Phage CRISPR-like regulatory RNAs silence bacterial adaptive and innate immunity

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Abstract: In prokaryotes, arrayed CRISPR RNAs (crRNAs) guide Cas proteins to destruct phage DNA/RNA, while solitary crRNA-like RNAs (crlRNAs) program Cas proteins for auto-regulation or to control abortive infection (Abi)-inducing toxins to back up CRISPR immunity. Here, we report that phages exploit crlRNA mimics to hijack these multi-layered host defenses. *Pseudomonas aeruginosa* phages use crlRNAs to thwart CRISPR-Cas immunity by inhibiting Cas expression, or to block Abi by silencing an unprecedented RNA toxin that features consecutive proline codons. Remarkably, the anti-CRISPR protein AcrIF24 selectively inhibits Cas proteins loaded with host crRNAs/crlRNAs, while allowing those complexed with viral crlRNAs to synergistically block Abi responses, as viral crlRNAs have co-adaptively evolved shorter spacer sequences. Furthermore, viral crlRNAs frequently organized as multiplexed arrays, mirroring the architecture of bacterial CRISPRs. Our findings showcase how phage RNAs hijack Cas proteins to silence multi-layered bacterial defenses, and highlight the delicate synergy between RNA and protein-based anti-CRISPR strategies.

Key words: CRISPR-Cas, abortive infection, anti-CRISPR, small RNA, RNA toxin

In prokaryotes, CRISPR-Cas systems provide adaptive immunity against invasive mobile genetic elements (MGEs), such as phages and plasmids. (*1-3*). These diverse systems are classified into two main groups (class 1 and class 2), encompassing six types (types I-VI) and over 30 subtypes (*4*). The CRISPR array consists of short "spacer" sequences derived from MGEs, interspersed between conserved direct repeats. Adjacent to CRISPR arrays are *cas* genes, which encode an effector complex of multiple Cas subunits (class 1) or a single-protein effector (class 2) (*4*). The long transcripts of CRISPR arrays are processed into small mature CRISPR RNA (crRNA) molecules, which direct the Cas effector to identify and cleave the invading nucleic acids. This process relies on a precise match between the crRNA spacer and the target "protospacer" sequence, found next to a specific 'protospacer adjacent motif' (PAM) (*5, 6*).

Recent research has uncovered the secondary function of Cas effectors in gene regulation, guided by a series of regulatory crRNA-like RNAs (crlRNAs) (*7-9*). Unlike traditional crRNAs, crlRNAs are encoded by solitary repeat units (SRUs) or mini-arrays and redirect Cas proteins to host promoter sequences for transcriptional repression. Importantly, the match between the crlRNA spacer sequence and the target protospacer is only partial, which allows target DNA binding without licensing cleavage (*7-9*). Our recent studies have systematically demonstrated the regulatory role of type I-B, I-E, I-F, and type V-A Cas effectors, which are directed by a crlRNA known as Cas-regulating (CreR) RNA (*10, 11*). CreR RNAs are typically encoded upstream of the Cas effector gene(s) and direct the Cas effector to repress its own promoter (see Fig. 1A for example). This autorepression has shown to reduce the risk of CRISPR autoimmunity while fine-tuning Cas expression to match the cellular abundance of endogenous crRNAs (which compete for Cas proteins with CreR), and up-regulating Cas expression when viral anti-CRISPR (Acr) proteins inactivate host Cas proteins *(10)*. Similarly, recent works have revealed that the majority of type II-A CRISPR-Cas systems encode a long isoform of the trans-activating crRNA (tracr-L) acting as a regulatory crlRNA, which re-directs Cas9 to auto-regulate its own expression, leading to a strong spike of Cas9 production when host Cas9 proteins are inhibited by Acr proteins (*12, 13*).

In addition, in some type I systems, CreR elements have shown to direct Cas proteins to transcriptionally inhibit a CreT toxin (CRISPR-repressed toxin) that is typically encoded next to the Cas effector genes (*8*). In these loci, CreR molecules are referred to as CreA (crRNA-resembling antitoxin) RNAs due to their antidote activity. These CRISPR-regulated toxin-antitoxin (CreTA) modules trigger cellular death/dormancy when *cas* genes are disrupted by transposable elements or when Cas proteins are compromised by Acr proteins encoded by MGEs. Indeed, we have recently demonstrated that CreTA systems safeguard CRISPR-Cas systems and expel Acr-encoding MGEs from the bacterial population, thus providing broad-spectrum anti-anti-CRISPR protection to CRISPR-Cas systems (*14*). This strategy is reminiscent of the immune guard hypothesis initially described in plants and increasingly recognized in prokaryotes, where the inhibition of one defense mechanism triggers the activation of another (*15-17*). However, little is known about how viruses overcome multilayered defenses.

In a recent study, Shmakov *et al.* identified viral crRNA-like sequences resembling CreR elements in bacteria, which show partial complementarity to *cas* operon promoters (*9*). These findings suggest that these phage-encoded elements may interfere with host CRISPR-Cas expression, yet their functions remained enigmatic. In this study, we experimentally demonstrated that diverse viral crlRNAs can re-direct host Cas proteins to robustly suppress the expression of Cas proteins or CreT toxins, thus exhibiting RNA-guided anti-CRISPR and anti-anti-anti-CRISPR functions, respectively. To distinguish these crlRNAs from their cellular counterparts (CreR and CreA), we refer to these viral Cas/CreT-repressing RNA anti-CRISPRs as Cracr RNAs, aligning with the nomenclature of Racrs (RNA anti-CRISPRs), recently demonstrated to sequester Cas proteins to form aberrant complexes (*18*). Remarkably, Cracr RNAs are frequently found stacked as CRISPR-like arrays in viruses to simultaneously block diverse CRISPR-Cas variants and/or their associated CreTAs. Moreover, we also uncovered the marked co-occurrence and functional synergy between type I-F Cracr RNAs and AcrIF24, an inhibitory protein that differentiates Cracrs from cellular crlRNAs and crRNAs based on their shorter spacer sequences. Therefore, our findings showcase the genetic diversity of viral anti-CRISPR elements and their mechanistic synergy, providing valuable insights for improving the efficacy of therapeutic phages and the safety of CRISPR-Cas tools.

Results

Two conserved Cracr RNAs encoded by *Pseudomonas aeruginosa* MGEs

To explore whether CreR-mediated Cas auto-repression is a strategy exploited by genetic parasites to suppress host Cas expression, we searched for MGE-encoded *creR* homologs across the genomes of the opportunistic pathogen *P. aeruginosa*. Bioinformatic screening of type I-F CRISPR-Cas loci across the *P. aeruginosa* genomes in the NCBI database revealed two conserved *creR* genes, *creRIF1* and *creRIF2*, located within the intergenic region between *cas2-3* and *csy1* (Fig. 1A). Notably, this intergenic region is only present in a fraction of *P. aeruginosa cas* operons and is absent in the well-studied PA14 I-F *cas* operon, likely explaining its late discovery. Blastn searches using *creRIF1* and *creRIF2* and manual exclusion of the hits within or nearby CRISPR-Cas loci revealed over 150 SRUs or mini-arrays that likely encode homologs of CreRIF1 and CreRIF2, which we referred to as candidate CracrIF1 and CracrIF2, respectively (Fig. 1B and Table S1). Notably, these

Cracr candidates are enriched on MGE sequences, primarily prophages and plasmids (Fig. 1B). Interestingly, while CracrIF1 RNAs prefer a 18-nt spacer-like sequence (ΨS), CracrIF2 prefer a 20-nt ΨS. We also noted high-level nucleotide conservation of the ΨS sequences, except for variations in nucleotides every 6th position (Fig. 1B), which are known not to base pair with the target DNA (*19, 20*). This marked sequence conservation indicates that *cracrIF1* or *cracrIF2* genes are mutationally constrained to maintain their base-paring potential to conserved DNA targets, suggesting their interaction with Cas proteins to carry out CreR-like functions.

Most of the identified *cracr* genes cluster with known Acr protein-coding genes, strongly suggesting their role in immune inhibition (Fig. 1B). While prophage IF *cracr* genes typically locate directly downstream of an *acrIF23-24* operon, in line with the findings of Shmakov et al (*9*). Plasmid-born *cracr* genes do not exhibit this pattern, though a co-occurring *acrIF1* gene was frequently observed (Fig. 1B). A systematic analysis of the genomic contexts of all available *acrIF24* genes revealed that nearly 88.76% of them are followed by *cracr* genes (fig. S1A), indicating a potential functional synergy between AcrIF24 and Cracr RNAs, as well as suggesting *acrIF24* may serve as a valuable marker to uncover further Cracr diversity. Facilitated by this marker, our systematic searches for *cracr* genes across taxa revealed diverse putative Cracr RNAs with 85 SRUs or mini-arrays that are carried by phages or MGEs from a series of non-*P. aeruginosa* bacteria (spanning 9 genera, and 7 orders of β-proteobacteria and γ-proteobacteria) (Table S2 and fig. S1B). These *cracr* genes typically share highly similar repeat sequences with a type I-F CRISPR array co-occurring within the same (or a closely-related) host genome, facilitating prediction of their ΨR and ΨS sequences. Notably, these *cracr* genes harbor different ΨS sequences, suggesting their ability to suppress a diversity of CRISPR-Cas loci. Accordingly, we predicted 8 CRISPR-Cas loci with their *cas* promoters likely being targeted by one of these *cracr* genes (Table S2). Altogether, our findings suggest that Cracr elements are widespread across diverse MGEs and are likely employed to silence host CRISPR-Cas immune functions.

CracrIF1 shows anti-CRISPR activity

The *Pseudomonas* phage vB_PaS_IME307 carries a *cracrIF1* gene (previously noticed by Shmakov *et al.* (*9*)) that is homologous to the *creRIF1* gene from *P. aeruginosa* NY5506, while the prophage in the *P. aeruginosa* strain P3-S8 contains a *cracrIF2* gene that is homologous to the *creRIF2* gene from *P. aeruginosa* strain Y010 (Fig. 1A). All the CRISPR repeat-like (ΨR) sequences of these Cracr candidates are highly similar to the consensus repeat of type I-F CRISPR arrays, except for the eight 3'-terminal nucleotides of their second ΨR (ΨR2) (fig. S2A). These sequences all contain internal palindromes indicative of a conserved RNA hairpin structure that is predicted to be recognized by the Csy4 endonuclease (Fig. 1B). Notably, the two *cracr* genes share 8 identical 3'-terminal nucleotides, which differ significantly from those of the two *creR* genes. In addition, the *cracr* genes have slightly shorter ΨS sequences (Fig. 1A). Therefore, it is likely that the ΨS and ΨR2 portions of the viral crlRNA genes (*cracr*) underwent adaptive evolution following their exaptation from the cellular genes (*creR*), while retaining the ability to interact specifically with host Cas proteins.

To functionally characterize the phage-encoded CracrIF1, we first confirmed its production from a plasmid in *P. aeruginosa* cells expressing the type I-F Cas system (fig. S2C). In PAO1 cells lacking a I-F CRISPR-Cas system, no small RNA products were detected by Northern blotting. However, the introduction of a plasmid expressing the PA14 Csy proteins (Csy1-Csy4) led to the detection of mature CracrIF1 RNAs, indicating specific binding and processing of CracrIF1 by host Cas proteins (fig. S2C). By constructing a chimeric *cas* operon (designated PA14-NY5506) composed of the complete set of PA14 *cas* genes and the NY5506 *cas* intergenic sequence including *creRIF1*, we also confirmed the production of mature CreRIF1 RNAs (fig. S2C). Notably, the CracrIF1 RNAs were observed to be slightly smaller than host CreRIF1 RNAs, consistent with their differing ΨS sizes (18-nt and 20-nt, respectively). Despite this small difference, the ΨS sequences of CracrIF1 and CreRIF1 share 17 identical nucleotides, enabling almost perfect base pairing (except the 6th nucleotide) with a target sequence upstream of the NY5506 *csy* operon (Fig. 2A). The presence of a canonical type I-F PAM, i.e., 5'-CC-3' further indicated that CracrIF1 and CreRIF1 both have the potential to direct the Csy complex to bind to this target site. Together, these data indicate that both CracrIF1 and CreRIF1 are effectively matured like endogenous crRNAs by Csy4 and contain a ΨS predicted to bind upstream of the *csy* operon.

To investigate whether the predicted binding of CreRIF1 and CracrIF1 to their target site impacts Cas expression, we first mapped the location of the transcription start site (TSS) of the NY5506 *csy* operon through 5' RACE analysis (fig. S3A) and predicted its -35 and -10 promoter elements (Fig. 2A). Interestingly, the *csy* transcripts have an unusually long 5' untranslated region (5'-UTR), later discovered to produce an RNA toxin (see below). To evaluate the potential *cys*-silencing effect of CreRIF1 and CracrIF1, we constructed a fluorescent mCherry reporter under the control of the *csy* promoter (P_{csy}) and confirmed its TSS through primer extension (consistent to the results of 5' RACE; fig. S3B). By measuring the fluorescence signal in PAO1 cell culture expressing the PA14 Csy proteins along with CracrIF1 or CreRIF1, we observed that CracrIF1 and CreRIF1 reduced the strength of P_{csy} by a factor of 14 and 21, respectively (Fig. 2B). Importantly, when we mutated three ΨS nucleotides (indicated in Fig. 1A) of either RNA regulator to disrupt its complementarity with P_{csy}, the inhibitory effects were no longer observed (Fig. 1C). Therefore, the phage-encoded CracrIF1 redirects host Cys complexes to specifically silence the *cys* operon, likely providing phages with a means to immunosuppress host cells.

To evaluate the anti-CRISPR effects of CracrIF1 in the context of infection, we isolated a siphophage infecting PAO1, ΦW8-1 (fig. S4A), and designed a crRNA targeting its tail tape measure protein gene (fig. S4B). In PAO1 cells containing the chimeric PA14-NY5506 *cas* operon, the phage-targeting crRNA resulted in a reduction of 3-4 logs in plaque forming units (PFUs) compared to a non-targeting control (Fig. 2C). However, the expression of CracrIF1 RNA rescued phage infectivity to the level of the non-targeting control, demonstrating its robust anti-CRISPR activity. Indeed, mutating the ΨS sequence (nucleotides 1-3; seed) of CracrIF1 completely reverted its immunosuppressive activity (Fig. 2C), underscoring the importance of base pairing with the P_{csy} target. Quantitative PCR further confirmed that CracrIF1 markedly reduced *csy* transcripts level (by approximately 75%; Fig. 2D). Notably, the target-binding requirement distinguishes Cracrs

212 from the recently reported Racr RNAs, which lack a ΨS and rely on the interaction between
213 their repeat portions and Cas proteins. We conclude that CracrIF1 transcriptionally inhibits
214 Cas proteins by mimicking the RNA-guided auto-regulatory function of host CreR RNAs,
215 presenting a distinct RNA-based anti-CRISPR mechanism exploited by phages.
216
217 **CracrIF2 does not show anti-CRISPR activity**
218 We then aimed to functionally characterize CracrIF2, which exhibits a strong genetic
219 association with *acr* genes, including *acrIF24* and *cracrIF1* (Fig. 1B). The ΨS sequences
220 of CracrIF2 and CreRIF2 share 17 identical nucleotides, forming extensive base pairings
221 to a sequence upstream of the Y010 *csy* operon. This predicted target is adjacent to a 5'-
222 CC-3' PAM sequence and includes mismatches at positions 6 and 12, which do not affect
223 target DNA recognition (Fig. 3A). Therefore, we posited that CracrIF2 exhibits anti-
224 CRISPR activity against the Y010 CRISPR-Cas system. However, we were unable to
225 obtain sufficient immune targeting against ΦW8-1 by the Y010 system in PAO1. Therefore,
226 we engineered a chimeric *cas* operon (designated as PA14-Y010) that comprises the
227 closely-related PA14 *cas* genes and the Y010 IGR between *cas2-3* and *csy1* (containing
228 *creRIF2* and its target). Unexpectedly, while this chimeric operon conferred robust
229 immunity against phage ΦW8-1 in PAO1 cells, expression of CracrIF2 RNAs did not
230 provide the phage with a replicative advantage, indicating the inability of CracrIF2 to
231 suppress immunity (Fig. 3B). To investigate the reasons for this lack of inhibitory activity,
232 we investigated the production and maturation of CracrIF2. Northern blotting revealed that
233 CracrIF2 RNAs (as well as CreRIF2 RNAs) were effectively matured in PAO1 cells
234 expressing the PA14 Csy proteins (fig. S5). Processing was further confirmed via small
235 RNA sequencing, which showed the characteristic 5' and 3' handles of endogenous
236 crRNAs (fig. S5D). Thus, we concluded that the failure of CrarIF2 to inhibit CRISPR
237 immunity could not be attributed to inappropriate Cracr RNA maturation.
238
239 To gain mechanistic insights into the lack of anti-CRISPR activity by CracrIF2, we
240 experimentally identified the promoter of the Y010 *csy* operon by mapping its TSS via 5'
241 RACE (fig. S6A). Surprisingly, we identified two TSS in this region, each preceded by an
242 evident promoter sequence (labeled in Fig. 3A). Interestingly, CracrIF2 targets the
243 promoter farther from *cys1*, potentially silencing a short cryptic gene in the intergenic
244 region before the *csy* operon, which may explain its lack of anti-CRISPR activity. Notably,
245 our previous research has demonstrated that certain CreR-like elements are part of minimal
246 TA systems, which encode crRNA-resembling antitoxins (CreA) that complex with Cas
247 proteins and guide them to inhibit the transcription of a small RNA toxin gene (designated
248 *creT* for CRISPR-repressed toxin) located within the intergenic regions between *cas* genes
249 (*8, 21*). Motivated by the architectural resemblance of CreA loci with the site targeted by
250 CracrIF2, we carefully examined the 5' UTR of the long *csy* transcripts. We noticed a mini-
251 ORF (of 15 nt) preceded by a putative Shine-Dalgarno (SD) sequence and followed by a
252 hairpin structure (Fig. 3A), reminiscent of the small RNA toxins we previously discovered
253 that sequester rare transfer RNAs using two or more consecutive rare codons (*8, 21*).
254 Despite failing to identify any rare codons within the mini-ORF, we used an inducible *tac*
255 promoter to express a series of differently truncated RNA transcripts and confirmed that
256 the SD sequence, mini-ORF, and hairpin structure cause toxicity in PA14 cells (fig. S7, A
257 and B). Through point mutations, we further confirmed the simultaneous requirement of

the three elements for toxicity (Fig. 3C). Therefore, Y010 *csy* transcription can be driven by both a promoter shared with a small RNA toxin (likely suppressed by CracrIF2 and CreRIF2), and also by an independent promoter closer to *csy1*, explaining why CracrIF2 fails to inhibit CRISPR immunity. For clarity, we designate the toxin as CreT and refer to CreRIF2 as CreA hereafter.

