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HIGHLIGHTS

- *Saccharomyces cerevisiae* is a robust and efficient host for cloning microbial genomes.
- Recent developments allow the efficient engineering of microbial genomes in yeast that can then be rescued using transplantation/transfection to yield modified bacteria/viruses.
- Overcoming bottlenecks is key to expand use of yeast as a factory and to offer new possibilities in the synthetic biology field.
- Rapid synthesis, assembly or modification of viral and bacterial genomes may be a critical factor to respond to emerging pathogens.

ABSTRACT

Yeast cells have long been used as hosts to propagate exogenous DNA**.** Recent progress in genome editing opens new avenues in synthetic biology. These developments allow the efficient engineering of microbial genomes in *Saccharomyces cerevisiae* that can then be rescued to yield modified bacteria/viruses. Recent examples show that the ability to quickly synthesize, assemble and/or modify viral and bacterial genomes may be a critical factor to respond to emerging pathogens. However, this process has some limitations. DNA molecules much larger than two megabase pairs are complex to clone, bacterial genomes have proven difficult to rescue, and the dual-use potential of these technologies must be carefully considered. Regardless, the use of yeast as a factory has enormous appeal for biological applications.

INTRODUCTION

Laboratory workhorses such as *Escherichia coli*, *Bacillus subtilis*, and *Saccharomyces cerevisiae* have proved invaluable since they have been used as hosts to propagate and edit genetic material of other organisms. Initially, relatively small DNA fragments were cloned but, over time, this size has gradually increased and now reach the megabase range, including complete microbial genomes of native or synthetic origin[1–4] .

In this review, we will discuss the ever-expanding use of yeast as an efficient propagating and editing factory for the genomes of various microbial species. This process involves the cloning or assembly of a full or partial genome into yeast, its engineering and rescue into a suitable recipient cell to rescue the designed function or live cells (**Figure 1**). This approach can be a novel method to study intractable organisms, genetically edit intractable organisms or build new living systems for basic and applied biology. Each component of the yeast factory cycle is detailed below. Potential barriers are discussed as well as the risks/benefits of such an approach.

IN-YEAST CLONING OF WHOLE, NATIVE, AND SYNTHETIC MICROBIAL GENOMES

Yeast has long been used as a host to clone DNA molecules, either as Yeast Artificial Chromosomes (YACs) or Yeast Centromeric plasmids (YCps), from a wide range of donor organisms. Many of the early examples involved cloning genomic DNA fragments from a range of eukaryotic[5,6] and prokaryotic species[7] as well as viruses[8,9] for genome analysis, including physical maps of complex genomes and gene function studies. However, several issues of chimeras and instability of some cloned heterogeneous DNA in yeast reduced its use, while vectors in bacterial systems such as cosmids and bacterial artificial chromosomes (BACs) gained favor for genome analysis and development of reverse genetics tools.

Over the past decade, yeast has re-emerged as an attractive genome engineering host, bolstered by a groundbreaking experiment to assemble and boot-up the first “synthetic cell”[1], and subsequently, by the cloning of several partial and full bacterial or eukaryotic genomes as well as assembly of viral genomes (**Table 1**).

Multiple approaches can now be used to clone large DNA fragments in yeast, including complete megabase-sized genomes. Depending on the characteristics of the donor organism or downstream applications, some approaches enable the cloning of native genomes whereas others permit the simultaneous cloning, editing or assembly of entire genomes from PCR-amplified, fully synthetic or TAR-cloned fragments. All of these methods require the presence of certain

