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SUPPLEMENTARY INFORMATION

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An anti-CRISPR protein disables type V Cas12a by acetylation

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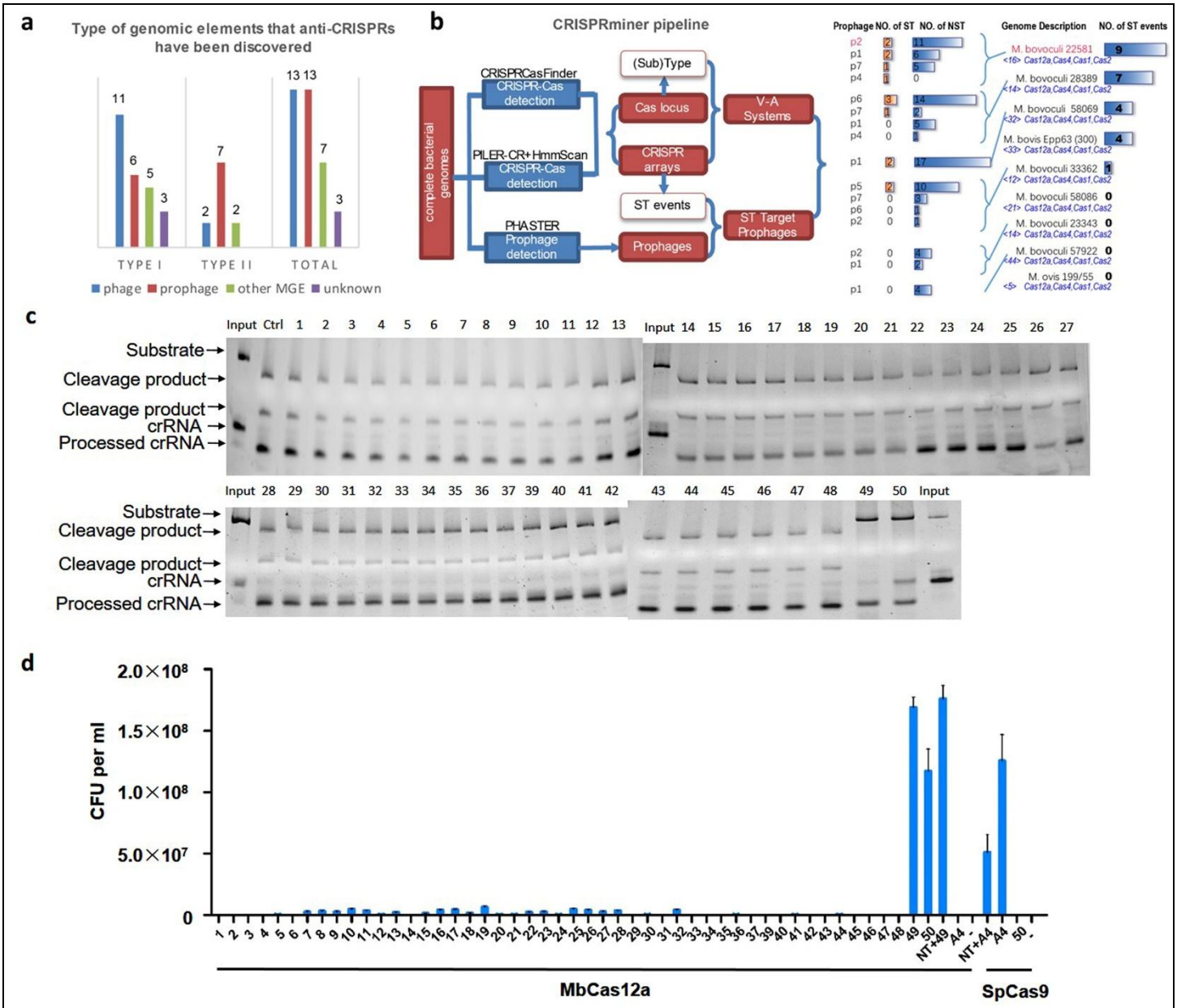
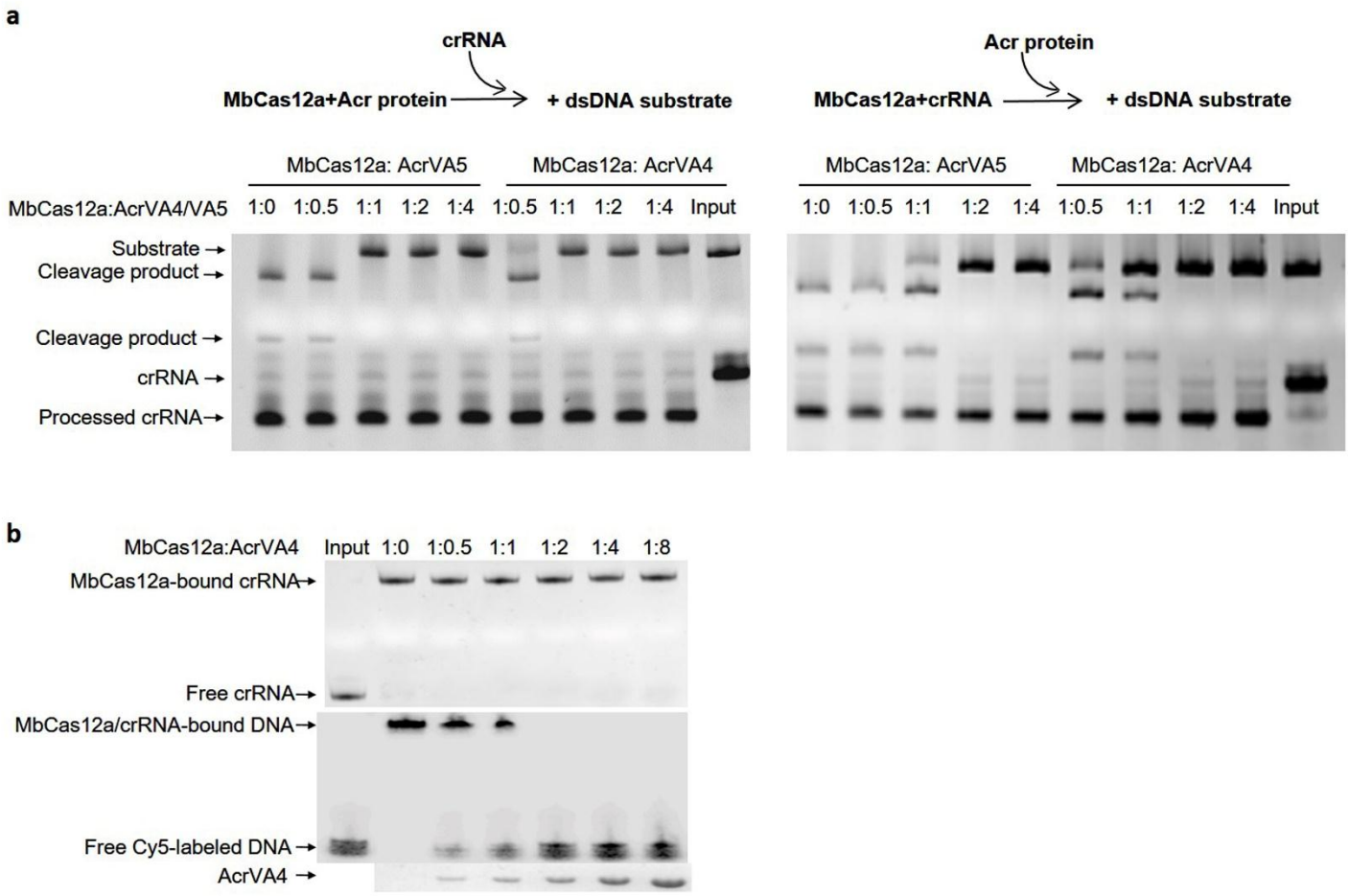


