

Assembly of functional microbial ecosystems: from molecular circuits to communities

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Abstract

Microbes compete and cooperate with each other via a variety of chemicals and circuits. Recently, to decipher, simulate, or reconstruct microbial communities, many researches have been engaged in engineering microbiomes with bottom-up synthetic biology approaches for diverse applications. However, they have been separately focused on individual perspectives including genetic circuits, communications tools, microbiome engineering, or promising applications. The strategies for coordinating microbial ecosystems based on different regulation circuits have not been systematically summarized, which calls for a more comprehensive framework for the assembly of microbial communities. In this review, we summarize diverse cross-talk and orthogonal regulation modules for *de novo* bottom-up assembling functional microbial ecosystems, thus promoting further consortia-based applications. First, we review the cross-talk communication-based regulations among various microbial communities from intra-species and inter-species aspects. Then, orthogonal regulations are summarized at metabolites, transcription, translation, and post-translation levels, respectively. Furthermore, to give more details for better design and optimize various microbial ecosystems, we propose a more comprehensive design-build-test-learn procedure including function specification, chassis selection, interaction design, system build, performance test, modeling analysis, and global optimization. Finally, current challenges and opportunities are discussed for the further development and application of microbial ecosystems.

Keywords: microbiome engineering; signal crosstalk; orthogonal regulations; cell-cell communication; synthetic microbial consortia; synthetic ecology

Highlights:

- Cross-talk circuits should be considered as valuable tools in synthetic biology.
- Optimization of circuits and communities should be conducted simultaneously.
- Propose a comprehensive procedure to enhance the existing DBTL cycle.
- Provide perspectives for developing promising consortia-based applications.

Introduction

Microbes always exist as communities (Sepich-Poore et al. 2021), where the use of diverse chemicals (Chen et al. 2018) and genetic devices (Kenny and Balskus 2018) is in competition among vari-

ous cells. In recent decades, to decipher (Wu et al. 2020), simulate (Shetty et al. 2022), or reconstruct (Cheng et al. 2022) natural microbial communities, numerous researchers have been engaged in engineering microbiomes with the help of artificial intelligence, multi-omics techniques (Wu et al. 2022a), and synthetic biology approaches (Kumar et al. 2022). Traditionally, due to the excessive metabolic burden, the unbalanced metabolic flux distribution limits the cell growth and productivity of a single-cultured microbe (Zhou et al. 2015). With a better understanding of microbial interactions, ecology, and evolution, more researchers are focusing on engineering different microbiomes. Although some consortia-based strategies, such as the cross-feeding control, can ease the key bottleneck of metabolic labor to a certain extent (Holtz and Keasling 2010), the imbalance of the resource allocation within the cell calls for new solutions (Hartline et al. 2021). Due to the dynamic changes of biological systems and the complexity of microbial communities, it is necessary to design corresponding control strategies to regulate, coordinate, and stabilize

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microbiomes for different applications (Li et al. 2022), such as easing the metabolic division of labor (Wang et al. 2022a), increasing genetic stability (Liao et al. 2019), resisting environmental disturbances (Xiao et al. 2020), and increasing the efficiency of bioprocesses (Shahab et al. 2020). The regulations for various microbial consortia can be broadly divided into cross-talk and orthogonal strategies (Wu et al. 2022b).

Cross-talk regulations exist widely in natural microbial ecosystems, such as cell-cell communications among human gut ecosystems (Moura-Alves et al. 2019). Gut microbes participate in a wide range of cross-talk communications through different signaling molecules and hormones, and the analysis of relevant pathways may serve as potential therapeutic targets (Li et al. 2019, Toda et al. 2019). For example, natural quorum sensing (QS) crosstalk, which includes signal crosstalk, promoter crosstalk, and combined crosstalk (Scott and Hasty 2016), is common among the communications of diverse gut microbes (Wu et al. 2022a). It is reported that QS language crosstalk is potentially helpful for the stability of a microbial ecosystem by using synthetic quorum-regulated lysis (Scott et al. 2017). Previously, our research group also found that QS crosstalk would benefit the improvement of isopropanol production in QS-based cocultivations (Wu et al. 2022b).

Orthogonal regulations are often utilized in developing multiple intercellular communication channels for reprogramming cells with minimizing signal interference on information dissemination, increasing the fidelity of signal transmission, and ultimately improving programming efficiency (Kylilis et al. 2018). Note that the predictable expression of synthetic gene circuits should reduce the internal and external noise as much as possible to achieve the stability and controllability of the system. Furthermore, more and more designs of the configurations are being proposed for engineering microbiomes by using different orthogonal communication channels to obtain more predictive and precise population dynamics (Lopatkin and Collins 2020). For example, Miano et al. have developed an inducible QS system mediated by p-coumaric acid, which expands the range of population dynamics, achieving the control of cargo release and population death (Miano et al. 2020). Therefore, many researchers have been engaged in constructing diverse orthogonal circuits or engineering microbiomes to perform expected cell behaviors, such as coaggregation bridging pattern formation (Glass and Riedel-Kruse 2018), population synchronization, and metabolic flux control (Wu et al. 2021c), etc.

Certainly, the increasing shifting of mono-culture synthetic biology to consortia-based synthetic ecology is leading to an intense effort into the development of a systematic framework to guide the design and synthesis of different genetic circuits and complex ecosystems simultaneously. While some typical reviews have been published, they have been separately focused on genetic circuit design (Hirschi et al. 2022, Yu et al. 2023), communication tools (Stallforth et al. 2023), microbiome engineering (van Leeuwen et al. 2023, Li et al. 2023b), or summaries for various applications (Tan et al. 2021, Jiang et al. 2023b). Furthermore, existing studies have often focused on developing various orthogonal strategies to construct circuits, while the quantitation or summary for cross-talk strategies is commonly neglected or ignored. Importantly, crosstalk is widely present in biosystems and plays an important role in the stability of the microbiome, which indicates that cross-talk regulations are indispensable for the assembly of microbial ecosystems.

This review aims to summarize diverse cross-talk and orthogonal regulation circuits for the *de novo* bottom-up assembling of

functional microbial ecosystems from molecular circuits to communities, thus promoting further consortia-based applications. First, we introduce the development of cross-talk and orthogonal regulation circuits in the advancing of synthetic biology toolboxes, respectively. Then cross-talk regulations are summarized at intra- and inter-species levels. Orthogonal regulations are reviewed from four aspects, i.e. metabolites, transcription, translation, and post-translation, respectively. Furthermore, considering the combinations of cross-talk and orthogonal key elements, we propose a more comprehensive design-build-test-learn (DBTL) cycle to provide more details for the *de novo* synthesis and optimization of various microbial ecosystems. Finally, we discuss current challenges and opportunities for the further development of microbial ecosystems.

Cross-talk regulation circuits

Microbes have adaptively evolved complex and highly interactive network configurations to cope with the complex and changeable environment (Faust and Raes 2012). Particularly, there are diverse cross-talk regulation circuits in the signaling processes mediated by different components, such as metabolites, genetic operons, and receptors (Zheng et al. 2022). In this section, because of space limitations, we mainly summarize intra- and inter-species cross-talk circuits based on different QS systems. The crosstalk mediated by other substances and mechanisms will be briefly discussed and we refer the reader to some other more comprehensive reviews in “Other crosstalk”.

Intra-species QS crosstalk

There are many binding and unintended binding events in the regulation of cell physiological activities due to the multitude of genes and regulators, which may lead to “promiscuous” crosstalk in the QS or some other communications. There are some typical QS autoinducers, such as acyl-homoserine lactones (AHLs), autoinducer 2 (AI-2), auto-inducing peptides (AIPs), and indole (Wang et al. 2020), etc. QS crosstalk includes diverse combinations of signals, receptors, and promoters. The “promiscuous” crosstalk could also be simply divided into three types, i.e. multiple signals going through one pathway, one signal going through multiple pathways, and a mixture of both.

As for the multiple signals going through one pathway, researchers have found some QS autoinducers analogs that inhibit the functions of the original QS signals, thus reducing the corresponding virulence and drug resistance without affecting the cell growths of pathogens (Ni et al. 2009). Specifically, with the help of high-throughput cell-free screening, Christensen et al. identified several AHLs synthase inhibitors that could serve as potential therapeutics for virulence and microbial infections (Christensen et al. 2013). In order to better use QS receptor-based crosstalk, we have developed a novel pipeline with similarity assessment and molecular docking. We constructed a QS interference database for different QS receptors termed as QSIDb (<http://www.qsidb.lbcj.net/>), which includes 633 reported and 73 073 expanded Quorum sensing interference molecules (QSIMs) (Wu et al. 2021a).

For one signal going through multiple pathways, there are also complex QS networks including different QS signal transduction pathways. For example, *Pseudomonas aeruginosa* has three interconnected QS systems, namely, LasR/I, RhlR/I, and PqsR/ABCDH (Fig. 1a). The biofilm formation of *P. aeruginosa* is cross-regulated by the above three QS systems. Note that even if some receptors are mutated, the highly interconnected multi-layered regulatory

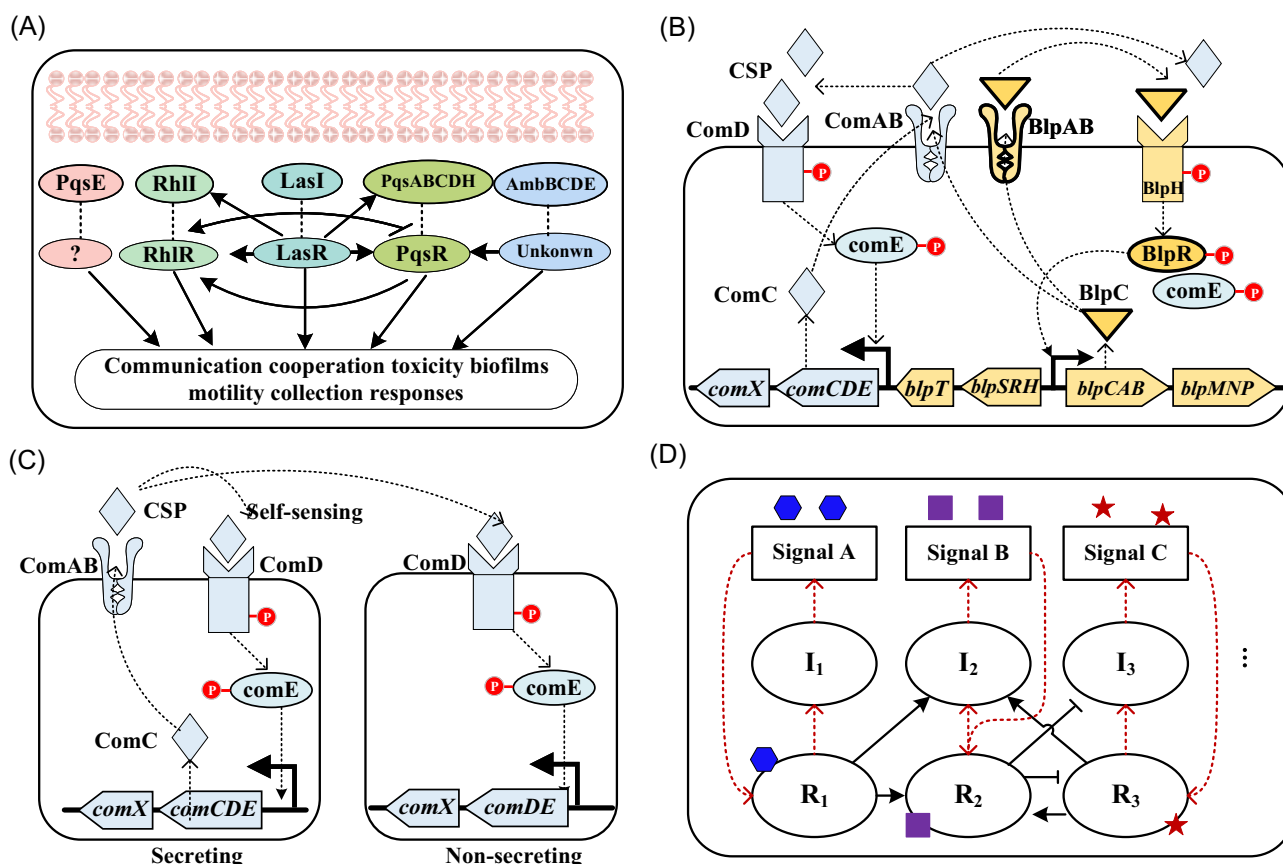


Figure 1. Illustration for different intra-species QS crosstalk. (A) QS interaction network in *P. aeruginosa*. Different signals from the *las*, *rhl*, *pqs*, and *amb* QS systems are recognized by the corresponding cytoplasmic transcription factors. (B) Diagram of QS crosstalk between ComQXP and Rap-Phr QS systems. Competence-stimulating peptide (CSP) binds to the histidine kinase receptor ComD, thereby phosphorylating the response regulator ComE, which increases transcription of *blpC* and the *blp* operon as well as the *com* QS systems. (C) CS-based quorum- and self-sensing crosstalk between secreting and non-secreting strains. (D) QS regulation network diagram composed of various QS synthases (denoted by “I”), signals and receptors (denoted by “R”).

pattern can generate alternative mechanisms to maintain the fitness of *P. aeruginosa* (Lee and Zhang 2015). The cross-talk activation or inhibition of the three aforementioned QS systems is beneficial to the colonization resistance of *P. aeruginosa* (Welsh et al. 2015). Bacteriocin production of *Streptococcus pneumoniae* is also regulated by *blp* and *com* QS systems. Miller et al. demonstrated that polymorphic QS leads to mismatches between AIPs and six different *blp* QS receptors, resulting in QS crosstalk, which is further demonstrated by *in vitro* experiments (Miller et al. 2018). Targeting characteristics of the combination of ComQXP and Rap-Phr QS systems (Fig. 1b) in *Bacillus subtilis*, Bareia et al. found that the autoinducer-secreting had a stronger QS response than the non-secreting cells (Fig. 1c) (Bareia et al. 2018).

Furthermore, “promiscuous” crosstalk includes different signals and receptors and is perhaps the most common phenomenon in microbial communications. Specifically, taking *lux*, *tra*, *las*, and *rpa* QS systems as examples, researchers have systematically investigated the dose-response characteristics of different QS circuits. The crosstalk of six common QS systems (*lux*, *las*, *tra*, *rpa*, *rhl*, and *cin*) was systematically investigated, and a software tool was developed for their quantitative analysis and design (Kylilis et al. 2018). Inducing by seven exogenously added AHLs, the activities of the corresponding QS receptors (RhlR, LuxR, LasR, BtaR1, QscR, CviR, BtaR2) have been measured in their native organisms and heterologous expression in *Escherichia coli* (Wellington and Greenberg 2019). Results showed that most of the QS receptors were re-

sponsive to at least one non-self AHL. Tekel et al. also conducted dose-dependent experiments to test the responses of LuxR, LasR, TraR, BjaR, and AubR receptors to a series of AHLs. The final results showed that most of them have a certain degree of crosstalk.

To sum up, the corresponding QS responses are determined by different QS crosstalk, which is controlled by complex QS networks that include different signal-receptor combinations (Fig. 1d).

Inter-species QS crosstalk

Microbial communities adapt to different environmental changes through different QS languages (Blackwell and Fuqua 2011). The inter-species QS crosstalk based on different signaling molecules mostly belongs to the signal crosstalk that is relevant to different phenotypes. In this section, we will provide a summary of inter-species crosstalk based on some typical QS signals, such as AHLs, AIPs, AI-2, indole, and diffusion signal factors (DSFs).

