation, competition, amensalism, and neutralism,[5] promoting a better understanding of natural microbial consortia.

Balagadde et al.[36] engineered a new predator–prey ecosystem, which is different from canonical predator–prey system in two aspects: first, the prey was an "antidote" rather than a food source; second, there was competition between predator and prey for nutrients, thus extinction, coexistence and oscillatory dynamics could be achieved under different conditions. Hu et al.[37] constructed a symbiotic *E. coli* ecosystem, in which the two members had resistance to ampicillin and kanamycin (Kan), and they showed a variety of subtle relationships at the different concentration of antibiotics. For example, with ampicillin at 4 mg/mL and kanamycin at 1 mg/mL, they rely on each other's resistance, but Kan[R] with high copy number have stronger degradation to antibiotics, thus dominating in the consortia.

However, in nature, even if a variety of interactions coexist, not every kind of interaction can be understood. To clarify the mechanism of cross-feeding, which is one of the common principles in ecosystems, many efforts have been made. Shou et al.[38] constructed a synthetic obligatory cooperative system, finding that the consistently increased its ability to survive, but the mechanisms underlying the stable cooperation were unknown. To make it clear, Zhang et al.[39] engineered a leucine (L) and lysine (K) auxotrophic community, and set the initial inoculation ratio to 1:1. Whenever OD reached 0.2 or so, the consortia would be inoculated into fresh medium for evolution, resulting a faster growth rate and lower mortality and improving the stability greatly. That was attributed to a virtuous circle: when L(K) had a higher productivity than K(L), K(L) dominated the population, correspondingly released more leucine, and therefore increased the proportion of L(K). Further, Pande et al.[40] studied the long-lasting cross-feeding interactions of non-cooperating auxotrophic *E. coli* consortium, finding a speedup growth rate under competition condition. The reason was that the fitness cost of overproducing amino acids was less compared to being provided by their partner. Even though the ratio reached 1:100, the weak strain wouldn't die but gradually restored to 1:1. At the same time, a much more difficult work was done by Mee et al.,[41] they devised a series of synthetic syntrophic communities (up to 14 members) to probe the complex interactions, finding that the amino acid biosynthesis has been broadly optimized to reduce individual metabolic burden, thus costly amino acid and common intermediates along branching pathways promoted stronger cooperative interactions.

On one hand, construction mechanism of ecosystem is gradually parsing and its capacity to endure fluctuant environment is stronger and stronger; on the other hand, why the biodiversity and stability is maintained is worth exploring, too. Kerr et al.[42] synthesized a three-member *E. coli* ecosystem, in which there were resistant cells (R), colicinogenic cells (C) and sensitive cells (S). According to the design, S could displace R, R could displace C (in both cases the former has a growth-rate advantage) and C could displace S (C kills S). The rock–paper–scissors relationship maintained just under static and localized growth condition. Once mixed together, only R lived to the end. The phenomenon is interesting, but the reasons are not clear. Few years later, Reichenbach et al.[43] did a deeper research on it in combination with spatial dispersal of static populations. Three members were arranged on a spatial lattice (Fig. A3), and the local reactions comprised selection and reproduction processes: selection was cyclic rock–paper–scissors, yielding an empty site (black); reproduction only occurred on empty neighboring sites. The mobility of individuals was realized by exchange of positions or occupation of an empty site. The competition between migration and local interactions could either promote or jeopardize biodiversity according to the value of mobility. At low values, the temporal development was dominated by interactions among neighbors, resulting in the long-term maintenance of species diversity; in contrast, when the values were high, spatial homogeneity caused biodiversity to disappear.

Another typical system is cooperator–cheater. In a two-member ecosystem established by Gore et al.,[44] cooperator hydrolyzed sucrose into monosaccharides, while cheater took advantage of them and invaded cooperator. However, the cooperator could also invade cheater cells. No matter which had absolute advantage in the initial inoculum, they would finally converge toward a steady state. This is a relationship of both competition and cooperation: when competing, they consumed glucose rapidly, forcing cooperator to express sucrose invertase to produce glucose; but high concentration of glucose inhibited gene expression in turn, and cooperator fell into the competition with cheater again. Based on the study above, Waite et al.[45] engineered a three-member ecosystem: two mutualist yeast strains and a cheater strain. Mutualists provided a limiting nutrient required by each other; however, the cheater used the nutrient secreted by mutualist but provided nothing in exchange. In a nutrient-limited environment, according to the different adaptive race between cheaters and cooperators, either could possibly destroy the other rapidly. But the final beneficiary were cooperators, allowing them to purge cheaters stochastically (Fig. A4). So, even though the selective pressure moldered the genetic variability impair to the evolution of cooperation, it did not molder the genetic variability necessary for the mutualism to persist. The capacity of mutualist partners to hitchhike with each other will help to maintain diversity in microbial communities.[46]

