COMMENTARY

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Holding a grudge

Persisting anti-phage CRISPR immunity in multiple human gut microbiomes

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The CRISPR (clustered regularly interspaced short palindromic repeats)/Cas (CRISPR-associated) system of bacteria and archaea constitutes a mechanism of acquired adaptive immunity against phages, which is based on genome-encoded markers of previously infecting phage sequences ("spacers"). As a repository of phage sequences, these spacers make the system particularly suitable for elucidating phage-bacteria interactions in metagenomic studies. Recent metagenomic analyses of CRISPRs associated with the human microbiome intriguingly revealed conserved "memory spacers" shared by bacteria in multiple unrelated, geographically separated individuals. Here, we discuss possible avenues for explaining this phenomenon by integrating insights from CRISPR biology and phage-bacteria ecology, with a special focus on the human gut. We further explore the growing body of evidence for the role of CRISPR/Cas in regulating the interplay between bacteria and lysogenic phages, which may be intimately related to the presence of memory spacers and sheds new light on the multifaceted biological and ecological modes of action of CRISPR/Cas.

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Introduction

Bacteriophages are known to play a crucial role in shaping the structure, diversity and evolution of microbial communities in various ecological niches.[1-4] The CRISPR (clustered regularly interspaced short palindromic repeats)/Cas (CRISPR-associated) system of bacteria and archaea constitutes a mechanism of acquired adaptive immunity against phages, which is based on genome-encoded markers of past infections.[5] The unique biology of this system has opened a window into the multifaceted interactions that take place in natural environments between microbial populations and their associated phages. At the same time, observation of these interactions raises intriguing questions about the underlying mechanistic activity of CRISPR/Cas. Here, we explore this reciprocity with a focus on the microbial community resident in the human gut.

CRISPR loci are composed of a succession of short repeat sequences separated by unique "spacer" sequences, usually sized 24–50 bp. While the mechanism of action of the CRISPR/Cas system is not yet fully characterized and some specifics vary by subtype, it generally entails three processes: incorporation of fragments of phage or plasmid genomes as novel spacers in bacterial CRISPR arrays; transcription and processing of the array into small RNAs (crRNAs) and formation of a crRNA/Cas protein complex that recognizes foreign nucleic acids through sequence complementarity and interferes with phage replication.[6-11]

Elucidation of the function of the CRISPR/Cas system has led to theoretical and experimental efforts at exploring the ecological impacts of CRISPR-mediated immunity, as well as the conditions under which its emergence would be favored.[12-14] However, this system provided an

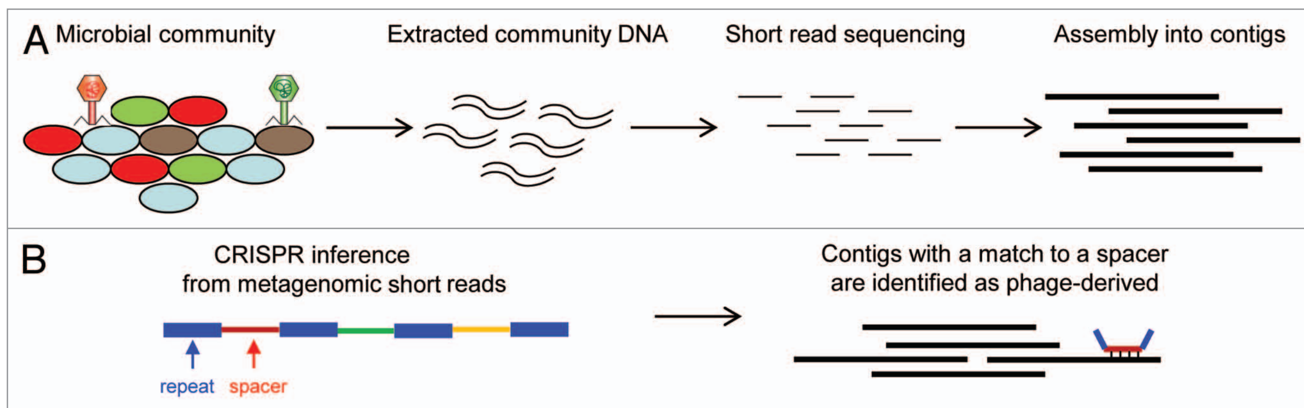

Figure 1. (**A**) A schematic of the process of metagenomic sequencing and assembly. Total DNA is extracted from a community of microorganisms, followed by sequencing and assembly into contigs belonging to the multiple organisms present. (**B**) Sequencing reads originating from CRISPR loci are identified and used to extract spacer sequences. Contigs in the metagenome found to match spacer sequences, but not a CRISPR repeat, are inferred to be of phage origin.

additional unexpected boon, as its modus operandi is especially well-suited to yield insight into phage-bacteria interactions in the context of metagenomic studies.

