Review

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Essential genes as antimicrobial targets and cornerstones of synthetic biology

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Essential genes are absolutely required for the survival of any living entity. Investigation of essential genes is therefore expected to advance tremendously our understanding of the universal principles of life. Determination of a minimal set of essential genes needed to sustain life also plays an important role in the emerging field of synthetic biology, whose goals include creation of a stringently controlled minimal cell with predesigned phenotypic traits. In addition, due to their indispensability for survival of bacteria, genes encoding essential cellular functions have great potential in medicine as promising targets for the development of novel antimicrobials. Here, we review recent advances in the investigation of essential genes, with emphasis on the practical applications in medicine and synthetic biology.

Essential genes of minimal genomes

Essential genes are absolutely necessary for the survival of an organism. Despite huge differences in size and gene composition, all so far sequenced genomes share a small core set of genes encoding key cellular functions: these are essential genes. Identification of this universal shared minimal set of essential genes needed to sustain a life form is expected to contribute enormously to our understanding of life at its simplest and fundamental level [1]. A broad spectrum of experimental and computational approaches has been used and a wide variety of naturally occurring near-minimal genomes, including those of bacterial endosymbionts [2–10], have been studied over the past few years to identify genes indispensable for life.

Determination of a minimal set of essential genes, besides having importance as one of the most fundamental questions of biology, also plays an important role in the emerging field of synthetic biology. One of the main aims of synthetic biology is to create a cell whose genome harbors the minimal set of essential genes [1,11,12]. Subsequent addition of other designed genes or whole metabolic pathways into such minimal cell would allow generation of stringently controlled living cells exhibiting only the desired phenotypic traits. Furthermore, investigation of essential genes is also of great importance for medicine, in particular, for the development of novel antimicrobials. Although there are many strategies to develop novel antibiotics, all share the same feature: antimicrobials must target processes that are indispensable for bacterial growth and survival during infection [13]. Essential genes are absolutely required for survival of the bacterial cell, therefore, gene products involved in essential cell functions belong to the most promising targets of novel antimicrobials.

In this review, we focus on practical applications of essential genes, namely for the development of novel antimicrobials and for the creation of minimal cells with desired features.

Search for the minimal genome

A variety of approaches (comparative genomics, saturation transposon mutagenesis, single-gene-specific mutagenesis, antisense RNA inhibition and experimental and targeted genome reductions) used over the past few years has led to the identification of variable sets of essential genes [4,14–34]. This variability is caused by inherent flaws in the methods used for the identification of minimal genomes. Furthermore, it seems that the outcome of essential gene identification analyses depends strongly on the type of the analysed cell. The set of essential genes identified in a particular bacterium differs to some extent from the set of essential genes identified in a eukaryotic cell or even from the set of essential genes identified in a different bacterial species. In light of these results it might seem appropriate to ask whether a universal minimal set of essential genes required for life actually exists [1].

Based on the outcome of numerous analyses [14–32,35–40], it seems that some genes are absolutely required for the survival of any life form; however, more work is needed, especially in order to assess which of the identified essential genes are universally required and which are indispensable only for the survival of a specific organism. For some of the unknown genes, their identification as an essential gene needs to be followed by investigation of its function. Due to their importance for life, essential genes cannot be analysed by standard mutagenesis, because mutants harbouring disruption in one of the essential genes are unable to grow and survive and consequentially cannot be isolated and examined. Therefore, in order to study a function of an essential gene, instead of disruption mutagenesis, the native promoter of the gene can be exchanged for another (e.g., rhamnose promoter), which is stringently regulated by conditional

