RESEARCH ARTICLE

A New Group of Phage Anti-CRISPR Genes Inhibits the Type I-E CRISPR-Cas System of *Pseudomonas aeruginosa*

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ABSTRACT **CRISPR-Cas systems are one of the most widespread phage resistance mechanisms in prokaryotes. Our lab recently identified the first examples of phage-borne anti-CRISPR genes that encode protein inhibitors of the type I-F CRISPR-Cas system of *Pseudomonas aeruginosa*. A key question arising from this work was whether there are other types of anti-CRISPR genes. In the current work, we address this question by demonstrating that some of the same phages carrying type I-F anti-CRISPR genes also possess genes that mediate inhibition of the type I-E CRISPR-Cas system of *P. aeruginosa*. We have discovered four distinct families of these type I-E anti-CRISPR genes. These genes do not inhibit the type I-F CRISPR-Cas system of *P. aeruginosa* or the type I-E system of *Escherichia coli*. Type I-E and I-F anti-CRISPR genes are located at the same position in the genomes of a large group of related *P. aeruginosa* phages, yet they are found in a variety of combinations and arrangements. We have also identified functional anti-CRISPR genes within nonprophage *Pseudomonas* genomic regions that are likely mobile genetic elements. This work emphasizes the potential importance of anti-CRISPR genes in phage evolution and lateral gene transfer and supports the hypothesis that more undiscovered families of anti-CRISPR genes exist. Finally, we provide the first demonstration that the type I-E CRISPR-Cas system of *P. aeruginosa* is naturally active without genetic manipulation, which contrasts with *E. coli* and other previously characterized I-E systems.**

IMPORTANCE **The CRISPR-Cas system is an adaptive immune system possessed by the majority of prokaryotic organisms to combat potentially harmful foreign genetic elements. This study reports the discovery of bacteriophage-encoded anti-CRISPR genes that mediate inhibition of a well-studied subtype of CRISPR-Cas system. The four families of anti-CRISPR genes described here, which comprise only the second group of anti-CRISPR genes to be identified, encode small proteins that bear no sequence similarity to previously studied phage or bacterial proteins. Anti-CRISPR genes represent a newly discovered and intriguing facet of the ongoing evolutionary competition between phages and their bacterial hosts.**

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The ubiquitous predation of bacteria by bacteriophages (phages) has resulted in the evolution of numerous bacterial mechanisms that protect against phage attack (1). One of the most widespread is the CRISPR-Cas (CRISPR stands for clustered regularly interspaced short palindromic repeat) system. This system utilizes small RNA molecules that act as sequence-specific guides for nuclease activity (2). Different types (i.e., I, II, and III) and subtypes (i.e., I-A, I-B, etc.) of CRISPR-Cas systems exist across bacterial and archaeal species. Most of these systems, including all type I systems described here, target DNA, while some type III systems target RNA (11). The sophisticated functional mechanisms of CRISPR-Cas systems combined with their ability to gain immunity to newly encountered phages has led to intensive study of these systems in recent years.

CRISPR loci consist of multiple semipalindromic DNA repeats of 21 to 48 nucleotides, interspersed with variable "spacer" sequences of similar length. The spacers comprise sequences that are complementary to mobile genetic elements, including phages and plasmids (3). In type I CRISPR-Cas systems, the CRISPR locus is transcribed as a precursor RNA molecule that is processed into single repeat-spacer units (mature CRISPR RNA [crRNA]) (4, 5). The mature crRNA is then bound by a complex of CRISPR-associated (Cas) proteins (2, 6). The CRISPR-Cas complex is able to recognize DNA sequences that are complementary to the crRNA and direct the destruction of the foreign DNA (7, 8).

Since CRISPR-Cas systems provide a potent defense mechanism against phage infection, one might expect phages to possess means of inhibiting these systems. We recently identified five different anti-CRISPR proteins that inhibit the type I-F CRISPR-Cas system of *Pseudomonas aeruginosa*. The genes encoding these proteins are located at the same genomic position within a group of closely related *Pseudomonas* phages (Fig. 1); they appear to be contained within a unique operon inserted between two highly conserved head morphogenetic genes. Intriguingly, greater than half of the genes within these putative anti-CRISPR operons did not mediate anti-CRISPR activity against the type I-F CRISPR-Cas system reported in our previous work (9). In the present study, we demonstrate that many of these previously uncharacter-

