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Engineering and modification of microbial chassis for systems and synthetic biology

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ABSTRACT

Engineering and modifying synthetic microbial chassis is one of the best ways not only to unravel the fundamental principles of life but also to enhance applications in the health, medicine, agricultural, veterinary, and food industries. The two primary strategies for constructing a microbial chassis are the top-down approach (genome reduction) and the bottom-up approach (genome synthesis). Research programs on this topic have been funded in several countries. The 'Minimum genome factory' (MGF) project was launched in 2001 in Japan with the goal of constructing microorganisms with smaller genomes for industrial use. One of the best examples of the results of this project is *E. coli* MGF-01, which has a reduced-genome size and exhibits better growth and higher threonine production characteristics than the parental strain [1]. The 'cell factory' project was carried out from 1998 to 2002 in the Fifth Framework Program of the EU (European Union), which tried to comprehensively understand microorganisms used in the application field. One of the outstanding results of this project was the elucidation of proteins secreted by *Bacillus subtilis*, which was summarized as the 'secretome' [2]. The GTL (Genomes to Life) program began in 2002 in the United States. In this program, researchers aimed to create artificial cells both *in silico* and *in vitro*, such as the successful design and synthesis of a minimal bacterial genome by John Craig Venter's group [3]. This review provides an update on recent advances in engineering, modification and application of synthetic microbial chassis, with particular emphasis on the value of learning about chassis as a way to better understand life and improve applications.

1. Introduction

With advances in the genomic revolution and the rise of systems biology, synthetic biology has experienced dramatic growth throughout the past decade in extent, expectation and output [4,5]. The engineering and modification of microbial chassis is accelerated by systems and synthetic biology for use in further fundamental research and applications in the biotechnological, pharmaceutical, biomedical and other fields [6]. In systems biology, an ideal chassis could be an organism harboring a simplified genome for full functionality and a metabolic network able to more effectively synthesize the desired products [5,7]. In synthetic biology, a chassis refers to an organism that houses and supports genetic components by providing the resources that allow them to function [8–10].

Attempts to construct a synthetic microbial chassis can be classified into two complementary and alternative approaches: top down and bottom up. Top-down approaches are also strategies to reduce genomes by removing unnecessary cellular genes to learn about genome architecture and improve its characteristics [11,12]. Based on the advent of large-scale DNA analysis, comparative analysis of genomes, especially those from diverse organisms, can often reveal genes that are indispensable for cellular lives and similar and/or distinctly different metabolic pathways [13]. Next, deletions can be achieved using different experimental strategies, including plasmid and linear DNA-mediated procedures and the use of site-specific recombinases, transposons, and the CRISPR/Cas system (Fig. 1). In the laboratory, such reductions have been pursued in *Escherichia coli* [1,14–21], *Streptomyces* [6,23–27], *Bacillus subtilis* [28–32], and *Pseudomonas putida* [33–35]. Some of these chassis displayed virtually unaffected physiological features, and some showed unexpected properties (Table 1). However, this approach also

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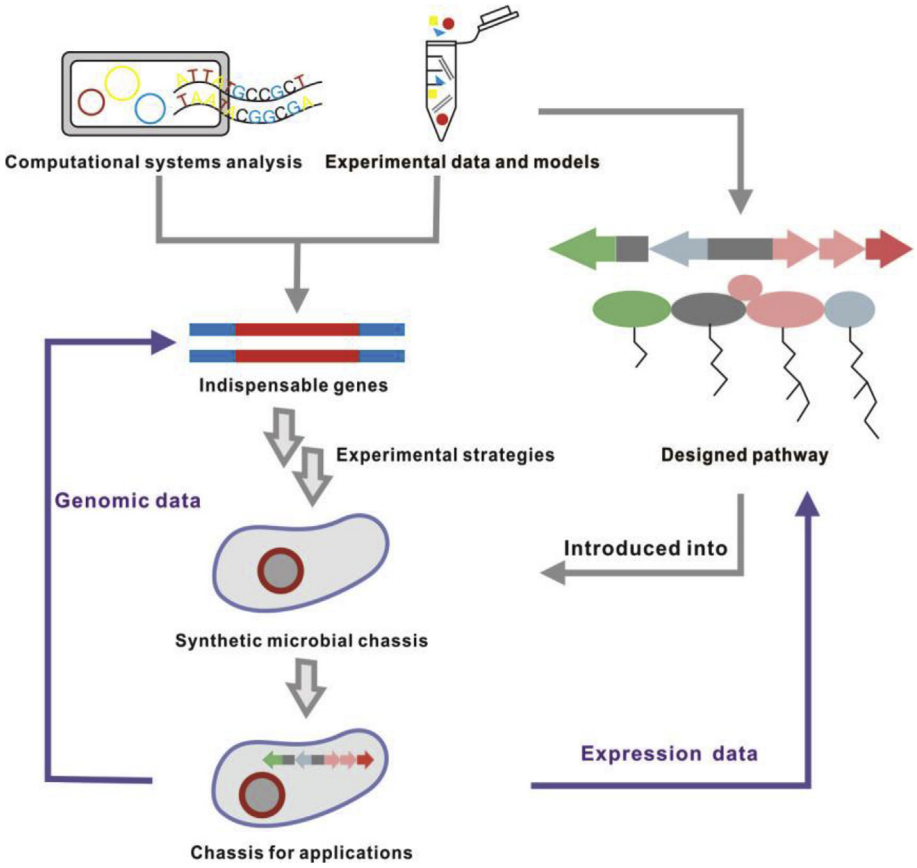

