Introduction

The ecology and evolution of microbial CRISPR-Cas adaptive immune systems

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Introduction

Over the past decade, the field of CRISPR-Cas research has received a lot of attention from the scientific community. While initially this mostly concerned microbiologists who were fascinated by the discovery that some bacteria encode RNA-guided adaptive immune systems, this rapidly spread to other scientific disciplines following the development of groundbreaking molecular biology tools [1], and more recently to the public domain where the societal and ethical implications and legislation surrounding CRISPR applications are being heavily debated. Some of the potential CRISPR applications that are currently being explored in the lab would involve the release of CRISPR genes into confined or open environments – for example, when CRISPR would be used to protect focal bacterial species against phage infections, when it is applied to suppress the spread of antimicrobial resistance or to control vectors of disease [2-4]. One component of the societal impacts of these applications entails an assessment of the potential risks associated with these strategies (e.g. [5-7]), which requires an understanding how these CRISPR-Cas behaves in an ecological context. In this special issue we explore this question, by examining the evolutionary history of CRISPR-Cas immune systems, where they occur naturally, when they evolve and how this impacts the spread and evolution of other DNA elements. Finally we return to the question how CRISPR-Cas may be exploited in an ecological context for the benefit of human health, and the ethical challenges that are associated with this.

CRISPR-Cas adaptive immune systems – a brief overview

CRISPR-Cas adaptive immune systems were discovered around 15 years ago [8-11], and are estimated to exist in approximately 50% of all bacterial genomes and roughly 90% of all archaeal genomes [12]. A CRISPR immunity phenotype is genetically encoded by a so-called CRISPR locus (Clustered Regularly Interspaced Short Palindromic Repeats) – an array of repetitive and unique sequences (repeats and spacers, respectively), both of which are typically around 30 nt in length. Spacers are derived from (foreign) genetic elements, such as plasmids and viruses, and provide immunity to re-infection based on recognition of the cognate sequence (known as "protospacer") [13-15]. Bacteria or archaea may carry a single linked array of spacers interspersed with repeats (one CRISPR locus) or multiple loci. CRISPR loci can evolve very rapidly due to insertion of new spacers and the occasional loss of spacers or deletion of the CRISPR locus itself, which can cause very closely related strains to carry unique combinations

of spacer sequences, known as a CRISPR allele. The overall length of CRISPR loci will increase and decrease with the acquisition and loss of spacers, and can vary from as little as a single spacer flanked by two repeats to hundreds of spacers and repeats [16]. Since new spacers are added at the so-called leader-end of the locus, which is the sequence that contains the CRISPR promoter, CRISPR loci form an inverse chronological record of previous infections from the leader to the trailer end of the locus [17]. The extent to which different strains share the same spacer sequences in the same order (usually at the trailer end of the CRISPR locus), is commonly used to define related allele groups (RAGs) as a measure of their evolutionary relatedness.

Apart from the genetic CRISPR memory, a functional CRISPR-Cas immune system also requires a set of CRISPR-associated genes (*cas* genes), which encode the protein machinery required for carrying out the immune response [18]. Cas operons vary in their *cas* gene composition and gene synteny, resulting in a classification of CRISPR-Cas systems into 2 classes, 6 Types and 33 subtypes [19-22]. These diverse CRISPR-Cas variants differ in many of their mechanistic details, which have been discussed elsewhere [23, 24], yet also have commonalities in the basic steps of the immune pathway. For example, two Cas proteins – Cas1 and Cas2 – are almost invariably part of CRISPR-Cas immune systems and are responsible for inserting new spacer sequences into CRISPR arrays, sometimes assisted by other Cas proteins (reviewed in [15, 25]). CRISPR transcripts are processed by either Cas proteins or housekeeping RNases [26], and the resulting processed CRISPR RNAs (crRNAs) are bound by Cas proteins to form a ribonucleoprotein complex that serves to detect and cleave complementary nucleic acid sequences [23] .

