The novel anti-CRISPR AcrIIA22 relieves DNA torsion in target plasmids and impairs SpyCas9 activity

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Running title: AcrIIA22 alters DNA torsion and protects plasmids from Cas9

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Abstract

To overcome CRISPR-Cas defense systems, many phages and mobile genetic elements encode CRISPR-Cas inhibitors called anti-CRISPRs (Acrs). Nearly all characterized Acrs directly bind Cas proteins to inactivate CRISPR immunity. Here, using functional metagenomic selection, we describe AcrIIA22, an unconventional Acr found in hypervariable genomic regions of clostridial bacteria and their prophages from human gut microbiomes. AcrIIA22 does not bind strongly to SpyCas9 but nonetheless potently inhibits its activity against plasmids. To gain insight into its mechanism, we obtained an X-ray crystal structure of AcrIIA22, which revealed homology to PC4-like nucleic-acid binding proteins. Based on mutational analyses and functional assays, we deduced that *acrIIA22* encodes a DNA nickase that relieves torsional stress in supercoiled plasmids. This may render them less susceptible to SpyCas9, which uses free energy from negative supercoils to form stable R-loops. Modifying DNA topology may provide an additional route to CRISPR-Cas resistance in phages and mobile genetic elements. *(150)*

Introduction

CRISPR-Cas systems in bacteria and archaea confer sequence-specific immunity against invading phages and other mobile genetic elements (MGEs)[1,2]. In response, MGEs can circumvent CRISPR-Cas systems by evading CRISPR immunity. In its simplest form, evasion requires only a single mutation within a CRISPR target site, which allows a phage or MGE to escape immune recognition[3]. However, CRISPR-Cas systems routinely acquire new spacer sequences corresponding to new sites within phage and MGE genomes[1]. This means that any single-site evasion strategy is likely to be short-lived. Thus, phages also employ forms of CRISPR-Cas evasion that are less easily subverted. For instance, some jumbophages assemble a proteinaceous, nucleus-like compartment around their genomes upon infection, allowing them to overcome diverse bacterial defenses, including CRISPR-Cas and restriction-modification (RM) systems[4,5]. Similarly, other phages decorate their DNA genomes with diverse chemical modifications such as the glucosylated cytosines used by phage T4 of *Escherichia coli*[6], which can prevent Cas nucleases from binding their target sequence.

MGEs may also overcome CRISPR-Cas systems by inactivating, rather than evading, CRISPR immunity. MGEs encode diverse CRISPR-Cas inhibitors called anti-CRISPRs (Acrs), which allow them to overcome CRISPR-Cas systems and infect otherwise immune hosts[7]. Most known Acrs bind Cas proteins and inhibit Cas activity by restricting access to target DNA, preventing necessary conformational changes, or inactivating critical CRISPR-Cas components[8,9]. The direct inactivation of Cas proteins by Acrs has proven an effective and widespread strategy for overcoming CRISPR immunity[10].

Recent genetic, bioinformatic, and metagenomic strategies have identified many Acrs that independently target the same CRISPR-Cas system[7-10]. Yet, most CRISPR-Cas systems are not inhibited by known Acrs[10]. Thus, many undiscovered strategies to inhibit or evade CRISPR-Cas systems likely exist in nature. Indeed, over half of the genes in an average phage genome have no known function[11]. To uncover new counter-immune strategies, we recently devised a high-throughput functional metagenomic selection to find genes that protect a target plasmid from *Streptococcus pyogenes* Cas9 (SpyCas9), the variant used most frequently for genome editing[12]. Our selection strategy was designed to reveal any gene capable of overcoming SpyCas9 activity in this system, regardless of mechanism. With this approach, we previously described a new

phage inhibitor of SpyCas9, called AcrIIA11, which exhibits broad-spectrum anti-Cas9 activity and is prevalent across human gut microbiomes[12].

Here, we describe *acrIIA22*, which was the second most common Acr candidate recovered from our original functional selection. *AcrIIA22* encodes a 54 amino acid protein that impairs SpyCas9 activity. We observe that homologs of *acrIIA22* are found in hypervariable loci in phage and bacterial genomes. Unlike most other Acrs, AcrIIA22 does not bind strongly to SpyCas9 *in vitro*. Instead, guided by an X-ray crystal structure of AcrIIA22, coupled with mutational and biochemical analyses, we show that AcrIIA22 encodes a DNA nickase. By nicking a supercoiled plasmid substrate and relieving its torsional stress, AcrIIA22 renders the target less susceptible to SpyCas9 activity. AcrIIA22 thus represents a novel mechanism of SpyCas9 evasion, which capitalizes on SpyCas9's preference for negative supercoils to efficiently form R-loops and cleave DNA[13-16]. Such a resistance mechanism could be accessible to diverse MGEs, providing a route to CRISPR-Cas tolerance in many genetic contexts.

Results

Functional selection reveals a novel anti-CRISPR protein, AcrIIA22

We recently carried out a functional selection for SpyCas9 antagonism, recovering clones from metagenomic libraries that could potently inhibit SpyCas9[12]. In this two-plasmid setup, we used an arabinose-inducible SpyCas9 on an expression plasmid to cleave the *kanamycin resistance* (*Kan^R*) gene of a second 'target' plasmid. We then grew cultures in SpyCas9-inducing conditions and measured the proportion of colony forming units (cfus) that remained kanamycin resistant (Figure 1A). This proportion is a measure of how many clones retained their target plasmid and, thus, how effectively that plasmid withstood SpyCas9 attack. In our previously published work, we describe AcrIIA11, a novel anti-CRISPR from a metagenomic clone named F01A_2 (Genbank ID MK637582.1), which was the most abundant clone after functional selection of a human fecal microbiome[12]. This functional selection also revealed another protective clone, F01A_4 (Genbank ID MK637587.1), which was the second most abundant contig following selection. Together, these two contigs (F01A_2 and F01A_4) accounted for >96% of the normalized read coverage.

The F01A_4 contig is 685 bp long, encodes three potential open reading frames (ORFs), and confers complete protection against SpyCas9, with plasmid retention equaling that of an uninduced SpyCas9 control (Figure 1B). To determine the genetic basis for SpyCas9 antagonism in this contig, we introduced an early stop codon into each of the three potential ORFs and analyzed how these mutations affected the contig's ability to protect a target plasmid from SpyCas9. We found that an early stop codon in *orf_1* reduced the proportion of *Kan^R* cfus by a factor of 10^5, matching the value observed for an empty vector control (Figure 1B). Furthermore, expression of *orf_1* alone was sufficient for SpyCas9 antagonism (Figure 1C), protecting a target plasmid from SpyCas9 cleavage as effectively as the potent SpyCas9 inhibitor, AcrIIA4. In this assay, *orf_1* was slightly toxic when singly expressed in *E. coli*, reducing growth rate by 7% (Supplemental Figure 1). Combined, our results indicate that *orf_1* completely accounts for the SpyCas9 protection phenotype of contig F01A_4.

One mechanism through which *orf_1* could apparently antagonize SpyCas9 in our functional assay would be by lowering its expression. To address this possibility, we carried out two experiments. First, we swapped the *spycas9* gene for *gfp* in our expression vector and asked whether *orf_1* induction impacted fluorescence output. We saw no change in fluorescence upon

orf_1 induction, indicating that *orf_1* neither suppressed transcription from our expression vector nor altered its copy number (Supplemental Figure 2). Second, we used Western blots to test whether *orf_1* expression impacted SpyCas9 protein levels through the course of a plasmid protection assay. We used a crRNA that did not target our plasmid backbone to ensure that *orf_1* expression remained high to maximize its potential impact on SpyCas9 expression levels. We observed that *orf_1* expression had no meaningful effect on SpyCas9 expression at any timepoint (Supplemental Figure 2). Thus, we conclude that *orf_1* does not impact SpyCas9's translation or degradation rate. Therefore, *orf_1* must act via an alternative mechanism to inhibit SpyCas9 activity. Based on these findings, we conclude that *orf_1* encodes a *bona fide* anti-CRISPR protein and hereafter refer to it as *acrIIA22*.

Next, we investigated whether *acrIIA22* could also allow phages to escape from SpyCas9 immunity (Supplemental Figure 3). We measured SpyCas9's ability to protect *E. coli* from infection by phage Mu, in the presence or absence of *acrIIA22*. As a control, we carried out similar phage infections in the presence or absence of the well-established SpyCas9 inhibitor, *acrIIA4*. As anticipated, SpyCas9 significantly impaired Mu when targeted to the phage's genome but not if a non-targeting CRISPR RNA (crRNA) was used. Consistent with previous findings[12], phage Mu could infect targeting strains equally well as non-targeting strains when *acrIIA4* was expressed, indicating that SpyCas9 immunity was completely abolished by this *acr*. In comparison, *acrIIA22* improved the infectivity of phage Mu by a factor of 100 to 1,000 across multiple experimental conditions (Supplemental Figure 3). We therefore conclude that *acrIIA22* only partially protects phage Mu from SpyCas9 whereas it completely protects plasmids against SpyCas9 cleavage.

AcrIIA22 homologs are found in hypervariable regions of bacterial and prophage genomes

AcrIIA22 is 54 amino acids in length and has no sequence homology to any protein of known function, including all previously described Acrs. We examined the distribution of *acrIIA22* homologs in NCBI's NR and WGS databases but found just seven hits, limiting our ability to make evolutionary inferences about its origins or prevalence. We therefore expanded our search to include IMG/VR, a curated database of cultured and uncultured DNA viruses[17], and assembly data from a meta-analysis of 9,428 diverse human microbiome samples[18]. With an additional 23 unique homologs from these databases, we found that the majority of *acrIIA22* homologs exist in either of two genomic contexts: prophage genomes (Figure 2A, Supplemental Figure 4A) or small, hypervariable regions of bacterial genomes, which we refer to hereafter as 'genomic islands' (Figure 2B, Supplemental Figure 4B). The original metagenomic DNA fragment from our selection, F01A_4, shared perfect nucleotide identity with one of these genomic islands (Figure 2B).

Because most *acr*s are found in phage genomes, we first examined the prophages that encoded AcrIIA22 homologs. These prophages were clearly related, based on many homologous genes and a similar genome organization (Supplemental Figure 4A). We found that these prophages had inserted into several different bacterial loci, including one site between the bacterial genes *purF* and *radC* (locus #3, Supplemental Figure 4A). This insertion site is nearly identical to the highly conserved sequences that flanked *acrIIA22*-encoding bacterial genomic islands (Supplemental Figure 4B). Based on this common insertion site, we hypothesize that the apparently bacterial genomic islands with *acrIIA22* homologs originated from a common prophage insertion at this locus. We speculate that the original *acrIIA22*-encoding bacterial genomic island resulted from the incomplete excision of an ancestral, *acrIIA22*-encoding prophage. Supporting

this hypothesis, *acrIIA22* homologs are always found at the end of prophage genomes, near their junction with a host bacterial genome (Figure 2A, Supplemental Figure 4A).

To better understand *acrIIA22*'s gene neighborhood, we again searched the assemblies of over 9,400 human microbiomes for more examples of these genomic islands[18]. We did not include *acrIIA22* as a query. Instead, we only considered contigs with ≥98% nucleotide identity to *purF* and *radC*, the conserved genes that flanked the genomic islands. This search yielded 258 contigs. Aligning these sequences revealed that each contig encoded a short, hypervariable region of small ORFs which was flanked by conserved genomic sequences (Figure 2B, Supplemental Figure 4B). In total, we observed 128 unique examples of these hypervariable loci, which displayed considerable gene turnover, resulting in 54 distinct gene arrangements among the 128 unique loci. Despite not being included in our search criteria, *acrIIA22* homologs were universally conserved in all 128 unique genomic islands. In contrast, no other gene was present in more than two-thirds of the 54 distinct gene arrangements (Figure 2B, Supplemental Figure 4C). Based on this finding, we infer that the arrival of *acrIIA22* preceded the diversification seen at this locus and has been retained despite the considerable gene turnover that has occurred subsequently.

Though most ORFs in these islands were of unknown function, many had close homologs in the genomes of nine representative *acrIIA22*-encoding phage (Supplemental Figures 4A, 4B, 4C). This suggests that phages continue to supply the genetic diversity seen at these hypervariable genomic loci. These rapid gene gains and losses likely occur as they do in other genomic islands, via recombination between this locus and related MGEs that infect the same host bacterium, without the MGE necessarily integrating into the locus[19]. Taken together, our data suggest that an incomplete prophage excision event left *acrIIA22* behind in a bacterial genomic locus, which then diversified via gene exchange with additional phage genomes (Figure 2C, Supplemental Figure 4D).

Like in the genomic islands (Figure 2B), we found *acrIIA22* homologs in hypervariable regions of prophage genomes, where they were consistently near the junction with a host bacterial genome (Supplemental Figure 4A). Thus, nearly all *acrIIA22*-encoding loci show signatures of frequent recombination. Despite this, we could find no gene consistently present within or outside of *acrIIA22*-encoding genomic islands that could account for their hypervariable nature (*e.g.* an integrase, transposase, recombinase, or similar function that is typically associated with locus-specific recombination[20]). Instead, *acrIIA22* was the only gene conserved at this locus. This conservation led us to speculate that *acrIIA22* might promote recombination, either alone or with other factors. If this were true, it could explain the high rates of gene exchange observed adjacent to the *acrIIA22* gene in phage and bacterial genomes (Figure 2, Supplemental Figure 4).

In total, we identified 30 unique *acrIIA22* homologs, 25 of which were predicted to originate from the unnamed clostridial genus, CAG-217 (Figure 3A). Because *acr*s are only beneficial to phages if they inhibit CRISPR-Cas activity, they are typically found only in taxa with a high prevalence of susceptible Cas proteins[9]. If AcrIIA22 functions naturally as an Acr, we would predict that Cas9-encoding, type II-A CRISPR-Cas systems like SpyCas9 would be common in CAG-217 bacteria. To test this idea, we examined 779 draft assemblies of CAG-217 genomes and found that 179 of the 181 predicted CRISPR-Cas systems were type II-A systems (the remaining two loci were Cas12-encoding, type V-A systems). This enrichment for Cas9 is particularly striking as *Clostridia* typically encode other CRISPR-Cas defenses and only rarely encode Cas9-based systems[21]. Moreover, prophages from CAG-217 encode 78 type II-A Acrs (homologs of AcrIIA7, AcrIIA17, and AcrIIA21), suggesting they are actively engaged in an arms

race with Cas9-based defenses in these bacteria. In one case, we found *acrIIA17* and *acrIIA22* homologs within one kilobase of each other in a prophage genome (Supplemental Figure 5)[22]. Phages often collect *acr* genes in the same genomic locus[23], commonly pairing narrow-spectrum *acr*s that act during lytic infection alongside broad-spectrum *acr*s that operate during lysogeny[24]. Together, these observations support our hypothesis that prophages encode *acrIIA22* homologs to inhibit type II-A CRISPR-Cas (Cas9) systems in CAG-217 genomes.

We next tested whether the ability to inhibit type II-A CRISPR-Cas systems was shared among *acrIIA22* homologs from CAG-217 bacteria. To do so, we selected *acrIIA22* homologs that spanned the phylogenetic diversity present among CAG-217 genomes (Figure 3A) and tested their ability to protect a target plasmid from SpyCas9 elimination. These analyses revealed that diverse *acrIIA22* homologs from CAG-217 bacteria (for example, sharing only 56.9% identity) could antagonize SpyCas9 activity at least partially (Figure 3B), reminiscent of the broad inhibition that has been previously observed for some other type II-A Acrs[12]. To determine if this anti-CRISPR activity extended beyond SpyCas9, we used a slightly modified plasmid protection assay (see methods) to test whether *acrIIA22* could inhibit other type II and type V CRISPR-Cas systems, as these were the only two CRISPR-Cas types present in CAG-217 genomes. Though *acrIIA22* could not inhibit any of the type V (Cas12-encoding) systems we tested, it did protect a target plasmid from two substantially diverged type II CRISPR-Cas systems, consistent with the high prevalence of Cas9-based systems among CAG-217 bacteria (Figure 3C). Such broad-spectrum inhibition can occur either by targeting a conserved feature of Cas9 or by inhibiting Cas9 via an indirect mechanism that it cannot easily evade.

AcrIIA22 functions via a non-canonical mechanism

Almost all characterized Acrs inhibit their cognate Cas proteins via direct binding without the involvement of additional co-factors. As a result, they exhibit strong inhibitory activity when tested *in vitro* (Supplemental Table 1). To determine if this was the case for AcrIIA22, we purified it from *E. coli* and asked whether it could bind and inhibit SpyCas9. To test for binding, we asked whether twin-strep-tagged AcrIIA22 co-precipitated with untagged SpyCas9 when mixed as purified proteins. Unlike with AcrIIA4, which binds strongly to SpyCas9 and inhibits its activity *in vitro*, we detected little to no binding between AcrIIA22 and SpyCas9, regardless of whether a single-guide RNA (sgRNA) was included or not (Supplemental Figure 6). We also observed that AcrIIA22 had no impact on SpyCas9's ability to cleave linear, double-stranded DNA (dsDNA), even when AcrIIA22 was included at substantial molar excess over SpyCas9 (Supplemental Figure 7). These results suggest that AcrIIA22 cannot bind and inhibit SpyCas9, at least in isolation. Thus, AcrIIA22 lacks the predominant biochemical activities exhibited by previous Acrs that have been mechanistically characterized.

We therefore considered the possibility that AcrIIA22 encodes an unconventional anti-CRISPR that acts via a non-canonical mechanism. However, the only AcrIIA22 homologs we could detect using BLAST were proteins of unknown function, which provided few clues about AcrIIA22 activity or biochemical mechanisms. Anticipating that structural homology might provide better insight into its mechanism of inhibition, we solved AcrIIA22's structure using X-ray crystallography. We first built a *de novo* model from AcrIIA22's primary sequence with Robetta[25]. We then used this model as a molecular replacement probe to solve its structure at 2.80Å resolution (PDB:7JTA). The asymmetric unit in AcrIIA22's crystal comprises two monomers stacked end-to-end, with each monomer folding into a four-stranded β-sheet (Figure 4A, Table 1). A DALI structure-structure search revealed that the AcrIIA22 monomer is similar to members

of the newly recognized PC4-like structural fold (Figure 4B, Supplemental Table 2). PC4-like proteins have independently evolved in all domains of life, typically adopt a β-β-β-β-α topology, and often homodimerize to bind diverse RNA and DNA species using variably positioned β-sheets[26].

Despite crystallizing as a homodimer, AcrIIA22 migrated from a size exclusion chromatography (SEC) column at an elution volume corresponding to a calculated mass approximately four times larger than its expected monomeric molecular weight (Figure 4B). This suggested that AcrIIA22 may oligomerize *in vivo*. Indeed, AcrIIA22 was predicted to form a stable tetramer when analyzed with PISA, a tool for inferring macromolecular assembles from crystal structures[27] (Figure 4C, Supplemental Figures 8A, 8B). This putative tetramer has a molecular mass consistent with that observed by SEC and comprises pairs of outward-facing, concave β-sheets. A series of hydrophobic interactions likely stabilize this configuration of β-sheets instead of the typical α-helical interactions seen in other PC4-like proteins, potentially explaining the absence of an α-helix in AcrIIA22 (Supplemental Figures 8C, 8D). Interestingly, many PC4-like proteins bind nucleic acids using similar concave β-sheets, and in some instances form higher-order oligomers as an obligate step for binding and/or unwinding DNA or RNA[26]. Consistent with this possibility, AcrIIA22's β-sheets orient along each outward face of the putative tetramer, resemble those in PC4-like proteins, and form a groove that could potentially accommodate a nucleic acid substrate (Figure 4D, Supplemental Figures 8A, 8B, 8E). Thus, AcrIIA22's structural and functional attributes led us to suspect that it could also interact with nucleic acids and potentially affect their topology.

Our tetramer model predicts that an interface at the C-terminus of AcrIIA22 is required for adjacent β-sheets to bind one another and form a grooved, oligomeric structure (Figures 4C, 4F). We reasoned that a two-residue, C-terminal truncation of AcrIIA22 would disrupt this interface (Figure 4F, Supplemental Figure 8G). To test this hypothesis, we examined the oligomeric state of this 2-aa AcrIIA22 deletion mutant by SEC. Consistent with our hypothesis, we found that the mutant AcrIIA22 complexes migrated at half the size of the wild-type complexes, corresponding to approximately twice AcrIIA22's molecular weight (Figure 4B). This suggests that the C-terminal interface is required to progress from a two to four-membered oligomer, consistent with our model. Moreover, we found that the 2-aa deletion mutant was also impaired for SpyCas9 antagonism in our plasmid protection assay (Supplemental Figure 9A). Thus, this C-terminal motif is necessary for protection from SpyCas9 and for higher-order oligomerization, suggesting that oligomerization may be necessary for AcrIIA22's anti-SpyCas9 activity.

AcrIIA22 is a DNA nickase that relieves torsion of supercoiled plasmids

Our structural analyses indicated that AcrIIA22 is a PC4-like nucleic acid-interacting protein. Like AcrIIA22, many of the known PC4-like proteins are encoded in phage genomes. Among these is AcrIIA22's closest structural relative in the PC4-like family: a predicted single-stranded binding (SSB) protein from phage T5 (Figure 4E)[28]. This putative SSB protein has been predicted to directly stimulate recombination during the recombination-dependent replication of phage T5's genome[29]. This prediction, together with our inference from genomic analyses (Figure 2, Supplemental Figure 4), led us to hypothesize that AcrIIA22 may have similar recombination-stimulating activity. Indeed, other PC4-like proteins have been observed experimentally to unwind duplex DNA, a function consistent with their proposed roles in transcription and recombination[26,30]. Therefore, we investigated whether AcrIIA22 might also similarly interact with duplexed DNA to affect its topology.

We investigated whether we could detect any biochemical effect of *acrIIA22* on a double-stranded DNA (dsDNA) plasmid *in vivo*. In this experiment, we compared two *acrIIA22* genotypes: the wild-type sequence and a null mutant with a single base pair change to create an early stop codon. We grew overnight cultures of plasmids expressing each genotype, purified plasmid DNA, and analyzed its topology using gel electrophoresis (Figure 5A). As is typical for plasmid purifications from *E. coli*, the plasmid encoding the null mutant was predominantly recovered in a supercoiled form. In contrast, AcrIIA22 expression shifted much of the target plasmid to a slowly migrating form, consistent with an open-circle conformation. These findings suggest that AcrIIA22 expression could relieve plasmid supercoiling, potentially via DNA nicking activity.

Though *acrIIA22* expression appeared to alter plasmid topology *in vivo*, DNA topology is a dynamic process, regulated by many competing factors and dependent on cellular physiology[31]. Thus, we could not attribute the observed change in plasmid conformation solely to AcrIIA22. To more directly investigate AcrIIA22's effect on plasmid topology, we purified an N-terminal, His6-tagged AcrIIA22 protein and examined its impact on a plasmid DNA substrate *in vitro*. By gel electrophoresis, we observed that AcrIIA22 shifted a supercoiled plasmid to a slowly migrating form in a time and concentration-dependent manner (Figure 5B, Supplemental Figure 10D). For comparison, we also treated a plasmid with the nickase Nb.BssSI, yielding a band that migrated at the same position as the putatively open-circle product generated via AcrIIA22 activity (Figure 5B). High concentrations of AcrIIA22 resulted in conversion of plasmids to a linearized DNA product, consistent with a nickase-like nuclease activity acting on both strands of DNA (Figure 5B). This nicking activity was strongly stimulated in the presence of Mn^{2+}, Co^{2+}, and Mg^{2+}, weakly with Ni^{2+} and Zn^{2+}, and not at all with Ca^{2+} (Supplemental Figure 11). To confirm that the observed gel-shift was the result of nicking activity and not protein-bound DNA, we purified an AcrIIA22-treated plasmid with phenol-chloroform and re-examined it by gel electrophoresis. We observed that the open-circle form of the plasmid persisted through purification, establishing it as the product of a *bona-fide* nickase (Supplemental Figure 11). Consistent with our *in vivo* observations (Supplemental Figure 9A), we found that the 2-aa deletion mutant was impaired for nicking activity relative to wildtype AcrIIA22 (Supplemental Figure 9B). These data suggested that *acrIIA22* may encode for a protein that nicks DNA.

No known nuclease has been previously characterized among the PC4-family proteins[26]. Therefore, to further test our hypothesis that AcrIIA22 nicks supercoiled plasmids, we performed several additional experiments. First, we re-purified an N-terminal, His6-tagged AcrIIA22 protein, but this time examined individual fractions for nicking activity. Consistent with AcrIIA22's hypothesized function, nicking activity correlated with AcrIIA22 concentration across these fractions (Supplemental Figures 10B, 10C); no co-purifying contaminant was detected via Coomassie stain (Supplemental Figure 10A). This nicking activity, however, was low enough that we could not eliminate the possibility that another protein, undetectable via Coomassie stain, might have co-purified with AcrIIA22 and could explained this behavior. Reasoning that different contaminating proteins would result from different purification strategies, we generated a new version of the AcrIIA22 protein and purified it via a C-terminal, twin-strep-tag. A more sensitive, silver-stained gel indicated that this AcrIIA22 preparation was also very pure (Supplemental Figure 10E). We subsequently confirmed that it nicked supercoiled plasmids with a specific activity of 5.1×10^{-7} nmol/min/mg (Figures 5C, Supplemental Figures 10G, 10H). This activity is comparable to other nickases involved in phage-bacterial conflicts (including SspB, which nicks at a rate of 8.9×10^{-7} nmol/min/mg)[32]. Notably, this specific activity is significantly higher than we observed for our original, N-terminal His6-tagged variant (compare AcrIIA22 concentrations in

Figures 5B and 5C). This difference in nicking activity is also reflected in plasmid protection phenotypes observed *in vivo*; only C-terminally tagged AcrIIA22, but not N-terminally tagged AcrIIA22, protected a plasmid from SpyCas9 attack (Supplemental Figure 10F). Thus, our studies find a strong correlation between AcrIIA22's nicking and plasmid protection activities.

If AcrIIA22 encoded a true nickase, we hypothesized that we might be able to abrogate this activity via point mutations in putative catalytic residues. Therefore, we searched for individual point mutations that impaired nicking activity *in vitro*. If such mutants existed, they would allow us to test our hypothesis that AcrIIA22 is a nickase. Reasoning that acidic amino acids were likely to be important catalytic residues[33], we individually changed each aspartic acid and glutamic acid in AcrIIA22 to an alanine. Hypothesizing that AcrIIA22's *in vitro* biochemical activity would correlate with its anti-Cas9 function *in vivo,* we tested whether these alanine variants still inhibited SpyCas9 in our plasmid protection assay. Of the 11 mutants tested, D14A stood out. This mutant showed clear SpyCas9-dependent plasmid loss, with a >250-fold reduction in plasmid retention compared to a wild-type AcrIIA22 control (Figure 6A).

Purification of the D14A mutant (via a C-terminal twin-strep tag) revealed that it displayed similar expression level, purification yield, oligomeric size distribution and solution behavior as wild-type AcrIIA22 (Figure 6B, Supplemental Figure 10E), indicating that the mutant protein is still properly folded. The D14A mutant was substantially impaired for nicking activity compared to the wild-type AcrIIA22 protein (Figures 6C, Supplemental Figure 10G), consistent with its diminished anti-Cas9 activity *in vivo* (Figure 6A). Unlike previous observations with the 2-aa deletion mutant (Figure 4B), the reduction in nicking for the D14A mutant is unlikely to be the result of oligomeric differences between it and wild-type AcrIIA22 (Figure 6B). Instead, we speculate that D14 may contribute to AcrIIA22's nicking activity, as two D14 residues from different AcrIIA22 monomers sit very near to one another in our proposed tetramer, such that they may be stabilized via the presence of a divalent cation under physiological conditions (Supplemental Figure 8F).

Our surveys of divergent AcrIIA22 homologs also revealed a naturally occurring AcrIIA22 homolog with diminished function *in vivo* (AcrIIA22a, Fig. 3B). Despite encoding for a protein that differs by only two amino acids from the original sequence (V3I and R30K), *acrIIA22a* was >450-fold less effective at protecting a plasmid from SpyCas9 than *acrIIA22* (Fig. 3B). We examined whether this loss of SpyCas9 protection correlated with loss of nicking activity, just like the D14A mutant. Upon purification, a twin-strep-tagged AcrII22a protein eluted with an SEC profile identical to that of AcrIIA22, suggesting a similar oligomeric state (Figure 6B). Yet, AcrIIA22a exhibited substantially less nicking activity that wild-type AcrIIA22 *in vitro* (Figure 6C). In our proposed AcrIIA22 tetramer, R30 likely forms a hydrogen bond with the C-terminus of a diagonal monomer, raising the possibility that the R30K variant alters the protein's conformational plasticity or mediates other allosteric effects (Supplemental Figure 8G). As with D14A, the partial loss of nicking activity seen for AcrIIA22a (Figure 6C) correlated with a partial loss of plasmid protection against SpyCas9 (Figure 3B). Thus, we describe two closely related AcrIIA22 variants, one engineered and one naturally occurring, whose nicking activity *in vitro* corresponds directly to plasmid protection *in vivo*. From these data, along with our other *in vitro* and *in vivo* findings, we conclude that *acrIIA22* encodes a nickase protein that relieves the torsional stress of supercoiled plasmids.

AcrIIA22's nicking activity indirectly impairs SpyCas9

Having established that AcrIIA22 is a DNA nickase, we next investigated how this biochemical activity may enable its inhibition of SpyCas9 without directly binding the Cas protein. We therefore tested the consequences of expressing AcrIIA22 on a target plasmid in the presence of SpyCas9. As before, we began by comparing overnight plasmid purifications of a target plasmid expressing AcrIIA22 and a null mutant with an early stop codon as a negative control. For both genotypes, we subjected the *acrIIA22*-encoding plasmid to SpyCas9 targeting during bacterial growth. We were unable to recover the negative control target plasmid after overnight growth, implying that this target plasmid was eliminated by SpyCas9 (Figure 7A). In contrast, SpyCas9 did not eliminate a target plasmid that expressed full-length AcrIIA22 (Figure 7A), consistent with AcrIIA22's capacity to protect against SpyCas9 (Figure 1C).

To be effective, a CRISPR-Cas system must eliminate its target at a faster rate than the target can replicate[34]. Our findings raised the possibility that AcrIIA22 modifies a target plasmid into a SpyCas9-resistant conformation to win this 'kinetic race' against SpyCas9, potentially shifting the equilibrium to favor plasmid persistence instead of elimination. To test this kinetic race model, we asked whether a plasmid that had been pre-treated with AcrIIA22 could resist digestion by SpyCas9 *in vitro*. Therefore, we purified the open-circle plasmid that resulted from AcrIIA22 pre-treatment and determined how efficiently it was cleaved by SpyCas9 compared to an unmodified, supercoiled plasmid (Figure 7B). SpyCas9 showed a clear preference for cleaving the supercoiled substrate versus the AcrIIA22-treated open-circle plasmid (Figures 7C, 7D, 7E), consistent with previous reports[13-16]. An open-circle plasmid pre-treated with the nickase Nb.BssSI was similarly recalcitrant to SpyCas9 digestion (Figures 7C, 7D). Taken together, our findings suggest that relieving DNA torsion provides the mechanistic explanation for AcrIIA22's ability to inhibit SpyCas9 *in vivo*.

Discussion

In this study, we identify and characterize *acrIIA22*, a previously undescribed gene that can antagonize SpyCas9. We show that AcrIIA22 homologs are common in genomes and prophages of CAG-217 bacteria, which have a high prevalence of Cas9 homologs. Using a combination of structural and biochemical studies, we show that AcrIIA22 acts by nicking supercoiled DNA to relieve torsional stress on a target plasmid, and that this activity correlates with protection against SpyCas9 *in vivo* and *in vitro*. This torsion-based model for SpyCas9 inhibition helps explain why AcrIIA22 protects plasmids more effectively than phage Mu against SpyCas9. Because plasmids are maintained as circular, extrachromosomal elements, they are more likely to undergo torsional change when nicked than the dsDNA genome of phage Mu, which is injected as linear DNA and spends significant time integrated into *E. coli*'s genome[35]. Additionally, linear DNA experiences much lower torsional stress and therefore is less susceptible than supercoiled plasmids to cleavage by SpyCas9[15]. This difference also likely explains why AcrIIA22 failed to protect a linear dsDNA substrate from SpyCas9 during our earlier *in vitro* experiments (Supplemental Figure 7).

