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1 **A nucleus-like compartment shields bacteriophage DNA**
2 **from CRISPR-Cas and restriction nucleases**
3
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All viruses require strategies to inhibit or evade the immunity pathways of cells they infect. The viruses that infect bacteria, bacteriophages (phages), must avoid nucleic-acid targeting immune pathways such as CRISPR-Cas and restriction endonucleases to replicate efficiently[1]. Here, we show that a jumbo phage infecting *Pseudomonas aeruginosa*, phage ΦKZ, is resistant to many immune systems *in vivo*, including CRISPR-Cas3 (Type I-C), Cas9 (Type II-A), Cas12 (Cpf1, Type V-A), and Type I restriction-modification (R-M) systems. We propose that ΦKZ utilizes a nucleus-like shell to protect its DNA from attack. Supporting this, we demonstrate that Cas9 is able to cleave ΦKZ DNA *in vitro*, but not *in vivo* and that Cas9 is physically occluded from the shell assembled by the phage during infection. Moreover, we demonstrate that the Achilles heel for this phage is the mRNA, as translation occurs outside of the shell, rendering the phage sensitive to the RNA targeting CRISPR-Cas enzyme, Cas13a (C2c2, Type VI-A). Collectively, we propose that the nucleus-like shell assembled by jumbo phages enables potent, broad spectrum evasion of DNA-targeting nucleases.

Phage infection and replication can cause bacterial death via cell lysis, necessitating protective immune systems to negate this great threat to bacterial viability[2,3]. Restriction-modification (R-M) and adaptive CRISPR-Cas (clustered regularly interspaced short palindromic repeats and CRISPR-associated genes) immunity detect and degrade phage nucleic acids to protect the host[1]. Recent CRISPR-Cas discovery efforts have led to the characterization of six distinct CRISPR-Cas types (I-VI) with independent mechanisms for CRISPR RNA (crRNA) biogenesis, surveillance complex assembly, substrate selection, and target degradation[4]. Type III and VI CRISPR-Cas systems target RNA[5,6], while Types I, II, and V predominantly target DNA[7-9]. Despite these differences, all characterized CRISPR-Cas systems function via crRNA-guides derived from the DNA-based CRISPR array, which stores the memory of past infections[10,11].

Phages that infect *Pseudomonas aeruginosa* avoid CRISPR-mediated destruction by encoding “anti-CRISPR" (Acr) proteins that inhibit the Type I-E and I-F CRISPR-Cas systems[12-14]. We sought to determine whether any *P. aeruginosa* phages are also resistant to the Type I-C CRISPR-Cas subtype, also present in *P. aeruginosa*[15]. This subtype is notable as it is the most minimal Type I system identified to date, is highly abundant in bacterial genomes[16], and is understudied relative to other subtypes. We identified an isolate in our lab encoding a Type I-C system, and designed and expressed a crRNA targeting related phages JBD30 and DMS3m, which encode AcrIF and AcrIE proteins, respectively. The successful crRNA-specific targeting of

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48 these phages by the Type I-C system demonstrated functional CRISPR immunity and that these
49 phages did not possess functional Type I-C Acr proteins (Fig. 1a). To test the sensitivity of other
50 phage families against the Type I-C system, we transferred the necessary *cas* genes (*cas3,*
51 *cas5, cas8, cas7*) into the chromosome of a commonly used, phage-sensitive lab strain (PAO1)
52 that naturally lacks CRISPR-Cas immunity. A small screen was then conducted, where crRNAs
53 were tested against phages from five taxonomic groups: JBD30, D3, ΦKZ, F8, and JBD68.
54 JBD30, D3, and JBD68 are distinct temperate siphophages while ΦKZ and F8 are distinct lytic
55 myophages. All phages succumbed to targeting, except ΦKZ (Fig. 1b, 1c). ΦKZ titer did not
56 decrease when exposed to ten different Type I-C crRNAs (Fig. 1c, Extended Data Fig. 1),
57 suggesting that it is completely resistant to this immune system of *P. aeruginosa*.
58
59 The ΦKZ genome possesses no homologs of *acr* genes[12-14,17,18] or anti-CRISPR associated
60 (*aca*) genes that have previously enabled identification of *acr* genes[14,17,18]. Moreover, ΦKZ is a
61 phage with no genetic tools currently available to manipulate it, rendering traditional knockout
62 approaches infeasible. Thus to determine the mechanism by which the ΦKZ phage resists Type
63 I CRISPR-Cas, we attempted to utilize the Type II-A CRISPR-Cas9 system from *Streptococcus*
64 *pyogenes* (SpyCas9) to knock out phage genes. SpyCas9 and sgRNAs were adapted for
65 expression and function in *P. aeruginosa,* leading to robust targeting of control phage JBD30,
66 but notably, ΦKZ replication and associated cell lysis was unaffected both in plate and liquid
67 assays (Fig. 2a). An additional eight sgRNA sequences also failed to target ΦKZ (Extended
68 Data Fig. 2a), suggesting that ΦKZ resists both Type I and II CRISPR immunity. Given the
69 ability of this phage to evade two unrelated CRISPR systems originating from distantly related
70 microbes, we considered that it may be generally resistant CRISPR-Cas immunity. To test this,
71 the Type V-A Cas12a (Cpf1) CRISPR-Cas system from *Moraxella bovoculi* was expressed in *P.*
72 *aeruginosa* and again, robust targeting of phage JBD30 was observed, but not of ΦKZ with any
73 of the nine crRNAs tested (Fig. 2b, Extended Data Fig. 2b). The ability of this phage to resist
74 CRISPR systems found in its natural host (Type I-C) and those not present in *Pseudomonas*
75 (Type II-A and V-A) suggests that this phage possesses a mechanism to enable "pan-CRISPR"
76 resistance.
77
78 Restriction-modification systems are the most common bacterial immune system in nature and
79 pose a significant impediment to phage replication[1]. To test whether ΦKZ is also resistant to
80 attack from restriction endonucleases, the phage was propagated on strain PAK, an isolate that
81 generates phages that are restricted by strain PAO1. When phage JBD30 was propagated on

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82 PAK, and then plated on strain PAO1, its titer was reduced by ~3 orders of magnitude (Fig. 2c),
83 an effect that was ameliorated in a PAO1 strain lacking the Type I R-M system (Δ*hsdR*).
84 However, when ΦKZ was propagated on PAK, its titer did not decrease on PAO1 (Fig. 2c).
85 Conversely, when propagated on PAO1, ΦKZ titer was not decreased on PAK, while JBD30
86 was again reduced in titer by ~3 orders of magnitude (Fig. 2c). This demonstrates that ΦKZ is
87 also recalcitrant to Type I restriction endonucleases *in vivo*.
88
89 Given the strong resistance to targeting, we considered whether DNA base modifications were
90 protecting this phage from enzyme targeting, as has been previously seen with phage T4 and
91 others[19-22]. Purified phage DNA, extracted from ΦKZ virions, was subjected to restriction
92 digestion reactions with a panel of restriction enzymes that are inhibited by glc-HmC moieties,
93 including HindIII, EcoRI, SacI, KpnI, NcoI, and EcoRI (ref. 22,23 and per New England Biolabs).
94 Additionally, MspJI was utilized, a modification-dependent restriction nuclease that requires 5-
95 HmC or 5-mC modified DNA for cleavage[24]. Except for SacI, which lacks a sequence
96 recognition motif in the ΦKZ genome, all enzymes tested cleaved ΦKZ gDNA, demonstrating
97 the absence of glc-HmC modifications (Fig. 3a). To determine whether the phage genome is a
98 substrate for CRISPR-Cas9-based cleavage, purified phage DNA was subjected to a cleavage
99 assay with two distinct crRNA sequences using a dual crRNA:tracrRNA-loaded SpyCas9
100 nuclease *in vitro*. Cas9 cleaved the phage genome in two locations, liberating the predicted 10
101 kb fragment from the much larger 280 kb genome (Fig. 3b). Notably, the two crRNA sequences
102 used here are the same sequence as crRNAs II-A, #1 and II-A, #2 used *in vivo* (Fig. 2a),
103 demonstrating that these crRNA sequences do not target phage *in vivo,* but are competent for
104 genome cleavage *in vitro.*
105
106 Recently it was shown that ΦKZ and ΦKZ-like phages infecting *Pseudomonas* sp. construct an
107 elaborate nucleus-like, proteinaceous compartment where phage DNA replicates, with PhuZ, a
108 phage tubulin homologue, centering the compartment within the host cell[25-29]. Proteins involved
109 in DNA replication and transcription localize inside the shell, while proteins mediating translation
110 and nucleotide synthesis are relegated to the cytoplasmic space, akin to the eukaryotic nucleus.
111 Given the rapid assembly of the shell and its apparent exclusion of select proteins, we
112 considered whether this structure was responsible for the resistance of ΦKZ to four unrelated
113 CRISPR-Cas and restriction nucleases. The Type II-A single protein effector Cas9 was chosen
114 as a representative immune system for ease of manipulation and imaging. *P. aeruginosa* cells
115 infected with ΦKZ were imaged with a Cas9 fluorescent antibody, revealing that the protein is

