Mature clustered, regularly interspaced, short palindromic repeats RNA (crRNA) length is measured by a ruler mechanism anchored at the precursor processing site

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Precise RNA processing is fundamental to all small RNA-mediated interference pathways. In prokaryotes, clustered, regularly interspaced, short palindromic repeats (CRISPR) loci encode small CRISPR RNAs (crRNAs) that protect against invasive genetic elements by antisense targeting. CRISPR loci are transcribed as a long precursor that is cleaved within repeat sequences by CRISPR-associated (Cas) proteins. In many organisms, this primary processing generates crRNA intermediates that are subject to additional nucleolytic trimming to render mature crRNAs of specific lengths. The molecular mechanisms underlying this maturation event remain poorly understood. Here, we defined the genetic requirements for crRNA primary processing and maturation in *Staphylococcus epidermidis*. We show that changes in the position of the primary processing site result in extended or diminished maturation to generate mature crRNAs of constant length. These results indicate that crRNA maturation occurs by a ruler mechanism anchored at the primary processing site. We also show that maturation is mediated by specific *cas* genes distinct from those genes involved in primary processing, showing that this event is directed by CRISPR/Cas loci.

conjugation | genetic interference | antisense RNA

Clustered, regularly interspaced, short palindromic repeat (CRISPR) sequences are present in ~40% of eubacterial genomes and nearly all archaeal genomes sequenced to date. CRISPR loci consist of short (~24–48 nt) repeats separated by similarly sized unique spacers (1–4). CRISPR systems protect against bacteriophage and plasmid infection by a genetic interference mechanism that relies on the identity between CRISPR spacers and the invading targets (5–7). CRISPR arrays are transcribed into a long precursor containing spacers and repeats that are processed into small CRISPR RNAs (crRNAs) by dedicated CRISPR-associated (Cas) endoribonucleases (6, 8, 9). crRNAs act as guides for a targeting complex (10–15) that cleaves the genetic material of the invading bacteriophage or plasmid (16).

In many prokaryotes, the biogenesis of mature crRNAs can be divided into two stages: (i) a primary cleavage of the crRNA precursor within repeat sequences that generates intermediate crRNAs containing a full spacer flanked by partial repeats, and (ii) a final maturation event where intermediate crRNAs are subject to additional nucleolytic digestion at one end. Repeat spacer arrays and their adjacent *cas* genes are classified into three CRISPR/Cas types (I–III) (17) that undergo different mechanisms of crRNA processing. In Types I and III CRISPR/Cas systems, primary processing is achieved by Cas6 endoribonucleases and results in crRNA intermediates with 5'-hydroxyl and 3'-phosphate or 2'-3'-cyclic phosphate ends (9, 10, 12, 13). Cleavage occurs 8 nt upstream of the beginning of the spacer sequence, leaving a 5' handle (6) or crRNA tag (18) on the 5' end. In Type III systems, crRNA intermediates are subject to additional nucleolytic attack at the 3' end, generating mature crRNAs with reduced length (18, 19). This final maturation step seems to be absent in Type I systems (10, 12, 13, 15). In Type II systems, a *trans*-encoded crRNA complementary to the precursor crRNA guides primary processing. In contrast to Type III systems, maturation occurs at the 5' end of the in-

termediate crRNA (20). Maturation is critical for the cleavage of the target nucleic acid, which occurs at a specific distance from the mature end (11, 16).

To date, little is known about the molecular mechanisms underlying crRNA maturation. Here, we studied crRNA biogenesis in *Staphylococcus epidermidis*, a bacterial pathogen harboring an active Type III CRISPR/Cas system that prevents the conjugation of multidrug-resistant plasmids (7). We found that the minimal functional sequence required for CRISPR interference consists of a single repeat spacer unit and that primary processing requires both sequence and structural elements within repeats. In contrast, we found that maturation does not depend on the sequence, length, or secondary structure of the intermediate crRNA. Instead, we show that maturation is linked to the primary processing site. We show that changes in the position of the 5' primary processing site result in extended or diminished maturation at the 3' end that generates mature crRNAs of constant length. These results suggest a ruler mechanism for crRNA maturation that uses the primary processing site as a reference point to measure the distance between both ends of the crRNA. Furthermore, we show that maturation is mediated by specific *cas* genes distinct from those genes involved in primary processing, establishing that this event is directed by CRISPR/Cas loci.