CracrIF2 transcriptionally inhibits a unique RNA toxin

To understand how the identified rare-codon-lacking CreT elicits toxicity, we deleted or inserted one or more nucleotides following its ATG start codon. Remarkably, toxicity persisted only when three nucleotides were deleted (fig. S7C). This underscores the important role of the downstream codons, which, interestingly, include two consecutive proline codons (CCT and CCG) except the stop codon (Fig. 3A). Subsequently, we constructed a series of variants with these two proline codons substituted by other synonymous or non-synonymous codons (Fig. 3D). Toxicity was maintained only when one or both proline codons were synonymously altered, indicating two consecutive proline codons at this position are critical for toxicity. Previous studies have shown that during protein translation, peptide bond formation is especially slow with proline, and two continuous proline codons can cause ribosomal stalling (*22, 23*). Therefore, we propose that this CreT is a novel small RNA toxin that exploits two highly and consecutively translated proline codons, presumably leading to severe ribosome stalling and eventual starvation.

The location of the target of CracrIF2 and CreA (CreRIF2) suggests that these crlRNAs may inhibit the promoter of *creT* (P_{creT}) rather than P_{csy} of Y010. To explore this hypothesis, we constructed fluorescent reporters by linking the P_{creT} DNA to *mCherry* and validated its TSS through primer extension (consistent to the results of 5' RACE; fig. S6B). By measuring the fluorescence signal, we demonstrated that both CracrIF2 and CreA reduced the activity of P_{creT} by a factor of 34 and 30, respectively, in the presence of PA14 Csy proteins (Fig. 3E). Complementary mutations further confirmed that their inhibitory effects relied on critical base-pairings to the target promoter (Fig. 3E). We also evaluated the *P. aeruginosa* CreTA module in *E. coli* cells. Introduction of a plasmid carrying the Y010 *creT-csy* operon and an IPTG-inducible *creA* gene into MG1655 cells resulted in severe growth defects under non-inducing conditions but not under inducing conditions (Fig. 3F), which demonstrated that the toxicity of the *P. aeruginosa* CreT in *E. coli* cells and the crucial antidote role of CreA. Notably, when another plasmid expressing CracrIF2 was also introduced into these cells, growth defects were not observed under either condition, highlighting its robust anti-CreTA activity (Fig. 3F). Taken together, our results demonstrate that CracrIF2 mimics the CreA to transcriptionally inhibit the CreT RNA toxin.

CracrIF2 exhibits anti-anti-anti-CRISPR activity

The targeting of CreTA by CracrIF2 raises intriguing questions about the evolutionary drivers of these inhibitory elements. Our recent study revealed that CreT toxins, which are normally suppressed by CRISPR-Cas systems, are activated by a variety of Acr proteins to induce abortive infection, ultimately killing infected cells to prevent further viral spread in the population (*14*). We have shown that this process can expel Acr-encoding MGEs from bacterial populations, thus providing a broad-spectrum anti-anti-CRISPR protection for

CRISPR-Cas. Therefore, we reasoned that phages may inhibit CreTA through CracrIF2 deployment as an anti-anti-anti-CRISPR strategy. To experimentally test this, we introduced the entire Y010 *cas* operon, including its *creTA* genes, into PAO1 cells and then expressed AcrIF24 (frequently linked with *cracr* genes) using the BAD promoter (P_{BAD}) (Fig. 4A). As expected, PAO1 cells exhibited growth defects when AcrIF24 was expressed through arabinose induction, but not when its expression was repressed with glucose, indicating that the *P. aeruginosa* CreT was activated by AcrIF24 expression. Consistently, growth defects were no longer observed when *creT* was mutated (Fig. 4A). However, growth defects were alleviated when CracrIF2 RNAs were expressed *in trans* from another plasmid, consistent with its anti-CreTA function (Fig. 4A). A similar rescuing effect was observed when *cracrIF2* was placed downstream of *acrIF24* to reproduce their natural genetic arrangement in phage genomes (fig. S7D). Our results demonstrate that CracrIF2 counteracts the anti-anti-CRISPR effect of CreTA against AcrIF24 protein deployment, thereby representing an unprecedented RNA-based anti-anti-anti-CRISPR mechanism.

To further explore the anti-anti-anti-CRISPR effect of CracrIF2 at the population level, we engineered two reporter plasmids, one containing *gfp* (pGFP) and the other containing *mCherry* (pMC), and introduced them in a 1:1 ratio to *P. aeruginosa* PAO1 cells expressing the PA14-Y010 *cas* operon (Fig. 4B). In our experiments, pMC was designed to encode no anti-CRISPR factors (negative control), the AcrIF24 protein alone, or both the AcrIF24 protein and the CracrIF2 RNA. By monitoring the green and red fluorescence, we showed that when only AcrIF24 was induced, pMC was swiftly eliminated from the *P. aeruginosa* bacterial populations (Fig. 4B). These results demonstrate a herd immunity process mediated by the Y010 CreTA module. However, the presence of CracrIF2 RNAs disrupted this herd immunity protection, as evidenced by the lack of pMC elimination in the bacterial population (Fig. 4B). Altogether, our data underscores that CracrIF2 exerts an anti-anti-anti-CRISPR effect in the presence of Acrs that would otherwise trigger a CreTA-mediated herd immunity response.

AcrIF24 differentiates CreA and Cracr by spacer size

Previous studies have revealed that AcrIF24 inhibits immunity by directly binding to Csy-crRNA complexes, preventing interaction with their DNA targets (*24*). Since Cracrs guide the Csy complex to hybridize with host immune promoter DNA targets, this raises questions about how AcrIF24 does not negatively affect the activity of Cracrs. Surprisingly, Cracrs show a strong genetic association with AcrIF24 (Fig. 1B), suggesting that these elements either synergize or are at least not functionally incompatible. To explore the potential impact of AcrIF24 on the RNA-guiding activity of CracrIF2 and CreA, we established an *mCherry*-based reporter assay in PAO1 cells expressing PA14 Csy proteins (Fig. 5A). Remarkably, while the ability of CreA to repress P_{creT} was significantly impaired by AcrIF24 (repression fold decreased from 30.6-fold to 1.88-fold; P =9.25e-07), the regulatory activity of CracrIF2 was almost unaffected (P = 0.08). By excluding Cracr and CreA components from the assay, we confirmed that AcrIF24 (which can also act as an Acr-associated transcriptional regulator (24)) does not independently regulate P_{csy} (Fig. 5A). These results reinforce the suspicion that AcrIF24 can discriminate between CracrIF2-guided and CreA-guided Csy complexes.

By comparing the sequences of the *cracrIF2* and *creA* elements, we hypothesized that discrimination by AcrIF24 could rely on the differing nucleotides in their ΨR sequences, their different ΨS sizes, or a combination of both (Fig. 5B). To explore the importance of the ΨR sequence identity, we substituted the ΨR sequences of *cracrIF2* with those of *creA* (Fig. 5B). However, the resulting mutant (*cracrIF2*-sub) was still able to effectively repress the targeted P_{creT} in the presence of AcrIF24 (Fig. 5C), thus ruling out a mechanism dependent on the ΨR sequence composition. Next, we extended the ΨS sequence of *cracrIF2* from 21 nt to 26 nt, matching the size of the ΨS in *creA* (Fig. 5B). In this experiment, Csy complexes guided by the ΨS-extended *cracrIF2* became significantly susceptible to AcrIF24 (Fig. 5C), suggesting that the ΨS size is mechanistically important for AcrIF24 to distinguish between CracrIF2-guided and CreA-guided Csy complexes. Indeed, in a survey of all identified type I-F *cracr* genes and their *creA* or *creR* counterparts (listed in Table S2), we noticed a significant difference in their ΨS sizes (P = 6.23E-08) (Fig. 5D). While Cracr RNAs prefer a ΨS size between 18-21 nt, CreA/CreR RNAs usually favor a ΨS sequence of 24-26 nt.

To further investigate the role of ΨS in AcrIF24 discrimination, we created a series of CracrIF2 derivatives with ΨS sequence lengths ranging from 18 nt to 26 nt. Our observations revealed that the regulatory effect became susceptible to AcrIF24 inhibition only when the spacer size exceeded 22 nt (Fig. 5E). Consistently, the regulatory effects of CracrIF1 (18 nt) and CreRIF1 (20 nt) on the NY5506 *csy* promoter were both resistant to AcrIF24 (fig. S8). This suggests that CreRIF1-mediated regulation of Cas proteins—and likely the CreT RNA toxin—does not sense the presence of this specific anti-CRISPR protein. Taken together, our results reveal a unique discriminatory mechanism by AcrIF24 to distinguish between host and phage-derived crlRNAs, depending on slight differences in their ΨS lengths.

AcrIF24 cannot bind Csy-Cracr complexes with fewer Csy3 subunits

The length of the crRNA has structural implications for type I interference complexes, determining the number of Csy3 subunits that assemble to form the backbone. Therefore, we hypothesized that the different spacer sizes of the crRNA, CreA, and Cracr elements—as confirmed via Northern blotting (fig. S5, A and B)—likely impact the number of Csy3 subunits in the resulting Csy complexes. To explore this, we purified the Csy complexes assembled with different backbone RNAs through affinity purification of His_6-Csy1. During size-exclusion chromatography (SEC), the elution peak of the Csy-crRNA complex appeared first, immediately followed by the Csy-CreA complex, with the Csy-CracrIF2 elution peak appearing last and revealing a flatter shape (Fig. 6A). This indicated a smaller and inconsistent ratio of Csy3 subunits in the Csy-CracrIF2 complex, in line with its shorter spacer. In agreement, native PAGE (polyacrylamide gel electrophoresis) analysis of the Csy-CracrIF2 complexes showed diffused bands, indicating the presence of a mixture of complexes with varying Csy3 ratios (fig. S5C). Furthermore, through SDS-PAGE, we confirmed that the Csy-CracrIF2 complex contained fewer Csy3 subunits compared to the complexes containing crRNA or CreA (Fig. 6B).

A previous study uncovered that AcrIF24 binds to the Csy-crRNA complex via its extensive interaction with five Csy3 subunits (*24*). These results led us to speculate that AcrIF24 may

be unable to bind to the Csy-CracrIF2 complex which has fewer Csy3 subunits. To investigate this, we performed pull-down assays using GST-labeled AcrIF24 to capture the different Csy complexes containing crRNA, CreA, or CracrIF2. Our results showed that Csy-crRNA and Csy-CreA complexes were effectively pulled down, while only a minimal amount of Csy-CracrIF2 complexes were captured (Fig. 6C). This suggests that AcrIF24 exhibits significantly lower binding affinity to Csy-CracrIF2 compared to Csy-crRNA and Csy-CreA complexes. Additionally, we investigated the target DNA binding specificity of different Csy complexes in the presence or absence of AcrIF24. Remarkably, AcrIF24 promoted Csy-crRNA and Csy-CreA to bind to non-target DNA, while Csy-CracrIF2 continued to specifically associate with target DNA regardless of the presence of AcrIF24 during the competition assay (Fig. 6D).

Using cryogenic electron microscopy (Cryo-EM), we sought to shed light on the structural and mechanistic nuances of the Csy complexes assembling with different RNA guides (Fig. 6E). Notably, the Csy-crRNA and Csy-CreA complexes contained six and five Csy3 subunits, respectively, while the Csy-Cracr complex primarily featured four Csy3 subunits. By comparing the structures to the previously reported Csy-Acr24 complex, we observed that the lack of Csy3.1 and Csy3.2, but not the lack of only Csy3.1, significantly reduced the interface between the Csy complex and AcrIF24 (Fig. 6F). Therefore, we conclude that the functional compatibility between CracrIF2 to AcrIF24 is a result of its shorter spacer size, which leads to the absence of two Csy3 subunits essential for AcrIF24 binding to the Csy complex. Together, these structural analyses underscore the unprecedented co-evolution of protein and RNA anti-CRISPRs, resulting in their functional synergy to effectively counter both adaptive and innate immune functions in bacteria.

A Cracr array displays both anti-CRISPR and anti-anti-anti-CRISPR activities

Interestingly, eight *P. aeruginosa* prophages and two closely-related plasmids were found to carry a combined structure featuring both *cracrIF1* and *cracrIF2* ΨS sequences (thus referred to as *cracrIF1/2*) (Fig. 1B). This stacked configuration resembles a two-spacer CRISPR array, which we hereafter refer to as Cracr array. By comparing the ΨR sequences of the Cracr array with those of the individual *cracrIF1* and *cracrIF2* genes (Fig. 7A), we observed significant homology and similar nucleotide variations in the first (ΨR1) and second (ΨR2) repeat sequences. Notably, ΨR2 of the Cracr array retains 8 conserved 3'-terminal nucleotides (5'-CTAAGAAA-3'), essential for generating a typical 8-nt 5' handle for its CracrIF2 RNA products. In contrast, the last ΨR (ΨR3) of the Cracr array shares 8 identical terminal nucleotides (5'-AGAAAGGC-3') with the ΨR2 (also the last ΨR) of *cracrIF1* and *cracrIF2* (Fig. 7A). Importantly, the starting nucleotides (5'-TATGA-3') of ΨR3 are significantly different from those of other ΨR sequences, and notably, the second ΨS of the Cracr array (18-nt) is 3 nucleotides shorter than the corresponding ΨS from standalone *cracrIF2* genes (21-nt) (Fig. 7A). Therefore, it appears that the *cracrIF1/2* Cracr array did not originate from a direct recombination event between *cracrIF1* and *cracrIF2*, but rather likely emerged from an integration event within the ΨR2 sequence of *cracrIF1* that is unrelated to standalone *cracrIF2* genes.

We next aimed to explore the potential multiplexed functionality of the identified Cracr array. Through Northern blotting, we confirmed that the Cracr array indeed produced both

CracrIF1 and CracrIF2 RNAs in PAO1 cells expressing the PA14 Csy proteins (fig. S9A). We then examined the potential anti-CRISPR function of the Cracr array by introducing it into PAO1 cells that contained a phage-targeting mini-CRISPR and the chimeric *cas* operon PA14-NY5506 (Fig. 7B). Upon infection by phage ΦW8-1, we observed that the Cracr array robustly suppressed the immune targeting ability of PA14-NY5506, with its effect relying on the seed nucleotides of its first rather than its second ΨS. Next, we introduced the Cracr array along with an inducible *acrIF24* gene into PAO1 cells that contained the Y010 *cas* operon (Fig. 7C). In this setup, when AcrIF24 was expressed, the Cracr array completely suppressed the anti-anti-CRISPR response (arresting cell growth) mediated by the Y010 *creTA* genes, and this suppression relied on its second rather than its first ΨS. Therefore, the Cracr array exhibited both anti-CRISPR and anti-anti-anti-CRISPR activities, which cooperatively confronted the targeted immunity and herd immunity pathways mediated by the host CRISPR-Cas.

Among the non-*P. aeruginosa cracr* genes, we were surprised to observe that a considerable fraction (35 out of 85) evolved arrays of at least two ΨS sequences, with ten arrays and two arrays even containing 3 and 4 different ΨS sequences, respectively (Table S2). Specifically, we investigated the three-spacer Cracr array from the *Burkholderia gladioli* strain BCC1711, and two related dual-spacer arrays from the *B. gladioli* strains BCC1700 and BCC1864 (fig. S9B). The two ΨS sequences of BCC1700 closely resemble the second and third ΨS of BCC1711, while the two ΨS sequences of BCC1864 are almost identical to the first and third ΨS of BCC1711. Together, our findings suggest that assembling individual *cracr* genes into a multiplex anti-CRISPR and/or anti-anti-anti-CRISPR array is likely a common strategy employed by different MGEs to confront different CRISPR-Cas variants.

Salmonella MGEs use Cracrs to silence host I-E CRISPR-Cas

We then wondered if Cracr elements could also target other CRISPR-Cas systems beyond the studied type I-F. For this purpose, we searched for putative *cracr* genes targeting I-E CRISPR-Cas by performing bioinformatic searches across MGE genomes with previously identified type I-E *creR* genes as querries. Using this approach, we discovered three putative Cracr RNAs encoded by *S. enterica* MGEs, including prophages and plasmids, which we named CracrIE1, CracrIE2, and CracrIE3 (fig. S10A). In the *S. enterica* subsp. salamae serovar 56:b:[1,5] strain SA20053897, one prophage contains a two-spacer array (namely *cracrIE1/2*) encoding CracrIE1 and CracrIE2, while another prophage contains a single-spacer gene (*cracrIE2*) encoding CracrIE2. In the *S. enterica* subsp. diarizonae strain CFSAN030538, a plasmid carries a dual-spacer array (*cracrIE3/2*) encoding CracrIE3 and CracrIE2. Therefore, *S. enterica* MGEs appear to also have developed individual and multiplexed Cracr elements.