Supplementary Figure 1

Bioinformatics approach for discovering anti-CRISPR candidates for the V-A CRISPR-Cas12a system (AcrVAs) and identification of anti-CRISPR genes.

a, Statistics of the type of genomic elements that anti-CRISPRs have been discovered to inhibit type I or II CRISPR-Cas system (based on the anti-CRISPRs collection reported in https://tinyurl.com/anti-CRISPR). **b**, CRISPRminer pipeline was used to predict the V-A CRISPR-Cas systems (including CRISPR arrays, Cas locus and their (sub)types), find spacers with identifiable proto-spacers target their own prophage regions (self-targeting, ST) or prophage regions on other genomes (non-self-targeting, NST). **c,** *In vitro* MbCas12a inhibition assay was used to identify the anti-CRISPRs from 49 purified proteins (49 candidates). MbCas12a was first incubated with anti-CRISPR candidates at 1:8 molar ratio. Then crRNA and dsDNA substrate were added and incubated. The reactions were stopped by adding loading buffer for denaturing gel and the reaction mixtures were run on TBE-Urea polyacrylamide gels and visualized by EB staining. Data shown are representative of three independent experiments. **d**, Plasmid-targeting assay with 49 gene products (candidates 1-50, except 38) from *M. bovoculi* 22581 prophage2 in *E. coli*. Survival (CFU: colony-forming unit) of