Metagenomics is a microbial community sequencing approach in which sequencing is performed on total DNA derived from a given habitat, followed by assembly of genomic segments (contigs) belonging to the multiple organisms present in the habitat.[15,16] Metagenomics can provide a comprehensive view of phage and bacterial population dynamics and co-evolutionary patterns, especially when performed over time. However, several challenges must be overcome. It is often difficult to classify assembled novel sequences from metagenomic samples as viral due to the enormous diversity of bacteriophage sequences and their corresponding sparse representation in the sequence databases.[17,18] Even when virus-like particles are filtered and sequenced directly,[19] it is often challenging to associate a virus with its host without supporting information.[20]

Andersson and Banfield first demonstrated how CRISPR can be used to address both these challenges.[21] They extracted CRISPR spacers from the metagenome of an acid-mine drainage biofilm dominated by a few bacterial and archaeal species, and attempted to match them to all assembled contigs from that metagenome. Contigs showing similarity to a spacer sequence but not to a CRISPR repeat were inferred with high confidence to be derived from mobile genetic elements, and further analysis confirmed most were of phage origin. In addition, a spacer match also revealed which prokaryotic species was infected by which phage, as the hosts harboring these spacers were known and spacers are derived from phages previously infecting the host. Variations on this general scheme for using CRISPR to interpret metagenomic data (**Fig. 1**) have been used to study microbiomes in the ocean,[22,23] hot springs[24-26] and the human body.[27-30]

There are yet more subtle ways in which CRISPR biology can inform on phage-bacteria ecology. New spacer integrations in CRISPR arrays generally take place in a unidirectional manner next to a sequence element just upstream of the repeat-spacer array ("leader"), as first inferred in *Yersinia pestis* and later verified experimentally.[5,31,32] While rare exceptions to this rule have been reported,[33,34] directional integration allows to distinguish between recent and historical spacer acquisitions with implications for elucidating temporal dynamics of phage-host interactions and reconstructing approximate array evolution when compared across strains.

A comparison of over 100 strains of *Streptococcus thermophilus* demonstrated several important principles for CRISPR array evolution (illustrated in **Fig. 2A**).[35] First, homologous CRISPR arrays showed great heterogeneity in spacer content at their leader-proximal end, where new spacers are integrated. The degree of heterogeneity was generally expected to correlate with the level of activity of the CRISPR locus in response to phage pressure. However, homologous arrays also showed conservation of "old" spacers in their leader-distal end (also termed trailer-end), despite a tendency for occasional internal spacer deletion, possibly due to homologous recombination between repeats. Additional studies reported qualitatively similar findings in this and other species.[31-33,36-39]

The Human Gut as a Case Study for Using CRISPR to Study Niche Ecology

We have recently examined phage-bacteria interactions in the human gut microbiomes of 124 unrelated Danish and Spanish individuals through the prism of CRISPR targeting.[28] In this work, motivated by the burgeoning interest in the impact of the gut microbiome on human health and disease,[40-43] we extracted a large spacer catalog from metagenomic sequences of gut-residing bacteria.[44] Using these spacers as "baits" to identify contigs derived from phage DNA, we detected almost 1,000 gut-associated phage genomes, many of which appeared in multiple individuals and also across gut samples taken from Americans and Japanese.[28]

Memory spacers in human gut bacteria. Most of the gut-residing phages we identified were targeted by CRISPR spacers extracted from gut microbiomes of multiple individuals.[28] This

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Figure 2. (**A**) An illustration of trailer-end clonality of CRISPR arrays. Each row depicts a CRISPR array from related strains, colored boxes represent different spacers. Leader-proximal spacers show great heterogeneity, whereas leader-distal spacers are conserved. (**B**) Trailer-end clonality found in the human gut across four individuals. A CRISPR array in the isolate gut bacterium Clostridium sp. L2-50[43] derived from an infant in the UK is shown at the top. CRISPR repeats are depicted as thick black boxes; spacers as colorful boxes. Reads from three Danish and Spanish samples were mapped onto the isolate array,[28,44] revealing preservation of the leader-distal spacers, hence termed memory spacers (in red), whereas colorful spacers represent presumably newly acquired spacers. When no reads were successfully mapped, the coloring is gray. (**C**) Four hypotheses are presented to explain the existence of shared spacers in bacterial strains present in samples across the globe: (I) they are recently derived from a common ancestor, (II) they are mechanistically shielded from loss by recombination due to their location at the trailer-end of the array, (III) they are part of a "frozen" CRISPR array, in which the rate of spacer insertion and deletion is extremely slow, (IV) they are derived from an ancient ancestor and selective pressure retains the spacers. Such pressure may result from repeated induction of lysogenized prophages.