Previous *in vitro* experiments indicate that Cas9 requires a higher degree of negative supercoiling than type I CRISPR-Cas systems to provide the free energy needed for R-loop formation[13]. Similarly, *in vivo* observations have shown that DNA supercoiling promotes the recruitment of SpyCas9 to its target site in bacteria[14]. Based on these published findings, we speculate that Cas9 may be particularly susceptible to changes in DNA torsion among CRISPR-Cas systems. Thus, factors that modify DNA torsion, like AcrIIA22, could provide a general means to protect against Cas9 or other enzymes with a strong preference for negative supercoils.

Taken together, our data implicate DNA topology as a new battleground in the evolutionary arms race between CRISPR-Cas systems and MGEs. Because DNA topology is dynamically regulated in phages, plasmids and other MGEs, many topology-modifying factors already exist in these genomes. Our findings suggest that at least some of these factors could have secondary effects on CRISPR-Cas activity and thus prove useful in the context of a molecular arms race[31,36]. For instance, though not studied in the context of bacterial defense systems, the fitness of phage T4 is improved via the expression of an accessory protein that modifies DNA supercoiling and the propensity of R-loops to form[37]. Other phages, such as the intrinsically Cas9-resistant phage T5[38], incorporate regular nicks into their genome, the function of which has eluded description for over 40 years[39]. Additionally, conjugative plasmids were recently shown to evade CRISPR-Cas in *Vibrio cholerae* by the action of homologs of the recombination proteins Redβ and λExo[40]. Based on our findings, we hypothesize that phages and MGEs targeted by Cas9 exploit factors that modify DNA topology as a general tactic to evade host immunity.

Functional selections like ours are biased towards identifying genes that work well in heterologous contexts. For example, even though AcrIIA22 is encoded on the genome of a genetically intractable bacterium, we could identify it using a functional metagenomic selection for SpyCas9 antagonism in *E. coli*. Although we have characterized its activities in *E. coli* and *in vitro*, we cannot be certain that AcrIIA22 functions similarly in its native context. Little is known about the life cycle of native CAG-217 phages, though many dsDNA phage genomes undergo circular, topologically-constrained stages during their replicative cycles[41], during which AcrIIA22 might act to specifically overcome Cas9 immunity. Alternatively, AcrIIA22 may enable Cas9 evasion as a secondary function related to some other activity. Comparative genomics (Figure 2) and structural homology to a proposed recombination-stimulating protein of phage T5 suggest a potential role for AcrIIA22 in recombination, a process which has recently been shown to promote CRISPR-Cas evasion[40].

Nevertheless, the heterologous behavior of AcrIIA22 in *E. coli* is clearly sufficient for SpyCas9 antagonism *in vivo* and its nicking activity can protect plasmids from SpyCas9 *in vitro*. Furthermore, AcrIIA22 mutants that are defective for nicking *in vitro* (Figure 6C, Supplemental Figure 9B) are orders of magnitude less effective at protecting a plasmid from SpyCas9 *in vivo* (Figures 3B, 6A, Supplemental Figure 9A). This indicates that modest changes in nicking activity can have major consequences for plasmid survival, which is consistent with our kinetic race model (Figure 7B) and previous observations that non-linear equilibrium dynamics determine whether an MGE withstands CRISPR-Cas immunity[34].

Our results suggest that other proteins that affect DNA torsion may also enable Cas9 antagonism. For example, in addition to AcrIIA22, the Nb.BssSI nickase was capable of protecting a plasmid from SpyCas9 *in vitro*. Yet, despite the regular occurrence of nickases in nature, functional selections for anti-Cas9 activity have not previously recovered such enzymes[12,42]. We speculate that AcrIIA22 was identified from a metagenomic library because it treads a balance between activity and toxicity in *E. coli*; its nicking activity is high enough to antagonize SpyCas9 in a kinetic race, but not so high that it would be toxic to the host cell (Supplemental Figure 1). Such a balance could result from the inherent activity of the enzyme or via some form of regulation, either direct or indirect. AcrIIA22's activity is probably also regulated in its native context to avoid secondary impacts on other essential processes. Potential forms of regulation include sequence preference, oligomerization, or transient interactions with Cas9 or other host factors (Figures 4B, 4C). Studies of other phage- and bacterial-encoded nickases may provide

456 further insight into whether AcrIIA22 proteins have additional properties that render them
457 especially well-suited to antagonize Cas9.

458 Is AcrIIA22 a true anti-CRISPR? AcrIIA22 lacks features that are typical of conventional Acrs,
459 such as the ability to bind Cas proteins or to inhibit CRISPR-Cas activity as a purified protein.
460 However, other Acr proteins also lack these features. For example, the well-characterized
461 SpyCas9 antagonist AcrIIA1 does not inhibit purified SpyCas9, but instead stimulates Cas9
462 degradation *in vivo*[24]. Similarly, AcrIIA7 does not appear to bind SpyCas9 but can nevertheless
463 inhibit it via an unknown mechanism *in vitro*[42]. Indeed, anti-CRISPR proteins are defined by a
464 common strategy and outcome rather than by a common biochemical mechanism. Our finding
465 that *AcrIIA22* is encoded by prophages as a single gene that strongly protects plasmids and
466 partially protects phages from SpyCas9 (Figure 3B, Supplemental Figure 3) makes it much more
467 similar to other Acrs[23] and distinct from non-canonical CRISPR-Cas evasion strategies like DNA
468 glucosylation[6].

469 Although it can protect phage Mu from SpyCas9, AcrIIA22 does not appear to provide the
470 same potency of Cas9 inhibition as some other characterized Acrs. However, potent inhibition is
471 not a pre-requisite for effective anti-CRISPR activity. In nature, multiple phages can cooperate to
472 overcome Cas9 immunity by each contributing some anti-CRISPR protein to overcome a common
473 foe[43,44]. These dynamics can favor weak anti-CRISPRs over strong ones, as the latter permits a
474 higher incidence of cheater phages (those without anti-CRISPRs) to persist in mixed phage
475 populations[45]. Thus, even in cases where AcrIIA22 only weakly inhibits Cas9 (Supplemental
476 Figure 3), it may nonetheless confer substantial benefit. Additionally, slowing down Cas9
477 cleavage could increase the time and probability for escape mutants to arise (*e.g.* Cas9 target-
478 site variants[1], deletion mutants[40]), allow for additional Acr expression[43,44], or permit further
479 genome replication to overwhelm CRISPR-Cas immunity[34]. This phenomenon – weak inhibition
480 giving rise to long-term resistance – is reproducibly observed in cases of strong selective
481 pressure. For instance, in the context of antibiotic resistance, the expression of QNR pentapeptide
482 proteins by many human pathogens can provide low-level drug tolerance, extend survival, and
483 allow time for additional mutations to develop that completely resist quinolone antibiotics[46].

484 As the use of functional metagenomics to study phage-bacterial conflicts grows more
485 common, many novel genes and mechanisms for CRISPR-Cas inhibition are likely to be
486 described[12,42]. Like AcrIIA22, which has no homology to any previously described anti-CRISPR
487 and lacks other genetic signatures used for *acr* discovery (*e.g.,* linkage with helix-turn-helix
488 transcription factors)[47,48], these new genes may not exhibit canonical Acr behaviors. It is inevitable
489 that these discoveries will lead to a more nuanced understanding of the arms race between
490 CRISPR-Cas systems and MGEs. These findings will also reveal undiscovered strategies for
491 molecular antagonism and new battlegrounds in the age-old conflict between bacteria and their
492 phages.

Methods

Plasmid protection assay

All plasmid protection assays were done in *Escherichia coli* (strain: NEB Turbo). As described previously[12], SpyCas9 was expressed via the arabinose-inducible promoter pBAD on a CloDF13-based plasmid marked with a spectinomycin resistance cassette. The SpyCas9 construct, called pSpyCas9_crA, was designed to eliminate a target vector with a kanamycin resistance cassette. This target vector also expressed a gene-of-interest (*e.g.,* an *acr*) via the doxycycline-inducible pLtetO-1 promoter (Supplemental Table 4). We induced expression from the target vector via depression of the TetR transcription factor with doxycycline (we generically named this vector pZE21_tetR; Supplemental Table 4). IPTG was used in samples with the target vector to ensure high levels of TetR expression (which was driven by the lac promoter) and thus inducible control of our gene of interest. Unless noted in Supplemental Table 5, all genes, including each alanine mutant depicted in Figure 6A, were synthesized by Synbio technologies and cloned directly into pZE21_tetR for functional testing.

Cultures of each sample were grown overnight at 37ºC with shaking at 220 rpm in lysogeny broth (LB; 10 g/L casein peptone, 10 g/L NaCl, 5 g/L ultra-filtered yeast powder) containing spectinomycin 50 µg/ml, kanamycin 50 µg/ml, and 0.5mM IPTG. These growth conditions kept both SpyCas9 and the gene of interest in uninduced states. The next morning, overnight cultures were diluted 1:50 into LB broth containing spectinomycin (at 50 µg/ml), kanamycin (at 50 µg/ml), 0.5mM IPTG, and doxycycline 100 ng/ml to induce the gene of interest. Cultures were grown at 37ºC on a roller drum to mid-log phase (for approximately 1.5 hours to OD600 of 0.3-0.6). Once cells reached mid-log phase, they were diluted to OD600 value of 0.01 into two media types: (a) LB containing spectinomycin 50 µg/ml, 0.5mM IPTG, and doxycycline 100 ng/ml, and (b) LB containing spectinomycin 50 µg/ml, 0.5mM IPTG, doxycycline 100 ng/ml, and 0.2% (L) arabinose. These media induced either the gene of interest alone, or both the gene of interest and SpyCas9, respectively. Each sample was grown in triplicate in a 96 well plate in a BioTek Cytation 3 plate reader. After 6 hours of growth at 37ºC with shaking at 220 rpm, each sample was diluted ten-fold and plated on two types of media: (a) LB spectinomycin 50 µg/ml + 0.5mM IPTG or (b) LB spectinomycin 50 µg/ml, kanamycin 50 µg/ml, 0.5mM IPTG. Plates were incubated at 37ºC overnight. Then, colonies were counted to determine the fraction of colony forming units (cfus) that maintained kanamycin resistance (and thus the target vector). All figures depicting these data show the log-transformed proportion of Kan^R/total cfu, with or without SpyCas9 induction. The growth curves in Supplemental Figure 1 match the experiment depicted in Figure 1C for the uninduced SpyCas9 samples. For the uninduced *orf_1* control samples, doxycycline was omitted from media throughout the experiment. Growth rates referenced in the text and in Supplemental Figure 1 were calculated using the slope of the OD600 growth curves during log phase, following a natural log transformation.

To test AcrIIA22 function against a panel of Cas9 and Cas12 orthologs in Figure 3C, we used a slightly modified, three-plasmid setup. As before, *spyCas9*, *nmCas9*, *fnCas12* and *lbCas12* were encoded in a CloDF13-based plasmid with a spectinomycin resistance cassette. Expression of the Cas effector was controlled by promoter J23100 and a theophylline riboswitch. The accompanying gRNAs were encoded in a separate set of plasmids called pDual4 under an arabinose expression system, in a p15A-based plasmid and a chloramphenicol resistance cassette (Supplemental Table 4). The gRNAs in the different pDual4 constructs were programmed to target the kanamycin-marked target plasmid in the same manner as

pSpyCas9_crA. All assays were done in *Escherichia coli* (strain: NEB Turbo) following the same plasmid protection assay described previously. However, in this case, we induced expression of the different Cas effectors and gRNAs, by adding 2 mM theophylline and 0.2% (L) arabinose, respectively, to the media.

Impact of AcrIIA22 on GFP expression

We swapped *spyCas9* for *egfp* in our CloDF13-based plasmid and co-expressed AcrIIA22 to determine if AcrIIA22 impacted expression from this construct. If AcrIIA22 influenced CloDF13's copy number or the transcription of *spyCas9*, we anticipated that it would also impact GFP levels in this construct (pCloDF13_GFP; Supplemental Table 4). To perform this experiment, we co-transformed pCloDF13_GFP and pZE21_tetR encoding *acrIIA22* into *E. coli* Turbo. Single colonies were picked into 4 ml of LB containing spectinomycin at 50 µg/ml ('spec50') and kanamycin at 50 µg/ml ('kan50') and 0.5mM IPTG and grown overnight at 37ºC shaking at 220rpm. The next morning the overnight culture was diluted 1:50 into both LB spec50 Kan50 + 0.5mM IPTG with or without doxycycline (to induce *acrIIA22*) and grown at 37ºC for about 1.5 hours to mid-log phase (OD600 0.2-0.6). The OD600 was measured, and all samples were diluted to OD600 of 0.01 in two media types: (a) LB spec50 + kan50 + 0.5mM IPTG + 0.2% arabinose (inducing *gfp* only) or (b) LB spec50 + kan50 + 0.5mM IPTG + 0.2% arabinose + 100ng/ml doxycycline (inducing *gfp* and *acrIIA22*). A volume of 200 µl of each sample was then transferred to a 96-well plate in triplicate and GFP fluorescence was measured every 15 minutes for 24 hours (GFP was excited using 485 nm light and emission detected via absorbance at 528 nm). In parallel, we included control samples that lacked the kanamycin-marked plasmid and varied whether doxycycline was added or not (at 100 ng/ml). In these control samples, we noticed that doxycycline slightly diminished GFP expression (it is possible that sub-lethal levels of the antibiotic may still depress translation). Thus, we normalized GFP fluorescence measurements in our experiment with AcrIIA22 to account for this effect in all samples containing doxycycline. These normalized fluorescence measurements are shown in Supplemental Figure 2B.

Western blots to determine AcrIIA22's impact on SpyCas9 expression

We grew overnight cultures of *E. coli* Turbo that expressed pSpyCa9_crNT and pZE21_tetR encoding a gene of interest (Supplemental Tables 4, 5) in LB spec50 + kan50 + 0.5mM IPTG. The next morning, we diluted these cultures 1:100 in 4ml of either (a) LB spec50 + kan50 + 0.5mM IPTG or (b) LB spec50 + kan50 + 0.5mM IPTG + 100 ng/ml doxycycline (to induce the gene of interest). We included samples that expressed either *acrIIA22* or *gfp* as a gene of interest. In all SpyCas9 constructs, we used a crRNA that did not target our plasmid backbone (pSpyCa9_crNT) to ensure that *acrIIA22* expression remained high and its potential impact on SpyCas9 expression levels would be most evident. All samples were grown for two hours at 37ºC to reach mid-log phase (OD600 0.3 to 0.5) and transferred into media that contained 0.2% arabinose to induce SpyCas9. At transfer, volumes were normalized by OD600 value to ensure that an equal number of cells were used (diluted to a final OD600 of 0.05 in the arabinose-containing medium). This second medium either contained or lacked 100 ng/ml doxycycline to control expression of *acrIIA22* or *gfp,* as with the initial media. Throughout this experiment, we included a control strain that lacked pZE21_tetR and only expressed SpyCas9. Kanamycin and doxycycline were omitted from its growth media. For this control strain, we also toggled the addition of arabinose in the second growth medium to ensure that positive and negative controls for SpyCas9 expression were included in our experiment. After three hours and six hours of SpyCas9 induction, OD600 readings were again taken and these values used to harvest an equal

number of cells per sample (at three hours, OD600 values were between 0.76 and 0.93 and 0.75ml to 0.9ml volumes harvested; at six hours 0.4ml was uniformly harvested as all absorbance readings were approximately 1.6).

All samples were centrifuged at 4100*g* to pellet cells, resuspended in 100 µl of denaturing lysis buffer (12.5 mM Tris-HCl, pH 6.8; 4% SDS), and passed through a 25 gauge needle several times to disrupt the lysate. Samples were then boiled at 100ºC for 10 minutes, spun at 13,000 rpm at 4ºC for 15 minutes and the supernatants removed and frozen at -20ºC. The next day, 12 µl of lysate was mixed with 4 µl of 4x sample buffer (200 mM Tris-HCl, 8% SDS, 40% glycerol, 200 mM DTT, and 0.05% bromophenol blue) and boiled at 100ºC for 10 minutes. Then, 10 µl sample was loaded onto a BioRad Mini-Protean "any KD Stain Free TGX" gel (cat. #4569035) and run at 150V for 62 minutes. To verify that equivalent amounts of each sample were run, gels were visualized on a BioRad chemidoc for total protein content. Protein was then transferred to a 0.2 µM nitrocellulose membrane using the Bio-Rad Trans-Blot Turbo system (25 V, 1.3 A for 10 min). We then washed membranes in PBS/0.1% Triton-X before incubating them with a mixture of the following two primary antibodies, diluted in in LI-COR Odyssey Blocking Solution (cat. #927–40000): (i) monoclonal anti-SpyCas9, Diagenode cat. #C15200229-50, diluted 1:5,000; (ii) polyclonal anti-GAPDH, GeneTex cat. # GTX100118, diluted 1:5,000. The GAPDH antibody served as a loading control and a second check to ensure equal protein levels were run. Membranes were left shaking overnight at 4ºC, protected from light. Then, membranes were washed four times in PBS/0.1% Triton-X (ten-minute washes) before they were incubated for 30 minutes at room temperature with a mixture of secondary antibodies conjugated to infrared dyes. Both antibodies were diluted 1:15,000 in LI-COR Odyssey Blocking Solution. To detect SpyCas9, the following secondary antibody was used: IR800 donkey, anti-mouse IgG, LI-COR cat# 926–32212. To detect GAPDH, IR680 goat, anti-rabbit IgG, LI-COR cat# 926-68071 was used. Blots were imaged on a LI-COR Odyssey CLx after three additional washes.

Phage plaquing assay

We grew overnight cultures of *E. coli* Turbo expressing pSpyCa9_crMu and pZE21_tetR encoding a gene of interest (Supplemental Tables 4, 5) at 37ºC in LB spec50 + kan50 + 0.5 mM IPTG. Genes of interest were either *acrIIA4*, *gfp*, or *acrIIA22*. The pSpyCas9 construct targeted phage Mu and was previously demonstrated to confer strong anti-phage immunity in this system[12]. A control strain expressing pZE21-tetR-*gfp* and SpyCas9_crNT (which encoded a CRISPR RNA that does not target phage Mu) was grown similarly. The next morning, all cultures were diluted 50-fold into LB spec50 + kan50 + 0.5 mM IPTG + 5 mM MgCl2 and grown at 37ºC for three hours. Then, doxycycline was added to a final concentration of 100 ng/ml to induce the gene of interest. Two hours later, SpyCas9 was induced by adding a final concentration of 0.2% w/v arabinose. Two hours after that, cultures were used in soft-agar overlays on one of two media types, discordant for arabinose, to either maintain SpyCas9 expression or let it fade as arabinose was diluted in top agar and consumed by the host bacteria (per Supplemental Figure S2). Top and bottom agar media were made with LB spec50 + kan50 + 0.5 mM IPTG + 5 mM MgCl2. In cases where SpyCas9 expression was maintained, arabinose was also added at a final concentration of 0.02% to both agar types. Top agar was made using 0.5% Difco agar and bottom agar used a 1% agar concentration. For the plaquing assay, 100 µl of bacterial culture was mixed with 3 ml of top agar, allowed to solidify, and ten-fold serial dilutions of phage Mu spotted on top using 2.5 µl droplets. After the droplets dried, plates were overturned and incubated at 37ºC overnight before plaques were imaged the following day.

Identification of AcrIIA22 homologs and hypervariable genomic islands

We searched for AcrIIA22 homologs in three databases: NCBI nr, IMG/VR, and a set of assembled contigs from 9,428 diverse human microbiome samples[18]. Accession numbers for the NCBI homologs are indicated on the phylogenetic tree in Figure 3A. We retrieved AcrIIA22 homologs via five rounds of an iterative PSI-BLAST search against NCBI nr performed on October 2nd, 2017. In each round of searching, at least 90% of the query protein (the original AcrIIA22 hit) was covered, 88% of the subject protein was covered, and the minimum amino acid identity of an alignment was 23% (minimum 47% positive residues; e-value ≤ 0.001). Only one unique AcrIIA22 homolog was identified in IMG/VR (from several different phage genomes) via a blastp search against the July, 2018 IMG/VR proteins database (using default parameters). This homolog was also found in other databases and its amino acid sequence is identical to that of AcrIIA22b (Figure 3A).

Most unique AcrIIA22 homologs were identified in the assembly data of over 9,400 human microbiomes performed by Pasolli and colleagues[18]. These data are grouped into multiple datasets: (i) the raw assembly data, and (ii) a set of unique species genome bins (SGBs), which were generated by first assigning species-level phylogenetic labels to each assembly and then selecting one representative genome assembly per species. We identified AcrIIA22 homologs using several queries against both databases. First, we performed a tblastn search against the SGB database using the AcrIIA22 sequence as a query, retrieving 141 hits from 137 contigs. A manual inspection of the genome neighborhoods for these hits revealed that most homologs originated from a short, hypervariable genomic island; some homologs were encoded by prophages. No phage-finding software was used to identify prophages; they were apparent from a manual inspection of the gene annotations that neighbored *acrIIA22* homologs (see the section entitled “Annotation and phylogenetic assignment of metagenomic assemblies” for details).

To find additional examples of AcrIIA22 homologs and of these genomic islands, we then queried the full raw assembly dataset. To do so without biasing for *acrIIA22*-encoding sequences, we used the *purF* gene that flanked *acrIIA22*-encoding genomic islands as our initial query sequence. Specifically, we used the *purF* gene from contig number 1 in Supplemental Table 3; its sequence is also in Supplemental Table 5. To consider only the recent evolutionary history of this locus, we required all hits have ≥98% nucleotide identity and required all hits to be larger than 15 kilobases in length to ensure sufficient syntenic information. From these contigs, we further filtered for those that had ≥98% nucleotide identity to *radC*, the gene which flanked the other end of *acrIIA22*-encoding genomic islands. Again, we used the variant from contig number 1 in Supplemental Table 3; its sequence is also in Supplemental Table 5. In total, this search yielded 258 contig sequences; nucleotide sequences and annotations for these contigs are provided in Supplemental Dataset 5. We then searched for *acrIIA22* homologs in these sequences using tblastn, again observing them in genomic islands and prophage genomes (which were assembled as part of the 258 contigs). In total, this search revealed 320 *acrIIA22* homologs from 258 contigs. The 258 genomic islands from these sequences were retrieved manually by extracting all nucleotides between the *purF* and *radC* genes. These extracted sequences were then clustered at 100% nucleotide identity with the sequence analysis suite Geneious Prime 2020 v1.1 to identify 128 unique genomic islands.

Altogether, our two searches yielded 461 AcrIIA22 sequences from these metagenomic databases that spanned 410 contig sequences. The 461 AcrIIA22 homologs broke down into two groups: 410 clustered with genomic island-like sequences whereas 51 clustered with prophage-

like homologs. In nature, the relative prevalence of AcrIIA22 in genomic islands or prophages may not be accurately reflected by these numbers because we never directly searched for prophage-encoded homologs. We then combined these 461 AcrIIA22 sequences with those from NCBI and IMG/VR and clustered the group on 100% amino acid identity to reveal 30 unique proteins. To achieve this, we used the software cd-hit[49] with the following parameters: -d 0 -g 1 -aS 1.0 -c 1.0. These 30 sequences were numbered to match one of their parent contigs (as indicated in Supplemental Table 3) and used to create the phylogenetic tree depicted in Figure 3A. For AcrIIA22 homologs found outside NCBI, the nucleotide sequences and annotations of their parent contigs can be found in Supplemental Datasets 1 and 2. For NCBI sequences, accession numbers are shown in Figure 3A. The gene sequences used in functional assays (Figure 3B) have been reprinted in Supplemental Table 5, for convenience.

Annotation and phylogenetic assignment of metagenomic assemblies

Contig sequences from IMG/VR, the Pasolli metagenomic assemblies, and some NCBI entries lacked annotations, making it difficult to make inferences about ***acrIIA22's*** genomic neighborhood. To facilitate these insights, we annotated all contigs as follows. We used the gene-finder MetaGeneMark[50] to predict open reading frames (ORFs) using default parameters. We then used their amino acid sequences in a profile HMM search with HMMER3[51] against TIGRFAM[52] and Pfam[53] profile HMM databases. The highest scoring profile was used to annotate each ORF. We annotated these contigs to facilitate genomic neighborhood analyses for *acrIIA22*; these are not intended to provide highly accurate functional predictions of their genes. Thus, we erred on the side of promiscuously assigning gene function; our annotations should therefore be treated with appropriate caution. A visual inspection of these annotated contigs made apparent several examples of *acrIIA22*-encoding prophages (we noticed 35-40 kilobase insertions in some contigs that were otherwise nearly identical to those without prophages). We were confident that these insertions were prophages because they contained mostly co-linear genes with key phage functions annotated. As a simple means to sample this phage diversity, we manually extracted nine examples of these prophage sequences (their raw sequences and annotated genomes can be found in Supplemental Datasets 3 and 4). Annotations were imported into the sequence analysis suite Geneious Prime 2020 v1.1 for manual inspection of genome neighborhoods.

We used the genome taxonomy database (GTDB) convention for all sequences discussed in this manuscript[54]. In part, this was because all *acrIIA22* genomes are found in clostridial genomes, which are notoriously polyphyletic in NCBI taxonomies (for instance, species in the NCBI genus *Clostridium* appear in 121 GTDB genera and 29 GTDB families)[55]. All SGBs that we retrieved from the Pasolli assemblies were assigned taxonomy as part of that work and were called Clostridium sp. CAG-217. Similarly, NCBI assemblies that encoded the most closely *acrIIA22* homologs to our original hit were assigned to the GTDB genus CAG-217[54,55]. The raw assembly data from the Pasolli database was not assigned a taxonomic label but was nearly identical in nucleotide composition to the CAG-217 contigs (Figure 2, Supplemental Figure 4, Supplemental Datasets 1 and 2). Therefore, we also refer to these sequences as originating in CAG-217 genomes but take care to indicate which assemblies have been assigned a rigorous taxonomy and which ones for which taxonomy has been inferred in this fashion (Supplemental Table 3).

Comparing genes in genomic islands to phage genomes

We first examined the annotated genes within each of the 128 unique genomic islands. Manual inspection revealed 54 unique gene arrangements that differed in gene content and orientation. We then selected one representative from each arrangement and extracted amino acid sequences from each encoded gene (n=506). Next, we collapsed these 506 proteins into orthologous groups by clustering at 65% amino acid using cd-hit with the following parameters: -d 0 -g 1 -aS 0.95 -c 0.65. These cluster counts were used to generate the histogram depicted in Supplemental Figure 4C. To determine which protein families may also be phage-encoded, we queried the longest representative from each cluster with at least two sequences against the database of nine CAG-217 phages described in the section entitled "Annotation and phylogenetic assignment of metagenomic assemblies". We used tblastn with default parameters to perform this search, which revealed that some proteins in the CAG-217 genomic islands have homologs in prophage genomes that are out-of-frame with respect to the MetaGeneMark annotations depicted in Supplemental Figure 4A.

Phylogenetic tree of AcrIIA22 homologs

The 30 unique AcrIIA22 homologs we retrieved were used to create the phylogeny depicted in Figure 3A. These sequences were aligned using the sequence alignment tool in the sequence analysis suite Geneious Prime 2020 v1.1. This alignment is provided as Supplemental Dataset 6. From this alignment, the phylogenetic tree in Figure 3A was generated using PhyML with the LG substitution model[56] and 100 bootstraps. Coloration and tip annotations were then added in Adobe Illustrator.

Identification of CRISPR-Cas systems and Acrs in CAG-217 assemblies

To determine the type and distribution of CRISPR-Cas systems and Acrs in CAG-217 genomes, we downloaded all assembly data for the 779 SGBs assigned to CAG-217 in Pasolli *et. al*[18] (bin 4303). We then predicted CRISPR-Cas systems for all 779 assemblies in bulk using the command line version of the CRISPR-Cas prediction suite, cctyper[57]. Specifically, we used version 1.2.1 of cctyper with the following options: --prodigal meta --keep_tmp. To identify type II-A Acrs, we first downloaded representative sequences for each of the 21 experimentally confirmed type II-A Acrs from the unified resource for tracking anti-CRISPRs[58]. We then used tblastn to query these proteins against the 779 CAG-217 genome bins and considered any hit with e-value better than 0.001 (which included all hits with >30% identity across 50% of the query). To check if these Acrs were present in *acrIIA22*-encoding phages, we performed an identical tblastn search, but this time using the set of nine *acrIIA22*-encoding prophages as a database.

Recombinant protein overexpression and purification

The AcrIIA22 protein and its mutants were codon optimized for *E. coli* (Genscript or SynBio Technologies) and the gene constructs were cloned into the pET15HE or pET15b plasmid[12] to contain an N-terminal, thrombin-cleavable 6XHistidine (His6) tag. These plasmids differ by only a few bases just upstream of the N-terminal thrombin tag. For purified, twin-strep tagged proteins, constructs were cloned into a modified pET15b that lacks the N-terminal tag but instead has a C-terminal twin-strep tag (Supplemental Table 4). Constructs were transformed and overexpressed in BL21 (DE3) RIL or BL21 (DE3) pLysS *E. coli* cells. A 10 mL overnight culture (grown in LB + 100 µg/mL ampicillin) was diluted 100-fold into the same media and grown at 37°C with shaking to an OD600 of 0.8 for His6-tagged constructs and 0.3 for twin-strep-tagged constructs. Expression was then induced with 0.5 mM IPTG. For His6-tagged constructs, the culture was

shaken for an additional 3 hours at 37°C; twin-strep-tagged constructs were induced at 16C for 22 hours. Cells were harvested by centrifugation and the pellet stored at -20°C.

Cell pellets for His6-tagged constructs were resuspended in 25 mM Tris, pH 7.5, 300 mM NaCl, 20 mM imidazole; twin-strep tagged constructs were resuspended in Tris 100nM 8.0 pH, 150mM NaCl, 1mM EDTA. Cells were lysed by sonication on ice. The lysate was centrifuged in an SS34 rotor at 18,000 rpm for 25 minutes, followed by filtering through a 5 µm syringe filter for the His6-tagged constructs and a 0.45 µM syringe filter for the twin-strep-tagged constructs.

To purify His6-tagged constructs, the clarified lysate was bound using the batch method to Ni-NTA agarose resin (Qiagen) at 4°C for 1 hour. The resin was transferred to a gravity column (Biorad), washed with >50 column volumes of Lysis Buffer, and eluted with 25 mM Tris, pH 7.5, 300 mM NaCl, 200 mM imidazole. The protein was diluted with 2 column volumes of 25 mM Tris, pH 7.5 and purified on a HiTrapQ column (GE Healthcare) using a 20 mL gradient from 150 mM to 1 M NaCl in 25 mM Tris, pH 7.5. Peak fractions were pooled, concentrated, and buffer exchanged into 200 mM NaCl, 25 mM Tris, pH 7.5 using an Amicon Ultra centrifugal filter with a 3,000 molecular weight cutoff (Millipore, UFC900324), then cleaved in an overnight 4°C incubation with biotinylated thrombin (EMD Millipore). Streptavidin agarose slurry (Novagen) was incubated with cleaved protein at 4°C for 30 minutes to remove thrombin. The sample was then passed through a 0.22 µm centrifugal filter and loaded onto a HiLoad 16/60 Superdex 200 prep grade size exclusion column (Millipore Sigma) equilibrated in 25 mM Tris, pH 7.5, 200 mM NaCl. The peak fractions were pooled, concentrated, and confirmed for purity by SDS-PAGE before use in most assays. Figure 4B depicts size exclusion chromatography data generated for thrombin-cleaved AcrIIA22 variants generated using a Superdex75 16/60 (GE HealthCare) column with 25 mM Tris, pH 7.5, 200mM NaCl. To correlate nicking activity with protein content across fractions (Supplemental Figure 10B), we collected 13 fractions that span the entire elution peak as well as fractions without AcrIIA22 protein. The protein gel shown in Supplemental Figures 10A and 10B was loaded with 5ul of each concentrated fraction.