Ecology and diversity of CRISPR-Cas immunity

CRISPR-Cas immune systems are unevenly distributed across taxa and environments. For example, only less than half of mesophilic bacteria encode CRISPR-Cas immune systems, compared to over 90% of bacterial thermophiles and archaea (both mesophilic and thermophilic) [11, 16, 27-29]. Moreover, some uncultured bacterial lineages are virtually devoid of CRISPR-Cas immune systems [30]. A recent computer learning approach suggested that abiotic factors such as oxygen levels and temperature are important predictors of whether microorganisms encode CRISPR-Cas immune systems [31]. However, the ecological drivers of CRISPR

distribution remain unclear. In this context, the key question is when CRISPR-Cas is favoured over alternative defense strategies [32]. Experimental, theoretical and correlational studies have suggested a role for viral abundance and diversity [27, 28, 33, 34], direct and indirect fitness costs of CRISPR-Cas immune systems (e.g. autoimmunity, reduced horizontal gene transfer, induced fitness costs, see below) [35-38], and epistasis with other host genes [39, 40].

In natural environments, whether that be the human lung, a hot spring or a fermenter, the ecological and evolutionary impact of CRISPR depends on the population level diversity of immune alleles. The diversity of alleles in the real world may reflect the previous history of interactions and can be used to predict whether infection epidemics will occur in the future. For example, the spread of a virulent virus can eliminate immune diversity from a population by eliminating all susceptible (non-immune) cells, resulting in a selective sweep of an individual strain with a matching CRISPR allele. In the future, this immunodominance across the CRISPR allele might make a population susceptible to the subsequent epidemic spread of a new virus or other mobile element in the population. Starting with a single strain of host or virus, diversity has been shown to evolve in experimental studies [33, 41] and to protect bacteria from virus invasion [42]. Data suggest that immune diversity shapes the evolution of viral pathogens by selecting for recombinant genotypes that are more likely to escape immunity [43] and, recombinant microbial CRISPR alleles that are more likely to increase immune profiles of a single strain [44, 45]. This inevitable interaction between viruses and mobile elements with immune diversity will thereby broadly impact dynamics of multiple microbial pathogens and link their dynamics through CRISPR.

Interestingly, studies of CRISPR diversity have observed differences in different microbial species from different environments. *Leptospirillum* species from acid mine drainage biofilms [43], and *Sulfolobus islandicus* strains from a single hot spring [46], Halophilic archaea from a saline pond [47], *Heloicobacter cinaedi [48]* and *Pseudomonas aeruginosa* from a single hospital [49] show diversity of CRISPR spacers co-exist. In contrast, *Pseudomonas aeruginosa* within a human lung, or *Prevotella* strains in a single gut sample are clonal with complete immunodominance at one time and place [49, 50]. These differences in diversity among CRISPR populations may result from difference in CRISPR dynamics or demographics (*e.g.* colonization bottle-necks [51]) and biological constraints (*e.g.* an inactive spacer acquisition machinery [52]). As CRISPR studies have mainly focused on mechanistic details, our understanding of microbial

population dynamics in real populations is surprisingly limited. Additional studies from a diverse range of CRISPR-containing organisms and their viruses, across a range of environments are needed to identify the basis for the differences in the genetic structure of CRISPR and predict how they impact epidemics and evolutionary dynamics in local populations.

Co-evolution between CRISPR hosts and viruses

Given the detailed molecular understanding of how CRISPR-Cas systems work, one can often predict resistance phenotypes of an individual on the basis of its genotype and that of its phage (or other foreign DNA element). Specifically, while bacteria gain resistance through the acquisition of spacers, phages and other mobile genetic elements can overcome immunity through the acquisition of "escape mutations" in (or sometimes near) the protospacer (e.g. [53, 54]). The fascinating details of the biology of CRISPR immunity and phage evasion challenge the classical theoretical framework used to understand host-parasite interactions. In particular, the Lamarckian ability of CRISPR to acquire new spacers allows the bacteria to accumulate a diverse range of resistance alleles against a focal phage [28, 55, 56]. This multiplicity of resistance within individual bacteria and/or within bacterial populations can overwhelm the evolutionary potential of the phage and drive it to extinction [42, 56-58]. Alternative mechanisms of escape are also possible and, recently, a number of different anti-CRISPR (Acr) systems have been described in different phages [59]. These Acr proteins have the ability to down-regulate the immunity of CRISPR-Cas systems and allow the phage to exploit CRISPR resistant bacteria. Interestingly, this immunosuppression has been shown to require the cooperation between multiple phages, where the first phages reduce the efficacy of CRISPR-Cas and allow subsequent infections to exploit the host [60, 61].