Prediction of their potential regulatory targets revealed that the three CracrIE RNAs each share a similar ΨS sequence with a *creR* gene (designated *creRIE1*, *creRIE2*, or *creRIE3*), and each shared ΨS sequence matches a target sequence located between the corresponding *creR* and the downstream *casA* gene (fig. S10B). Note that we suggest referring to these cellular crlRNA genes as *creR,* rather than *creA,* until the cognate *creT* pair gene is identified. To explore its putative transcriptional repression activity, we introduced a

plasmid containing the *cracrIE1/2* array into *E. coli* MG1655 cells, along with a second plasmid expressing GFP and a third plasmid over-expressing the *E. coli* Cascade proteins (fig. S10C). When GFP expression was driven by the putative promoter sequence targeted by *creRIE1* (from the *S. enterica* strain FDAARGOS_717), the fluorescence signal was significantly silenced by *cracrIE1/2* (fig. S10D), which was dependent on the integrity of the first ΨS. Similarly, when GFP expression was controlled by the putative promoter sequence targeted by *creRIE2* (from strain CFSAN001011), fluorescence was suppressed by *cracrIE1/2,* unless its second ΨS was mutated (fig. S10E). Our results demonstrate that the *cracrIE1/2* array can transcriptionally target two distinct type I-E CRISPR-Cas variants. However, the exact physiological activity of this array—whether it acts as an anti-CRISPR or an anti-anti-anti-CRISPR—requires further investigation.

Discussion

Disabling host defense systems is a crucial strategy employed by genetic invaders to ensure their survival and proliferation. Phages and plasmids have evolved various mechanisms to counteract bacterial CRISPR-Cas immunity, exemplified by over one hundred identified Acr protein families (*25, 26*) and the recent discovery of Racrs (*18*). However, the mechanisms allowing phages to evade the typically multilayered defenses of bacteria remain poorly understood. In this study, we introduce a distinct category of anti-CRISPR RNAs, known as Cracr RNAs, which suppress the expression of Cas proteins or a CRISPR-regulated toxin (CreT) by mimicking the regulatory crRNA-like RNAs (crlRNAs) of CRISPR-Cas.

Our previous work has shown that CRISPR-Cas systems use regulatory crlRNAs, like CreR and CreA, to modulate adaptive and herd immunity, respectively. CreR directs Cas proteins to target their own promoter and boosts Cas protein levels in the presence of Acr inhibitors (*10*). CreA directs Cas proteins to inhibit the promoter of the CreT toxin and elicits abortive infection when Acr proteins inhibit the Cas surveillance complex, ultimately providing herd immunity against Acr-encoding MGEs (*14*). CreTA elements thereby provide an anti-anti-CRISPR layer for CRISPR immunity. Remarkably, this study further unveils another layer of complexity to the MGE-CRISPR arms race, demonstrating how viral crlRNAs mimic cellular RNAs to counteract both the adaptive and herd immunity mediated by CRISPR-Cas systems (Fig. 7D). Viral mimics of CreR RNAs, like CracrIF1, exhibit anti-CRISPR function by robustly inhibiting Cas expression. Conversely, mimics of CreA RNAs, such as CracrIF2, can suppress CreT, undermining herd immunity and showing an unprecedented anti-anti-anti-CRISPR activity.

We further show that multiple Cracr RNAs, exhibiting both anti-CRISPR and anti-anti-anti-CRISPR activities, are frequently stacked within the same transcriptional unit. The structural resemblance between viral Cracr arrays and bacterial CRISPR arrays underscores the multiplexing potentials of both defensive and anti-defensive small RNAs. Such multivalent properties of Cracr arrays may play a crucial role as a phage bet-hedging strategy to overcome CRISPR-Cas systems in different hosts or to cooperatively thwart multilayered defenses. In addition, Cracr RNAs often work in tandem with protein anti-CRISPRs, at least for type I-F, where CracrIF RNAs are genetically linked to proteins like AcrIF23 and AcrIF24. While AcrIF23 disrupts the Cas2-3 nuclease, AcrIF24 inhibits the

binding of the Csy complex to DNA targets (*24*). Given that target promoter binding is crucial for Cracr RNA function, the co-locaiton of Cracrs with AcrIF24 was initially paradoxical. However, we discovered that Csy-Cracr complexes can remarkably evade AcrIF24 inhibition by utilizing shorter spacer sequences, leading to the absence of two Csy3 subunits necessary for AcrIF24 binding. (Fig. 6, E and F). These results showcase the delicate co-evolution of RNA and protein anti-CRISPR strategies. Furthermore, the identification of CRISPR-safeguarding CreTA elements, as well as the common genomic clustering of various defense genes into so-called "defense islands", indicate that defense systems may collaborate or protect each other, offering a multi-layered defense strategy (*27*). In contrast, our discovery of viral Cracrs, their clustering into anti-defense Cracr arrays, and the elegant functional synergy with anti-CRISPR proteins, strongly suggests that phages in parallel counter-evolve multi-layered anti-defense strategies. This intricate co-evolutionary dance between phages and bacteria showcases an ongoing arms race for survival, with each side developing sophisticated strategies to outmaneuver the other.

In summary, our findings uncover how phages hijack the RNA-guided properties of Cas effectors, developing RNA-based anti-CRISPRs or anti-anti-anti-CRISPRs to silence CRISPR immunity. These elements can functionally cooperate with each other or with Acr proteins to effectively inhibit the adaptive and/or herd immune capabilities of different CRISPR-Cas variants. Together, these discoveries highlight the genetic and mechanistic diversity of viral anti-CRISPR elements and offer valuable insights for developing CRISPR-resistant therapeutic phages and more effective payload delivery vectors.

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Author contributions
M. L. conceived and supervised the project with valuable suggestions from R.P.-R., M.L, R.P.-R., Y.F., C.L., R.W., H.Z., and W.W. designed the experiments. J.Y., C.L., M.R.-M., and R.P.-R. performed the bioinformatics analyses and predicted Cracr genes. C. L. and R.W. generated strains and plasmids. C.L. performed phage infection assays. C.L. and R.W. conducted the efficiency of plaque assays and liquid culture growth assays. C.L., R.W. and J.D. performed the fluorescence measurement. C.L. J.X and S.X. performed RNA-seq and primer extension assays. C.L. conducted the 5' RACE assays. H.Z, T.Z., and Q.X. provided the phage and genome sequences. Z.L, X.D., and L.G. conducted protein purification. Y.F. and W.W. resolved proteins Cryo-EM structure. Z.L performed EMSA and protein pull-down assays. F.C. performed RT-qPCR. M.L., C.L., R.W., Z.L., and W.W. analyzed data. M.L. prepared the original draft. M. L., R.P.-R., and Y. F. finalized the manuscript with input from all authors.

Competing interests: The authors declare no competing interests.

Data and code availability: Data are publicly available. The atomic coordinates for the cryo-EM structures of type I-F cascade complexed with crRNA (PDB 9JPH), CreA (PDB 9JPG) and CracrIF2 (PDB 9JPF) were deposited to the PDB (www.rcsb.org). The cryo-EM density maps reported in this study for the type I-F cascade complexed with crRNA (EMD-61700), CreA (EMD-61699) and CracrIF2 (EMD-61698) were deposited to the EM Data Bank. The raw data for the RNAseq experiments in fig. S5D were deposited to the NCBI with the BioProject accession number PRJNA1173583. All additional data reported in this paper will be shared by the lead contact upon request. This paper does not report original code.

Supplementary Materials

Materials and Methods

Figs. S1 to S10

Tables S1 to S4

Figures

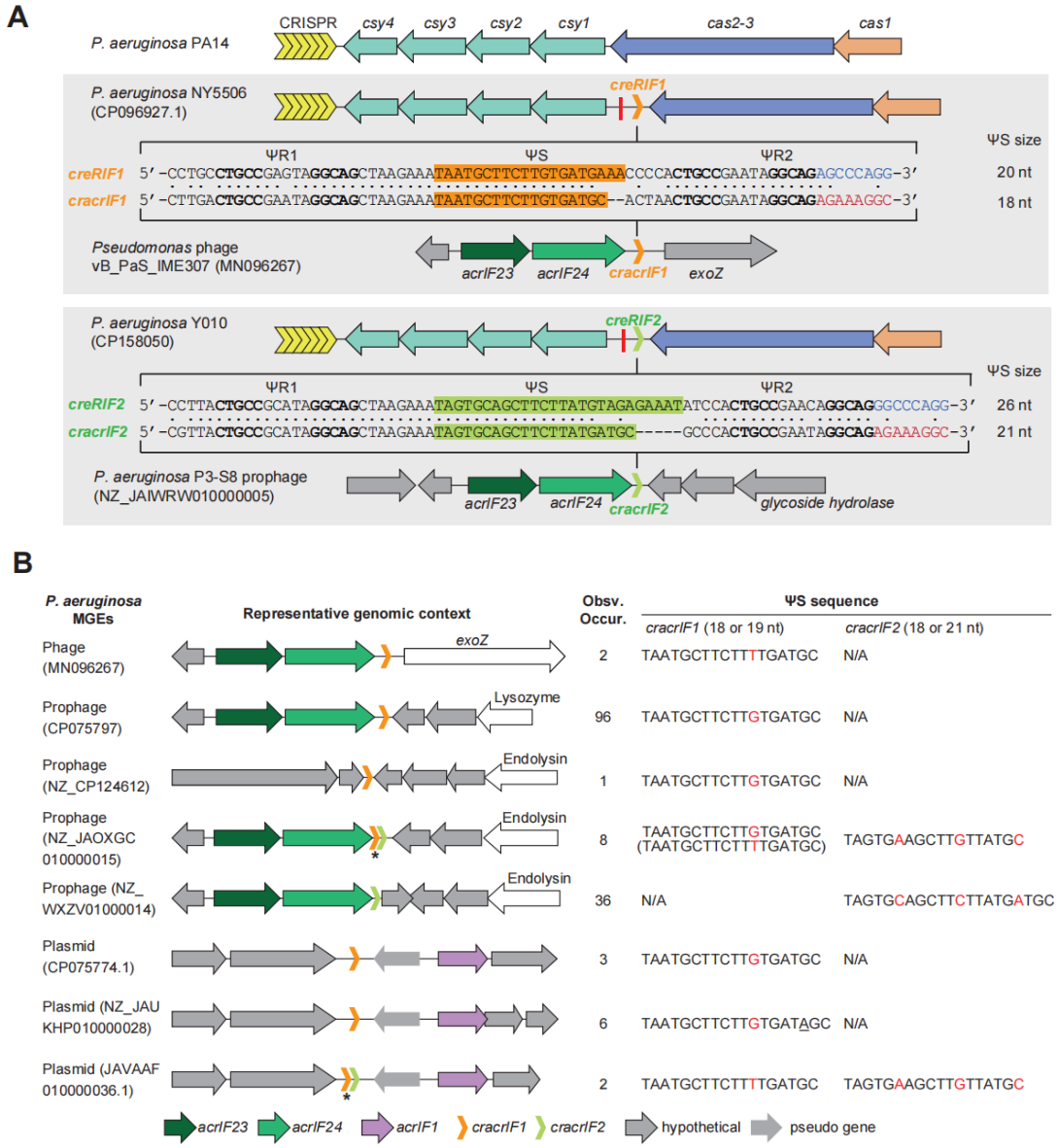

Fig. 1. Two conserved *P. aeruginosa cracr* genes and their *creR* homologs.
(**A**) Illustration showing the homology between representative *cracrIF1* or *cracrIF2* and their *creR* homologs. Dots indicate the identical nucleotides. Eight differing terminal nucleotides are highlighted in red (*cracr*) or blue (*creR*). Palindromic nucleotides within each ΨR sequence are bolded. The size of ΨS is provided for each crlRNA gene. The red vertical bar represents the target sequence of *cracr* and *creR*. The well characterized PA14 *cas* operon is also included for comparison. (**B**) Occurrences of *cracrIF1* and *cracrIF2* genes in *P. aeruginosa* MGEs and their varied genomic contexts. Single ΨS nucleotides displaying polymorphism are highlighted in red. An asterisk (*) denotes a CRISPR-like array encoding both CracrIF1 and CracrIF2 RNAs.

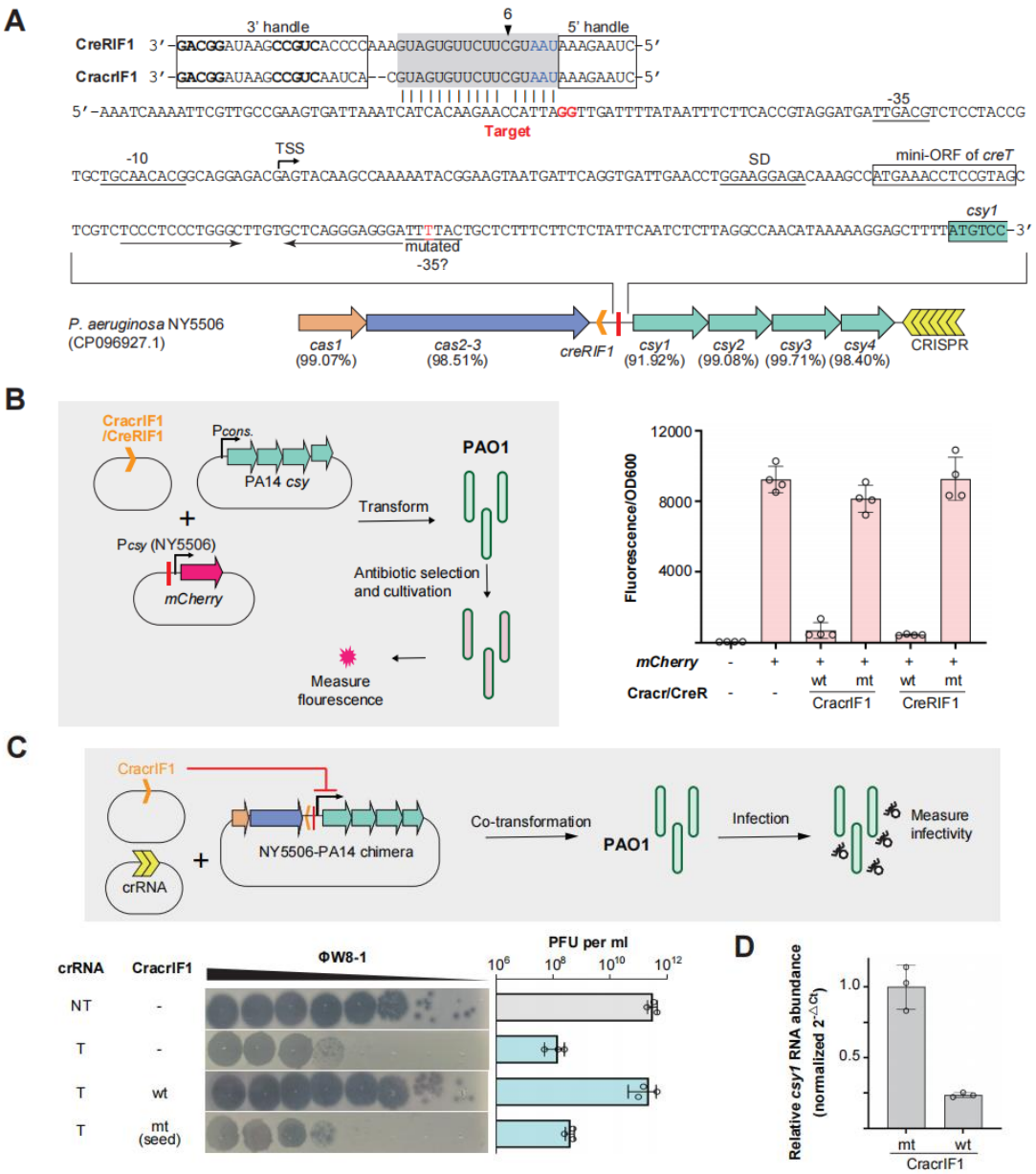

Fig. 2. CracrIF1 displays anti-CRISPR activity against the NY5506 CRISPR-Cas system. (**A**) Illustration showing the targeting potential of CracrIF1 and CreRIF1 to the *csy* promoter (P_{csy}) of *P. aeruginosa* NY5506. Nucleotides complementary to PAM are highlighted in red. Mismatch at position 6 (respective to PAM) is indicated. The identical ΨS nucleotides shared by CracrIF1 and CreRIF1 are shaded in gray. Blue nucleotides were mutated in panels **B** and **C**. Nucleotides of 5' and 3' handles are framed. Palindromic nucleotides within 3' handle are in bold. TSS was determined by 5' RACE (see fig. S3). Functional elements of a cryptic CreT RNA toxin, including an SD sequence, a mini-ORF, and a pair of inverted repeats (labeled with two convergent arrows), are also indicated. A red thymine (T) likely has inactivated a -35 element (underlined). Sequence similarities shared by PA14 and NY5506 Cas proteins are noted. (**B**) Fluorescence intensity from PAO1 cells with an *mCherry* gene under the control of the NY5506 P_{csy}. Three seed nucleotides (highlighted in blue in panel **A**) of CracrIF1 and CreRIF1 were mutated (mt). wt, wild-type. Error bars, mean±s.d. (n=4). (**C**) Plaque forming units (PFU) of ΦW8-1 infecting PAO1 cells with a phage-targeting (T) or non-targeting (NT) crRNA, a chimeric *cas* operon targeted by CracrIF1, and either an empty vector (-) or its derivative expressing wild-type

620 (wt) or seed-mutated (mt) CracrIF1 RNAs. The *cas* operon of PA14 was modified by incorporating the
621 *cas* intergenic sequence from NY5506 (containing the target of CracrIF1). Error bars, mean±s.d. (n=3).
622 (**D**) The RNA level of *csy1* relative to *cas3* in PAO1 cells expressing the wild-type or mutated CracrIF1.
623 Error bars, mean±s.d. (n=3).
624

