The quest for the minimal bacterial genome

Esteban Martínez-García and Víctor de Lorenzo*

Systems and Synthetic Biology Program, Centro Nacional de Biotecnología (CNB-CSIC), C/Darwin 3, 28049, Madrid, Spain

Short title: Small genomes: Biology meets Biotechnology

*Correspondence to: Víctor de Lorenzo (vdlorenzo@cnb.csic.es)

Centro Nacional de Biotecnología (CNB-CSIC)
Campus de Cantoblanco
28049 Madrid, Spain
Tel: (+34 91) 585 45 36
Fax: (+34 91) 585 45 06
Abstract

The onset of techniques for both editing extant bacterial chromosomes and synthesizing long DNA sequences has enabled addressing the question on the smallest set of genes and biological functions that are required for running an operative cell. But this is not only a fundamental scientific endeavour: simpler genomes could be easier to understand and eventually reprogram for the sake of diverse applications. This has fostered efforts to eliminate apparently useless -if not annoying DNA segments from many biotechnologically relevant strains as well as attempts to (re)write complete genomes à la carte. Yet, the smallest number of genes that enable given functionalities do vary depending on the ultimate agenda, as both natural and engineered small-size genomes afford genetic simplicity by engaging in a suite of complex interactions with the surrounding medium.

Introduction

The size of naturally-occurring bacterial genomes varies from the few hundreds of genes found in endosymbionts of phloem-feeding insects [1] to the >11000 genes of the soil-dwelling myxobacterium Sorangium cellulosum [2,3]. A detailed comparative inspection suggests that extant prokaryotes may combine a core of persistent genes and biological functions that allow autonomous life (what has been called the paleome [4]) with others for thriving in specific environments: the cenome [5,6]. In between lay a large number of DNA sequences that deal with orchestrating expression of the right genes at the adequate time and location as well as others considered genuinely parasitic or useless for the performance of the bacterium's biological program –in the Laboratory or in the fermentor. The growing data on sequenced genomes [3] enable the pursuit of streamlined microorganisms (specifically bacteria) that keep their whole functionality while being erased of non-essential or detrimental traits. Yet, until the onset of current technologies for genome editing and large-scale DNA synthesis, this endeavour was mostly theoretical. On one hand, comparative bioinformatic analyses of bacterial genomes helped to identify (not without difficulties [7]) universal gene sets present in all living systems [8]. On the other hand, inspection of some of the smallest natural genomes (e.g. endosymbionts) have provided interesting hints on the same question [9,10•,11,12]. But it was only after the establishment of efficient methods for iterative genomic deletions [13,14] that the matter could start to be tested
experimentally, first in *E. coli* [15-18] and then in a suite of microrganisms (see below). The technologies adopted to this end, the bacterial species to be targeted and the set of functions deemed to be desirable in the thereby genome of edited/reduced strains vary considerably depending on the final intention of the exercise. If the purpose is to identify the smallest number of genes that bring about Life (whatever definition we make of it) then the figures may be lowered by reducing genomic complexity to the very minimum at the expense of more dependence on the growth medium [19,20]. When the objective is simplifying bacteria for making them easier to engineer (one of the ambitions of contemporary synthetic biology [21]) one must also pursue a higher degree of robusteness and autonomy that increase the number of genes at stake. Moreover, while contemporary biotechnology demands stable and highly productive strains, genetic drift has always been a major obstacle for long-term production processes. This becomes more acute under harsh bioreactor conditions such as extreme pH and temperature, osmotic pressure and challenging substrates. Applications under industrial conditions (e.g. redox biocatalysis) thus ask for maintenance of a large number of stress-tolerance, metabolic flexibility and heftiness traits that could hardly be found in bacteria with a very small gene set [22-24,25•,26,27]. The sections below summarize some highlights in each of these different tracks and discuss possible avenues in the field. Note that we focus only on bacteria (Fig. 1). Although yeasts are often used as surrogate hosts for assembling synthetic bacterial genomes [28-31,32••,33•], they have not yet been subject themselves to the massive minimization efforts that we have witnessed in the bacterial species in this article. Yet, novel methods for deep editing of yeasts genomes (including rational chemical synthesis and genome reduction) have been reported in recent years [34-36].

