CRISPR Interference Limits Horizontal Gene Transfer in Staphylococci by Targeting DNA

Luciano A. Marraffini and Erik J. Sontheimer*

Horizontal gene transfer (HGT) in bacteria and archaea occurs through phage transduction, transformation, or conjugation, and the latter is particularly important for the spread of antibiotic resistance. Clustered, regularly interspaced, short palindromic repeat (CRISPR) loci confer sequence-directed immunity against phages. A clinical isolate of Staphylococcus epidermidis harbors a CRISPR spacer that matches the nickase gene present in nearly all staphylococcal conjugative plasmids. Here we show that CRISPR interference prevents conjugation and plasmid transformation in S. epidermidis. Insertion of a self-splicing intron into nickase blocks interference despite the reconstitution of the target sequence in the spliced mRNA, which indicates that the interference machinery targets DNA directly. We conclude that CRISPR loci counteract multiple routes of HGT and can limit the spread of antibiotic resistance in pathogenic bacteria.

Clustered, regularly interspaced, short palindromic repeat (CRISPR) loci are present in ~40% of eubacterial genomes and nearly all archaeal genomes sequenced to date and consist of short (~24 to 48 nucleotides) repeats separated by similarly sized unique spacers. They are generally flanked by a set of CRISPR-associated (cas) protein-coding genes (3-5). The CRISPR spacers and repeats are transcribed and processed into small CRISPR RNAs (crRNAs) (4, 6-8) that specify acquired immunity against bacteriophage infection by a mechanism that relies on the strict identity between CRISPR spacers and phage targets (3, 4).

The rise of hospital- and community-acquired methicillin- and vancomycin-resistant Staphylococcus aureus (MRSA and VRSA, respectively) is directly linked to the horizontal transfer of antibiotic resistance genes by plasmid conjugation (9, 10). S. aureus and S. epidermidis strains are the most common causes of nosocomal infections (11-13), and conjugative plasmids can spread from one species to the other. Although the S. epidermidis strain American Type Culture Collection (ATCC) 12228 (14) lacks CRISPR sequences, the clinically isolated strain RP62a (15) contains a CRISPR locus (Fig. 1A and fig. S1A) that includes a spacer (spc1) that is homologous to a region of the nickase (nes) gene found in all sequenced staphylococcal conjugative plasmids (fig. S1B), including those from MRSA and VRSA strains (9, 16, 17).

To test whether spc1 prevents plasmid conjugation into S. epidermidis RP62a, we disrupted the sequence match by introducing nine silent mutations into the nes target in the conjugative plasmid pG0400 (18), generating pG0(mut) (Fig. 1B) (19). We tested whether both wild-type and mutant pG0400 transferred from S. aureus strain RN4220 (20) into either of the two S. epidermidis strains (Fig. 1D and fig. S1C). Although the conjugation frequency of both plasmids was similar for the CRISPR-negative ATCC 12228 strain, only pG0(mut) was transferred into the CRISPR-positive RP62a strain and with a frequency similar to that of wild-type pG0400 in the control ATCC 12228 strain. These results indicate that CRISPR interference can prevent plasmid conjugation in a manner that is specified by sequence identity between a spacer and a plasmid target sequence.

To test this conclusion more rigorously, and to determine whether the CRISPR sequences themselves are responsible for the observed interference, we deleted the four repeats and three spacers present in the RP62a locus in order to generate the isogenic Δcrispr strain LAM104 (Fig. 1A) and tested its ability to act as a recipient for the conjugative transfer of pG0400. Again, wild-type RP62a was refractory to pG0400 transfer, whereas the conjugation efficiency for the LAM104 strain was similar to that obtained for S. epidermidis ATCC 12228 (Fig. 1D and fig. S1C). pG0(mut) transfer was similar in both strains. To restore interference in the Δcrispr mutant, we transformed LAM104 with plasmid pCRISPR or pCRISPR-L (Fig. 1C). Both of these plasmids contain the RP62a CRISPR repeats and spacers downstream of an isopropyl-β-thiogalactopyranoside (IPTG)-inducible pro-
dis
of pG0(I2) to were also used as donors LAM104 transconjugants
nes
interference with spliced
pG0(wt) and mutant LAM104 as re-
cipients for pG0(wt) and pG0(I2). To test for in-
currence with spliced nes mRNA, RP62a and LAM104 transconjugants
were also used as donors of pG0(I2) to S. epidermi-
dis ATCC 12228.

Fig. 2. CRISPR interference requires an intact target sequence in plasmid DNA but not mRNA. (A) Disruption of the pG0400 nes target sequence with the orf142-I2 self-splicing intron, generating the conjugative plasmid pG0(I2). (B) Con-
jugation efficiency was measured as in Fig. 1D with RP62a and the Δcrispr mutant LAM104 as recipients for pG0(wt) and pG0(I2). To test for interference with spliced nes mRNA, RP62a and LAM104 transconjugants
were also used as donors of pG0(I2) to S. epidermi-
dis ATCC 12228.

Fig. 3. Plasmid transformation is subject to CRISPR interference. (A) Introduction of the wild-type and mutant nes target sequences (mutations highlighted in gray) into the plasmid pC194 (Δ, direct insertion; i, inserted insertion). The origin of replication (ori) and protein-coding genes are indicated. Stem loops
denote the rep and cat transcriptional terminators. (B) RP62a and the Δcrispr mutant LAM104 were transformed in triplicate, with the plasmids described in (A). Transformation efficiency was calculated as CFU/μg of DNA (mean ± SD).

