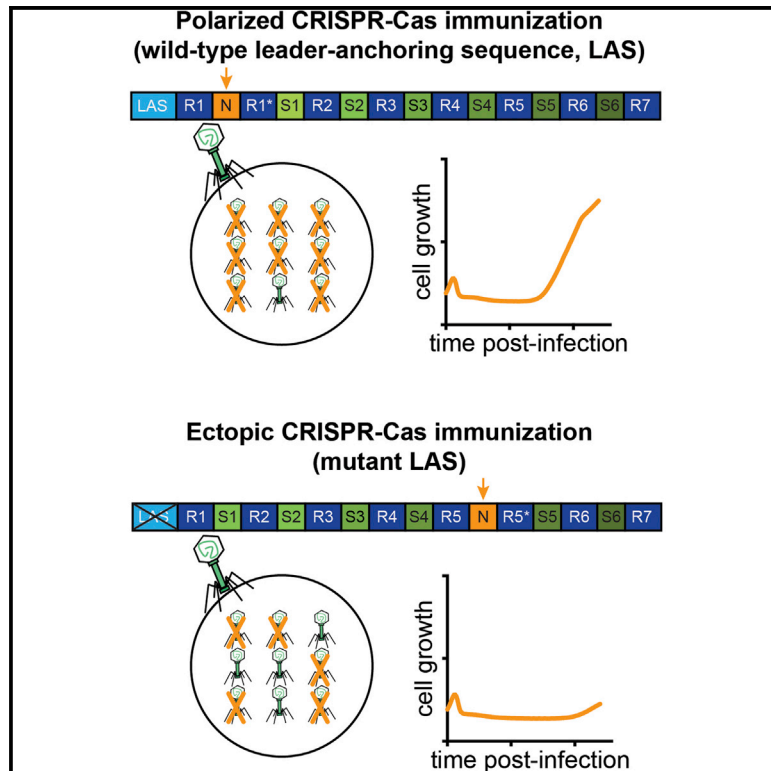


Molecular Cell

CRISPR-Cas Systems Optimize Their Immune Response by Specifying the Site of Spacer Integration

Graphical Abstract



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In Brief

The organization of immunological memories in the CRISPR immune system is tightly controlled. The CRISPR leader specifies the site of spacer integration to prioritize immunity against the latest viral infection, thereby protecting the host against its most immediate threat.

Highlights

- Type II CRISPR spacers are predominantly added to the leader-end of the array
- A conserved sequence within the leader specifies the site of spacer integration
- Leader mutations lead to spacer insertion at alternate positions within the array
- Polarized spacer integration gives rise to a more robust CRISPR immune response

CRISPR-Cas Systems Optimize Their Immune Response by Specifying the Site of Spacer Integration

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SUMMARY

CRISPR-Cas systems defend prokaryotes against viruses and plasmids. Short DNA segments of the invader, known as spacers, are stored in the CRISPR array as immunological memories. New spacers are added invariably to the 5' end of the array; therefore, the first spacer matches the latest foreign threat. Whether this highly polarized order of spacer insertion influences CRISPR-Cas immunity has not been explored. Here we show that a conserved sequence located immediately upstream of the CRISPR array specifies the site of new spacer integration. Mutation of this sequence results in erroneous incorporation of new spacers into the middle of the array. We show that spacers added through polarized acquisition give rise to more robust CRISPR-Cas immunity than spacers added to the middle of the array. This study demonstrates that the CRISPR-Cas system specifies the site of spacer integration to optimize the immune response against the most immediate threat to the host.

INTRODUCTION

Prokaryotes are faced with the perpetual threat of invasion by foreign nucleic acids through phage infection and horizontal gene transfer. Clustered regularly interspaced short palindromic repeats (CRISPR) loci and CRISPR-associated (Cas) proteins comprise a heritable and adaptive immune system that protects bacteria and archaea from phage (Barrangou et al., 2007) and plasmid (Marraffini and Sontheimer, 2008) infections. Immunological memories of these infections are stored in the CRISPR array as short spacer sequences that intercalate between repeats and specify the targets of CRISPR-Cas immunity. Upon infection, new spacer sequences matching the genome of the invading DNA are added to the 5' end of the CRISPR array (Barrangou et al., 2007). Therefore, the CRISPR locus constitutes a molecular fossil record of infections in which the first spacer matches the most recent foreign threat, whereas downstream spacers correspond to older infections. The CRISPR array of repeats and spacers is first transcribed as a long precursor that is

processed at the repeat sequences to generate small, mature, CRISPR RNAs (crRNAs) (Brouns et al., 2008; Carte et al., 2008; Deltcheva et al., 2011). These associate with and direct RNA-guided Cas nucleases to their targets, known as protospacers, in the genome of the invader (Gasiunas et al., 2012; Jinek et al., 2012; Jore et al., 2011; Samai et al., 2015). Cleavage of the viral or plasmid target DNA prevents infection (Garneau et al., 2010; Marraffini and Sontheimer, 2008).