yeast genetic elements, including an autonomously replicating sequence (ARS) a centromere and a selection marker in order to replicate and maintain the cloned DNA. An ARS is not necessarily required for genomes with low G+C% (<40%) as the AT-rich consensus motif may naturally occur in their sequence (**Figure 1A**). These elements can be added before cloning, as a plasmid integrated in a bacterial genome. Then, the newly marked genome is isolated and transferred intact into yeast spheroplasts using the conventional yeast transformation procedure[10,11] or by fusing the bacterial cell to yeast[12] (**Figure 1B**). The advantage of this approach is the selection of vector insertion sites that do not interfere with bacterial viability, which is convenient for genomes that are meant to be transplanted into a recipient cell to produce live cells. Another approach, TAR-cloning, exploits yeast's ability to efficiently recombine DNA fragments if they contain ends (~60 bp) that are homologous to a target sequence. In this case, the genome is isolated, linearized *in vitro* by a restriction enzyme or using the CRISPR-Cas9 system and co-transformed into yeast together with a linear yeast vector containing homology sequences[13–16] (**Figure 1B**). A variation of this approach is CReasPy-Cloning which enables the simultaneous cloning and engineering of megabase-sized genomes in yeast[17] (**Figure 1B**). The TAR-cloning approach can be extended so that the yeast transformation is carried out with multiple overlapping fragments, either PCR-amplified, synthetic or previously TAR-cloned (**Figure 1C**), allowing for genome-wide engineering of microbial genomes.

Using these methods, many bacterial and viral genomes, both native and synthetic, have been cloned or assembled in yeast. Key examples are shown in **Table 1**. For future target genomes, certain considerations can be factored into the choice of the cloning method. These include whether the organism is cultivable, transformable and/or has genetic tools. If the organism has all of these characteristics, then any of the outlined approaches can be used. For other organisms lacking one or more characteristics or for large scale editing, the *in vitro* or assembly methods are more appropriate.

IN-YEAST GENOME ENGINEERING

Over the last decade, the cost of DNA synthesis has drastically reduced, almost reaching the 0.01$/base bar. Such low costs have enabled the engineering of organisms with fully synthetic DNA, with recent examples of re-coded or re-organized genomes[3,4]. As a result, genome editing can now be performed by assembly of synthetic fragments in yeast. This approach remains nonetheless costly at the megabase scale and may be excessive for small, localized editing tasks. Therefore, depending on the need, it may be more appropriate to use one of the

many genome engineering tools already available in yeast to modify the native cloned genome. Particular examples are TREC[18], CRISPR-Cas9[19–22] and Cre-Lox[23] (**Figure 1D**). The first system was developed for the scar-less edition of mycoplasma genomes cloned in yeast[24,25], and was later improved in TREC-IN[26,27]. Cas9, the well-known and broadly used RNA-guided endonuclease, has been adapted to a wide array of organisms, including yeast[28]. Due to the very high efficiency of this system, it opened the door for marker-less genome edition, with the ability to delete, add or replace genomic loci in the kbp range. Given its efficiency, it has become the engineering method of choice for precisely altering genomes cloned in yeast[29,30]. Finally, Cre-Lox has also been extensively used for targeted editing, but interestingly, it is the basis of SCRaMbLE, a system enabling massive chromosome rearrangements to produce strains with large genotypic diversity[31,32]. Regarding microbial genomes cloned in yeast, the Cre-Lox system was notably used during the construction of the "minimal cell"[2,33].

RESCUE OF GENOMES CLONED IN YEAST: TRANSPLANTATION, TRANSFECTION OR *IN VITRO* APPROACHES

Once a microbial genome has been modified in yeast, it can be "rescued" using various approaches. For this review, "rescue" is defined as the process by which the cloned genome isolated from yeast is converted into the biological entity it encodes.

Since viruses are generally simpler systems, they are relatively easy to rescue (**Figure 1E, right panel**). In many cases, viruses can be reconstituted by transfecting their modified genomes or fusion into host cells[34–38]. For RNA viruses, the modified genomes can be transcribed *in vitro* using purified RNA Polymerase and the resulting RNAs transfected into host cells[39]. Significantly, the Noireaux laboratory has shown the capacity to package bacterial viruses *in vitro* using the TXTL system[40]. In other cases, it is necessary to use helper genes or viruses to boot-up the recombinant genomes[41–43].

