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Discovery of widespread type I and type V CRISPR-Cas inhibitors

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Bacterial CRISPR-Cas systems protect their host from bacteriophages and other mobile genetic elements. Mobile elements, in turn, encode various anti-CRISPR (Acr) proteins to inhibit the immune function of CRISPR-Cas. To date, Acr proteins have been discovered for type I (subtypes I-D, I-E, and I-F) and type II (II-A and II-C) but not other CRISPR systems. Here, we report the discovery of 12 *acr* genes, including inhibitors of type V-A and I-C CRISPR systems. AcrVA1 inhibits a broad spectrum of Cas12a (Cpf1) orthologs—including MbCas12a, Mb3Cas12a, AsCas12a, and LbCas12a—when assayed in human cells. The *acr* genes reported here provide useful biotechnological tools and mark the discovery of *acr* loci in many bacteria and phages.

The discovery of bacterial CRISPR-Cas systems that prevent infection by bacterial viruses (phages) has opened a paradigm for bacterial immunity while yielding exciting tools for targeted genome editing. CRISPR systems destroy phage genomes, and in turn, phages express anti-CRISPR (Acr) proteins that directly inhibit Cas effectors (*1*, *2*). Six distinct types (I to VI) of CRISPR systems are spread widely across the bacterial world (*3*), but Acr proteins have only been discovered for type I and II CRISPR systems (*1*, *3*–*6*). Given the prevalence and diversity of CRISPR systems, we predict that Acr proteins against other types await discovery.

Acr proteins do not have conserved sequences or structures and only share their relatively small size, making de novo prediction of *acr* function challenging (*6*). However, *acr* genes often cluster together with other *acr* genes or are adjacent to highly conserved Acr-associated genes (*aca* genes) in "*acr* loci" (*7*, *8*). In this work, we sought to identify *acr* genes in bacteria and phages that are not homologous to previously identified *acr* or *aca* genes.

Acr proteins were first discovered in *Pseudomonas aeruginosa*, inhibiting type I-F and I-E CRISPR systems (*1*, *9*). *P. aeruginosa* strains also encode a third CRISPR subtype (type I-C), which lacks known inhibitors (*10*). We engineered *P. aeruginosa* to target phage JBD30 with type I-C CRISPR-Cas (fig. S1A) and used it in parallel with existing type I-E (strain SMC4386) and I-F (strain PA14) CRISPR strains to screen for additional *acr* candidates.

Homologs of *aca1* were searched for in *Pseudomonas* genomes, and seven gene families not previously tested for Acr function were identified upstream of *aca1* (Fig. 1A). Three genes inhibited the type I-E CRISPR-Cas system (*acrIE5-7*), one inhibited type I-F (*acrIF11*), restoring the plaquing of a targeted phage, and two genes had no inhibitory activity (*orf1* and *orf2*) (Fig. 1B, fig. S1B, and tables S1 and S2). Another gene exhibited dual I-E and I-F inhibition, and domain analysis revealed a chimera of previously identified *acrIE4* and *acrIF7* (*acrIE4-F7*). No type I-C inhibitors were identified. The type I-F inhibitor *acrIF11* was commonly represented in both the *P. aeruginosa* mobilome and in more than 50 species of diverse Proteobacteria (fig. S2 and table S2). *acrIF11* is often associated with genes that encode DNA-binding motifs, which we have designated *aca4* to *aca7* (fig. S2 and tables S1, S3, and S4). To confirm that these *aca* genes can be used to facilitate *acr* discovery, we used *aca4* to discover an additional *Pseudomonas* Acr, *acrIF12* (Fig. 1, A and B).

Given the widespread nature of *acrIF11*, we next used it to discover Acr proteins against CRISPR systems in which they have not yet been found: type I-C, a minimal class 1 system, and type V-A CRISPR-Cas12a (Cpf1), a class 2 single effector system that has high efficiency in genome editing (*11*–*13*). To find AcrIC and AcrVA proteins, we first searched for genomes that encode CRISPR spacers that match a target protospacer elsewhere in the same genome (Fig. 2A). The tolerance of this "self-targeting" in viable bacteria indicates potential inhibition of the CRISPR system (*4*) because genome cleavage would result in bacterial death.

