

REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE (DD-MM-YYYY) 19-02-2013		2. REPORT TYPE R&D Status Report (Quarterly)		3. DATES COVERED (From - To) 17-11-2012 to 19-02-2013	
4. TITLE AND SUBTITLE Construction of a Bacterial Cell that Contains Only the Set Of Essential Genes Necessary to Impart Life				5a. CONTRACT NUMBER HR0011-12-C-0063	
				5b. GRANT NUMBER NA	
				5c. PROGRAM ELEMENT NUMBER NA	
6. AUTHOR(S) John Glass Tony Yee				5d. PROJECT NUMBER NA	
				5e. TASK NUMBER NA	
				5f. WORK UNIT NUMBER NA	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) J. Craig Venter Institute 9704 Medical Center Drive Rockville, MD 20850				8. PERFORMING ORGANIZATION REPORT NUMBER HR0011-12-C-0063.3	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) DARPA, MTO Prog: Living Foundries ATGC 675 N Randolph St Arlington, VA 22203				10. SPONSOR/MONITOR'S ACRONYM(S) DARPA	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S) NA	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution is unlimited					
13. SUPPLEMENTARY NOTES NA					
14. ABSTRACT We are working towards the goal of creating a bacterium that contains only the set of genes that are essential for life. Toward that end, we have continued to delete genes and gene clusters from the starting genome (<i>M. mycoides</i> JCVI-syn1.0). To date, the smallest viable genome is 779 kbp, representing a ~30% reduction. The transplanted cells were later found to grow slowly compared to wild type, after being transferred from solid media to liquid. We have made several additional single gene deletions to this genome, but we have begun to explore adding genes back into the 779 kbp genome in order to restore growth rates. We have also made progress on the design and synthesis of a minimal genome (483 kbp). Transplantation experiments using the previously reported 1/8th minimized genome molecules are complete. One of the 1/8th molecules proved to be viable. A more conservative design of a minimal genome (623kbp) has been completed, and synthesis is underway.					
15. SUBJECT TERMS NA					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES 5	19a. NAME OF RESPONSIBLE PERSON Tony Yee
a. REPORT UU	b. ABSTRACT UU	c. THIS PAGE UU			19b. TELEPHONE NUMBER (include area code) 301 795 7133



Construction of a Bacterial Cell that Contains Only the Set of Essential Genes Necessary to Impart Life

Report Title: R&D Status Report (Quarterly)
Report Number: HR0011-12-C-0063.3
Reporting Period: November 17, 2012 to February 19, 2013
Contract No.: HR0011-12-C-0063
Performing Organization: J. Craig Venter Institute
9704 medical Center Drive
Rockville, MD 20850
USA
Author of Report: John Glass, Anthony Yee
Principal Investigators: Hamilton Smith, Clyde Hutchison

Abstract

We are working towards the goal of creating a bacterium that contains only the set of genes that are essential for life. Toward that end, we have continued to delete genes and gene clusters from the starting genome (*M. mycoides* JCVI-syn1.0). To date, the smallest viable genome is 779 kbp, representing a 30% reduction. The transplanted cells were later found to grow slowly compared to wild type, after being transferred from solid media to liquid. We have made several additional single gene deletions to this genome, but we have begun to explore adding genes back into the 779 kbp genome in order to restore growth rates.

We have also made progress on the design and synthesis of a minimal genome (483 kbp). Transplantation experiments using the previously reported 1/8th minimized genome molecules are complete. One of the 1/8th molecules proved to be viable. A more conservative design of a minimal genome (623 kbp) has been completed, and synthesis is underway.

Sponsored by
Defense Advanced Research Projects Agency
Microsystems Technology Office (MTO)
Program: Living Foundries: Advanced Tools and Capabilities for Generalizable Platforms (ATCG)
Issued by DARPA/CMO under Contract No: HR0011-12-C-0063

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Summary

The goal of the project is to create a cell that contains only the set of genes that are essential for life under ideal laboratory conditions. We are working to minimize *Mycoplasma mycoides* JCVI-syn1.0 (the synthetic version of *Mycoplasma mycoides* subsp *capri*) using two approaches:

- Top Down: remove genes and clusters of genes one (or a few) at a time, proceeding only if the reduced strain is viable, with a reasonable growth rate
 - o The genome has been reduced to 779 kbp and found to be viable
 - This strain proved to be slow growing after transfer to liquid media
 - Growth appeared to be comparable to the wild type strain on plates
 - The change could be due to cell membrane structure
 - More single gene deletions have been made, but were also slow growing
 - We have started to add genes back in order to restore the growth rate
- Bottom Up: design our best guess as to the content of a minimal genome (483 kbp) and synthesize it from oligonucleotides
 - o One of the 1/8th genome molecules has been found to be viable
 - o Deletion experiments were performed in parallel to 1/8th molecule synthesis
 - Based on transposon studies, 146 clusters were expected to be non-essential
 - Twenty-six of these clusters yielded no transplants or slow growth
 - Some of these deletions were deleted in the design of the 483 kbp genome
 - o We have designed a more conservative genome (683 kbp), which is being synthesized at SGI in 1/8th molecules

Introduction

The goal of this research project is to build a minimal bacterial cell. The pursuit of a minimized cell is critical to the advancement of biology, both as a pathway for understanding the basic requirements for replication and as a chassis for creating an optimized platform for any number of possible applications. Ham Smith recently listed his top ten reasons for why we need to build and characterize a minimal cell:

1. To define minimal components for life
2. To provide a minimal platform for systems biology – a comprehensive description of cell behavior
3. To discover as yet unknown functions required for life
4. To provide a platform for modularization of cell processes
5. To examine the plasticity of gene arrangements in a minimal genome
6. To examine *ori* locations and multi-*ori* constructions on cell viability
7. To determine what genes control the cell cycle and the rate of cell doubling
8. To examine the feasibility of going from the minimal cell to more complex cells by adding back genetic functions
9. To change energy metabolism from one carbon source to another
10. To go where no man has gone before

Chuck Merryman offered a different, also very compelling rationale for our work and why it is vital to U.S. biological science. Chuck noted that cells are catalysts. They transform A into B - often in ways that conventional chemical or enzymatic catalysts cannot approach.

1. The catalyst itself will make more catalyst for you. Any scale is attainable.
2. If a catalyst is 'poisoned' it can self-repair. It is essentially immortal.
3. If the environment changes, the catalyst adjusts on the fly to maximize output.
4. From a single A (eg glucose), there are millions and millions of B's to choose from (*arguendo*).

All this comes at a price: Evolution and natural selection have ensured that B isn't just any product. It is another cell (by happenstance some byproducts are useful and produced at reasonable levels, for example: ethanol).

The genetic 'cassette' that defines and orchestrates the production of new cells [in all cells] is the minimal cell. In other words, the minimal cell is the genetic architecture that ensures another cell is life's fundamental product. In a perfect production system we would have absolute control over this cassette. Defining what the cassette is the first step. We know control is possible. More or less, animals do it to their cells all the time.

In a perfect production system, the minimal-cell cassette would initially function as nature intended and make copies of itself as fast as possible. Then, replication would turn off; however, unlike most saturated cultures, metabolic activity would not be slowed. Instead, metabolism would continue to run at full speed but it would be redirected towards the synthesis of a different, desired product (B). At the same time, cell maintenance (a sub-cassette of the replicative cassette) would remain responsive and perform necessary repairs. A perfect production system would be little more than supporting infrastructure for a sack of enzymes (and a corresponding balanced chemical equation) that transforms A into the desired product B. The product and all byproducts would exit the system to avoid equilibrium. The system would respond to environmental vagaries to maximize the output of B. If a cell became extensively damaged and was no longer able to function, it would be recycled and replaced. In essence, the culture itself would become immortal. It would continue to pump out product B as long as you supplied A. ('Perfect' is a boundary system of course. There are less perfect systems that are still useful. Nevertheless, imperfect systems benefit from coming as close as possible to the boundary).