For modified bacterial genomes, the rescue is more difficult, due in part to larger genome size, more complicated pathways, and cellular structure. One possibility to rescue a whole genome is to isolate intact edited microbial chromosomes from yeast and transfer them into recipient cells (**Figure 1E, left panel**)[10,44–46]. This process is known as Genome Transplantation (GT) and yields live cells driven by the donor recombinant genomes. It is for now limited to a small set of mycoplasma species.

For non-mycoplasma bacterial species, it is convenient to use yeast to clone and/or engineer large sub-genomic fragments and then integrate them into native target bacterial genomes for desired applications. For example, Fredens *et al.* have used assembly of synthetic *E. coli* 100kb fragments in yeast as an intermediate to generate an *E., coli* strain that uses only 61 codons for protein synthesis[3], instead of the native 64 codons. A similar approach was used by Lau et al. to recode large segments of *Salmonella typhimurium* genome's, using iterative genomic integration of 10-25 kb chunks assembled in yeast [47].

BOTTLENECK AND FUTURE DEVELOPMENTS

Although potentially extremely powerful, the in-yeast cloning and editing of microbial genomes comes with a few drawbacks and bottlenecks.

Based on previous experience, we expect that the cloning of genomes in yeast to be more readily achievable compared to the subsequent rescue of the genomes. In addition, viral genomes have also proven much easier to clone and rescue than their bacterial counterpart.

In-yeast cloning. For bacterial genomes, the nature of the cloned DNA, as well as its genetic content should be taken into consideration. First, size might matter. To date, the *Haemophilus influenza* and the *Spiroplasma citri* chromosomes are the largest DNA molecules cloned in yeast (1.8Mb)[12,45]. However, it is still not yet clear whether much larger genomes such as *Bacillus subtilis* (4.2 Mb) can be transformed intact in yeast. Approaches that allow the construction of a genome inside the yeast cell[48] or based on bacterial/yeast fusion could alleviate this problem[12]. Moreover, results from the SC2.0 consortium and others suggest that replicating up to a 12Mbp chromosome may not be an issue other than, potentially, the cumulative size of the yeast and the cloned genome[49,50]. With this in mind, using a yeast cell with a minimized genome could be key for increasing the amount of "cargo" DNA that it could carry. The G+C% of the cloned genome also appears to be a relevant problem. While the cloning of the A+T rich mycoplasma genomes, (0.58 to 1.8Mb; G+C%<40%) is routine, bacterial genomes with much higher G+C% require adding an ARS to the target genomes for maintenance in yeast[4,48]. Another issue is ectopic expression of the cloned genome that may be toxic to yeast[51]. This can be solved by empirical identification of the culprit toxic gene(s), or through the engineering of new host cells that are genetically isolated from their cargo (e.g. using orthogonal promoters and RBS, or having a non-standard genetic code). Lastly, the presence of repeat sequences in target genomes may present issues in yeast, especially if they are in the overlapping homologous

sequences during TAR assembly. However, in our experience, if the repeat sequences are buried within the fragments or genomes to be assembled, they have not caused problems[10,11,35,36].

In-yeast engineering. Current methods are effective to perform a few modifications at a time. TAR assembly alleviates this issue to some extent but it is somewhat limited by the number of fragments that can be used as well as the efficiency of homologous recombination. A potential improvement may be the use of yeast mutants impaired in competing repair pathways, such as non-homologous end-joining, as engineering hosts for microbial genomes. Another possibility is the use of other yeasts as hosts, such as *Schizosaccharomyces pombe*, *Yarrowia lipolytica*, *Pichia pastoris* and *Kluyveromyces marxianus*. In addition, development of improved technology should increase the speed and widen the scale of microbial genome engineering in yeast[31,52–54].

