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Inactivation of CRISPR-Cas systems by anti-CRISPR proteins in diverse bacterial species

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CRISPR-Cas systems provide sequence-specific adaptive immunity against foreign nucleic acids[1,2]. They are present in approximately half of all sequenced prokaryotes[3] and are expected to constitute a major barrier to horizontal gene transfer. We previously described nine distinct families of proteins encoded in *Pseudomonas* phage genomes that inhibit CRISPR-Cas function[4,5]. We have developed a bioinformatic approach that enabled us to discover additional anti-CRISPR proteins encoded in phages and other mobile genetic elements of diverse bacterial species. We show that five previously undiscovered families of anti-CRISPRs inhibit the type I-F CRISPR-Cas systems of both *Pseudomonas aeruginosa* and *Pectobacterium atrosepticum*, and a dual specificity anti-CRISPR inactivates both type I-F and I-E CRISPR-Cas systems. Mirroring the distribution of the CRISPR-Cas systems they inactivate, these anti-CRISPRs were found in species distributed broadly across the phylum Proteobacteria. Importantly, anti-CRISPRs originating from species with divergent type I-F CRISPR-Cas systems were able to inhibit the two systems we tested, highlighting their broad specificity. These results suggest that all type I-F CRISPR-Cas systems are vulnerable to inhibition by anti-CRISPRs. Given the widespread occurrence and promiscuous activity of the anti-CRISPRs described here, we propose that anti-CRISPRs play an influential role in facilitating the movement of DNA between prokaryotes by breaching the barrier imposed by CRISPR-Cas systems.

Horizontal gene transfer (HGT) is a major driving force of bacterial evolution[6,7]. However, many HGT events are maladaptive. Bacteria have therefore evolved numerous mechanisms to destroy or silence foreign DNA. One of the most widespread of these defences is the CRISPR-Cas system, which confers adaptive, sequence-specific immunity against mobile genetic elements (MGEs) including bacteriophages (phages) and plasmids[1,2,8,9]. This adaptive immunity is powerful, as it allows organisms to build a memory of earlier invasions and deploy targeted defence strategies when related invaders return. CRISPR-Cas systems employ small CRISPR RNAs (crRNAs), often matching foreign genetic sequences, that guide CRISPR-associated (Cas) proteins to recognize and destroy DNA, or in some cases, RNA[1,10–13]. These adaptive defence systems seem likely to pose a major barrier to HGT, but evolutionary studies have implied that the global impact of CRISPR-Cas on HGT is minimal[14]. Despite the prevalence of CRISPR-Cas systems, HGT occurs frequently and is ongoing within diverse bacterial niches[15,16]. Why is HGT still pervasive among organisms possessing these sophisticated adaptive defence systems? One possible answer is the widespread existence of MGE-encoded mechanisms that inhibit CRISPR-Cas systems. In support of this hypothesis, phages infecting *Pseudomonas aeruginosa* were found to encode diverse families of proteins that inhibit the CRISPR-Cas systems of their host through several distinct mechanisms[4,5,17,18]. However, homologues of these anti-CRISPR proteins were found only within the *Pseudomonas* genus. Here, we describe a bioinformatic approach that allowed us to identify five novel families of functional anti-CRISPR proteins encoded in phages and other putative MGEs in species spanning the diversity of Proteobacteria.

The nine previously characterized anti-CRISPR protein families possess no common sequence motifs, so we used genomic context to search for novel anti-CRISPR genes. A highly conserved gene encoding a putative transcriptional regulator (Pfam family HTH_24) was found downstream of all known phage anti-CRISPR gene loci[4,5] and was absent in related phages lacking anti-CRISPRs. We conducted a BLAST search with this protein, which we refer to as anti-CRISPR-associated protein 1 (Aca1), and analysed the proteins encoded by genes immediately upstream of putative Aca1 homologues. Proteins were identified as candidate anti-CRISPRs if they were encoded on the same strand as the Aca1 homologue and were less than 200 amino acids long, because previously described anti-CRISPRs range from 50 to 139 residues in length. A predicted promoter was identified upstream of each putative anti-CRISPR-*aca1* gene pair, similar to the arrangement of the known anti-CRISPR operons. In this way, we identified four distinct putative anti-CRISPR gene families present in a variety of *Pseudomonas* prophages unrelated to previously described phages bearing anti-CRISPRs (Fig. 1a). To facilitate the description of anti-CRISPR genes, we have established a naming convention whereby anti-CRISPRs targeting a type I-F CRISPR-Cas system are referred to as AcrF, and different numbers are given to each family. The species from which a particular gene originated may be designated by a subscript. The numbering of new functional AcrF families discovered here begins at 6, because five families were discovered in our previous work[4].

