

1 **A minimal CRISPR-Cas3 system for genome engineering**

2 Bálint Csörgő<sup>1,2\*</sup>, Lina M. León<sup>1\*</sup>, Ilea J. Chau-Ly<sup>3</sup>, Alejandro Vasquez-Rifo<sup>4</sup>, Joel D. Berry<sup>1</sup>,  
3 Caroline Mahendra<sup>1</sup>, Emily D. Crawford<sup>1,5</sup>, Jennifer D. Lewis<sup>3,6</sup>, Joseph Bondy-Denomy<sup>1,7,8</sup>

4 <sup>1</sup>Department of Microbiology and Immunology, University of California, San Francisco, 94143, San Francisco, CA, USA

5 <sup>2</sup>Genome Biology Unit, European Molecular Biology Laboratory, 69117 Heidelberg, Germany

6 <sup>3</sup>Department of Plant and Microbial Biology, University of California, Berkeley, 94720, Berkeley, CA, USA

7 <sup>4</sup>Program in Molecular Medicine, University of Massachusetts Medical School, 01605, Worcester, MA, USA

8 <sup>5</sup>Chan-Zuckerberg Biohub, 94158, San Francisco, CA, USA

9 <sup>6</sup>Plant Gene Expression Center, United States Department of Agriculture, Albany, CA 94710, USA

10 <sup>7</sup>Quantitative Biosciences Institute, University of California, San Francisco, 94143, San Francisco, CA, USA

11 <sup>8</sup>Innovative Genomics Institute

12

13 \* Equal contribution

14

15 **Abstract**

16 CRISPR-Cas technologies have provided programmable gene editing tools that have  
17 revolutionized research. The leading CRISPR-Cas9 and Cas12a enzymes are ideal for  
18 programmed genetic manipulation, however, they are limited for genome-scale interventions.  
19 Here, we utilized a Cas3-based system featuring a processive nuclease, expressed  
20 endogenously or heterologously, for genome engineering purposes. Using an optimized and  
21 minimal CRISPR-Cas3 system (Type I-C) programmed with a single crRNA, large deletions  
22 ranging from 7 - 424 kb were generated in *Pseudomonas aeruginosa* with high efficiency and  
23 speed. By comparison, Cas9 yielded small deletions and point mutations. Cas3-generated  
24 deletion boundaries were variable in the absence of a homology-directed repair (HDR) template,  
25 and successfully and efficiently specified when present. The minimal Cas3 system is also  
26 portable; large deletions were induced with high efficiency in *Pseudomonas syringae* and  
27 *Escherichia coli* using an “all-in-one” vector. Notably, Cas3 generated bi-directional deletions  
28 originating from the programmed cut site, which was exploited to iteratively reduce a *P.*  
29 *aeruginosa* genome by 837 kb (13.5%) using 10 distinct crRNAs. We also demonstrate the utility  
30 of endogenous Cas3 systems (Type I-C and I-F) and develop an “anti-anti-CRISPR” strategy to  
31 circumvent endogenous CRISPR-Cas inhibitor proteins. CRISPR-Cas3 could facilitate rapid  
32 strain manipulation for synthetic biological and metabolic engineering purposes, genome  
33 minimization, and the analysis of large regions of unknown function.

34

## 35 Introduction

36 CRISPR-Cas systems are a diverse group of RNA-guided nucleases<sup>1</sup> that defend prokaryotes  
37 against viral invaders<sup>2,3</sup>. Due to their relatively simple architecture, gene-editing applications have  
38 focused on Class 2 CRISPR systems<sup>4</sup> (i.e. Cas9 and Cas12a), but Class 1 systems hold great  
39 potential for gene editing technologies, despite being more complex<sup>5-7</sup>. The signature gene in  
40 Class 1 Type I systems is Cas3, a 3'-5' ssDNA helicase-nuclease enzyme that, unlike Cas9 or  
41 Cas12a, degrades target DNA processively<sup>5,6,8-13</sup>. This property of CRISPR-Cas3 systems raises  
42 the possibility of its development as a tool for large genomic deletions.

43  
44 Organisms from all domains of life contain vast segments of DNA that are poorly characterized,  
45 of unknown function, or may be detrimental for fitness. In prokaryotes, these include prophages,  
46 plasmids, and mobile islands, while in eukaryotes, large regions of repetitive sequences and non-  
47 coding DNA are poorly characterized. Methods for generating programmable and rapid large  
48 genomic deletions are needed for the study and engineering of these regions, however these are  
49 currently inefficient<sup>14</sup>. A methodology that allows for targeted large genomic deletions in any host,  
50 either with precisely programmed or random boundaries, would be broadly useful<sup>15</sup>.

51  
52 Type I systems are the most prevalent CRISPR-Cas systems in nature<sup>1</sup>, which has enabled the  
53 use of endogenous CRISPR-Cas3 systems for genetic manipulation via self-targeting. This has  
54 been accomplished in *Pectobacterium atrosepticum* (Type I-F)<sup>16</sup>, *Sulfolobus islandicus* (Type I-  
55 A)<sup>17</sup>, in various *Clostridium* species (Type I-B)<sup>18-20</sup>, and in *Lactobacillus crispatus* (Type I-E)<sup>21</sup>,  
56 leading to deletions as large as 97 kb amongst the self-targeted survivor cells<sup>16</sup>. Additionally, two  
57 recent studies have repurposed Type I systems for use in human cells, including the  
58 ribonucleoprotein (RNP) based delivery of a Type I-E system<sup>22</sup>, and the use of I-E and I-B systems  
59 for transcriptional modulation<sup>23</sup>. Here, we repurposed a Type I-C CRISPR system from  
60 *Pseudomonas aeruginosa* for genome engineering in microbes. Importantly, by targeting the  
61 genome with a single crRNA and selecting only for survival after editing, this tool is a counter-  
62 selection-free approach to programmable genome editing. CRISPR-Cas3 is capable of efficient  
63 genome-scale modifications currently not achievable using other methodologies. It has the  
64 potential to serve as a powerful tool for basic research, discovery, and strain optimization.

## 65 66 Results

### 67 ***Implementation and optimization of genome editing with CRISPR-Cas3***

68 Type I-C CRISPR-Cas systems utilize just three *cas* genes to produce the crRNA-guided  
69 Cascade surveillance complex that can recruit Cas3: *cas5*, *cas8*, and *cas7* (Figure 1A), making it  
70 a minimal system<sup>24,25</sup>. A previously constructed *Pseudomonas aeruginosa* PAO1 strain (PAO1<sup>IC</sup>)  
71 with inducible *cas* genes and crRNAs<sup>26</sup> was used here to conduct targeted genome manipulation.  
72 The expression of a crRNA targeting the genome caused a transient growth delay (Figure 1B),  
73 but survivors were isolated after extended growth. By targeting *phzM*, a gene required for  
74 production of a blue-green pigment (pyocyanin), we observed yellow cultures (Figure 1C) for 16  
75 out of 36 (44%) biological replicates (18 recovered isolates from two independent *phzM* targeting

76 crRNAs). PCR of genomic DNA confirmed that the yellow cultures had lost this region, while blue-  
77 green survivors maintained it (Supplementary Figure 1). Three of these PAO1<sup>IC</sup> deletion strains  
78 were sequenced, revealing deletions of 23.5 kb, 52.8 kb, and 60.1 kb, and each one was bi-  
79 directional relative to the crRNA target site (Figure 1D). This demonstrated the potential for Type  
80 I-C Cas3 systems to be used to induce large genomic deletions with random boundaries  
81 surrounding a programmed target site.

82 To determine the *in vivo* processivity of the Cas3 enzyme, we targeted 2 of the 16 extended non-  
83 essential (XNES) regions >100 kb in length (Supplementary Table 1) identified from a transposon  
84 sequencing (TnSeq) data set<sup>27</sup>. The frequency of deletions generated by crRNAs targeting XNES  
85 1 and XNES 2 (along with additional targeting of *phzM*, which is found in XNES 15) was quantified  
86 revealing that 20-40 % of the surviving colonies had deletions (Figure 2A). To understand how  
87 cells lacking large deletions had survived self-targeting, three possibilities were considered: i) a  
88 *cas* gene mutation, ii) a PAM or protospacer mutation, or iii) a mutation to the plasmid expressing  
89 the crRNA. Three non-deletion survivors from each of the six self-targeting crRNAs had functional  
90 *cas* genes when the self-targeting crRNA was replaced with a phage targeting crRNA  
91 (Supplementary Figure 2A), and target sequencing revealed no mutations. PCR-amplification and  
92 sequencing of the crRNA-expressing plasmids isolated from the survivors revealed the primary  
93 escape mechanism: recombination between the direct repeats, leading to the loss of the spacer  
94 (Supplementary Figure 2B). The resulting bands from an additional 17 survivors that lacked  
95 deletions were also ~60 bp shorter (Supplementary Figure 2C), consistent with the loss of one  
96 repeat and spacer.

97 Spacer excision was successfully prevented by engineering a modified repeat (MR), with six  
98 mutated nucleotides in the stem and three in the loop of the second repeat (Figure 2B), disrupting  
99 homology between the two direct repeats. A phage-targeting crRNA with this new design targeted  
100 phage as well or better than the same crRNA with unmodified repeats (Supplementary Figure  
101 3A). Using the same self-targeting spacers designed against *phzM*, XNES 1, and XNES 2 with  
102 the MR resulted in a robust increase in editing efficiencies to 94-100% for the six tested crRNAs  
103 (Figure 2A) and spacer excision was no longer detected. 211 of 216 (98 %) total survivor cells  
104 had large deletions based on PCR screening (i.e. > 1 kb), while the remaining 5 had inactive  
105 CRISPR-Cas systems when tested with the phage-targeting crRNA (Supplementary Figure 3B).

106 The processivity of Cas3 could likely lead to unintended deletions of neighboring essential genes,  
107 if targeting is initiated nearby. To assess the phenotype of such an event, we intentionally targeted  
108 an essential gene, *rpIQ* (a 50S ribosomal subunit protein<sup>28</sup>). Two different MR crRNAs targeting  
109 *rpIQ* led to a severely extended lag time compared to non-essential gene targeting. Only 8 out of  
110 36 *rpIQ*-targeting biological replicates grew after 24 hours, compared to the transient growth delay  
111 of ~12 hours when targeting non-essential genes (Supplementary Figure 4A). Subsequent  
112 analysis of these 8 survivor cultures with phage targeting assays revealed non-functional *cas*  
113 genes (Supplementary Figure 4B). Importantly, no spacer excision events were detected in this  
114 experiment or among the 216 replicates screened above. This experiment highlights the  
115 robustness of the deletion method, as the outcome of essential gene versus non-essential gene  
116 targeting is noticeably distinct.