Rescue of viral genomes. For the most part, rescue of viral genomes is not a major concern. However, there are still a few viruses, such as African swine fever virus (ASFV), whose genomes are not infectious or for which there are no known helper genes or helper viruses to reconstitute live virus from recombinant genomes[55]. In addition, novel dangerous viruses may emerge as humans encroach into new environments for which reverse genetics would need to be developed. Thus, for these types for viruses, generalized methods to boot them up would need to be developed.

Rescue of bacterial genomes. Currently, the most broadly applicable strategy is to transfer sections of the engineered genome back to the original cell and proceed in an incremental manner to completely replace the original genome[3,47]. Alternatively, GT can be attempted to transfer in one step the entirety of the engineered genome. However, this strategy has only been achieved for a small cluster of *Mollicutes* and appears to have multiple hurdles that limit its broad application. First, the recipient cell should be closely related to the donor genome, in order to process and replicate the donor's genetic information[10,45]. Therefore, to apply GT to other species, one needs to develop a specific set of recipient cells and transplantation methods. The recipient cell may also be engineered to remove a number of natural systems that might limit the efficiency of GT. For instance, secreted or membrane-bound nucleases[56], internal defense mechanisms against foreign DNA, such as restriction-modification systems[57–59] or CRISPR-Cas9[60,61], may degrade unprotected donor genomes prior to or after entry in the recipient cell. A recipient cell with a strong recombination activity may be problematic GT, as it could lead to increased frequency of illegitimate exchanges between the donor and recipient genomes, leading to transfer of the selection marker to the recipient' genome or the emergence of chimeric

chromosomes and thus, to hybrid cells rather than the desired outcome. Using ghost cells devoid of the resident DNA as recipient cells or using DNA-damaging agents to make the resident genome non-functional for recombination may overcome this issue. Another important concern is that DNA uptake may be limited by transformation efficiency and cell surface structure. To bypass these obstacles, improvement of methods to make spheroplasts/protoplasts in target organisms may be used to remove cell walls to increase DNA uptake. In addition, other DNA transfer methods, such as conjugation, can be used to transfer a genome from the donor species to the recipient.

CONCLUSIONS AND PERSPECTIVES: BENEFITS AND RISKS OF SUCH TECHNOLOGIES

The combination of genome transplantation/transfection and genome engineering in yeast is an exciting approach to manipulate synthetic and native genomes. This approach could be of importance for genetically intractable yet, medically and industrially important organisms, such as *Chlamydia*, *M. leprae* and *Clostridia* and ASFV for which it would provide convenient tools to better understand their biology. However, there are still many unanswered questions regarding the process of GT and at a lower degree back transfection. More investigation in understanding the process would facilitate its expansion to other organisms.

Moreover, the ability to quickly synthesize or modify viral or bacterial genomes might be a critical factor to respond to emerging pathogens[62–64]. Indeed, while acquiring genomic information is now a matter of days due to (meta)genome sequencing, creating new microbial strains is much longer. These new strains can be used as vaccines, or to decipher the virulence of pathogens. The design of such strains is often not the most limiting step but rather, the actual manufacturing of the modified biological entity. However, progress made in DNA synthesis, and now extremely short turnaround times of commercial suppliers, suggest that this bottleneck may soon disappear. Recently, it was shown that only 30 days were necessary to go from a publicly released sequence of SARS-CoV2 to a functional, rescued recombinant virus, using yeast to assemble synthetic DNA fragments[39]. This example highlights the potential of in-yeast cloning and editing, and indicates that it can be highly beneficial to the global population.

Nevertheless, as discussed elsewhere, advances in synthetic genomics methods, including methods described herein, raise several dual-use concerns[36,64,65]. A number of measures can be adopted to ensure biological control: some are inherent to the organism (engineered

auxotrophy; use a non-standard genetic code) while other devices can be added (genetically encoded kill-switches, incorporation of unnatural amino acids into essential proteins…).
In conclusion, while it is clear that budding yeast is a powerful engineering factory, there is still room for improvement to fulfil its use for synthetic biology applications.