However, even if phage lack *acr* genes, CRISPR immunity is far from being the silver bullet against phages because it carries several different fitness costs. Some fitness costs are likely to result from the production of an effective interference against invading pathogens and mobile genetic elements [33, 62]. Some other costs are associated with self-targeting and auto-immunity [35, 36]. Also, when CRISPR targets mobile genetic elements or prophages that carry some adaptive mutation for the bacteria, CRISPR immunity may also be viewed as a form of self-targeting, since immunity directly harms the bacterial host. For instance, when CRISPR targets beneficial plasmids carrying antibiotic resistance, this immunity can be counter selected

and lead to the loss of CRISPR loci [37, 63-65], potentially explaining why many bacteria lack CRISPR-Cas immune systems. Note, however, that apart from a few studies [66], the experimental study of the coevolution between CRISPR immunity and phage evasion is often limited to a few generations. The monitoring of longer coevolution experiments in the laboratory or in natural environments are very welcome to better understand the coevolutionary dynamics driven by CRISPR immunity (see several papers in this special issue).

Application of CRISPR in an ecological context

While CRISPR-mediated targeting of antibiotics resistance plasmids may be maladaptive for individual bacteria (see above), it was soon realized that this feature of CRISPR systems may be exploited to limit the spread of antibiotics resistance [67-69], and more generally to engineer microbial communities. As the majority of bacteria do not encode end-joining mechanisms to repair double-strand DNA breaks, CRISPR-based technologies are particularly useful for either sequence-specific killing of pathogenic bacteria or removal of accessory genes (e.g. antimicrobial resistance, virulence, etc.). Synthetic CRISPR systems can be delivered to a target bacterium using so-called phagemids, which are replication-deficient bacteriophage particles, [67, 69], or through conjugative delivery of CRISPR systems [67, 70], but research is still in its infancy and considerable challenges are associated with these approaches [3].

CRISPR-mediated ecological engineering has also been explored in higher organisms, where it has raised major interest given its potential use in pest and invasive species control and reducing vector-borne diseases. Ecological engineering can be achieved by CRISPR-based genome engineering of wild animals followed by introducing these engineered animals in a local wild population. However, this method is likely to be effective only if the target population is relatively small (see Buchthal *et al* in this issue). A more powerful, but also riskier approach is to apply synthetic CRISPR-based gene drives [4, 71, 72]. These elements spread with super-Mendelian inheritance through a population and can disrupt genetic loci or facilitate the spread of genetically linked genes. CRISPR-based gene drives have been successfully used in confined laboratory settings in a range of organisms, including yeast, fruit flies, mosquitoes and, very recently, mice [73-76], and some first insights into the evolutionary dynamics of these drives, including evolution of resistance, are emerging [77-80].

For all these ecological engineering approaches, there is a clear need to better understand the potential risks of these gene drives and whether there is any appetite in society for these types of interventions. For example, what is the durability of these interventions, what are their long-term ecological consequences for (microbial) communities, and what countermeasures can be taken to limit or reverse gene drive spread? Being able to predict risks and benefits will be one important factor to get support from local communities, and for ethical and legislative approval. Recognizing the urgent need for new guidelines, scientists from a range of disciplines, including ethicists, social scientists and biologists, are working together to develop improved safety recommendations for CRISPR-based ecological engineering technologies [6, 81, 82], and ethical guidelines and the involvement of the public at an early stage in the development of these applications [81, 83, 84].

Aims of the theme issue

This theme issue aims to examine when and where CRISPR-Cas immune systems are important, how this impacts the coexistence and coevolution between bacteria and phages, and how this ties in with more practical and ethical considerations concerning the application of CRISPR-based technologies in an ecological context. The studies presented in this issue use a wide range of different approaches, from bioinformatics and metagenomics correlational studies to experimental and theoretical analyses, to answer an equally wide range of questions all somehow linked to CRISPR ecology and evolution: from understanding the basic principles of CRISPR evolution in bacteria to community engagement studies to examine if CRISPR-based applications can be applied in the real world to limit the spread of Lyme disease. The theme issue highlights the need to not only understand how CRISPR works in an ecological context, but also to engage with important practical issues when translating this knowledge to real-world applications to improve human health.

