Critical Reviews in Biotechnology

ISSN: 0738-8551 (Print) 1549-7801 (Online) Journal homepage: http://www.tandfonline.com/loi/ibty20

High molecular weight DNA assembly in vivo for synthetic biology applications

Mario Juhas & James W. Ajioka

To cite this article: Mario Juhas & James W. Ajioka (2016): High molecular weight DNA assembly in vivo for synthetic biology applications, Critical Reviews in Biotechnology, DOI: 10.3109/07388551.2016.1141394

To link to this article: http://dx.doi.org/10.3109/07388551.2016.1141394

Published online: 10 Feb 2016.

Critical Reviews in Biotechnology

http://informahealthcare.com/bty
ISSN: 0738-8551 (print), 1549-7801 (electronic)

Crit Rev Biotechnol, Early Online: 1–10
 DOI: 10.3109/07388551.2016.1141394

Taylor & Francis
Taylor & Francis Group

REVIEW ARTICLE

High molecular weight DNA assembly *in vivo* for synthetic biology applications

Mario Juhas and James W. Ajioka

Department of Pathology, University of Cambridge, Tennis Court Road, Cambridge, UK

Abstract

DNA assembly is the key technology of the emerging interdisciplinary field of synthetic biology. While the assembly of smaller DNA fragments is usually performed *in vitro*, high molecular weight DNA molecules are assembled *in vivo* via homologous recombination in the host cell. *Escherichia coli*, *Bacillus subtilis* and *Saccharomyces cerevisiae* are the main hosts used for DNA assembly *in vivo*. Progress in DNA assembly over the last few years has paved the way for the construction of whole genomes. This review provides an update on recent synthetic biology advances with particular emphasis on high molecular weight DNA assembly *in vivo* in *E. coli*, *B. subtilis* and *S. cerevisiae*. Special attention is paid to the assembly of whole genomes, such as those of the first synthetic cell, synthetic yeast and minimal genomes.

Keywords

Bacillus subtilis, Escherichia coli, genome assembly, homologous recombination, minimal genome, Saccharomyces cerevisiae, synthetic cell, synthetic yeast

History

Received 23 September 2015
Revised 3 December 2015
Accepted 3 December 2015
Published online 4 February 2016

Introduction

The emerging interdisciplinary field of synthetic biology aims to build novel biological systems and devices or to re-design existing biological systems for useful purposes. DNA assembly is among the key synthetic biology technologies. A number of novel DNA assembly methods developed in the last few years have paved the way for the engineering of high molecular weight DNA molecules, including whole genomes.[1–11]

Whole genome engineering and assembly is a multistep process. Shorter DNA fragments are usually assembled with *in vitro* methods, such as Gibson isothermal assembly,[12] SLIC: sequence and ligase independent cloning,[13] CPEC: circular polymerase extension cloning,[5] SLiCE: seamless ligation cloning extract,[6] OGAB: ordered gene assembly in *Bacillus subtilis*,[14–17] and LCR: ligase cycling reaction.[18] SLIC combines *in vitro* homologous recombination with single-strand annealing to assemble DNA fragments. SLIC is very efficient even at low DNA concentrations, particularly when utilizing RecA-catalysed homologous recombination.[13] Gibson isothermal assembly utilizes a cocktail of three enzymes, namely T5 exonuclease, Phusion DNA polymerase and Taq ligase to assemble multiple overlapping DNA molecules in a single-step isothermal reaction.[12] CPEC has been used for the high-throughput cloning of complex combinatorial DNA libraries recently. Unlike Gibson isothermal assembly, CPEC requires only a single enzyme, Phusion DNA polymerase, for a single-step DNA assembly *in vitro* in a vector of choice.[5,19] SLiCE is very cost-effective as it utilizes bacterial cell extracts for DNA assembly *in vitro*.[6] Both standard laboratory strains and strains with properties enhanced by genetic modification can be used as the source of the cell extract in SLiCE.[6] OGAB utilizes the plasmid transformation system of *B. subtilis* and ligation by T4 DNA ligase in the presence of 150 mM NaCl and 10% polyethylene glycol for DNA assembly.[14] Unlike previous methods, OGAB does not require circular ligation products but needs tandem repeat ligation products. OGAB permits assembly of multiple DNA fragments with very high efficiency and fidelity.[14,17] The degree of variability in the molar concentration of DNA fragments to be assembled was identified as the main cause affecting the DNA assembly efficiency in OGAB.[17] A modified OGAB method utilizing an equimolar DNA mixture has been used successfully for one-step assembly of over 50 DNA fragments recently.[17]

Short DNA fragment assembly is critical for virtually all synthetic biology (and increasingly for molecular biology in general). As high molecular weight DNA molecules are difficult to handle *in vitro*, they are usually assembled *in vivo* by homologous recombination in the host cell. The Gram-negative bacterium *Escherichia coli*, the Gram-positive

Address for correspondence: Mario Juhas, Department of Pathology, University of Cambridge, Tennis Court Road, CB2 1QP Cambridge, UK. Tel: +44 (0)1223 333923. E-mail: mj417@cam.ac.uk

bacterium *Bacillus subtilis* and yeast *Saccharomyces cerevisiae* are the most frequently used synthetic biology ''workhorses'' and hosts for DNA assembly *in vivo*.[20–25] A number of genomes have been assembled to date, including the entire 583 kb, 1.08 Mb, and 3.5 Mb genomes of *Mycoplasma genitalium, Mycoplasma mycoides*[26,27] and *Synechocystis* PCC6803 [28], respectively. The *M. mycoides* genome assembly led to the construction of the first cell (dubbed ''Synthia''), controlled solely by a chemically synthesized genome. Furthermore, the genome assembly of a eukaryote, the yeast *S. cerevisiae*, is currently underway.[29–34] Recent advances in DNA assembly also have implications for the bottom-up assembly of minimal genomes.

This review sums up the latest synthetic biology advances, with particular emphasis on high molecular weight DNA assembly *in vivo* in *E. coli*, *B. subtilis* and *S. cerevisiae*. Special attention is paid to the assembly of whole genomes, including those of ''Synthia'', ''Synthetic Yeast 2.0'' and minimal genomes.

BASIC and PaperClip: the latest *in vitro* DNA assembly techniques

Short DNA fragments are usually assembled with *in vitro* techniques that are crucial for virtually all synthetic biology. A number of *in vitro* DNA assembly methods have been developed recently.[1,2,4–6,12] In addition to Gibson isothermal assembly, SLIC, CPEC, SLiCE, OGAB, and LCR, recently developed methods for short DNA fragments assembly include Golden Gate,[35,36] MASTER: methylation-assisted tailorable ends rational method,[37] Golden Braid,[38] MODAL: modular overlap directed assembly with linkers,[39] and MoClo,[40] which have been reviewed elsewhere.[41] The Golden Gate and MASTER methods rely on type II restriction enzymes and MspJI endonuclease, respectively. In addition, MASTER requires PCR amplification of the DNA fragments. Due to these requirements, Golden Gate and MASTER are not the most suitable methods for high molecular weight DNA assembly.[37] Golden Braid and MoClo are improvements of the traditional Golden Gate method for standardized assembly of higher molecular weight DNA molecules.[38,40] Allowing assembly of larger, multigene pathways, Golden Braid and MoClo are still not used for the construction of whole genomes. For excellent summaries of the above techniques, see the following reviews.[3,41]

