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14. ABSTRACT Work has continued on the Top Down approach to genome minimization. Because the smallest genome to date grows too slowly to be used in future experiments, we have conducted additional transposon studies and used the data to construct a new, fast growing parental strain. The effort to modularize the genome has been initiated. We have made a modular map of the genome by grouping genes according to function. In the future, we will modularize the genome as much as possible, although we expect that there will be some general genome layout requirements that will make full modularization impossible. Preliminary work aimed at genome complementation has been conducted. A plasmid system for quickly adding back deleted genes will allow us to quickly examine which gene(s) within a deleted cluster might be causing growth defects or cell death.					
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Construction of a Bacterial Cell that Contains Only the Set of Essential Genes Necessary to Impart Life

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Performing Organization:	J. Craig Venter Institute 9704 medical Center Drive Rockville, MD 20850 USA
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Abstract

Work has continued on the Top Down approach to genome minimization. Because the smallest genome to date grows too slowly to be used in future experiments, we have conducted additional transposon studies and used the data to construct a new, fast growing parental strain.

The effort to modularize the genome has been initiated. We have made a modular map of the genome by grouping genes according to function. In the future, we will modularize the genome as much as possible, although we expect that there will be some general genome layout requirements that will make full modularization impossible.

Preliminary work aimed at genome complementation has been conducted. A plasmid system for quickly adding back deleted genes will allow us to quickly examine which gene(s) within a deleted cluster might be causing growth defects or cell death.

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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 kb and found to be viable but too slow growing for continued use as a parental strain for additional deletions
 - o A new transposon study was conducted and genes were re-categorized as Essential (E), Non-essential (NE), or Impaired (I)
 - The use of the new "I" category should allow us to minimize the chance of encountering unexpected synthetic effects such as slow growth
 - o A new parental strain (848 kb) was assembled from 1/8 genome molecules that were reduced using data from the new transposon study
 - Grows at an approximately normal rate
- Bottom Up: design our best guess as to the content of a minimal genome (483 kb) and synthesize it from oligonucleotides
 - o We have designed a more conservative genome (683 kb)
 - o Synthesis is ongoing at SGI in 1/8th molecule sections

We have also designed a preliminary modular map of the *Mycoplasma mycoides* JCVI-syn1.0 genome, based on the previously reported Hail Mary Genome. The map is presented in the body of this report.

Introduction

The goal of this research project is to build a minimal bacterial cell that contains only the genes that are required for life in ideal laboratory conditions. 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.

The *Mycoplasma mycoides* JCVI-syn1.0 genome has been successfully reduced from 1078 kb to 779 kb. While the 779 kb genome is viable, its growth characteristics are such that we cannot continue to use this genome for further deletions. The growth rate is far too slow to allow follow up experiments at an acceptable pace. Although attempts to restore the growth rate were moderately successful, we opted to perform a new round of transposon work aimed at refining our prediction of the effects of gene deletion. Using data from the new transposon study, we have assembled a new parental strain (848 kb).

Synthesis of a redesigned minimal genome (683 kb) is ongoing at SGI. We expect that many of the 1/8 genome segments from this design will prove to be viable once synthesis is complete.

A preliminary modular map of the genome has been designed and is presented below.

Methods, Assumptions and Procedures

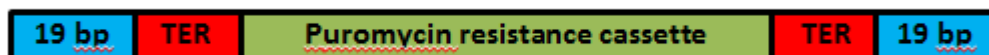
TOP DOWN APPROACH

The plan here was to start with the full size 1078 kb *M. mycoides* JCVI-syn1.0 synthetic genome. We previously reported the development of an 8-piece strategy to rapidly build genomes containing multiple deletions. Using this strategy, we produced a series of functional reduced genomes, including the smallest 779 kb genome (~30% reduction). Transplanted cells carrying this genome grew reasonably well on agar plates, but very slowly in liquid media. The team suspects that there is a problem with the cell membrane, and that the transplanted cells are very fragile.

Four cluster deletions were evaluated for causation of the aforementioned growth characteristics. Experiments were conducted to add each of these four clusters back into the genome. Two of these strains grew faster than the other two, but none grew at a high enough rate for continued use as a parental strain for additional deletions. Because cell doubling time is closely tied to the speed with which the team can perform iterative experiments, it would have been counterproductive to continue with the 779 kb genome.