The requirement for nes transcription, splicing, and translation in the donor cell during conjugation (16), and our ability to obtain RP62a transconjugants with the intron-containing plasmid, allowed us to test the capacity of the CRISPR system to target intact spliced nes mRNA by using RP62a as a pG0(I2) donor. pG0(I2) conjugative transfer was just as efficient from RP62a as from the isogenic Δcrispr strain LAM104 (Fig. 2), which indicates that spliced functional nes mRNA (which must be present for conjugation to occur) is not target-
d during CRISPR interference. Reverse transcription polymerase chain reaction as-
says confirmed the splicing of the nes pre-
mRNA in RP62a cells carrying pG0(I2) (fig. S3C). Although our observations do not for-
mally exclude an RNA targeting event that is somewhat restricted to nascent, transient, un-
spliced transcripts, they provide strong evidence that DNA rather than mRNA is the likely crRNA target during CRISPR interference.
CRISPR activity against phage and conjugative plasmid DNA molecules suggests that CRISPR systems may also prevent plasmid DNA transformation. We therefore introduced pG0 wt and pG0 (mut) nes-target and -flanking sequences (200 base pairs) in either orientation into the staphylococcal plasmid pC194 (23), generating pNes (wt) and pNes (mut), respectively (Fig. 3A). Flanking DNA was included in the inserts to ensure the presence of any sequences outside of the target that may contribute to CRISPR interference (24). Plasmids were transformed by electroporation into wild-type RP62a and isogenic ΔcrrISP LAM104 strains. pC194 and both pNes (mut) plasmids were transformed into both strains, whereas the pNes (wt) plasmids were transformed only into the ΔcrrISP mutant (Fig. 3B). We also performed pNes (wt)/pNes (mut) mixed transformations of RP62a or LAM104 strains to test interference in an internally controlled fashion. Again, only pNes (mut) plasmids were recovered from RP62a transformants, whereas pNes (wt) and pNes (mut) plasmids were found in LAM104 transformant colonies (Fig. S4). It remains to be established whether natural transformation, which involves the uptake of a single DNA strand (25), is subject to CRISPR interference. Nonetheless, our experiments suggest that CRISPR systems can counteract multiple routes of plasmid transfer.

These transformation data provide additional evidence that crRNAs target DNA molecules. First, interference occurred regardless of the insert orientation in pNes (wt); this, combined with the lack of compelling evidence for CRISPR-derived double-stranded RNA (fig. S2) (4, 6, 7), is consistent with spc1 targeting either DNA strand rather than a unidirectional transcript. Second, the target sites in the pNes (wt) and pNes (mut) plasmids are located between the transcriptional terminators of the rep and cat genes (Fig. 3A) (23, 26, 27). This minimizes the likelihood that this region of the plasmid is even transcribed, which is consistent with its dispensability for plasmid maintenance (23, 28).

Altogether, these data provide strong functional evidence that CRISPR interference acts at the DNA level and therefore differs fundamentally from the RNA interference (RNAi) phenomenon observed in eukaryotes and with which CRISPR activity was originally compared (29). A DNA targeting mechanism for CRISPR interference implies a means to prevent its action at the encoding CRISPR locus itself, as well as other potential chromosomal loci, such as prophage sequences. Little information exists to suggest how crRNAs would avoid targeting “self” DNA, although the role of flanking sequences during CRISPR interference (24) could contribute to target specificity. From a practical standpoint, the ability to direct the specific addressable destruction of DNA that contains any given 24- to 48-nucleotide target sequence could have considerable functional utility, especially if the system can function outside of its native bacterial or archaeal context. Furthermore, our results demonstrate that CRISPR function is not limited to phage defense, but instead encompasses a more general role in the prevention of HGT and the maintenance of genetic identity, as with restriction-modification systems. A primary difference between restriction-modification and CRISPR interference is that the latter can be programmed by a suitable effecter crRNA. If CRISPR interference could be manipulated in a clinical setting, it would provide a means to impede the ever-worsening spread of antibiotic resistance genes and virulence factors in staphylococci and other bacterial pathogens.

References and Notes

Nascent RNA Sequencing Reveals Widespread Pausing and Divergent Initiation at Human Promoters

Leighton J. Core,* Joshua J. Waterfall,* John T. Lis†

RNA polymerases are highly regulated molecular machines. We present a method (global run-on sequencing, GRO-seq) that maps the position, amount, and orientation of transcriptionally engaged RNA polymerases genome-wide. In this method, nuclear run-on RNA molecules are subjected to large-scale parallel sequencing and mapped to the genome. We show that peaks of promoter-proximal polymerase reside on ~30% of human genes, transcription extends beyond messenger RNA 3’ cleavage, and antisense transcription is prevalent. Additionally, most promoters have an engaged polymerase upstream and in an orientation opposite to the annotated gene. This divergent polymerase is associated with active genes but does not elongate effectively beyond the promoter. These results imply that the interplay between polymerases and regulators over broad promoter regions dictates the orientation and efficiency of productive transcription.

Transcription of coding and noncoding RNA molecules by eukaryotic RNA polymerases requires their collaboration with hundreds of transcription factors to direct and control polymerase recruitment, initiation, elongation, and termination. Whole-genome microarrays and ultra-high-throughput sequencing technologies enable efficient mapping of the distribution of transcription factors, nucleosomes, and their modifications, as well as accumulated RNA transcripts throughout genomes (1, 2), thereby providing a global correlation of factors and transcription states. Studies using the chromatin immunoprecipitation assay coupled to genomic DNA microarrays (ChIP-chip) or to high-throughput sequencing (ChIP-seq) indicate that RNA polymerase II (Pol II) is present at disproportionately higher amounts near the 5’ end of many eukaryotic

Website: www.sciencemag.org