The latest *in vitro* DNA assembly methods not reviewed elsewhere include BASIC: biopart assembly standard for idempotent cloning[7] and PaperClip. [8] BASIC and PaperClip were developed to address the main limitations of the previous techniques, such as reliance on PCR and homology between the ends of neighboring parts in Gibson isothermal assembly,[12] SLIC,[13] CPEC,[5] and SLiCE.[6] Furthermore, methods such as Golden Gate,[35,36] Golden Braid,[38] and MoClo [40] rely on digestion with restriction endonucleases and cloning into specific vectors. [8] BASIC relies on digestion of parts with type II S restriction endonucleases, followed by ligation to oligonucleotide linkers with long single strand overhangs which regulate the order in which the individual DNA fragments assemble into a final construct.[7] In the initial step of BASIC, linkers are attached onto each end of the DNA fragment/parts by type II restriction endonuclease-mediated digestion and ligation. Unligated linkers are removed and DNA fragments harboring linkers are assembled into a final construct.[7] BASIC is highly accurate with confirmed accuracy of nearly 100% for four part assemblies and above 90% for seven part assemblies. The main advantages of BASIC over other methods includes utilization of standard reusable parts, idempotent cloning, automatability, size independence and parallel assembly.[7] PaperClip is an extremely flexible DNA assembly method that allows multi-part DNA assembly from existing libraries cloned into virtually any plasmid.[8] PaperClip only requires design of four reusable short oligonucleotides, dubbed ''Clips'', for each DNA fragment/part to determine the order in which the DNA fragments are assembled into a final construct.[8] PaperClip is a very efficient method that does not rely on digestion with restriction endonucleases. DNA fragments in PaperClip can be assembled by various methods, including PCR and recombination using cell extracts. Furthermore, while other methods using bridging oligonucleotides for multipart DNA assemblies, such as LCR,[18] usually require synthesis of new oligonucleotides for each assembly, ''Clips'' in PaperClip can be reused in any assembly utilizing that particular DNA part.[8] It was shown that the assembly of six DNA parts in any order can be accomplished in several hours with PaperClip.[8]

In vivo DNA assembly in yeast

Homologous recombination is the homology-based transfer of genetic information between two DNA fragments.[42] Due to its high rate of homologous recombination, *S. cerevisiae* is the preferred chassis for the simultaneous assembly of multiple DNA fragments.[43] In addition to DNA assembly, homologous recombination in *S. cerevisiae* is often used for the integration of foreign DNA into the chromosome.[44,45]

Homologous recombination in yeast can efficiently join DNA fragments that have at least 40 bp of overlapping sequence.[33] An improved method for multi-fragment DNA assembly in *S. cerevisiae* utilizes 60 bp synthetic recombination sequences that are non-homologous with the yeast genome to enhance reliability, flexibility and accuracy of the yeast homologous recombination for assembly of plasmids.[46] The synthetic recombination sequences are non-homologous with the yeast genome to avoid interference and recombination with the host cell genomic DNA. Separation of the survival elements of the plasmid backbone (selection marker and yeast episome) into two DNA fragments flanked by 60 bp synthetic recombination sequences led to a 100 fold decrease in the number of false positive transformants as compared to previous methods (such as using linearized plasmid backbone). Using this approach, nine parts were assembled into a 21 kb plasmid with an accuracy of 95%.[46]

An improved DNA assembly method, dubbed RADOM (rapid assembly of DNA overlapping multifragments), has been developed recently [47] (Figure 1). RADOM combines

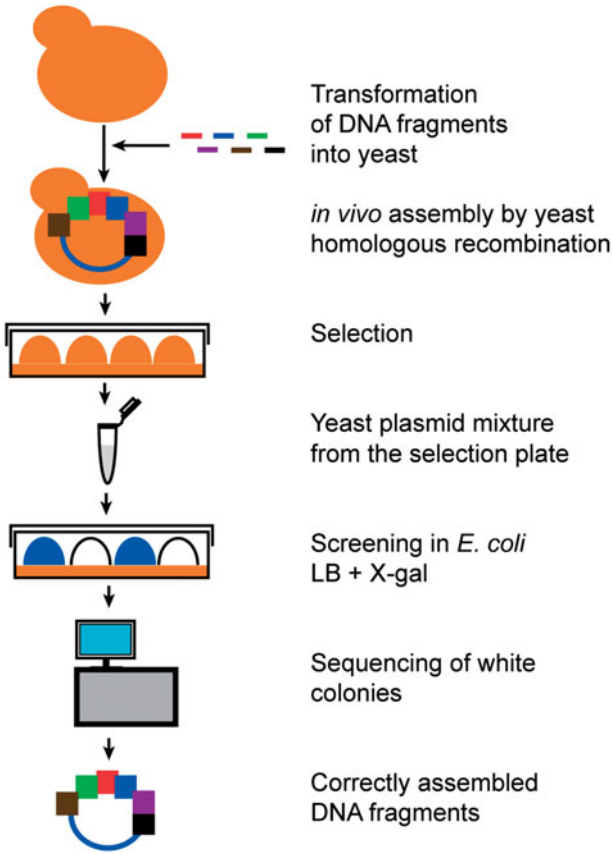

Figure 1. Rapid assembly of DNA overlapping multifragments (RADOM) in yeast. The figure shows the key steps of the improved DNA assembly method exploiting homologous recombination in yeast and screening in *E. coli* for rapid DNA assembly. First, DNA fragments are assembled in yeast. Then, the mixture comprising the assembled plasmids from the entire yeast population is transformed into *E. coli.* Transformants are analyzed by blue-white screening on plates with X-gal. Blue colonies indicate an empty vector. White colonies are subjected to colony PCR and sequencing to identify the correctly assembled DNA fragments.

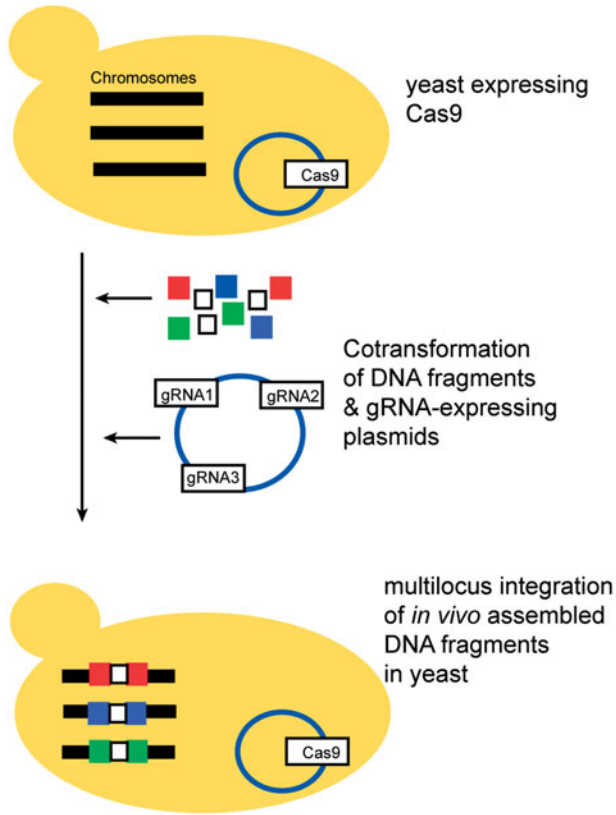

Figure 2. Cas9-mediated genomic integration of DNA fragments assembled in yeast. The figure shows the main steps of the CasEMBLR method for the Cas9-facilitated integration of *in vivo* assembled DNA fragments into the *S. cerevisiae* chromosome at multiple loci. CasEMBLR uses the CRISPR/Cas9 system to generate double strand breaks to increase the efficiency of DNA integration into the yeast chromosome by homologous recombination. DNA fragments with 50 bp overlaps and plasmids expressing guide RNAs (gRNAs) are co-transformed into Cas9-expressing *S. cerevisiae*. DNA fragments are assembled and integrated into the targeted chromosomal loci by homologous recombination.