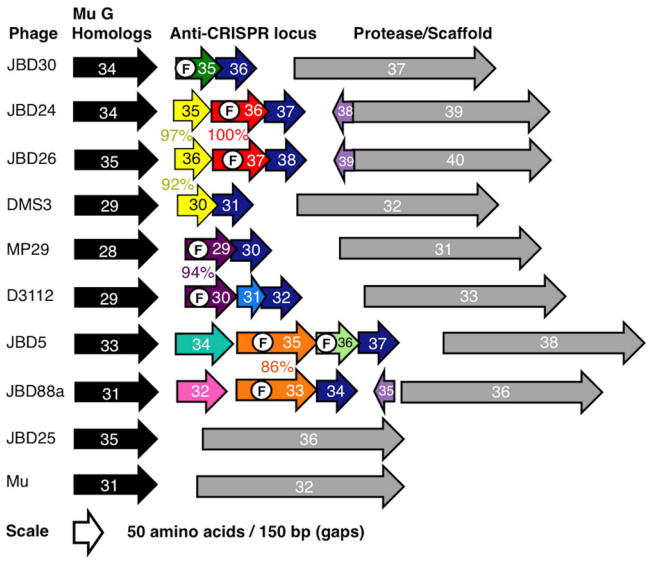

FIG 1 Anti-CRISPR phages encode type I-F anti-CRISPR genes and other uncharacterized open reading frames at a conserved genomic location. All of the phages shown are similar to *E. coli* phage Mu in terms of organization of this genomic region. The anti-CRISPR region is found between the gene homologous to gene G from Mu (black boxes) and the protease/scaffold gene (gray boxes). The genes included in the anti-CRISPR region are represented by colored boxes. The genes with the letter F in a white circle were previously shown to have anti-CRISPR activity against the type I-F CRISPR-Cas system of *P. aeruginosa* strain PA14. Genes sharing high sequence identity are indicated by boxes of the same color, and the percent identity of the encoded proteins is shown. The gene boxes and intergenic gaps are drawn proportionally according to the size marker shown. The 3′ conserved gene homologous to JBD30-*36* is present in all anti-CRISPR-encoding phages, and all homologs shown are greater than 90% identical in amino acid sequence. The related *P. aeruginosa* phage JBD25 and *E. coli* phage Mu lacking the anti-CRISPR region are shown at the bottom of the figure for comparison.

ized genes mediate anti-CRISPR activity directed against a different subtype of CRISPR-Cas system, the type I-E system of *P. aeruginosa*. This work reveals a second group of anti-CRISPR proteins comprising four distinct families and provides the first *in vivo* characterization of a naturally active type I-E CRISPR-Cas system.

RESULTS

Two *P. aeruginosa* strains possess active type I-E CRISPR-Cas systems. To determine whether phage genes could mediate anti-CRISPR activity against the type I-E system of *P. aeruginosa*, it was imperative to identify strains in which this type of CRISPR-Cas system was active. A previous study identified several strains of *P. aeruginosa* that likely encode intact type I-E CRISPR-Cas systems; however, the activity of these systems was not tested (10). To determine whether these *P. aeruginosa* strains contain active type I-E systems, we used a plasmid transformation efficiency assay described in our previous work (9). In this assay, the transformation efficiency of a plasmid containing a "protospacer" sequence complementary to a CRISPR spacer within a given strain is compared to an empty-vector control. We synthesized and cloned three protospacer sequences, protospacer sequence 1 (PS1), PS2, and PS3, that were complementary to CRISPR spacers found within five type I-E-containing strains (10) (Fig. 2A; see Fig. S1 in the supplemental material). We flanked each of these protospacer sequences with a protospacer adjacent motif (PAM), which is required for targeting (11, 12). Since PAM sequences for the *P. aeruginosa* type I-E system had not been experimentally determined, we used the PAM sequence 5′-CAT-3′, an experimentally validated PAM for the *Escherichia coli* type I-E system (7), or the sequence 5′-CTT-3′, a consensus PAM based on the alignment of putative type I-E protospacers found in *P. aeruginosa* phage genomes (12) (Fig. 2B).

By testing strains using the plasmid transformation assay, we ultimately identified two strains, *P. aeruginosa* SMC4386 and SMC4389, that displayed 100-fold to 2,000-fold reductions in transformation efficiency of protospacer-containing plasmids (Fig. 2C). These results implied that these strains possess active type I-E systems. The plasmids that contained the PS1 and PS2 protospacers flanked by the PAM sequence 5′-CTT-3′ transformed both strains poorly, which is expected because both strains possess corresponding CRISPR spacers. Since only strain SMC4386 possessed a CRISPR spacer complementary to PS3, the plasmid bearing the PS3 sequence displayed reduced transformation efficiency only in this strain. We sequenced the type I-E CRISPR-associated (*cas*) genes of strain SMC4386 and found that all of the type I-E *cas* genes were present and that the Cas protein sequences were greater than 93% identical to the homologous proteins encoded in the genome of PA2192, a fully sequenced *P. aeruginosa* strain that contains a complete type I-E system (10).

Interestingly, both strain SMC4386 and strain SMC4389 targeted plasmids containing the PAM sequence 5′-CTT-3′, but not 5′-CAT-3′ (Fig. 2C), which is the most favored PAM sequence for the *E. coli* type I-E system. Therefore, the *P. aeruginosa* and *E. coli* type I-E systems clearly possess different PAM specificities (7, 13). To further define this specificity, we determined the transformation efficiencies of plasmids containing the PS2 protospacer flanked by a panel of different 3-nucleotide PAM sequences (Fig. 2D). Although 5′-CTT-3′ elicited the strongest inhibition of transformation, any PS2 construct with a PAM sequence of 5′-NTT-3′ resulted in at least a 100-fold decrease in transformation efficiency, while 5′-CCT-3′ exhibited a 10-fold reduction. Plasmids bearing other PAM sequences retained transformation efficiencies equal to the empty-vector control.