For two additional proteins, we also performed similar Ni-NTA-based purifications of His6-tagged constructs, with small deviations from the protocol described in the preceding paragraph. Recombinant AcrIIA4 was purified similarly to other His6-tagged Acr proteins but with the following deviations, as previously described[12]. IPTG was used at 0.2 mM and cells were harvested after 18 hours of induction at 18°C. Thrombin cleavage also occurred at 18°C. This untagged version was used to help generate Supplemental Figure 6. Peak fractions for all proteins were pooled, concentrated, flash frozen as single-use aliquots in liquid nitrogen, and stored at −80°C. SpyCas9 was expressed in *E. coli* from plasmid pMJ806 (addgene #39312) to contain a TEV-cleavable N-terminal 6XHis-MBP tag and was purified as described previously[12] with sequential steps of purification consisting of Ni-NTA affinity chromatography, TEV cleavage, Heparin HiTrap chromatography, and SEC. The protein was stored in a buffer consisting of 200 mM NaCl, 25 mM Tris (pH 7.5), 5% glycerol, and 2 mM DTT. Again, peak fractions were pooled, concentrated, and flash frozen as single-use aliquots.

We also purified AcrIIA22 and AcrIIA4 constructs with a C-terminal twin-strep tag. The protein was expressed and lysed as described above and purified according to the manufacturer's guidelines (IBA Life Sciences, Inc.). Clarified lysates were passed over Strep-Tactin-Sepharose resin using a gravity filtration column. The flow through was passed over the resin a second time. The column was washed with a minimum of 20 column volumes of buffer W, followed by elution in buffer E (150 mM NaCl, 100 mM Tris, pH 8.0 mM, 1 EDTA, 2.5 mM desthiobiotin). The eluted

protein was purified over a HiTrapQ column (GE Healthcare) using a 40 mL gradient from 150 mM to 0.5 M NaCl in 25 mM Tris, pH 7.5. Peak fractions were pooled and then purified again via size exclusion chromatography with a Biorad Enrich SEC650 10x300mm column in 150mM NaCl, 25 mM Tris, pH 7.5. These elution data are shown for AcrIIA22 and its variants in Figure 6B. Fractions were collected across the elution peak and confirmed for purity via silver stain (Supplemental Figure 10E), per manufacturer's recommendations (Thermo Fisher Cat. No. 24612). For these proteins, we chose fraction number four to carry forward, as it eluted at approximately four times the monomer's molecular weight, consistent with our proposed tetramer, which is depicted in Figure 4C. Protein was then concentrated and flash frozen as single-use aliquots for later use.

X-ray crystallography and structural analyses

An AcrIIA22 crystal was grown using 14mg/mL protein via the hanging drop method using 200mM ammonium nitrate, 40% (+/-)-2-methyl-2,4-pentanediol (MPD, Hampton Research), 10mM MgCl2 as a mother liquor. Diffraction data was collected at the Argonne National Laboratory Structural Biology Center synchrotron facility (Beamline 19BM). Data was processed with HKL2000 in space group P4332, then built and refined using COOT[59] and PHENIX[60]. The completed 2.80Å structure was submitted to the Protein Data Bank with PDB Code 7JTA. The detailed PDB validation report is provided (Supplemental Dataset 7). We submitted this finished coordinate file to the PDBe PISA server (Protein Data Bank Europe, Protein Interfaces, Surfaces and Assemblies; http://pdbe.org/pisa/) which uses free energy and interface contacts to calculate likely multimeric assemblies[27]. The server calculated tetrameric, dimeric and monomeric structures to be thermodynamically stable in solution. The tetrameric assembly matches the molecular weight expected from the size exclusion column elution peak and is the most likely quaternary structure as calculated by the PISA server. The tetramer gains -41.8 kcal/mol free energy by solvation when formed and requires an external driving force of 3.1 kcal/mol to disassemble it according to PISA ΔG calculations.

sgRNA generation

The single-guide RNA (sgRNA) for use in *in vitro* experiments was generated as described previously[12]. We made the dsDNA template via one round of thermal cycling (98°C for 90 s, 55°C for 15 s, 72°C for 60 s) in 50 µl reactions. We used the Phusion PCR polymerase mix (NEB) containing 25 pmol each of the following two oligo sequences; the sequence that binds the protospacer on our pIDTsmart target vector is underlined:

(i) GAAATTAATACGACTCACTATAGGTAATGAAATAAGATCACTACGTTTTAGAGCT AGAAATAGCAAGTTAAAATAAGGCTAGTCCG

(ii) AAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTAT TTTAACTTGC.

The dsDNA templates were then purified using an Oligo Clean and Concentrator Kit (ZymoResearch) before quantification via the Nanodrop. Single-guide RNA (sgRNA) was transcribed from this double-stranded DNA (dsDNA) template by T7 RNA polymerase using Megashortscript Kit (Thermo Fisher #AM1354). Reactions were then treated with DNAse, extracted via phenol-chloroform addition and then chloroform addition, ethanol precipitated,

resuspended in RNase free water, quantified by Nanodrop, analyzed for quality on 15% acrylamide/TBE/UREA gels, and frozen at −20°C.

Pulldown assay using twin-strep-tagged AcrIIA22 and AcrIIA4

The same buffer, consisting of 200 mM NaCl, 25 mM Tris (pH 7.5), was used for pulldowns and to dilute proteins. As a precursor to these assays, 130 pmol SpyCas9 and sgRNA were incubated together at room temperature for 15 minutes where indicated. SpyCas9, with or without pre-complexed sgRNA, was then incubated with 230 pmol AcrIIA4 or 320 pmol AcrIIA22 for 25 minutes at room temperature. Subsequently, 50 µl of a 10% slurry of Strep-Tactin Resin (IBA Lifesciences #2-1201-002), which was pre-equilibrated in binding buffer, was added to the binding reactions, and incubated at 4°C on a nutator for 45 minutes. Thereafter, all incubations and washes were carried out at 4°C or on ice. Four total washes of this resin were performed, which included one tube transfer. Washes proceeded via centrifugation at 2000 rpm for one minute, aspiration of the supernatant with a 25-gauge needle, and resuspension of the beads in 100 µl binding buffer. Strep-tagged proteins were eluted via suspension in 40 µl of 1x BXT buffer (100 mM Tris-Cl, 150 mM NaCl, 1 mM EDTA, 50 mM Biotin, pH 8.0) and incubated for 15 min at room temperature. After centrifugation, 30 µl of supernatant was removed and mixed with 4X reducing sample buffer (Thermo Fisher). Proteins were then separated by SDS PAGE on BOLT 4–12% gels in MES buffer (Invitrogen) and visualized by Coomassie staining.

SpyCas9 linear DNA cleavage assay

All SpyCas9 cleavage reactions using linear DNA were performed in cleavage buffer[61] (20mM Tris HCl (pH7.5), 5% glycerol, 100mM KCl, 5mM MgCl2, 1mM DTT). In preparation for these reactions, all proteins were diluted in 30 mM NaCl / 25 mM Tris, pH 7.4 / 2.7mM KCl, whereas all DNA and sgRNA reagents were diluted in nuclease-free water. Where indicated, SpyCas9 (0.36 µM) was incubated with sgRNA (0.36 µM) for 10 minutes at room temperature. Before use, sgRNA was melted at 95°C for five minutes and then slowly cooled at 0.1 °C/s to promote proper folding. SpyCas9 (either pre-complexed with sgRNA or not, as indicated in Supplemental Figure 7) was then incubated for 10 minutes at room temperature with AcrIIA4 (2.9 µM) or AcrIIA22 at each of the following concentrations: [23.2, 11.6, 5.8, and 2.9 µM]. As substrate, the plasmid pIDTsmart was linearized by restriction digest and used at a final concentration of 3.6 nM. The reaction was initiated by the addition of this DNA substrate either in isolation or in combination with sgRNA (0.36 µM) as indicated in Supplemental Figure 7. Reactions were immediately moved to a 37ºC incubator and the reaction stopped after fifteen minutes via the addition of 0.2% SDS/100 mM EDTA and incubation at 75ºC for five minutes. Samples were then run on a 1.5% TAE agarose gel at 120V for 40 minutes. Densitometry was used to calculate the proportion of DNA cleaved by SpyCas9; band intensities were quantified using the BioRad ImageLab software v5.0.

***In vivo* assay to assess impact of AcrIIA22 on plasmid topology**

In all experiments, cultures were first grown overnight at 37ºC with shaking at 220 rpm in LB with 0.5mM IPTG and, if included, spectinomycin at 50 µg/mL, and kanamycin at 50 µg/mL. For each sample with a SpyCas9-expressing plasmid (*e.g.* Figure 7A), overnight cultures were grown with spectinomycin and kanamycin and diluted 1:50 into LB with 0.5mM IPTG,

spectinomycin (at 50 µg/mL), and, where indicated, doxycycline (at 100 ng/mL, to induce *acr*s). Cultures were grown at 37°C with shaking at 220 rpm. If required, 0.2% (L)-arabinose was added after two hours of growth to induce *spyCas9* expression. The next morning, cultures were centrifuged at 4100*g* and plasmids purified using a miniprep kit (Qiagen). We measured the concentration of dsDNA in each miniprep using the Qubit-4 fluorometer and the associated dsDNA high sensitivity assay kit (Invitrogen). For each sample with a SpyCas9-expressing plasmid, 150ng of DNA was digested with the restriction enzyme HincII (NEB) per manufacturer's recommendations, except that digests were incubated overnight before being stopped by heating at 65°C for 20 minutes. This restriction enzyme will cut once, only in the SpyCas9 plasmid, to linearize it. This allowed us to visualize the SpyCas9 plasmid as a single band, which allowed us to identify bands from *acrIIA22*-encoding undigested plasmids more easily. It also served as an internal control for plasmid DNA that is unaffected by SpyCas9 targeting or AcrIIA22 expression (Supplemental Figure 2). Following restriction digest, 30ng of sample was analyzed via gel electrophoresis using a 0.7% TAE-agarose gel run at 120V for 30 minutes.

In samples that lacked a SpyCas9-expressing plasmid (*e.g.* Figure 5A), overnight cultures were grown with kanamycin and diluted into LB. Where required, 0.5mM IPTG and doxycycline at 100 ng/mL were added to induce the gene of interest. The next morning, cultures were centrifuged at 4100*g* and plasmids purified using a miniprep kit (Qiagen). The concentration of dsDNA in each miniprep was measured using the Qubit-4 fluorometer and the associated dsDNA high sensitivity assay kit (Invitrogen). Then, 30ng of purified plasmid was directly analyzed by gel electrophoresis using a 0.7% TAE-agarose gel run at 120V for 30 minutes.

***In vitro* AcrIIA22 plasmid nicking assay**

Except for the divalent cation experiment, all reactions were performed using NEB buffer 3.1 (100 mM NaCl, 50 mM Tris-HCl, pH 7.9, 10 mM MgCl2, 100 µg/mL BSA). To determine cation preference, the same reaction buffer was re-created, but MgCl2 was omitted. All proteins were diluted in 130 mM NaCl, 25 mM Tris, pH 7.4, 2.7 mM KCl. DNA was diluted in nuclease-free water. In the cation preference experiment, 60 µM His6-AcrIIA22 and 6 nM of purified pIDTsmart plasmid DNA were used. All other reactions were set up with AcrIIA22 constructs and concentrations indicated in figure panels and captions. In the cation preference experiment (Supplemental Figure 11A), reactions were started by adding 10 mM of the indicated cation. All other reactions were initiated via the addition of 2 nM pIDTsmart plasmid DNA. In these cases, reactions were immediately transferred to a 37°C incubator. At 0.5, 1, 2, 4, 6, or 20-hour timepoints, a subset of the reaction was removed and run on a 1.5% TAE agarose gel at 120V for 30 minutes. For the fractionation experiment depicted in Supplemental Figure 10B, 5ul of each concentrated fraction was used in a 15ul reaction volume and the reaction was incubated for 24 hours at 37°C. For the cation preference experiment, only the 2-hour timepoint was considered and the reaction was stopped via the addition of NEB loading buffer and 100 mM EDTA. In this case, DNA was visualized on a 1% TBE gel run for 60 minutes at 110V. Densitometry was used to calculate the proportion of DNA in each topological form via band intensities quantified using the BioRad ImageLab software v5.0.

SpyCas9 cleavage kinetics assay

All cleavage reactions were performed in the cleavage buffer[61] containing 20mM Tris HCl (pH7.5), 5% glycerol, 100mM KCl, 5mM MgCl2, 1mM DTT. In preparation for these reactions, all proteins were diluted in 30 mM NaCl / 25 mM Tris, pH 7.4 / 2.7mM KCl, whereas all DNA and sgRNA reagents were diluted in nuclease-free water.

Purified pIDTsmart plasmid was pre-treated with either AcrIIA22, the nickase Nb.BssSI (NEB), or no enzyme. For the AcrIIA22 pre-treatment, 3.1 µg of plasmid was incubated with 230 µM AcrIIA22 and the plasmid nicked as described previously. Plasmid nicking with Nb.BssSI was carried out via manufacturer's recommendations (NEB). Both reactions were incubated at 37 ^{o}C for 2 hours. To isolate the nicked plasmid, samples were then run on a 1.5% agarose gel for 2 hours and the open-circle form of the plasmid was excised and purified using the Zymo Research Gel DNA Recovery Kit. Untreated plasmid was also purified via gel extraction. Plasmid yield was quantified using a Nanodrop.

To determine SpyCas9's substrate preference, we incubated each pre-treated plasmid substrate with SpyCas9 and assayed for the appearance of a linearized plasmid as indication of SpyCas9 digestion. In all cases, SpyCas9 was used at a final concentration of 0.32 µM. All reaction components except dsDNA were added on ice, following which SpyCas9 was complexed with equimolar levels of its sgRNA for ten minutes at room temperature. Before addition to the reaction, sgRNA was melted at 95°C for five minutes and then slowly cooled at 0.1 °C/s to promote proper folding. To begin the reaction, DNA substrate was added to the reaction mix at a final concentration of 2 nM and the samples moved immediately to 37 ^{o}C. At each timepoint, a subset of the reaction was removed, and digestion stopped with 0.2% SDS/100 mM EDTA and by incubating at 75^{o}C for 5 minutes. Samples were run on a 1.5% TAE gel at 120V for 40 minutes and densitometry was used to calculate the proportion of DNA in each topological form via band intensities quantified with the BioRad ImageLab software v5.0.

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Competing Interests

All authors declare no significant competing financial, professional, or personal interests that might have influenced the performance or presentation of the work described in this manuscript.

Main Figure Captions

Figure 1. **Functional selection reveals a metagenomic contig encoding a novel SpyCas9 inhibitor.** (**A**) A plasmid protection assay was used to reveal SpyCas9 inhibition. In this assay, plasmids without SpyCas9 inhibitors are cleaved by Cas9 and do not give rise to Kan^R colonies, whereas those encoding inhibitors withstand SpyCas9 attack and yield Kan^R colonies. (**B**) The contig F01A_4 protects a plasmid from SpyCas9 attack but an early stop codon in *orf_1 (Δ1)* eliminates this phenotype. Stop codons in *orf_2* or *orf_3* (*Δ2* and *Δ3*) have no effect. Thus, we conclude that *orf_1* is necessary for inhibition of SpyCas9. Asterisks depict statistically significant differences in plasmid retention between the indicated genotype and an empty vector control in SpyCas9-inducing conditions (Student's t-test, $p<0.002$, $n=3$); ns indicates no significance. All p-values were corrected for multiple hypotheses using Bonferroni's method. (**C**) Expression of *orf_1* (which we name *acrIIA22*) is sufficient for SpyCas9 antagonism, protecting a plasmid as effectively as *acrIIA4*. Asterisks are as in panel B but relate to the GFP negative control rather than to an empty vector. The individual numerical values that underlie the summary data in this figure may be found as supporting information file SI_Data.

Figure 2. ***AcrIIA22* homologs are found in hypervariable regions of prophage and bacterial genomes in the unnamed clostridial genus, CAG-217.** (**A**) We show a schematic representation of an *acrIIA22* homolog embedded in a prophage genome, which is integrated into a bacterial genome (contig #57). We can delineate precise boundaries of the inserted prophage based on comparison to a near-identical bacterial contig (contig #55). Prophage genes are colored by functional category, according to the legend at the top. Bacterial genes are colored light gray. (**B**) Homologs of *acrIIA22* are depicted in diverse genomic islands, including Contig #1, whose sequence includes a portion identical to F01A_4, the original metagenomic contig we recovered. All *acrIIA22* homologs in these loci are closely related but their adjacent genes are different, unrelated gene families (depicted by different colors). Genomic regions flanking these hypervariable islands, including genes immediately adjacent to these islands (*purF* and *radC*, in bold outlines), are nearly identical to one another (≥98% nucleotide identity). Contigs are numbered to indicate their descriptions in Supplemental Table 3, which contains their metadata, taxonomy, and sequence retrieval information. All sequences and annotations may also be found in Supplemental Datasets 1 and 2. (**C**) We propose an evolutionary model for the origin of the *acrIIA22*-encoding hypervariable genomic islands depicted in panel B. We propose that *acrIIA22* moved via prophage integration into a bacterial genomic locus but remained following an incomplete prophage excision event. Its neighboring genes subsequently diversified via horizontal exchange with additional phage genomes without these phage genomes inserting into the locus. Supplemental Figure 4 depicts a more detailed version of the genomic data underlying this model.

Figure 3. **Several AcrIIA22 homologs in the CAG-217 clostridial genus can inhibit SpyCas9.** (**A**) A phylogeny of all unique AcrIIA22 homologs identified from metagenomic and NCBI databases. Phylogenetic classifications were assigned corresponding to the GTDB naming convention (Methods). Prophage sequences are shaded brown and homologs from hypervariable bacterial genomic islands are shaded yellow. Sequences obtained from NCBI are labeled with protein accession numbers. In other cases, AcrIIA22 homologs are numbered to match their contig-of-origin (Supplemental Table 3). In some cases, more than one AcrIIA22 homolog is found on the same contig ('gi' or 'p' indicates its presence in a hypervariable genomic island or prophage genome, respectively). Circles at nodes indicate bootstrap support ≥ 0.75. Dashed boxes separate sequences identified from CAG-217 versus *Eubacterium_R* bacterial genera. Filled

green circles indicate homologs that were tested for their ability to inhibit SpyCas9 in the plasmid protection assay in panel B. These homologs have been named with 'a', 'b', or 'c' suffixes to distinguish them from the original AcrIIA22 metagenomic hit; their amino acid identity to the original hit is shown in parentheses. (**B**) Several homologs of AcrIIA22 in CAG-217 genomes inhibit SpyCas9. Asterisks depict statistically significant differences in plasmid retention under SpyCas9-inducing conditions between the indicated sample and a null mutant with an early stop codon in *acrIIA22*, as indicated in the legend at right (ns indicates no significance; $p > 0.05$). All p-values were corrected for multiple hypotheses using Bonferroni's method (Student's t-test, n=3). (**C**) AcrIIA22 inhibits divergent Cas9 proteins from Streptococcus pyogenes (SpyCas9) or *Neisseria meningitidis* (NmCas9) but not Cas12 proteins from *Lachnospiraceae bacterium* (LbCas12) or *Francisella novicida* (FnCas12). As in panel B, green bars indicate samples with expression of the indicated Cas nuclease while unexpressed controls are depicted with gray lines. For Cas-expressing samples, significance was determined via a Student's t-test (n=3) and denoted as follows: '*', $p \leq 0.05$; '**' $p \leq 0.001$; 'ns' no significance. Due to slight differences in the plasmid protection assay in panel C compared to panel B, A22 was re-tested against SpyCas9 to confirm activity (Methods). The individual numerical values that underlie the summary data in this figure may be found as supporting information file SI_Data.

Figure 4. AcrIIA22 is an oligomeric PC4-like protein. (**A**) AcrIIA22's crystal structure reveals a homodimer of two four-stranded β-sheets. (**B**) AcrIIA22 elutes as an oligomer that is approximately four times the predicted molecular mass of its monomer, which is 7 kDa. The gray, dashed trace depicts protein standards of the indicated molecular weight, in kDa. The orange trace depicts the elution profile of a two-amino acid C-terminal AcrIIA22 truncation mutant that is predicted to disrupt oligomerization. (**C**) Ribbon diagram of a proposed AcrIIA22 tetramer which requires binding between anti-parallel β-strands at the C-termini of AcrIIA22 monomers to form extended, concave β-sheets. The putative oligomerization interface is indicated by the regions highlighted in yellow and the dashed box, and is detailed further in panel F. Each monomer in the proposed tetramer is labeled with lower-case Roman numerals (i-iv). (**D**) β-sheet topology and orientation in AcrIIA22 (blue) resemble that of PC4-like family proteins (in gray, PDB:4BG7 from phage T5). (**E**) A monomer of AcrIIA22 (in blue, PDB:7JTA) is structurally similar to a PC4-like single-stranded DNA binding protein, which is proposed to promote recombination in phage T5 (in gray, PDB:4BG7, Z-score=6.2, matched residues 15%), except for a missing C-terminal alpha helix. (**F**) A putative oligomerization interface between the C-termini of two AcrIIA22 monomers from panel (C) is shown in more detail. Dashed lines indicate potential hydrogen bonds between the polypeptide backbones. This interface occurs twice in the putative tetramer, between red-hued and blue-hued monomers in panel C.

Figure 5. AcrIIA22 nicks supercoiled plasmids *in vivo* and *in vitro*. (**A**) Gel electrophoresis of plasmids purified from overnight *E. coli* cultures expressing either acrIIA22, or a null mutant with an early stop codon, or neither. Compared to the null mutant, more plasmid runs in a slowly migrating, open-circle conformation (OC) rather than supercoiled plasmid (SC) with the wild-type *acrIIA22* allele, suggesting that *acrIIA22* may impact plasmid topology. %SC indicates the percentage of DNA in the supercoiled form for each sample. (**B**) N-terminally His6-tagged AcrIIA22 nicks supercoiled plasmids *in vitro*. (**C**) C-terminally twin-strep-tagged AcrIIA22 nicks supercoiled plasmids *in vitro* with higher specific activity than shown in panel B (compare protein concentrations). Original, uncropped versions of images depicted in figure may be found in the supporting information file, SI_raw_images.

Figure 6. **Impaired nicking activity of AcrIIA22 variants *in vitro* correlates with lower SpyCas9 inhibition *in vivo***. (**A**) Alanine mutagenesis of acidic amino acid residues (glutamic acid or aspartic acid) in AcrIIA22 reveals that D14 is important for plasmid protection against SpyCas9. Asterisks depict statistically significant differences in plasmid retention under SpyCas9-inducing and non-inducing conditions, per the legend at right. The D14A mutant is significantly impaired, the E4A mutant is slightly impaired, whereas all other mutants are not impaired for plasmid protection against SpyCas9 compared to an uninduced control. All p-values were corrected for multiple hypotheses using Bonferroni's method (Student's t-test, n=3). (**B**) AcrIIA22 (black), AcrIIA22a (dark gray), and a D14A mutant (light gray) all elute with similar oligomer profiles via SEC. The dashed trace depicts protein standards of the indicated molecular weight, in kDa. (**C**) AcrIIA22a and the D14A mutant are impaired for nicking relative to AcrIIA22. All experiments were performed in triplicate, with standard deviations indicated by dashed lines (in most cases, the data points obscure these error bars). Asterisks denote cases where AcrIIA22 is significantly different than both AcrIIA22a and the D14A mutant after correcting for multiple hypotheses (Student's t-test, n=3, Bonferroni correction). A single asterisk (*) means that adjusted p-values for both comparisons are below 0.05. A double asterisk (**) means that adjusted p-values are both below 0.005. Supplemental Figures 10G and 10H show representative gels for these nicking experiments. The individual numerical values that underlie the summary data in this figure may be found as supporting information file SI_Data.

Figure 7. Nicking by AcrIIA22 protects plasmids from SpyCas9 *in vivo* and *in vitro*. (**A**) Gel electrophoresis of plasmids purified from overnight *E. coli* cultures expressing either wildtype *acrIIA22* or a mutant with an early stop codon ('null'). In these cultures, SpyCas9 was expressed from a second plasmid, which was linearized via a unique restriction site before electrophoresis. The *acrIIA22*-encoding plasmids are indicated with the 'pTarget' label. OC, open-circle; SC, supercoiled. The '%pTarget' figure indicates the fraction of total DNA attributable to pTarget, quantified by densitometry analysis. In cases with complete pTarget elimination, all DNA comes from the SpyCas9 expression plasmid, and thus these bands are more pronounced. However, in the presence of wildtype acrIIA22, pTarget is protected from SpyCas9-mediated cleavage and makes up 43% of total plasmid DNA. (**B**) We present a schematic of the experimental design for the data depicted in panel C. The experiment tests whether SpyCas9 preferentially cleaves a supercoiled or open-circle plasmid target *in vitro*. Though both plasmid substrates will be linearized following SpyCas9 cleavage, linear DNA will accumulate more readily with a preferred substrate. (**C**) Plasmid purifications from overnight cultures were either left unmodified or pre-treated with one of two nickase enzymes, AcrIIA22 or Nb.BssSI, following which each substrate was digested with SpyCas9 *in vitro*. The percentage of DNA in the linear form is quantified below the gel, which indicates complete SpyCas9 cleavage. Linear, open-circle (OC), and supercoiled (SC) plasmid forms are indicated along with the left of the gel, and reaction components below the gel. SpyCas9 cuts DNA strands sequentially; incomplete digestions with supercoiled substrates produce open-circle plasmids if only one strand has been cleaved (*e.g.* lane 5). Pre-nicked plasmids, by either AcrIIA22 or Nb.BssSI, are less susceptible to linearization via SpyCas9 cleavage. (**D**) Endpoint measurements indicate that SpyCas9 more efficiently linearizes supercoiled plasmids than substrates nicked with either AcrIIA22 or Nb.BssSI (Student's t-test, n=3). (**E**) A time course experiment demonstrates that SpyCas9 more efficiently linearizes supercoiled plasmids than AcrIIA22-treated substrates. An asterisk (*) denotes significant differences between AcrIIA22-treated and untreated substrates (Student's t-test, $p < 0.05$, n=3).

The individual numerical values and original images for the data presented in this figure may be found in the supporting information files SI_Data and SI_raw_images, respectively.

Supplemental Figure Captions

Supplemental Figure 1. ***Orf_1* (*acrIIA22*) confers mild toxicity in *E. coli*.** Growth rates with *orf_1* induction (green) are 7% lower than those without *orf_1* induction (orange). The cfu data shown in Figure 1C were generated from the same experiment depicted here (samples were removed after six hours of growth to determine these cfu counts). Thus, these data demonstrate that anti-SpyCas9 activity occurs under conditions with mild *orf_1* toxicity. Growth curves are shown for samples without SpyCas9 induction to ensure that *orf_1* toxicity is not mitigated due to elimination of its plasmid. Points indicate averages from three replicates. Standard deviations at each timepoint are so small that the error bars do not exceed the bounds of the data point. The individual numerical values that underlie the summary data in this figure may be found as supporting information file SI_Data.

Supplemental Figure 2. ***Orf_1 (acrIIA22*) does not impact SpyCas9 expression.** (**A**) A schematic description of the experimental design shown in panel (**B**) is presented. If ORF_1 prevented transcription from pCas9 or altered its copy number, we would expect expression of the *orf_1* gene to deplete the level of green fluorescence observed from a construct that replaces the *spycas9* gene with *gfp*. (**B**) Fluorescence measurements for the experiment depicted in panel A show that ORF_1 does not impact GFP expression throughout an *E. coli* growth curve. Points indicate averages from three replicates, error bars indicate standard deviation. A western blot shows no depletion of SpyCas9 expression as a function of ORF_1 or GFP expression in growing *E. coli* cultures at three hours (**C**) or six hours **(D)**. As an internal control, GAPDH expression was also detected. The individual numerical values and original images for the data presented in this figure may be found in the supporting information files SI_Data and SI_raw_images, respectively.

Supplemental Figure 3. **AcrIIA22 only modestly protects Mu phages against SpyCas9.** Mu phage fitness was measured by plaquing on *E. coli* in the presence of *gfp*, *acrIIA22*, or *acrIIA4* via serial ten-fold dilutions. Bacterial clearing (black) occurs when phage Mu overcomes SpyCas9 immunity and lyses *E. coli*. In (**A**) and in (**B**), SpyCas9 with a Mu-targeting crRNA confers substantial protection against phage Mu relative to a non-targeting (n.t.) control, in both conditions tested. These conditions are depicted at left, with the only difference being whether SpyCas9 was only expressed in liquid growth prior to phage infection (panel A) or expressed both in liquid media and in solid media throughout infection (panel B). When expressed from a second plasmid, the positive control *acrIIA4* significantly enhances Mu fitness by inhibiting SpyCas9 in all conditions *in trans*. Though *acrIIA22* confers protection against SpyCas9 compared to *gfp* (negative control), this effect is milder than with *acrIIA4* and dependent on SpyCas9 expression.

Supplemental Figure 4. ***AcrIIA22* homologs are found in hypervariable regions of prophage and bacterial genomes in the CAG-217clostridial genus.** (**A**) Homologs of *acrIIA22* are depicted in three related prophage genomes, integrated at three different genomic loci, revealed by a comparison of prophage-bearing contigs (#57, #56, #37) relative to unintegrated contigs (#55, #58, #17 respectively), which are otherwise nearly identical. Prophage genes are colored by functional category, according to the legend at the left of panel A. Genes immediately adjacent to *acrIIA22* (solid boxes) vary across phages, despite strong relatedness across much of the prophage genomes. Bacterial genes are colored gray, except for contig #17, which is also depicted in panel B, below. (**B**) Homologs of *acrIIA22* are depicted in diverse genomic islands,

including Contig #1, whose sequence includes a portion that is identical to the original metagenomic contig we recovered (F01A_4). All *acrIIA22* homologs in these loci are closely related but differ in their adjacent genes, which often have homologs in the prophages depicted in panel A (dashed boxes). Bacterial genomic regions flanking these hypervariable islands are nearly identical to one another and to prophage integration locus #3, as shown by homology to contig #17 from panel A. Contigs are numbered to indicate their descriptions in Supplemental Table 3, which contains their metadata, taxonomy, and sequence retrieval information. All sequences and annotations can also be found in Supplemental Datasets 1 and 2. (**C**) We tabulate the prevalence of various protein families (clustered at 65% amino acid identity) in a set of 54 unique genomic islands. Each of these islands is flanked by the conserved genes *purF* and *radC* but contains a different arrangement of encoded genes. Domain-level annotations are indicated below each protein family (unk; unknown function). Gene symbols above each protein family are colored and lettered to indicate their counterparts or homologs in panels A and B. The phage capsid icon indicates sequences with homologs in prophage genomes. (**D**) An evolutionary model for the origin of the *acrIIA22*-encoding hypervariable genomic islands depicted in panel B is shown. This panel is reprinted from Figure 2C, for continuity. We propose that *acrIIA22* moved via a phage insertion into a bacterial genomic locus, remained following an incomplete prophage excision event, and its neighboring genes subsequently diversified via horizontal exchange with additional phage genomes. The individual numerical values that underlie the summary data in this figure may be found as supporting information file SI_Data.