yeast homologous recombination with blue/white screening in *E. coli*, to reduce the time and labor required for screening for correctly assembled DNA fragments. Blue/white screening is enabled by disruption of the α-complementation process by linearization of the plasmid backbone within *lacZα*. In contrast to other yeast assembly methods, plasmids from the entire population of yeast transformants are extracted and then transformed into *E. coli*. Blue/white screening differentiates between plasmids harboring assembled DNA fragments and empty vectors, thus allowing rapid screening of the whole pool of the plasmid-borne DNA assemblies (Figure 1). The enhanced screening efficiency of RADOM significantly reduces labor and time required for DNA assembly, particularly in hierarchical assembly projects. RADOM has been used to assemble a number of 3 kb and 10 kb yeast chromosome fragments.[47]

DNA integration into the chromosome is preferable for metabolic engineering and construction of large enzymatic pathways.[44] The recently developed CasEMBLR method allows marker-free integration of DNA fragments into the yeast chromosome at multiple loci (Figure 2). The efficiency of chromosomal integration by homologous recombination can be greatly increased by introducing a double strand break into the genome.[44,48,49] CasEMBLR exploits the CRISPR/Cas9 system to generate double strand breaks at targeted integration sites to increase efficiency of homologous recombination-mediated targeting of DNA fragments into the yeast chromosome (Figure 2). Plasmids expressing guide RNAs (gRNAs) and DNA fragments to be assembled and integrated into the chromosome with 50 bp overlaps are co-transformed into yeast harboring a plasmid constitutively expressing Cas9. Cas9 generates double strand breaks at integration sites targeted by gRNAs and DNA fragments are assembled and integrated into the yeast chromosome at targeted sites by homologous recombination. CasEMBLR was used to integrate the 15 part DNA assembly encoding the carotenoid pathway into three targeted sites and the 10 part DNA assembly encoding the tyrosine pathway into two targeted loci.[44]

Furthermore, yeast homologous recombination has recently been used to assemble conditional shuttle vectors for yeast chromosomal integration.[50] The autonomous replication sequence and centromere of the constructed shuttle vectors are flanked by *loxP* sites targeted by Cre site-specific recombinase. In the extrachromosomal form, shuttle vectors can be used as backbones for yeast homologous recombination-mediated DNA assembly. Expression of

Cre recombinase initiates excision of the autonomous replication sequence and centromere-harboring cassette which leads to integration of the vector into the chromosome at a targeted locus.[50]

In vivo DNA assembly in *B. subtilis*

The rod-shaped Gram-positive bacterium *B. subtilis* is a frequently used host for a number of biotechnology and synthetic biology applications.[20,51–53] *B. subtilis* is naturally competent and readily transformable with extracellular DNA. Extracellular DNA can be imported into the cytoplasm of *B. subtilis* cells in a single stranded form via *B. subtilis* transformation machinery. The imported DNA is then integrated into the *B. subtilis* chromosome by RecA-mediated homologous recombination.[54–57] The natural competence allows utilization of the whole *B. subtilis* genome as a vector in the *B. subtilis* genome (BGM) vector system. [56] BGM is a novel cloning system allowing stable integration of large DNA fragments into the *B. subtilis* chromosome by homologous recombination. BGM encompasses the entire 4.2 Mb *B. subtilis* genome and can support integration of large DNA

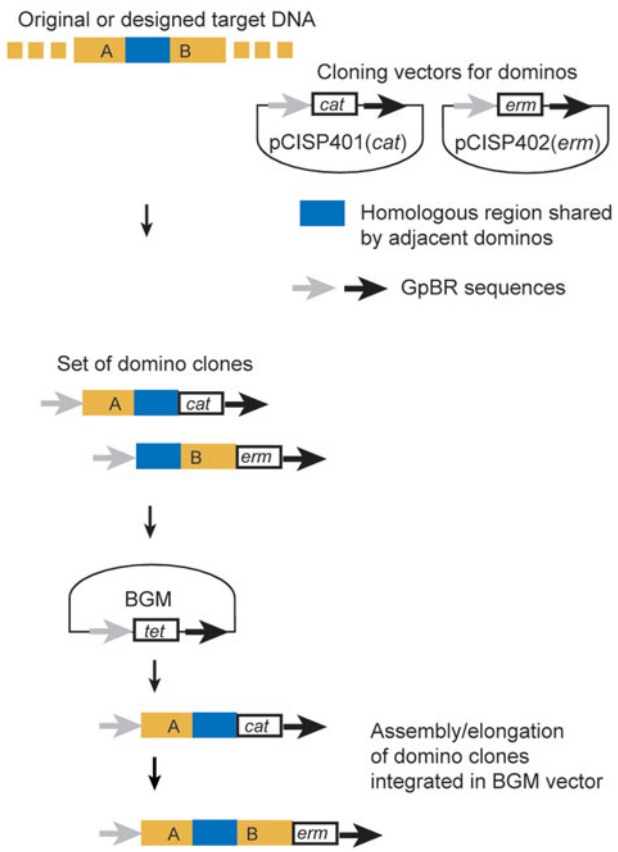

Figure 3. DNA assembly by the ''domino method'' in the BGM vector. The figure depicts the key steps of the ''domino method'' for DNA assembly in the *B. subtilis* genome (BGM) vector. Domino clones are prepared in pBR322-based plasmids pCISP401(*cat*) and pCISP402(*erm*), which are identical, with the exception of the antibiotic selection markers. The first domino (domino A) harboring chloramphenicol resistance marker (*cat*) integrates into GpBR (genomic pBR322 sequences) locus of BGM by homologous recombination at the two halves of the pBR322 sequences, thus replacing the tetracycline resistance gene (*tet*). The second domino (domino B) harboring erythromycin resistance marker (*erm*) integrates downstream of domino A. Alternative use of *cat* and *erm* permits unlimited elongation with additional dominos.[28]

fragments.[28,58,59] Up to 3.5 Mb of *Synechocystis* PCC6803 DNA were stably integrated into BGM by the ''inchworm method'', which relies on high quality long (over 100 kb) DNA templates. The improved ''domino method'' using homologous recombination between overlapping sequences does not require purified long DNA templates and facilitates stable DNA integration into BGM (Figure 3). Furthermore, the ''domino method'' is very flexible as the configuration of integrated DNA can be easily determined by choosing the first and last dominos in an assembly. Dominos are prepared in pBR322-based plasmids and integrated into the GpBR (genomic pBR322 sequences) locus of BGM by homologous recombination. Alternative use of two antibiotic selection markers allows unlimited rounds of domino elongation (Figure 3). The ''domino method'' has been used to clone the 16.3 kb mouse mitochondrial genome and the 134.5 kb rice chloroplast genome into BGM.[28] The BGM has also been successfully used for the manipulation and reconstruction of genomic DNA for mouse transgenesis.[59] Notably, these studies also revealed difficulty in cloning the ribosomal RNA gene fragments of foreign genomes into *B. subtilis*.[28] To avoid undesirable recombination between homologous sequences within the BGM vector, an inducible *recA* expression BGM vector (iREX) has been developed recently.[56] In iREX, the endogenous *recA* was exchanged for a xylose-inducible *recA* expression cassette. Homologous recombination and integration of DNA fragments in iREX is therefore strictly controlled by xylose in the growth medium.[56] The BGM vector has many advantages over the alternative tools for high molecular weight DNA manipulation, such as bacterial artificial chromosomes (BACs) and yeast artificial chromosomes (YACs). BACs are easy to manipulate; however, their integration capacity is considerably smaller than that of BGM (up to 300 kb). Although YACs can support integration of larger DNA fragments than BACs (up to 2 Mb) they are difficult to manipulate and prone to chimerism.[56] Due to large cloning capacity (over 3 Mb) and established genome editing strategies, such as DNA insertions, inversions and deletions,[56,59] BGM is a promising tool for manipulating high molecular weight DNA. The recently developed iREX further improves stability of the integrated DNA by suppressing undesired recombination between DNA fragments containing homologous sequences.[56]