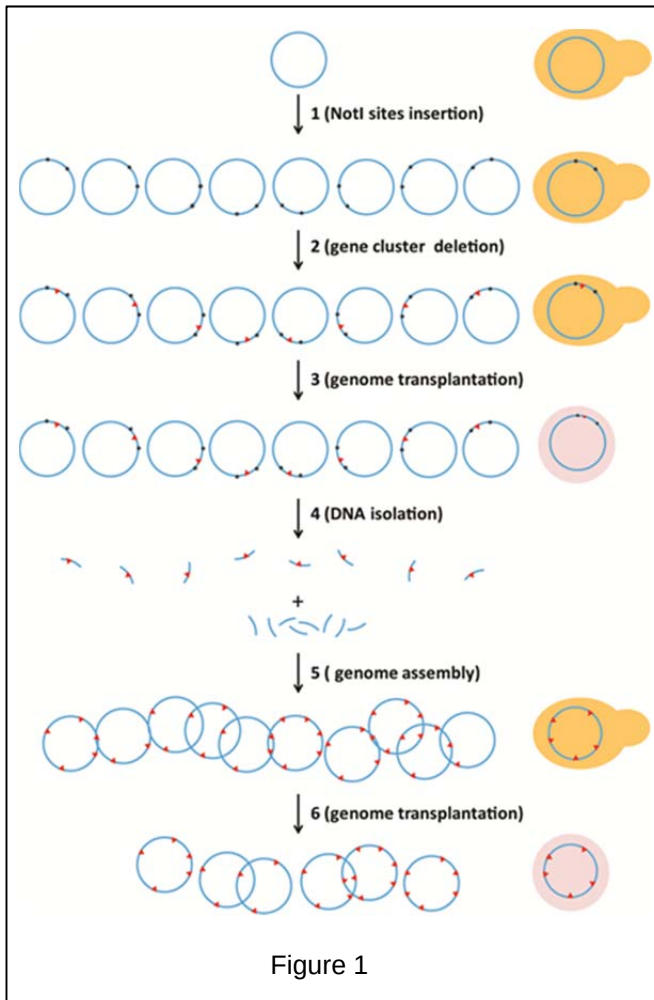
Until we build a minimal cell and understand how the genetic cassette that encodes life works, the sub-functions of replication, repair, and overall metabolic activity will remain intertwined and beyond our full control. For the time being we have ignored the other cassettes used to build and keep a cell running with a simple feedstock A. They are not in a minimal cell. These manufacturing cassettes will be fully or mostly comprised of genes that we will layer back onto a minimal cell chassis in various combinations. It will be that knowledge of the minimal cell that will enable us to model and control the added A to B manufacturing machinery.

Even as we labor at our top down and bottom up approaches to construct a minimal cell other scientific teams are working to have the infrastructure in place to make use of our minimal cell. Several years ago the JCVI began collaborating with Stanford systems biologist Markus Covert to develop a comprehensive computational model of a near minimal cell that was much more sophisticated than previous cellular modeling efforts such as the E-Cell (Tomita, Hashimoto et al. 1999). The first installment of that Stanford-JCVI effort at cellular modeling was reported in *Cell* last summer (Karr, Sanghvi et al. 2012). Based on previous JCVI efforts using transposon mutagenesis of near minimal cells that identified about 100 essential genes whose function was unknown and that were conserved in many bacterial and eukaryotic genomes (Glass, Assad-Garcia et al. 2006) we have enlisted a number of academic labs to try and determine the functions of the proteins encoded by those genes. By the old one gene – one enzyme – one graduate student – one Ph.D. approach cellular roles can now attributed to at least 8 different genes. The NIH is now reviewing large program project proposals to use modern omics approaches to determine unknown gene function (including one from the JCVI). Biological science is waiting for, getting ready for, anxious for a minimal cell to use as a research platform. DARPA aims to pave the way for the next 30-100 years of US science by making initial investments. The minimal cell is key to a lot of what biology and in particular synthetic biology hopes to accomplish.

Methods, Assumptions and Procedures

TOP DOWN APPROACH

The plan here was to start with a 1079 kb with the full size viable *M. mycoides* JCVI-syn1.0 synthetic genome. We began to remove clusters of genes we thought to be non-essential. Initially we did this in single experimental steps. This involved using the TREC approach we developed to cleanly eliminate specific regions of the genome that was parked in a yeast cell as a yeast centromeric plasmid (YCP). We would then boot up that partially minimized YCP using genome transplantation into a suitable bacterial recipient cell. Later we began using Yo Suzuki's Green Monster technique to remove multiple clusters at once from the YCP. The Green Monster method leaves GFP markers in the YCP, so it is not suitable for making the final molecule, but it does show us deletions that can be made without loss of cell viability or growth rate. After learning what groups of presumably non-essential gene clusters to target, we use TREC (Noskov, Segall-Shapiro et al. 2010) to seamlessly make deletions. At each step re-test for viability. Only proceed to the next step if the preceding construction is viable and the doubling time is approximately normal.