AHLs-mediated inter-species crosstalk

AHLs vary in their sensitivity to receptor activation and inhibition not only for crosstalk between QS systems within a single organism, but also for inter-species crosstalk, thus leading to the intricate AHL-based communications in natural communities (Decho et al. 2011). Specifically, Dulla and Lindow found that 3-oxohexanoyl-homoserine lactone (3OC6) from other species would induce the QS crosstalk for relieving the virulence of *Pseu-*

domonas syringae pv. *syringae* (Pss), thus leading to fewer lesions for leaves (Dulla and Lindow 2009). Hosni et al. also identified that AHLs produced by two other bacteria (*Pantoea agglomerans* and *Erwinia toletana*) would activate the inter-species QS crosstalk that aggravated the knot disease caused by *P. savastanoi* pv. *savastanoi* (Psv) (Hosni et al. 2011). Similarly, Chandler et al. demonstrated that AHLs-based eavesdropping guided the inter-species competition between *Burkholderia thailandensis* and *Chromobacterium violaceum* (Chandler et al. 2012). Furthermore, AHL-based eavesdropping through promiscuous receptors can also be applied to mediate various inter-species sense-kill systems for the removal of pathogens. For instance, as illustrated in Fig. 2a, the LasI/LasR-type QS system was often used to construct the sense-kill circuits for the removal of *P. aeruginosa*. The engineered *E. coli* was modified to specifically sense QS signal molecules of *P. aeruginosa*. When QS signal molecules reached a certain density, they would induce the activation of the lysis protein to release pyosin S5, which effectively inhibited the density of *P. aeruginosa* (Hwang et al. 2017).

AIPs-mediated inter-species crosstalk

The AIP-mediated crosstalk regulation of the *agr* QS system is also of great significance in an inter-species niche competition and the expression of density-dependent virulence factors. AIPs can specifically activate cognate receptors and compete for binding to non-homologous receptors, which is promising for the development of probiotic therapies (Piewngam and Otto 2020). Specifically, Borrero et al. constructed a heterologous monitoring circuit in the probiotic *Lactococcus lactis* by sensing the concentration of the pheromone cCF10, a typical AIP, for inhibiting the cell growth of the *Enterococcus faecalis*, which is responsible for enterococcal infections (Borrero et al. 2015). Piewngam et al. explored the mechanism of the probiotic *Bacillus* sp. inhibiting the *agr* QS system of *Staphylococcus aureus* (Piewngam et al. 2018). They found that the fengycin and AIP from *Bacillus* have similar chemical structures, so they competed for binding to the extracellular domain of AgrC, thus leading to the fengycin-mediated inhibition of QS-related virulence and colonization (Fig. 2b). Piewngam et al. utilized the spores of the probiotic *Bacillus subtilis* to inhibit the activity of fecal streptococci regulator (*fsr*) in *E. faecalis*. Note that the *fsr* QS system can be used by *E. faecalis* to produce protease GelE that disrupts the integrity of the intestinal epithelium, thereby blocking its translocation from the gut to the bloodstream and subsequent systemic infection (Piewngam et al. 2021).

AI-2-mediated inter-species crosstalk

AI-2, a common inter-species microbial language, can mediate various microbial communications (Galloway et al. 2011, Meng et al. 2022), such as the interaction between *E. coli* and *Vibrio harveyi* (Xavier and Bassler 2005). Hsiao et al. discovered the AI-2-based crosstalk between *Ruminococcus obeum* and *Vibrio cholera*, and the pathogenicity of the latter would be reduced by the former (Hsiao et al. 2014). Interestingly, AI-2 from engineered *E. coli* can also be manipulated to restore the streptomycin-induced imbalance of the cell density ratio between *Bacteroidetes* and *Firmicutes*, thus relieving gut dysbiosis (Fig. 2c) (Thompson et al. 2015). Recently, in addition to the LuxP and LsrB families, Zhang et al. have also identified a third type of AI-2 receptor that includes a dCACHE domain, such as PctA and TlpQ from *P. aeruginosa*, KinD from *B. subtilis*, and diguanylate cyclase rPHK15-Z16 from *Rhodopseudomonas palustris* (Zhang et al. 2020). Note that the widespread existence of dCACHE domains in various receptors indicates that AI-2-based crosstalk is among a large number of prokaryotic species. Furthermore, there are more than 680 microbes in the rumen including AI-2

synthase- or receptor-encoding genes, which suggests the universal intra- and inter-species crosstalk among rumen microbes (Liu et al. 2022d).

Indole-mediated inter-species crosstalk

Indole, another common QS signal, plays an important role in diverse inter-species interactions, which are relevant to biofilm development (Sethupathy et al. 2020) and persister formation (Vega et al. 2012), etc. Note that gut microbes are often exposed to indole whether they produce indole or not, which indicates the universal indole-based crosstalk among human gut microbiota (Lee and Lee 2010). It is reported that the indole-based crosstalk could be either beneficial or detrimental for the corresponding cell activities. Specifically, with the help of indole produced by *E. coli*, Nicole et al. found that the antibiotic tolerance of *Salmonella typhimurium* (which does not natively produce indole) would increase in response to indole-based crosstalk (Vega et al. 2013) (Fig. 2d). Similarly, Lee et al. realized that indole-based crosstalk would inhibit cell growth and decrease the motility of the non-indole-producing *Agrobacterium tumefaciens* (Lee et al. 2015). To gain a better understanding of the role of indole in microbial communities, please refer to a good review of indole-related mechanisms and physiologies, as well as the major gaps and contradictions in this field (Zarkan et al. 2020).

DSFs-mediated inter-species crosstalk

DSFs, another common QS signal, not only mediate intra-species crosstalk but also play an important role in inter-species interactions (Deng et al. 2011). There are many types of DSFs with different chain lengths and branching, which are often from *Xanthomonas campestris*. DSFs were also produced by many other microbes, such as *P. aeruginosa*, *Burkholderia cenocepacia*, *Xylella fastidiosa*, *Lysobacter enzymogenes*, and *Streptococcus mutans* (Zhou et al. 2017). Therefore, it is not surprising that there are many DSF-mediated inter-species crosstalk in microbial communities. For example, when coculturing *Stenotrophomonas maltophilia* and *P. aeruginosa*, the DSFs from the former can be sensed by the kinase PA1396 of the latter by affecting its biofilm formation and polymyxin tolerance (Ryan et al. 2008). It is also reported that DSF-related crosstalk might lead to polymicrobial infections including *Burkholderia*, *Stenotrophomonas*, and *P. aeruginosa* (Twomey et al. 2012). There is DSF-mediated inter-species crosstalk between *Xanthomonas* and *Bacillus* species bacteria in biological ecosystems, where DSFs from the former interfere with morphological transition and sporulation of the latter (Deng et al. 2016). Furthermore, DSFs-mediated crosstalk can also target HilD, an AraC-type transcription regulator, to repress the virulence of *Salmonella enterica* (Golubeva et al. 2016). Specifically, An et al. discovered that two of five transmembrane helices of PA1396 are required for DSFs sensing, and developed synthetic DSF analogs to mediate crosstalk for modulating or inhibiting biofilm formation and antibiotic tolerance for *P. aeruginosa* (Fig. 2e) (An et al. 2019).

Other crosstalk

Except for QS signals, some other substances and mechanisms can also mediate the intra-species or inter-species crosstalk. For example, the interactions between transcription factors (TFs) and corresponding targets have a certain non-specificity, which may lead to various intra-species crosstalk (Todeschini et al. 2014). To investigate unintended crosstalk, Maerkl and Quake developed a high-throughput microfluidic platform to measure properties and molecular interactions. They took advantage of the

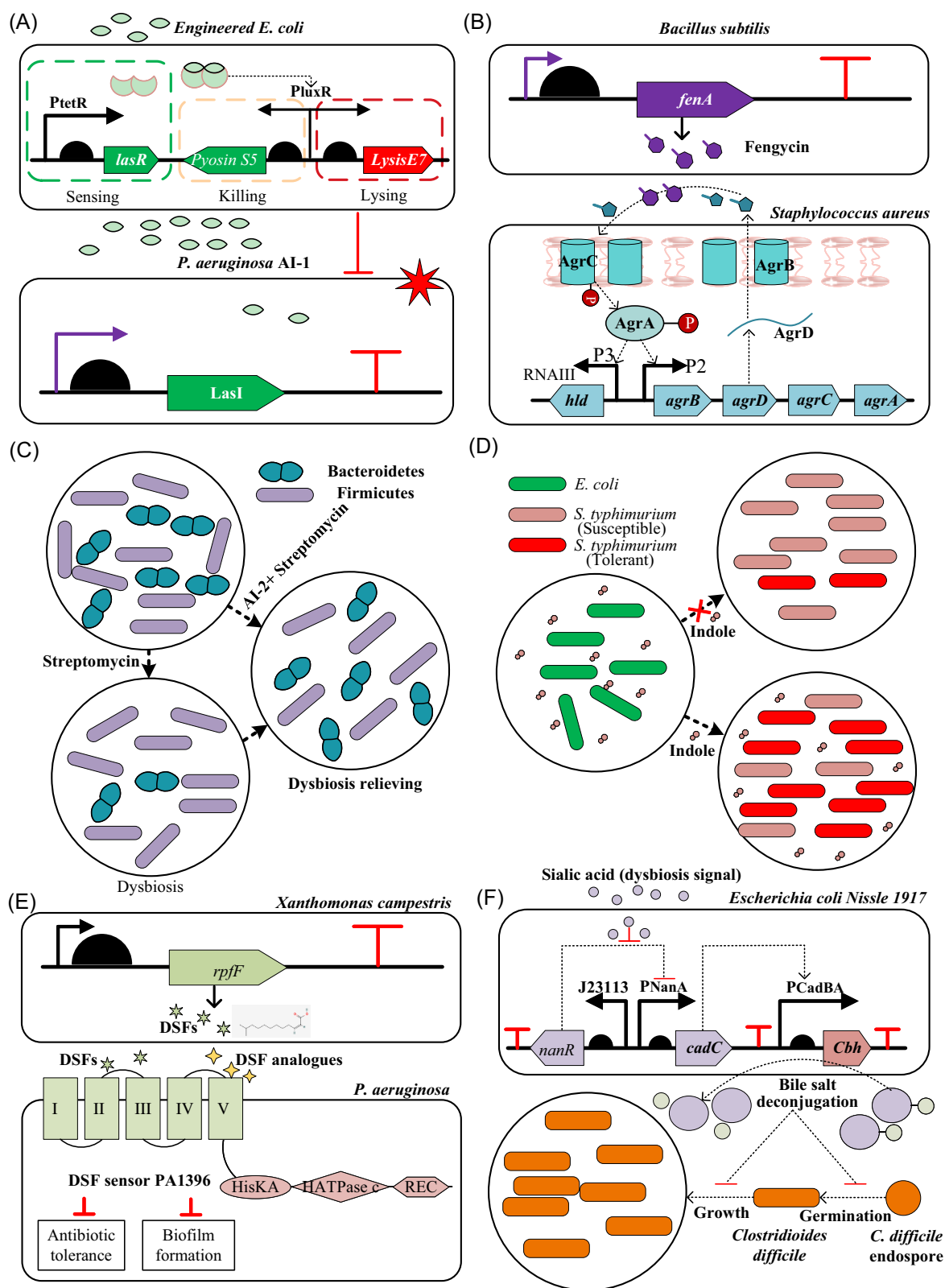


Figure 2. Illustration for different inter-species QS crosstalk. (A) AHL-based inter-species sense-kill systems for the removal of *P. aeruginosa*. Engineered *E. coli* was designed to sense the QS signaling molecule unique to *P. aeruginosa*, lysing to release the toxin, pyocin S5, when a specific density was reached. (B) Fengycin-mediated inhibition of the *agr* QS system (*agrABCD*) of *Staphylococcus aureus*. Fengycin produced by the probiotic *B. subtilis* can antagonize the *agr* system, inhibiting the virulence and the ability of *S. aureus* to colonize in mice. (C) *Escherichia coli*-derived AI-2 can restore the streptomycin-induced imbalance of the cell density ratio between Bacteroidetes and Firmicutes. (D) Pathogenic *S. typhimurium* encountering indole from *E. coli* to enhance its own tolerance to antibiotics. (E) Histidine kinase PA1396 auto-phosphorylation to DSFs and analogs to inhibit the biofilm formation and antibiotic tolerance of *P. aeruginosa*. (F) Bile salt-based crosstalk inhibits the germination and growth of *C. difficile* with an engineered *E. coli* Nissle 1917. Sialic acid can remove the repression of the inducible promoter P_{CadBA} by the *nanR* repressor and induce the efficient expression of the activation therapeutic module through the *CadC* signal amplification module.

high-throughput nature of the microfluidic platform to measure the DNA binding energy landscape of four eukaryotic TFs using molecular interaction mechanisms (Maerkl and Quake 2007). With the global analysis of protein phosphorylation in yeast, Ptacek et al. found that there is extensive crosstalk in the two-component signaling systems, a microbial dominant signaling modality including a sensor histidine kinase and a response regulator (Ptacek et al. 2005).

Certainly, some other metabolites can also mediate inter-species crosstalk, which is meaningful for the stability maintenance of microbial communities and the analysis of microbe–host interactions. Specifically, the crosstalk mediated by indole derivatives can also affect the development of microbial biofilm and virulence. 7-hydroxyindole (Lee et al. 2009), Indole-3-acetaldehyde (Kim et al. 2011), and 7-benzoyloxyindole (Lee et al. 2013) have been verified for the inhibition of the virulence of *P. aeruginosa*, *E. coli* O157:H7, and *S. aureus*, respectively. Note that human-derived primary bile salts can also be sensed by various gut microbes, and transformed into secondary bile acids, the dysregulation of which will lead to *Clostridium difficile* infection (CDI).

To sum up, the intra- and inter-species crosstalk mediated by diverse signals or metabolites are universal in various microbial ecosystems, and can be utilized for virulence reduction, growth interference, antibiotic resistance elimination, and pathogens inhibition. For example, to help patients with CDI, Koh et al. have engineered a genetic circuit to encode a sensor, amplifier, and actuator in *E. coli* Nissle 1917 (Fig. 2f) to restore the metabolism of bile salt to limit the germination of endospores and vegetative cell growth of *C. difficile* (Koh et al. 2022). Note that further details can be referred to in some excellent reviews or databases (Sam et al. 2017) that have summarized the extensive crosstalk in diverse signaling networks of prokaryotes (Qin et al. 2020), eukaryotes, and even microbe–host interactions (Zheng et al. 2022).

Orthogonal regulation circuits

The modularization of synthetic toolboxes requires independent functions of each component to prevent the unwanted interactions that may cause malfunction (Lu et al. 2019). Therefore, accumulating research has been devoted to developing various orthogonal channels for the construction of stable and multifunctional gene regulation networks (Costello and Badran 2021). In this section, to give a broad illustration of different orthogonal regulation strategies, we will outline the circuits or elements based on the levels of metabolites, transcription, translation, and post-translation modification, respectively.

Metabolite response biosensors

Microbes have evolved complex regulatory mechanisms to sense the changes in the concentration of diverse metabolites to adapt to different environments. This indicates that metabolites play an important role in the regulation of different genes expression and phenotypes (Rinschen et al. 2019). Therefore, many metabolite-based orthogonal sensors were developed by introducing intermediate metabolites or by-products. The responses of targets and their corresponding homologous promoters were often applied to adaptively adjust the activation or inhibition of genes, with easy-detection phenotypes (such as the fluorescence intensity) as the outputs for various sensors (Jung et al. 2021). Note that biosensors were often developed to balance the metabolic flux and improve the efficiency of production (Jung et al. 2021). Recently, in order to further expand the versatility and orthogonality of metabolite-

based biosensors in microbial communication, Du et al. also exploited some metabolites, such as 2,4-Diacetylphosphoglucitol (DAPG), methylenomycin furan, and naringenin to design a genetic toolbox for orthogonal multi-channel communications and biological computations (Du et al. 2020). Similarly, many researchers have also constructed diverse metabolite-based orthogonal sensors, such as naringenin, isoprene, and tyrosine, etc. To sum up, diverse metabolite response biosensors were developed in different microbes for the design of applications. Here, in Table 1, we summarize some metabolite-based orthogonal sensors for different functions.

