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Abstract	In any discipline, simple systems are desirable for understanding the basic workings in the groups of systems they represent. In biology, cells are established as units for life, but even the simplest cells in nature are complex systems with uncharacterized functions beyond simply living. Making a minimal model cell in the laboratory was therefore contemplated and then achieved. Many pioneering efforts, including those at Keio University, shaped the process. This endeavor created the synthetic genomics field and a worldwide community to understand the cell and explore its applications in biotechnology and medicine.
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［招待論文：総説・レビュー論文］

20 Years of the Minimal Cell's Rise from Theory to Practice

Yo Suzuki
Assistant Professor, Synthetic Biology and Bioenergy Group, J. Craig Venter Institute
Correspondence to: ysuzuki@jcvi.org

J. Craig Venter
Founder, Chairman, and Chief Executive Officer, J. Craig Venter Institute

Abstract: In any discipline, simple systems are desirable for understanding the basic workings in the groups of systems they represent. In biology, cells are established as units for life, but even the simplest cells in nature are complex systems with uncharacterized functions beyond simply living. Making a minimal model cell in the laboratory was therefore contemplated and then achieved. Many pioneering efforts, including those at Keio University, shaped the process. This endeavor created the synthetic genomics field and a worldwide community to understand the cell and explore its applications in biotechnology and medicine.

サイエンスでは単純な実験系を使うことで、飛躍的に研究が進むことが多い。細胞は生命の単位と考えられるが、自然界に存在する最も単純な細胞でさえ、人の理解を超える未解明の機能を持った複雑な系を構築している。そこで生命を解析するためのモデルとして、実験室での最小細胞作成が検討され、その目標が達成された。この過程で慶應義塾大学は先駆的な役割を果たした。この研究は合成ゲノミクスという新分野を生み出し、最小細胞を通じて生命を理解し、産業と医学に応用するための世界的な動きにつながった。

Keywords: synthetic genomics, synthetic biology, synthetic cell
合成ゲノミクス、合成生物学、人工細胞

1　Naturally minimal cells

Harold Morowitz's pursuit of a simple cell for understanding life has identified mycoplasmas as model organisms (Morowitz, 1984). Mycoplasmas are small cells with the smallest genomes of all known bacteria that are culturable in an axenic condition. Morowitz's group uncovered some fundamental molecular constraints

about cellular life. His foresight is noteworthy, as the original review (Morowitz, 1984) delineated what were to come including genome sequencing, computational modeling, and the development of defined culture media. Arguably, the work is still ongoing 38 years later.

Mycoplasmas were considered to be useful for determining the minimal number of genes required for life (Peterson et al., 1991), but were initially studied as natural organisms containing whole, yet uncharacterized, genomes. *Mycoplasma genitalium* has the smallest genome among all mycoplasma species examined. Its genome sequence therefore was of great interest and was one of the first to be determined completely (Fraser et al., 1995). However, the sequence did not reveal functions for many genes, given the limited information available at the time. Studies of whole mycoplasma cells led to Luis Serrano's pioneering systems biology work (e.g., Guell et al., 2009). Those studies generally showed the surprising complexity of the organisms, despite their limited genetic content.

2　Theoretically minimal cells

Starting around 1995, a research focus emerged to establish minimal genomes as concepts, departing from the constraints of natural organisms, through abstraction, as well as clever genetic experiments and bourgeoning bioinformatics. The 20-year progression toward minimal cells coincided with 20 years of incessant technology development efforts at Institute for Advanced Biosciences (IAB) at Keio University, which enhanced research at J. Craig Venter Institute (JCVI) and in the systems biology and synthetic biology fields at large.

In 1995, Mitsuhiro Itaya published a study describing systematic mutagenesis in *Bacillus subtilis* (Itaya, 1995). Mutagenesis studies to estimate the number of genes required for a given biological process were common, but Itaya had a unique perspective. He analyzed 79 randomly generated mutants and found that 73 were viable (Table 1); only six sequences tested were considered to be essential for life. Itaya went on to make multi-mutants with up to 33 loci simultaneously disrupted. This experiment suggested that synergistic lethal phenotypes among randomly

selected mutations are rare. He estimated that all the essential genes needed for life can be contained within roughly 350 kb, 8% (6/73) of the 4.2-Mb wild-type genome size in *B. subtilis*.