Some anti-CRISPR phages possess genes that inactivate the type I-E system. In our previous work, we described eight phages bearing genes that inhibit the *P. aeruginosa* type I-F CRISPR-Cas system (Fig. 1). These phages are temperate, so lysogenic strains can be isolated in which the phage genome is integrated into the host genome in the form of a prophage. The anti-CRISPR genes are transcribed from the prophage, thereby inactivating the CRISPR-Cas systems in lysogenic strains containing these phage genomes (9). To determine whether these anti-CRISPR phages encode genes able to inhibit the type I-E system, we created five *P. aeruginosa* SMC4386 lysogens that each contained a different anti-CRISPR prophage. We tested these lysogens using the transformation efficiency assay with the PS2 protospacer coupled with the 5′-CTT-3′ PAM. In contrast to SMC4386 lacking a prophage, the lysogens of phages JBD5, JBD24, DMS3, and JBD26 could be transformed with ~100% efficiency compared to the empty vector. The MP29 lysogen was transformed inefficiently at a level similar to that of the unlysogenized strain (Fig. 3A). These data indicate that each of these phages except MP29 possesses genes that inhibit the type I-E CRISPR-Cas system.

Each of these phage genomes possesses a gene at the 3′ end of

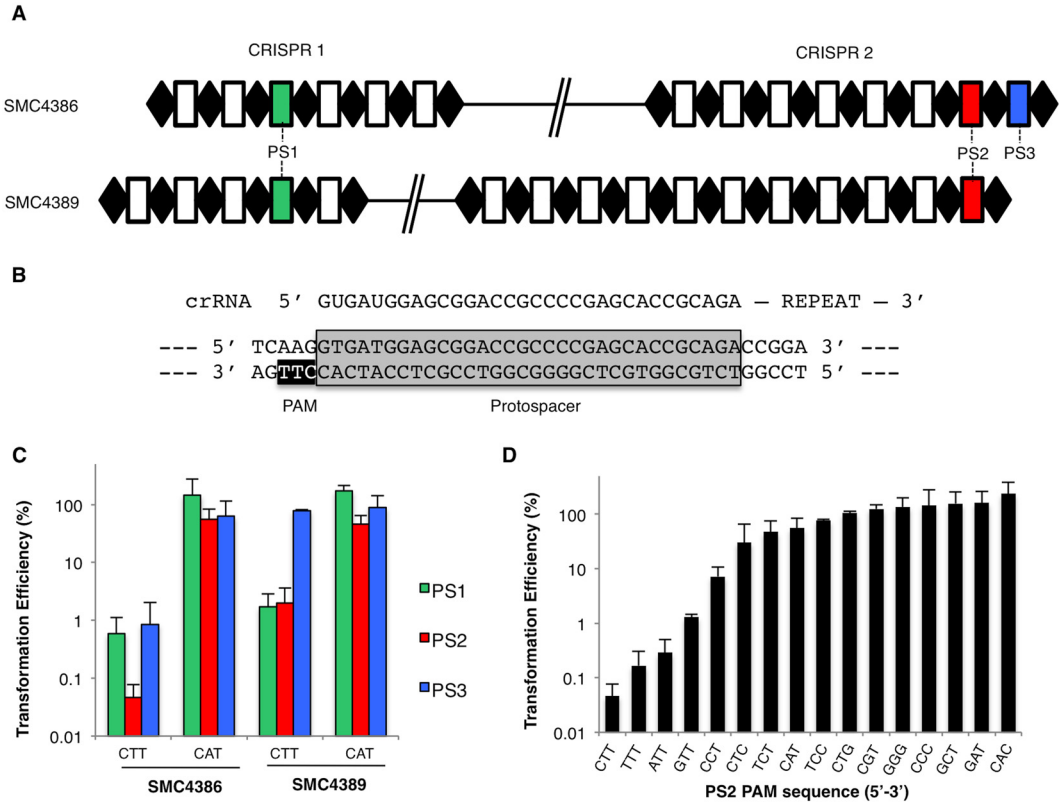

FIG 2 The type I-E CRISPR-Cas systems of *P. aeruginosa* strains SMC4386 and SMC4389 are active. (A) The CRISPR loci from strains SMC4386 and SMC4389, sequenced by Cady et al. (10), are shown. CRISPR repeat sequences (black diamonds) and CRISPR spacers (white boxes) are indicated. CRISPR 1 and CRISPR 2 loci are separated by a line segment, representing the location of the *cas* genes between the two loci. The direction of transcription for both loci is from left to right. The following three protospacer sequences were constructed: PS1, corresponding to SMC4386 CRISPR 1 spacer 3 (CR1_sp3) and SMC4389 CR1_sp4; PS2, corresponding to SMC4386 CR2_sp7 and SMC4389 CR2_sp11; and PS3, corresponding to SMC4386 CR2_sp8 and containing no match within SMC4389 CRISPR loci. (B) Representative CRISPR RNA (crRNA), protospacer (gray box), and protospacer adjacent motif (PAM) (black box) are shown. (C) Transformation efficiencies of the PS1, PS2, and PS3 plasmids. Here, transformation efficiency is defined as the number of transformants per nanogram of the protospacer construct, represented as a percentage of the number of transformants per nanogram of empty vector in the same experiment. Error bars represent standard deviations of three biological replicates. (D) The PAM recognition specificity of the SMC4386 type I-E CRISPR-Cas system is assessed. The transformation efficiency of the PS2 protospacer flanked by the PAM sequence indicated on the *x* axis is shown. Error bars represent standard deviations of three biological replicates.