The shrinking genome of *E. coli*

Given its long-time status as a reference in Molecular Biology and Microbiobial Biotechnology, it cannot come as surprise that *E. coli* has been the subject of different efforts to reduce its genome to the smallest possible size. One key development to this end was the establishment in 1999 of a method for seamless and iterative genome editing (insertions, deletions, allelic replacements). The procedure was based on repair of double stranded breaks in the chromosome caused by *in vivo* expression of the meganuclease I-Scel targeting a cognate sequence inserted in the genome of interest through homologous recombination [13]. The method has been later adapted to generate
genome variants with a growing number of deletions [15,17,18] that reach now about 20% of the starting chromosomal size [37•]. An alternative procedure relied on recombination between homologous sequences placed in distant locations of the genome through plasmid co-integration [38]. In this case, the genome of *E. coli* could be reduced by >29%. More recently [39•] reported yet another promising method for genomic deletions based on the clustering of multiple essential genes present in the target region in an *E. coli*-S. *cerevisiae* shuttle vector. This affords removal of larger chromosomal segments (~80-205 kb) containing essential and nonessential genes while placing back the cloned essential genes by recombination through I-SceI cleavage as in [13]. Conspicuous morphological properties of such genome-reduced *E. coli* strains included changes in cell length and width and a different distribution of the nucleoid [38]. But the most interesting traits rely in their physiological stability and robustness as hosts of heterologously expressed genes [40-43]. This is explained by the availability of more cell resources for production of recombinant proteins and the limitation of mutations caused by insertion sequences and other endogenous causes of instability e.g prophages. On the downside, *E. coli* strains with a reduced genome appear to decrease the carrying capacity for foreign DNA [44].

In a subsequent screwturn, the technological revolution in prokaryotic genome editing brought about by Multiplex Automated Genomic Engineering (MAGE; [45,46,47•,48,49,50•,51,52•]) and CRISPR/Cas9-based methods ([53]; reviewed in [54] and [55]) and their combination [56] provides users with a panoply of choices reducing the *E. coli* genome even further. A recent development designated CasHRA (Cas9-facilitated Homologous Recombination Assembly) composes directly large circular DNAs in a one-step in vivo assembly process set in yeast [33]. The large circular DNAs are simultaneously hosted in *Saccharomyces cerevisiae* by protoplast fusion, where they are cleaved by RNA-guided Cas9 nuclease to release the linear DNA segments for subsequent assembly by homologous recombination. This strategy has been claimed to ease the production of a 1.03 Mb Minimal Genome of *E. coli* that contained 449 essential genes and 267 important growth genes. However, attempts to transplant this large DNA construct to *E. coli* failed thus far [33] and it is thus impossible to know whether such small complement of genes may sustain a viable *E. coli* cell. In reality, there could be factual limit in the genomic reduction of *E. coli* (up to ~ 30%) and in a fashion that may not be related to the essentiality of the genes by separate. It is possible that genomic DNA acts as also an inner scaffold that provides the coordinates for placing given functions in specific
locations of the cell [57]. Some combinations of large deletions could offset the relative positions of such coordinates and result in non-viable cells. In fact, the organization of genomic segments could encode a physical representation of the cell shape [58-60]. This issue and the corresponding design principles need to be revisited for high-scale engineering bacterial genomes [5,61].