We have developed an 8-piece strategy to rapidly build genomes containing multiple deletions. This has increased the rate at which we can remove genes by a factor of 8. We have produced a series of functional reduced genomes, including the smallest 779-kb genome (~30% reduction).

The process is combinatorial genome assembly. As shown in Figure 1: 1) Two NotI restriction sites (black dots) were introduced into neutral sites in each of eight genomes, such that digestion at these sites would release eight genomic fragments that overlap with neighboring fragments by 200 bp for assembly of the whole genome. 2) Seamless gene or gene cluster deletion (red triangle) was carried out by TREC method on 8 yeast strains in parallel. 3) Genomes were isolated from yeast and transplanted into recipient cells. 4) DNA from each *M. mycoides* strains was digested with NotI, and the genomic fragment was isolated after gel electrophoresis. 5) The deleted and wild-type versions of the 8 pieces were combinatorially assembled in yeast. 6) Assembled genome was screened for completeness, isolated, and transplanted into recipient cells. Transplantation yielded viable *M. mycoides* with a variety of genotypes.

This development will be a huge accelerator as we continue the Top Down approach. Importantly it can be general tool for genome scale engineering through combinatorial genome synthesis that is fully amenable to automation.

BOTTOM UP APPROACH

As noted in our last quarterly report, we designed what we expected to be a near minimal genome based on our combined transposon mutagenesis analysis of genes individually dispensable, and based on an incomplete directed mutagenesis study that removed continuous clusters of genes we expected to be non-essential. That designed genome, which we refer to as the “Hail Mary Genome” or HMG, was comprised of 483 kbp (see “Hail Mary” reference). The HMG was synthesized by Synthetic Genomics, Inc. (SGI) in 8 segments that overlapped their adjacent segments by 200 bp. To determine whether each of these segments was individually viable we constructed 8

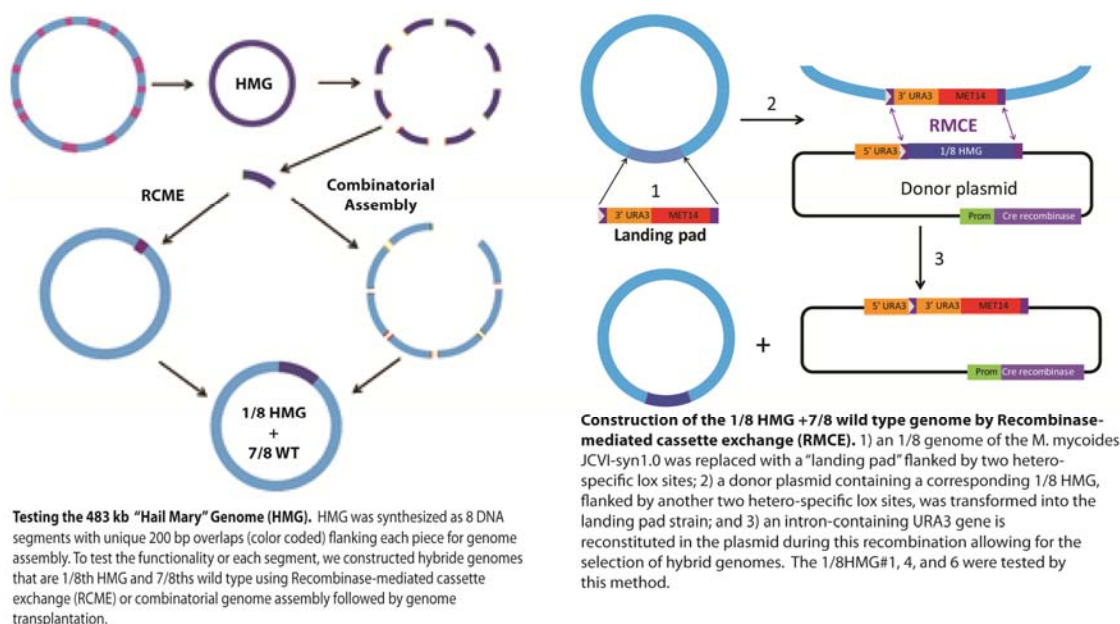


Figure 2

different genomes. One eighth of each genome was an HMG and seven eighths were wild type (Figures above). The pieces were assembled by Recombinase mediated cassette exchange.