All previously identified anti-CRISPR proteins inhibited either the type I-E or type I-F CRISPR-Cas systems of *P. aeruginosa*[4,5], so we tested one representative from each new family for activity in strains of *P. aeruginosa* possessing active type I-E or I-F systems. Anti-CRISPR genes were expressed in these strains on their own from a plasmid using an inducible promoter, and their ability to support replication of a CRISPR-targeted phage was assessed[4,5]. AcrF6$_{Pae}$, a putative anti-CRISPR from a prophage region in *P. aeruginosa* (Fig. 1a), completely inhibited the type I-F CRISPR-Cas system, as demonstrated by a 10^6-fold increase in

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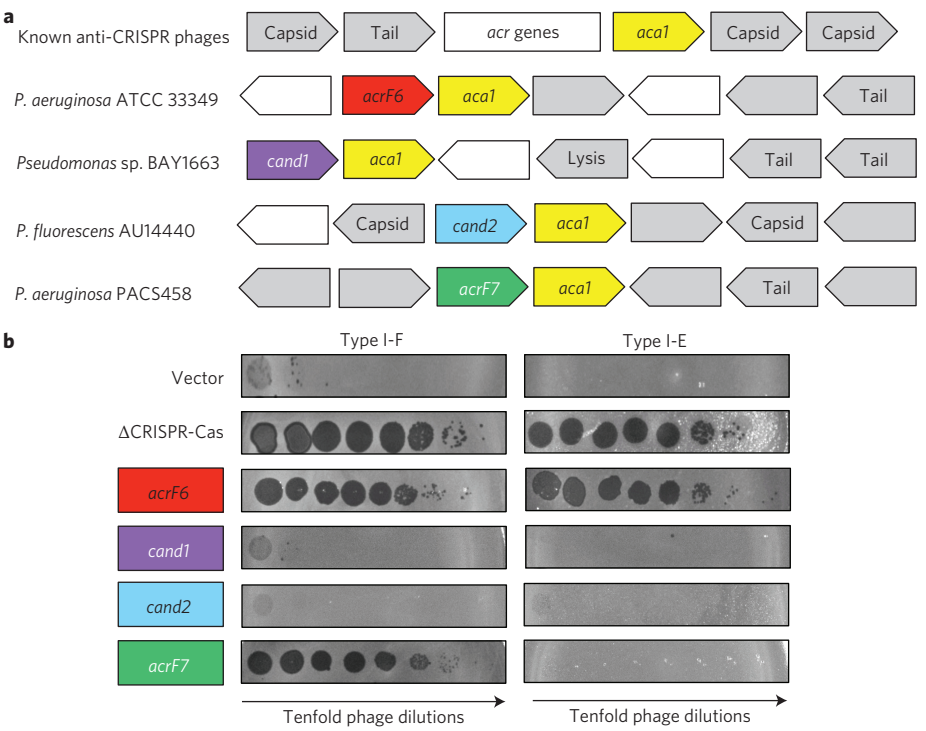

Figure 1 | Discovery and characterization of *aca1*-associated anti-CRISPR genes. a, Representative genome regions from Aca1 BLAST results with candidate anti-CRISPR genes (coloured) identified upstream. Arrows representing genes are not drawn to scale. Genes coloured in light grey are associated with mobile DNA, and gene functions are annotated (when known) to illustrate the unique genomic contexts of anti-CRISPRs. The putative functions of the proteins encoded by some open reading frames are shown to highlight the distinct genomic contexts of these anti-CRISPR genes. These functions are defined as follows: 'Capsid' is involved phage capsid morphogenesis; 'Tail' is involved in phage tail morphogenesis; 'Lysis' is required for cell lysis; 'Ter' denotes a phage terminase; 'Int' denotes an integrase; 'Transp' denotes a transposase; 'Mobil' is involved in mobilization of plasmids and conjugative elements; 'cand' denotes candidate anti-CRISPR gene. **b**, Putative anti-CRISPRs shown in **a** were assayed for biological activity. Tenfold dilutions of lysates of CRISPR-sensitive phages were applied to bacterial lawns of *P. aeruginosa* strain UCBPP-PA14 (possessing a type I-F CRISPR-Cas system) or *P. aeruginosa* strain SMC4386 (possessing a type I-E CRISPR-Cas system). As indicated, some strains carried plasmids expressing the anti-CRISPR genes or candidate anti-CRISPR genes. Clearing of the bacterial lawn indicates phage replication. Strains UCBPP-PA14 ΔCRISPR-Cas and SMC4386 ΔCRISPR-Cas are included as positive controls for loss of I-F and I-E CRISPR-Cas activity, respectively. The replication efficiency of a CRISPR-insensitive phage was unchanged following expression of each candidate gene (Supplementary Fig. 1a). Photographs are representative images from three biological replicates.