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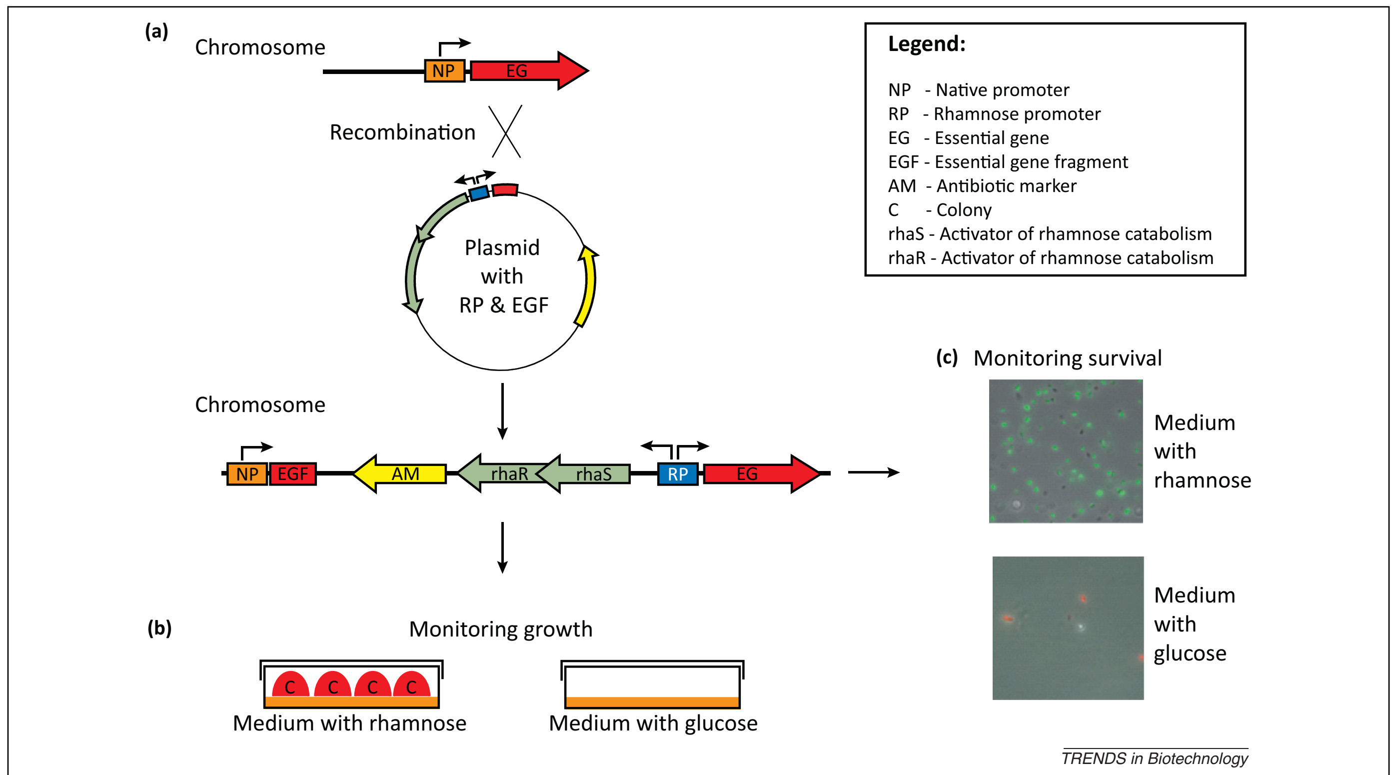

Figure 1. Elucidating functions of essential genes. Instead of standard mutagenesis, functions of essential genes can be investigated with the help of conditional mutants. Here, the native promoter of an essential gene is exchanged for a promoter whose expression can be easily regulated by the composition of the growth medium (e.g., rhamnose inducible promoter). The constructed conditional mutants are only able to grow and survive in the presence of the substrate (e.g., rhamnose), and the phenotypic traits regulated by the investigated essential gene can be analysed by altering the concentration of the substrate in the growth medium [57]. **(a)** Rhamnose inducible promoter together with a short DNA sequence spanning the start region of the target gene is introduced into the cell on a plasmid. By homologous recombination the native promoter of the investigated essential gene is exchanged for the rhamnose inducible promoter. **(b)** The constructed conditional mutant with the essential gene under the control of the rhamnose promoter grows on the medium with rhamnose, but not when rhamnose is supplemented with glucose. **(c)** To investigate essentiality of the gene for cell survival, cells are stained with the BacLight Live/Dead viability stain, grown on the medium with rhamnose or glucose, and examined by fluorescence microscopy. Green fluorescence indicates viable cells, whereas dead cells are fluorescent red. If the investigated gene is essential, conditional mutants survive in the medium with rhamnose but not when rhamnose is supplemented with glucose.