## 117 **Comparison of Cas3 and Cas9 based editing**

118 To determine whether large deletions are a direct consequence of the Cas3 enzyme, we  
119 compared self-targeting outcomes to an isogenic strain expressing the non-processive  
120 *Streptococcus pyogenes* Cas9, PAO1<sup>IIA</sup>. Two crRNAs (as sgRNAs) that partially overlapped with  
121 the crRNAs used for PAO1<sup>IC</sup> were targeted to *phzM* (Figure 2E, Supplementary Figure 5). PCR  
122 and sequencing analysis of these surviving cells revealed that deletions larger than 1 kb are a  
123 rare occurrence (5.6 % assayed survivor cells, n = 72) compared to 98.6 % with PAO1<sup>IC</sup> (Figure  
124 2E). The more common modes of survival were small deletions between 0.2 – 0.5 kb in length  
125 (19.4 % of all survivors), or 1-3 bp protospacer/PAM deletions/mutations (25 %). Whole-genome  
126 sequencing (WGS) of two large deletion survivors selected for by Cas9 showed lesions of 5 kb  
127 and 23 kb around the target site, respectively. The frequent isolation of small deletions from  
128 targeting with the non-processive SpyCas9 directly implicates Cas3's enzymatic activity as the  
129 cause of large deletions.

130 The direct relationship between Cas3 nuclease-helicase activity and survival via large deletions  
131 led us to hypothesize that its processive ssDNA nuclease activity may promote recombination.  
132 To test this, we provided a repair template with ~500 bp of the upstream and downstream regions  
133 flanking the desired deletion to enable homology directed repair (HDR). We chose 0.17 kb and  
134 56.5 kb deletions around *phzM* and a 249 kb deletion within XNES8 for the programmed deletions  
135 (Supplementary Figure 6). The recombination efficiencies were significantly higher with Cas3 than  
136 with Cas9 (Figure 2F). The 249 kb deletion was incorporated in 22 % of the Cas3-generated  
137 survivors, compared to 0% using Cas9 ( $\chi^2$  (1, N = 72) = 9,  $p = 2.7E-03$ ). The 56.5 kb deletion had  
138 an efficiency of 61 % vs. 5.5 % ( $\chi^2$  (1, N = 72) = 25,  $p = 5.73E-07$ ), and the 0.17 kb deletion had  
139 an efficiency of 100 % vs. 39 % when targeting with Cas3 or Cas9, respectively ( $\chi^2$  (1, N = 72) =  
140 31.68,  $p = 1.82E-08$ ). These data support the hypothesis that Cas3 enhances recombination at  
141 cleavage sites.

### 142 **Rapid genome minimization of *P. aeruginosa* using CRISPR-Cas3 editing**

143 Large deletions with undefined boundaries provide an unbiased mechanism for genome  
144 streamlining, screening, and functional genomics. To demonstrate the potential for Cas3, we  
145 aimed to minimize the genome of *P. aeruginosa* through a series of iterative deletions of the XNES  
146 regions (Figure 3A). Six XNES regions (including XNES 15, carrying *phzM*) were iteratively  
147 targeted in six parallel lineages (Figure 3B), resulting in 35 independent deletions (WGS revealed  
148 no deletion at XNES 2 in one of the strains). Deletion efficiency remained high (> 80 %) throughout  
149 each round of self-targeting (Supplementary Figure 7). WGS of these 6 multiple deletion strains  
150 ( $\Delta 6_1$  -  $\Delta 6_6$ ) revealed that no two deletions had the exact same coordinates, highlighting the  
151 stochastic nature of the process. The smallest isolated deletion was 7 kb and the largest 424 kb  
152 (mean: 92.9 kb, median: 58.2 kb). Of note, 4 genes (PA0123, PA1969, PA2024, and PA2156)  
153 previously identified as essential<sup>27</sup> were deleted in at least one of the lineages. Most deletions  
154 appeared to be resolved by flanking microhomology regions (Supplementary Table 2), implicating  
155 alternative-end joining<sup>29</sup> as the dominant repair process.

156 To minimize the genome further, one of the already reduced strains was subjected to 4 additional  
157 rounds of deletions at XNES regions for a total of 10 genomic deletions ( $\Delta 10$ , Figure 3B). Whole-  
158 genome sequencing of the  $\Delta 10$  strain showed a genome reduction of 849 kb (13.55 % of the  
159 genome). Generation of large deletions resulted in a growth defect in some cases, with

160 significantly slower growth in 3 of the 6 deletions strains ( $\Delta 6_1$ ,  $\Delta 6_3$ , and  $\Delta 6_4$ ), with the other 3  
161 growing normally (Figure 3C).  $\Delta 10$  also displayed a decrease in fitness, showing a ~15 % increase  
162 in doubling time compared to the parent strain. The general subtlety of the growth defects was  
163 likely bolstered by the selection of fast-growing colonies at each round of deletion.

#### 164 **CRISPR-Cas3 editing in distinct bacteria**

165 To enable expression of this system in other hosts, we constructed an all-in-one vector  
166 (pCas3cRh) carrying the I-C specific crRNA with a modified repeat sequence and *cas3*, *cas5*,  
167 *cas8*, and *cas7* (Supplementary Figure 8A). As a pilot experiment, we transformed wild-type  
168 PAO1 with a non-targeting crRNA and crRNAs targeting *phzM* and *XNES2*. Induction of the  
169 targeting crRNAs induced editing efficiencies between 95-100 % (Supplementary Figure 8B-D).

170 Having verified that pCas3cRh was functional, we tested this system in the model organism  
171 *Escherichia coli* K-12 MG1655. crRNAs were designed to target *lacZ* or its vicinity (Figure 4A),  
172 where it is flanked by non-essential DNA (124.5 kb upstream, 22.4 kb downstream).  
173 Transformations were plated directly on inducing media containing X-gal and scored using  
174 blue/white screening. Depending on the crRNA used, directly targeting *lacZ* or 30 kb upstream  
175 yielded 51-90% or 82-85% editing efficiencies, respectively (Figure 4B). 95 of the 96 LacZ (-)  
176 survivors assayed by PCR showed an absence of the *lacZ* region. crRNAs downstream of *lacZ*,  
177 however, had reduced efficiency as they approached the essential gene, *hemB*. *frmA* targeting  
178 (13 kb downstream of *lacZ*) had lower editing efficiencies (21-25 %) and *yaiS* (18 kb downstream  
179 of *lacZ*) even lower (2%). This drop in efficiency was independent of the strand being targeted  
180 (and therefore the predicted strand for Cas3 loading and 3'-5' translocation), confirming the  
181 importance of Cas3 bi-directional deletions. Indeed, WGS of selected  $\Delta lacZ$  cells revealed bi-  
182 directional deletions ranging from 17.5-106 kb encompassing the targeted region (Figure 4C).

183 Next, we tested Cas3-mediated editing in the plant pathogen *Pseudomonas syringae* pv. *tomato*  
184 DC3000, which does not naturally encode a CRISPR-Cas system<sup>30</sup>. *P. syringae* encodes many  
185 non-essential virulence effector genes whose activities are difficult to disentangle due to their  
186 redundancy<sup>31</sup>. We designed crRNAs targeting four chromosomal virulence effector clusters (IV,  
187 VI, VIII, and IX), or one plasmid cluster (pDC3000<sup>32</sup>, cluster X) in *P. syringae* strain DC3000. Two  
188 clusters (IV and IX) shared identical sequences that could be targeted simultaneously using a  
189 single crRNA. Expression of targeting crRNAs led to a noticeable growth delay compared to non-  
190 targeted controls (Figure 4D). PCR analysis of surviving cells showed editing efficiencies of 67-  
191 92% (Supplementary Figure 9A). *In planta* and *in vitro* growth assays of three deletion mutants  
192 effectively recapitulated the phenotypes of previously described cluster deletion polymutants<sup>32</sup>  
193 (Figure 4E, Supplementary Figure 9B-G). Targeting cluster X cured the 73 kb plasmid and  
194 simultaneous cluster IV and IX targeting led to dual deletions in 8 out of 12 survivors, with a  
195 sequenced representative having 68.5 kb and 55.3 kb deletions, respectively, at the expected  
196 target sites. The effector cluster VI Cas3-derived mutant had a more severe growth defect *in vitro*  
197 and *in planta* than the control mutant (Figure 4E, Supplementary Figure 9B,9E). This large  
198 deletion (100.1 kb in size) likely impacted general fitness (Figure 4F), demonstrating one  
199 drawback of large deletions, but this can be easily overcome by assessing *in vitro* growth of  
200 multiple isolates. Using our portable minimal system, we achieved three new applications: the  
201 single-step deletion of large virulence regions, multiplexed targeting, and plasmid curing. Overall,

202 we have demonstrated I-C CRISPR-Cas3 editing to be a generally applicable tool capable of  
203 generating large genomic deletions in three distinct bacteria.

#### 204 **Repurposing endogenous CRISPR-Cas3 systems for gene editing**

205 Type I CRISPR-Cas3 systems are the most common CRISPR-Cas systems in nature<sup>1</sup>. Therefore,  
206 many bacteria have a built-in genome editing tool to be harnessed. We first tested the  
207 environmental isolate from which our Type I-C system was derived. Introduction of the self-  
208 targeting *phzM* crRNA led to the isolation of genomic deletions at the targeted site according to  
209 PCR analysis in 0-30% of survivors when wild-type repeats flanked the spacer and 30-60% of  
210 survivors when modified repeats were used (Supplementary Figure 10A). Whole-genome  
211 sequencing of two of these isolates revealed 33.7 (wild-type repeat) and 39 kb (MR) deletions of  
212 the target gene and surrounding regions (Figure 5A). Additionally, HDR-based editing with a  
213 single construct was again efficacious, with 7/10 survivors acquiring the specific 0.17 kb deletion  
214 (Supplementary Figure 10B). Together, these experiments demonstrate the capacity for different  
215 forms of genome editing using a single plasmid and an endogenous CRISPR-Cas system.