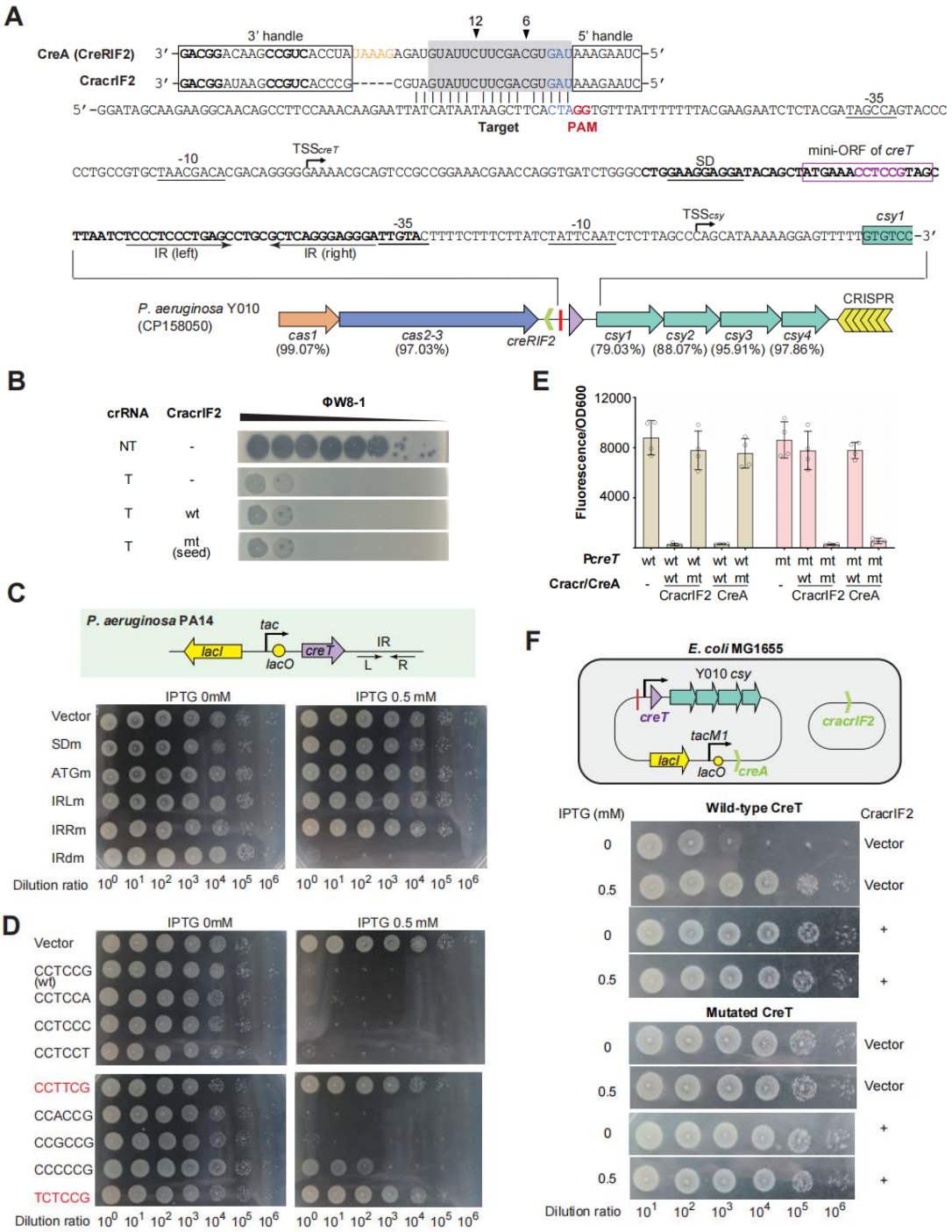

Fig. 3. CracrIF2 transcriptionally inhibits a distinct CreT toxin.

(**A**) Illustration showing the targeting potential of CracrIF2 and CreA (CreRIF2) to the promoter of the CreT toxin (P_{creT}) from *P. aeruginosa* Y010. Nucleotides complementary to PAM are in red. Mismatches at positions 6 and 12 (respective to PAM) are indicated. The identical ΨS nucleotides shared by CracrIF2 and CreA are shaded in gray, with the extra nucleotides in CreA shown in orange. Blue nucleotides were mutated in panels **B** and **E**. Nucleotides of 5' and 3' handles are framed. Palindromic nucleotides within 3' handle are in bold. TSS information was obtained by 5' RACE (see fig. S6). The sequence of *creT* is bolded. IR, inverted repeats. Sequence similarities shared by PA14 and Y010 Cas proteins are noted. (**B**)

PFUs of ΦW8-1 infecting PAO1 cells with a phage-targeting (T) or non-targeting (NT) crRNA, a chimeric *cas* operon (PA14-Y010) targeted by CracrIF2, and either an empty vector (-) or its derivative expressing wild-type (wt) or seed-mutated (mt) CracrIF2 RNAs. (**C**) Growth of PA14 cells expressing a series of CreT mutants (controlled by an IPTG-inducible *tac* promoter containing the *lacO* operator). (**D**) Synonymous and non-synonymous (highlighted in red) mutation analyses of the two consecutive proline codons of CreT. The wild-type (wt) sequence is CCTCCG. (**E**) Fluorescence intensity from PAO1 cells containing a P_{creT}-controlled *mCherry* gene. Three seed nucleotides (highlighted in blue in panel **A**) of CracrIF2 and CreA were mutated (mt). wt, wild-type. Error bars, mean±s.d. (n=4). (**F**) Growth of PAO1 cells with a plasmid containing the Y010 *csy* operon, *creT* (controlled by its native promoter), and an IPTG-inducible *creA*, along with a second plasmid expressing CracrIF2 RNAs (or its empty vector control).

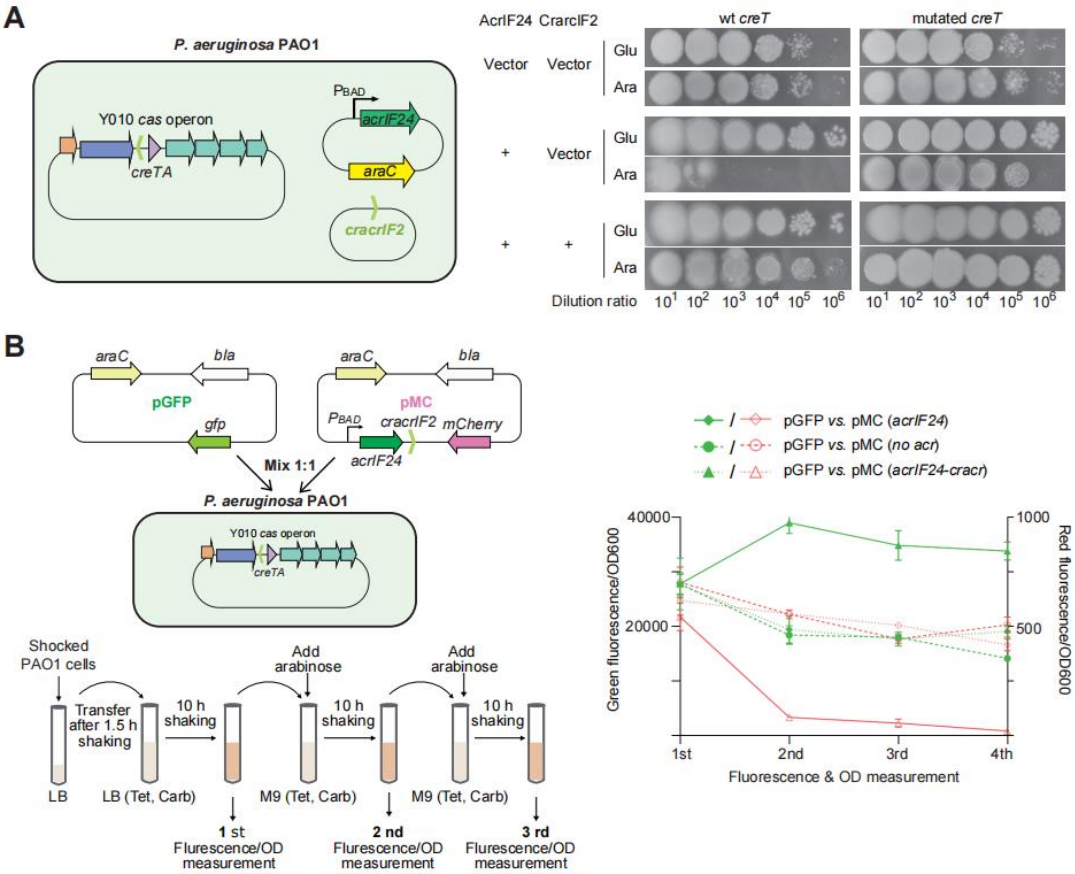

Fig. 4. CracrIF2 shows anti-anti-anti-CRISPR activity against Y010 CRISPR-Cas system. (**A**) Schematic representation (left) and results (right) of the assay to evaluate the ability of CracrIF2 RNAs to alleviate the CreTA cytoxicity that is triggered by AcrIF24 expression. AcrIF24 expression is controlled by the arabinose-inducible BAD promoter (P_{BAD}), with glucose used to suppress any basal activity of P_{BAD}. In the mutated *creT* control, the two critical proline codons were removed. (**B**) Schematic representation (left) and results (right) of the assay to assess the anti-anti-anti-CRISPR function of CracrIF2. PAO1 cells carrying the Y010 *cas* operon were electrotransformed with two reporter plasmids (pGFP and pMC, mixed in a 1:1 ratio). M9, a minimal medium. Tet, tetracycline; Carb, carbenicillin. Error bars, mean±s.d. (n=3).

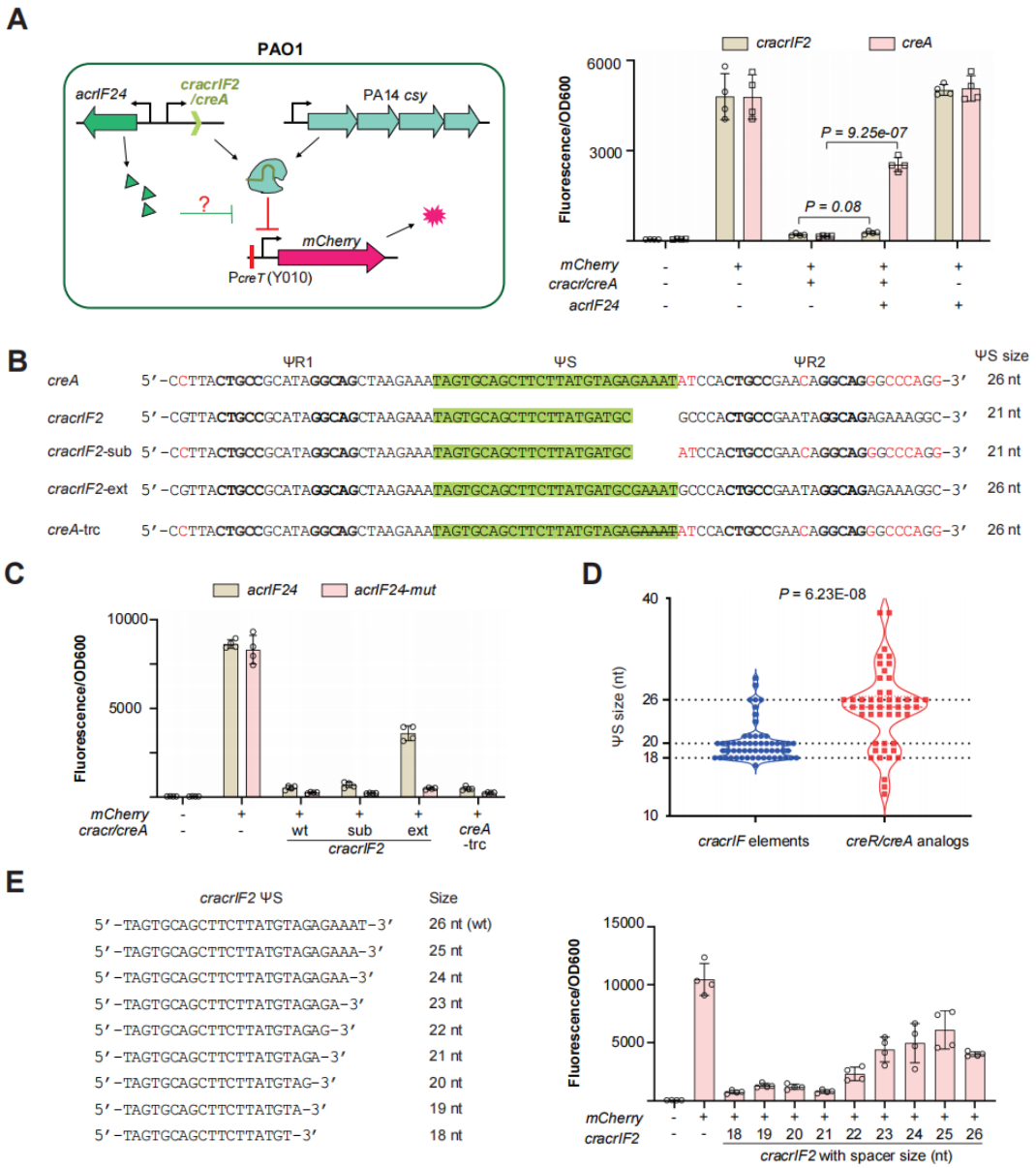

Fig. 5. AcrIF24 differentiates between CracrIF2 and CreA (CreRIF2) based on their differing spacer sizes.

(**A**) Fluorescence intensity of PAO1 cells containing a plasmid expressing mCherry under the control of the Y010 *creT* promoter (targeted by its corresponding CreA and the viral CracrIF2), a second plasmid expressing PA14 Csy proteins, and a third plasmid expressing CracrIF2 or CreA with or without the co-expression of AcrIF24. (**B**) Illustration showing the sequence differences between *creA*, *cracrIF2* and their ΨR-substituted (sub), ΨS-extended (ext), or ΨS-truncated (trc) mutants. (**C**) The inhibitory effects of the mutated CracrIF2 or CreA on their target promoter in the presence of AcrIF24 or its mutant. (**D**) Distribution of the spacer size of all discovered *cracrIF* and related *creR/creA* elements. (**E**) Impact of spacer size on the regulatory activity of CracrIF2 RNAs in the presence of AcrIF24. Error bars, mean± s.d. (n=4). *P* values were calculated from two-sided *t*-test.

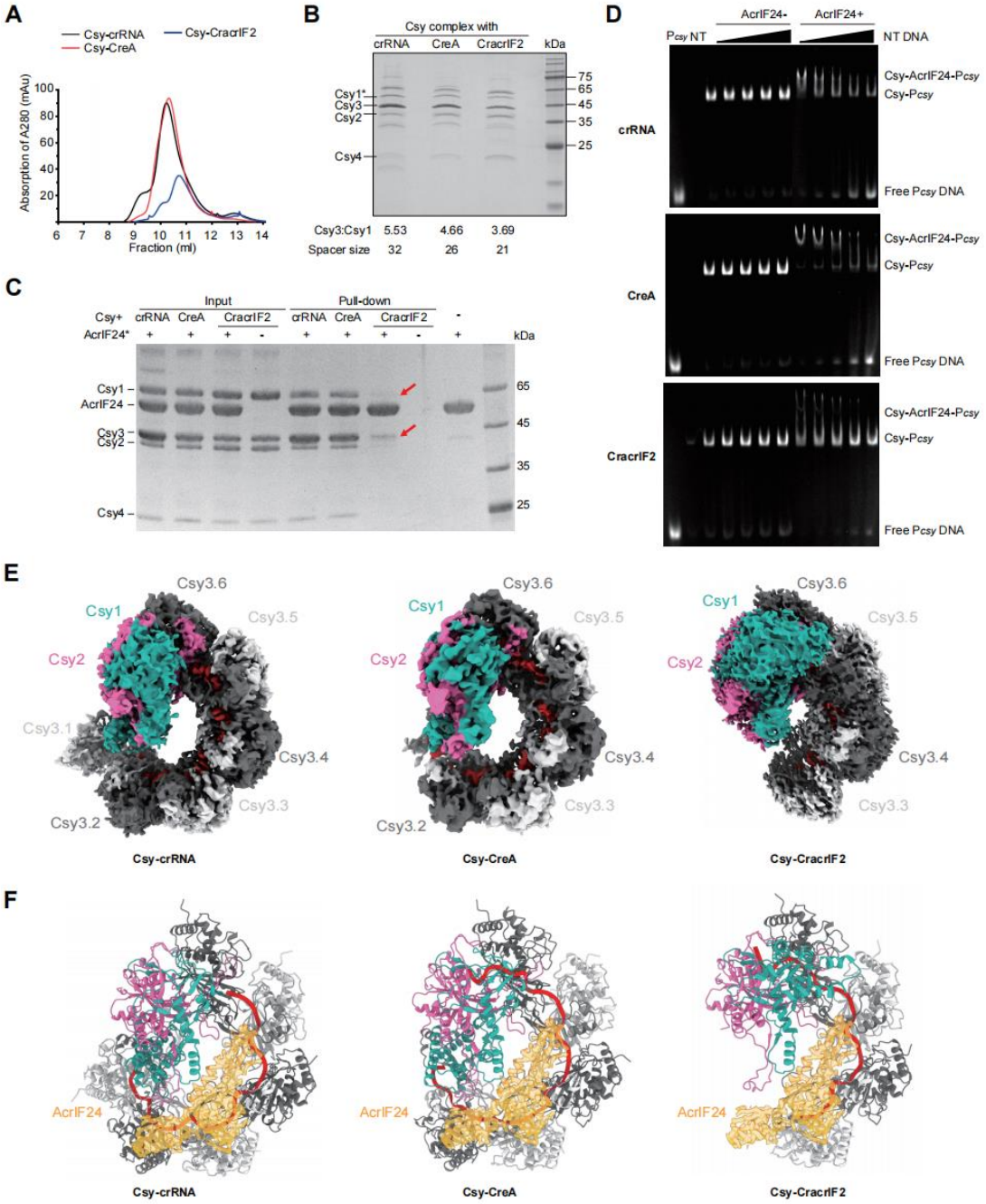

Fig. 6. The interaction of AcrIF24 with Csy complexes assembled with crRNA, CreA, or CracrIF2. (**A**) SEC traces show co-expressed Csy1-4 proteins (with His*6 tagged Csy1) with crRNA, CreA, or CracrIF2 in *E. coli*. mAU, milliabsorbance units. (**B**) SD-PAGE analysis of peak fractions purified by SEC. The molar ratio of Csy3 and Csy1 was estimated based on band intensities and molecular weights. (**C**) GST-labeled AcrIF24 pull-down experiments with Csy proteins bound to crRNA, CreA, or CracrIF2. Red arrows indicate the inefficiently captured Csy1 and Csy3 proteins in the CracrIF2 assay. (**D**) Competition assay with non-target DNA (0, 0.15, 0.3125, 0.625, or 1.25 μM) and FAM-labeled target P_{csy} DNA (0.04 μM) for 0.8 μM Csy-crRNA, Csy-CreA, or Csy-CracrIF2 complexes in the absence or presence of AcrIF24 proteins (9.6 μM). (**E**) Overall structures of the Csy complex assembled with crRNA, CreA, or CracrIF2. The Csy3 subunits are numbered according to the canonical Csy complex with crRNA. (**F**) Structural superimposition between Csy-AcrIF24 and Csy-crRNA, Csy-CreA, or Csy-