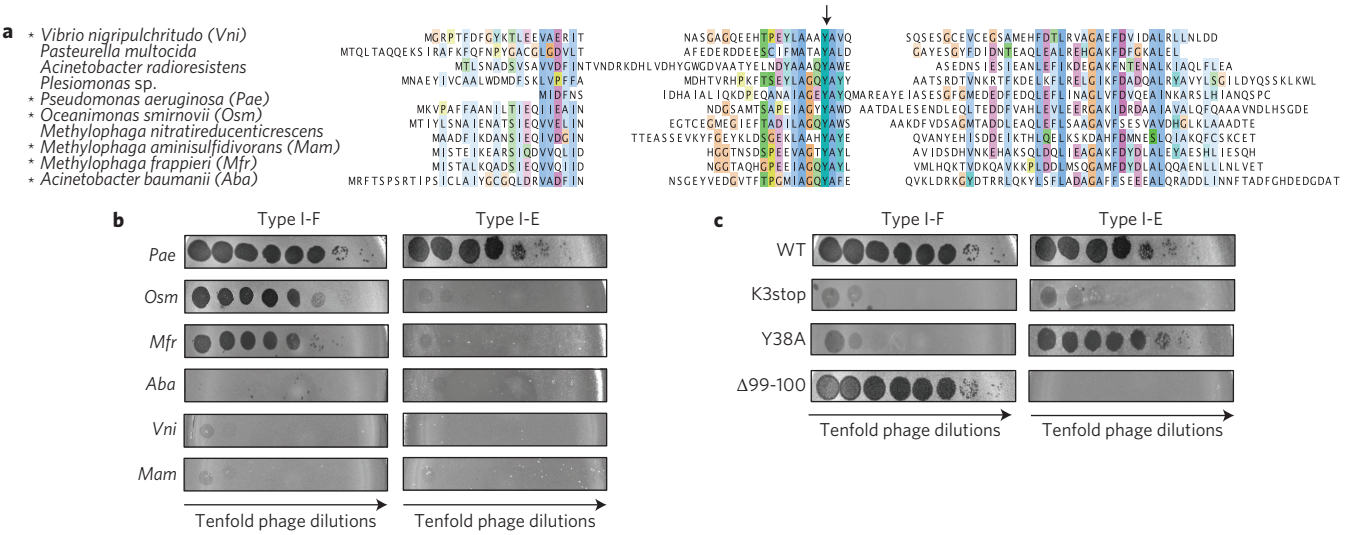

Figure 2 | Investigation of AcrF6$_{Pae}$ dual anti-CRISPR activity. a, Sequence alignment of AcrF6 homologues coloured with the ClustalX scheme. The homologues tested are indicated with an asterisk. The conserved tyrosine residue (Y38 of AcrF6$_{Pae}$) is indicated by an arrow. **b**, Phage spotting experiments as performed in Fig. 1b were carried out on strains bearing plasmids expressing homologues of AcrF6 originating from the indicated species (see **a** for three-letter species codes). **c**, Phage spotting experiments as in Fig. 1b were performed on strains expressing the indicated AcrF6$_{Pae}$ mutants. K3stop indicates a mutation where the third codon of AcrF6$_{Pae}$, which normally encodes lysine, was mutated to a stop codon. The replication efficiency of a CRISPR-insensitive phage was constant in all experiments (Supplementary Fig. 1b,c). Photographs are representative of assays on three biological replicates.

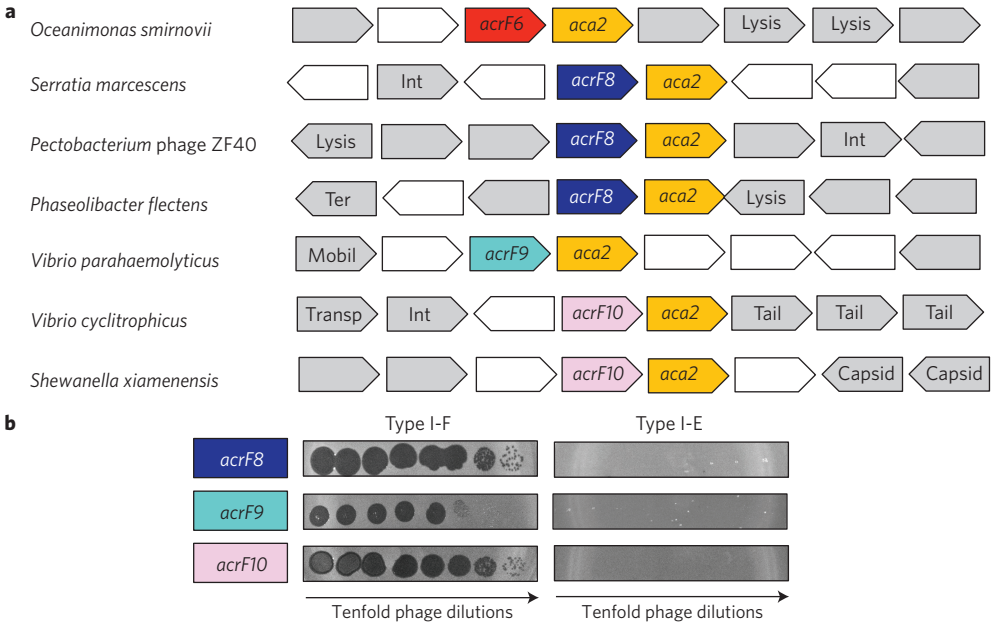

Figure 3 | Discovery and characterization of *aca2*-associated anti-CRISPR genes. a, Genomic contexts of Aca2 BLAST hits that were found in mobile DNA with candidate anti-CRISPR genes upstream. **b**, Putative anti-CRISPRs shown in **a** were assayed for biological activity. Phage spotting experiments as performed in Fig. 1b were carried out on strains bearing plasmids expressing each indicated anti-CRISPR candidate gene. The replication efficiency of a CRISPR-insensitive phage was unchanged following expression of each candidate gene (Supplementary Fig. 1d). Photographs are representative images from three biological replicates tested.

