Report

Current Biology

Parasite Exposure Drives Selective Evolution of Constitutive versus Inducible Defense

Highlights

- Constitutive and inducible defenses evolve at the two extremes of parasite exposure
- Surface modification of bacteria has a constitutive (fixed) fitness cost
- CRISPR-Cas has an inducible (infection-dependent) fitness cost
- Successful prediction of ecological conditions where CRISPR-Cas immunity evolves

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In Brief

Hosts have many defenses that protect against parasites. In nature, two distinct defense strategies are found: constitutive defenses (always active) and inducible defenses (triggered by parasites). Here, Westra et al. show that parasite exposure drives the evolution of these two distinct strategies.

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Report

Parasite Exposure Drives Selective Evolution of Constitutive versus Inducible Defense

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SUMMARY

In the face of infectious disease, organisms evolved a range of defense mechanisms, with a clear distinction between those that are constitutive (always active) and those that are inducible (elicited by parasites) [1]. Both defense strategies have evolved from each other [2], but we lack an understanding of the conditions that favor one strategy over the other. While it is hard to generalize about their degree of protection, it is possible to make generalizations about their associated fitness costs, which are commonly detected [3–5]. By definition, constitutive defenses are always "on," and are therefore associated with a fixed cost, independent of parasite exposure [4, 5]. Inducible defenses, on the other hand, may lack costs in the absence of parasites but become costly when defense is elicited [6] through processes such as immunopathology [7]. Bacteria can evolve constitutive defense against phage by modification/masking of surface receptors [8, 9], which is often associated with reduced fitness in the absence of phage [10]. Bacteria can also evolve inducible defense using the CRISPR-Cas (clustered regularly interspaced short palindromic repeat, CRISPR associated) immune system [11], which is typically elicited upon infection [12–14]. CRISPR-Cas functions by integrating phage sequences into CRISPR loci on the host genome [15]. Upon re-infection, CRISPR transcripts guide cleavage of phage genomes [16–20]. In nature, both mechanisms are important [21, 22]. Using a general theoretical model and experimental evolution, we tease apart the mechanism that drives their evolution and show that infection risk determines the relative investment in the two arms of defense.

RESULTS AND DISCUSSION

To examine factors that can explain the relative investment in constitutive and inducible defenses, we constructed a mathematical model of hosts and obligately killing parasites that incorporates the evolution of immunity and epidemiology. Our model relies on two assumptions concerning the costs associated with immunity: (1) constitutive defense is associated with a fixed (constitutive) cost that is independent of parasite levels and (2) inducible defense is associated with an infection-induced cost that increases with increasing frequencies of infection. This is incorporated in our model as follows: constitutive defense prevents infection and comes with a constitutive cost such that high defense reduces host birth rate. Inducible defense increases recovery rate and involves an induced immunopathological cost such that increased defense increases death rate.

First, we investigated how resource availability affects the relative investment in constitutive and inducible defenses. Resource availability is an ecologically relevant factor that, among a range of possible effects, impacts population density, which in turn impacts parasite abundance. Evolutionary analysis (see the Supplemental Experimental Procedures) clearly shows that constitutive defense is selected for in high resource environments, whereas inducible defense is favored at low resources (Figure 1A). We hypothesize that this effect emerges from the epidemiology of the system with more infections under nutrient-rich conditions, which leads to higher emergent costs to inducible defenses. As such, our model, along with predictions of more specific previous theory [23, 24], suggests that infection risk may be a general mechanism that determines the relative investment in constitutive and inducible defense.

We tested this theoretical prediction using bacteria and their phages. As a model system we used *Pseudomonas aeruginosa* PA14, which has a type I-F CRISPR-Cas (clustered regularly interspaced short palindromic repeat, CRISPR associated) system (Figure S1A) [25], and phage DMS3vir [26], a mu-like phage [27]. First, we examined the evolution of bacteriophage immunity mechanisms under high- and low-resource conditions. We challenged wild-type (WT) *P. aeruginosa* PA14 with 10^4 plaque-forming units (pfu) NTϕ (non-targeted phage; DMS3vir), which is not a priori targeted by the WT type I-F CRISPR-Cas system [26]. In agreement with the theoretical model, bacteria primarily evolved immunity by loss of the phage receptor (loss of the pilus; surface modification [*sm*]) in nutrient-rich medium (LB) at 3 days post-infection (dpi) but evolved immunity by CRISPR-Cas in nutrient-limited medium (M9 supplemented with 0.2% glucose) (Figure 1B).