Orthogonal transcription regulation

Various regulation strategies often require the expression of multiple active components in a single cell at different time scales (Rollié et al. 2012). The prosperity and development of synthetic biology has provided a series of engineered biological elements for gene transcription regulations. In this section, we will focus on the summary of orthogonal circuits at the transcription level, including RNA polymerases (RNAPs), RNA-based switches, CRISPR-based, and DNA-binding regulations.

RNAPs-based regulations

It is one of the important checkpoints to control the function of gene circuits and cell behaviors by RNAPs. The orthogonal T7 RNAP has previously been screened to develop a set of resource allocators that can attract RNAP to specific promoters to produce orthogonal gene expression (Segall-Shapiro et al. 2014). The output of each controller was presented under the control of homologous promoters to express orthogonal σ fragments, which were applied to control the transcription intensity of the system. T7 RNAP has also been applied to construct a modular and programmable system for the activation of multiple orthogonal transcription regulations in *E. coli* (Hussey and McMillen 2018), as well as semi-synthetic organisms (Zhang et al. 2017, Feldman et al. 2019). Furthermore, the essential components of RNAPs (σ factors and anti- σ -factors) were often combined to conduct transcription regulations. For example, based on the extensive investigation of the extracytoplasmic function σ s and the corresponding anti- σ s, Rhodius et al. studied their orthogonality on the -35 and -10 binding domains of different promoters, which were applied to construct diverse synthetic genetic switches in *E. coli* (Rhodius et al. 2013). Similarly, Bervoets et al. found that three heterologous σ factors (σ^B , σ^F , and σ^W) from *Bacillus subtilis* show mutual and host orthogonality in *E. coli* strains (Fig. 3a). By creating promoter libraries, they developed a sigma factor toolbox for orthogonal gene expressions with a wide range of transcription initiation frequencies, tunable multiple outputs in response to different signals (Bervoets et al. 2018). Note that more details about the advances and potential applications of RNAPs can be referred to in another good review (Wang et al. 2022b).

RNA-based switch

Toehold switches are a highly orthogonal and specific new type of RNA nano-device that can detect any trigger RNA sequence to activate downstream gene translation (Green et al. 2014). Toehold switches can be used to construct multi-input cellular logic gates to perform biological calculations. Recently, some researchers further deployed the scalability of toehold switch variants and developed an orthogonal paper-based diagnostic method for sensing various viruses and gut microbiota (Cao et al. 2021). Furthermore, Kim et al. designed some powerful translation-repressing

Table 1. Details of different orthogonal metabolite-based biosensors.

Metabolites	Functions	References
4-hydroxybenzoic acid (pHBA)	Detection of benzoic acid derivatives	(Castaño-cerezo et al. 2020)
3-hydroxypropionic acid (3-HP)	Biosensor for accelerating evolution	(Seok et al. 2021)
3-hydroxypropionic acid (3-HP)	Improve biochemical production	(Kang et al. 2022)
p-coumaroyl-CoA	Biosensor for dynamic regulation	(Liu et al. 2022a)
L-2-Hydroxyglutarate (L-2-HG)	Biosensor for carbon starvation response	(Kang et al. 2021)
2,4-Diacetylphosphoroglucinol (DAPG)	Orthogonal optimized small-molecule sensors	(Meyer et al. 2019)
Cuminic acid (Cuma)	Orthogonal optimized small-molecule sensors	(Meyer et al. 2019)
3-oxohexanoyl-homoserine lactone (3OC6)	Orthogonal optimized small-molecule sensors	(Meyer et al. 2019)
Vanillic acid	Orthogonal optimized small-molecule sensors	(Meyer et al. 2019)
Isopropyl- β -D-thiogalactoside (IPTG)	Orthogonal optimized small-molecule sensors	(Meyer et al. 2019)
Anhydrotetracycline HCl (aTc)	Orthogonal optimized small-molecule sensors	(Meyer et al. 2019)
aTc	Monitor the quantitative relationship between sialic acid and its corresponding enzyme	(Lim et al. 2017)
l-Arabinose	Orthogonal optimized small-molecule sensors	(Meyer et al. 2019)
Choline chloride	Orthogonal optimized small-molecule sensors	(Meyer et al. 2019)
Naringenin	Orthogonal optimized small-molecule sensors	(Meyer et al. 2019)
3,4-Dihydroxybenzoic acid (DHBA)	Orthogonal optimized small-molecule sensors	(Meyer et al. 2019)
Sodium salicylate	Orthogonal optimized small-molecule sensors	(Meyer et al. 2019)
N-(3-hydroxytetradecanoyl)-l-homoserine lactone (3OHC14)	Orthogonal optimized small-molecule sensors	(Meyer et al. 2019)
Acrylic acid	Orthogonal optimized small-molecule sensors	(Meyer et al. 2019)
Erythromycin	Orthogonal optimized small-molecule sensors	(Meyer et al. 2019)
D-2-hydroxyglutarate	Quantitative detection of D-2-HG as a biomarker in many cancers	(Xiao et al. 2021)

riboregulators (also termed as toehold repressors and three-way junction repressors) with sensing and logic capabilities. The high-performance translation repressors were designed to induce the opening of the hairpin structure variants (Fig. 3b). The dynamic range of the toehold repressor was improved by the forward automation process, and the mechanism of the three-way junction repressor was determined by high-throughput RNA structure analysis. Using the above two highly specific and highly orthogonal inhibitors, a four-input logic gate was realized and correctly evaluated in *E. coli* (Kim et al. 2019). As summarized by other researchers, we also believe that the sensitivity, stability, and compatibility of the ecosystems based on the RNA switches will be improved gradually.

CRISPR-based regulations

The bacterial CRISPR-CAS defense was massively applied to the flexible and modular transcription regulations. The nuclease-null CRISPR-Cas (dCas) proteins were validated to regulate the corresponding process orthogonally. Note that the dCas9 protein has a RNA-guided gene targeting property, which is very important for the regulation of externally induced genes (Yu et al. 2021). dCas9 protein can also bind to the downstream of RNAP, thus blocking the corresponding transcription process. Ligand-activated or inhibited single-guide RNA (sgRNA) can be used for constructing a multi-layered CRISPR loop to provide an orthogonal and modular transcription regulation strategy for programming bacterial cells (Dong et al. 2018). Note that the working principle of the intracellular controller is to respond to a burden increase by reducing the gene expression rate of the synthetic target proteins. Specifically, Ceroni et al. constructed a genetic circuit by using a dCas9-based feedback controller to respond to burden (Fig. 3c). Results showed that microbes equipped with the dCas9-based feedback controller could realize robust growth and higher protein yield in batch production (Ceroni et al. 2018). Yang et al. have established a dual-function dynamic regulation strategy by using CRISPR in-

terference (CRISPRi) to couple with antisense RNAs. They used muconic acid (MA) as the input signal to couple antisense RNA to construct a sensor. Then MA sensor was applied to dynamically regulate the phosphoenolpyruvate metabolic node (EP module) in *E. coli* to distribute the carbon flux for native metabolism (such as cell growth and maintenance) and MA production (NC module) (Fig. 3d) (Yang et al. 2018). Some other researchers have also developed some multi-stable and dynamic CRISPRi-based circuits with high predictability, orthogonality, and low metabolic burden (Santos-Moreno et al. 2020). Another powerful tool based on CRISPR activation (CRISPRa) has also emerged for creating orthogonal synthetic gene circuits for diverse functions in microbial cells (Liu et al. 2019). Furthermore, orthogonal CRISPRa and CRISPRi systems can be further coupled for simultaneous transcription upregulation of a subset of target genes while downregulating another subset, thus gaining the control of gene regulatory networks, signaling pathways, and cellular processes (Martella et al. 2019). As a powerful tool for microbial regulations, researchers have also conducted more comprehensive summaries and discussions for CRISPR-based regulations (McCarty et al. 2020, Nishida and Kondo 2021).

DNA-binding regulations

There are some engineered regulators based on orthogonal DNA binding elements, such as transcription activator-like effectors (TALEs) (Leben et al. 2022), selected TFs, and engineered promoters. For example, Segall-Shapiro et al. redesigned promoters for maintaining constant levels of expression at any copy number, due to the metabolic burden being affected by the copy number of the vector and the location of the genome. In order to achieve stable gene expression intensity and minimize the impact of foreign perturbation, an incoherent feedforward loop (iFFL) of a TALE was engineered into *E. coli* promoters, which had near-identical expression in different genome locations and plasmids (Fig. 3e) (Segall-Shapiro et al. 2018). Some selected TFs are also

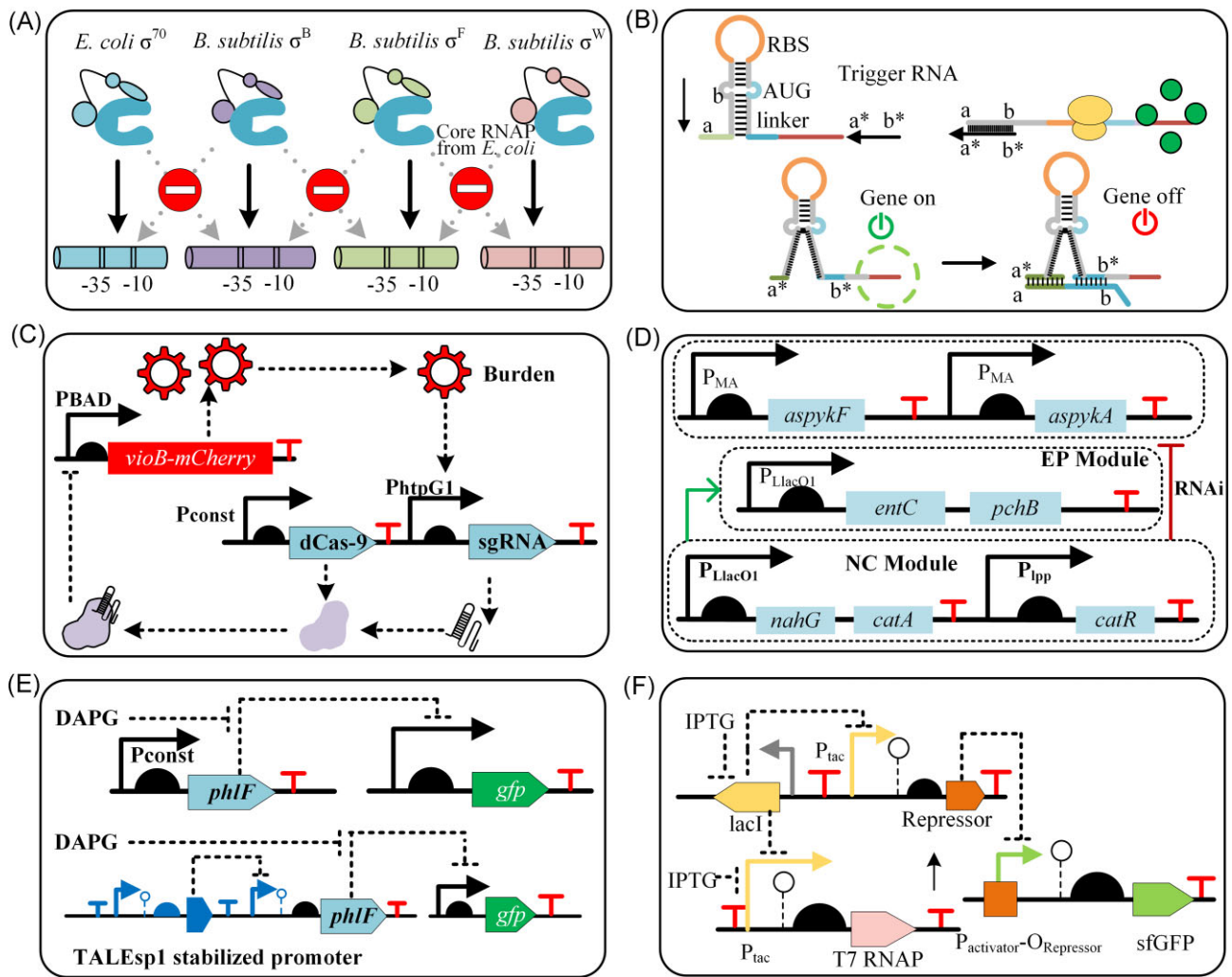


Figure 3 Illustrations for orthogonal transcription regulations based on different tools. (A) Orthogonal regulations of *E. coli* RNAP and *Bacillus subtilis* sigma factors without crosstalk. (B) Illustration for translation-repressing riboregulators. Translation regulation by the binding of ribosome binding site (RBS) and start codon and two single-stranded domains *a** and *b**. (C) Illustration for a biomolecular feedback controller. The *htpG1* promoter drives the expression of CRISPR sgRNA, which in turn directs binding of dCas9 to target *pBAD* promoter to inhibit transcription of *VioB-mCherry*. (D) Muconic acid (MA) promotes the expression of the phosphoenolpyruvate metabolic node (EP module) and decreases the carbon flux into the TCA cycle via RNAi. (E) The DAPG-induced PhIF repressor was regulated using either the constitutive promoter (*Pconst*) or the TALEsp1 stabilized promoter. (F) Molecular implementation of transcription incoherent-feedforward-loop networks, in which the *LacI* is induced by IPTG; *ci434*, T7 RNAP, and sfGFP are the repressor, activator, and the output, respectively.

used as DNA-binding regulators and are regarded as “gatekeepers” for various genes expression (Collins et al. 2006). Mutation of ligand binding domain residues can effectively enhance the specificity of inducers without changing the function of allosteric TFs. The method of coupling computer prediction models and high-throughput screening can improve the design and selection of high-quality TFs (Jha et al. 2015). For example, Meyer et al. developed a directed evolution and screening strategy, and obtained 12 high-performance sensors with low background and crosstalk, and amplified the available orthogonal transcription regulators (Meyer et al. 2019). Furthermore, Zong et al. conducted rational engineering on biological systems at the transcription level by insulated promoter design and operator optimization. Results showed that the combinatorial promoters with insulated transcription elements had good performances on mean errors and success rate for encoding NOT-gate functions. They also combined their optimized insulated transcription elements to be a four-node network (Fig. 3f) with iFFL topology, and verified its complex functions (Zong et al. 2017). There are some other excellent

reviews that have provided more details for the transcriptional regulation circuits (Soutourina 2018, Cramer 2019, Maucourt et al. 2020).

Orthogonal translation regulations

Synthetic biological regulations design can also be conducted at the translation level, which has a shorter time scale than the transcription level (Fink et al. 2019). In this section, we provide an overview of different orthogonal translation regulations, including ribosomes, riboswitches, and aminoacyl-tRNA synthetase (aaRS)-tRNA pairs based on non-canonical amino acids (ncAAs).

Ribosomes

Orthogonal ribosomes have become a new mode of translation level regulation strategy.

