A new life in a bacterium through synthetic genome: a successful venture by craig venter

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The first synthetic genome, a stripped down version of a natural organism, is only the beginning. I now want to go further. My company Synthetic Genomics Inc., is already trying to develop cassettes-modules of genes-to turn an organism into a biofactory that could make clean hydrogen fuel from sunlight and water or soak up more carbon dioxide. From there I want to take us far from shore into unknown waters, to a new phase of evolution, to the day when one DNA-based species can sit down at a computer to design another. I plan to show that we understand the software of life by creating true artificial life. And in this way I want to discover whether a life decoded is truly a life understood [1].

– A Life Decoded
By J. Craig Venter

Early events culminating to the idea of minimal genome

Ever since the discovery of double helical structure of DNA by Watson and Crick in 1953, for nearly two decades there had been no remarkable development in different methodologies and systems for manipulating the ‘natural’ software of a living cell. However, in early 1970s methods were discovered to manipulate DNA. In reality transformation

methods, cloning vectors and several enzymes including restriction endonucleases became available to manipulate DNA in early 1970s. The quest to synthesize genes was fulfilled in 1977 when the first gene ‘insulin’ was synthesized, cloned and expressed in *E. coli* [2,3]. Since then scientists believed that synthesis of large stretches of oligonucleotides and genes was possible. A pioneer effort of Craig Venter and his colleagues have now made it realistic what appeared to be impossible a few years ago. In this review we trace back the sequence of events and the enormous effort that Craig Venter and his team have put in the past few decades leading to successful synthesis of a genome that could replicate in a bacterium (Table 1).

A revolutionary idea of shotgun sequencing of whole genomes

The concept of cloning individual genes as witnessed during 1970s and 1980s began to change during 1990s. In this context upgrading of DNA sequencing techniques especially Sanger’s method coupled with the development of shotgun sequencing approach seem to have played a very significant role. As a consequence to these developments, genomes of several viruses and bacteria were completed between 1990–2000. Although bacteriophage ϕ X174 (5386bp), was the first to be sequenced in 1977 [4], the bacterial genomic era appears to have begun with the whole genome sequencing of a parasitic bacterium *Haemophilus influenzae* by Craig Venter and his colleagues in 1995 [5]. In fact *H. influenzae* genome was the first genome in which Venter and his team tested the concept of whole genome shot-gun sequencing and assembly algorithm whereby a genome sequence was reconstructed by using a computer [5].

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Table 1 Chronological achievements of Craig Venter and his team towards realizing the creation of synthetic bacterial cell

Year	Journey towards the Synthetic Cell
1995	The first complete genome sequencing of <i>H. influenza</i> using whole genome shotgun approach. The genome consisted of 1,830,140 base pairs in a single circular chromosome with 1740 protein-coding genes, 58 transfer RNA genes tRNA, and 18 other RNA genes [5]
1995	Second complete bacterial genome to be sequenced <i>Mycoplasma genitalium</i> G37 containing the minimum gene complement [6]
2003	Generating a Synthetic Genome by Whole Genome Assembly: phiX174 Bacteriophage from Synthetic Oligonucleotides [12]
2005	Identification of essential genes in <i>Mycoplasma genitalium</i> as a minimal bacterium [8]
2007	Intact genomic DNA from <i>Mycoplasma mycoides</i> was transplanted into <i>Mycoplasma capricolum</i> cells by polyethylene glycol-mediated transformation [16]
2008	Complete chemical synthesis, assembly and cloning in <i>Escherichia coli</i> of a <i>Mycoplasma genitalium</i> genome [14]
2008	One step assembly of 25 overlapping fragments in yeast for complete synthetic <i>Mycoplasma genitalium</i> genome [17]
2009	Transplantation of synthesized genome (cloned and engineered in yeast) of <i>Mycoplasma mycoides</i> into <i>Mycoplasma capricolum</i> to produce a viable <i>M. mycoides</i> cell [19]
2010	Cloning whole bacterial genomes (<i>Mycoplasma genitalium</i> (0.6 Mb), <i>Mycoplasma pneumoniae</i> (0.8 Mb) and <i>Mycoplasma mycoides</i> subspecies <i>capri</i> (1.1 Mb) in yeast <i>Saccharomyces cerevisiae</i> [20]
2010	Synthetic Bacterial Cell with a Chemically Synthesized Genome [18]