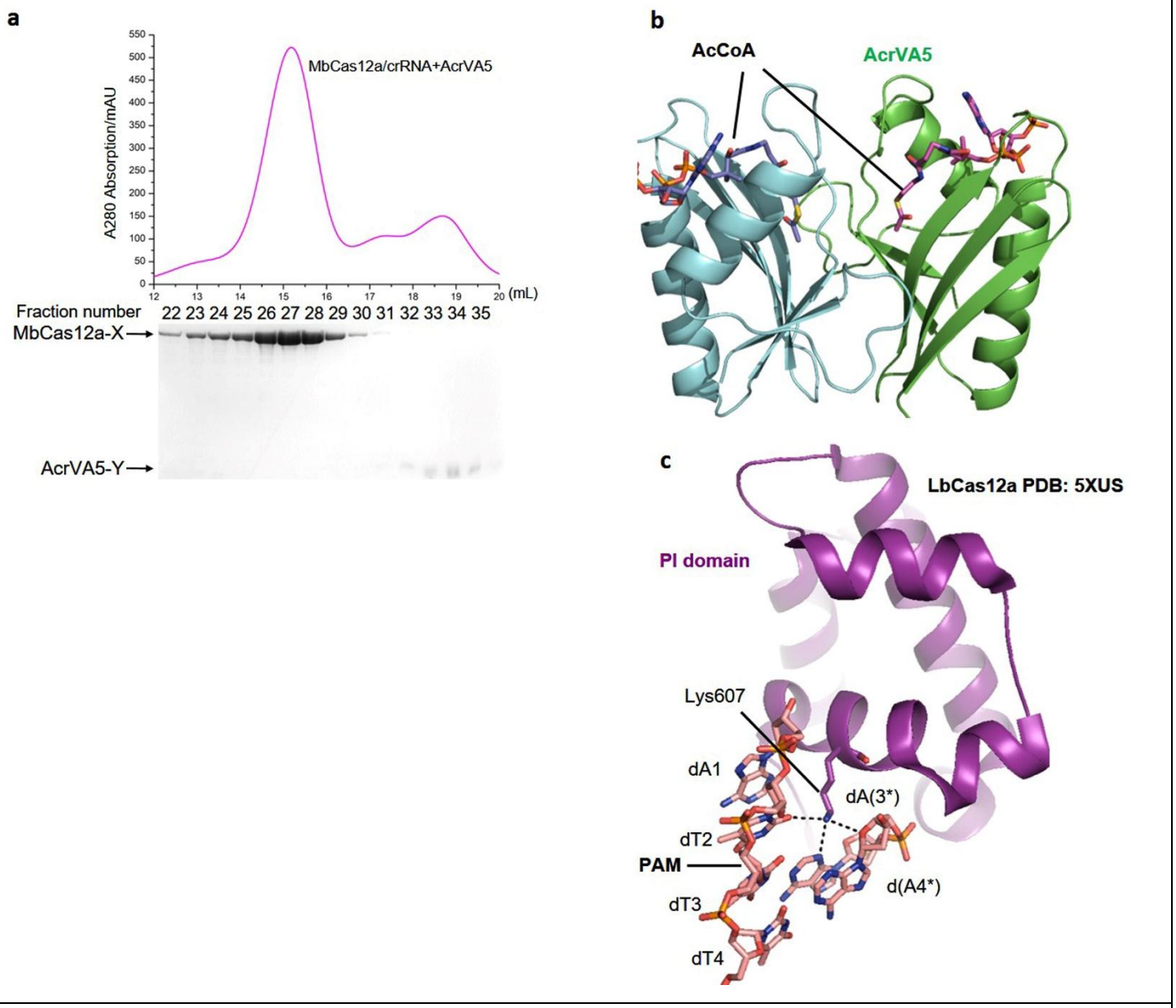
bacteria was calculated in the presence of candidate and targeted plasmids. Non-targeted plasmid co-transformed with candidate 49 (NT+49), targeted plasmid co-transformed with AcrIIA4 (A4), and only targeted plasmid (-) were controls of the assay. The inhibitory effects of AcrIIA4 and candidate 50 against SpCas9 were also tested by four groups: non-targeted plasmid co-transformed with AcrIIA4 (NT+A4), targeted plasmid co-transformed with AcrIIA4 (A4), targeted plasmid co-transformed with candidate 50 (50) and only targeted plasmid (-). Error bars represent the mean ± SD of three biological replicates.



Supplementary Figure 2

Inhibition of MbCas12a by AcrVA4 and AcrVA5.