By redesigning the specific recognition between the small ribosomal subunit ribosomal mRNA and the corresponding mRNA leader sequence, the modified orthogonal ribosomal machinery

can only translate specific mRNAs, achieving independent parallelism with the strain's endogenous translation mechanism. Specifically, the orthogonal 16S subunit and wild type 50S subunit of orthogonal ribosomal translation only recognize the particular mRNA, which can be used as a completely orthogonal translation. An and Chin developed an orthogonal transcription by T7 RNA polymerase and orthogonal ribosomes (O-ribosomes) to construct a simple logic gate, which realizes the adjustment of the response time of different input systems in the similar AND gate system in the natural regulatory network (An and Chin 2009). It was also reported that the covalent linkage of circularly permuted rRNAs could eliminate the heterogenous ribosome, and improve the cellular orthogonality for ribosomes (Fried et al. 2015). Furthermore, the use of screening and engineering approaches, as well as combinations with some other tools, can increase the orthogonality and the application scope of ribosomes. For example, Carlson et al. applied an evolutionary approach to engineer ribosomes with tethered subunits to minimize the association of covalently linked ribosomal subunits with their native counterparts (Fig. 4a), thus obtaining orthogonal ribosomes that enable faster cell growth and protein expression (Carlson et al. 2019). It is reported that the combination of orthogonal ribosomes and dynamic resource allocators could reduce heterologous expression and gene crosstalk (Darlington et al. 2018). There is a more comprehensive review, which can be referred to obtain more details for the assembly and repair of ribosomes (Yang and Karbstein 2024).

Riboswitches

With the deepening of the understanding of the regulation and structural principles of ribosomal switches, a series of orthogonally responsive native and non-native ribosomal switches continue to be applied to gene regulations in different bacteria. For example, through targeted mutagenesis and comprehensive screening strategies, Dixon et al. successfully created an orthogonal RNA regulatory element-riboswitch, which was applied to construct a multi-component dual-promoter co-expression system and a synthetic operator system (Dixon et al. 2012). By combining consistent *E. coli* ribosome binding site (RBS) and anti-RBS libraries, Kent and Dixon adopted a high-throughput method based on fluorescence-activated cell sorting to identify riboswitches with the maximal protein expression (Kent and Dixon 2019). Then four endogenous *E. coli* stress response promoters were designed as riboswitch regulators, which were induced by orthogonal ribosome-specific ligands (Fig. 4b). Furthermore, riboswitches can be used as RNA-based intracellular sensors that control gene expression (Hossain et al. 2020). The rate or opening of translation is controlled by cleavage or blocking of the corresponding RBS. For example, Xiu et al. developed the first naringenin-responsive biosensor based on the RNA riboswitch, which was scanned by flow cytometer and used to screen *E. coli* strains with real-time detection of metabolite products (Xiu et al. 2017). In view of the crosstalk in the induced expression system, Robinson et al. used a structure-guided chemical genetic screening method to successfully identify excellent chimeric ligands for orthogonal riboswitches (Robinson et al. 2014). They used the corresponding ligand to regulate the dose-dependent expression of the physiologically important *cheZ* gene required for the movement of *E. coli*. Riboswitches can also be used to specifically respond to exogenous small molecules to achieve self-cleavage regulation of artificial cells. Specifically, the histamine-specific corresponding ribosome switches were isolated by *in vitro* screening, then encapsulated in artificial cells of phospholipid vesicles for the characterization of a self-destructive kill-switch (Fig. 4c) (Dwidar et al. 2019). Therefore, orthogonal

riboswitches can precisely regulate the expression of bacterial genes and provide new modular and orthogonal regulatory components for functions verification and synthetic biology toolboxes (Kavita and Breaker 2023, Salvail and Breaker 2023).

ncAAs-based aaRS/tRNA pairs

Note that ensuring the orthogonality of ncAAs-based aaRS/tRNA pairs is the key to their translation level regulation, which includes orthogonal tRNA engineering and orthogonal aaRS engineering (Hammerling et al. 2020). The orthogonality of tRNA requires that tRNA can only be specifically recognized by the corresponding tool enzyme and cannot cross-talk with other endogenous aaRS. Therefore, the aaRS/tRNA pairs can be selected and modified from evolutionarily distant organisms to reduce the probability of cross-reactivity. For example, tRNA^{Pyl} has been rationally evolved with six nucleotide changes to improve the efficiency for incorporating ncAAs at the termination codon, such as UAG codons in fluorescence protein (Fan et al. 2015). Note that pyrrolysyl-tRNA synthetase/pyrrolidine tRNA pairs (PylRS/tRNA^{Pyl}) have high orthogonality in a variety of biological systems and are suitable as a universal codon expansion tool for modification in different microbial ecosystems (Suzuki et al. 2017). To improve the orthogonality of tRNA, rational design and a directed evolution strategy have been effectively used to modify and optimize tRNA by the manipulation of the variable loop region (Willis and Chin 2018), such as elongation factor-Tu (EF-Tu) (Fan et al. 2015) and elongation factor P (EF-P) (Hammerling et al. 2020) (Fig. 4d).

Naturally, the amino-acid binding pocket of aaRS can specifically recognize the corresponding amino acid, so as to ensure the orthogonality of aaRS to the substrate. To improve orthogonality and efficiency, different types of aaRS have been used for modification, and several tool enzymes for gene codon extension, and diverse aaRS/tRNA pairs engineering has been successfully developed, enabling the specific introduction of more than 200 ncAAs into biological proteins (Vargas-Rodriguez et al. 2018). The orthogonality of aaRS/tRNA pairs requires that the heterologous aaRS tools can specifically recognize exogenously added non-native amino acids (Fig. 4e) (de la Torre and Chin 2021). Furthermore, through the in-depth analysis and mining of bioinformatics, some new PylRS/tRNA^{Pyl} pairs have been discovered, which can be used to develop the same type of mutually orthogonal non-natural amino acid coding tools. For example, Willis and Chin created several new PylRS/tRNA^{Pyl} pairs that are mutually orthogonal to other existing aaRS/tRNA pairs (Willis and Chin 2018). Based on the computation and analysis of millions of sequences, Cervettini et al. identified 243 candidate tRNAs in *E. coli*, obtained 71 orthogonal tRNAs, discovered five orthogonal pairs, and characterized a matrix of 64 orthogonal aaRS/tRNA specificities with the help of their proposed approach (Fig. 4f) (Cervettini et al. 2020).

To sum up, genetic code expansion has been engaged in the use of orthogonal aaRS that can specifically recognize ncAAs and tRNA in different microbes (Sisila et al. 2022). Recently, massive genomic and metagenomic data have become an important resource for the development of new orthogonal aaRS/tRNA pairs (de la Torre and Chin 2021). Future efforts will be engaged in integrating codon expansion using the aforementioned mutually orthogonal aaRS-tRNA pairs, synthetic bases (Fischer et al. 2020), orthogonal mRNA design (Dunkelmann et al. 2021), and some other strategies to decode diverse ncAAs to be incorporated into a protein. More details of the advances and applications of genetic code expansion and ncAAs can be referred to in some other good reviews (Ishida et al. 2024, Yi et al. 2024).

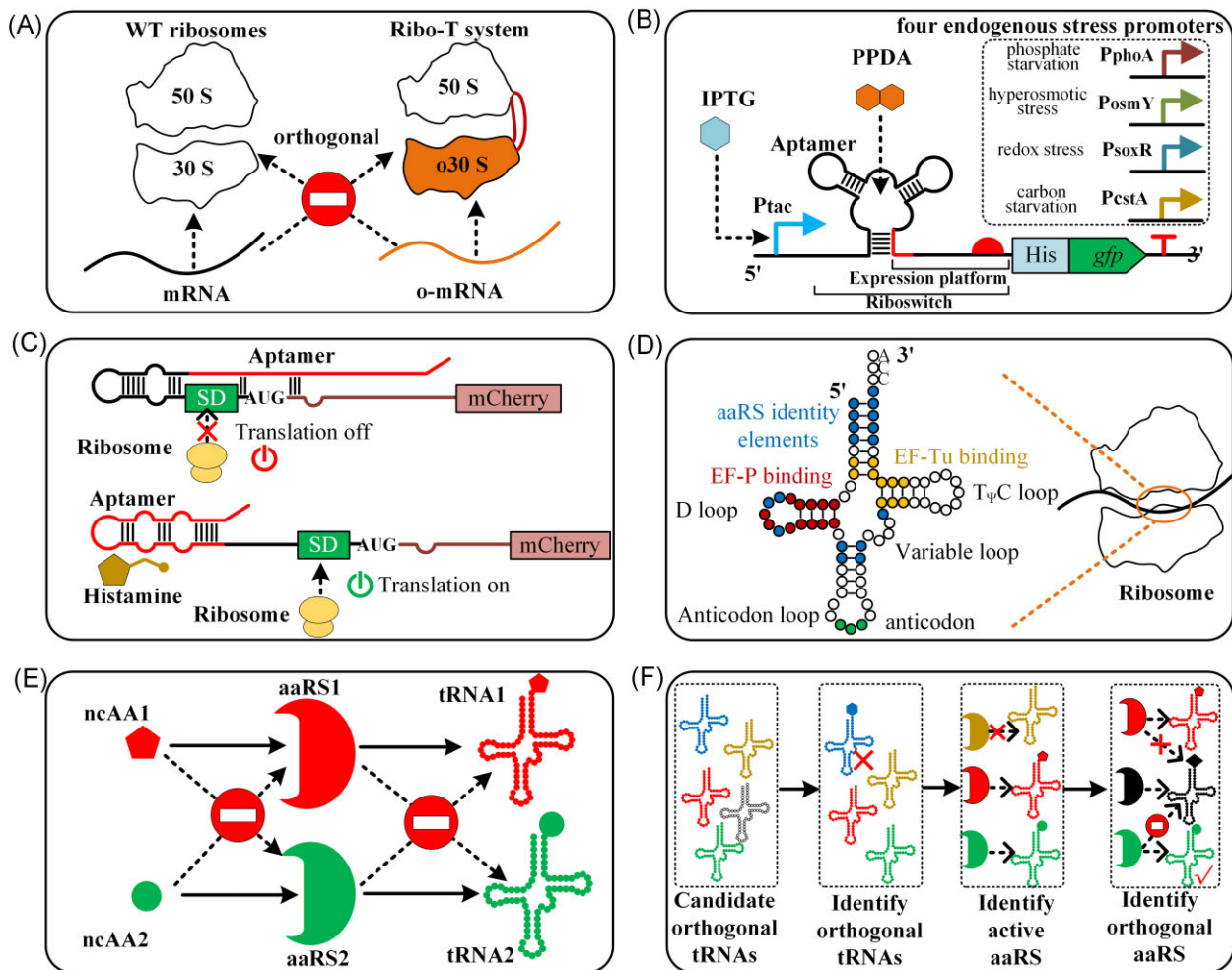


Figure 4. Illustrations for orthogonal translation regulations based on different tools. (A) Orthogonal function evolved for the wild type ribosome and fully orthogonal ribosome (Ribo-T) system. (B) Schematic overview of [4,5-d] pyrimidine-2,4-diamine (PPDA) responsive orthogonal riboswitch. (C) Illustration of the histamine-specific corresponding ribosome switches. Histamine binding to aptamer disrupts the secondary structure of RNA and activates the translation state. (D) Techniques to engineer tRNA including aaRS identity elements, EF-Tu binding, EF-P binding, and four loops. (E) Schematic overview of the orthogonal pairs of ncAA, aaRS, and tRNA. ncAAs are recognized by orthogonal aaRSs, loaded onto orthogonal tRNAs. (F) A pipeline to identify orthogonal aaRS-tRNA pairs. First, the method of calculation or experiment was used to produce a series of candidate tRNAs, and then orthogonal tRNAs were experimentally confirmed. Subsequently, the active homologous synthases were screened and their orthogonality to each other was confirmed.

Orthogonal protein regulations

In addition to genetic circuits based on transcription and translation levels, there are many biomolecular regulations based on post-translation modification, such as protein circuits. For example, the viral proteases that specifically recognize and cleave short peptide targets provide the basic elements for protein circuits (Chung and Lin 2020). Specifically, using the diversity and programmability of viral proteases, a combinable protein system (circuits of hacked orthogonal modular proteases, CHOMP) was developed (Fig. 5a) for the construction of different synthetic logics (Gao et al. 2018). In order to regulate protein circuits more quickly and accurately, Fink et al. obtained split-protease-cleavable orthogonal-CC-based (SPOC) logic circuits, which could quickly respond to small molecule inducers within a few minutes (Fink et al. 2019). The complete activation of functional enzymes can be achieved when the other recombinant enzyme has stronger matching ability and paired implementation (Fig. 5b). The CHOMP and SPOC logic circuits are both modular and expandable; the former relies on protein degradation mechanisms, while the latter logic circuits rely on protease cleavage, which

has a faster response rate. A cooperatively induced protein heterodimer (CIPHR), used as the basic input of the protein circuit, was constructed by adjusting the binding strength of protein heterodimers (Fig. 5c). These elements are based on the affinity between proteins and do not depend on the intracellular environment. Therefore, CIPHR was programmable and portable, which has been verified in yeast, T cells, cell-free, and other systems (Chen et al. 2020).

With the enhancement of computing and design capabilities, protein circuits will have a wider range of applications. As one of the important circuits, the development of protein sensors and switches is still limited and challenging (Yu et al. 2018). Note that Quijano-Rubio et al. have designed and constructed a general class of protein biosensor from scratch (Quijano-Rubio et al. 2021). The protein sensor consists of two protein components: the “lucCage” part and the “lucKey” part. The “lucCage” part consists of a cage-like domain and a luciferase fragment containing a target-binding motif and cleavage (similar to latch structure), and the “lucKey” part consists of a fragment complementary to the open state lucCage binding bond peptide and luciferase (similar