In vivo DNA assembly in *E. coli*

Like *B. subtilis* and *S. cerevisiae*, the Gram-negative bacterium *E. coli* is a frequently used chassis for a plethora of biotechnology and synthetic biology applications.[20,60–63] However, unlike yeast and the naturally transformable bacteria, such as *B. subtilis*, *E. coli* is not readily transformable with linear DNA fragments.[64] Linear dsDNA in *E. coli* is degraded by the ATP-dependent exonuclease, RecBCD.[65] The Red recombinase system of the bacteriophage lambda, which mediates homologous recombination between linear and circular DNA molecules, is frequently used for DNA integration into *E. coli*. The lambda Red recombinase system utilizes three proteins, namely Gam, Beta and Exo. Exo is a 5′-3′ exonuclease that binds to dsDNA ends and generates 3′ ssDNA overhangs by digesting 5′ DNA ends. Beta binds to the

ssDNA overhangs generated by Exo and facilitates their annealing to the complementary ssDNA in the cell.[65] The Gam protein inhibits exonuclease RecBCD binding to dsDNA ends.[64,66]

The Rac prophage was recently used for large DNA assembly in *E. coli*.[67] The RecET recombination system of the Rac prophage is functionally analogous to the Red system of the bacteriophage lambda (RecE and RecT are analogous to Exo and Beta, respectively). The truncated version of RecE, consisting of just the C terminus starting at residue 588 in combination with RecT has been previously used for recombineering in *E. coli* between linear and circular DNA molecules.[68] A recent study revealed that full length RecE along with RecT, mediates efficient homologous recombination between two linear DNA molecules.[67] This analysis also showed that the lambda Red system is more suitable for recombination between linear and circular DNA molecules, while the Rac RecET system is more suited for recombination between two linear DNA molecules. The Rac prophage-borne RecE/RecT system was used to clone ten megasynthetase gene clusters from *Photorabdus luminescens* that varied in size from 10 to 52 kb into *E. coli* expression vectors.[67]

DNA integration into the *E. coli* chromosome has many advantages over maintenance on plasmids.[69] A number of lambda Red recombinase-based methods for *E. coli* chromosomal integration have been developed recently. This includes a series of knock-in/knock-out (KIKO) vectors for lambda Red recombinase-mediated integration at well-defined loci, namely *arsB, lacZ,* and *rsbA-rsbR* encoding an arsenite transporter, β-galactosidase and a ribose metabolism operon, respectively.[70] Lambda Red recombinase mediates homologous recombination between two KIKO-borne homologous insertion sequences flanking a multiple cloning site and an antibiotic resistance marker removable by flippase-mediated recombination. KIKO-vectors were used to integrate 5.4 kb, 7.3 kb and 11.3 kb DNA fragments into the *E. coli* chromosome.[70]

Another recently developed method combines lambda Red-mediated homologous recombination and bacteriophage φ80 Int-mediated site-specific recombination.[71–73] DNA fragments with the removable antibiotic resistance marker flanked by I-SceI endonuclease target sites are introduced into specialized CRIM (conditional-replication, integration and modular) vectors harboring an *attP* site.[71] Integration is targetted into an artificial *attB* site previously introduced into the chromosome by lambda Red- mediated homologous recombination.[71] This method was used to integrate an 8 kb DNA fragment into the *E. coli* chromosome.[71]

The recently constructed plasmid pSB1K3(FRTK) can easily accept virtually any genetic circuit for *E. coli* chromosomal integration. First, the DNA fragment is cloned into pSB1K3(FRTK) next to the kanamycin resistance cassette flanked by Flippase recombinase target (FRT) sites and amplified with sequences homologous to the target loci on the *E. coli* chromosome.[74] Next, the IPTG-inducible lambda Red recombinase system on plasmid pKM208 mediates DNA integration into the *E. coli* chromosome. Finally, plasmid pCP20-borne Flp recombinase ''flips out'' the kanamycin resistance cassette from the chromosome. Integration of synthetic genetic circuits into the *E. coli* chromosome can mitigate against many problems associated with their introduction on plasmids, such as variable copy numbers, higher metabolic burden and required antibiotic selection pressure. However, there is only limited information available on the suitable integration target sites. Ideally, integration target sites should be well-characterized, conserved, non-essential and highly expressed. *E. coli* flagellar genes fulfill all these criteria. pSB1K3(FRTK) has been used to integrate synthetic genetic circuits into a number of loci in the *E. coli* flagellar regions 1, 2, 3a and 3b.[74–76] This led to the identification and validation of suitable integration targets in the *E. coli* flagellar regions, which support high integration efficiency and expression of integrated genetic circuits.[74–76]

Genome assembly

Recent advances in DNA assembly have paved the way for the construction of whole genomes. Almost the entire 3.5 Mb chromosome of *Synechocystis* PCC6803 has been assembled using the *B. subtilis* genome (BGM) vector.[28] The lambda Red recombination system was used to assemble a big part of the *Haemophilus influenzae* genome in *E. coli*.[77] Furthermore, synthetic genomes of mycoplasmas have been completed recently and the assembly of the first eukaryotic genome and minimal genomes are currently underway.

''Synthia'': the first cell controlled by a chemically synthesized genome

The 583 kb *M. genitalium* chromosome was the first bacterial genome assembled entirely from chemically synthesized DNA fragments.[27] This was followed by the chemical synthesis and assembly of the entire 1.08 Mb *M. mycoides* genome.[26] Activation of the synthetic *M. mycoides* genome in the host cell led to the first synthetic cell JCVI-syn1.0, dubbed ''Synthia'' (Figure 4).[26] The initial steps of the *M. mycoides* genome construction were performed *in vitro*. Oligonucleotides were chemically synthesized and 1 kb overlapping DNA fragments were assembled using Gibson isothermal assembly.[26] Then, 10 kb and subsequently 100 kb DNA fragments were generated by yeast homologous recombination. 100 kb DNA fragments were cloned into BACs and the whole *M. mycoides* chromosome was assembled in *S. cerevisiae* using yeast homologous recombination (Figure 4). The transplantation of the assembled *M. mycoides* chromosome into *M. capricolum* generated cells controlled solely by the synthetic *M. mycoides* genome [26,78] (Figure 4). The transplantation was further improved by direct transfer of whole genomes from bacteria to yeast by fusion of bacterial cells with yeast spheroplasts.[79]

Synthetic yeast 2.0

The synthesis of the *S. cerevisiae* genome (12 Mb in 16 chromosomes) is currently underway in a project dubbed ''Synthetic Yeast 2.0'' (Sc2.0) [29–32] Sc2.0 is the biggest ongoing joint synthetic biology project that involves research groups from a number of institutions worldwide. These are working simultaneously on the synthesis and assembly of yeast chromosomes. The assembly of parts of yeast