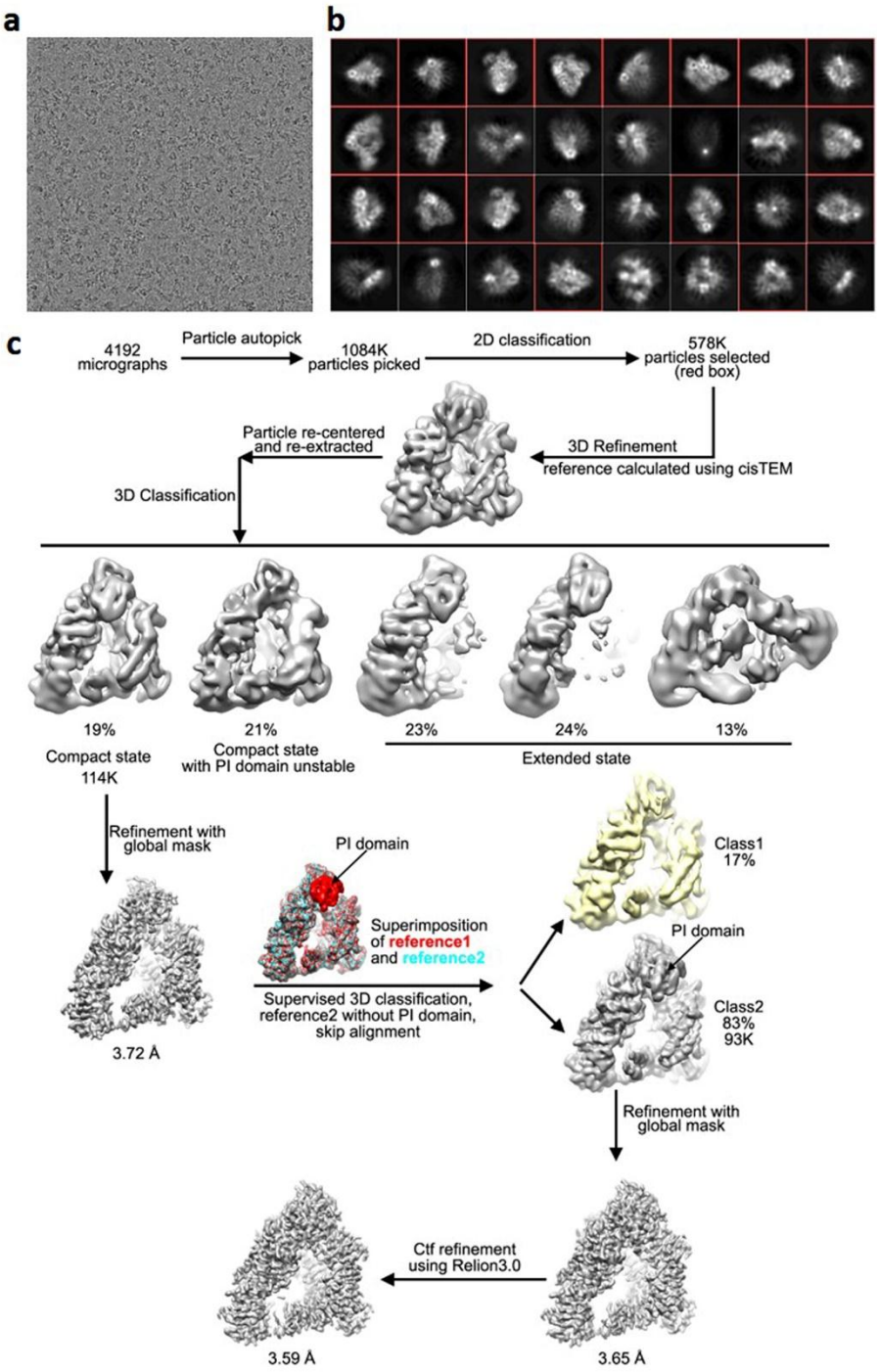
a, Inhibition assays for MbCas12a by AcrVA4 and AcrVA5. Left panel: crRNA was added to buffer containing MbCas12a following addition of AcrVA4 or AcrVA5. Right panel: MbCas12a was pre-incubated with crRNA before AcrVA4 or AcrVA5 was added. After reaction, the reaction mixtures were loaded onto 8% denature polyacrylamide gels. Molar ratios of an anti-CRISPR protein and MbCas12a are shown at the top of each lane. **b,** Electrophoretic mobility shift assay (EMSA) was used to test the binding of MbCas12a with crRNA or dsDNA in the presence of anti-CRISPR protein AcrVA4. MbCas12a or MbCas12a (D874A/E968A)-crRNA complex was first incubated with increasing amounts of AcrVA4, then crRNA (upper panel) or fluorophore-labelled dsDNA (middle panel) was added and incubated. Nucleic acid-bound MbCas12a complexes were resolved from free nucleic acid by native gel electrophoresis. CrRNA was visualized by ethidium bromide staining (upper panel), and dsDNA was visualized by a fluorescence imaging machine (600 nm) (middle panel). Coomassie blue staining of AcrVA4 at different molar ratios was shown in lower panel. Molar ratios of MbCas12a:AcrVA4 are shown at the top of each lane. Data shown is representative of three independent experiments.



Supplementary Figure 3

The structures of AcrVA5 and the PAM-interacting domain from LbCas12a.

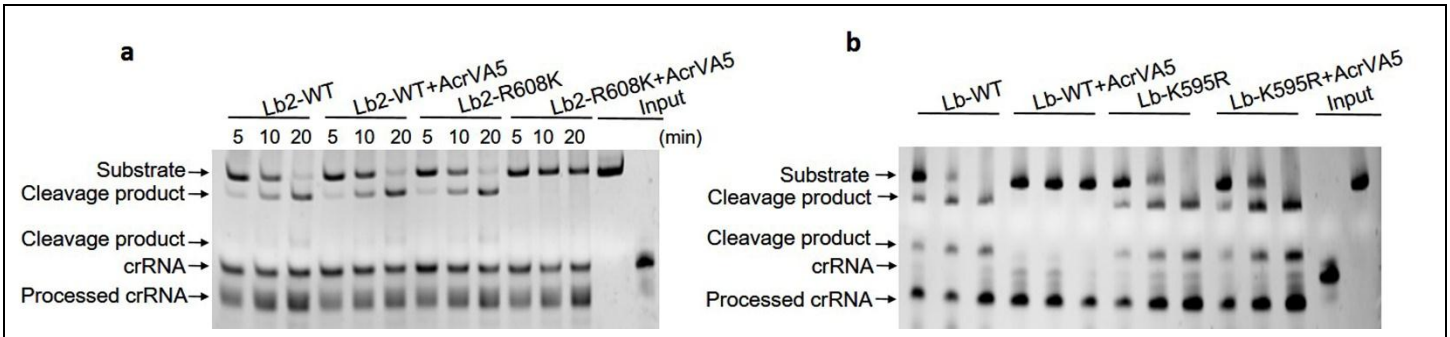
a, Shown is the result of gel filtration experiment after the reaction of AcrVA5 and crRNA-bound MbCas12a. Top, UV absorbance (280 nm) peaks from size-exclusion chromatography. Bottom, Coomassie blue staining following SDS–PAGE of the peak fractions from gel filtration shown on the top. **b**, The overall structure of the AcCoA-bound AcrVA5 complex shown in cartoon, and two AcCoA molecules are shown as sticks. The two AcrVA5 molecules in the structure were colored cyan (left) and green (right), respectively. **c**, Detailed interactions of the PAM base pairs with Lys607. Hydrogen bonds are shown as dashed lines. Recognition of the 5'-TTTA-3' PAM by LbCas12a (PDB: 5XUS).