CRISPR-sensitive phage plaque formation resulting from expression of this protein (Fig. 1b). AcrF6$_{Pae}$ also inhibited the type I-E system, a remarkable result that will be discussed in the following paragraph. AcrF7, a putative anti-CRISPR from a different *P. aeruginosa* strain, displayed activity against only the type I-F system, while the remaining two anti-CRISPR candidate genes (Fig. 1a, *cand1* and *cand2*) failed to inhibit either CRISPR-Cas system (Fig. 1b). In summary, searching for homologues of Aca1 yielded two new anti-CRISPR proteins encoded by *P. aeruginosa* prophages that are unrelated to previously described anti-CRISPRs[4,5].

The ability of AcrF6$_{Pae}$ to inhibit both type I-E and I-F systems was unexpected. Despite mechanistic similarities between these CRISPR-Cas systems, their interference complexes are composed of distinct sets of Cas protein components with little detectable sequence similarity[3]. Using PSI-BLAST searches, we identified ten diverse AcrF6 homologues (Fig. 2a) that were encoded in a wide variety of Gammaproteobacteria in prophages, putative conjugative elements and regions not predicted to be horizontally transferred. Five homologues of AcrF6$_{Pae}$ were tested for anti-CRISPR activity in *P. aeruginosa*. The anti-CRISPRs from *Oceanimonas smirnovii* (AcrF6$_{Osm}$) and *Methylophaga frappieri* (AcrF6$_{Mfr}$) both inhibited the type I-F system, despite sharing only 35 and 27% pairwise sequence identity, respectively, with AcrF6$_{Pae}$ (Fig. 2b). Unlike AcrF6$_{Pae}$, AcrF6$_{Osm}$ and AcrF6$_{Mfr}$ did not inhibit the type I-E system, nor did any of the other homologues tested (Fig. 2b). Notably, these proteins represent the first functional anti-CRISPRs identified outside of the *Pseudomonas* genus.

To elucidate the basis of the dual activity of AcrF6$_{Pae}$, we introduced mutations to identify regions important for each anti-CRISPR activity. The introduction of a stop codon at the third codon of the open reading frame (K3stop) resulted in a loss of both types of anti-CRISPR activity, indicating that the same protein was responsible for both activities. Sequence alignment identified a central A-[G/A]-X-Y-A-[W/F/Y] motif that was highly conserved in all AcrF6 homologues (Fig. 2a). A single alanine substitution of the invariable tyrosine residue in this motif of AcrF6$_{Pae}$ (Y38A) abrogated anti-type I-F activity without affecting anti-type I-E activity (Fig. 2c). The greatest diversity in the AcrF6 homologues was observed at the C-termini of these proteins (Fig. 2a) and a C-terminal truncation of two residues from AcrF6$_{Pae}$ (Δ99-100) abolished type I-E anti-CRISPR activity without reducing anti-type I-F activity (Fig. 2c). These findings showed that the two anti-CRISPR activities of AcrF6$_{Pae}$ are mediated by distinct functional regions, and only the region responsible for anti-type I-F activity is a conserved feature of this family. The existence of the AcrF6$_{Pae}$ dual specificity anti-CRISPR, where a type I-F anti-CRISPR protein appears to have acquired a short sequence at its C-terminus allowing it to also inhibit the type I-E system, emphasizes the evolutionary advantages that must accrue from anti-CRISPR activities.

Our analysis of the AcrF6 homologues revealed that the gene encoding the active anti-CRISPR AcrF6$_{Osm}$ lay upstream of a gene encoding a HTH protein from a sequence family distinct from Aca1 (Pfam family DUF1870) (Fig. 3a). Hypothesizing that this HTH protein might also be anti-CRISPR associated, we identified putative anti-CRISPR genes directly upstream of genes encoding homologues of this protein, which we refer to as Aca2. In this way, three additional families of putative anti-CRISPRs were identified (Fig. 3a). Functional assays demonstrated that each of these three families included proteins that inhibited the type I-F CRISPR-Cas system of *P. aeruginosa* (Fig. 3b). These proven Aca2-associated anti-CRISPRs and their homologues were found among distantly related proteobacterial genera, including *Delftia* (Betaproteobacteria), *Desulfobulbus* (Delta/Epsilonproteobacteria) and several diverse Gammaproteobacterial genera (for example, *Vibrio*, *Pectobacterium* and *Shewanella*) (Supplementary Table 1). In total, our bioinformatic approach based on anti-CRISPR-associated genes allowed us to identify five novel families of type I-F anti-CRISPR genes, denoted AcrF6, AcrF7, AcrF8, AcrF9 and AcrF10, which are widely distributed across the Proteobacterial phylum.