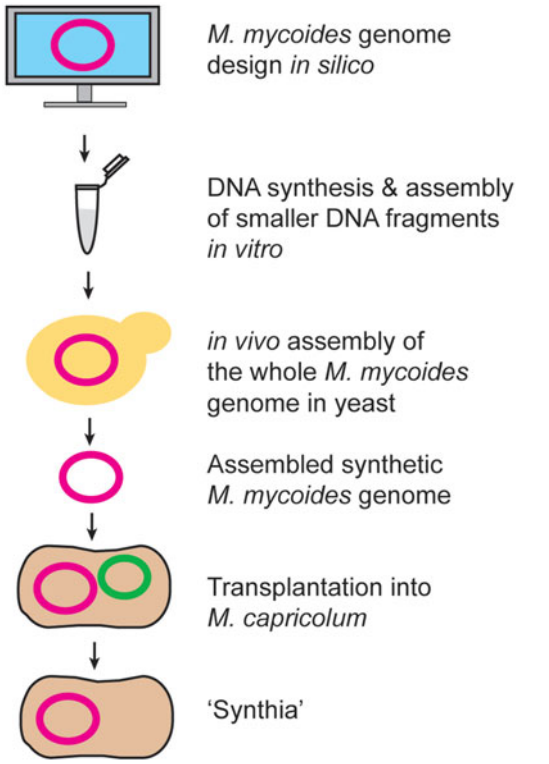

Figure 4. Generation of the first cell controlled by the synthetic genome. The genome of *M. mycoides* was first designed *in silico*. The smaller DNA fragments (up to 10 kb) were synthesized and assembled *in vitro*. The larger DNA fragments (100 kb) and the whole *M. mycoides* genome (1.08 Mb) were assembled using yeast homologous recombination. The chemically synthesized and assembled *M. mycoides* genome was transplanted into *M. capricolum* cells. Selective pressure was used to eliminate the native *M. capricolum* genome. The resulting JCVI-syn1.0 cell (dubbed ''Synthia'') was controlled by a chemically synthesized *M. mycoides* genome.

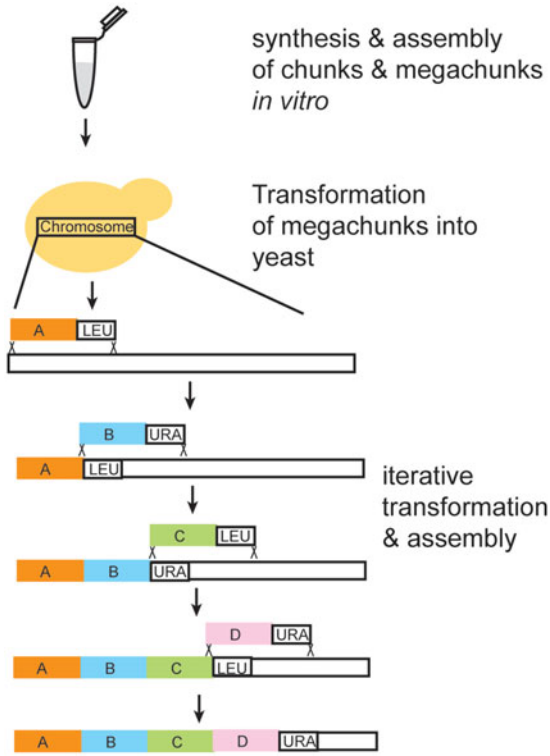

Figure 5. Synthetic yeast chromosome assembly. The Figure shows the key steps of *S. cerevisiae* chromosome assembly. Chunks (DNA fragments of approximately 6–10 kb length) are synthesized and assembled into megachunks (*ca*. 30–50 kb) *in vitro*. Megachunks are then transformed into yeast cells where they integrate into the native yeast chromosome by homologous recombination. Megachunks carry a selectable auxotrophic marker, such as *leu2* or *ura3* that allow selection for the successful recombinants. The yeast chromosome assembly is iterative, thus generating a number of hybrid chromosomes composed of a mosaic of natural and synthetic parts.

chromosomes IX and VI, in addition to the whole of chromosome III, was published recently.[30,34] The smaller fragments of the synthetic yeast chromosome III dubbed ''minichunks'' (2–3 kb) and ''chunks'' (approximately 8–10 kb) were assembled *in vitro* and then integrated into the native chromosome III using yeast homologous recombination.[34] This ultimately led to the exchange of the native chromosome III for its synthetic counterpart. The ''chunks'' method can be easily modified for use in any naturally competent bacteria to replace the native chromosome with its synthetic counterpart. An alternative method utilizes longer DNA fragments, ''megachunks'' (30–50 kb) assembled from the commercially synthesized ''chunks'' *in vitro* (Figure 5). Megachunks are integrated into the yeast chromosome by homologous recombination. The assembly of the yeast chromosome is a multistep iterative process where each ''chunk'' or ''megachunk'' is integrated into the chromosome and exchanged for the native fragment. This generates a number of hybrid chromosomes composed of a mosaic of natural and synthetic parts.[33]

Although significantly smaller (273 kb) than the native chromosome III (317 kb) the synthetic chromosome III is functional.[34] The difference between the size of the synthetic and the native chromosome III is the result of a number of modifications. These include deletion of redundant parts, such as horizontally acquired genetic elements, and removal of all tRNA genes. Replacement of TAG stop codons with TAA stop codons allows incorporation of non-natural amino acids using the freed TAG codon.[80,81] Furthermore, 34 bp loxP recombination sites introduced downstream of all known non-essential genes mediate Cre recombinase-induced genome rearrangements dubbed SCRaMBLE (synthetic chromosome rearrangement and modification by loxP-mediated evolution).[30,34] SCRaMBLE allows easy modifications of the synthetic yeast genome by directed evolution. For instance, addition of amino acids into the growth medium can result in the deletion of genes required for their biosynthesis. Furthermore, cultivation in a rich medium could lead to the deletion of all non-essential genes, thus generating the first minimal eukaryotic genome.

Minimal genomes

Recent advances in DNA assembly are also important for the bottom-up construction of genomes composed solely of essential genes (minimal genomes).[82] Investigation of essential genes is crucial for understanding the fundamental principles of life. Essential genes are usually classified into two groups: ''core'' and ''accessory''. The ''core'' essential genes are considered to be those that are required for all living organisms, while the ''accessory'' essential genes are required for individual cell types, species or under specific growth conditions.[83] As the ''accessory'' essential genes are

indispensable in specific organisms and environments, they are promising antimicrobial targets.[83–85] The universally required ''core'' essential genes are considered to be the building bricks of minimal cell factories.[11,20] Unlike the top-down approach that aims to generate minimal genomes by deleting non-essential genes, the bottom-up approach aims to synthesize and assemble minimal genomes from scratch. It is assumed that ''truly'' minimal genomes consist of around 300 ''core'' essential genes; however, their exact composition remains unknown. Furthermore, a number of essential genes are hypothetical open reading frames with unknown function.[86–94] The unknown genes constitute 9.8% of general *Pseudomonas aeruginosa* essential gene set required for growth in three primary growth conditions (minimal medium, sputum, LB medium). Furthermore, unknown genes constitute 13.4% of highly confirmed *P. aeruginosa* essential genes identified as essential in four genome-wide analyses.[83,92] Identification and an in-depth analysis of essential genes and their interactions is the prerequisite for constructing viable minimal genomes. Therefore, at this time, the top-down minimization of natural genomes, such as those of *E. coli* and *B. subtilis* appears to be more realistic.[20] Alternatively, both approaches could be combined. The genomes of mycoplasmas, which are the smallest genomes among known organisms capable of independent growth in the laboratory, can be chemically synthesized and assembled using methods described above and their non-essential genes deleted.[11] This can be conducted with the recombinase-mediated cassette exchange (RMCE) system utilizing Cre recombinase for the replacement of the native DNA fragment with its synthetic counterpart.[95] RMCE was used to replace a 100 kb native DNA fragment from *M. mycoides* with its synthetic counterpart in yeast.[95] Information from genome-wide transposon mutagenesis studies can lead to prediction and removal of redundant regions by progressively clustering deletions using meiotic recombination between bacterial genomes in yeast (yeast sexual cycling).[96] This method was used to reduce the original *M. mycoides* genome by approximately 10%.[96] To avoid damaging the circular *M. mycoides* genomes during cell division, *M. mycoides* genomes were linearized prior to deletion of the targeted regions and then circularized via homologous recombination in yeast. Transplantation of the circularized genomes into recipient cells generated viable mycoplasma cells. The reduction of the original *M. mycoides* genome by 10% did not negatively affect growth or viability in a rich medium. This might be due to the compensatory mutations that accumulated in the background or the activity of undeleted homologous genes; however, this will require further investigation.