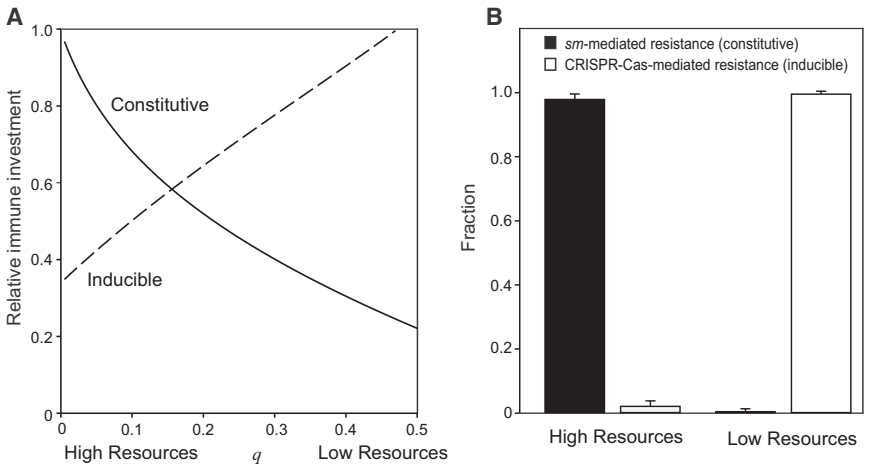

Figure 1. Resource Availability Determines the Evolution of Constitutive and Inducible Defenses

(A) High resources (low *q*) select for high constitutive defense, whereas poorer resources select for lower constitutive and higher inducible defense.

(B) Fraction of WT that acquired *sm*-mediated immunity and CRISPR-Cas-mediated immunity at 3 dpi with 10^4 pfu NTϕ (n = 6 in all treatments). Error bars correspond to 95% confidence intervals (CIs). Bacteria were grown in LB (high resources) or M9 (low resources).

See also Figure S1.

To formally check for CRISPR-mediated immunity, we sequenced both CRISPR loci (CRISPR1 and CRISPR2) of 144 independent WT clones that were infected in nutrient-limited medium with 10^4 pfu NTϕ and that were isolated at 3 and 7 dpi (from six replicate experiments). CRISPR2 was more active than CRISPR1 (Figures 2A–2C) and the number of acquired spacers increased over time (Figure 2C). Mapping of novel spacers onto the phage genome revealed clustering of protospacers around position 28,000 on the phage genome. Protospacers upstream and downstream of this position were typically located on the positive and negative strand, respectively (Figure 2D), a typical feature of primed spacer acquisition in type I-F CRISPR-Cas systems [28] that most likely resulted from the partial match between spacer 1 of CRISPR2 and gene 42 (position 27,616–28,572) on the phage genome [29]. Sequence analysis showed that nearly all acquired spacers were unique (97.2% ± 2.5% at 3 dpi and 97.9% ± 3.1% at 7 dpi; Table S1). In agreement with earlier studies [30], protospacers were invariably flanked by a GG protospacer-adjacent motif (PAM) (Figure 2E). PAM sequences play an important role both in spacer acquisition [31, 32] and in the interference stage of the immune response [33]. These data corroborate previously reported molecular features of acquired immunity by type I-F CRISPR-Cas systems.

