CRISPR Immunity Drives Rapid Phage Genome Evolution in *Streptococcus thermophilus*

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ABSTRACT Many bacteria rely on CRISPR-Cas systems to provide adaptive immunity against phages, predation by which can shape the ecology and functioning of microbial communities. To characterize the impact of CRISPR immunization on phage genome evolution, we performed long-term bacterium-phage (Streptococcus thermophilus-phage 2972) coevolution experiments. We found that in this species, CRISPR immunity drives fixation of single nucleotide polymorphisms that accumulate exclusively in phage genome regions targeted by CRISPR. Mutation rates in phage genomes highly exceed those of the host. The presence of multiple phages increased phage persistence by enabling recombination-based formation of chimeric phage genomes in which sequences heavily targeted by CRISPR were replaced. Collectively, our results establish CRISPR-Cas adaptive immunity as a key driver of phage genome evolution under the conditions studied and highlight the importance of multiple coexisting phages for persistence in natural systems.

IMPORTANCE Phages remain an enigmatic part of the biosphere. As predators, they challenge the survival of host bacteria and archaea and set off an “arms race” involving host immunization countered by phage mutation. The CRISPR-Cas system is adaptive: by capturing fragments of a phage genome upon exposure, the host is positioned to counteract future infections. To investigate this process, we initiated massive deep-sequencing experiments with a host and infective phage and tracked the coevolution of both populations over hundreds of days. In the present study, we found that CRISPR immunity drives the accumulation of phage genome rearrangements (which enable longer phage survival) and escape mutations, establishing CRISPR as one of the fundamental drivers of phage evolution.

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**RESULTS** Deep sequencing of host-phage co-culture in a time series. DNA was extracted from a subset of four experimental series. We used lytic phage 2972 MOIs that trigger novel CRISPR spacer acquisition in *S. thermophilus* and are commonly used to generate bacteriophage-insensitive mutants in dairy starter cultures (9). Thus, we used a phage-to-host abundance ratio of 2 (MOI-2) in two replicate experiments (MOI-2A and MOI-2B) and also a phage-to-host abundance ratio of 10 (MOI-10). Samples from these three experimental series, and from an *S. thermophilus* culture that was not inoculated with phage, were collected periodically over time until the loss of the phage; the sampling period ranged up to 232 days. The combination of the unprecedented large amount of samples and data processed in this work shows that, in a closed system, this bacterium rapidly acquires a highly diverse set of CRISPR spacers that drive the evolution of the phage over extended time periods to extinction.

**Bacte**ria-phage interactions influence microbial community structure, ecology, and evolution and are therefore important determinants of the overall functioning of natural environments (1–3). Phages rely on specific hosts for replication and are a major cause of bacterial cell death; thus, phages impact carbon and other nutrient cycles, as well as microbial population levels, turnover, and diversity (4). In the face of this pressure, bacteria have evolved sophisticated phage defenses, such as the CRISPR-Cas adaptive immune system (5), which provides sequence-specific interference against invasive nucleic acids (6, 7).

Previous studies showed that upon exposure to phage, the lactic acid bacterium model strain *Streptococcus thermophilus* DGCC7710 readily acquired spacers in two of its four CRISPR loci (CRISPR1 and CRISPR3, both classified as type IIA) to build adaptive immunity against lytic phage (8, 9).

Here, we investigate the genomic response of a phage to rapid diversification of the CRISPR spacer immunity in the host population by exposing *S. thermophilus* DGCC7710 grown in milk medium to infective lytic phage 2972 at multiplicities of infection (MOI) that trigger a novel CRISPR spacer acquisition in this system. Samples from three experimental series, and from a control series (*S. thermophilus* culture that was not inoculated with phage), were collected periodically over time until the loss of the phage; the sampling period ranged up to 232 days. The combination of the unprecedented large amount of samples and data processed in this work shows that, in a closed system, this bacterium rapidly acquires a highly diverse set of CRISPR spacers that drive the evolution of the phage over extended time periods to extinction.
MOI-0, MOI-2A, MOI-2B, and MOI-10 samples were subjected to Illumina metagenomic sequencing (165 Gb of data from 36 samples) (see Table S1 in the supplemental material). Host and phage counts were monitored over the course of the experiments (see Materials and Methods). After 5 weeks of co-culture (day 35), we were unable to detect phage particles in the MOI-2A experiment (Fig. 1A). In contrast, the phage and host coexisted for 232 days in the MOI-2B experiment (Fig. 1A) and for 195 days in the MOI-10 experiment (Fig. 1A).