Additionally, his team used for the first time a combination of methods that included random coverage of genome, the paired end sequencing strategy, a blend of mathematics and new computational tools and pragmatism in the form of novel methods to fill up the gaps. This marked the first full fledged demonstration that shotgun method could be used to read an entire genome. Even as the sequencing of *Haemophilus influenzae* was in the final stages of closure and annotation, Venter began to think of sequencing a second genome, primarily to demonstrate to the world the power of the shotgun method and to show that *H. influenzae* genome was not a fluke. The second published complete genome sequence of *Mycoplasma genitalium*, a parasitic bacterium that lives in the genital tract is thus also credited to Venter and his team. The bacterium interestingly seems to have the smallest genome of 582970 bp, and found to contain merely 480 coding genes [6]. The sequencing of the genome of *M. genitalium* was based on number of methods that were used for *H. influenzae*, but apart from having the smallest genome; its sequencing had something more to offer. It also led to the development of a new software, algorithms and approaches to determine for the first time in history, interpreting specific genes in the genome what we today refer to as annotations. Not only this work validated the methods developed earlier by Venter and his team for sequencing *H. influenzae* genome [5] but also led to the transformation of analog versions of biology into the digital world of computer.

The concept of a minimal genome

Hence, the complete sequencing of the first two parasitic bacteria also played a major role in defining the minimal

gene concept. These two genomes though belonging to different groups of parasitic bacteria, gave a clear indication that the genes they shared were most likely necessary for their survival. *Mycoplasma genitalium* thus became an excellent model organism to show efficient use of its limited amount of DNA. But decoding this limited amount of DNA led researchers to initiate yet another cumbersome job of quizzing out essential genes of a minimal bacterium. This was again accomplished by Venter and his colleagues through global transposon mutagenesis into genes causing their disruption [8]. These gene disrupted mutants were then isolated followed by characterization of non-essential protein coding genes to elucidate that 382 of the 482 protein encoding genes and 43 structural RNA genes could sustain a viable synthetic cell which was presumptively named as *Mycoplasma laboratorium* [7]. But of all the essential genes there were around 111 (constituting 28% of all the essential genes) of unknown function of which the majority were found to be essential suggesting that all the basic molecular mechanisms underlying cellular life still remain an unsolved mystery. In any case these studies reflected that the *Mycoplasma genitalium* genome has the minimal set of genes needed to sustain bacterial life.

Synthesis of minimal genome

As mentioned above, a minimal genome refers to the possession of minimum number of genes needed for life by an organism. On the way to better understanding of the essential set of genes for a self-replicating cellular life, random whole genome transposon mutagenesis was carried out in *Mycoplasma genitalium* to inactivate one gene per cell. In

this way, nearly 380 genes were described and a hypothesis was laid to generate a synthetic chromosome in order to test the viability of these genes in a cell [8]. But before jumping to the synthesis of a minimum genome, there was still a need to think upon the strategy that had been adopted decades before when DNA of poliovirus was synthesized in laboratory. Before Venter and his team could begin to materialize this idea they had the background work before them in which the chemical sequence and genetic map of Poliovirus had already been decoded [9,10] and thus by *in-vitro* chemical-biochemical means it was synthesized by assembling oligonucleotides of plus and minus strands [11]. However accomplishing this task by the previous workers took months and thus it became essential to resort to ways which could replace this slow process with a dynamic one. Thus an improved strategy for synthesis of multi-gene segments was adopted by Venter's team as a step towards synthesis of a cellular genome. In order to test the feasibility of the new method, bacteriophage ϕ X174 (5,386 bp) was chosen because it was supposed to be non-hazardous to human, plants or animals and thus its synthesis would not have ethical issues or potential risks associated with the science of synthetic genomics [12]. Φ X174 with a genome size of ~5,386bp with just 11 genes [4] would be a simpler job to work on before switching on to a more complex system of a bacterium (Table 2).