Our model suggests that differential phage exposure might be the driver of investment into constitutive versus inducible immunity; hence, we examined bacterial and phage population densities in the two types of growth media. In M9, bacteria reached 10-fold lower densities compared to LB in the absence of phage (Figure S1B) and had longer generation times (57 ± 6 min in M9 versus 28 ± 2 min in LB). Furthermore, phage accumulation was slower in M9 compared to LB, and phage densities were approximately 100-fold lower throughout the measurements (Figure S1C). Lower host and parasite densities reduce the infection risk (determined by the product of their densities; see the Supplemental Experimental Procedures). When we varied parasite production (burst size) in our model, we found that higher parasite production selected for constitutive defense (Figure S1D), which further indicated that infection risk may drive the evolution of the immune strategies.

To experimentally verify that CRISPR-Cas and *sm*-mediated immunity indeed results from different phage exposures (and not from other differences between the LB and M9 growth media), we increased phage exposure in M9. Strikingly, a gradual increase in phage exposure from 10^4 to 10^7 and 10^9 pfu NTϕ was associated with decreased evolution of CRISPR-Cas-mediated immunity and increased *sm*-mediated immunity (Figure 3A; $F_{2,15} = 81.4$, $p < 0.0001$). Evolution of *sm* further increased when adding 10^9 pfu NTϕ at every daily transfer (Figure S2A; $F_{1,10} = 11.98$, $p = 0.006$). Finally, when infected with 10^7 pfu NTϕ, the WT strain evolved higher levels of *sm*-mediated immunity when grown in 60 μl volume (high phage density) compared to 6 ml (low phage density, but same number of phages) (Figure 3B; $F_{1,10} = 25.2$, $p = 0.0005$; Figure S2B; $F_{1,10} = 12.02$, $p = 0.006$). These data clearly show that infection risk directly impacts the relative investment in constitutive and inducible defenses.

We next measured the relative fitness associated with either immunity mechanism by competing the WT strain against a *sm* mutant in the presence of Tϕ (targeted phage; DMS3mvir), which is targeted by the WT CRISPR-Cas system (i.e., both strains are resistant) [26]. An increase in the frequency of one bacterial strain over another serves as a direct measure of relative fitness. In agreement with the data above, CRISPR-Cas-mediated immunity provided a fitness advantage over *sm*-mediated immunity when phage exposure was low (relative fitness > 1 upon infection with 10^4 and 10^7 pfu Tϕ), and vice versa when exposure was high (relative fitness < 1 upon infection with 10^9 pfu Tϕ) (Figure 3C; $T_5 = 5.98$, $T_5 = 11.52$, $T_5 = 430$; $p < 0.005$ in all cases; Figure S2C).

There are three possible causes for the competitive disadvantage of CRISPR-Cas versus surface modification at high phage exposure: CRISPR-Cas could (1) provide partial immunity, (2) be overcome more readily by phage evolution, or (3) be associated with greater fitness costs than constitutive immunity, as assumed in our model. We first examined whether differences exist in the associated resistance levels by exposing bacteria with the two types of immunity to high concentrations of phage. In agreement with the previously reported high survival rates upon phage exposure of CRISPR immune bacteria [33], we did not detect any mortality in either case (Figure S2D), which suggested that the immunity levels were not different. Although we cannot exclude the possibility that differences in mortality rates exist that fall within the error of the method, further experiments suggest that partial immunity is unlikely to explain our results.