Based on the cas gene content, CRISPR-Cas systems are classified into six types (I–VI) and 19 subtypes (Makarova et al., 2015; Shmakov et al., 2015). The polarity of spacer incorporation, i.e., the addition of new spacers in the first position of the CRISPR array, is a feature of all CRISPR-Cas types studied so far. At the molecular level, the process of spacer acquisition has been mostly studied using the type I-E system from *Escherichia coli* MG1655 (Datsenko et al., 2012; Yosef et al., 2012). The Cas1-Cas2 complex from *E. coli* serves as an integrase, catalyzing a nucleophilic attack by the incoming spacer at the first repeat (Arslan et al., 2014; Nuñez et al., 2014; Nuñez et al., 2015). During this concerted cleavage-ligation reaction, the spacer is added to the array and the repeat is duplicated simultaneously. The integration host factor (IHF) protein directs the addition of spacers into the first position of the CRISPR array (Nuñez et al., 2016). IHF is a histone-like bacterial protein conserved in Gram-negative organisms that binds an AT-rich region immediately upstream of the type I-E CRISPR locus, known as the leader sequence (Jansen et al., 2002), creating the proper DNA topology for Cas1-Cas2-mediated spacer integration at the first repeat. Type II-A CRISPR-Cas systems also display a stringently polarized spacer acquisition process (Barrangou et al., 2007; Heler et al., 2015). However, most of these systems are present in Gram-positive bacteria, which lack IHF homologs. In these systems, the leader sequence is also important for spacer acquisition (Wei et al., 2015); however, how the polarity of this process is achieved is not clear. More importantly, the physiological significance of polarized spacer acquisition, a fundamental feature of CRISPR-Cas immunity, has not been explored in any CRISPR type.

Here, we studied these fundamental problems of spacer acquisition in the type II-A CRISPR-Cas system of *Streptococcus pyogenes* SF370. Consistent with previous studies, we found that deletions of the array-proximal region of the leader abolish spacer integration at the leader-end of the CRISPR array. However, these deletions do not abolish all spacer acquisition activity. Instead, leader mutations result in the erroneous

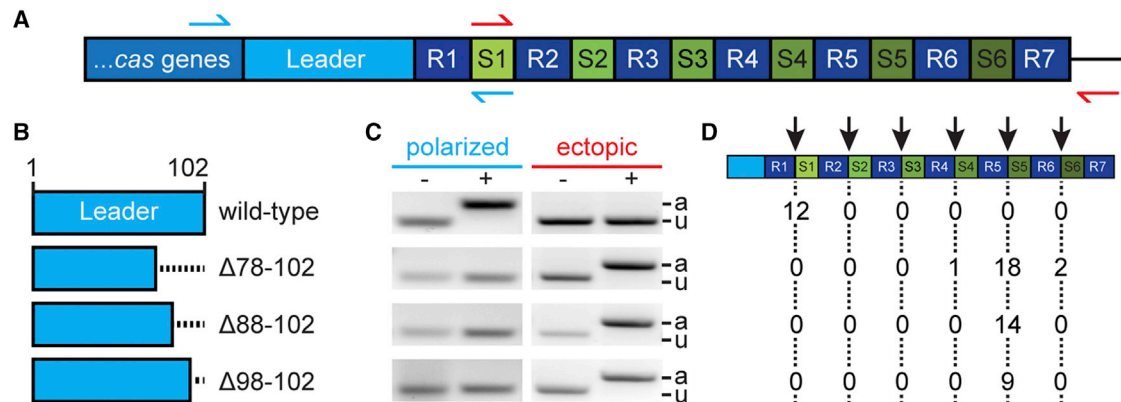


Figure 1. Deletions within the Leader Sequence Result in Ectopic Spacer Integration

(A) Type II-A CRISPR locus from *Streptococcus pyogenes* SF370. A 102 bp leader sequence separates the *cas* genes from the CRISPR array, which contains seven repeats (R1–7) flanking six spacers (*spc1*–6). Blue and red arrows indicate primers used to detect spacer integration at the leader-end (polarized) or at the middle of the array (ectopic), respectively.

(B) Deletions of the leader sequence analyzed in this study.

(C) PCR-based detection of polarized or ectopic spacer integration using the primers described in (A). DNA for PCR was extracted from colonies obtained from cultures incubated with (+) or without (–) phage ΦNM4γ4. PCR products were separated by agarose gel electrophoresis and stained with ethidium bromide. A gel image representative of many PCRs is shown. The size of the PCR product reflects the presence (a, “adapted”) or absence (u, “unadapted”) of integration of new spacers.

(D) Position of spacer integration events (marked by the black arrows) after Sanger sequencing of PCR products obtained in (C) for cells infected with phage carrying different leader sequence deletions. Numbers in table represent totals from three replicates.

integration of new spacers into the middle of the array, a phenomenon we term ectopic spacer integration. Further interrogation revealed that a short and conserved sequence at the 3' end of the leader dictates the site of spacer integration. By uncoupling the requirement for the leader during spacer acquisition from its role in positioning spacer integration, we were able to investigate the physiological significance of spacer order within the CRISPR array. We determined that wild-type, polarized spacer integration provides a fitness advantage over ectopic spacer acquisition due to higher levels of host protection provided by spacers in the leader-end of the array. This increased level of immunity is particularly critical during high titers of phage, like those that occur during CRISPR immunization. Our results demonstrate that polarized spacer acquisition ensures robust immunity against the latest invader, and thereby the most immediate threat to the host.

