Title: Translation-dependent downregulation of Cas12a mRNA by an anti-CRISPR protein

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Abstract:
Bacterial CRISPR-Cas systems cleave the genomic DNA of bacteriophage and other mobile genetic elements. Many temperate phages encode anti-CRISPR (Acr) proteins that use diverse and innovative mechanisms to inhibit CRISPR-Cas and stabilize lysogeny. We previously reported that a *Moraxella bovoculi* prophage encodes two anti-CRISPR proteins, AcrVA1 and AcrVA2, that inhibit MbCas12a. Whereas AcrVA1 inactivates CRISPR-Cas12a complexes by cleaving the CRISPR RNA (crRNA), the mechanism and utility of AcrVA2 have remained unclear. Here, we show that AcrVA2 unexpectedly inhibits Cas12a biogenesis by binding to the nascent Cas12a polypeptide and triggering degradation of its mRNA. Mutations in the first 15 amino acids of Cas12a abolish binding, downregulation, and inhibition by AcrVA2, while altering the Cas12a codon sequence and promoter has no effect. These co-encoded anti-CRISPRs therefore use complementary strategies to recognize conserved features in Cas12a and inhibit its DNA targeting and biogenesis. Beyond *Moraxella,* AcrVA2 is broadly distributed across numerous bacterial clades, including where Cas12a systems are not found, suggesting that this novel mechanism of gene regulation may target other proteins.

Main Text
Bacterial viruses (phages) are the most abundant biological entities on earth (*1*). The intense selective pressure that phages impose on bacteria has led to the evolution of a wide array of bacterial defense systems, including restriction enzymes and CRISPR-Cas (*2*). CRISPR-Cas systems are found in 40% of bacteria and 90% of archaea, where they function as adaptive immune systems against phage and other mobile genetic elements (*3*). When phage invade the cell, short DNA fragments are acquired from their genome and incorporated into a CRISPR array, which is transcribed and processed into CRISPR RNAs (crRNAs). These crRNA molecules form interference complexes with Cas protein(s) that identify complementary sequences in phage and target them for cleavage. Because CRISPR-Cas systems are readily programmable and have high specificity and targeting efficiency, they have been adapted as gene editing platforms in various cell types and tissues (*4*).

In response to this pressure, phages have evolved proteins, called anti-CRISPRs, to block CRISPR-Cas targeting (*5*). Most known anti-CRISPRs inhibit cleavage by binding CRISPR-Cas complexes directly and preventing them from binding their targets or changing conformation. However, some anti-CRISPRs enzymatically modify CRISPR-Cas complexes using novel or co-opted protein functions. The broad-spectrum CRISPR-Cas12a (Type V-A) inhibitor AcrVA1, for example, cleaves crRNA using a non-canonical nuclease motif (*6*, *7*).

AcrVA1 was found encoded on a prophage within *Moraxella bovoculi*, where the native CRISPR-Cas12a system encodes multiple spacers against the phage (*8*, *9*). Because cleavage of an integrated prophage is generally lethal for both the phage and bacterium, their stable coexistence indicates that Cas12a is effectively inhibited. Interestingly, *acrVA1* was found encoded next to another Cas12a inhibitor, *acrVA2*, that is notably large (~1 kb) and widely distributed across diverse classes of Proteobacteria (*8*). AcrVA2 potently inhibits MbCas12a (*Moraxella bovoculi*) in bacteria but not in human cells, suggesting it has a different mode of

action, but its role and mechanism have remained unclear. Likewise, it is not clear why this phage encodes multiple inhibitors against the same nuclease.

Here we show that AcrVA2 inhibits Cas12a biogenesis by recognizing conserved residues in the Cas12a nascent polypeptide and downregulating its mRNA before translation is complete. Given that *acrVA2* homologs are widely distributed and its key residues are conserved, this novel mechanism of gene regulation may be widespread in prokaryotes and co-opted for different forms of microbial and molecular antagonism. The dual strategies employed here to inhibit CRISPR-Cas biogenesis (i.e. AcrVA2) and targeting (i.e. AcrVA1) likely allow phage to survive during infection and maintain a stable coexistence with the bacterial host.