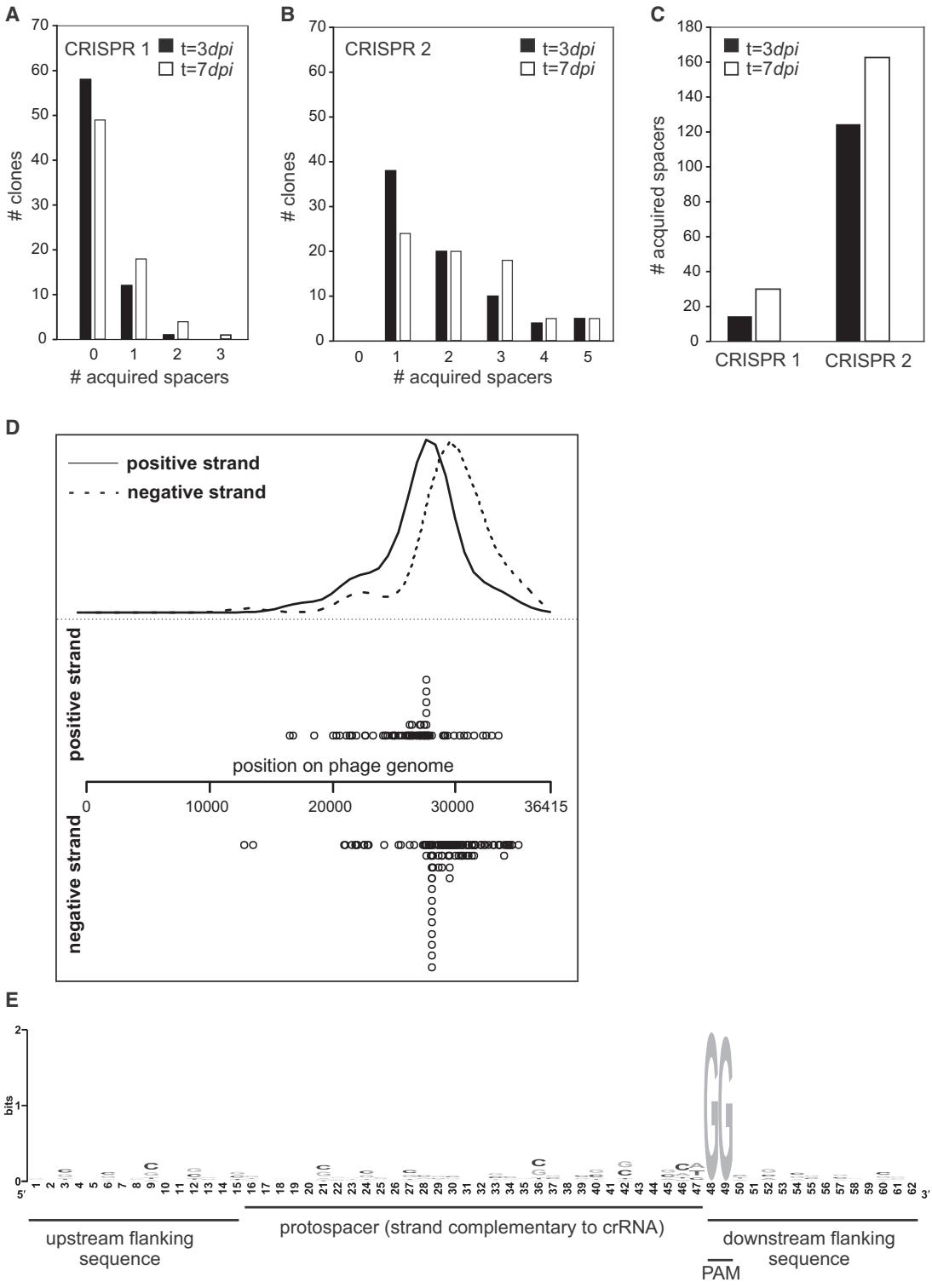

Figure 2. Molecular Analysis of Spacer Acquisition

(A–C) PCR-based analysis of the number of spacers acquired by WT in CRISPR1 and CRISPR2 at 3 and 7 dpi with NTϕ.

(D) Mapping of protospacer locations on either strand of the NTϕ genome. Of the acquired spacers, 67% ± 8% (3 dpi) and 59% ± 11% (7 dpi) target the negative strand of the phage, revealing a strand bias at 3 dpi (T_5 = 4.14; p = 0.0089), but not at 7 dpi (T_5 = 1.50; p = 0.19).

(E) Weblogo of protospacers and 15 bp flanking sequence reveals a protospacer-adjacent motif (PAM), generated using the WebLogo online software (http://weblogo.berkeley.edu). Bacteria were grown in M9.

See also Figure S2.

