An anti-CRISPR viral ring nuclease subverts type III CRISPR immunity

Previously, we identified in the archaeon Sulfolobus solfataricus a family of cellular enzymes—referred to hereafter as the CRISPR-associated ring nuclease I (Crn1) family—that degrades cA₄ molecules and deactivates the cA₄-dependent RNase Csx1⁶. This enzyme is thought to act by mopping up cA₄ molecules in the cell without compromising the immunity provided by the type III CRISPR system. In the absence of such a mechanism to remove cyclic oligoadenylates (cOAs) following the clearance of viral infections, cells could be pushed towards dormancy or cell death under inappropriate circumstances⁷–¹⁰. Unsurprisingly, viruses have responded to the threat of the CRISPR system by evolving a range of anti-CRISPR (Acr) proteins, which are used to inhibit and overcome the cell’s CRISPR defences using a variety of mechanisms (reviewed in ref. ¹¹). Acrs have been identified for many of the CRISPR effector subtypes, and number more than 40 families²⁰.

Here we investigate the DUF1874 protein family, which is conserved and widespread in a variety of archaeal viruses and plasmids, bacteriophages and prophages (Extended Data Fig. 1), for an Acr function. Structures are available for several members of the DUF1874 family, including gp29 from Sulfolobus islandicus rod-shaped virus 1 (SIRV1)¹⁷ and B16 from Sulfolobus turreted icosahedral virus (STIV)⁹. The structures reveal an intriguing dimeric structure, with a large central pocket flanked by conserved residues. B16 is also known to be important for normal virus replication kinetics, as deletion of the gene results in a marked ‘small plaque’ phenotype²⁹, consistent with an Acr function.

DUF1874 is a type III anti-CRISPR, AcrIII-1

To investigate a possible Acr function of DUF1874, we deleted the genes for the type I-A CRISPR system in Sulfolobus islandicus M.16.4, so that it had only a type III-B system for defence²⁰ (Extended Data Fig. 2). We challenged this strain with the archaeal virus SSeV (Fig. 1a), a lytic virus isolated from Kamchatka, Russia, that has an exact CRISPR-spacer match of 100% in M.16.4, as well as several other potentially active CRISPR spacers. SSeV lacks a duf1874 gene and failed to form plaques on a lawn of S. islandicus M.16.4 with type III-B CRISPR defence unless the effector gene csx1 was deleted (Fig. 1a and Extended Data Fig. 2). However, the same cells expressing the SIRV1 gp29 gene from a plasmid were readily infected, giving rise to plaque formation. These data are consistent with the hypothesis that SIRV1 gp29 functions as an Acr specific for the type III CRISPR defence.

To explore this possibility further, we used a recently developed recombinant type III CRISPR system from Mycobacterium tuberculosis; this system allows the effector protein downstream of cOAs to be swapped in order to provide effective immunity based on either cA₄ or cA₆ signalling²⁰ (Fig. 1). We then transformed strains capable of cA₄- or cA₆-based immunity with a plasmid that was targeted for interference owing to a match in its tetracycline-resistance gene to a spacer in the CRISPR array. We observed efficient interference (lack of plasmid transformation) after one day for either strain in the absence of the duf1874 gene from bacteriophage THSA-485A (Fig. 1c, d). However, the presence of the duf1874 gene on the

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The CRISPR system in bacteria and archaea provides adaptive immunity against mobile genetic elements. Type III CRISPR systems detect viral RNA, resulting in the activation of two regions of the Cas10 protein: an HD nuclease domain (which degrades viral DNA)¹² and a cyclase domain (which synthesizes cyclic oligoadenylates from ATP)³–⁵. Cyclic oligoadenylates in turn activate defence enzymes with a CRISPR-associated Rossmann fold domain⁶, sculpting a powerful antiviral response⁷–¹⁰ that can drive viruses to extinction⁸. Cyclic nucleotides are increasingly implicated in host–pathogen interactions¹¹–¹³. Here we identify a new family of viral anti-CRISPR (Acr) enzymes that rapidly degrade cyclic tetra-adenylate (cA₄). The viral ring nuclease AcrIII-1 is widely distributed in archaeal and bacterial viruses and in proviruses. The enzyme uses a previously unknown fold to bind cA₄ specifically, and a conserved active site to rapidly cleave this signalling molecule, allowing viruses to neutralize the type III CRISPR defence system. The AcrIII-1 family has a broad host range, as it targets cA₄-signalling molecules rather than specific CRISPR effector proteins. Our findings highlight the crucial role of cyclic nucleotide signalling in the conflict between viruses and their hosts.
Fig. 1 | DUF1874 is an anti-CRISPR protein specific for cA4 signalling. a, SSeV infection assay, showing that gp29 (a duf1874 gene from Sirv1) can neutralize the type III-B CRISPR system in S. islandicus. We challenged S. islandicus RJW007Δtype I-A or RJW007Δtype I-AΔcsx1 mutant strains with SSeV, in the presence or absence of duf1874 (Sirv1 gp29) expressed on a replicative plasmid. Plaques were observed when csx1 was deleted, or when the resistant strain expressed duf1874 (n = 3 biological replicates) (Extended Data Fig. 2d). b, Diagram showing the recombinant M. tuberculosis type III-A CRISPR interference system established in E. coli. By swapping the native ancillary nuclease Csm6 for Csx1, the system can be converted from cA6 to cA4-mediated antiviral immunity. c, Plasmid transformation assay (after one day’s growth), using a plasmid with a match to a spacer in the CRISPR array. If the plasmid is successfully targeted by the CRISPR system, fewer transformants are expected. Plasmids with or without the duf1874 gene were targeted successfully when Csx1 (Csm6)-mediated antiviral signalling was active. By contrast, cells using a Csx1 (Csm6)-based system reduced transformation only when duf1874 was not present, suggesting that DUF1874 was effective in neutralizing Csx1-based CRISPR interference. The control strain lacked CSoA-dependent ribonucleases. These results are representative of two biological replicates, with four technical replicates each (n = 8). d, Colony counts for transformants visible after one and four days’ growth in the presence or absence of DUF1874 and the indicated effector proteins. DUF1874 antagonizes Csx1 but not Csm6-mediated immunity. Data are mean and s.d. from two biological replicates with four technical replicates each (n = 8).