Supplemental Figure 5. **Genomic proximity of *acrIIA22* homologs to other *acr* genes.** An *acrIIA22*-encoding prophage like the one depicted in Figure 2A and those in Supplemental Figure 4A is shown. This prophage encodes for a homolog of the previously described SpyCas9 inhibitor *acrIIA17* within one kilobase of an *acrIIA22* homolog. Sequence relatedness between the depicted *acrIIA17* gene and the originally discovered acrIIA17 is shown[22]. Because phages often encode multiple *acr*s in the same locus, the co-localization of *acrIIA17* with *acrIIA22* is consistent with the latter gene functioning natively to inhibit CRISPR-Cas activity. Prophage genes are colored by functional category, per the legend and as in Supplemental Figure 4A. Contigs are numbered to indicate their descriptions in Supplemental Table 3, which contains their metadata, taxonomy, and sequence retrieval information. All sequences and annotations can also be found in Supplemental Datasets 1 and 2.

Supplemental Figure 6. **AcrIIA22 does not strongly bind SpyCas9.** SpyCas9 and sgRNA were pre-incubated before mixing with a twin-strep (TS) tagged AcrIIA22 or AcrIIA4. SpyCas9 without sgRNA was also used. Strep-Tactin pulldowns on AcrIIA4 also pulled down SpyCas9 pre-incubated with sgRNA, as previously reported[12]. Similar pulldowns with AcrIIA22 indicate little to no interaction with SpyCas9, regardless of whether sgRNA was used. These images depict total protein content visualized by Coomassie stain. Reaction components are indicated below the gel image. Asterisks (*) and dagger (†) symbols indicate AcrIIA4 and AcrIIA22 protein bands that run at slightly different positions than expected due to gel distortion. Original, uncropped versions of images depicted in figure may be found in the supporting information file, SI_raw_images.

Supplemental Figure 7. **AcrIIA22 does not protect linear DNA from SpyCas9 cleavage.** (**A**) A schematic cartoon depicts the experiment in panel (B). SpyCas9 was pre-incubated with sgRNA targeting linear DNA. Then, Acr candidates were added. Subsequently, cleavage reactions were performed, and the DNA products visualized by gel electrophoresis. (**B**) We show the products of the reactions described in panel A for the inhibitors AcrIIA22 and AcrIIA4. SpyCas9 activity is

greatly inhibited by AcrIIA4 but unaffected by AcrIIA22, as indicated by the proportion of cleaved DNA product. Reaction components are depicted atop the gel image, with molar equivalents relative to SpyCas9 indicated. The percent of DNA substrate cleaved by SpyCas9 is quantified below each lane. (**C**) We perform a similar experiment as in panel A, except candidate Acrs were incubated with SpyCas9 before sgRNA addition. Reactions were begun via the simultaneous addition of sgRNA and linear dsDNA instead of just dsDNA. (**D**) The products of the reactions described in panel C for AcrIIA22 and AcrIIA4 inhibitors are shown. SpyCas9 activity is inhibited by AcrIIA4 but unaffected by AcrIIA22, as indicated by the proportion of cleaved DNA product. The data depicted in this figure are not directly comparable to those in figure 7, due to methodological differences and because the preparations of SpyCas9 used in each experiment exhibited different activities. Original, uncropped versions of images depicted in figure may be found in the supporting information file, SI_raw_images.

Supplemental Figure 8. **AcrIIA22 resembles a PC4-like protein.** (**A**) We present a ribbon diagram of a proposed AcrIIA22 tetramer, which requires binding between anti-parallel β-strands at the C-termini of AcrIIA22 monomers to form extended, concave β-sheets. This putative oligomerization interface is indicated by the regions highlighted in yellow. Each monomer in the proposed tetramer is labeled with lower-case Roman numerals (i-iv). (**B**) Space filling model of the tetrameric AcrIIA22 structure from panel A, with relative charge depicted, highlighting a groove (dashed line with arrowhead) that may accommodate nucleic acids (based on analogy to other PC4-like proteins). (**C**) AcrIIA22 monomers (i) and (ii) from the tetramer in panel A likely interact via a series of hydrophobic interactions, as indicated by the predominantly non-polar sidechains colored in yellow. The boxed region highlights residue D14, which is important for nicking activity and plasmid protection against SpyCas9, and is enlarged in panel F. (**D**) In conventional PC4-like family proteins, such as the putative single-stranded DNA binding protein from phage T5 depicted in gray (PDB:4BG7), the same topology of outward facing, concave β-sheets are instead stabilized via interactions between opposing α-helices (depicted in opaque light blue). (**E**) An overlay of β-sheets from AcrIIA22 (blue, PDB:7JTA) and the phage T5 PC4-like protein (gray, PDB:4BG7) illustrates their similar topologies. (**F**) Two D14 residues in loop regions of AcrIIA22 monomers (i) and (ii) are in close proximity. These residues are important for nicking activity and may bind divalent cations in cells under physiological pH. (**G**) A close view of a putative salt bridge between R30 of monomers (i) / (ii) and the peptide backbone of the C-terminus of monomers (iv) / (iii), respectively. AcrIIA22 monomers are colored as described in panel A.

Supplemental Figure 9. A 2-aa truncation mutant of AcrIIA22 is impaired for SpyCas9 inhibition and nicking activity. (**A**) An *in vivo* plasmid protection assay. Asterisks depict statistically significant differences in plasmid retention under SpyCas9-inducing conditions with either wild-type AcrIIA22, a null mutant with an early stop codon, a 2-aa truncation, or a negative control *gfp* gene (adj. $p < 0.005$, Student's t-test, n=3). The truncation mutant retains mild but severely impaired activity, as it protects a plasmid from SpyCas9 more effectively than a null mutant ($p = 0.012$) or GFP control ($p = 0.015$). All p-values were corrected for multiple hypotheses using Bonferroni's method. (**B**) The 2-aa truncation mutant is impaired for nicking *in vitro*, relative to wild-type AcrIIA22. In both cases, 25μM of protein was used following NiNTA-based purification of an N-terminal, His6-tagged construct. An asterisk (*) denotes significant differences between AcrIIA22-treated and untreated substrates (Student's t-test, $p < 0.05$, n=3). Standard deviations are indicated by dashed lines (in most cases, the data points obscure these error bars). The individual numerical values that underlie the summary data in this figure may be found as supporting information file SI_Data.

Supplemental Figure 10. **AcrIIA22 nicks supercoiled plasmids.** (**A**) A Coomassie stain of an N-terminally His6-tagged AcrIIA22 construct shows no co-purifying proteins. (**B**) The nicking activity for this protein preparation (bottom) correlates with the intensity of the Coomassie-stained protein band across purification fractions (top). In each lane, supercoiled (SC) plasmid DNA represents the un-nicked fraction whereas open circle (OC) and linear DNA have been nicked at least once. (**C**) This panel is a quantification of the experiment depicted in panel B across all 13 fractions collected. (**D**) His6-AcrIIA22 nicks supercoiled plasmids in a time and concentration dependent manner. A decrease in the proportion of supercoiled plasmid DNA indicates nicking activity, as depicted in Figure 5B. (**E**) A silver stain of a C-terminally twin-strep-tagged AcrIIA22 construct shows no co-purifying proteins. Equal volumes of each protein fraction were loaded in each lane, for all samples. Fraction 4 was concentrated and used for all *in vitro* experiments. (**F**) A C-terminal, but not N-terminal twin-strep tag is compatible with AcrIIA22's ability to protect a target plasmid from SpyCas9 elimination *in vivo*. Statistically significant differences in plasmid retention between SpyCas9-inducing and non-inducing conditions were determined via a Student's t-test (n=3); '**' indicates p≤0.001. All p-values were adjusted for multiple hypotheses using the Bonferroni correction. (**G**) The D14A mutation in AcrIIA22 impairs nicking activity. Over time, the wild-type AcrIIA22-twin-strep construct consistently converts a higher fraction of plasmid DNA from its supercoiled (SC) form to an open-circle (OC) conformation than a D14A mutant. Control plasmids include a miniprepped sample and sample pre-treated with the commercial nickase, Nb.BssSI. Reaction times are indicated to the right of each image. (**H**) AcrIIA22a (Figure 3B) is impaired for nicking activity relative to AcrIIA22. As in panel G, both constructs were purified via C-terminal twin-strep tags. The individual numerical values and original images for the data presented in this figure may be found in the supporting information files SI_Data and SI_raw_images, respectively.

Supplemental Figure 11. Divalent cations influence AcrIIA22's nicking activity. (**A**) We present the impact of different divalent cations on AcrIIA22's nicking activity, which is highest with Mg^{2+}, Mn^{2+}, and Co^{2+}. OC, open-circle plasmid form. SC, supercoiled plasmid. (**B**) The open-circle plasmid product persists through phenol-chloroform extraction following AcrIIA22 treatment, indicating that it directly results from AcrIIA22's nicking activity.

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Table 1. Structural features of AcrIIA22.

Data collection	
Space Group	P4332
Cell Dimensions	
a, b, c (Å)	128.56, 128.56, 128.56
α, β, γ (°)	90.0, 90.0, 90.0
Resolution (Å)	50.00 - 2.80
R_{merge}	0.106 (0.906)
I/σ_I	17.4 (2.6)
Completeness (%)	98.7 (100.0)
Redundancy	10.4 (10.7)
CC 1/2	0.837
Refinement	
No. Reflections	9334
R_{work} (R_{free}) (%)	22.2 (24.6)
No. Complex in ASU	2
No. atoms	
Protein	810
Heteroatoms	50
Water	3
B-factor	82.82
R.m.s deviations	
Bond lengths (Å)	0.003
Bond angles (°)	0.610
Ramachandran	
Preferred (%)	98.15
Allowed (%)	1.85
Outliers (%)	0

Other supplemental materials.

Supplemental Table 1. Whether known anti-CRISPRs can bind Cas proteins or inhibit their cleavage activity as purified proteins.

Supplemental Table 2. PC4-like proteins with structural homology to AcrIIA22.

Supplemental Table 3. Descriptions of all sequences used in this study. All sequences and annotations are also available as supplemental data.

Supplemental Table 4. Plasmids used in this study.

Supplemental Table 5. Gene sequences used in this study.

SI_Data. All raw data for main and supplemental figures depicted in this study (as a spreadsheet).

SI_raw_images. Full gel images for all cropped gels depicted in this study, compiled into a .pdf document.

Supplemental Dataset 1. 68 contigs sequences referenced in the manuscript with Pfam, TIGRFAM, and AcrIIA22 homolog annotations (in genbank format).

Supplemental Dataset 2. 68 contigs sequences referenced in the manuscript (in fasta format).

Supplemental Dataset 3. Nine AcrIIA22-encoding prophage sequences referenced in the manuscript with Pfam, TIGRFAM, and AcrIIA22 homolog annotations (in genbank format).

Supplemental Dataset 4. Nine AcrIIA22-encoding prophage sequences referenced in the manuscript (in fasta format).

Supplemental Dataset 5. All metagenomic contigs with ≥98% nucleotide identity to *acrIIA22*-associated genes, *purF* and *radC*. Pfam, TIGRFAM, and AcrIIA22 homolog annotations are also provided (file in genbank format).

Supplemental Dataset 6. Amino acid sequence alignment of 30 AcrIIA22 homologs (in fasta format).

Supplemental Dataset 7. The detailed PDB validation report for AcrIIA22's crystal structure.

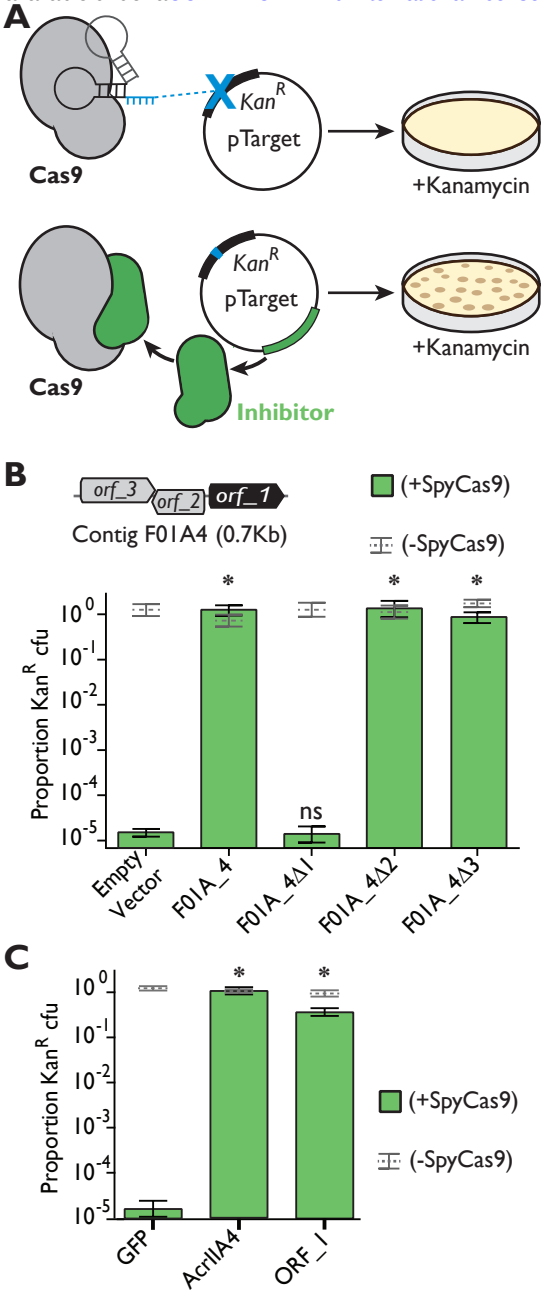


Figure 1. **Functional selection reveals a metagenomic contig encoding a novel SpyCas9 inhibitor.** (**A**) A plasmid protection assay was used to reveal SpyCas9 inhibition. In this assay, plasmids without SpyCas9 inhibitors are cleaved by Cas9 and do not give rise to Kan^R colonies, whereas those encoding inhibitors withstand SpyCas9 attack and yield Kan^R colonies. (**B**) The contig F01A_4 protects a plasmid from SpyCas9 attack but an early stop codon in *orf_1 (Δ1)* eliminates this phenotype. Stop codons in *orf_2* or *orf_3* (*Δ2* and *Δ3*) have no effect. Thus, we conclude that *orf_1* is necessary for inhibition of SpyCas9. Asterisks depict statistically significant differences in plasmid retention between the indicated genotype and an empty vector control in SpyCas9-inducing conditions (Student's t-test, $p<0.002$, n=3); ns indicates no significance. All p-values were corrected for multiple hypotheses using Bonferroni's method. (**C**) Expression of *orf_1* (which we name *acrIIA22*) is sufficient for SpyCas9 antagonism, protecting a plasmid as effectively as *acrIIA4*. Asterisks are as in panel B but relate to the GFP negative control rather than to an empty vector. The individual numerical values that underlie the summary data in this figure may be found as supporting information file SI_Data.

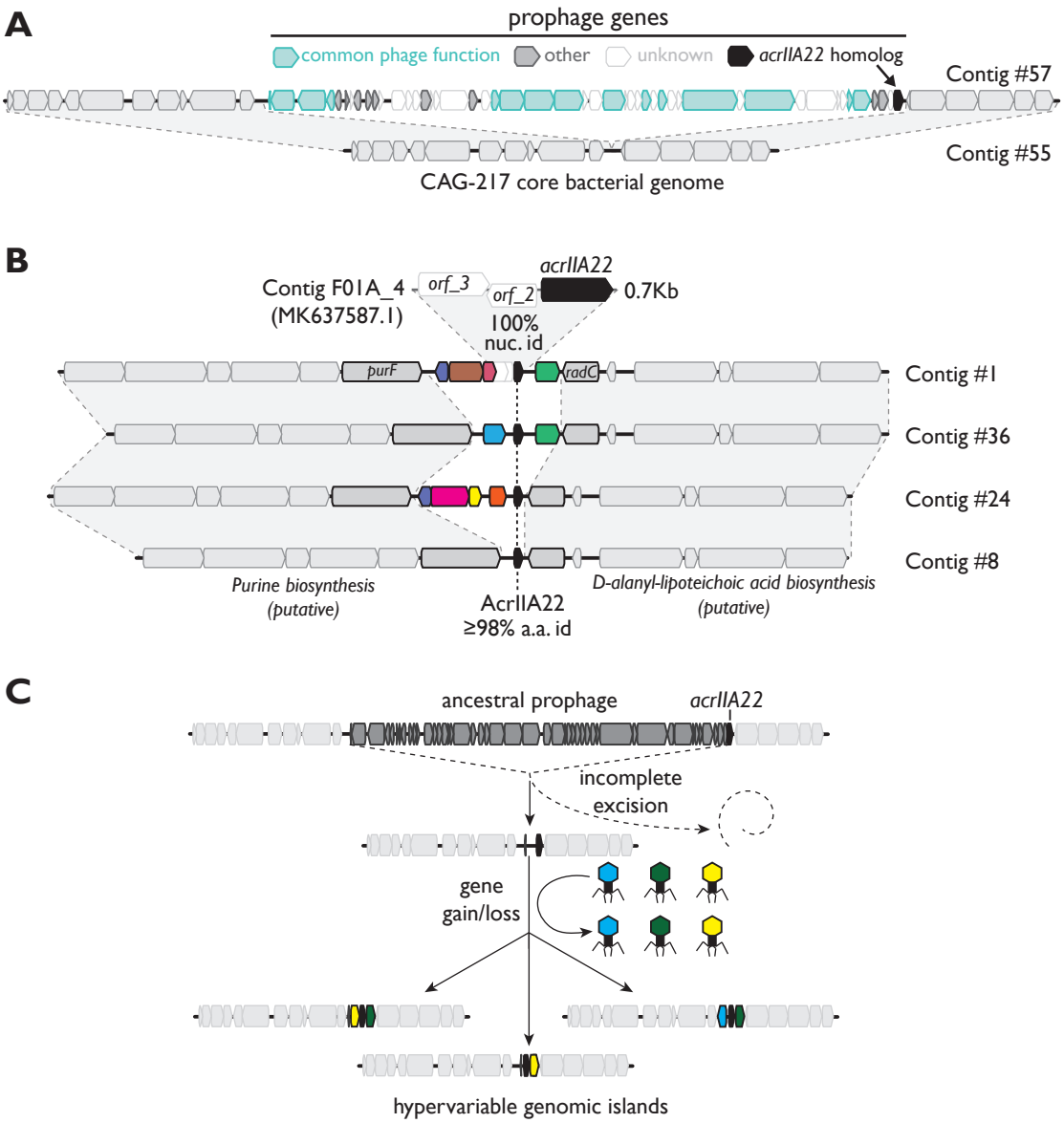


Figure 2. ***AcrIIA22* homologs are found in hypervariable regions of prophage and bacterial genomes in the unnamed clostridial genus, CAG-217.** (**A**) We show a schematic representation of an *acrIIA22* homolog embedded in a prophage genome, which is integrated into a bacterial genome (contig #57). We can delineate precise boundaries of the inserted prophage based on comparison to a near-identical bacterial contig (contig #55). Prophage genes are colored by functional category, according to the legend at the top. Bacterial genes are colored light gray. (**B**) Homologs of *acrIIA22* are depicted in diverse genomic islands, including Contig #1, whose sequence includes a portion identical to F01A_4, the original metagenomic contig we recovered. All *acrIIA22* homologs in these loci are closely related but their adjacent genes are different, unrelated gene families (depicted by different colors). Genomic regions flanking these hypervariable islands, including genes immediately adjacent to these islands (*purF* and *radC*, in bold outlines), are nearly identical to one another (≥98% nucleotide identity). Contigs are numbered to indicate their descriptions in Supplemental Table 3, which contains their metadata, taxonomy, and sequence retrieval information. All sequences and annotations may also be found in Supplemental Datasets 1 and 2. (**C**) We propose an evolutionary model for the origin of the *acrIIA22*-encoding hypervariable genomic islands depicted in panel B. We propose that *acrIIA22* moved via prophage integration into a bacterial genomic locus but remained following an incomplete prophage excision event. Its neighboring genes subsequently diversified via horizontal exchange with additional phage genomes without these phage genomes inserting into the locus. Supplemental Figure 4 depicts a more detailed version of the genomic data underlying this model.

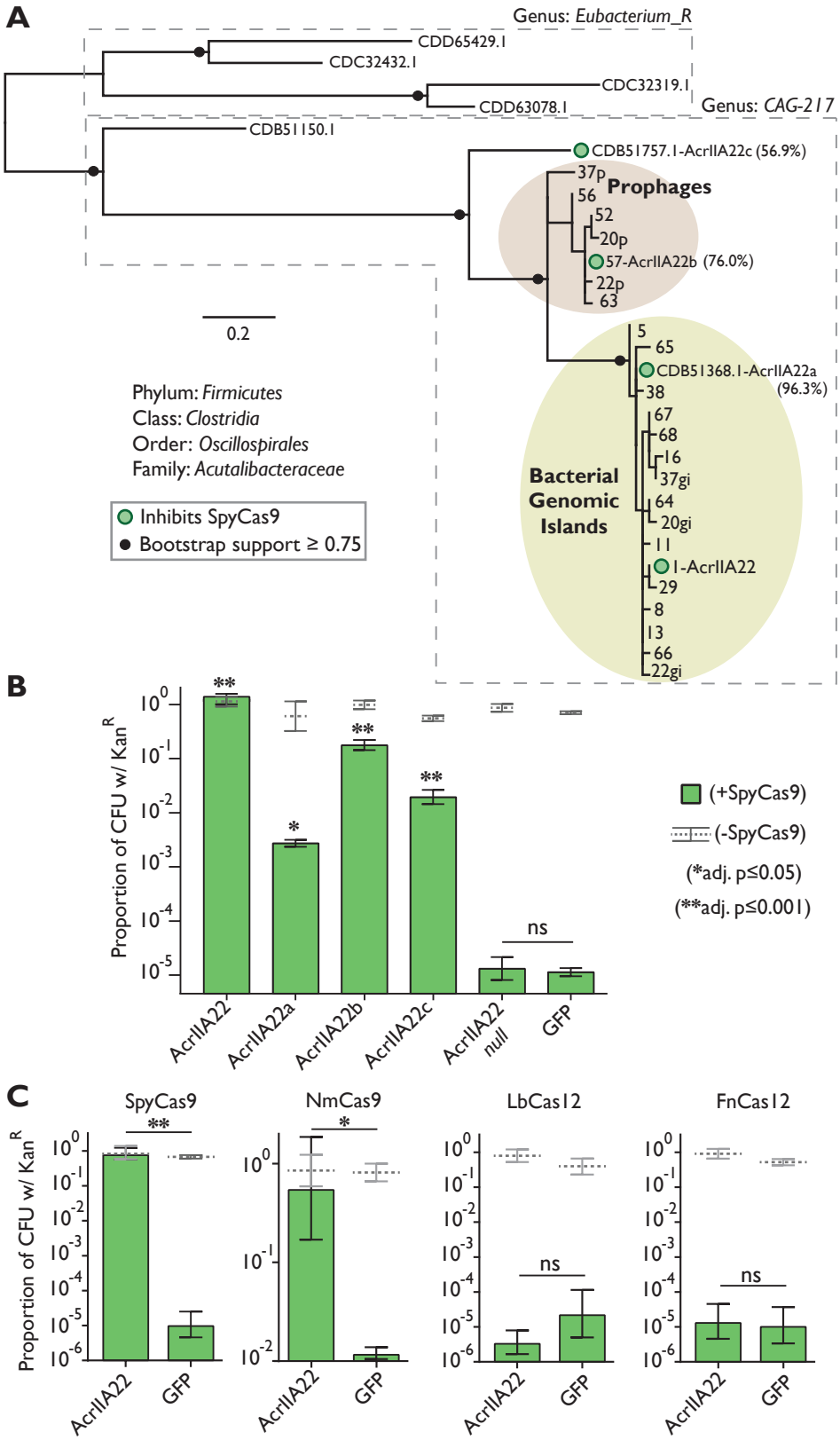


Figure 3. **Several AcrIIA22 homologs in the CAG-217 clostridial genus can inhibit SpyCas9.** (**A**) A phylogeny of all unique AcrIIA22 homologs identified from metagenomic and NCBI databases. Phylogenetic classifications were assigned corresponding to the GTDB naming convention (Methods). Prophage sequences are shaded brown and homologs from hypervariable bacterial genomic islands are shaded yellow. Sequences obtained from NCBI are labeled with protein accession numbers. In other cases, AcrIIA22 homologs are numbered to match their contig-of-origin (Supplemental Table 3). In some cases, more than one AcrIIA22 homolog is found on the same contig ('gi' or 'p' indicates its presence in a hypervariable genomic island or prophage genome, respectively). Circles at nodes indicate bootstrap support ≥ 0.75. Dashed boxes separate sequences identified from CAG-217 versus *Eubacterium_R* bacterial genera. Filled green circles indicate homologs that were tested for their ability to inhibit SpyCas9 in the plasmid protection assay in panel B. These homologs have been named with 'a', 'b', or 'c' suffixes to distinguish them from the original AcrIIA22 metagenomic hit; their amino acid identity to the original hit is shown in parentheses. (**B**) Several homologs of AcrIIA22 in CAG-217 genomes inhibit SpyCas9. Asterisks depict statistically significant differences in plasmid retention under SpyCas9-inducing conditions between the indicated sample and a null mutant with an early stop codon in *acrIIA22*, as indicated in the legend at right (ns indicates no significance; $p > 0.05$). All p-values were corrected for multiple hypotheses using Bonferroni's method (Student's t-test, n=3). (**C**) AcrIIA22 inhibits divergent Cas9 proteins from Streptococcus pyogenes (SpyCas9) or *Neisseria meningitidis* (NmCas9) but not Cas12 proteins from *Lachnospiraceae bacterium* (LbCas12) or *Francisella novicida* (FnCas12). As in panel B, green bars indicate samples with expression of the indicated Cas nuclease while unexpressed controls are depicted with gray lines. For Cas-expressing samples, significance was determined via a Student's t-test (n=3) and denoted as follows: '*', $p \leq 0.05$; '**' $p \leq 0.001$; 'ns' no significance. Due to slight differences in the plasmid protection assay in panel C compared to panel B, A22 was re-tested against SpyCas9 to confirm activity (Methods). The individual numerical values that underlie the summary data in this figure may be found as supporting information file SI_Data.

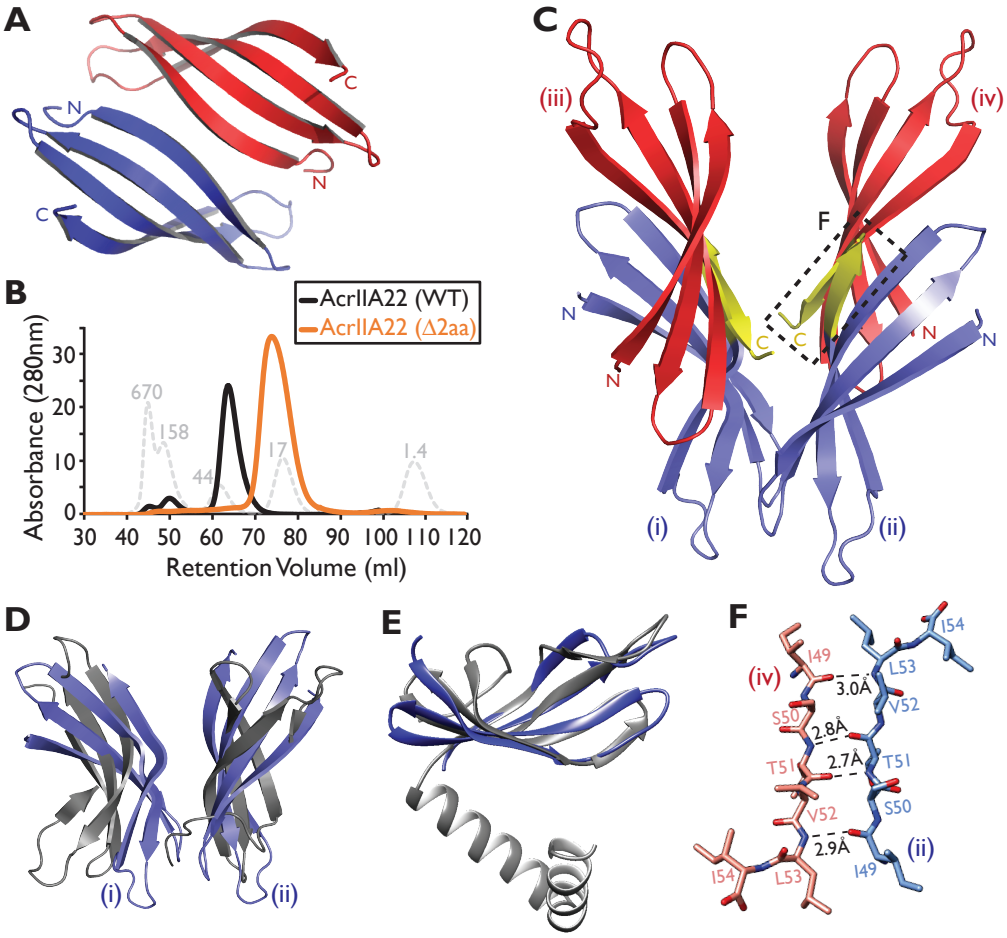


Figure 4. AcrIIA22 is an oligomeric PC4-like protein. (**A**) AcrIIA22's crystal structure reveals a homodimer of two four-stranded β-sheets. (**B**) AcrIIA22 elutes as an oligomer that is approximately four times the predicted molecular mass of its monomer, which is 7 kDa. The gray, dashed trace depicts protein standards of the indicated molecular weight, in kDa. The orange trace depicts the elution profile of a two-amino acid C-terminal AcrIIA22 truncation mutant that is predicted to disrupt oligomerization. (**C**) Ribbon diagram of a proposed AcrIIA22 tetramer which requires binding between anti-parallel β-strands at the C-termini of AcrIIA22 monomers to form extended, concave β-sheets. The putative oligomerization interface is indicated by the regions highlighted in yellow and the dashed box, and is detailed further in panel F. Each monomer in the proposed tetramer is labeled with lower-case Roman numerals (i-iv). (**D**) β-sheet topology and orientation in AcrIIA22 (blue) resemble that of PC4-like family proteins (in gray, PDB:4BG7 from phage T5). (**E**) A monomer of AcrIIA22 (in blue, PDB:7JTA) is structurally similar to a PC4-like single-stranded DNA binding protein, which is proposed to promote recombination in phage T5 (in gray, PDB:4BG7, Z-score=6.2, matched residues 15%), except for a missing C-terminal alpha helix. (**F**) A putative oligomerization interface between the C-termini of two AcrIIA22 monomers from panel (C) is shown in more detail. Dashed lines indicate potential hydrogen bonds between the polypeptide backbones. This interface occurs twice in the putative tetramer, between red-hued and blue-hued monomers in panel C.

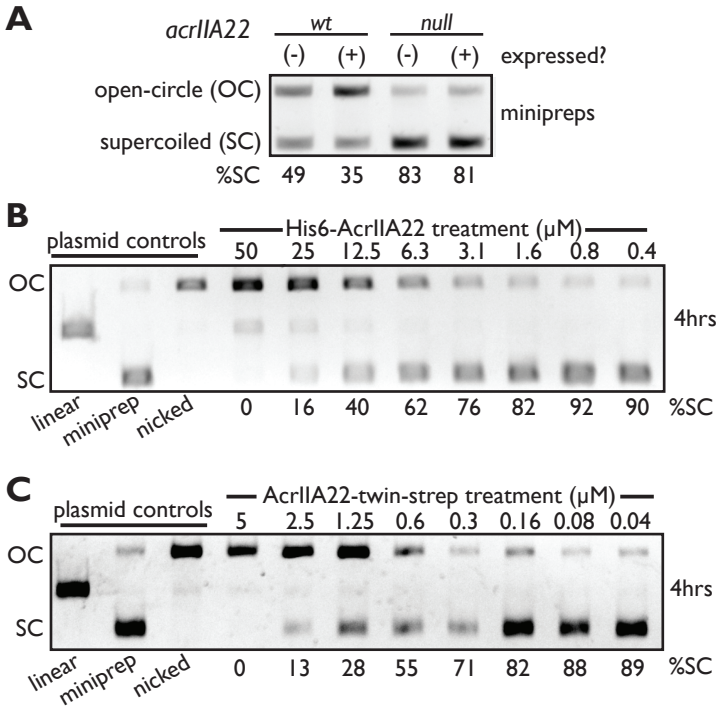


Figure 5. AcrIIA22 nicks supercoiled plasmids *in vivo* and *in vitro*. (**A**) Gel electrophoresis of plasmids purified from overnight *E. coli* cultures expressing either acrIIA22, or a null mutant with an early stop codon, or neither. Compared to the null mutant, more plasmid runs in a slowly migrating, open-circle conformation (OC) rather than supercoiled plasmid (SC) with the wild-type *acrIIA22* allele, suggesting that *acrIIA22* may impact plasmid topology. %SC indicates the percentage of DNA in the supercoiled form for each sample. (**B**) N-terminally His6-tagged AcrIIA22 nicks supercoiled plasmids *in vitro*. (**C**) C-terminally twin-strep-tagged AcrIIA22 nicks supercoiled plasmids *in vitro* with higher specific activity than shown in panel B (compare protein concentrations). Original, uncropped versions of images depicted in figure may be found in the supporting information file, SI_raw_images.