Table 1 Frequencies of viable mutants in various species

Species	Non-lethal	Normal growth	Reference	Note
Bacillus subtilis	92%		Itaya, 1995	
Escherichia coli	93%	"Vast majority"	Baba et al., 2006	
Saccharomyces cerevisiae	83%	60%	Winzeler et al., 1999	80% of non-lethal's have synergistic growth phenotypes (Tong et al., 2004)
Mycoplasma genitalium	21%		Glass et al., 2006	
Mycoplasma mycoides JCVI-syn1.0	73%	48%	Hutchison et al., 2016	Non-lethal: nonessential & quasi-essential categories; normal growth: nonessential category; > 13% (54/432) of non-lethal's are presumed to be involved in synergistic lethality
Mycoplasma mycoides JCVI-syn3.0 (minimal cell)	25%	8%	Hutchison et al., 2016; this study	Non-lethal: nonessential & quasi-essential categories; normal growth: nonessential category

The size of this estimated essential fraction is just under the smallest mycoplasma genome sizes, giving validity to Itaya's suggestion of a link to the minimal genome. The low frequency of essential genes in *B. subtilis* was surprising, but was later supported by similar observations with systematic gene knockout studies in *Escherichia coli* (Baba et al., 2006) and *Saccharomyces cerevisiae* (Winzeler et al., 1999; Table 1). However, a set of essential genes identified with any single-gene inactivation technique would not be sufficient for enabling life at least for *B. subtilis* or any of other typical microbes researched.

What is the difference between such an essential gene set and a gene set of a viable minimal genome? A major difference would be that the "essential" set is

missing functionally redundant genes. The evidence comes from a double knockout study in *S. cerevisiae*, where 80% of non-lethal single-gene deletions were shown to have at least one gene deletion partner to exhibit a synergistic growth defective phenotype (Tong et al., 2004). Because non-lethal single-gene deletions are a large fraction of the genome (Winzeler et al., 1999; Table 1), the majority of the genes in the genome would be involved in essential life processes. The specific synergistic lethal combinations would rarely be found randomly because the search space for combinatorial genotypes is large. However, the prevalence of genes involved in synergistic lethal interactions would limit the extent of maximal genome reduction to a fewfold for typical microbes and less for mycoplasmas.

After the predecessor of JCVI completed sequencing the first two bacterial genomes, the genomes of *Haemophilus influenzae* (Fleischmann et al., 1995) and *M. genitalium* (Fraser et al., 1995), Eugene Koonin and Arcady Mushegian determined through bioinformatics which *M. genitalium* genes were conserved in *H. influenzae* (Mushegian and Koonin, 1996). Considering the large evolutionary distance between the two organisms, the genes conserved between them had to be essential and collectively sufficient for life. They proposed a 256-gene minimal genome. This genome contained 22 genes that were restored from *M. genitalium* to fill the gaps in pathways, to account for possible cases where genes with unrelated sequences evolved to perform the same function in organisms compared. A caveat would be that if genes with functions that are unknown fall into this so-called nonorthologous gene displacement category, they would be missed in their approach. Nevertheless, this study greatly motivated the quest for minimal genomes. The mycoplasma-centric approach likely enabled them to predict a small gene count for a minimal genome.

In 1999, Masaru Tomita developed the first computational platform to simulate whole-cell behaviors (Tomita et al., 1999). This system termed E-CELL was a perfect match with the simple biological program in *M. genitalium* revealed by the genome sequencing project. Only known molecular mechanisms were included, and processes such as DNA replication and cell division were omitted in the simulation. Nevertheless, insights were gained into the life and death of the cell. A research

trajectory was proposed to conduct a model-guided study to understand genes of unknown function. The spirit of this approach was passed onto multiple computer modeling studies (e.g., Breuer et al., 2019; Karr et al., 2012).

By 1999, Hutchison et al. developed a method to determine the sequences around transposon insertions in a high-throughput manner and applied it to the genomes of *M. genitalium* and *M. pneumoniae* to identify genes that can be disrupted (Hutchison et al., 1999). This was followed by a study where mycoplasma cells with a transposon insertion were isolated, archived, and then used to determine the site of transposon insertion (Glass et al., 2006). These efforts established that 382 of the 482 genes (79%) are likely to be essential in *M. genitalium* (Table 1). This is a large fraction when compared to earlier results from other organisms, leaving little room for synergistic lethal interactions. Redundant genes would have had to be much more than proportionally reduced in the evolution of *M. genitalium* and closely related mycoplasmas, when the yeast result is used as a reference. It is understandable that redundant genes were first to be lost when a selective pressure was applied in nature to favor genome reduction. The first multi-gene knockout attempt resulting in a ~10% reduction of the genome size in *M. mycoides* did not reveal any synergistic interactions, supporting the view that synergistic lethal interactions are rare in mycoplasmas (Suzuki et al., 2015). The shortage of genetic redundancy might have facilitated the construction of viable reduced genomes described below.