Results:

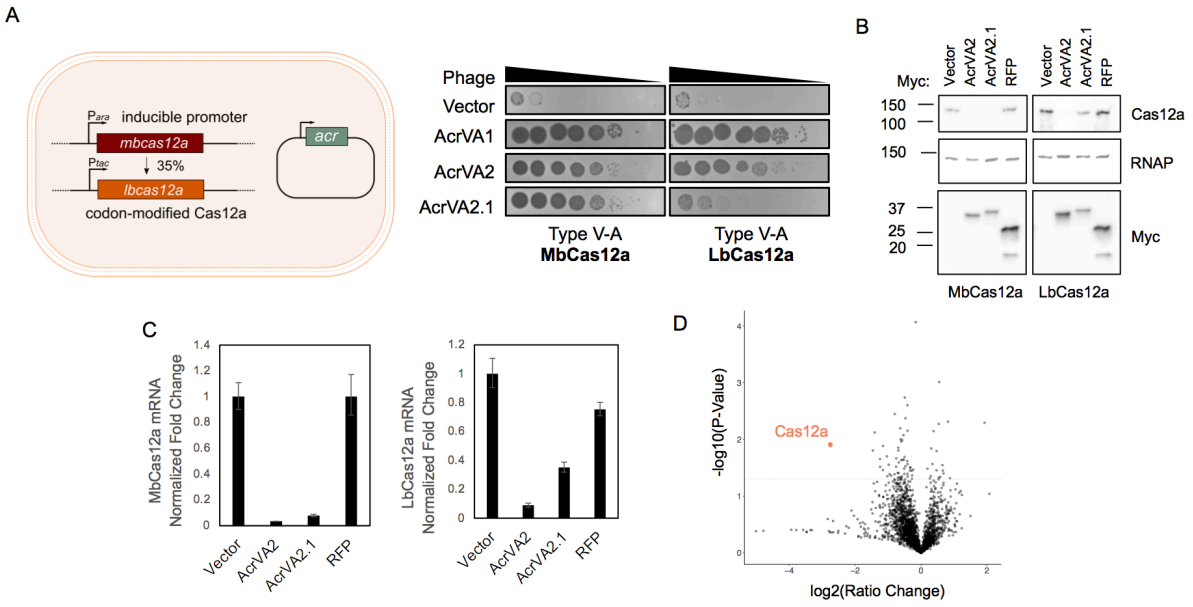

Figure 1. AcrVA2 specifically downregulates mRNA and protein of divergent Cas12a orthologs. (A) Left: Schematic of *Pseudomonas aeruginosa* strains engineered to express MbCas12a or codon-modified LbCas12a from inducible promoters and *acr* genes from plasmids. Right: Phage plaque assay with ten-fold serial dilutions of phage to assess CRISPR-Cas12a inhibition. (B) Western blot on bacterial lysates expressing myc-tagged anti-CRISPR or control proteins and Cas12a. RNAP, RNA polymerase (loading control). (C) qRT-PCR on mRNA from bacterial lysates expressing Cas12a and anti-CRISPR protein or control. Error bars indicate standard deviation. (D) Volcano plot for transcriptomic analysis. mRNA was extracted from bacteria expressing MbCas12a and AcrVA2 or controls. log2(Ratio Change) is the mean expression level for samples expressing AcrVA2 or AcrVA2.1 relative to controls (see methods for details). Each dot represents one gene. Dotted line indicates p-value of 0.05.

Although many CRISPR-Cas inhibitory mechanisms have been discovered, Acr proteins typically inhibit Cas proteins through a direct interaction that prevents target DNA binding or cleavage (*5*). However, when we tested AcrVA2 for MbCas12a binding and inhibition *in vitro*, we saw no inhibition of cleavage (fig. S1), consistent with its inability to inhibit Cas12a in human cells (*8*). This result suggested that AcrVA2 may inhibit Cas12a upstream of ribonucleoprotein (RNP) complex formation. To test this, we used strains of *Pseudomonas aeruginosa* in which plasmid-borne AcrVA2 robustly inhibits MbCas12a or LbCas12a expressed from the

chromosome via different inducible promoters (Fig. 1A). Intriguingly, AcrVA2 drastically reduced mRNA and protein levels for both MbCas12 and LbCas12a, which share 35% amino acid identity. The impact of AcrVA2 (and AcrVA2.1, an ortholog with 84% identity) on MbCas12a and LbCas12a expression correlated well with their ability to inhibit both orthologs (i.e. AcrVA2.1 was less active against LbCas12a).

