Ten Years of Anti-CRISPR Research

It is a remarkable pleasure for us to present this special issue on anti-CRISPRs, which marks the tenth anniversary of the publication describing their discovery.[1] The fact that "anti-CRISPR" is now a field, with more than 250 publications on the subject, that warrants a whole issue of JMB is amazing to us. One has to be surprised to make such a discovery! In addition, as is the case with so many discoveries, we did not set out with the goal of discovering anti-CRISPRs. Since the actual route to discovery was too convoluted to fully relate in any of our previous publications, we decided to use this opportunity to tell the whole story. We feel that it exemplifies the most fun and exciting part of science: when you start a project, you never know where it will lead.

CRISPR was not on our minds at all when Joe started his Ph.D. work at the University of Toronto. Joe's project was to characterize a large group of temperate phages that he isolated from a collection of *Pseudomonas aeruginosa* (*Pae*) strains. He noticed that when these phages were integrated into their host genomes in the form of prophages, the lysogenic bacteria became highly resistant to many other phages.[2] We became intrigued by the anti-phage immune systems that might be mediating this resistance.

At some time during 2011, we realized that the *Pae* strain with which we were performing our studies (called PA14) encoded a CRISPR-Cas system. Although this was before the start of the CRISPR-Cas9 genome editing revolution,[3] CRISPR was already an exciting enough topic to pique our interest. Fortunately, George O'Toole's lab at Dartmouth University had already done some work on the type I-F CRISPR system in PA14 (known as the *Yersinia* subtype at that time). Surprisingly, they found that this system did not block phage replication even when the infecting phages had exact matches to CRISPR spacers encoded in the PA14 genome.[4] They also detected processed CRISPR RNA molecules, so the system should have been functional. We came up with a hypothesis that some prophages might activate the PA14 CRISPR-Cas system, which could impart phage resistance to lysogens. Joe went to work in Steve Lory's lab at Harvard Medical School during the summer of 2011 to learn how to do RNA sequencing as a means to find out which genes were expressed from our prophages of interest. By happy coincidence, while in Boston, Joe met Kyle Cady, the graduate student who was working on CRISPR in George O'Toole's lab at the Boston Bacterial Meeting. Kyle had generated PA14 CRISPR array and *cas* gene deletion strains, which he kindly provided to Joe so that he could bring them home with him in the fall of 2011.

Once back in Toronto, Joe quickly proved that our idea that prophages were activating the CRISPR-Cas system was wrong. However, he did notice that the presence of the CRISPR-Cas system slightly attenuated the replication of a few phages. This got us to thinking that the CRISPR-Cas system might actually have some anti-phage activity. We then remembered that Joe had isolated at least 30 distinct phages that could not replicate on strain PA14 but replicated well on other *Pae* strains. With the revelatory idea that some of these phages might be blocked by the PA14 CRISPR-Cas system, Joe quickly tested their replication on the mutant PA14 strain lacking its CRISPR arrays. He found three phages that could replicate on the PA14 ΔCRISPR strain, but not on the wild-type strain. This result demonstrated that the CRISPR-Cas system of PA14 did indeed act as an anti-phage system. We immediately called George and Kyle to tell them the exciting news and found that they had just made the same discovery by creating a perfect match between the DMS3 phage and a native spacer, which we wrote up with them in a collaborative paper.[5]

Now that we had a functioning CRISPR-Cas system and phages that were sensitive to it, our attention turned back to prophages. We wondered again whether the presence of prophages might affect CRISPR-Cas activity, but as an inhibitor rather than activator. To address this, Joe tested the replication of three CRISPR-sensitive phages on the 44 different PA14 lysogens that he had already investigated. Stunningly, Joe found three lysogens on which the CRISPR-sensitive phages were able to replicate. This result immediately told us that the prophages in these strains must express an inhibitor of the CRISPR-Cas system. We have often spoken of the exhilaration that we felt staring at the phage-induced zones of clearing on those three plates because we knew

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immediately that it was a big discovery. After this initial experiment, it took less than two months to identify five anti-CRISPR gene families, and seven months later the paper describing this work was submitted.

