Dear Dr. Glass,

We have completed a natural selection experiment with *M. mycoides* JCVI-syn1.0 and *M. mycoides* JCVI-syn3B. We evolved 6 replicates of each strain for ~2000 generations on SP4 medium. Although we observed the syn3B ancestor to have about 50% decreased fitness compared to the syn1.0 ancestor, we found that the evolved syn3B lines recovered much of that fitness deficit, ending at around 80% fitness. The fitness of the syn1.0 lines increased by about the same absolute amount, ending at around 130% fitness.

Next, we will analyze pooled population genomic sequence data from the evolved populations to investigate the genetic pathways that underly the evolved increases in fitness. As we begin with this, we wondered about which reference genomes to use.

For JCVI-syn1.0, it appears that there is an NCBI reference genome for exactly the strain that was sent to us ([link](https://www.ncbi.nlm.nih.gov/nuccore/CP002027)), synthesized by Gibson et al. 2010. Is this the correct strain that was sent to us?

For JCVI-syn3B, we have not found a sequence on NCBI, although we do see JCVI-syn3.0 from Hutchison et al. 2016 ([link](https://www.ncbi.nlm.nih.gov/nuccore/CP014940.1)) and JCVI-syn3A directly submitted by Dr. Glass ([link](https://www.ncbi.nlm.nih.gov/nuccore/CP016816.2)). Is there a syn3B reference genome sequence that you could provide to us, corresponding to the *M. mycoides* JCVI-syn3B strain that your team sent to us? In addition, is there a syn3B genome annotation you could provide?

Sincerely,

Dear Dr. Glass,

Thank you for explaining the process to generate constructs. Jay and I have discussed which constructs we think would be most efficient for making new inferences. In short, there are 6 constructs that would be very interesting to test. There are also 4 additional constructs we could perhaps use down the line if all goes well.

First, we would like to recreate, in the ancestral background, 6 mutations (3 in JCVI-syn1.0, and 3 in JCVI-syn3B) which occurred during our natural selection experiment and which believe are adaptive. The 6 mutations are:

1. Syn1.0 ftsZ E315\* (GAA-->TAA)

2. Syn1.0 MMSYN1\_0641 P183L (CCT-->CTT)

3. Syn1.0 lpdA E557G (GAA-->GGA)

4. Syn3B ftsZ E315\* (GAA-->TAA)

5. Syn3B pyrG P48L or D47Y or G297D (CCA-->CTA or GAT-->TAT or GGT-->GAT) (Each of these mutations occurred in one line; whichever mutation is easiest to construct will work!)

6. Syn3B atpD G155R (GGA-->AGA)

We would then use our competition assay to measure the fitness of each constructed strain. With these 6 mutations, we would be able to verify that 3 of the putatively adaptive, frequently observed mutations are truly adaptive.

If all goes well with this verification, perhaps later on it would be interesting to test the fitness effect of each mutation in the other strain background as well. For example, we observed mutation 2 and mutation 3 in adapting JCVI-syn1.0 populations, but never in adapting JCVI-syn3B populations. Perhaps this is simply because the mutation never occurred for syn3B populations. But a more interesting explanation could be that the mutation, while beneficial in a syn1.0 background, is not beneficial in a syn3B background. In other words, there may be epistatic effects of genome minimization. And it would likewise be interesting to test mutations 5 and 6 in a JCVI-syn1.0 background.

Please let us know if these 6 constructs sound doable and if there is any additional information you would need. We are also happy to set up a call to discuss first if that would be useful.

We look forward to continue working with you!

Very respectfully,

Roy

Dear John,

Thank you for the information. We can certainly run our experiments with a small number of different mutants. At this point we are thinking perhaps 6 in total, 3 engineered in JCVI-syn1.0 and 3 engineered in JCVI-syn3B.

One thing to note is that the mutations we observe during the evolution and which we would like to re-engineer are not typically whole-gene disruptions or deletions. The 6 mutations are each a single SNP. Would it be possible to engineer single-nucleotide mutations? Would the labor burden also change?

If SNPs are not possible, we would still be interested in a 0522 knockout mutant in both JCVI-syn1.0 and JCVI-syn3B (this gene is one of the non-essentials removed from syn3.0 but added back to syn3A and syn3B).

And then we could either offer to set up a call if any of those things sound good to him, or we could leave that up in the air until we hear more about the SNP engineering.