Viral Diversity Threshold for Adaptive Immunity in Prokaryotes

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ABSTRACT Bacteria and archaea face continual onslaughts of rapidly diversifying viruses and plasmids. Many prokaryotes maintain adaptive immune systems known as clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated genes (Cas). CRISPR-Cas systems are genomic sensors that serially acquire viral and plasmid DNA fragments (spacers) that are utilized to target and cleave matching viral and plasmid DNA in subsequent genomic invasions, offering critical immunological memory. Only 50% of sequenced bacteria possess CRISPR-Cas immunity, in contrast to over 90% of sequenced archaea. To probe why half of bacteria lack CRISPR-Cas immunity, we combined comparative genomics and mathematical modeling. Analysis of hundreds of diverse prokaryotic genomes shows that CRISPR-Cas systems are substantially more prevalent in thermophiles than in mesophiles. With sequenced bacteria disproportionately mesophilic and sequenced archaea mostly thermophilic, the presence of CRISPR-Cas appears to depend more on environmental temperature than on bacterial-archaeal taxonomy. Mutation rates are typically severalfold higher in mesophilic prokaryotes than in thermophilic prokaryotes. To quantitatively test whether accelerated viral mutation leads microbes to lose CRISPR-Cas systems, we developed a stochastic model of virus-CRISPR coevolution. The model competes CRISPR-Cas-positive (CRISPR-Cas+) prokaryotes against CRISPR-Cas-negative (CRISPR-Cas−) prokaryotes, continually weighing the antiviral benefits conferred by CRISPR-Cas immunity against its fitness costs. Tracking this cost-benefit analysis across parameter space reveals viral mutation rate thresholds beyond which CRISPR-Cas cannot provide sufficient immunity and is purged from host populations. These results offer a simple, testable viral diversity hypothesis to explain why mesophilic bacteria disproportionately lack CRISPR-Cas immunity. More generally, fundamental limits on the adaptability of biological sensors (Lamarckian evolution) are predicted.

IMPORTANCE A remarkable recent discovery in microbiology is that bacteria and archaea possess systems conferring immunological memory and adaptive immunity. Clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated genes (CRISPR-Cas) are genomic sensors that allow prokaryotes to acquire DNA fragments from invading viruses and plasmids. Providing immunological memory, these stored fragments destroy matching viral and plasmid DNA. CRISPR-Cas systems also provide adaptive immunity, keeping up with mutating viruses and plasmids by continually acquiring new DNA fragments. Surprisingly, less than 50% of mesophilic bacteria, in contrast to almost 90% of thermophilic bacteria and archaea, maintain CRISPR-Cas immunity. Using mathematical modeling, we probe this dichotomy, showing how increased viral mutation rates can explain the reduced prevalence of CRISPR-Cas systems in mesophiles. Rapidly mutating viruses outrun CRISPR-Cas immune systems, likely decreasing their prevalence in bacterial populations. Thus, viral adaptability may select against, rather than for, immune adaptability in prokaryotes.

Received 16 October 2012 Accepted 22 October 2012 Published 4 December 2012


Editor Stephen Giovannoni, Oregon State University

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serially target and incorporate 30- to 84-bp DNA fragments from invading viruses and plasmids between CRISPR repeat sequences (16–18). These CRISPR-incorporated fragments are known as “spacers,” whereas the corresponding viral or plasmid sequences are termed “protospacers.”

Spacers make CRISPR-Cas an adaptive immune system, immunizing bacterial and archaeal hosts against subsequent invasions by viruses or plasmids with matching protospacers (16–18). In many ways, analogous to the RNA interference system of eu-karyotes (19), spacer-mediated immunity is RNA guided. The CRISPR locus is first transcribed into a single long RNA sequence; this “pre-CRISPR RNA” is then cleaved into individual spacer repeat units by a complex of Cas proteins (20–24). Aided by additional Cas proteins, the single bound spacer senses and degrades cognate protospacers, inactivating invading viruses or plasmids (12, 18, 20, 22, 25–27). Viruses can evade CRISPR-Cas through minimal changes in targeted protospacer regions. In several experiments, single protospacer mutations have rendered CRISPR-Cas ineffectual (16, 28–30). Conversely, hosts have regained antiviral immunity through new spacer additions (28, 29, 31, 32), driving potential coevolutionary arms races between mutating virus and spacer-incorporating host.

Previously, we combined metagenomic time series data with a mathematical model to track the arms race between CRISPR spacer incorporation and viral protospacer mutation across a multiyear period in an acid mine drainage system (33). To focus on spacer/protospacer coevolution, the previous mathematical model assumed that all prokaryotes contained CRISPR-Cas loci. Similarly, the metagenomic reconstructions targeted CRISPR-Cas regions, limiting most of our analysis to CRISPR-Cas-positive (CRISPR-Cas+) hosts. In actuality, however, less than half of all sequenced bacteria contain CRISPR-Cas loci (15).