the putative anti-CRISPR operon encoding a protein with greater than 94% identity to JBD30-*36* (Fig. 1). We previously determined that these genes do not confer anti-CRISPR activity against the I-F system (9). All five of the lysogenic strains of SMC4386 tested here encode a JBD30-*36* homolog irrespective of their ability to inhibit the type I-E system. These findings suggest that the JBD30-*36* protein does not mediate anti-type I-E activity. Phage MP29, which did not mediate anti-type I-E activity, encodes only a JBD30-*36* homolog and a type I-F anti-CRISPR protein in this region. However, the four phages exhibiting type I-E anti-CRISPR activity (JBD5, JBD24, DMS3, and JBD26) all possess genes in their anti-CRISPR regions encoding proteins of unassigned function in addition to the JBD30-*36* homolog and any type I-F anti-CRISPR genes (Fig. 1). We postulated that these additional genes were the source of the observed type I-E anti-CRISPR activity.

Plasmid-based expression of four distinct genes found in anti-CRISPR phages mediates inhibition of the *P. aeruginosa* type I-E system. To aid in identifying the genes responsible for the anti-CRISPR effect observed in lysogenic strains, we searched for a phage that was inhibited by the type I-E system of *P. aeruginosa* SMC4386. We examined the genome sequences of *P. aeruginosa* phages in our collection to identify putative protospacers. Phage JBD8 has a protospacer sequence identical to a spacer in one of the type I-E CRISPR loci of strain SMC4386 flanked by a CTT PAM sequence. We tested the ability of JBD8 to form plaques on wild-type SMC4386 and the lysogenic strains of SMC4386 described above with an inactivated type I-E system (Fig. 3A). Phage JBD8 was not able to form plaques on wild-type SMC4386, but it was able to form plaques robustly when this strain was lysogenized by anti-CRISPR phage JBD5 (Fig. 3B). These data indicate that phage JBD8 is targeted by the type I-E system, and the ability of this phage to form plaques was restored in the presence of the JBD5 anti-CRISPR activity.

Once a phage that was targeted by the type I-E CRISPR-Cas system of strain SMC4386 was isolated, we were able to test directly the type I-E anti-CRISPR activity of individual genes from phage anti-CRISPR regions by expressing them from plasmids. Assays were performed utilizing plasmids expressing representatives of each unique family of genes of unassigned function within the phage anti-CRISPR regions (Fig. 1). As shown in Fig. 3C, expression of putative anti-CRISPR genes from phages JBD88a, DMS3, D3112, and JBD5 allowed CRISPR-sensitive phage JBD8

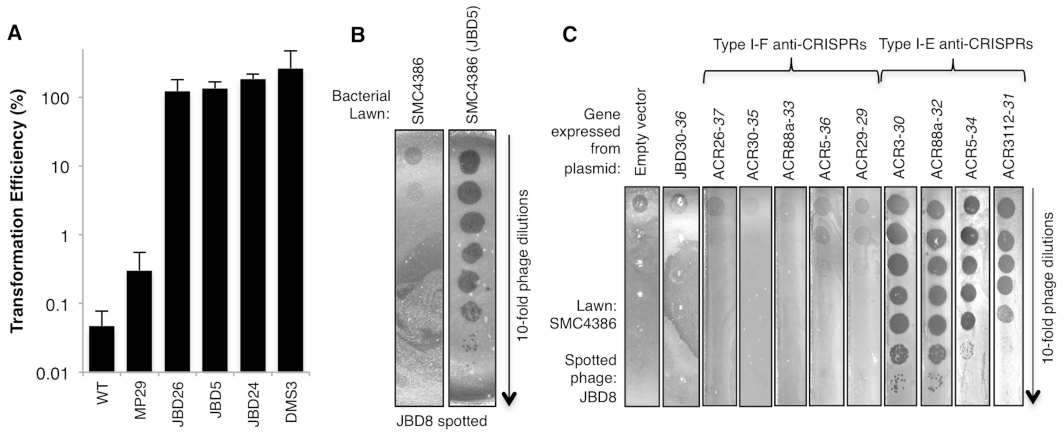

FIG 3 *P. aeruginosa* SMC4386 lysogenic strains display type I-E anti-CRISPR activity that is mediated by four unique genes. (A) Transformation efficiency assays using the PS2 construct with CTT PAM in wild-type (WT) SMC4386 or SMC4386 lysogens of the indicated anti-CRISPR phages are shown. The transformation efficiency of the protospacer/PAM construct is normalized to that of the empty vector and indicated as a percentage. Error bars represent standard deviations of biological replicates ($n \geq 2$). (B) Tenfold serial dilutions of a lysate of phage JBD8 were spotted on lawns of wild-type strain SMC4386 or strain SMC4386 harboring a JBD5 prophage. (C) Tenfold serial dilutions of a lysate of a CRISPR-sensitive phage, JBD8, were spotted on lawns of SMC4386 expressing individual putative anti-CRISPR genes from a plasmid.