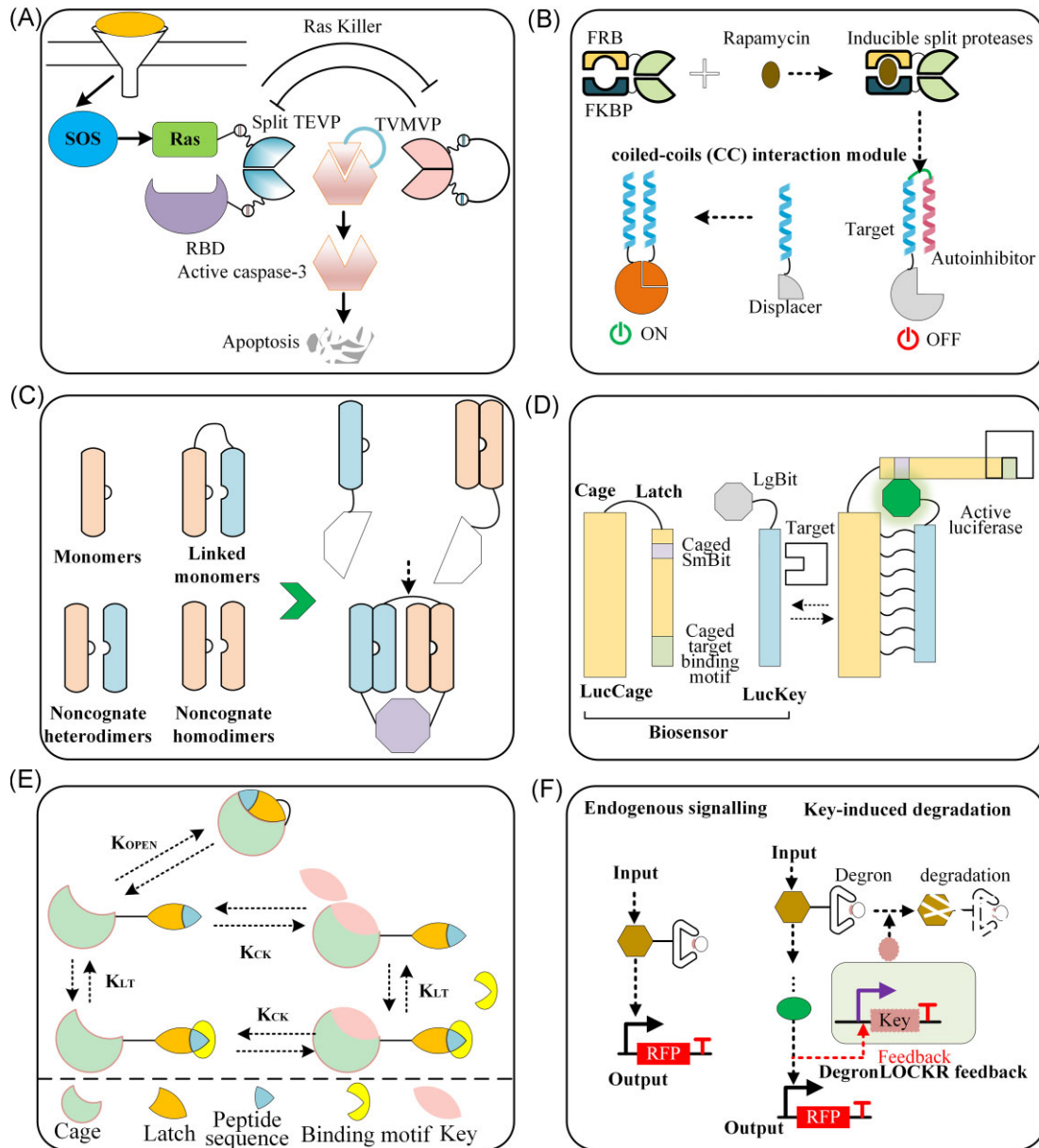


Figure 5. Orthogonal regulatory strategies based on protein circuits. (A) Illustration of the CHOMP circuit. SOS activates Ras, causing it to bind RBD, reconstituting RasTEVP. TVMVP cleavage detaches Casp3 and reduces its ability by membrane-localized TEVP. (B) Scheme of chemically inducible split proteases with rapamycin based on a coiled-coils (CC) interaction module. The complementary split fragments of the protease were fused to a domain pair, the proteolytic activity was obtained after the addition of the inducer rapamycin, and the leucine zipper was removed to activate the luciferase reconstitution. (C) Schematic overview of the basic input of the protein circuit by adjusting the binding strength of the dimer. The inactivation operation was performed by separating the formed dimer through competitive binding, and the activation operation was performed by connecting two non-interacting monomers and recombining the fused split protein domains. (D) Schematic mechanism of multi-state biosensors. The binding of the target and key allows the reconstitution of SmBiT and LgBiT for luciferase activity. (E) Illustration of the LOCKR system. The protein switch is composed of a cage and latch with a functional motif, which is in thermodynamic equilibrium state. (F) Schematic diagram of the DegronLOCKR-induced degradation, which consists of the designer degronSwitch and inducer protein (the "key").

to key structure) (Fig. 5d). The switch state of the sensor only depends on the thermodynamic coupling of the analyte and sensor activation, and its sensitivity depends on the target binding domain and the change of free energy after target binding. Similarly, Langan et al. cleverly used the adjustable spiral structure in a wide dynamic range to design a protein switch with a model of the lock-key structure (Langan et al. 2019). The external key competes with the door latch to bind to the cage. Before unlocking, the functional peptide of the door latch is in a state of inactivation, and the external key is inserted (by adjusting its thermodynamic pa-

rameters) to activate the function peptide (Fig. 5e). Furthermore, in another work, the degradation determinant (degron) was designed as a cage structure, and the degradation process of the protein was coupled with the switch state of the protein molecule (Ng et al. 2019). Different functional peptides can be adjusted to design different LOCKRs, indicating that LOCKR has plug-and-play modularity. The feedback loop mediated by DegronLOCKR not only can realize the control of the endogenous pathway and synthesis circuit, it can also change the affinity of the key (Fig. 5f).

Compared with regulation at the DNA level, protein-based circuits have the advantages of fast response, direct coupling to endogenous pathways, and no need to integrate the genome of cells (Gao et al. 2018). In this section, we only conduct a brief browsing of protein circuits; for more details of the design of different protein circuits, please refer to an extensive review (Chen and Elowitz 2021).

To sum up, although the engineering ability of a single species has made extraordinary advances, the engineering of microbial ecosystems often fails to achieve their stability and robustness. Whether the above-mentioned cross-talk and orthogonal regulatory tools and circuits are suitable for the targeting behavior of cells mainly depends on the application fields and related requirements, such as the sensitivity, selectivity, dynamic range, host adaptability, and other factors. Future efforts will be engaged in developing a comprehensive cycle for the *de novo* design and optimization of various microbial ecosystems from the assembling of various cross-talk and orthogonal regulatory tools by considering the global characteristics, controllability, and robustness, etc. All the above efforts introducing the cross-talk and orthogonal circuits based on various levels, such as metabolites, transcription, translation, and post-translation, provide the experimental basis (a summary of the different circuits is listed in the following “Build module”) for the precise regulations of different microbial ecosystems.

Assembling of microbial ecosystems

Naturally occurring microbial ecosystems are functional and stable (Giri et al. 2020). Members of microbial communities exhibit robustness and emergent properties in response to environmental changes. Although multi-omics and high-throughput techniques have made rapid progress in capturing microbiota phenotypes and metabolic functions, little is known about the corresponding interaction mechanisms. The use of bottom-up construction of microbial ecosystems is expected to be used to explore the ecological knowledge of microbiota through modular rational design and assembly. To design microbial ecosystems, the DBTL cycle has been proposed to guide and promote microbial community engineering (Lawson et al. 2019). However, more details for the guidance of designing microbial ecosystems based on DBTL cycles are lacking. Considering the complexity of various practical application conditions, such as the human gut and soil environment, the construction of controllable, stable, and robust microbial ecosystems must be the basis and direction of future microbial applications. To better design and optimize various microbial ecosystems, we propose a more comprehensive DBTL (cDBTL) procedure that includes function specification, chassis selection, interactions design, system build, performance test, modeling analysis, and global optimization (Fig. 6). Herein, to gain a better understanding of microbial interaction mechanisms and optimized design of microbial ecosystems, thus achieving the desired engineering functions, we provide an introduction (with details of each step) to the design and optimization of functional microbial ecosystems.

Design module

Synthetic microbial ecosystems can function as low complexity systems to further understanding of the composition and interactions of the microbiota. To further realize their stability and functional activities, we propose that the design module of the micro-

bial ecosystems should specifically include the steps of function specification, chassis selection, and interaction design.

Function specification

Because of their unique advantage of division of labor, microbial ecosystems have been extensively used in bio-computing, bio-manufacturing, bio-therapy, and bio-remediation, etc. In this section, we will only give a brief introduction for the updated and specific function.

The integration of circuits can realize different bio-computing with accurate perception and calculation for various biological networks (Liu et al. 2020c). For example, Müller et al. combined the sensor-sender and receiver-digitizer cells to develop the fragrance-programmable analog-to-digital converter for remote control of digital gene expression (Fig. 7a) (Müller et al. 2017). With the expansion of orthogonal communication toolkit, Du et al. deployed a three-input AND-XOR logic gate in seven *E. coli* strains based on the signal transmission of 3OC6, salicylate, *p*-coumaroyl-HSL (pC), and DAPG (Fig. 7b) (Du et al. 2020). Shin et al. distributed a biocomputing process into seven *E. coli* strains that acquire four different signals, i.e. 3OC6, N-(3-hydroxytetradecanoyl)-l-homoserine lactone (3OHC14), anhydrotetracycline (aTc), and isopropyl- β -D-1-thiogalactopyranoside (IPTG), to realize the binary-coded digit to 7-segment decoder for clocks and calculators (Fig. 7c) (Shin et al. 2020). In another study, inspired by structural similarity between multi-cellular networks and artificial neural networks, Li et al. conducted a neural-like calculation in microbial consortia to sense the weights of various group induction signals and recognize patterns (Fig. 7d) (Li et al. 2021). These studies showed the programmability of neuron-like bio-computing in different microbial ecosystems.

Recently, several studies have been carried out among various microbial ecosystems to achieve various bio-manufacturing products, such as biofuels (Zhang et al. 2021), short-chain fatty acids (SCFAs), and other value-added chemicals (Sgobba and Wendisch 2020). For example, Li et al. distributed the two upstream branches (the synthesis pathway of the caffeic acid and salvianic acid A) and downstream modules of the synthetic pathway of rosmarinic acid to three strains of *E. coli*, and optimized the external conditions such as mixed carbon source and inoculation ratio to increase the stability and synthesis ability of the co-culture system (Fig. 8a) (Li et al. 2019). Compared with single-strain strategy, the yield of rosmarinic acid produced by co-culture of three strains of *E. coli* increased by 38 times (172 mg/L). Recently, Li et al. have also developed a stable system of co-culture of three strains by using a carbon source to reduce competition independently and cross-feeding multiple metabolites to strengthen internal connection (Li et al. 2022). DmpR (responsive to caffeic acid), a self-regulating biosensor, was introduced to dynamically regulate GdhA that successfully regulated the composition of the microbial community, which was demonstrated by the biosynthesis of silybin/isosilybin (Fig. 8b). Furthermore, with the help of the design for metabolic niche, various SCFAs were produced under the synergistic effect of various heterogeneous consortia with the lignin as input carbon source, as well as the acetic acid and lactic acid being the intermediate carbon source (Shahab et al. 2020) (Fig. 8c). Jiang et al. constructed a synergistic consortium including *Trichoderma asperellum* and *Lactobacillus paracasei* to boost lactic acid conversion from lignocellulose (Fig. 8d) (Jiang et al. 2023a). As stated above, the mechanism understanding of metabolic function accelerates the applications of microbial ecosystems, and provides feasible solutions for global problems of environmental governance and energy crisis.

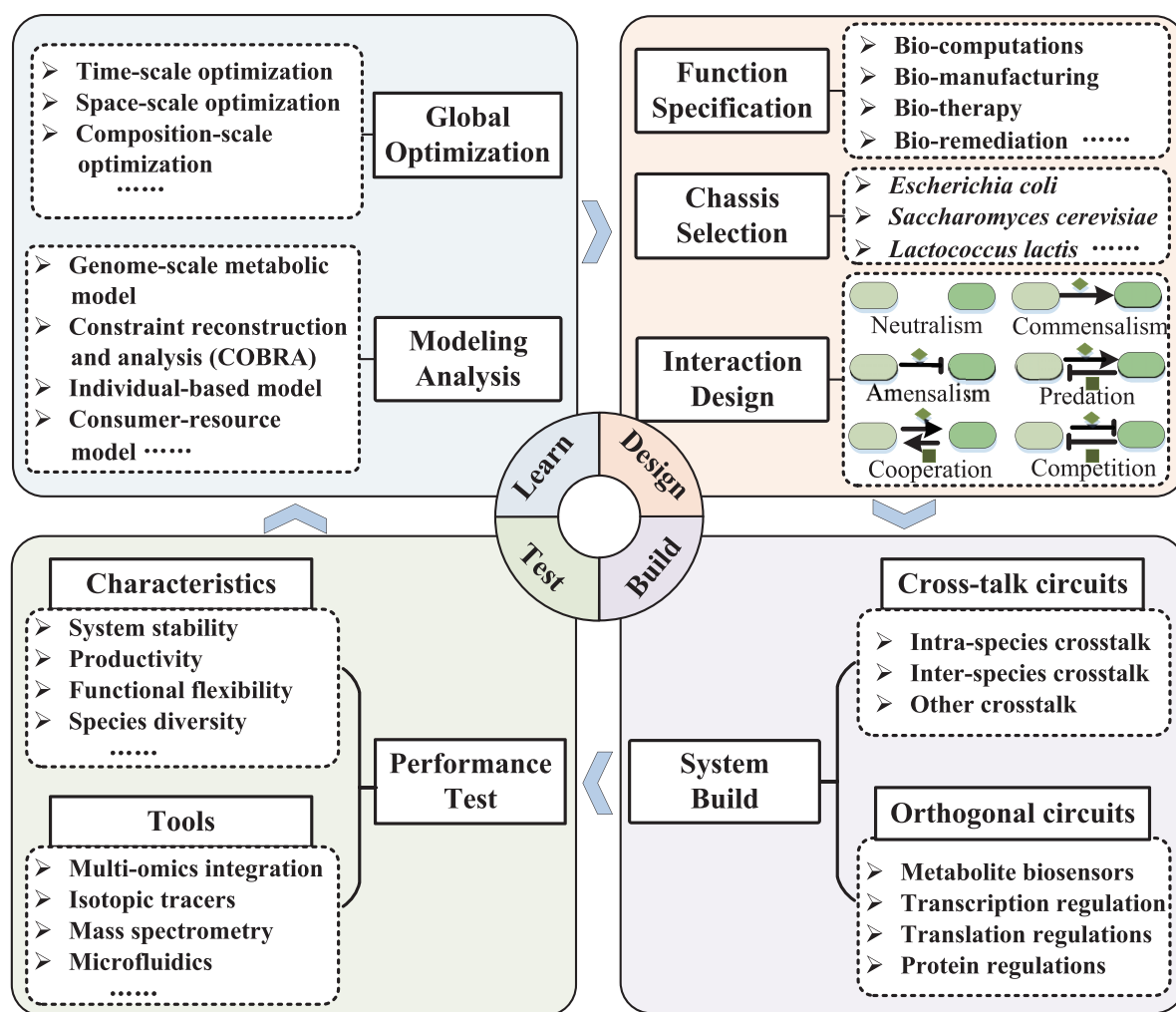


Figure 6. Illustration of the comprehensive DBTL (cDBTL) procedure including Design module (function specification, chassis selection, and interactions design), Build module (system build), Test module (performance test), and Learn module (modeling analysis and global optimization).

With the deepening of the microbial community, many studies have found that combinations of well characterized strains for bio-therapies not only can reduce side effects (such as the removal of drug-resistant bacteria), they also make full use of the advantages of each strain (Tan et al. 2021). For example, lactic acid bacteria have the advantages of biological safety and metabolic diversity (Liu et al. 2022c), and have been used to construct synthetic microbial ecosystems in recent years. Specifically, Mao et al. designed the gene loop of *L. lactis*, so that it can be applied to the diagnosis and colonization resistance of *V. cholerae* (Mao et al. 2018). With the help of hydrogel consisting of the poly (ethylene glycol) diacrylate and chitosan (CS), Li et al. have also developed a two-strain consortium (*Synechococcus elongatus* and *L. lactis*) as a photoautotrophic living material to promote skin wound healing (Li et al. 2023a). In the *S. elongatus*-*L. lactis* consortium, *S. elongatus* PCC7942 was provided to produce sucrose by photosynthesis, while the *L. lactis* was designed to use the intermediate sucrose for cell growth and secreting the functional biomolecules. Therefore, exploiting some more consortia-based bio-therapies for different diseases will be an important area for future medical applications of microbial communities.

Bio-remediation engineering can be guided by synthetic biology and engineering principles to realize bio-remediation, such as plastic bio-degradation and wastewater treatment (Wei et al.