3　Birth of synthetic genomics

With hundreds of deletions to make in a genome, a top-down approach to conduct engineering on the wild-type genome would be cumbersome. The initial success with the bottom-up approach of synthesizing and activating the genome of the bacteriophage ΦX174 (Smith et al., 2003) set a stage for a conceptually similar approach for bacterial genomes. Gibson et al. developed a hierarchical assembly method and generated a whole genome of *M. genitalium* from synthetic DNA fragments (Gibson et al., 2008). The final genome was established in yeast as a plasmid, but they were unable to activate it to produce any bacterial cell.

Preceding this development, Itaya had undertaken a unique approach to genome engineering. First, parts of the genome of *B. subtilis* were gradually replaced with genomic fragments from related *B. subtilis* var. natto within *B. subtilis* cells (Itaya and Matsui, 1999). The recipient organisms acquired phenotypes related to the production of natto, a traditional food in Japan. This is a partial conversion of an organism, as the resulting organism had some of the old genome sequences, as well as old functions. Expanding on this work, Itaya then introduced a whole 3.5-Mb cyanobacterial genome into the 4.2-Mb genome of *B. subtilis* to generate a 7.7-Mb hybrid genome within *B. subtilis* (Itaya et al., 2005). The organism with the hybrid genome was propagated as *B. subtilis*, but was also tested for growth in cyanobacterium medium and culturing condition. This marked the first attempt to activate a whole genome of a species that is different from the host in the laboratory.

Lartigue et al. succeeded in transferring the natural *M. mycoides* genome from *M. mycoides* to *M. capricolum* recipient cells and obtaining *M. mycoides* cells (Lartigue et al., 2007). They then showed that an equivalent *M. mycoides* genome maintained in yeast also rendered *M. mycoides* cells upon transplantation (Lartigue et al., 2009). This breakthrough discovery cleared a path for the first synthetic cell. The essentially full-length *M. mycoides* genome was generated from synthetic DNA fragments via the hierarchical assembly strategy, established in yeast, and transplanted to the *M. capricolum* recipient cells to generate *M. mycoides* cells having an entirely synthetic genome (Gibson et al., 2010). The watermarks introduced in the genome confirmed that the generated cells were a genuine JCVI product derived from the synthetic genome.

The origin of this cell lineage is unusual. It is not a *M. mycoides* cell. The initial cytoplasm was from the recipient *M. capricolum*, but was quickly replaced by products made from instructions in the new genome after the transplantation event. This new genome came from yeast, but had originated in a DNA synthesizer, based on the design in a computer. This challenges Louis Pasteur's theory, "Omne vivum ex vivo (all life from life)." The escape from this constraint promised great flexibility in genome sequences that can be manipulated to create reduced genomes or massively

reorganize genes within genomes (Hutchison et al., 2016; Venter et al., 2022).

Genome transplantation has been expanded to a useful mycoplasma-like organism *Mesoplasma florum* (Baby et al., 2018). *M. capricolum* has been a dependable recipient with a capability to take in mega-sized DNA, without deleterious homologous recombination activity (Labroussaa et al., 2019). At this stage of development, the main constraint seems to be that the cytoplasm and the cellular chassis of the recipient need to be compatible with the incoming genome. Understanding the molecular mechanisms of genome activation would be important for further expanding the range of organisms that can be engineered with synthetic genomics. The approach to gradually replace a native genome with a synthetic genome using synthetic DNA pieces has been practiced in organisms such as *E. coli* (Fredens et al., 2019) and *S. cerevisiae* (Annaluru et al., 2014).

4　Practically minimal cells

To make a minimal cell, we used repeated cycles to design, build, and test cells having reduced genetic content. The design relied on gene essentiality data with Hutchison's transposon bombardment method (Hutchison et al., 1999) adapted for next-generation sequencing to identify genes to discard. An improved hierarchical assembly of synthetic DNA was then conducted to generate the next version of the genome. The test was to identify viable organisms after genome transplantation. A modular eight-segment construction enabled acquisition of viable organisms with subsets of reduced segments in the wild-type background, providing materials for the next cycles of reduction. After three design-build-test cycles, we established the synthetic bacterium JCVI-syn3.0, "a working approximation of a minimal cell" (Hutchison et al., 2016). The genome had 473 genes in 531.5 kb, smaller than the *M. genitalium* genome and 50% smaller than the *M. mycoides* wild-type genome. The resulting cell has the highest fraction of the genes characterized that has been reported for any organism.