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116 indeed excluded from the shell during phage infection (Fig. 4a). DAPI staining reveals the phage
117 DNA inside the nucleus-like shell, while the host genome is rapidaly degraded during infection.
118 As a control, a protein previously shown to be internalized in the shell, ORF152, was imaged
119 revealing co-localization with the DAPI-positive phage nucleus. While the rules for protein
120 internalization in the shell are currently unknown, these data and work from the initial shell
121 studies[28,29] suggest that the default localization for large host-encoded proteins is to be
122 excluded from the shell.
123
124 While ΦKZ DNA is protected from CRISPR-Cas and restriction endonucleases, the mRNA is not
125 afforded this same luxury, as it leaves the confines of the shell to be translated in the cytoplasm.
126 We therefore envisaged that an RNA-targeting CRISPR-Cas system would provide immunity to
127 ΦKZ even if localized in the cytoplasm. To test this, we adapted the RNA-guided RNA nuclease
128 Cas13a (C2c2, Type VI-A) from *Listeria seeligeri*[5,30] for phage targeting in *P. aeruginosa*. By
129 targeting several different transcripts, three LseCas13 spacers were found (two targeting tail
130 gene *gp146* and one targeting the shell gene *gp054*) that decreased ΦKZ plaquing efficiency by
131 10-1000-fold (Fig. 4b, Extended Data Fig. 3). Corroborating the plaquing results, LseCas13 also
132 provided protection of *P. aeruginosa* in liquid cultures, with strong bacterial growth at phage
133 inputs that kill cultures with a non-targeting spacer (Fig. 4c). The sensitivity of ΦKZ to RNA-
134 targeting but not DNA-targeting underscores the role of the shell in broad spectrum resistance
135 to DNA-cleaving enzymes and also provides the first evidence of a DNA phage being inhibited
136 by Cas13.
137
138 Evasion of the endogenous *P. aeruginosa* Type I CRISPR-Cas system by ΦKZ suggests that
139 these jumbo phages are likely to pervasively avoid this system in nature. Supporting this
140 hypothesis, our analysis of >4,000 *P. aeruginosa* non-redundant spacers (Type I-C, I-E, and I-F)
141 reported by van Belkum et al. (2015) found no spacers against ΦKZ, or its jumbo phage
142 relatives ΦPA3, PaBG, KTN4, and PA7 (Table 1). This is in contrast to the many spacer
143 matches from each system against diverse *P. aeruginosa* phages, such as those assayed in our
144 screen and those encoding anti-CRISPR proteins (Table 1). Finally, given the efficacy of the
145 RNA-targeting CRISPR-Cas13 system, we propose that perhaps these CRISPR systems are
146 well-suited to target the mRNA of DNA phages when the DNA is inaccessible (i.e. due to base
147 modifications or physical segregation). However, the rules governing the ability of Cas13 to limit
148 the replication of DNA phages remain to be elucidated as only 3/11 LseCas13a crRNAs tested
149 targeted ΦKZ and 0/6 were effective at targeting phage JBD30 (Extended Data Fig. 3).

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150
151 Here, we searched for CRISPR-Cas resistant phages by designing crRNAs against them and
152 testing their efficacy. This screen identified jumbo phage ΦKZ (genome size: 280,334 bp[31]) as
153 resistant to the Type I-C CRISPR system, and subsequently to Type II-A and Type V-A single
154 effector nucleases Cas9 and Cas12. ΦKZ is also recalcitrant to the Type I restriction-
155 modification system of *P. aeruginosa*. Despite this apparent resistance *in vivo,* ΦKZ genomic
156 DNA is sensitive to restriction enzymes and Cas9 cleavage *in vitro*. We propose that the
157 assembly of a proteinaceous compartment to house the replicating phage DNA creates a
158 physical protective barrier resulting in the resistance of phage ΦKZ to DNA-cleaving enzymes
159 (Fig. 4d). Although this shell-like structure has only been documented among the jumbo phages
160 of *Pseudomonas*[28,29], we consider that physical occlusion of phage genomic DNA through this
161 and other mechanisms may comprise a novel route to immune system evasion in bacteria.
162
163 The pan-resistance of ΦKZ to DNA-targeting enzymes provides an explanation for the elaborate
164 and impressive shell structure, and suggests that the phage DNA may never be exposed to the
165 cytoplasm. Other hypotheses to describe the shell's existence remain to be addressed,
166 including protection from phage-derived nucleases that degrade the bacterial genome or as a
167 mechanism to spatially restrict the large phage genome during replication and packaging.
168 Regardless, we conclude that the nucleus-like shell provides a strong protective barrier to DNA-
169 targeting immune pathways. We expect that simple CRISPR-based screens, such as the one
170 conducted here, may reveal many other fascinating mechanisms that phages have evolved to
171 ensure their replicative success when faced with immune systems to overcome.
172

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173

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179
180 ΦKZ, JBD30, JBD68, D3, and F8 were provided by Alan Davidson's lab. Phage DMS3m was a
181 gift from the O'Toole lab and Jason M. Peters and Carol A. Gross provided the *S. pyogenes*
182 *cas9* expression plasmid for integration in the PAO1 chromosome. pTE4495
183 (MbCpf1/MbCas12a) was a gift from Ervin Welker (Addgene plasmid # 80339), and LseCas13a
184 (Addgene plasmid #83486) is a gift from Jennifer Doudna.
185

186 **Author Contributions:**

187 S.D.M. conducted restriction-modification experiments, constructed Cas13 strains and
188 conducted associated experiments with that strain and the Cas12 strain, including all liquid
189 infection assays, and prepared figures. J.D.B. constructed and conducted experiments with
190 Cas9 and Cas12 expressing strains, and conducted *in vitro* digestion assays. L.M.L. conducted
191 Type I-C Cas3 experiments. J.B.-D. conceived of the project, conducted Cas3 and Cas9
192 experiments, supervised all experiments, and wrote the manuscript together with S.D.M. E.S.N.
193 conducted microscopy experiments, under the supervision of D.A.A. All authors edited the
194 manuscript.
195

