Use of Crispr Cas9 in Improving Transformation Integrity in *Methanosarcina acetivorans*

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Abstract

A principal hurdle with which many biologists have struggled is the ability to alter the genetics of the organisms they study. Years have been spent studying a handful of model organisms for their ease of use, availability, and developed tools which have allowed scientists to alter their genetics. The discovery of CRISPR, Clustered Regularly Interspaced Short Palindromic Repeats, fundamentally broadened the previously limited scope by which researchers could study their respective lab organisms. CRISPR-Cas9 is a protein found in *Streptococcus pyogenes*, a sometimes pathogenic bacterium found on human skin. Cas9, a dual RNA-guided DNA endonuclease enzyme, functions as an adaptive viral defense mechanism for microorganisms. Cas9 utilizes guide RNAs (gRNA) which recognize the DNA sequences of non-native DNA to cleave the nucleotide sequence and protect the organism from infection. CRISPR-Cas9 is currently being used to transform the genomes of a wide variety of organisms due to its wide availability, precision of DNA cuts, and ease of use.

The Buan lab studies methanogens, methane-producing archaea found in landfills, ocean sediment, and guts around the world. These organisms have been difficult to study due to their sensitivity to oxygen and their high genome copy numbers, possessing up to 50 copies of their genome in one cell. Buan lab has several strains of *Methanosarcina acetivorans* (Figure 1) which have been transformed to possess a recombination site (attB/P) on all chromosomes. These replacements were achieved by inactivating a native hypoxanthine phosphoribosyltransferase (hpt) gene which activates toxic purine analogs such as 8-aza-2,6-diaminopurine (8ADP) that cause termination when incorporated by DNA polymerase. By replacing the hpt gene with attP or attB and selecting for 8ADP resistance, fully transformed strains can be obtained (Figure 2). However, using these attachment sites to integrate novel genes along with puromycin resistance has had shortcomings. Complete integration of a plasmid across all chromosomes is less efficient and as a result integrated plasmids can be lost over time. Our lab is investigating whether Cas9 can be used to target untransformed chromosomes such that strains maintain the integrated plasmid in all chromosomes. Expanding the Cas9 genetic toolbox for methanogens will be helpful in future studies of the metabolism and genetics of these organisms.

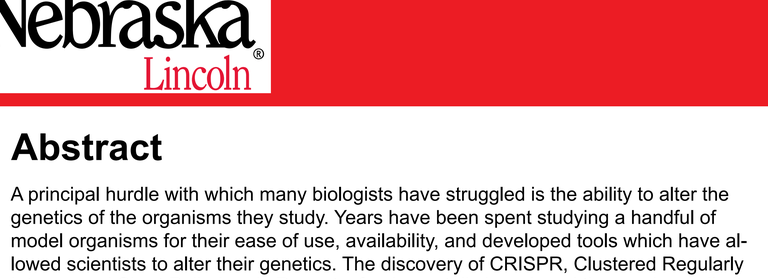

Figure 1. Within samples taken from the ocean sediment near La Jolla, California (left) the methanogenic archaea *Methanosarcina acetivorans* (right) was discovered.

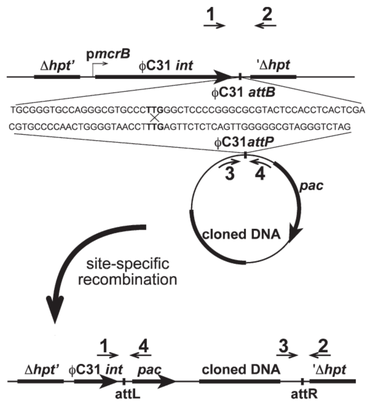

Figure 2. A recombination site has been inserted into the genome of our lab strain methanogens. Site-specific recombination is the method by which genes are inserted into the genome of methanogens.

Protein Purification of Cas9 Protein

A plasmid encoding for the expression of Cas9-6xHis was obtained from AddGene (Figure 4) and transformed into a BL21-derivative strain of *Escherichia coli*. The protein purification proceeds as follows:

1) The culture is induced using 0.1 mM IPTG and incubated at 20°C and 215 rpm for 16 hours

2) The sonicated lysate is applied to a 5 mL FastFlow His-Trap column and separated using an HPLC

3) Cas9 is eluted using an increasing concentration of imidazole with fractions collected throughout this concentration gradient

4) Samples from these fractions are separated using SDS-PAGE in a 7.5% gel at 200V for 40 minutes and stained (Figure 3)

5) Fractions 18-22 are pooled together in a 100kDa MWCO Protein Concentrator and buffer exchanged using Dialysis Buffer

6) Protein concentrate is subjected to a Bradford assay in order to determine the Cas9 concentration

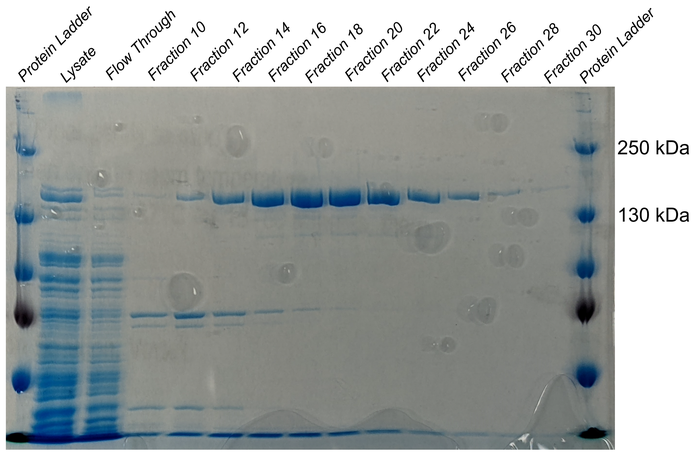

Figure 3. An SDS-PAGE gel showing the separation of Cas9 from cell lysate. The size of Cas9 is approximately 159 kDa; in the gel one can see an abundance of Cas9 between the bands representing 130 kDa and 250 kDa.