Fig. 1. Schematic illustration of engineering and modification of synthetic microbial chassis using a top-down strategy. The advent and use of computational systems analysis and experimental data and models can often reveal genes that are indispensable for cellular life. Subsequently, synthetic chassis can be generated by removing non-essential genes and then be verified in downstream applications. Genomic data in applications can be of further benefit for optimizing chassis and pathways.

comes with limitations inherent to the starting organism and is largely empirical and time consuming [36].

Conversely, bottom-up approaches try to construct chassis that could plausibly self-assemble into artificial chassis from scratch [37]. The *de novo* synthesis of long DNA sequences containing complex gene compositions can be achieved due to advances in DNA synthesis, sequencing technologies and transplantation. *De novo* synthesis methods for long DNA molecules and even the whole genome are primarily based on the use of polymerase chain reaction (PCR) technology to assemble pools of overlapping short oligonucleotides (Fig. 2). Those techniques allowed for the complete reconstruction of a whole genome and a new synthetic microbial chassis (Table 1) [38,39].

In the present review, we focus on advances in the research on synthetic microbial chassis and their potential in systems and synthetic biology frameworks to emphasize the value of learning about chassis as a way to understand the universal principles of life and to enhance applications.

2. Top-down approach in systems and synthetic biology

2.1. *Escherichia coli*

The peculiar characteristics of *E. coli* such as its clear genetic background, ease of handing, and potential for industrial and medical applications make it an important host for engineering chassis [40]. *E. coli* K-12 is one of the most thoroughly analyzed organisms and is the choice of chassis for genetic, biochemical and metabolic research. Most *E. coli* chassis were derived from two closely related K-12 strains, MG1655 and W3110 [41,42]. Since the first report of a genome-reduced *E. coli* strain that was published in 2002 [18], the deleted genome size has been improved from 5.6 to 38.9%.

To construct multiple-deletion series (MDS) strain 12, a rapid and straightforward method was developed [18]. A PCR-generated DNA fragment was inserted into the genome by λ red-type homologous recombination, followed by a double-strand break (DSB)-stimulated recombinational repair process, resulting in a scarless deletion. The genome of 12 deletion strains (MD1 to MD12) were generated from the MG1655 genome by removing most of the potentially "selfish" DNA (cryptic prophages, phage remnants, and insertion sequences [ISs]) and a large fraction of genes of unknown functions. The growth characteristics and transformability of MDS12 were essentially identical to those of wild-type MG1655. Beginning with strain MDS12, nearly 100 proposed deletions (20% of the K-12 genome) were identified by a series of genomic sequence comparisons and subsequently deleted via λ red-type homologous recombination [19]. The resulting strains, MDS41, 42, and 43, were confirmed to be IS-free, with total deleted lengths of 662.6, 663.3 and 708.3 kb, respectively. Their growth rates were similar to that of their parent, MG1655. The electroporation efficiencies of MDS42 equaled or exceeded those of the purchased strains used for transformation. However, the latest study confirmed that genome reductions in MDS42 did not stabilize the chassis under metabolic stress [20]. For longer-term applications or for proposed environmental biotechnologies, further chassis modifications are required. It would be of great use to introduce phosphorothioate (PT) modification, in which the non-bridging oxygen in the phosphate moiety of the DNA sugar-phosphate backbone is replaced by sulfur, to protect oligonucleotides against nuclease degradation [43–45]. DNA PT modification consists of two parts: DndABCDE functions as the M component of a restriction-modification (R-M) system that protects cells against invading foreign DNA, whereas DndFGH, together as the R component, recognize and hydrolyze non-PT-protected foreign DNA [46,47]. The function of some proteins related to PT modification has been investigated to unravel the biological function of the DNA PT system [48]. Recently, PT modification has been considered to be versatile player involved in epigenetic gene regulation and in the maintenance of cellular redox homeostasis, which could help maintain the stability of a simplified chassis

Table 1
Characteristics of synthetic microbial chassis.