The downregulation of LbCas12a was surprising, given that its codons are modified from the native sequence and it shares only 35% amino acid identity with MbCas12a. Furthermore, we found that AcrVA2 also downregulates both human codon-modified and native MbCas12a equally well (Fig. 1, B and C, fig. S2). The codon-modified sequence of MbCas12a was used for the rest of this study.

The ability of AcrVA2 to downregulate divergent *cas12a* transcripts expressed from non-native promoters and featuring dramatic nucleotide changes prompted us to assess its specificity. Transcriptomic analysis revealed that *cas12a* was the only expressed gene that was significantly downregulated by AcrVA2 (Fig. 1D), while a hypothetical protein (PA3431) and a PpiC-type peptidyl-prolyl cis-trans isomerase (PA3871) were upregulated for reasons that are unclear. A closer analysis of the *cas12a* open reading frame revealed that reads were reduced by AcrVA2 at the 5' end and were barely detectable for the latter 75% of the gene (fig. S3). Altogether, these results demonstrate that AcrVA2 specifically downregulates divergent and codon-modified Cas12a orthologs independently of the promoter and codon sequence.

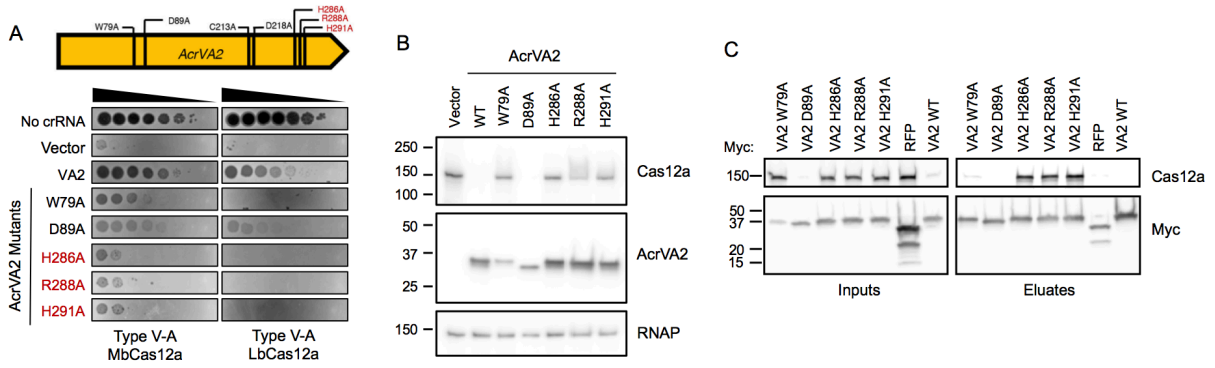

Figure 2. Inactive AcrVA2 mutants stably bind Cas12a protein. (A) Phage plaque assay on strains expressing Cas12a and wildtype or mutant AcrVA2. Ten-fold serial dilutions of phage were plated on bacterial lawns to assess Cas12a inhibition. (B) Western blot on bacterial lysates expressing AcrVA2 point mutants and Cas12a. RNAP, RNA polymerase (loading control). (C) Immunoprecipitations on myc-tagged AcrVA2 H286A or GST control from bacterial lysates. Samples were resolved by SDS-PAGE and probed via Western blot.

AcrVA2 does not appear to have any conserved domains or catalytic residues that suggest a molecular mechanism for this RNA downregulation. The crystal structure of AcrVA2 revealed three distinct domains but likewise did not resemble any known enzymes (*10*). To identify amino acids in AcrVA2 that are important for its function, we mutated residues that are highly conserved across diverse AcrVA2 orthologs, including FinQ from *E. coli*, a distant homolog (*8*). Interestingly, mutating W79 and D89 caused a moderate loss of function, while mutating

residues H286A, R288A, and H291A in the C-terminal region of the protein abolished AcrVA2 activity and restored Cas12a protein (Fig. 2, A and B) and mRNA (fig. S3).

The inability of AcrVA2 mutants to fully downregulate Cas12a in bacteria enabled us to ask whether these two proteins interact *in vivo*. Immunoprecipitation of myc-tagged AcrVA2 mutants (H286A, R288A, H291A) showed stable co-precipitation with Cas12a (Fig. 2C). AcrVA2^{W79A}, on the other hand, abrogated downregulation of Cas12a but did not yield as robust an interaction as the C-terminal mutants, suggesting that this residue is important for the interaction with Cas12a or for AcrVA2 stability. Overall, these results demonstrate that AcrVA2 interacts with Cas12a protein in a manner that is not directly inhibitory.