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FIGURE LEGEND

Figure 1: Overview of the in yeast cloning and editing process and associated techniques. (A)Specific genetic elements derived from yeast must be added to the target genomes to ensure proper replication and segregation. (B) Multiple strategies can be used to introduce the yeast element in the genome, and to introduce the genome in the yeast cells. (C) The target genome can be fully synthesized chemically or biochemically and assembled in yeast cells. (D) A wide

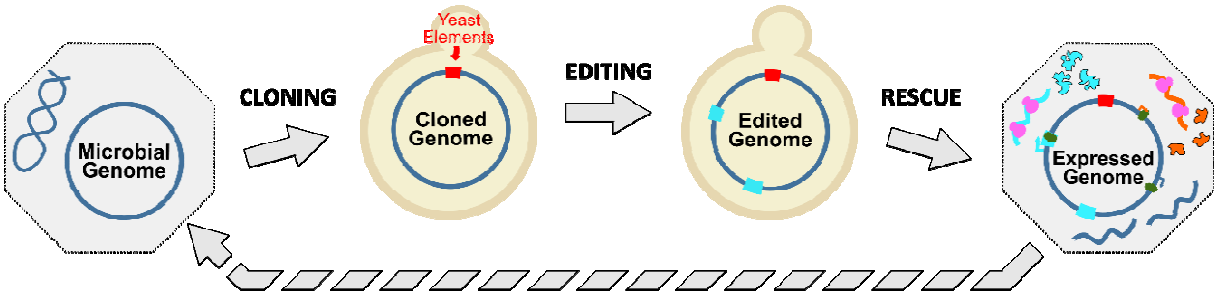
array of tools are available in yeast to perform genome editing. (E) Biological entities can be reconstituted from the edited microbial genomes using diverse strategies depending on their viral or bacterial nature.

