Exploring the ecological function of CRISPR-Cas virus defense

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ABSTRACT
Virus-host interaction is a key process in understanding the ecology and evolution of life. The study of the CRISPR-Cas RNA-guided adaptive immune systems of bacteria and archaea has added to our understanding of the virus defense mechanisms of microorganisms. The molecular details of the CRISPR-Cas systems are well explored and have allowed development a new generation of gene editing tools. However, the actual role and importance of CRISPR-Cas virus defense in nature is complex to study and have attracted less attention. Metagenomic analysis of microbial populations and the study of viruses-host systems in the laboratory have begun to unravel this question. Key findings in the field are described, with focus on recent developments.

Introduction
Like most other cells and organisms, bacteria and archaea need to handle the threat of viral infection that kill the organism or reduce their fitness. It matters little how successful an organism is at other aspects of its life if it cannot deal with its viruses. That fact contributes to the multitude of anti-virus defense mechanisms we see in nature. The defenses can be divided into innate systems, which recognize certain pre-set features to limit infection, and adaptive systems, that are able to learn to recognize threats that were previously not recognized. Microorganisms contain a variety of innate defense mechanisms, such as preventing DNA injection, cleaving certain sequences using restriction-modification systems, preventing phage proliferation by bacteriophage exclusion, and even committing altruistic suicide by abortive infection systems to prevent viruses to spread in a population.1,2 However, for a long time we humans, along with other vertebrates, had the only known adaptive immune system. The presence of an adaptive immune system in microorganisms was unanticipated when what is now known as the CRISPR-Cas systems were suggested in 2005-5 and subsequently demonstrated in 2007.6 The system is based on storing fragments of genetic material from viruses in a locus called Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) in the cells genome. The transcribed CRISPR RNA (crRNA) is used to guide CRISPR associated (Cas) proteins to destroy the virus’ genetic material.7 In the many hundreds of articles since published, detailed molecular understanding of the CRISPR-Cas systems have been generated: how the system learns to recognize new viruses, which the components of the systems are and how they are produced, how the virus is recognized and destroyed,8 what countermeasures the viruses use,9 and which non-immunity processes CRISPR-Cas components participate in.10 The diversity of the system is explored and today there are 6 basic types described along with numerous subtypes.11,12

A key question that has been surprisingly peripheral over the years is the actual function and importance of the system in nature. In sequenced genomes, convincing CRISPRs have been found in slightly less than half (45%) of bacteria and almost all (85%) archaea.13 The numbers may be interpreted as CRISPR-Cas systems being important, though not essential. However, CRISPR-Cas systems have evolved from mobile genetic elements,14 and appear to have retained a capability to horizontally transferring themselves between cells (though this have not yet been demonstrated experimentally). This mobility is the reason for CRISPR-Cas phylogeny not being very related to the family tree of the organisms they inhabit.15 The mobility also means that prevalence is not a good indicator of importance, as CRISPR-Cas systems can be regarded as selfish genetic elements. Further, a recent investigation of uncultured microorganisms headed by...
Jillian Banfield indicate that in some major bacterial lineages, only 10% contain CRISPR-Cas systems. The authors suggest that restriction enzymes serve as the most important virus defense in the studied lineages. The finding underlines that that investigation of natural communities are important for understanding the importance of CRISPR-Cas systems.

**Phage-host dynamics in natural communities**

The dynamics of CRISPR-Cas immunity was first demonstrated in another study by Jillian Banfields team where virus-host interaction in acid mine drainage biofilms in California was investigated. The extreme environment of the chosen site was found to harbor only a few species of microorganisms, which allowed an in-depth study normally prevented by the complexity of microbial ecosystems. Using large-scale sequencing of DNA directly from environmental samples (at least in terms of what possible at the time), cellular and viral genome sequences could be reconstructed. By matching CRISPR spacers and virus genomes it was clear that only the most recently acquired resistance was useful. Viruses quickly acquired mutations that circumvented the defenses, so called “escape virus” (or “escape phage”), by introducing changes in the regions targeted by the spacer. In the case of the acid mine drainage viral community, recombinatory shuffles of virus genomes played an important role in generating escape viruses.

**Experiments on phage-host interaction in the laboratory**

Early experiment on the role of CRISPR-Cas in virus-host interaction in laboratory settings was published in 2013 using the bacterial dairy workhorse *Streptococcus thermophilus* and its D2972 phage. *S. thermophilus* is convenient for such studies as most phage-resistance is generated by the CRISPR-Cas system. By following the bacteria-phage co-evolution in the population it was clear that CRISPR spacer diversification and phage evolution was very quick, and it was also discovered that certain regions of the phage genome were preferred as targets for the CRISPR-Cas system. Another study on the same species demonstrated that in addition to the expected competition between CRISPR-Cas immunity and phage escape mutants, there were some unanticipated results: phages could establish themselves in a culture containing one (but not 2) spacers targeting it, bacteria without CRISPR-Cas immunity persist in cultures despite presence of a large amount of phage, and bacteria with 2 spacers targeting a phage could still not establish themselves in a population of phage-sensitive bacteria. The authors conclude that for a full understanding of the interaction of phage and their host bacteria, a model beyond a simple iterative process of CRISPR-Cas immunity and phages escaping it is needed.