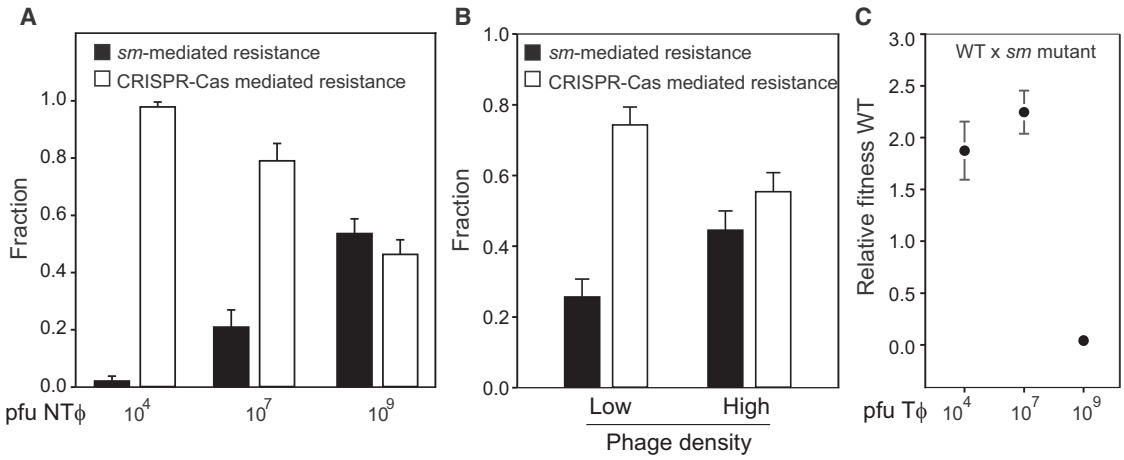

Figure 3. Selection for Constitutive and Inducible Defenses Depends on Parasite Exposure

(A) Fraction of WT that acquired *sm*-mediated immunity and CRISPR-Cas-mediated immunity at 3 dpi with varying pfu NTϕ as indicated (n = 6 in all treatments).

(B) Fraction of WT that acquired *sm*-mediated immunity and CRISPR-Cas-mediated immunity at 3 dpi upon exposure to 10^7 pfu NTϕ while grown in 6 ml (low phage density) or 60 μl (high phage density) (n = 6 in all treatments).

(C) Relative fitness of WT after competing 3 days with *sm* mutant in the presence of varying pfu Tϕ, as indicated (n = 6 in all treatments).

Error bars correspond to 95% CIs. Bacteria were grown in M9. See also Figures S2 and S3.

CRISPR-mediated immunity led to a 2-fold reduction in phage titers after 2 hr (Figure S2E), along with a latent period of 80 min and a phage burst size of approximately 88 ± 8 (data not shown), suggesting that at least 99.4% of the bacteria with CRISPR-mediated immunity are phage resistant (fraction of phage present after a single infection round = burst size × fraction of sensitive bacteria).

As a result of the sequence specificity of CRISPR-Cas-mediated immunity, phages can readily evolve to overcome this type of immunity [33, 34]. As genetic diversity increases with the phage population size, this could contribute to reduced fitness associated with CRISPR-Cas at high phage exposure. Phages that carry point mutations that result in escape from CRISPR-Cas could indeed be readily detected (Figure S2F), while we could not detect pili-binding phages that overcome *sm*. To examine this in detail, we performed competition experiments between *sm* mutants and CRISPR-Cas-immune bacteria that have multiple spacers targeting multiple Tϕ loci (bacteriophage-insensitive mutant 1 [BIM1] and BIM2; Table S1). Because multiple spacers of BIM1 and BIM2 target Tϕ (two and three spacers, respectively), the phage has a reduced probability of escaping the immune response by mutation. Crucially, we found that CRISPR-Cas-mediated immunity was associated with a fitness disadvantage (relative fitness < 1) when exposed to 10^9 pfu Tϕ, regardless of the number of target loci (Figure S2G [competition in M9]: WT, T_5 = 466; BIM1, T_5 = 11.7; BIM2, T_5 = 11.6; $p < 0.0001$ in all cases; Figure S2H [competition in LB]: WT, T_5 = 63.5; BIM1, T_5 = 21.5; BIM2, T_5 = 8.4; $p < 0.0001$ in all cases), and phage escaping BIM1 and BIM2 could not be detected (data not shown). This, along with the data shown in Figure 3B, indicates that under these conditions the differential evolution of CRISPR-Cas versus *sm* is primarily driven by phage exposure rather than by phage genetic diversity.