196 **Competing Interests:**

197 None to declare

198

199 **Materials & Correspondence:**

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201

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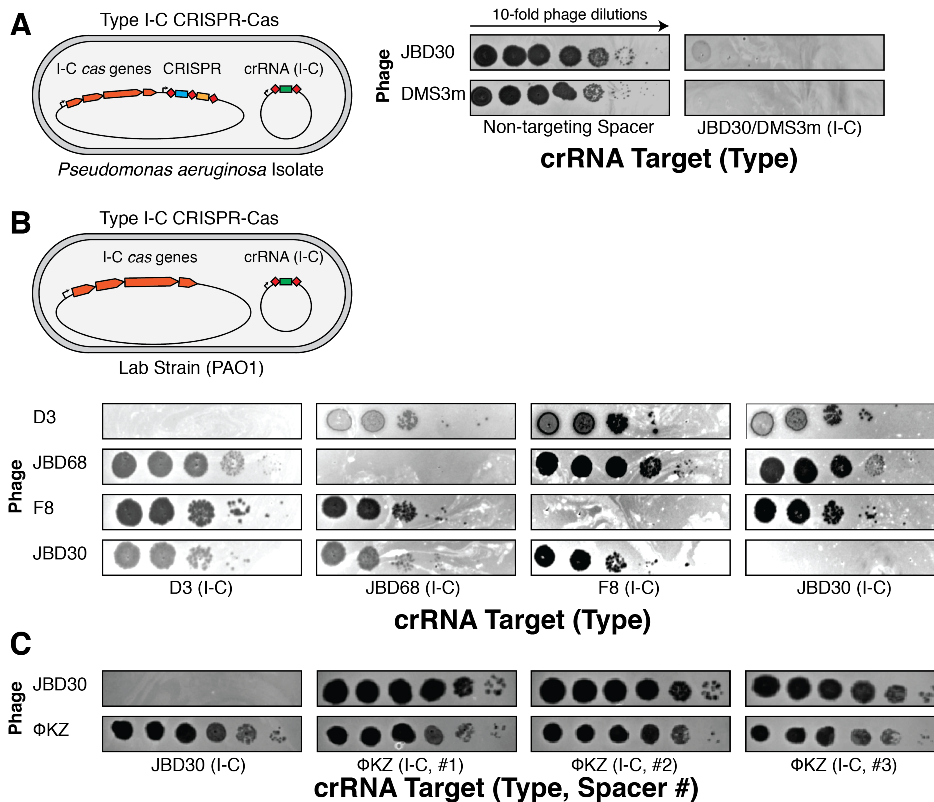
202 **Figures:**



203
204 **Figure 1: Identification of a phage that resists *P. aeruginosa* Type I-C CRISPR-Cas**
205 **immunity. a,** Phages JBD30 and DMS3m were spotted in ten-fold serial dilutions on a lawn of a
206 *P. aeruginosa* isolate naturally expressing the I-C *cas* genes, and an engineered crRNA to
207 target both phages. Dark clearings in the lawn represent phage replication. **b,** Strain PAO1 was
208 engineered to express the I-C *cas* genes and crRNAs targeting the indicated phages, and
209 plaque assays were conducted as in Fig. 1a. **c,** PAO1 strains expressing crRNAs engineered to
210 target phage JBD30 and phage ΦKZ (I-C, #1-#3) were subjected to a plaque assay as in Fig.
211 1a.
212
213

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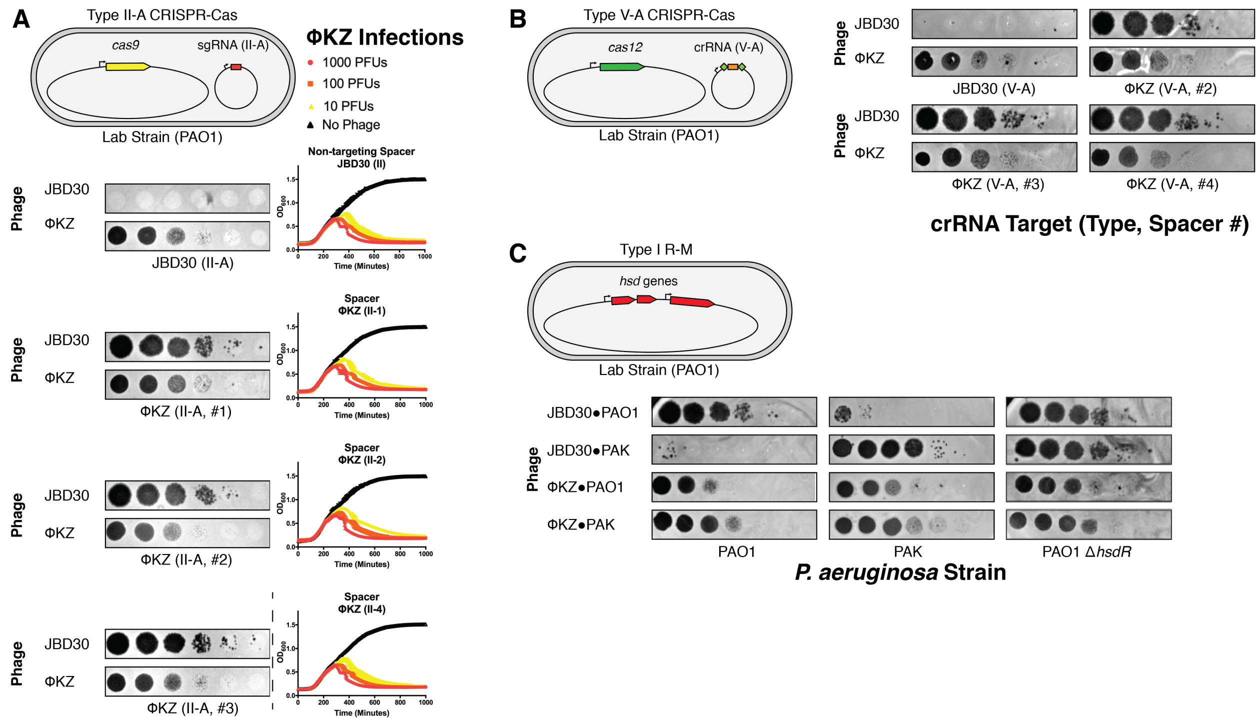

214 **crRNA Target (Type, Spacer #)**

215 **Figure 2: Jumbo phage ΦKZ resists targeting by heterologous Type II-A and V-A**
216 **CRISPR-Cas systems and and endogenous R-M system. a,** Strain PAO1 was engineered to
217 express the Type II-A Cas9 protein and single guide RNAs (sgRNAs) targeting the indicated
218 phage. Plaque assays were conducted as in Figure 1a and growth curves were conducted,
219 monitoring the OD600 of PAO1 cells infected with the indicated number of ΦKZ plaque forming
220 units (pfu). **b,** Strain PAO1 was engineered to express the Type V-A Cas12a protein and
221 crRNAs against the indicated phage. Plaque assays conducted as in Figure 1a. **c,** The
222 endogenous Type I R-M system (*hsdRSM*) in strains PAO1 and PAK was assayed using
223 phages propoagated on PAO1 or PAK as indicated (e.g. JBD30•PAO1 was first propagated on
224 strain PAO1). Together with an isogenic PAO1Δ*hsdR* knockout, all strains were subjected to a
225 plaque assay as in Figure 1a.
226