Here we investigate why only ~45% of sequenced bacterial genomes maintain CRISPR-Cas systems, in contrast to the over 90% of sequenced archaeal genomes that are CRISPR-Cas+. The relative dearth of bacterial CRISPR-Cas systems appears especially surprising given the extensive diversity of lytic viral bacteria (34, 35) against which CRISPR-Cas would be expected to provide critical adaptive immunity.

One potential driver of the dichotomous prevalence of CRISPR-Cas between bacteria and archaea could be that most sequenced archaea are thermophiles, whereas most sequenced bacteria are mesophiles. Recent biophysical models show that mutations are more likely to be lethal in thermophilic environments, because high temperatures reduce protein stability (36–40). With an increased cost to mutation, thermophilic genomes are predicted to have lower mutation rates than mesophilic genomes (38, 40). The results of several experiments match these predictions, reporting substantially reduced genomic mutation rates in archaeal and bacterial thermophiles (41–43). A further indicator of the increased cost of mutation in thermophiles is that the average ratio of nonsynonymous to synonymous substitutions, i.e., the dNd/s ratio, averaged across thousands of pairs of orthologous genes, drops from 0.14 in mesophiles to 0.09 in thermophiles (44).

These predictions and measurements indicate that viruses infecting thermophiles are afforded fewer viable protospacer mutations to evade CRISPR-Cas targeting. With viable viral mutation rates reduced, each CRISPR-incorporated spacer provides antiviral immunity for a longer period of time. Armed with more beneficial spacers, the entire CRISPR-Cas system would provide greater immunity in mutationally constrained thermophilic environments. We thus hypothesized that decreased viral mutation rates select for the increased presence of CRISPR-Cas in thermophiles, explaining the disproportionate presence of CRISPR-Cas in archaea.

To quantitatively test the hypothesis that decreased viral mutation rates increase the prevalence of CRISPR-Cas, we developed a population genetic model in which hosts with and without CRISPR-Cas compete under pressure from mutating, lytic viruses. CRISPR-Cas+ hosts serially acquire antiviral spacers, but CRISPR-Cas− hosts also comes with a parameterized fitness cost. Weighing the fixed fitness cost of CRISPR-Cas against its changing immunological benefit, the model calculates the evolutionary stability of CRISPR-Cas across the parameter space. In agreement with the thermophilicity hypothesis, simulations capture striking phase transitions in which CRISPR-Cas is highly prevalent at reduced viral mutation rates but eradicated once viral mutation rates surpass cost-dependent thresholds. Thus, increasing viral adaptability appears to depress host immune adaptability.

RESULTS

CRISPR-Cas is disproportionately present in bacterial and archaeal thermophiles. The basic premise of our thermophilicity hypothesis is that, by reducing viral mutation rates, increased environmental temperatures increase the prevalence of CRISPR-Cas. Thus, a high frequency of CRISPR-Cas is predicted for the minority of bacteria that are thermophiles. To test this prediction, we sampled a representative set of 383 bacterial and archaeal genomes from the collection of all fully sequenced prokaryotes (Materials and Methods). Only one sequence per genus was generally sampled, increasing statistical independence. We then analyzed the sampled genomes for the presence of putatively functional CRISPR-Cas loci, using established bioinformatics methods (45, 46).

In agreement with the prediction of the thermophilicity hypothesis, approximately 90% of bacterial thermophiles possess CRISPR-Cas systems, whereas only 46% of bacterial mesophiles are CRISPR-Cas+ hosts (Fig. 1, top). Archaeal thermophiles are also more likely to contain CRISPR-Cas than are archaeal mesophiles. Across all prokaryotes, thermophilicity and the presence of CRISPR-Cas are highly correlated (P < 10−11 by Fisher’s exact test). Multivariate logistic regression verifies that the strong correlation between thermophilicity and the presence of CRISPR-Cas exists independent of whether the thermophiles are archaea or bacteria (P < 10−6). In addition to the strong environmental correlation, there is a weak correlation between archaeal-bacterial taxonomic affiliation and CRISPR-Cas presence (P = 0.02). To test whether the presence of CRISPR-Cas hinges more strongly on thermophilic environment or on archaeal taxonomic affiliation, we used the Akaike information criterion (AIC), a model selection method (47). The AIC computes relative goodness of fit for statistical models, with a lower AIC indicating a better fit. Computing the AIC values shows that the presence of CRISPR-Cas is better predicted by thermophilic environment alone (AIC = 479) than by archaeal taxonomy alone (AIC = 509).

