Investigating the Inhibitory Range of Type II-A Cas9 Inhibitors
Blake D. McMahon¹, Kyle E. Watters², Jennifer A. Doudna³,4,5,6
1Diablo Valley College, 2U.C. Berkeley Department of Molecular and Cell Biology, U.C. Berkeley Department of Chemistry,
3Howard Hughes Medical Institute, 4Lawrence Berkeley National Laboratory, 5Innovative Genomics Initiative

Abstract
The field of genome engineering was revolutionized by the discovery of CRISPR (clustered regularly interspaced short palindromic repeats) systems, adaptive immune systems found in bacteria and archaea to defend against phage infection. CRISPR systems cleave foreign DNA or RNA using a CRISPR associated (Cas) protein guided by a RNA strand called gRNA, which allows the system to target highly specific sequences. The most commonly used Cas protein, Cas9, is now being used as a genome editing tool in a variety of organisms. Specifically, the Cas9 homolog from the Type II-A CRISPR system in Streptococcus pyogenes is most frequently used. Recently, four anti-Cas9 proteins from bacteriophages were discovered to inhibit the CRISPR system in Listeria monocytogenes [1], two of which were shown to inhibit the Cas9 from S. pyogenes. However, little is known about the diversity of Cas9 proteins that these anti-CRISPR proteins can inhibit. Therefore, we tested the inhibition range of the known Type II-A anti-CRISPRs with multiple Cas9 homologs using a cleavage assay. By investigating the ability of these anti-CRISPRs to inhibit a variety of Type II-A Cas9 homologs, this work will set the stage for future efforts to develop broadly inhibiting Cas9 anti-CRISPR proteins.

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Contact Information
Blake McMahon - Email: blakemcmahon1ne@me.com - Phone: (925) 719-6498

References

Type II-A anti-CRISPR Homologs

Homology tree showing the relative distance of Cas9 homologs from L. monocytogenes Cas9.

Homologs were chosen by selecting close and distant evolutionary relatives to the Cas9 L. monocytogenes. This distance was determined based on the similarity between the Cas9 coding sequences.

Type II-A Cas9 CRISPR Systems

Bacteria and archaea possess adaptive immune systems that allow cells to defend against invasive genetic material. CRISPR loci are genomic sequences which store previously detected viral DNA elements, and which code for Cas (CRISPR associated) proteins [2]. Type II-A CRISPR systems work in three stages. In the first stage, viral DNA is cleaved by the proteins Cas1 and Cas2. The resulting viral DNA fragment is inserted into the CRISPR locus in the cell’s genome. Transcription of the CRISPR locus produces pre-cRNA. In the second stage, pre-cRNA is processed into crRNA which functions as a guide for the Cas9 protein. In the third stage, crRNA binds to Cas9, creating an RNA-guided complex that targets and destroys invading viral DNA.

The activity of the Cas9 protein can be harnessed for genome engineering by generating a single stranded guide RNA (sgRNA) that directs the Cas9 protein to cleave areas of interest in a host’s genome [3].

Objectives
- Purify homologs of the recently discovered Cas9 anti-CRISPRs AcrIa2 and AcrIa4
- Test ability of anti-CRISPR proteins to inhibit DNA cleavage in vitro by Type II-A Cas9 homologs
- Determine what the most distant relative to the Listeria monocytogenes Cas9 each anti-CRISPR homolog can inhibit and what relative concentrations are required

In Vitro Cleavage Assay

Two Cas9 homologs from Listeria innocua (Lin) and Streptococcus pyogenes (Spy) were directed to cleave a target strand of DNA while in the presence of acrIa1 (anti-CRISPR type II-A protein) 1, 2, and 4. The resulting products were run on an agarose gel for imaging, and band intensity was measured to determine the percentage of DNA cleaved in each assay. SpyCas9 was inhibited by acrIa1 and acrIa4. AcrIa2 has been shown to inhibit SpyCas9, but inconsistencies among the control assays for SpyCas9 prevent us from confirming this conclusion. LinCas9 was inhibited by acrIa2 and acrIa4 at all three concentration ratios. On the contrary, acrIa1 does not appear to inhibit cleavage activity in LinCas9. This result challenges the prevailing hypothesis regarding acrIa1’s mode of inhibition, which postulates that this anti-CRISPR inhibits via nucleic acid binding, rather than protein binding.

Conclusion
- Anti-CRISPR Type II-A4 appears to be the best candidate for a broadly inhibiting Type II-A anti-CRISPR, as it had the greatest effect in vitro
- Anti-CRISPR Type II-A2 effectively inhibited L. innocua Cas9, but needs to be tested on S. pyogenes Cas9
- Anti-CRISPR Type II-A1 did not inhibit L. innocua Cas9, but was able to inhibit target cleavage the more distant S. pyogenes Cas9 homolog

Next Steps
- Purify five more Cas9 homologs
- Lactobacillus gasseri
- Streptococcus thermophilus
- Staphylococcus aureus
- Neisseria meningitidis
- Actinomyces naeslundii
- Purify Type II-A anti-CRISPR homologs
- 2b, 2c, 2d, 4b Found in Listeria monocytogenes
- 3b, found in Listeria virus AS11
- 3c, found in Streptococcus cuniculi
- 4b, found in Streptococcus pyogenes
- Perform in vitro cleavage assays to test cleavage efficacy of Cas9 homologs in the presence anti-CRISPR homologs

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