216 We next evaluated the feasibility of repurposing other Type I systems, using the naturally active  
217 Type I-F systems<sup>33</sup> encoded by laboratory strain *P. aeruginosa* PA14, and the clinical strain *P.*  
218 *aeruginosa* z8. Plasmids with Type I-F specific crRNAs were expressed, targeting various  
219 genomic sites for deletion (Supplementary Table 3). HDR templates (600 bp arms on average)  
220 were included in the plasmids to generate deletions of defined coordinates ranging from 0.2 to  
221 6.3 kb. Overall, at 5 different genomic target sites in strain z8 and 2 sites in PA14, we observed  
222 desired deletions in 29 - 100 % of analyzed survivor colonies (Figure 5B). This demonstrates a  
223 high recombination efficiency and the feasibility of repurposing other Type I CRISPR-Cas3  
224 systems in the manner we employed for the minimal I-C system.

225 Finally, one potential impediment to the implementation of any CRISPR-Cas bacterial genome  
226 editing tool is the presence of anti-CRISPR (*acr*) proteins that inactivate CRISPR-Cas activity<sup>34</sup>.  
227 In the presence of a prophage expressing AcrIc1 (a Type I-C anti-CRISPR protein<sup>26</sup>) from a native  
228 *acr* promoter, self-targeting was completely inhibited, but not by an isogenic prophage expressing  
229 a Cas9 inhibitor AcrIIA4<sup>35</sup> (Figure 5C). To attempt to overcome this impediment, we expressed  
230 *aca1* (anti-CRISPR associated gene 1), a direct negative regulator of *acr* promoters, from the  
231 same construct as the crRNA. Using this repression-based “anti-anti-CRISPR” strategy, CRISPR-  
232 Cas function was re-activated, allowing the isolation of edited cells despite the presence of *acrIc1*  
233 (Figure 5C and Supplementary Figure 10C). In contrast, simply increasing *cas* gene and crRNA  
234 expression did not overcome AcrIc1-mediated inhibition (Figure 5C). Therefore, using anti-anti-  
235 CRISPRs presents a viable route towards enhanced efficiency of CRISPR-Cas editing and  
236 necessitates continued discovery and characterization of anti-CRISPR proteins and their cognate  
237 repressors.

#### 238 **Discussion**

239 By repurposing a minimal CRISPR-Cas3 system as both an endogenous and heterologous  
240 genome editing tool, we show that hurdles to generating large deletions can be overcome. We  
241 obtained high efficiencies after modifying a repeat sequence to prevent spacer loss. Using only a  
242 single crRNA, we isolated deletions as large as 424 kb without requiring the insertion of a

243 selectable marker or HDR templates guiding the repair process. Additionally, the I-C system  
244 appears to produce bi-directional deletions, similar to what was previously observed with the I-F  
245 CRISPR-Cas3 system<sup>36,37</sup>, but not with type I-E<sup>9,10,22</sup>. CRISPR-Cas3 presents a genome editing  
246 tool useful for the targeted removal of large elements (e.g. virulence clusters, plasmids) and also  
247 for unbiased screening and genome streamlining. As a long-term goal of microbial gene editing  
248 has been genome minimization<sup>38,39,57</sup>, we used our optimized CRISPR-Cas3 system to generate  
249 ten iterative deletions, achieving >13 % genome reduction of the targeted strain. This spanned  
250 only 30 days while maintaining editing efficiency, a great improvement over previous genome  
251 reduction methods<sup>40</sup>. Some basic microbial applications of Cas3 include studying chromosome  
252 biology (e.g. replicore asymmetry<sup>41</sup>), virulence factors<sup>42</sup>, and the impact of the mobilome.

253 An important outcome of this work is the enhanced recombination observed at cut sites when  
254 comparing Cas3 and Cas9 directly. The potential for Cas3 to be more recombinogenic through  
255 the generation of exposed ssDNA may be advantageous for both programmed knock-outs and  
256 for programmed knock-ins. The direct comparison between Cas3 (large deletions) and Cas9  
257 (small deletions) presented here confirms the causality of Cas3 in the deletion outcomes.

258 Our study has revealed some of the benefits and challenges of working with CRISPR-Cas3. While  
259 some of the iteratively edited strains demonstrated slight growth defects, the Cas3 editing  
260 workflow shows high potential for genome minimization efforts. Since many distinct deletion  
261 events are generated, screening various isolates for fitness benefits or defects is possible, and  
262 one can proceed with the strain that has the desired fitness property. Additionally, if the location  
263 of essential genes within an organism's genome is not fully described, this is not an impediment  
264 as editing efficiency drops precipitously when targeting near essential genes. Finally, despite our  
265 success at transplanting the minimal Type I-C system, it remains to be seen whether the approach  
266 will be limited by differences in DNA repair mechanisms. Indeed, in *E. coli* and *P. syringae*, larger  
267 regions of homology, such as 34 bp long REP sequences were observed, indicating the role of  
268 RecA-mediated homologous recombination<sup>43</sup> in the repair process. Meanwhile in *P. aeruginosa*,  
269 the borders of the deletions showed either small (4-14 bp) micro-homologies or no noticeable  
270 sequence homology. The former implies a role for alternative end-joining<sup>29</sup>, while the latter non-  
271 homologous end-joining<sup>44</sup> in the repair process. Downstream studies are required to dissect the  
272 roles of each mechanism in the deletion generation process for better predictable deletion  
273 outcomes.

274 CRISPR-Cas3 is an especially promising tool for use in eukaryotic cells as it would facilitate the  
275 interrogation of large segments of non-coding DNA, much of which has unknown function<sup>45</sup>.  
276 Additionally, it was recently shown that Cas9-generated "gene knockouts" (i.e. small indels  
277 causing out-of-frame mutations) frequently encode pseudo-mRNAs that may produce protein  
278 products, necessitating methods for full gene removal<sup>46,47</sup>. Encouragingly, a Type I-E CRISPR-  
279 Cas system was recently delivered to human cells as a ribonucleoprotein (RNP) complex and  
280 resulted in large deletions<sup>22</sup>, demonstrating the feasibility of Cas3-mediated editing in human  
281 cells. Overall, the intrinsic properties of Cas3 make it a promising tool to fill a void in current gene  
282 editing capabilities. Employing Cas3 to make large genomic deletions will facilitate the  
283 manipulation of repetitive and non-coding regions, having a broad impact on genetics research  
284 by providing a tool to probe genomes *en masse*, as well as the capability to rapidly streamline  
285 genomes for synthetic biology.



286

## 287 **Methods**

### 288 *Bacterial strains, plasmids, DNA oligonucleotides, and media*

289 A previously described<sup>26</sup> environmental strain of *Pseudomonas aeruginosa* was used as a  
290 template to amplify the *four cas* genes of the Type I-C CRISPR-Cas system genes (*cas3*, *cas5*,  
291 *cas7*, and *cas8*). The genes were cloned into the pUC18-mini-Tn7T-LAC vector<sup>48</sup> using the SacI-  
292 PstI restriction endonuclease cut sites in the order *cas5*, *cas7*, *cas8*, *cas3* to generate the plasmid  
293 pJW31 (Addgene number: 136423). This vector was introduced into *Pseudomonas aeruginosa*  
294 PAO1<sup>49</sup>, inserting the *cas* genes into the chromosome, following previously described methods<sup>50</sup>.  
295 Following integration, the excess sequences, including the antibiotic resistance marker, were  
296 removed via Flp-mediated excision as described previously<sup>50</sup>. The resulting strain, dubbed  
297 PAO1<sup>IC</sup>, allowed for inducible expression of the I-C system through induction with isopropyl  $\beta$ -D-  
298 1 thiogalactopyranoside (IPTG). An isogenic strain carrying Cas9 derived from *Streptococcus*  
299 *pyogenes* was constructed in the same fashion, resulting in the strain PAO1<sup>IIA</sup>. For experiments  
300 to test the system in *Pseudomonas syringae*, we employed the previously characterized strain  
301 DC3000<sup>30</sup>. *E. coli* editing experiments were conducted with strain K-12 MG1655<sup>51</sup>.

302 To achieve genomic self-targeting of the I-C CRISPR-Cas strains, crRNAs designed to target the  
303 genome were expressed from the pHERD20T and pHERD30T shuttle vectors<sup>52</sup>. So-called “entry  
304 vectors” pHERD20T-ICcr and pHERD30T-ICcr were first generated by cloning at the EcoRI and  
305 HindIII sites an annealed linear dsDNA template carrying the I-C CRISPR-Cas system repeat  
306 sequences flanking two BsaI Type IIS restriction endonuclease recognition sites. Additionally, a  
307 preexisting BsaI site in a non-coding site of the pHERD30T and pHERD20T plasmids was  
308 mutated using whole-plasmid amplification so it would not interfere with the cloning of the  
309 crRNAs<sup>26</sup>. Oligonucleotides with repeat-specific overhangs encoding the various spacer  
310 sequences were annealed and phosphorylated using T4 polynucleotide kinase (PNK) and cloned  
311 into the entry vectors using the BsaI sites. For experiments using Cas9, sgRNAs were expressed  
312 from the same pHERD30T vector, with the sgRNA construct cloned using the same restriction  
313 sites as with the I-C crRNAs.

314 The all-in-one vector pCas3cRh (Addgene number 133773) is a derivative of the pHERD30T-IC  
315 plasmid, with the 4 I-C system genes cloned downstream of the crRNA site. This was achieved  
316 by amplifying the genes *cas3*, *cas5*, *cas8*, and *cas7* in two fragments with a junction within *cas8*  
317 designed to eliminate an intrinsic BsaI site with a synonymous point mutation. The amplified  
318 fragments were cloned into pHERD30T-IC using the Gibson assembly protocol<sup>53</sup>. Finally, to guard  
319 against potential leaky toxic expression, we replaced the *araC*-Para<sub>BAD</sub> promoter with the  
320 rhamnose-inducible *rhaSR*-Prh<sub>BAD</sub> system<sup>54</sup>. The sequence for *rhaSR*-Prh<sub>BAD</sub> was amplified  
321 from the pJM230 template<sup>54</sup>, provided by the lab of Joanna B. Goldberg (Emory University), and  
322 cloned into the pHERD30T-IC plasmid to replace *araC*-Para<sub>BAD</sub> using Gibson Assembly (New  
323 England Biolabs). Without induction, transformation efficiencies of targeting constructs of  
324 assembled pCas3cRh were on average 5-10-fold lower when compared to non-targeting controls  
325 (Supplementary Figure 8C), indicating residual leakiness of the I-C system.

326 The *aca1*-containing vector pICcr-*aca1* is a derivative of the pHERD30T-ICcr plasmid, with *aca1*  
327 cloned downstream of the crRNA site under the control of the pBAD promoter. The *aca1* gene  
328 was cloned from *P. aeruginosa* phage DMS3m.