AcrVA2 binds apoCas12a *in vitro* (fig. S1) and co-purifies with a fragment (residues 620-636) from Cas12a (*10*). However, a triple mutation in AcrVA2 (E98A/D129A/D195A) that was previously shown to break this specific interaction did not affect downregulation or inhibition in our *in vivo* assays (fig. S4), indicating that interaction at this site in Cas12a is not essential for inhibition.

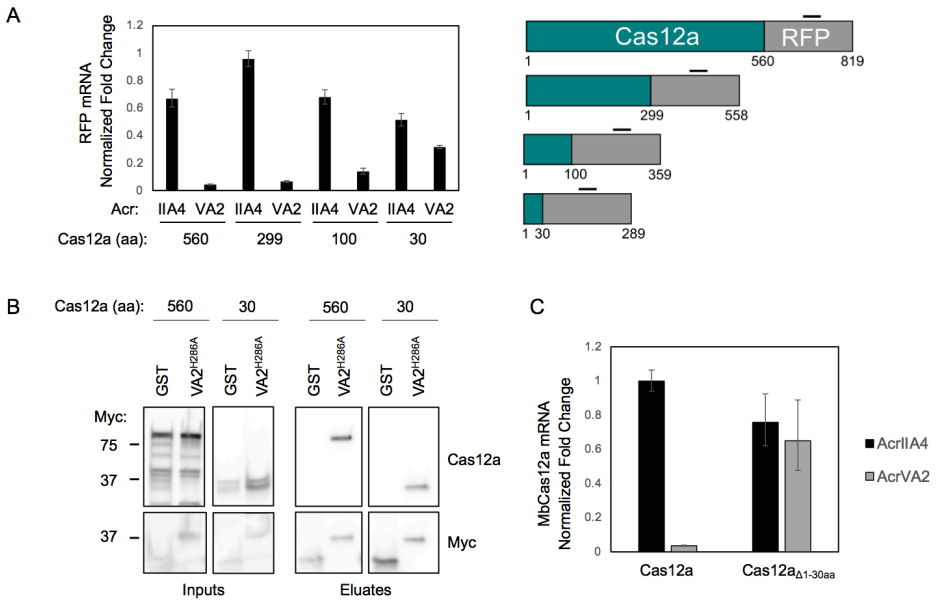

Figure 3. The N-terminal region of Cas12a is required for downregulation and is stably bound by AcrVA2 mutants. (A) Left: qRT-PCR on mRNA from bacteria expressing Cas12a truncated from the C-terminal end and fused to RFP. Numbers indicate amino acid length from the N-terminal end. Right: Schematic of MbCas12a C-terminal truncations fused to RFP. Numbers indicate amino acid residues. Dash indicates site probed by RFP-specific primers for qRT-PCR. Error bars indicate standard deviation. (B) Immunoprecipitations on myc-tagged AcrVA2 H286A or GST control from bacterial lysates. Samples were resolved by SDS-PAGE and probed via Western blot. (C) qRT-PCR on mRNA from bacteria expressing wildtype Cas12a or Cas12a lacking the first 30 amino acids (and provided with a start codon).

To find the region of Cas12a that is sufficient for downregulation and interaction, we truncated MbCas12a from the C-terminus and fused the remaining fragments to RFP. Probing RFP mRNA revealed that AcrVA2 requires only the first 100 amino acids (~1/14th) of Cas12a to

trigger mRNA downregulation (Fig. 3A). Although the first 30 amino acids of Cas12a were insufficient for downregulation, this region stably co-precipitated with AcrVA2^{H286A}, suggesting that AcrVA2 recognizes and binds a sequence within this region (Fig. 3, A and B). Conversely, a Cas12a mutant lacking the N-terminal 30 a.a. was well expressed but was no longer downregulated by AcrVA2 (Fig. 3C). Altogether, these data indicate that AcrVA2 binds near both the N-terminus and the PID of Cas12a, yet only the N-terminal region of Cas12a is necessary and sufficient for AcrVA2-induced downregulation.

