VIROLOGY

How phages defeat CRISPR

As more phage-derived Cas9 inhibitors are discovered, their detailed characterization is a prerequisite to using them as research tools.

In the evolutionary arms race, bacteria found a powerful way to defend against viruses. Their CRISPR–Cas immune system incorporates short strands of invading phage DNA into the bacterial genome and uses those sequences to guide a Cas nuclease to destroy the phage during subsequent infections. But the phages do not stand idly by as their genomes are being cleaved to bits.

As researchers began to discover in 2013, phages acquire genes encoding anti-CRISPR proteins (Acrs) to inactivate the CRISPR system. Phage biologist Sylvain Moineau from the Université Laval in Canada asked why some phages no longer respond to CRISPR. "We studied how phages found a way to bypass the CRISPR system," he said. A team led by Jennifer Doudna from the University of Berkeley pursued a similar question. "We were interested in how viruses fight back," she said, "but also interested in ways one might take advantage of those strategies in viruses for technology purposes."

These independent teams took two very different paths in the pursuit of answers.

Moineau's group pursued a 'phage first' approach. They tested five virulent phages that had been shown to infect industrial *Streptococcus thermophilus*, a strain that expresses a similar type II-A CRISPR–Cas9 system as that of *Streptococcus pyogenes* and is widely used to modify mammalian genomes. Of the five phage strains, one was resistant to CRISPR-based immunity even if a spacer against a conserved region in the phage genome was inserted in the CRISPR locus.

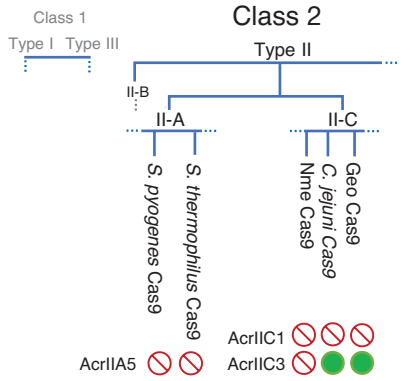
Postdoctoral fellow Alexander Hynes and research assistant Genevieve Rousseau set out to identify the phage gene responsible for blocking CRISPR. "They cloned over 80% of the phage genome before we stumbled on the right gene," recalled Moineau. The 'right gene' turned out to be *acrIIA5*, which encodes a 140-amino-acid protein with a predicted coiled-coil motif. It blocked the nuclease activity of Cas9, including that of Cas9 from *S. pyogenes*.

The team is currently working on understanding the mechanism of AcrIIA5, and many questions about its function and mode of action remain. "What is the consequence for the phage whether they have or do not have such a protein? An obvious one is to fight CRISPR, but are there others?" Moineau asked.

A collaborative effort headed by Doudna focused on exactly this question of mechanism. The teams concentrated on two Acrs that had been cloned from *Neisseria meningitidis*, a bacterial strain expressing type II-C CRISPR–Cas9. AcrIIC1 and AcrIIC3 robustly inhibited Nme Cas9, but their sequences are very different from each other, which led the researchers to believe that the proteins may have distinct molecular mechanisms to inhibit CRISPR.

Lucas Harrington and Kevin Doxzen, former and current graduate students in the Doudna lab, developed biochemical assays to test the specificities of the two Acrs and solved the structure of AcrIIC1 to confirm that the proteins' host selectivity and mechanisms of action were indeed different. AcrIIC3 prevented Cas9 from binding its target DNA and was specific for *N. meningitidis*, while AcrIIC1 inhibited cleavage of DNA bound to several Cas9 orthologues. This hints at an interesting potential role of Acrs in the phage. "Anti-CRISPR inhibitors that are working like C1 could be turning the CRISPR system into a regulatory pathway rather than a destructive pathway," explained Doudna.

Now that some of their mechanistic details are understood, Doudna and her collaborators are looking to develop these proteins as research tools and to evolve or design even broader inhibitors and potentially arrive at a universal Cas9 inhibitor. To further mine bacterial databases for anti-CRISPR proteins, Doudna's team is working on a pipeline to computationally identify and then test anti-CRISPR candidates.



Specific and broad-range anti-CRISPR proteins continue to be discovered for various types of Cas9.

Conceptually, these Cas9 inhibitors could be valuable for research; AcrIIC1, for example, could make the design of dead Cas9, a protein that still binds but not longer cuts its target, obsolete.

There is, however, some skepticism in the research community regarding the practical value of these inhibitors. Doudna shared some of that skepticism, saying, "I also wonder how useful these are going to be. Clearly their activity is valuable, but how to implement them is trickier." Open questions range from how to deliver the inhibitors to how to time and control their activities. Doudna said that an important goal is to understand the fundamental biology of how these inhibitors operate. She concluded, "We hope this understanding will be useful in terms of developing technology."

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RESEARCH PAPERS

Hynes, A.P. *et al.* An anti-CRISPR from a virulent streptococcal phage inhibits *Streptococcus pyogenes* Cas9. *Nat. Microbiol.* http://dx.doi.org/10.1038/s41564-017-0004-7 (2017).

Harrington, L.B. *et al.* A broad-spectrum inhibitor of CRISPR-Cas9. *Cell* **170**, 1224–1223.e15 (2017).