plasmid reduced immunity for cA6-mediated, but not cA4-mediated, CRISPR defence. This observation supports the hypothesis that DUF1874 acts as an Acr against cA6-mediated type III CRISPR defence. We therefore propose the collective name AcrIII-1 for this family. The -1 in place of the subtype reflects the fact that AcrIII-1 will inhibit any type III CRISPR subtype that utilizes cA4 molecules for defence22. We also found that, after four days of growth, Csm6-mediated immunity was lost, regardless of the presence of DUF1874. This could indicate that alternative mechanisms exist to remove cA6, (Fig. 1d and Extended Data Fig. 3).

AcrIII-1 degrades cA4 rapidly
To explore the mechanism of action of the AcrIII-1 family, we cloned and expressed two family members in Escherichia coli: the Sirv1 gp29 protein and the Yddf protein, encoded by an integrative and conjugative element (ICE). Bsl1, from Bacillus subtilis22 (Extended Data Fig. 1b). We found that both proteins possess potent ring nuclease activity, rapidly degrading cA6 to generate linear di-adenylate (ApA-P) with a cyclic 2’-3’-phosphate (ApA-P) (Fig. 2b and Extended Data Fig. 4). With a catalytic rate exceeding 5 min⁻¹, the Acr enzyme is at least 60-fold more active than the cellular ring nuclease Crn1 from S. solfataricus. Both Sirv1 gp29 and Yddf show a strong preference for cA6 over cA4, with the latter being degraded very slowly by comparison (Extended Data Fig. 4). We showed previously that the type III-D CRISPR effector of S. solfataricus generates cA4 in proportion to the amount of cognate target RNA present14. By varying the target RNA input and following cA6 levels and Csx1 activity, we compared the abilities of Crn1 and AcrIII-1 to destroy the signalling molecule and deactivate the ancillary defence nucllease Csx1. In keeping with its low turnover number, Crn1 was effective at degrading cA6 and thus deactivating Csx1 only at the lowest levels of target RNA (Fig. 2c). By contrast, AcrIII-1 degraded cA6 completely at the highest target RNA concentration examined, preventing Csx1 activation. We investigated the ability of each enzyme to prevent Csx1 activation over a range of cA6 concentrations spanning four orders of magnitude (Extended Data Fig. 4e). Crn1 (2 µM) provided protection only up to 5 µM cA6, but 2 µM of AcrIII-1 provided complete protection at the highest level of cA6 tested (500 µM). Thus, AcrIII-1 has the potential to destroy large concentrations of the second messenger cA4 rapidly, preventing activation of Csx1.

Structure and mechanism of AcrIII-1
The structure of AcrIII-1 is unrelated to that of proteins with the CRISPR-associated Rossmann fold (CARF) domain—the only protein family known thus far to bind cA4. To elucidate the mechanism of cA4 binding and cleavage by AcrIII-1, we co-crystallized an inactive variant (H47A) of Sirv1 gp29 with cA6, and solved the structure to 1.55 Å resolution...
that the binding site is preformed. Fig. 3 | Structure of AcrIII-1 bound to cA 4.