probably reflects the prevalent association of these phages with human gut bacteria. Interestingly, many of these cases involved not only targeting of the same phage but also through the same spacer sequence. Approximately 22% (11,421/52,267) of all spacers extracted in the study were shared across two or more unrelated individuals, often between Danish and Spanish samples, with up to 20 different individual gut microbiomes harboring the same spacer (**Table S1**). Our findings may in fact represent an underestimate of the extent of shared spacers as a result of variation in sequencing depth between samples. CRISPR spacers shared across the microbiomes of multiple individuals have also been reported in other recent studies.[29]

Furthermore, in cases where we were able to fully or partially reconstruct CRISPR arrays in our study, we detected conservation of blocks of "historical," trailer-end spacers in bacteria from

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multiple individuals, which also extended to sequenced isolates in the databases (**Fig. 2B**).[28] The conservation of spacer blocks as compared with sequenced isolates allowed us to pinpoint the bacterium to which the array belonged and, thus, link a phage targeted by a spacer in that array to its host.

The sharing of spacers among gut bacteria of geographically separated, unrelated individuals is puzzling, as it suggests an unexpected degree of uniformity among multiple parallel ecological niches, in each of which the microbiome is evolving separately. In the following, we examine various avenues for explaining the phenomenon of spacer sharing and how well they fit available evidence.

One possible explanation is that bacterial strains inhabiting different individuals share a recent common ancestor (**Fig. 2C**). Under this scenario, spacer sharing among geographically separated individuals emerges as a result of the ongoing spread of clones of gut bacteria carrying the same CRISPR array, which occurs on a timescale faster than that of spacer turnover. However, the observation that spacer sharing mainly occurs in trailer-end spacers runs counter to the possibility of frequent transmission of gut bacteria. Rather, it is in line with an ancient population bottleneck, either caused by phage pressure or by unrelated niche-adaptation, which fixed patterns of trailer-end spacers in gut bacterial strains that have since become globally distributed. These pre-existing patterns may thus become established in individuals around the world during bacterial colonization of the infant gut or during community shifts in the adult (e.g., after antibiotics usage).[45-47] This is supported by the finding that strains of prevalent gut bacterial species remain stable in an individual over time periods much exceeding the time measured for spacer turnover in the environment.[21,48]

The apparent historical nature of the shared spacers means that they represent a persistent long-term memory of viral infection, and so we dub them "memory spacers." This persistence of memory spacers across multiple individuals then begs explanation in light of the rapid evolution of CRISPR arrays and the frequent deletions in prokaryotic genomes.[49] A possible answer is that memory spacers are shielded from deletion for mechanistic reasons related to the molecular biology of CRISPR arrays (**Fig. 2C**). For example, mutations in the flanking repeat sequences have been preferentially observed at the trailer-end of CRISPR arrays and might interfere with recombination-mediated spacer loss.[35,38,50] Loss of memory spacers could also be limited by the transcriptional orientation of CRISPR arrays. With transcription generally starting at the leader-end, expression of leader-proximal spacers can be markedly higher compared with trailer-end spacers. This can reduce the likelihood of recombination events involving the latter, especially in longer arrays.[32,46-48]

Alternatively, CRISPR arrays harboring memory spacers may be "frozen," such that spacers are inserted and deleted very slowly (**Fig. 2C**). This is in line with findings on CRISPR in *Escherichia coli*, where the rate of spacer insertion and spacer deletion was found to be exceptionally low.[51] However, the majority of bacterial strains in the human gut is comprised of *Bacteroidetes* and *Firmicutes*,[52] and memory spacers in our study were found in CRISPR arrays of 55 different gut bacteria reference genomes, often belonging to these prevalent phyla (**Table S1**). While the rate of spacer insertion is not conclusively determined in these strains, it is unlikely that CRISPR arrays in so many different species are frozen, as evidenced by the diversity of leader-proximal ends of CRISPR arrays that harbor memory spacers.[28,39]