*S. thermophilus* DGCC7710 CRISPR spacer analysis. A time series metagenomic analysis was used to document the inventory of spacer sequences added to each locus across the experimental series. There was underrepresentation or no representation of some potential spacer sequences predicted from the phage genome (proto-spacers), consistent with findings of a prior study (10). Interestingly, we detected acquisition of spacers into the type IE CRISPR4 locus, as well the type IIA CRISPR1 and CRISPR3 loci (Fig. 1B). We observed that spacers mapped unevenly over the phage genome (Fig. 2A).

Although CRISPR4 was recently shown to be biochemically active in vitro (11, 12), and the associated Cas proteins have been induced in the presence of phages (13), spacer acquisition has not been reported previously. Intriguingly, none of the 71 new spacer sequences added to CRISPR4 targeted phage 2972. However, one newly acquired CRISPR4 spacer targeted other *S. thermophilus* phages, hinting at the presence of several additional low-abundance phages in the experimental system. The majority of the CRISPR4 spacers targeted unidentified DNA.

In the control series (MOI-0), both CRISPR1 and CRISPR3 acquired one unique new spacer, although the culture was not inoculated with phage. These spacers target chromosomal regions. Host chromosomal targeting was also detected in the three other experimental series. Overall, 0.01%, 0.03%, and 0.02% of newly acquired spacers in series MOI-2A, MOI-2B, and MOI-10, respectively, targeted the host genome (consistent with a previous report of a frequency of ~4 × 10⁻⁴ self-targeting immunization events [10]), and each spacer was only detected at one time point.

Using information about host genome sampling depth and the number of spacers sequenced, we estimated the average length of each CRISPR array at each genomically sampled time point. Results showed that over the course of the experiments, locus length fluctuated, with selection of variants with up to ~11 new spacers in CRISPR1. Typically, CRISPR3 variants were estimated to have
had one or two newly acquired spacers, although evidence pointed to selection for variants with up to five new spacers at some time points. In contrast, the type IIIA CRISPR2 and CRISPR4 typically lacked newly acquired spacers (see Table S2 in the supplemental material). The four CRISPR loci did not exhibit wild-type parental CRISPR spacer loss, as previously documented (14, 15).

Sensitivity of the approach. A subset of spacers acquired in CRISPR1 and CRISPR3 in the MOI-2B and MOI-10 series, representing 13.5% of the total set of 160,000, did not target phage 2972, but instead had sequence identity to phage 2766, a closely related phage family used in other experiments in the same laboratory (see Table S3 in the supplemental material). The four CRISPR loci did not exhibit wild-type parental CRISPR spacer loss, as previously documented (14, 15).

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Phage mutations and mutation rates. In silico analyses revealed the presence of many fixed (100%) or nearly fixed (70 to 99%) single nucleotide polymorphisms (SNPs) in the phage genome (e.g., ~0.3% of sites over ~1,600 bacterial generations in the MOI-2B experiment). Notably, fixed SNPs were detected only in regions targeted by CRISPR spacers (proto-spacers) or in their associated proto-spacer adjacent motifs (PAMs) (Fig. 2B). Prior lower-scale experimental studies (16–18) had established that mutations in these regions allow the phage to evade the CRISPR-Cas machinery that is responsible for recognition and cleavage of the cognate phage sequence. The localization of fixed SNPs to exclusively proto-spacers and PAMs over the extended periods of time surveyed in this study demonstrates a strong and direct link between CRISPR targeting and phage genome evolution.