a, Superimposition of the apo SIRV1 gp29 structure (salmon) and the same protein in complex with cA 4 (purple), highlighting the movement of the loop and α-helix upon cA 4 binding. cA 4 is shown coloured by element. b, Surface representation of the structure of SIRV1 gp29 (purple) in complex with cA 4, emphasizing the complete burial of the ligand. c, Surface representation of the apo structure of SIRV1 gp29 (salmon) with cA 4 in the position observed in the structure of the complex, indicating that the binding site is preformed. d, Structure of cA 4 bound to SIRV1 gp29. The cA 4 molecule makes symmetrical interactions with each monomer of AcrIII-1 (Extended Data Fig. 5). Arginine R85 on the loop from one monomer interacts with the distant half of the cA 4 molecule and appears to ‘lock’ the closed dimer. Other important interactions are made with main-chain L92, I69 and N8, and side-chain R66, N8, Q81, S11, T50, S49 and N13, most of which are semi or fully conserved (Extended Data Figs. 1, S), suggesting that they have important roles in cA 4 binding and/or catalysis in this whole family of enzymes. At two positions, on opposite sides of the ring, the 2′-hydroxyl of the ribose is positioned correctly for in-line attack on the phosphodiester bond, consistent with the observed bilateral cleavage (Fig. 3d). The catalytic power of the AcrIII-1 family probably derives from active-site residues that position the 2′-hydroxyl group for in-line nucleophilic attack, stabilize the transition state and protonate the oxyanion leaving group. For the AcrIII-1 family, the absolutely conserved residue H47 is suitably positioned to act as a general acid and fulfill the latter role (Fig. 3d). To test this hypothesis, we assayed variant H47A of AcrIII-1. The variant enzyme suffered a more than 2,500-fold decrease in catalytic power, which could be partially reversed by chemical rescue with 500 mM imidazole in the reaction buffer (Extended Data Fig. 6). We also noted that two active-site histidine residues (H47A and H47A′, from each monomer of the dimer; modelled on the basis of the position of the alanine side chain in the H47A variant crystallized with cA 4, and coloured to represent residues from different monomers) are in suitable positions to act as the general acid, protonating the oxyanion leaving group. The corresponding ribose sugars have 2′-hydroxyl groups suitably positioned for in-line nucleophilic attack on the phosphodiester bond. In the cA 4 ligand, carbon atoms are shown in green, phosphates in orange, oxygens in red and nitrogens in blue.

(Fig. 3 and Extended Data Table 1). The complex reveals a molecule of cA 4 bound at the dimer interface. Comparison of the cA 4-bound and apo structures reveals a substantial movement of a loop (comprising residues 82–94) and subsequent α-helix to bury cA 4 within the dimer. These loops adopt variable or unstructured conformations in the various apo protein structures. Once bound, the ligand is completely enclosed by the protein—a considerable accomplishment when one considers the relative sizes of protein and ligand (Fig. 3b). Superimposition of the cA 4 ligand on the apo-protein structure reveals that the binding site is largely preformed, with the exception of the mobile loops that form the lid (Fig. 3c). The overall change is like two cupped hands catching a ball, with the loops (fingers) subsequently closing around it. The cA 4 molecule makes symmetrical interactions with each monomer of AcrIII-1 (Extended Data Fig. 5). Arginine R85 on the loop from one monomer interacts with the distant half of the cA 4 molecule and appears to ‘lock’ the closed dimer. Other important interactions are made with main-chain L92, I69 and N8, and side-chain R66, N8, Q81, S11, T50, S49 and N13, most of which are semi or fully conserved (Extended Data Figs. 1, S), suggesting that they have important roles in cA 4 binding and/or catalysis in this whole family of enzymes. At two positions, on opposite sides of the ring, the 2′-hydroxyl of the ribose is positioned correctly for in-line attack on the phosphodiester bond, consistent with the observed bilateral cleavage (Fig. 3d). The catalytic power of the AcrIII-1 family probably derives from active-site residues that position the 2′-hydroxyl group for in-line nucleophilic attack, stabilize the transition state and protonate the oxyanion leaving group. For the AcrIII-1 family, the absolutely conserved residue H47 is suitably positioned to act as a general acid and fulfill the latter role (Fig. 3d). To test this hypothesis, we assayed variant H47A of AcrIII-1. The variant enzyme suffered a more than 2,500-fold decrease in catalytic power, which could be partially reversed by chemical rescue with 500 mM imidazole in the reaction buffer (Extended Data Fig. 6). We also noted that the conserved residue E88, situated on the tip of the loop that covers the binding site, is positioned close to the H47 residue of the opposite subunit. When mutated to alanine, the catalytic rate was reduced by 84-fold to 0.064 min⁻¹ (Extended Data Fig. 6b), consistent with a role for E88 in positioning H47 and/or increasing the pKa of the catalytic histidine residue to enhance catalysis.

By targeting a key signalling molecule, a single AcrIII-1 enzyme should have broad utility in the inhibition of endogenous cA 4-specific type III CRISPR systems in any species. Of the CRISPR ancillary nucleases studied to date, most are activated by cA 4; activation by cA 6 appears to be limited to certain bacterial phyla, including the Firmicutes and Actinobacteria. Recently, a type III Acr (AcrIII1β) has been reported that appears to function by binding and inhibiting the type III-B effector complex. Two other Acr proteins with enzymatic functions have been described: AcrVA1, which catalyses CRISPR RNA (crRNA)-mediated cleavage of Cas12a27, and AcrVA5, which acetylates the site in Cas12a that senses the protospacer-adjacent motif (PAM) of target DNA28. These and other Acrs target a protein (or protein/nucleic acid complex), implying a requirement for specific interactions that could be evaded by sequence variation. This is not a limitation of AcrIII-1.

