Biology Paper Review

Creating Bacterial Strains from Genomes That Have Been Cloned and Engineered in Yeast

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Overview

The goal of this paper is to showcase research regarding the cloning of a bacterial genome in yeast. The researchers cloned the genome of *Mycoplasma mycoides* in yeast and then transplanted it into the species *M. capricolum*, another species of bacteria, to produce an *M. mycoides* cell. The benefit of cloning the genome in yeast is that modification of the genome can be made using yeast-specific machinery, and thus modifications that cannot occur using bacterial-machinery alone are now possible (Lartigue *et al.*, 2009).

Presentation of the Data

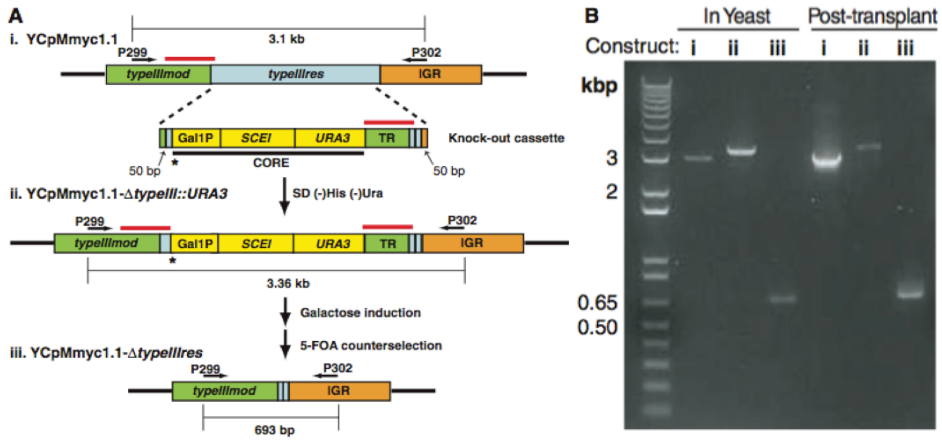

Figure 1. Generation of Type III restriction enzyme deletions (Lartigue *et al.*, 2009).

Panel A shows how, after being inserted into yeast, the Type III restriction enzyme is deleted from the *M. mycoides* genome (YCpMmyc1.1). Section *i.* shows the normal *M. mycoides* genome at the Type III restriction enzyme locus. Below this is a knockout cassette made up of a CORE, which itself contains the Gal1 promoter, an endonuclease called SCEI, and URA3 which marks the insertion of the cassette, and a tandem repeat (TR) sequence. Using homologus recombination, this sequence replaces the *typeIIIres* gene, with the result shown in section *ii*. Genomes that were homologously recombined were selected for by being grown on (-)His and (-)Ura medium. Section *iii.* shows the genome after deletion of the cassette. This occurs when the Gal1 promoter is induced, which leads to endonuclease (SCEI) production, and cleaving at the site marked by * in figure *ii*. Homologous recombination occurs again at the TR sequence marked by red lines in section *ii.*, and genomes with the presence of URA3 are selected against using 5-FOA.

Panel B is a gel electrophoresis of genomes between PCR primers P299 and P302 (black arrows in Panel A*ii.* and A*iii.*) with and without the knockout cassette. Construct *i* is the normal YCpMmyc1.1 clone in yeast. Constructs *ii* and *iii* are clones with and without the knockout cassette respectively. Lane 1 shows a molecular weight marker. Lanes 2 and 5 show construct *i* at a molecular weight of

about 3-kb. Lanes 3 and 6 show construct *ii*, with a molecular weight similar, but a bit greater than construct *i*. Lanes 4 and 7 show construct *iii*, with a molecular weight of about 650-bp. This makes sense since the construct without the cassette should be the smallest. Lanes 2-4 show the constructs in yeast and lanes 5-7 show the constructs post-transplant. This gel shows that the constructs are the proper molecular weights and remain these weights in yeast and after they are transplanted into *M. capricolum*.