Towards the boundary biology-chemistry: Mycoplasma and L-forms

Since the first reports [62] on the small genome size of *Mycoplasma genitalium* in 1995 (525 genes) this bacterium and other members of the same genera have become a most useful testbed for exploring the minimal number of components that empower a living cell. In fact, some *Mycoplasma* strains are thus far the most and best know biological systems. Virtually all aspects of their components and their interactions have been examined and quantified in extraordinary detail [63,64•,65-67]. This wealth of information includes the tridimensional distribution of their proteome [68] and a whole-cell computational model [69] able to predict complex genotype-phenotype relationships. Being already small-genome bacteria, *Mycoplasma* variants have been the favourite starting point for a further reduction of its genetic and functional complement to the very minimum required for Life. Milestones in the way include inspection of the essentiality of each of the genes by transposon mutagenesis [70,71], complete chemical synthesis of the *M. genitalium* genome [29,30], generation in the Laboratory of a strain entirely controlled by a chemically synthesized genome [72]. In a subsequent and spectacular breakthrough, the complete design and synthesis of a bacterial chromosome derived from *M. mycoides* with the lowest number of genes that support autonomous life has been recently reported [73••]. This last achievement included a ~ 50% reduction of the net genome size (from ~1079 kbp to 531 Kbp) in respect to its parental strain and the streamlining of the essential biological information down to 473 genes. Genome reduction was performed through a cycle of rational design, full genome synthesis, genome transplantation and testing. Besides the phenomenal technical muscle deployed in the work of Hutchison *et al.* [73••] the results are a treasure trove for understanding the key components of live systems and guide future endeavours in *Mycoplasma* and other bacteria of more immediate biotechnological interest.

One outstanding piece of information in [73••] is the essentiality of 149 genes in thereby minimized strain with altogether unknown functions and which need to be tackled for any future engineering of
live systems from first principles. Perhaps some of them run high-level functions related to local
information gathering for creating a locally ordered environment while obeying the second law of
thermodynamics - a sort of biological Maxwell demons [74]. This situation asks for creative approaches
to determine unknown roles of unknown genes. For instance, by examining the functions necessary
for the performance of Mycoplasma as a physical chassis but before considering the structures that
drive them in action [75•] was able to temptatively annotate more than 60 genes of the list of
unknowns [74]. A second aspect of [73••] is the variation in size and shape of the corresponding cells,
perhaps related to the effect of offsetting genomic segments mentioned above. In any case [73••] is
bound to remain as a landmark in the pursuit of bacteria with the smallest number of genes for the
sake of both fundamental science and biotechnological applications. Yet, even the smallest
Mycoplasma derivative engineered until now may still be far from recreating the transition from prebiotic
chemical systems to functional cells that had to occur at some point in evolution. Mutations in Bacillus
subtilis were reported in 2009 that cause formation of the so-called L-forms [76]. These are bacteria
that lack a wall and propagate through an FtsZ-independent division mechanism [77••] based on
membrane blebbing and extrusion. Along the line [78] showed that an excess of membrane synthesis
is sufficient to drive L-form division. This means that simple biophysical processes can support the
type of cell proliferation during the early times of biological evolution [79]. There could therefore be
room still for decreasing the number of genes necessary for independent bacterial life by replacing the
cell division machinery by some physico-chemical alternatives that could have been instrumental at
the onset of life in Earth [80].

The pursuit of a smaller but still fully functional genome may also adventure in reducing the apparently
redundant number of codes that extant cells deploy for their functioning. For instance, one could
envision engineering bacteria that lack one or more of the 20 canonical amino acids, thereby resulting
in potential savings in genes for their biosynthesis and loading into proteins (tRNAs, amino acid-tRNA
synthetases etc). In a remarkable article Pezo et al [81] managed to reassign the Trp codon UGG to
His, what eliminated tryptophan from Escherichia coli. This demonstrated that the genetic code can
indeed be evolved to have less components [82]. Along the line, redundant codons have been
eliminated or reassigned for the sake of simplifying and streamlining bacterial genomes
[50,51,83•,84•]. Furthermore, the number of ribosomal RNA operons, which are present in several
copies in bacterial genomes can be reduced, as not all of them appear to be necessary for growth in
non-changing environments [85].