A key finding in this work was the need to include quasi-essential genes that were revealed after transposon bombardment by analyzing populations at two stages

of outgrowth. They appeared dispensable in the early population, but essential in the later population. (Some of these had been classified as nonessential in earlier experiments.) They may be needed for fast growth or only in limited occasions (e.g., with the gene products made before gene disruption lasting multiple generations or with the genes needed to withstand any irregular processes during the culture). However, including quasi-essential genes in subsequent genome constructs ensured viable genomes. It may be that having multiple slow-growth deletions compounded to make the growth rate too slow to be detected in our transplantation assay.

A thorough analysis of functionally redundant gene combinations was not conducted during the construction of the JCVI-syn3.0 cells, but a partial search identified roughly 26 genes that were possibly involved in synergistic lethal interaction (Hutchison et al., 2016). Of the 432 singly nonessential genes in the starting genome, 26 (or 52 if one redundant partner is assumed to be present for each of the 26 genes; Table 1) makes a small fraction, consistent with the rarity of synergistic gene interactions in mycoplasmas.

Loss of functional redundancy during evolution probably correlated with massive genome reduction in mycoplasmas, but more studies are needed to understand the molecular basis. Mycoplasmas import many nutrients such as amino acids and nucleotides from the growth media. Removing biosynthetic pathways would greatly promote genome reduction and loss of redundancy. Elimination of the cell wall probably helped remove complicated interactions among gene products. Small cell size might have allowed mycoplasmas to delete many structural proteins involving complex regulation. For several reasons, and as predicted from earlier theoretical considerations, mycoplasmas might have been especially suited for the genome minimization work.

The JCVI-syn3.0 cell had 149 genes (32%) that were uncharacterized (Hutchison et al., 2016). As many of them are essential for life, studying these genes could uncover new paradigms for biology in mycoplasmas and beyond. Collaboration between Zaida Luthey-Schulten and the JCVI group is making good progress on this front (Breuer et al., 2019; manuscript in preparation). Experimental functional

characterization is required when sequences do not reveal any useful information about their functions. For this purpose, collaboration involving Tomita's group led to the development of CRISPRi as a tool for gene inactivation (Mariscal et al., 2018). Mycoplasmas are not ancient cells; they recently evolved in a clade of Gram-positive bacteria. However, they seem to possess multiple characteristics expected for the primordial cell (Morowitz, 1984). Adding to this list, a recent microfluidics study showed that JCVI-syn3.0 cells exhibit variable elongated or enlarged morphologies attributable to defective cell division, but with DNA replication and the general growth rate not severely affected (Pelletier et al., 2021). This characteristic may be expected for a primordial cell without a well-developed cell division machinery. It is intriguing to think that minimal cells kept basic functions inherited from the primordial cell through evolution and engineered deletion.

With the JCVI cell distributed to 60 laboratories around the world (J. Glass, personal communication), the field is expanding in multiple directions. An exciting development is to advance minimal cell research toward the industrial realm. As synthetic genomics makes introducing new pathways easier and faster, we will be able to generate numerous genetic modules for desired properties found in any organism and test their fit to the minimal cell (Venter et al., 2022). Such an effort would lead to minimal cell factories or devices that can be precisely controlled due to the cell's simplicity. Michael Guarnieri's US Department of Energy-funded project at National Renewable Energy Laboratory (Arnolds et al., 2021) recognizes JCVI-syn3.0 as a future platform for bioproduction. In this collaboration, we are developing biocontainment strategies for the JCVI cell. The cell requires many nutrients in the environment for growth, and they are unlikely to escape. However, the situation may change after various genetic parts are added to the cells. The minimal cell enables imagining a powerful biocontainment strategy on a blank slate. This could involve recoding the genome, mitigating the effects of mutations, or developing a killing mechanism that is distanced from industrially relevant cellular processes. Currently, JCVI-syn3.0 is a cell factory to convert glucose to lactate. Our collaboration with SyntheticGestalt, an artificial intelligence company based in Japan, aims to produce

various chemicals with computer model-guided metabolic engineering. Introduction of metabolic pathways such as those for amino acids would make minimal cells more robust and less reliant on media ingredients, reducing the costs of culturing. With multi-gene engineering, synergy among genetic parts can be incorporated in bioproduction or in any engineered cell behavior such as biocontainment to increase the efficiency of the bioprocess of interest. Whole collections of minimal cells tailored to specific purposes may assume critical roles in future biobased industries.

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Conflicts of interest

The authors declare no competing interests.

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