The Gram-negative bovine pathogen *Moraxella bovoculi* (*14*, *15*) is a Cas12a-containing organism (*11*) in which four of the seven genomes feature type V-A self-targeting (table S5), and one strain (58069) also features self-targeting by type I-C (table S6). Although no previously described *acr* or *aca* genes were present in this strain, an *acrIF11* homolog was found in phages that infect the human pathogen *Moraxella catarrhalis* (*16*), a close relative of *M. bovoculi*. Genes adjacent to *acrIF11* in *M. catarrhalis* had homologs in the self-targeting *M. bovoculi* strains (Fig. 2B), and together, these genes were selected as candidate *acr* genes. Each gene was first tested against the type I-C and I-F systems introduced above because both subtypes are found in *Moraxella*. Gene *AAX09_07415* (now *acrIC1*) inhibited the type I-C system, explaining the tolerance of self-targeting

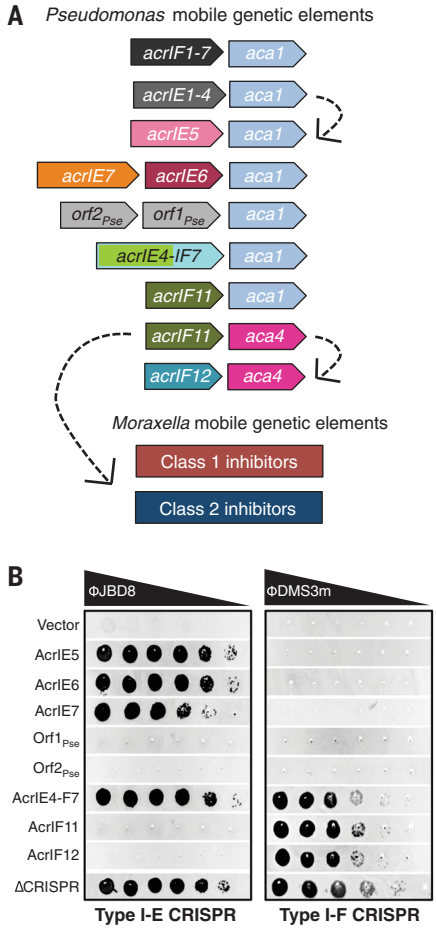

Fig. 1. The discovery of a widespread type I inhibitor. (**A**) Schematic of type I-E and type I-F Acrs with Acr-associated (*aca1* and *aca4*) genes in *Pseudomonas* sp. mobile genetic elements, with dotted lines indicating the "guilt-by-association" relationships used to discover new *acr* genes in *Pseudomonas* sp. and *Moraxella* sp. from known *acr* genes (top two rows). (**B**) Phage plaque assays to assess CRISPR-Cas inhibition. Tenfold serial dilutions of a type I-E or type I-F CRISPR-targeted phage (JBD8 or DMS3m, respectively) titered on lawns of *P. aeruginosa* with naturally active type I-E or type I-F CRISPR-Cas systems expressing candidate inhibitors. Δ*CRISPR* strains measure phage replication in the absence of CRISPR immunity (top row).