RESULTS

Deletions within the Leader Sequence Result in Ectopic Spacer Integration

The type II-A CRISPR system of *S. pyogenes* SF370 contains four *cas* genes, a *tracrRNA* gene, and six spacers (*spc1*–6) in the CRISPR array (Deltcheva et al., 2011) (Figure 1A). Immediately upstream of the first repeat there is a 102 bp, AT-rich sequence known as the leader (Jansen et al., 2002). We previously studied the roles of the different *cas* genes in spacer acquisition by cloning this CRISPR-Cas system on the pC194 staphylococcal plasmid and using *Staphylococcus aureus* RN4220, a genetically tractable Gram-positive organism without an endogenous CRISPR-Cas locus, as the host (Kreishirth et al., 1983). Here we used this experimental setup to

investigate the function of the leader sequence. We created three strains containing different deletions of 25, 15, or 5 bp at the 3' end of the leader sequence (Figure 1B). Cultures of these mutant strains as well as a wild-type control were infected with the staphylococcal lytic phage ΦNM4γ4 (Heler et al., 2015) during exponential growth, at a multiplicity of infection (MOI) of 1 virus per bacterium. After 24 hr, DNA was isolated from the surviving cells in each culture and used for PCR analysis of the CRISPR locus with primers that amplify the leader-end and thus detect the acquisition of new spacers in the first position of the CRISPR array (Heler et al., 2015) (Figure 1A, blue arrows). All three deletions prevented any detectable insertion of new spacers in this position (Figure 1C). This is in agreement with the results obtained with the type II-A CRISPR1 locus of *Streptococcus thermophilus* DGCC7710 (Wei et al., 2015). All of the leader deletion strains tested gave rise to bacteriophage-resistant mutants, however, which suggested that the CRISPR-Cas immune response could still be functional. We hypothesized that spacer acquisition could occur in other positions of the CRISPR array and therefore used a different set of primers to detect the incorporation of new spacers in any position (Figure 1A, red arrows). Surprisingly, we were able to observe bona fide spacer acquisition events in the middle of the CRISPR array (Figure 1C). Upon sequencing of many of these PCR products, we determined that, in all three leader deletion mutants, the majority of spacer integration events occurred at the fifth repeat, positioning the new spacer between *spc4* and *spc5* (Figure 1D). We term this phenomenon, in which new spacers are added into the middle of the array, “ectopic” spacer integration. These experiments provide direct, in vivo evidence that the leader is involved in specifying the site of spacer integration.

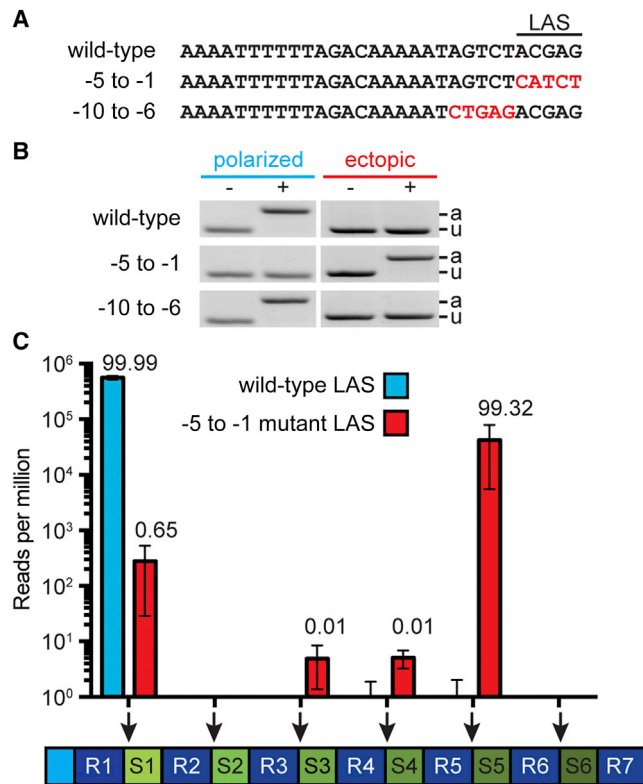


Figure 2. A Sequence within the Leader Specifies the Site of Spacer Integration

(A) Mutations were introduced at the 3' end of the leader to define the leader-anchoring sequence (LAS).

(B) Strains containing the leader mutations described in (A) were infected with phage Φ NM4 γ 4 and analyzed by PCR for polarized and ectopic spacer integration.