mutagenesis [41]. The expression of an essential gene can then be tightly controlled by the amount of inducer (e.g., rhamnose) added to the growth medium (Figure 1). Identification and assessing functions of essential genes have great implications in synthetic biology. Knowledge of the composition of the universal minimal genome is the first crucial step for the construction of a universal minimal cell to which any desired phenotypic features can be added. By contrast, in medicine, universally essential genes usually cannot be exploited as antimicrobial targets because any drug inhibiting such a function in a bacterium would likely also have a negative effect on its human homologue. Here, the identification of specific accessory essential genes of particular cell types could be more valuable, because their investigation could lead to novel antimicrobials active only against specific bacterial species or orders.

Essential genes in medicine

In medicine, infectious diseases remain a major worldwide cause of morbidity and mortality. This problem has worsened with the emergence of multi-antibiotic resistant bacteria and the failure of pharmaceutical companies to develop antibiotics with truly novel modes of action. As a consequence, resistance against antibiotics is rising, while antibiotic discovery and development are on the decline [42,43]. Not long after penicillin was introduced in the early 1940s, bacteria developed resistance to it. Nowadays, many common bacteria have acquired resistance to multiple antibiotics, making some infections virtually impossible to eradicate [44]. Novel antimicrobials are therefore needed to combat infections. Although there are many strategies to develop novel antibiotics, they all share one feature: antimicrobials must target processes that are indispensable for bacterial growth and survival during infection [13]. Due to their indispensability for survival of all living organisms, including bacteria, essential genes are considered to be the best targets for development of novel antimicrobials.

Although there is no general consensus as to what the exact gene composition of the universal minimal genome could be, it is widely accepted that genes encoding basic biological processes are most likely to be essential [1]. These genes, which are present even in the near-minimal genomes of bacterial endosymbionts, include genes involved in DNA replication, transcription and RNA processing, aminoacyl-tRNA formation, protein folding, cell membrane biosynthesis, as well as genes encoding translation factors and ribosomal subunits. Many of the antibiotics currently used target these basic cellular functions. Among those interfering with nucleic acid synthesis are quinolones targeting DNA gyrase [45] and rifamycins inhibiting RNA synthesis by binding to RNA polymerase

[46]. Antibiotics targeting protein biosynthesis usually inhibit the activity of the ribosome at different stages of protein synthesis [47]. Good examples of these include tetracyclines, which block protein translation by binding to a ribosomal subunit, and puromycin that inhibits protein synthesis by mimicking tRNA [47,48]. Aminoacyl-tRNA synthetases were shown to be inhibited by the broad-spectrum antifungal agent, 5-fluoro-1,3-dihydro-1-hydroxy-2,1-benzoxaborole, by trapping tRNA in the editing site. The trapping of tRNA in the editing site inhibits synthesis of aminoacyl-tRNAs and consequentially blocks protein synthesis [49].