to form plaques with 10^3- to 10^5-fold-higher efficiency than on cells containing empty vector. In contrast, plasmids expressing any one of five different type I-F anti-CRISPR genes, or gene JBD30-*36* (homologs of which are found at the 3′ ends of all anti-CRISPR regions) had no effect on JBD8 plaquing. These results demonstrate that four distinct families of genes mediate anti-CRISPR activity against the type I-E CRISPR-Cas system of *P. aeruginosa*. We designate the proteins encoded by these anti-CRISPR genes as ACR88a-32, ACR3-30, ACR3112-31, and ACR5-34

Although the type I-E anti-CRISPR genes did not inhibit the *P. aeruginosa* type I-F system as assayed in our previous study (9), we tested whether they might inhibit the distantly related type I-E CRISPR system of *E. coli* (see Fig. S3A in the supplemental material). For this purpose, we used an inducible type I-E CRISPR-Cas system in *E. coli* strain K-12, engineered to target phage M13 (14). The system consists of a targeting strain expressing CRISPR-Cas machinery and crRNA targeting M13 and an isogenic nontargeting strain that lacks the locus encoding crRNA. We expressed each of the type I-E anti-CRISPR proteins from a plasmid in these strains and examined the ability of phage M13 to form plaques. The targeting strain reduced the efficiency of plating of M13 by ~10^5-fold compared to the efficiency of plating of the nontargeting strain; we observed no change in the efficiency of plating upon expression of any anti-CRISPR gene (Fig. S3B). This result was not due to lack of protein expression in *E. coli*, since we examined induced lysates using SDS-PAGE followed by Coomassie staining and confirmed that all four anti-CRISPR proteins were robustly expressed (Fig. S3C). These data show that the anti-CRISPR proteins encoded by *P. aeruginosa* phages are not active against the type I-E system in *E. coli*.

Type I-E anti-CRISPRs function during the infection process to protect phage from CRISPR-Cas-mediated inhibition. We used derivatives of phage DMS3m (9) to assess the requirement of a type I-E anti-CRISPR gene for lytic phage replication. Phage DMS3m possesses a protospacer matching *P. aeruginosa* SMC4386 CRISPR 1 spacer 3 that is flanked by the PAM CTT; thus, it should be targeted by the type I-E CRISPR-Cas system of strain SMC4386. However, phage DMS3m also has an active type I-E anti-CRISPR gene (gene *30*), encoding ACR3-30 (Fig. 3C), that is expected to protect it from inhibition by the CRISPR-Cas system. As shown in Fig. 4, wild-type DMS3m is able to form plaques robustly on strain SMC4386. In contrast, a mutant phage in which the DMS3m type I-E anti-CRISPR gene was replaced

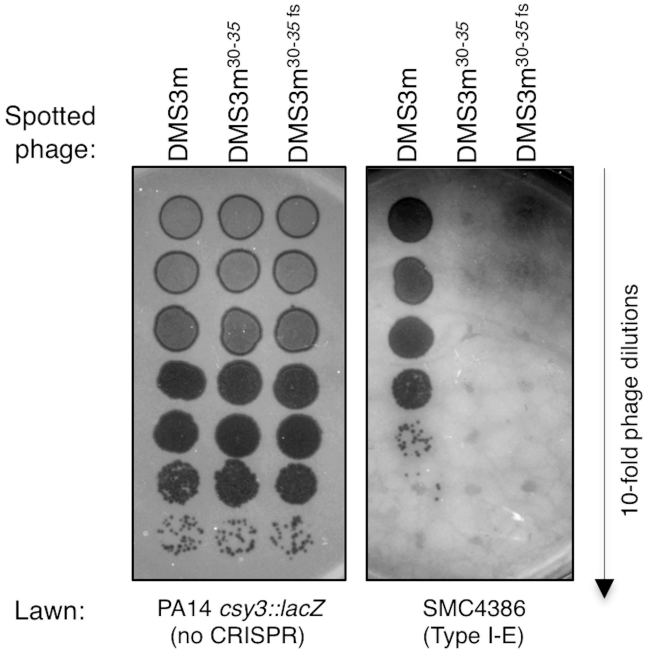

FIG 4 Anti-CRISPRs work during the process of infection to protect a targeted phage. Tenfold serial dilutions of lysates of three phages were spotted on either *P. aeruginosa* strain PA14 *csy3*::*lacZ* (a strain with no type I-E or type I-F CRISPR-Cas activity) or strain SMC4386. Phage DMS3m contains protospacer matches for the type I-E system of strain SMC4386. DMS3m$^{30\text{-}35}$ is an isogenic phage with its I-E anti-CRISPR ACR3-*30* replaced by a type I-F anti-CRISPR ACR30-*35*. The DMS3m$^{30\text{-}35fs}$ phage encodes a frameshifted version of ACR30-*35*.