Phylogenetic analysis of AcrIII-1

The gene encoding AcrIII-1 is found in representatives of at least five distinct viral families, making it one of the most widely conserved of all archaeal virus proteins (Extended Data Fig. 1 and Supplementary Data 1). The distribution of AcrIII-1 in archaea is sporadic but covers most of the main lineages (Supplementary Data 1), and is typically adjacent to open reading frames (ORFs) from mobile genetic elements rather than CRISPR loci. A good example is the STIV integrated into S. acidocaldarius genomes. AcrIII-1 is also present in several bacteriophages of the order Caudovirales, and there are many instances of acrIII-1 genes...
in sequenced bacterial genomes, with homologues found in the Firmicutes, Cyanobacteria, Proteobacteria, Actinobacteria and other phyla (Supplementary Data 1). Maximum likelihood phylogenetic analysis of the AcrIII-1 proteins suggests multiple horizontal gene transfers between unrelated viruses, as well as between bacteria and archaea (Extended Data Figs. 7–9). Sometimes the acrIII-1 gene is clearly part of an integrated mobile genetic element, such as the yadV gene in *B. subtilis*29. However, in other species (*n* = 49) the gene is associated with cellular type III CRISPR systems. In *Marinitoga piezophila*, AcrIII-1 is fused to a cOA-activated HEPN RNase of the Csx1 family. Given that both active sites are conserved, this fusion protein may have cA4-degradative RNase activity coupled with a cA4-degradative ring nuclease, thus providing an explicit linkage between the AcrIII-1 family and the type III CRISPR system. In this context the enzyme is likely to be acting as a host-encoded ring nuclease, like Csnl, rather than an Acr. We therefore propose the family name of Csn2 (CRISPR-associated ring nuclease 2) to cover DUF1874-family members that are associated with type III CRISPR systems (Extended Data Fig. 8).

**Cyclic nucleotides in defence systems**

AcrIII-1 is, to our knowledge, the first Acr to be predicted to have functional roles in both ‘offense and defence’. It remains to be determined whether the acrIII-1 gene arose in viruses and was appropriated by cellular type III systems, or vice versa. However, the extremely broad distribution of acrIII-1 and limited distribution of crn2 suggests the former. Adoption of an anti-CRISPR protein as a component of a cellular CRISPR defence system seems counterintuitive. However, the enzyme could have been harnessed for a role in defence by putting it under tight transcriptional control so that it is expressed at appropriate times or levels. The unprecedentedly wide occurrence of this Acr across many archaeal and bacterial virus families reflects the fact that this enzyme degrades a key signalling molecule to subvert cellular immunity. This makes it very hard for cells to evolve counter-resistance, other than by switching to a different signalling molecule. Recent discoveries have highlighted the existence of diverse cellular defence systems involving cyclic nucleotide signalling in bacteria20–13. It is possible that cOAs, and the enzymes that metabolize them, have functions that extend beyond type III CRISPR systems. The identification here of a new class of cA4-binding proteins highlights the potential for further discoveries in this area.

**Online content**

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41586-019-1909-5.

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BamHI sites.

For cloning, we purchased synthetic genes (g-blocks) from IDT, and by using a modified plasmid integration and segregation knockout strategy, in line with the methodology developed in ref. 34, the resultantly type I-A deletion mutant (RJW007 Δtype I-A) was then used as a parental strain to further delete the csx1 gene. The mutant strain RJW007 Δtype I-A Δcsx1 mutant via electroporation as described, generating strains expressing and not expressing SIRV1 gp29, respectively.

Viral quantification

The genome sequence of SSeV is available in GenBank (accession code MN33972). To calculate the titre of SSeV, we co-incubated 100 µl diluted virus (10−5, 10−6 and 10−7) with 500 µl supernatant at different dilutions (10°, 10−1, 10−2, 10−3, 10−4, 10−5 and 10−6) in a Falcon tube at 76–78 °C for 1 h without shaking. The titre was then loaded onto a HisTrap FF column (GE Healthcare) and eluted with wash buffer containing 50 mM Tris-HCl pH 7.5, 0.5 M NaCl, 30 mM imidazole and 10% glycerol. Unbound protein was washed away with 20 column volumes of wash buffer, before elution of histidine-tagged protein using a linear gradient (holding at 10% for three column volumes, and 50% for three column volumes) of elution buffer containing 50 mM Tris-HCl pH 7.5, 0.5 M NaCl, 0.5 M imidazole and 10% glycerol. We carried out SDS–polyacrylamide gel electrophoresis (PAGE) to identify fractions containing the protein of interest, and pooled and concentrated relevant fractions using a 10 kDa molecular mass cut-off centrifugal concentrator (Merck). The histidine tag was removed by incubating concentrated protein overnight with tobacco etch virus (TEV) protease (1 mg per 10 mg protein) while dialysing in buffer containing 50 mM Tris-HCl pH 7.5, 0.5 M NaCl, 30 mM imidazole and 10% glycerol at room temperature. The protein with histidine tag removed was isolated using a 5 ml HisTrap FF column, eluting the protein using four column volumes of wash buffer. Histidine-tag-removed protein was further purified by size-exclusion chromatography (S200 16/60; GE Healthcare) in buffer containing 20 mM Tris-HCl pH 7.5, 0.125 M NaCl using an isocratic gradient. After SDS–PAGE, fractions containing protein of interest were concentrated and protein was aliquoted and stored at −80 °C. We generated variant enzymes using the QuickChange site-directed mutagenesis kit as per the manufacturer’s instructions (Agilent Technologies), and purified them as for the wild-type proteins.