Supplementary Figure 4

Workflow of the data processing of the AcrVA5-acetylated MbCas12a in complex with crRNA.

a, A representative cryo-EM image of MbCas12a-crRNA complex. **b**, Representative class averages of 2D classification of auto-picked particles. Particles included for further processing were indicated by red boxes. **c**, Workflow of the image processing, including unsupervised and supervised 3D classification, 3D refinement and CTF refinement. See more details in Methods.



Supplementary Figure 5

AcrVA5 inhibits the Cas12a strain with lysine but not arginine for recognition of PAM.

a, Time course of cleavage inhibition assays were used to test the inhibition activities of AcrVA5 against WT and R608K mutant Lb2Cas12a. The reactions were stopped by adding loading buffer for denaturing gel and the reaction mixtures were run on TBE-urea polyacrylamide gels and visualized by ethidium bromide staining. Lb2Cas12a from strain *Lachnospiraceae bacterium COE1* denotes a close LbCas12a ortholog (45% identity). **b**, Time course of cleavage inhibition assays were used to test the inhibition activities of AcrVA5 against WT and K595R mutant LbCas12a. The reactions were stopped by adding loading buffer for denaturing gel and the reaction mixtures were run on TBE-urea polyacrylamide gels and visualized by ethidium bromide staining.