329 All oligonucleotides used in this study were obtained from Integrated DNA Technologies. For a  
330 complete list of all DNA oligonucleotides and a short description, see Supplementary Table 4.

331 *P. aeruginosa* and *E. coli* strains were grown in standard Lysogeny Broth (LB): 10 g tryptone, 5 g  
332 yeast extract, and 10 g NaCl per 1 L dH<sub>2</sub>O. Solid plates were supplemented with 1.5 % agar. *P.*  
333 *syringae* was grown in King's medium B (KB): 20 g Bacto Proteose Peptone No. 3, 1.5 g K<sub>2</sub>HPO<sub>4</sub>,  
334 1.5 g MgSO<sub>4</sub>•7H<sub>2</sub>O, 10 ml glycerol per 1 L dH<sub>2</sub>O, supplemented with 100 µg/ml rifampicin. The  
335 following antibiotic concentrations were used for selection: 50 µg/ml gentamicin for *P. aeruginosa*  
336 and *P. syringae*, 15 µg/ml for *E. coli*; 50 µg/ml carbenicillin for all organisms. Inducer  
337 concentrations were 0.5 mM IPTG, 0.1 % arabinose, and 0.1 % rhamnose. For transformation  
338 protocols, all bacteria were recovered in Super optimal broth with catabolite repression (SOC): 20  
339 g tryptone, 5 g yeast extract, 10mM NaCl, 2.5 mM KCl, 10 mM MgCl<sub>2</sub>, 10 mM, MgSO<sub>4</sub>, and 20  
340 mM glucose in 1 L dH<sub>2</sub>O.

341

#### 342 *Bacterial transformations*

343 Transformations of *P. aeruginosa*, *E. coli*, and *P. syringae* strains were conducted using standard  
344 electroporation protocols. 10 ml of overnight cultures were centrifuged and washed twice in an  
345 equal volume of 300 mM sucrose (20 % glycerol for *E. coli*) and suspended in 1 ml 300 mM  
346 sucrose (20 % glycerol for *E. coli*). 100 µl aliquots of the resulting competent cells were  
347 electroporated using a Gene Pulser Xcell Electroporation System (Bio-Rad) with 50 – 200 ng  
348 plasmid with the following settings: 200 Ω, 25 µF, 1.8 kV, using 0.2 mm gap width electroporation  
349 cuvettes (Bio-Rad). Electroporated cells were incubated in antibiotic-free SOC media for 1 hour  
350 at 37 °C (28 °C for *P. syringae*), then plated onto LB agar (KB agar for *P. syringae*) with the  
351 selecting antibiotic, and grown overnight at 37 °C (28 °C for *P. syringae*). Cloning procedures  
352 were performed in commercial *E. coli* DH5α cells (New England Biolabs) or *E. coli* XL1-Blue (QB3  
353 Macrolab Berkeley), according to the manufacturer's protocols.

354

#### 355 *Construction of recombinant DMS3m acr phages*

356 The isogenic DMS3m *acrIIA4* and *acrIC1* phages were constructed using previously described  
357 methods<sup>55</sup>. A recombination cassette, pJZ01, was constructed with homology to the DMS3m *acr*  
358 locus. Using Gibson Assembly (New England Biolabs), either *acrIC1* or *acrIIA4* were cloned  
359 upstream of *aca1*, and the resulting vectors were used to transform PAO1<sup>IC</sup>. The transformed  
360 strains were infected with WT DMS3m, and recombinant phages were screened for. Phages were  
361 stored in SM buffer at 4 °C.

362

#### 363 *Isolation of PAO1<sup>IC</sup> lysogens*

364 PAO1<sup>IC</sup> was grown overnight at 37 °C in LB media. 150 µl of overnight culture was added to 4 ml  
365 of 0.7 % LB top agar and spread on 1.5 % LB agar plates supplemented with 10 mM MgSO<sub>4</sub>. 5  
366 µl of phage, expressing either *acrIC1* or *acrIIA4* were spotted on the solidified top agar and plates  
367 were incubated at 30 °C overnight. Following incubation, bacterial growth within the plaque was  
368 isolated and spread on 1.5 % LB agar plate. After an overnight incubation at 37 °C, single colonies  
369 were assayed for the prophage. Confirmed lysogens were used for genomic targeting  
370 experiments.

371

372 *Genomic targeting*

373 *Pseudomonas aeruginosa*

374 Genomic self-targeting of *P. aeruginosa* PAO1<sup>C</sup> was achieved by electroporating cells with  
375 pHERD30T (or pHERD20T) expressing the self-targeting spacer of choice. Cells were plated onto  
376 LB agar plates containing the selective antibiotic, without inducers, and grown overnight. Single  
377 colonies were then grown in liquid LB media containing the selective antibiotic, as well as IPTG  
378 to induce the genomic expression of the I-C system genes, and arabinose to induce the  
379 expression of the crRNA from the plasmid. The *aca1*-containing crRNA plasmids do not need  
380 additional inducers, as the pBAD promoter controls *aca1*. Cultures were grown at 37 °C in a  
381 shaking incubator overnight to saturation, then plated onto LB agar plates containing the selecting  
382 antibiotic, as well as the inducers, and incubated overnight again at 37 °C. The resulting colonies  
383 were then analyzed individually using colony PCR for any differences at the targeted genomic  
384 site compared to a wild-type cell. gDNA was isolated by resuspending 1 colony in 20 µl of H<sub>2</sub>O,  
385 followed by incubation at 95 °C for 15 min. 1-2 µl of boiled sample was used for PCR. The primers  
386 used to assay the targeted sites were designed to amplify genomic regions 1.5 - 3 kb in size. In  
387 the event of a PCR product equal to or smaller than the wild-type fragment (as was often observed  
388 when analyzing Cas9-targeted cells), Sanger sequencing (Quintara Biosciences) was used to  
389 determine any modifications of the targeted sequences. In some cases, additional analysis of the  
390 crRNA-expressing plasmids of the surviving colonies was also performed, by isolating and  
391 reintroducing the plasmids into the original I-C CRISPR-Cas strain, where functional self-targeting  
392 could be determined based on a significant increase in the lag time of induced cultures,  
393 characteristic of self-targeting events.

394 *Escherichia coli*

395 Genomic self-targeting of *E. coli* was conducted in a similar fashion as *P. aeruginosa*, except  
396 using the pCas3cRh all-in-one vector. Electrocompetent *E. coli* cells were transformed with  
397 pCas3cRh expressing a crRNA targeting the genome. Individual transformants were selected and  
398 grown in liquid LB media containing the selecting antibiotic (gentamicin) overnight without any  
399 inducers added. The overnight cultures were then plated in the presence of inducer and X-gal to  
400 screen for functional *lacZ* (LB agar + 15 µg/ml gentamicin + 0.1 % rhamnose + 1 mM IPTG + 20  
401 µg/ml X-gal) and blue/white colonies were counted the next day.

402 *Pseudomonas syringae*

403 Electrocompetent *P. syringae* cells were also transformed with pCas3cRh plasmids targeting  
404 selected genomic sequences. Initial transformants were plated onto KB agar + 100 µg/ml  
405 rifampicin + 50 µg/ml gentamicin plates, and incubated at 28 °C overnight. Single colony  
406 transformants were then selected and inoculated in KB liquid media supplemented with rifampicin,  
407 gentamicin, and 0.1 % rhamnose inducer, and grown to saturation in a shaking incubator at 28  
408 °C. Cultures were finally plated onto KB agar plates with rifampicin, gentamicin, and rhamnose  
409 and incubated at 28 °C. Individual colonies were finally assayed with colony PCR to determine  
410 the presence of deletions at the targeted genomic sites.

411

## 412 *Iterative genome minimization*

413 Iterative targeting to generate multiple deletions in the *P. aeruginosa* PAO1<sup>C</sup> strain was carried  
414 out by alternating the pHERD30T and pHERD20T plasmids each expressing different crRNAs  
415 targeting the genome. Each crRNA designed to target the genome was cloned into both the  
416 pHERD30T plasmid, which confers gentamicin resistance, as well as the pHERD20T plasmid,  
417 which confers carbenicillin resistance. After first transforming and targeting with a pHERD30T  
418 plasmid expressing a specific crRNA, deletion candidate isolates were transformed with a  
419 pHERD20T expressing a crRNA targeting a different genomic region. As the two plasmids are  
420 identical with the exception of the resistance marker, this eliminated the necessity for curing of  
421 the original plasmid to be able to target a different region. For the next targeting event, the  
422 pHERD30T plasmid could again be used, this time expressing another crRNA targeting a different  
423 genomic region. In this manner, pHERD30T and pHERD20T could be alternated to achieve  
424 multiple deletions in a rapid process. At each new transformation step, the cells were checked for  
425 any residual resistance to the given antibiotic from a previous cycle. Additionally, functionality of  
426 the CRISPR-Cas system of the edited cells could be determined through the introduction of a  
427 plasmid expressing crRNA targeting the D3 bacteriophage<sup>34</sup>, then performing a phage spotting  
428 assay to see if phage targeting was occurring or not.

429

## 430 *Measurement of growth rates*

### 431 *Pseudomonas aeruginosa*

432 Growth dynamics of various strains were measured using a Synergy 2 automated 96-well plate  
433 reader (Biotek Instruments) and the accompanying Gen5 software (Biotek Instruments).  
434 Individual colonies were picked and grown overnight in 300  $\mu$ l volumes of LB in 96-well deep-well  
435 plates at 37 °C. The grown cultures were then diluted 100-fold into 100  $\mu$ l of fresh LB in a 96-well  
436 clear microtitre plate (Costar) and sealed with Microplate sealing adhesive (Thermo Scientific).  
437 Small holes were punched in the sealing adhesive for each well for increased aeration. Doubling  
438 times were calculated as described previously<sup>56</sup>.

### 439 *Pseudomonas syringae*

440 To test bacterial growth *in planta*, we used the *Arabidopsis thaliana* ecotype *Columbia* (Col-0),  
441 which has previously been shown to be susceptible to infection by *P. syringae* DC3000. Plants  
442 were grown for 5-6 weeks in 9h light/15h darkness and 65 % humidity. For each inoculum, we  
443 measured bacterial growth in 10 individual Col-0 plants. Four leaves from each plant were  
444 infiltrated at OD<sub>600</sub> = 0.0002, and cored with a #3 borer. The four cores from each plant were then  
445 ground, resuspended in 10 mM MgCl<sub>2</sub> and plated in a dilution series on selective media for colony  
446 counts at both the time of infection and 3 days post-infection.