Conclusions

DNA assembly is the key synthetic biology technology. Advances in DNA assembly over the last few years have paved the way for the construction of whole genomes. While the assembly of smaller DNA fragments is usually performed *in vitro*, whole genomes are assembled using homologous recombination in the host cell. Recently developed techniques, such as BASIC and PaperClip, highlighted in this review, address some of the limitations of the previous *in vitro* assembly techniques, such as reliance on PCR, vectors and homology between neighboring parts and reusability of bridging oligonucleotides.[8] However, it is important to keep in mind that small fragment DNA assembly and DNA assembly *in vivo* are often used for distinct purposes. Although small fragment DNA assembly is, at this time, not used for assembly of whole genomes, it is critical for virtually all synthetic biology (and increasingly for molecular biology in general). Furthermore, recent methods, such as OGAB utilizing an equimolar DNA mixture can be used for one-step assembly of unprecedentedly large numbers of DNA fragments.[17] Over 50 DNA fragments were successfully assembled by this method and the computer simulations indicate that as many as 100 DNA fragments can be assembled at once if the concentration fluctuation of DNA fragments can be kept within 3.3%.[17]

E. coli, *B. subtilis* and *S. cerevisiae* are among the most frequently used hosts for DNA assembly *in vivo*. A number of whole genomes have been constructed exploiting homologous recombination in *B. subtilis* and *S. cerevisiae*, and phage recombinase systems in *E. coli*, such as lambda Red system and bacteriophage φ80 Int-mediated site-specific recombination. Bacteriophages can be also used to mobilize large pre-assembled and integrated DNA fragments. Flanking DNA fragments with a phage packaging recognition signal (*pac* site) [97–99] may speed up the building of large genomes. Recently developed or improved systems for manipulation of high molecular weight DNA, such as BGM vector or iREX described above have many advantages over the traditional tools, such as BACs and YACs. However, a few issues remain, such as increasing the efficiency of cloning the ribosomal RNA gene fragments of foreign genomes into BGM.[28]

An exciting area of synthetic biology is aimed at the bottom-up construction of minimal genomes. However, assembly of viable minimal genomes will require better understanding of essential genes and their interactions. The universal minimal genome has not been identified yet and it appears that the essential gene set depends critically on the environment the organism is grown in, and issues such as genome stability, which determine the essentiality of genes. Recent *P. aeruginosa* genomic analyses highlight the evolution of the definition of an, ''essential'' gene from the traditional to the context-dependent.[83,92,100,101] Furthermore, *B. subtilis* and *E. coli* share only approximately half of their essential genes.[20] Interestingly, a large proportion of essential genes are hypothetical with unknown function (as determined, for example, in *P. aeruginosa*).[83,92] Better understanding of essential genes and progress in DNA assembly will aid construction of custom-made genomes. This in turn will lead to generating well-characterized cells endowed with properties relevant for healthcare, biomanufacturing and energy production.

Acknowledgements

Cordial thanks to all researchers who contributed to these advances in DNA assembly. M.J. and J.W.A. are supported by the Engineering and Physical Sciences Research Council (EPSRC).

Declaration of interest

There are no conflicts of interest associated with this manuscript.

References

[1] Kosuri S, Eroshenko N, Leproust EM, et al. Scalable gene synthesis by selective amplification of DNA pools from high-fidelity microchips. Nat Biotechnol. 2010;28:1295–1299.

[2] Matzas M, Stähler PF, Kefer N, et al. High-fidelity gene synthesis by retrieval of sequence-verified DNA identified using high-throughput pyrosequencing. Nat Biotechnol. 2010;28:1291–1294.

[3] Merryman C, Gibson DG. Methods and applications for assembling large DNA constructs. Metab Eng. 2012;14:196–204.

[4] Blake WJ, Chapman BA, Zindal A, et al. Pairwise selection assembly for sequence-independent construction of long-length DNA. Nucleic Acids Res. 2010;38:2594–2602.

[5] Quan J, Tian J. Circular polymerase extension cloning for high-throughput cloning of complex and combinatorial DNA libraries. Nat Protoc. 2011;6:242–251.

[6] Zhang Y, Werling U, Edelmann W. SLiCE: a novel bacterial cell extract-based DNA cloning method. Nucleic Acids Res. 2012;40: e55

[7] Storch M, Casini A, Mackrow B,, et al. BASIC: a new biopart assembly standard for idempotent cloning provides accurate, single-tier DNA assembly for synthetic biology. ACS Synth Biol. 2015;4(7):781–7

[8] Trubitsyna M, Michlewski G, Cai Y, et al. PaperClip: rapid multi-part DNA assembly from existing libraries. Nucleic Acids Res. 2014;42:e154

[9] Juhas M, Davenport PW, Brown JR, et al. Meeting report: The Cambridge BioDesign TechEvent – synthetic biology, a new ''age of wonder''? Biotechnol J. 2013;8:761–763

[10] Seo SW, Yang J, Min BE, et al. Synthetic biology: tools to design microbes for the production of chemicals and fuels. Biotechnol Adv. 2013;31(6):811–7

[11] Juhas M. On the road to synthetic life: the minimal cell and genome-scale engineering. Crit Rev Biotechnol. 2015;1–8. Epub ahead of print.

[12] Gibson D, Young L, Chuang R, et al. Enzymatic assembly of DNA molecules up to several hundred kilobases. Nat Methods. 2009;6:343–345.

[13] Li MZ, Elledge SJ. Harnessing homologous recombination in vitro to generate recombinant DNA via SLIC. Nat Methods. 2007;4:251–256.

[14] Tsuge K, Matsui K, Itaya M. One step assembly of multiple DNA fragments with a designed order and orientation in *Bacillus subtilis* plasmid. Nucleic Acids Res. 2003;31:e133.

[15] Nishizaki T, Tsuge K, Itaya M, et al. Metabolic engineering of carotenoid biosynthesis in *Escherichia coli* by ordered gene assembly in *Bacillus subtilis*. Appl Environ Microbiol 2007;73: 1355–1361.

[16] Tsuge K, Matsui K, Itaya M. Production of the non-ribosomal peptide plipastatin in *Bacillus subtilis* regulated by three relevant gene blocks assembled in a single movable DNA segment. J Biotechnol. 2007;129:592–603.

[17] Tsuge K, Sato Y, Kobayashi Y, et al. Method of preparing an equimolar DNA mixture for one-step DNA assembly of over 50 fragments. Sci Rep. 2015;5:10655.

[18] de Kok S, Stanton LH, Slaby T, et al. Rapid and reliable DNA assembly via ligase cycling reaction. ACS Synth Biol 2014;3(2):97–106

[19] Quan J, Tian J. Circular polymerase extension cloning of complex gene libraries and pathways. PLoS One. 2009;4:e6441.

[20] Juhas M, Reuß DR, Zhu B, et al. *Bacillus subtilis* and *Escherichia coli* essential genes and minimal cell factories after one decade of genome engineering. Microbiology. 2014;160:2341–2351.

[21] Blount BA, Weenink T, Ellis T. Construction of synthetic regulatory networks in yeast. FEBS Lett. 2012;586:2112–2121.