Top-down Approach			
Name	**Deletion**	**Characteristics**	**References**
Escherichia coli			
MDS12	376 kb (8.1%)	Similar growth characteristics and transformability	Kolisnychenko et al. [18]
MDS42	663 kb (14.3%)	Higher electroporation efficiency	Pósfai et al. [19]
MGF-01	1030 kb (22.2%)	Higher final cell density (1.5-fold), higher L-threonine production (2.4-fold)	Mizoguchi et al. [50]
DGF-327	1380 kb (30.9%)	No insertion sequences (ISs), no auxotrophy phenotype, and better growth fitness and cell yield	Hirokawa et al. [52]
DGF-298	1670 kb (37.4%)	No insertion sequences (ISs), no auxotrophy phenotype, and better growth fitness and cell yield	Hirokawa et al. [52]
BLK01-16	410 kb (9%)	The loss of Ap^R plasmids, high cellular and genomic stability, and improved transformability	Umenhoffer et al. [54]
Streptomyces			
S. avermitilis SUKA17	1670 kb (18.5%)	Normal growth rate, stable genome, higher streptomycin and cephamycin C (2.7-fold) production	Komatsu et al. [26]
S. coelicolor M1152 & M1154	173 kb (2%)	Chassis for efficient heterologous gene expression	Gomez-Escribano et al. [61]
Streptomyces sp. FR-008 LQ3	150 kb (2.1%)	Chassis for heterologous expression of desired products	Liu et al. [62]
Pseudomonas putida			
407.1-Δ_2	272 kb (4.4%)	Similar or better growth	Leprince et al. [69]
407.3-Δ_2	457 kb (7.4%)	Similar or better growth	Leprince et al. [69]
Bacillus subtilis			
Δ6	320 kb (7.7%)	Normal growth rate	Westers et al. [30]
MG1M	991 kb (23.5%)	No marked morphological change	Ara et al. [32]
MGB874	874 kb (20.7%)	Remarkable improvement in the productivity of extracellular cellulase (1.7-fold) and protease (2.5-fold)	Morimoto et al. [77]
Schizosaccharomyces			
S. pombe	500 kb (4.0%)	Enhanced human growth hormone production (30-fold)	Giga-Hama et al. [84]
Saccharomyces cerevisiae			
MFY1158	472 kb (3.9%)	Sensitive to low temperatures; higher glycerol production (2.0-fold)	Murakami et al. [88]
MFY1160	531 kb (4.4%)	Slightly higher ethanol and glycerol production	Murakami et al. [88]
MFY1162	281 kb (2.3%)	Higher ethanol production (1.8-fold)	Murakami et al. [88]
SY14	–	Only one chromosome, nearly identical transcriptome and similar phenome profiles	Shao et al. [91]
n = 2 strain	–	Carrying two chromosomes, modest transcriptomic changes and without major growth defects	Luo et al. [92]

Bottom-up Approach			
Name	**Genome Size**	**Strategy**	**References**
Mycoplasma genitalium JCVI-1.0	583 kb	Overlapping "cassettes" of 5–7 kb were assembled from chemically synthesized oligonucleotides and joined by *in vitro* recombination to produce intermediate assemblies of approximately 24 kb, 72 kb ("1/8 genome"), and 144 kb ("1/4 genome"), which were assembled to form a complete synthetic genome by transformation-associated recombination cloning in the yeast *Saccharomyces cerevisiae*	Gibson et al. [108,109]
Mycoplasma mycoides JCVI-syn1.0	1.08 Mb	After design and synthesis, syn1.0 genome was transplanted into a *M. capricolum* recipient cells to create new *M. mycoides* cells	Gibson et al. [98]
Mycoplasma mycoides JCVI-syn3.0	531 kb	Starting from syn1.0 genome, JCVI-syn3.0 was generated through three more cycles of design, synthesis, and testing, with retention of quasi-essential genes	Hutchison et al. [3]
Saccharomyces cerevisiae			
SynIII	273 kb	BB synthesis, assembly of minichunks, direct replacement of native yeast chromosome III, TAG/TAA stop codon substitutions and introduction of loxPsym sites and PCRTags	Annaluru et al. [116]
Ring SynV	536 kb	A clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR associated protein 9 (Cas9)-based method	Xie et al. [113]

for further studies and actions [49].

Another representative reduced-genome *E. coli* strain, MGF-01, was constructed using a λ Red recombination system and the negative selection marker *sacB* (a markerless deletion method) [50]. With a comparative analysis of the genome sequences of *E. coli* K-12 MG1655 (accession no. U00096) [41] and *Buchnera* sp. APS (accession no. NC002528) [51], 103 regions were selected, and the genome size was reduced by 22% (1.03 Mb) [1]. MGF-01 grew to a final cell density in M9 minimal medium that was 1.5 times higher than that of W3110red. Furthermore, MGF-01 also exhibited a 2.4-fold increase in L-threonine production. With further reduction of the genome of MGF-01, the strains DGF-327 and DGF-298 were constructed without any ISs and had reduced genome sizes of 3.27 and 2.98 Mb, respectively [52]. During the mutant construction, intrinsic mutations in *ilvG* and *rph* were functionally restored to enhance initial growth after inoculation. Both strains showed no auxotrophy phenotype and had better growth fitness, especially during the initial growth phase, and a better cell yield in a rich medium than the wild-type K-12 strain.