Results

Upstream Repeat Elements Direct crRNA Primary Processing. *S. epidermidis* RP62a (21) contains a Type III CRISPR/Cas system with four direct repeats, three spacers, and nine *cas* genes (Fig. 1A and Fig. S1). crRNAs derived from the first spacer (*spc1* crRNAs) are generated by cleavage of the precursor within the first and second direct repeat (DR) sequences at the base of a putative stem loop structure (7) (Fig. S24). *Spc1* crRNAs mediate CRISPR interference against conjugation by targeting a region of the *nickase* gene present in staphylococcal conjugative plasmids (7). We studied crRNA processing in vivo in *S. epidermidis* Δ *crispr* (7) by introducing plasmids carrying different mutations of the CRISPR array. We used primer extension analysis and conjugation assays to measure primary processing and CRISPR function, respectively (details about mutations and conjugation efficiencies of all constructs used in this study can be found in Tables S1 and S2, respectively).

To define the repeat sequences required for CRISPR function, we generated constructs carrying different deletions within repeats flanking *spc1* (Fig. 1 and Fig. S1). These deletions showed that primary processing requires upstream, but not downstream, repeat

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Fig. 1. Upstream repeat elements direct crRNA processing. (A) Organization of the *S. epidermidis* RP62A CRISPR locus. Leader (black), repeats (blue), spacers (colored), and *cas/csm* genes (gray) are indicated. (B) Organization of CRISPR direct repeats (DR1–4) and spacers (*spc1*–3) in the plasmid pCRISPR and various deletion mutants. Primers P1 and P2 were used in primer extension assays to detect *spc1* and *spc2* crRNAs, respectively. (C) Primer extension analysis of *S. epidermidis* Δ*crispr* strains harboring the plasmids shown in B. Extension products on *spc1* (42 nt) and *spc2* (37 nt) crRNAs and 5S rRNA (26 nt) are indicated. M, DNA size marker. (D) *spc1*-Directed CRISPR interference against the staphylococcal conjugative plasmid pG0400. *S. epidermidis* Δ*crispr* recipients containing different plasmids were used as recipients. Conjugation was carried out by filter mating in triplicate; the values (in cfu/mL; mean ± SD) obtained for recipients and transconjugants are shown. Conjugation efficiency (Conj. Eff.) was calculated as the transconjugants/recipients ratio. (E) DR1 sequence with predicted hairpin-forming nucleotides underlined. Primary processing (arrow) occurs 8 nt upstream of *spc1*, generating a crRNA tag in the 5' end of intermediate and mature crRNAs. (F) Primer extension analysis of DR1 mutants. Results for CRISPR interference against pG0400 are shown as + (interference occurs) and – (interference is abrogated). Nucleotides essential for efficient crRNA processing and CRISPR interference appear in red.

sequences. Deletions of the 3' half of DR1 and DR2 [mutants DR1 (3'del) and DR2 (3'del)] eliminated processing of *spc1* and *spc2* crRNAs, respectively (Fig. 1B and C and Fig. S1), but not of others, indicating that primary processing of each repeat spacer unit is independent. Deletions of the 5' half of DR1 and DR2 [mutants DR1 (5'del) and DR2 (5'del)] allowed processing but at a reduced efficiency that is not sufficient to provide CRISPR interference (Fig. 1B–D and Fig. S1). We attribute this diminished processing to the elimination of the stem loop structure in the crRNA precursors derived from these constructs (see below). A construct containing only DR1-*spc1* sequences [DR2–4 (del)] produced WT levels of crRNA and complemented the CRISPR deficiency of the Δ*crispr* strain, indicating that this unit is the

minimal unit required to support CRISPR interference (additional deletions in DR1 abrogated processing and function in this minimal construct) (Fig. S1 and Tables S1 and S2).