447 To test bacterial growth *in vitro*, we used both KB and plant apoplast mimicking minimal media  
448 (MM)<sup>57</sup>. Overnight cultures were prepared from single colonies of each strain, washed, and diluted  
449 to OD<sub>600</sub> = 0.1 in 96-well plates using either KB or MM. Plates were incubated with shaking at 28  
450 °C. OD<sub>600</sub> was measured over the course of 24-25 hours using an Infinite 200 Pro automated  
451 plate reader (Tecan). Statistical analysis determined significantly different groups based on  
452 ANOVA analysis on the day 0 group of values and the day 3 group of values. Significant ANOVA  
453 results ( $p < 0.01$ ) were further analyzed with a Tukey's HSD post hoc test to generate adjusted p-

454 values for each pairwise comparison. A significance threshold of 0.01 was used to determine  
455 which treatment groups were significantly different.

456

#### 457 *Bacteriophage plaque (spot) assays*

458 Bacteriophage plaque assays were performed using 1.5 % LB agar plates supplemented with 10  
459 mM MgSO<sub>4</sub> and the appropriate antibiotic (gentamicin or carbenicillin, depending on the plasmid  
460 used to express the crRNA), and 0.7 % LB top agar supplemented with 0.5 mM IPTG and 0.1 %  
461 arabinose inducers added covering the whole plate. 150 µl of the appropriate overnight cultures  
462 was suspended in 4 ml molten top agar poured onto an LB agar plate leading to the growth of a  
463 bacterial lawn. After 10-15 minutes at room temperature, 3 µl of ten-fold serial dilutions of  
464 bacteriophage was spotted onto the solidified top agar. Plates were incubated overnight at 30 °C  
465 and imaged the following day using a Gel Doc EZ Gel Documentation System (BioRad) and Image  
466 Lab (BioRad) software. The following bacteriophage were used in this study: bacteriophage  
467 JBD30<sup>34</sup>, bacteriophage D3<sup>58</sup>, and bacteriophage DMS3m<sup>59</sup>.

468

#### 469 *Whole-genome sequencing*

470 Genomic DNA for whole-genome sequencing (WGS) analysis was isolated directly from bacterial  
471 colonies using the Nextera DNA Flex Microbial Colony Extraction kit (Illumina) according to the  
472 manufacturer's protocol. Genomic DNA concentration of the samples was determined using a  
473 DS-11 Series Spectrophotometer/Fluorometer (DeNovix) and all fell into the range of 200-500  
474 ng/µl. Library preparation for WGS analysis was done using the Nextera DNA Flex Library Prep  
475 kit (Illumina) according to the manufacturer's protocol starting from the tagment genomic DNA  
476 step. Tagmented DNA was amplified using Nextera DNA CD Indexes (Illumina). Samples were  
477 placed overnight at 4 °C following the tagmented DNA amplification step, then continued the next  
478 day with the library clean up steps. Quality control of the pooled libraries was performed using a  
479 2100 Bioanalyzer Instrument (Agilent Technologies) with a High Sensitivity DNA Kit (Agilent  
480 Technologies). The majority of samples were sequenced using a MiSeq Reagent Kit v2 (Illumina)  
481 for a 150 bp paired-end sequencing run using the MiSeq sequencer (Illumina). *P. syringae* and  
482 Cas9-generated *P. aeruginosa* deletion strains were sequenced using a NextSeq 500 Reagent  
483 Kit v2 (Illumina) for a 150 bp paired-end sequencing run using the NextSeq 500 sequencer  
484 (Illumina).

485 Genome sequence assembly was performed using Geneious Prime software version 2019.1.3.  
486 Paired read data sets were trimmed using the BBDuk (Decontamination Using Kmers) plugin  
487 using a minimum Q value of 20. The genome for the ancestral PAO1<sup>IC</sup> strain was de novo  
488 assembled using the default automated sensitivity settings offered by the software. The  
489 consensus sequence of PAO1<sup>IC</sup> assembled in this manner was then used as the reference  
490 sequence for mapping all of the PAO1<sup>IC</sup> strains with multiple deletions. As a control, the  
491 sequences were also mapped to the reference *P. aeruginosa* PAO1 sequence (NC\_002516) to  
492 verify deletion border coordinates. Coverage of these sequenced strains ranged from 66 to 143-  
493 fold, with an average of 98.3-fold. The sequenced *P. aeruginosa* environmental strains were also  
494 mapped to the PAO1 (NC\_002516) reference, while the sequenced *E. coli* strains were mapped  
495 to the *E. coli* K-12 MG1655 reference sequence (NC\_000913). Finally, sequenced *P. syringae*  
496 strains were mapped to the *P. syringae* DC3000 (NC\_004578) reference sequence, along with

497 the pDC3000A endogenous 73.5 kb plasmid sequence (NC\_004633). All of the remaining  
498 sequenced strains had > 100-fold coverage. All deletion junction sequences were manually  
499 verified by the presence of multiple reads spanning the deletions, containing sequences from both  
500 end boundaries.

501 WGS data was visualized using the BLAST Ring Image Generator (BRIG) tool<sup>60</sup> employing  
502 BLAST+ version 2.9.0. In several cases, short sequences were aligned within previously  
503 determined large deletions at redundant sequences such as transposase genes. Such  
504 misrepresentations created by BRIG were manually removed to reflect the actual sequencing  
505 data.

#### 506 **Data availability**

507 Raw whole-genome sequencing data associated with Figures 1D, 3B, 4A, 4C, 4F, and 5A) has  
508 been uploaded to GenBank (Submission number SUB6598604, accession number pending) and  
509 is also available, along with bacterial strains, upon request from the corresponding author.

510 Correspondence and requests for strains should be addressed to J.B.D. ([joseph.bondy-](mailto:joseph.bondy-denomy@ucsf.edu)  
511 [denomy@ucsf.edu](mailto:joseph.bondy-denomy@ucsf.edu)). *Pseudomonas aeruginosa* strains available for laboratories with BSL-2  
512 clearance.

513

#### 514 **Acknowledgements**

515 We thank Joanna B. Goldberg (Emory University) for providing the plasmid pJM230, and Adair  
516 Borges (UCSF) for providing pAB04 to clone Type I-F crRNAs. We thank the Bondy-Denomy lab  
517 for productive conversations pertaining to this project.

#### 518 **Funding**

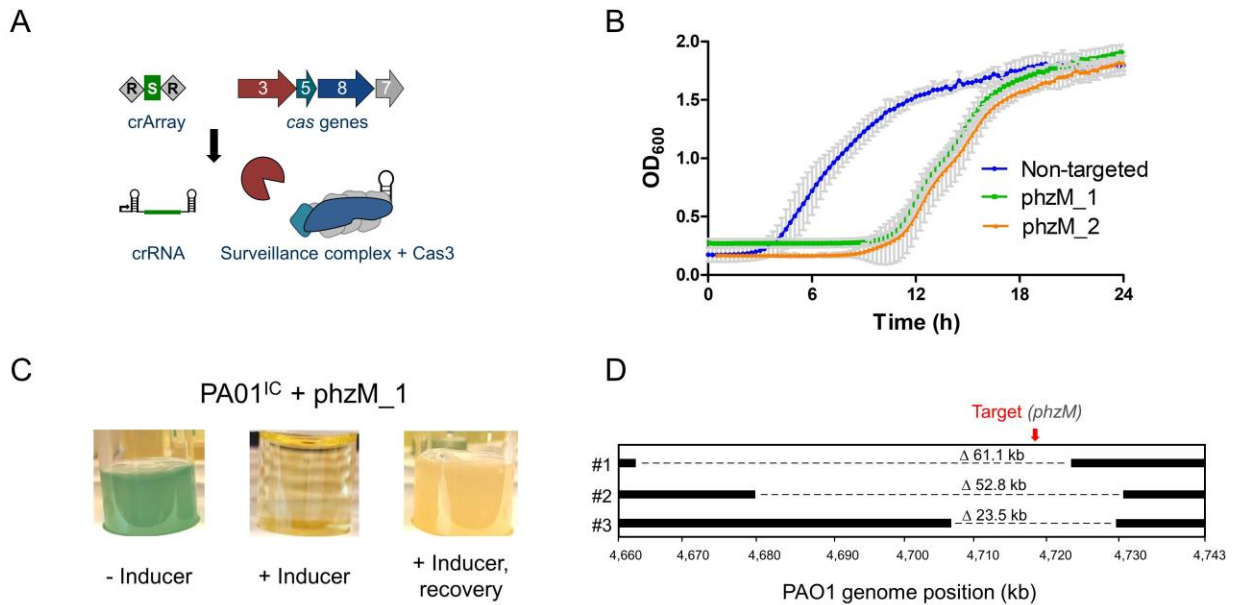
519 B.C. is supported by the Eötvös National Scholarship of Hungary and a Marie Skłodowska-Curie  
520 Actions Individual Global Fellowship (number 844093) of the Horizon 2020 Research Program of  
521 the European Commission. L.L. is supported by the HHMI Gilliam Fellowship for Advanced Study  
522 and the UCSF Discovery Fellowship. Research on plant immunity in the Lewis laboratory is  
523 supported by the USDA ARS 2030-21000-046-00D and 2030-21000-050-00D (J.D.L.), and the  
524 NSF Directorate for Biological Sciences IOS-1557661 (J.D.L.). I.J.C. is supported by a Grace  
525 Kase fellowship from UC Berkeley and the NSF Graduate Research Fellowship Program. A.V.R.  
526 is supported by funding from the Pew Charitable Trusts. E.D.C. is funded by the Chan Zuckerberg  
527 Biohub. CRISPR-Cas3 projects in the Bondy-Denomy Lab are supported by the UCSF Program  
528 for Breakthrough Biomedical Research funded in part by the Sandler Foundation and an NIH  
529 Director's Early Independence Award DP5-OD021344.

#### 530 **Competing Interests:**

531 J.B.-D. is a scientific advisory board member of SNIPR Biome and Excision Biotherapeutics and  
532 a scientific advisory board member and co-founder of Acrigen Biosciences.