After discovering the first group of anti-CRISPRs, the next question was how to find more of them. We were able to find four gene families encoding anti-CRISPRs inhibiting the *Pae* type I-E CRISPR-Cas system because they were in the same phage operons as those encoding the original type I-F anti-CRISPRs.[6] However, sequence-based searching with the anti-CRISPR proteins did not reveal any new anti-CRISPR genes outside of a small group *Pae* phages. The key to finding many more anti-CRISPR families was uncovered by April Pawluk in our group who exploited the presence of conserved transcriptional regulators (anti-CRISPR associated, or Aca proteins) associated with anti-CRISPR genes. Using what has become known as the "guilt by association" method, April discovered new anti-CRISPR families by testing proteins encoded adjacent to *aca* genes. This led to the discovery of diverse families of anti-CRISPRs blocking type I-F systems,[7] and ultimately to three families of anti-CRISPRs inhibiting CRISPR-Cas9 systems.[8] Subsequently, the guilt by association approach combined with the identification of self-targeting spacers, led to the discovery of the first *S. pyogenes* Cas9 inhibitors[9] and the first anti-CRISPR blocking Cas12.[10] Since then, more than 90 families of anti-CRISPRs that block 13 different types of CRISPR-Cas systems have been discovered. In this issue, Makarova et al. review the computational approaches that have been used to predict anti-CRISPRs.[11] Since computational procedures may not be capable of finding all anti-CRISPRs, purely experimental approaches are also needed. Forsberg provides a general review of anti-CRISPR discovery including approaches involving functional selections, which allow the discovery of anti-CRISPRs that are atypical in sequence and/or genomic location.[12]

The other initial question relating to anti-CRISPRs was how they work. We provided the first *in vitro* analyses of anti-CRISPR mechanisms, demonstrating inhibition of DNA-binding or cleavage,[13] and the first structure of an anti-CRISPR protein.[14] These studies were quickly followed by many more structural and mechanistic studies of anti-CRISPRs. Studies pertaining to type I, type II, and type V anti-CRISPRs are reviewed here by Yin et al.,[15] Hwang and Maxwell,[16] and Marino,[17] respectively. A new research paper describing the mechanism of an anti-CRISPR that blocks type II-C CRISPR-Cas systems is also included.[18] The final question that is covered in this issue is how anti-CRISPRs affect the ecology and evolution of phages. Pons et al. review the complexities involved in attempting to understand the impact of anti-CRISPRs on the evolution of phages and CRISPR-Cas systems.[19]

The ongoing battle between hosts and parasites is often described as an "evolutionary arms race" where mutations conferring an advantage to one adversary are countered by compensatory mutations in the other. These arms races are ubiquitous in biology, with the battle between viruses and anti-viral immune systems being a prime example. The anti-CRISPR versus CRISPR-Cas struggle is now probably the most intensively studied interaction involving an immune system and its virally encoded proteins inhibitors. As such anti-CRISPRs will serve as a paradigm for future studies on similar systems. Of particular relevance, a hugely exciting development in recent years has been the discovery of dozens of new bacterial anti-phage systems, many of which are directly related to human innate immune systems.[20–23] Phage proteins that inactivate these systems are also being rapidly discovered,[24–26] and future studies on these proteins will certainly be guided by the accumulated knowledge pertaining to anti-CRISPRs. Another important aspect of anti-CRISPRs is their potential to improve genome editing technologies.[27,28] Finally, the potential of phages for curing antibiotic-resistant bacterial infections is starting to be realized.[29–31] Since CRISPR-Cas systems are found in ~40% of bacterial genomes,[32] arming phages with Acrs will be crucial for the design of robust phage-based therapeutics. For all these reasons, we feel that this JMB issue on anti-CRISPRs is timely and will stimulate important and impactful future studies.

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