(C) Analysis of the site of spacer integration using next-generation sequencing. Liquid cultures harboring a wild-type or mutant LAS were infected at an MOI of 1, and the DNA isolated from surviving cells at the end of infection was used for PCR amplification of the entire array. The expanded PCR amplicons were purified from the gel and used for MiSeq next-generation sequencing. Bars show the number of normalized reads for the integration of new spacers in each possible position of the CRISPR array (marked by the black arrows). Mean \pm SEM of three replicates are reported. Percentage of spacer integration events is indicated above each bar.

See also Figure S1.

A Leader-Anchoring Sequence Specifies the Site of Integration for New Spacers

To investigate the leader sequences that specify the site of integration of new spacers in more detail, we introduced A-C and G-T transversion mutations in the 10 bp at the 3' end of the leader. We tested two mutants, one with mutations in the -5 to -1 region of the leader and one with mutations in the -10 to -6 region (Figure 2A). PCR analysis after phage infection with each set of primers used in Figure 1 showed that only the -5 to -1 leader mutant resulted in ectopic spacer integration (Figure 2B). We have therefore termed this region of the leader the “leader-anchoring sequence,” or LAS. This sequence, especially the 3'-end GAG, is highly conserved in related type II-A CRISPR systems (Chylinski et al., 2013) (Figure S1A). To

precisely determine the effect of the LAS on the position of spacer integration within the CRISPR locus, we performed next-generation sequencing of the PCR products containing the full array obtained after infection of wild-type and LAS mutant cultures. We found that while wild-type cells displayed polarized spacer integration almost exclusively (>99.999% of new spacers were integrated into the first repeat; Figure 2C), LAS mutant bacteria acquired new spacers at different positions in the array, with only ~0.65% of the integration events occurring at the first repeat and greater than 99% at the fifth repeat, positioning the new spacer between *spc4* and *spc5* (Figure 2C).

We wondered whether the sequence of *spc4* could work as a “pseudo-LAS” upstream of the fifth repeat that would direct the integration of new spacers in the absence of the wild-type LAS upstream of the first repeat. Comparison of the LAS and *spc4* sequences indicated that there is very little homology between them (Figure S1B). However, it is possible that different sequences could serve as anchors for spacer integration. To test this, we switched the positions of *spc2* and *spc4* and determined the location of newly acquired spacers. As expected, switching the spacers did not affect polarized acquisition in the strain harboring the wild-type LAS (Figure S1C). However, all of the LAS mutant, phage-resistant colonies tested contained the new spacer integrated in the third repeat, i.e., immediately downstream of the new location of *spc4* in this strain (Figure S1D). In addition, the combination of the LAS mutation and the deletion of *spc4* prevented the detection of spacer integration (Figure S1E). These results suggest that in the absence of a proper LAS, other sequences within the type II CRISPR array (*spc4* in the case of the *S. pyogenes* CRISPR-Cas system) can anchor spacer integration. Altogether, our experiments show that short sequences immediately upstream of repeats are able to specify the site of spacer integration. In particular, a short conserved sequence within the CRISPR leader immediately upstream of the first repeat, here named the LAS, specifies the acquisition of new spacers in the first position of the array with high fidelity.

The LAS Provides a Competitive Advantage during Spacer Acquisition

Our experiments with LAS mutant cells showed that the CRISPR-Cas immune response does not absolutely require the addition of new spacers in the first position. In spite of this, all CRISPR-Cas systems studied so far display an invariable specificity for spacer integration in this position. We wondered if polarized spacer acquisition provided an advantage versus ectopic acquisition. To test this, we carried out a series of competition analyses between strains that acquire spacers in different positions within the CRISPR array. First, we performed a pairwise competition assay between strains harboring wild-type or mutant LAS. Each of the two naive strains were grown to exponential phase and mixed in a 1:1 ratio. One aliquot of the mixed culture was uninfected as a control, while another was infected with phage Φ NM4 γ 4 at an MOI of 1. The cultures were grown for 24 hr and then streaked onto agar plates. DNA from individual colonies (originating from cells that survived phage infection) was used for PCR and Sanger sequencing to determine the population composition after the experiment (16