An intriguing feature of the newly identified anti-CRISPRs was their ability to inhibit the CRISPR-Cas system of *P. aeruginosa*, despite

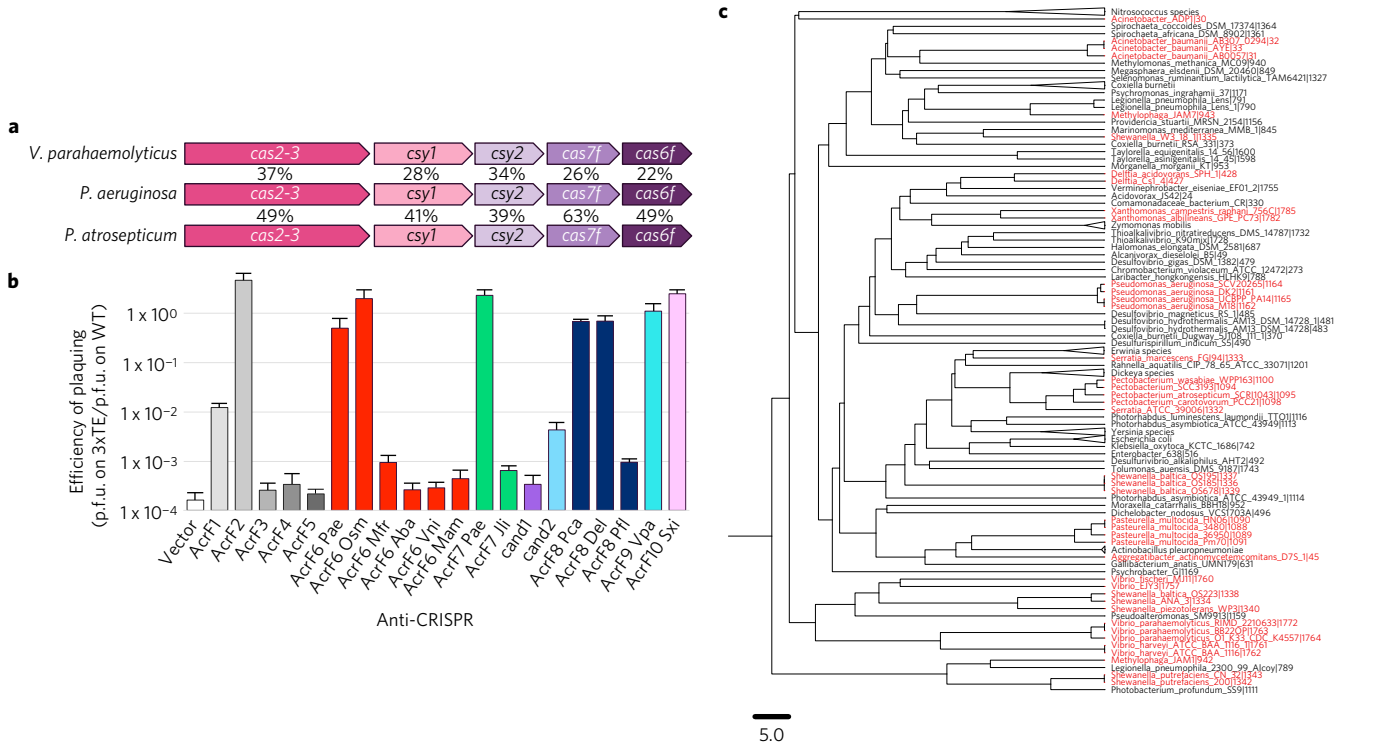

Figure 4 | Newly identified anti-CRISPRs impact diverse type I-F CRISPR-Cas systems. a, Gene arrangement and pairwise percent identities for type I-F Cas proteins between *V. parahaemolyticus* and *P. aeruginosa*, and between *P. atrosepticum* and *P. aeruginosa*. These comparisons are representative of the levels of diversity among type I-F systems relevant to this work. **b**, Relative efficiency of plaquing of phage ϕTE on lawns of *P. atrosepticum* cells expressing each of the indicated candidate anti-CRISPR genes from a plasmid. The relative efficiency is calculated by dividing the p.f.u. of phage ϕTE on *P. atrosepticum* 3xTE cells (where the CRISPR-Cas system targets ϕTE) by the p.f.u. on wild-type *P. atrosepticum* (where the CRISPR-Cas system does not target the phage). Anti-CRISPRs are colour-coded according to Figs 1 and 3. Error bars represent standard deviation among five replicates. **c**, Sequence similarity dendogram of type I-F CRISPR-Cas interference components developed by Makarova and co-authors.[3] A sequence similarity score was calculated by comparing all proteins involved in the interference process (Csy1, Csy2, Cas7f (Csy3), Cas6f (Csy4) and Cas2-3)[3]. CRISPR-Cas systems are coloured red if they belong to a genus encoding a member of a validated type I-F anti-CRISPR protein family.