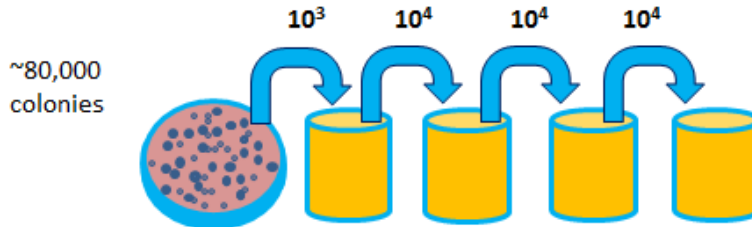
It was clear from this line of experiments that the transposon analysis performed early in this project was not sufficiently informative to detect possible downstream synthetic effects. To that end, we have performed a new round of transposon analysis and sequencing:

- Step 1. Construct Tn5 transposon (Epicentre) containing 19 bp mosaic ends, terminator sequences and selectable marker.

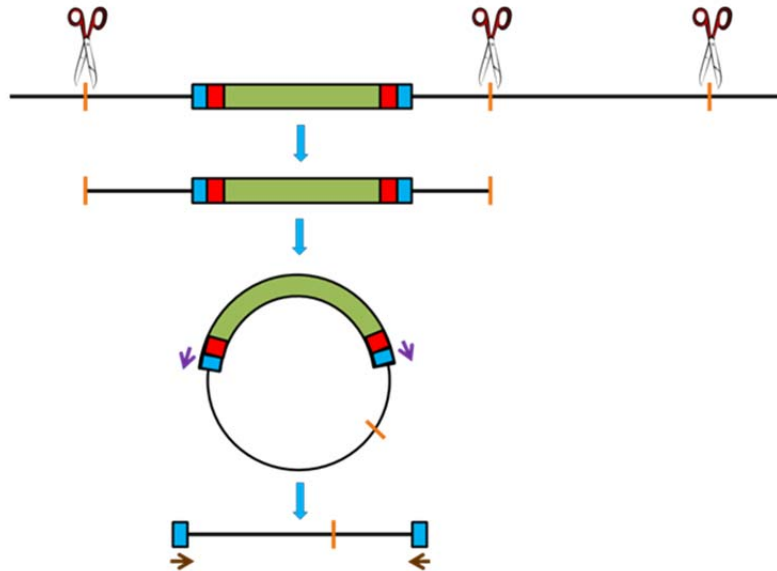


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Step 2. Introduce transposome into *Mycoplasma mycoides* JCVI-Syn1.0 R-M (minus) strain by polyethylene glycol (PEG) transformation method. Collect resistant colonies and prepare two libraries. The first is analyzed prior to serial passaging. The second is serially propagated for four passages to select for fast growers.



Step 3. Isolate genomic DNA, shear or cut by restriction, ligate to circularize fragments, PCR amplify specific fragments and sequence these fragments using Ion Torrent.



Comparison of data between the two libraries allows us to re-categorize genes into three categories: Essential (E), Non-Essential (NE) and Impaired Growth (I). Previously, we had only used the E vs. NE designations, which makes no provision for synthetic effects such as a slowed growth rate. A summary of the new deletion information is appended to this report in an Excel file.