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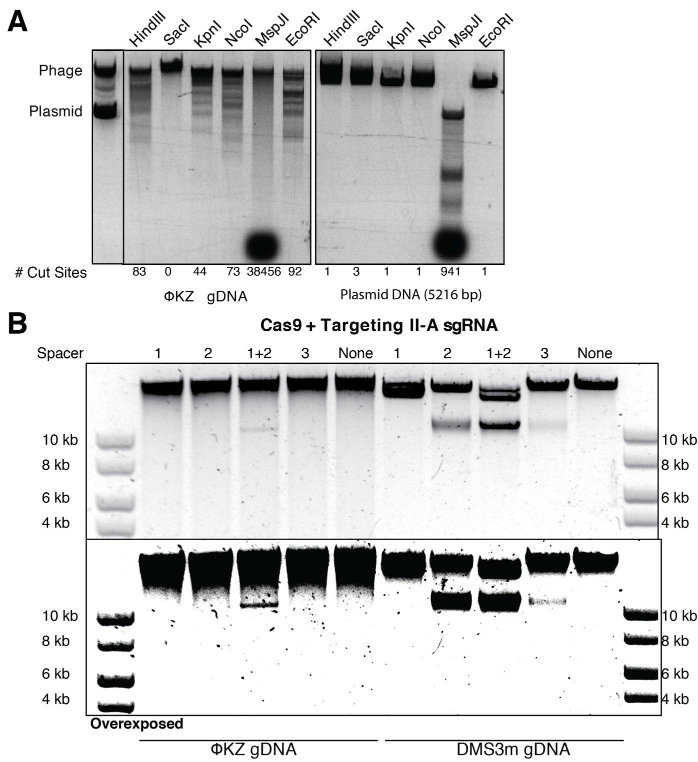

228
229 **Figure 3: ΦKZ genomic DNA is sensitive to restriction enzymes and Cas9 *in vitro*. a,** ΦKZ
230 genomic DNA and plasmid DNA were subjected to digestion with the indicated restriction
231 enzymes *in vitro*. The first lane contains purified phage and plasmid DNA run together. The
232 number of cut sites for each enzyme is shown at the bottom of the gels. **b,** ΦKZ and phage
233 DMS3m genomic DNA were digested *in vitro* using Cas9 loaded with crRNA:tracrRNA targeting
234 the indicated phage. The bottom gel is the same as the top gel, however the image was
235 overexposed to enhance faint bands. Products were visualized on a 0.7% agarose gel,
236 visualized with SYBR Safe nucleic acid stain.
237
238

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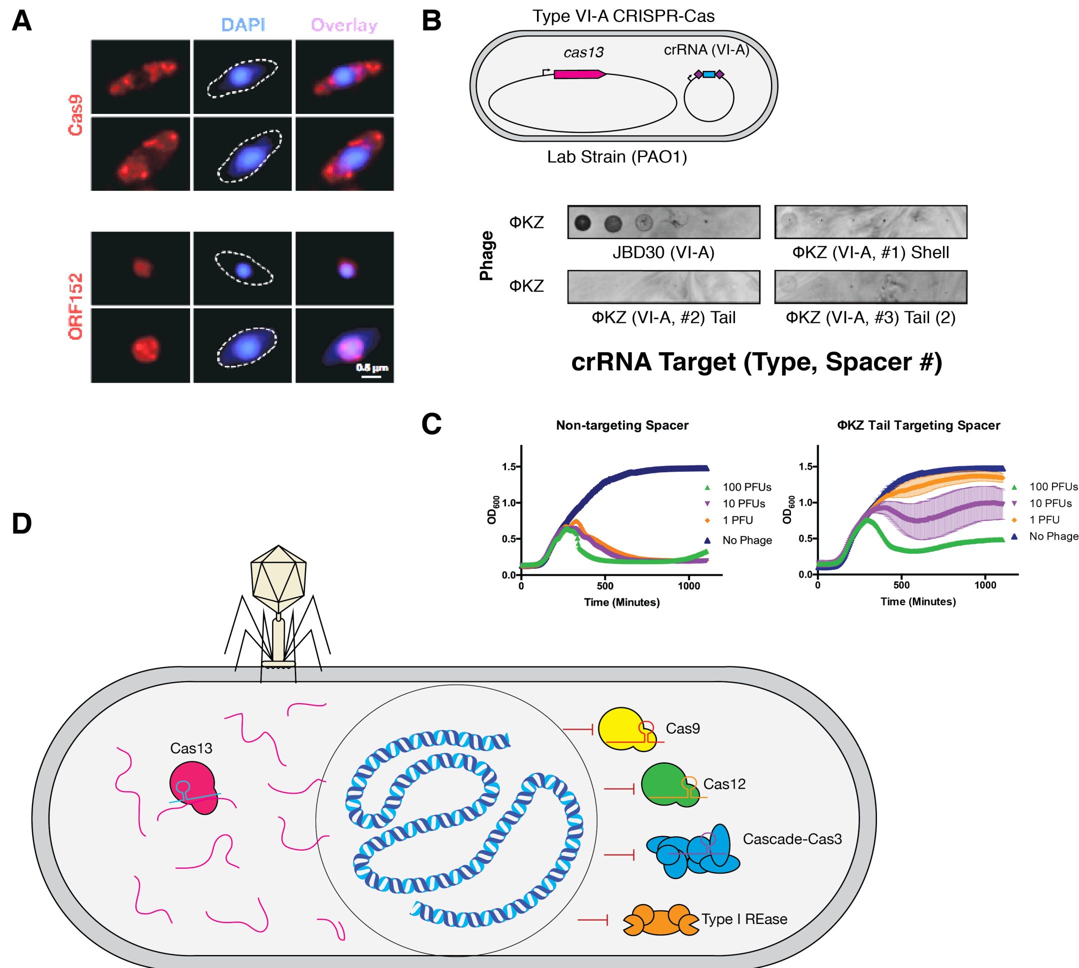

239
240 **Figure 4: Phage ΦKZ DNA is protected from Cas9 and other DNA targeting enzymes, but**
241 **is sensitive to RNA-targeting Cas13. a,** Fluorescence microscopy of *P. aeruginosa*,
242 immunostained for Cas9 (top panels), or Myc-ORF152 (bottom panels). DAPI stain shows the
243 phage DNA within the shell. **b,** Strain PAO1 expressing LseCas13a and a crRNA targeting the
244 indicated phage. Plaque assays conducted as in Figure 1a. **c,** Growth curves measuring the
245 OD600 of PAO1 infected with the indicated number of ΦKZ plaque forming units (PFUs). **d,** A
246 model summarizing the ΦKZ nucleus-like shell excluding Cas9, Cas12, Cascade-Cas3 (Type I-
247 C, and Type I restriction endonucleases (REase), while the mRNA (red) is exported and can be
248 targeted by Cas13.
249

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250 **Tables:**

251 **Table 1: ΦKZ and ΦKZ-like phages have no natural spacers matching their genomes from**
252 **a natural collection of >4000 *P. aeruginosa* spacers.**
253 The total number of Type I CRISPR spacers with a perfect match to the indicated P. aeruginosa
254 phages assayed in this study and previous CRISPR-Cas studies. The experimental sensitivity of
255 each phage to the indicated subtypes are shown. AcrIE3 and AcrIF1 are I-E and I-F anti-
256 CRISPR proteins, respectively. * indicates that all spacers have mismatches (≤4) to the F8
257 genome.