2.3. Applications of synthetic microbial consortia composed of single species

Although the construction and analysis of synthetic microbial consortia are booming, little achievement is gained in application of single species. That is probably because the main purpose for designing single-species consortia is exploring the natural relationships, and for improving the production efficiency which is difficult for single strain, so harsh tasks such as degrading toxic pollutant are out of the capacity of single-species consortia. Moreover, model strains have limited scopes, and are difficult to adapt to the complex and dynamic environments due to their low biodiversity. In addition, single species have similar background, which may impair the labor division and further confine them to few applications.

Using xylose-selective and glucose-selective *E. coli* strains, Eiteman et al.[47] designed a simultaneous multi-sugar fermentation consortium that consumed substrate more quickly, saving about 15% time than the wild-type. Shin et al.[48] constructed a binary microbial consortium, in which two *E. coli* strains hydrolyzed xylan and produced ethanol orderly, converted xylan hemicellulose to ethanol with a yield about 55% of the theoretical value. Chen et al.[49,50] constructed a minicellulosome yeast consortium that three members secreted different dockerin-tagged cellulases and the other member secreted a trifunctional scaffolding protein to match these cellulases,

reaching a titer of 1.87 g/L (Fig. A5). Similarly, Tan et al.[51] used nearly the same system obtaining ethanol at 1.138 g/L. The difference in productivity may be due to the different cellulases utilized and the un-optimized ratio. Two years later, they[52] conducted tough work in enzyme–enzyme synergy, enzyme–proximity synergy, cellulose–enzyme–cell synergy, and the length of anchoring miniscaffolding, improving the titer to 1.412 g/L.

These studies devoted to the development of renewable lignocellulose with fast, effective and cost-effective methods. Though problems still exist in growth rate match, low production, long fermentation period, and so on, they are worth being popularized for a wider range of substrates utilization, such as arabinose, or even inhibitors frequently found in lignocellulosic hydrolysates, microbes combination, and more biorefinery products, such as *cis,cis*-muconic acid and 4-hydroxybenzoic acid.[53]

3. Synthetic microbial consortia composed of two species

Current synthetic microbial consortia composed of two species can be divided into four types: bacteria–bacteria, fungi–bacteria, archaea–bacteria and other microbes with bacteria. The design and construction principles are more diverse than single species, and the investigation focuses more on the material and cofactor communications between different strains. Although the mechanism has not been fully elucidated, the systems are extensively used.

3.1. Design and construction of synthetic microbial consortia composed of two species

When designing a two-species microbial consortium, the first to consider is how they coexist, and then the constructing principle to communicate across species, which probably results in more special mechanisms than single species, thus it is worth uncovering the signal molecules and their working mechanisms.

E. coli and *Bacillus megaterium* consortium was a QS system, but the difference was that the cell–cell communication relied on autoinducing peptides (AIP). To produce and secrete mature AIP, *E. coli* cells expressed the AgrD and AgrB proteins; to recognize AIP, *B. megaterium* cells expressed the two-component AgrCA. Then monomer AgrA activated gene expression and AgrC catalyzed AIP-dependent phosphorylation.[54]

For an auxotrophic consortium,[55] engineered *E. coli* need glutamine, while what nutrients *Dictyostelium discoideum* need is unknown. To make it clear, 16 amino acids and 5 vitamins were tested in monoculture, the results were further selected in co-culture, and finally lipoic acid was found to be the best one.

In addition to relationships relying on nutrition, detoxifying can also be used as the basis for communication. When cultivated alone, *E. coli* absorbed glucose to produce acetate as end-metabolite, which inhibited the downstream pathway. But, rationally engineering *Acinetobacter baylyi* which was acetate dependent, resulted in an increase in biomass and proteins of *E. coli*.[56]

Another kind of communication mechanism is electron transfer. For example, under anoxic conditions, lactate permease of sulfate-reducing species dehydrogenized lactate and produced H+, which was further reduced into H_2 with the help of electron carrier and hydrogenase, then hydrogenotrophic methanogens reacted with H_2 and CO_2 to produce CH_4.[57,58] Rotaru et al.[59] designed another methanogenesis consortium, in which *Geobacter metallireducens* and *Methanosaeta harundinacea* exchanged electrons not only by acetate molecule which is a typical way, but also directly via electrically conductive pili (Fig. B1).