To further analyze the environmental dependence of CRISPR-Cas, we estimated the distribution of the number of spacers per CRISPR-Cas+ host in mesophilic and thermophilic environments (Fig. 1, bottom; see Fig. S1 in the supplemental material). On average, thermophiles possess a greater number of CRISPR...
(iii) Step 3. CRISPR-Cas+ hosts can lose CRISPR-Cas or delete spacers, and CRISPR-Cas− hosts can gain CRISPR-Cas. Independent of the virus-host encounters and mutations in steps 1 and 2, all host strains can undergo homologous recombination or plasmid-driven HGT. This leads to spacer deletions and acquisitions and losses of entire CRISPR-Cas systems. When a CRISPR-Cas+ host is chosen to delete spacers, two random spacers in its CRISPR locus are sampled. The new mutant deletes all spacers between the two chosen spacers. When a CRISPR-Cas+ host loses CRISPR-Cas, the entire CRISPR-Cas locus is deleted, with all attendant spacers. Finally, when a CRISPR-Cas− host acquires CRISPR-Cas by HGT, the CRISPR-transferring donor strain is chosen by randomly sampling all host strains. If the chosen donor strain lacks CRISPR-Cas, the mutant receives a functional CRISPR-Cas locus but no spacers. Thus, the model continually reintroduces CRISPR-Cas into CRISPR-Cas− populations, testing the stability of CRISPR-Cas− populations to mutational invasion by CRISPR-Cas+ strains. Accordingly, at each point in the parameter space, we can average the prevalence of CRISPR-Cas across large numbers of iterations, instead of averaging the results of many independent simulations (the continual reintroduction of CRISPR-Cas fits an ergodic assumption). As in step 2, all host mutants are generated independently at an initial abundance of 1.

(iv) Step 4. Selection for immune hosts and infective viruses. Selection modulates the frequencies of host and viral strains according to the fitness functions defined below. This frequency adjustment is performed only for the “parent” strains that under-
went the virus-host encounters in step 1: mutants from steps 2 and 3 remain at frequencies of 1/N, where N is the respective host or viral population size.

To increase in fitness, viruses need to productively infect hosts, whereas hosts need to immunize themselves without paying too high a fitness cost. Because viruses mutate only in productive encounters and hosts incorporate spacers only if they survive infection, mutations are concentrated in immune hosts and infective viruses. In the next step, more mutations can occur if CRISPR-Cas+ hosts delete spacers or lose entire CRISPR-Cas loci. Similarly, CRISPR-Cas− hosts can acquire CRISPR-Cas loci. In the fourth step, selection modifies strain frequencies according to equations 1 and 2 in the text. Finally, each iteration ends with the model randomly sampling approximately fixed numbers of hosts and viruses.

FIG 2  Model of virus-host coevolution. Schematic of a representative model iteration. Host and viral populations are divided into strains labeled B, and B', (B stands for bacteria) and Vi and V'j (V stands for virus), respectively. Host strains are shown as ovals and viral strains as hexagons; bars within the strains reflect host spacers and viral protospacers. Identical spacer and protospacer sequences have the same colored bar. Host strains lacking CRISPR-Cas are shown as empty ovals. In the first step of a model iteration, host and viral strains encounter one another. In the next step, mutations occur (single and double quotation marks). Because CRISPR-Cas hosts only in productive encounters and hosts incorporate spacers only if they survive infection, mutations are concentrated in immune hosts and infective viruses. In the next step, more mutations can occur if CRISPR-Cas+ hosts delete spacers or lose entire CRISPR-Cas loci. Similarly, CRISPR-Cas− hosts can acquire CRISPR-Cas loci. In the fourth step, selection modifies strain frequencies according to equations 1 and 2 in the text. Finally, each iteration ends with the model randomly sampling approximately fixed numbers of hosts and viruses.
(for viral mutants) where $N_v$ stands for the total virus population, is then deducted from the frequency of the parent strain. When the frequency of a parent strain falls below 0, it is cleared from the model.

(v) **Step 5. Sampling.** Collecting all parent and mutant strains from the previous steps, we multiply the host and viral strain frequencies by the total population sizes, $N_h$ and $N_v$, of the host and viral populations. This yields an abundance for each strain (with an abundance of 1 for each mutant). We then sample (with replacement) an average of $N_h$ hosts and $N_v$ viruses. Sampled hosts and viruses remain in the model, whereas unsampled strains are removed.

Sampling mimics genetic drift by challenging new mutants that arise at the low abundance of 1 with stochastic extinction, regardless of their fitness. Further, sampling randomly removes old strains which selection has reduced in abundance. Thus, mutation creates diversity, whereas selection and sampling limit diversity, allowing the model to implement mutation-selection-drift balance.

Finally, at the end of an iteration, host and viral strain frequencies are renormalized to ensure that both sum to 1. The model then returns to step 1 in all but the final iteration.