Yeast strain	Genome	Methylation treatment	Number of transplants (colonies or plugs)	
			M. capricolum RE(–)	Wild-type *M. capricolum*
VL6-48N	YCpMmyc1.1	Untreated	37 ± 3	0
		M. capricolum extracts	32 ± 13	9 ± 4
		M. mycoides extracts	15 ± 8	22 ± 8 [13 ± 4]* [10 ± 4]†
		Mock-methylated	34 ± 17	0
		M. mycoides purified methylases	20 ± 17	13 ± 10
W303a	YCpMmyc1.1	Untreated	22 ± 5	Not done
	YCpMmyc1.1-*ΔtypeIIIres::URA3*	Untreated	52 ± 10	Not done
	YCpMmyc1.1-*ΔtypeIIIres*	Untreated	52 ± 12	Not done
	YCpMmyc1.1-Δ500kb	Untreated	0	Not done

*Yeast plugs were cleared of yeast genomic DNA by digestion with a cocktail of Asi SI, Rsr II, and Fse I followed by pulsed-field gel electrophoresis. †Yeast plugs were cleared of yeast genomic DNA by using pulsed-field gel electrophoresis.

Table 1. Transplantation of *M. mycoides* YCp genomes from yeast into wild-type and RE(-) *M. capricolum* recipient cells (Lartigue *et al.*, 2009).

This table shows the number of effective transplants of *M. mycoides* into *M. capricolum*. The researchers first tested *M. mycoides* that had been manipulated in yeast strain VL6-48N. The genome used in this yeast strain was the normal YCpMmyc1.1 genome – the Type III restriction enzyme site had not been deleted. The genome was either untreated, methylated with *M. capricolum* extracts, methylated with *M. mycoides* extracts, mock-methylated or methylated with *M. mycoides* purified methylases. Methylation was done to protect the donor DNA. Each of the treatments was inserted into both *M. capricolum* whose restriction enzyme had been deactivated, and wild-type *M. capricolum*.

The first half of the chart shows that *M. capricolum* whose restriction enzyme had been deactivated was more likely to take up *M. mycoides* successfully. It also shows that mock-methylation does nothing to prevent the wild-type *M. capricolum* from chewing up *M. mycoides* genomic DNA. Overall, methylation protected some transplants, but deactivating *M. capricolum*'s restriction enzyme was the best way to ensure uptake of *M. mycoides* DNA.

In the bottom half of the table, the researchers tested *M. mycoides* that had been manipulated in yeast strain W303a. They tested three different manipulations in RE(-) *M. capricolum*, using the original YCpMmyc1.1 as a control. The three manipulated genomes were YCpMmyc1.1-Δ*typeIIIres::URA3* (the original with knockout cassette, as seen in Fig 1A*ii*.), YCpMmyc1.1-Δ*typeIIIres* (the original without Type III restriction enzyme or knockout cassette, as seen in Fig 1A*iii*.), and YCpMmyc1.1-Δ500kb (the original with a 500 kb deletion which lacks many essential genes but retains the YCp element and *tetM*). The control had about half as many successful transplants as YCpMmyc1.1-Δ*typeIIIres::URA3* and YCpMmyc1.1-Δ*typeIIIres*, while the 500 kb deletion product had no successful transplants, which is expected with the loss of essential genes. The data is consistent with the fact that loss of restriction enzyme functions in the donor and the receiver leads to greater success in transplantation.

