Investigating the Inhibitory Range of Type II-A Cas9 Inhibitors

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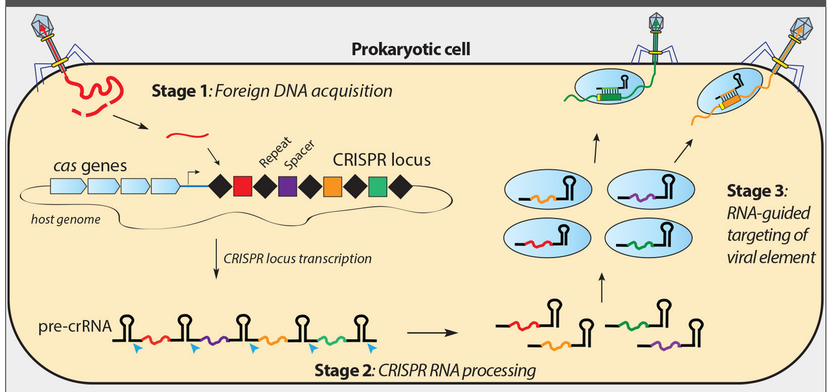
Abstract

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 an 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-CRISPR 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-CRISPRs can inhibit. Therefore, we tested the inhibitory 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.

Type II-A 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-crRNA. In the second stage, pre-crRNA 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 cleaves 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].

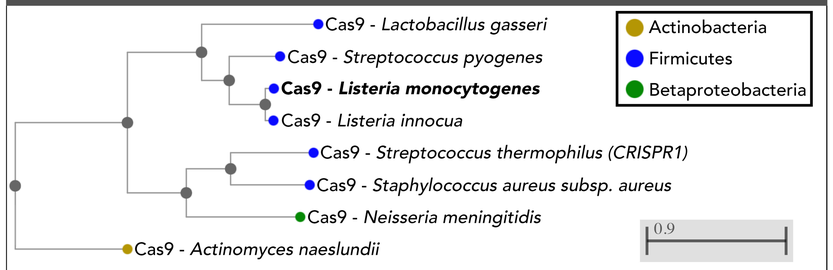
Objectives

- **Purify homologs of the recently discovered Cas9 anti-CRISPRs AcrIIA2 and AcrIIA4**
- **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**

References

1. Rauch, B. J., Silvis, M. R., Hultquist, J. F., Waters, C. S., McGregor, M. J., Krogan, N. J., & Bondy-Denomy, J. Inhibition of CRISPR-Cas9 with Bacteriophage Proteins. *Cell* **168**, 150-158 (2017).
2. Sternberg, S. H., Haurwitz, R. E., & Doudna, J. A. Mechanism of substrate selection by a highly specific CRISPR endoribonuclease. *RNA* **18**, 661-672 (2012).
3. Jinek, M., Chylinski, K., Fonfara, I., Hauer M., Doudna, J. A., & Charpentier, E. A Programmable Dual-RNA–Guided DNA Endonuclease in Adaptive Bacterial Immunity. *Science* **337**, 816-821 (2012).

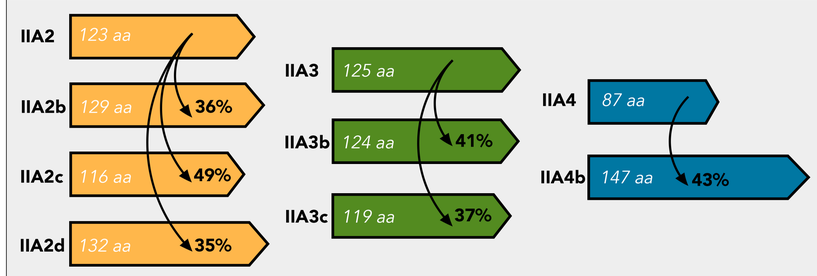
Cas9 Homologs



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 anti-CRISPR Homologs



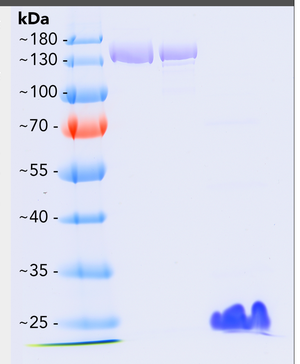
The percent identity (the extent to which protein sequences are related) of anti-CRISPR homologs selected for assay.