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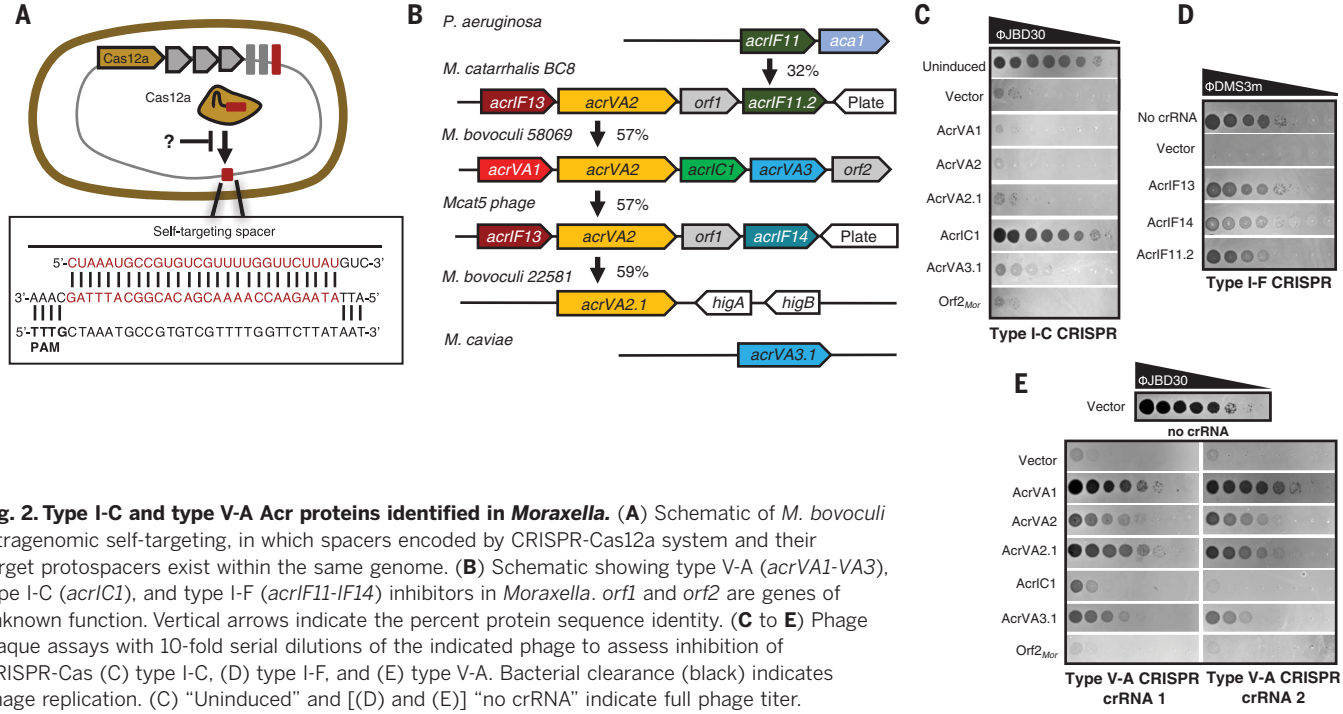

Fig. 2. Type I-C and type V-A Acr proteins identified in *Moraxella*. (**A**) Schematic of *M. bovoculi* intragenomic self-targeting, in which spacers encoded by CRISPR-Cas12a system and their target protospacers exist within the same genome. (**B**) Schematic showing type V-A (*acrVA1-VA3*), type I-C (*acrIC1*), and type I-F (*acrIF11-IF14*) inhibitors in *Moraxella*. *orf1* and *orf2* are genes of unknown function. Vertical arrows indicate the percent protein sequence identity. (**C** to **E**) Phage plaque assays with 10-fold serial dilutions of the indicated phage to assess inhibition of CRISPR-Cas (C) type I-C, (D) type I-F, and (E) type V-A. Bacterial clearance (black) indicates phage replication. (C) "Uninduced" and [(D) and (E)] "no crRNA" indicate full phage titer.