Specially, physical connection nanotubes can also be the media for communication between species, but the two cells have to reside in proximity and they both need to have nanotubes.[60,61]

Double-species microbial consortia have more choices to combine various species together. But this particularity makes it less regular. Development of these principles can just be horizontal to accumulate more fresh ideas, rather than be longitudinal to form scientific systems.

3.2. Analysis of synthetic microbial consortia composed of two species

Different from single species, analysis methods for interactions between two species are mainly metabolic profiling, metabolomics, genomics, proteomics, and dynamics analysis. The reasons for the difference may be because that though single species are reengineered to play many roles in the ecosystem, their similarities in genetic background and metabolic pathways limit the analysis at the macroscopic level. Zhou et al.[62] investigated the metabolic cooperation mechanism of *B. megaterium* and *Ketogulonicigenium vulgare*, finding that *K. vulgare* converted peptides into amino acid for *B. megaterium*, and the latter provided erythrose, erythritol, and other metabolites for the former. At the same time, Ma et al.[63] integrated proteomic and metabolomics analysis of the consortium, revealing the fact that the unselfish *B. megaterium* facilitated *K. vulgare* in purine biosynthesis and energy production. The community dynamics were further investigated by Du et al.[12] illuminating that *B. megaterium* promoted *K. vulgare*'s TCA cycle, nucleotide and amino acids metabolisms. Whether the latter promoted or inhibited the former depended on if it provided amino acids or biosynthesized 2-keto-L-gulonic acid (2-KLG) (Fig. B2). To improve the production of 2-KLG, a further study focused on cofactor balance. Du et al.[64] found that overexpressing sorbose/sorbosone dehydrogenase together with the cofactor pyrroloquinoline quinone in the consortium could significantly enhance the production of 2-KLG.

3.3. Applications of synthetic microbial consortia composed of two species

Either construction and analysis or their application, *E. coli* and *S. cerevisiae* are far from the demand for two-species synthetic microbial consortia. More strains should be taken into account. However, the dependence of certain interactions on regulation of external environments just improved the specific indicators marginally. Reengineering these strains at the molecular level is also important.

In energy, biofuels are attracting more and more attentions. Usually, the substrates are lignocellulosics or its hydrolysate, and the products are ethanol, butanol and so on. At present, bioethanol research has obtained certain achievements in processing techniques, such as control of oxygen transfer,[17] batch cells recycling fermentation,[65] and development of new strain combination.[66,67] Among these approaches, one-pot method aided with cellulase-inducing substrate satisfied short time and high production at the same time. In studies on butanol, Minty et al.[68] reached a titer of 1.88 g/L isobutanol through rigorous calculation and supplemented by experimental verification, but the mathematic process was cumbersome, and ultimately, yield was not high. For its isomer butanol,[69] consolidated bioprocessing was used for simultaneous saccharification and fermentation of cellulose by complementary organisms under mesophilic conditions, resulting in a butanol concentration of 3.73 g/L. A higher titer 5.5 g/L was obtained from pretreated rice straw, and when exogenous cellulase was added into the consortium, up to 6.9 g/L butanol was produced.[70]