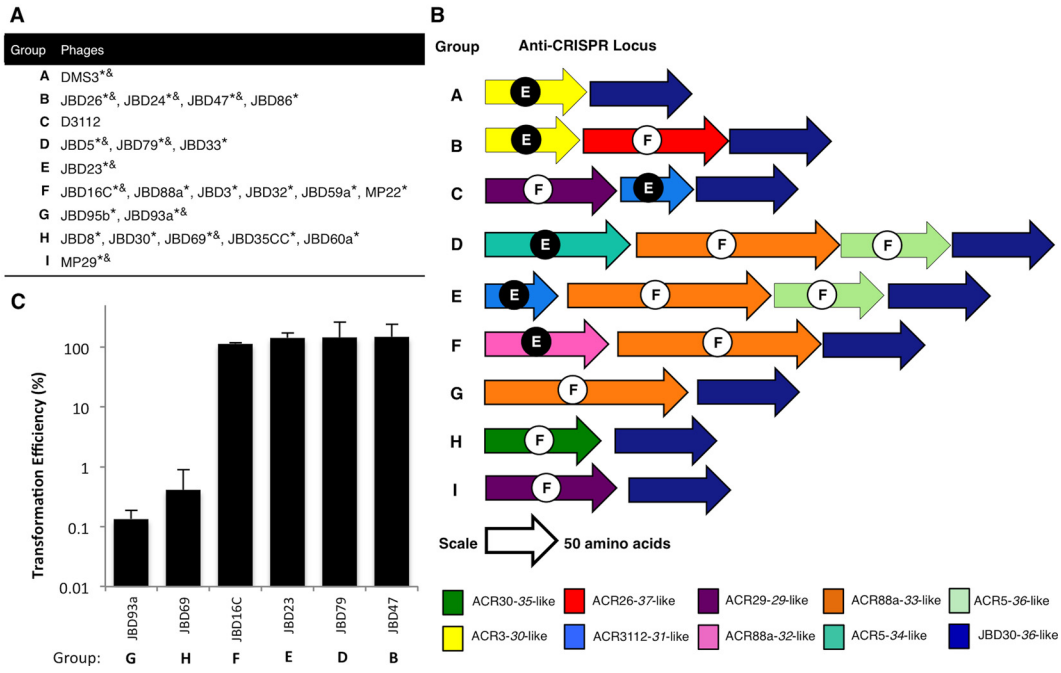

FIG 5 Anti-CRISPRs are widespread in *P. aeruginosa* Mu-like phages. (A) Transformation efficiency assays using PS2 with the CTT PAM construct are shown for a variety of *P. aeruginosa* SMC4386 lysogens. The anti-CRISPR gene regions of these phages have been sequenced, and their sequence groups are indicated. Prophages tested for type I-E anti-CRISPR activity as prophages in *P. aeruginosa* SMC4386 are indicated by an ampersand, and prophages tested for type I-F anti-CRISPR activity as prophages in *P. aeruginosa* PA14 are indicated by an asterisk. (B) The anti-CRISPR region sequence groups are defined. The boxes are drawn in proportion to the scale bar shown. Colored boxes indicate high sequence identity to one of the four type I-E anti-CRISPR genes (indicated by a white E on a black circle) or one of the five type I-F anti-CRISPR genes (indicated by a black F on a white circle). All anti-CRISPR-encoding phages have a conserved gene shown in blue, which does not have anti-CRISPR activity against the type I-E or I-F systems (3). (C) The phages belonging to each of the anti-CRISPR region sequence groups defined in panel B are listed.

with a type I-F anti-CRISPR gene (DMS3m$^{30\text{-}35}$ [9]) was not able to form plaques on SMC4386. A version of DMS3m containing a frameshifted version of the type I-F anti-CRISPR gene (DMS3m$^{30\text{-}35fs}$) was also unable to form plaques on SMC4386. All three phages infected an *P. aeruginosa* strain lacking CRISPR-Cas activity with equal efficiency (Fig. 4). These results demonstrate that gene *30*, the type I-E anti-CRISPR gene of phage DMS3m, functions during lytic infection to inhibit the type I-E CRISPR-Cas system of SMC4386 and, thus, allow robust phage replication within this strain.

Anti-CRISPR genes are widespread in Mu-like *P. aeruginosa* phages. We detected both type I-E and I-F anti-CRISPR genes at a single defined genomic location in a number of closely related Mu-like *P. aeruginosa* phages. To extend these findings, we assessed the prevalence and diversity of anti-CRISPR genes in a larger collection of related phages. By sequencing the regions of these phages where anti-CRISPR genes were expected to be located, we identified 15 additional phages that possess an anti-CRISPR region at the same genomic position as the characterized anti-CRISPR phages (Fig. 1). Of these newly identified phages, nine had type I-E and I-F anti-CRISPR genes and six had only type I-F anti-CRISPR genes. Combining these data with our previous anti-CRISPR phage analyses, we have identified a total of 15 phages with both type I-E and I-F anti-CRISPRs, eight with type I-F anti-CRISPR genes, and one phage with only a type I-E anti-CRISPR gene (Fig. 5A and B). We constructed lysogenic strains using many of these phages in *P. aeruginosa* strain SMC4386 (to assay for type I-E anti-CRISPR activity) or strain PA14 (to assay for type I-F anti-CRISPR activity). Utilizing the plasmid transformation efficiency assay to test lysogens of the anti-CRISPR phages, we were able to detect *in vivo* type I-E anti-CRISPR activity for eight phages (Fig. 3A, 5A, and C), and type I-F anti-CRISPR activity for a total of 22 phages (Fig. 5A) (9). It should be noted that this was not an exhaustive study, so the relative proportions of the anti-CRISPR activities against each subtype may not be indicative of how widespread each is in nature.