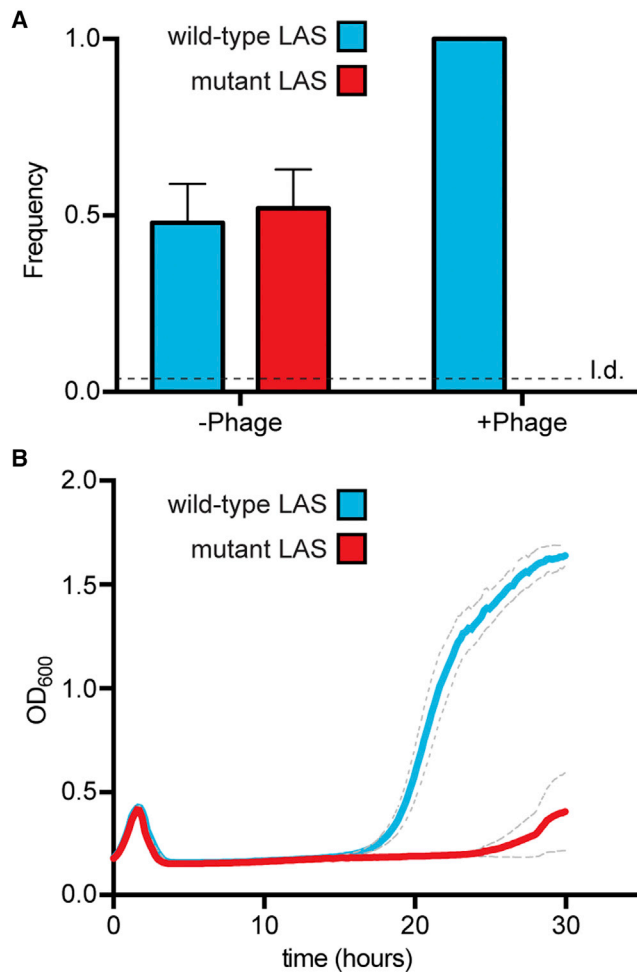


Figure 3. The LAS Confers a Fitness Benefit during CRISPR-Cas Immunity

(A) Analysis of culture composition following direct competition between strains harboring the wild-type or mutant LAS. Strains were mixed in a 1:1 ratio and infected with phage Φ NM4 γ 4 at an MOI of 1. Once the infection completed, the cultures were streaked onto a plate and colonies were picked to determine their LAS by Sanger sequencing ($n = 16$ per condition per replicate, 96 colonies tested in total). Mean \pm SEM of three replicates are reported. L.d., limit of detection.

(B) Growth of cultures infected with phage Φ NM4 γ 4 followed by the measurement of optical density at 600 nm (OD_{600}). Cells containing a wild-type or mutant LAS were infected, and their OD_{600} was followed over time. While most cells die after infection, a small fraction can acquire new spacers and resume growth after viral clearance through CRISPR-Cas immunity. Mean \pm SEM (gray dotted line) of three replicates are reported.

See also Figure S2.

colonies were analyzed per replicate; 48 colonies total; Figure 3A). The control showed a 1:1 ratio of wild-type to LAS mutant cells in the absence of phage infection, indicating that there is no intrinsic selective advantage for any of the strains (16 colonies were analyzed per replicate; 48 colonies total). In contrast, in the presence of phage only the strain harboring the wild-type leader was able to generate resistant colonies, suggesting a strong fitness defect for the LAS mutant cells. As

expected, all of the cultures only contained new spacers in the first position of the CRISPR array (Figure S2A). This fitness defect was also observed during monoculture phage challenges, where the survival of cells is measured as the optical density of the culture after addition of phage (Figure 3B). In this experiment, naive cells succumb to viral infection and the optical density decreases dramatically. However, cultures can regain growth upon acquisition of new spacer sequences that direct phage destruction. While cells harboring a wild-type leader sequence restart growing at ~ 16 hr, LAS mutants take ~ 25 hr to regrow.

Interestingly, when preparing the samples for next-generation sequencing (Figure 2C), we noticed an additional PCR product from the LAS mutant sample, smaller than both the naive and adapted CRISPR arrays present in both populations (Figure S2B). Sequencing of these PCR products revealed the presence of CRISPR loci that had lost the first four repeat-spacer units to relocate the new spacer in the first position (Figure S2C). This result shows that, in the event of ectopic adaptation, there is selective pressure to reposition new spacers to the leader-end of the array. Altogether, these experiments demonstrate that by enabling the acquisition of new spacers in the first position of the CRISPR array, the LAS confers a selective advantage during the CRISPR-Cas immune response.

Spacers in the First Position of the CRISPR Array Provide a More Robust Immune Response

There are at least two possible explanations for the fitness advantage of cells harboring a wild-type LAS observed in Figure 3. One scenario is that *spc4* has poor LAS properties and spacer integration at position 5 (in the LAS mutant) is less efficient than at the first position (in the wild-type CRISPR locus). Another possibility is that spacers integrated into repeat 5 provide a weaker CRISPR immune response and take more time to clear the virus and regrow. To explore the first scenario, we replaced *spc4* with a 30 bp fragment of the leader containing the LAS (Figure 4A). In this way, the LAS is present upstream of both the first and fifth repeats, and there is an optimal LAS for the acquisition of spacers in the first or fifth position of the CRISPR array. This set up allowed us to perform an “intracellular competition experiment” in which integration at repeat 1 (the wild-type position) and repeat 5 (the ectopic position) should have similar rates. We first corroborated that, in the absence of a wild-type LAS upstream of the first repeat, the replacement of *spc4* for the LAS resulted in the exclusive acquisition of new spacers in position 5 (Figure S3A). This also demonstrates that the LAS positively directs spacer acquisition, and therefore that the particular LAS mutations that we introduced are not inhibitory for spacer incorporation. Next, we infected cells harboring a duplicated LAS and analyzed the surviving colonies (Figure 4A). Spacer acquisition events were detectable at both leader-repeat junctions, but spacers integrated into the first repeat were highly enriched after phage selection (in fact, ectopic spacer integration was observed in only 1 of 6 replicates). These data show that polarized spacer acquisition is favored over ectopic spacer acquisition even when both integration events are mediated by the same, wild-type, LAS. Although it is still possible that upstream leader sequences not included in the 30 bp LAS spacer have a minimal positive effect on the