To determine the precise sequence requirements for primary processing, we mutated each of the 36 nt of DR1 to their Watson–Crick complement (Fig. 1E and F). We found that mutations of the bases surrounding the cleavage site (nucleotides 26–31, GGGACG) eliminated processing and accumulation of *spc1* crRNA as well as interference against the conjugative plasmid pG0400. In the crRNA precursor, bases G25–G28 would pair with C13–C16 in the putative stem loop structure of *S. epidermidis* repeats (Fig. S24). Interestingly, mutation of C13 resulted in lower levels of *spc1* crRNA that do not support interference in vivo (Fig. 1E and F and Table S1), and the same phenotype was obtained for the combined mutation of C14–16G (Fig. S2), suggesting that formation of an RNA hairpin is important for processing. This finding was corroborated by compensatory mutations in positions 13 and 28; although the G28A mutation eliminated primary processing, the double mutant C13T-G28A partially restored it (Fig. S2). The double mutant C13G-G28C, however, showed no processing, suggesting that purines are better tolerated than pyrimidines at position 28. Altogether, these data indicate that recognition of both the GGGACG sequence and the stem loop structure is needed for efficient primary processing and CRISPR interference.

Maturation Is Independent of the Sequence, Structure, or Length of the Intermediate crRNA. In *S. epidermidis*, primary processing produces an intermediate crRNA of 71 nt, and additional nucleolytic cleavage of the 3' end generates smaller mature crRNAs (Fig. 2A and Fig. S3). To determine whether 3'-end maturation relies on a specific sequence or hairpin structure present downstream of *spc1*, we performed Northern blots of crRNAs containing different mutations in this region (Figs. 1B and 2B and Fig. S4). We analyzed three classes of mutants: (i) containing different sequences immediately downstream of *spc1* [DR2 (5'del), DR2–4 (del), and DR2 (1–11)], (ii) lacking the hairpin (HP) structure in DR2 [DR2 (5'del), DR2–4 (del), and DR2 (no HP)], and (iii) producing longer intermediate crRNAs [DR2 (5'del), DR2 (3'del), DR2–4 (del), and DR2(G28C)]. Strikingly, all mutants produced two mature crRNA species of WT length and abundance that supported CRISPR interference against plasmid conjugation (Fig. 2C and Table S2). Although the intermediates generated by primary processing vary in length accordingly with the introduced mutations in DR2 (Fig. S4D), the mature crRNAs maintained a constant size.

To precisely measure the length of the mature species, we captured crRNAs with a biotinylated oligonucleotide complementary to *spc1* (Fig. 2D). All intermediate crRNAs were reduced to mature crRNAs with precise lengths of 43 and 37 nt compared with ladders generated by alkaline hydrolysis or RNase T1 cleavage of a synthetic *spc1* crRNA. Together, these results indicate that 3'-end maturation does not depend on the sequence, structure, or length of the intermediate crRNA. Interestingly, the ladder RNAs required 3'-end dephosphorylation to match the electrophoretic mobility of the mature crRNAs (Fig. S5B), indicating that mature crRNAs likely contain 3'-hydroxyl groups as opposed to intermediate species that display 3'-phosphate or 2'-3'-cyclic phosphate ends (9, 10, 12, 13). To confirm a 3'-OH group on mature crRNAs, we showed that both the 43- and 37-nt species are suitable substrates for T4 RNA ligase-mediated addition of [5'-³²P]Cp to the 3' end (Fig. S5C). The difference in the chemical nature of the 3' end between intermediate and mature crRNAs suggests that primary processing and final maturation are achieved by distinct catalytic mechanisms.