2020). Many researchers have conducted bio-degradation by top-down screening or engineered synthetic microbial communities. For example, Wang et al. studied the community succession of plastsphere of polyethylene film (Wang et al. 2023). Results showed that the community combination of *Rhodobacter* sp. Rs and *Bacillus aryabhattai* 5-3 had high polyethylene mulching film (PMF) degradation efficiency (Fig. 9a). Phenanthrene is a polycyclic aromatic hydrocarbon with carcinogenic effects. Zhang et al. rationally distributed 17 key genes of phenanthrene degradation into three *E. coli* strains, thus contributing to degrading consortium, which had synergistic functions and clear division of labor (Fig. 9b) (Zhang et al. 2022). After optimizing the degradation conditions, 90.66% phenanthrene degradation was achieved within 21 days. Furthermore, microbial communities provide an economical and eco-friendly option, as well as an excellent paradigm for pollutant treatment capacity. For instance, to realize the bio-degradation of acetacetanilide (AAA) in hypersaline wastewater, a synthetic consortium, including *Paenarthrobacter*, *Rhizobium*, *Rhodococcus*, *Delftia*, and *Nitrateductor*, was developed to treat AAA wastewater with different water quality characteristics (Fig. 9c) (Zhang et al. 2023). To bio-degrade the palm oil mill effluent (POME), Islam et al. utilized the response methodology to evaluate the performances of the two-strain inoculated microbial fuel cells, including *Klebsiella variicola* and *P. aeruginosa*

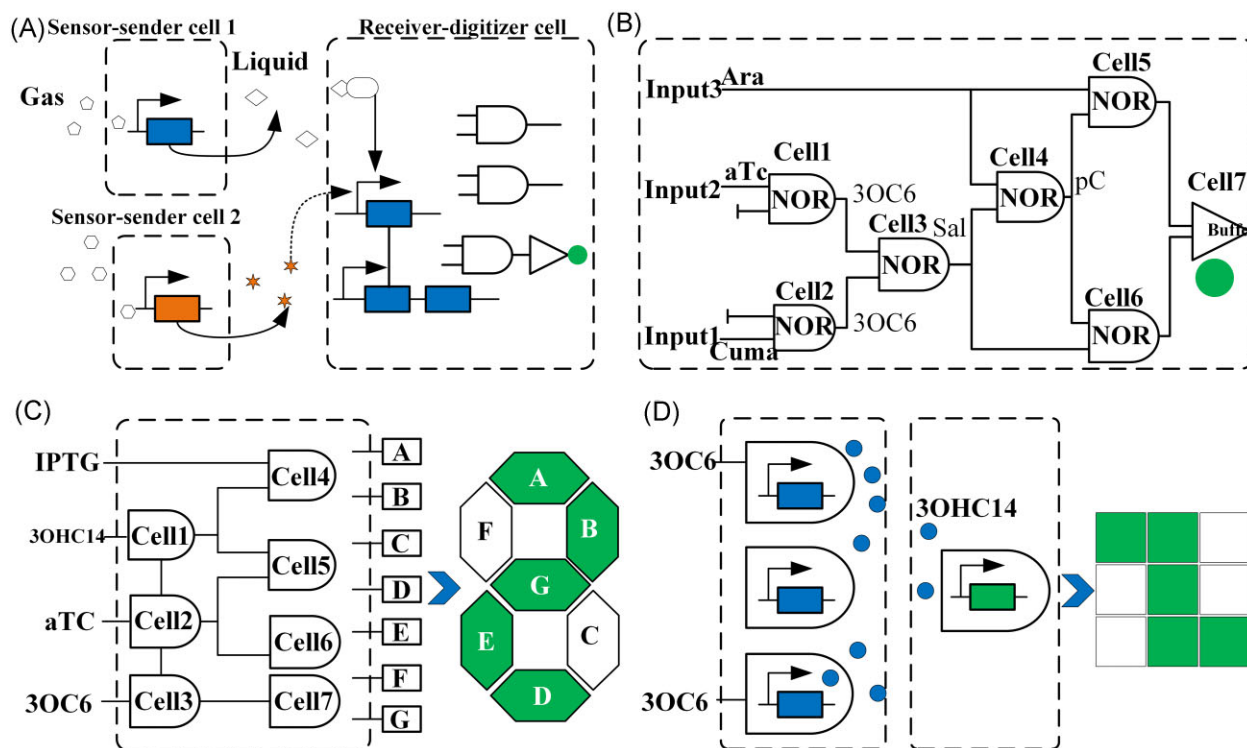


Figure 7. Illustrations of the assembling of microbial ecosystems for bio-computing. (A) Diagram of the fragrance-programmable analog-to-digital converter with Boolean expression logic, including sampling-and-quantization module, gas-to-liquid transducer, and the digitizer module with signal amplifier. (B) Diagram of 7-strain AND-XOR biocomputing circuit with three inputs and four orthogonal channels, i.e. anhydrotetracycline HCl (aTc), cuminic acid (Cuma), N-(3-Oxohexanoyl)-L-homoserine lactone (3OC6), and p-coumaroyl-HSL (pC). (C) Schematic diagram of the 7-strain digital display with four signal inputs, i.e. IPTG, N-(3-hydroxytetradecanoyl)-L-homoserine lactone (3OHC14), aTc, and 3OC6. (D) Schematic diagram of a QS-based perceptron network among two *E. coli* strains that based on the sending and receiving 3OC6 and 3OHC14 QS molecules.

(Fig. 9d). Therefore, different microbial ecosystems will provide us with important selections and strategies to face the major challenges of environmental safety.

As stated above, the rapid development of biotechnology in synthetic biology has expanded the engineering ability of microbial ecosystems, which can effectively realize diversified functions in different fields, including but not limited to the aforementioned bio-computing, bio-manufacturing, bio-therapy, and bio-remediation (Sgobba and Wendisch 2020, Jiang et al. 2023b). However, most of the applications were based on the cocktail of microbes, and metabolic division of biological pathways for the distribution of functions. The defined microbial communities should be designed and optimized more rationally based on some other strategies, such as QS devices, metabolite-based sensors, and chassis selection.

Chassis selection

It is reported that the diversity of synthetic biology circuits and the applicability of different tools are suitable for different chassis cells (Calero and Nikel 2019). With the development of synthetic biology, *E. coli*, *S. cerevisiae*, *Bacillus subtilis*, *Lactococcus lactis*, *Corynebacterium glutamicum*, and *Streptomyces* have acted as common microbial chassis to produce a bounty of products (Liu et al. 2020b). There are many efficient tools to engineer the aforementioned chassis strains and they have well-studied genomes and fast growth rates. Note that the understanding of chassis strains is still limited, and much remains unclear in the dynamics and properties of heterologous modules, thus leading to the difficulty in achieving the applications of optimal chassis. An ideal chassis should be an organism harboring the availability of genomic se-

quences and a relatively comprehensive metabolic network (Vickers et al. 2010). Moreover, a good chassis supports various genetic manipulations and modifications to scale up its application fields (Adams 2016). Chassis selection relies on microbial physiological characteristics, such as the tolerance to heat and high concentrations of products (Choi et al. 2019). Especially in the process of chemical production using toxic substrates or intermediates, the host's tolerance to environmental stress is an important condition for efficient production. In addition, many conventional chassis strains are not ideal hosts for bioproduction due to the lack of synthetic tools, low yields, slow growth rates, or vulnerability to environmental changes. In light of the inherent drawback and bottleneck of model chassis strains in heterologous expression of complex products, engineering non-model microbes to efficiently biosynthesize metabolites has attracted increasing attention. Therefore, the engineering and selection of chassis microorganisms are practically indispensable for metabolic engineering (Volk et al. 2023). To have a better design for microbial ecosystems, it is essential to select and deal with the basic problems of chassis cells, such as identifying optimal genetic modules, selecting a suitable host, investigating the metabolic network, and mastering genetic editing tools. More details can be referred to in other reviews that focus on the engineering and selection of chassis strains for natural products (Xu et al. 2020) or secondary metabolites (Liu et al. 2022b).

Interactions design

The investigation of microbial interactions of a community is a key issue in understanding the stability maintenance. Note that microbial interactions can be divided into six types: mutualism

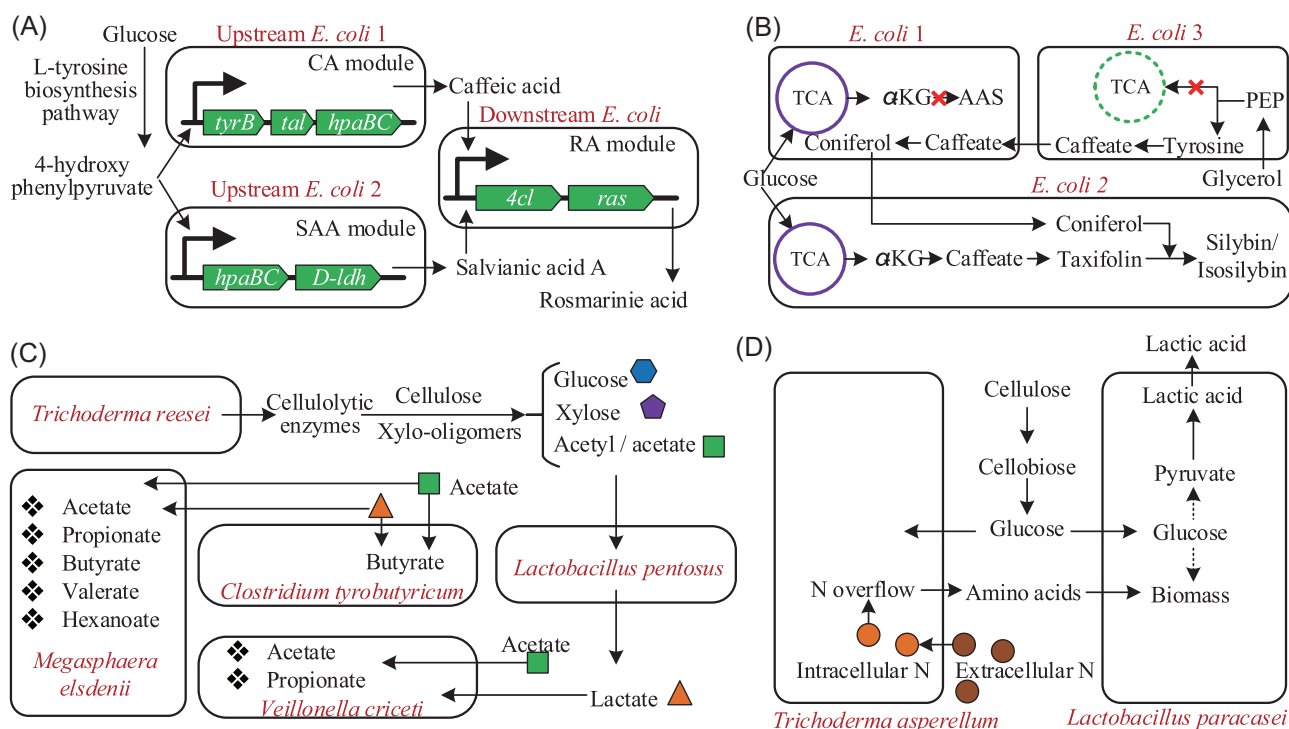


Figure 8. Illustrations of the assembling of microbial ecosystems for bio-manufacturing. (A) Diagram of division of labor for rosmarinic acid (RA) production, including caffeic acid (CA), salvianolic acid A (SAA), and RA module. (B) Schematic of the carbon metabolic flow in the three-strain ecosystem to produce silybin/isosilybin. *glpK* gene encoding glycerol kinase and glutamate synthesis genes were knocked out in strains 1 and 2, while genes involved in the glucose utilization pathway (*glk ptsG manXYZ*) and entry into the TCA cycle were knocked out in strain 3. (C) Schematic of the carbon metabolic flow in a microbial consortium-based consolidated bioprocessing strategy for the production of short-chain fatty acids from lignocellulose using lactate and acetate as central intermediates. (D) Schematic of the compartmentalization strategy on carbon metabolic and nitrogen metabolic flow for boosting lactic acid conversion from lignocellulose via consolidated bioprocessing.

(+/+), favoritism (+/0) or favoritism (-/0), parasitism or predation ($\pm$), competition (-/-) and neutrality (0/0) (Faust and Raes 2012). These interactions can have different impacts on related species, thus determining the corresponding community morphology (Liu et al. 2020a). For example, Zhou et al. redesigned the competitive relationship through carbon source allocation (xylose and acetate) as a mutualistic interaction between the two species (Fig. 10a) (Zhou et al. 2015). Specifically, glucose was initially used as the common substrate for the co-culture of *E. coli* and *Saccharomyces cerevisiae*, but the growth of *E. coli* was seriously affected by the ethanol produced by yeast. Therefore, xylose and acetate were added as carbon sources for *E. coli* and *S. cerevisiae*, respectively, and the co-culture interaction between them was changed, and the yield of target products was effectively improved. To investigate how interaction variability shapes succession of synthetic microbial communities, Liu et al. designed synthetic microbial consortia with three strains of *Lactococcus lactis* (named as Kp, C α , and C β) induced by environmental pH to quantitatively capture the dynamic changes of different communities (Fig. 10b) (Liu et al. 2020a). Liao et al. designed reciprocal inhibitory circuits mediated by toxin-antitoxin (TA) pairs in three *E. coli* strains, with each strain producing its own TA pair and the specific toxin against the next strain, similar to the rock-paper-scissors interaction model (Fig. 10c) (Liao et al. 2019). The same QS signaling molecules were applied to synchronize and coordinate the cell growth of different populations. This unique interaction pattern ensures genetic stability and species diversity of gene circuit function (Liao et al. 2020). Kong et al. conducted a systematic work for the design of

microbial consortia with six defined social interactions to establish social-interaction engineering, which was recognized as an effective and valuable route for microbial ecosystem programming (Fig. 10d) (Kong et al. 2018). Therefore, designing specific interaction relationships for synthetic microbial consortia will contribute to exploring the underlying mechanisms and potential applications.

Build module

The diverse toolbox of synthetic biology with new genetic elements enables us to engineer microorganisms with increasingly complex functional levels and increasing numbers of species. The biotechnology of modular assembly of gene regulatory elements and encoded synthetic enzymes is also constantly being updated. Building experiments for the regulations of microbial ecosystems is mainly based on various biological cross-talk or orthogonal circuits (such as genetic and protein circuits; more specific details have been listed in the aforementioned “Cross-talk regulation circuits” and “Orthogonal regulation circuits”) to achieve the corresponding regulations based on metabolic utilizations or communications. Note that, in order to better select and apply different circuits for various functions, we have summarized the characteristics, such as the chassis cells, advantages, drawbacks, and the potential applications of the aforementioned toolkits, in Table 2. For more of the latest regulations and synthesis pathways of assembly strategies, readers can refer to an extensive review (Young et al. 2021).

Table 2. Summarization of the characteristics of the cross-talk and orthogonal regulation strategies.