originating in diverse bacterial species. Five of the six non-*Pseudomonas* species harbouring active members of anti-CRISPR protein families (Supplementary Table 1) encoded their own type I-F CRISPR-Cas systems. Although the anti-CRISPRs from these species efficiently inhibited the type I-F CRISPR-Cas system of *P. aeruginosa*, the individual Cas proteins from these systems were extremely diverse (ranging from 22 to 68% pairwise identity with *P. aeruginosa* Cas proteins). For example, AcrF9 from *Vibrio parahaemolyticus* abrogated type I-F CRISPR-Cas activity in *P. aeruginosa*, even though the Cas proteins of these two species share only 22–37% pairwise identity (Fig. 4a). Assuming that these anti-CRISPRs inhibit CRISPR-Cas activity within their species of origin, their ability to inhibit the *P. aeruginosa* system implies a broad specificity. To further examine anti-CRISPR specificity, we tested the activities of these anti-CRISPR proteins in *Pectobacterium atrosepticum*, which has an active type I-F CRISPR-Cas system[19–21] distinct from *P. aeruginosa* (Cas proteins share 39–63% pairwise sequence identity; Fig. 4a and Supplementary Table 2). We discovered that representatives from all five of the new type I-F anti-CRISPR families and one of the previously identified anti-CRISPR families (AcrF2) completely blocked CRISPR-Cas targeting of phage ϕTE in *P. atrosepticum* (Fig. 4b). When we examined previously described anti-CRISPRs for which the mechanisms of inhibition are known[17], we discovered that anti-CRISPR specificity could not be predicted by the sequence similarity of the target Cas proteins. For example, AcrF2 was active in both type I-F systems, and was previously shown to inhibit the CRISPR-Cas system through an interaction with the Csy1–Csy2 heterodimer[17]. The Csy1 and Csy2 proteins from *P. aeruginosa* and *P. atrosepticum* share only 41 and 39% sequence identity, respectively (Fig. 4a). By contrast, AcrF1 targets Cas7f (Csy3) (ref. 17), the most conserved subunit between *P. aeruginosa* and *P. atrosepticum* (63% identity), but it did not efficiently inhibit the *P. atrosepticum* system (Fig. 4a,b). Overall, these data highlight the promiscuous activities of anti-CRISPR proteins, and illustrate the difficulty of predicting whether a particular anti-CRISPR will inhibit a CRISPR-Cas system of interest.

A recent bioinformatic study showed that 30% of 282 *P. aeruginosa* strains with type I-E or I-F CRISPR-Cas systems possessed anti-CRISPR genes that would inactivate these systems[22]. To estimate the potential influence of anti-CRISPR activity on all known type I-F CRISPR-Cas systems, we used a recently published protein sequence similarity dendrogram that included all of these systems[3]. We identified CRISPR-Cas systems on the dendrogram that belonged to a genus encoding a member of a validated type I-F anti-CRISPR protein family (Fig. 4c). With this approach, we discovered that type I-F anti-CRISPRs are encoded in prophages and MGEs of bacteria spanning the diversity of type I-F CRISPR-Cas systems. Given the promiscuous activity of anti-CRISPRs described here, these data suggest that all type I-F CRISPR-Cas systems are probably susceptible to inhibition by anti-CRISPRs. It is also notable that members of each anti-CRISPR gene family were found in varied genomic contexts, including different orientations and genomic positions in several unrelated prophage families, as well as in other types of MGEs. For example, two very closely related AcrF10 family members (79% protein sequence identity) were found in different genomic positions in two unrelated phages, one infecting *Shewanella* and

one infecting *Vibrio* (Fig. 3a). Thus, even closely related anti-CRISPRs have the potential to be disseminated to diverse lineages of bacteria through recombination into different MGE vectors, explaining how anti-CRISPRs have spread so broadly and emphasizing the evolutionary advantage that must be conferred by these proteins.