**Cost-benefit threshold for CRISPR-Cas systems.** To analytically derive the dependence of CRISPR-Cas prevalence on experimentally measurable parameters, we calculated when CRISPR-Cas is under positive selection in a given model iteration. According to equation 1, the selection equations set the frequency of a host strain to be its fraction of all immune encounters, and CRISPR-Cas+ strains have their immune encounters reduced by a cost. Thus, the postselection prevalence of CRISPR-Cas is the sum of the cost-reduced fractions of immune encounters for all CRISPR-Cas+ host strains. When the postselection prevalence of CRISPR-Cas is greater than its preselection prevalence, CRISPR-Cas is under positive selection in the model. As derived in the supplemental material, CRISPR-Cas is under positive selective when:

$$C < f_{\text{BC}} \left( \frac{1}{P_0} - 1 \right)$$

Here $C$ is the fitness cost of maintaining CRISPR-Cas, whereas $f_{\text{BC}}$ is the probability that a randomly chosen CRISPR-Cas+ host strain shares at least one spacer with a randomly chosen viral strain. Given the extremely small failure rate measured for CRISPR-Cas systems (16, 30), this is effectively the probability of CRISPR-Cas providing immunity in a model iteration. Conversely, $P_0$ is the fraction of viral encounters that a host strain survives when CRISPR-Cas is absent or fails.

Because $f_{\text{BC}}$ is a nonlinear function dependent on all model parameters, equation 3 is not in itself predictive. However, $f_{\text{BC}}$ can be divided into measurable components, yielding a predictive threshold for CRISPR-Cas in a simplified setting. For simplicity, we assume that the CRISPR-Cas+ strains initially lack spacers against the current viruses and that the number of protospacers per virus is one.

Lacking protective spacers in advance, $f_{\text{BC}}$ is the combined probability that a CRISPR-Cas+ host survives viral infection due to innate resistance, adds a spacer, and then encounters a virus with a protospacer matching the acquired spacer. With the three events independent, $f_{\text{BC}} = (P_0)(P_{\text{add}})(\Sigma_i \Sigma_j s_i s_j)$, where $P_0$ is the probability of innate resistance and $P_{\text{add}}$ is the probability of adding a spacer in an immune encounter ($P_0$). $P_{\text{add}}$ is thus the probability that a spacer addition occurs. The probability of the acquired protospacer being protospacer $i$ is $s_i$, where $s_i$ is the fraction of viruses containing protospacer $i$. Because the next virus encountered will also contain protospacer $i$ with probability $s_i$, once a spacer addition occurs, $\Sigma_i s_i^2$ is the probability that the next virus encountered has the same protospacer. Critically, the $s_i$ values (i.e., the protospacer frequencies) are directly measurable in both laboratory and metagenomic samplings.

In addition to being measurable, $\Sigma_i s_i^2$ has an immediate connection to a well-studied measure of population diversity known as the Simpson diversity index $D$ (56) that is defined to equal $1 - \Sigma_i s_i^2$, i.e., the probability that two randomly sampled protospacers are distinct. Combining equation 3 with the decomposition of $f_{\text{BC}}$, in the simplified model setting, selection promotes CRISPR-Cas+ strains when:

$$C < (P_{\text{add}})(1 - D)(1 - P_0) \quad (4)$$

Two key predictions arise from this inequality. First, equation 4 is a cost-benefit threshold that quantifies when the cost of CRISPR-Cas is less than the immunological benefit conferred by CRISPR-Cas. The immunological benefit of CRISPR-Cas reflects its ability to acquire protospacers shared by many viruses together with the likelihood that competing, innate resistance mechanisms are nonprotective. Second, this inequality predicts that, if viral diversity is sufficiently high, CRISPR-Cas cannot rise in frequency. As protospacer diversity gets large, $1 - D$ approaches 0, whereas $P_{\text{add}}$ and the $1 - P_0$ term cannot surpass 1. Thus, for any nonnegligible cost, high viral diversities are predicted to purge CRISPR-Cas from a population, irrespective of the spacer addition rate.

**CRISPR-Cas emerges only at intermediate levels of innate resistance.** Inequalities 3 and 4 appear to imply that increasing the probability of innate resistance ($P_0$) decreases the selective advantage of CRISPR-Cas (i.e., reduces the maximal cost at which CRISPR-Cas can be maintained). However, this is not always the correct interpretation. Increasing $P_0$ also decreases viral diversity ($D$) by decreasing the frequency of productive encounters in which the viruses can mutate. Thus, when increasing $P_0$ increases the $1 - D$ term more than it decreases the $1 - P_0$ term, increasing $P_0$ actually promotes CRISPR-Cas+ strains. Increasing innate immunity offers a second advantage to CRISPR-Cas by providing more immune encounters to prime the CRISPR locus with spacers against new viruses.

While initial increases in innate immunity promote CRISPR-Cas+ strains, inequalities 3 and 4 show that CRISPR-Cas+ strains will be selected against when $P_0$ increases to become close to 1. Inequality 3 shows that even perfect CRISPR-Cas systems, with immunity against 100% of the viruses, cannot evolve when $P_0 > 2/3$ (at the parameterized CRISPR-Cas cost of 0.5). This is because there is no benefit to maintaining a costly CRISPR-Cas system when the cost-free alternative (innate immunity) provides almost complete protection.