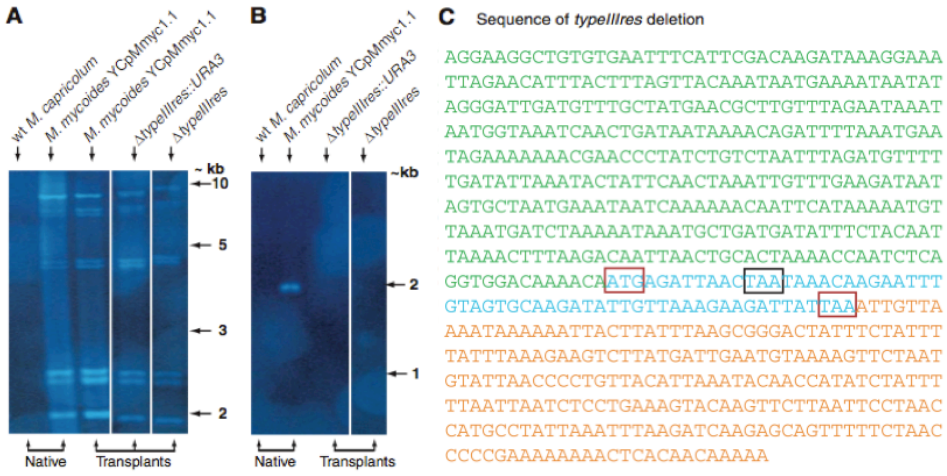

Figure 2. Southern blot analysis of *M. mycoides* transplants (Lartigue *et al.*, 2009).

Panel A shows that every transplant product, when digested with HindIII restriction enzyme, bears the same IS1296 pattern as the original, untransplanted *M. mycoides* YCpMmyc1.1 genome. The researchers digested wild-type *M. capricolum* (as a negative control), untransplanted *M. mycoides* YCpMmyc1.1 (as a positive control), and transplanted YCpMmyc1.1 (Fig1A*i.*), *M. mycoides* YCpMmyc1.1-Δ*typeIIIres::URA3* (Fig1A*ii.*) and YCpMmyc1.1-Δ*typeIIIres* (Fig1A*iii.*). They then probed everything with IS1296 and each experimental matched the positive control in banding pattern. This shows that each transplant product maintained some level stability in its new environment.

Panel B is a control showing the presence of absence of the Type III restriction enzyme gene. The researchers probed genomic DNA from wild-type *M. capricolum*, *M. mycoides* YCpMmyc1.1 (Fig1A*i.*), *M. mycoides* YCpMmyc1.1-Δ*typeIIIres::URA3* (Fig1A*ii.*) and YCpMmyc1.1-Δ*typeIIIres* (Fig1A*iii.*) with the *typeIIIres* gene sequence. They found that only the original YCpMmyc1.1 genome contained the Type III restriction enzyme, which is expected since the two deletion products had it removed and the wild-type *M. capricolum* does not contain the gene. This shows that the two transplant products maintained stability in *M. capricolum*.

Panel C is a sequence of the YCpMmyc1.1-Δ*typeIIIres M. mycoides* genome transplant (Fig1A*iii.*). The sequencing was done to verify that the Type III restriction enzyme gene had been deleted. The start and stop codons for this gene remain however, and these are boxed in red. The black box surrounds the stop codon of the *typeIIImod* gene. The text color is the same as that used in Fig 1A.

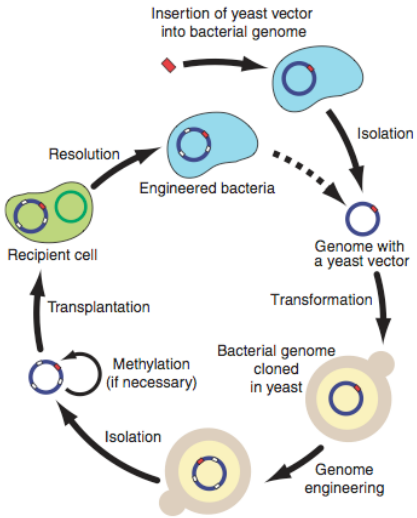

Figure 3. Moving a bacterial genome into yeast, engineering it, and installing it back into a bacterium by genome transplantation (Lartigue *et al.*, 2009).

This figure shows the steps researchers use to reboot a bacterial genome. First a yeast vector is transformed into a bacterial genome (YCp into *M. mycoides* in our case), which in turn is cloned into yeast. This allows the bacterial genome to be modified using yeast genetic machinery, such as a *typeIIIres* deletion. The genome is then transplanted into a recipient cell, in our case *M. capricolum*, which takes on the identity of the donated genome, or *M. mycoides*.