Radiolabelled cA₄ cleavage assays

We generated cOA by incubating 120 µg Sulfolobus solfataricus (Sso) type III-D (Csm) complex with 5 nM α-32P-ATP, 1 mM ATP, 120 mM A26 RNA target and 2 mM MgCl₂ in Csx1 buffer containing 20 mM 2-(N-morpholino)ethanesulfonic acid (MES) pH 5.5, 100 mM K-glutamate, 1 mM dithiothreitol (DTT) and three units SUPERase•In Inhibitor for 2 h at 70 °C in a 100 µl reaction volume. We extracted cOA through phenol/chloroform (Ambion) extraction followed by chloroform extraction (Sigma-Aldrich), with storage at −20 °C.

For single-turnover kinetics experiments, we assayed AcrIII-1 SIRV1 gp29 and variants (4 µM protein dimer) for radiolabelled cA₄ degradation by incubating with 1/400 diluted 32P-labelled SsoCsm cOA (roughly 200 nM cA₄, generated in a 100 µl cOA-synthesis reaction as above) in Csx1 buffer supplemented with 1 mM EDTA at 50 °C. We incubated AcrIII-1 Yddf (8 µM dimer) with cOA in buffer containing 20 mM MES pH 6.0, 100 mM NaCl, 1 mM DTT, 1 mM EDTA and three units SUPERase•In Inhibitor at 37 °C. We incubated Csm Y08.82.36 host20 (ten-fold concentrated) without shaking at 76–78 °C for 30 min. Afterwards, we transferred the virus-infected cells into a glass test tube containing 5 ml of prewarmed sucrose-yeast extract (SY) and 0.8% gelrite mixture, and plated onto SY plates. The plates were put into a plastic bag, and incubated for two days at 76–78 °C. We counted plaques in plates with proper virus dilutions, and determined the titre of SSeV to be 4.96 × 10⁹ plaque-forming units (PFUs) per millilitre.

SSeV infection of S. islandicus with or without type III CRISPR

We carried out the SSeV infection assay as described, with minor modifications. In brief, approximately 6 × 10⁴ cells of S. islandicus M16.4 cells taken from the exponential stage were spun down at 4,000 r.p.m. for 12 min, and resuspended in 1 ml of arabinose-tryptone (AT) medium. The suspensions were then co-incubated with 20 ml of fresh AT medium or SSeV supernatant at different dilutions (10°, 10−1, 10−2, 10−3, 10−4, 10−5 and 10−6) in a Falcon tube at 76–78 °C for 30 min. Afterwards, we transferred the virus-infected cells into a glass test tube containing 5 ml of prewarmed sucrose-yeast extract (SY) and 0.8% gelrite mixture, and plated onto SY plates. The plates were put into a plastic bag, and incubated for two days at 76–78 °C. We counted plaques in plates with proper virus dilutions, and determined the titre of SSeV to be 4.96 × 10⁹ plaque-forming units (PFUs) per millilitre.
For TLC, we spotted 1 µl of radio-labelled product 1 cm from the bottom of a 20 × 20 cm silica gel TLC plate with fluorescence indicator 254 nm (Supelco Sigma-Aldrich). We placed the TLC plate in a sealed glass chamber prewarmed and humidified at 37 °C containing 0.5 cm of a running buffer composed of 30% water, 70% ethanol and 0.2 M ammonium bicarbonate. pH 9.2. The temperature was lowered to 35 °C and the buffer was allowed to rise along the plate through capillary action until the migration front reached 17 cm. The plate was air dried and sample migration was visualized by phosphor imaging.

To examine degradation of CA, and CA, by AcrIII-1 proteins, we incubated unlabelled CA, or CA, (450 µM, BIOLOG Life Science Institute, Bremen, Germany) with SIRV1 gp29 or YddF (40 µM dimer), in reaction buffers described above, at 70 °C and 37 °C, respectively. Reactions were quenched at the indicated time points and prepared for TLC as above. We visualized reaction substrate and products, which block fluorescence of the indicator on the plate, under shortwave UV light (254 nm) and photographed the plates using a 12-megapixel/1.8-aperture camera.

For kinetic analysis, we quantified CA, cleavage using the Bio-Formats plugin of ImageJ as distributed in the Fiji package and fitted the data to a single exponential curve ($y = m_1 + m_2x(1 - \exp(-m_3x))$; $m_1 = 0.1$, $m_2 = 1$ and $m_3 = 1$) using Kaleidagraph (Synergy Software), as before. We obtained the CA, cleavage rate by the H74A variant in the absence ofimidazole by linear fit. Raw data for kinetic analyses are available in Supplementary Data 2.