Overview of the papers

This theme issue is broadly divided into four areas that deal respectively with (i) the evolutionary history and relative importance of CRISPR-Cas immune systems, (ii) the role of CRISPR-Cas immune systems during bacteria-phage or Archaea-virus coevolution in real environments, (iii) understanding the drivers and consequences of coevolution in controlled lab environments, and

(iv) the way CRISPR may be harnessed to remove infectious diseases in real environments, and the associated considerations that need to be taken into account.

A) Evolutionary history and relative importance of CRISPR-Cas immune systems

One of the key questions in the field of CRISPR-Cas is how these sophisticated immune systems evolved in the first place, and how the enormous diversity of CRISPR Classes, Types and subtypes emerged in this process. Koonin & Makarova (ref) use comparative genomics to bring the evolutionary history of this system in focus. They suggest a key role for a Cas1-related transposase in the early evolution of the "Adaptation" module of the system, followed by evolution of increased complexity through a series of gene duplication and displacement events, recruitment of genes with nuclease activities and in some cases signal transduction genes to form the "Interference" module of the system. While a cloud of uncertainty inevitably surrounds such analyses, their work provides a plausible explanation for the way in which a highly complex adaptive immune system may have evolved. A next obvious question is then, why do not all bacteria and Archaea carry this adaptive immune system. This is a recurrent question, and one factor that clearly matters is how well CRISPR-Cas immune systems performs (in terms of the benefits it provides) relative to alternative immune strategies. For example, many bacteria and archaea encode restriction-modification systems or they can mutate receptors that phages use to attach to the cell surface. So why put up with CRISPR immunity, is the question that is raised by Levin and co-workers (ref). Using mathematical modeling they demonstrate that in theory the conditions where CRISPR is favoured over these alternative defenses is restricted. In other words, microbes have many options available to defend themselves against phages, and CRISPR may simply not always be the best one. However, the benefits of CRISPR are not only determined by the presence or absence of phage, but also by the genetic context of these immune systems, as Aude Bernheim and her colleagues show using bioinformatics approaches (ref), and as Anne Chevallereau and co-workers show using experimental manipulations (ref). Specifically, Bernheim et al find evidence for both positive and negative epistatic interactions between CRISPR-Cas subtypes and double stranded DNA repair pathways in bacteria. This therefore suggests that the distribution of CRISPR-Cas subtypes across bacterial species is shaped by the genetic context, but the underlying mechanism for this association is unclear. Earlier work from the same team demonstrated that one *cas* gene can inhibit the NHEJ pathway, which conceivably

can be maladaptive, explaining why these systems hardly ever co-occur in the same genome [39]. Chevallereau et al experimentally examined the impact of *mutS*, which is part of the DNA mismatch repair system, on the evolution of CRISPR resistance by *Pseudomonas aeruginosa* against its phage DMS3vir. They found that due to the resulting increase in mutation supply, bacteria were much more likely to evolve resistance through mutation of the phage receptor, suggesting no selective benefit of carrying CRISPR-Cas immune systems in this host genetic background. Together, these studies show how CRISPR-Cas immune systems may have evolved in the first place, and how natural selection for these systems not only depends on the presence or absence of phage, but also on the presence of alternative defense mechanisms as well as the genetic context of the CRISPR-Cas system.