Length of Mature crRNAs Is Measured from the 5' Primary Processing Site. In the above experiments, the sequence, length, and/or secondary structure of the 3' end of the crRNA intermediate was

Fig. 2. Maturation does not depend on the sequence, structure, or length of the intermediate crRNA. (A) Processing and maturation of crRNAs in *S. epidermidis*. Primary processing of consecutive direct repeats (black triangles) yields an intermediate crRNA (71 nt) that is further trimmed at the 3' end (white triangle) to yield mature crRNAs. The probe used to detect *spc1* crRNAs by Northern blot is shown. (B) Alterations downstream of *spc1* were introduced to determine the effect of sequence or structural elements on crRNA maturation. Sequences are highlighted according to Fig. 1A. Mutations are shown in red. HP, hairpin. (C) Northern analysis of strains containing different mutant constructs using a radiolabeled probe antisense to *spc1*. The intermediate and mature crRNA species are indicated. Detection of 5S rRNA is shown as control. M, DNA size marker. (D) Mature crRNAs were captured with a biotinylated probe antisense to *spc1*. crRNAs were radiolabeled at their 5' end and resolved by denaturing PAGE alongside RNA ladders generated by alkaline hydrolysis (OH) and RNase T1 digestion (T1) of a synthetic RNA with the sequence tag-*spc1*-DR2 (Fig. S5A).

modified, whereas the 5' end of the crRNA intermediate was maintained constant. The mature crRNA length also remained constant; therefore, we hypothesized that the length of mature crRNA could be determined by a mechanism that measures the distance from the 5'-end primary processing site. To test this hypothesis, we moved the primary processing sequence within DR1 by deleting or inserting nucleotides in the 8-nt crRNA tag (Fig. 3A). In this way, we modified the sequence and length of the 5' end of the intermediate crRNA but kept its 3' end constant. The engineered crRNAs exhibited corresponding shortened or lengthened 5' tags in primer extension assays (Fig. 3B). In addition, Northern blots showed intermediate crRNAs of different sizes that correlated with the deletions or insertions introduced in the crRNA tag region (Fig. 3C). In striking contrast, the length of the mature crRNAs remained constant at 43 and 37 nt (Fig. 3C and D). For reasons that are still unclear, the mature species in these mutants exist in different proportions. Importantly, sequence analysis of both mature species confirms that extension or reduction of the 5' tag results in a corresponding reduction or extension of the 3' end that maintains the crRNA length constant (Fig. S6 and Table S3). Altogether, these results show that maturation is achieved by a mechanism that measures the overall

Fig. 3. crRNA maturation is anchored at the upstream primary processing site. (A) The primary processing site was changed by deletions (dashes) or insertions (shaded sequences) in the crRNA tag of DR1. (B) Primer extension analysis of crRNA tag mutants shown in A. The different *spc1* crRNA extension products are indicated by asterisks. Extension of 5S rRNA is shown as control. M, DNA size marker. (C) Northern blot analysis of crRNA tag mutants shown in A. Intermediate and mature crRNAs of altered and constant lengths, respectively, are indicated. Detection of 5S rRNA is shown as control. M, DNA size marker. (D) Separation of crRNA tag mutants shown in A with single nucleotide resolution as in Fig. 2D. The slight differences in electrophoretic mobility of the captured mature crRNAs correlate with differences in their molecular weight (Table S3).

crRNA length using the 5'-end primary processing site as the reference point.

Primary Processing and Maturation Are Mediated by Different *cas* Genes. To study the enzymatic machinery responsible for crRNA maturation, we integrated the CRISPR/Cas locus of *S. epidermidis* RP62a (21) (Fig. 1A) into the *lipase* gene (*geh*) of *S. aureus* RN4220 (22) using pCL55 (23) (Fig. S7). The resulting strain produced mature crRNAs of sizes similar to those crRNAs observed in *S. epidermidis* but with reduced levels that cannot support CRISPR interference against pG0400 (Fig. 4 and Fig. S7).

