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

Connor Hines, Laura Kirshenbaum, Sean Carr, Nicole R. Buan

Department of Biochemistry, University of Nebraska-Lincoln

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. The 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 possess 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.

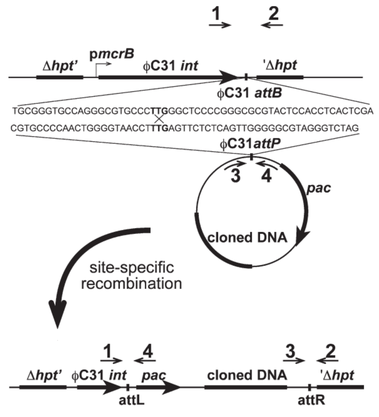

Figure 2. A recombination site has been inserted into the genome of our lab strain methanogens. Site-specific recombination is the method by which genes are inserted into the genome of methanogens.

gRNA Design

Two guide RNAs were designed for the purpose of directing Cas9 to the attP site on the untransformed *M. acetivorans* genome. After inducing a doublestranded break at the integration site there are several possibilities which could occur:

1. Homologous Recombination does not occur and cut genomes are degraded over time.
2. Homologous Recombination utilizes the transformed genomes as templates, carrying the inserted gene onto the cut genomes.
3. Non-Homologous Endjoining repairs the genome, insertions or deletions at the cut site can prevent repeated Cas9 interactions.

Outcomes 1 & 2 would be ideal as the resulting transformants would have full integration of the plasmid in all genomes. Outcome 3 would present additional challenges for progression of the project.

The two gRNAs designed to direct Cas9 to the attP site were generated after sequencing the area of the genome where the attP site is located. The site CHOPCHOP was used to analyze the sequence for protospacer adjacent motif (PAM) sites and return the sequences which possessed the highest likelihood of being cleaved by Cas9. From these sequences two were chosen and ordered from ThermoFisher.

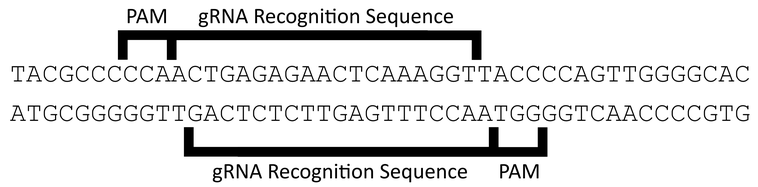

Figure 3. The DNA sequence from a genomic extract of our *M. acetivorans* showing the attP integration site and the areas which were predicted by CHOPCHOP to have high cutting efficacy with Cas9.

qPCR to assess Cas9 Cleavage Efficacy

Before using Cas9 to transform *M. acetivorans* we wanted to make sure the gRNAs and Cas9 would introduce double stranded breaks in the sequences they were designed to cleave. A protocol to assess Cas9 activity was created using an Opentrons robot to assemble the reactions in a 96-well plate format. Using SYBR Green, the qPCR was designed to amplify the cut regions using primers located 100bp upstream of the cut site.

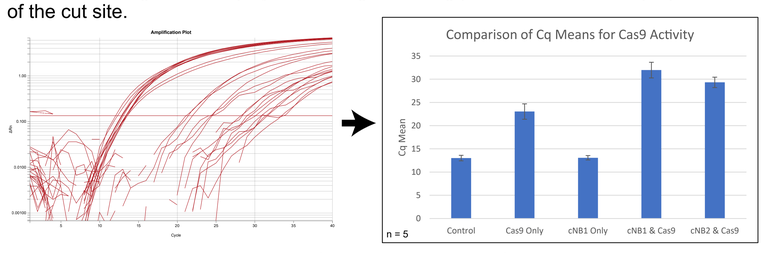

Figure 4. The qPCR returns data showing fluorescence at the end of each cycle. The cycle at which the fluorescence exceeds the set threshold, 0.133, is the Cq. These averages can be compared on a bar graph to display how long each sample took to reach 0.133. The samples containing both the gRNA and Cas9 took significantly longer to reach the Cq in comparison to the controls.