533

534

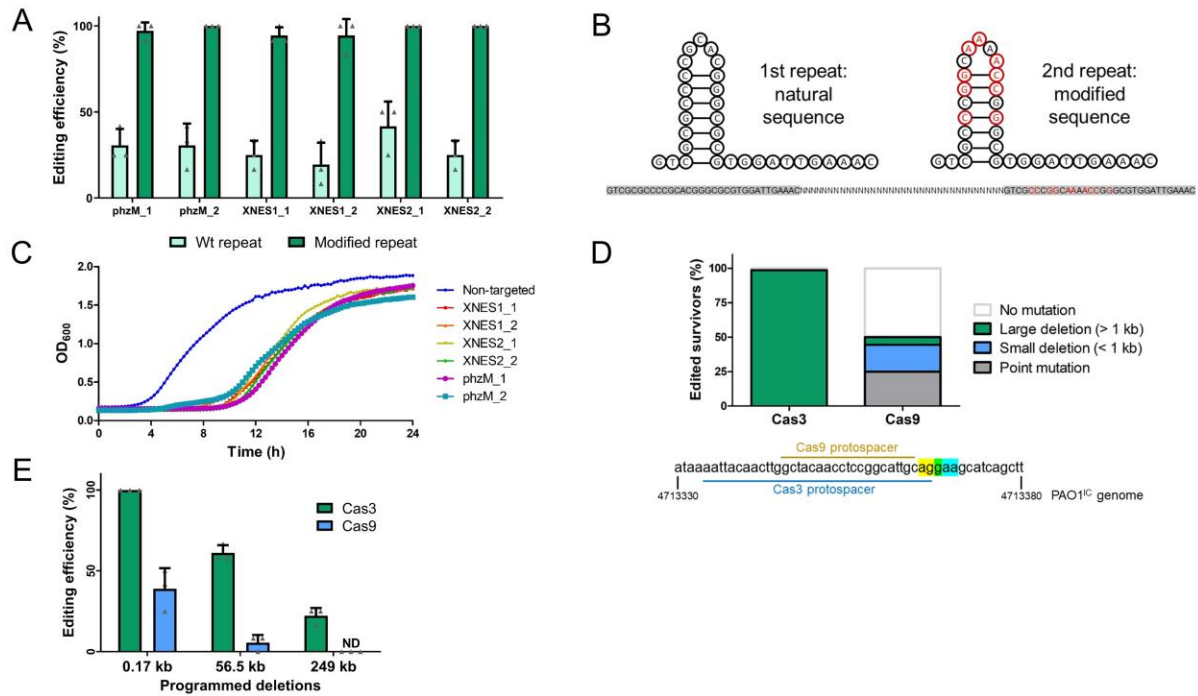


535

536

537 **Figure 1. A)** A schematic of the Type I-C *cas* gene operon and CRISPR array. The surveillance  
 538 complex is made up of Cas proteins (Cas5<sub>1</sub>:Cas8<sub>1</sub>:Cas7<sub>7</sub>) and one crRNA, which recruits Cas3  
 539 upon target DNA recognition. **B)** Growth curves of 2 PAO1<sup>IC</sup> strains expressing different crRNAs  
 540 targeting *phzM* (green and orange) compared to a non-targeting strain (blue). Values are the  
 541 mean of 8 biological replicates each, error bars indicate SD values. **C)** Cultures resulting from  
 542 *phzM* targeting, in the absence of inducer (-ind), presence (+ind), and after recovery. **D)** Whole-  
 543 genome sequencing of three PAO1<sup>IC</sup> self-targeted survivor strains. Bars indicate boundaries of  
 544 deletions; red arrow indicates genomic position of targeted sequences.

545



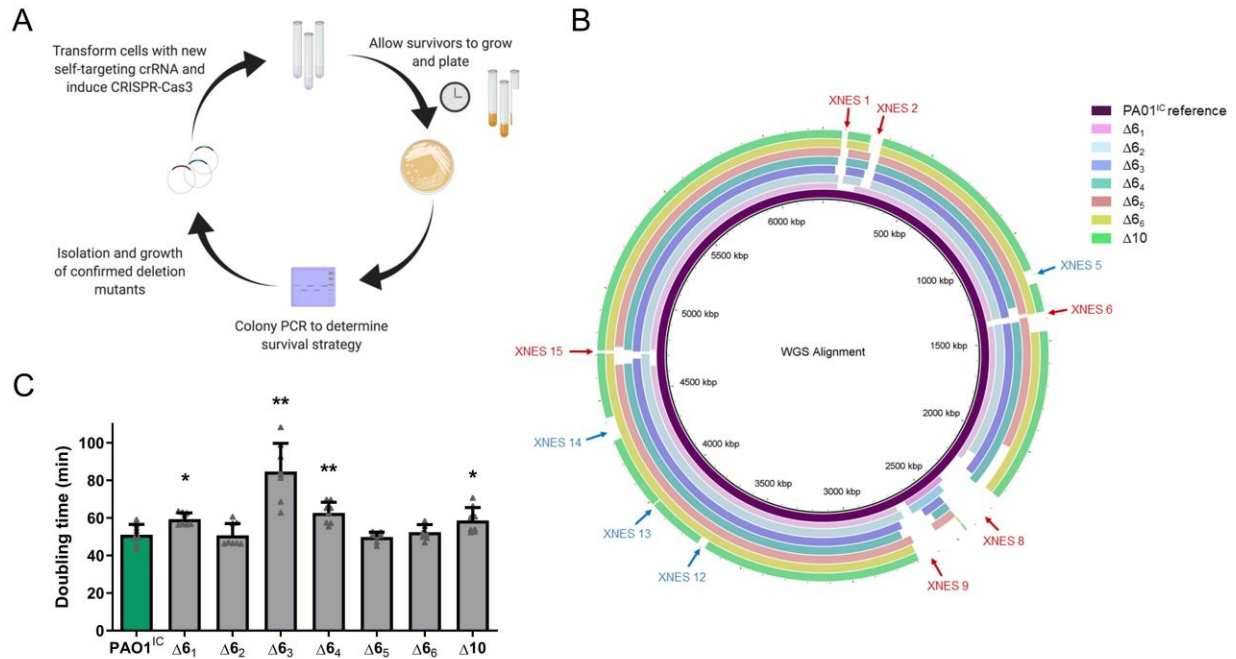
546

547

548 **Figure 2. A)** Percentage of survivors with a genomic deletion at the location targeted. Six different  
 549 crRNA constructs with either wild-type (Wt) repeat sequences (light green) or with the second  
 550 repeat being modified (dark green). Values are means of 3 biological replicates each, where 12  
 551 individual surviving colonies were assayed per replicate, error bars show SD values. **B)** Sequence  
 552 and structure of natural and modified repeat sequences. Specifically engineered modified  
 553 nucleotides shown in red; repeat sequences highlighted in gray with an arbitrary intervening  
 554 spacer sequence. **C)** Growth curves of PAO1<sup>IC</sup> strains expressing distinct self-targeting crRNAs  
 555 flanked by modified repeats. Non-targeting crRNA expressing control is marked in blue. Values  
 556 depicted are averages of 4 biological replicates each. **D)** Gene editing outcomes for distinct  
 557 survivor cells targeted with either a Type II-A SpyCas9 system or a Type I-C Cas3 system (n=72).  
 558 **E)** Percentage of survivors with the specific deletion size present (0.17 kb, 56.5 kb, or 249 kb)  
 559 using homologous repair templates with the Cas3 system (green) or the SpyCas9 system (blue).  
 560 Values are means of 3 biological replicates each, where 12 individual surviving colonies were  
 561 assayed per replicate, error bars show SD values, ND: not detected.

562





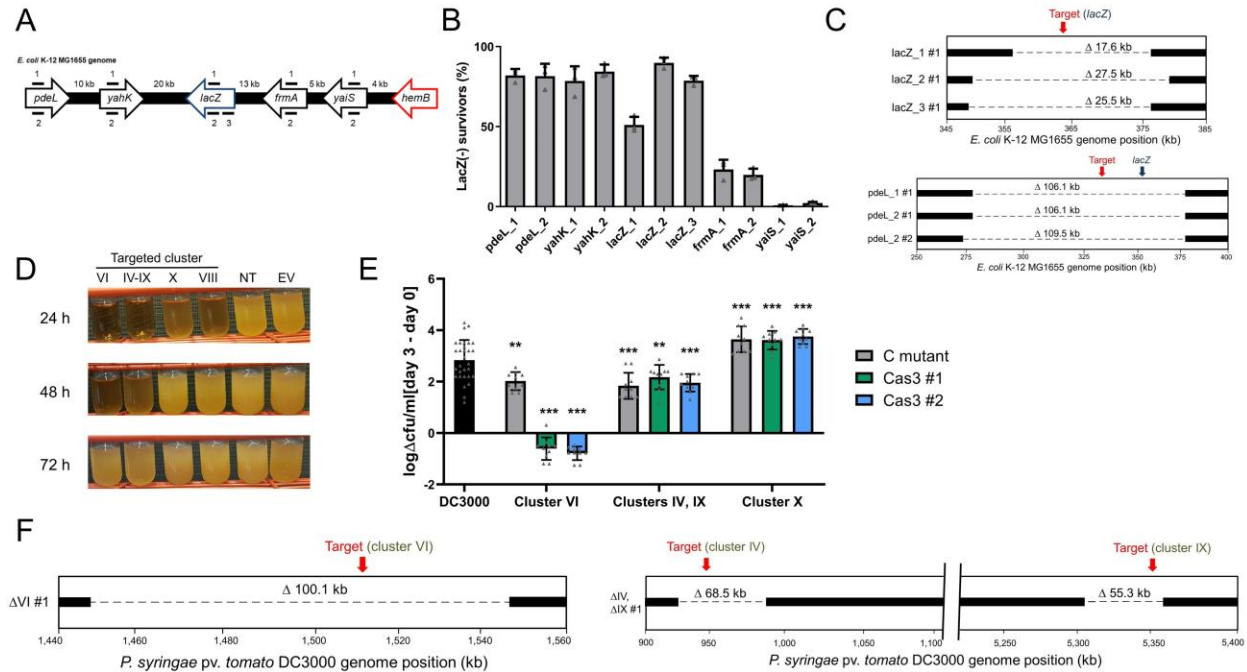
563

564

565 **Figure 3. A)** Schematic overview of the iterative deletion generating process. **B)** Whole-genome  
566 sequences of six PAO1<sup>IC</sup> strains that have been iteratively targeted at six distinct genomic  
567 positions and one (derived from strain  $\Delta 6_6$ ) with ten total deletions ( $\Delta 10$ ) aligned to the parental  
568 *P. aeruginosa* PAO1<sup>IC</sup> strain. The first six targeted sites are marked with red arrows, and the final  
569 four are marked with blue arrows. **C)** Calculated doubling times of the seven genome-reduced  
570 strains (strains  $\Delta 6_1 - 6_6$  with six deletions,  $\Delta 10$  with ten) compared to the parent PAO1<sup>IC</sup> strain  
571 (green). Values are means of 8 biological replicates, error bars represent SD values, \* p < 0.05,  
572 \*\* p < 0.01, paired T-test compared to PAO1<sup>IC</sup>.