We created in-frame deletions of each *cas* gene and measured the resulting accumulation and maturation of crRNAs by primer extension, Northern blot analysis, and isolation using an oligonucleotide antisense to *spc1* (Fig. 4A and B and Fig. S7). Mature crRNAs were detected in *cas1*, *cas2*, and *cas6* mutants, indicating that the proteins encoded by these genes do not participate in crRNA biogenesis. In contrast, crRNAs were not detected in

Fig. 4. Primary processing and maturation are mediated by different *cas* genes. (A) Primer extension analysis of *spc1* crRNAs generated in WT and *cas/csm* in-frame deletion strains. Extension of 5S rRNA is shown as control. M, DNA size marker. (B) Northern blot of *spc1* crRNAs in WT and mutant strains. Intermediate and mature crRNAs are indicated. Detection of 5S rRNA is shown as control. M, DNA size marker. The multiple deletion mutant $\Delta cas1/2\Delta csm2/3/5/6$ only expresses Cas10, Csm4, and Cas6.

cas10, *csm4*, or *cas6* mutants. Cas6 is responsible for primary processing in *Pyrococcus furiosus* (8, 24), and therefore, the *S. epidermidis* homolog most likely has the same function. However, the presence of *cas6* alone is insufficient for crRNA accumulation (Fig. S7E). Cas10 and Csm4 may be required either to activate or assist Cas6 or for the stability of crRNAs. Interestingly, only the 71-nt intermediate crRNA was detected in strains with single deletions of *csm2*, *csm3*, or *csm5* or strains containing only *cas10*, *csm4*, and *cas6* (Fig. 4B, $\Delta cas1/2\Delta csm2/3/5/6$ lane). These results reveal that Csm2, Csm3, and Csm5 mediate crRNA maturation and that maturation and primary processing are different stages of crRNA biogenesis.

Discussion

Small antisense RNAs that guide genetic interference mechanisms must undergo nucleolytic processing to generate the mature molecules found in functional ribonucleoprotein complexes (25). crRNAs are no exception, requiring multiple cleavage events and dedicated enzymatic machinery for their biogenesis. Although emerging evidence has begun to uncover the protein and nucleic acid requirements for crRNA primary processing (6, 8–11, 13), little is known about the mechanism underlying crRNA maturation. Here, we report on the molecular mechanisms underlying crRNA biogenesis in vivo in the Type III CRISPR/Cas system of the human bacterial pathogen *S. epidermidis*.

In *S. epidermidis*, the sequences surrounding the primary cleavage site are absolutely required for precursor processing (Fig. 1). In addition, the RNA hairpin formed by repeat sequences enhances crRNA processing, and it is necessary for the generation of appropriate levels of crRNAs to protect the cell against conjugative plasmids. We speculate that the stem and loop structure in the repeats enhances the binding and/or nucleolytic activity of the enzyme performing the precursor cleavage. The low levels of crRNAs in mutants that lack this structure cannot prevent conjugation, possibly because a critical level is required to target a multicopy plasmid. The repeat requirements for the generation of crRNA intermediates in *S. epidermidis* are in direct contrast to those intermediates found for the Type III system of *P. furiosus*. In this archaeon, sequences ~13–20 nt upstream of the primary cleavage site are required for Cas6 binding and therefore, are essential for primary processing (8, 24). Additionally, there seems to be no requirement for a stem loop structure in the repeat. These

data highlight the mechanistic differences among CRISPR/Cas systems, even within those systems belonging to the same type.

In *S. epidermidis*, maturation generates two crRNA species, 43- and 37-nt long, after nucleolytic attack of the 3' end of a 71-nt-long intermediate (Fig. 2). Maturation occurs in diverse CRISPR/Cas types. In the Type III CRISPR/Cas system present in *P. furiosus*, maturation produces 39- and 45-nt-long crRNAs from 69-nt-long intermediate crRNAs (18). In the Type II system of *Streptococcus pyogenes*, 66-nt intermediates are trimmed at the 5' end to generate a 42-nt-long mature species, and there is additional trimming at the 5' end of some crRNAs that results in 39- to 42-nt-long species (20). In *P. furiosus*, both mature crRNAs can direct the cleavage of the target nucleic acid in vitro (11). The presence of two mature species in Type III systems is intriguing; however, additional work is needed to determine if both species have equivalent functions in vivo. In this study, we showed that maturation is independent of the sequence, length, and secondary structure of the degraded region of the intermediate crRNA (Fig. 2). Instead, maturation occurs by a mechanism that maintains the length of mature crRNAs using the primary processing site as the reference point for length measurement (Fig. 3). This mechanism is reminiscent of the miRNA processing pathway in which a DGCR8-RNA interaction precisely positions the primary-miRNA (pri-miRNA) for Droscha cleavage (26), and of miRNA and siRNA pathways in which Dicer, itself a molecular ruler, produces small RNAs of precise lengths (27).