Results and Discussion

BOTTOM UP APPROACH

As previously reported, a minimal genome was designed by the project team. The minimal genome was designed in 1/8th genome molecules, so that smaller sections could be tested for viability. Since the last reporting period, all eight of the 1/8th genome sections were used in transplantation experiments. Of the 8 segments only #2 has yielded a living cell. That partial HMG cell grew slowly.

In the interim between the design and synthesis of the HMG we completed our evaluation of all 146 of the *M. mycoides* gene clusters we expected to be non-essential based on transposon studies. Twenty six of those individual cluster deletions yielded no transplants or the resulting cells grew much slower than wild type cells. Some of those 26 gene clusters were deleted in the HMG.

Conservative Minimal Genome was designed based on cluster deletions tested for viability.

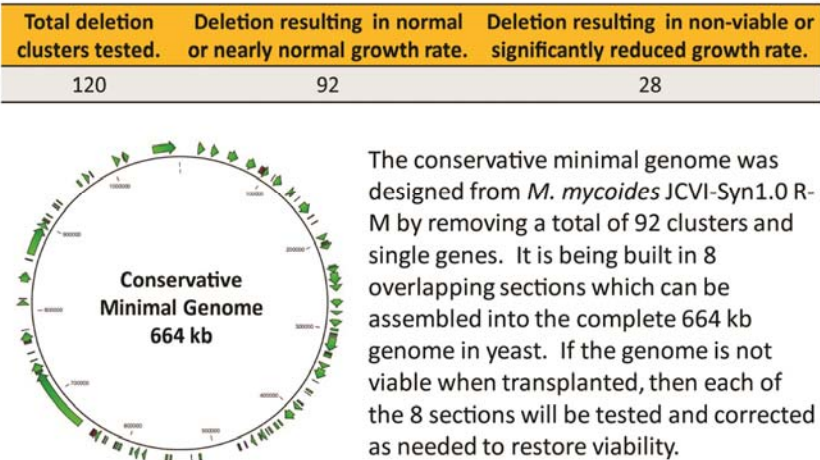
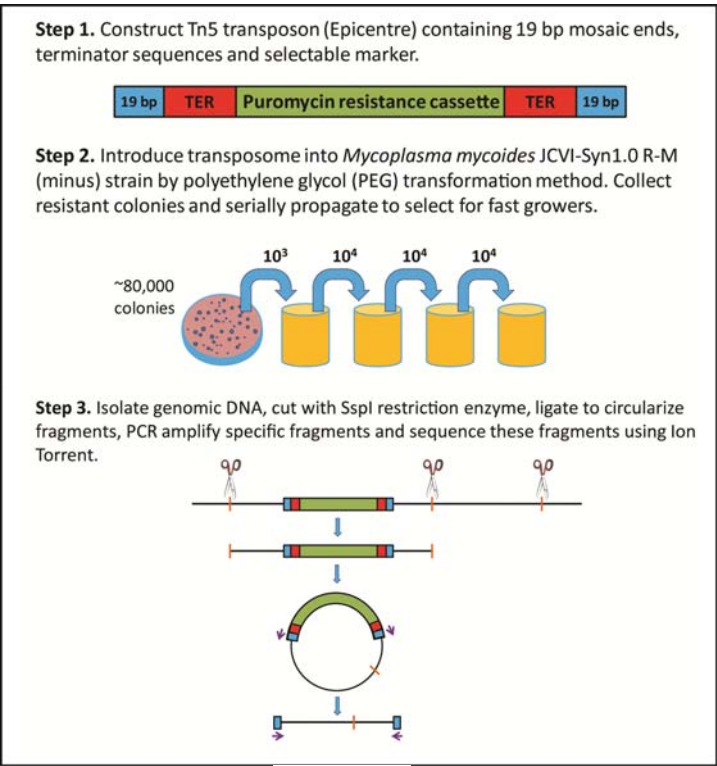


Figure 3

As a result of our unexpected findings about these 28 deleterious mutations we redesigned our bottom-up approach to make what we call the “Conservative Genome” or CG. This 623 kbp genome is currently being synthesized at SGI, again in 8 overlapping segments.