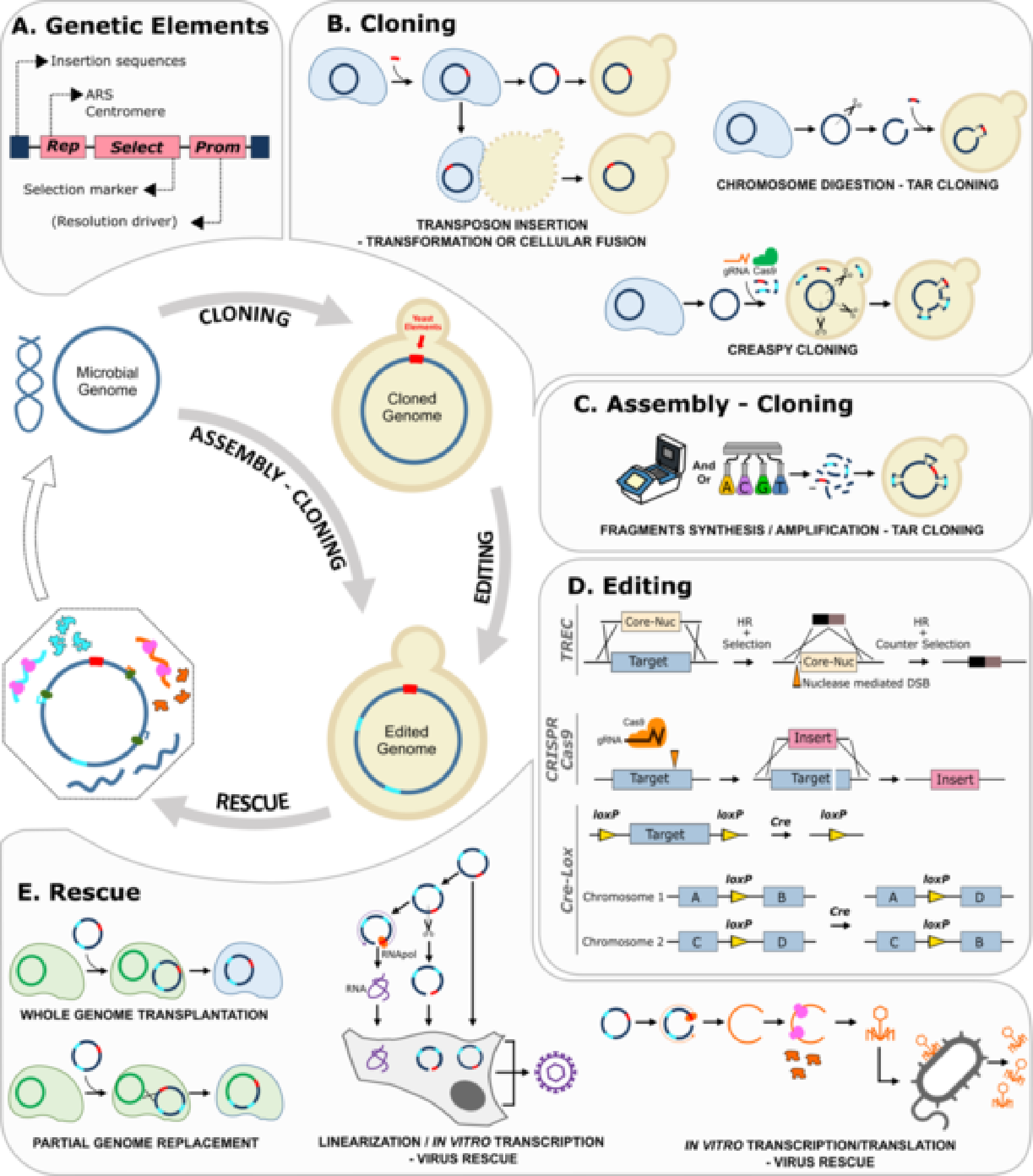
Table 1: Key examples of microbial genomes cloned or edited in yeast *