Our work also defines the minimal requirements for the production of functional crRNAs. In *S. epidermidis*, the minimal CRISPR functional unit consists of only one repeat followed by one spacer, because (i) upstream, but not downstream, repeat sequences are required for primary processing, (ii) each repeat spacer unit is processed independently, and (iii) no specific sequences are required downstream of the spacer for the final maturation step. Additionally, crRNAs with both deletions (tag – 2 nt) and insertions (tag + 4 nt) in the tag sequence were able to perform CRISPR interference (Table S2), showing that the strict 8-nt tag conservation observed in all crRNAs of Type I and III CRISPR/Cas systems (6, 7, 9, 18) is not required for CRISPR function. Failure to prevent plasmid conjugation by other tag mutants may be related to a minimal tag length required to license interference (19) (in mutants with shorter tags) or a decrease in spacer/target match (in mutants carrying longer tags and shorter spacers) (Table S3). These findings are important for the bioengineering of CRISPR immunity in bacteria for industrial and synthetic biology applications and could be extended to other CRISPR/Cas systems (Fig. S8).

Genetic analysis of crRNA biogenesis in *S. epidermidis* reveals that primary processing and maturation are distinct steps in this pathway (Fig. 4). crRNA maturation and abundance were not altered in *cas1*, *cas2*, and *csm6* mutants, indicating that the proteins encoded by these genes do not participate in crRNA biogenesis. This finding is expected, because Cas1 and Cas2 are thought to be involved in the acquisition of new spacer sequences (1–3). Csm6 may also participate in spacer acquisition; however, it is more likely to be involved in target recognition or cleavage (28). In contrast, we found that *cas6*, *cas10*, and *csm4* are required for the accumulation of crRNAs in vivo. It has been observed that unprocessed crRNA precursors are degraded by cellular nucleases (29), and therefore, we interpret the absence of primer extension products and Northern blot signals in these mutants as lack of primary processing of the precursor. The Cas6 homolog from *P. furiosus* cleaves synthetic RNA molecules containing repeat sequences (8, 24) and most likely, performs the endoribonucleolytic cleavage of the crRNA precursor in *S. epidermidis*. One possibility is that Cas10 and Csm4 either activate Cas6 activity or protect crRNAs from cellular nucleases.

In contrast to the absence of crRNAs observed in *cas6*, *cas10*, and *csm4* mutants, only the 71-nt intermediate could be detected

in cells lacking *csm2*, *csm3*, and/or *csm5*. This result indicates that these genes mediate crRNA maturation. In addition, our data cannot rule out the possibility that Cas10, Csm4, and/or Cas6 are also involved in crRNA maturation. Supporting a direct role in maturation, Csm3, Csm4, and Csm5 belong to the repeat-associated mysterious proteins (RAMP) family that possesses an RNA recognition motif and catalytic histidine, which suggests the potential for ribonuclease activity (17). Alternatively, Csm2, Csm3, and/or Csm5 could recruit cellular ribonucleases or trigger a rearrangement of a processing complex that results in the exposure of the 3' end of the intermediate crRNA to cellular nucleases.

In summary, our results show that Cas proteins mediate a molecular ruler mechanism that determines the length of mature crRNAs using the primary cleavage site in upstream repeat sequences as the anchor point.

Materials and Methods

Bacterial Strains and Growth Conditions. *S. epidermidis* [RP62a (21) and Δ crispr (7)] and *S. aureus* [RN4220 (22) and OS2 (30)] strains were grown in brain heart infusion media (Difco). When required, the medium was supplemented with antibiotics: neomycin (15 μ g/mL) for selection of *S. epidermidis* Δ crispr, chloramphenicol (10 μ g/mL) for selection of pCRISPR- and pCL55-based plasmids, and mupirocin (5 μ g/mL) for selection of pG0400.