Phage	# spacers	Type I CRISPR sensitivity	Reference
DMS3m	75	I-C: sensitive I-E: resistant (AcrIE3) I-F: sensitive	This study, ref. 13,33
JBD30	51	I-C: sensitive I-E: sensitive I-F: resistant (AcrIF1)	This study, ref. 12,13
JBD18	51	I-F: sensitive	Ref. 33
JBD25	46	I-F: sensitive	Ref. 33
JBD68	28	I-C: sensitive	This study
D3	49	I-C: sensitive	This study
F8	3*	I-C: sensitive	This study
ΦKZ	0	I-C: resistant	This study
phiPA3	0	not assayed	
PaBG	0	not assayed	
KTN4	0	not assayed	
PA7	0	not assayed	

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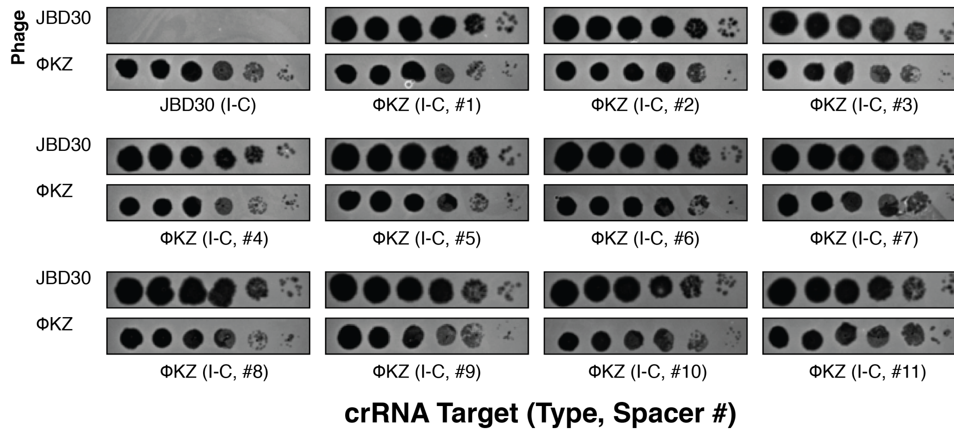
260 **Extended Data Figures**



261 **crRNA Target (Type, Spacer #)**

262 **Extended Data Figure 1: Phage ΦKZ is resistant to Type I-C CRISPR-Cas immunity.**
263 *P. aeruginosa* strain PAO1 expressing the Type I-C *cas* genes and crRNAs programmed to
264 recognize the indicated phage. One crRNA targeting JBD30 is shown, while 11 distinct crRNAs
265 are directed towards ΦKZ. Phages are spotted in ten-fold serial dilutions (left to right) on a lawn
266 of the indicated PAO1 strain.
267

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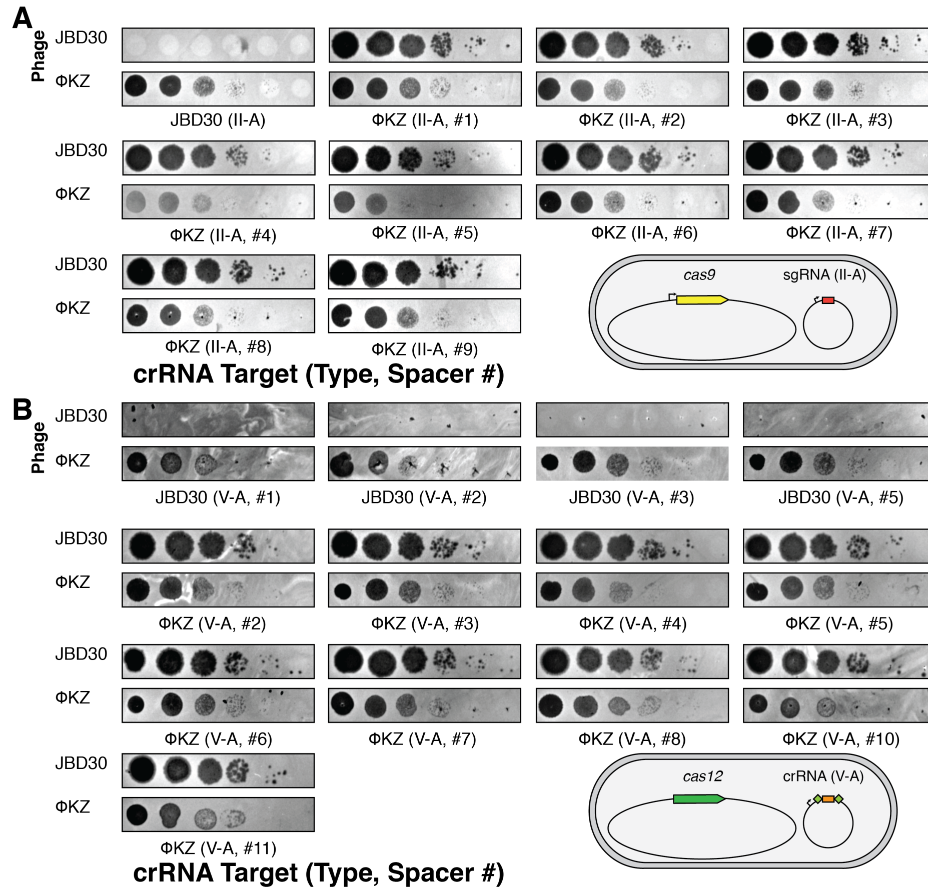

268
269 **Extended Data Figure 2: Phage ΦKZ is resistant to Type II-A and V-A CRISPR-Cas**
270 **immunity.** *P. aeruginosa* strain PAO1 expressing the (**a**) Type II-A Cas9 or (**b**) Type V-A
271 Cas12a effectors and sgRNAs/crRNAs programmed to recognize the indicated phage. Phages
272 are spotted in ten-fold serial dilutions (left to right) on a lawn of the indicated PAO1 strain.

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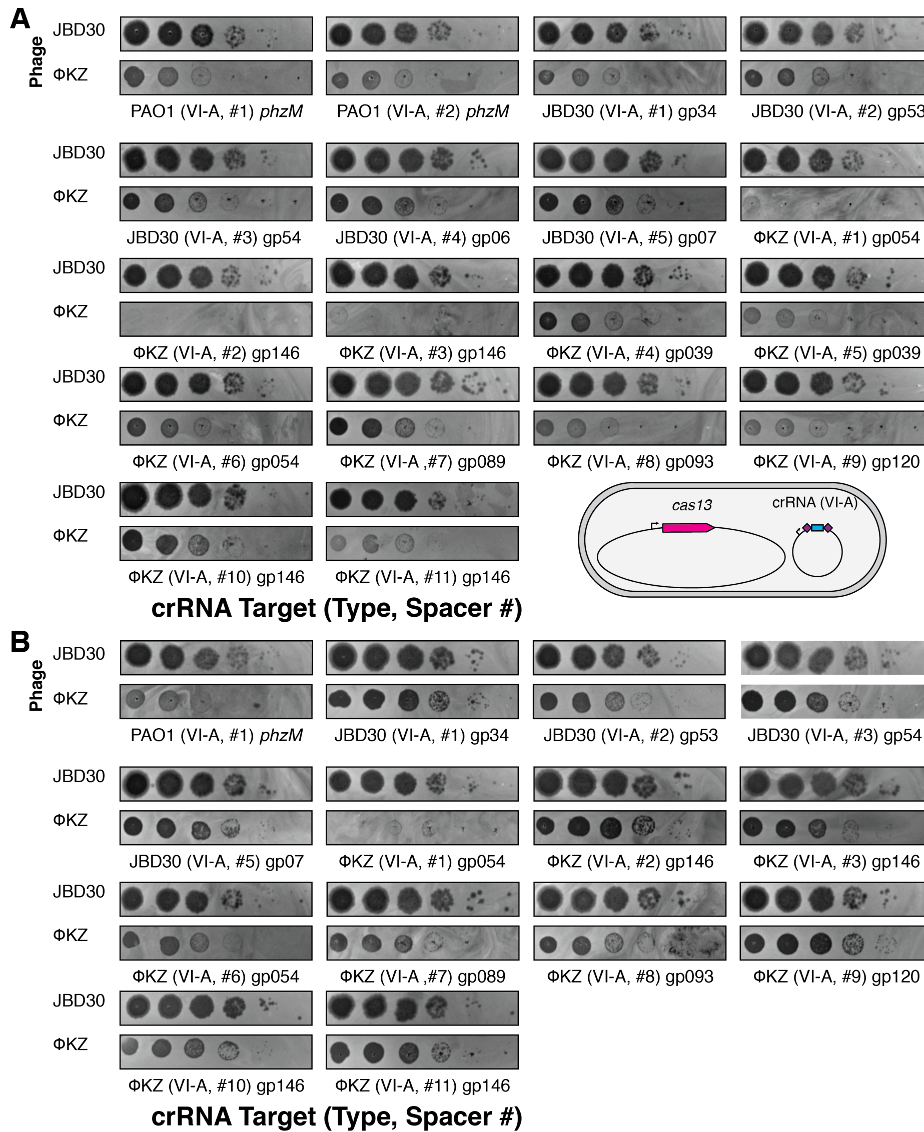

274
275 **Extended Data Figure 3: Phage ΦKZ is sensitive to Type VI-A CRISPR-Cas immunity.**
276 *P. aeruginosa* strain PAO1 expressing the Type VI-A (**a**) LseCas13a or (**b**) LshCas13a effectors
277 and crRNAs programmed to recognize the indicated phage. Phages are spotted in ten-fold
278 serial dilutions (left to right) on a lawn of the indicated PAO1 strain.