From the test tube to the fermenter and the environment

The panoply of technologies that have enabled the generation in the Laboratory of E. coli and Mycoplasma strains with smaller genomes has been echoed by similar - if less dramatic efforts in other bacteria (Table 1). Alas, the rules for genome reduction that may have been learnt from these two species cannot be instantly projected to other microorganisms. While these model systems may be enough for tackling the fundamental biological question on the minimal list of genes and functions necessary for life, they are also insufficient for operating under harsh industrial-scale conditions [25,26,86]. Despite the fact that E. coli is one of the favourite workhorses for heterologous protein and pathway expression in modern biotechnology, its lifestyle is such that its default genetic and biochemical programs may not be able to endure the physicochemical stress associated to redox catalysis, organic solvents and toxic intermediates. This may be fixed in some cases by importing stress-tolerance traits from other bacteria [87,88]. Yet, it would be preferable to have alternative default chassis that are already endowed with some of such stress-enduring qualities. In the case of Mycoplasma, its natural niches (and thus its default chassis) limit possible applications thus far to in vivo delivery of bioactive compounds or antigens to animals and humans for the sake of vaccination [89] or other medical uses (see e.g. http://www.mycosynvac.eu/). Both E. coli and Mycoplasma can be surely upgraded to meet more demanding industrial-scale specifications. But in the meantime other bacteria of biotechnological interest have benefitted from many of the concepts and tools that emerged in the way towards minimizing the genome of such model microbes.

The next microbe in line for genome minimization has been Bacillus subtilis [90]. Strains of this species are not only basic models of the biology of Gram-positive bacteria, but also agents of a large number of biotechnological processes, in particular involving protein secretion [91]. The background for removing DNA segments from the chromosome of this bacterium was the earlier identification of essential genes done through both computational methods and wet experimentation [92,93,94•]. The 4.2-Mb genome of B. subtilis strain 168 (4106 genes, only 271 indispensable for growth in rich medium) has been subject to recurrent minimization efforts: first a 7.7% genome reduction [92] and then an approx. 20% of the total DNA. Deletions involved all prophage and prophage-like sequences
and a suite of genes and gene clusters deemed not to be of interest for industrial performance [95]. As was the case with *E. coli*, the resulting strain showed much higher protein production yields [95,96], a phenomenon that seems to be connected to favourable changes in the carbon flux through central metabolic pathways [97]. The availability of genetic tools for editing *B. subtilis'* genome has improved considerably in recent times [94,98,99] and the number of strains with further reductions is expected to increase in the near future [100-103]. Some updates on this front can be followed in http://www.minibacillus.org/project.

Corynebacteria belong also the elite of bacteria useful in industrial biotechnology. Although some species and strains of this Gram-positive genus are known for their pathogenicity, others (e.g. *C. glutamicum*) have been long appreciated for production of amino acids and, more recently, of a good number of added value products and bioactive molecules [104-106]. Although genetic technologies for making large genomic deletions inspired in Posfai’s procedure (see above) were available as early as 2005 [107], the impact of erasing large segments of the *C. glutamicum* genome of industrially important traits had to await until quite later. [108] reported the construction of a prophage-free variant of *C. glutamicum* with a 6% reduced genome. The resulting strain did better under SOS-response conditions, displayed an increased transformation efficiency and delivered >30% of a cloned heterologous protein. In a more recent development [109•] determined that ca. 20% of the *C. glutamicum* genome is dispensable and could in principle be eliminated for having a streamlined Cell Factory. But surprisingly, some combinations of deletions -which were perfectly viable by separate, were detrimental when entered together in the same strain -thus limiting the overall reduction effort. This phenomenon (which has been observed in other cases, see above) suggests again that there may be a level of tridimensional cellular organization that uses genomic coordinates as reference and which may be affected when the relative position of the DNA sequences involved are altered [61,110]. This issue is still controversial and needs more research to be sorted out [111].