BOTTOM UP APPROACH

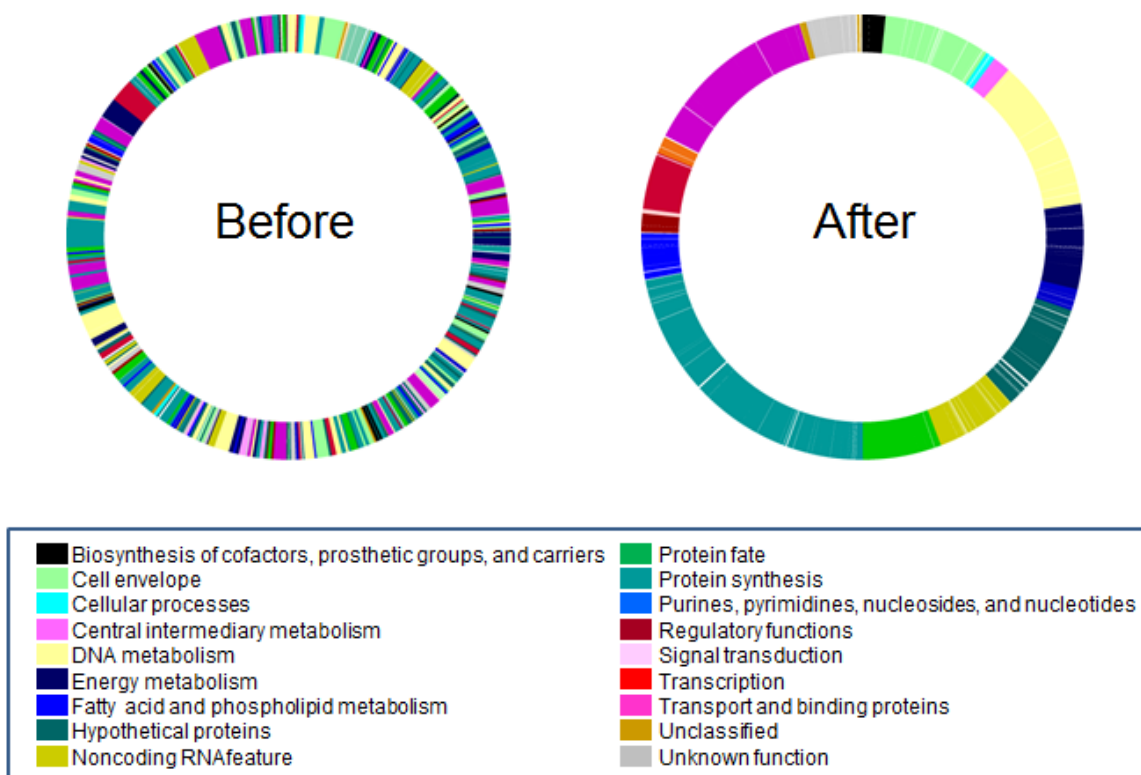
The 623 kb “Conservative Genome” or CG is currently being synthesized at SGI in 8 overlapping segments. We have redesigned the Hail Mary Genome (HMG) to add back in 28 genes or gene clusters that we discovered to be deleterious during our examination of 120 gene clusters that were predicted by the original transposon studies to be non-essential.

GENOME COMPLIMENTATION

We have begun initial experiments aimed to enable genetic complementation to restore desirable phenotypes to deletion mutants. As we have previously seen, interactive effects between deleted genes or gene clusters cannot always be predicted. A plasmid system capable of replication in both *M. mycoides* and in the genome transplantation recipient cell, *M. capricolum* would be a powerful tool to help de-convolute growth-retarding synthetic effects. Results are *very* preliminary at this time; however, the initial results suggest that a plasmid transformed into the recipient cell can be detected as a free plasmid following genome transplantation.

MODULARIZATION

A preliminary, modular map of the genome has been constructed and is shown below:



The modular map is based on the 483 kb Hail Mary Genome, and should be near the size and gene content of the final minimal genome. The process behind construction of the map can be thought of as analogous to defragmentation. We have categorized genes by function and plan to group them accordingly to the greatest extent possible.