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279 **Methods**

280 **Bacterial growth and genetic manipulation**

281 Strains, plasmids, phages, and spacer sequences used in this study are listed in Supplemental
282 Tables 1-4. *Pseudomonas aeruginosa* strain PAO1 was grown in LB at 37 °C with aeration at
283 225 RPM. When necessary plating was performed on LB agar with carbenicillin (200 µg/ml) or
284 gentamicin (50 µg/ml). Gene expression was induced by the addition of L-arabinose (0.1% final)
285 and/or isopropyl β-D-1-thiogalactopyranoside (IPTG, 0.5 mM or 1 mM final). For chromosomal
286 insertions at the attTn7 locus, *P. aeruginosa* cells were electroporated with the integrating
287 vector pUC18T-lac and the transposase expressing helper plasmid pTNS3, and selected on
288 gentamicin. Potential integrants were screened by colony PCR with primers PTn7R and PglmS-
289 down. Electrocompetent cell preparations, transformations, integrations, selections, plasmid
290 curing, and FLP recombinase mediated marker excision with pFLP were performed as
291 described previously[32].

292

293 **Phage growth and DNA extraction**

294 Phage growth was conducted in LB at 37 °C with PAO1 as a host. Growth curves were
295 conducted in a Biotek Synergy plate reader at 37 °C with orbital shaking set to maximum speed.
296 Phage stocks were diluted and stored in SM buffer[33] and used for routine plaquing assays. For
297 high titer lysates to generate phage DNA, plates with a high number of plaques were flooded
298 with SM buffer and collected[33]. The lysates were subsequently DNase treated and filtered
299 through a 0.22 µm filter. Phage DNA was extracted with the Wizard Genomic DNA Purification
300 Kit (Promega). DNA restriction assays were performed according to standard NEB protocols
301 and restriction fragments were assessed by agarose gel electrophoresis.

302

303 **Type I-C CRISPR-Cas system expression in *P. aeruginosa* PAO1**

304 Type I-C CRISPR-Cas function was tested by electroporating a strain containing I-C *cas* genes
305 with pHERD30T plasmids encoding crRNAs that target phages. To express this system
306 heterologously in PAO1, the four effector *cas* genes (*cas3-5-8-7*) were cloned into pUC18T-lac
307 and inserted in the PAO1 chromosome as described above. After removal of the gentamicin
308 marker, this strain was electroporated with the same pHERD30T crRNA-encoding plasmids to
309 confirm function upon IPTG/arabinose induction.

310

311 **crRNA cloning and expression**

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312 All crRNAs used here, were cloned into established entry vectors in the pHERD30T
313 background. After removing a pre-existing BsaI cut site in the vector by mutagenesis, a pseudo-
314 CRISPR array (i.e. repeat-spacer-repeat for Type I, V, VI, or a sgRNA scaffold for Type II) was
315 then cloned into the vector, where the spacing sequence possessed two inverted BsaI digest
316 sites, to facilitate scarless cloning of new spacers. Desired spacer sequences were ordered as
317 two complementary oligonucleotides that generate sticky ends when annealed, to be cloned into
318 the entry vector, which was BsaI digested. Spacer oligonucleotides were PNK treated,
319 annealed, and ligated into the entry vector.
320
321 **Streptococcus pyogenes (Spy) Cas9 and sgRNA expression in *P. aeruginosa***
322 The *S. pyogenes Cas9* gene was cloned into a pUC18T-Ara integration vector and then inserted
323 into the attTn7 locus of PAO1. A single guide RNA scaffold was constructed based on a
324 previous design[34] with internal BsaI cut sites to enable insertion of pre-annealed oligos for
325 scarless sgRNA design. This sgRNA scaffold was amplified with primers p30T-gRNA_BsaI and
326 p30T-gRNA_BsaI_rev. The resulting product was inserted into the pHERD30T vector via
327 Gibson assembly following backbone (pJW1) amplification by inverse PCR with primers
328 gRNA_BsaI-p30T and gRNA_BsaI-p30T-rev. The sgRNA scaffold was positioned into pJW1 so
329 that following BsaI cleavage the spacer insert +1 position would conincide with the pBAD TSS
330 +1 position. The resulting plasmid, pJB1, was BsaI digested (NEB) followed by ligation of
331 indicated pre-annealed oligos. Table 3 contains a complete list of all target sequences. The
332 sequence of the sgRNA construct with BsaI site locations is shown in Supplemental Table 3.
333
334 **Cas9 in vitro cleavage**
335 Cas9-based phage genome cleavage *in vitro* was conducted with purified Cas9 protein (NEB
336 #M0386S), and the Cas9-gRNA-tracrRNA based cleavage reaction was then performed using
337 according to the manufacturer's (NEB) instructions. Cas9 crRNAs (Supplemental Table 3) were
338 ordered as Alt-R CRISPR-Cas9 crRNAs from IDT and utilized without further modification. The
339 tracrRNA was amplified using primers tracrRNA-FOR and tracrRNA-REV from a plasmid
340 (pBR62). The tracrRNA was produced through a T7 RNAP reactions using dsDNA encoding the
341 tracrRNA downstream of a T7 RNAP promoter. Cas9 protein (NEB) was combined with pre-
342 annealed crRNA and tracRNA complex at a 1:1 molar ratio. The reaction was performed at 37
343 °C for 4 hrs with 300 ng of ΦKZ or DMS3 genomic DNA and the products were assessed by
344 agarose gel electrophoresis. Two Cas9 guides were selected that would cleave at pos. 158,649
345 and 168,715 of the ΦKZ genome to liberate an ~10 kb fragment.

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346

347 **Cas12a and crRNA design for PA expression**

348 The humanized allele of the *cpf1* gene of *Moraxella bovoculli* (MBO_03467, KDN25524.1) was
349 sub-cloned from pTE4495 (Addgene) into pUC18T-lac using primers pUC_cpf1_F and
350 pUC_cpf1_R and inserted in the PAO1 chromosome as described above. A Cpf1 repeat-
351 spacer-repeat pseudo-CRISPR array was synthesized as oligonucleotides, annealed, and
352 ligated into a pHERD30T vector, digested with NcoI and HindIII. Spacer sequences were cloned
353 into the resulting vector (pJB2) following BsaI digestion and ligation of pre-annealed spacer
354 oligonucleotide pairs.