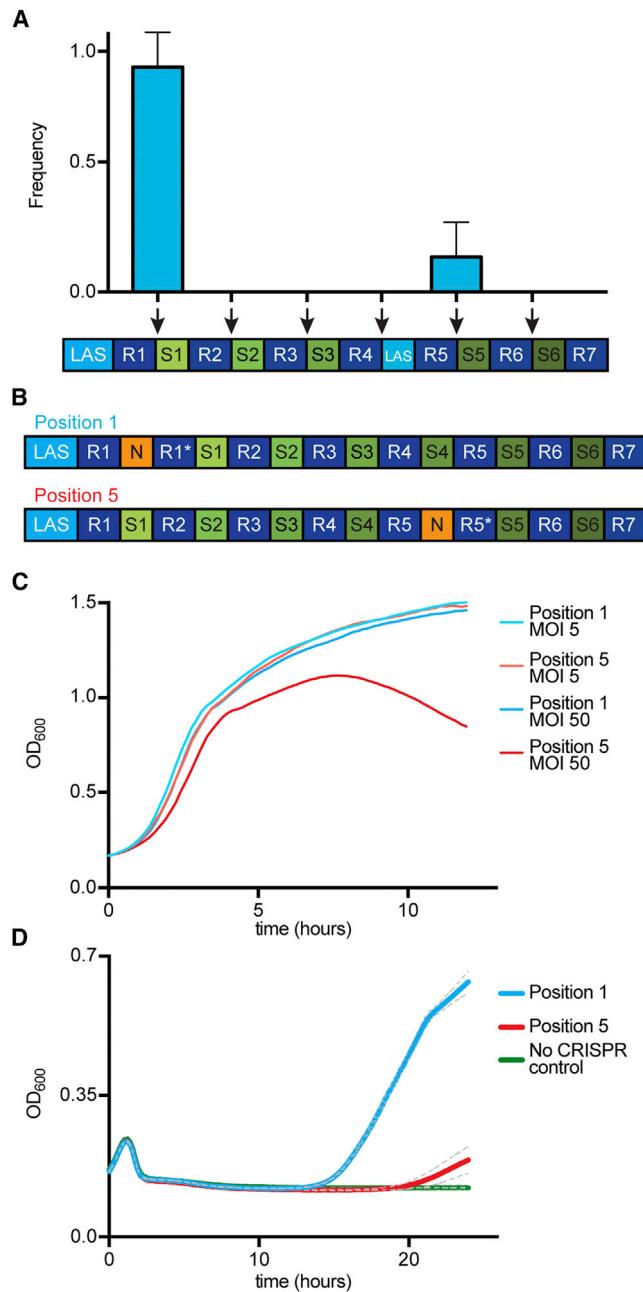


Figure 4. Leader-End Spacers Provide More Robust Immunity than Spacers in the Middle of the CRISPR Array

(A) Analysis of the site of spacer integration in a strain harboring two copies of the LAS, upstream of the first and fifth repeat. A culture was infected with Φ NM4 γ 4 at an MOI of 1. The CRISPR array from surviving cells was amplified and subjected to Sanger sequencing to determine the position of integration (marked by the black arrows) of new spacers. Mean \pm SEM of six replicates are reported.

(B) Two strains were engineered to test the levels of CRISPR-Cas immunity provided by the same spacer sequence located in the first (Position 1 strain) or fifth (Position 5 strain) position. In addition, the *cas1* gene was mutated to prevent the acquisition of new spacers.

(C) Growth of Position 1 and Position 5 cultures infected with phage Φ NM4 γ 4 at MOI 5 or 50 followed by the measurement of optical density at 600 nm (OD_{600}). Mean of three replicates is reported.

efficiency of spacer acquisition, this result suggests that the selective advantage of the wild-type CRISPR-Cas system is not due to a low rate of spacer incorporation in LAS mutant cells, but rather reflects a difference in the level of immunity provided from different positions within the CRISPR array.