Yet another possible explanation is that the retention of memory spacers across individuals is a consequence of phage selective pressure. Weinberger et al. have recently studied conservation of spacers at the trailer-end of an archaeal CRISPR array using an elegant combination of computer simulation and metagenomic reconstruction from an acid-mine drainage biofilm.[53] They suggested that trailer-end clonality can result from sweeps of immunogenically favored strains, and that memory spacers are not subsequently lost since they confer a selective advantage against repeatedly induced prophages or phages re-introduced to the niche. Furthermore, a recent analysis of CRISPR spacers in archaea showed that trailer-end spacers are much more likely to match viral genomes and particularly those with broad host ranges.[54]

Consistent with this model, our data identify common phages associated with gut bacteria of geographically distant human populations.[28] Moreover, phages associated with human gut bacteria appear to be predominantly lysogenic and at least some components of an individual's gut phage cohort show marked stability over time.[28,55-58] Thus, it is possible that there is an evolutionary advantage for gut bacterial strains to retain spacers that would provide them protection against the repeated induction of prophages, or against phages that are consistently present in many human guts (**Fig. 2C**). However, while the model of Weinberger et al. considers a single geographic locale, we observed spacer sharing across multiple individuals (i.e., multiple gut niches in parallel). This suggests that the clonal sweeps that yielded trailer-end clonality could not have occurred in each individual separately, as that would have led to different trailer-end patterns in each gut. Rather, the sweep likely occurred in an ancestral CRISPR array that became globally distributed, as discussed above.

The riddle of the establishment and maintenance of memory spacers is not yet fully resolved, and its answer may involve elements from all possible explanations discussed above. Several lines of evidence could prove particularly enlightening in the future. One would entail clarification of the mechanisms and timescales of spacer gain and loss, both through mechanistic studies[59] and through higher-resolution characterization of spacer evolution over time in conditions closely approximating natural environments.[60] In addition, as deeper and more complete catalogs of the CRISPR spacer repertoire associated with various body sites are produced, it would become possible to compare the prevalence and persistence of memory spacers across niches and correlate observed spacer sharing with the specific characteristics of each site.[27,29]

Phage lysogeny co-occurring with CRISPR targeting. The finding that CRISPR targets lysogenic phages, which integrate into the host genome, raises a more general issue that is increasingly

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gaining attention in CRISPR research. Co-occurrence of an immunity spacer and the targeted phage in the same genome might lead to degradation of the bacterial genome and cell death, as demonstrated by Edgar and Qimron.[61] We and others have previously reported on self-targeting of core bacterial genes by CRISPR, and demonstrated that such cases of inadvertent auto-immunity often lead to loss or silencing of the CRISPR/Cas system in order to protect the cell.[62,63] However, targeting of lysogenic phages cannot be construed as mere error in the context of the human gut, nor do CRISPR/Cas systems of gut bacteria necessarily show signs of inactivation. How then can this phenomenon be explained?

The most parsimonious scenario is that the spacer may reside in one sub-population of cells belonging to a given strain, while the lysogenized phages reside in a different sub-population (**Fig. 3A**). Under these circumstances, spacers against lysogenic phages serve to maintain diversity in the host strain population and limit the proportion of cells susceptible to prophage induction and lysis. This interpretation seems to be favored with respect to the role of CRISPR/Cas in regulating the mobilome of the gut bacterium *Streptococcus agalactiae*,[39] and is akin to other instances of defense strategies serving to maintain population diversity by compartmentalizing host susceptibility such as have been documented in the ocean.[64]

Alternatively, a spacer and an integrated phage may actually coexist in the same cell. Coexistence may be facilitated by the host silencing its own CRISPR/Cas system under "normal" conditions (**Fig. 3B**). Indeed, it has been shown that the expression of several *cas* genes as well as the CRISPR array can be repressed, with the system undergoing upregulation upon signs of phage infection.[65,66] Under this model, CRISPR spacers targeting the lysogenic phage would become lethal only when a prophage is induced, sacrificing the host but limiting the spread of the phage to adjacent cells. This hypothesis is supported by recent studies showing that type IIIB CRISPR/Cas requires active transcription of the DNA target in order to degrade it.[67] An immune response only against an induced prophage would be in line with the coupling of anti-viral defenses and "altruistic" suicide recently proposed as a fundamental theme of prokaryotic immune systems.[68] Targeting of the induced prophage might actually prevent lysis and cell death under certain circumstances, as demonstrated in *E. coli*.[61]