573

574

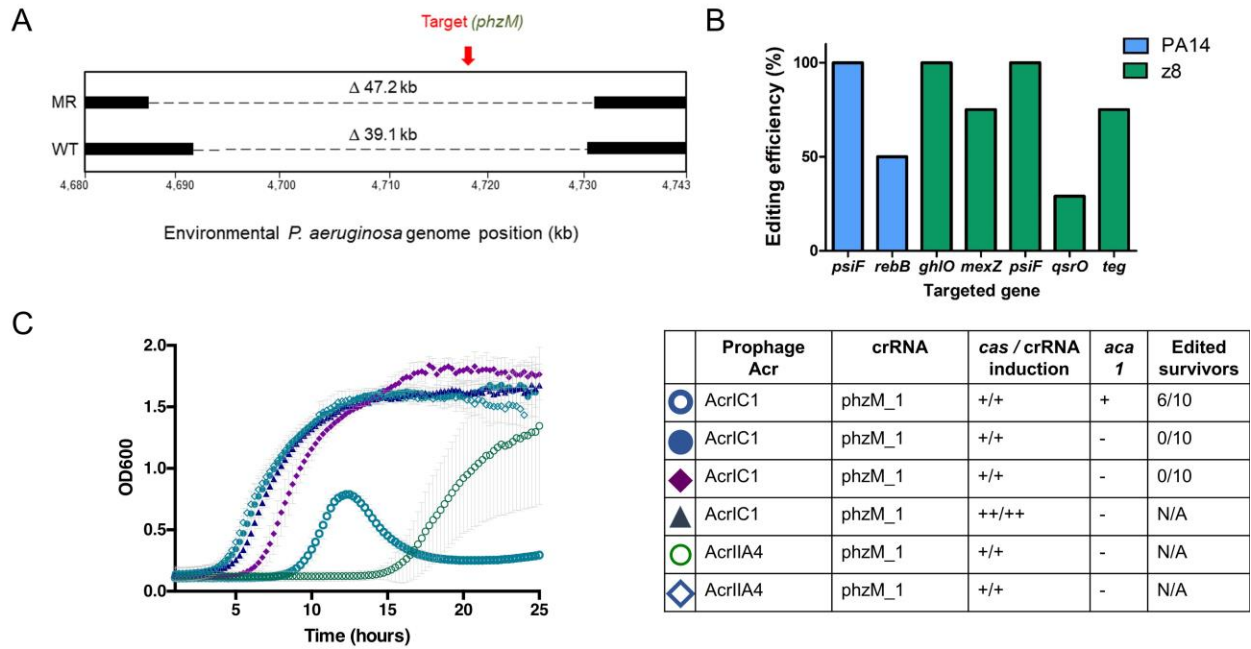


575

576

577 **Figure 4. A)** Schematic of the crRNA targeted sites in the *E. coli* MG1655 genome at the *lacZ*  
578 locus. **B)** *lacZ* deletion efficiencies using distinct crRNAs targeting the *E. coli* K-12 MG1655  
579 chromosome. Efficiencies calculated based on LacZ activity. Values are averages of 3 biological  
580 replicates, error bars represent standard deviations. **C)** Whole-genome sequencing of an *E. coli*  
581 deletion mutant targeted 30 kb upstream of *lacZ* at *pdeL*. **D)** Growth of *P. syringae* DC3000 strains  
582 expressing the I-C system and distinct crRNAs. Constructs VI, IV-IX, and VIII target *P. syringae*  
583 DC3000 non-essential chromosomal genes, non-targeting crRNA (NT), empty vector (EV). **E)**  
584 Bacterial growth of deletion mutants in *Arabidopsis thaliana*. Values are differences in colony  
585 forming units (cfu) / ml counted on day 0 of the experiment and day 3, shown on a logarithmic  
586 scale. The wild-type DC3000 strain is shown in black, while gray bars represent previously  
587 constructed polymutant control (C) strains of the different clusters (labeled at bottom), and  
588 green and blue bars show deletion mutants generated using Cas3 (two isolated strains for  
589 each targeted cluster, #1, #2). Values shown are means of 10 biological replicates each (30  
590 for DC3000), error bars show SD values, \*\* p < 0.01, \*\*\* p < 0.005, ANOVA analysis (see  
591 methods). **F)** Whole-genome sequencing of *P. syringae* deletion mutants. Left panel shows  
592 virulence cluster VI targeting, while right panel shows virulence cluster IV and IX targeting with a  
593 single crRNA, as the clusters share sequence identity.

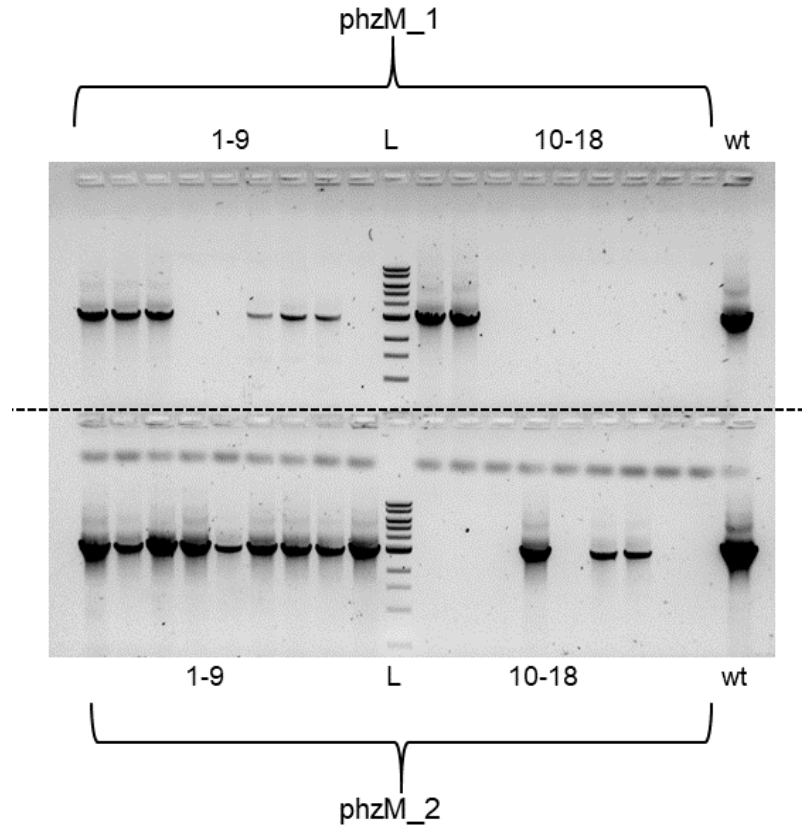
594



595

596

597 **Figure 5. A)** Schematic of whole genome sequencing of an environmental isolate of PAO1 with  
 598 an endogenous Type I-C system. Two survivors were isolated post-targeting using either WT  
 599 direct repeats flanking the spacer, or modified repeats. **B)** Editing efficiencies at targeted genomic  
 600 sites using homologous templates in a laboratory (PA14) and clinical (z8) strain of *P. aeruginosa*.  
 601 See Supplementary Table 3 for additional details. **C)** Growth curves of PAO1<sup>IC</sup> lysogenized by  
 602 recombinant DMS3m phage expressing *acrIIA4* or *acrIC1* from the native *acr* locus. CRISPR-  
 603 Cas3 activity is induced with either 0.5mM (+) or 5mM (++) IPTG and 0.1% (+) or 0.3% (++)  
 604 arabinose. Edited survivors reflect number of isolated survivor colonies missing the targeted gene  
 605 (*phzM*). Each growth curve is the average of 10 biological replicates and error bars represent SD.

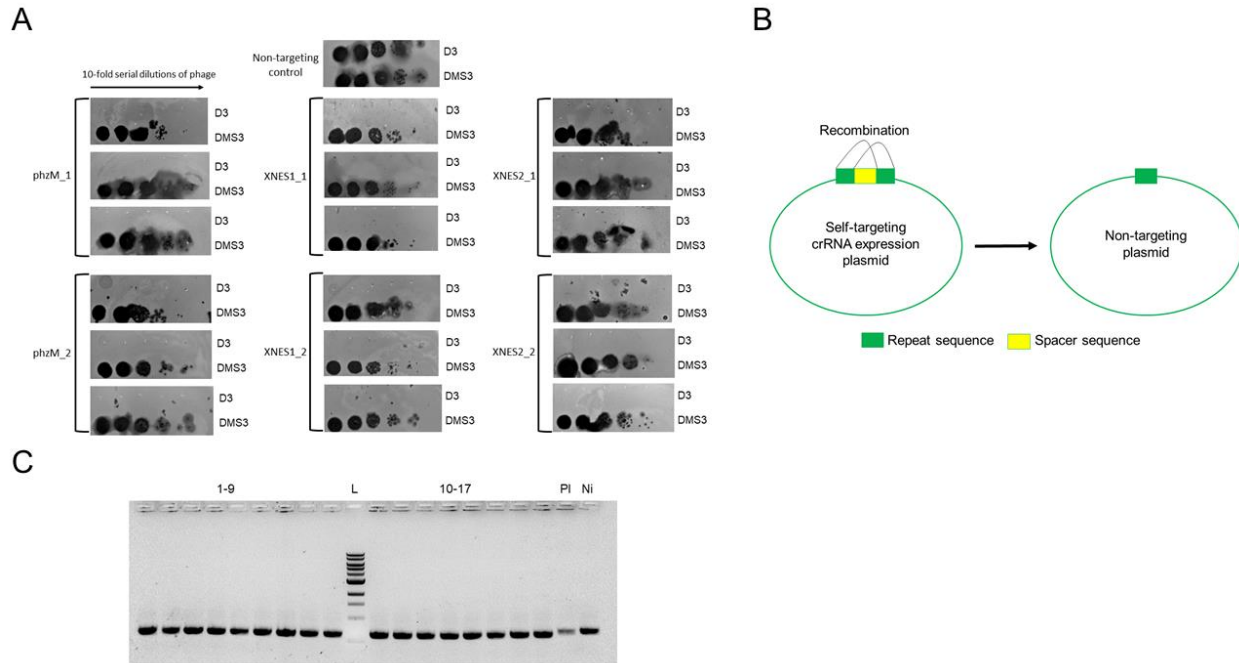


606

607

608 **Supplementary Figure 1.** PCR amplification of a 3 kb genomic fragment flanking the *phzM* gene  
609 targeted using two different crRNAs, *phzM\_1* and *phzM\_2*. Colony PCRs were performed on 18  
610 biological replicates of self-targeted strains for each crRNA. The PAO1<sup>IC</sup> parental strain is used  
611 as a positive control (wt). L indicates a 1 kb DNA ladder.