References

Figure 2 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.

This work was supported by the Nebraska Public Power District through the Nebraska Center for Energy Sciences Research at the University of Nebraska-Lincoln.

Homology Directed Repair Approach

Using the endogenous mechanisms of Homology-Directed Repair (HDR) a gene could be inserted into the *M. acetivorans* genome using Cas9. A plasmid with the gene of interest flanked by two areas with homology to the area on the genome being targeted by Cas9 could potentially disrupt an endogenous gene with the gene of interest. After investigating various metabolic pathways the gene MA1966, asparagine synthetase, was chosen for the potential of creating an L-asparagine auxotroph. The front of MA1966 was analyzed using CHOPCHOP for potential cut sites and our chosen site would introduce a double stranded break within the start codon of MA1966 potentially eliminating the need for a promoter on the plasmid. The gRNA was included on the plasmid under the control of a promoter which would allow for expression within *M. acetivorans*. Numerous Restriction Sites were included to confer a high degree of modularity to the plasmids.

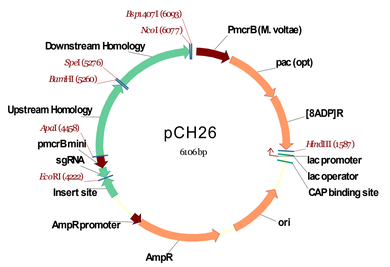

Figure 5. Plasmid map of pCH26 which contains a gRNA for MA1966 and Upstream and Downstream Homologies for the areas flanking the cut site.

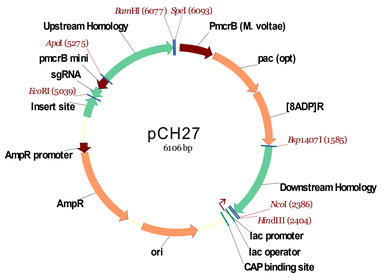

Figure 6. Plasmid map of pCH27 which is similar to pCH26 however the inserted sequence includes both puromycin and 8ADP resistance cassettes.

Using Cas9 to Transform Methanogens

To transform methanogens, DOTAP encapsulates plasmids and proteins in micelles which when applied to a culture of *M. acetivorans* fuse with the membrane, depositing the plasmid and/or protein into the cytoplasm. Using DOTAP we can either transfer gRNA loaded Cas9 alone or unloaded Cas9 together with a gRNA expressing plasmid (Figures 5 and 6). The double stranded breaks introduced by the Cas9 on untransformed chromosomes should lead to its degradation by the cell (Figure 6). These two approaches have respective advantages and drawbacks the top path of Figure 7 requires two transformations in order to achieve an isolate with complete plasmid integration however unlike the bottom path of Figure 7 it is not dependent on HDR or L-asparagine supplementation.

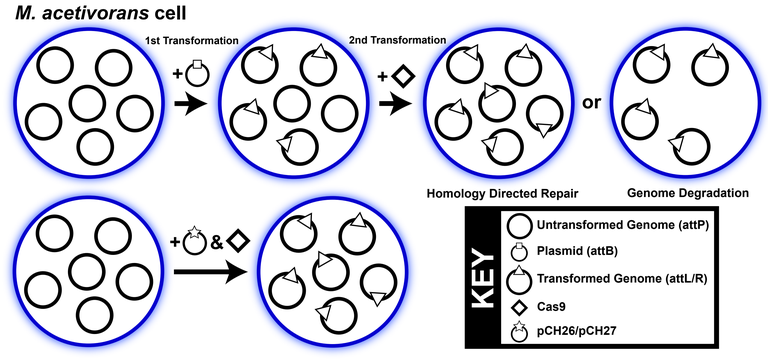

Figure 7. A simplified diagram of our two approaches to Cas9 mediated transformation of *M. acetivorans*. The top lane involves an initial transformation with a attB containing plasmid which will integrate onto the genome followed by a subsequent transformation with gRNA loaded Cas9 in order to achieve full integrants through Homologous Recombination or genome degradation. The bottom lane combines the plasmid and Cas9 in a single transformation in order to achieve full integrants through HDR.