We calculated the rates of accumulation of CRISPR escape mutations in the phage genome in the MOI-2A, MOI-2B, and MOI-10 series experiments to be $4 \times 10^{-6}$, $1 \times 10^{-6}$, and $2 \times 10^{-6}$ substitutions per nucleotide per host generation, respectively. In comparison, the rate of SNP fixation in the host genome, calculated by comparing the genome sequence at the last time point to that of the reference genome draft, was $4 \times 10^{-7}$ and $8 \times$
10^{-10} fixed substitutions per nucleotide per generation for the MOI-2B and MOI-10 series, respectively. Intriguingly, not a single fixed SNP was observed in the host genome in the MOI-0 or the MOI-2A series (in which the phage was lost by day 35).

Previously, we noted that spacers that were anomalously highly represented in the CRISPR-Cas locus of *S. thermophilus* tended to be clustered on the phage genome (10). Here, we report a correlation between such regions and elevated rates of SNP fixation in the phage genome (Fig. 2C and D). Mutations that could enable phage to escape CRISPR silencing could occur in the PAM (19), the ~7-bp proto-spacer “seed” region adjacent to the PAM, which drives target sequence recognition and cleavage for the interference step (16, 19–21), or elsewhere in the proto-spacer region. In the large data set generated by this experiment, we noted that fixed SNPs statistically significantly accumulated in the PAMs relative to the nonseed proto-spacer region by factors of 1.86, 11.65, and 3.50 in the MOI-2A, MOI-2B, and MOI-10 experiments, respectively. SNPs were also preferentially fixed in the seed regions relative to the nonseed proto-spacer region by factors of 2.06, 7.05, and 2.22 in the MOI-2A, MOI-2B, and MOI-10 experiments, respectively (see Table S4 in the supplemental material).

**Reconstruction of phage genomes and recombination events.** A second mechanism of escape of CRISPR immunity was identified by comparing phage genomes reconstructed from metagenomic data with the parental viral genomes (the original 2972 phage and later-introduced phage 2766) (Fig. 3). At most time points, the samples were surprisingly dominated by phages with genomes that were chimeras of the 2972 and 2766 genotypes (Fig. 3 and 4). Strikingly, at least 16 different recombination boundaries were identified (see Table S5 in the supplemental material). Notably, the genome sequence blocks from phage 2766 replaced blocks of phage 2972 that were specifically highly targeted by the set of novel CRISPR spacers acquired in the host genome at that time point (Fig. 2 and 4).

A phylogenetic tree built using all the reconstructed phage genomes demonstrated the dynamic nature of the phage genomes over the time scales of the MOI-2A, MOI-2B, and MOI-10 experiments (Fig. 5) and allowed us to detect the presence of the second phage (2766) from early stages of the sampling for MOI-2B and MOI-10 (around day 4 and day 15, respectively).

**Phage loss and phage "rescue."** Because the assessment of the hypothesis that sustainable phage coexistence with a single host with active CRISPR-Cas systems requires the presence of multiple phage types, we conducted two additional experiments. First, we tested the hypothesis that phage loss was predictable in the single-phage MOI-2A experiment. To do this, we reinoculated samples archived from time points preceding phage loss and repeatedly observed the disappearance of the phage population (Fig. 6). In a second series of experiments, we added a second phage (recovered from the series MOI-2B_41) to a sample archived from a time point before the loss of the phage in the MOI-2A experiments to determine whether the second phage population could “rescue” the first. Results showed that an increase in phage genotypic diversity could extend the phage-versus-host “arms race” (see Fig. S1 in the supplemental material).

**DISCUSSION**

Although the CRISPR-Cas system has been extensively described as a resistance mechanism against phages, it has remained unclear how phage genomes evolve in response to CRISPR immunity. We measured phage evolutionary rates by applying metagenomics time-series analyses to samples from long-term experiments that included *S. thermophilus* DGCC7710 and phage 2972. Interestingly, we identified a migrant phage in two of the experimental
series. Such events probably occur commonly in long-term experiments but would normally be undetectable without long-term monitoring and deep sequencing. By implication, metagenomic analysis of host CRISPR spacer complements provided a sensitive method by which potentially very rare phages in the environment of a bacterial culture can be detected. This finding has implications for bacterial source tracking applications.