Recently, CRISPR/Cas-assisted multiplex automated genome engineering (MAGE) was developed for rapid editing of host genomes, and its usefulness was demonstrated in *E. coli* BL21(DE3), a strain routinely used for high-level plasmid-based recombinant protein production [53,54]. The same deletions in MDS strains [19] were transferred into a chosen recipient genome (BL21) within months using P1 transduction-aided genome shuffling. Then, the novel CRISPR/Cas-assisted MAGE method rapidly abolished the 30 remaining copies of ISs by inserting and selecting for an in-frame TAATAGG (combined double-stop and frame-shift mutations) insertion. All 9 resident prophages and 50 active ISs were deleted from the genome of BL21 (DE3). The engineered strains have three major improvements. First, regarding the loss of Ap^R plasmids, the chassis facilitates the use of this common selection marker for plasmid maintenance. Second, BLK16 and especially BLK17

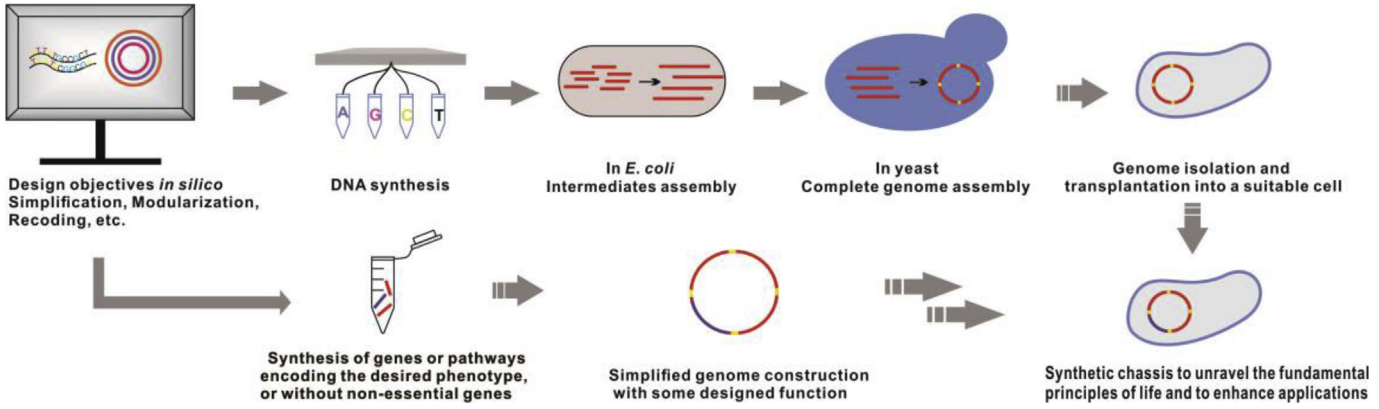

Fig. 2. Construction of synthetic microbial chassis by a bottom-up strategy. The first row shows genome building by means of synthesis and cloning in *E. coli* and yeast and testing for viability by means of genome transplantation. The second row shows the further design of genes, pathways or genomes with a desired phenotype, followed by the use of the same methods to construct optimal synthetic chassis.

demonstrate high cellular and genomic stability and are recommended for chromosomal or plasmid-based high-fidelity expression of heterologous enzymes or complete pathways. Finally, BLK16 unexpectedly showed improved transformability.

2.2. Streptomyces

A prominent property of the *Actinomycetes* and in particular *Streptomycetes* is the ability to produce novel natural products that have proven value in human and veterinary medicine and agriculture [55,56]. During the postgenomic era, the vast (meta) genomic data collected reveal that *Actinomycetes* has large genomes that encode multiple secondary metabolite biosynthetic gene clusters, most of which remain cryptic [55]. However, most of these strains have been shown to be recalcitrant to genetic manipulation. Thus, it is necessary to develop some versatile model chassis as hosts to express heterologous secondary metabolite pathways, especially for the activation of cryptic gene clusters to aid in drug discovery. Such hosts, without redundant secondary gene clusters, were predicted to remove competing carbon and nitrogen sinks, increasing precursor availability and the productivity of heterologous cloned gene clusters [57,58].

Streptomyces avermitilis is a producer of the anthelmintic agent avermectin with a linear chromosome of 9.02 Mb [59]. Comparative analysis of three published *Streptomyces* genomes, *Streptomyces coelicolor* A3 (2), *Streptomyces griseus* and *Streptomyces avermitilis*, revealed a conserved core region of 6.28–6.50 Mb, where the genes are essential for growth [26]. The left and right subtelomeric regions are 2 Mb and 0.5 Mb in length, respectively, and contain strain-specific genes, genes encoding secondary metabolite biosynthesis and no essential genes. More than 1.4 Mb of subtelomeric regions was deleted using general homologous recombination or site-specific recombination (Cre-*lox*P) techniques to obtain a series of multiple-deletion mutants whose chromosomes correspond to 83.12–81.46% of the wild-type chromosome. The mutants could grow on minimum medium without additional supplements, suggesting that there are no essential genes in the deletion regions. Furthermore, these mutants did not produce any of the major endogenous secondary metabolites and were successfully used as chassis for efficient heterologous gene expression at higher levels than those of the native producers, resulting in the production of exogenous natural and unnatural metabolites [27].