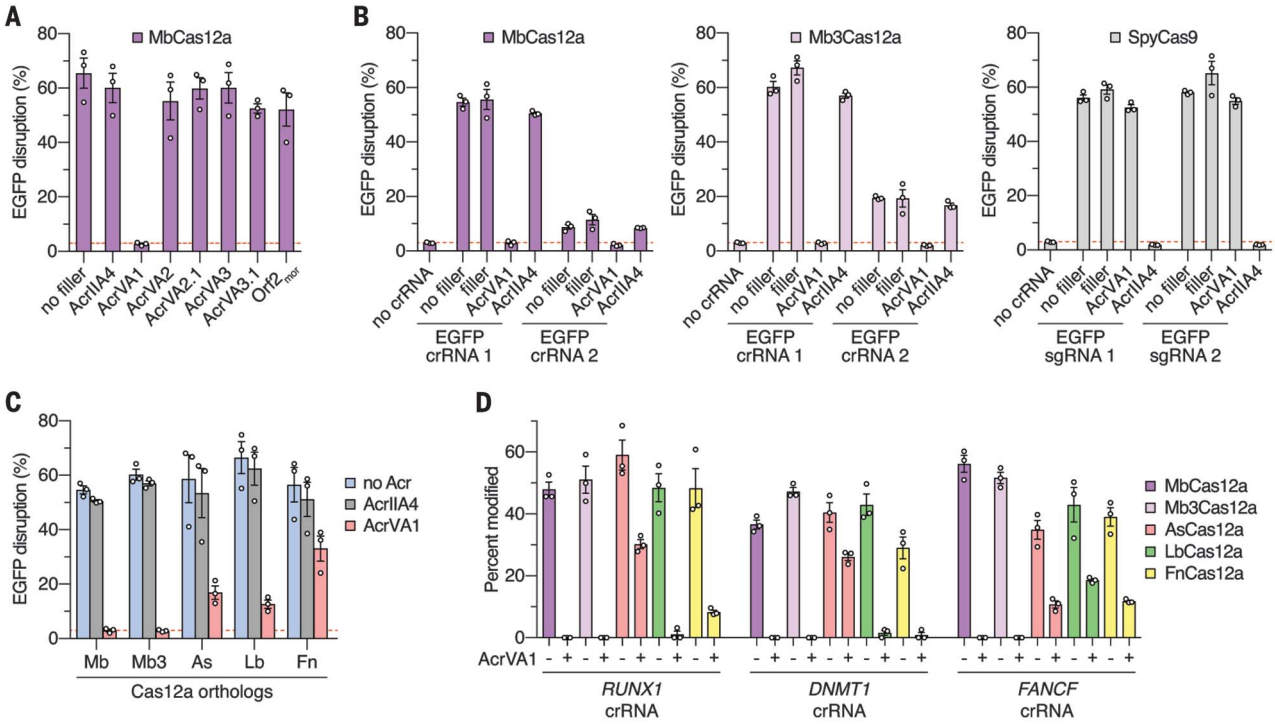

Fig. 3. AcrVA1 blocks Cas12a-mediated gene editing in human cells. (**A** to **C**) Human cell U2-OS-EGFP disruption experiments to assess AcrVA-mediated inhibition of Cas12a activities. (A) Inhibition of MbCas12a activity with various AcrVA constructs; the "no filler" condition contained only plasmids for Cas12a and crRNA expression. (B) Comparisons between the inhibitory activities of AcrVA1 and AcrIIA4 against MbCas12a, Mb3Cas12a, and SpyCas9. Controls that use "filler" plasmid in lieu of Acr plasmids were included in order to equalize amounts of DNA. (C) Assessment of AcrVA1 activity against Cas12a orthologs, with AcrIIA4 used as control. Background EGFP disruption is indicated by the red dashed line; error bars indicate SEM for $n = 3$ independent biological replicates. (**D**) Inhibition of activity of Cas12a orthologs against endogenous sites in human cells (*RUNX1*, *DNMT1*, or *FANCF* genes). Gene modification assessed by means of T7E1 assay 72 hours after transfection; error bars indicate SEM for $n = 3$ independent biological replicates.

in strain 58069 (Fig. 2C). Additionally, gene *E9U_08473* (*acrIF13*) from the *M. catarrhalis* BC8 prophage completely inhibited I-F function, as did AKI27193.1 (*acrIF14*), which is found in phage Mcat5 at the same genomic position as that of *acrIF11* in BC8 (Fig. 2, B and D). These Acr proteins possess broad spectrum activity; the type I-C and I-F systems in *Moraxella* and *Pseudomonas* only share an average pairwise identity of 30 and 36%, respectively (fig. S3)