We are developing tools (Figure 4) to enable genetic complementation to restore desirable phenotypes to deletion mutants. This will be a plasmid capable of replication in both *M. mycoides*



and in the genome transplantation recipient cell, *M. capricolum*. Its first planned use will be to determine what specific gene deletions caused the 1/8th HMG#2-7/8th wild type genome to grow slowly. We know that 2 of the gene clusters whose deletion caused much slower cell growth were for gene clusters not included in HMG segment #2. Using expression of those gene clusters or individual genes from a plasmid we expect to be able to restore a more rapid growth phenotype to the 1/8th HMG#2-7/8th wild type mutant. Thus we may be able to winnow the number of genes we need to include in our minimal gene set from the 5 genes contained in the 2 growth retarding cluster deletions to perhaps just 2 genes. This plasmid system will be a powerful tool as we begin to make final gene deletions in our genome minimization process.

Figure 4

MAPPING NON-ESSENTIAL GENES

Tn5 insertion map.

Tn5 insertion sites (black lines) were mapped to *M. mycoides* JCVI-Syn1.0 R-M (minus) genome. Open reading frames are highlighted in blue, tested cluster deletions that produced viable cells with nearly normal or normal growth rates (green).

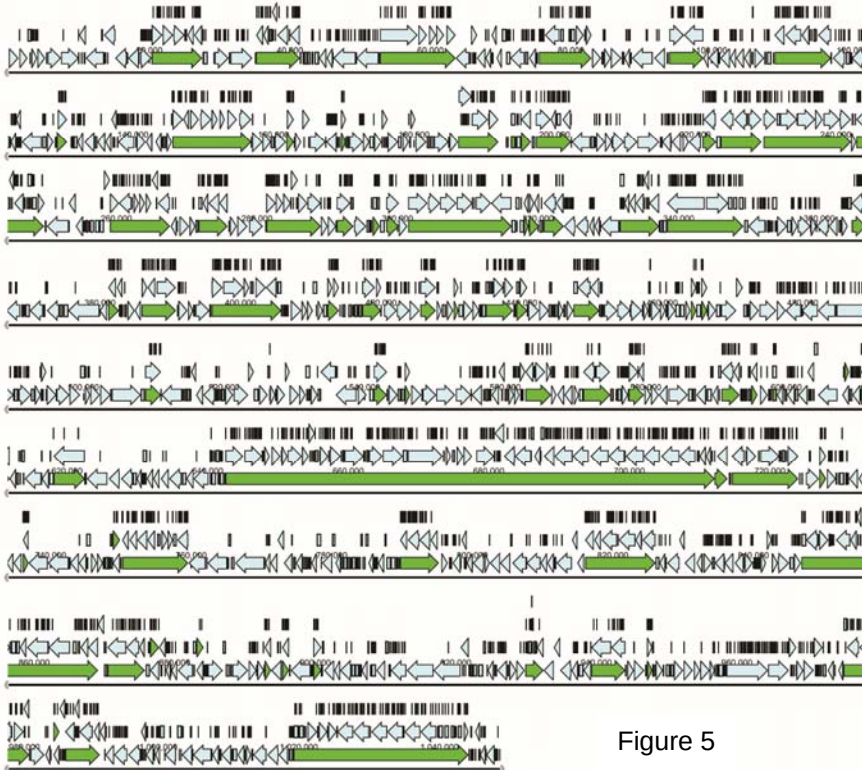


Figure 5

Illumina sequencing we reported previously. This approach eliminates most insertions in genes we would almost certainly expect to be essential. A new Tn5 transposon map is shown.

Because the Tn5 mapping of non-essential genes is now better, faster, and less expensive we recently tested the method on our partially reduced 928 kb genome. We did this right after transposon bombardment and then again after the cells had undergone ~40 cell divisions. What we will examine here is what genes are non-essential in the short term as opposed to what genes are non-essential for sustained survival. The analysis of these data will be presented in our next progress report.

We believe that our efforts to enhance methods for non-essential gene determination will be valuable for groups that may want to eliminate non-essential genetic baggage from production organisms so that they are more easily modeled.