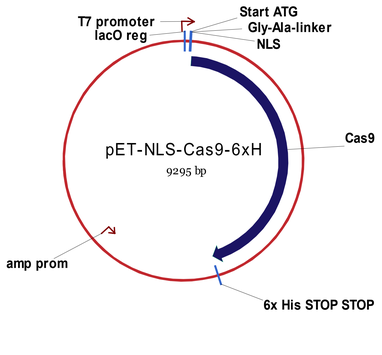

Figure 4. A plasmid map of the pET vector containing Cas9 under the the control of the IPTG inducable lacO regulon.

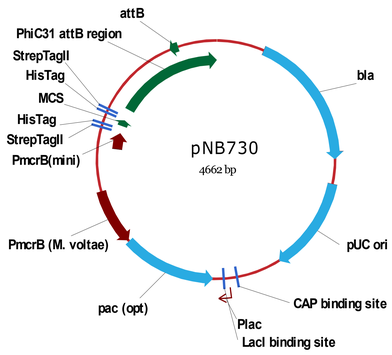

Figure 5. A plasmid map of the a vector used to transform Methanogens with an attP site in their genome.

References

Figure 1 from Guss, A. M., et al. (2008). New methods for tightly regulated gene expression and highly efficient chromosomal integration of cloned genes for Methanosarcina species. Archaea, 2(3), 193-203.
Figures 6 and 8 created with BioRender.com

Using Cas9 to Transform Methanogens

To transform methanogens, DOTAP encapsulates plasmids in micelles which when applied to a culture of *Methanosarcina acetivorans* fuse with the membrane, depositing the plasmid into the cytoplasm. The same approach may apply to transformation using Cas9. The double stranded breaks introduced by the Cas9 on untransformed chromosomes should lead to its degradation by the cell (Figure 6).

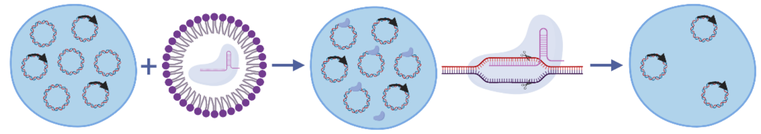

Figure 6. Cas9 with sgRNA encapsulated in DOTAP micelles fuse with *M. acetivorans* cells depositing the Cas9 in the cytoplasm where it will induce double stranded breaks in all untransfromed genomes (no black arrow) of the cell resulting an a cell with only transformed genomes (black arrow present).

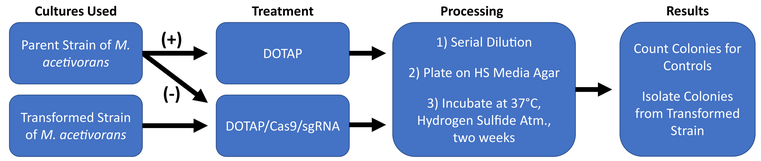

Figure 7. The workflow for assessing the efficacy of a Cas9 Transformation. Positive (+) and negative (-) controls utilize an untransformed strain of M. acetivorans and the colony counts on plates can be compared to assess efficacy. The previously transformed strain of M. acetivorans can also be used to determine how many copies of the untransformed genome remain.

Following the workflow in Figure 7, we expect to see fewer colonies on the negative control plates in comparison with the positive control plates. Colonies from the previously transformed strain will be cultivated and their genomic DNA will be analyzed using a MinION and the copy numbers from a housekeeping gene will be compared to the inserted gene. Higher copy numbers are expected for the inserted gene post-Cas9 transformation compared to pre-Cas9 transformation.

In order to target the attB or attP site, gRNA was ordered from Integrated DNA Technologies with those sequences (Figure 2). Tests of the efficacy of Cas9 to induce double stranded breaks on pNB730 (Figure 5) will be conducted according to the procedure below with expected results in Figure 8.

1) Assemble reactions of Cas9, sgRNA, and pNB730
2) Incubate at 37°C for extended periods of time
3) Release Cas9 from DNA using 1% SDS
4) Aliquot into qPRC reaction using a primer with homology for attB

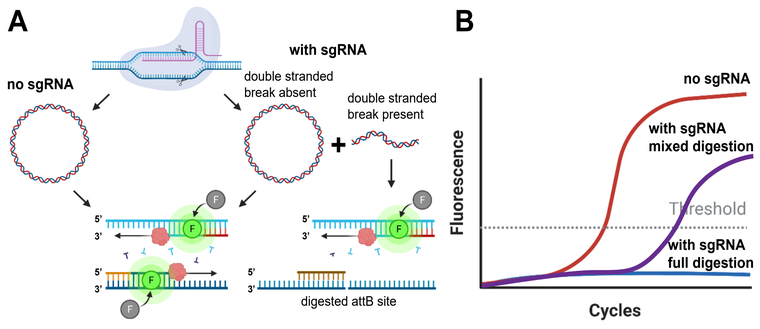

Figure 8. A) Without sgRNA, no linearization is expected of the plasmid however with sgRNA there will be a mixed population of linarized and circular plasmids. **B)** Using a primer slightly upstream of the attB cut site PCR should amplify the region containing attB of circular plasmids (red) however the length of transcripts from PCR should be reduced by the introduction of a double stranded break at the attB site resulting in diminished fluroescence (purple and blue).

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