355

356 **Cas13a and crRNA design for PA expression**

357 The wild type allele of the *cas13* gene of *Listeria seeligeri* and *Leptotrichia shahii* were sub-
358 cloned from p2CT-His-MBP-Lse_C2c2_WT and p2CT-His-MBP-Lsh_C2c2_WT (Addgene) into
359 pUC18T-lac. LseCas13 and Lsh Cas13 were inserted in the PAO1 chromosome as described
360 above. An Lse and an Lsh Cas13a repeat-spacer-repeat pseudo-CRISPR array were
361 synthesized as oligonucleotides, annealed, and ligated into a pHERD30T vector, digested with
362 NcoI and EcoRI. Spacer sequences were cloned into the resulting vectors (pSDM057 and
363 pSDM070, respectively) following BsaI digestion and ligation of pre-annealed spacer
364 oligonucleotide pairs. crRNA expression vectors were introduced into PAO1 tn7::$cas13^{Lse}$ and
365 PAO1 tn7::$cas13^{Lsh}$. The resulting strains were grown to saturation in LB at 37 °C. 4 mL of 0.7%
366 agar, 10 mM $MgSO_4$, 1 mM IPTG molten top agar were seeded with 100 µL saturated culture
367 and spread on 20 mL 10 mM $MgSO_4$, 50 µg/mL gentamicin, 0.1% (L)-arabinose, 1 mM IPTG LB
368 agar plates. 2.5 µL 10-fold serial dilutions of bacteriophage JBD30 and ΦKZ were spotted on
369 plates. Plates were incubated at 37 °C overnight and were imaged on the following day.

370

371 **Restriction-Modification Assay**

372 The PAO1 *hsdR* gene (PA2732) was knocked out using CRISPR-Cas9 and a targeted sgRNA.
373 PAO1, PAK, and PAO1 Δ*hsdR* were grown to saturation in LB at 37 °C. 4 mL of 0.7% agar, 10
374 mM $MgSO_4$ molten top agar were seeded with 100 µL saturated culture and spread on 20 mL
375 10 mM $MgSO_4$ LB agar plates. 2.5 µL 10-fold serial dilutions of bacteriophage JBD30 and ΦKZ
376 propagated on strain PAO1 and PAK were spotted on plates. Plates were incubated at 37°C
377 overnight and were imaged the following day.

378

379 **Immunofluorescence**

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380 Sample Growth

381 5 mL overnight cultures of a strain expressing Cas9 and an sgRNA targeting ΦKZ (SDM065)
382 and a strain expressing cMyc-ORF152 (bESN27) were grown at 30 °C in LB media with
383 gentamicin. A 1:30 back-dilution of the overnight culture into LB was grown at 30 °C for 1 h.
384 Protein and guide expression was induced with 0.1% arabinose and 0.5 mM IPTG, respectively.
385 After 1 h of expression, an aliquot of uninfected cells was fixed while the remaining cultures
386 were infected with ΦKZ using MOI 1.5. Infected cell aliquots were collected and fixed at 60 mpi.
387

388 Fixation

389 This protocol was adapted from ref. 35. Samples were fixed with 5X Fix Solution (12.5%
390 paraformaldehyde, 150 mM KPO4, pH 7.2) and incubating for 15 minutes at room temperature
391 followed by 20 minutes on ice. Samples were then washed in PBS 3 times and finally
392 resuspended in GTE (50 mM glucose, 10 mM EDTA, pH 8.0, 20 mM Tris-HCl, pH 7.65) with 10
393 ug/mL lysozyme. Resuspended cells were transferred to polylysinated coverslips and dried.
394 Once dry, coverslips were incubated in cold methanol for 5 minutes followed by cold acetone for
395 5 minutes. Cells were rehydrated by a rinse in PBS followed by a 3-minute incubation in PBS +
396 2% BSA blocking solution. Cells were incubated with a 1:50 dilution of primary antibody (Cas9
397 (7A9-3A3): sc-517386 or cMyc (9E10): sc-40) in PBS + 2% BSA for 1 hr followed by 3, 7 minute
398 washes in fresh PBS + 2% BSA. Coverslips were then incubated in the dark for 1 hr with
399 secondary antibody (goat anti-mouse Alexa Fluor 555, Life Technologies A-21424) diluted 1:500
400 in PBS + 2% BSA. DAPI was added for the final 10 minutes of the incubation. Cells were
401 washed in PBS 3 times for 7 minutes. Coverslips were then placed on slides using mounting
402 media (v/v 90% glycerol, v/v 10%Tris pH 8.0 and w/v 0.5% propyl-gallate) and sealed with clear
403 nail polish.
404

405 Microscopy and Analysis

406 Images were collected using a Zeiss Axiovert 200M microscope.

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501 **Supplementary Information**

502 **Strains, Plasmids, and Oligonucleotides**

503 **Supplemental Table 1. Phage and Strains**

Name	**Source**	**Reference**
ΦKZ	Davidson Lab	31
DMS3m	O'Toole Lab	33
JBD30	Davidson Lab	36
F8	Davidson Lab	Unpublished Accession: DQ163917
D3	Davidson Lab	37
JBD68	Davidson Lab	36
	Genotype	**Reference**
JW31	PAO1 tn7::I-C/Cas3^{PA}	This study
JB10	PAO1 tn7::cas9^{Spy}	This study
JB90	PAO1 tn7::cas12a^{Mb}	This study
SDM084	PAO1 tn7::cas13^{Lse}	This study
SDM020	PAO1 Δ*hsdR*	This study

504

505

506 **Supplemental Table 2. Plasmids**

Name	Information	Ref.
pHERD30T	Arabinose inducible, gentamicin resistant shuttle vector	38
pUC18T-Lac	IPTG inducible, Amp^{R}/Gent^{R}, Tn7 integrative plasmid with FRT sites flanking gentamicin cassette	39
pTNS3	Expresses tnsABCD for flipping out cassette flanked by FRT sites	40
pBR62	Contains Spy Cas9 tracrRNA sequence	This study
pJW1	pHERD30T with BsaI site at pos. 235 GAGACC mutated to GTGACC	This study
pJW13	pJW1 with Type I-C pseudo-CRISPR array for spacer cloning	This study
pJB1	pJW1 with Type II-A sgRNA backbone at the +1 TSS of pBAD	This study
pJB2	pJW1 with Type V-A pseudo-CRISPR array for spacer cloning	This study
pSDM057	pJW1 with Type VI-A (LseCas13a) pseudo-CRISPR array for spacer cloning	This study
pSDM070	pJW1 with Type VI-A (LshCas13a) pseudo-CRISPR array for spacer cloning	This study

507

508 **Supplemental Table 3. Oligonucleotides, g-blocks, crRNAs**

Name	Sequence	Notes

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I-C_BsaI_for	gatccGTCGCGCCCCGCACGGGCGCGTGGATTGAAACgagacc*TCTCTGGACAAA*ggtctcGTCGCGCCCCGCACGGGCGCGTGGATTGAAACa	Underlined regions indicate location of BsaI sites
I-C_BsaI_rev	agcttGTTTCAATCCACGCGCCCGTGCGGGGCGCGACgagacc*TTTGTCCAGAGA*ggtctcGTTTCAATCCACGCGCCCGTGCGGGGCGCGACg	Underlined regions indicate location of BsaI sites
sgRNA scaffold sequence	ccatagagaccACGTACGTACggtctcAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTT	Underlined regions indicate location of BsaI sites
p30T-gRNA_BsaI	ctctactgtttctccatccatagagaccacgtacgtacg	
p30T-gRNA_BsaI_rev	gcccaaaaaaacgggtccgggcaggataggtgaag	
gRNA_BsaI-p30T	atggagaaacagtagagagttgc	
gRNA_BsaI-p30T-rev	acccgttttttgggctag	
Cpf1_crRNA_top	catgaaatttctactgtttgtagatGgagaccTCTCTGGACAAAggtctcGaaatttctactgtttgtagat	Underlined regions indicate location of BsaI sites
Cpf1_crRNA_bottom	agctatctacaaacagtagaaatttCgagaccTTTGTCCAGAGAggtctcCatctacaaacagtagaaatt	Underlined regions indicate location of BsaI sites
tracrRNA-FOR	gaaattaatacgactcaactatagaaaacagcatagcaagttaaata	For T7 RNAP in vitro rxn
tracrRNA-REV	aaaaaaagcacccgactcggtgccac	For T7 RNAP in vitro rxn
PTn7R	cacagcataactggactgatttc	Ref. 39
PglmS-down	gcacatcgggcgacgtgctctc	Ref. 39
LseCas13a crRNA Top	catggggtaagagactacctctatatgaaagaggactaaaaccgagaccacgtacgtacggtctccggtaagagactacctctatatgaaagaggactaaaacg	Underlined regions indicate location of BsaI sites
LseCas13a crRNA Bottom	aattcgttttagtcctctttcatatagaggtagtctcttaccggagaccgtacgtacgtggtctcggttttagtcctctttcatatagaggtagtctcttaccc	Underlined regions indicate location of BsaI sites
LshCas13a crRNA Top	catggggatttagaccaccccaatatcgaaggggactaaaaccgagaccacgtacgtacggtctccggatttagaccaccccaatatcgaagggggactaaaacg	Underlined regions indicate location of BsaI sites
LshCas13a crRNA Bottom	aattcgttttagtccccttcgatattggggtggtctaaatccggagaccgtacgtacgtggtctcggttttagtccccttcgatattggggtggtctaaatccc	Underlined regions indicate location of BsaI sites
Alt-R CRISPR-Cas9 crRNA		
KZ (IV) – 1	rGrArArUrCrUrGrCrUrArArUrArArGrGrU rUrCrArGrUrUrUrUrArGrArGrCrUrArUrGrC rU	Pos. 158,649