**The importance of different anti-virus strategies**

A key question for understanding the dynamics of virus defense is the relative importance of different anti-virus mechanisms. Which system is most important and under what conditions? When comparing constitutively active (like constitutively costly receptor mutations) and inducible systems (like temporarily costly CRISPR-Cas systems), which is most useful? This question was addressed by Edze Westra, Angus Buckling and coworkers, using a combination of theoretical modeling and experimental evolution. As a model system they used *Pseudomonas aeruginosa* bacteria and its DMS3vir phage. Unlike *S. thermophilus*, *P. aeruginosa* frequently become resistant to phage not just by using its CRISPR-Cas system, but also by mutating the receptor that the phage uses for infection. Under nutrient-rich conditions about a hundred times more phage were produced than under poor conditions. The authors demonstrated that the flood of infections during nutrient-rich conditions makes a constitutive defense favorable, while the CRISPR-Cas system is favored under nutrient-poor conditions as the cells then only rarely encounter a phage.

**Role of diversity in CRISPR-Cas immune systems**

Having addressed the issue of balancing constitutive and inducible virus defense, the Westra and Buckling teams turned their attention to the role of immune system diversity. The basic question, as described in the article by van Houte et al. came from the observation that while phage readily generates escape mutants, the DMS3vir phages still became extinct after some time in *P. aeruginosa* cultures. Studies on disease and parasites in plants and animals demonstrate that genetic diversity improves the resistance of a population. Could the phage onslaught result in a diversity of CRISPR-Cas immunity sufficient to overpower escape phage development? The teams set up a study to examine the relationship between CRISPR diversity and the systems effectiveness by mixing and infecting cultures consisting of different number of clonal strains, where each strain had a different spacer matching the phage but were otherwise identical. The cultures ranged from a single strain to mixtures of 48 different strains. Phages readily evolved escape mutants and established themselves in single-strain cultures, but found life increasingly difficult the more diversity there was in the culture, and in mixtures of 24–48 strains the phages quickly
became extinct. The fitness of the population increased with increasing diversity in the presence of phage. The most diverse cultures could even outcompete a receptor mutant that was constitutively phage resistant. The reason was demonstrated to be that phages are unable to generate mutants resistant to all clones in a mixture. In the most diverse cultures, no escape phages were detected at all. In diverse cultures, an escape phage may be able to infect some strains, but eventually the phage will encounter a resistant cell that will destroy the phage. The sensitive clones are probably prevented from being wiped out in a mixture of strains by the effect known as herd immunity, where disease is unable to spread between a few sensitive individuals in a majority of resistant individuals. To confirm that the interplay between diversity and immunity was not limited to the type I-F CRISPR-Cas system of *P. aeruginosa*, the role of diversity in type II-A system on infection of *S. thermophilus* by virus 2972 in was tested. The result was essentially the same as for *P. aeruginosa* though phage infection was more persistent, probably due to the lower rate of spacer acquisition in *S. thermophilus*.

**The effect of anti-CRISPR proteins**

The ability of a population with high diversity in CRISPR-Cas immunity to successful protect themselves from viral escape mutants may be a selection pressure driving the evolution of other anti-CRISPR strategies. Though anticipated after the discovery of the CRISPR-Cas immune system, it took several years for the first cases of phage-encoded anti-CRISPR proteins to be described. Indeed, a DMS3vir phage encoding an anti-CRISPR protein affected high-diversity CRISPR populations in the same manner as CRISPR monocultures, demonstrating that the usefulness of CRISPR-Cas immunity is basically nullified. What impact anti-CRISPR proteins have on the evolution of CRISPR-Cas system is not clear, but they likely contribute to the evolution of diversity in CRISPR-Cas systems and explain why many microorganisms have several different systems.

**Summary**

The findings described above demonstrate that microbial model systems can be used to describe the functional role of virus defense systems. The small but increasing number of studies in the field has provided an increase in our understanding of the interaction between viruses and their hosts, a key process in evolution, ecology and population biology. We will hopefully see more investigation of virus-host interaction in the wake of the above work, e.g. determining the role of predator-prey models such as the killer-the winner hypothesis and co-evolution models such as the Red Queen hypothesis. The use of laboratory experiments of phage-host interaction is valuable for hypothesis driven research and is an important addition to direct analysis of natural populations with their higher degree of complexity in species composition and virus interaction.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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