In summary, in this work we describe five new anti-CRISPR protein families and provide the first evidence that anti-CRISPRs are widespread across the phylum Proteobacteria. Representatives from all five of the new anti-CRISPR families possess broad activity within a CRISPR-Cas subtype, inhibiting both *P. aeruginosa* and *P. atrosepticum* CRISPR-Cas systems, despite originating from species with divergent type I-F systems. The anti-CRISPRs discovered here surely represent only the tip of the iceberg with respect to the full diversity of anti-CRISPRs in existence. Our approach inherently overlooks any anti-CRISPR gene families not associated with *aca*-like genes[4,5], as well as any anti-CRISPRs that may function in different types of CRISPR-Cas systems or distantly related type I-F and I-E systems. False-negative results in this initial survey could arise from inadequate protein expression or stability. Given our success in discovering new anti-CRISPRs, even using the focused approach described here, we expect that a large-scale unbiased functional screen would uncover large numbers of new anti-CRISPRs against a variety of CRISPR-Cas system types. A wide distribution of anti-CRISPRs with a broad spectrum of activity would render many CRISPR-Cas systems inactive, providing an explanation for the high frequency of HGT[15,16], despite the prevalence of these sophisticated defence mechanisms[3,14]. Because many HGT events involve the transfer of antibiotic resistance and virulence factors, especially among human-associated bacteria[12], anti-CRISPRs have the potential to significantly influence bacterial pathogenesis.

Methods

Bioinformatic analysis. BLASTp searches for Aca1 were conducted with the sequence of gene product *36* from *Pseudomonas* phage JBD30 (accession code YP_007392343) as the query. Homologues of AcrF6 were found using iterative PSI-BLAST[23] and transitive homology, and the protein sequence alignment of the AcrF6 family was created using MAFFT[24]. BLASTp searches for Aca2 were conducted with WP_019933869.1 from *Oceanimonas smirnovii* as the query. All protein alignments were constructed using MAFFT and visualized using JalView[25]. The sequence similarity dendogram in Fig. 4c is a FigTree visualization of a portion of the interference dendogram Newick file provided in ref. 3, on which we coloured in red the clades belonging to a genus encoding a member of a validated anti-CRISPR protein family (Supplementary Table 1).

***P. aeruginosa* strains and bacteriophages.** *P. aeruginosa* strains UCBPP-PA14 and SMC4386 were grown in lysogeny broth (LB) medium and supplemented with 50 μg ml^{-1} when necessary to maintain pHERD30T[26] plasmids. Further information on these strains and their derivatives is provided in Supplementary Table 3. CRISPR-sensitive and CRISPR-insensitive bacteriophages for each type of *P. aeruginosa* CRISPR-Cas system are described in detail in the '*P. aeruginosa* bacteriophage plaque assays' section and in Supplementary Table 3.

Cloning of candidate anti-CRISPR genes. The coding sequences for candidate anti-CRISPRs were synthesized by Genscript. Sequences were flanked with NcoI and HindIII restriction sites and synthesized in tandem gene arrays in pUC57. Synthesized plasmid DNA was digested with NcoI and HindIII enzymes, and the resulting product mixture was subcloned into pHERD30T[26] predigested with the same two enzymes. Isolated plasmid DNA was sequenced to confirm the presence of individual gene inserts. Several genes were synthesized with a 6xHis tag at either the N- or C-terminus for downstream applications. Codon optimization was employed for genes that were found in non-*Pseudomonas* species, and synonymous mutations were introduced to remove incompatible restriction endonuclease sites when necessary. DNA sequences are provided in Supplementary Table 4.

***P. aeruginosa* bacteriophage plaque assays.** Bacteriophage plaque assays were conducted to assess CRISPR-Cas system activity as described previously for the type I-F[4,27] and type I-E[5] systems of *P. aeruginosa*. Briefly, *P. aeruginosa* cells containing pHERD30T[26] plasmid expressing candidate anti-CRISPR genes were mixed with soft LB agar and poured onto LB agar plates containing 10 mM $MgSO_4$, 50 μg ml^{-1} gentamicin and 2 mM arabinose to induce expression from the p_{BAD} promoter of the plasmid. Tenfold serial dilutions of a CRISPR-targeted phage (DMS3m for I-F; JBD8 for I-E, as described previously[4,5]) and a non-targeted phage (DMS3 for I-F; JBD93a for I-E, as described previously[4,5]) were spotted on the surface. Plates were incubated overnight at 30 °C. Our previous work has shown that these assays are very specific in that a type I-F anti-CRISPR will not be active against the type I-E system and vice versa[4,5]. We have also previously found that a very low expression of anti-CRISPRs (that is, without addition of inducer) is sufficient for biological activity.

Site-directed mutagenesis. Complementary oligonucleotides comprising the codon to be mutated plus fifteen nucleotides in both directions were synthesized by Eurofins Genomics. PCR was carried out using Pfu DNA polymerase and template plasmid DNA. Subsequently, the template was digested using DpnI, and ethanol precipitation was used to concentrate the resultant PCR product before transforming *E. coli* DH5α. Mutations were confirmed by DNA sequencing.

***P. atrosepticum* CRISPR assays.** *P. atrosepticum* 3xTE was transformed with plasmids (pHERD30T[26]) expressing the different anti-CRISPRs, and overnight cultures were used to pour top agar plates (100 μl culture added to 3 ml LB with 0.35% agar, containing 30 μg ml^{-1} gentamicin) onto LB agar plates (containing 30 μg ml^{-1} gentamicin). Tenfold serial dilutions of phage ϕTE lysates (in phage buffer: 10 mM Tris-HCl pH 7.4, 10 mM $MgSO_4$ and 0.01% wt/vol gelatin) were made, and 10 μl was spotted on the dried top agar plates and incubated overnight at 25 °C. The efficiency of plaquing (EOP) was determined by calculating the plaque-forming units per ml (p.f.u.) of the *P. atrosepticum* 3xTE cells (expressing the different anti-CRISPRs) divided by the p.f.u. of the wild-type *P. atrosepticum*. All experiments were repeated five times.