Because of the limited tools available for the genetic manipulation of *Moraxella* sp., the remaining genes were tested for type V-A Acr function in *P. aeruginosa* PAO1 engineered to express MbCas12a and a CRISPR-RNA (crRNA) that targets *P. aeruginosa* phage JBD30. Two distinct crRNAs were used, showing strong reduction of titer by more than four orders of magnitude (Fig. 2E). The first gene in the *M. bovoculi* 58069 *acr* locus, *AAX09_07405* (*acrVA1*), restored phage titers nearly to levels seen with the crRNA-minus control, indicating that it robustly inhibits Cas12a. This is in good agreement with the independent discovery of AcrVA1 reported in a companion paper (*17*). The adjacent gene, *acrVA2*, also inhibited targeting, as did its ortholog (*acrVA2.1*) (Fig. 2E). An additional gene from this locus, *acrVA3*, possessed subtle anti-Cas12a activity but was toxic to cells and adversely affected JBD30 phage growth independently of Cas12a (fig. S5). We therefore tested an ortholog with 43% sequence identity, *B0181_04965* (*acrVA3.1*), which showed stronger Cas12a inhibition with no toxicity or adverse effects on phage growth (Fig. 2E and fig. S5). Surprisingly, *acrVA3.1* also showed partial restoration of phage titer during type I-C targeting, suggesting that it may inhibit the type I-C as well as type V-A system (Fig. 2, C and E). Although these two CRISPR subtypes do not share any protein components, a dual-specificity inhibitor may use distinct protein-interaction interfaces or modulate an undiscovered host process required for CRISPR immunity. We used the Acr "key" *acrIF11* to unlock *acr* loci that encode seven distinct *acr* genes inhibiting type I-C, I-F, and V-A CRISPR. Below, we focus on the evolutionary analysis of type V-A inhibitors and their function in mammalian cells.

The gene *acrVA1* encodes a 170–amino acid protein found only in *Moraxella* sp. and *Eubacterium eligens* (fig. S6), both type V-A CRISPR-containing organisms. By contrast, *acrVA2* (322 amino acids) and *acrVA3* (168 amino acids) orthologs are found broadly distributed throughout multiple classes of bacteria. For example, *acrVA2* orthologs are present in *Lachnospiraceae* and *Leptospira* (fig. S7), which contain type V-A CRISPR, as well as in *Moraxella, Leptospira,* and *Lactobacillus* phages. Distant orthologs of *acrVA2* were also identified on plasmids and conjugative elements in *Escherichia coli* (fig. S7), although the importance of a bacterium that lacks type V-A CRISPR encoding a putative *acrVA* gene is unknown. Orthologs of *acrVA3* were identified in many Proteobacteria and in *Eubacterium* and *Clostridium* species, which encode type V-A CRISPR (fig. S8).

Given the inhibitory effect of *acrVA1-3.1* on MbCas12a in bacteria, we sought to determine whether AcrVA proteins could block MbCas12a activity in human cells. Human U2-OS–enhanced green fluorescent protein (EGFP) cells (*18*) transiently expressing MbCas12a, *EGFP*-targeting crRNA, and human codon-optimized *acrVA1-3.1* were assessed for EGFP disruption by using flow cytometry. Coexpression of MbCas12a and crRNA resulted in ~60 to 70% disruption of *EGFP* expression relative to background (Fig. 3A). AcrVA1 expression reduced *EGFP* disruption to background levels, indicating inhibition of MbCas12a, whereas the other *acrVA* genes showed little evidence of activity here (Fig. 3A). We additionally found that *acrVA1* inhibited another Cas12a ortholog (Mb3Cas12a) while having no impact on SpyCas9 editing in the same assay (Fig. 3B). Titration of the Acr plasmid relative to the Cas12a expression plasmid revealed comparable dose-dependent responses to inhibition between MbCas12a or Mb3Cas12a with AcrVA1 and SpyCas9 with AcrIIA4 (fig. S9). Furthermore, AcrVA1 was found to be a broad-spectrum inhibitor of other commonly used Cas12a orthologs (*11*), providing strong inhibition of AsCas12a and LbCas12a and modest inhibition of FnCas12a (Fig. 3C).