Critique of the Data

Lartigue *et al.* presents an excellent case for genome rebooting. They successfully demonstrate the steps shown in figure 3 by transplanting *M. mycoides* into *M. capricolum*. The researchers first show the three constructs that they create in yeast and transplant into *M. capricolum* (Fig 1A). They successfully demonstrate that the pre- and post-transplant molecular weights of each construct are the same (Fig 1B). However, the gel representing this lacks both a negative control and a loading control. The loading control would be especially helpful to perhaps explain the reason why the band in lane 4 appears so much brighter than the others.

The researchers go on to report the number of successful transplants of the constructs in yeast strain VL6-48N based on methylation and whether or not *M. capricolum* restriction enzyme is active or not (Table 1). This yields impressive results, showing that many colonies grow successfully when *M. capricolum* restriction enzyme is inactive. They also tested transplantation of the varying constructs in yeast strain W303a, using both a positive and negative control, which yielded similar results (Table 1). Overall, they showed that deactivating, or removing completely, the restriction enzymes from the donor DNA or the receiving organism, leads to an increase in colony growth.

The only complaint with table 1 is that it is just that, a table. I would prefer to see actual data, such as Petri plates with colonies of growth. This would strengthen the data in the table, rather than force the reader to assume the data is legitimate.

After reporting this data, the researchers compare the IS1296 pattern of an untransplanted *M. mycoides* genome with IS1296 patterns of the three constructs, and they appear more or less identical (Fig 2A). This is strong evidence that *M. capricolum* has taken up the constructs effectively, and is presented well, with the wild-type *M. capricolum* as a negative control. A loading control could strengthen this figure however, and may help to explain why the YCpMmyc1.1-Δ*typeIIIres* band seems much less apparent than the rest.

The researchers go on to show that the *typeIIIres* gene is present only in the untransplanted YCpMmyc1.1 (Fig 2B). They compare this to only two of the three constructs transplanted into *M. capricolum*. I feel they should show that the *typeIIIres* gene is also present in transplanted YCpMmyc1.1. Another missing element is a loading control. In this figure, the lack of a loading control seriously impacts the effectiveness of the figure. While a band exists in lane 2, it is uncertain whether the amount of DNA loaded is the reason why there are no other bands.

The final figure, which shows the method is an excellent summery of the way the researchers manipulated the bacterial genome. Though this is minor, I feel that this figure should be toward the beginning of the paper. It gives a great overview of the method and would be more helpful as the first figure than the last one. The only major complaint is with the final step in the figure, labeled "resolution." I am unsure how the recipient cell's original plasmid, shown before "resolution," disappears after "resolution." The method is stated in neither the figure legend, nor in the paper.

Future Work in Genome Rebooting

Though it may seem obvious, I feel that re-running several gels using the controls I mentioned in the "critique" section would strengthen this paper. I also feel that the paper could benefit from a figure showing the colonies referenced in table 1, and greater explanation of the "resolution" step in figure 3. In fact, there could be an entire paper written about the resolution step. What happens if the original plasmid is not deleted? Is there interference between the genomes? Whose genes are expressed and whose are silenced?

There is much else that can be done to further the research on genome rebooting. Early in the paper, the authors mention "the complete chemical synthesis of the 580-kb *M. genitalium* genome"

(Lartigue *et al.*, 2009). Though the authors choose *M. mycoides*, it would be an interesting experiment to reboot the synthetic *M. genitalium* genome in another bacterium like *M. capricolum*. Success with a completely synthetic organism would be a major step using genome rebooting.

In figure 3, it is quickly mentioned: "after cloning, the repertoire of yeast genetic methods is used to create insertions, deletions, rearrangements, or any combination of modifications in the bacterial genome" (Lartigue *et al.*, 2009). However, this paper only experiments with deletion. Future work in this area should stretch the yeast genetic machinery to its breaking point, experimenting with various modifications. An insertion would be as easy as using homologous recombination, as done in this paper. Instead of inserting a construct that cuts itself out, one could use any desired gene. The researcher's favorite gene (RFG) would need to homologously recombine with a non-essential gene for the construct to work correctly. The RFG construct would need a promoter region as well as a marker that could be selected for, much like the researchers in the current paper used URA3. This is but one example of the many things yeast genetic machinery can accomplish.

References

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