Finally, *Pseudomonas putida*, a soil bacterium which was first consider an ideal vehicle for delivering engineered biodegradative activities to polluted sites [112] has more recently been revisited as an ideal platform for industrial redox biocatalysis involving organic solvents [113-116]. The reason for such a property is to be found in the many solvent extrusion pumps [117] and the core metabolic network of this bacterium, that is geared towards production of reducing power [118••,119•]. As soon
as genetic tools for seamless and iterative elimination of DNA segments became available [120], the
strain of reference \((P.\ putida\ KT2440)\) was subject to a series of directed genomic deletions that
erased phages, flagella and a suite of instability determinants (approximately 4.3 % of the
chromosome), which resulted in much more stable, robust and productive derivatives [121•,122].
These operations were facilitated by the availability of a large collection of vectors for deep
engineering of the \(P.\ putida\) genome deposited in the so-called SEVA (Standard European Vector
Architecture) repository [123]. An alternative method for random deletions of large DNA segments \textit{in vivo} caused a 7.4% reduction of the \(P.\ putida\) genome size [124,125], but this approach is alas limited
by the neighbourhood of essential and non-essential genes in the same chromosomal regions.
CRISPR and MAGE-based methods (see above) are currently being developed (e.g. [126•]) for further
editing of \(P.\ putida\) aimed at increasing its value in industrial biotechnology.

The growing list of genome-reduced microorganisms

Other bacterial species well established as Cell Factories of industrial interest are currently undergoing
different genome minimization efforts that echo the approaches and motivations discussed above
(Table 1). \textit{Streptomyces} stands above them in their capacity to produce antibiotics and bioactive
secondary metabolites. While the genomic sizes found in specimens of this genus vary extraordinarily
-including some with a naturally reduced genetic complement [127], some strains of \textit{Streptomyces
avermitilis} have been subject to deliberate deletion of non-essential segments [128,129] for improving
expression of endogenous and heterologous pathways. Any step in such a direction depends on the
availability of suitable genetic tools and DNA deletion and insertion methods. Several other bacterial
groups are receiving considerable attention as possible subjects of a deep genetic and genomic
editing -including chromosomal size reduction. Just to mention some of them, reader is directed to
recent advances in cyanobacteria e.g. \textit{Synechococcus} [130,131], \textit{Synechocystis} [132,133] and related
photosynthetic bacteria of great potential for energy and biofuel production [134] that could benefit from
application of CRISPR-based genetic tools [135]. Along the same lines, other microbial types such as
Clostridia [136] and \textit{Bacteroides} [137], which were traditionally difficult to edit genetically, can now be
included in the group of those amenable to genome reduction and deep rational engineering. Lastly,
because of its unusually fast growth (~ 10 min doubling time) and the ease of genetic manipulation,
the marine bacterium \textit{Vibrio natriegens} has been recently advocated as the genomic chassis of choice.
for all types of synthetic biology endeavours [138,139]. Different efforts to edit/streamline/reduce the two-chromosome \textit{V. natriegens} genome for the sake of specific biotech applications (i.e. generation of the so-called Vmax strains) are going on at the time of writing this article (see e.g. \url{http://goo.gl/K5YEMj}). And many others with additional bacterial species will surely follow.

\section*{Outlook}

Two distinct agendas with different methodological approaches converge in the endeavour of generating genomes that are smaller and simpler than those which appear in their natural bacterial host (Fig. 1). First, there is a fundamental drive to understand what one could call \textit{minimal life} on the basis of known cases of already existing small genomes, the further deletion of remaining non-essential functions and the chemical synthesis of the corresponding DNA. The resulting entities (e.g. the \textit{Mycoplasma} strain called JCVI-syn3.0 [73]) embody an amazing wealth of information on such a question, including identification of a large number of essential genes with unknown functions. But as discussed above, although such minimal cells have a potential for medical applications they are not yet \textit{bona fide} Cell Factories that can be used in Industrial Biotechnology in the near future. The second agenda stems not from a basic interest in understanding Life, but from a more mundane concern in improving biotechnological processes, which are often afflicted by the unpredictability of the live biological components of the corresponding industrial flowcharts. In these cases, the approach starts with an already useful strain that is growingly streamlined for a better performance, but without considering genome reduction \textit{per se} as the ultimate objective. Instead, the aim is at erasing limitations or flaws within the bacterial genomes of interest, a sort of \textit{remove what bothers and leave the rest} [110]. Still, there are no rules for such genome reducing/streamlining exercises and each new species under consideration may behave differently in that respect. But despite these flaws, the two parallel agendas have been and will continue inspiring each other if they are to fulfil the promise of synthetic biology in the way towards a knowledge-based bio-economy [140,141].