B) Patterns of spacer acquisition in an ecological context

One of the key questions in the field of CRISPR ecology and evolution is what drives the generation and maintaince of CRISPR diversity. Using mathematical modeling, Bradde et al show that spacer acquisition rates can be constrained by a cost of autoimmunity (ref). Their model assumes that higher spacer acquisition rates not only increase the benefits of phage resistance, but also the costs of autoimmunity due to spacer sampling from the bacterial genome. This results in selection for an optimum spacer acquisition rate that maximizes bacterial survival (a balance of phage resistance and autoimmunity), which in turn depends on factors that influence the infection risk, such as bacterial and phage population sizes. In addition to this theoretical approach, several papers in this issue apply comparative population studies to describe spacer diversity in natural environments, and to identify the factors that drive this diversity. Lopatina et al. (ref) examine the CRISPR loci of populations of the bacterium Thermus in different geographical locations in Chile, Italy and Russia. They demonstrate that within a single population of the bacterium Thermus more spacers are shared among strains than among distant populations. The authors infer from the pattern that local dynamics between hosts and viruses define the local diversity although the shared spacers among populations indicate some evidence for gene flow. Similarly, Pauly et al. (ref) show patterns of persistent local diversity within a single hot spring of *Sulfolobus islandicus.* Pauly also shows that these patterns vary for different viruses indicating that virus lifestyle impacts immune diversity and explore different mechanisms of CRISPR escape from different viruses through mutation or virus genome

replacement. Finally, Hoikkala et al. review the importance of CRISPR-Cas immune systems in acquaculture, and the observed dynamics of coevolution of a bacterial fish pathogen and its phage. They discuss how CRISPR diversity of bacterial strains in these environments can be exploited for tracking the epidemiology of bacterial pathogens, and for phage therapy interventions (ref). Their review highlights how a better understanding of CRISPR-diversity may be used to stabilize and shape healthy microbial populations and prevent invasions of bacterial pathogens into acquaculture environments. Collectively, these studies provide novel insights into the generation, maintenance and potential application of spacer diversity in natural environments.

C) Coevolution between CRISPR-Cas immune systems and phages in the lab

Experiments carried out in controlled environments in the lab allow to explore the details of the coevolutionary processes between CRISPR systems and phages. Common et al (ref) carried out a coevolution experiment between *Streptococcus thermophilus* and a lytic phage over 30 days. The monitoring of phenotypic and genotypic changes in both the bacteria and the phage across time confirmed the emergence of an antagonist arms race that often results in the extinction of the phage populations. These extinctions suggest that this coevolution is asymmetric because the phage cannot cope with the accumulation of escape mutations. The existence of a fitness cost associated with most escape mutations was confirmed by a separate study by Chabas et al (ref) on the same biological model. In addition, Chabas et al. found a dramatic variation in the speed at which the phage can escape different CRISPR mediated resistance alleles. The resistance induced by some spacers can be easily bypassed by the rapid emergence of escape mutations, while the resistance induced by other spacers is much more durable. The measurement of the phage mutation rate using Luria-Delbruck fluctuation tests allowed Chabas et al to suggest that this variation in resistance durability is likely to be driven by heterogeneity in the mutation rate across different protospacers. The evolution of escape mutations was also studied by Watson et al (ref) in two lytic phages infecting CRISPR resistant *Pectobacterium atrosepticum*. This study contrasted the evolution of the phage against single or multiple spacers. When the resistance was mediated by a single spacer, most escape mutations were due to a single point mutation in the PAM or the seed sequence. When the resistance is due to multiple spacers, most phages escaped with deletions in genes encoding structural proteins. These mutations were viable but affect the

morphology and the fitness of the virus, which further support the existence of a cost of escape and an asymmetry in the coevolutionary arms race. The details of phage adaptation was also studied by McKitterick et al (ref) in a fascinating system where the phage ICP1 uses a fully functional CRISPR-Cas system to down-regulate bacterial immunity of *Vibrio cholerae* mediated by a phage inducible chromosomal island-like element (PLE). High-throughput sequencing allowed the authors to monitor the acquisition of new spacers against PLE in the CRISPR of ICP1, which provide quantitative resistance against PLE that depends on the number and sequence of spacers. Furthermore, even spacers not targeting the PLE but instead targeting the small chromosome of the bacterium could still reduce bacterial immunity if the chromosomal targeting occurred close to the PLE integration site. Collectively, these different experiments in the lab shed a new light on the interference mediated by CRISPR and by the costs associated with different mutations allowing phages to escape bacterial immunity.