684 CracrIF2. Only AcrIF24 monomer is shown for the Csy-AcrIF24 structure.
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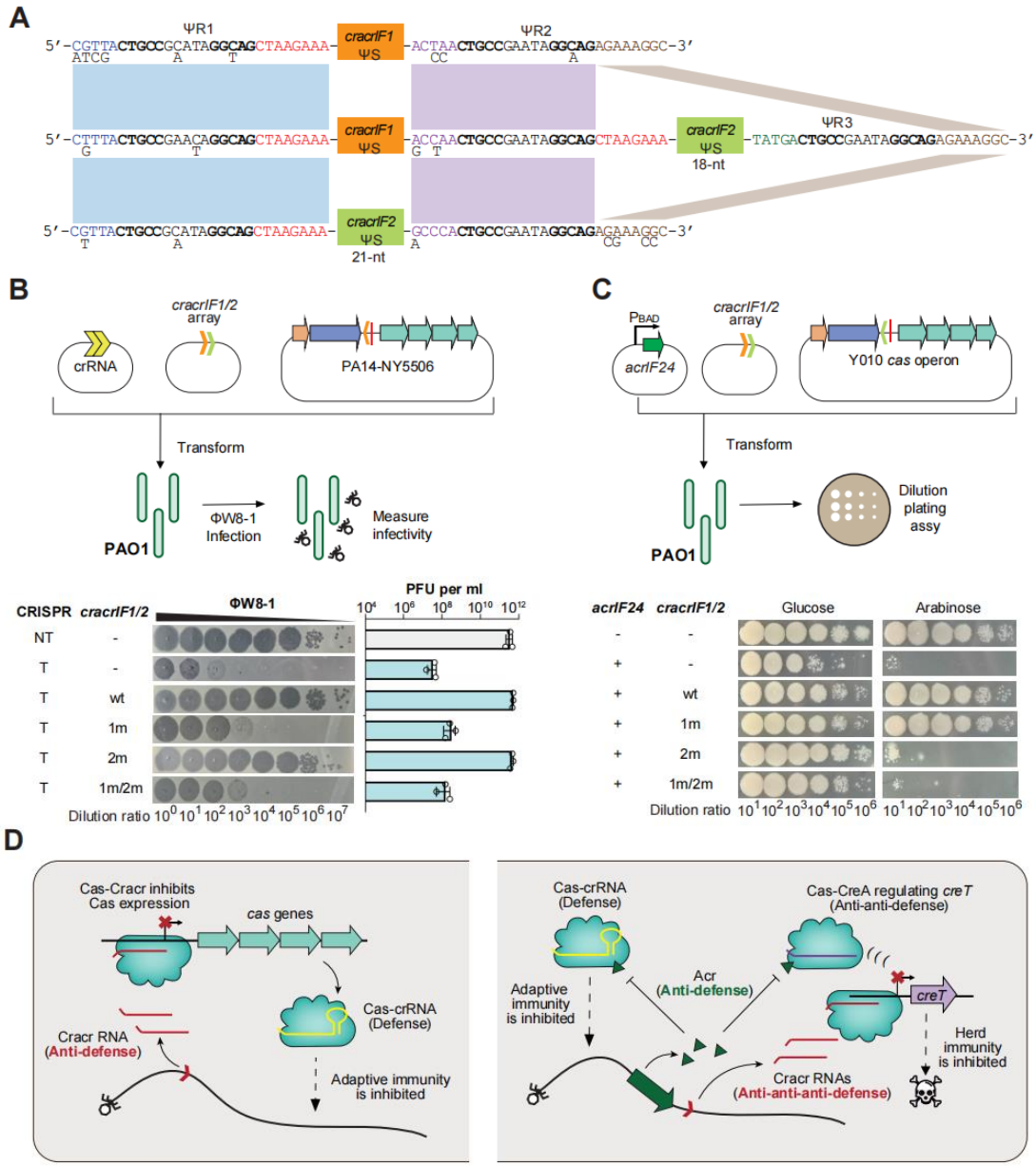

Fig. 7. A Cracr array shows both anti-CRISPR and anti-anti-anti-CRISPR activities.
(**A**) Illustration showing the homology among ΨR sequences from the *cracrIF1/2* array and the standalone *cracrIF* genes. Nucleotide substitutions observed for each ΨR unit are indicated below the sequence. Signature 5'-end nucleotides for each ΨR are highlighted in blue (ΨR1), purple (ΨR2), and green (ΨR3 of the array). Conserved nucleotides producing a 5'-handle on mature Cracr RNAs are highlighted in red. Palindromic nucleotides within each ΨR are shown in bold. (**B**) PFUs of ΦW8-1 infecting PAO1 cells with a phage-targeting (T) or non-targeting (NT) crRNA, a chimeric *cas* operon (PA14-NY5506) targeted by CracrIF1, and either an empty vector (-) or a vector expressing the Cracr array (+). The two ΨS sequences of the Cracr array were individually mutated (1m and 2m) or simultaneously mutated (1m/2m). Error bars, mean±s.d. (n=3). (**C**) Growth of PAO1 cells containing the Y010 *cas* operon, the Cracr array, and an P_{BAD}-controlled *acrIF24* gene. Glucose or arabinose was used to inhibit or induce AcrIF24 expression. (**D**) Model illustrating the modes of action (anti-CRISPR or anti-anti-anti-CRISPR) of Cracr RNAs targeting the promoter of Cas proteins or a CreT toxin.

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Materials and Methods

Bacterial stains and growth conditions

The bacterial strains used in this study are shown in Table S3. *P. aeruginosa* strains (PA14 and PAO1) and *E. coli* strains (DH5α, Mg1655, BL21(DE3)) were cultured at 37°C in lysogeny broth (LB) shaken at 200 rpm or LB-Agar (LBA) plates containing 1.2% (w/v) agar. When applicable, antibiotics were added at the following concentration: ampicillin (Amp), 100 mg L^{-1}; chloramphenicol (Cm), 25 mg L^{-1}; streptomycin (Str), 50 mg L^{-1}; tetracycline (Tet), 50 mg L^{-1}; kanamycin (Kan), 50 mg L^{-1}; carbenicillin (Car), 300 mg L^{-1} for *P. aeruginosa* and 100 mg L^{-1} for *E. coli*; gentamicin (Gen), 50 mg L^{-1} for *P. aeruginosa* and 15 mg L^{-1} for *E. coli*; isopropyl β-D-1-thiogalactopyranoside (IPTG), 500 μM; L-arabinose (Ara), 0.3% (w/v); Glucose (Glu), 0.5% (w/v).

Phage purification and titration

The phage used in this study are listed in Table S3. 1 mL of overnight host culture was inoculated into 100 mL LB and incubated for one hour. Thereafter, a single plaque containing the phage from a top agar was transferred to the culture and incubated overnight. Centrifugation (4000 rpm for 10 min at 4°C) then separates the phage from the cellular debris. The supernatant was further sterilized by filtration using a 0.22 μm filter and stored in a sterile tube. Last, the phage titre was determined by pipetting 5 μL drops of serial dilutions of the phage stock in SM buffer (100 mM $CaCl_2$, 8 mM $MgSO_4$, 50 mM Tris-HCl pH 7.5, 0.01% w/v gelatin) onto an LBA overlay (0.5% w/v) seeded with 200 μL host overnight culture, plaques were counted after incubation overnight. The *P. aeruginosa* phage (ΦW8-1, PQ030834) was amplified on PAO1, and the phages were stored in SM buffer over chloroform.

DNA isolation and manipulation

The oligonucleotides used in this study are listed in Table S3. The DNA polymerases, restriction enzymes, Gibson Assembly mix and T4 DNA ligase were obtained from Vazyme or New England Biolabs. DNA form agarose gels or PCR was purified using the E.Z.N.A.® Gel Extraction Kit (OMEGA). PCR, Restriction digest, Gibson Assembly, ligations and *E. coli* transformations following the standard guidelines. Plasmid DNA was extracted from overnight cultures using the EasyPure Plasmid Miniprep Kit (Transgen), and confirmed by Sanger sequencing is necessary.

Plasmids and their construction details are listed in Table S3. The pRSF1010 and pVS1 related plasmids were introduced into *P. aeruginosa* strains by conjugation with *E. coli* S17 strains. In brief, the overnight cultures of PAO1 and *E. coli* S17-λpir containing the plasmid were mixed in a 1:2 (recipient: donor) ratio for conjugation. The mixed cells were centrifuged at 7000 rpm for 2 min to pellet the cells, then 100 μl of LB resuspended the pellet. The mixed cultures were spotted onto a 0.45 μM sterile filter membrane in the LBA plates and incubated at 37°C for 10 h. Conjugation spots were collected and resuspended in LB, then conjugation cultures were plated on Pseudomonas Isolation Agar (BD Difco) plates supplemented with responsive antibiotics. Other plasmids were introduced into *P. aeruginosa* or *E. coli* strains by electroporation using standard techniques.

Bioinformatics search for Cracrs in databases

Known CreR sequences of types IF and IE were utilized as queries for BLASTn

879 (v2.5.0+) searches against the NCBI RefSeq, IMG/VR, and IMG/PR databases. The
880 BLASTn search was performed with the following parameters: -reward 1, -penalty -2, -
881 gapopen 4, -gapextend 2, -word_size 6, -dust no, and -evalue 10. CRISPR-Cas-Typer was
882 used to identify and eliminate CRISPR-Cas systems in the genomes corresponding to these
883 BLASTn hits. Finally, the palindromic structures containing typical CRISPR repeat
884 sequences were identified as Cracr by manual screening.
885 Due to the observed co-location pattern between AcrIF24 and Cracr IF, a further
886 search for potential Cracr IF sequences was conducted. Initially, the AcrIF24 profile HMM
887 was used to identify AcrIF24 homologs in the three databases (NCBI RefSeq, IMG/VR,
888 IMG/PR). From these results, 1.5kb upstream and downstream intergenic regions were
889 extracted, ensuring that regions were at least 40bp in length. A BLASTn search was then
890 carried out within these intergenic regions using known CracrIF/CreR sequences as queries,
891 with the following parameters: -reward 1, -penalty -2, -gapopen 5, -gapextend 2, -
892 word_size 7, -dust no, and -evalue 10.

893 Construction of Phylogenetic Tree

894 The AcrIF24 profile HMM was used to search the NCBI RefSeq, IMG/VR, and
895 IMG/PR databases, retaining results with at least 90% sequence coverage. To construct an
896 evolutionary tree for AcrIF24 and *cracr* genes, the retrieved sequences were first aligned
897 using MAFFT (v7.525) with the FFT-NS-1000 algorithm. A phylogenetic tree was then
898 constructed from the aligned sequences using FastTree (v2.1.11), employing gamma-
899 distributed site rates and the WAG evolutionary model. Taxonomic orders are represented
900 by colored rectangles outside the tree. Mobile genetic element (MGE) origins are indicated
901 by symbols at the branch tips, with circles for phages and squares for plasmids.

902 Expression of CracrIF and related constructs

903 For the experiments presented in Fig. 2C, 2D, 3B, 3F and fig. S2B, S5, B and D,
904 CracrIF1 (MN096267: 40,013-40,171bp), CracrIF2 (NZ_JAIWRW010000005: 191,361-
905 191,521bp), CracrIF1/2-array (NZ_JAOXGH010000015: 4,931-5,124) and their
906 variants were expressed from the *Escherichia-Pseudomonas* (ColE1-pRO1600) shuttle
907 vector pRGPDuo4 either under the control of its wild-type promoter. For the fluorescence
908 measurement in Fig. 2B, 3C, 5A, 5C and 5E, CracrIF1 (MN096267: 40,013-40,129bp),
909 CreR1 (CP096927.1: 2,821,988-2,822,107bp), CracrIF2 (NZ_JAIWRW010000005:
910 191,361-191,476bp), CreA2 (CP158050: 2,825,629-2,825751bp) and their variants were
911 expressed with the predicted TSS downstream of the P_{TAC} promoter. CracrIF mutations
912 were cloned by PCR using mismatched primers and overlap PCR. Detailed information on
913 the CracrIF sequences is provided in Table S1, and the primer sequence information is
914 provided in Table S3.

915 Plaque assay

916 Triplicate cultures of hosts carrying either a phage targeting spacer or non-targeting
917 control, and containing a plasmid expressing a candidate Cracr, Cracrmut or empty vector
918 control were grown overnight. Then, 150 μL of the culture was mixed into 3 mL of molted
919 top agar (0.7%, w/v) supplemented with 10 mM $MgSO_4$ and corresponding antibiotics, and
920 the mixture was poured over an LBA plate containing with 10 mM $MgSO_4$ and antibiotics
921 as needed. After allowing the top agar to solidify at room temperature, 2.5 μL drops of 10-
922 fold serial dilutions of the phage reservoir were spotted on the surface. The plates were

923 incubated upright at 37°C overnight. Plaques were counted and expressed as the plaque
924 forming units (PFU) mL^{-1}.

925 Fluorescence measurement

926 To evaluate the activity of *csy* or *creT* promoter, PCR amplified the promoter
927 sequence (containing the promoter and targeted sequence) and its variants, the *mCherry*
928 gene, respectively, and then were cloned on the pVS1 plasmid. The plasmid was then
929 introduced into the PAO6 strains (PAO1 strain carries the *csy1-4* operon expressed with
930 P_{J23116}.) along with another plasmid expressing Cracr or CreA.

931 For the experiments presented in Fig. 5, A, C and E and fig. S8, we constructed the
932 constitutive promoter J23119 to express the AcrIF24 protein in the reverse transcriptional
933 orientation to that of CracrIF or CreR/CreA, thus avoiding any potential interference. The
934 constructed plasmids were introduced into PAO6 strains for subsequent fluorescence
935 measurements.

936 To evaluate the activity of *casA* promoter, PCR amplified the promoter sequence
937 (containing the promoter and targeted sequence) and its variants, the *gfp* gene, respectively,
938 and then were cloned on the pET28a plasmid. The plasmid was then introduced into the *E.*
939 *coli* MG1655::P_{tacm2}-CasA-E (MG1655::Cas) strains along with another pACYC plasmid
940 expressing CracrIE or CreRIE.

941 Three or more biological replicates were included in each experimental set. The
942 transformants were inoculated into 3 mL of LB supplemented with corresponding
943 antibiotics, and incubated overnight. 200 μL of the cultures for measurement of
944 fluorescence intensity and optical density at 600 nm (OD600) using the Synergy H4 Hybrid
945 multimode microplate reader (BioTeck). For each biological replicate, the ratio of
946 fluorescence to OD600 was determined, and the mean and standard deviation were
947 subsequently computed. To conduct the statistical analysis, a two-tailed Student's t-test was
948 applied to ascertain the P values.

949 RNA isolation

950 Overnight cultures were diluted 1:100 into 10 mL LB supplemented corresponding
951 antibiotics, and grown at 37°C until logarithmic phase. Centrifuge 2 mL of culture at 7000
952 rpm for 2 min at 4°C to pellet the cells, then the total RNA was extracted from the pellet
953 using E.Z.N.A. Bacterial RNA kit (Omega), following the standard guidelines. Residual
954 genomic DNA was removed using DNase I (Thermo Fisher Scientific) according on the
955 manufacture's instruction. RNA concentration were determined using a NanoDrop One
956 Spectrophotometer (Thermo Fisher Scientific).

957 5' RACE

958 To identify the transcription start-site (TSS) of the *csy1*, 5' RACE was used to
959 identify the TSS of the mRNA transcript using the template-switching enzyme (New
960 England Biolabs). In brief, 1μg of the total RNA was mixed with 1mM *csy1*-specific
961 reverse primer, the mixture was incubated at 85°C for 5 min and then immediately placed
962 on ice. Afterwards, a template-switching reverse-transcription reaction was used to
963 generate cDNAs with a universal sequence of choice (introduced by a template-switching
964 oligonucleotide) attached to the 3' end of the cDNA (New England Biolabs). The
965 transcripts of mRNA were identified by PCR amplification with primers that bind upstream
966 from the *csy1*-RT-primers site and in the template-switching oligonucleotide, respectively.

967 PCR products were verified on a gel and verified by Sanger sequencing (PAO1_Y010,
968 PAO1 strain carries the CRISPR-Cas system from PA_Y010) or cloned into the pET28a
969 vector (PAO1_NY5506, PAO1 strain carries the CRISPR-Cas system from PA_NY5506),
970 then plasmids were isolated from individual colonies and subjected to Sanger sequencing.

971 Primer extension analysis

972 The 5′-FAM-labeled mCherry-specific primer as the reverse transcription primer.
973 30 μg of the total RNA was mixed with 2.5 μg of the labeled primer, and reverse
974 transcription was performed using 200 U of the M-MLV reverse transcriptase (Promega,),
975 following the standard guidelines. The extension products were screened using the ABI
976 3730xl DNA Analyzer (Thermo Fisher Scientific), and the results were visualized using
977 Peak Scanner Software v1.0.

978 Small RNA sequencing and analysis

979 A total of 50 μg of RNA was treated with T4 polynucleotide kinase (New England
980 Biolabs) according to the manufacture's protocol. The RNA was purified using the phenol-
981 chloroform method and then precipitated with an equal volume of isopropanol and 0.1
982 volume of 3M sodium acetate. The RNA molecules ranging from 30 to 300 nt were selected
983 to construct a small RNA library with the NEXTFLEX Small RNA-Seq Kit (Bioo
984 Scientific) and then subjected to Illumina HiSeq sequencing (paired-end, 150-bp reads).
985 The raw data was processed to remove adapters. The resulting reads were mapped to the
986 *CracrIF2* or *creA2* sequence using custom Perl scripts.