Recent work has shown that the *trans*-translation machinery is essential for the survival of *Helicobacter pylori*. The ubiquitous bacterial *trans*-translation is an important quality control mechanism, which uses a small RNA, SsrA, and a protein cofactor, SmpB, to release ribosomes stalled on defective mRNAs and to add a specific tag sequence to aberrant polypeptides to direct them to degradation pathways. It has been shown that both *ssrA* and *smpB* are essential in *H. pylori*, and therefore represent excellent targets for the development of novel antibiotics [50]. The cell cycle is another potential target. Determination of essential cellular genes, *ccrM* and *menH*, that regulate the bacterial cell cycle has led to the development of potential broad spectrum antibiotics: borinic esters that are now in clinical trials [51]. The cells wall is an essential structure for the majority of bacteria. The cell wall protects the cell from damage and osmotic lysis, and as such, is the target of our most potent antimicrobials [52]. Among the most well-known antibiotics that interfere with the cell wall synthesis is penicillin, which inhibits synthesis of the major component of the cell wall: peptidoglycan [53]. Recently, a lead compound of the family of peptidomimetic antibiotics, based on the antimicrobial peptide protegrin I, has been synthesised, which shows potent activity against *Pseudomonas* spp. Biochemical and genetic examinations led to the identification of *lptD* (formerly *ostA*) as the target of peptidomimetics [54]. This gene is widely distributed among Gram-negative bacteria, including *Escherichia coli*, in which it is involved in the biogenesis of the outer membrane [55,56]. Investigations are currently under way to analyse in detail the essential role of *lptD* for cell survival [56].

Essential genes can be divided into two categories: core essential genes, which are universally required for every living cell, and accessory essential genes, indispensable for the survival of specific cell types. Accessory essential genes have the most important implications in medicine as potential drug targets in bacteria belonging to particular evolutionary lineages. Several approaches can be used to identify these species- or order-specific essential genes. A relatively fast and reliable method exploits comparative genomics approach to analyse genomes of phylogenetically closely related organisms in order to identify accessory essential genes [57]. The comparison of genomes of phylogenetically related organisms allows a more reliable prediction of essential genes than those previously determined for very distantly related organisms. This method avoids major drawbacks of the comparative genomics: lower sequence homologies between phylogenetically unrelated organisms and nonorthologous genes encoding the same essential cellular functions [1]. Recently, this method has been applied for the determination of essential genes of *Burkholderia cenocepacia* [57] (Figure 2). It has led to the identification of a number of novel essential genes, many of them without known homologues in databases. Some of these novel accessory essential genes may have the potential to be exploited as targets of antimicrobials against members of the order *Burkholderiales*.

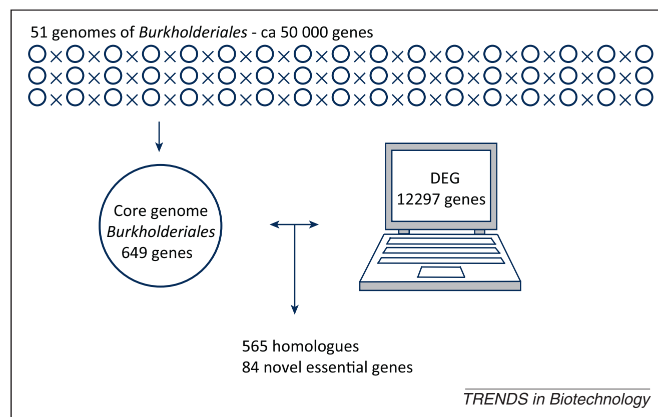

Figure 2. *In silico* identification of essential genes of *Burkholderiales*. The comparison of genomes of phylogenetically related organisms allows a reliable prediction of species- or order-specific essential genes, avoiding major comparative genomics drawbacks, such as low homologies between phylogenetically unrelated organisms and nonorthologous genes encoding the same essential cellular functions. Recently, application of this method has led to the identification of essential genes of *Burkholderiales*, including a number of novel essential genes with no homologues in the database of essential genes (DEG) [75–77].

Essential genes in synthetic biology

The identification of the minimal set of essential genes required to support life has interesting implications in the emerging field of synthetic biology and *in vitro* systems [58]. One of the goals of synthetic biology is to synthesise a living cell 'chassis', which can be amended with properties predesigned for various tasks, ranging from gene therapy to biofuels production and biodegradation [59]. It is unclear however, whether minimal genomes constitute the best chassis, because robust and rapid growth and access to multiple pathways seem to benefit from larger genomes. However, because the exact composition of the minimal genome remains elusive, direct comparison will require further investigation.