Homologs selected for assay were distant relatives to anti-CRISPRs IIA2, IIA3, and IIA4 found in *L. monocytogenes*. Only protein-binding homologs were selected for

Homolog Purification

The Cas9 homologs from *Streptococcus pyogenes* (Spy) and *Listeria innocua* (Lin) were purified. Anti-CRISPR type II-A2 from *Listeria monocytogenes* was the only anti-CRISPR purified. AcrIIA1, and AcrIIA4 were sourced from within the Doudna Lab.

A four-step purification process was used. The proteins were expressed in *E. coli* bound by MBP (maltose binding protein) to a polyhistidine (6x His) sequence tag. Nickel-charged affinity resin was used to separate the protein from the cell lysate. Controlled cleavage of the MBP-His tag was accomplished using TEV protease (Tobacco Etch Virus nuclear-inclusion-a endopeptidase). The protein was run through a second nickel resin column to remove the cleaved MBP-His tag. The Cas9 homologs were bound to heparin, and eluted into fractions via ion exchange. AcrIIA2 was bound to an anion exchange column, and eluted into fractions via ion exchange. Size exclusion filtration was the final purification step. The purified samples were concentrated and flash frozen for storage at -80°C until needed.



Coomassie-stained gel of proteins purified.

From left to right: Protein Ladder, SpyCas9, LinCas9, AcrIIA2

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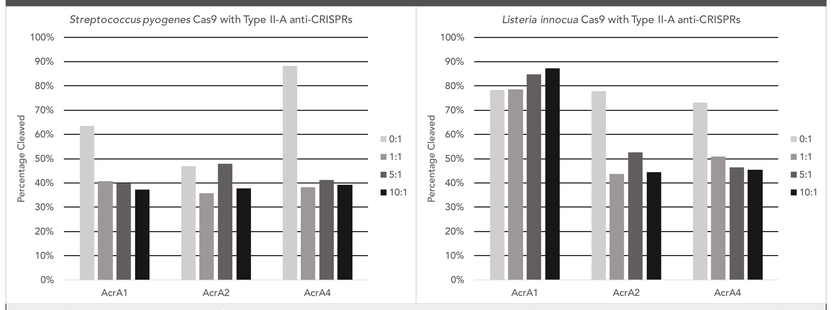
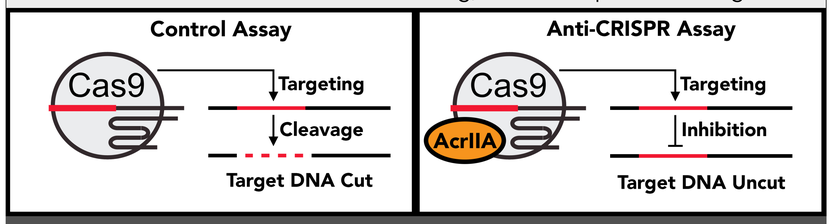
In Vitro Cleavage Assay



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 acrIIA (anti-CRISPR type II-A proteins) 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 acrIIA1, and 4. AcrIIA2 has been shown to inhibit SpyCas9, but inconsistencies among the control assays for SpyCas9 prevent us from confirming this conclusion. LinCas9 was inhibited by acrIIA2 and acrIIA4 at all three concentration ratios. On the contrary, acrIIA1 does not appear to inhibit cleavage activity in LinCas9. This result challenges the prevailing hypothesis regarding acrIIA1'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 retested 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 thermophiles*
  - *Staphylococcus aureus*
  - *Neisseria meningitides*
  - *Actinomyces naeslundii*
- Purify Type II-A anti-CRISPR homologs
  - 2b, 2c, 2d, 4b Found in *Listeria monocytogenes*
  - 3b, found in *Listeria* virus A511
  - 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

Acknowledgments

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