Within the inventory of over 160,000 spacers recovered across all the samples, we detected extensive novel spacer acquisition in the CRISPR1 and CRISPR3 loci, as well as rare acquisition events for the CRISPR4 locus, a novel type I system for which we observed activity for the first time. The fastest locus expansion rate occurred in CRISPR1, with addition of one spacer added almost every generation early in the experiment. Only one spacer every ~7 generations was added to CRISPR3 over the same time period. Both CRISPR2 and CRISPR4 typically lacked any newly acquired spacers, although 71 CRISPR4 spacers were detected across all the experiments. We attribute the unusual detection of spacer acquisition in CRISPR4 to the long experimental timescale and the deep sequencing used in this study. The bulk of the CRISPR4 spacers targeted unidentified DNA (probably trace milk-associated sequences). Spacers targeting the bacterial genome were also identified, but they were both rare and lethal (10, 22, 23).

We measured the rate of SNP fixation in the host genome by comparing the bacterial genome sequence at the last time point to that of the reference draft genome. The numbers of fixed substitutions per nucleotide per generation for the MOI-2B and MOI-10 series were very low (4 × 10⁻⁹ and 8 × 10⁻¹⁰, respectively). Thus, the host genome evolves much more slowly than the phage genome counterpart. Intriguingly, not even one fixed SNP was observed in the host genome in the MOI-0 or the MOI-2A series (from which the phage was lost by day 35 and no immigrant phage 2766 was present). This observation hints that the presence of phages may accelerate host genome evolution.

To ensure persistence, we postulated that phage genomes must circumvent spacer targeting at rates that are comparable to those of spacer acquisition. We noted that SNPs are preferentially fixed in the PAM relative to the nonseed proto-spacer region. This is consistent with previous findings (9, 19) and the observation that Cas9 first binds a PAM within the phage DNA prior to interrogation of adjacent phage DNA by the CRISPR spacer sequence (24). SNPs are also preferentially fixed in the seed regions relative to the nonseed proto-spacer regions. Given the role of the PAM in spacer sampling from the phage genome and in subsequent phage genome targeting and cleavage by Cas9, and also the significant role of the seed sequence for targeting (16, 20), this result further elaborates the strong link between CRISPR immunity and phage genome evolution demonstrated in the present study. The apparent
selection for phage genomes with PAM compared to “seed” mutations may arise because these mutations can prevent both spacer acquisition and interference (whereas seed sequence mutations only impact the latter).

The phage persistence in the two experiments that contained multiple phages, but not in the experiment with a single phage (MOI-2A) (Fig. 1), may not be a coincidence. We identified a second mechanism by which phage escape CRISPR immunity that involves formation of chimeric genomes (e.g., involving the 2972 and 2766 genotypes in experiments MOI-2B and MOI-10). Phage genome mosaicism has been widely reported (25), and a link between recombination and phage evasion of CRISPR immunity was suggested previously, based on metagenomic analysis of natural phage population sequence information (26). The findings of the current study directly constrain the rate at which selection for (potentially rare) recombination events can occur and experimentally demonstrate recombination as a mechanism by which phages specifically circumvent CRISPR-conferred immunity.

Phage loss was predictable and reproducible in experiments that contained only a single phage (MOI-2A experiment). The “tipping point” probably occurs when a sufficiently high level of host immune diversity is achieved. Addition of a second, distinct phage type (from the series MOI-2B_41) extended phage persistence and, thus, the time period of phage-host coevolution. The increased phage predation pressure may dilute the ability of *S. thermophilus* to assemble sufficient immunity to eliminate any single phage population. Because all these experiments eventually ended with phage loss (Fig. 1 and 6; see also Fig. S1 in the supplemental material), continual immigration of new phage populations may be important to extend the bacteria-phage arms race indefinitely in natural systems.