Types	Strategies	Chassis	Advantages	Limits	Applications
Cross-talk regulation	AHLs	Prokaryotic (G+/G-)	- Diversified and modular circuits - Easy to transform and utilize	- The intensity of crosstalk is affected by external culture conditions and internal expression intensity - Time and intensity of activation regulation are difficult to optimize	- Bio-computing - Bio-manufacturing - Bio-therapy - Bio-remediation - Others
	AIPs	Prokaryotic (G+)	- The mechanism is clear - Modular communication circuit	- Not portable - Exogenous natural products may block the AIP binding pathway	- Bio-computing - Bio-therapy
	AI-2	Prokaryotic and Eukaryotic	- Clear transduction pathway - Regulate many phenotypes	- Unstable structure of signals - It is difficult to quantitatively measure and add exogenously	- Bio-therapy - Bio-manufacturing
	Indole	Prokaryotic (G+/G-)	- Use indole to gain survival advantage - Easy access	- Concentration gradients lead to pleiotropic effects - Mechanism is not yet fully understood.	- Bio-therapy - Bio-remediation
	DSFs	Prokaryotic and Eukaryotic	- Diversity - Clear signal transduction pathway	- The interaction mechanism with plants is not yet fully understood - Few practical applications	- Bio-therapy - Bio-manufacturing
Orthogonal regulation	Others	Prokaryotic and Eukaryotic	- Diversity - Helpful for the analysis of microbe–host interactions	- Mechanism is not yet fully understood - Most of them are only qualitative analyses but not quantitative measurements	- Bio-therapy - Bio-manufacturing
	Biosensors	Prokaryotic and Eukaryotic	- Portable - User-defined - Diversity	- Poor versatility - Time-consuming and labor-intensive optimization process - Difficult to accurately quantify, and highly dependent on metabolic pathways	- Bio-computing - Bio-manufacturing - Bio-therapy - Bio-remediation - Others

Table 2. Continued

Types	Strategies	Chassis	Advantages	Limits	Applications
Types	RNAPs	Prokaryotic (G+/G-)	• High expression level • Strict regulation of genes • Simple operation	• Irrational distribution will lead to high metabolic burden on the host • Relatively narrow range	• Biocomputing • Bio-manufacturing
	RNA-based switches	Prokaryotic (G+/G-)	• High specificity • High affinity binding to various metabolites	• Time-consuming and costly for screening • Relatively low compatibility	• Bio-computing • Bio-manufacturing • Bio-therapy
	CRISPRs	Prokaryotic and Eukaryotic	• Versatility • Wide host applicability • High compatibility • Realize multi-stable and dynamic control	• Potential off-target effects • High expression causing cytotoxicity • Restricted target genes and larger proteins • Increase metabolic burden	• Bio-computing • Bio-manufacturing • Bio-therapy • Bio-remediation • Others
Types	DNA-binding regulations	Prokaryotic and Eukaryotic	• Modularization • Stabilize genes expression • High compatibility	• Lack of rational guidance from models • Trial-and-error optimization design is time-consuming and laborious	• Bio-computing • Bio-manufacturing • Bio-therapy
	Ribosomes	Prokaryotic (G+/G-)	• Strong orthogonality • Rapid regulation	• Limited to specific chassis organisms • Low screening efficiency	• Bio-manufacturing
	Riboswitches	Prokaryotic and Eukaryotic	• High dynamic range • Low crosstalk • Composability	• Single input type (trigger RNA) • Strictly designed nucleic acid sequence	• Bio-computing • Bio-therapy
Types	ncAAs-based aaRS/tRNA pairs	Prokaryotic and Eukaryotic	• Strong specificity • Diverse functions • High sensitivity	• Difficult to design and screen ncAAs are costly and difficult to synthesize • Limited dynamic range of regulation	• Bio-manufacturing • Bio-therapy • Bio-safety.
	Protein circuits	Prokaryotic and Eukaryotic	• Versatility • Flexibility • Rapid response (minutes)	• Lack composability capabilities • require <i>de novo</i> design or optimization • Poor portability	• Bio-computing • Bio-manufacturing • Bio-therapy

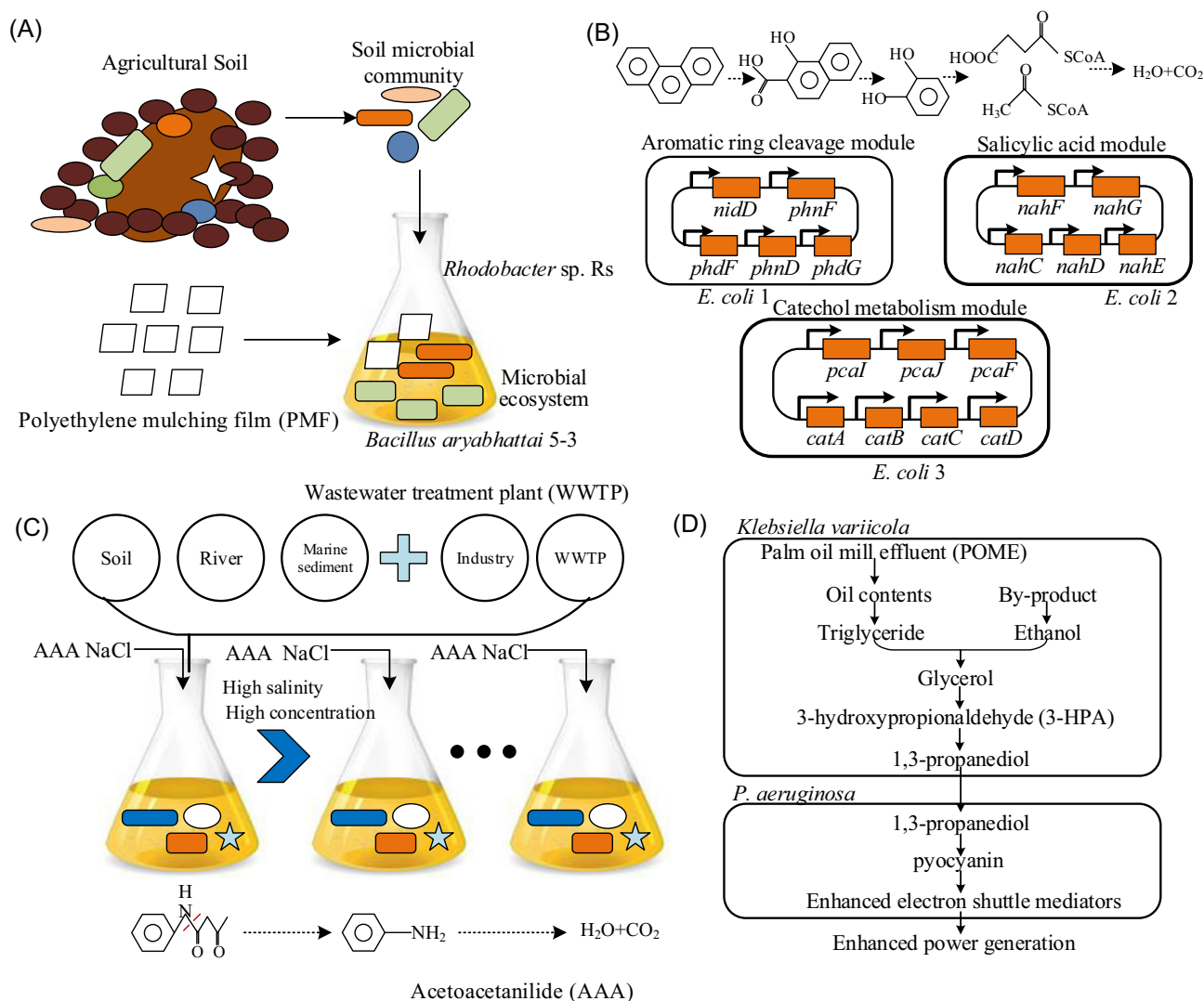


Figure 9. Illustrations of the assembling of microbial ecosystems for bio-remediation. (A) Schematic diagram of polyethylene mulching film (PMF) with the combination of *Rhodobacter* sp. Rs and *Bacillus aryabhattai* 5-3. (B) Illustration of division of labor in the degradation of phenanthrene in a consortium consisting of three *E. coli* strains. (C) Diagram of consortia-based bio-degradation of acetoacetanilide (AAA) in the treatment of wastewater, which is from industry, using a wastewater treatment plant (WWTP), etc. (D) Schematic of coculture of *Klebsiella variicola* and *P. aeruginosa* in bio-degrading palm oil mill effluent (POME) for a better performance of microbial fuel cells.

Test module

The test module for the assembly of microbial ecosystems involves using different tools to measure target phenotypes and evaluating characteristics to determine the efficacy of the design and build modules. Note that the tools for measuring target phenotypes have been comprehensively summarized in an extensive review (Lawson et al. 2019), which includes different high-throughput phenotypic screening technologies, such as multi-omics integration, isotopic tracers, mass spectrometry imaging, and microfluidics, etc. Therefore, in this section, we only introduce the summary for evaluating characteristics, including system stability, productivity, functional flexibility, and species diversity, etc., to determine the engineering efficacy.

Resilience is an important index of the stability of the microbial community, including elasticity and ecological amplitude. The former refers to the recovery rate of the microbial community, while the latter refers to the maximum deviation that the consortia can recover. Coyte et al. evaluated the stability of the microbial ecosystem from three aspects, namely, the possibility of the consortia

returning to the original state after a small disturbance, dynamic characteristics when facing specific disturbance, and the response time required for recovery (Coyte et al. 2015). A new microbial interaction model was developed by combining the consumption and production of the corresponding resources. Then the corresponding Jacobian matrix was used for determining whether the equilibrium was locally stable according to the eigenvalues of the matrix (Butler and O'Dwyer 2018). Di and Yang discussed the effects of different factors on the stability of microbial ecosystems in combination with substrate utilization and microbial interactions, and evaluated the effects from three aspects (Di and Yang 2019). The first is how quickly the consortium can recover and converge to the corresponding steady state after deviating from the stable state. The second is the range of the corresponding operating parameters when the consortium reaches a steady state. The third is to evaluate whether there will be multiple steady states or whether there will be stable oscillation behavior.

Except for the stability test, some other characteristics, such as diversity, productivity, and flexibility, were also focused on by

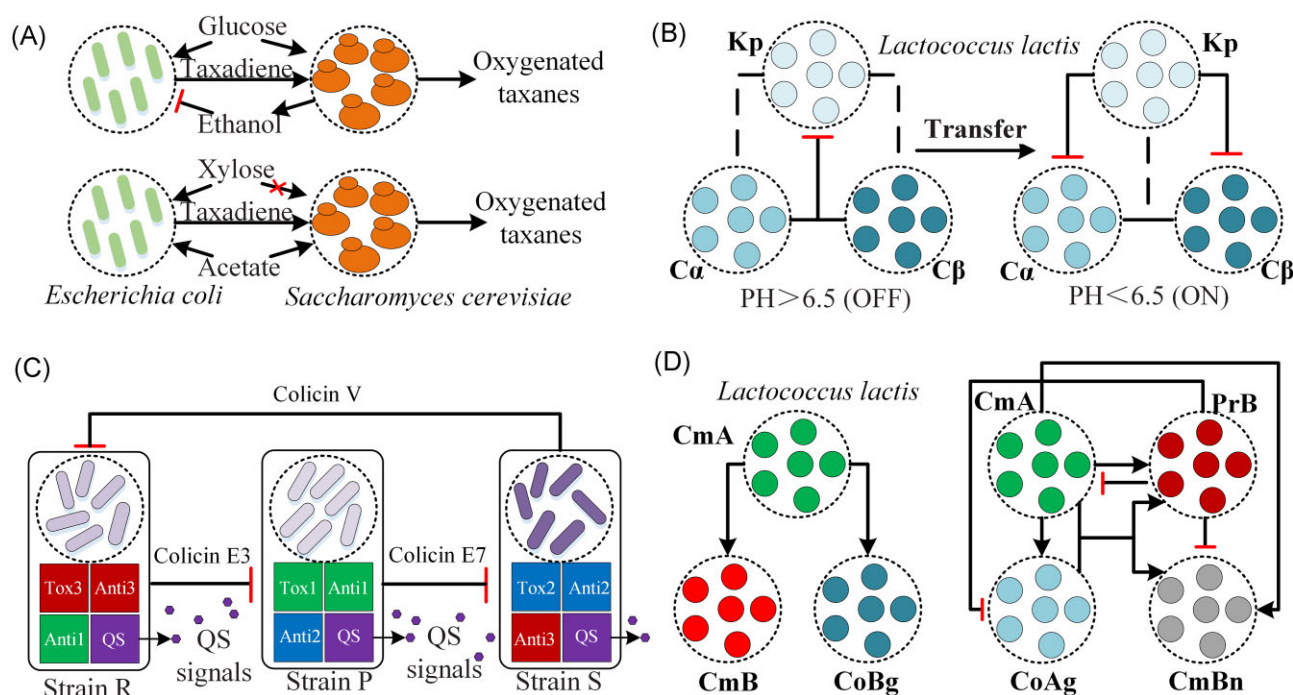


Figure 10. Microbial interactions design for different synthetic microbial consortia. (A) Interaction design of *E. coli*-*S. cerevisiae* consortium for production of oxygenated taxanes through the utilization of xylose and acetate. (B) Interaction design of the *L. lactis* α - β -Kp community in different pH conditions. The pH-response promoter dynamically realizes its switches from inactivation to activation with pH reduction. (C) Interaction topology of a rock-paper-scissors consortium. Each *E. coli* strain could kill or be killed by one of the other two strains by producing its own toxin-antitoxin pair and the toxin, such as the colicin E3, E, and V, against the next strain. (D) Interaction diagram of three- and four-strain consortia composed of different *L. lactis* strains. For example, there are commensalism relationships among CmA, CmB, and CmBn, as well as the complex interaction consisting of the two commensalism strains (CmA and CmBn), a predation strain (PrB), and a cooperation strain (CoAg).

some other researchers. For example, recently, Hu et al. proposed that the behavior of microbial ecosystems can be predicted by mastering only two community-scale control variables, i.e. ecological diversity and microbial interactions (Hu et al. 2022). They found that an increase in the number of species and the average interspecies interactions caused the microbial ecosystem to undergo phase transitions among three distinct dynamical phases, from a stable equilibrium of all species to a stable coexistence of some species, and finally to a continuous oscillation of species numbers over time. Productivity is one of the important indicators of interest in the field of metabolic engineering (Anesiadis et al. 2008). Therefore, Di and Yang have conducted a comprehensive analysis of productivity and stability for two-strain and three-strain communities, and found that there is a certain trade-off between the two objectives in synthetic microbial consortia (Di and Yang 2019). Besides, Libusha Kelly proposed that microbes can tune their functional output based on signals from surroundings, which was termed as functional flexibility (Segrè et al. 2023). They pointed out that understanding of the functional flexibility of the microbial community will help us identify the desired functions. In short, when assembling a specific consortium, we need to test as much as possible about its community-level characteristics in order to gain more knowledge for better optimizing the microbial ecosystems and realizing the specific function.

Learn module

The Learn module of the cDBTL cycle is essential for increasing the efficiency of design, build, and test modules. The new knowledge from the Learn module is evolved from the modeling analysis and specific optimization, which will be incorporated into the subsequent DBTL cycle.

Modeling analysis

The quantitative characterization and analysis of microbial ecosystems by mathematical models in the Learn module provide a unique perspective for deepening the understanding of the underlying physical and molecular mechanisms (Goryachev 2011). Recently, many researchers have reported that genome-scale metabolic models (GEMs) can provide more comprehensive perspectives for the deciphering and design of microbial communities. The combination of GEMs and a large amount of available omics data will provide better analysis for processing and designing microbial communities (Esvap and Ulgen 2021, Kim et al. 2022). Flow balance analysis (FBA), the specific modeling method of the GEMs, is usually used to predict the flux distribution and gene phenotype, which is often realized based on constraint reconstruction and analysis (or COBRA) (Heirendt et al. 2019). For example, Dukovski et al. developed a platform for the computation of microbial ecosystems in time and space (COMETS), which compartmentalizes and encapsulates metabolic models of different species into a given metabolic environment (Dukovski et al. 2021). Specifically, COMETS uses dynamic FBA to simulate the spatial distribution of a microbial community, and also introduces a diffusion model to predict the proportion of species, as well as the temporal and spatial dynamics of the community (Harcombe et al. 2014). OptCom, a metabolic model used to optimize the production of consortia, was developed to realize the co-cultivation of *D. vulgaris* and *M. maripaludis* to produce hydrogen energy (Zomorodi and Maranas 2012). SteadyCom, a metabolic model used to improve the stability of microbial communities, could be applied to solve the problem of unbalanced metabolic flux distribution (Chan et al. 2017). Moreover, FLYCOP provides a universal framework and high-precision kinetic metabolism models, which

could flexibly achieve multi-objective optimization, predict bacterial population ratio and amino acid secretion rate of different strains (García-Jiménez et al. 2018). For more details regarding the application of GEMs in understanding and designing interactions within microbial communities, please refer to our recent review (Wu et al. 2024).