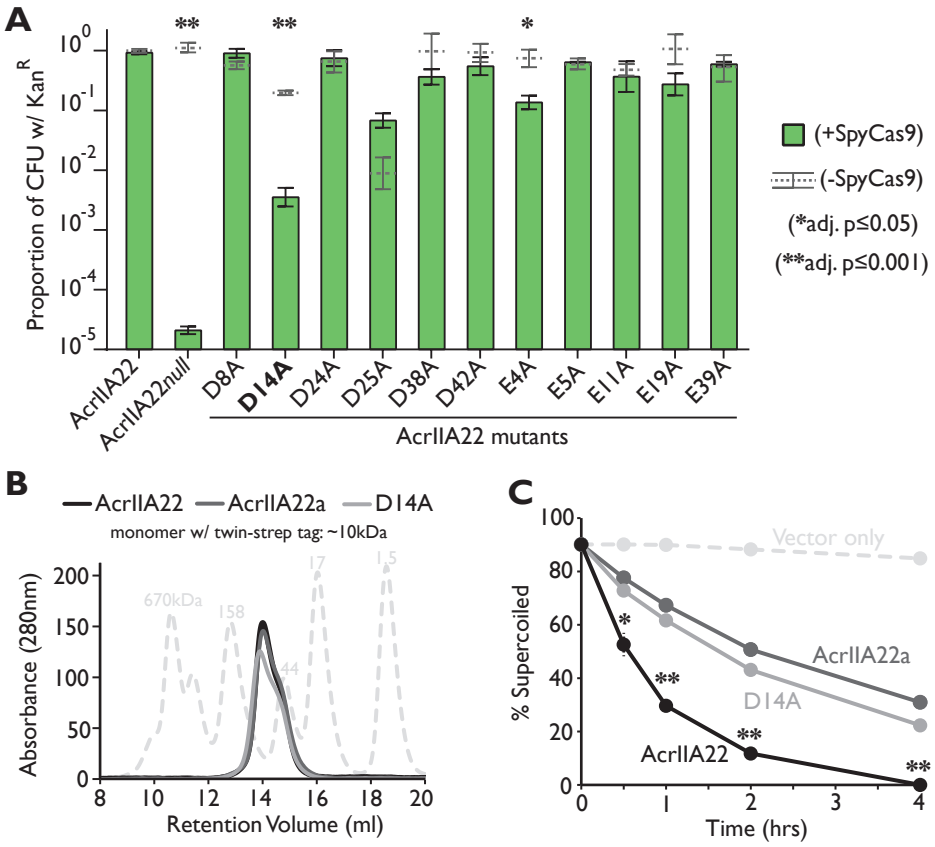


Figure 6. **Impaired nicking activity of AcrIIA22 variants *in vitro* correlates with lower SpyCas9 inhibition *in vivo***. (**A**) Alanine mutagenesis of acidic amino acid residues (glutamic acid or aspartic acid) in AcrIIA22 reveals that D14 is important for plasmid protection against SpyCas9. Asterisks depict statistically significant differences in plasmid retention under SpyCas9-inducing and non-inducing conditions, per the legend at right. The D14A mutant is significantly impaired, the E4A mutant is slightly impaired, whereas all other mutants are not impaired for plasmid protection against SpyCas9 compared to an uninduced control. All p-values were corrected for multiple hypotheses using Bonferroni's method (Student's t-test, n=3). (**B**) AcrIIA22 (black), AcrIIA22a (dark gray), and a D14A mutant (light gray) all elute with similar oligomer profiles via SEC. The dashed trace depicts protein standards of the indicated molecular weight, in kDa. (**C**) AcrIIA22a and the D14A mutant are impaired for nicking relative to AcrIIA22. All experiments were performed in triplicate, with standard deviations indicated by dashed lines (in most cases, the data points obscure these error bars). Asterisks denote cases where AcrIIA22 is significantly different than both AcrIIA22a and the D14A mutant after correcting for multiple hypotheses (Student's t-test, n=3, Bonferroni correction). A single asterisk (*) means that adjusted p-values for both comparisons are below 0.05. A double asterisk (**) means that adjusted p-values are both below 0.005. Supplemental Figures 10G and 10H show representative gels for these nicking experiments. The individual numerical values that underlie the summary data in this figure may be found as supporting information file SI_Data.

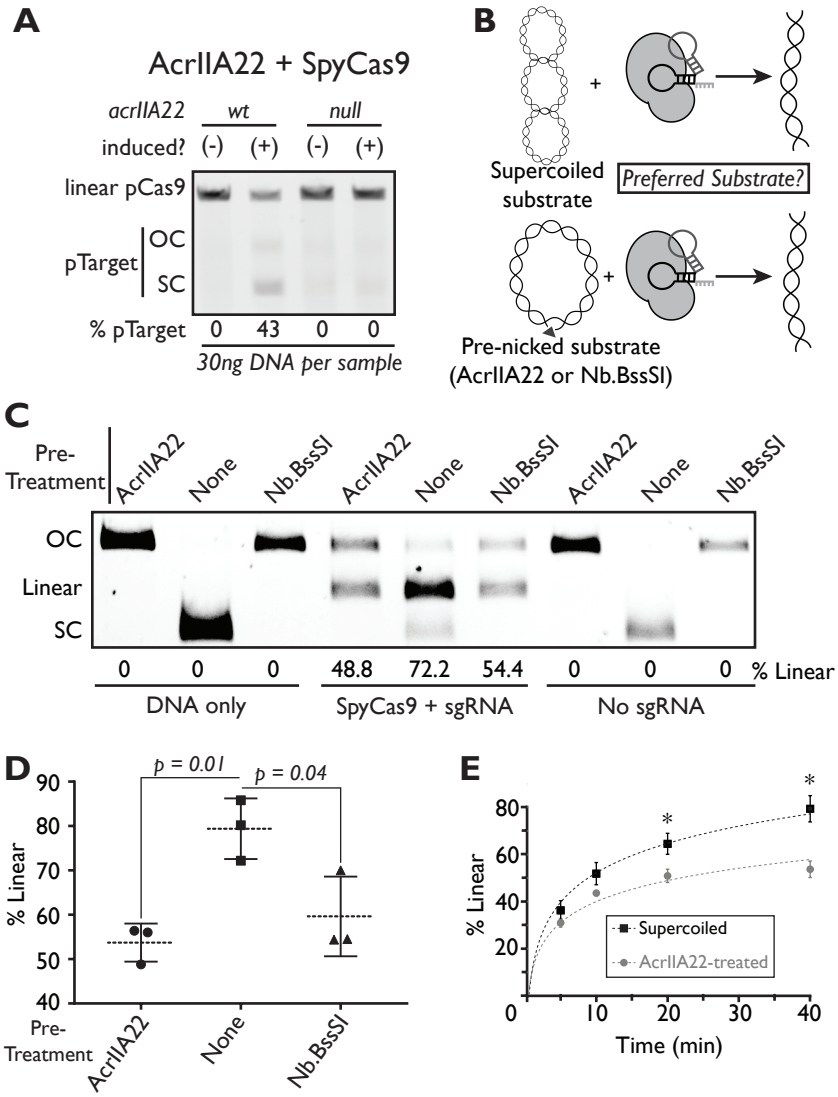
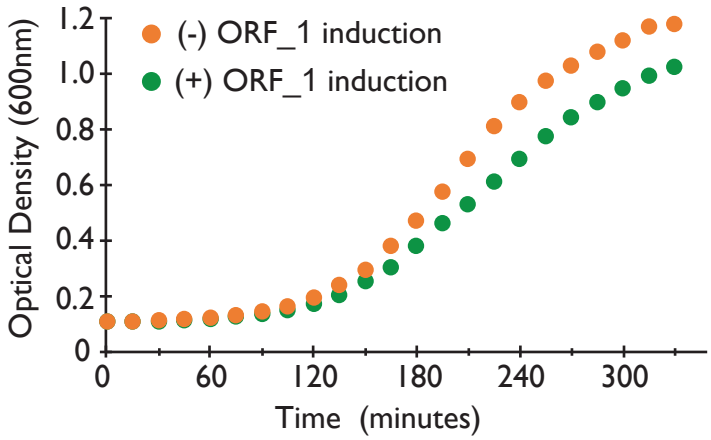
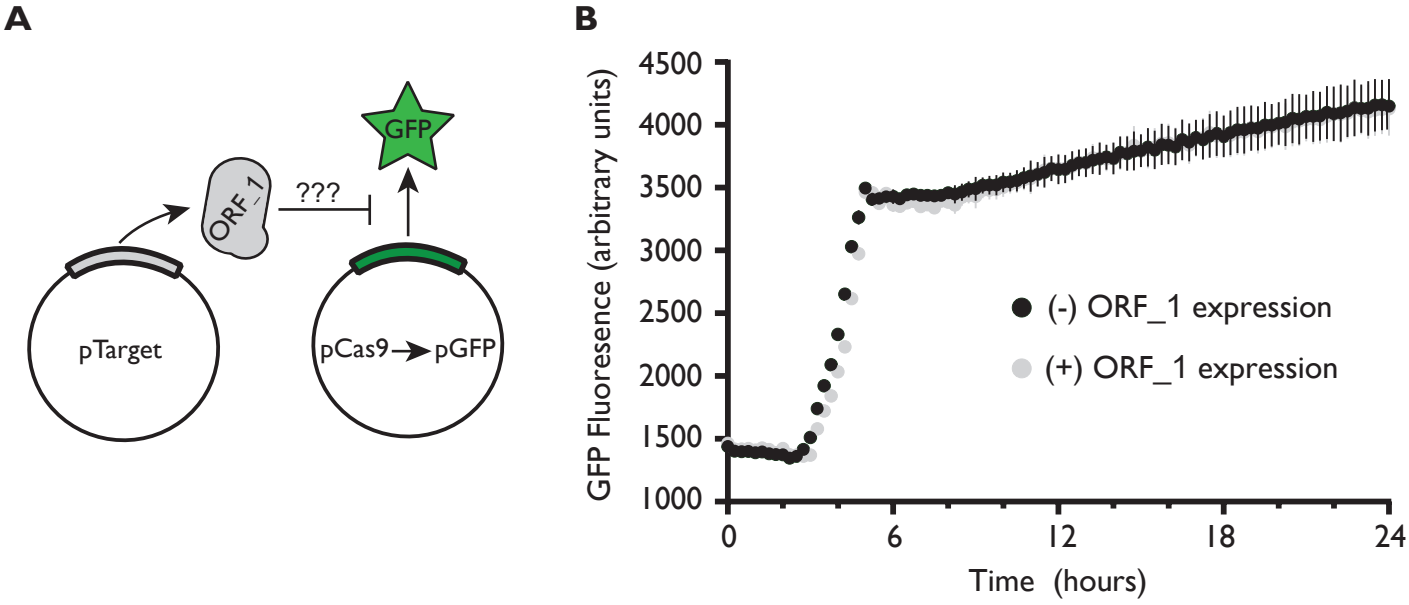
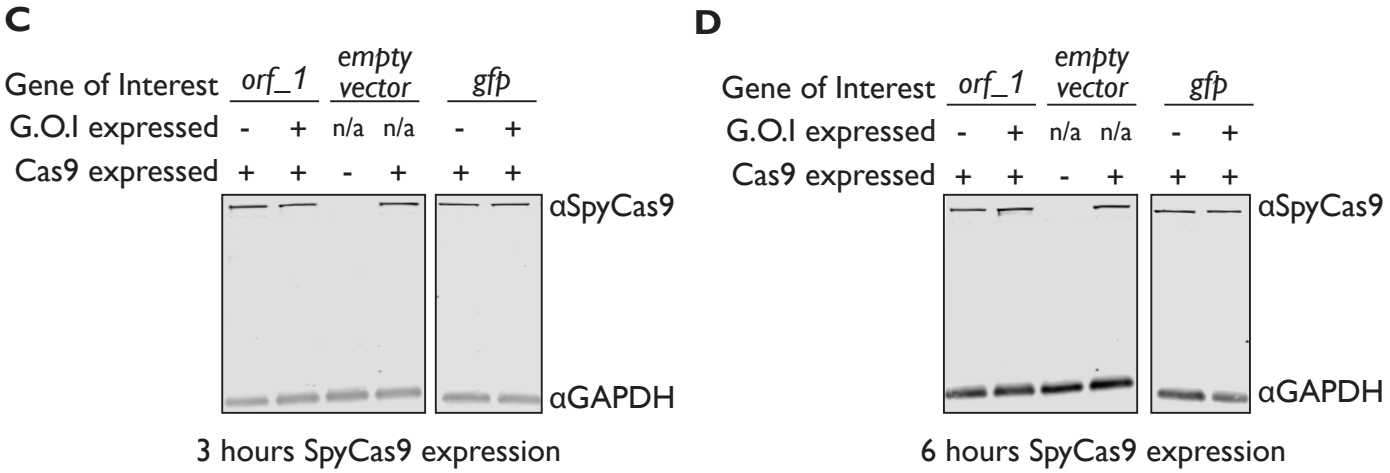


Figure 7. Nicking by AcrIIA22 protects plasmids from SpyCas9 *in vivo* and *in vitro*. (**A**) Gel electrophoresis of plasmids purified from overnight *E. coli* cultures expressing either wildtype *acrIIA22* or a mutant with an early stop codon ('null'). In these cultures, SpyCas9 was expressed from a second plasmid, which was linearized via a unique restriction site before electrophoresis. The *acrIIA22*-encoding plasmids are indicated with the 'pTarget' label. OC, open-circle; SC, supercoiled. The '%pTarget' figure indicates the fraction of total DNA attributable to pTarget, quantified by densitometry analysis. In cases with complete pTarget elimination, all DNA comes from the SpyCas9 expression plasmid, and thus these bands are more pronounced. However, in the presence of wildtype acrIIA22, pTarget is protected from SpyCas9-mediated cleavage and makes up 43% of total plasmid DNA. (**B**) We present a schematic of the experimental design for the data depicted in panel C. The experiment tests whether SpyCas9 preferentially cleaves a supercoiled or open-circle plasmid target *in vitro*. Though both plasmid substrates will be linearized following SpyCas9 cleavage, linear DNA will accumulate more readily with a preferred substrate. (**C**) Plasmid purifications from overnight cultures were either left unmodified or pre-treated with one of two nickase enzymes, AcrIIA22 or Nb.BssSI, following which each substrate was digested with SpyCas9 *in vitro*. The percentage of DNA in the linear form is quantified below the gel, which indicates complete SpyCas9 cleavage. Linear, open-circle (OC), and supercoiled (SC) plasmid forms are indicated along with the left of the gel, and reaction components below the gel. SpyCas9 cuts DNA strands sequentially; incomplete digestions with supercoiled substrates produce open-circle plasmids if only one strand has been cleaved (*e.g.* lane 5). Pre-nicked plasmids, by either AcrIIA22 or Nb.BssSI, are less susceptible to linearization via SpyCas9 cleavage. (**D**) Endpoint measurements indicate that SpyCas9 more efficiently linearizes supercoiled plasmids than substrates nicked with either AcrIIA22 or Nb.BssSI (Student's t-test, n=3). (**E**) A time course experiment demonstrates that SpyCas9 more efficiently linearizes supercoiled plasmids than AcrIIA22-treated substrates. An asterisk (*) denotes significant differences between AcrIIA22-treated and untreated substrates (Student's t-test, $p < 0.05$, n=3). The individual numerical values and original images for the data presented in this figure may be found in the supporting information files SI_Data and SI_raw_images, respectively.

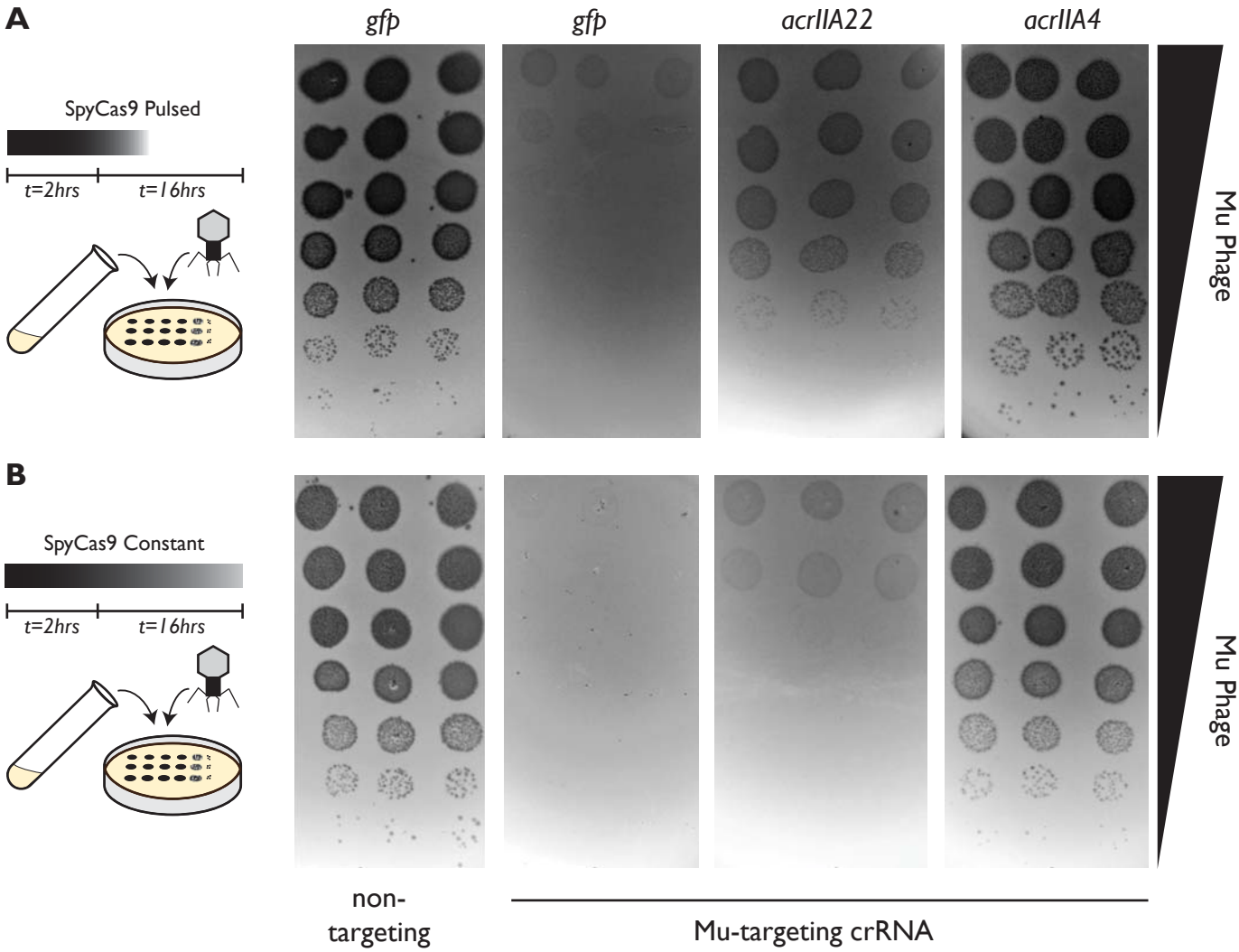


Supplemental Figure 1. ***Orf_1* (*acrIIA22*) confers mild toxicity in *E. coli*.** Growth rates with *orf_1* induction (green) are 7% lower than those without *orf_1* induction (orange). The cfu data shown in Figure 1C were generated from the same experiment depicted here (samples were removed after six hours of growth to determine these cfu counts). Thus, these data demonstrate that anti-SpyCas9 activity occurs under conditions with mild *orf_1* toxicity. Growth curves are shown for samples without SpyCas9 induction to ensure that *orf_1* toxicity is not mitigated due to elimination of its plasmid. Points indicate averages from three replicates. Standard deviations at each timepoint are so small that the error bars do not exceed the bounds of the data point.

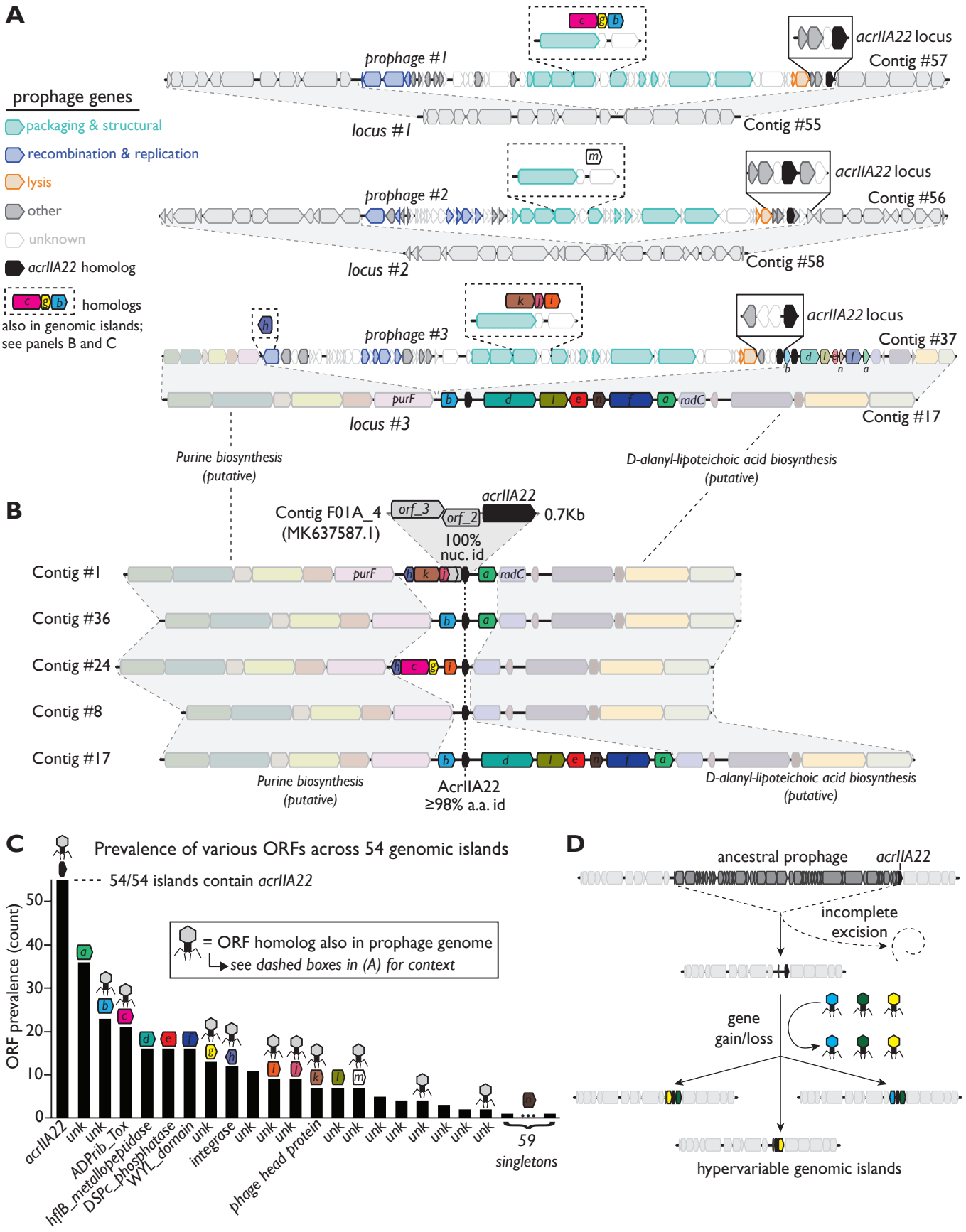




Supplemental Figure 2. ***Orf_1 (acrIIA22)* does not impact SpyCas9 expression.** (**A**) A schematic description of the experimental design shown in panel (**B**) is presented. If ORF_1 prevented transcription from pCas9 or altered its copy number, we would expect expression of the *orf_1* gene to deplete the level of green fluorescence observed from a construct that replaces the *spycas9* gene with *gfp*. (**B**) Fluorescence measurements for the experiment depicted in panel A show that ORF_1 does not impact GFP expression throughout an *E. coli* growth curve. Points indicate averages from three replicates, error bars indicate standard deviation. A western blot shows no depletion of SpyCas9 expression as a function of ORF_1 or GFP expression in growing *E. coli* cultures at three hours (**C**) or six hours **(D)**. As an internal control, GAPDH expression was also detected.

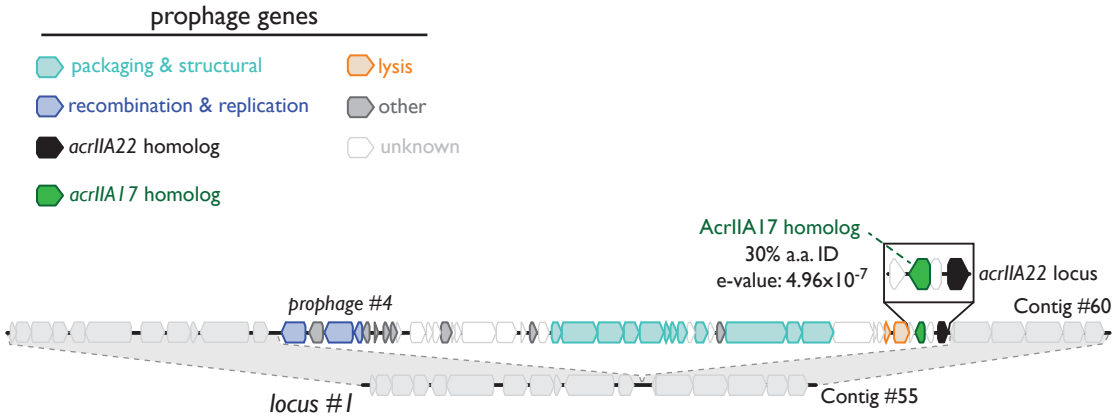


Supplemental Figure 3. **AcrIIA22 only modestly protects Mu phages against SpyCas9.** Mu phage fitness was measured by plaquing on *E. coli* in the presence of *gfp*, *acrIIA22*, or *acrIIA4* via serial ten-fold dilutions. Bacterial clearing (black) occurs when phage Mu overcomes SpyCas9 immunity and lyses *E. coli*. In (**A**) and in (**B**), SpyCas9 with a Mu-targeting crRNA confers substantial protection against phage Mu relative to a non-targeting (n.t.) control, in both conditions tested. These conditions are depicted at left, with the only difference being whether SpyCas9 was only expressed in liquid growth prior to phage infection (panel A) or expressed both in liquid media and in solid media throughout infection (panel B). When expressed from a second plasmid, the positive control *acrIIA4* significantly enhances Mu fitness by inhibiting SpyCas9 in all conditions *in trans*. Though *acrIIA22* confers protection against SpyCas9 compared to *gfp* (negative control), this effect is milder than with *acrIIA4* and dependent on SpyCas9 expression.

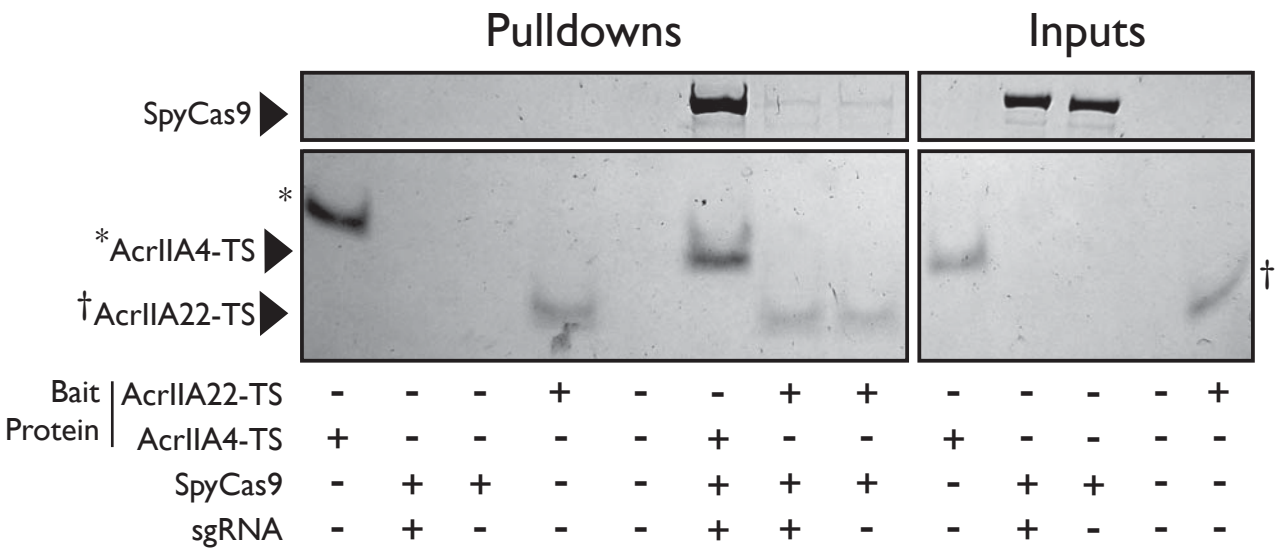


Supplemental Figure 4.