Streptomyces coelicolor is a model strain that was used in the development of the earliest genetic recombinant techniques for heterologous gene transfer [60]. Four antibiotic gene clusters were deleted from *Streptomyces coelicolor* M145 (a derivative of the wild-type strain A3(2) lacking plasmids SCP1 and SCP2) by homologous recombination. Next, the introduction of *rpoB*[S433L] (conferring resistance to rifampicin) and in addition *rpsL*[K88E] (conferring streptomycin resistance) yielded *Streptomyces coelicolor* M1152 (Δ*act* Δ*red* Δ*cpk* Δ*cda rpoB* [C1298T]) and M1154 (Δ*act* Δ*red* Δ*cpk* Δ*cda rpsL*[A262G]). Although biomass yields obtained using M1152 and M1154 are less than those of M145 in liquid culture, both strains grow similarly to the parental strain after being inoculated from seed cultures. The study showed M1154 was observed to often provide the highest levels of antibiotic production, and M1152 sporulates better than the wild-type strain, facilitating strain preservation and manipulation [61]. *Streptomyces* sp. FR-008 is a fast-growing microorganism that is a potential industrial production chassis, producing a considerable amount of the macrolide candicidin via a modular polyketide synthase [62]. First, the 7.26-Mb length of the *Streptomyces* sp. FR-008 genome was confirmed using PacBio sequencing and transcriptome analyses, which indicated that this genome was one of the smallest sequenced *Streptomyces* genomes. A mutant (LQ3) was then constructed by deletion of three endogenous polyketide synthase (PKS) gene clusters comprising 150 kb of the chromosome. LQ3 has a much more stable and streamlined genome and allows for simpler and more efficient separation and purification processes for biotechnological applications.

2.3. Other prokaryotes

Pseudomonas putida, a ubiquitous gram-negative soil bacterium, meets many of the criteria for the generation of synthetic biology chassis, including its metabolic diversity, robustness and ease of manipulation [63–66]. In addition, *Pseudomonas* species naturally produce multifarious secondary metabolites, including rhamnolipids, terpenoids, polyketides, and nonribosomal peptides and other amino acid-derived compounds [34,35,67]. *P. putida* KT2440 is the best-characterized saprophytic laboratory *Pseudomonas*, and its entire chromosome sequence has been available since 2002 [33,68]. Based on this strain, four double-deletion mutants, with up to 7.4% of a single genome deleted over two cycles, were constructed using a combinatorial method. This recyclable three-step excision method is based on a combination of customized mini-Tn5 transposons and the FLP-*FRT* site-specific recombination system [69]. Strains 407.1-Δ_2 and 407.3-Δ_2 showed similar or better growth than the wild-type strain in LB medium, with up to 1.4-fold higher final cell densities than TEC1.

Comamonas is a genus of gram-negative species that often contributes to bioremediation and material cycling due to its ability to sense and pursue xenobiotic compounds through chemotaxis [70,71]. *Comamonas testosteroni* CNB-1 is a common *Comamonas* strain that is a highly promising candidate for use as a versatile and programmable chassis owing to genomic and proteomic information obtained for this bacterium, as well as its genetic and biochemical backgrounds [71–73].

C. testosteroni CNB-1 harbors the plasmid pCNB1, a 91-kb indigenous plasmid that can interfere with the metabolic performance of the host and with the transformation efficiency and expression of exogenous plasmids. Thus, pCNB1 was successfully eliminated using a novel plasmid curing strategy that included the use of a rare-cutting homing endonuclease, a selectable marker and a counter-selection system. The generation of a plasmid-free strain was subsequently verified to increase the ability to genetically manipulate the *C. testosteroni* CNB-1 chromosome using a PCR-based *Cre-lox*P system [74].

Bacillus subtilis, a nonpathogenic gram-positive bacterium, is well known for its superior capacity for protein secretion [28]. In addition, the wide range of natural two-component systems and quorum-sensing systems present in *Bacillus* species have made them preferred chassis organisms for the industrial production of a variety of products, including chemicals, biopolymers, and proteins [2,28,75,76]. The resulting strain *B. subtilis* Δ6, lacking two prophages (SPβ, PBSX), three prophage-like regions, and the largest polyketide synthase (*pks*) operon, was constructed using stepwise deletion [30]. Thus, the genome was reduced by 7.7% with the elimination of 332 genes. Metabolic flux analysis, proteomics, and specific assays for protein secretion, competence development, sporulation, and cell motility showed that removal of dispensable genes may pave the way towards an optimized *Bacillus* cell factory [30]. The MG1M strain was constructed by deletion of 11 phage, phage-like and antibiotic-production-related gene regions for approximately 1 Mb (991 kb) [32]. The MG1M strain, which is deleted of approximately 24% of the *B. subtilis* genome, showed no marked morphological changes but did exhibit changes in the expression of extracellular proteins, with a notable increase in heterologous amylase secretion [32]. Another novel *B. subtilis* strain, MGB874, lacked 874 kb (20%) of the sequence from the original *B. subtilis* 168 by step-by-step genome deletions [77]. The *upp* (encoding uracil-phosphoribosyltransferase) cassette and 5-fluorouracil (5-FU) selection were applied to remove the drug-resistant markers used to introduce primary deletions. MGB874 cells and further systematic analysis are used in industry due to their remarkable improvement in the productivity of extracellular cellulase (1.7-fold) and protease (2.5-fold) [77].