We examined the second possibility by directly comparing the levels of CRISPR immunity provided by polarized and ectopic spacer acquisition. We engineered two CRISPR-Cas systems containing the same spacer sequence (GTGTTCTCTCAATC CATTCTATTGCT) in two different positions within the array (Figure 4B), one mimicking polarized spacer integration (“Position 1”) and the other mimicking ectopic spacer integration (“Position 5”). To prevent additional immunization events, spacer integration was abrogated in both strains by the introduction of an inactivating mutation (E220A) in the Cas1 integrase (Heler et al., 2015; Wiedenheft et al., 2009). Both strains were grown to exponential phase and infected with phage Φ NM4 γ 4 at two MOIs, 5 and 50, and growth was monitored by measuring the optical density of the cultures (Figure 4C). Position 1 and Position 5 strains exhibited comparable levels of immunity at an MOI of 5. In contrast, at an MOI of 50, the Position 5 strain showed a severe growth defect. A similar result was obtained when phage propagation was measured on plates containing Position 1 or Position 5 cells, seeded with 10-fold serial dilutions of the phage Φ NM4 γ 4 stock. Whereas the number of plaques originating from phage escapers (usually harboring mutations in the target sequence that make them refractory to CRISPR-Cas immunity; Deveau et al., 2008) were similar for both strains (Figure S3B), Position 5 plates showed an inhibition of growth zone (most notable at the 10^{-3} dilution, Figure S3C), suggestive of some level of phage propagation due to a poor CRISPR-Cas defense. Altogether these experiments demonstrate that while spacers in any location of the array confer some level of immunity to the host, positioning the immunity-conferring spacer at the leader-end of the array enables a more robust immunity at higher titers of phage.

CRISPR-Cas immunization is a rare event, calculated to happen only in 1 in 10^7 cells of the infected population in our experimental set up (Heler et al., 2015). As a consequence of this, most cells in the culture succumb to viral infection, creating very high titers of phage. We estimated that the small fraction of cells that is able to acquire a new spacer against the phage face an extremely high MOI (on the order of 10,000). Given the results obtained in Figure 3A, we speculated that the position of the targeting spacer in the CRISPR array could be critical for the CRISPR immune response under these extreme phage stresses. To test this, we developed an assay that simulates the CRISPR immunization process in which a small proportion of CRISPR-immune cells (already harboring a phage-targeting spacer sequence in either position 1 or position 5) is mixed with a majority of non-CRISPR cells that enables exponential phage

(D) Simulation of CRISPR immunization with Position 1 and Position 5 strains. Position 1 or Position 5 cells were diluted with cells lacking CRISPR-Cas in a 1:10,000 ratio and infected with phage Φ NM4 γ 4 at an MOI of 1. Cell growth that results from CRISPR-Cas immunity was monitored by optical density measurements at 600 nm (OD_{600}) for 24 hr. Cells lacking a CRISPR-Cas system were used as control. Mean \pm SEM (gray dotted line) of three replicates is reported. See also Figure S3.

propagation. In this assay, the time at which the culture resumes growth after viral infection is delayed proportionately to the fraction of CRISPR-immune cells (Figure S3D). When the Position 1 CRISPR-immune strain is tested, growth resumes at ~15 hr (Figure 4D). In stark contrast, when the Position 5 CRISPR-immune strain is used, growth resumes at ~20 hr. This pattern mirrors the growth curves produced during CRISPR immunization of wild-type and LAS mutant cultures (Figure 3B). Together, these results demonstrate that cells in which spacers are integrated ectopically suffer a severe growth defect due to compromised immunity.

DISCUSSION

A hallmark feature of CRISPR-Cas systems is the integration of short viral spacer sequences into the 5' end of the CRISPR locus (Barrangou et al., 2007). However, the physiological significance of this highly polarized process has remained unknown. Here, we studied this problem in the type II-A CRISPR-Cas system of *S. pyogenes*. We found that a short sequence immediately upstream of the first CRISPR repeat, which we called the leader-anchoring sequence or LAS, is required for the exclusive insertion of spacer sequences in the first position of the CRISPR array. Mutations in the LAS result in the integration of new spacers in the middle of the array, a phenomenon we called ectopic spacer integration. The phenotype of the LAS mutant allowed us to determine the importance of ordered spacer addition during the CRISPR immune response against phage infection. We found that polarized spacer integration bestows the host with a competitive advantage by positioning the new spacer in the first position of the array, where spacers provide more robust CRISPR-Cas immunity. Since the first spacer derives from the most recent invader, polarized spacer acquisition allows CRISPR-Cas systems to prioritize immunity against the most immediate threat to the host.

Given that Cas9 acts as a single-turnover enzyme (Sternberg et al., 2014), each crRNA molecule that directs the cleavage of one invading phage cannot be re-used to cleave a second viral genome. Therefore, the abundance of a targeting crRNA could be critical during CRISPR immunization, when cells are challenged by an extremely high number of phages. This is because upon infection of a naive bacterial population, the viral titers rise to extraordinary levels due to the initially unconstrained transmission of the virus. In this exceptional condition in which a few newly immunized cells are infected by thousands of phages at the same time, the abundance of the crRNA guide produced from the new spacer could be decisive for the success of the CRISPR-Cas immune response. A higher abundance of leader-end crRNAs has been observed in many CRISPR-Cas systems (Deltcheva et al., 2011; Elmore et al., 2013; Nickel et al., 2013; Randau, 2012; Richter et al., 2012). Importantly for our study, the *S. pyogenes* type II-A CRISPR-Cas system produces higher levels of *spc1* crRNA than the other crRNAs derived from downstream spacers (Deltcheva et al., 2011). Further supporting this scenario, we found that the levels of the Φ NM4 γ 4-targeting crRNA produced from Position 1 is ~2-fold higher than when it originates from Position 5 during exponential growth (Figure S3E). We believe that it is conceivable that the same spacer

sequence integrated in the first or a more downstream position of the CRISPR array could produce different levels of mature crRNA due to asymmetric transcription and/or differential processing of the crRNA precursor. The molecular mechanisms that lead to the uneven distribution of crRNAs, and how small differences in crRNA abundance affect the CRISPR immune response, will require further investigation, in type II and other CRISPR types.