Next, we examined whether *sm* and CRISPR-Cas are associated with constitutive and inducible costs of immunity, as assumed in the model. To examine whether *sm* is associated with a constitutive fitness cost, we competed two independent *sm* mutants (*sm* and *sm2*; Figures S3A and S3B) against WT *P. aeruginosa* PA14. In agreement with previous studies [35], *sm* (loss of the pilus, the phage receptor) was associated with a fitness cost (relative fitness WT > 1) in the absence of phage (constitutive cost of immunity; Figures 4A and S3C; *sm*, T_5 = 4.99; *sm2*, T_4 = 9.31; $p < 0.005$ in both cases). Nutrient availability can directly impact on the cost associated with immunity [36], suggesting that the observed CRISPR evolution under nutrient-limited conditions could be purely resource driven. However, we found that the *sm*-associated constitutive cost was independent of resource availability (M9 versus LB) (Figure S3D; *sm*, $F_{1,10}$ = 1.76; *sm2*, $F_{1,10}$ = 0.36; $p > 0.20$ in both cases).

The inducible cost associated with CRISPR-Cas-mediated immunity was apparent from our data showing that the relative fitness of WT versus *sm* decreases with increasing phage exposure (Figures 3C, S3E, and S3F; Kruskal-Wallis Test, $p < 0.005$ in both cases); this is because the inducible cost increases with increasing phage density due to CRISPR-Cas being elicited more frequently. By contrast, we found no constitutive cost of CRISPR-Cas-mediated immunity. When the WT *P. aeruginosa* strain was competed in the absence of phage against a knockout mutant (CRISPR-KO; *P. aeruginosa csy3::lacZ*, carrying a non-functional CRISPR-Cas system [26]), we found that the WT and CRISPR-KO strains had a similar fitness (Figure 4B; relative fitness = 1 T_5 = 1.87; $p = 0.12$ at 5 days), showing that a functional CRISPR-Cas immune pathway has no detectable fitness cost when parasites are absent. To examine the fitness consequences of encoding *cas* genes and CRISPR loci on the genome, we also competed the CRISPR-KO strain against an isogenic strain from which the entire CRISPR-Cas system has been deleted (*P. aeruginosa* ΔCRISPR-Cas [29]). Surprisingly, the ΔCRISPR-Cas strain had a lower fitness (relative fitness < 1) than the *P. aeruginosa csy3::lacZ* strain (Figure S3G; T_5 = 38.3;

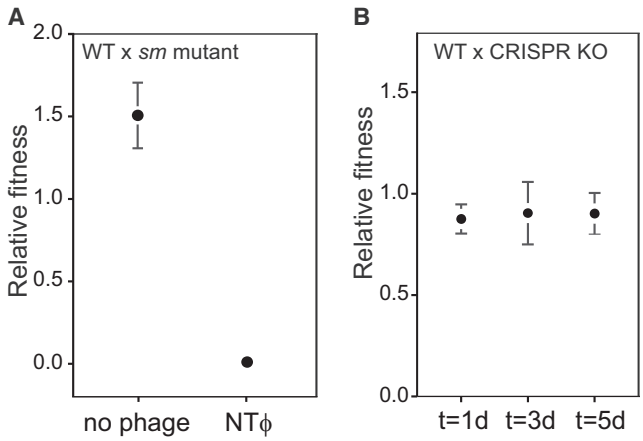

Figure 4. Surface Modification and CRISPR-Cas Are Associated with a Constitutive and Inducible Cost of Immunity

(A) Relative fitness of WT after competing 1 day with *sm* mutant in the presence or absence of phage as indicated (n = 6 for each treatment).