2.4. Eukaryotes

Among all known microbial bioactive compounds (22,500) [78], 38% are from fungi. Thus, it is necessary to generate novel yeast chassis for the production of high-value secondary metabolites used as antibiotics, other medicines, toxins, pesticides, and animal and plant growth factors [79,80].

The fission yeast *Schizosaccharomyces pombe*, with the ability to proliferate by fission and sharing many molecular, genetic and biochemical features with higher eukaryotes, such as plants and animals, is distinguishable from other yeasts [81]. *S. pombe* has gradually become an attractive host to express an increasing number of membrane and secretory proteins and cytoplasmic proteins [82]. However, the degradation of the recombinant gene products by host-specific proteases in *S. pombe* has a significant impact on the productivity and secretion efficiency of heterologous proteins [83]. The construction of multiple protease-deficient chassis is an effective method to avoid these problems [81]. Based on the *S. pombe* genomic database (http://www.genedb.org/genedb/pombe/), over 200 genes are known to be related to proteolytic bioprocesses [84]. Fifteen multiple-deletion mutant strains were constructed by the successful deletion of seven protease genes using repeat fusion PCR-based gene disruption and 5-fluoroorotic acid-mediated ura4-negative selection. The production of a proteolysis-sensitive model protein, the human growth hormone (hGH), was enhanced up to approximately 30-fold in the resultant mutants compared with the control.

Saccharomyces cerevisiae [85], the first eukaryotic genome to be completely sequenced, is among the most widely used model organisms to understand mammalian genes and for use in biotechnological processes ranging from bioethanol to insulin production [86]. A large knowledge base and considerable expertise with this fungus in both academia and industry make it an attractive chassis for producing new products [87]. To identify essential genes for yeast growth and to efficiently produce ethanol, a computer program was developed to predict the deletable regions in the yeast genome. Next, PCR-mediated chromosome splitting (PCS) was carried out to manipulate the yeast genome on a large scale, with mutants losing approximately 5% of the genome. The deletion mutants (MFY1158, MFY1160, and MFY162) provided increased yields of ethanol and glycerol while showing levels of resistance to various stresses that were nearly equivalent to those of the parental strain [88]. Modularized chassis of *Saccharomyces cerevisiae* were also designed to produce different compounds for industrial interest by combining precursor and cofactor availability and promoter exchange to construct an inducible pathway and resistance mechanisms to cope with biomolecule production [80].

The great achievements in *Saccharomyces cerevisiae* were driven by various advances in chromosome engineering. In 2011 [89], the two largest chromosomes (IV and XII) of budding yeast *Saccharomyces cerevisiae* were fused by homologous recombination in vivo, producing a 3.2-Mb long compound chromosome that does not impair cell growth. Further fusions were achieved in 2014, in which four chromosomes (VII–V–XV–IV) of *Saccharomyces cerevisiae* were fused to generate a 4.3-Mb long chromosome with the longest yeast chromosome arm (3.7 Mb) [90].

Recently, a biologically functional SY14 with only one chromosome was constructed by successive end-to-end fusion of sixteen native chromosomes (I–XVI) of *Saccharomyces cerevisiae* BY474 with the deletion of 15 centromeres, 30 telomeres and 19 long repeats [91]. The first chromosome fusion strain, SY0, was constructed by CRISPR–Cas9-mediated fusion of chromosomes VII and VIII. Another fourteen successive rounds of chromosome fusion were successfully carried out that resulted in the production of a series of strains (SY1–SY14) using the same pairwise fusion strategy. This precise and efficient fusion method uses both the CRISPR–Cas9 cleavage system and the robust homologous recombination activity of yeast. The global three-dimensional structure of the chromosome in SY14 has dramatically changed due to the loss of the majority of the interchromosomal interactions. Nevertheless, the chromosomes in SY14 and wild-type yeast cells have nearly identical transcriptomes. Regardless of the reduced growth across environments, competitiveness, gamete production and viability, the giant single chromosome can support cell life. At almost the same time, an $n = 2$ strain carrying two chromosomes was generated by fusing the sixteen-chromosomes of *Saccharomyces cerevisiae* BY474 using CRISPR–Cas9 and showed slight transcriptomic changes without major defects [92]. However, an $n = 1$ strain failed to be constructed by multiple strategies. This result could be due to the different orders of chromosome fusion and centromere position, which may affect the final successful fusion of a single chromosome, or it may depend on whether the redundant copies of telomere-associated long repetitive sequences located on different chromosomes were deleted or not. However, this research noted two trends. First, the number of chromosomes could impact spore viability, especially when the number dropped below sixteen. For twelve chromosomes, sporulation was reduced markedly, reaching less than 10% of its wild-type value. Another trend is that yeast sporulation was arrested when a cross between a sixteen-chromosome strain and an eight-chromosome strain showed greatly decreased full tetrad formation and less than 1% sporulation, where no viable spores could be recovered. These results show that eight chromosome-chromosome fusion events are sufficient to isolate strains reproductively. Chassis with deleted and fused genomes can be a vital model to unravel the fundamental principles of genomes and to use in different kinds of applications.