While the LAS is critical for the integration of new spacers into the first repeat, we have found that the *spc4* sequence can also specify the addition of spacers into the repeat that follows it. In addition, the mutations inserted in the LAS (–5 to –1) do not completely abrogate the integration of spacers in the first position of the CRISPR array (Figure 2C). These results suggest that other sequences or sequence motifs can perform the LAS function. Moreover, it is possible that upstream sequences within the leader could contribute to spacer integration, though such sequences were not detectable in a related Type II-A CRISPR system (Wei et al., 2015). We propose that the Cas1-Cas2 integrase complex samples the nucleotides immediately upstream of the repeat and that the wild-type LAS provides the optimal sequence for anchoring the complex, biasing its activity toward the leader-end of the array. In the type I CRISPR-Cas system, binding of IHF to the leader creates the required DNA topology for spacer integration at the 5' end of the array. Given the absence of IHF homologs in Gram-positive bacteria (including the host used in our studies, *S. aureus*), it is possible that there are other factors that perform a similar function for type II CRISPR-Cas systems (Wei and Terns, 2016). Alternatively, the type II Cas1-Cas2 complex could be sufficient to catalyze polarized spacer integration without a requirement for additional host factors (Rollie et al., 2015). Additional work employing biochemical and structural techniques will address these questions.

The prioritization of the CRISPR-Cas immune response against the most recent invader has been proposed as a bet-hedging strategy (Weinberger et al., 2012). This is analogous to the mammalian adaptive immune response, where effector T cell populations and antibody titers against a virus are highest immediately post-immunization and gradually decrease over time (Murphy et al., 2012). By devoting more resources to defending the host against the most recent infection, immune systems provide robust protection against the infectious agents that are most likely to be present at high titers. This is an efficient way to conserve resources while still providing robust immunity against the most threatening attackers. For CRISPR-Cas systems, the benefit of deprioritizing immunity against past invaders would be 2-fold. First, during infection of a population that has been immunized in the past, i.e., harboring the invader-matching spacer in the middle of the array, transmission of the re-infecting virus will be immediately contained and viral titers will remain low. In this situation, the CRISPR-Cas immune response does not need to be at peak levels to efficiently protect the population, as shown in Figure 4C in which a spacer in position 5 provides full immunity in conditions of low MOI. Second, it has been reported that phage mutations in target sequences are highly abundant in viral populations and are selected for their ability to enable the escape from CRISPR-Cas immunity (Andersson and Banfield,

2008; Deveau et al., 2008). Therefore, there is a high probability that spacers in the middle of the array would not be able to provide immunity against re-infecting, mutated phages. In this scenario, maintaining full expression of these spacers would also be wasteful. Interestingly, in a scenario of re-infection by high titers of a non-mutated virus, an old spacer sequence can regain full potency through its repositioning as the first spacer sequence (similar to our results in Figure S2). Recombination and deletion within the CRISPR array have been extensively described in natural populations (Andersson and Banfield, 2008; Sun et al., 2013) and could represent a functional, rather than accidental, feature of CRISPR-Cas loci. Our results ascribe a physiological role to the establishment and preservation of the timeline of infection that is a hallmark of CRISPR-Cas immune systems and further our understanding of the selective pressures that guide the evolution of CRISPR systems.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions

Cultivation of *S. aureus* RN4220 (Kreiwirth et al., 1983) was carried out in brain-heart infusion (BHI) or heart infusion (HI) media (BD) at 37°C. When applicable, media was supplemented with chloramphenicol (10 µg/mL) or spectinomycin (250 µg/mL) to ensure maintenance of the pC194-derived (Horinouchi and Weisblum, 1982) or pLZ12-derived (Perez-Casal et al., 1991) plasmids, respectively.

Simulation of CRISPR Immunization

Overnight cultures were diluted 1:100 into fresh BHI supplemented with appropriate antibiotics and 5 mM CaCl₂. These cultures were then grown to an OD₆₀₀ of 0.2–0.4, upon which they were normalized to OD₆₀₀ = 0.2. Position 1 and Position 5 strains were diluted 1:10,000 into the sensitive strain RN4220 in triplicate. The mixed cultures were infected with phage ΦNM4γ4 at an MOI of 1. Growth of the cultures was detected measuring OD₆₀₀ using plate reader.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, and one table and can be found with this article online at <http://dx.doi.org/10.1016/j.molcel.2016.08.038>.

AUTHOR CONTRIBUTIONS

J.M. performed all experiments in this paper. L.A.M. and J.M. wrote the manuscript.

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