Plasmid Construction. pCRISPR-based plasmids were generated using PCR to amplify pCRISPR (19) using primers containing the desired mutation. PCR products were 5'-phosphorylated using T4 Polynucleotide Kinase (New England Biolabs) and then circularized using T4 DNA ligase (New England Biolabs). The reaction mix was transformed into *S. aureus* OS2 (30). Plasmids were isolated, and their mutations were corroborated by sequencing and transformed into *S. epidermidis* Δ crispr as described previously (7). Primer sequences and their uses are shown in Tables S4 and S5, respectively.

Strain Construction. Construction of *S. aureus* RN4220::CRISPR/cas strain was performed by integrating pCL55 (23) derivatives containing WT or *cas/csm*

mutant versions of the *S. epidermidis* CRISPR/cas locus (Table S6) into the *geh* locus of *S. aureus* RN4220 (22). Full details are in SI Materials and Methods.

Conjugation. Conjugation was carried out by filter mating as previously described elsewhere (7). Corroboration of the presence of the desired plasmid in transconjugants and recipients was achieved by extracting DNA of at least two colonies, performing PCR with suitable primers (L6/L50, L70/L71, and L86/L87; specific for *S. epidermidis* RP62a, pG0400, and pCRISPR, respectively), and sequencing the resulting PCR product.

Primer Extension. Page-purified primers P1, P2, P3, and PrrfA (for *spc1*, *spc2*, and *spc3* crRNAs and 5S rRNA, respectively) (Table S4) were used in primer extension reactions that were performed as described in ref. 7.

Northern Blot. Oligonucleotide probes antisense to *spc1*, *spc2*, and *spc3* crRNAs and 5S rRNA (Probe1, Probe2, Probe3, and PrrfA, respectively) (Table S4) were used. Northern blots were performed as described in ref. 19.

CRISPR RNA Capture. 50 μ g of total RNA and 2 ng of 5'-biotinylated PAGE-purified oligonucleotide antisense to *spc1* (5'-CTTTGACTGATGATTATATCTTCGGCATACGT) were combined in annealing buffer (5 mM Tris-HCl, pH 8.3, 75 mM KCl, 1 mM EDTA) and heated to 95 °C for 1 min and 48 °C for 45 min. Samples were then mixed with 20 μ L streptavidin-coated magnetic beads (Thermo) and incubated at room temperature for 1 h. Beads were separated from the supernatant, washed three times in annealing buffer, and resuspended in 30 μ L annealing buffer. The samples were heated to 95 °C for 2 min and then placed on ice to release the bound RNA. The RNA was labeled using γ -[³²P]ATP and T4 polynucleotide kinase (New England Biolabs) and resolved on a 10% denaturing polyacrylamide gel. The gel was exposed to a storage phosphor screen and visualized using a Typhoon 9400 PhosphorImager.