Although the experiments described above identified 15 new phages bearing anti-CRISPR genes, no novel anti-CRISPR gene families were discovered. Each newly sequenced anti-CRISPR region possessed closely related homologs (greater than 90% protein sequence identity) of one or more of the type I-F and type I-E anti-CRISPR proteins previously identified (Fig. 5B). Remarkably, homologs of the nine different families of type I-E and I-F anti-CRISPR genes appeared in nine different combinations in various phages (Fig. 5A and B), indicating that these genes have been "mixed and matched" during the evolution of this family of phages. All of the phages possessed a gene homologous to JBD30-*36* downstream of their anti-CRISPR genes, and all possessed a very similar DNA sequence upstream of their anti-CRISPR genes that appears to be a promoter, as judged by the presence of regions matching −10 and −35 transcription initiation sequences.

Active anti-CRISPR genes are found in *P. aeruginosa* mobile elements. Searches for homologs of the proteins encoded by the type I-E anti-CRISPR genes mostly yielded closely related sequences encoded in phages or prophages similar to the other anti-

CRISPR phages. However, homologs of type I-E anti-CRISPR ACR5-34 were found in several *P. aeruginosa* strains in nonprophage regions. Bioinformatic analysis of proteins encoded in the vicinity of these putative bacterial anti-CRISPR genes indicated that these regions encompass mobile DNA elements (i.e., several genes encode homologs of proteins involved in DNA transfer and conjugation [see Fig. S4A in the supplemental material]). Interestingly, one of these regions encodes homologs of both ACR5-34 and ACR5-35, a type I-F anti-CRISPR. These findings coupled with our previous discovery of type I-F anti-CRISPR genes in other *P. aeruginosa* mobile elements (9) suggests that anti-CRISPR genes may often protect other mobile DNA elements besides phages from destruction by CRISPR-Cas systems. To confirm that non-phage-encoded anti-CRISPR genes are functional, we demonstrated that two homologs of phage-encoded type I-F anti-CRISPRs (ACR30-35 and ACR5-35) and one homolog of a type I-E anti-CRISPR (ACR5-34) possess anti-CRISPR activity using a standard phage plating assay (Fig. S4C and S4D).

DISCUSSION

Phages and their bacterial hosts exert profound influences on the environment, the human microbiome, and the spread of disease. Thus, understanding the systems that affect the "arms race" between bacteria and phages is of crucial importance (15). Since CRISPR-Cas systems are key players in the phage-bacterium battle, inhibitors of these systems, if widespread, could have a large impact on bacterial and phage populations. Our recent discovery of type I-F anti-CRISPR genes in *P. aeruginosa* phages (9) raised new questions, such as whether anti-CRISPR genes are prevalent in many genomes and whether anti-CRISPR genes may inhibit other types of CRISPR-Cas systems. In this paper, we describe the discovery of an additional group of four distinct phage-borne anti-CRISPR genes that inhibit the type I-E CRISPR-Cas system of *P. aeruginosa*. As was observed for the type I-F anti-CRISPRs, these genes encode unique proteins with homologs in other closely related *P. aeruginosa* phages and in *Pseudomonas* mobile genomic elements.

The type I-E anti-CRISPR genes were almost always found adjacent to type I-F anti-CRISPR genes in closely related phages. These anti-CRISPR gene clusters all contain a conserved putative promoter region at their 5′ end and a conserved gene at their 3′ end. The latter gene encodes a protein that does not possess anti-CRISPR activity (Fig. 3C) (9) and contains a helix-turn-helix DNA-binding domain (Pfam accession no. PF13412). This protein may be a regulator of this putative anti-CRISPR operon. A surprising aspect of the anti-CRISPR gene clusters is that we have identified nine distinct arrangements of various type I-E and type I-F anti-CRISPR genes, suggesting a process by which these genes have reassorted multiple times through horizontal gene transfer. Since the anti-CRISPR proteins lack sequence similarity to any other protein families, it is difficult to trace their evolutionary origin. However, the occurrence of these genes in a variety of arrangements in both *P. aeruginosa* phages and mobile DNA elements implies that they provide a meaningful fitness advantage.

Another important contribution of this work is identifying the first examples of bacterial strains with naturally active type I-E CRISPR-Cas systems. Although the type I-E system of *E. coli* has been intensively studied (6, 13, 16–19), it is not active in wild-type *E. coli* strains. The type I-E CRISPR-Cas systems of all *E. coli* strains investigated are transcriptionally repressed and do not encode crRNAs with complementarity to characterized phages or plasmids. For these and other reasons, it has been estimated that the *E. coli* type I-E system likely has not been functional for greater than 250,000 years (20, 21). The *P. aeruginosa* type I-E system is highly divergent from the *E. coli* system (see Fig. S3A in the supplemental material). One result of this divergence is that the PAM specificity of the two systems is distinct; the PAM sequence 5′-CAT-3′ of the *E. coli* system is not recognized by the *P. aeruginosa* system, and the *E. coli* 5′-CCT-3′ and 5′-CTC-3′ PAM sequences (7, 13) are only weakly targeted by the *P. aeruginosa* system. Our characterization of the distinctive *P. aeruginosa* type I-E system will open new avenues for comparative structural and functional studies. In addition, directed engineering or experimental evolution may allow the development of anti-CRISPRs to inhibit the *E. coli* system using the *P. aeruginosa* anti-CRISPRs as starting material.