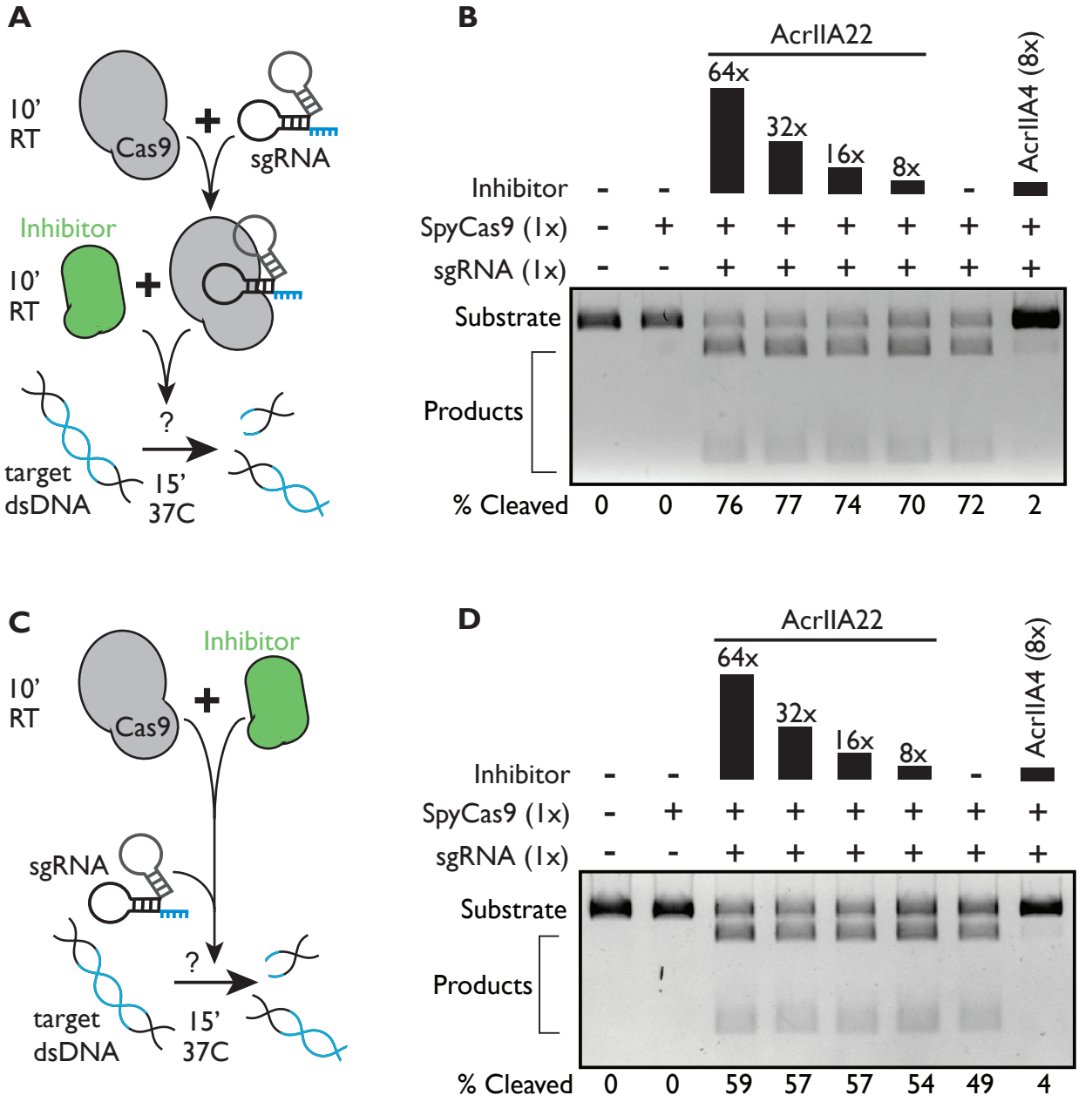
Supplemental Figure 4. ***AcrIIA22* homologs are found in hypervariable regions of prophage and bacterial genomes in the CAG-217clostridial genus.** (**A**) Homologs of *acrIIA22* are depicted in three related prophage genomes, integrated at three different genomic loci, revealed by a comparison of prophage-bearing contigs (#57, #56, #37) relative to unintegrated contigs (#55, #58, #17 respectively), which are otherwise nearly identical. Prophage genes are colored by functional category, according to the legend at the left of panel A. Genes immediately adjacent to *acrIIA22* (solid boxes) vary across phages, despite strong relatedness across much of the prophage genomes. Bacterial genes are colored gray, except for contig #17, which is also depicted in panel B, below. (**B**) Homologs of *acrIIA22* are depicted in diverse genomic islands, including Contig #1, whose sequence includes a portion that is identical to the original metagenomic contig we recovered (F01A_4). All *acrIIA22* homologs in these loci are closely related but differ in their adjacent genes, which often have homologs in the prophages depicted in panel A (dashed boxes). Bacterial genomic regions flanking these hypervariable islands are nearly identical to one another and to prophage integration locus #3, as shown by homology to contig #17 from panel A. Contigs are numbered to indicate their descriptions in Supplemental Table 3, which contains their metadata, taxonomy, and sequence retrieval information. All sequences and annotations can also be found in Supplemental Datasets 1 and 2. (**C**) We tabulate the prevalence of various protein families (clustered at 65% amino acid identity) in a set of 54 unique genomic islands. Each of these islands is flanked by the conserved genes *purF* and *radC* but contains a different arrangement of encoded genes. Domain-level annotations are indicated below each protein family (unk; unknown function). Gene symbols above each protein family are colored and lettered to indicate their counterparts or homologs in panels A and B. The phage capsid icon indicates sequences with homologs in prophage genomes. (**D**) An evolutionary model for the origin of the *acrIIA22*-encoding hypervariable genomic islands depicted in panel B is shown. This panel is reprinted from Figure 2C, for continuity. We propose that *acrIIA22* moved via a phage insertion into a bacterial genomic locus, remained following an incomplete prophage excision event, and its neighboring genes subsequently diversified via horizontal exchange with additional phage genomes.



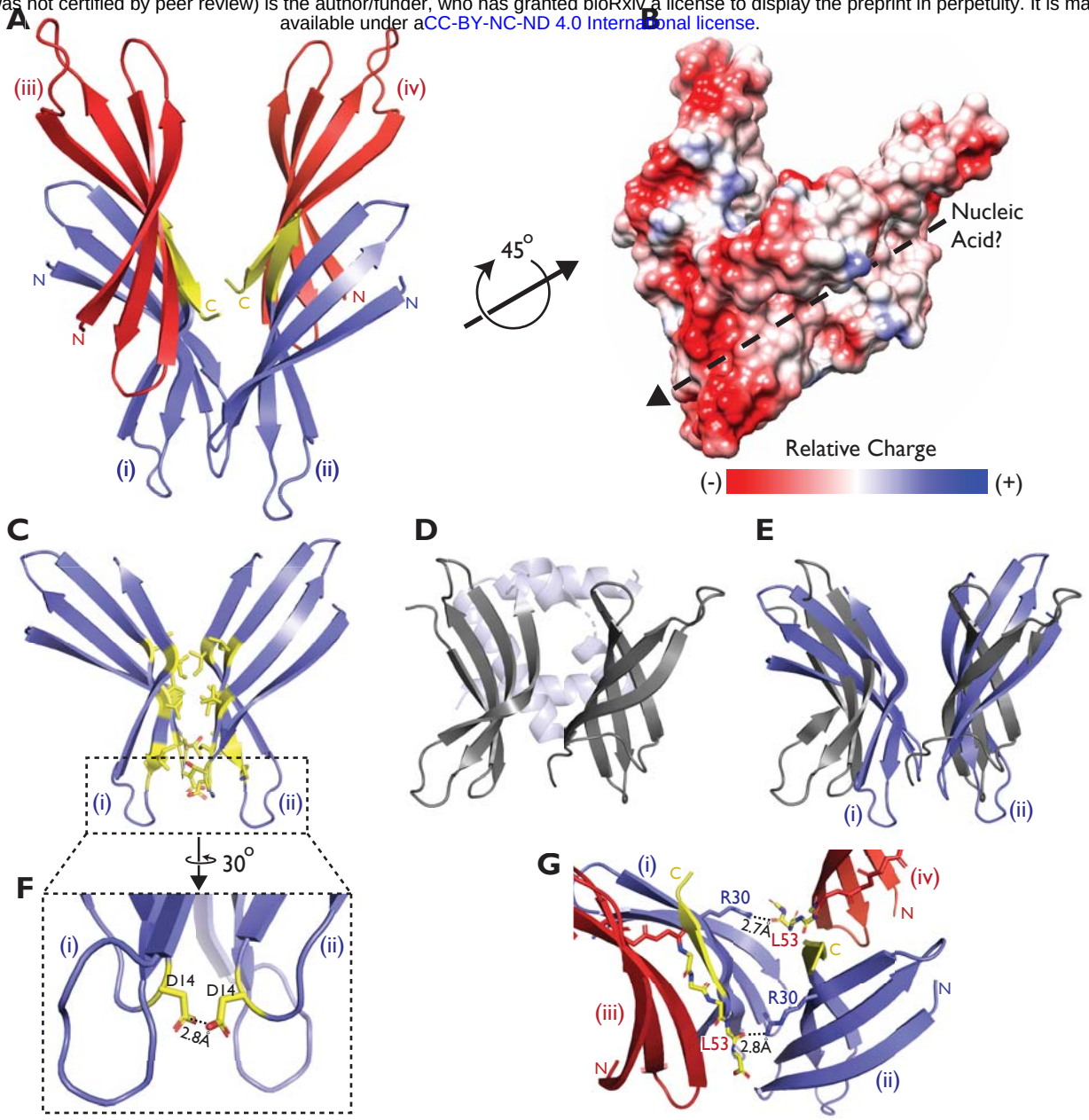
Supplemental Figure 5. **Genomic proximity of *acrIIA22* homologs to other *acr* genes.** An *acrIIA22*-encoding prophage like the one depicted in Figure 2A and those in Supplemental Figure 4A is shown. This prophage encodes for a homolog of the previously described SpyCas9 inhibitor *acrIIA17* within one kilobase of an *acrIIA22* homolog. Sequence relatedness between the depicted *acrIIA17* gene and the originally discovered acrIIA17 is shown[22]. Because phages often encode multiple *acr*s in the same locus, the co-localization of *acrIIA17* with *acrIIA22* is consistent with the latter gene functioning natively to inhibit CRISPR-Cas activity. Prophage genes are colored by functional category, per the legend and as in Supplemental Figure 4A. Contigs are numbered to indicate their descriptions in Supplemental Table 3, which contains their metadata, taxonomy, and sequence retrieval information. All sequences and annotations can also be found in Supplemental Datasets 1 and 2.



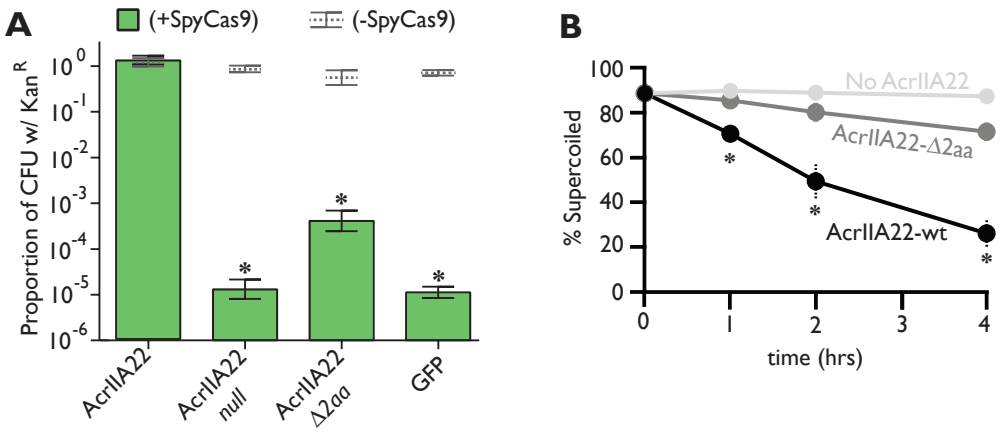
Supplemental Figure 6. **AcrIIA22 does not strongly bind SpyCas9.** SpyCas9 and sgRNA were pre-incubated before mixing with a twin-strep (TS) tagged AcrIIA22 or AcrIIA4. SpyCas9 without sgRNA was also used. Strep-Tactin pulldowns on AcrIIA4 also pulled down SpyCas9 pre-incubated with sgRNA, as previously reported[12]. Similar pulldowns with AcrIIA22 indicate little to no interaction with SpyCas9, regardless of whether sgRNA was used. These images depict total protein content visualized by Coomassie stain. Reaction components are indicated below the gel image. Asterisks (*) and dagger (†) symbols indicate AcrIIA4 and AcrIIA22 protein bands that run at slightly different positions than expected due to gel distortion.



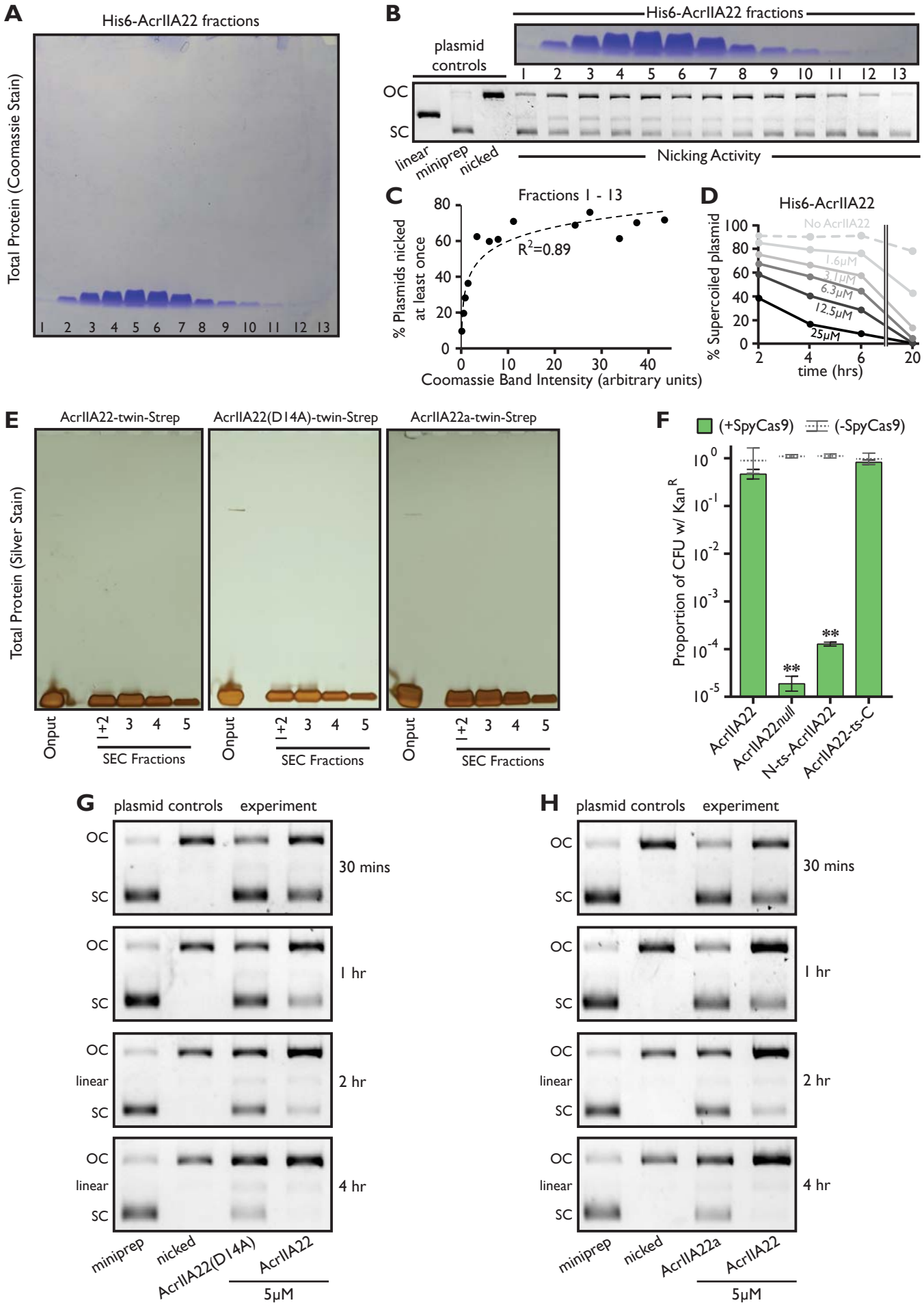
Supplemental Figure 7. **AcrIIA22 does not protect linear DNA from SpyCas9 cleavage.** (**A**) A schematic cartoon depicts the experiment in panel (B). SpyCas9 was pre-incubated with sgRNA targeting linear DNA. Then, Acr candidates were added. Subsequently, cleavage reactions were performed, and the DNA products visualized by gel electrophoresis. (**B**) We show the products of the reactions described in panel A for the inhibitors AcrIIA22 and AcrIIA4. SpyCas9 activity is greatly inhibited by AcrIIA4 but unaffected by AcrIIA22, as indicated by the proportion of cleaved DNA product. Reaction components are depicted atop the gel image, with molar equivalents relative to SpyCas9 indicated. The percent of DNA substrate cleaved by SpyCas9 is quantified below each lane. (**C**) We perform a similar experiment as in panel A, except candidate Acrs were incubated with SpyCas9 before sgRNA addition. Reactions were begun via the simultaneous addition of sgRNA and linear dsDNA instead of just dsDNA. (**D**) The products of the reactions described in panel C for AcrIIA22 and AcrIIA4 inhibitors are shown. SpyCas9 activity is inhibited by AcrIIA4 but unaffected by AcrIIA22, as indicated by the proportion of cleaved DNA product. The data depicted in this figure are not directly comparable to those in figure 7, due to methodological differences and because the preparations of SpyCas9 used in each experiment exhibited different activities.



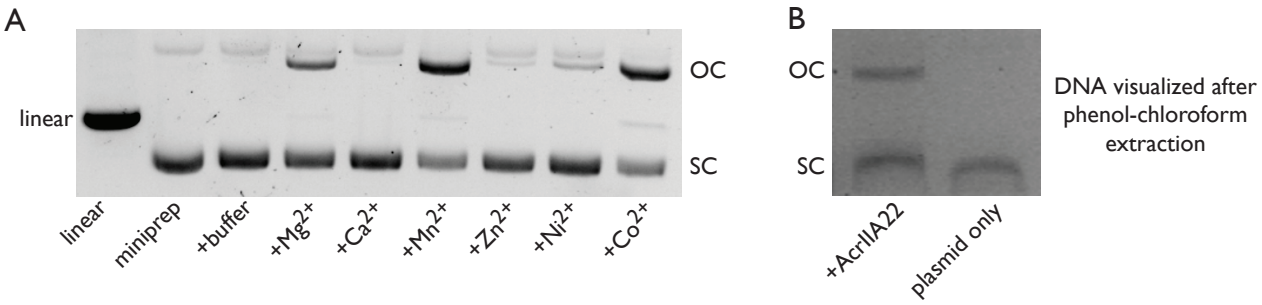
Supplemental Figure 8. **AcrIIA22 resembles a PC4-like protein.** (**A**) We present a ribbon diagram of a proposed AcrIIA22 tetramer, which requires binding between anti-parallel β-strands at the C-termini of AcrIIA22 monomers to form extended, concave β-sheets. This putative oligomerization interface is indicated by the regions highlighted in yellow. Each monomer in the proposed tetramer is labeled with lower-case Roman numerals (i-iv). (**B**) Space filling model of the tetrameric AcrIIA22 structure from panel A, with relative charge depicted, highlighting a groove (dashed line with arrowhead) that may accommodate nucleic acids (based on analogy to other PC4-like proteins). (**C**) AcrIIA22 monomers (i) and (ii) from the tetramer in panel A likely interact via a series of hydrophobic interactions, as indicated by the predominantly non-polar sidechains colored in yellow. The boxed region highlights residue D14, which is important for nicking activity and plasmid protection against SpyCas9, and is enlarged in panel F. (**D**) In conventional PC4-like family proteins, such as the putative single-stranded DNA binding protein from phage T5 depicted in gray (PDB:4BG7), the same topology of outward facing, concave β-sheets are instead stabilized via interactions between opposing α-helices (depicted in opaque light blue). (**E**) An overlay of β-sheets from AcrIIA22 (blue, PDB:7JTA) and the phage T5 PC4-like protein (gray, PDB:4BG7) illustrates their similar topologies. (**F**) Two D14 residues in loop regions of AcrIIA22 monomers (i) and (ii) are in close proximity. These residues are important for nicking activity and may bind divalent cations in cells under physiological pH. (**G**) A close view of a putative salt bridge between R30 of monomers (i) / (ii) and the peptide backbone of the C-terminus of monomers (iv) / (iii), respectively. AcrIIA22 monomers are colored as described in panel A.



Supplemental Figure 9. A 2-aa truncation mutant of AcrIIA22 is impaired for SpyCas9 inhibition and nicking activity. (**A**) An *in vivo* plasmid protection assay. Asterisks depict statistically significant differences in plasmid retention under SpyCas9-inducing conditions with either wild-type AcrIIA22, a null mutant with an early stop codon, a 2-aa truncation, or a negative control *gfp* gene (adj. $p < 0.005$, Student's t-test, n=3). The truncation mutant retains mild but severely impaired activity, as it protects a plasmid from SpyCas9 more effectively than a null mutant ($p = 0.012$) or GFP control ($p = 0.015$). All p-values were corrected for multiple hypotheses using Bonferroni's method. (**B**) The 2-aa truncation mutant is impaired for nicking *in vitro*, relative to wild-type AcrIIA22. In both cases, 25μM of protein was used following NiNTA-based purification of an N-terminal, His6-tagged construct. An asterisk (*) denotes significant differences between AcrIIA22-treated and untreated substrates (Student's t-test, $p < 0.05$, n=3). Standard deviations are indicated by dashed lines (in most cases, the data points obscure these error bars).



Supplemental Figure 10. **AcrIIA22 nicks supercoiled plasmids.** (**A**) A Coomassie stain of an N-terminally His6-tagged AcrIIA22 construct shows no co-purifying proteins. (**B**) The nicking activity for this protein preparation (bottom) correlates with the intensity of the Coomassie-stained protein band across purification fractions (top). In each lane, supercoiled (SC) plasmid DNA represents the un-nicked fraction whereas open circle (OC) and linear DNA have been nicked at least once. (**C**) This panel is a quantification of the experiment depicted in panel B across all 13 fractions collected. (**D**) His6-AcrIIA22 nicks supercoiled plasmids in a time and concentration dependent manner. A decrease in the proportion of supercoiled plasmid DNA indicates nicking activity, as depicted in Figure 5B. (**E**) A silver stain of a C-terminally twin-strep-tagged AcrIIA22 construct shows no co-purifying proteins. Equal volumes of each protein fraction were loaded in each lane, for all samples. Fraction 4 was concentrated and used for all *in vitro* experiments. (**F**) A C-terminal, but not N-terminal twin-strep tag is compatible with AcrIIA22's ability to protect a target plasmid from SpyCas9 elimination *in vivo*. Statistically significant differences in plasmid retention between SpyCas9-inducing and non-inducing conditions were determined via a Student's t-test (n=3); '**' indicates $p \leq 0.001$. All p-values were adjusted for multiple hypotheses using the Bonferroni correction. (**G**) The D14A mutation in AcrIIA22 impairs nicking activity. Over time, the wild-type AcrIIA22-twin-strep construct consistently converts a higher fraction of plasmid DNA from its supercoiled (SC) form to an open-circle (OC) conformation than a D14A mutant. Control plasmids include a miniprepped sample and sample pre-treated with the commercial nickase, Nb.BssSI. Reaction times are indicated to the right of each image. (**H**) AcrIIA22a (Figure 3B) is impaired for nicking activity relative to AcrIIA22. As in panel G, both constructs were purified via C-terminal twin-strep tags.



Supplemental Figure 11. Divalent cations influence AcrIIA22's nicking activity. (**A**) We present the impact of different divalent cations on AcrIIA22's nicking activity, which is highest with Mg^{2+}, Mn^{2+}, and Co^{2+}. OC, open-circle plasmid form. SC, supercoiled plasmid. (**B**) The open-circle plasmid product persists through phenol-chloroform extraction following AcrIIA22 treatment, indicating that it directly results from AcrIIA22's nicking activity.

Supplemental Table 1. Whether all known anti-CRISPRs can bind Cas proteins or inhibit their cleavage activity as purified proteins.

Acr	Binds cognate Cas protein?	Inhibit as pure proteins?	References
AcrIIA1	Yes	No	(Osuna et al., 2020)
AcrIIA2	Yes	Yes	(Jiang et al., 2019; Liu et al., 2019)
AcrIIA3	unknown	unknown	(Rauch et al., 2017)
AcrIIA4	Yes	Yes	(Dong et al., 2017; Shin et al., 2017; Yang and Patel, 2017)
AcrIIA5	Yes	Yes	(An et al., 2020; Garcia et al., 2019; Song et al., 2019)
AcrIIA6	Yes	Yes	(Fuchsbauer et al., 2019)
AcrIIA7	No	Yes	(Uribe et al., 2019)
AcrIIA8	Yes	Yes	(Uribe et al., 2019)
AcrIIA9	Yes	Yes	(Uribe et al., 2019)
AcrIIA10	Yes	Yes	(Uribe et al., 2019)
AcrIIA11	Yes	Yes	(Forsberg et al., 2019)
AcrIIA12	probable	Yes	(Eitzinger et al., 2020; Osuna et al., 2020)
AcrIIA13	unknown	Yes	(Watters et al., 2020)
AcrIIA14	unknown	Yes	(Watters et al., 2020)
AcrIIA15	unknown	Yes	(Watters et al., 2020)
AcrIIA16	Yes	Yes	(Mahendra et al., 2020)
AcrIIA17	Yes	No	(Mahendra et al., 2020)
AcrIIA18	Yes	No	(Mahendra et al., 2020)
AcrIIA19	Yes	No	(Mahendra et al., 2020)
AcrIIA20	unknown	Yes	(Eitzinger et al., 2020)
AcrIIA21	unknown	Yes	(Eitzinger et al., 2020)
AcrIIA22	**No**	**No**	**This study**
AcrIIA23	unknown	unknown	(Varble et al., 2020)
AcrIIC1	Yes	Yes	(Pawluk et al., 2016)
AcrIIC2	Yes	Yes	(Pawluk et al., 2016)
AcrIIC3	Yes	Yes	(Pawluk et al., 2016)
AcrIIC4	Yes	Yes	(Lee et al., 2018)
AcrIIC5	Yes	Yes	(Lee et al., 2018)
AcrVA1	Yes	Yes	(Knott et al., 2019b; Watters et al., 2018; Zhang et al., 2019)
AcrVA2	unknown	unknown	(Marino et al., 2018)
AcrVA3	unknown	unknown	(Marino et al., 2018)

AcrVA4	Yes	Yes	(Knott et al., 2019a; Knott et al., 2019b; Watters et al., 2018; Zhang et al., 2019)
AcrVA5	transiently	Yes	(Knott et al., 2019b; Watters et al., 2018; Zhang et al., 2019)
AcrVIA1(Lse)	Yes	Yes	(Meeske et al., 2020)
AcrVIA1(Lwa)	Yes	unknown	(Lin et al., 2020)
AcrVIA2	Yes	unknown	(Lin et al., 2020)
AcrVIA3	Yes	unknown	(Lin et al., 2020)
AcrVIA4	Yes	unknown	(Lin et al., 2020)
AcrVIA5	Yes	unknown	(Lin et al., 2020)
AcrVIA6	Yes	unknown	(Lin et al., 2020)
AcrVIA7	unknown	unknown	(Lin et al., 2020)
AcrIB1	unknown	unknown	(Lin et al., 2020)
AcrIC1	unknown	unknown	(Leon et al., 2020)
AcrIC2	probable	unknown	(Leon et al., 2020)
AcrIC3	unknown	unknown	(Leon et al., 2020)
AcrIC4	probable	unknown	(Leon et al., 2020)
AcrIC5	probable	unknown	(Leon et al., 2020)
AcrIC6	unknown	unknown	(Leon et al., 2020)
AcrIC7	probable	unknown	(Leon et al., 2020)
AcrIC8	probable	unknown	(Leon et al., 2020)
AcrID1	Yes	unknown	(He et al., 2018)
AcrIE1	Yes	unknown	(Pawluk et al., 2017)
AcrIE2	unknown	unknown	(Pawluk et al., 2014)
AcrIE3	probable	unknown	(Stanley, 2018)
AcrIE4	unknown	unknown	(Pawluk et al., 2014)
AcrIE5	unknown	unknown	(Pawluk et al., 2014)
AcrIE6	unknown	unknown	(Pawluk et al., 2014)
AcrIE7	unknown	unknown	(Pawluk et al., 2014)
AcrIE4-IF7	unknown	unknown	(Marino et al., 2018)
AcrIE8	unknown	unknown	(Pinilla-Redondo et al., 2020)
AcrIF1	Yes	unknown	(Bondy-Denomy et al., 2015; Chowdhury et al., 2017; Guo et al., 2017)
AcrIF2	Yes	unknown	(Bondy-Denomy et al., 2015; Chowdhury et al., 2017; Guo et al., 2017)
AcrIF3	Yes	unknown	(Bondy-Denomy et al., 2015; Wang et al., 2016a; Wang et al., 2016b)
AcrIF4	Yes	unknown	(Bondy-Denomy et al., 2015)
AcrIF5	unknown	unknown	(Bondy-Denomy et al., 2013)

AcrIF6	Yes	Yes	(Zhang et al., 2020)
AcrIF7	Yes	unknown	(Hirschi et al., 2020)
AcrIF8	Yes	Yes	(Zhang et al., 2020)
AcrIF9	Yes	Yes	(Hirschi et al., 2020; Zhang et al., 2020)
AcrIF10	Yes	unknown	(Guo et al., 2017)
AcrIF11	unknown	unknown	(Marino et al., 2018)
AcrIF12	unknown	unknown	(Marino et al., 2018)
AcrIF13	unknown	unknown	(Marino et al., 2018)
AcrIF14	unknown	unknown	(Marino et al., 2018)
AcrIF15	probable	unknown	(Pinilla-Redondo et al., 2020)
AcrIF16	unknown	unknown	(Pinilla-Redondo et al., 2020)
AcrIF17	unknown	unknown	(Pinilla-Redondo et al., 2020)
AcrIF18	probable	unknown	(Pinilla-Redondo et al., 2020)
AcrIF19	unknown	unknown	(Pinilla-Redondo et al., 2020)
AcrIF20	unknown	unknown	(Pinilla-Redondo et al., 2020)
AcrIF21	unknown	unknown	(Pinilla-Redondo et al., 2020)
AcrIF22	unknown	unknown	(Pinilla-Redondo et al., 2020)
AcrIF23	unknown	unknown	(Pinilla-Redondo et al., 2020)
AcrIF24	unknown	unknown	(Pinilla-Redondo et al., 2020)
AcrIII-1	No (degrades CA4 second messenger)	No	(Athukoralage et al., 2020)
AcrIIIB1	Yes	unknown	(Bhoobalan-Chitty et al., 2019)

Supplemental Table 2. PC4-like proteins with structural homology to AcrIIA22

Structural Homolog		Function	Similarity to AcrIIA22			
PDBID	Name	DNA/RNA Binding*	Zscore	r.m.s.d.	n-align	% A.A. ID
4bg7	PC4 putative transcriptional coactivator p15	DNA	6.2	2.5	54	15
3k44	*D. melanogaster* Pur-α	DNA/RNA	5.9	2.6	47	9
5fgp	Pur-α repeat I and II from *D. melanogaster*	DNA/RNA	5.6	2.1	48	8
3n8b	Pur-α from *B. burgdorferi*	DNA/RNA	5	2.8	48	6
2gje	Mitochondrial RNA Binding Protein (*T. brucei*)	RNA	4.9	2.5	52	8
5zkl	Protein of unknown function SP_0782, *S. pneumoniae*	DNA	4.7	3.6	52	12
5fgo	*D. melanogaster* Pur-α repeat III	No info	4.5	2.7	44	14
1pcf	Replication & transcription cofactor PC4 CTD	DNA	4.5	2.5	45	7
2ltt	Putative Uncharacterized Protein YDBC	DNA	4.5	2.8	50	12
4bhm	MoSub1-DNA PC4 transcription cofactor	DNA	3.9	2.8	45	4
3cm1	SSGA-like sporulation specific cell division protein	No info	2.8	3.7	47	13
1l3a	Transcription factor PBF-2 (P24, WHY1)	DNA	2.8	5	48	8
4ntq	Anti-toxin CdiI, *E. cloacae*	No info	2.7	3	49	12
3n1k	WHY2 transcription factor, *S. tuberosum*	DNA	2.6	2.8	52	4

*RNA/DNA binding data from (Janowski and Niessing, 2020).

Supplemental Table 3. All sequences used in this study. Sequence names and databases are indicated. All sequences and annotations are also available as supplemental data. Sequences retrieved from Pasolli *et al.* refer to the following study: (Pasolli et al., 2019).