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- Deveau H, Garneau JE, Moineau S (2010) CRISPR/Cas system and its role in phage-bacteria interactions. *Annu Rev Microbiol* 64:475–493.
- Horvath P, Barrangou R (2010) CRISPR/Cas, the immune system of bacteria and archaea. *Science* 327:167–170.
- Marraffini LA, Sontheimer EJ (2010) CRISPR interference: RNA-directed adaptive immunity in bacteria and archaea. *Nat Rev Genet* 11:181–190.
- Terns MP, Terns RM (2011) CRISPR-based adaptive immune systems. *Curr Opin Microbiol* 14:321–327.
- Barrangou R, et al. (2007) CRISPR provides acquired resistance against viruses in prokaryotes. *Science* 315:1709–1712.
- Brouns SJ, et al. (2008) Small CRISPR RNAs guide antiviral defense in prokaryotes. *Science* 321:960–964.
- Marraffini LA, Sontheimer EJ (2008) CRISPR interference limits horizontal gene transfer in staphylococci by targeting DNA. *Science* 322:1843–1845.
- Carte J, Wang R, Li H, Terns RM, Terns MP (2008) Cas6 is an endoribonuclease that generates guide RNAs for invader defense in prokaryotes. *Genes Dev* 22:3489–3496.
- Haurwitz RE, Jinek M, Wiedenheft B, Zhou K, Doudna JA (2010) Sequence- and structure-specific RNA processing by a CRISPR endonuclease. *Science* 329:1355–1358.
- Gesner EM, Schellenberg MJ, Garside EL, George MM, Macmillan AM (2011) Recognition and maturation of effector RNAs in a CRISPR interference pathway. *Nat Struct Mol Biol* 18:688–692.
- Hale CR, et al. (2009) RNA-guided RNA cleavage by a CRISPR RNA-Cas protein complex. *Cell* 139:945–956.
- Jore MM, et al. (2011) Structural basis for CRISPR RNA-guided DNA recognition by Cascade. *Nat Struct Mol Biol* 18:529–536.
- Sashital DG, Jinek M, Doudna JA (2011) An RNA-induced conformational change required for CRISPR RNA cleavage by the endoribonuclease Cse3. *Nat Struct Mol Biol* 18:680–687.
- Semenova E, et al. (2011) Interference by clustered regularly interspaced short palindromic repeat (CRISPR) RNA is governed by a seed sequence. *Proc Natl Acad Sci USA* 108:10098–10103.
- Wiedenheft B, et al. (2011) RNA-guided complex from a bacterial immune system enhances target recognition through seed sequence interactions. *Proc Natl Acad Sci USA* 108:10092–10097.
- Garneau JE, et al. (2010) The CRISPR/Cas bacterial immune system cleaves bacteriophage and plasmid DNA. *Nature* 468:67–71.
- Makarova KS, et al. (2011) Evolution and classification of the CRISPR-Cas systems. *Nat Rev Microbiol* 9:467–477.
- Hale C, Kleppe K, Terns RM, Terns MP (2008) Prokaryotic silencing (psi)RNAs in *Pyrococcus furiosus*. *RNA* 14:2572–2579.
- Marraffini LA, Sontheimer EJ (2010) Self versus non-self discrimination during CRISPR RNA-directed immunity. *Nature* 463:568–571.
- Deltcheva E, et al. (2011) CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III. *Nature* 471:602–607.
- Gill SR, et al. (2005) Insights on evolution of virulence and resistance from the complete genome analysis of an early methicillin-resistant *Staphylococcus aureus* strain and a biofilm-producing methicillin-resistant *Staphylococcus epidermidis* strain. *J Bacteriol* 187:2426–2438.
- Kreiswirth BN, et al. (1983) The toxic shock syndrome exotoxin structural gene is not detectably transmitted by a prophage. *Nature* 305:709–712.
- Lee CY, Buranen SL, Ye ZH (1991) Construction of single-copy integration vectors for *Staphylococcus aureus*. *Gene* 103:101–105.
- Wang R, Preamplume G, Terns MP, Terns RM, Li H (2011) Interaction of the Cas6 ribonuclease with CRISPR RNAs: Recognition and cleavage. *Structure* 19:257–264.
- Bartel DP (2004) MicroRNAs: Genomics, biogenesis, mechanism, and function. *Cell* 116:281–297.
- Han J, et al. (2006) Molecular basis for the recognition of primary microRNAs by the Drosha-DGCR8 complex. *Cell* 125:887–901.
- Macrae IJ, et al. (2006) Structural basis for double-stranded RNA processing by Dicer. *Science* 311:195–198.
- Makarova KS, Aravind L, Wolf YI, Koonin EV (2011) Unification of Cas protein families and a simple scenario for the origin and evolution of CRISPR-Cas systems. *Biol Direct* 6:38.
- Pougach K, et al. (2010) Transcription, processing and function of CRISPR cassettes in *Escherichia coli*. *Mol Microbiol* 77:1367–1379.
- Schneewind O, Model P, Fischetti VA (1992) Sorting of protein A to the staphylococcal cell wall. *Cell* 70:267–281.