The apparent fitness advantage provided by type I-E and I-F anti-CRISPR genes indicates that both of these CRISPR-Cas systems are naturally active in many strains of *P. aeruginosa*. The common occurrence of anti-CRISPR genes in prophages found in a diverse collection of *P. aeruginosa* isolates further suggests that phage-borne anti-CRISPR genes often suppress the activity of these systems in natural settings. Since anti-CRISPR genes protect not only the phage that encodes them but also any incoming foreign DNA that might be naturally targeted by the CRISPR-Cas system, undiscovered anti-CRISPR genes may explain difficulties in demonstrating natural *in vivo* CRISPR-Cas function in various organisms and system subtypes. Our discovery of anti-CRISPR genes in mobile genetic elements other than phages suggests that anti-CRISPR genes may play a significant general role in lateral gene transfer events by allowing incoming foreign DNA to bypass CRISPR-Cas systems.

In summary, we have shown that phage-encoded anti-CRISPRs are an important mechanism by which *P. aeruginosa* phages evade both type I-E and type I-F CRISPR-Cas activities encoded by their bacterial host. Similar phenomena may be at play in a wide variety of organisms and types of CRISPR-Cas systems.

MATERIALS AND METHODS

Bacterial and phage growth. All bacterial and phage growth was performed as previously described (9). Phage lysogens in *P. aeruginosa* strain SMC4386 were created by streaking cells from the center of a phage plaque to single colonies. The presence of a prophage was confirmed by the development of resistance to superinfection by the phage in question and the production of that phage from the lysogenic strain.

Transformation efficiency assay. Oligonucleotides encoding 32-nucleotide CRISPR spacers with five nucleotides upstream and downstream (encompassing the PAM sequence), flanked by NcoI and HindIII sites were synthesized and cloned into pHERD30T (22). Known concentrations of each of the protospacer-containing plasmids were used to transform *P. aeruginosa* via electroporation. One milliliter of overnight culture was collected by centrifugation, washed twice with 300 mM sucrose, and resuspended in 100 μl of sucrose. Aliquots of cells (100 μl) were transformed with 15 to 50 ng of plasmid DNA, diluted in 1 ml Luria broth, and incubated for 40 min at 37°C to allow for recovery. Serial dilutions of each transformation reaction were plated on LB agar containing gentamicin (50 μg/ml) and incubated overnight at 37°C. The following day, the colonies were counted and normalized according to the dilution factor and amount of plasmid DNA introduced during electroporation. The data were expressed as a percentage of colonies transformed with the empty-vector pHERD30T in the same experiment.

***P. aeruginosa* plaque assays.** One hundred fifty microliters of overnight culture grown in LB was added to 3 ml LB agar containing 10 mM $MgSO_4$, 1.5% agar, and top plated on LB agar plates containing 50 μg/ml gentamicin, 10 mM $MgSO_4$, and 0.7% agar. Tenfold serial dilutions of phage lysates were spotted on the surface. The plates were incubated overnight at 30°C, and the numbers of plaques were counted.

***E. coli* M13 plaque assays.** *E. coli* strains BW40114 and BW40119 contain an isopropyl-β-D-thiogalactopyranoside (IPTG)- and arabinose-inducible type I-E CRISPR-Cas system, and strain BW40119 contains a crRNA spacer that targets phage M13 (14). These cells were transformed with pHERD30T plasmids expressing each anti-CRISPR gene. Overnight cultures were diluted 1:100 into LB with 15 μg/ml gentamicin, grown to an optical density at 600 nm (OD_{600}) of 0.6, and then induced with both 1 mM arabinose and 1 mM IPTG. After 3-h induction, cells were pelleted by centrifugation, resuspended in 3 ml soft agar, and then poured onto thick LB agar plates containing 1 mM arabinose and 1 mM IPTG. Serial dilutions of M13 phage were spotted on the surface. After incubation overnight at 30°C, plaques visible at the lowest dilution were counted, and the efficiency of plating (EOP) was calculated for each anti-CRISPR construct and for the pHERD30T empty vector in both strains.

Confirmation of anti-CRISPR expression in *E. coli*. *E. coli* strain BW40119 (14) transformed with various anti-CRISPR constructs in pHERD30T were subcultured 1:100 into LB containing the appropriate antibiotic, grown for 4 h, and then either induced with 3 mM arabinose or inhibited with 0.2% glucose. After 2 h of induction, 1 ml of culture was collected by centrifugation, and cells were resuspended in 100 μl SDS running buffer and analyzed by SDS-PAGE on a 15% Tris-Tricine gel, followed by Coomassie staining.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00896-14/-/DCSupplemental.

Figure S1, JPG file, 0.1 MB.
Figure S2, JPG file, 0.2 MB.
Figure S3, JPG file, 0.1 MB.
Figure S4, JPG file, 0.1 MB.

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