With simple analytics implying that innate immunity has competing effects on the evolution of CRISPR-Cas, we designed a model simulation to directly probe the prevalence of CRISPR-Cas as a function of the level of innate immunity (see Fig. S2 in the supplemental material). The model simulations confirm that innate immunity must be increased above a basal, “priming” threshold to maintain CRISPR-Cas. Similarly, CRISPR-Cas loci are lost...
from populations at extremely high levels of innate resistance. Only at intermediate levels of innate immunity does CRISPR-Cas dominate populations.

**High viral mutation rates overwhelm CRISPR-Cas systems.**

While the prevalence of CRISPR-Cas nonmonotonically depends on the probability of innate immunity, inequalities 3 and 4 predict a simpler dependence of the prevalence of CRISPR-Cas on the level of viral mutation. Increasing the viral mutation rate lowers the probability that a host’s spacers match future viral protospacers, directly decreasing the benefit of CRISPR-Cas. To test whether this decreased immunological benefit purges CRISPR-Cas from host populations, we simulated the model across thousands of iterations at gradually increasing viral mutation rates.

At low viral mutation rates, all hosts maintain CRISPR-Cas immunity (Fig. 3a; see Fig. S3 in the supplemental material). Because viral diversity is depressed at low viral mutation rates (Fig. S3 and Fig. S4), few spacers are required to provide immunity against the entire viral population. With spacer deletion outpacing spacer addition in the model (see Table S1 in the supplemental material), CRISPR loci delete all but the few antiviral spacers that selection maintains. The CRISPR loci are thus kept small at low viral mutation rates. As the viral mutation rate increases, CRISPR loci gradually increase in length, requiring more and more spacers to maintain immunity against an increasingly diverse viral population. The model predicts that CRISPR loci contain hundreds of spacers per locus at intermediate viral mutation rates, matching the largest experimentally observed CRISPR-Cas systems (Fig. 1, bottom). Further increases in the rate of viral mutation cannot be matched with further increases in CRISPR lengths. Beyond a viral mutation rate threshold, average locus lengths plunge to zero and CRISPR-Cas is purged from populations. Thus, viral mutation overwhelms the CRISPR-Cas system.

Before the viral mutation rate $P_{v\text{-}mut}$ is increased to the point that it purges CRISPR-Cas from host populations, an intermediate viral mutation regime emerges ($0.001 < P_{v\text{-}mut} < 0.003$) in which the average prevalence of CRISPR-Cas is often strictly between 0 and 1 (Fig. 3a). Two explanations for this intermediate CRISPR-Cas prevalence are conceivable: either mixed CRISPR-Cas+ and CRISPR-Cas− populations coexist in individual iterations, or the model oscillates between entirely CRISPR-Cas+ and entirely CRISPR-Cas− iterations, yielding an intermediate time-averaged prevalence. To discriminate between these cases, we analyzed all individual iterations of the 600 simulations in which $0.001 < P_{v\text{-}mut} < 0.003$. Only 0.2% of the individual model iterations contained mixed CRISPR-Cas+ and CRISPR-Cas− populations (see Fig. S5 in the supplemental material). Thus, at intermediate viral mutation rates (i.e., the separatrix points), hosts occasionally undergo rapid phase transitions between 100% CRISPR-Cas− and 100% CRISPR-Cas+ states (Fig. S4, middle panel). Across thousands of model iterations, the average prevalence of CRISPR-Cas can thus fall anywhere between 0 and 1, depending on the residence times at the CRISPR-Cas+ and CRISPR-Cas− quasi-steady states.

The viral mutation parameter probed above is not the sole determinant of viral diversity. Because viral mutants emerge only in productive virus-host encounters, the probability of a viral mutation is the product of the parameterized viral mutation rate and the probability that virus-host encounters are productive. We denote this product the “effective” viral mutation rate. Similarly, the effective host spacer addition rate is the product of the host spacer addition rate and the probability that a random virus-host encounter is immune (nonproductive). Plotting the effective viral mutation rate and the effective spacer addition rate as functions of the parameterized viral mutation rate reveals an initial inverse symmetry: as the viral mutation parameter increases, slow increases in the effective viral mutation rate match slow decreases in the effective host spacer addition rate (Fig. 3b). These changes in the effective adaptation rates are initially buffered because a still-functioning CRISPR-Cas system keeps most encounters immune (see Fig. S3 in the supplemental material). However, when the viral mutation parameter increases to an intermediate level, a narrow regime emerges in which both the effective spacer addition rate and effective viral mutation rate are nonnegligible. This is the regime of most intensive coevolution, in which hosts frequently
reflect coexisting CRISPR-Cas 0% CRISPR-Cas efficiently high (the cost and the viral mutation rate (Fig. 4). track the average prevalence of CRISPR-Cas as a function of both immunity and cost. We thus ran new simulations to

Although Fig. 3 shows that CRISPR is lost at high viral mutation rates shows that all but a constant (innate immunity) fraction of encounters are productive absent CRISPR-Cas. 