To initiate with the genome synthesis of Φ X174, Venter and his team [12] tried assembling 5-6kb segments from chemically synthesized oligonucleotides but contamination of the truncated species posed the biggest hurdle as this could lead to assembly errors and mutations in the final product i.e. double stranded RF (replicative form). Therefore, a new strategy was applied wherein a single pool of chemically synthesized oligonucleotides was assembled within 3 short steps comprising oligonucleotide purification analysis, ligation and polymerase chain assembly. Initially, to avoid contamination of incorrect chain length molecules, these pooled oligonucleotides were gel purified. These were then ligated under stringent annealing conditions (55°C) which could probably remove incorrect pairing. Also factors like incomplete oligonucleotide phosphorylation and sequence errors that interfere with the process made

obtaining a full length ϕ X174 genome by simple ligation still a distant dream. Unequal concentration of the ligation mixture led to termination of growing assemblies. For these reasons, further assembly of oligonucleotides was accomplished by polymerase chain assembly. This process imitated the normal thermocycling polymerase reaction but did not involve primer pairs in excess to the template [13]. Thus the task was successfully completed and the first synthetic genome of a virus was created. This monumental undertaking of using a hybrid approach of improved oligonucleotide synthesis in combination with the above mentioned strategy helped Venter and his group pave the way to a faster and accurate synthesis of genomes of organisms like *Mycoplasma genitalium* to understand in depth the concept of minimal cellular life.

Synthetic creation of *Mycoplasma genitalium* genome

After achieving success in synthesizing a viral genome using a methodology with key features like accuracy and rapidity, it was time for Venter and his colleagues to take the next major leap in synthetic genomics i.e. moving from virus to bacterium. While this meant dealing with genomes nearly 600 times larger to reveal the ability of a cell to survive with a minimum genome, there were still many barriers in achieving this goal like devising new methods for the assembly of the DNA segments. As the DNA segments get longer, they become more difficult to work with and this time Venter's team was eyeing something high [14]. With the previous strategy they had an expertise in assembling 5-6 kb segments but their present target was nearly 582Kb. The initial steps of assembly were carried out by cloning DNA fragments in *E. coli* [14]. Although *E. coli* is considered an efficient organism in reference to transformation but Venter's group could not obtain any clones containing DNA fragments even half the size of *Mycoplasma genitalium* genome [14]. Focus was then shifted from *E. coli* to yeast as it accommodated large foreign DNA molecules well, where the fragments were assembled together by homologous recombination, a unique system used by yeast to

Table 2 Comparative account of functional prediction and sequence conservation in: Φ X174, *H. influenza* & *M. genitalium* [8]

	No. of proteins		
	<i>Bacteriophage ΦX174</i>	<i>Haemophilus influenzae</i>	<i>Mycoplasma genitalium</i>
Broad Functional prediction	4	1079	313
General Functional prediction	6	330	94
Sequence Conservation only	10	177	17
Non functional prediction	-	128	44

repair damaged DNA [15]. Therefore, DNA molecules that integrated into the yeast chromosomes during transformation exploited the property of homologous recombination, which led to assembly of DNA fragments thereby helping in construction of a synthetic genome [14,15,16]. After this initial study of available transformation systems that could solve the problem of larger assemblies, Venter's group moved on to create a synthetic version of the *Mycoplasma genitalium* genome [14].

Their work began with the generation of 101 chemically synthesized oligonucleotide cassettes ranging in the size of 5–7kb, containing approximately 80–360bp overlapping regions at the ends [14]. To differentiate the natural genome from the synthetic one a few of the cassettes contained watermark sequences which were unique identifiers that could not be translated into peptides and were created by short insertions or substitutions. The synthesis of this genome involved a five stage assembly process. Initially the 6kb (5–7kb) fragments of DNA were assembled in tetrads forming 25 sub-assemblies of 24kb each (A-series assemblies). Further 8 large stretches of about 72kb were assembled from these 25 sub-assemblies (forming B-series assemblies). Two of these 1/8 fragment of genome were joined together generating a larger fragment of approximately 145kb representing 1/4 fragment of whole genome (C-series assemblies). The first three steps of assembly were carried out in *E. coli* using *in vitro* recombination in order to generate large amount of DNA for the next step and for sequence validation at each step of assembly. Thereafter, for the last two phases assembly was carried out in *Saccharomyces cerevisiae* using Transformation Recombination Cloning (TAR) by exploiting the homologous recombination system to assemble 1/4 fragment of genome to generate the final stretch of DNA of approximately 5,80,000 bp forming the entire synthetic genome. The reason for shifting from *E. coli* to yeast was the ability of Yeast Artificial Chromosome (YAC) to support DNA fragments in Mbp in comparison to *E. coli* which as mentioned before proved unstable in handling such large amounts of DNA [14].