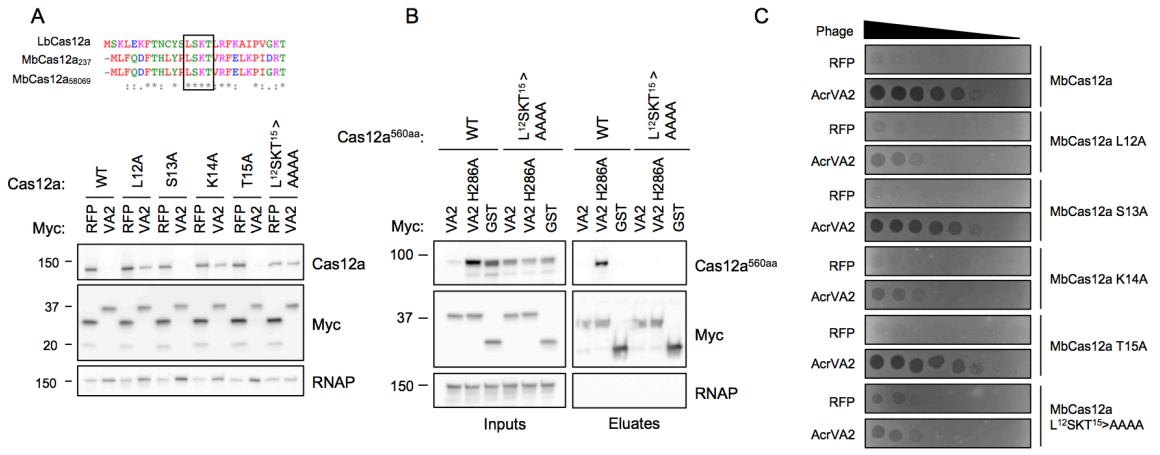

Figure 4. Mutations in the first 15 amino acids of Cas12a abrogate binding, downregulation, and inhibition by AcrVA2. (A) Top: Alignment of N-terminal polypeptide sequences from Cas12a orthologs that are susceptible to AcrVA2. Bottom: Western blot on bacterial lysates expressing MbCas12a mutants and myc-tagged AcrVA2 or RFP. RNAP, RNA polymerase (loading control). (B) Immunoprecipitations on myc-tagged AcrVA2 or GST control from bacterial lysates. Samples were resolved by SDS-PAGE and probed via Western blot. (C) Phage plaque assay on strains expressing wildtype or mutant MbCas12a and AcrVA2 or RFP control. Ten-fold serial dilutions of phage were plated on bacterial lawns to assess Cas12a cleavage activity and inhibition.

The first 30 amino acids of Cas12a are part of the wedge (WED) domain, which interacts directly with crRNA and features many conserved residues, including an LSKT sequence (residues 12-15 in MbCas12a) (*11*). Mutating either L12 or K14 alone to alanine diminished AcrVA2-mediated downregulation, while mutating all four of these residues abolished it (Fig. 4A). As seen with codon-modified versions of Cas12a (Fig. 1B and 1C and fig. S2), synonymous mutations at this site had no effect on downregulation, showing that the amino acid sequence—rather than nucleic acid sequence—of Cas12a is the recognized substrate (fig. S5). Consistent with a role for the translated polypeptide, omitting the start codon from Cas12a prevented its translation into polypeptide and dramatically decreased its downregulation by AcrVA2 (fig. S6).

We next assessed whether MbCas12a$^{LSKT>AAAA}$ interacts with AcrVA2^{H286A} or wildtype AcrVA2. Because AcrVA2 binds multiple regions in MbCas12a (amino acids 1-30 and amino acids 620-636), we used truncated MbCas12a^{560aa} that lacks the PID binding site. Interestingly, truncated MbCas12a protein levels are higher when co-expressed with AcrVA2^{H286A}, presumably due to the stabilizing effect from their interaction (Fig. 3B and 4B). The LSKT>AAAA mutations in the

MbCas12a polypeptide sequence abrogated both the increased protein expression and interaction, consistent with this site being important for interaction.

We next tested the ability of full-length MbCas12a$^{LSKT>AAAA}$ to target phage. We found that it mildly lost function relative to wild type Cas12a, but was no longer susceptible to inhibition by AcrVA2 (Fig. 4C). The degree of inhibition against the quadruple and single amino acid mutants correlated closely with the levels of downregulation. Altogether, this data demonstrates that AcrVA2 recognizes and interacts with the Cas12a N-terminal polypeptide to drive its mRNA downregulation and inhibition.