[22] Dikicioglu D, Pir P, Oliver SG. Predicting complex phenotype-genotype interactions to enable yeast engineering: *Saccharomyces cerevisiae* as a model organism and a cell factory. Biotechnol J. 2013;8:1017–1034.

[23] Li M, Borodina I. Application of synthetic biology for production of chemicals in yeast *Saccharomyces cerevisiae*. FEMS Yeast Res. 2014. Epub ahead of print.

[24] Redden H, Morse N, Alper HS. The synthetic biology toolbox for tuning gene expression in yeast. FEMS Yeast Res. 2014. Epub ahead of print.

[25] Dai Z, Liu Y, Guo J,, et al. Yeast synthetic biology for high-value metabolites. FEMS Yeast Res. 2014. Epub ahead of print.

[26] Gibson D, Glass J, Lartigue C, et al. Creation of a bacterial cell controlled by a chemically synthesized genome. Science. 2010; 329:52–56.

[27] Gibson D, Benders G, Andrews-Pfannkoch C, et al. Complete chemical synthesis, assembly, and cloning of a *Mycoplasma genitalium* genome. Science. 2008;319:1215–1220.

[28] Itaya M, Fujita K, Kuroki A, et al. Bottom-up genome assembly using the *Bacillus subtilis* genome vector. Nat Methods. 2008;5: 41–43.

[29] Perkel JM. Genome engineering: writing a better genome. Biotechniques. 2012;53:21–35.

[30] Dymond JS, Richardson SM, Coombes CE, et al. Synthetic chromosome arms function in yeast and generate phenotypic diversity by design. Nature. 2011;477:471–476.

[31] Nawy T. Yeast 2.0. Nat Methods. 2011;8:895.

[32] Enyeart PJ, Ellington AD. Synthetic biology: a yeast for all reasons. Nature. 2011;477:413–414.

[33] Jovicevic D, Blount BA, Ellis T. Total synthesis of a eukaryotic chromosome: Redesigning and SCRaMbLE-ing yeast. Bioessays. 2014;36:855–860.

[34] Annaluru N, Muller H, Mitchell LA, et al. Total synthesis of a functional designer eukaryotic chromosome. Science. 2014;344: 55–58.

[35] Engler C, Kandzia R, Marillonnet S. A one pot, one step, precision cloning method with high throughput capability. PLoS One. 2008;3:e3647.

[36] Engler C, Gruetzner R, Kandzia R, et al. Golden gate shuffling: a one-pot DNA shuffling method based on type IIs restriction enzymes. PLoS One. 2009;4:e5553.

[37] Chen WH, Qin ZJ, Wang J, et al. The MASTER (methylation-assisted tailorable ends rational) ligation method for seamless DNA assembly. Nucleic Acids Res. 2013;41:e93.

[38] Sarrion-Perdigones A, Falconi EE, Zandalinas SI, et al. GoldenBraid: an iterative cloning system for standardized assembly of reusable genetic modules. PLoS One. 2011;6:e21622.

[39] Casini A, MacDonald JT, De Jonghe J, et al. One-pot DNA construction for synthetic biology: the Modular Overlap-Directed Assembly with Linkers (MODAL) strategy. Nucleic Acids Res. 2014;42:e7.

[40] Weber E, Engler C, Gruetzner R, et al. A modular cloning system for standardized assembly of multigene constructs. PLoS One. 2011;6:e16765.

[41] Chao R, Yuan Y, Zhao H. Recent advances in DNA assembly technologies. FEMS Yeast Res. 2014. Epub ahead of print.

[42] Lisby M, Rothstein R. Cell biology of mitotic recombination. Cold Spring Harb Perspect Biol. 2015;7:a016535

[43] Muller H, Annaluru N, Schwerzmann JW, et al. Assembling large DNA segments in yeast. Methods Mol Biol. 2012;852:133–150.

[44] T Jakociunas, AS Rajkumar, J Zhang, et al. CasEMBLR: Cas9-Facilitated Multiloci Genomic Integration of in Vivo Assembled DNA Parts in *Saccharomyces cerevisiae*. ACS Synth Biol. 2015;4(11):1226–1234

[45] Shao Z, Zhao H. Construction and engineering of large biochemical pathways via DNA assembler. Methods Mol Biol. 2013;1073:85–106.

[46] Kuijpers NG, Solis-Escalante D, Bosman L, et al. A versatile, efficient strategy for assembly of multi-fragment expression vectors in *Saccharomyces cerevisiae* using 60 bp synthetic recombination sequences. Microb Cell Fact. 2013;12:47.

[47] Lin Q, Jia B, Mitchell LA, et al. RADOM, an efficient in vivo method for assembling designed DNA fragments up to 10 kb long in *Saccharomyces cerevisiae*. ACS Synth Biol. 2015;4:213–220.

[48] Kuijpers NG, Chroumpi S, Vos T, et al. One-step assembly and targeted integration of multigene constructs assisted by the I-SceI meganuclease in *Saccharomyces cerevisiae*. FEMS Yeast Res. 2013;13:769–781.