**High costs and rapid viral mutation eradicate CRISPR-Cas.** Although Fig. 3 shows that CRISPR is lost at high viral mutation rates due to the loss of antiviral immunity (i.e., benefit), inequalities 3 and 4 predict that the prevalence of CRISPR-Cas is a function of both immunity and cost. We thus ran new simulations to track the average prevalence of CRISPR-Cas as a function of both the cost and the viral mutation rate (Fig. 4).

As shown in Fig. 4a, when the cost of CRISPR-Cas is sufficiently high ($C > \sim 8$), CRISPR-Cas cannot persist for any viral mutation rate. Matching these simulations, inequality 3 shows that the maximal cost at which even 100% immunogenic CRISPR-Cas systems can evolve is $C = 9$ (with $P_c$ parameterized to equal 0.1). Conversely, at very low costs, CRISPR-Cas will be maintained in populations, even for the high viral mutation rates at which CRISPR-Cas provides almost no immunity (Fig. 4b). Thus, sufficiently increasing either the cost or the viral mutation rate takes host populations from entirely CRISPR-Cas + to entirely CRISPR-Cas −.

To better understand how the loss of CRISPR-Cas both drives and is driven by increased viral diversity, we also tracked how viral diversity varies with the viral mutation rate and CRISPR-Cas cost (Fig. 4c). To quantify viral diversity, the Shannon diversity index (56) of the viral protospacers was calculated during each model iteration. Similar to Simpson’s diversity index, the Shannon index reflects the unpredictability of a randomly chosen viral protospacer. Mathematically, the Shannon index is defined to equal $-\sum_i s_i \log(s_i)$, where $s_i$ denotes the fraction of viruses containing protospacer $i$. Providing a reliable metric of viral diversity, the Shannon index sums to 0 when the viruses are all identical (e.g., in the absence of viral mutation). The Shannon index then increases as increasing viral mutation diversifies the viral protospacer population (Fig. 4c). Importantly, the Shannon index can also increase when the viral mutation rate is kept constant. This occurs when the cost of CRISPR-Cas is increased to the point that CRISPR-Cas is purged from host populations, offering the viruses new productive encounters in which to mutate (Fig. 4c).

**Rapid spacer addition cannot preserve CRISPR-Cas at high viral mutation rates.** One might expect that CRISPR-Cas systems can maintain immunity against rapid viral mutation by simply incorporating spacers at a higher rate. To test whether accelerated spacer addition can preserve CRISPR-Cas loci at high viral muta-
tion rates, we systematically tracked CRISPR-Cas prevalence as a function of both the viral mutation rate and the host spacer addition rate. While viral mutation rates are kept low, CRISPR-Cas increased spacer addition maintains CRISPR-Cas immunity against increased viral mutation. However, once the rate of viral mutation surpasses a (cost-dependent) threshold, CRISPR-Cas is purged from host populations even when the rate of spacer addition far outpaces the rate of viral mutation (see Fig. S6 in the supplemental material). With hosts unlikely to encounter the same viral protospacers twice, increasing the rate of spacer addition is of little benefit.

**DISCUSSION**

Despite the ubiquity of lytic prokaryotic viruses, less than 50% of bacteria maintain CRISPR-Cas adaptive immune systems. Here we formulate a testable hypothesis to explain the relative dearth of adaptive immunity in bacteria. Using comparative genomics, we first report that the absence of CRISPR-Cas in bacteria is highly temperature dependent. While the majority of bacteria are mesophilic and contain CRISPR-Cas at the relatively low prevalence of 45%, bacterial thermophiles are 88% CRISPR-Cas+. Both theory and experimental results indicate that mesophilic genomes possess higher mutation rates than thermophilic genomes (38, 41–44). We wondered whether the increased viral mutation rates of mesophiles were sufficient to explain the low prevalence of CRISPR-Cas in mesophilic environments. To test this hypothesis, we developed an evolutionary model to analyze how the prevalence of CRISPR-Cas varies as the viral mutation rate and other basic parameters are varied. Model analytics and simulations support the viral mutation hypothesis, capturing how CRISPR-Cas is purged from host populations as viral mutation rates increase above cost-dependent thresholds. By mutating rapidly, viruses undermine the key benefit of CRISPR-Cas, immunological memory. In other words, hosts gain little fitness advantage from CRISPR-Cas storing viral sequences never again encountered.