Another research direction toward energy is the microorganism electrogenesis, and the electron donors are various. Venkataraman et al.[71] engineered a bioelectrochemical systems from *Enterobacter aerogenes* and *Pseudomonas aeruginosa*, and the former provided 2,3-butanediol by hydrolyzing glucose, which improved the electrogenesis
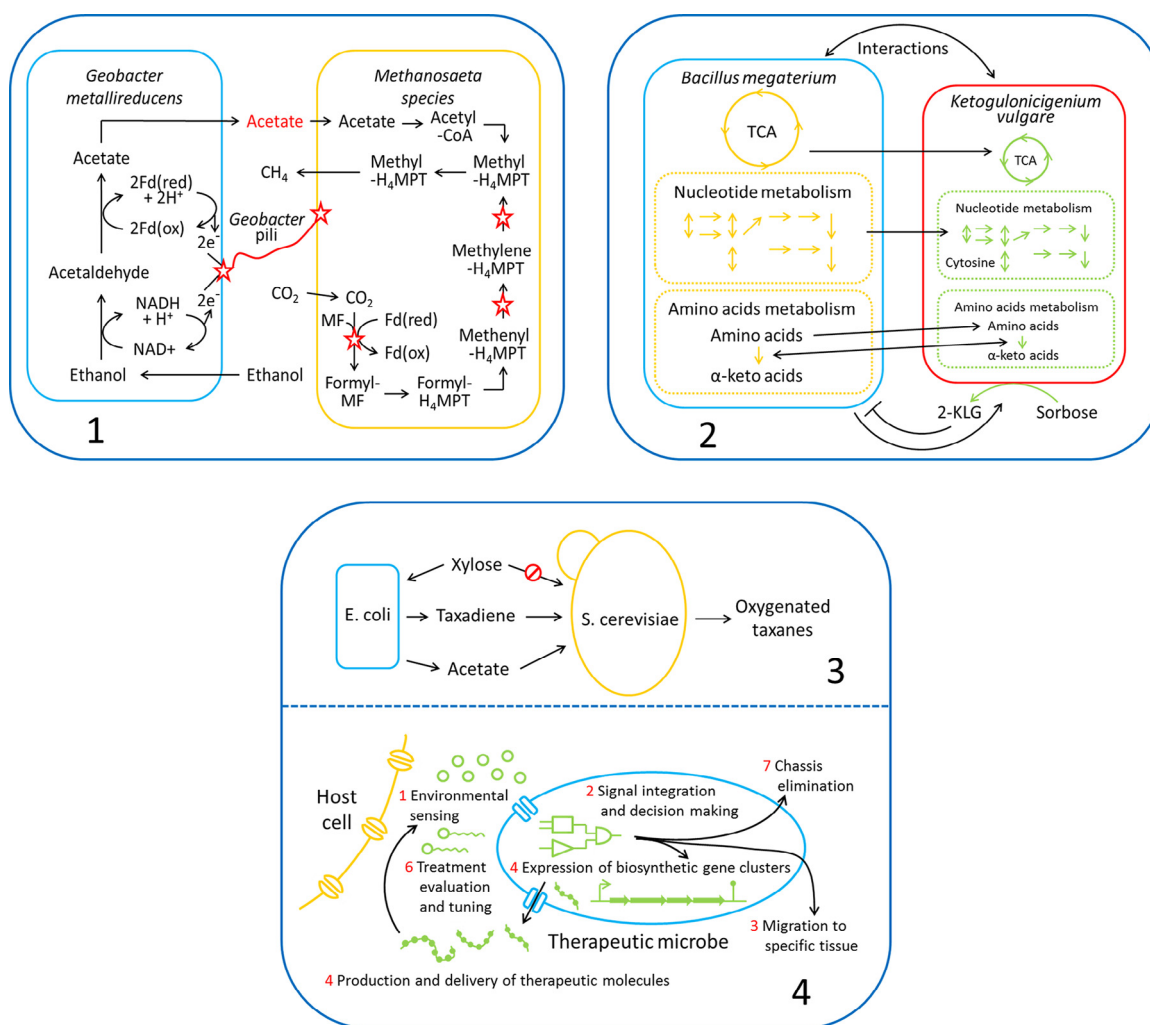
Fig. B. Design, analysis and application of synthetic microbial consortia composed of two species. (1) Electron transfer between *G. metallireducens* and *M. harundinacea*. (2) Metabolites exchange between *B. megaterium* and *K. vulgare*. (3) Distribute oxygenated taxanes metabolic pathway into *E. coli* and *S. cerevisiae* consortium. (4) Bacterial cell therapy systems in the future. Part 1 is adapted by permission from *Energy & Environmental Science*[59] ©. Part 2 is adapted by permission from *Metabolomics*[12] ©. Part 3 is adapted by permission from Macmillan Publishers Ltd: *Nature Biotechnology*[20] ©. Part 4 is adapted by permission from *ACS Synthetic Biology*[81] ©.