Supplementary Data Set 1 | Uncropped Gel Image

Figure 1a

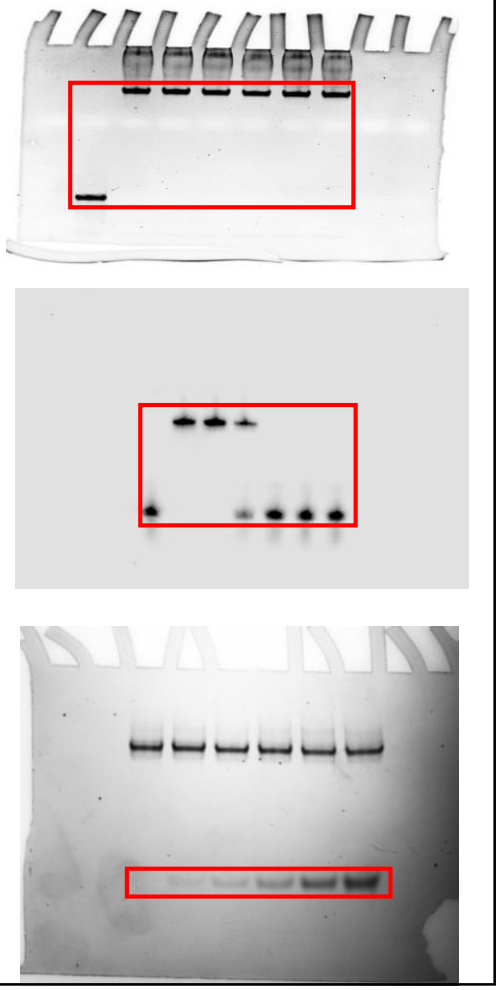


Figure 1b

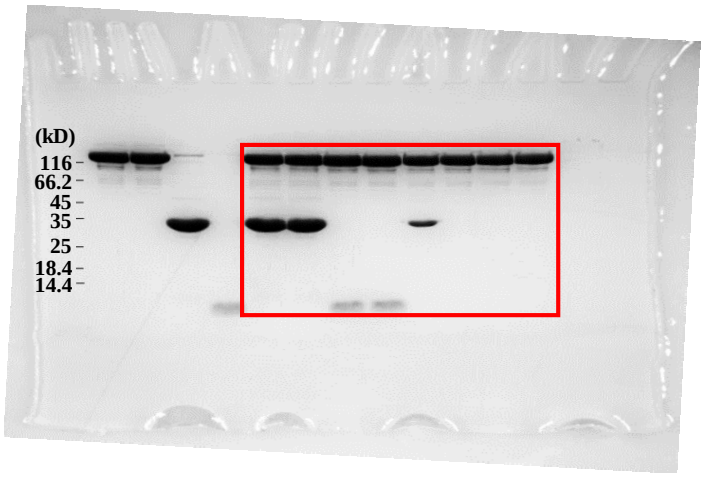


Figure 1d

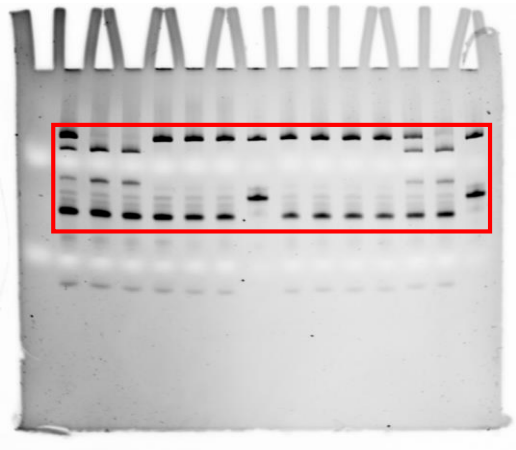
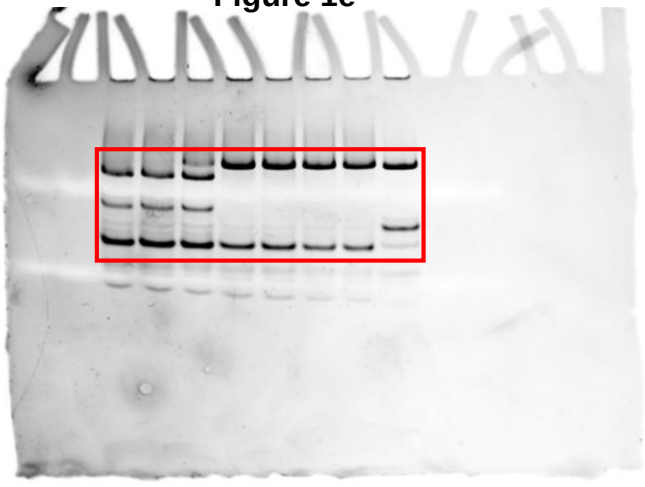
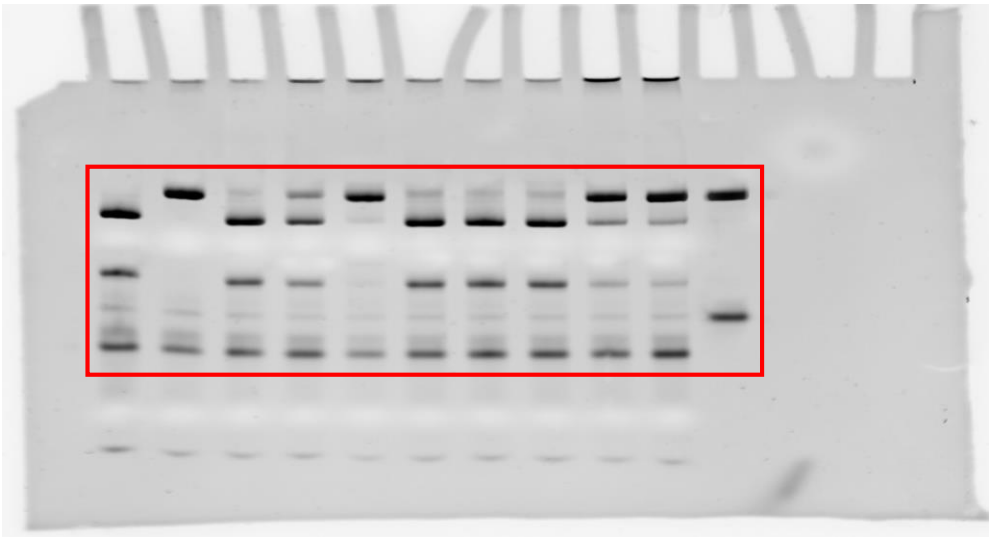