D) Implications of CRISPR-Cas for human health and wellbeing

Natural CRISPR-Cas systems can have an important impact on human health by for example altering the spread of antimicrobial resistance and virulence genes [85]. However, even though experimental studies show that CRISPR-Cas forms an important barrier for horizontal gene transfer, correlational studies have shown mixed results [63, 86]. Here, Shereen et al (ref) examine if the presence of anti-CRISPR genes may impact the correlation between CRISPR-Cas immune systems and antibiotic resistance genes (ARGs) genes. They find a very high degree of variation between bacterial species in the abundance of CRISPR-Cas systems and no correlation with ARG for most species. However, for *Pseudomonas aeruginosa* they found a positive correlation between anti-CRISPR and ARG genes, indicating that anti-CRISPRs may facilitate the spread of clinically relevant genes such as those encoding antibiotic resistance or virulence factors in the face of CRISPR-Cas immune systems. These genes may be removed using CRISPR-based ecological engineering technologies. Although this is still a very young field the developments have moved fast and scientists now have a variety of CRISPR-based tools at hand to alter microbiome composition and function, and use them as next-generation antimicrobials. Ramachandran & Bikard (ref) provide a thorough overview of the these various CRISPR-based applications to alter the microbiome, including its application in editing bacteria and phage genomes, controlling their gene expression using CRISPRi, killing pathogenic bacteria using

CRISPR-based antimicrobials and removing antibiotic resistance genes or other virulence determinants.

While these CRISPR-based ecological engineering approaches have great potential to solve ecological problems, there are many technological, regulatory, societal and ethical challenges. In this issue, De Graeff et al (ref) provide a thorough overview of the arguments reported in the scientific literature for and against genome editing in animals, including gene drives, which relate to human health, efficiency, risks and uncertainty, animal welfare, animal dignity, environment and public acceptability. The authors also pointed out that the ethical debate on genome editing in animals is predominantly shaped by biomedical and veterinary scientists, and less so by ethicists and social scientists. They argue that involvement of ethicists and social scientists from the very early research stages of technology development may help facilitate responsible governance of animal genome editing applications [87]. Furthermore, there is a need for engagement with the public to address amongst others concerns around equity of access (who will benefit from the new technology?) and the commercialization of the technology (will businesses prioritize profit-making over providing a safe public good?). Buchthal et al (ref) provide an excellent example of involving the public in decision-making on CRISPR-based ecological engineering. 'Mice against Ticks' is an exciting new community-guided ecological engineering project aimed at reducing the incidence of tick-borne diseases vectored by mice on the islands of Nantucket and Martha's Vineyard off the US east coast. Their idea is to introduce CRISPR-edited mice that are heritably resistant to ticks and/or tick-borne disease in the islands' local mouse population, which is hypothesized to disrupt the disease transmission cycle. From the stages of conception of the project the local communities of both islands have been actively involved in all decision-making. Although at a relatively early stage still, successes and challenges from this exciting pilot project will provide future ecological engineering projects with highly valuable insights on how to set up community-driven projects.

Conclusion and outlook

While our mechanistic understanding of CRISPR-Cas immune systems has raced ahead, our understanding of the evolutionary ecology of these fascinating immune systems is still relatively limited. As outlined above, this issue fills some important gaps in our knowledge of the distribution and impact of CRISPR immune systems, but there are also many outstanding

questions that need to be answered. For example, why are there so many different CRISPR-Cas variants, what are their costs and benefits, and how does this depend on the environment? Why do so many bacteria not encode CRISPR-Cas immune systems, and what drives their loss? Why is it that in the lab bacteria that encode CRISPR-Cas systems often evolve receptor-based resistance against their phages? Is this because of a lack of ecological realism, widespread anti-CRISPR strategies of phages, or are as yet unknown phage life history traits important for the evolution of CRISPR resistance? As argued in this introduction, a better understanding of the principles that govern evolution of CRISPR-Cas immune systems and their coevolution with mobile genetic elements can help to inform applications of CRISPR-based technologies in real environments. Apart from this quest for a better understanding of CRISPR ecology and evolution, contributions in this issue also highlight the need for community engagement by CRISPR biologists and participation in debates surrounding the ethics and legislative aspects of these technologies, and provide some excellent examples of how this can be done.

Competing interests

The authors declare no competing interests.

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