Contig No.	Sequence Name	How Used?	NCBI Nuc. ID	Pasolli et al SGB?	Pasolli et al Raw Assembly?	Pasolli Reconstructed Genome Name	SGB id	GTDB Taxonomy	External Data Available at:
1	4303_LiJ_2014__V1.UC63-0__bin.67_NODE_112_length_95405_cov_4.60675	Figure 2, find gene functions from 54 unique genomic loci; Figure 3 Acr Seq	n/a	Yes	No	LiJ_2014__V1.UC63-0__bin.67	4303	d__Bacteria; p__Firmicutes_A; c__Clostridia; o__Oscillospirales; f__Acutalibacteraceae; g__CAG-217	http://segatalab.cibio.unitn.it/data/Pasolli_et_al.html
2	Bengtsson-PalmeJ_2015__TRAVELRES9_NODE_4_length_538580_cov_9.43148	find gene functions from 54 unique genomic loci	n/a	No	Yes	n/a	n/a	d__Bacteria; p__Firmicutes_A; c__Clostridia; o__Oscillospirales; f__Acutalibacteraceae; g__CAG-217 (inferred)	http://segatalab.cibio.unitn.it/data/Pasolli_et_al.html
3	ChengpingW_2017__AS9raw_NODE_922_length_27664_cov_3.49089	find gene functions from 54 unique genomic loci	n/a	No	Yes	n/a	n/a	d__Bacteria; p__Firmicutes_A; c__Clostridia; o__Oscillospirales; f__Acutalibacteraceae; g__CAG-217 (inferred)	http://segatalab.cibio.unitn.it/data/Pasolli_et_al.html
4	CosteaPI_2017__SID713B025-11-0-0_NODE_4_length_351620_cov_7.46108	find gene functions from 54 unique genomic loci	n/a	No	Yes	n/a	n/a	d__Bacteria; p__Firmicutes_A; c__Clostridia; o__Oscillospirales; f__Acutalibacteraceae; g__CAG-217 (inferred)	http://segatalab.cibio.unitn.it/data/Pasolli_et_al.html
5	BritoIL_2016__M1.64.ST_NODE_47_length_140472_cov_9.49805	find gene functions from 54 unique genomic loci; Figure 3 Acr Seq	n/a	No	Yes	n/a	n/a	d__Bacteria; p__Firmicutes_A; c__Clostridia; o__Oscillospirales; f__Acutalibacteraceae; g__CAG-217 (inferred)	http://segatalab.cibio.unitn.it/data/Pasolli_et_al.html
6	BritoIL_2016__M2.57.ST_NODE_3_length_405636_cov_14.0428	find gene functions from 54 unique genomic loci	n/a	No	Yes	n/a	n/a	d__Bacteria; p__Firmicutes_A; c__Clostridia; o__Oscillospirales; f__Acutalibacteraceae; g__CAG-217 (inferred)	http://segatalab.cibio.unitn.it/data/Pasolli_et_al.html
7	BritoIL_2016__WL.14.ST_NODE_13_length_259523_cov_10.8408	find gene functions from 54 unique genomic loci	n/a	No	Yes	n/a	n/a	d__Bacteria; p__Firmicutes_A; c__Clostridia; o__Oscillospirales; f__Acutalibacteraceae; g__CAG-217 (inferred)	http://segatalab.cibio.unitn.it/data/Pasolli_et_al.html
8	ChengpingW_2017__AS67raw_NODE_2_length_439177_cov_9.00174	Figure 2, find gene functions from 54 unique genomic loci; Figure 3 Acr Seq	n/a	No	Yes	n/a	n/a	d__Bacteria; p__Firmicutes_A; c__Clostridia; o__Oscillospirales; f__Acutalibacteraceae; g__CAG-217 (inferred)	http://segatalab.cibio.unitn.it/data/Pasolli_et_al.html
9	CM_madagascar__A90_04_1FE_NODE_125_length_81453_cov_9.00904	find gene functions from 54 unique genomic loci	n/a	No	Yes	n/a	n/a	d__Bacteria; p__Firmicutes_A; c__Clostridia; o__Oscillospirales; f__Acutalibacteraceae; g__CAG-217 (inferred)	http://segatalab.cibio.unitn.it/data/Pasolli_et_al.html
10	CM_madagascar__V12_01_2FE_NODE_5_length_202628_cov_9.50435	find gene functions from 54 unique genomic loci	n/a	No	Yes	n/a	n/a	d__Bacteria; p__Firmicutes_A; c__Clostridia; o__Oscillospirales; f__Acutalibacteraceae; g__CAG-217 (inferred)	http://segatalab.cibio.unitn.it/data/Pasolli_et_al.html
11	CosteaPI_2017__SID713A046-11-0-0_NODE_322_length_69000_cov_4.32987	find gene functions from 54 unique genomic loci; Figure 3 Acr Seq	n/a	No	Yes	n/a	n/a	d__Bacteria; p__Firmicutes_A; c__Clostridia; o__Oscillospirales; f__Acutalibacteraceae; g__CAG-217 (inferred)	http://segatalab.cibio.unitn.it/data/Pasolli_et_al.html
12	CosteaPI_2017__SID713A045-11-0-0_NODE_78_length_64886_cov_3.97493	find gene functions from 54 unique genomic loci	n/a	No	Yes	n/a	n/a	d__Bacteria; p__Firmicutes_A; c__Clostridia; o__Oscillospirales; f__Acutalibacteraceae; g__CAG-217 (inferred)	http://segatalab.cibio.unitn.it/data/Pasolli_et_al.html
13	CosteaPI_2017__SID713A004-11-0-0_NODE_1_length_647860_cov_14.1013	find gene functions from 54 unique genomic loci; Figure 3 Acr Seq	n/a	No	Yes	n/a	n/a	d__Bacteria; p__Firmicutes_A; c__Clostridia; o__Oscillospirales; f__Acutalibacteraceae; g__CAG-217 (inferred)	http://segatalab.cibio.unitn.it/data/Pasolli_et_al.html
14	CosteaPI_2017__peacemaker-11-60-	find gene functions from 54 unique genomic loci	n/a	No	Yes	n/a	n/a	d__Bacteria; p__Firmicutes_A; c__Clostridia; o__Oscillospirales; f__Acutalibacteraceae; g__CAG-217 (inferred)	http://segatalab.cibio.unitn.it/data/Pasolli_et_al.html

	0_NODE_48_length_49378_cov_15.5445								
15	CosteaPI_2017__SID713A063-11-90-0_NODE_2082_length_16960_cov_2.98527	find gene functions from 54 unique genomic loci	n/a	No	Yes	n/a	n/a	d__Bacteria; p__Firmicutes_A; c__Clostridia; o__Oscillospirales; f__Acutalibacteraceae; g__CAG-217 (inferred)	http://segatalab.cibio.unitn.it/data/Pasolli_et_al.html
16	CosteaPI_2017__SID713A088-11-0-0_NODE_89_length_113297_cov_5.77445	find gene functions from 54 unique genomic loci; Figure 3 Acr Seq	n/a	No	Yes	n/a	n/a	d__Bacteria; p__Firmicutes_A; c__Clostridia; o__Oscillospirales; f__Acutalibacteraceae; g__CAG-217 (inferred)	http://segatalab.cibio.unitn.it/data/Pasolli_et_al.html
17	CosteaPI_2017__SID713A062-11-0-0_NODE_38_length_192196_cov_4.03099	Find gene functions from 54 unique genomic loci	n/a	No	Yes	n/a	n/a	d__Bacteria; p__Firmicutes_A; c__Clostridia; o__Oscillospirales; f__Acutalibacteraceae; g__CAG-217 (inferred)	http://segatalab.cibio.unitn.it/data/Pasolli_et_al.html
18	CosteaPI_2017__SID713B051-11-0-0_NODE_14_length_298619_cov_7.20988	find gene functions from 54 unique genomic loci	n/a	No	Yes	n/a	n/a	d__Bacteria; p__Firmicutes_A; c__Clostridia; o__Oscillospirales; f__Acutalibacteraceae; g__CAG-217 (inferred)	http://segatalab.cibio.unitn.it/data/Pasolli_et_al.html
19	FengQ_2015__SID31872_NODE_2_length_392843_cov_5.93617	find gene functions from 54 unique genomic loci	n/a	No	Yes	n/a	n/a	d__Bacteria; p__Firmicutes_A; c__Clostridia; o__Oscillospirales; f__Acutalibacteraceae; g__CAG-217 (inferred)	http://segatalab.cibio.unitn.it/data/Pasolli_et_al.html
20	FengQ_2015__SID530258_NODE_5_length_350476_cov_17.595	find gene functions from 54 unique genomic loci; Figure 3 Acr Seq	n/a	No	Yes	n/a	n/a	d__Bacteria; p__Firmicutes_A; c__Clostridia; o__Oscillospirales; f__Acutalibacteraceae; g__CAG-217 (inferred)	http://segatalab.cibio.unitn.it/data/Pasolli_et_al.html
21	FengQ_2015__SID530373_NODE_21_length_272157_cov_9.73468	find gene functions from 54 unique genomic loci	n/a	No	Yes	n/a	n/a	d__Bacteria; p__Firmicutes_A; c__Clostridia; o__Oscillospirales; f__Acutalibacteraceae; g__CAG-217 (inferred)	http://segatalab.cibio.unitn.it/data/Pasolli_et_al.html
22	HeQ_2017__SZAXPI029561-52_NODE_1_length_502752_cov_8.09488	find gene functions from 54 unique genomic loci; Figure 3 Acr Seq	n/a	No	Yes	n/a	n/a	d__Bacteria; p__Firmicutes_A; c__Clostridia; o__Oscillospirales; f__Acutalibacteraceae; g__CAG-217 (inferred)	http://segatalab.cibio.unitn.it/data/Pasolli_et_al.html
23	HeQ_2017__SZAXPI029575-90_NODE_229_length_94918_cov_3.79903	find gene functions from 54 unique genomic loci	n/a	No	Yes	n/a	n/a	d__Bacteria; p__Firmicutes_A; c__Clostridia; o__Oscillospirales; f__Acutalibacteraceae; g__CAG-217 (inferred)	http://segatalab.cibio.unitn.it/data/Pasolli_et_al.html
24	KarlssonFH_2013__S463_NODE_1_length_570037_cov_16.3973	Figure 2, find gene functions from 54 unique genomic loci	n/a	No	Yes	n/a	n/a	d__Bacteria; p__Firmicutes_A; c__Clostridia; o__Oscillospirales; f__Acutalibacteraceae; g__CAG-217 (inferred)	http://segatalab.cibio.unitn.it/data/Pasolli_et_al.html
25	LiJ_2014__O2.UC12-1_NODE_323_length_49995_cov_5.04395	find gene functions from 54 unique genomic loci	n/a	No	Yes	n/a	n/a	d__Bacteria; p__Firmicutes_A; c__Clostridia; o__Oscillospirales; f__Acutalibacteraceae; g__CAG-217 (inferred)	http://segatalab.cibio.unitn.it/data/Pasolli_et_al.html
26	LiJ_2014__V1.FI02_NODE_274_length_84286_cov_3.49253	find gene functions from 54 unique genomic loci	n/a	No	Yes	n/a	n/a	d__Bacteria; p__Firmicutes_A; c__Clostridia; o__Oscillospirales; f__Acutalibacteraceae; g__CAG-217 (inferred)	http://segatalab.cibio.unitn.it/data/Pasolli_et_al.html
27	LiJ_2017__H1M413815_NODE_71_length_81514_cov_18.301	find gene functions from 54 unique genomic loci	n/a	No	Yes	n/a	n/a	d__Bacteria; p__Firmicutes_A; c__Clostridia; o__Oscillospirales; f__Acutalibacteraceae; g__CAG-217 (inferred)	http://segatalab.cibio.unitn.it/data/Pasolli_et_al.html
28	LiJ_2017__H2M514909_NODE_68_length_69076_cov_10.283	find gene functions from 54 unique genomic loci	n/a	No	Yes	n/a	n/a	d__Bacteria; p__Firmicutes_A; c__Clostridia; o__Oscillospirales; f__Acutalibacteraceae; g__CAG-217 (inferred)	http://segatalab.cibio.unitn.it/data/Pasolli_et_al.html
29	LiuW_2016__SRR3992969_NODE_1149_length_18999_cov_8.45033	find gene functions from 54 unique genomic loci; Figure 3 Acr Seq	n/a	No	Yes	n/a	n/a	d__Bacteria; p__Firmicutes_A; c__Clostridia; o__Oscillospirales; f__Acutalibacteraceae; g__CAG-217 (inferred)	http://segatalab.cibio.unitn.it/data/Pasolli_et_al.html

30	LiuW_2016__SRR3992984_NODE_127_length_61384_cov_18.0593	find gene functions from 54 unique genomic loci	n/a	No	Yes	n/a	n/a	d__Bacteria; p__Firmicutes_A; c__Clostridia; o__Oscillospirales; f__Acutalibacteraceae; g__CAG-217 (inferred)	http://segatalab.cibio.unitn.it/data/Pasolli_et_al.html
31	LiuW_2016__SRR3993014_NODE_8_length_143441_cov_89.3981	find gene functions from 54 unique genomic loci	n/a	No	Yes	n/a	n/a	d__Bacteria; p__Firmicutes_A; c__Clostridia; o__Oscillospirales; f__Acutalibacteraceae; g__CAG-217 (inferred)	http://segatalab.cibio.unitn.it/data/Pasolli_et_al.html
32	QinJ_2012__NOM001_NODE_179_length_28679_cov_2.87521	find gene functions from 54 unique genomic loci	n/a	No	Yes	n/a	n/a	d__Bacteria; p__Firmicutes_A; c__Clostridia; o__Oscillospirales; f__Acutalibacteraceae; g__CAG-217 (inferred)	http://segatalab.cibio.unitn.it/data/Pasolli_et_al.html
33	QinJ_2012__T2D-050_NODE_25_length_192521_cov_10.1129	find gene functions from 54 unique genomic loci	n/a	No	Yes	n/a	n/a	d__Bacteria; p__Firmicutes_A; c__Clostridia; o__Oscillospirales; f__Acutalibacteraceae; g__CAG-217 (inferred)	http://segatalab.cibio.unitn.it/data/Pasolli_et_al.html
34	VatanenT_2016__G78791_NODE_43_length_22491_cov_6.98654	find gene functions from 54 unique genomic loci	n/a	No	Yes	n/a	n/a	d__Bacteria; p__Firmicutes_A; c__Clostridia; o__Oscillospirales; f__Acutalibacteraceae; g__CAG-217 (inferred)	http://segatalab.cibio.unitn.it/data/Pasolli_et_al.html
35	XieH_2016__YSZC12003_35392_NODE_87_length_196476_cov_13.3023	find gene functions from 54 unique genomic loci	n/a	No	Yes	n/a	n/a	d__Bacteria; p__Firmicutes_A; c__Clostridia; o__Oscillospirales; f__Acutalibacteraceae; g__CAG-217 (inferred)	http://segatalab.cibio.unitn.it/data/Pasolli_et_al.html
36	XieH_2016__YSZC12003_35563_NODE_11_length_353850_cov_18.1068	Figure 2, find gene functions from 54 unique genomic loci	n/a	No	Yes	n/a	n/a	d__Bacteria; p__Firmicutes_A; c__Clostridia; o__Oscillospirales; f__Acutalibacteraceae; g__CAG-217 (inferred)	http://segatalab.cibio.unitn.it/data/Pasolli_et_al.html
37	XieH_2016__YSZC12003_36005_NODE_238_length_99923_cov_5.91259	Figure 2, find gene functions from 54 unique genomic loci, source of orf1-encoding phage genome; Figure 3 Acr Seq	n/a	No	Yes	n/a	n/a	d__Bacteria; p__Firmicutes_A; c__Clostridia; o__Oscillospirales; f__Acutalibacteraceae; g__CAG-217 (inferred)	http://segatalab.cibio.unitn.it/data/Pasolli_et_al.html
38	XieH_2016__YSZC12003_36794_NODE_1_length_781521_cov_10.2961	find gene functions from 54 unique genomic loci, source of orf1-encoding phage genome; Figure 3 Acr Seq	n/a	No	Yes	n/a	n/a	d__Bacteria; p__Firmicutes_A; c__Clostridia; o__Oscillospirales; f__Acutalibacteraceae; g__CAG-217 (inferred)	http://segatalab.cibio.unitn.it/data/Pasolli_et_al.html
39	XieH_2016__YSZC12003_37133_NODE_3_length_676817_cov_24.9073	find gene functions from 54 unique genomic loci	n/a	No	Yes	n/a	n/a	d__Bacteria; p__Firmicutes_A; c__Clostridia; o__Oscillospirales; f__Acutalibacteraceae; g__CAG-217 (inferred)	http://segatalab.cibio.unitn.it/data/Pasolli_et_al.html
40	XieH_2016__YSZC12003_37322_NODE_5_length_601737_cov_115.712	find gene functions from 54 unique genomic loci	n/a	No	Yes	n/a	n/a	d__Bacteria; p__Firmicutes_A; c__Clostridia; o__Oscillospirales; f__Acutalibacteraceae; g__CAG-217 (inferred)	http://segatalab.cibio.unitn.it/data/Pasolli_et_al.html
41	XieH_2016__YSZC12003_37399_NODE_3_length_598430_cov_49.9887	find gene functions from 54 unique genomic loci	n/a	No	Yes	n/a	n/a	d__Bacteria; p__Firmicutes_A; c__Clostridia; o__Oscillospirales; f__Acutalibacteraceae; g__CAG-217 (inferred)	http://segatalab.cibio.unitn.it/data/Pasolli_et_al.html
42	XieH_2016__YSZC12003_37878_NODE_8_length_402183_cov_76.149	find gene functions from 54 unique genomic loci	n/a	No	Yes	n/a	n/a	d__Bacteria; p__Firmicutes_A; c__Clostridia; o__Oscillospirales; f__Acutalibacteraceae; g__CAG-217 (inferred)	http://segatalab.cibio.unitn.it/data/Pasolli_et_al.html
43	YuJ_2015__SZAXPI003435-11_NODE_1_length_772218_cov_11.5924	find gene functions from 54 unique genomic loci	n/a	No	Yes	n/a	n/a	d__Bacteria; p__Firmicutes_A; c__Clostridia; o__Oscillospirales; f__Acutalibacteraceae; g__CAG-217 (inferred)	http://segatalab.cibio.unitn.it/data/Pasolli_et_al.html
44	YuJ_2015__SZAXPI015230-16_NODE_32_length_174349_cov_17.3543	find gene functions from 54 unique genomic loci	n/a	No	Yes	n/a	n/a	d__Bacteria; p__Firmicutes_A; c__Clostridia; o__Oscillospirales; f__Acutalibacteraceae; g__CAG-217 (inferred)	http://segatalab.cibio.unitn.it/data/Pasolli_et_al.html
45	ZeeviD_2015__PNP_DietIntervention_11_NODE_16_le	find gene functions from 54 unique genomic loci	n/a	No	Yes	n/a	n/a	d__Bacteria; p__Firmicutes_A; c__Clostridia; o__Oscillospirales; f__Acutalibacteraceae; g__CAG-217 (inferred)	http://segatalab.cibio.unitn.it/data/Pasolli_et_al.html

	ngth_97163_cov_10.0000_ID_22997								
46	ZeeviD_2015__PNP_Main_234_NODE_10_length_202229_cov_13.9987_ID_180729	find gene functions from 54 unique genomic loci	n/a	No	Yes	n/a	n/a	d__Bacteria; p__Firmicutes_A; c__Clostridia; o__Oscillospirales; f__Acutalibacteraceae; g__CAG-217 (inferred)	http://segatalab.cibio.unitn.it/data/Pasolli_et_al.html
47	ZeeviD_2015__PNP_Main_294_NODE_20_length_208110_cov_20.9981_ID_106095	find gene functions from 54 unique genomic loci	n/a	No	Yes	n/a	n/a	d__Bacteria; p__Firmicutes_A; c__Clostridia; o__Oscillospirales; f__Acutalibacteraceae; g__CAG-217 (inferred)	http://segatalab.cibio.unitn.it/data/Pasolli_et_al.html
48	ZeeviD_2015__PNP_Main_390_NODE_33_length_137723_cov_10.9985_ID_46475	find gene functions from 54 unique genomic loci	n/a	No	Yes	n/a	n/a	d__Bacteria; p__Firmicutes_A; c__Clostridia; o__Oscillospirales; f__Acutalibacteraceae; g__CAG-217 (inferred)	http://segatalab.cibio.unitn.it/data/Pasolli_et_al.html
49	ZeeviD_2015__PNP_Main_578_NODE_20_length_138741_cov_8.9988_ID_132563	find gene functions from 54 unique genomic loci	n/a	No	Yes	n/a	n/a	d__Bacteria; p__Firmicutes_A; c__Clostridia; o__Oscillospirales; f__Acutalibacteraceae; g__CAG-217 (inferred)	http://segatalab.cibio.unitn.it/data/Pasolli_et_al.html
50	ZeeviD_2015__PNP_Main_741_NODE_13_length_214417_cov_12.0572_ID_91679	find gene functions from 54 unique genomic loci	n/a	No	Yes	n/a	n/a	d__Bacteria; p__Firmicutes_A; c__Clostridia; o__Oscillospirales; f__Acutalibacteraceae; g__CAG-217 (inferred)	http://segatalab.cibio.unitn.it/data/Pasolli_et_al.html
51	ZellerG_2014__CCIS03857607ST-4-0_NODE_542_length_35291_cov_2.7674	find gene functions from 54 unique genomic loci	n/a	No	Yes	n/a	n/a	d__Bacteria; p__Firmicutes_A; c__Clostridia; o__Oscillospirales; f__Acutalibacteraceae; g__CAG-217 (inferred)	http://segatalab.cibio.unitn.it/data/Pasolli_et_al.html
52	ZellerG_2014__CCIS22958137ST-20-0_NODE_40_length_181493_cov_7.91373	find gene functions from 54 unique genomic loci, source of orf1-encoding phage genome; Figure 3 Acr Seq	n/a	No	Yes	n/a	n/a	d__Bacteria; p__Firmicutes_A; c__Clostridia; o__Oscillospirales; f__Acutalibacteraceae; g__CAG-217 (inferred)	http://segatalab.cibio.unitn.it/data/Pasolli_et_al.html
53	XieH_2016__YSZC12003_35635_NODE_109_length_156568_cov_5.12141	find gene functions from 54 unique genomic loci	n/a	No	Yes	n/a	n/a	d__Bacteria; p__Firmicutes_A; c__Clostridia; o__Oscillospirales; f__Acutalibacteraceae; g__CAG-217 (inferred)	http://segatalab.cibio.unitn.it/data/Pasolli_et_al.html
54	ZeeviD_2015__PNP_Main_85_NODE_182_length_52997_cov_7.0000_ID_133080	find gene functions from 54 unique genomic loci	n/a	No	Yes	n/a	n/a	d__Bacteria; p__Firmicutes_A; c__Clostridia; o__Oscillospirales; f__Acutalibacteraceae; g__CAG-217 (inferred)	http://segatalab.cibio.unitn.it/data/Pasolli_et_al.html
55	4303_HeQ_2017__SZAXPI029570-85__bin.1_NODE_2_length_608092_cov_26.3259	Figure 2	n/a	Yes	No	HeQ_2017__SZAXPI029570-85__bin.1	4303	d__Bacteria; p__Firmicutes_A; c__Clostridia; o__Oscillospirales; f__Acutalibacteraceae; g__CAG-217	http://segatalab.cibio.unitn.it/data/Pasolli_et_al.html
56	4303_CosteaPI_2017__SID713B074-11-90-0__bin.57_NODE_18_length_238289_cov_5.37382	Source of orf1-encoding phage genome; Figure 3 Acr Seq	n/a	Yes	No	CosteaPI_2017__SID713B074-11-90-0__bin.57	4303	d__Bacteria; p__Firmicutes_A; c__Clostridia; o__Oscillospirales; f__Acutalibacteraceae; g__CAG-217	http://segatalab.cibio.unitn.it/data/Pasolli_et_al.html
57	Clostridiales_bacterium_isolate_CIM:MAG_317_1 contig_8085	Figure 2, source of orf1-encoding phage genome	QALM010000002.1	No	No	n/a	n/a	d__Bacteria; p__Firmicutes_A; c__Clostridia; o__Oscillospirales; f__Acutalibacteraceae; g__CAG-217 (inferred)	NCBI Genbank
58	TPA_asm:_Ruminococcaceae_bacterium_isolate_UBA8277_contig_226	Figure 3 Acr Seq	DPDR010001 0.1	No	No	n/a	n/a	d__Bacteria; p__Firmicutes_A; c__Clostridia; o__Oscillospirales; f__Acutalibacteraceae; g__CAG-217 (inferred)	NCBI Genbank
59	KarlssonFH_2013__S424_NODE_2_length_526279_cov_9.22761	Source of orf1-encoding phage genome	n/a	No	Yes	n/a	n/a	d__Bacteria; p__Firmicutes_A; c__Clostridia; o__Oscillospirales; f__Acutalibacteraceae; g__CAG-217 (inferred)	http://segatalab.cibio.unitn.it/data/Pasolli_et_al.html

60	XieH_2016__YSZC12003_36696_NODE_1_length_776477_cov_39.8546	Source of orf1-encoding phage genome, AcrIIA17 encoding phage (figure S5)	n/a	No	Yes	n/a	n/a	d__Bacteria; p__Firmicutes_A; c__Clostridia; o__Oscillospirales; f__Acutalibacteraceae; g__CAG-217 (inferred)	http://segatalab.cibio.unitn.it/data/Pasolli_et_al.html
61	XieH_2016__YSZC12003_37308R1_NODE_3_length_717276_cov_26.9646	source of orf1-encoding phage genome	n/a	No	Yes	n/a	n/a	d__Bacteria; p__Firmicutes_A; c__Clostridia; o__Oscillospirales; f__Acutalibacteraceae; g__CAG-217 (inferred)	http://segatalab.cibio.unitn.it/data/Pasolli_et_al.html
62	ZellerG_2014__CCIS88007743ST-4-0_NODE_31_length_210910_cov_8.07406	source of orf1-encoding phage genome	n/a	No	Yes	n/a	n/a	d__Bacteria; p__Firmicutes_A; c__Clostridia; o__Oscillospirales; f__Acutalibacteraceae; g__CAG-217 (inferred)	http://segatalab.cibio.unitn.it/data/Pasolli_et_al.html
63	4303_QinN_2014__LD-22__bin.75_NODE_22_length_329763_cov_10.7401	Figure 3 Acr Seq	n/a	Yes	No	QinN_2014__LD-22__bin.75	4303	d__Bacteria; p__Firmicutes_A; c__Clostridia; o__Oscillospirales; f__Acutalibacteraceae; g__CAG-217	http://segatalab.cibio.unitn.it/data/Pasolli_et_al.html
64	ZellerG_2014__CCMD25963797ST-21-0_NODE_9_length_356111_cov_10.1715	Figure 3 Acr Seq	n/a	No	Yes	n/a	n/a	d__Bacteria; p__Firmicutes_A; c__Clostridia; o__Oscillospirales; f__Acutalibacteraceae; g__CAG-217 (inferred)	http://segatalab.cibio.unitn.it/data/Pasolli_et_al.html
65	ZellerG_2014__CCIS41222843ST-4-0_NODE_17_length_267133_cov_14.7383	Figure 3 Acr Seq	n/a	No	Yes	n/a	n/a	d__Bacteria; p__Firmicutes_A; c__Clostridia; o__Oscillospirales; f__Acutalibacteraceae; g__CAG-217 (inferred)	http://segatalab.cibio.unitn.it/data/Pasolli_et_al.html
66	FengQ_2015__SID530168_NODE_20_length_224404_cov_6.02914	Figure 3 Acr Seq	n/a	No	Yes	n/a	n/a	d__Bacteria; p__Firmicutes_A; c__Clostridia; o__Oscillospirales; f__Acutalibacteraceae; g__CAG-217 (inferred)	http://segatalab.cibio.unitn.it/data/Pasolli_et_al.html
67	FengQ_2015__SID530041_NODE_7_length_421742_cov_9.32571	Figure 3 Acr Seq	n/a	No	Yes	n/a	n/a	d__Bacteria; p__Firmicutes_A; c__Clostridia; o__Oscillospirales; f__Acutalibacteraceae; g__CAG-217 (inferred)	http://segatalab.cibio.unitn.it/data/Pasolli_et_al.html
68	FengQ_2015__SID31223_NODE_13_length_228767_cov_7.50553	Figure 3 Acr Seq	n/a	No	Yes	n/a	n/a	d__Bacteria; p__Firmicutes_A; c__Clostridia; o__Oscillospirales; f__Acutalibacteraceae; g__CAG-217 (inferred)	http://segatalab.cibio.unitn.it/data/Pasolli_et_al.html

Supplemental Table 4. Plasmids used in this study. Supplemental Table S5 indicates genes expressed from pZE21_tetR.

Plasmid	crRNA promoter, sequence (5'-3')	Notes	Refs	Purpose
pZE21_tetR	n/a	Contains tetR behind pLac promoter for inducible expression of candidate Acrs. Targeted by crRNA_A; PAM = AGG.	(Forsberg et al., 2019)	Expressing genes to test *in vivo* anti-CRISPR activity
pSpyCas9 _crA	pJ23100, GTTCATTCAGGGCACCGGAC	Arabinose-inducible SpyCas9 with pZE21 targeting pZE21_tetR	(Forsberg et al., 2019)	Target pZE21_tetR for elimination with SpyCas9
pSpyCas9 _crMu	pJ23100, GTAATACTTGTCCCGCAAAG	Mu-targeting spacer for phage Mu immunity testing. Otherwise identical to pSpyCas9_crA	(Forsberg et al., 2019)	Phage Mu immunity testing
pSpyCa9_crNT	pJ23100, GAACGAAAAGCTGCGCCGGG	non-targeting spacer used as control. Otherwise identical to pSpyCas9_crA	(Forsberg et al., 2019)	Phage Mu immunity testing, Western blots
pCloDF13_GFP	pJ23100, GAACGAAAAGCTGCGCCGGG	*eGFP* gene replaces *spyCas9* in pSpyCas9_crA		Measure generic protein expression from pSpyCas9 expression vector
pIDTsmart	n/a	Plasmid used for *in-vitro* SpyCas9 digestion. Sequence available at: https://www.idtdna.com/pages/products/genes-and-gene-fragments/custom-gene-synthesis		Plasmid template for *in-vitro* nuclease reactions
pET15b/HE	n/a	Novagen Cat. No. 69661-3; pET15 variants 'b' and 'HE' differ only by a few bases upstream of the N-terminal thrombin cut site		Protein purification
pSpyCas9_Fig3C	n/a	J23100 promoter expressing a theophylline inducible SpyCas9, used in Figure 3C	(Uribe et al., 2019)	Testing AcrIIA22 activity against a panel of Cas9 and Cas12 effector nucleases
pNmCas9_Fig3C	n/a	J23100 promoter expressing a theophylline inducible NmCas9, used in Figure 3C	(Uribe et al., 2019)	Testing AcrIIA22 activity against a panel of Cas9 and Cas12 effector nucleases
pLbCas12_Fig3C	n/a	J23100 promoter expressing a theophylline inducible LbCas12, used in Figure 3C	(Uribe et al., 2019)	Testing AcrIIA22 activity against a panel of Cas9 and Cas12 effector nucleases
pFnCas12_Fig3C	n/a	J23100 promoter expressing a theophylline inducible FnCas12, used in Figure 3C	(Uribe et al., 2019)	Testing AcrIIA22 activity against a panel of Cas9 and Cas12 effector nucleases
pDual4_Spy	P_{BAD}, GTTCATTCAGGGCACCGGAC	Arabinose inducible gRNA for SpyCas9 targeting pZE21	(Uribe et al., 2019)	Testing AcrIIA22 activity against a panel of Cas9 and Cas12 effector nucleases
pDual4_Nm	P_{BAD}, GAACACGGCGGCATCAGAGC	Arabinose inducible gRNA for NmCas9 targeting pZE21	(Uribe et al., 2019)	Testing AcrIIA22 activity against a panel of Cas9 and Cas12 effector nucleases
pDual4_Lb	P_{BAD}, TCAAGACCGACCTGTCCGGTGCCCTGAATG	Arabinose inducible gRNA for LbCas12 targeting pZE21	(Uribe et al., 2019)	Testing AcrIIA22 activity against a panel of Cas9 and Cas12 effector nucleases
pDual4_Fn	P_{BAD}, TCAAGACCGACCTGTCCGGTGCCCTGAATG	Arabinose inducible gRNA for FnCas12 targeting pZE21	(Uribe et al., 2019)	Testing AcrIIA22 activity against a panel of Cas9 and Cas12 effector nucleases

Supplemental Table 5. Gene sequences used in this study.