\section*{Acknowledgements}

This work was supported by the CAMBIOS Project of the Spanish Ministry of Economy and Competitiveness (RTC-2014-1777-3), the EVOPROG (FP7-ICT-610730), ARISYS (ERC-2012-ADG-

The authors declare that there are no conflicts of interest.

References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

• of special interest

•• of outstanding interest


The paper reports an original method for genome editing and eventual minimization using yeast as a mothership.


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Thorough inspection and deployment of the minimal number of genes that originate a living system. Probably a landmark in the history of Biology.


Evidence that the division machinery of bacteria is not necessary for cell viability


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Table 1. Major bacterial species subject to genome reduction efforts

<table>
<thead>
<tr>
<th>Species</th>
<th>Purpose</th>
<th>Technology</th>
<th>References</th>
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</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em></td>
<td>Optimization of Cell Factories</td>
<td>Double-stranded break repair</td>
<td>[15,17,18,37].</td>
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<td></td>
<td>Genome structure and organization</td>
<td>Intra-genomic recombination</td>
<td>[38].</td>
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<td></td>
<td>Exploration of essential genes</td>
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<td>[39].</td>
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<td></td>
<td>Determination of minimal genetic complement</td>
<td>Cas9-facilitated recombination</td>
<td>[33].</td>
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<td></td>
<td>Multiplexing genome editing</td>
<td>CRISPR/Cas9-based editing</td>
<td>[56].</td>
</tr>
<tr>
<td></td>
<td>Codon emancipation</td>
<td>Multiplexed genome engineering</td>
<td>[83,84].</td>
</tr>
<tr>
<td><em>Mycoplasma genitalium</em></td>
<td>Programmable Cells</td>
<td>Chemical genome synthesis</td>
<td>[72].</td>
</tr>
<tr>
<td><em>Mycoplasma mycoides</em></td>
<td>Reaching the limits of live systems</td>
<td>Chemical genome synthesis</td>
<td>[73].</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>Increasing Cell Factory predictability</td>
<td>Forced intra-genomic recombination</td>
<td>[92,100-103].</td>
</tr>
<tr>
<td></td>
<td>Enhancing heterologous expression and redox catalysis</td>
<td>Double-stranded break repair</td>
<td>[121,122].</td>
</tr>
<tr>
<td><em>Pseudomonas putida</em></td>
<td>Improving genetic programmability</td>
<td>Transposon-mediated random deletions</td>
<td>[124,125].</td>
</tr>
<tr>
<td><em>Streptomyces avermitilis</em></td>
<td>Expression of heterologous pathways</td>
<td>Homologous recombination and/or Cre/loxP-based recombination</td>
<td>[128,129].</td>
</tr>
<tr>
<td><em>Vibrio natriegens</em></td>
<td>Optimization of SynBio chassis</td>
<td>Chemical synthesis, recombination, CRISPR/Cas9-based manipulations</td>
<td>[138,139].</td>
</tr>
</tbody>
</table>
Figure 1. Strategies for minimizing/streamlining bacterial genomes. The starting point is always naturally-occurring strain of a given species, often selected on the basis of its biotechnological interest, unique properties and/or ease of genetic manipulation. Identification of essential genes can guide either the chemical synthesis of the cognate DNA (and its further implantation in a surrogate cytoplasm) or their stepwise random/directed deletion \textit{in vivo}. Depending on the final purpose of the exercise, the process can stop either at the stage where improvement of desirable traits is reached – or all the way down to the size limit that allows having a viable cell.