We have improved the Tn5 transposon bombardment process we initially reported on in our previous progress reports. The Tn5 was re-made so that there would be no possibility of transcripts that crossed the transposon being translated to produce partial gene products that would falsely indicate a gene was non-essential. We did this by flanking the puromycin antibiotic resistance marker located in the transposon with transcriptional terminators on both side (red boxes in the figure above). We are also now using Ion Torrent sequencing to identify the locations of transposon insertions. This approach is both faster and less expensive than the

BIOLOGICAL PARCEL SERVICE – COLLABORATION WITH MIT’S NEIL GERSHENFELD

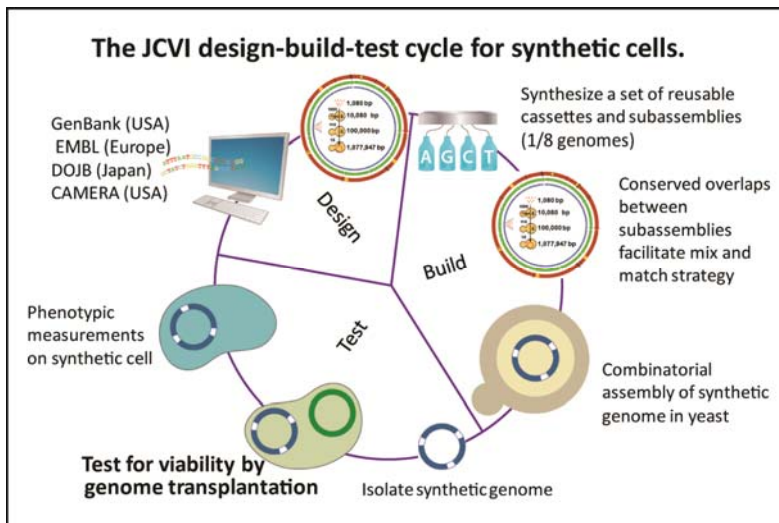
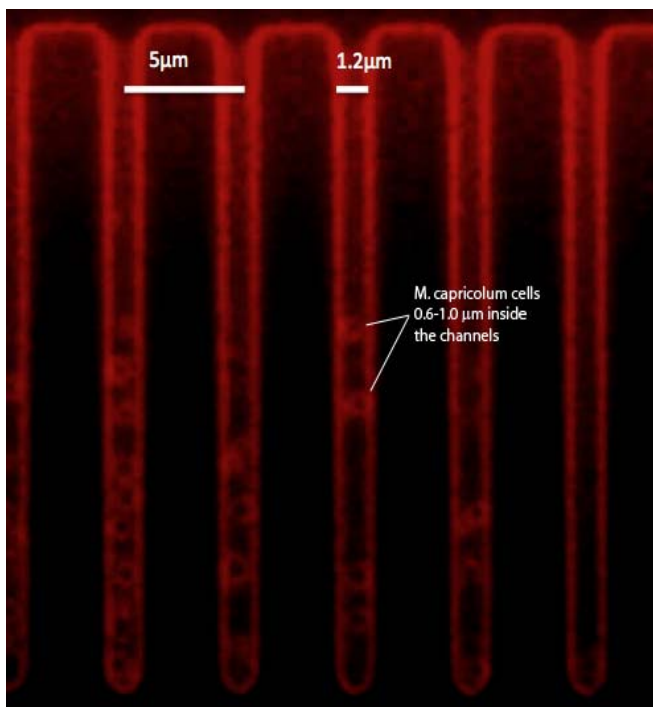


Figure 6

Interactions between John Glass and Neil Gershenfeld through DARPA Living Foundries activities fostered this collaboration. We hope to develop a new technology that will address the current major issue in the JCVI approach for building synthetic cells, Genome Transplantation. Genome transplantation is a method for installing an isolated bacterial genome into a suitable recipient cell so that it is booted up by the enzymatic machinery of the recipient cell and produces daughter cells with the genotype and phenotype encoded by the transplanted genome (Lartigue, Glass et al. 2007). Currently the process has only been done reproducibly to transplant *M. mycoides* genomes into *M. capricolum* recipient cells. For this application, genome transplantation reliable and robust in expert hands, it takes a great deal of experience to learn how to pipet whole bacterial genomes without shearing them and how to judge that recipient cells have reached an exact state of competence that will result in a successful transplantation.