Gene Name	Sequence	Notes
acrIIA22wt	atggtagtagaagagacgcgggatttagccgaaactgcggattgtgtagtgatcgaagccattttagtggatgacggattgcgttacagacagctttctgtcggcatcaaagacgaaaacggcgacattattcgtatcgtccctatttcaaccgttc*ctgatc*tag	The italicized six base pairs were deleted in the Δ2aa truncation mutant via Q5 site-directed mutagenesis (NEB).
acrIIA22-null	atggtagtagaagagacgcgggatttagccgaaactgcggattgtgtagtgatcgaagccattt**A**agtggatgacggattgcgttacagacagctttctgtcggcatcaaagacgaaaacggcgacattattcgtatcgtccctatttcaaccgttctgatctag	Mutation to introduce early stop codon via Q5 site-directed mutagenesis (NEB). Indicated in bold, capitalized, underline
acrIIA22a	atggtcatagaagagacgcgggatttagctgaaactgcggattgtgtagtgatcgaagccattttagtggatgacggattgcgttacaaacagctttccgtcggcatcaaagacgaaaacggtgacattattcgtatcgtccctatttcaaccgttctgatctag	Same amino acid sequence as NCBI protein CDB51368.1; synthesized by GenScript and cloned into pZE21_tetR
acrIIA22b	atgattgtggaagatacgaaagatttggttgaaactgcggactatgtgatcatcgaagctgttttagtggatgatggattgcgttacaaacaactttctgttggcattaaagccaaaaatggtgacattatccgcataattccaatatcgacaatgctgatgtaa	Synthesized by GenScript and cloned into pZE21_tetR
acrIIA22c	atgaaaatgattgtggaagatacgaaagatctggtagaaacggacgattatgtaatcattgaagcgactttgtcagagggcgatttgttgtttgtgcaaattgccgtgggcattcgcaacgaagtgggcgacattgttcgtattattcccatttccaccaacccaatctaa	Same amino acid sequence as NCBI protein CDB51757.1; synthesized by GenScript and cloned into pZE21_tetR
ts-acrIIA22	atg***tggagtcatccacaatttgagaag****ggaggaggcagtggaggaggcag****tggaggaagtgcctggagccacccgcagttcgaaaaa****ggcagtggtggtggtagtggtggagga*aatggtagtagaagagacgcgggatttagccgaaactgcggattgtgtagtgatcgaagccattttagtggatgacggattgcgttacagacagctttctgtcggcatcaaagacgaaaacggcgacattattcgtatcgtccctatttcaaccgttctgatctag	N-terminal twin-strep (ts) tagged AcrIIA22. The tag is indicated in bold italics, linker regions are only italicized; synthesized by GenScript and cloned into pZE21_tetR for functional testing.
acrIIA22-ts	atggtagtagaagagacgcgggatttagccgaaactgcggattgtgtagtgatcgaagccattttagtggatgacggattgcgttacagacagctttctgtcggcatcaaagacgaaaacggcgacattattcgtatcgtccctatttcaaccgttctgatc*ggaggaggcagtggaggaggcagtggaggaagtgcct****tggagtcatccacaatttgagaag*** *ggaggaggcagtggaggaggcagtggaggaagtgcct****tggagccacccgcagttcgaaaaa***tag	C-terminal twin-strep (ts) tagged AcrIIA22. The tag is indicated in bold italics, linker regions are only italicized; synthesized by GenScript and cloned into pZE21_tetR or pET15 for functional testing or protein purification, respectively.
purF	atgttcgatagtttgcacgaggaatgcggtgttttcggcgtatttgaaaatcagaccactacggtggcccagacggcgtatctggctctgtttgccttgcagcacagagggcaggagagttgcggcattgccgtgaatgacgacggcgtgtttcgccaccatcggggcgacggactggtgccggatgtgtttagcaaggagcagctggctgccctgggtacaggtaatatggccatcggtcatgtgcgctactccaccaccggcggcaaaaacgccaacaatattcagcccctggtcattcgccatattaagggtaatttggcggtggcacataaccggcaatttggtaaacgccccggagctgcgccgccagtttgagctgaagggcgccatttttcacggcacatcggacaccgagtccattgcctattctattgtagaggagcgcctgcacagtaagagcacggaagaggccatcgaaaaaatcatgccccggctgcaaggggcattctcttgcgtggtgatgactgccaccaaactcattgcgtttcgtgacccaacggctttcggcctctttgcctgggtaagactgcggacgatgcttatgtggtggcgtcggagagttgtgcgctggattccatcggcgcccactttgtgcggaatattgctcccggcgagatcgttgtgatcagcaaggatggcgtgcgctctattaccacccattgcggcggactacgccacatttgtgtgtttgagtacatctattttgctcggccggacagtgtgattgagggcgtgtctgtgcagcacgccagaatgcgggccggtgcgtacctggcgaaggaacacccggtagacgcggatattgtcatcggcgtgccggacagcggcctggacgccgccttgggctatgcccaggagagcggcattccttacggtattggatttatcaagaaccgctacatcggccgcagctttattcagcctacccaaggtcagcgtgaggacgcggtgaagatcaagctgaatgtactgcgagagaatatcaagggcaagcgggtggtgatgatcgatgactctattgttcgcggcaccaccagcgctcggattgtcagcctgctgcgagaggccggcgccaccgaggtgcatatgcgggtttctgcccctccgtttcggcatccttgcttctttggaacggacattgatagcgaagaaaacctgattgcatgcaaatttcacgaaatttctgaaatttcttgccaattaggggttgacagcttggggtatcttagtgtaaaatctactcacgaacttgcgaaggagtccggattcgatttttgcgacggttgcttcaccggccattatcccatccccaccccgaagcaacagtccaaggataagtttgaggaaaagctgaatcagttctcctcttactaccaggtcttggattaa	Flanks *acrIIA22*-encoding bacterial genomic islands. Used as bait to retrieve additional examples of this locus for genomic and evolutionary analyses.
radC	atgcgtgccgcttatctgcaaggcggcggcgacgctatgccggaccaccagttgctggaattgctgctgtccatcagcattccccgcagagatgtaaagcccattgcctatgcgctcattaaccgcttcggctcgctggagcaggtgtttgccgccggcgcagcagatctgcaacaagtgccgggcgtcggcgaacagaccgccgtacagattctgctggtacgggatctgaaccggcggatccatcaaaatcaaaacaaaccggtcaagcacctgacagatgccacccagtcctgcgcctacttttccaatctgttacgggacaaaaccgccgagcaggtgtacttggtcacccctggacggcagtgccaaaatcctgcaaacccacgccgtaggcagcggcagcgtcaacctggcctctgtggatcagcgcactttgatggaacatattctgcgagacaacgccaacgctgttatgctggcacacaaccatcccggcggcaaggcccagccctctgcgcaggatctggaattcaccattcgtctgctttccattctgcgttccattcatgtgcagctgctggatcatattatcgtcagtcctaccgacacctactccatgcgcagcgacccggagtacggcagcttcttcaccgtcaaataa	Flanks *acrIIA22*-encoding bacterial genomic islands. Used as bait to retrieve additional examples of this locus for genomic and evolutionary analyses.
acrIIA4	atgaatattaacgatttgatccgtgagattaagaataaggattatactgtcaaattgtccgggacagattccaattctattacacaattaatcatccgtgtgaataacgatggtaatgagtatgtcatctctgaatcagaaaacgagagcatcgtagaaaagttcatcagtgccttcaagaacgggtggaaccaagagtatgaagatgaggaggaattttacaatgatatgcagacaattacgcttaaatcagaattgaattaa	Discovered by (Rauch et al., 2017); synthesized by GenScript and cloned into pZE21_tetR
spyCas9	atggataagaaatactcaataggcttagatatcggcacaaatagcgtcggatgggcggtgatcactgatgaatataaggttccgtctaaaaagttcaaggttctgggaaatacagaccgccacagtatcaaaaaaaatcttataggggctcttttatttgacagtggagagacagcggaagcgactcgtctcaaacggacagctcgtagaaggtatacacgtcggaagaatcgtatttgttatctacaggagatttttttcaaatgagatggcgaaagtagatgatagtttctttcatcgacttgaagagtctttttttggtggaagaagacaagaagcatgaacgtcatcctatttttggaaatatagtagatgaagttgcttatcatgagaaatatccaactatctatcatctgcgaaaaaaattggtagattctactgataaagcggatttgcgcttaatctatttggccttagcgcatatgattaagtttcgtggtcatttttttgattgagggagatttaaatcctgataatagtgatgtggacaaactatttatccagttggtacaaacctac	The sequence was amplified from Addgene plasmid #48645 (Esvelt *et al.*, 2013) for use in this study as described in (Forsberg *et al.*, 2019).

	aatcaattatttgaagaaaaccctattaacgcaagtggagtagatgctaaagcgattctttctgcacgattgagtaaatcaagacgattagaaaatctcattgctcagctcccc ggtgagaagaaaaatggcttatttgggaatctcattgctttgtcattgggtttgacccctaattttaaatcaaattttgatttggcagaagatgctaaattacagctttcaaaagata cttacgatgatgatttagataatttattggcgcaaattggagatcaatatgctgatttgtttttggcagctaagaatttatcagatgctattttacttttcagatatcctaagagtaaatac tgaaataactaaggctcccctatcagcttcaatgattaaacgctacgatgaacatcatcaagacttgactcttttaaaagctttagttcgacaacaacttccagaaaagtataa agaaatctttttttgatcaatcaaaaaacggatatgcaggttatattgatgggggagctagccaagaagaatttttataaatttatcaaaccaatttttagaaaaaatggatggtact gaggaattattggtgaaactaaatcgtgaagatttgctgcgcaagcaacggacctttgacaacggctctattccccatcaaattcacttgggtgagctgcatgctatttttgagaa gacaagaagacttttatccatttttaaaagacaatcgtgagaagattgaaaaaatcttgacttttcgaattccttattatgttggtccattggcgcgtggcaatagtcgttttgcatgg atgactcggaagtctgaagaaacaattaccccatggaattttgaagaagttgtcgataaaggtgcttcagctcaatcatttattgaacgcatgacaaacttttgataaaaatcttc caaatgaaaaagtactaccaaaacatagtttgctttatgagtattttacggtttataacgaattgacaaaggtcaaatatgttactgaaggaatgcgaaaaccagcatttctttc aggtgaacagaagaaagccattgttgatttactcttcaaaacaaatcgaaaagtaaccgttaagcaattaaaagaagattatttcaaaaaaatagaatgttttgatagtgttg aaatttcaggagttgaagatagatttaatgcttcattaggtacctaccatgatttgctaaaaattattaaagataaagatttttggataatgaagaaaatgaagatatcttagagg atattgttttaacattgaccttatttgaagatagggagatgattgaggaaagacttaaaacatatgctcacctctttgatgataaggtgatgaaacagcttaaacgtcgccgttat actggttggggacgtttgtctcgaaaattgattaatggtattagggataagcaatctggcaaaacaatattagatttttgaaatcagatggttttgccaatcgcaattttatgcagc tgatccatgatgatagtttgacatttaaagaagacattcaaaaagcacaagtgtctggacaaggcgatagtttacatgaacatattgcaaatttagctggtagccctgctattaa aaaaggtattttacagactgtaaaagttgttgatgaattggtcaaagtaatggggcggcataagccagaaaatatcgttattgaaatggcacgtgaaaatcagacaactcaa aagggccagaaaaattcgcgagagcgtatgaaacgaatcgaagaaggtatcaaagaattaggaagtcagattcttaaagagcatcctgttgaaaatactcaattgcaaa atgaaaagctctatctctattatctccaaaatggaagagacatgtatgtggaccaagaattagatattaatcgtttaagtgattatgatgtcgatcacattgttccacaaagtttcct taaagacgattcaatagacaataaggtcttaacgcgttctgataaaaatcgtggtaaatcggataacgttccaagtgaagaagtagtcaaaaagatgaaaaactattggag acaacttctaaacgccaagttaatcactcaacgtaagtttgataatttaacgaaagctgaacgtggaggtttgagtgaacttgataaagctggttttatcaaacgccaattggtt gaaactcgccaaatcactaagcatgtggcacaaattttggatagtcgcatgaatactaaatacgatgaaaatgataaacttattcgagaggttaaagtgattaccttaaaatct aaattagtttctgacttccgaaaagatttccaattctataaagtacgtgagattaacaattaccatcatgcccatgatgcgtatctaaatgccgtcgttggaactgctttgattaag aaatatccaaaacttgaatcggagtttgtctatggtgattataaagtttatgatgttcgtaaaatgattgctaagtctgagcaagaaataggcaaagcaaccgcaaaatatttctt ttactctaatatcatgaacttcttcaaaacagaaattacacttgcaaatggagagattcgcaaacgccctctaatcgaaactaatggggaaactggagaaattgtctgggata aagggcgagattttgccacagtgcgcaaagtattgtccatgccccaagtcaatattgtcaagaaaacagaagtacagacaggcggattctccaaggagtcaattttaccaa aaagaaattcggacaagcttattgctcgtaaaaaagactgggatccaaaaaaatatggtggttttgatagtccaacggtagcttattcagtcctagtggttgctaaggtggaa aaagggaaatcgaagaagttaaaatccgttaaagagttactagggatcacaattatggaaagaagttcctttgaaaaaaatccgattgacttttagaagctaaaggatata aggaagttaaaaaagacttaatcattaaactacctaaatatagtctttttgagttagaaaacggtcgtaaacggatgctggctagtgccggagaattacaaaaaggaaatga gctggctctgccaagcaaatatgtgaatttttatatttagctagtcattatgaaaagttgaagggtagtccagaagataacgaacaaaaacaattgtttgtggagcagcataa gcattatttagatgagattattgagcaaatcagtgaattttctaagcgtgttattttagcagatgccaatttagataaagttcttagtgcatataacaaacatagagacaaaccaat acgtgaacaagcagaaaatattattcatttatttacgttgacgaatcttggagctcccgctgcttttaaatattttgatacaacaattgatcgtaaacgatatacgtctacaaaaga agttttagatgccactcttatccatcaatccatcactggtctttatgaaacacgcattgatttgagtcagctaggaggtgactga	
nmCas9	atggccgccttcaagcccaaccccatcaactacatcctgggcctggacatcggcatcgccagcgtgggctgggccatggtggagatcgacgaggacgagaaccccatc tgcctgatcgacctgggtgtgcgcgtgttcgagcgcgctgaggtgcccaagactggtgacagtctggctatggctcgccggcttgctcgctctgttcggcgccttactcgccgg cgcgctcaccgccttctgcgcgctcgccgcctgctgaagcgcgagggtgtgctgcaggctgccgacttcgacgagaacggcctgatcaagagcctgcccaacactccttg gcagctgcgcgctgccgctctggaccgcaagctgactcctctggagtggagcgccgtgctgctgcacctgatcaagcaccgcggctacctgagccagcgcaagaacga gggcgagaccgccgacaaggagctgggtgctctgctgaagggcgtggccgacaacgcccacgccctgcagactggtgacttccgcactcctgctgagctggccctgaa caagttcgagaaggagagcggccacatccgcaaccagcgcggcgactacagccacaccttcagccgcaaggacctgcaggccgagctgatcctgctgttcgagaag cagaaggagttcggcaacccccacgtgagcggcggcctgaaggagggcatcgagaccctgctgatgacccagcgccccgccctgagcggcgacgccgtgcagaag atgctgggccactgcaccttcgagccagccgagcccaaggccgccaagaacacctacaccgccgagcgcttcatctggctgaccaagctgaacaacctgcgcatcctg gagcagggcagcgagcgcccctgaccgacaccgagcgcgccacccctgatggacgagccctacccgcaagagcaagctgacctacgcccagggcccgcaagctgct gggtctggaggacaccgccttcttcaagggcctgcgctacggcaaggacaacgccgaggccagcaccctgatggagatgaaggcctaccacgccatcagccgcgccc tggagaaggagggcctgaaggacaagaagagtcctctgaacctgagccccgagctgcaggacgagatcggcaccgccttcagcctgttcaagaccgacgaggacat caccggccgcctgaaggaccgcatccagcccgagatcctggaggccctgctgaagcacatcagcttcgacaagttcgtgcagatcagcctgaaggccctgcgccgcat cgtgccccctgatggagcagggcaagcgctacgacgaggcctgcgccgagatctacggcgaccactacggcaagaagaacaccgaggagaagatctacctgcctcct atccccgccgacgagatccgcaaccccgtggtgctgcgcgccctgagccaggcccgcaaggtgatcaacggcgtggtgcgccgctacggcagccccgcccgcatcca catcgagaccgcccgcgaggtgggcaagagcttcaaggaccgcaaggagatcgagaagcgccaggaggagaaccgcaaggaccgcgagaaggccgccgccaa gttccgcgagtacttccccaacttcgtgggcgagcccaagagcaaggacatcctgaagctgcgcctgtacgagcagcagcacggcaagtgcctgtacagcggcaagga gatcaacctgggccgcctgaacgagaagggctacgtggagatcgaccacgccctgcccttcagccgcacctgggacgacagctgacctacgcccaggcccgcaagctgct cagcgagaaccagaacaagggcaaccagacccccctacgagtacttcaacggcaaggacaacagccgcgagtggcaggagttcaaggcccgcgtggagaccagcc gcttcccccgcagcaagaagcagcgcatcctgctgcagaagttcgacgaggacggcttcaaggagcgcaacctgaacgacacccgctacgtgaaccgcttcctgtgcc agttcgtggccgaccgcatgcgcctgaccggcaagggcaagaagcgcgtgttcgccagcaacggccagatcaccaacctgctgcgcggcttctggggcctgcgcaag gtgcgcgccgagaacgaccgccaccacgccctggacgccgtggtggtggcctgcagcaccgtggccatgcagcagaagatcacccgcttcgtgcgctacaaggagat gaacgccttcgacggtaaaaccatcgacaaggagaccggcgaggtgctgcaccagaagacccacttcccccagccctgggagttcttcgcccaggaggtgatgatccg cgtgttcggcaagcccgacggcaagcccgagttcgaggaggccgacacccccgagaagctgcgcaccctgctggccgagaagctgagcagccgccctgaggccgtg cacgagtacgtgactcctctgttcgtgagccgcgcccccaaccgcaagatgagcggtcagggtcacatggagaccgtgaagagcgccaagcgcctggacgagggcgt gagcgtgctgcgcgtgccctgacccagctgaagctgaaggacctggagaagatggtgaaccgcgagcgcgagcccaagctgtacgaggccctgaaggcccgcctg gaggcccacaaggacgaccccgccaaggccttcgccgagcccttctacaagtacgacaaggccggcaaccgcacccagcaggtgaaggccgtgcgcgtggagcag gtgcagaagaccggcgtgtgggtgcgcaaccacaacggcatcgccgacaacgccaccatggtgcgcgtggacgtgttcgagaagggcgacaagtactacctggtgcc	The sequence was amplified from Addgene plasmid #48646 (Esvelt *et al.*, 2013). The sequence of NmCas9 was cloned in place of the cas9 gene in pSpyCas9_Fig3C.

	catctacagctggcaggtggccaagggcatcctgcccgaccgcgccgtggtgcagggcaaggacgaggaggactggcagctgatcgacgacagcttcaacttcaagtt cagcctgcaccccaacgacctggtggaggtgatcaccaagaaggcccgcatgttcggctacttcgccagctgccaccgcggcaccggcaacatcaacatccgcatcca cgacctggaccacaagatcggcaagaacggcatcctggagggcatcggcgtgaagaccgccctgagcttccagaagtaccagatcgacgagctgggcaaggagatc cgcccctgccgcctgaagaagcgccctcctgtgcgctaa	
lbCas12	atgtcaaagctcgagaaattcaccaactgttattcgttgagcaaaacactgcggtttaaagcgattccagtcggcaagactcaagagaatatagacaataagcggctgttg gtggaagatgaaaagcgcgcggaagactacaaaggggtgaagaagttgttggacagatactacctctcttttatcaatgatgtcttgcactcaatcaaattgaagaatctga acaactacatctccctcttcagaaagaaaacaaggacagaaaaggagaataaggaacttgaaaatttggagatcaatctgaggaaagagatcgcgaaagcctttaaag gcaacgaaggatacaaaagtctgttcaagaaggatataattgagacaattttgccagagttcctcgatgacaaggacgagattgcgctggtcaattcgttcaacggattcac aacagcattcacaggcttctttgataatcgggaaaatatgttctctgaggaggcaaagtccacttctattgcgttcaggtgtatcaatgagaatctcactaggtacatttccaaca tggatatctttgagaaggttgacgcaatttttgacaagcacgaagttcaggagattaaggagaagatcctcaattccgattatgacgttgaggacttcttcgaaggtgagttttt aatttcgtgctcactcaagagggtatcgacgtgtataatgcgatcatcggtgggttcgtgactgagtccggtgaaaagattaagggattgaacgagtatatcaaccttacaac caaaagacgaaacagaagctgccaaagttcaagcctctttacaaacaggttctttcagaccgcgagtcactctcgttctatggggagggctacacttcggatgaggaagtc ctggaggtgttcaggaatactctcaataagaattcggagattttctcttctataaaaaaactggaaaagttgtttaagaattttgacgaatactctagcgccggcatatttgtgaa aaacggcccggccatatcaacgataagtaaagatatcttcggcgaatggaacgtgatcagagacaaatggaacgcggagtatgacgatattcacctgaagaagaagg ctgtcgtaacggagaagtacgaggatgatcgcaggaaaagcttcaaaaagatccggaagtttcagcctggaacagttgcaggagtatgctgacgccgatcttagcgtcgtc gagaagttgaaggagataatcatccaaaaggtcgacgagatatataaagtctatggatcaagtgaaaaactgttcgacgccgacttcgttttggagaagtccctgaagaa gaacgacgctgttgttgccattatgaaggatctgctcgacagcgtgaagagtttcgagaactatattaaggctttttcggggaggggaaggagactaacagagatgagtcct tctacggagacttcgtcctcgcgtacgatatactccttaaggtagaccacatctacgacgcaatcagaaattacgtgacacaaaagccgtacagcaaggacaagttcaaa ctctacttccagaacccccagttcatgggcggctgggacaaggacaaggaaacggattacagggctacgatcctgaggtatggttcaaaatactacttggcgattatggac aagaagtacgccaagtgtctccagaagattgacaaagacgatgtcaatggcaattatgagaagatcaactacaagctgcttccgggtccgaacaagatgctcccaaagg ttttcttcagcaagaaatggatggcctactataacccaagcgaggacatccagaagatttataagaacggtacgttcaagaagggcgcatgttcaatcttaacgactgtca caagctgatcgacttcttcaaagactcaattagccggtacccaaagtggtctaacgcctatgacttcaacttttcggaaaccgagaagtacaaggatatagccggattttata gagaggtggaagagcagggctacaaggtgtcattcgagtccgccagcaagaaggaagtggacaagctcgtggaagagggtaagctctacatgttccagatttataataa agactttagcgataagagccacgggacacctaatctccacacaatgtatttcaagctgctcttcgacgagaataaccacggccaaatcaggttgtcaggaggggctgaact cttcatgcggcgcgctagccttaagaaggaggagcttgtagtccaccctgcgaatagtccaattgcgaataagaacccggacaatcctaaaaagactacaacattgagct acgacgtgtacaaggataagaggttttccgaggatcagtacgagctccacatcccgattgcgatcaacaagtgcccaaagaatattttcaagataaacacagaggtgcgt gtactcctgaagcatgacgacaatccttacgtcattgggattgatcggggcgagaggaacctcctctatattgtggtggtggacgggaaggggaacatagtcgaacagtac tcccttaacgaaataattaacaatttcaacggcatccgtatcaagaccgactaccattcgttgctggacaagaaggagaaggagagatttgaggcgcggcaaaattggac aagtatcgagaacatcaaggaactcaaagcaggttatatctctcaagttgtgcataagatatgcgagctggttgagaagtatgacgcagtgatcgctcttgaggacctcaac tcgggctttaagaattctagagttaaagtggagaagcaggtctatcaaaagttcgagaagatgcttatagataagctcaactacatggtcgataagaaatcgaacccatgtg ccaccggcggcgcactcaaaggttaccaaataacaaacaaattcgagtccttcaaatcgatgagtactcagaatgggttcatatttttatataccggcgtggcttacgtctaag atcgacccgtcaactggttttgtcaacctgttgaagacgaaatacacgtccattgccgattcgaaaaagttcatatctagttttgatcgtattatgtacgtcccagaggaagatctt ttcgagtttgctctcgactacaaaaactttccgcggaccgatgcggattacattaaaaaatggaaactctattcgtacggcaacagaatcaggatttttcgcaaccctaagaag aataacgtctttgattgggaggaagtttgcttgactagcgcgtacaaggagctctttaataagtatggcattaactaccaacagggtgatatcagagcactgctttgcgaacaa tctgacaaggctttctactcatccttcatggctttgatgagcctgatgctccagatgagaaattcaattacaggcagaaccgacgtggatttcttgatctccccggttaaaaattct gatggcatcttttacgatagcaggaactatgaagcgcaagagaatgcgattctgccaaaaaatgcagacgccaacggtgcctataacatcgccaggaaagtcctgtggg cgatcggccagttcaaaaaggccgaagacgaaaaattggacaaggtcaaaatcgctatcagcaacaaagagtggctggagtatgctcagacatccgtaaagcattaa	The amino acid sequence was obtained from Uniprot accession no A0A182DWE3 and codon optimized for expression in *E.coli*. The DNA sequence was synthetized by Twist bioscience and cloned in place of the cas9 gene in pSpyCas9.
fnCas12	atgtcaatttatcaagaatttgttaataaatatagtttaagtaaaactctaagatttgagttaatcccacagggtaaaacacttgaaaacataaaagcaagaggtttgattttaga tgatgagaaaagagctaaagactacaaaaaggctaaacaaataattgataaatatcatcagtttttatagaggagatattaagttcggtttgtattagcgaagatttattacaa aactattctgatgtttatttttaaacttaaaaagagtgatgatgataatctacaaaaagatttataaagtgcaaaagatacgataaagaaacaaatatctgaatatataaaggac tcagagaaatttaagaatttgtttaatcaaaaccttatcgatgctaaaaaagggcaagagtcagatttaattctatggctaaagcaatctaaggataatggtatagaactattta aagccaatagtgatatcacagatatagatgaggcgttagaaataatcaaatcttttaaaggttggacaacttatttttaagggttttcatgaaaatagaaaaaatgtttatagtag caatgatattcctacatctattatttataggatagtagatgataatttgcctaaatttctagaaaataaagctaagtatgagagtttaaaagacaaagctccagaagctataaact atgaacaaattaaaaaagatttggcagaagagctaaccttgatattgactacaaaacatctgaagttaatcaaagagtttttttcacttgatgaagtttttgagatagcaaacttt aataattatctaaatcaaagtggtattactaaatttaatactattattggtggtaaatttgtaaatggtgaaaatacaaagagaaaaggtataaatgaatatataaatctatactca cagcaaataaatgataaaacactcaaaaaatataaaatgagtgttttatttaagcaaattttaagtgatacagaatctaaatcttttgtaattgataagttagaagatgatagtga tgtagttacaacgatgcaaagttttatgagcaaatagcagcttttaaaacagtagaagaaaaatctattaaagaaacactatctttattatttgatgatttaaaagctcaaaaac ttgatttgagtaaaatttatttaaaaatgataaatctcttactgatctatcacaacaagtttttgatgattatagtgttattggtacagcggtactagaatatataactcaacaaatag cacctaaaaatcttgataaccctagtaagaaagagcaagaattaatagccaaaaaaactgaaaaagcaaaatacttatctctagaaactataaagcttgccttagaagaa tttaataagcatagagatatagataaacagtgtaggtttgaagaaatacttgcaaactttgcggctattccgatgatatttgatgaaatagctcaaaacaaagacaatttggca cagatatctatcaaatatcaaaatcaaggtaaaaaagacctacttcaagctagtgcggaagatgatgttaaagctatcaaggatcttttagatcaaactaataatctcttacat aaactaaaaatatttcatattagtcagtcagaagataaggcaaatattttagacaaggatgagcatttttatctagtatttgaggagtgctactttgagctagcgaatatagtgcct ctttataacaaaattagaaactatataactcaaaagccatatagtgatgagaaatttaagctcaattttgagaactcgactttggctaatggttgggataaaaataaagagcct gacaatacggcaattttatttatcaaagatgataaatattatctgggtgtgatgaataagaaaaataacaaaatatttgatgataaagctatcaaagaaaataaaggcgagg gttataaaaaaattgtttataaacttttacctggcgcaaataaaatgttacctaaggttttcttttctgctaaatctataaaatttttataatcctagtgaagatatacttagaataagaa atcattccacacatacaaaaaatggtagtcctcaaaaaggatatgaaaaatttgagtttaatattgaagattgccgaaaatttatagatttttataaacagtctataagtaagcat ccggagtggaaagattttggatttagattttctgatactcaaagatataattctatagatgaattttatagagaagttgaaaatcaaggctacaaactaacttttgaaaatatatca gagagctatattgatagcgtagttaatcagggtaaattgtacctattccaaatctataataaagatttttcagcttatagcaaagggcgaccaaatctacatacttttatattggaa	The sequence was amplified from Addgene plasmid #69973 (Zetsche *et al*., 2015). The sequence of FnCas12 was cloned in place of the cas9 gene in pSpyCas9_Fig3C.

	agcgctgtttgatgagagaaatcttcaagatgtggtttataagctaaatggtgaggcagagctttttatcgtaaacaatcaatacctaaaaaaatcactcacccagctaaaga ggcaatagctaataaaaacaaagataatcctaaaaaagagagtgtttttgaatatgatttaatcaaagataaacgctttactgaagataagtttttctttcactgtcctattacaat caattttaaatctagtggagctaataagtttaatgatgaaatcaatttattgctaaaagaaaaagcaaatgatgttcatatattaagtatagatagaggtgaaagacatttagctt actatactttggtagatggtaaaggcaatatcatcaaacaagatactttcaacatcattggtaatgatagaatgaaaacaaactaccatgataagcttgctgcaatagagaa agatagggattcagctaggaaagactggaaaaagataaataacatcaaagagatgaaagagggctatctatctcaggtagttcatgaaatagctaagctagttatagagt ataatgctattgtggtttttgaggatttaaattttggatttaaaagagggcgtttcaaggtagagaagcaggtctatcaaaagttagaaaaaatgctaattgagaaactaaactat ctagttttcaaagataatgagtttgataaaactgggggagtgcttagagcttatcagctaacagcacctttgagacttttaaaaagatgggtaaacaaacaggtattatctact atgtaccagctggttttacttcaaaaatttgtcctgtaactggttttgtaaatcagttatatcctaagtatgaaagtgtcagcaaatctcaagagttctttagtaagtttgacaagattt gttataaccttgataagggctattttgagtttagttttgattataaaaactttggtgacaaggctgccaaaggcaagtggactatagctagctttgggagtagattgattaactttag aaattcagataaaaatcataattgggatactcgagaagtttatccaactaaagagttggagaaattgctaaaagattattctatcgaatatgggcatggcgaatgtatcaaag cagctatttgcggtgagagcgacaaaaagtttttgctaagctaactagtgtcctaaatactatcttacaaatgcgtaactcaaaaacaggtactgagttagattatctaatttca ccagtagcagatgtaaatggcaatttctttgattcgcgacaggcgccaaaaaatatgcctcaagatgctgatgccaatggtgcttatcatattgggctaaaaggtctgatgcta ctaggtaggatcaaaaataatcaagagggcaaaaaactcaatttggttatcaaaaatgaagagtattttgagttcgtgcagaataggaataactaa	

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