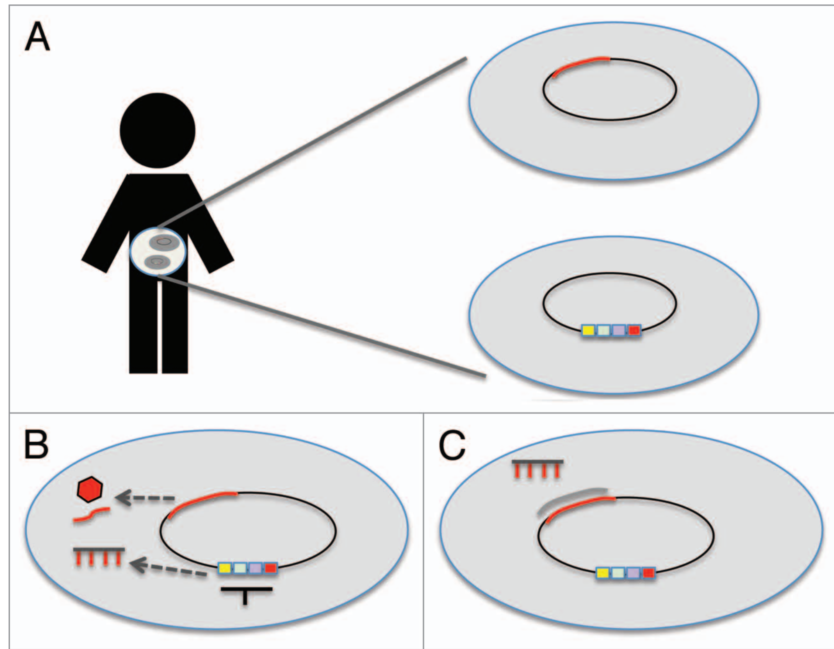

Figure 3. Three scenarios explain how CRISPR spacers (colored boxes) may coexist with their targeted lysogenized prophages (red line): (**A**) Bacterial cells exclusively contain either lysogens or the targeting CRISPR array. (**B**) CRISPR arrays are silenced, and perhaps activated only upon prophage induction (dashed arrows). (**C**) The prophage shields itself from CRISPR/Cas.

Conversely, the prophage itself could be the one responsible for silencing the CRISPR/Cas system as a means of evading host defenses (**Fig. 3C**). Indeed, temperate phages of *Pseudomonas aeruginosa* were recently shown to carry genes that inactivated the CRISPR/Cas system of the host bacterium while these phages were integrated into its genome.[69] The bacterial H-NS transcriptional repressor was also found borne on a phage genome, possibly leading to silencing of CRISPR/Cas in *Candidatus Accumulibacter phosphatis*.[70] Additional mechanisms can likely serve to shield the prophage from the effects of the CRISPR/Cas system, such as evasive mutations in the proto-spacer adjacent motif.[71,72]

Perhaps the most intriguing possibility is that the CRISPR/Cas system may be involved in regulating prophage induction or gene expression. Some evidence to support this comes from studies into the CRISPR/Cas system of *P. aeruginosa* and its role in mediating the effect of a lysogenic phage on the biofilm forming behavior of the bacteria.[73] Indeed, almost all CRISPR spacers that matched database sequences in clinical isolates of *P. aeruginosa* targeted prophages,[74] and CRISPR/Cas has been shown to limit growth of lysogenic phages in this species.[75] Another intriguing finding revealed a role for the Cas protein complex in inhibition of a defective prophage through repeat-directed cleavage of a prophage RNA transcript, independent of spacer targeting, in the haloarchaeon *Haloferax mediterranei*.[76]

Taken together, these findings provide strong impetus to the concept that in some ecological niches, the role of CRISPR/Cas may be much more multi-faceted than previously appreciated. Rather than a mere acute response to abort infection, it may exert ongoing, more subtle and diverse effects mediating the relationship between bacteria and their associated mobile genetic elements. Further insight into the mechanistic aspects of CRISPR/Cas interaction with lysogenized phages could lead to better understanding of the effects of CRISPR-mediated immunity in niches dominated by lysogenic phages, such as the human gut, and would provide

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valuable clues into the tantalizing possibility of a role for CRISPR/Cas in gene regulation. As discussed in the previous section, the repeated induction of prophages could also be an important element contributing to the ongoing retention of CRISPR spacers shared across multiple individuals.

Conclusion

CRISPR biology is uniquely suited to shed light on many aspects of phage-bacteria interactions. Sound ecological interpretations depend heavily on understanding of the underlying biological mechanisms shaping CRISPR arrays. Conversely, ecological observations provide data against which hypotheses on CRISPR biology may be evaluated, while raising intriguing questions that motivate research into mechanistic aspects. This interplay will surely continue to play a prominent role in the study of these remarkable systems.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental material may be found here: www.landesbioscience.com/journals/rnabiology/article/23929/

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