3. Bottom-up approach in systems and synthetic biology

In the last few years, synthetic biology has brought increased attempts to employ rational engineering principles and workflows to construct and program large-scale user-defined pathways and even whole organisms [37,93,94]. The major achievements in fast and inexpensive DNA synthesis and assembly techniques [3,83,95–98], "booting-up" of the synthetic DNA in the host cell [99] and novel low- and high-throughput genome-engineering techniques [100–107] have paved the way for the engineering and modification of synthetic microbial chassis. Therefore, some of the most pressing problems can be solved, such as healthcare; food production; the generation of renewable, superior and cleaner energy; and the production of biomaterials.

3.1. Strategies for chassis in prokaryotes

Fast and efficient DNA synthesis and assembly technologies such as Gibson assembly, Golden Gate assembly and DNA assembly generated a demand for *de novo* synthesis of the whole genome. A contiguous 32-kb polyketide synthase gene cluster, the largest piece of man-made DNA, was synthesized in 2004 by the use of PCR technology to assemble pools of overlapping short oligonucleotides [39]. Although there were some inconveniences, such as expensive oligonucleotide synthesis and high error rates on a genomic scale during the PCR steps, this achievement was also a gateway to fabricating artificial cells [37]. At the beginning of 2008, the first complete genome of an organism, a 582,970-bp *Mycoplasma genitalium* genome, was synthesized by Venter and colleagues and is a milestone considered 'the dawn of synthetic genomics' [37]. The genes of the resulting synthetic genome, named *M. genitalium* JCVI-1.0, are almost identical to the genes of wild-type *M. genitalium* G37. They differ by one gene, MG408, which was disrupted by an antibiotic marker to block pathogenicity and allow for selection [108]. A total of 101 overlapping "cassettes" of approximately 5–7 kb were chemically synthesized and verified by sequencing. Intermediate fragments of approximately 24 kb, 72 kb ("1/8 genome"), and 144 kb ("1/4 genome") were assembled by *in vitro* recombination and then cloned into *E. coli* as bacterial artificial chromosomes (BACs). However, there were some difficulties in carrying out the assembly of whole synthetic genomes in *E. coli*. For this reason, the full-length genome was assembled in *Saccharomyces cerevisiae* by transformation-associated recombination (TAR) cloning.

With advanced methods of whole-synthetic-genome assembly and genome transplantation, the creation of a bacterial cell controlled by a chemically synthesized genome became possible [98]. The 1.08-Mb genome of *Mycoplasma mycoides* JCVI-syn1.0, based on computer-designed genome sequences, was designed, synthesized, assembled and then transplanted into *Mycoplasma capricolum* recipient cells to create new *M. mycoides* cells that are controlled only by the synthetic chromosome. The assembly of the synthetic *M. mycoides* genome was accomplished through a combination of *in vitro* enzymatic strategies and in vivo recombination in yeast [97,108]. To transplant the synthetic genome, the restriction barrier was broken by methylating the donor DNA or by simply disrupting the recipient cell's restriction system [109]. JCVI-syn1.0 has the expected phenotypic properties and is capable of continuous self-replication.

With the syn1.0 genome as a starting point, the 531-kb genome JCVI-syn3.0, containing 473 genes that encode 438 proteins and 35 annotated RNAs, was designed and constructed by removing 42 additional genes [3]. This synthetic genome is smaller than that of any known organism that can be grown in axenic culture. First, Tn5 mutagenesis was used to identify essential, quasi-essential, and non-essential genes. The collected essential gene information was used to design and produce a minimal genome, but it failed to construct a viable genome. Then, a class of quasi-essential genes was verified to not be essential genes but needed for robust growth. Finally, through three cycles of design, synthesis and testing, a viable minimal genome, JCVI-syn3.0, including 149 genes with unknown function, was constructed. Recently, the putative functions of some of these 149 unknown genes were identified [110], which will benefit understanding of the minimal genome. JCVI-syn3.0 is a versatile chassis for investigating the core functions of life and exploring whole-genome design.

3.2. Strategies for the chassis in eukaryotes

The Synthetic Yeast Genome Project or Sc2.0 project is an ambitious plan aiming to generate the synthetic genome for a eukaryotic model organism to serve as a chassis for facilitating genetic studies of eukaryotic chromosomes [111]. Several synthetic chromosomes, including the synthetic yeast chromosome arm (synIXR) and the entirely synthetic chromosome (synII, synIII, synV, synVI, synX, synXII), have been shown to function in yeast [112–115].