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KZ (IV) – 2	rUrCrArCrCrArCrGrCrArUrUrArCrArUr rCrArGrGrUrUrUrArGrArGrCrUrArUrGrC rU	Pos. 168,715
DMS3m (IV) - 1	rGrCrC rGrArC rArUrU rUrUrC rCrArG rUrUrG rGrCrG rUrUrU rUrArG rArGrC rUrArU rGrCrU	Pos. 17,751
DMS3m (IV) - 2	rUrCrA rCrGrA rCrGrA rCrCrC rArGrA rArGrC rGrUrG rUrUrU rUrArG rArGrC rUrArU rGrCrU	Pos. 28,033

Supplemental Table 4. Spacer sequences

Phage (Type, #)	**Sequence**
JBD30 (I-C)	AACCTCGCGGCATCCGCAACAACAACCCCGGCAA
D3 (I-C)	ACGATTGCGGACATGGCAGGCTGCCGCTGCTGGA
F8 (I-C)	GCCAATCGGCCGATAGATGAAGCTGTGGAGGGTC
JBD68 (I-C)	AGCGGCGTGAGGTTGGACCTTGCTGCCGACCATT
ΦKZ (I-C, #1)	TGGACTAACAAATACGCCTATATATTCCGATCCT
ΦKZ (I-C, #2)	GAACTTGTATTTAACCCAAAGGTTTTTAAATGGT
ΦKZ (I-C, #3)	CCCATTTATTATTTTCTTTATTTATCCAACCGTA
ΦKZ (I-C, #4)	TAAAAAGAAAATTATATAAATAGTATATTATGTG
ΦKZ (I-C, #5)	TTTACATTCTTCTAAACTAATATTTAATTCATCT
ΦKZ (I-C, #6)	CATTATCATCTACCTCTTTTAATTTATCTTTAAT
ΦKZ (I-C, #7)	CAAAAGGATTATTTGATGTTGTGGTGAAAGAAAA
ΦKZ (I-C, #8)	GCCATTTTCTTTCACCACAACATCAAATAATCCT
ΦKZ (I-C, #9)	AGATAATGGGGATATTTTGTATTTTGATAACAAG
ΦKZ (I-C, #10)	TACTGGTACCCATAGAAGTTATATTTTACCAGCT
ΦKZ (I-C, #11)	ATTGAAATCAAGTAAAATCTCAAATGGAATCTGT
JBD30 (II-A)	GGCATCCGCAACAACAACCC
ΦKZ (II-A, #1)	GAATCTGCTAATAAGGTTCA
ΦKZ (II-A, #2)	TCACCACGCATTACATCAGG
ΦKZ (II-A, #3)	AAATTATATTAATCACAATG
ΦKZ (II-A, #4)	ATATATTCCGATCCTTATCC
ΦKZ (II-A, #5)	AAACATCCTCATGATAACCA
ΦKZ (II-A, #6)	AGCAGTAGCTTGAGTTTGAA
ΦKZ (II-A, #7)	TTTTAGATGAAGTAAAAAAG
ΦKZ (II-A, #8)	CTATTACCATTTTCGTCAAT
ΦKZ (II-A, #9)	CTCATTTTTTATTCCTACGT
ΦKZ (II-A, #10)	AGAGAACTGTTTAAACACAA
JBD30 (V-A, #1)	CGTTTTACCCACTGAGCGAACGC
JBD30 (V-A, #2)	GCCCGTTTCGATACCGCACATA
JBD30 (V-A, #3)	TACCGCGCCGCCCTTCTGGAGGA
JBD30 (V-A, #4)	AAGGTGCCGCACGGTGTCCCAGT
JBD30 (V-A, #5)	CGCATGACTCTCTATATGGGGCC
ΦKZ (V-A, #1)	TCACCACGCATTACATCAGGTGG
ΦKZ (V-A, #2)	GAATCTGCTAATAAGGTTCATGG
ΦKZ (V-A, #3)	AATAGGAATATAGCTATGCTAAT
ΦKZ (V-A, #4)	TGTGTCTCTTTTCCAAATGCTTT
ΦKZ (V-A, #5)	GGGATTCAACTATTGGAAAAGCA
ΦKZ (V-A, #6)	GCTAGTTGTTCATCAAATGATGA
ΦKZ (V-A, #7)	CTCATTAATAACAGATACTTTGT
ΦKZ (V-A, #8)	CTGGGCATTAATGACGATATATC
ΦKZ (V-A, #9)	TGCTCTAGTCCATACAGAAT

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ΦKZ (V-A, #10)	TTACAGCCTCGTCAGACAGGTAA
ΦKZ (V-A, #11)	CCTAATGCATTCCATTTAAATAC
PAO1 (VI-A, #1)	TCAAATTACGCGCAGCAGCAAGAT
PAO1 (VI-A, #2)	CGGCCTGCAGGATGGCCTTGGTCA
JBD30 (VI-A, #1)	GCGGCCAGCCCGGCCCCTCGTCCA
JBD30 (VI-A, #2)	GGCGCGTTGATGCGGACCTGGCCA
JBD30 (VI-A, #3)	AATGCCCTTCTCCCGGCAAACCGT
JBD30 (VI-A, #4)	TACCCGCAAGTTGTTGAGGGCTGA
JBD30 (VI-A, #5)	GGTGCCGGCCGGCTTGATGCCCAT
ΦKZ (VI-A, #1)	GCAGGAGCAGTAGCTTGAGTTTGA
ΦKZ (VI-A, #2)	TCATTAGTTTCAACCCAGTATGAA
ΦKZ (VI-A, #3)	ACATAATCTTCAAATGCAGAAGCC
ΦKZ (VI-A, #4)	AACCAGCACCACCACAAAAGTAAA
ΦKZ (VI-A, #5)	AGAATTACTAGTGCATTTAGTACT
ΦKZ (VI-A, #6)	AACGATGTAAGGAGTGAACTGCGG
ΦKZ (VI-A, #7)	TAACAGCTTGTAGATAATAACCAG
ΦKZ (VI-A, #8)	GTCTGCAATAGTCTTCGGATTATT
ΦKZ (VI-A, #9)	AGGTTAGCATTGGAGTTACCCATT
ΦKZ (VI-A, #10)	CAGCACCTTTGGAAACTACCCAAT
ΦKZ (VI-A, #11)	GCTTCCATAGTAACTAAGTATGCT

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