Two approaches can be used to achieve the goal of a minimal genome: top-down and bottom-up. The top-down approach aims to simplify further existing cells by eliminating nonessential genes, whereas the bottom-up approach attempts to create an artificial cell by synthesising all its essential components [33].

Top-down

The simplification of existing cells in the top-down approach can be performed either randomly by cultivating cells under the conditions that favour gene loss or by targeted genome reductions using a wide variety of experimental approaches. The targeted approach of genome reductions has been applied to several microorganisms in recent years, including *E. coli*, *Bacillus subtilis*, *Corynebacterium glutamicum* and

Mycoplasma genitalium [24,26,27,60,61]. For instance, in *E. coli*, targeted deletions allowed removal of up to 30% of the genome without any detectable defect in cell viability [17,27,28,38]. Interestingly, in some cases, the targeted deletion of parts of the genome has led to the improvement of the cell, including increased genome stability and transformation efficiency and higher plasmid and protein yields [26,27]. Even more intriguingly, many of the genes removed without any consequences for cell survival by targeted genome reductions were hitherto considered to be essential, based on the results of saturation transposon mutagenesis analyses [33,62]. This observation is in good agreement with the fact that transposon mutagenesis tends to overestimate the number of essential genes by counting genes as essential if they slow down growth without arresting it completely [1].

Recently, a construction of the first partially synthetic eukaryotic chromosome of yeast *Saccharomyces cerevisiae* has been reported [63]. The synthetic arm of the *S. cerevisiae* chromosome harbours the synthetic chromosome rearrangement and modification by loxP- mediated evolution-system (SCRaMbLE), which can induce massive gene rearrangements of the host genome [63]. This system could lead to generation of *S. cerevisiae* cells encoding specific phenotypic traits and to minimisation of the yeast genome by removal of nonessential genes.

Bottom-up

The major challenges of the bottom-up synthesis of the artificial minimal cells included for a long time the fast, cheap and reliable synthesis of long DNA fragments, their