Figure 1e



Continuation Supplementary Data Set 1 | Uncropped Gel Image

Figure 2c



Continuation Supplementary Data Set 1 | Uncropped Gel Image

Figure 4b

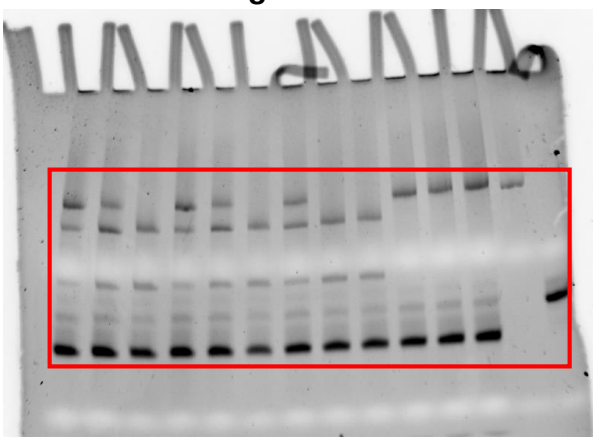
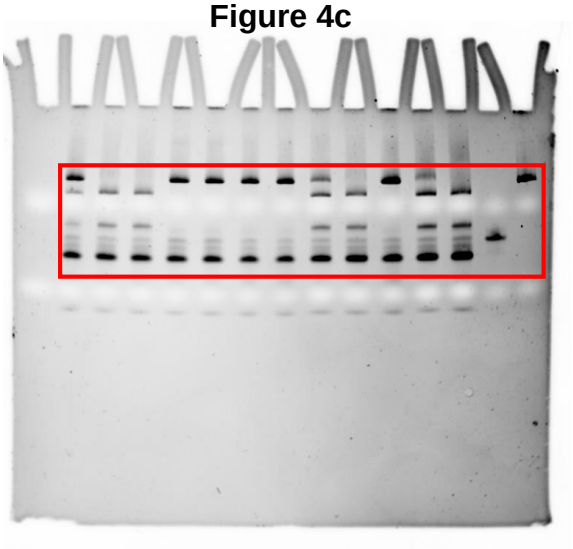
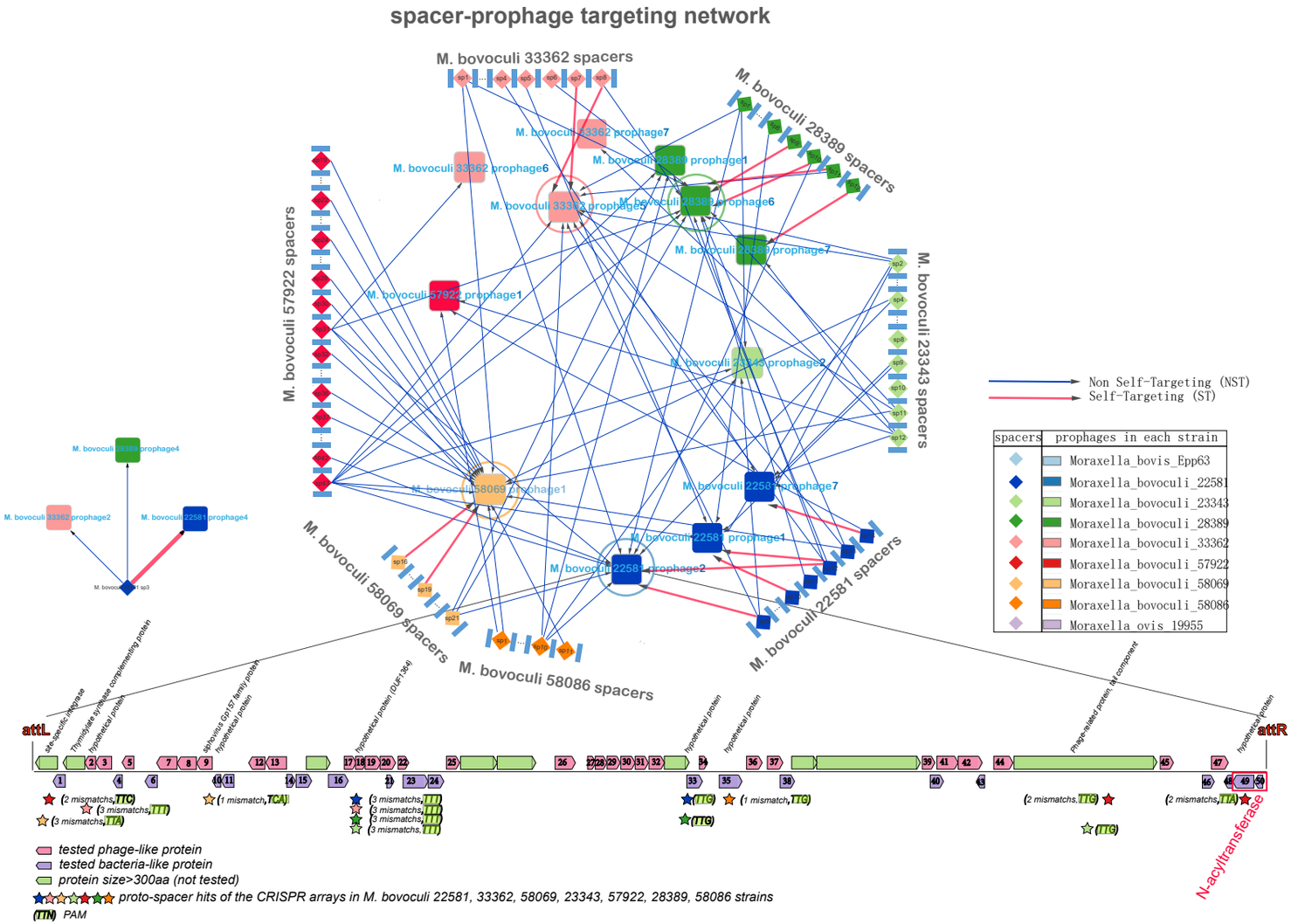


Figure 4c



Supplementary Note 1



Supplementary Note 1. Selection of candidate region for V-A anti-CRISPRs screening. prophage targeting network was built based on a comprehensive targeting analysis between the spacers from M. bovoculi V-A CRISPR arrays and the prophage regions on these genomes. The prophage regions identified as the hot spots of spacer attacking were highlighted by circles. The prophage2 on M. bovoculi strain 22581 was selected as the candidate region for anti-CRISPRs screening. 50 gene products (candidates 1-50) from prophage2 were tested for the inhibition of MbCas12a-mediated dsDNA cleavage, which resulted in the identification of candidate 49 and candidate 50 as novel AcrVAs. The ORFs marked pink, purple and green denote tested phage-like proteins, tested bacteria-like proteins and untested proteins (size > 300 aa) in this region, respectively. The locations of the targeting proto-spacers are marked using asterisks together with annotation of the mismatches and PAM sequences.