987 Northern bolt analysis

988 To detect transcription and processing products of CracrIF, biotin-labeled probes
989 were used for northern bolt analysis. In brief, RNA extracted from overnight cultures in
990 triplicate. RNA was mixed with an equal volume of RNA Gel Loading Dye (Thermo Fisher
991 Scientific), heated to 85 ℃ for 5 min and then cooled on ice for 2 min. Then, 10 μg total
992 RNA from each sample was resolved by denaturing 10% TBE-Urea PAGE and was
993 electrotransferred on a Biodyne B nylon membranes (Pall). Membranes were UV-
994 crosslinked, then pre-hybridized 1 hour at 42 ℃, followed by the addition of biotin-labelled
995 ssDNA probe and incubate overnight at 42 ℃. The signal was detected using the
996 Chemiluminescent Nucleic Acid Detection Module Kit (Thermo Fisher Scientific),
997 according to the manufacturer's protocol. The membrane was imaged using the Tanon 5200
998 Multi chemiluminescent imaging system (Tanon Science & Technology). Each blotting
999 assay was performed with two biological replicates, and a representative result was
1000 provided.

1001 RT-qPCR

1002 The total RNA was treated with DNase I (New England Biolabs) according to the
1003 manufacturer's instructions. cDNA was synthesized using M-MLV Reverse Transciptase
1004 (Promega) and quantified using the SYBR FAST qPCR kit (Kapa Biosystems) on an
1005 Applied Biosystems ViiA^{TM} 7 Real-Time PCR System. The triplicates were analyzed for
1006 each assay based on three independent samples. The primers listed in Table S3.

1007 Dilution plating assay

1008 For the experiments presented in Fig. 3D, 3E and fig. S7. the *creT* and its variants

1009 were cloned into IPTG-inducible vectors, transformed into *P. aeruginosa* PA14 cells and
1010 selected on LB agar plates containing appropriate antibiotic. An individual colony was
1011 inoculated in LB broth containing the appropriate antibiotic, and cultures were allowed to
1012 grow overnight on an incubator shaker to stationary phase. For the experiments presented
1013 in Fig. 3F, the Y010 *creT-csy* operon (CP158050: 2,821,349-2,825,633) and an IPTG-
1014 inducible *creA* gene were cloned into a plasmid with a broad-host-range replication of the
1015 RSF1010 origin of replication. Subsequently, the plasmid was transformed into *E. coli*
1016 MG1655 cells along with another plasmid carrying either the CracrIF2 gene or an empty-
1017 vector control, and selected on LB agar plates containing IPTG and appropriate antibiotic.
1018 An individual colony was inoculated in LB broth containing IPTG and appropriate
1019 antibiotic, and cultures were allowed to grow overnight on an incubator shaker to stationary
1020 phase. 10-fold serial dilutions of these cultures were prepared in LB broth, and 2.0 μL of
1021 these dilutions were spotted on LB agar plates containing the appropriate antibiotic and
1022 with or without 0.5 mM IPTG.

1023 CracrIF2 re-suppresses AcrIF24-destroying CreTA module

1024 To demonstrate the anti-anti-anti-CRISPR function of *cracrIF2* and *cracrIF1&2*-
1025 array, carrying CracrIF2 or CracrIF1&2-array and their variants were transformed into the
1026 PAO1_Y010 cells containing L-arabinose-induced expression of AcrIF24, and selected on
1027 LB agar plates containing glucose and appropriate antibiotic. An individual colony was
1028 inoculated in LB broth containing glucose and appropriate antibiotic, and cultures were
1029 allowed to grow overnight on an incubator shaker to stationary phase. 10 fold serial
1030 dilutions of these cultures were prepared in LB broth, and 2.0 μL of these dilutions were
1031 spotted on LB agar plates containing the appropriate antibiotic and with glucose or L-
1032 arabinose.

1033 Stability of the AcrIF24-CracrIF2 plasmid in CreTA- containing strains

1034 For the experiments presented in Fig. 4B, the GFP reporter plasmid (pGFP) and
1035 another mCherry reporter plasmid (pMC) carrying either the L-arabinose-induced *acrIF24*
1036 or the *acrIF24-cracrIF2* were mixed in equal proportions (500ng: 500ng) and transferred
1037 into the PAO1_Y010 strains. After incubation in LB medium for 1.5 h, the culture was
1038 inoculated into fresh LB medium appropriate antibiotic, and fluorescence was measured
1039 after 10 h of incubation. Subsequently, the culture was sub-inoculated (1:100 dilution) in
1040 M9 rich medium (M9 medium supplemented with 10% LB medium) appropriate antibiotic
1041 and L-arabinose. This process was repeated three more times and fluorescence
1042 measurements were taken each time. Each set of tests contained three biological replicates.

1043 Protein expression and purification

1044 Plasmids encoding Csy1-Csy4 and crRNA/Cracr/CreA were co-transformed into *E.*
1045 *coli* strain BL21(DE3). The proteins were expressed in *E. coli* strain BL21 (DE3) and
1046 induced by 0.2 mM IPTG when the cell density reached an OD_{600nm} of 0.8. After growth at
1047 18℃ for 12 h, the cells were harvested, resuspended in lysis buffer (50 mM Tris-HCl pH
1048 8.0, 300 mM NaCl, 10 mM imidazole and 1 mM PMSF) and lysed by sonication. The cell
1049 lysate was centrifuged at 20,000 g for 50 min at 4℃ to remove cell debris. The cleared
1050 lysate was affinity purified using a 5 mL HisTrap™ FF (Cytiva) column equilibrated in
1051 wash buffer containing 50 mM Tris-HCl pH 8.0, 300 mM NaCl, 30 mM imidazole, and
1052 eluted using a gradient against elution buffer (lysis buffer containing 500 mM imidazole).

Elution fractions were concentrated and further purified using a Superdex-200 Increase 10/300 GL (GE Healthcare) column equilibrated with a buffer containing 10 mM Tris-HCl pH 8.0, 200 mM NaCl, 5 mM dithiothreitol. The purified protein was analyzed by SDS-PAGE. To purify AcrIF24, the ORF from *P. aeruginosa* isolate B51 (DAFOPW010000042: 27,513-28,199) was constructed into pGEX-6P-1 plasmid for fusion expression with GST-tag, and subsequently transferred into *E. coli* strain BL21(DE3). GST-AcrIF24 was induced by 0.1 mM IPTG when the cell density reached an OD600nm of 0.8 and then growth at 18°C for 12 h. The purification procedure is the same as cascade except that the GSTrap™ HP (Cytiva) column, lysis buffer (20 mM Hepes pH 7.5, 500 mM NaCl, 1 mM PMSF) and elution buffer (20 mM Hepes pH 7.5, 500 mM NaCl, 10 mM reduced glutathione, 1mM dithiothreitol). The fusion protein was then digested with PreScission protease at 4°C overnight to remove GST-tag.

Cryo-EM sample preparation and data acquisition

4 μL aliquots of cascade complexed with crRNA/CracrIF2/CreA2 protein at a concentration of 4/4.5/1.5 mg/mL were respectively applied to discharged 300-mesh Quantifoil R1.2/1.3 grids (Quantifoil, Micro Tools GmbH, Germany). Grids were blotted for 4.5 s and plunged into liquid ethane using an FEI Mark IV Vitrobot operated at 8°C and 100% humidity.

Cryo-EM data were collected on a 300 kV Titan Krios G3 equipped with a Gatan K3 detector and a GIF Quantum energy filter (slit width of 20 eV). The defocus values ranged from -1.3 μm to -1.8 μm. Each stack of 32 frames was exposed for 2.56 s, and the exposure time of each frame was 0.08 s. The micrographs were automatically collected with the AutoEMation program (*28*) in super-resolution counting mode with a binned pixel size of 0.8374 Å. The total dose of each stack was about 50 e^{-} $Å^{-2}$. The alignment and summation of all 32 frames in each stack were performed using the whole-image motion correction program MotionCor2 (*29*).

Cryo-EM data processing

All cryo-EM data were processed using cryoSPARC (*30*). Contrast transfer function (CTF) parameters were estimated using patch-CTF. Blob picker was used for initial templates generation via 2D classification. Template picker was then used for all particle-picking tasks. For the cascade-crRNA, 732,730 particles were extracted from 928 micrographs. Subsequent two-dimensional (2D) classification, multi-class ab-initio reconstruction, nonuniform refinement and local refinement of the best class were performed, resulting in the resolution of 3.11 Å. Following the same workflow, 2,226,978 particles were extracted from 2,954 micrographs and 1,246,776 particles were extracted from 2,388 micrographs. Subsequently, 146,897 particles were used to resolve the structure of cascade-Cracr at 2.90 Å resolution, and 122,663 particles were used to resolve the structure of cascade-CreA at 3.32 Å resolution, respectively. All structures were determined with C1 symmetry. Resolution is reported using the gold-standard Fourier shell correlation with 0.143 cutoff. See Table S4 for data collection, refinement and validation statistics.

Cryo-EM model building and refinement

The initial models were derived using AlphaFold3 (*31*). We assigned predicted models into our density maps by UCSF ChimeraX (*32*). The models were further optimized by Coot (*33*). The final models of all datasets were refined against the corresponding maps

using PHENIX (*34*) in real space with secondary structure and geometry restraints. The structures were validated through examination of the Clash scores, Molprobity scores and statistics of the Ramachandran plots by PHENIX (Table S4). All the figures were created in PyMOL (*34*) and UCSF ChimeraX.

Electrophoretic mobility shift assay (EMSA)

For EMSA experiments, the His6-tag was tagged to the Csy1 protein, protein purification was performed as described above. To generate dsDNA, the FAM-labelled strand was heated to 98°C in the presence of a twofold excess of an unlabeled complementary strand and allowed to slowly return to room temperature. Specifically, non-target dsDNA without FAM labelling was generated by the same method described above, except that both strands were unlabeled. All full-length single-stranded DNA molecules were synthesized by Tsingke (Beijing, China)**.** Cascade-DNA binding reactions were performed in binding buffer (20 mM HEPES, pH 7.5, 1 mM $MgCl_2$, 150 mM NaCl and 5% glycerol) at 37°C for 15 min. The concentrations of Cascade, dsDNA and AcrIF24 are indicated in the Fig. legends. After the appropriate incubation, the reactions were resolved on native PAGE (6%). The free dsDNA and shifted protein-DNA complexes was detected on a Tanon-5200 Multi instrument (Tanon Science & Technology Co. Ltd.).

CreA-3'FAM:

GCCTTCCAAACAAGAATCACCATAAAAAGCTTCACTAGGCGTTTATTTTT

non-target:

GGCGTTTATGATTTAGTAGGTAATTGGAATTCTGTTGCTTCTATTACTCC

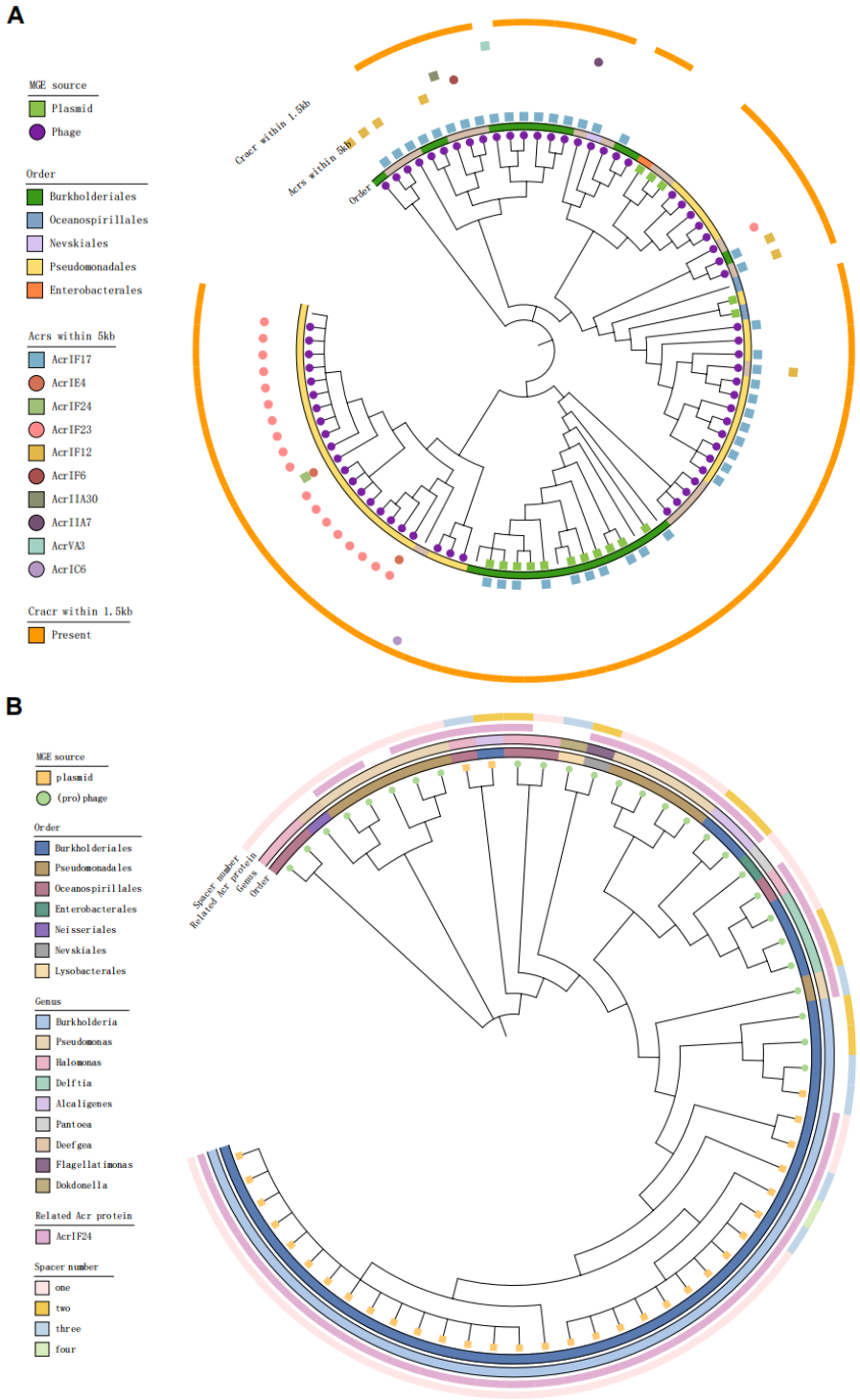
GST pull-down assay

The GST-AcrIF24 fusion protein was mixed with Cascade-crRNA, Cascade-CreA2 and Cascade-CracrIF2, respectively, according to the molecular weight of 1:2 with Mag-MK GST Fusion Protein Purification beads (BBI: C650031). At the same time, only cascade with magnetic beads were used as a negative control. The mixture was gently incubated at 4°C for 3h and then the supernatant was discarded. Then 1 ml pre-cooled wash buffer (20 mM Hepes pH 7.5, 150 mM NaCl, 1% Triton X-100) was added along the wall to wash the magnetic beads, and the washing was repeated for 4 times. Finally, elution buffer (20 mM Hepes pH 7.5, 500 mM NaCl, 10 mM reduced glutathione, 1mM dithiothreitol) was used to eluate the products, the products were tested using SDS-PAGE.

Quantification and statistical analyses

The number of replicates is specified in the associated figure legends. Each replicate represents a biological replicate of the specified experiment. Two-tailed *t* test was performed for statistical analyses. P-values above 0.05 were considered non-significant. Statistical comparisons for the transformation assays relied on log values, which assumes the samples are normally distributed on a log scale.