**Deactivation of HEPN nucleases by ring nucleases**

In the absence or presence of Crn1 Sso2081 (2 µM dimer) or AcrIII-1 SIRV1 gp29 (2 µM dimer), we incubated 4 µg S. solfataricus Csm complex (roughly 140 nM Csm carrying crRNA targeting A26 RNA target) with A26 RNA target (50 nM, 20 nM, 10 nM, 5 nM, 2 nM or 0.5 nM) in buffer containing 20 mM MES pH 6.0, 100 mM NaCl, 1 mM DTT and three units SUPERase-In Inhibitor supplemented with 2 mM MgCl2 at 70 °C for 60 min. We added 5’-3’ labelled A1 RNA (5’-AGGGA-UUAUUUGUUUUGUUCUCUAAACUAUAGCUGAUUGCGAAGA-3’) and 0.5 µM dimer SsoCsxl to the reaction at 60 min, and allowed the reaction to proceed for a further 60 min before quenching by adding phenol chloroform. We visualized A1 RNA cleavage by phosphor imaging after denaturing PAGE. A control reaction incubating SsoCsxl with A1 RNA in the absence of COA was carried out to determine SsoCsxl background activity. We visualized CA, synthesis by Csm in response to A26 target RNA, and subsequent CA, degradation in the presence of Crn1 Sso2081 or AcrIII-1 SIRV1 gp29, by adding 5 nM α-32P-ATP with 0.5 mM ATP at the start of the reaction. Reactions were quenched at 60 min with phenol chloroform, and CA, degradation products were visualized by phosphor imaging following TLC. We also carried out a control reaction incubating Csm with ATP and α-32P-ATP in the absence of A26 target RNA, quenching the reaction after 60 min.

We determined the CA, degradation capacity of AcrIII-1 SIRV1 gp29 and of the Crn1 enzyme Sso2081 by incubating 2 µM dimer of each enzyme with 500–0.5 µM unlabelled CA, (BIOLOG Life Science Institute, Bremen, Germany) in Csx1 buffer at 70 °C for 20 min before introducing SsoCsxl (0.5 µM dimer) and 3’-5’ labelled A1 RNA (50 nM). The reaction was left to proceed for a further 60 min at 70 °C before quenching by adding phenol chloroform. Deproteinized products were separated by denaturing PAGE to visualize RNA degradation.

**Phosphorylation from a reprogrammed type III system**

Plasmids pCsm1-5 ΔCsm6 and pCRISPR_T etR were transformed into the C43 containing pCsm1-5 ΔCsm6 and pCRISPR_TetR were transformed by heat shock with 100 ng of pRAT-Duet target plasmid containing different combinations of COA-dependent nuclease and viral ring nuclease. After outgrowth at 37 °C for 2 h, cells were collected and resuspended in 200 µL LB. A series of tenfold dilutions was applied onto LB agar containing 100 µg ml−1ampicillin and 50 µg ml−1 spectinomycin to determine the cell density of the recipient cells and onto LB agar additionally containing 25 µg ml−1 tetracycline, 0.2% (w/v) bile-lactose and 0.2% (w/v)-1-arabinose to determine the cell density of viable transformants. Plates were incubated at 37 °C for 16–18 h; further incubation was carried out at room temperature. Colonies were counted manually and corrected for dilution and volume to obtain colony-forming units (CFUs) per milliliter. Raw data for plasmid counts are available in Supplementary Data 3.

**Phosphorylation analysis**

AcrIII-1 homologues were collected by using gp29 (NP_666617) of SIRV1 as a query and running two iterations ($E = 1 \times 10^{-5}$) of PSI-BLAST against the non-redundant protein database at the National Center for Biotechnology Information (NCBI). Sequences were aligned using PROMALS3D. Redundant sequences (95% identity threshold) and sequences with a mutated active-site residue H47 were removed from the alignment. Poorly aligned low information content) positions were removed using the get 0.2 function of Trimal. The final alignment contained 124 positions. The maximum likelihood phylogenetic tree was constructed using PhyML with automatic selection of the best-fit substitution model for a given alignment. The best model identified by PhyML was LG + G + I. We assessed branch support using aBayes implemented in PhyML, and visualized the tree using iTOL.

**Crystallization**

The AcrIII-1H74A variant was concentrated to 10 mg ml−1, incubated at 293 K for 1 h with a 1.2 M excess of CA, and centrifuged at 13,000 r.p.m. for 10 min before crystallization. Sitting drop vapour diffusion experiments were set up at the nanolitre scale using commercially available
and in-house crystallization screens and incubated at 293 K. Crystals appeared in various conditions, but those used for data collection grew from 40% 2-methyl-2,4-pentanediol, 5% polyethylene glycol 8000 and 0.1 M sodium cacodylate, pH 6.5. Crystals were harvested and transferred briefly into cryoprotectant containing mather liquor with 20% glycerol immediately before cryo-cooling in liquid nitrogen. We used the H47A variant to avoid cleavage of the cA4 substrate during co-crystallization. The position of the active-site histidine was inferred from the structure of the apo-protein.

Data collection and processing

X-ray data were collected from two crystals at 100 K, at a wavelength 0.9686 Å, on beamline I24 at the Diamond Light Source, to 1.49 Å and 1.60 Å resolution. Both data sets were automatically processed with Xia241, using XDS and XS SCALE42. The data were merged in Aimless43 and the overall resolution truncated to 1.55 Å. The data were phased by molecular replacement using Phaser44, with a monomer from PDB file 2X4I stripped of water molecules as the search model. Model refinement of Acrl1 was achieved by iterative cycles of REFMACS45 in the CCP4 suite46 and manual manipulation in COOT47. Electron density for cA4 was clearly visible in the maximum likelihood/σA-weighted Fobs−Fcalc electron-density map at 3σ. The coordinates for cA4 were generated in ChemDraw (Perkin Elmer) and the library was generated using acedrg51, before fitting of the molecule in COOT. Model quality was monitored throughout using Molprobity52 (score 1.13; centile 99). Ramachandran statistics were 98.5% favoured, 0% disallowed. Data and refinement statistics are shown in Table 1.