capacity by 2-fold compared with taking glucose as substrate directly. Rosenbaum et al.[18] compared the bioelectrochemical performance of *Shewanella oneidensis* in a pure-culture and in a co-culture with the homolactic acid fermenter *Lactococcus lactis*, enabling the system to convert glucose into current with a coulombic efficiency of ~17%, which was a very low efficiency. Similar microbial fuel cells consisted of *E. coli* and *S. oneidensis* were designed to figure out the process.[72] The former fermented glucose to metabolites such as formate as electron donor for the latter, and the latter secreted flavin mediator to facilitate the former respiration on electrodes. Most importantly, flavin mediator promoted electron movement to the external charge collecting electrode to derive energy in MFCs. Qu et al.[73] further improved the system. Nonexoelectrogen (*E. coli*) consumed oxygen to provide *Geobacter sulfurreducens* a hypoxic environment, and acetate was added for *G. sulfurreducens*, leading to a coulombic efficiency about 85%, but process for such a good result was not explored. To make up this shortage, analyses of metabolites were conducted to explore the process.[74] Results showed that succinate played a key role in current variation. High level succinate decreased current density while removal of succinate was responsible for the increased current.

In natural products production, Zhou et al.[20] distributed oxygenated taxanes and metabolic pathway into a *E. coli* and *S. cerevisiae*

consortium. The fast-growing *E. coli* contained a taxadiene-producing module, and the *S. cerevisiae* expressed cytochrome P450s because of its excellent protein expression machinery. Through avoiding substrate competition and optimizing the module components, the consortium produced up to 33 mg/L oxygenated taxanes (Fig. B3). In addition to artificial pathways, natural pathways can also be regulated in synthetic consortia. For example,[75] when co-cultivated, *Aspergillus niger* was rapidly colonized by *Streptomyces coelicolor*, which caused production of cyclic dipeptide cyclo(Phe-Phe) and 2-hydroxyphenylacetic acid in response to colonization. Further on, biotransformation studies with *o*-coumaric acid and caffeic acid resulted in the production of the novel compounds, which was analyzed by NMR-based metabolomics, proposing as an efficient approach to find new natural products.[76]

In healthcare, to kill the pathogenic *P. aeruginosa*, many consortia were synthesized with nearly the same mechanism:[77–79] the engineered *E. coli* recognized and migrated toward *P. aeruginosa*, secreted toxin, and finally destroyed them. Further, Volzing et al.[80] designed a consortium aiming at Gram-negative bacteria in which *L. lactis* produced and secreted heterologous antimicrobial peptides against pathogenic *E. coli* and *Salmonella*. In the future, drug delivery systems consisting of synthetic cells will be autonomous microbial "physicians" as envisioned by Fischbach et al.[81] Bacterial

cell therapy systems integrate the capacities to diagnose human disease, take appropriate treatment and bring it into effect, and self-eliminate when disease is alleviated (Fig. B4).

In the environment, most studies focused on harmful pollutants' degradation. During the process of wastewater management with consortia, purification is not the only purpose, and turning waste into wealth is much more meaningful. Products from wastewater are various, such as lipid,[82–84] hydrogen,[84] electricity,[74,85] etc. On the other hand, toxic compounds degradation is also an attractive field. Through proteomic analysis, Fazzini et al.[19] compared pure cultures with a co-culture of *Pseudomonas reinekei* and *Achromobacter xylosoxidans* under simultaneous chemical and oxidative stress, and the latter showed enhanced biodegradation of 4-chlorosalicylate. Chen et al.[86] mixed *Pseudomonas* sp. XM-01 and *Acinetobacter* sp. XM-02 to degrade alkane hydrocarbon and crude oil, showing a higher capability than the pure culture.

As for high-profile new energy development, researches on it emerge endlessly. However, the feasibility of industrialization amplification is still to be improved. Especially the strains have to satisfy with the time-, cost- and labor-intensive challenges.[87] Drug production, human health and environmental management are confined to laboratory scale, while in practice, there are many problems to be considered: whether the complex metabolites of pharmaceutical producing microbes in the process of drug extraction have residues; whether the toxins are harmful to human body during pathogenic microorganism killing; whether new contaminants are introduced while treating the poisonous waste with microbial consortia *in situ*, etc. In these aspects, if we can draw lessons from the concept of green chemistry, new research fields may be developed in the future.

4. Synthetic microbial consortia composed of multiple species

Fewer studies are carried out on synthetic microbial consortia composed of multiple species due to their complicated interactions. Therefore, taking natural microbial ecosystems as blueprints,[6,10,88] starting from their ecological structures, then studying the interactions among the members, enhancing stability and adaptability of synthetic systems, are keys to open the door of multiple-species synthetic microbial consortia.