1136 **Fig. S1.**



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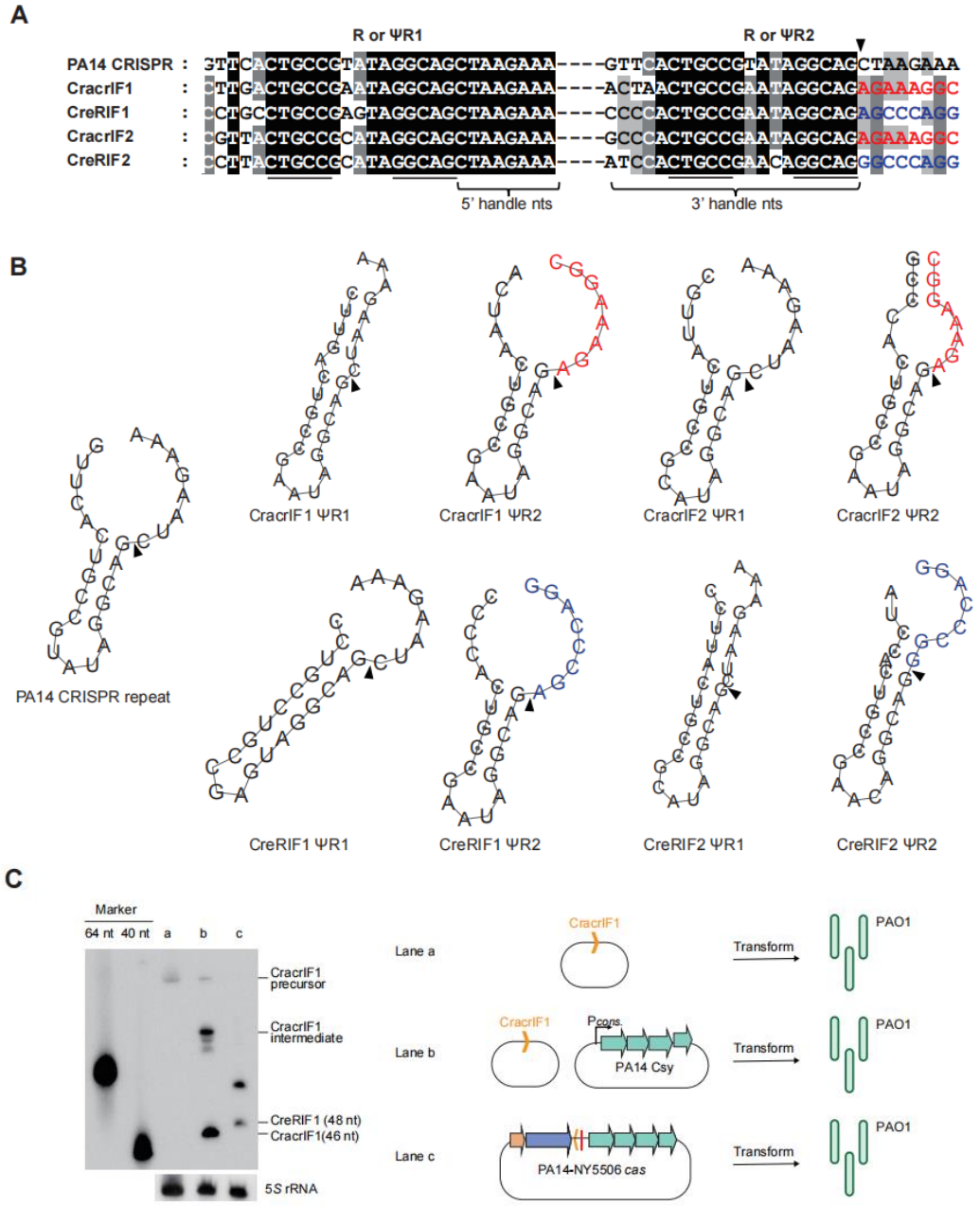
1139 **Fig. S1. Genetic association of *acrIF24* to *cracr* genes.**

1140 (**A**) All *acrIF24* genes from the NCBI RefSeq, IMG/VR and IMG/PR databases were
1141 retrieved and utilized to construct a phylogenetic tree following the removal of redundancy.
1142 Associating *cracr* genes were identified by searching the upstream and downstream 1.5 kb
1143 sequences for each *acrIF24* gene. Other *acr* genes located within a 5 kb distance from each

1144 *acrIF24* gene were also examined. (**B**) Distribution of non-*P. aeruginosa cracr* genes
1145 across different bacterial taxa. Note that *cracr* genes carried by phages (see Table S2) were
1146 not included in this analysis.
1147

1148 **Fig. S2.**



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1151 **Fig. S2. Sequence and structure conservation among the repeats from *cracr*, *creR*, and**
1152 **CRISPR, and the maturation of CracrIF1 and CreRIF1.**
1153 (**A**) Alignment of the ΨR sequences (ΨR1 and ΨR2) from *cracrIF1*, *cracrIF2*, *creRIF1*,
1154 *creRIF2* to the typical repeat sequence (R) from the PA14 CRISPR array. Nucleotides (nts)
1155 producing a 5' or 3' handle on mature RNAs are indicated. Palindromic nucleotides are
1156 underlined. (**B**) Predicted RNA structures of each repeat. Triangles indicate the processing
1157 sites by Csy4. ΨR2 nucleotides that evidently differ between *cracrIF* and *creRIF* genes are
1158 highlighted in red and blue, respectively. **(C)** Northern blotting analysis of CracrIF1 and
1159 CreRIF1 RNAs (using the same probe due to their high sequence similarity). Experimental
1160 design is shown on the right. 5*S* rRNA served as the internal control. Biotin-labeled single-

1161 stranded oligos (40 nt or 64 nt) served as markers.

Fig. S3.

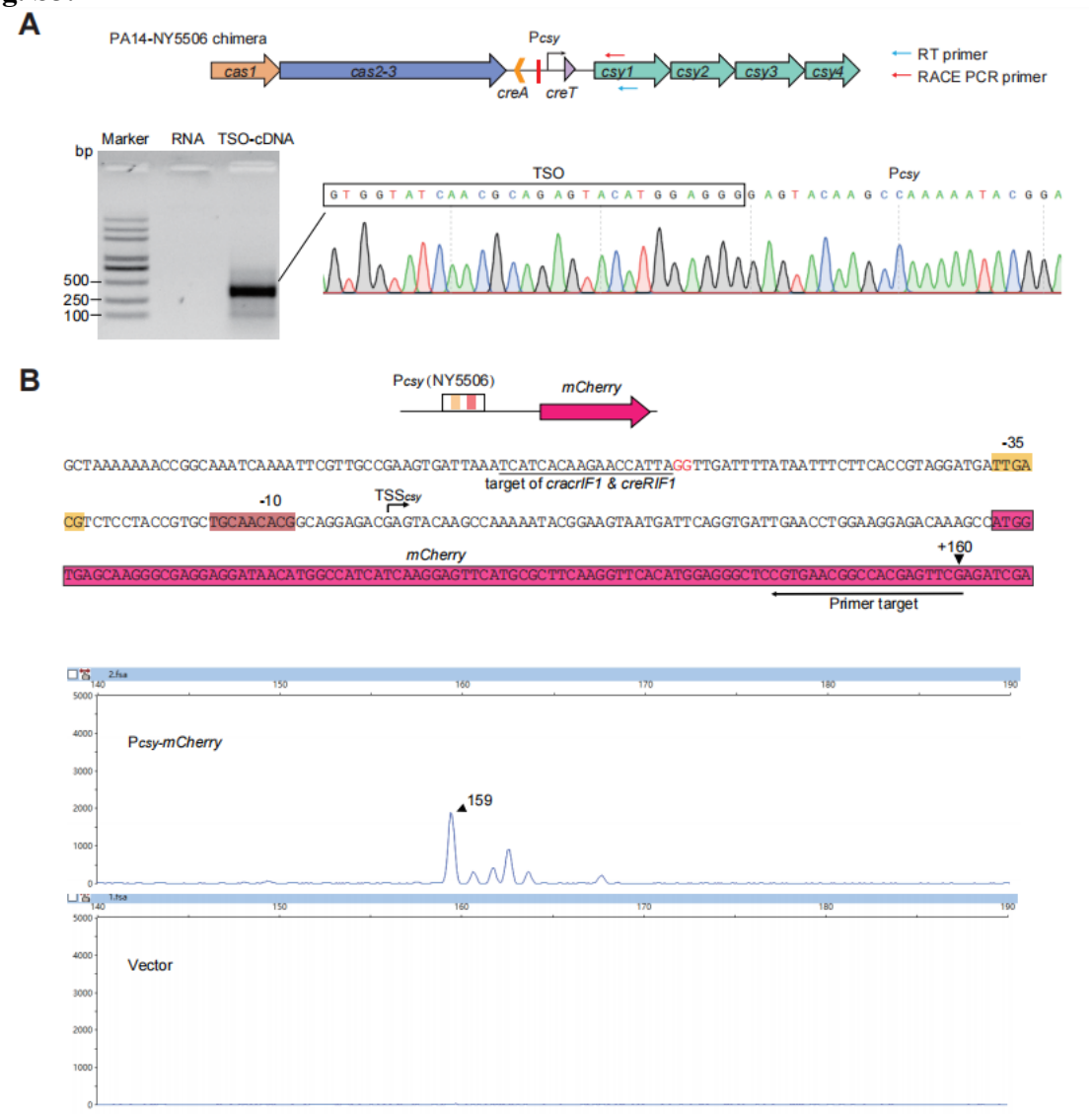
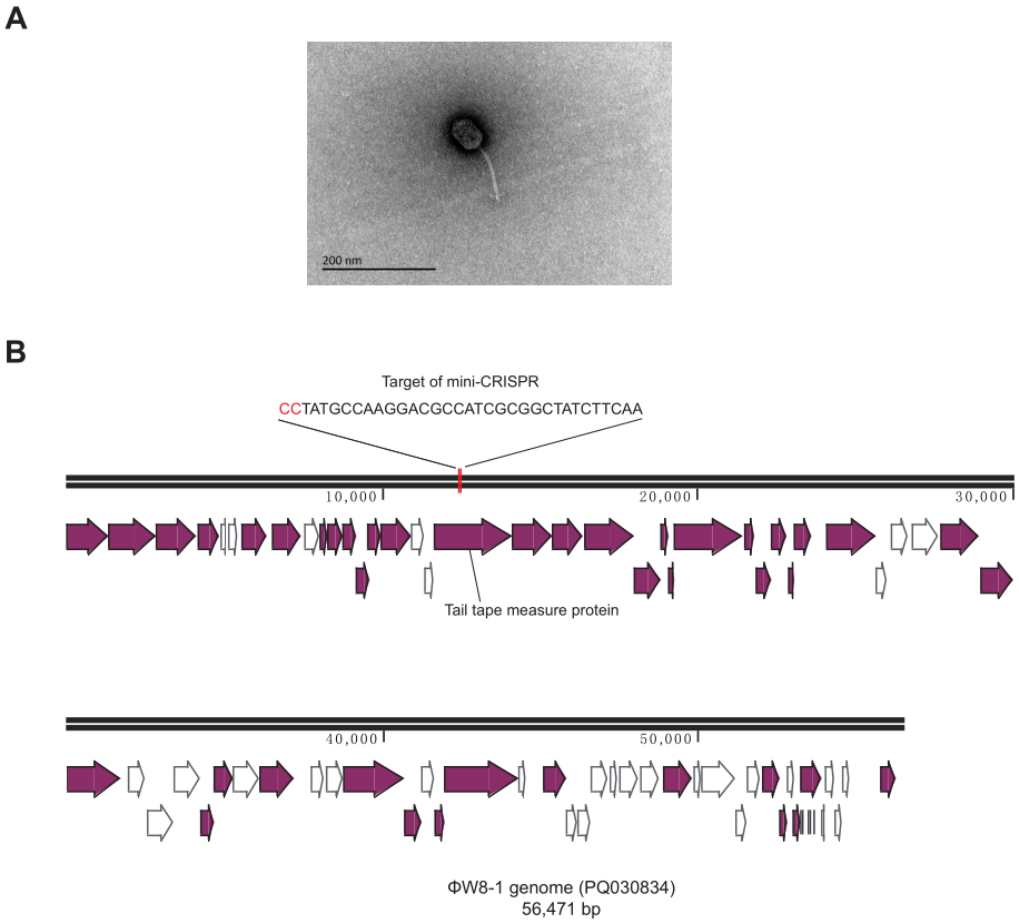


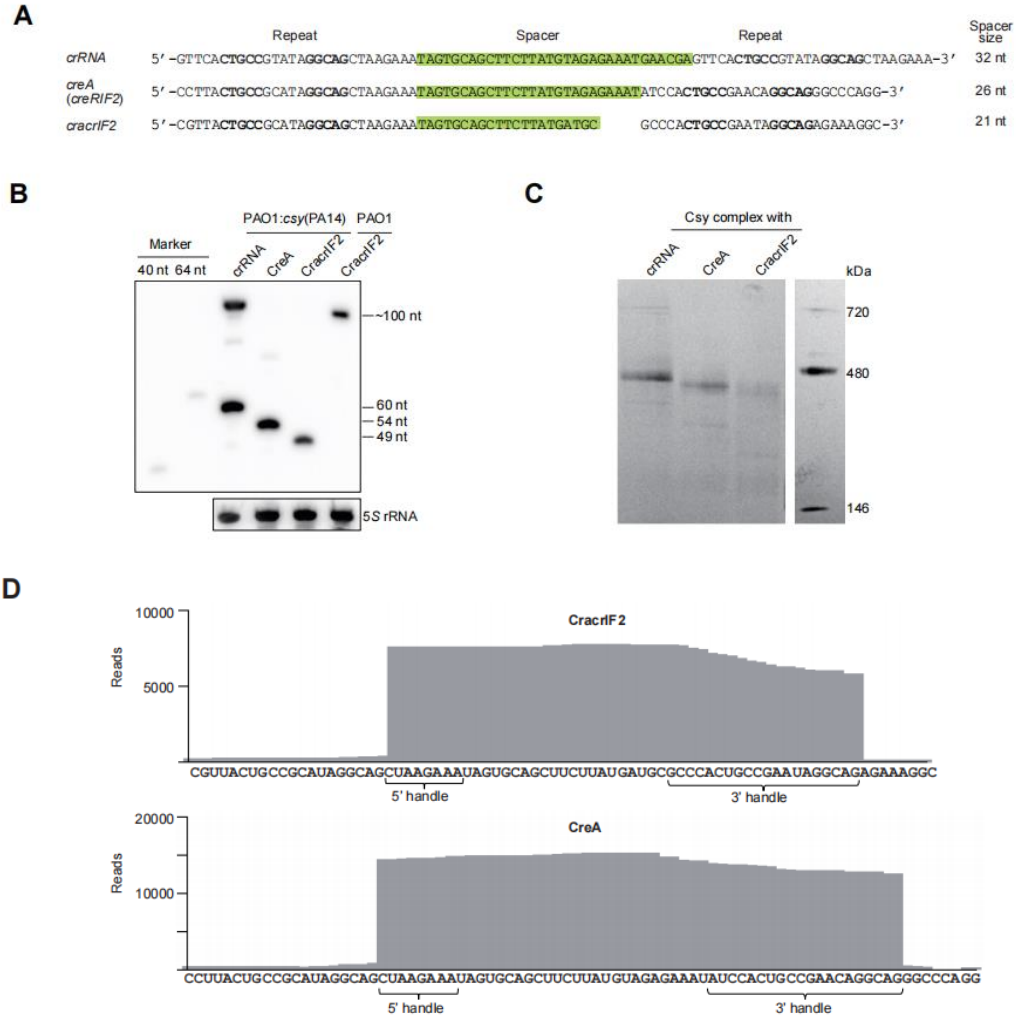
Fig. S3. Characterization of the NY5506 *csy* promoter (P_{csy}).
(**A**) The transcription start site (TSS) of NY5506 *csy* transcripts were determined by 5' RACE. Note that, for convenience, the chimeric *cas* operon PA14-NY5506 used for phage infection experiments was also used for this assay. Complementray DNA (cDNA) was generated by reverse transcription (using RT primer), and subsequently used as the template for PCR reactions with a forward primer against the template switching oligo (TSO) and a reverse primer against *csy1* sequence (RACE PCR primer). DNase-treated RNA samples were used as the negative control. **(B)** Primer extension assay to confirm the TSS of NY5506 P_{csy}. The P_{csy} DNA was first linked to an *mCherry* gene and then introduced into *P. aeruginosa* PAO1 cells, of which total RNA was extracted for primer extension. The primer was designed against the *mCherry* RNA transcript and 5'-labeled by FAM (see the Methods part). The cDNA products of primer extension assay were then subjected to fragment size analysis. PAO1 cells containing an empty vector served as a negative control. Promoter elements -10 and -35 are indicated. The target sequence of CracrIF1 or CreRIF1 RNAs are underlined, with the complementary nucleotides of PAM shown in red.

1179 **Fig. S4.**



1180
1181 **Fig. S4. Characterization of the ΦW8-1 phage infecting PAO1 cells.**
1182 (**A**) Electron-microscopy of the ΦW8-1 phage. The 200 nm scale bar is given. (**B**) The
1183 genome of ΦW8-1 (with the GenBank ID PQ030834) and design of the mini-CRISPR
1184 targeting the gene of tail tape measure protein. The PAM nucleotides are shown in red.
1185
1186

1187 **Fig. S5.**



1188

1189

1190 **Fig. S5. Analysis of CracrIF2, CreA (CreRIF2), and a cognate crRNA, as well as**
1191 **the Csy complexes bound to different guide RNAs.**
1192 **(A)** Illustration of the sequence differences between *creA*, *cracrIF2*, and a synthetic
1193 mini-CRISPR encoding a cognate crRNA. The spacer region of crRNA was designed
1194 by appending five extra nucleotides (not complementary to the shared target site) to that
1195 of *creRIF2*, resulting a typical spacer size of 32 nt. (**B**) Northern blotting analysis of
1196 mature CreA, CracrIF2, and the cognate crRNA, demonstrating different sizes. These
1197 RNA molecules were expressed in PAO1 cells expressing the Csy proteins of PA14,
1198 and the total RNA samples were subjected to the blotting assay. As a control, PAO14
1199 cells without Csy proteins producing the precursor RNAs of CracrIF2 were also
1200 included. 5*S* rRNA served as the internal control. Biotin-labeled single-stranded oligos
1201 (40 nt or 64 nt) served as markers. (**C**) Native PAGE analysis of the Csy complexes
1202 bound to CracrIF2, CreA, or the cognate crRNA. The Csy proteins and guide RNAs
1203 were co-expressed in *E. coli* BL21(DE3) and subsequently co-purified for analysis. (**D**)
1204 The 5' and 3' termini of CracrIF2 and CreA RNAs determined by small RNA
1205 sequencing.
1206

Fig. S6.