Sample size and randomization

No statistical methods were used to predetermine sample size. The experiments were not randomized and the investigators were not blinded to allocation during experiments and outcome assessment.

Reporting summary

Further information on research design is available in the Nature Research Reporting Summary linked to this paper.

Data availability

The structural coordinates and data have been deposited in the Protein Data Bank (PDB) with deposition code 6SCF. The genome sequence of the SSeV virus has been submitted to GenBank with accession code MN53972. Raw data are available in the Supplementary Information for the plasmid immunity analysis presented in Fig. 1 and Extended Data Fig. 3, and the kinetic analysis presented in Fig. 2 and Extended Data Figs. 5, 6.

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Author contributions

J.S.A. designed experiments and carried out enzyme assays and analysis; S.A.M. carried out structural biology; C.Z. constructed the plasmid immunity assay; S.A.M. and J.S.A. contributed to data analysis and writing.

Competing interests

The University of St Andrews has filed a patent application (UK Patent Application 1902256.S, “Novel enzyme for phage therapy”), filed 19 February 2019, on which J.S.A. and M.F.W. are inventors. The other authors declare no competing interests.

Additional information

Supplementary information is available for this paper at https://doi.org/10.1038/s41586-019-1909-5. Correspondence and requests for materials should be addressed to T.M.G. or M.F.W.

Peer review information

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Extended Data Fig. 1 | Multiple sequence alignment of DUF1874-family members, and purity of DUF1874 and CRISPR ancillary enzymes used in biochemical assays. a, This multiple sequence alignment includes the Acrl-I proteins from the archaeal viruses Sirv1, Stiv, Afv3, Arvi1, Svrf, Smv4 and Atv, the Ipecb1 protein Yddf from B. Subtilis, the bacteriophage proteins from Thermoanaerobacterium phage ThsA-485A, Synechococcus phage Scbwm1, Fusobacterium phage Fnu1 and Hydrogenobaculum phage 1, and the Cm2 protein from Crenothrix polyspora. Conserved residues H47, R66, R85 and E88 are indicated by asterisks. Light and dark grey shading indicate regions of partial and strong sequence conservation, respectively.

b, SDS–PAGE of Sirv1 gp29 (wild-type, H47A and E88A variants), Yddf, the Cm1 enzyme Sso2081, and the Csx1 enzyme Sso1389. The gel is representative of two or more biological replicates.
Extended Data Fig. 2 | Construction of RJW007 Δtype I-A and RJW007 Δtype I-AΔcsx1 mutant strains. a, Genomic context of the CRISPR system in the genetic host (S. islandicus RJW007) and in mutant strains. A1 and A2 denote two different CRISPR arrays, the orientations of which are indicated with arrows. b, PCR verification of Δtype I-A mutants. A representative Sulfolobus transformant with integrated type I-A knockout plasmid was grown in dextrin-tryptone liquid medium, and the cell cultures were plated on dextrin-tryptone plates containing 5-fluoroorotic acid (5-FOA, 50 µg mg⁻¹), uracil (20 µg ml⁻¹), and agamatine (1 mg ml⁻¹). Seven randomly selected 5-FOA-resistant (5-FOA R) colonies were screened using the primers that bind outside of the flanking homologous regions to confirm the type I-A deletion. A representative Δtype I-A mutant was further colony purified for subsequent experiments. The expected sizes of the PCR products amplified from the genomic DNA of the parental strain (referred to wild type, wt) and the Δtype I-A mutant are 8,380 base pairs (bp) and 3,001 bp, respectively. The minus symbol denotes a negative control (using water as the template for PCR). L, log₂ DNA ladder (NEB). Seven biological replicates were screened. c, PCR analysis of the RJW007 Δtype I-AΔcsx1 mutant and its parental strain RJW007 Δtype I-A using primers that anneal to the outside of the flanking homologous regions of csx1, generating amplicons of 2,312 bp and 3,650 bp, respectively. Minus symbol, negative control (using water as the template for PCR). L, Gene Ruler Express DNA ladder (Thermo Scientific). The experiment carried out once. d, Plaque counts for the three strains tested (n = 3 biological replicates).
Extended Data Fig. 3 | Effect of DUF1874 on plasmid immunity provided by a heterologously expressed *M. tuberculosis* type III-A CRISPR system, providing cas4- or cas6-mediated immunity. Unprocessed images of sample plates are shown for all replicates (two biological replicates with four technical replicates each; n = 8). Cell-culture dilutions are indicated above the plates.
Extended Data Fig. 4 | Substrate preference of the AcrIII-1 proteins SIRV1 gp29 and YddF, and effective range of cA₄ degradation. a–d. TLC images visualizing (under 254 nm UV light) cA₄ and cA₆ (450 µM) degradation by SIRV1 gp29 (a, b) and YddF (c, d) over time (in minutes). Both AcrIII-1 enzymes display a clear preference for cA₄ over cA₆. All TLC images are representative of three technical replicates. e. Denaturing PAGE showing activation of Csx1 (0.5 µM dimer) by the indicated amounts (500–0.5 µM) of HPLC-purified cA₄, and its subsequent deactivation when either AcrIII-1 or Crn1 (2 µM dimer) was present to degrade cA₄. The AcrIII-1 enzyme degraded 100-fold more cA₄ than did Crn1. The control reaction (C) shows RNA incubated with Csx1 in the absence of cA₄ (n = 3 technical replicates). For gel source data, see Supplementary Fig. 1.
Extended Data Fig. 5 | Structure of SIRV1 gp29 bound to cA₄