4.1. Construction and analysis of synthetic microbial consortia composed of multiple species

Bacteria, fungi and mammalian cells can coexist in a programmed condition. *S. cerevisiae* (sender) metabolizes glucose to volatile acetaldehyde, which triggers the expression of sBLA in human embryonic kidney cells (receiver); the production of sBLA decreases ampicillin levels and promotes *E. coli* proliferation. Though *E. coli* competes nutrients and finally impairs mammalian cells, the consortium doesn't last long; the new communication mechanism across kingdoms provides a new insight into constructing synthetic ecosystems.[88]

Kim et al.[89] created a new cultivation method by using a microfluidic device. Three wild-type soil bacteria are separated in different culture wells, while their metabolites can communicate through the channel of polycarbonate membrane (Fig. C1). In a nutrient-poor medium, there are cellulose/N_2/penicillin/as carbon source/nitrogen source/antibiotic pressures, which are utilized by *Paenibacillus curdlanolyticus*/*Azotobacter vinelandii*/*B. licheniformis*. The viability and biodiversity can maintain very well at intermediate separation distances compared with far away or well-mixed conditions. These findings provide new perspective for multispecies communities that require microscale spatial structure for stability, and the approach for controlling spatial structure may enable harnessing synthetic multispecies communities in the laboratory.

Another method for studying microbial consortia is DNA microarray technology. Scholten et al.[90] designed a four-member anaerobic community; lactate and propionate are the growth-limiting substrates, and can be oxidized to H_2 by two syntrophic strains, and then H_2 is converted into methane by two methanogens. Through 24-mer probes, the metabolic response of different strains to a 1 h exposure to air is detected. *Syntrophobacter fumaroxidans* up-regulates several genes involved in reactive oxygen species detoxification; *Methanospirillum hungatei* up-regulates heat shock protein to grasp ATP protecting cells against oxidative toxicity, while the other two seem to be less sensitive to the oxidative stress. With new analytical method, this work indicates that microarrays could provide novel viewpoint on the metabolism within microbial consortia.
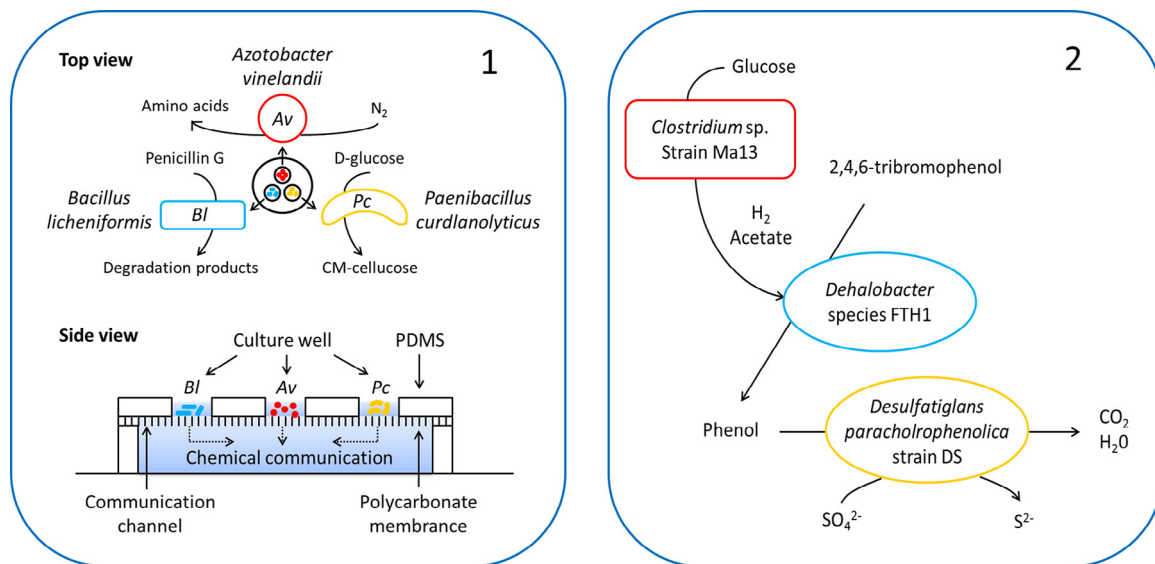

Fig. C. Design, analysis and application of synthetic microbial consortia composed of multiple species. (1) Relationships of *P. curdlanolyticus*/*A. vinelandii*/*B. licheniformi* and microfluidic device for their cultivation. (2) Relationships of the 2,4,6-tribromophenol degradation system. Part 1 is adapted by permission from *Proceedings of the National Academy of Sciences*[89] ©. Part 2 is adapted by permission from *Bioresource Technology*[91] ©.