Except for the GEMs, some other ecosystem modeling approaches, such as Lotka-Volterra (GLV), individual-based, and consumer–resource models, are also imperative for establishing the quantitative link between community structure and function. For example, the classical GLV, a minimal dynamical systems model of microbial communities, is a representative species-only population-level models with pairwise interactions. Note that the GLV model is relatively simple to construct because its parameters can be inferred from temporal or steady-state data of the community (Liu 2023). The construction of comprehensive metabolic models, such as individual-based and consumer-resource models, is inseparable from the application of microbiome tools, dynamic process control and monitoring. Specifically, van den Berg et al. provided a more detailed summary of how to select the appropriate model according to the characteristics of the need to predict (van den Berg et al. 2022). It should be pointed out that there is a trade-off between the number of parameters in the model of microbiota and the difficulty of obtaining experimental characterization parameters. The current multi-omics technology supplements and enriches the quantity and quality of existing models to a certain extent. The transcriptomics and proteomics provide model data of the gene expression process for building accurate models based on individuals, and enhancing the predictability of the assembling of various microbial ecosystems. Metabolomics under different environmental factors is conducive to the regulation of gene expression intensity and improves the long-term stability of the consortia. Due to limited space, for more details of mathematical modeling on the analysis of microbial community, readers can refer to some other excellent reviews (Colarusso et al. 2021, Esvap and Ulgen 2021, San León and Nogales 2022, van den Berg et al. 2022).

Global optimization

Certainly, the assembling of microbial ecosystems is not a simple combination of strains, but a comprehensive construction and optimization for different objectives (such as stability, robustness, diversity, productivity, and so on) with consideration of both internal interaction rules and external environmental changes. For example, Wortel et al. used the enzyme-flux cost minimization model to show that the trade-off between growth and yield is jointly determined by internal metabolic kinetics and external environmental conditions, so the global optimization of the system requires comprehensive consideration of multi-dimensions (Wortel et al. 2018). It should be pointed out that temporal characteristics (Ronda and Wang 2022), and spatial distribution (Ozgen et al. 2018), would affect the stability of the microbial community, which calls for optimization globally for the assembly of the microbial community. Therefore, optimization of the internal and external factors of the microbial community can be reflected by the common microbial composition, time, space, and their combination (Grandel et al. 2021) (more details in Fig. 11). The control mechanisms of the three dimensions were usually highly correlated, interdependent, and could be used in combination. Specifically, the QS-based consortium is time- and density-dependent in essence; the corresponding functions will only be activated when the population reaches a certain density. This indicates that the timing and composition optimization of the QS-based synthetic

consortium is a basic requirement for the assembling of different microbial ecosystems. Recently, we proposed and constructed QS language “interpreter” ecosystems in the linear and circular three *E. coli* strains, as well as optimizing them by combining strain-level microscopic and bulk-level macroscopic measurements in response to dramatic environmental changes (Wu et al. 2024).

Microbial ecosystems can be also dynamically manipulated and constructed with spatial distribution and multiple interaction topologies. For example, Shahab et al. designed a reasonable distribution of different strains according to different oxygen demands to achieve efficient production of lignocellulosic to SCFAs (Shahab et al. 2020). The study showed that the self-assembly of microbial ecosystems based on adhesion (Glass and Riedel-Kruse 2018) and the diffusion gradient of signal molecules (Boehm et al. 2018) would have a certain influence on the spatial control of different strains. Note that the rapid development of biological materials (An et al. 2023) has greatly enriched the regulations of synthetic microbial consortia, such as the use of hydrogels, 3D printing materials, and micro-encapsulation reactors, etc. In addition, the cultivation of the initial conditions (such as nutrition distribution, proportion of bacteria and total biomass, etc.) will interact with external conditions, by affecting the metabolism and growth fitness of community members. The initial conditions of the community are also important to improve the genetic stability of the microbial community so that different gene circuits can function better at different time scales (Ronda and Wang 2022). Furthermore, the differences in the external environment will cause different populations to use different resources in the system, thus enhancing the local internal interactions and avoiding the overall collapse of the ecosystem. Therefore, in the optimization of synthetic microbial consortia, different interactions should be designed, and environmental adaptation and spatial coordination of microbes should be carried out to improve their environmental adaptability. In the Learn module, efficient prediction of possible interactions and the potential structure of the community with the aid of different models are important for guiding the optimization of the function and stability of microbial ecosystems.

To sum up, the unclear internal interaction rules among microbes and the evolutionary mechanism of external environment adaptation greatly limit the rational design and wider application of microbial ecosystems (Lopatkin and Collins 2020). Relevant researchers have developed some good chassis strains, and constructed several co-culture systems to achieve the corresponding objectives and specific functions. However, the studies were relatively independent and scattered, and most of them only carried out simple combinations of strains through metabolic interactions or QS-based communications, without conducting quantitative, controllable, and stability analysis, let alone the optimization of the corresponding systems. Moreover, the influence of external environment on the dynamic characteristics of microbial ecosystems was not considered. Therefore, it is a good choice to assemble microbial ecosystems by following our proposed cDBTL procedure to select chassis strains, design internal interactions, build the corresponding ecosystems with cross-talk or orthogonal circuits, test performances, analyze relevant dynamics, and optimize ecosystems from composition, time, and space dimensions.

Concluding remarks and future perspectives

In summary, the design and optimization of complex ecosystems not only greatly improve the understanding of microbial inter-

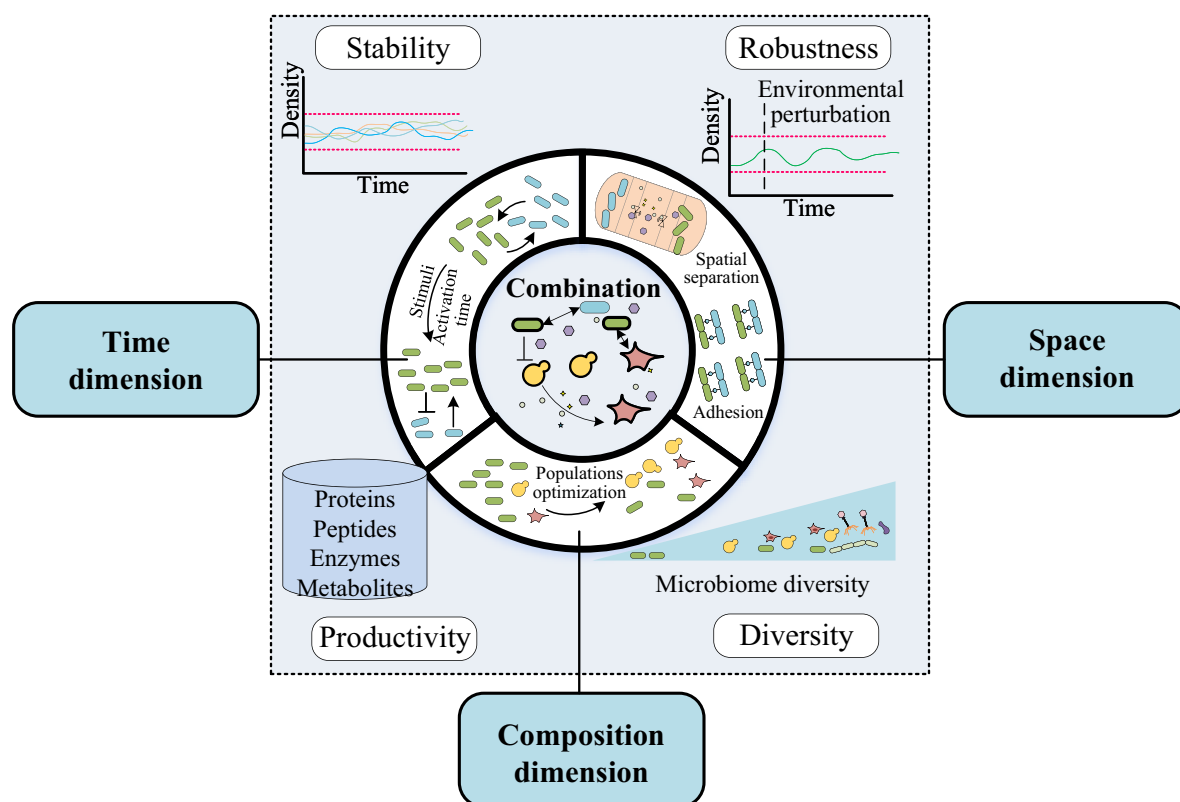


Figure 11. Schematics of global optimization for different objectives, such as productivity, stability, robustness, and diversity from time dimension, space dimension, composition dimension, and their combination. The time-based optimizations are often conducted using different activation moments (white left). The space-based optimizations are often conducted using adhesion and spatial separation (white right). The composition-based optimizations are often conducted by controlling different initial conditions for different populations (white below).

actions, but also transform them into various environmentally friendly biological products that can meet the needs of society. We envision that the *de novo* assembly of user-defined functional microbial ecosystems from molecular circuits to communities will be more widely used under the guidance of our proposed cDBTL procedure. Furthermore, the principle of bottom-up modular assembly contributes to a deep understanding of the deep-seated mechanisms between the structure and function of synthetic microbial communities. Finally, in view of the fact that there are many available cross-talk and orthogonal circuits for the design and construction of microbial ecosystems, various performances and modelings for the testing and learning of their dynamics and characteristics, future consortia-based biological applications will gradually enter the rapid development stage. There will be more challenges for further developing bottom-up assembly of functional ecosystems.

The development of a cross-talk toolkit

Compared with the traditional single-species culture, the microbial community has higher resistance and recovery ability in dealing with the invasion of heterogeneous strains and environmental disturbance (Lindemann et al. 2016). To obtain a predictable manner, the microbial consortia need to be rationally assembled by reducing the retrospective effect among modules, minimizing the interaction between heterogeneous loops and the host, and ensuring maximum signal transmission stability and fidelity. Therefore, in the past decades, most researches on microbial engineering were conducted based on the development and optimization of orthogonal channels to regulate different systems, without or

avoiding considering the existence of signal crosstalk. However, signal crosstalk is abundant in natural microbial communities. Moreover, bacteria have retained a large number of highly orthogonal signal transduction systems in the long-term evolution but also produced a large number of crosstalk regulation mechanisms (more details in “Cross-talk regulation circuits” and “Orthogonal regulation circuits”). Previously, our theoretical simulations for QS cross-talk communication showed that the combined application of most QS systems can effectively promote the rational allocation of metabolic flux among multiple strains (Wu et al. 2022b). However, compared with the ubiquitous QS system in nature, the number of well-characterized cross-talk elements is still the tip of the iceberg, which calls for more mining and quantitative characterization. At present, the molecular mechanisms of cross-talk regulations have not been fully and clearly characterized and understood. We propose some limitations hindering the development of a cross-talk toolkit: (i) the challenges involved in solving the cross-talk mechanisms and influencing factors behind the diversity of natural communities; (ii) quantifying the strength of crosstalk under given environmental conditions; and (iii) how to introduce and evaluate cross-talk regulation circuits for a specific function.

Simultaneous optimization of gene circuits and communities

The continuously developing orthogonal and cross-talk control systems are being rationally designed for constructing various functional microbial ecosystems with high stability and strong predictability. As a medium for sensing and transmitting signals, orthogonal and cross-talk modules with clear functions and

stable properties can be used to design and assemble different kinds of circuits to encode the functions of cells, just as electronic computers use strippable, modular standard components to perform complex logic operations. Note that most circuits in the past were designed, constructed, and optimized based on single-culture applications. At the same time, diverse microbial ecosystems were also designed, constructed, and optimized for many consortia-based applications at the community level. However, the synthesis of different genetic circuits and complex ecosystems was currently separated, which should be a unified whole. The optimization of gene circuits in single strains (local optimization) may not be suitable for the goals of the whole microbial community (global optimization). Therefore, synthetic ecosystems with reduced complexity and predictable circuit functions should be established from scratch with the optimization of gene circuits and communities simultaneously. Accordingly, we provide some difficulties for the simultaneous synthesis of genetic circuits and communities: (i) what principle should be adopted to model and design the synthesis of circuits and community; (ii) the challenges involved in determining the overall optimization objective, stability, productivity or both; and (iii) difficulties in the experimental fitting for some genetic circuits and chassis cells of microbial ecosystems.

The execution of the cDBTL cycle

Certainly, we need to select suitable chassis cells and design the specific interaction types to construct the microbial ecosystems based on the expected functional requirements. For example, the non-model strain *Yarrowia lipolytica*, with strong lipid accumulation capacity and excellent physiological tolerance, can be selected according to different functional demands acting as the tailored strain in different microbial ecosystems (Park and Ledesma-Amaro 2023). When constructing a synthetic microbial consortium for metabolic engineering, we tend to introduce symbiotic or cooperative interactions into the target ecosystem to maintain maximum metabolic flux flow to the target product, as well as the system stability. At the same time, the assembling of microbial communities must also take into account the inseparable natural evolution and external controllable conditions on the time and space scale. The predictable output of the expected function can be achieved to a certain extent through the optimization and expansion of the model. Therefore, to obtain quantitative relationships in ecology, different mathematical models should be combined with experiments to get better performances. According to the expected functional requirements, after designing and selecting specific components, the assembly of microbial ecosystems needs to meet the controllability of the systems, as well as system biosafety. Biosafety-controlled methods include the use of toxin-antitoxin systems, regulation of responses to specific environmental factors, the addition of amino acids essential for growth or unnatural amino acids, and gene mutation suppression.

Further applications of microbial ecosystems

In general, consortia-based applications in various fields are just emerging, and many cases are at a preliminary stage. The research of bio-computing focuses on the logic gates encoded by cells with multi-level inputs, which only cares about the performances of the accurate functional outputs, but not the specific molecular mechanism. Consortia-based bio-manufacturing has been extended into the production of natural products, biofuels, commodity chemicals, and so on. However, there are still great challenges in controlling the coordination of microbial ecosystems to

maintain the stability, density control desirably, respond to environmental changes timely, and achieve efficient production. Great progress has been made in the diagnosis of engineering bacteria *in vivo*, while the functional stability and genetic stability of chassis, as well as the necessary biological control, are difficult to be widely used in clinic. Because of the highly complex metabolic environment in the organism, the robust design of the sensor against the change of homeostasis and the interference of analog molecules must be considered. Note that there are few cases of interaction-based design and construction of synthetic consortia for corresponding medical applications. This may be because microbial stabilization and editing are inherently challenging, not to mention carrying them out in the complex environment of gut system. Similarly, most of the microbial ecosystems were not properly designed and optimized for the bio-remediation, and were constructed simply based on cocktail composition. In order to expand application fields, it is necessary not only to increase the efficiency of gene or protein circuits, but also to understand and realize the coordination of the corresponding microbial community. Indeed, the activity and abundance of protein can be quickly adjusted by signal processing and transduction through highly orthogonal combinable and detachable protease elements (Chen and Elowitz 2021). Furthermore, some communication-based regulations, such as QS molecular mechanisms, can realize the dynamic control of various microbial ecosystems (Wu et al. 2021b). To rationally engineer and design the complex communications, the QS systems in non-model microorganisms (e.g. human gut microbes) are not well studied experimentally, leading to another gap that needs to be addressed. Thanks to the excellent performance of effective orthogonal and cross-talk circuits, as well as diverse interaction networks [such as the QS-based communication network (QSCN)], consortia-based applications will be quickly developed in the future. Finally, we envision that future understanding and applications for microbial ecosystems lie in the development of co-culture techniques (Cao et al. 2023), genome editing of non-model gut microbiota, such as *Bacteroides* (Zheng et al. 2022), construction of comprehensive interaction networks (such as QSCN) (Wu et al. 2022a), the combination of top-down deciphering approaches and some other data-driven techniques.

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