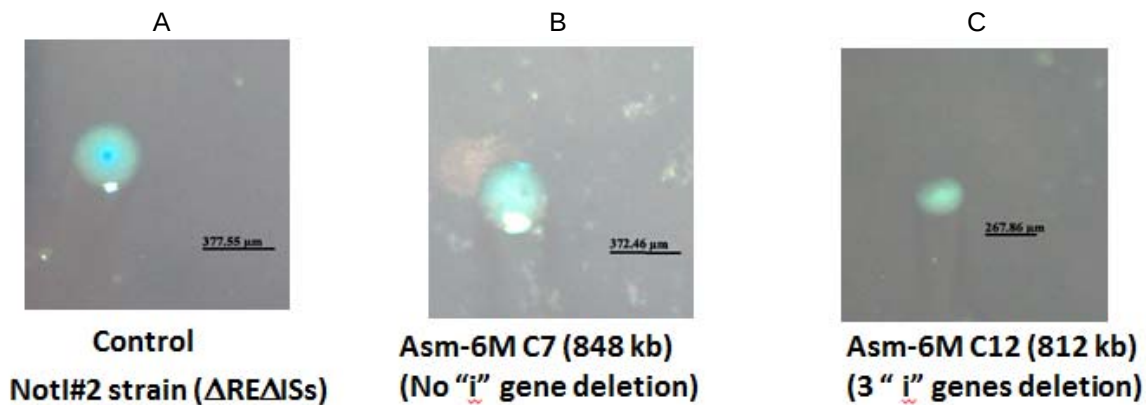
Results and Discussion

TOP DOWN APPROACH

We have used the new transposon information to make gene cluster deletions in the 1/8th genome segments. This approach has thus far been successful, as we have assembled and transplanted an 848-kb genome that has essentially the same growth rate as the *M. mycoides* syn 1.0 genome:

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Three genomes in this experiment were transplanted into recipient cells. Images of the colonies were captured three days after transplantation. Restriction systems and insertion sequences were deleted in the control genome (A), which we know grows at the same rate as the *M. mycoides* JCVI-syn1.0 genome. (B) Deletions of non-essential (NE) genes were made in Asm-6M C7. (C) Asm-6M C12 is the same as Asm-6M C7, except three additional gene clusters, each containing genes that if deleted would cause impaired growth (I) were deleted.

This illustrates the additional predictive power added by the new transposon studies and the re-categorization of genes into the E, NE and I bins. As expected, the Asm-6M C7 did not exhibit a significant reduction in growth rate. In addition to those deleted in Asm-6M C7, the following genes, predicted to cause impaired growth, were deleted in Asm-6M C12:

- Oligopeptide ABC transporter, ATP-binding component
- ABC transporter, ATP-binding protein, putative
- Putative lipoprotein

As predicted, Asm-6M C12 exhibited a significantly reduced growth rate compared to the control and Asm-6M C7. Genome Asm-6M C7 is currently the smallest genome with a high growth rate and will be used as the parental strain for further deletions.

MODULARIZATION

It should be stressed that modularization of the genome will be pursued in earnest in later phases of the minimal cell project. We would want only essential genes in the final modules, and we have already seen that growth defects can and do occur when deleted genes interact in unexpected fashion. If we were to fully modularize the genome at this point in time, we would have no choice but to include many non-essential genes. In addition, it might not be possible to fully modularize all functional gene groups because of general genome design requirements.

We are moving ahead with synthesis of a tRNA module. So far, construction has proven to be difficult because the complex secondary structures of the tRNAs is interfering with the synthesis process. Subsequent functions that we may attempt to modularize include the tRNA synthetases and the glycolytic enzymes.

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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.

Due: Month 6; Status - complete

Task 2: Delete up to 27 large gene clusters

We have reduced the genome size of *M. mycoides* JCVI-syn1.0 from 1079 kb to 779 kb through the deletion of some 30 clusters, representing a ~30% reduction. We are moving forward with an 848 kb genome because it grows at a higher rate than the smaller 779 kb version.

Due: Month 12; Status – complete

Task 3: Construct a preliminary modular map of the genome

The design of a modular map of the genome is complete and is presented in this report

Due: Month 12; Status – complete

Planned Activities for the Next Reporting Period

1. Continue the Top Down minimization of our synthetic genome (near term) and conduct a new transposon study on the minimized genome (within approximately 4-6 months)
2. We will complete synthesis and test the 683 kb Conservative Genome as part of our Bottom Up strategy.
3. Continue development of genome complementation strategies
 - a. Plasmid based system capable of propagation in both donor and recipient cells
 - b. Possibly develop a landing pad strategy for insertion of genes directly into yeast cloned mycoplasma genomes

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	\$708,902	86%	N/A	\$826,256	N/A
Task 3	\$43,487	\$43,487	100%	\$43,487	\$43,487	N/A
Cumulative	\$1,175,389	\$1,058,035	90%	N/A	\$1,175,389	N/A

There is no management reserve or unallocated resources. The financial data presented is current through March 31, 2013.

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.