Generation of ϕTE-resistant *P. atrosepticum* strain. A phage ϕTE-resistant *P. atrosepticum* SCRI1043 strain was constructed by exploiting the process of rapid spacer acquisition from a primed plasmid (containing phage ϕTE sequences), as described previously[28,29]. Briefly, plasmid pPF718 was constructed by cloning a 2 kb fragment of the ϕTE genome[30] (part of a gene encoding a virion structural protein), using primers PF1635 and PF1636, into the primed vector, pPF712 (strains and plasmids are listed in Supplementary Table 3 and primers in Supplementary Table 5). Plasmid pPF712 was constructed as follows. The Tc^R gene was amplified from pTRB31 (ref. 29) using primers PF1634 (contain primed protospacer) and PF1364, digested with NcoI and XhoI, and ligated with pQE-80LoriT-GFP that was previously cut with BspHI and XhoI. Plasmid pPF718 was conjugated into wild-type *P. atrosepticum* cells, and expanded CRISPR arrays of plasmid-interfering colonies were screened for acquisition of phage ϕTE-targeting spacers[29]. For the experiments described in the following section, a clone (hereafter referred to as '*P. atrosepticum* 3xTE'; strain PCF188) was selected that harboured two different phage ϕTE-targeting spacers in CRISPR1 and one in CRISPR2 (two of the three CRISPR arrays in *P. atrosepticum*), the sequences of which are listed in Supplementary Table 6.

Generation of *P. aeruginosa* SMC4386 ΔCRISPR-Cas strain. Complementary oligonucleotides encoding a crRNA designed to target near the beginning of the *cse1* gene of the type I-E CRISPR-Cas system in *P. aeruginosa* strain SMC4386 were annealed and ligated into pHERD30T[26]. SMC4386 cells transformed with this plasmid had greatly reduced transformation efficiency as compared to empty vector (approximately 10^{-6}), indicating that the endogenous CRISPR-Cas system was efficiently using this crRNA to target the genome of the strain, resulting in cell death. We grew colonies that were transformed by the crRNA-expressing plasmid, presuming that some of them may have mutations or deletions in *cse1*. We isolated and performed genome sequencing on one mutant that lost all detectable CRISPR-Cas activity against CRISPR-sensitive bacteriophages. Following sequencing, we found that a genomic deletion of ~125 kb had removed the entire CRISPR-Cas system, including all *cas* genes and both CRISPR loci. This strain was used as a ΔCRISPR-Cas control for type I-E CRISPR-Cas activity assays in strain SMC4386.

Accession codes. *P. aeruginosa* strains used in this study: UCBPP-PA14 (NC_008463.1) and SMC4386 (LOQZ00000000). *P. atrosepticum* strain used in this study: SCRI1043 (BX950851.1). Candidate anti-CRISPR genes tested in this study: $AcrF6_{Pae}$ (KQJ68297.1), AcrF6Osm (WP_019933870.1), AcrF6Mfr (WP_014702809.1), AcrF6Aba (EXB32219.1), AcrF6Vni (CCN39710.1), AcrF6Mam (WP_007146432.1), AcrF7Pae (ACD38920.1), AcrF7Jli (WP_034755374.1), AcrF8Pec (AFC22483.1), AcrF8Del (KEH13790.1), AcrF8Pfl (WP_036985738.1), AcrF9Vpa (WP_031500045.1), AcrF10Sxi (KEK29119.1), cand1 (EXF45286.1) and cand2 (WP_044274829.1). The results from anti-CRISPR activity assays for each of these proteins are provided in Figs 1b, 2b, 3b and 4b and Supplementary Table 1. The accession numbers for anti-CRISPR homologues not tested in this study are listed in Supplementary Table 1.

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Author contributions

A.P. designed experiments, performed bioinformatic analysis, performed experiments, analysed data and wrote the manuscript. R.H.J.S. designed and performed *Pectobacterium* experiments and wrote parts of the manuscript. C.T. performed *Pectobacterium* experiments. B.N.J.W. performed *Pectobacterium* experiments and wrote parts of the manuscript. S.S. assembled and uploaded to NCBI the genome sequences of *P. aeruginosa* strains SMC4386 and SMC4386 ΔCRISPR-Cas. P.C.F. designed *Pectobacterium* experiments and wrote parts of the manuscript. K.L.M. supervised experiments and wrote the manuscript. A.R.D. supervised experiments and wrote the manuscript.

Additional information

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Competing interests

The authors declare no competing financial interests.