Organism	Size (Mbp)	% G+C	Genetic code	Cloning strategy **	Rescue strategy	References*
Mollicutes						
Mycoplasma genitalium	0,58	32	Non standard	Synthesis & Assembly	N/A	[66]
Mycoplasma mycoides subsp. *capri*	1,1	24	Non standard	Cloning	Transplantation	[10]
Mycoplasma pneumoniae	0,81	41	Non standard	Cloning	N/A	[11,17]
JCVI Syn 1.0	1,1	24	Non standard	Synthesis & Assembly	Transplantation	[1]
Acholeplasma laidlawii	1,5	32	Universal	Cloning	N/A	[51]
JCVI Syn 3.0	0,53	24	Non standard	Synthesis & Assembly	Transplantation	[2]
Mycoplasma mycoides subsp. *mycoides*	1,2	24	Non standard	Cloning	Transplantation	[45]
Mycoplasma capricolum subsp. *capricolum*	1,1	25	Non standard	Cloning	Transplantation	[45]
Mycoplasma leachii	1	24	Non standard	Cloning	Transplantation	[45]
Mycoplasma putrefaciens	0,8	27	Non standard	Cloning	Transplantation	[45]
Spiroplasma citri	1,8	26	Non standard	Cloning	N/A	[45]
Mycoplasma hominis	0,66	27	Non standard	Cloning	N/A	[67]
Mesoplasma florum	0,79	27	Non standard	Cloning	Transplantation	[46]
Mycoplasma capricolum subsp. *capripneumoniae*	1	24	Non standard	Cloning	Transplantation	Pers. com 2020
Mycoplasma feriruminatoris	1,2	24	Non standard	Cloning	Transplantation	Pers. com. 2019
Proteobacteria						
Haemophilus influenzae	1,8	38	Universal	Cloning	N/A	[12]
Escherichia coli (reduced genome)	1,03	51	Universal	Synthesis & Assembly	N/A	[48]
Escherichia coli (recoded genome)	3.98	N/A	Universal	Synthesis & Assembly	Partial replacement	[68]
Salmonella typhimurium (recoded genome)	4.47	N/A	Universal	Synthesis & Assembly	Partial replacement	[47]
Escherichia coli (recoded genome)	3,98	N/A	Universal	Synthesis & Assembly	Partial replacement	[3]
Caulobacteur ethensis 2.0 (reduced/recoded genome)	0,78	57	Universal	Synthesis & Assembly	N/A	[4]
Cyanobacteria						
Prochlorococcus marinus	1,6	31	Universal	Cloning	N/A	[69]
Synechococcus elongatus (fragments)	2,7	55	Universal	Cloning	N/A	[70]
Diatoms						
Phaeodactylum tricornutum Chromosome 25	0,5	48	Universal	Cloning	N/A	[71]
Phaeodactylum tricornutum Chromosome 26	0,44	48	Universal	Cloning	N/A	[71]
Viruses						
Dengue virus type 2	0,011	46	Universal	Cloning	RNA transfection	[72]
MERS-CoV	0,029	41	Universal	Synthesis & Assembly	DNA transfection	[73]
AcMNPV	0,14	45	Universal	Synthesis & Assembly	DNA transfection	[74]
HCMV	0,23	49	Universal	TAR cloning & Assembly	DNA transfection	[35]
Herpes simplex virus type 1	0,15	68	Universal	TAR cloning & Assembly	DNA transfection	[36]
Horsepox Virus	0,21	33	Universal	Synthesis & Assembly #	DNA transfection	[42]
SARS-CoV-2	0,03	38	Universal	Synthesis & Assembly	RNA transfection	[39]
MHV	0,032	42	Universal	Synthesis & Assembly	RNA transfection	[39]
MERS-CoV	0,03	41	Universal	Synthesis & Assembly	N/A	[39]
HCoV-229E	0,027	38	Universal	Synthesis & Assembly	N/A	[39]
ZIKA virus	0,011	51	Universal	Synthesis & Assembly	N/A	[39]
Human RSV-B	0,015	34	Universal	Synthesis & Assembly	N/A	[39]

** Sorting is done by year of publication*
*** Cloning refers to any method described in the text*
 # The assembly was performed in mammalian cells

Graphical Abstract




A. Genetic Elements
Insertion sequences
ARS
Centromere
Rep
Select
Prom
Selection marker
(Resolution driver)
B. Cloning
TRANSPOSON INSERTION
- TRANSFORMATION OR CELLULAR FUSION
CHROMOSOME DIGESTION - TAR CLONING
gRNA Cas9
CREASPY CLONING
CLONING
Microbial Genome
Cloned Genome
ASSEMBLY - CLONING
EDITING
Edited Genome
RESCUE
C. Assembly - Cloning
And
Or
A
C
G
T
FRAGMENTS SYNTHESIS / AMPLIFICATION - TAR CLONING
D. Editing
TREC
Core-Nuc
Target
HR
+
Selection
Core-Nuc
Nuclease mediated DSB
HR
+
Counter Selection
CRISPR Cas9
Cas9
gRNA
Target
Insert
Target
Insert
Cre-Lox
loxP
Target
loxP
Cre
loxP
Chromosome 1
A
loxP
B
Cre
A
loxP
D
Chromosome 2
C
loxP
D
C
loxP
B
E. Rescue
WHOLE GENOME TRANSPLANTATION
PARTIAL GENOME REPLACEMENT
RNApol
RNA
LINEARIZATION / IN VITRO TRANSCRIPTION
- VIRUS RESCUE
IN VITRO TRANSCRIPTION/TRANSLATION
- VIRUS RESCUE