The Gershenfeld team at the MIT Center for Bits and Atoms (CBA) has developed nanofluidics approaches that we hope will lead to better more efficient and reproducible genome transplantation that no longer requires “magic hands” and can be done using a broad variety of bacterial chromosomes. The ambition of this project, which we call the “**Biological Parcel Service**”, is to precisely localize donor genomes and recipient cells in nanofluidics channels. Once so contained we hope to use physical and chemical approaches to force donor genomes into the recipient cells. This approach will be an extension of work done previously by CBA graduate student James Pelletier (Pelletier, Halvorsen et al. 2012). To initiate the collaboration CBA scientists Pelletier and Andreas Mershin fabricated nanofluidics channels that they brought to the JCVI team in Rockville, MD on December 21, 2012 to do some initial experiments. That day the MIT and JCVI teams were able to insert *M. capricolum* cells in 1.5 µm channels and then lyse those cells to release the genomic DNA. Since then the MIT group has work with killed cells sent to them by the JCVI team to further improve the insertion of the cells into the channels and then subsequently lyse

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those cells. As we become more skilled at insertion of cells into channels we will also begin working using live cells at the JCVI (the MIT lab cannot work with cells requiring BSL-2 isolation) to apply genome transplantation experimental conditions to the cells and isolated genomes inside the channels. The nanofluidics approaches will allow more rapid testing of different experimental conditions and direct microscopic examination of the tests. The goal will be to show sufficient progress to allow us to compete for Living Foundries funding to pursue this avenue of research. The teams have ambitions of adapting this and other nanotechnology methods used at the CBA to solve synthetic biology problems.

Conclusions

Tasks from the Statement of Work for Year 1:

Task 1: Complete a detailed global Tn5 transposon mutagenesis insertion map.

The Tn5 transposon insertion map was submitted with the initial quarterly report. The appended Excel file shows what *M. mycoides* genes can be disrupted by transposons and which ones cannot. The method has advanced greatly over our initial efforts at using transposons to identify non-essential genes.

Due: Month 6; Status - completed: Month 3

Task 2: Delete up to 27 large gene clusters

Using our top down approaches, we have reduced the genome size of *M. mycoides* JCVI-syn1.0 from 1079 kbp to 779 kbp through the deletion of some 30 clusters, representing a ~30% reduction. In this process we have identified some genes that we expected to be non-essential but proved to be needed at least in the genomes from which we had already eliminated a large number of genes. This reduced organism grows somewhat slower than the wild type cell and we are exploring re-introducing some genes to see if we can restore the wild type growth rate (~60 minutes per cell cycle).

Due: Month 12; Status – complete (but we will continue to reduce the genome as much as possible throughout Year 1).

Task 3: Construct a preliminary modular map of the genome

The design of a modular map of the genome is underway.

Due: Month 12; Status – in progress

Although our HMG bottom up approach proved to be non-viable genome, we found that one of the 1/8th HMG segments was viable when added to an otherwise wild type 7/8th genome.

We are synthesizing a Conservative Genome that has 623 kb. Based on our observations so far about other deletions, we have high expectations that this genome or at least most of it will prove to be viable.

The Biological Parcel Service collaboration with Neil Gershenfeld's team at MIT offers some hope of expanding the scope of genome transplantation. This could make it much more practical to boot up synthetic genomes for a broad range of other bacterial species and better enable many other efforts in synthetic biology such as genome recoding and expanding the genetic code.

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Planned Activities for the Next Reporting Period

1. We will offer analyses of non-essential genes needed for sustained growth as opposed to short term cellular survival.
2. We will continue Top Down minimization of our synthetic genome
3. We complete synthesis and test the 683 kb Conservative Genome as part of our Bottom Up strategy.
4. We begin efforts at genome functional modularization.
5. We will continue pilot efforts on the Biological Parcel Service project.

Program Financial Status

	Planned Expend	Actual Expend (Cumulative to Date)	% Budget Completion	At Completion	Latest Revised Estimate	Remarks
Task 1	\$305,646	\$305,646	100%	\$305,646	\$305,646	Completed
Task 2	\$826,256	\$413,145	50%	N/A	\$826,256	N/A
Task 3	\$43,487	-	0.0%	N/A	\$43,487	N/A
Cumulative	\$1,175,389	\$718,791	61%	N/A	\$1,175,389	N/A

There is no management reserve or unallocated resources.

Based on the currently authorized work:

- Is current funding sufficient for the current fiscal year? Yes
- What is the next fiscal year funding requirement at current anticipated levels? \$1,214,151.00
- Have you included in the report narrative any explanation of the above data and are they cross-referenced? Not applicable; current funding is sufficient for the current fiscal year.

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