Discussion

In this study, we have shown that AcrVA2 inhibits Cas12a biogenesis by recognizing its N-terminal polypeptide and triggering mRNA degradation. Multiple lines of evidence support this: first, AcrVA2 downregulates mRNA of divergent Cas12a orthologs independently of the promoter and codon sequence. Second, the N-terminal region of Cas12a is necessary and sufficient for this downregulation and stably binds non-functional AcrVA2 mutants. Finally, amino acid mutations (but not synonymous mutations) near the N-terminus of Cas12a abolish binding, downregulation, and inhibition by AcrVA2. The most straightforward model is that AcrVA2 recognizes the nascent polypeptide of Cas12a and triggers destruction of its mRNA before translation is complete. Although surprising, this strategy enables AcrVA2 to recognize a conserved region in Cas12a and destroy it before it is fully expressed.

Inhibiting biogenesis is presumably ineffective against pre-existing Cas12a present in the cell. (For the experiments shown here, Cas12a and AcrVA2 were induced simultaneously.) However, the prophage encoding *acrVA2* in *Moraxella bovoculi* also encodes *acrVA1*, which inactivates crRNA-loaded Cas12a complexes. The dual strategies employed by these co-encoded anti-CRISPRs to inactivate crRNA-loaded Cas12a complexes (i.e. AcrVA1) and suppress Cas12a expression (i.e. AcrVA2) likely inhibit Cas12a targeting more effectively than either strategy alone. Because the prophage encoding these *acrs* in *Moraxella bovoculi* is targeted by multiple spacers in the endogenous CRISPR-Cas12a system, constitutive Cas12a inactivation is required for stable coexistence of the prophage and survival of its host. Dual mechanisms that reduce expression and inactivate complexes have also been observed previously for Cas9 anti-CRISPRs (*12*, *13*). Anti-CRISPR function is also important during lysogeny for preventing the acquisition of self-targeting spacers that drive the loss of prophages and CRISPR-Cas, likely generating a strong need for CRISPR inhibition by symbiotic MGEs (*14*).

Some ribonucleases have previously been reported to bind the ribosomal aminoacylation (A)-site and cleave mRNAs in response to stress (*15*). The nascent chain of DnaA was also shown to modulate translation elongation in response to nutrient availability (*16*). To our knowledge, AcrVA2 is the first example in prokaryotes of a protein triggering specific mRNA degradation upon recognizing the translated polypeptide sequence. The broad distribution of AcrVA2 across diverse classes of proteobacteria—oftentimes where Cas12a is not present—and the conservation of key residues suggests that this mechanism may be widespread against different substrates (*8*) (fig. S8). In support of this, the protein FinQ (an AcrVA2 homolog) was previously

shown to downregulate transfer genes through an unknown mechanism and inhibit conjugation of F-plasmid in *E. coli (17–19)*. FinQ is present on F-like and I-like plasmids, suggesting it may use a similar antagonistic strategy in inter-MGE warfare. Some megaphages also encode distant orthologs of AcrVA2 where residues essential for its downregulatory mechanism are conserved (*20*) (fig. S8).

It remains unclear how AcrVA2 triggers Cas12a mRNA destruction. A similar mechanism has been demonstrated through multiple studies for tubulin autoregulation in mammalian cells (*21–25*), but the mechanism for mRNA degradation has also not yet been reported. In theory, AcrVA2 may trigger Cas12a mRNA downregulation by 1) cleaving the mRNA, 2) stalling the ribosome, or 3) terminating transcription, all of which would likely lead to the ribosome stalling at the end of the mRNA fragment (i.e. as a non-stop complex). Bacteria resolve non-stop complexes primarily via *trans*-translation, in which the transfer-messenger RNA (tmRNA) mimics tRNA to allow translation to resume while providing a short coding sequence that tags the polypeptide for degradation (*26*). The 3'-5' exonuclease RNase R is then recruited to degrade the mRNA after its release from the ribosome. The factors and pathways involved in this fascinating mechanism will need to be elucidated in future studies.

The arms race between bacteria and phage has yielded many exciting tools and key biological discoveries for gene editing and gene regulation. Here, we show a novel strategy for CRISPR-Cas regulation that may be pervasive in microbial antagonism. The insights from this work can be applied to achieve constitutive long-term inactivation of nucleases during gene editing and enable protein-specific gene regulation in bacteria. As we explore the amazing microbial diversity in nature, many more discoveries doubtless await.

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Competing interest: UCSF has filed a patent on the use of inhibitors of CRISPR-Cas12a, on which NDM and JBD are listed as inventors.

Data and material availability: Plasmids and strains will be made readily available upon request.