Last, to determine whether AcrVA1 could inhibit Cas12a-mediated modification of endogenous loci in human cells, U2-OS cells were cotransfected with plasmids expressing *acrVA1*, Cas12a, and crRNAs that target endogenous genes (*RUNX1*, *DNMT1*, or *FANCF*) and assessed for gene disruption by means of T7 endonuclease I (T7E1) assay. We found that AcrVA1 completely inhibited gene disruption by MbCas12a and Mb3Cas12a, with modest to strong inhibition of As-, Lb-, and FnCas12a orthologs (Fig. 3D and fig. S10).

Here, we report the discovery of a broadly distributed type I-F Acr protein (AcrIF11) that served as a marker for *acr* loci and led to the identification of type I-C and V-A CRISPR inhibitors. One of these *acrVA* genes (*acrVA1*) potently inhibits Cas12a in bacteria and in human cells, providing a new tool for Cas12a regulation. Our findings show that mobile genetic elements can tolerate bacteria with more than one CRISPR-Cas type by possessing multiple Acr proteins in the same locus. The strategy described here enabled the identification of many widespread Acr proteins, which may prove useful in future Acr discovery.

REFERENCES AND NOTES

1. J. Bondy-Denomy, A. Pawluk, K. L. Maxwell, A. R. Davidson, *Nature* **493**, 429–432 (2013).
2. J. Bondy-Denomy *et al.*, *Nature* **526**, 136–139 (2015).
3. E. V. Koonin, K. S. Makarova, F. Zhang, *Curr. Opin. Microbiol.* **37**, 67–78 (2017).
4. B. J. Rauch *et al.*, *Cell* **168**, 150–158.e10 (2017).
5. A. Pawluk *et al.*, *Cell* **167**, 1829–1838.e9 (2016).
6. A. L. Borges, A. R. Davidson, J. Bondy-Denomy, *Annu. Rev. Virol.* **4**, 37–59 (2017).
7. A. Pawluk, A. R. Davidson, K. L. Maxwell, *Nat. Rev. Microbiol.* **16**, 12–17 (2018).
8. A. Pawluk *et al.*, *Nat. Microbiol.* **1**, 16085 (2016).
9. A. Pawluk, J. Bondy-Denomy, V. H. W. Cheung, K. L. Maxwell, A. R. Davidson, *MBio* **5**, e00896, e00896–e14 (2014).
10. A. van Belkum *et al.*, *MBio* **6**, e01796–e15 (2015).
11. B. Zetsche *et al.*, *Cell* **163**, 759–771 (2015).
12. I. Fonfara, H. Richter, M. Bratovič, A. Le Rhun, E. Charpentier, *Nature* **532**, 517–521 (2016).
13. B. P. Kleinstiver *et al.*, *Nat. Biotechnol.* **34**, 869–874 (2016).
14. J. A. Angelos, P. Q. Spinks, L. M. Ball, L. W. George, *Int. J. Syst. Evol. Microbiol.* **57**, 789–795 (2007).
15. A. M. Dickey *et al.*, *Vet. Res.* **47**, 31 (2016).
16. A. Ariff *et al.*, *BMC Genomics* **16**, 860 (2015).
17. K. E. Watters, C. Fellmann, H. B. Bai, S. M. Ren, J. A. Doudna, *Science* **362**, 236–239 (2018).
18. D. Reyon *et al.*, *Nat. Biotechnol.* **30**, 460–465 (2012).

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SUPPLEMENTARY MATERIALS

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Materials and Methods
Figs. S1 to S10
Tables S1 to S11
References (*19*–*26*)

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Discovery of widespread type I and type V CRISPR-Cas inhibitors

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Cas12 inhibitors join the anti-CRISPR family

Bacteria and their phages continually coevolve in a molecular arms race. For example, phages use anti-CRISPR proteins to inhibit the bacterial type I and II CRISPR systems (see the Perspective by Koonin and Makarova). Watters *et al.* and Marino *et al.* used bioinformatic and experimental approaches to identify inhibitors of type V CRISPR-Cas12a. Cas12a has been successfully engineered for gene editing and nucleic acid detection. Some of the anti-Cas12a proteins identified in these studies had broad-spectrum inhibitory effects on Cas12a orthologs and could block Cas12a-mediated genome editing in human cells.

Science, this issue p. 236, p. 240; see also p. 156