Although our theoretical model shows that increased viral mutation rates are sufficient to explain the reduced prevalence of CRISPR-Cas in mesophilic bacteria, other hypotheses are plausible. For example, CRISPR-Cas might be more beneficial in thermophilic environments because high temperature settings might be closed off from their surroundings with limited inflow of new, diverse viruses. This viral immigration hypothesis is essentially equivalent to the viral mutation hypothesis: increasing immigration rates will have the same qualitative effect as increasing mutation rates. We focus on mutation rather than immigration because mutation rates are readily measurable in the laboratory and because previous data have already measured reduced thermophilic mutation. Another counterhypothesis might argue that unique genetic barriers specifically inhibit acquisition of CRISPR-Cas by bacteria. However, CRISPR-Cas is commonly found on mobile plasmids and widely distributed in diverse bacteria and archaea (15), undermining this argument. Finally, increased CRISPR-Cas costs, rather than decreased immunological benefits, might be implicated in the reduced frequency of CRISPR-Cas in mesophiles. These cost-driven hypotheses are compatible with the results of our model. Figure 4 shows that both high costs and high viral mutation rates purge CRISPR-Cas from populations.

One recent study suggesting an increased cost for CRISPR-Cas in mesophiles reports that bacterial CRISPR-Cas loci have a disproportionate number of self-targeting spacers in comparison to archael CRISPR-Cas loci (57). Thus, increased autoimmune costs might limit CRISPR-Cas in mesophilic bacteria. However, unlike viral mutation rates, one wonders why the frequency of self-targeting spacers would be temperature dependent. An alternative explanation for the high prevalence of self-targeting spacers in mesophiles is that they represent the effects, not the causes, of CRISPR-Cas failure at moderate temperatures. Self-targeting spacers might indicate bacteria abandoning the immune function of CRISPR-Cas, arguably because it fails to provide robust antiviiral immunity in mesophiles, instead coopting CRISPR-Cas for RNA interference (RNAi)-like gene regulation. Two studies have already addressed this possibility, with differing conclusions (53, 58), making further investigations required.

A similar cost-driven hypothesis assumes that mesophiles more frequently require DNA uptake via HGT, which CRISPR-Cas can block. Thus, the HGT hypothesis argues that mesophiles disproportionately lack CRISPR-Cas to disproportionately acquire HGT. However, there is little evidence for reduced HGT in thermophilic communities. Genomic screens have captured frequent genetic transfer among thermophiles, even between archaea and bacteria (59). Further, a basic assumption of the HGT hypothesis is that CRISPR-Cas actually blocks significant amounts of beneficial HGT in nature. Although a previous study has captured a dearth of CRISPR-Cas within *Enterococcus faecalis* strains with horizontally acquired drug resistance modules (55), no inverse CRISPR-HGT correlation has been shown at the interspecies scale in which demographic biases are better accounted for. Among the 383 species studied in this work, we found no significant difference in the presence of plasmids between the CRISPR-Cas+ and CRISPR-Cas− genomes (*P* = 0.27 by Fisher’s exact test). In fact, a recent study reports a positive CRISPR-HGT correlation, finding an increased prevalence of CRISPR-Cas systems in competent bacteria than in noncompetent bacteria (58). Future studies will need to disentangle what correlation, if any, exists between CRISPR-Cas and HGT.

Whether a CRISPR-HGT anticorrelation exists, an important evolutionary issue must be resolved. Unlike spacers that protect against deadly viruses, there seems to be no selective benefit to acquiring spacers that block beneficial plasmids. Thus, the experimental studies demonstrating that CRISPR-Cas blocks beneficial HGT are often forced to artificially engineer CRISPR-Cas loci with the deleterious spacers blocking critical plasmids and DNA. It is worth asking whether these deleterious spacers would naturally rise to high frequencies and thus present real costs to CRISPR-Cas+ hosts in nature. Either way, these experimental studies find little selection against CRISPR-Cas+, spacer controls, implying that beneficial HGT may select against spacers but not the CRISPR-Cas system.

The present hypothesis assumes that thermophilic viruses have reduced mutation rates, although previous experiments noting reduced thermophilic mutation have tracked only the mutation rates of thermophilic hosts (41–44). Our claim is premised on the fact that both thermophilic host and virus share the same environmentally driven mutational constraints. Supporting this assumption, in mesophilic environments, host and virus have been measured to have virtually identical per-genome mutation rates (60). With thermophilic hosts measured to have mutation rates an order of magnitude lower than those measured for both mesophilic host and virus (42), we infer that thermophilic viruses also possess reduced mutation rates. Further, a data-driven biophysical study
limits plasticity than mesophilic viruses do (38).

Thus, the results of initial experimental and genomic assays are compatible with many of the assumptions of our model. However, to show that in nature viral diversity is limited at high temperatures, better metagenomic resolution is required. Fortunately, next-generation deep-sequencing methods should enable more-detailed gauges of viral nucleotide diversity as a function of temperature. Moreover, the prediction of our model that viral mutability overwhelms CRISPR-Cas is directly testable in the laboratory through challenge experiments with increasingly mutagenized viruses.