(B) Relative fitness of WT after competition of 1, 3, and 5 days with the CRISPR-KO strain in the absence of phage (n = 6 at each time point).

Error bars correspond to 95% CIs. Bacteria were grown in LB. See also Figure S4.

$p < 0.0001$ at 5 days), which indicates that components of CRISPR-Cas provide a fitness benefit in the absence of phage. Perhaps *P. aeruginosa* PA14 CRISPR-Cas functions in processes other than immunity, akin to the CRISPR loci and *cas* genes of other bacterial species [37]. Taken together, our data show that *sm* and CRISPR-Cas are associated with constitutive and inducible costs of immunity.

Our results suggest that the most likely cause for the cost of immunity associated with CRISPR-Cas is either an increase in mortality rate or reduced replication. Increased mortality could result from autoimmunity [38–40]. In addition, spacer acquisition requires opening of the bacterial genome (reviewed in [11]) and erratic incorporation events are potentially lethal. The high number of spacer duplication events that we observed suggests that this step may be error prone (Table S1 and data not shown). Reduced replication rates could result from resources being allocated to defense that would otherwise be invested in replication. The reason why surface modification is costly is more intuitive since phage receptors are often important fitness determinants of bacteria [41]. Pili, the phage receptors in our experimental system, are important for bacterial motility [42] and biofilm formation [43]. Loss of pili has a clearly detectable cost in a natural environment, soil (data not shown). Although motility and biofilm formation are not expected to be important in an in vitro laboratory environment, our fitness analyses show that bacteria lacking pili suffered a significant competitive cost.

In nature, high parasite densities may contribute to the existence of non-functional or degenerated CRISPR-Cas systems. This may explain, along with the suggestion that the CRISPR-Cas system is less effective in the face of rapidly evolving viruses, why half of the bacteria lack CRISPR-Cas, whereas the system is almost ubiquitous among Archaea, which typically have lower population densities and less exposure to viruses [44]. Indeed, our study shows that carrying a functional CRISPR-Cas system provides a selective advantage under nutrient-limited conditions in the presence of NTϕ (relative fitness > 1; Figure S4A; 10^4 pfu, $T_5 = 5.0$; 10^7 pfu, $T_5 = 6.7$; 10^9 pfu, $T_5 = 4.7$; $p < 0.006$), but not under nutrient-rich conditions (relative fitness = 1; Figure S4B; $T_5 = 0.44$; $p = 0.67$). Our findings also help to explain the apparent discrepancy between laboratory studies and correlational studies of natural populations. The latter are typically associated with lower bacterial and phage densities and typically report highly diverse CRISPR loci that are consistent with a key role in conferring phage immunity [21, 45, 46]. By contrast, the predominantly reported mechanism of evolving phage immunity under (nutrient-rich) laboratory conditions is by modification or masking of surface receptors [41], even when a CRISPR-Cas adaptive immune system is present [26] and despite the large competitive cost generally associated with surface modification [10, 35].

More generally, the theory and the data together suggest that parasite exposure is likely to be a key factor in driving the evolution of constitutive and inducible defenses in nature. This suggests that organisms living together in large populations or parasite-rich conditions are more likely to evolve constitutive defenses, whereas low-parasite conditions select for inducible defenses. Given that most species have both types of defenses, constitutive defenses are likely to be directed at the more abundant parasite species, while inducible defenses protect against the rarer parasites. It will be fascinating to see whether changes in parasite abundance can explain the observed evolution of constitutive defense from inducible defense [2], and vice versa [47].

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2015.01.065.

AUTHOR CONTRIBUTIONS

E.R.W., S.v.H., and A. Buckling designed experiments. E.R.W., S.v.H., B.M., S.O.-B., and J.M.B. performed the experiments. A. Best and M.B. performed the theoretical analyses. E.R.W., S.v.H., M.B., and A. Buckling analyzed the data and wrote the manuscript. J.B.-D. and A.D. supplied phages and bacterial strains and provided feedback throughout the project.

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