4.2. Applications of synthetic microbial consortia composed of multiple species

Recently, Li et al.[91] developed an anaerobic mineralized microbial consortia, in which *Clostridium* fermented glucose to supply hydrogen and acetate as reducing agent; then *Dehalobacter* reduced 2,4,6-tribromophenol to phenol; finally *Desulfatiglans* mineralized phenol into CO_2 (Fig. C2). Another application study was property characterization of biosurfactants rhamnolipids.[92] In fact, all of the three members had the capacity to produce rhamnolipids, and their mixture didn't show an advantage over single strain or combination of any two strains in production. That may be related to the quorum sensing control mechanism among them; however, the authors didn't concentrate on this. But the product of mixture had much more complex structure, which resulted in better emulsification ability, surface activity, phenanthrene solubilization in a comprehensive angle. Brethauer et al.[93] engineered a consolidated bioprocessing from lignocellulose to ethanol in a batch membrane reactor, in which the process was similar to the one-pot method. Each member has a clear division of labor: *Trichoderma reesei* secreted cellulolytic enzyme; *Scheffersomyces stipitis* metabolized xylose; and *S. cerevisiae* produce ethanol with a 67% yield. The system makes a breakthrough of higher conversions and yields in shorter runtimes compared two-species consortia.[17,66] The most important is that the consortia are proven to be industrially robust, and thus have a good prospect of industrialization.

5. Conclusions and perspectives

Synthetic microbial consortia are getting more and more attentions with the rapid progress of synthetic biology. Academic authorities including Ron Weiss, Frances Arnold, Pam Silver are all working on the system design and construction, robust research, and dynamic stability analysis, etc. As one of the important aspects in synthetic microbial consortia, signals delivery and metabolites exchange are the basic principles and guidelines, which are reflected in QS[21,30] and cross-feeding[39–41] systems. Ecosystems not only contain interaction among strains but also between microbial consortia and external environment, and are wildly used in natural relations simulation.[36,37] Evolution is important as well, and is mainly a supporting method in studies such as biodiversity and stability.[42–45] In this review, current synthetic microbial consortia are divided into three types according to their composition complexity, which are single species, two species or multiple species. Due to their relatively simple relationships, synthetic microbial consortia composed of single species have relatively more thorough understanding about the mechanisms. There are much more principles proposed to describe two species consortia, due to there much more complicated interactions. As for multiple species, breakthroughs have not been made yet; the supplementary metabolites or other signaling chemicals provided by other microbes in the communities are still mysterious.[94]

Hence there is a phenomenon that with the increase of species, principles for design and analysis of microbial consortia are getting more special and novel, but the mechanisms are getting less understood, and the strains are more inclined to wild type rather than genetically engineered in application. Several challenges need to be overcome for uncovering the veil of multispecies consortia, the foremost of these is to optimize media compositions that satisfy all the multiple species, which influence population dynamics and further metabolism greatly.[5] Moreover, we need to take advantage of design and computational tools that take biological variability, uncertainty and evolution into account to provide rational guidance.[1] In addition, more novel approaches should be introduced into this field such as micro bead encapsulation,[95] ^{13}C-metabolic flux analysis,[96] synthetic biofilm for sequential layering of microbes.[97] Further, multispecies consortia finally put into application need to strengthen the robustness, especially in environment management or lignocellulose degradation in which survival conditions are tough for microbes, that is probably the reason why few genetically engineered strains are employed. One good solution to this may be to combine modified strains with natural microbial consortia derived strains together and evolve them to obtain a viability-improved synthetic microbial consortia.[98]

Therefore, a series of problems need to be addressed urgently, such as: how to expand research objects, jump out of the confine of model microbes; how to build functional pathways in the existing non-model systems with valuable applications; how to deeply illuminate ecological structures, interaction patterns, fluctuating environmental and evolutionary stresses[94] of multiple species, in order to develop more powerful systems and broaden the application scope and depth? Correspondingly, the research methods and train of thoughts can't be limited into synthetic biology, which should also be combined with a systems biology approach. We envision that with the rational guidance of engineering principles, the advantages of synthetic microbial consortia over single strains enable multiple species make breakthrough in design and application of genetically engineered consortia in the future.

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