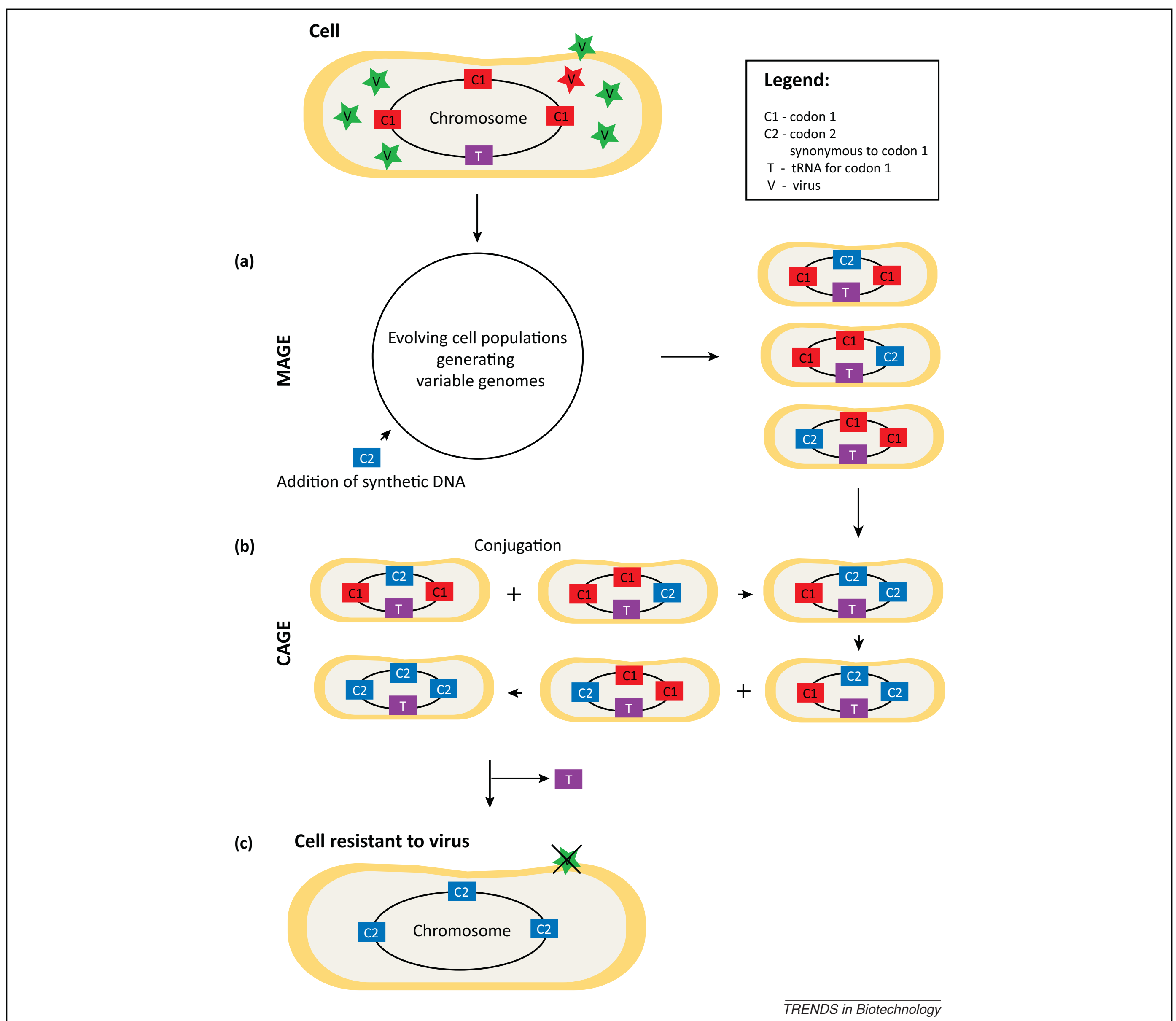

Figure 3. Creation of virus-resistant cells by changing the genetic code of the cell. Recently developed techniques of synthetic biology can be used to generate cells in which all codons (C1) have been exchanged for synonymous codons (C2), thus prohibiting invading viruses from using the translational machinery of the cell. **(a)** Multiplex automated genome engineering (MAGE) can be used for continuous introduction of synonymous codons (C2) to targeted genome locations. **(b)** Subsequently, conjugative assembly genome engineering (CAGE) can be used to combine MAGE-generated changes in order to generate larger genome constructs (currently up to 4.7 Mbp). **(c)** Finally, eliminating the corresponding tRNA gene (T) of the cell for original codon (C1) should render the cell immune to viruses. Due to their indispensability for life, changing codons of essential genes has proved to be the most difficult part of the whole procedure [72,74].