[49] Storici F, Lewis LK, Resnick MA. In vivo site-directed mutagenesis using oligonucleotides. Nat Biotechnol. 2001;19:773–776.
[50] Chou CC, Patel MT, Gartenberg MR. A series of conditional shuttle vectors for targeted genomic integration in budding yeast. FEMS Yeast Res. 2015;15.
[51] Manabe K, Kageyama Y, Morimoto T, et al. Improved production of secreted heterologous enzyme in *Bacillus subtilis* strain MGB874 via modification of glutamate metabolism and growth conditions. Microb Cell Fact. 2013;12:18.
[52] Hao T, Han B, Ma H, et al. In silico metabolic engineering of *Bacillus subtilis* for improved production of riboflavin, Egl-237, (R,R)-2,3-butanediol and isobutanol. Mol Biosyst. 2013; 9:2034–2044.
[53] Commichau FM, Alzinger A, Sande R, et al. Overexpression of a non-native deoxyxylulose-dependent vitamin B6 pathway in *Bacillus subtilis* for the production of pyridoxine. Metab Eng. 2014;25:38–49.
[54] Yadav T, Carrasco B, Serrano E, et al. Roles of *Bacillus subtilis* DprA and SsbA in RecA-mediated genetic recombination. J Biol Chem. 2014;289:27640–27652.
[55] Shi T, Wang G, Wang Z, et al. Establishment of a markerless mutation delivery system in *Bacillus subtilis* stimulated by a double-strand break in the chromosome. PLoS One. 2013;8: e81370.
[56] Ogawa T, Iwata T, Kaneko S, et al. An inducible recA expression *Bacillus subtilis* genome vector for stable manipulation of large DNA fragments. BMC Genomics. 2015;16:209.
[57] Carrasco B, Yadav T, Serrano E, et al. *Bacillus subtilis* RecO and SsbA are crucial for RecA-mediated recombinational DNA repair. Nucleic Acids Res. 2015;43:5984–5997.
[58] Watanabe S, Shiwa Y, Itaya M, et al. Complete sequence of the first chimera genome constructed by cloning the whole genome of *Synechocystis* strain PCC6803 into the *Bacillus subtilis* 168 genome. J Bacteriol. 2012;194:7007.
[59] Iwata T, Kaneko S, Shiwa Y, et al. *Bacillus subtilis* genome vector-based complete manipulation and reconstruction of genomic DNA for mouse transgenesis. BMC Genomics. 2013;14:300.
[60] Ajikumar PK, Xiao WH, Tyo KE, et al. Isoprenoid pathway optimization for Taxol precursor overproduction in *Escherichia coli*. Science. 2010;330:70–74.
[61] Park SJ, Lee TW, Lim SC, et al. Biosynthesis of polyhydroxyalkanoates containing 2-hydroxybutyrate from unrelated carbon source by metabolically engineered *Escherichia coli*. Appl Microbiol Biotechnol. 2012;93:273–283.
[62] Yim H, Haselbeck R, Niu W, et al. Metabolic engineering of *Escherichia coli* for direct production of 1,4-butanediol. Nat Chem Biol. 2011;7:445–452.
[63] Zhou L, Niu DD, Tian KM, et al. Genetically switched D-lactate production in *Escherichia coli*. Metab Eng. 2012;14: 560–568.
[64] Datsenko KA, Wanner BL. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc Natl Acad Sci USA. 2000;97:6640–6645.
[65] Copeland NG, Jenkins NA, Court DL. Recombineering: a powerful new tool for mouse functional genomics. Nat Rev Genet. 2001;2:769–779.
[66] Murphy KC. The lambda Gam protein inhibits RecBCD binding to dsDNA ends. J Mol Biol. 2007;371:19–24.
[67] Fu J, Bian X, Hu S, et al. Full-length RecE enhances linear-linear homologous recombination and facilitates direct cloning for bioprospecting. Nat Biotechnol. 2012;30:440–446.
[68] Zhang Y, Buchholz F, Muyrers JP, et al. A new logic for DNA engineering using recombination in *Escherichia coli*. Nat Genet. 1998;20:123–128.
[69] Marcellin E, Chen WY, Nielsen LK. Understanding plasmid effect on hyaluronic acid molecular weight produced by *Streptococcus equi* subsp. *zooepidemicus*. Metab Eng. 2010;12: 62–69.
[70] Sabri S, Steen JA, Bongers M, et al. Knock-in/Knock-out (KIKO) vectors for rapid integration of large DNA sequences, including whole metabolic pathways, onto the *Escherichia coli* chromosome at well-characterised loci. Microb Cell Fact. 2013; 12:60.
[71] Ublinskaya AA, Samsonov VV, Mashko SV, et al. A PCR-free cloning method for the targeted φ80 Int-mediated integration of any long DNA fragment, bracketed with meganuclease recognition sites, into the *Escherichia coli* chromosome. J Microbiol Methods. 2012;89:167–173.
[72] Kuhlman TE, Cox EC. Site-specific chromosomal integration of large synthetic constructs. Nucleic Acids Res. 2010;38:e92.
[73] Yang J, Sun B, Huang H, et al. High-efficiency scarless genetic modification in *Escherichia coli* by using lambda red recombination and I-SceI cleavage. Appl Environ Microbiol. 2014;80: 3826–3834.
[74] Juhas M, Evans LD, Frost J, et al. *Escherichia coli* Flagellar Genes as Target Sites for Integration and Expression of Genetic Circuits. PLoS One. 2014;9:e111451.
[75] Juhas M, Ajioka JW. Identification and validation of novel chromosomal integration and expression loci in *Escherichia coli* flagellar region 1. PLoS One. 2015;10:e0123007.
[76] Juhas M, Ajioka JW. Flagellar region 3b supports strong expression of integrated DNA and the highest chromosomal integration efficiency of the *Escherichia coli* flagellar regions. Microb Biotechnol. 2015;8:726–738.
[77] Gibson DG. Programming biological operating systems: genome design, assembly and activation. Nat Methods. 2014;11: 521–526.
[78] Itaya M. A synthetic DNA transplant. Nat Biotechnol. 2010;28: 687–689.
[79] Karas BJ, Jablanovic J, Sun L, et al. Direct transfer of whole genomes from bacteria to yeast. Nat Methods. 2013;10(5):410–2
[80] Isaacs FJ, Carr PA, Wang HH, et al. Precise manipulation of chromosomes in vivo enables genome-wide codon replacement. Science. 2011;333:348–353.
[81] Mandell DJ, Lajoie MJ, Mee MT, et al. Biocontainment of genetically modified organisms by synthetic protein design. Nature. 2015;518:55–60.
[82] Glass JI. Synthetic genomics and the construction of a synthetic bacterial cell. Perspect Biol Med. 2012;55:473–489.
[83] Juhas M. *Pseudomonas aeruginosa* essentials: an update on investigation of essential genes. Microbiology 2015;161:2053–2060
[84] Juhas M, Eberl L, Church GM. Essential genes as antimicrobial targets and cornerstones of synthetic biology. Trends Biotechnol. 2012;30:601–607.
[85] Werneburg M, Zerbe K, Juhas M, et al. Inhibition of lipopolysaccharide transport to the outer membrane in *Pseudomonas aeruginosa* by peptidomimetic antibiotics. Chembiochem. 2012; 13:1767–1775.
[86] Glass J, Assad-Garcia N, Alperovich N, et al. Essential genes of a minimal bacterium. Proc Natl Acad Sci USA. 2006; 103:425–430.
[87] Pósfai G, Plunkett Gr, Fehér T, et al. Emergent properties of reduced-genome *Escherichia coli*. Science. 2006;312: 1044–1046.
[88] Langridge GC, Phan MD, Turner DJ, et al. Simultaneous assay of every *Salmonella Typhi* gene using one million transposon mutants. Genome Res. 2009;19:2308–2316.
[89] Moya A, Gil R, Latorre A, et al. Toward minimal bacterial cells: evolution vs. design. FEMS Microbiol Rev. 2009;33: 225–235.
[90] Juhas M, Stark M, von Mering C, et al. High confidence prediction of essential genes in *Burkholderia cenocepacia*. PLoS One. 2012;7:e40064
[91] Juhas M, Eberl L, Glass JI. Essence of life: essential genes of minimal genomes. Trends Cell Biol. 2011;21:562–568.
[92] Lee SA, Gallagher LA, Thongdee M, et al. General and condition-specific essential functions of *Pseudomonas aeruginosa*. Proc Natl Acad Sci USA. 2015;112(16):5189–94.
[93] Turner KH, Wessel AK, Palmer GC, et al. Essential genome of *Pseudomonas aeruginosa* in cystic fibrosis sputum. Proc Natl Acad Sci USA. 2015;112:4110–4115.
[94] Yu B, Sung B, Koob M, et al. Minimization of the *Escherichia coli* genome using a Tn5-targeted Cre/loxP excision system. Nat Biotechnol. 2002;20:1018–1023.
[95] Noskov VN, Ma L, Chen S, et al. Recombinase-mediated cassette exchange (RMCE) system for functional genomics studies in *Mycoplasma mycoides*. Biol Proced Online. 2015;17:6.

[96] Suzuki Y, Assad-Garcia N, Kostylev M, et al. Bacterial genome reduction using the progressive clustering of deletions via yeast sexual cycling. Genome Res. 2015;25: 435–444.

[97] Quiles-Puchalt N, Carpena N, Alonso JC, et al. Staphylococcal pathogenicity island DNA packaging system involving cos-site packaging and phage-encoded HNH endonucleases. Proc Natl Acad Sci USA. 2014;111:6016–6021.

[98] Oliveira L, Tavares P, Alonso JC. Headful DNA packaging: bacteriophage SPP1 as a model system. Virus Res. 2013;173: 247–259.

[99] Zhang L, Sun Y, Chang L, et al. A novel method to produce armored double-stranded DNA by encapsulation of MS2 viral capsids. Appl Microbiol Biotechnol. 2015;99: 7047–7057.

[100] Gallagher LA, Shendure J, Manoil C. Genome-scale identification of resistance functions in *Pseudomonas aeruginosa* using Tn-seq. MBio. 2011;2:e00315–e00310.

[101] Skurnik D, Roux D, Aschard H, et al. A comprehensive analysis of in vitro and in vivo genetic fitness of *Pseudomonas aeruginosa* using high-throughput sequencing of transposon libraries. PLoS Pathog. 2013;9:e1003582.