By directly and quantitatively linking phage genome modification to the selective pressure associated with CRISPR-Cas immunity, this study establishes CRISPR adaptive immunity of *S. thermophilus* as a fundamental driver of phage genome evolution. When present and active, CRISPR-mediated immunity influences
the accumulation of point mutations and selects for advantageous homologous recombination events between genetically related phages. The selective advantage of reduced susceptibility to CRISPR silencing could explain why recombination is a common evolutionary phenomenon in natural phage populations.

MATERIALS AND METHODS

Model system: overview of the experimental design, sampling scheme, and sequence analysis pipeline. *Streptococcus thermophilus* strain DGCC7710 and co-culture of DGCC7710 with phage 2972 were grown, incubated, transferred, and stored as described in reference 13. We used the procedures from this study for the determination of CFU and PFU and detection of phage 2766. The *Streptococcus thermophilus* host was cultured over time (MOI-0 series) or challenged with lytic phage 2972 at two different multiplicities of infection (host/phage ratios of 1:2 for the MOI-2 series [MOI-2A and MOI-2B] and 1:10 for the MOI-10 series) (Fig. 1). Then, samples were transferred daily as a 1% (vol/vol) inoculum. Host counts (in CFU per milliliter) and phage counts (in PFU per milliliter) were monitored over time, and samples obtained at various time points were subjected to DNA extraction (50-ng minimum) and subsequently used as the templates for library preparation and deep sequencing using 100-bp paired-ends sequencing with the Illumina system. (Selection of the time points was based on dynamically interesting growth curve fluctuations of culture/co-culture experiments distributed across time and covering a wide range of host:virus ratios.)

Metagenomic sequencing. DNA samples were subsequently used as the templates for library preparation and deep sequencing by using 100-bp paired-ends sequencing with the Illumina system. A total of 165 Gb of sequence data were generated and subjected to bioinformatic analyses, including host CRISPR locus spacer detection, host and phage genome assembly, and comparison with wild-type sequences for the host chromosome, phage 2972, and phage 2766 sequences. Finally, postassembly as well as comparative analyses were performed to identify SNPs, indels, and recombination events.

Computational analyses. Total genomic DNA (bacterium and phage) extracted at various time points from all experimental series was sequenced using Illumina high-throughput technology at the WM Keck Center for Comparative and Functional Genomics (University of Illinois at Urbana—Champaign) (for details, see Table S1 in the supplemental material). Reads were quality filtered by trimming both ends, using sickle (a program available at the GitHub, a Web-based Git repository hosting service) and deposited the sequence information with NCBI (see “Nucleotide sequence accession numbers,” below). Only paired reads were used in the assemblies. Assemblies were evaluated using idba_ud (27) and default parameters.

Phage genomes were reconstructed from each sample separately as follows. First, all reads that belonged to the genome of *S. thermophilus*
DGCC7710 were removed based on alignment to the reference genome using Bowtie (28). Remaining reads were assembled using Velvet (29) with parameters were adjusted to the expected coverage, considering the number of reads and the length of phage 2972. Only a subset of the reads was used when the expected coverage exceeded ~200×. For phage genome analysis, we used miassembly procedures described previously (30).

SNPs were identified by using a program that takes read mapping information as input and computes base calling for every position on the target genome. C++ source code for the program is available at https://github.com/CK7/SNPs.

Spacer sequences were extracted using a custom Ruby script (included in SOM) that searched for each of the exact repeat sequences from each of the CRISPR loci, as well as their reverse complement sequences in the full read set. We grouped the spacers that shared 85% in length and 85% sequence identity to avoid overrepresentation of spacer types due to sequencing errors.

Nucleotide sequence accession numbers. The sequences identified in this work have been deposited with NCBI under Bioproject number PRJNA275232 and SRA main accession number SRP055779.

SUPPLEMENTAL MATERIAL
Supplemental material for this article may be found at http://mbio.asm.orglookup/supp/doi:10.1128/mBio.00262-15/-/DCSupplemental.

Table S1, PDF file, 0.1 MB.
Table S2, PDF file, 0.1 MB.
Table S3, PDF file, 0.05 MB.

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