To overcome the tedious process of generating multiple assemblies of the entire synthetic genome in a single step [17], 25 different overlapping DNA fragments (A-series) were transformed in yeast cells such that a single yeast cell would have atleast one representative of these 25 DNA segments. The clones were then screened for the presence of correct assemblies of DNA fragments i.e. complete synthetic genome using multiplex PCR. One such cell was called as *Mycobacterium mycoides* JCVI-1.0 (after the name of J. Craig Venter Institute) comprising 590,011bp of the synthetic genome. It had a size slightly higher than the natural as it included the vector sequence too [17]. The

creation of JCVI-1.0 thus became a landmark in the history of synthetic genomics.

Creation of a cell with a synthetic genome: genesis of life

The succeeding step before Craig Venter and his group was then to transplant this artificial genome into a cell and understand the intricate functioning mechanism of a minimum genome [18]. But since *Mycoplasma genitalium* was a slow growing bacterium and took weeks to grow, the team had to switch to other faster growing and previously sequenced *Mycoplasma mycoides subspecies capri* (GM12) as a donor and *Mycoplasma capricolum subspecies capricolum* (CK) as a recipient and started working on the creation of a *M. mycoides* strain from a genome that had been cloned and engineered in a yeast model system [Fig 1]. For this, *M. mycoides* was transformed [19] with a vector containing a yeast auxotrophic marker, a yeast centromere and a Yeast autonomously replicating sequence for its propagation in yeast as a Yeast Centromeric Plasmid (YCp). In one of the clones the entire vector got integrated into the genome and was denoted as YCpMmyc1.1. This (YCpMmyc1.1) was then isolated from *M. mycoides* and transformed into Yeast spheroplasts in which it grew robustly as a Yeast Centromeric Plasmid [19]. To test whether deletions occur during routine propagation in yeast, clones were analyzed for completeness and size by multiplex PCR and CHEF (Clamped Homogenous Electric Fields). This indicated that the bacterial genome was stable in yeast. Thereafter this whole genome transplanted into yeast was sequenced for accuracy which provided a definitive demonstration of stability in yeast system. In the next step, YCpMmyc1.1 was isolated from yeast and transplantation was attempted into wild type *Mycoplasma capricolum*. However, no transplants were recovered; the principle obstacle was the presence of a restriction endonuclease in the recipient *Mycoplasma capricolum* that degraded the unmethylated YCpMmyc1.1 (donor DNA) isolated from yeast. To overcome this restriction barrier, there was a need to either inactivate the restriction endonuclease of *Mycoplasma capricolum* and integrate puromycin resistance marker into the coding region of the gene or methylate the donor DNA (YCpMmyc1.1) isolated from yeast using *Mycoplasma capricolum* extracts. Hence, using the second available remedy YCpMmyc1.1- Δ typeIIIres strain was created [19].

With this framework, like that of *M. genitalium*, *M. mycoides* genome was digitally chopped up into 1,100 pieces, each about 1,080 base-pairs with a sticky end of 80bp [18]. These were commercially synthesized and were

Fig. 1 Cloning of *Mycoplasma mycoides* genome in yeast, engineering it and transforming it into *Mycoplasma capricolum* to create a viable engineered bacterium [19].

designed to contain *NotI* restriction sites at their termini, and recombined in the presence of vector elements to allow for growth and selection in yeast. Thereafter, yeast model system was used to assemble these DNA pieces into a synthetic genome in a three step strategy by transformation and homologous recombination. In the first stage, cassettes and a vector were recombined in yeast and transferred to *E. coli*. These 10kb assembly pools and their respective cloning vectors thus generated were transformed into yeast to generate 11 cassettes of 100kb each. But these could not be stably maintained in *E. coli* so the recombined DNA had to be extracted. In preparation for the final step the assembly intermediates were purified by anion exchange chromatography, enriched by agarose plugs and analyzed by Field inversion gel electrophoresis followed by transformation into yeast spheroplasts to stitch together the 100kb assemblies (Fig. 2).