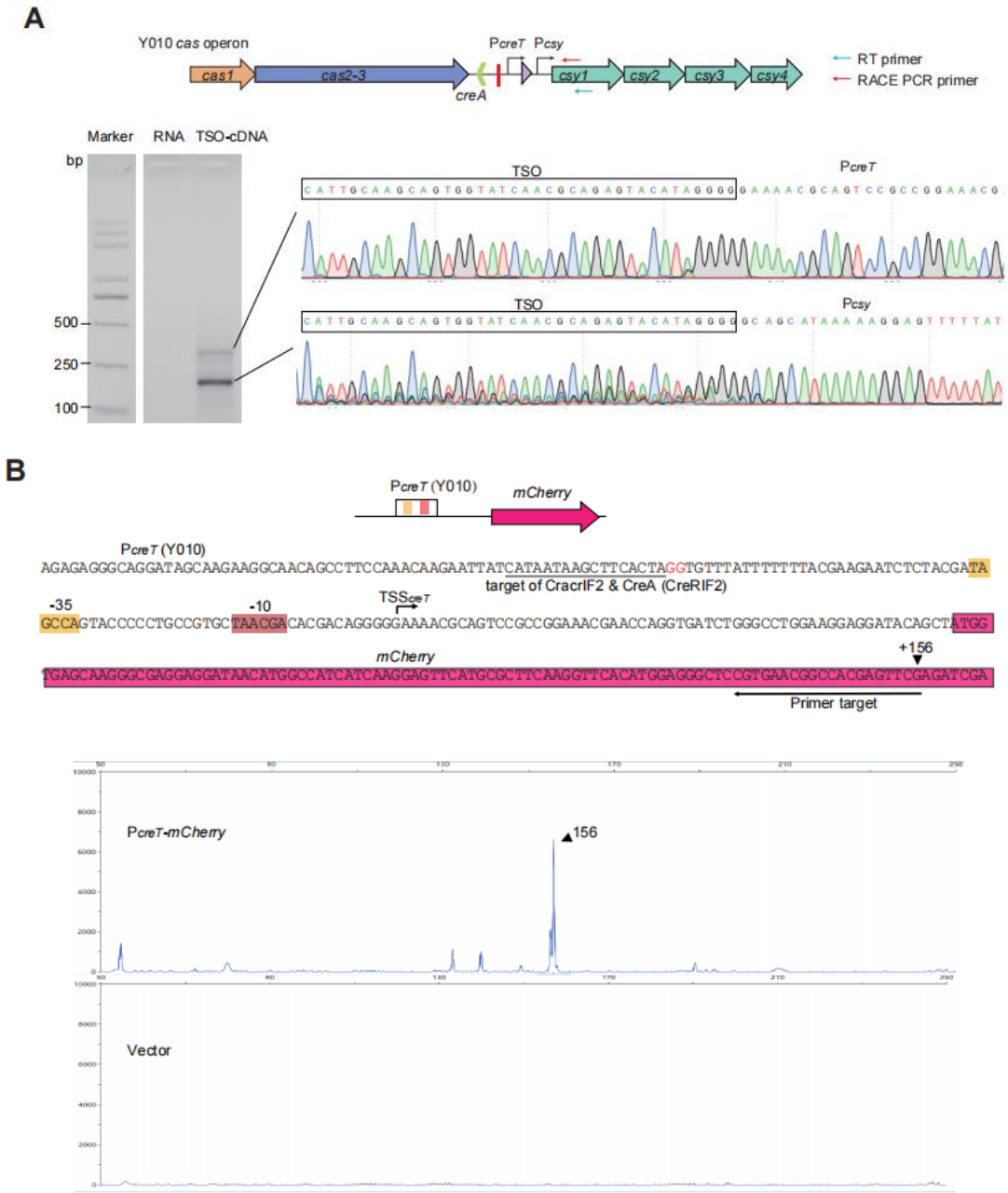


Fig. S6. Characterization of the *csy* promoter (P_{csy}) and *creT* promoter (P_{creT}) of NY010.

(**A**) Two transcription start sites of *csy* transcripts were determined by 5' RACE. cDNA was generated by reverse transcription (using RT primer), and subsequently used as the template for PCR reactions with a forward primer against the template switching oligo (TSO) and a reverse primer against *csy1* sequence (RACE PCR primer). DNase-treated RNA samples were used as the negative control. **(B)** Primer extension assay to confirm the TSS of Y010 P_{creT}. The P_{creT} DNA was first linked to an *mCherry* gene and then introduced into *P. aeruginosa* PAO1 cells, of which total RNA was extracted for primer extension. The primer was designed against the *mCherry* RNA transcript and 5'-labeled

1221 by FAM (see the Methods part). The cDNA products of primer extension assay were
1222 then subjected to fragment size analysis. PAO1 cells containing an empty vector served
1223 as a negative control. Promoter elements -10 and -35 are indicated. The target sequence
1224 of CracrIF2 or CreA RNAs are underlined, with the complementary nucleotides of PAM
1225 shown in red.
1226

Fig. S7.

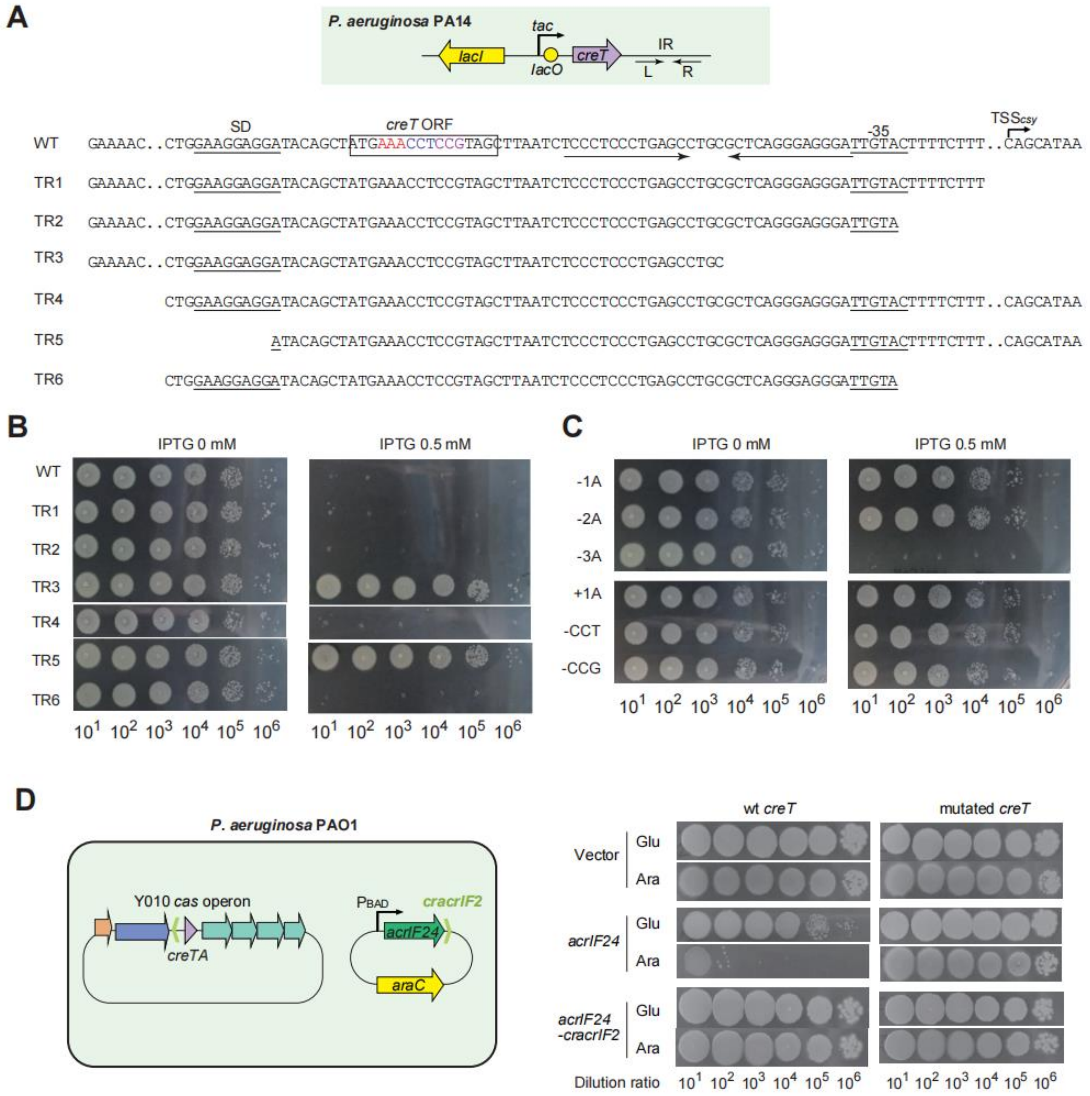
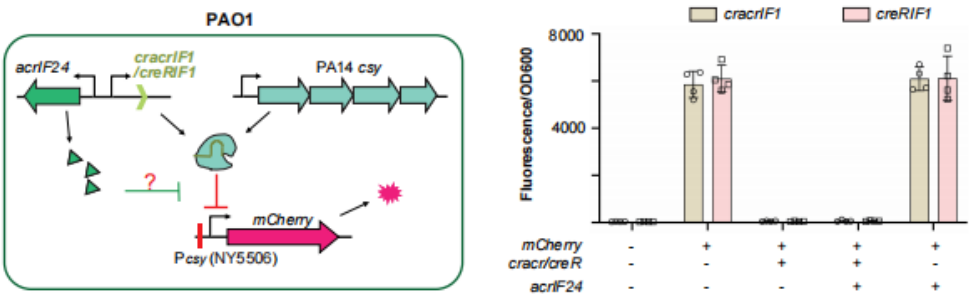


Fig. S7. Characterization of the CreT RNA toxin.

(**A**) Illustration showing the differently truncated DNA sequences (TR1-TR6) used to determine the minimal requirement to produce the toxicity of CreT. The critical elements, including a Shine-Dalgarno (SD) sequence, a mini-ORF, and a pair of inverted repeats (IR, labeled with two convergent arrows), are indicated along the wild-type (WT) sequence. The downstream -35 promoter element and TSS of *csy* genes are also denoted. The red nucleotides (AAA) were subjected to frameshift mutation analyses (panel **C**). The two proline codons (CCT and CCG) are highlighted in blue and purple, respectively. (**B**) Growth of PA14 cells expressing differently truncated *creT* genes. (**C**) Growth of PA14 cells when the mini-ORF of *creT* was differently mutated. The one (-1A), two (-2A), or all (-3A) the three nucleotides of the AAA codon (highlighted in red in panel **A**) were deleted for frameshift analysis. Addition of an extra adenine was also performed (+1A). The two proline codons were individually deleted in the -CCT and -CCG mutants. (**D**)Schematic representation (left) and results (right) of the assay to evaluate the ability of CracrIF2 RNAs to alleviate the CreTA cytoxicity

that is triggered by AcrIF24 expression. AcrIF24 was individually expressed or co-transcribed with CracrIF2 under the control of the arabinose-inducible BAD promoter (P_{BAD}), with glucose used to suppress any basal activity of P_{BAD}. In the mutated *creT* control, the two critical proline codons were removed.

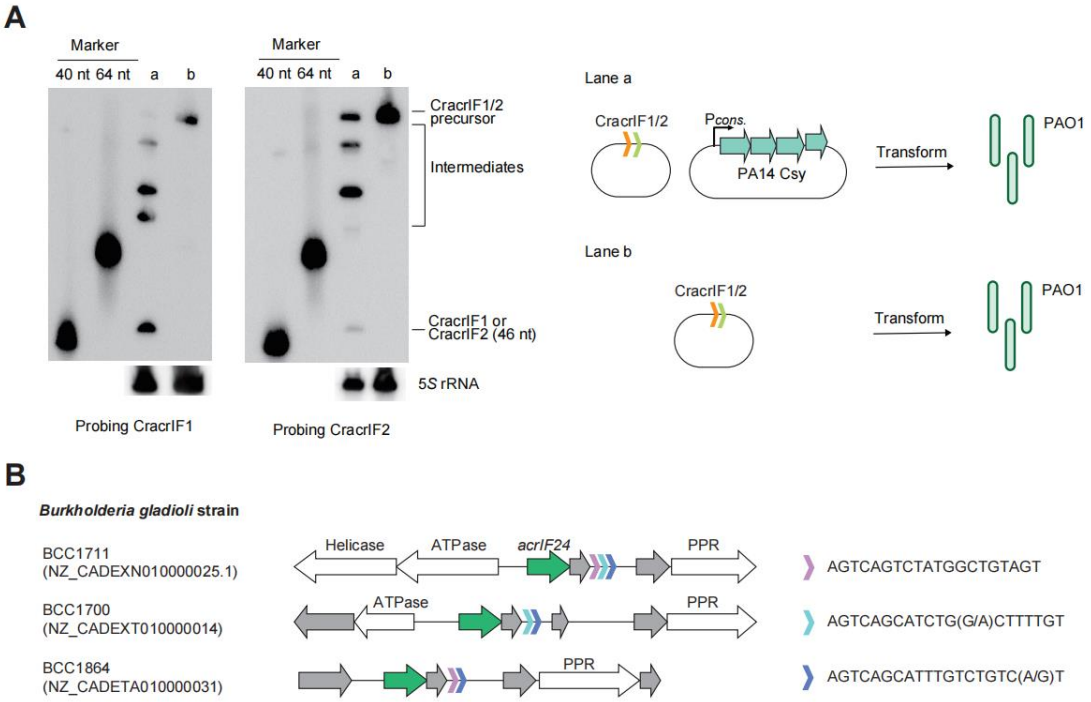
1251 **Fig. S8.**



1252

1253 **Fig. S8. Both CrcrIF1 and CreRIF1 are resistant to AcrIF24.**
1254 Fluorescence intensity of PAO1 cells containing a plasmid expressing mCherry under
1255 the control of the Y5506 *csy* promoter (targeted by its corresponding CreRIF1 and the
1256 viral CracrIF1), a second plasmid expressing PA14 Csy proteins, and a third plasmid
1257 expressing CracrIF1 or CreRIF1 with or without the co-expression of AcrIF24.
1258
1259
1260

1261 **Fig. S9.**



1262

1263 **Fig. S9. Cracr RNAs production from the *cracrIF1/2* array and other type I-F**
1264 **Cracr arrays from *Burkholderia gladioli* genomes.**
1265 (**A**) Northern blotting analysis of the CracrIF1 and CracrIF2 RNAs produced from their
1266 Cracr array. Experimental design is shown on the right. 5*S* rRNA served as the internal
1267 control. Biotin-labeled single-stranded oligos (40 nt or 64 nt) served as markers. (**B**)
1268 Three Cracr arrays carried by *B. gladioli* prophages. The nucleotide sequence of each
1269 ΨS is given on the right.
1270
1271

Fig. S10.

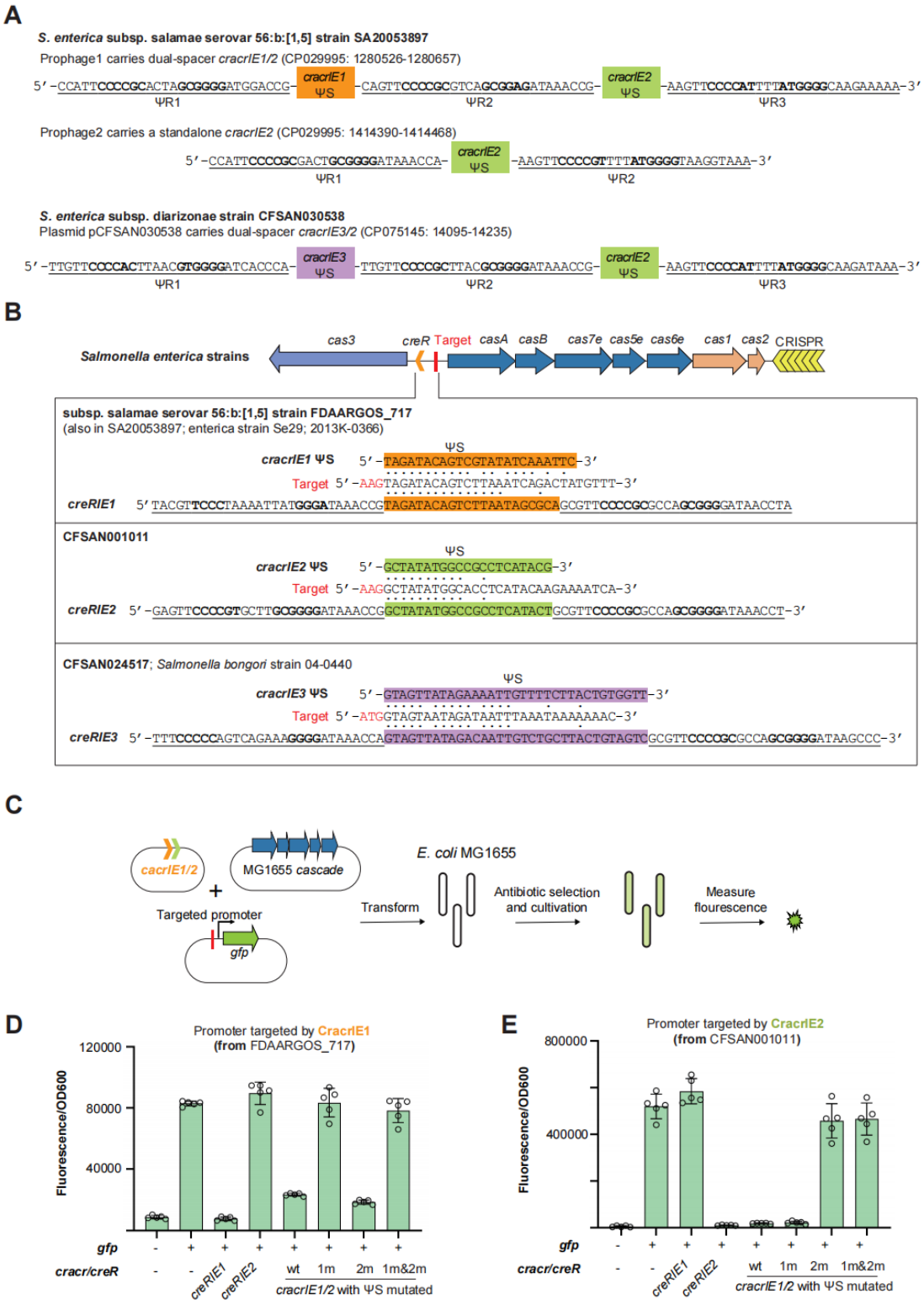


Fig. S10. Type I-E Cracr RNAs encoded by *Salmonella* MGEs and their regulatory activities.

(**A**) Three related *cracr* genes or arrays carried by a prophage and a plasmid from *Salmonella*. The palindromic nucleotides within each ΨR sequence are shown in bold. (**B**) CRISPR-Cas systems that are potentially targeted by CracrIE1, CracrIE2, or

1280 CracrIE3. The PAM nucleotides are shown in red. Dots indicate identical nucleotides.
1281 (**C**) Illustration showing the experimental design to measure the inhibitory effect of the
1282 *cracrIE1/2* array on putative target promoters. (**D**) and (**E**) Green fluorescence intensity
1283 from *E. coli* cells with a *gfp* gene under the control of a putative promoter sequence that
1284 is targeted by CracrIE1 (D) or CracrIE2 (E). The first (1m) and second (2m) ΨS
1285 sequences of *cracrIE1/2* were individually mutated or simultaneously mutated
1286 (1m&2m). wt, wild-type. Error bars, mean±s.d. (n=5).
1287