Supplementary Table 1. Plasmids used in the study

Plasmid	Description	Source or reference
pBBR1-Cas9	Harboring CRISPR-SpCas9 system, Kan^r	[1]
pCmr	Plasmid for testing inhibition of MbCas12a cleavage in *E. coli*. Cm^r	[2]
pBBR1-MbCas12a	Derived from pBBR1-Cas9, harboring CRISPR-MbCas12a system, Kan^r	this study
pCandidate 1 to 50	Expression plasmid of 49 candidates except 38 in *E.coli*. Amp^r	this study
pAcrIIA4	Expression plasmid of AcrIIA4 in *E.coli*. Amp^r	lab stock
pMbCas12a	Expression plasmid of MbCas12a in *E.coli*. Amp^r	this study
pMb2Cas12a	Expression plasmid of Mb2Cas12a in *E.coli*. Amp^r	this study
pLbCas12a	Expression plasmid of LbCas12a in *E.coli*. Amp^r	lab stock
pLb2Cas12a	Expression plasmid of Lb2Cas12a in *E.coli*. Amp^r	this study
pAcrVA4 (candidate 49)	Expression plasmid of AcrVA4 in *E.coli*. Amp^r	this study
pAcrVA5 (candidate 50)	Expression plasmid of AcrVA5 in *E.coli*. Amp^r	this study
pMbcas12a(K635R mutant)	Expression plasmid of pMbcas12a(K635R mutant) in *E.coli*. Amp^r	this study
pMb2cas12a(R625K mutant)	Expression plasmid of pMb2cas12a(R625K mutant) in *E.coli*. Amp^r	this study
pMbcas12a(D874A/E968A mutant)	Expression plasmid of pMbcas12a(D874A/E968A mutant) in *E.coli*. Amp^r	this study
pLbCas12a(K595R mutant)	Expression plasmid of pLbCas12a(K595R mutant) in *E.coli*. Amp^r	this study
pLb2Cas12a(R608K mutant)	Expression plasmid of pLb2Cas12a(R608K mutant) in *E.coli*. Amp^r	this study
pAcrVA5(K2A mutant)	Expression plasmid of pAcrVA5 (K2A mutant) in *E.coli*. Amp^r	this study
pAcrVA5(K29A mutant)	Expression plasmid of pAcrVA5 (K29A mutant) in *E.coli*. Amp^r	this study
pAcrVA5(R30A mutant)	Expression plasmid of pAcrVA5 (R30A mutant) in *E.coli*. Amp^r	this study
pAcrVA5(G32R mutant)	Expression plasmid of pAcrVA5 (G32R mutant) in *E.coli*. Amp^r	this study
pAcrVA5(G34R mutant)	Expression plasmid of pAcrVA5 (G34R mutant) in *E.coli*. Amp^r	this study
pAcrVA5(S35A mutant)	Expression plasmid of pAcrVA5 (S35A mutant) in *E.coli*. Amp^r	this study
pAcrVA5(Y57A mutant)	Expression plasmid of pAcrVA5 (Y57A mutant) in *E.coli*. Amp^r	this study
pAcrVA5(S74A mutant)	Expression plasmid of pAcrVA5 (S74A mutant) in *E.coli*. Amp^r	this study

Amp^r, ampicillin resistant; Cm^r, chloramphenicol resistant; Kan^r, kanamycin resistant.

Supplementary Table 2. Nucleic acid sequences used in the study.

RNA sequence	**Sequence (5' to 3')**
MbCas12a_crRNA	GGGUCUAACGACCUUUUAAAUUUCUACUGUUUGUAGAUCUGAUGG UCCAUGUCUGUUACUC
LbCas12a_crRNA	GGAAUUUCUACUAAGUGUAGAUCUGAUGGUCCAUGUCUGUUACUC
dsDNA substrate sequence	
MbCas12a_tarDNA_PAM	TTCCTGATGGTCCATGTCTGTTACTC
MbCas12a _tarDNA	GAGTAACAGACATGGACCATCAGGAA
RNA transcription assay DNA temple	
MbCas12a_crRNA_DNA templet	ATGTAATACGACTCACTATAGGGTCTAACGACCTTTTAAATTTCTACT GTTTGTAGATCTGATGGTCCATGTCTGTTACTC
LbCas12a_crRNA_DNA templet	ATGTAATACGACTCACTATAGGAATTTCTACTAAGTGTAGATCTGAT GGTCCATGTCTGTTACTC
DNA cleavage assay primer	
tar_BamH1_5	GATCCTTCCTGATGGTCCATGTCTGTTACTCG
tar_EcoR1_3	AATTCGAGTAACAGACATGGACCATCAGGAAG
pUC18_5	ATTCAGGCTGCGCAACTGTTGGGA
pUC18_3	TTTATGCTTCCGGCTCGTATGTTG
Cleavage assay DNA sequence	
pUC18_ substrate	ATTCAGGCTGCGCAACTGTTGGGAAGGGCGATCGGTGCGGGCCTCTT CGCTATTACGCCAGCTGGCGAAAGGGGGATGTGCTGCAAGGCGATTA AGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGAC GGCCAGTGCCAAGCTTGCATGCCTGCAGGTCGACTCTAGAGGATCCT TCCTGATGGTCCATGTCTGTTACTCGAATTCGTAATCATGGTCATAGC TGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATAC GAGCCGGAAGCATAAA
pCmr target site Sequence	
For MbCas12a	**TTTA**TTCACATTCTTGCCCGCCTGATG
For SpCas9	CCATTGGGATATATCAACGG**TGG**
pCmr target site primer	
MbCas12a_cmr_f	agatTTCACATTCTTGCCCGCCTGATG
MbCas12a_cmr_r	tgctCATCAGGCGGGCAAGAATGTGAA
SpCas9_cmr_f	agatCCATTGGGATATATCAACGG
SpCas9_cmr_r	tgctCCGTTGATATATCCCAATGG
constitutive promoter sequence for sgRNA	

CTAGGTTTATACATAGGCGAGTACTCTGTTATGGAGTCAGATCTTAGC