Screening for the complete genome [18] was done using multiplex PCR with 11 primer pairs, designed to span each of the eleven 100kb assembly junctions. Also restriction analyses were done using *AscI* and *BssHII* as their sites of cleavage were present in the watermarks.

To confirm the functionality of the 100kb assembly intermediates, semi-synthetic genomes (mixing natural pieces with synthetic ones) were constructed and transplanted. Viable colonies were produced after transplantation assuring that the synthetic fraction contained no lethal mutations. However, inviability of one of the clones helped detect an error in insertion of a single nucleotide in one of the assemblies that affected its viability which led to a frameshift mutation in *dnaA*- a gene essential for replication. This delayed the project by three months. Error free assembly was re-constructed to produce an error free sMmYCp235 yeast strain clone with the complete synthetic genome of *M. mycoides*.

Finally for distinguishing the synthetic genome from the natural one, two analyses were performed. First, four primer pairs were designed specific to each of the four watermarks such that they produced amplicons in a single multiplex PCR reaction. Secondly, restriction patterns obtained with *AscI* and *BssHII* was checked for consistency with a transplant produced from a synthetic *M. mycoides* genome.

Eventually one of the yeast cells out of the lot had a complete synthetic genome with designed watermarks [18]. The synthetic genome from the sMmYCp235 yeast clone was

Fig. 2 Assembly of the synthetic *Mycoplasma mycoides* genome (JCVI-syn1.0) [18].

subsequently transformed into restriction minus, fast growing *M. capricolum* recipient cells. Various genome combinations were transplanted to finally achieve a blue colony selected on SP4 medium containing tetracycline and X-gal. DNA of this colony was sequenced and it was established that it was manufacturing proteins characteristic of *M. mycoides* instead of *M. capricolum*. It matched the intended design, new polymorphisms, an *E. coli* transposon insertion and an 85bp duplication site. The cells were self replicating and capable of logarithmic growth [18].

Even after realizing the dream of artificial life, Craig Venter states –

“There is still an ocean of great science left for me to explore [1]”

Applications and ethical issues

Synthetic biology, an innovative and highly promising blend of science and engineering aims to construct novel entities

and redesign existing ones, has revolutionized the way biotechnology is being done today. Fast and cheaper DNA sequencing and synthesis with parallel developments in the field of bioinformatics has allowed rapid design, fabrication and testing of systems. The behavior of molecules and their activity inside living cells can be probed and it will serve to highlight relevant intracellular physics. Potential benefits of synthetic biology include development of low cost drugs, vaccines, antibiotics, production of chemicals and energy, detection through biosensors, transformation, bioremediation or biodegradation through engineered bacteria. After creating the first synthetic cell, a future aim of Venter’s team is to build up the algae genome with numerous variations designed for growth to make super productive organisms. Biodiesel production is a great boost to biofuel manufacturing and the synthetic cell made holds great promise for large scale biofuel production. The creation of ‘Synthia’ [18] is a milestone in the field of biotechnology, and its application in medicine will result in saving lives and improving human health. Creating life is the most extreme form of

control over other organisms and also bestows a new status and responsibility on scientists and society.

With the emergence of new technology we have to take a pragmatic look at both the benefits as well as the risks due to deliberate or accidental damage. The invention has raised numerous questions of ethics, bio-safety, bio-security and intellectual property. Focus on three basic issues is a necessity: bioterrorism, worker safety and protection of the environment in the vicinity of research labs. The issue in general is not really creation of the organism but at the level of perceived controllability and feeling of safety. Robust regulations for the safe use of recombinant DNA technology need to be in place, and hands-on monitoring is required to ensure that the regulations are followed. It is also important to create governance systems which are sustainable, forward looking and dynamic and which allow innovations in synthetic biology to emerge. Past experiences from GM crops and stem cells have shown the importance of public debate and discussion, a similar platform needs to be created for synthetic biology too. Part of the challenge will be to develop a common, widely understood language for discussing ethical and social, as well as technical aspects of synthetic biology.

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