a, b, Orthogonal views of SIRV1 gp29 dimer in complex with cA₄. The protein monomers are coloured purple and gold, with catalytic residue H47 from the apo structure shown in salmon. cA₄ is shown as a spacefill model, with green, blue, red and orange representing carbon, nitrogen, oxygen and phosphorus atoms, respectively. Conserved residues (Extended Data Fig. 1) in the AcrIII-1 family are indicated and discussed in the text. c, Interactions between each monomer of the SIRV1 dimer (orange and blue), with cA₄ shown in green. d, Diagram showing the interaction between SIRV1 gp29 and cA₄. Dotted lines represent hydrogen bonds, with distances annotated. Spheres represent water molecules.
Extended Data Fig. 6 | Single-turnover cA₄ cleavage by SIRV1 gp29 and variants, and chemical rescue with imidazole. a, Phosphorimage of TLC visualizing cA₄ cleavage by SIRV1 gp29 H47A (4 µM dimer, 50 °C) in the presence or absence of 500 mM imidazole, over time. The rate of cA₄ cleavage to generate A₃>P and A₂>P was calculated by quantifying densiometric signals from the phosphorimage (n = 3 technical replicates). b, Plot comparing the single-turnover rates of cA₄ by SIRV1 gp29, its E88A variant and its H47A variant, in the presence or absence of imidazole. Cleavage of cA₄ by the H47A variant can be partially restored when the reaction is supplemented with 500 mM imidazole. Data are mean and s.d. (n = 3 technical replicates). For gel source data, see Supplementary Fig. 1.
Extended Data Fig. 7 | Maximum likelihood phylogeny of AcrIII-1 homologues. The maximum likelihood phylogenetic tree was constructed with automatic selection of the best-fit substitution model for a given alignment (LG + G + I). Red circles indicate 95–100% branch support, as assessed using aBayes implemented in PhyML. The scale bar represents the number of substitutions per site. Branches and labels are colour coded: red, archaea; black, bacteria; blue, bacteria and archaea in which AcrIII-1 homologues are associated with CRISPR loci; green, archaeal viruses and plasmids; orange, bacteriophages.
Extended Data Fig. 8 | Genomic context of crn2 genes in selected bacteria. Type III CRISPR loci in the bacterial species *Crenothrix polyspora*, *Methylovulum psychrotolerans*, *Methylomagnum ishizawai*, *Thioalkalivibrio sulfidiphilus* and *Marinitoga piezophilia* are shown, with genes labelled and colour coded. The crn2 gene is shown in pale yellow with a bold outline; CRISPRs are indicated by small black arrowheads; and unrelated/hypothetical genes are shown as small white arrows. The sizes and orientations of genes are not reflected. Ago, Argonaute; CARF, CRISPR-associated Rossman fold; CARF-RelE, CARF domain fused to the RelE toxin; DUF1887, predicted CARF nuclease; RT, reverse transcriptase.
Extended Data Fig. 9 | CRISPR-associated AcrIII-1 homologues. Genomic neighbourhoods were analysed using the enzyme function initiative–genome neighbourhood tool (EFI-GNT) against the Pfam profile database53. Gene annotations are colour coded according to the key at the right.
Extended Data Table 1 | Data collection and refinement statistics for AcrIII-1 in complex with cA₄

| Data collection                                      | AcrIII-1 with cA₄ |
|------------------------------------------------------|-------------------|
| Space group                                          | P1                |
| Cell dimensions                                      |                   |
| a, b, c (Å)                                          | 49.8, 51.7, 85.6  |
| α, β, γ (°)                                          | 80.2, 89.7, 83.4  |
| Resolution (Å)                                       | 50.63-1.55 (1.58-1.55) * |
| R_{sym} or R_{merge}                                  | 0.12 (0.36)       |
| I / σI                                               | 12.3 (1.7)        |
| Completeness (%)                                     | 98.6 (92.4)       |
| Redundancy                                           | 2.9 (1.8)         |

**Refinement**

| Resolution (Å) | 84.26-1.55 |
|----------------|------------|
| No. reflections | 113882    |
| R_{work} / R_{free} | 0.20 / 0.25 |

No. atoms

- Protein: 7,365
- Ligand/ion: 352
- Water: 595

B-factors

- Protein: 20.2
- Ligand/ion: 13.3
- Water: 30.7

R.m.s. deviations

- Bond lengths (Å): 0.012
- Bond angles (°): 1.64

*Values in parentheses are for the highest-resolution shell. R, residual factor; I, intensity.
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The structural coordinates and data have been deposited in the Protein Data Bank with deposition code 6SCF.

Raw data is available for the plasmid immunity analysis presented in figure 1.

Raw data is available for the kinetic analysis presented in figure 2 and extended data figure 6.
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