Beyond offering a testable hypothesis for the absence of CRISPR-Cas in many bacteria, this work has a more general evolutionary implication. At an abstract level, CRISPR-Cas is a genomic sensor that seeks to directly acquire beneficial mutations in response to a stochastically changing environment (i.e., the virome). Studying the prevalence of CRISPR-Cas can thus provide insight into the conditions under which Lamarckian, directed adaptation is favored in evolution. Seminal analytic work by Kussell and Leibler (61) provides the required mathematical framework by analytically deriving a sensor cost threshold above which genomic sensors are deleterious to their hosts. Surprisingly, Kussell and Leibler’s threshold predicts that the sensor cost threshold increases as the Shannon diversity index (i.e., entropy) of the environmental states increases. In other words, the more a cell requires a sensor because of environmental unpredictability, the more a cell can pay for the sensor. Thus, the results of Kussell and Leibler are opposite to the conclusions that we derive from inequalities 3 and 4 and obtain in the simulations.

To explain the dichotomy between the predictions of our model and those of Kussell and Leibler, we note that the assumptions of Kussell and Leibler are unlikely to apply to adaptive immune systems such as CRISPR-Cas. For analytic tractability, Kussell and Leibler required a model in which the environment remains constant while the population adapts to it. Rapid viral mutation is likely to render this separation of time scales inapplicable in the context of virus-host coevolution. More importantly, Kussell and Leibler’s model assumes that sensors always perfectly adapt to the environment, whatever the environmental entropy. In our model, the efficacy of the sensor is directly reduced by increased environmental entropy (Fig. 4b). Thus, when sensor performance hinges on the difficulty of the sensing task at hand (i.e., environmental entropy), we infer inversion of the predictions of Kussell and Leibler. Future work will aim to capture how this phase transition arises as the assumptions of immediate and perfect sensor performance are relaxed.

A final question can be posed. If CRISPR-Cas sensors are unable to confer antiviral immunity against high levels of viral diversity, why have bacteria and archaea been unable to evolve fitter alternatives over billions of years? In contrast, in just about 500 million years, vertebrates have evolved an adaptive immune system that prefabricates immunity against virtually any viral variant. In principle, an analogous preemptive system could have evolved in prokaryotes, with CRISPR-Cas systems generating unlimited repertoires of random spacers, while keeping in place the necessary Cas and genetic machinery to target and cleave matching foreign sequences. However, no prokaryote is known to possess a genome larger than 13 Mb (62). With more than 50 bp contained in each spacer repeat unit, the vertebrate mode of preemptive adaptive immunity is unlikely to be feasible in compact single-celled microbes. With no way to fit billions of randomly generated spacer sequences in a single prokaryotic cell, perhaps the best microbes can do is to adaptively chase the diversifying viral population, trying to stay apace.

MATERIALS AND METHODS

Comparative genomics of CRISPR-Cas. Bacterial and archaeal genome sequences were downloaded from the NCBI FTP site ftp.ncbi.nih.gov/ genomes/Bacteria/ in March 2010. At that time, 978 bacterial and 77 archaenal genomes were available. A representative set of 383 genomes (45) used in this work includes the largest genome from each genus (as defined by the NCBI taxonomy database) with greater than 500 annotated protein-coding genes. Exceptions were made for the genus Shigella that was considered to be identical to Escherichia and the genera Escherichia and Bacillus that also included the model genomes Escherichia coli strain K-12 substrain MG1655 and Bacillus subtilis strain 168. Ecological information (environment and growth temperature) was obtained from the NCBI Complete Microbial Genomes Web page (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi). cas genetic loci were identified using the PSI-BLAST profiles (45), while the number of CRISPR repeats was determined using the PILER-CR program (46). Statistical analyses of the data were performed in R version 2.14.

Mathematical model. The mathematical model (see the supplemental material for the full algorithm) was programmed in MatLab. To probe multidimensional parameter space, thousands of simulations were run in parallel on NIH’s Helix compute cluster and Harvard Medical School’s Orchestra cluster.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00456-12/-/DCSupplemental.

Figure S1, PDF file, 0.1 MB.
Figure S2, PDF file, 0.1 MB.
Figure S3, PDF file, 0.1 MB.
Figure S4, PDF file, 1 MB.
Figure S5, PDF file, 0.1 MB.
Figure S6, PDF file, 0.1 MB.
Table S1, DOC file, 0.1 MB.
Text S1, DOC file, 0.1 MB.

ACKNOWLEDGMENTS

We are grateful to David Kristensen, Sergey Kryazhimskiy, Mateusz Pluciński, Eugene Shakhnovich, and Leor Weinberger for modeling and statistical insights, to Azat Badretdin for help identifying CRISPR-Cas loci in the sequenced genomes and to Chanan Reitblat and Adriana Schulz for expert technical assistance. We are grateful for the financial support from NIH grant AI072360 (to M.S.G.) and an NIH F32 Postdoctoral Fellowship (to A.D.W.). A.E.L., Y.I.W., and E.V.K. were supported by intramural funding from the U.S. Department of Health and Human Services (National Library of Medicine, NIH). We thank the Kavli Institute of Theoretical Physics (KITP) for hosting a cross-disciplinary workshop at which this collaboration began. We declare that we have no conflicts of interest.

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