The first Sc2.0 synthetic chromosome, designated synIII and having a functional 272,871-bp chromosome, is based on the 316,617-bp native *Saccharomyces cerevisiae* chromosome III [116]. According to fitness, genome stability, and genetic flexibility principles, the "designer" sequence was designed by editing the native sequence *in silico*. Then, three major steps were used to construct synIII, including building block (BB) synthesis, assembly of minichunks, and direct placement of native yeast chromosome III with pools of synthetic minichunks. The synIII genome was minimized by removing redundant parts such as subtelomeric regions, transfer RNAs, introns, transposons and silent mating sites. Other changes include TAG/TAA stop codon substitutions that allow the incorporation of non-natural amino acids using the freed TAG codon and the introduction of loxPsym sites and PCRTags that allow for easy modifications of the synthetic yeast genome by directed evolution [116]. These changes did not significantly impact the fitness, transcriptome or replication of *Saccharomyces cerevisiae*.

Project Sc2.0 built a software framework, BioStudio, to generate chromosomal designs, which enables the use of a common assembly strategy from nucleotide to genome scales and enforces version control to systematically track edits [117]. A set of rules was applied while designing each chromosome, similar to some changes in the construction of synIII [116]. The design rules were developed to generate over one-third of the yeast chromosomes, with only minimal problems encountered, testifying to the soundness of design. Chunks are in turn bounded by restriction enzyme (RE) recognition sites otherwise absent from the 10-kb segment. The smaller fragments, dubbed "minichunks" (2–3 kb) and "chunks" (approximately 8–10 kb), were assembled into the longer DNA fragments, "megachunks" (30–50 kb), by RE cutting and ligation *in vitro*, and the megachunks were subsequently integrated into the native genome, replacing the corresponding wild-type segment. Recently, Sc2.0 design principles have been extended due to the construction of ring synV chromosomes [113]. SynV (536,024 bp) of *Saccharomyces cerevisiae* was chemically synthesized, assembled and incorporated according to Sc2.0 principles. All mutations were corrected by means of a CRISPR–Cas9-based method. A functional circular synV (ring_synV) derivative was constructed in yeast by precisely joining the chromosome ends (telomeres) at specified coordinates. It provides a model to study genomic rearrangement, ring chromosome evolution, and human ring chromosome disorders. An important stride in generating a completely synthetic yeast is cells carrying two and three completely synthetic chromosomes that compared with the wild-type cells, have no differential phenotype or genome architecture [117]. The Sc2.0 project has facilitated the ability to engineer genomes to better understand life and has also provided a model to construct chassis for applications.

4. Conclusions

To engineer and modify synthetic microbial chassis for industrial application and understanding the universal principles of life, numerous research groups around the world have made efforts to develop

highly efficient strategies for low- and high-throughput genome engineering, whole-genome synthesis, assembly and "booting-up" methods. Biological design from both top-down and bottom-up perspectives provides a useful framework for proceeding towards this goal.

The identification of non-essential genes is absolutely vital for engineering and modifying microbial chassis, which has contributed to an understanding of which genes are essential for cellular properties and how many genes are necessary to support cellular life. In general, a number of computational analyses were firstly employed to define the core essential genes for sustaining life. Essential genes are generally involved in basic metabolism, cell wall metabolism, cell division and DNA metabolism. Apart from these processes, non-essential genes, including cryptic prophages, phage remnants, insertion sequences [ISs], some unknown genes and abundant metabolic pathways were subsequently identified or deleted by a wide variety of experimental approaches. Finally, analysis of experimental data can facilitate an understanding of some unknown genes or pathways and allow for further engineering. Some of the chassis that were constructed by reducing existing redundant genomes have shown enhanced genome stability, increased production of industrial products or both.

The basic process of the top-down method to construct and engineer chassis is to determine the indispensable genes by combining computational system analysis, experimental data and models such as those of metabolic, regulatory and signal networks. Then, a chassis is constructed and modified by deleting the nonessential genes using various strategies. After the interaction between the biosynthesis pathway and the genome in the chassis is analyzed, a suitable biosynthesis pathway is designed and introduced into the chassis for industrial application. Further feedback data on the genomic identification and repository of natural biological parts can help significantly advance synthetic biology applications (Fig. 1). Recently, advances in genome fusion technologies not only contribute to a better strategy for engineering versatile chassis for research and applications but also make important strides forward possible.

To synthesize chassis employing the bottom-up approach, genetic circuits were easily designed by computational automation, constructed by low-cost DNA synthesis, assembled and then transplanted to create a bacterial cell controlled by a chemically synthesized genome. Due to further improvements in both software and DNA synthesis technology, current Sc2.0 synthesis costs average approximately US$0.10 per base pair. Of course, the total cost of the Sc2.0 project, including direct and associated indirect costs, will be considerably higher [117].

The investigation of chassis had already provided detailed information on gene networks and interactions. These results will speed up the construction of customized industrial chassis for the production of useful pharmaceuticals and chemicals, bioremediation of environmental toxins, and the creation of renewable energy sources.

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Appendix A. Supplementary data

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