assembly to whole genomes, and their 'booting up' in a cell. Work over the past few decades led to several crucial advances in the *de novo* synthesis of long DNA sequences, which paved the way for the synthesis of artificial minimal genomes [33]. Recently, the bottom-up approach has been applied successfully to construct a complete 3.5-Mbp recombinant genome of *Synechocystis* PCC6803 using the *B. subtilis* genome vector [64]. Neither this recombinant genome nor smaller synthetic organelle genomes are so far functional. Similarly, a not yet functionally tested 582 970-bp genome of *M. genitalium* has been constructed [65]. Finally, last year, two advances in synthetic biology concerning synthesis of long DNA fragments and the booting up of genomes in a cell converged when the whole 1.08-Mbp *Mycoplasma mycoides* genome was synthesised and transplanted into *Mycoplasma capricolum*, thus generating *M. mycoides* cells controlled by the transplanted synthetic genome [66]. In that study, chemically synthesised oligonucleotides were used to assemble overlapping cassettes of 1-kb fragments, which were then joined by *in vitro* recombination to produce intermediate assemblies. These were cloned as bacterial artificial chromosomes in *E. coli* and subsequently assembled in the yeast *S. cerevisiae* into a complete synthetic genome and transplanted into *M. capricolum* [65]. The resulting bacterium contained the entire synthetic genome without any detectable traces of the recipient DNA and was phenotypically identical to the donor strain. During assembly in yeast, the *M. mycoides* genome has been modified using the yeast genetic system, thus generating *M. mycoides* strain that could not be created with tools currently available for this bacterium [67]. Therefore, one potential application of this method is modification of those hard-to-manipulate bacteria that are suitably close to be efficiently transformed by the genomes assembled in yeast. However, it is still too soon to predict if the method described above can be used for transplantation of synthetic minimal genomes in general, because the efficiency for transforming *M. mycoides* is very low (1 in 10 million), thus making combinatorial genomics via genomes assembled in yeast very challenging. The synthetic 1.08-Mbp *M. mycoides* genome was almost identical to the genome of the original wild type. One wrong base in an essential gene rendered the genome inactive and despite sequencing of the 1-kbp DNA fragments used for the assembly, considerable manual detective work was required to identify the detrimental mutation. An additional 19 accidental differences between the designed genome and the final genome were not fixed. The cost for the entire process was approximately 1$ per bp, a price that has not changed significantly for the past 6 years. Alternatives for bringing down the cost include synthesis from oligonucleotide chips (as low as 10 kbp/$) [68–70]. Cost of making many small or radical changes genome-wide can be reduced by multiplex automated genome engineering (MAGE) (Figure 3), with efficiencies close to 25%, and with co-selection 98%, allowing up to eight changes per MAGE cycle [71–73]. Large and complex genome constructs can be combinatorially tested and assembled at the 4.7-Mbp scale using hierarchical conjugative assembly genome engineering (CAGE) (Figure 3) [74]. The ability to make these genome-wide changes simply and with the 'system running' seems to be a huge advantage over booting-up strategies that require multiple expensive intermediates not subject to purifying selections. MAGE has been used to make up to 4 billion genomes per day [72]. This suggests that the next big challenge of synthetic biology is developing clever systems not just for robust growth, but also for radical genome changes that aim at producing useful products. As an example, changing the translational genetic code is an enormous challenge, because the codons of all essential genes need to be changed. However, one payoff could be multi-virus resistant cells (Figure 3) [74], because one of the few things that diverse viruses have in common is the assumption of a highly conserved translation machinery of the host.

Concluding remarks

Investigation of essential gene components of minimal genomes represents one of the most rapidly advancing fields of research, and provides invaluable insights into the basic functions required to sustain life. Examination of essential genes has proved to be an interesting avenue in medicine for the identification of important antimicrobials targets. Here, comparison of genomes of phylogenetically related organisms could lead to identification of novel essential genes that might be exploited as potential antimicrobial targets of drugs against bacteria belonging to particular evolutionary lineages [1,57]. Furthermore, analysis of essential genes over recent years has led to great advances in the field of synthetic biology, and we might be not far away from the day when we will be able to create easily controllable minimal cells with desired phenotypic traits. One of the main advantages of the future minimal genomes and minimal cells will be the complete understanding of all their genes and interactions between them, and the resulting stringent control of the outcome of any biological process in these cells. Changing the translational genetic code, including codons of essential genes, could lead to generation of cells resistant to viruses or incapable of survival outside the laboratory environment.

Nevertheless, several challenges remain. The first challenge is to identify the exact composition of the universal minimal genome, its design, assembly, transplantation and booting up in the host cell (if it turns out that universal minimal genome exists). Another challenge lies in the development of systems capable of robust growth and extensive genome changes, allowing rapid directed evolution to generate cells with desired phenotypic traits.

Disclosure statement

There are not any conflicts of interest related to this manuscript.

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