612



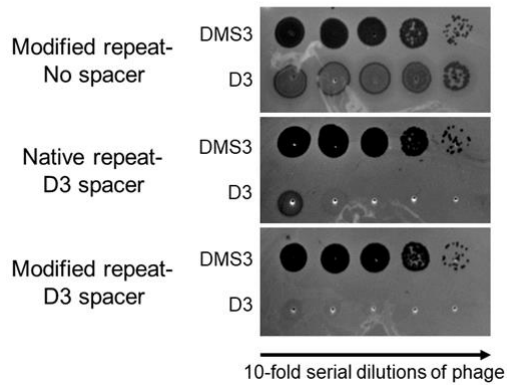
613

614

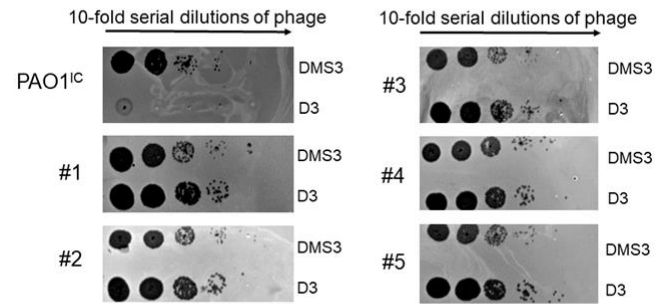
615 **Supplementary Figure 2. A)** Phage targeting assays with survivors that had no discernable  
616 deletion of the crRNA-targeted genomic site. Strains were transformed with a D3 phage-targeting  
617 crRNA to assay for IC CRISPR-Cas3 activity. Three unique survivors were isolated from six self-  
618 targeting assays for a total of 18 survivors. Control is a non-targeting crRNA. **B)** Schematic of  
619 spacer excision events where the two direct repeats recombine, resulting the loss of the targeting  
620 spacer. **C)** PCR amplification of the crRNA sequence from plasmids isolated from 17 non-deletion  
621 self-targeted survivors. PI indicates the original plasmid as the PCR template, Ni indicates a  
622 sample where the crRNA was not induced, L indicates a 1 kb DNA ladder.

623

A



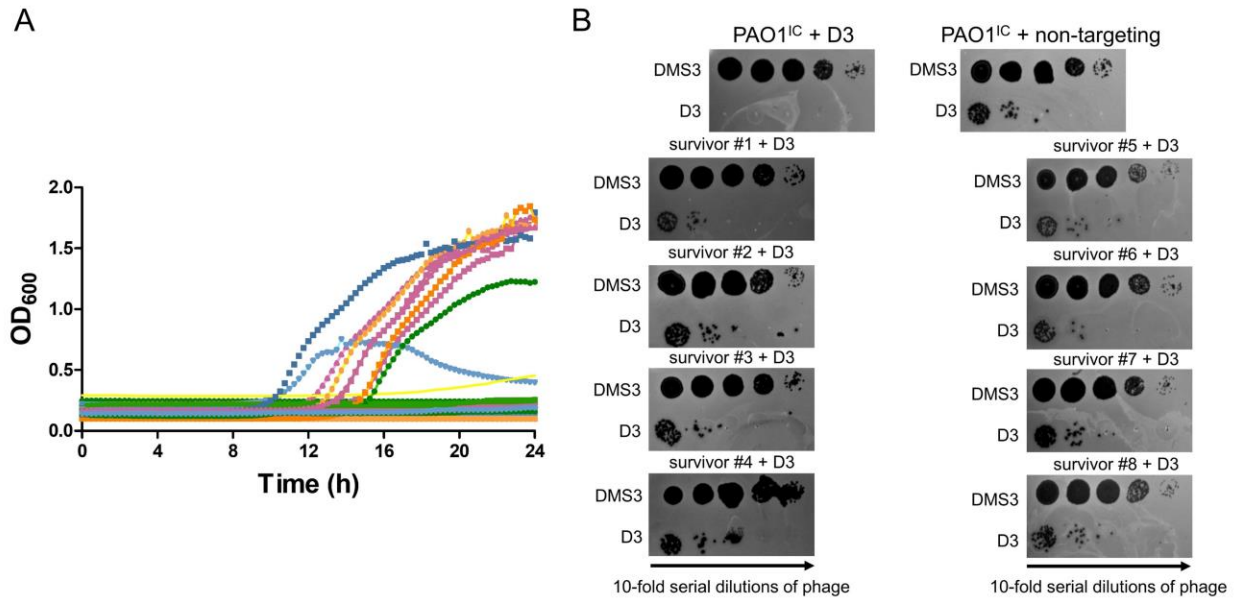
B

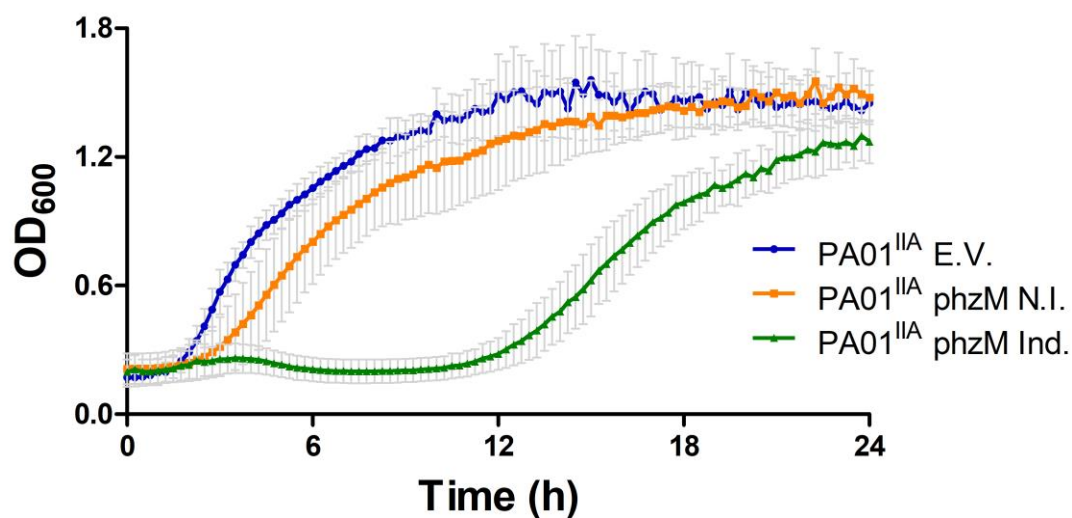


624

625

626 **Supplementary Figure 3. A)** Phage-targeting assay showing the activity of the modified repeat  
627 crRNA constructs. Ten-fold serial dilutions of DMS3 phage and D3 phage were spotted on lawns  
628 of PAO1<sup>IC</sup> expressing either empty vector (top), a crRNA targeting D3 with WT direct repeats  
629 (middle), or a crRNA targeting D3 with modified repeats (bottom). **B)** Phage targeting assay of  
630 five non-deletion self-targeting survivors expressing a D3 phage targeting crRNA. Unsuccessful  
631 targeting of phage indicates a non-functional CRISPR-Cas system in these strains. The parental  
632 PAO1<sup>IC</sup> strain with a functional CRISPR-Cas system was used as a control.





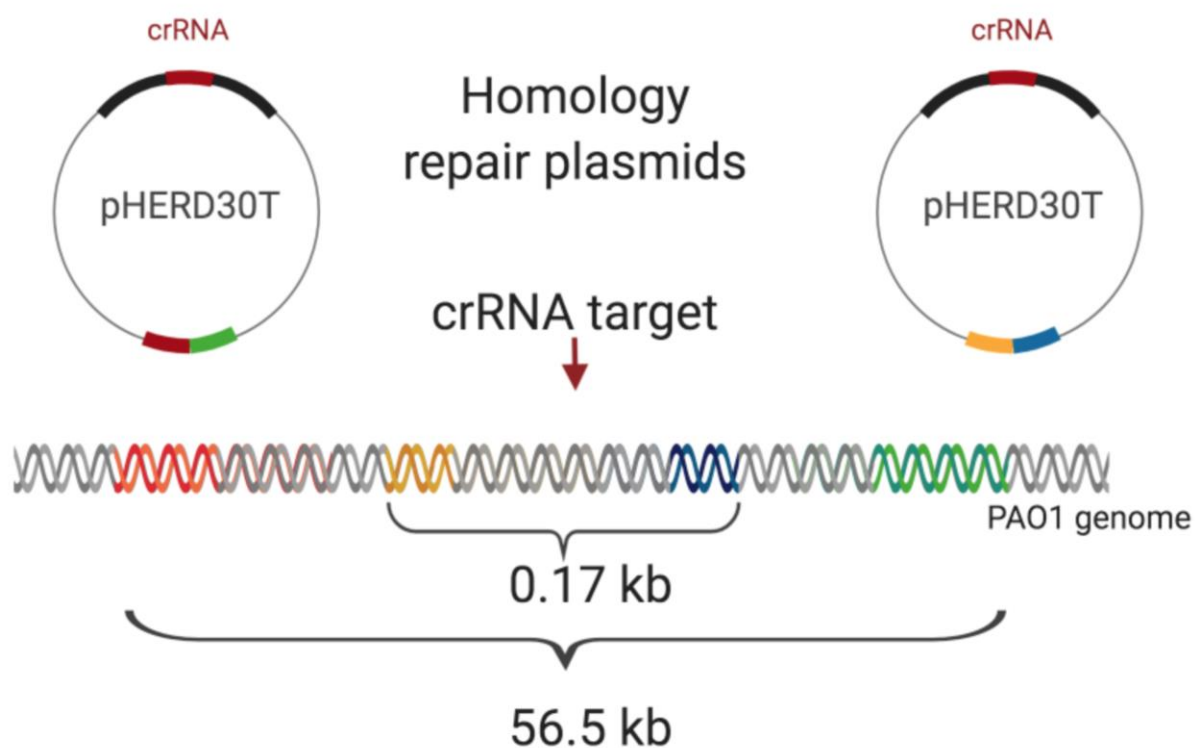
641

642

643 **Supplementary Figure 5.** Growth of self-targeting strains of PAO1<sup>IIA</sup> expressing a self-targeting  
644 crRNA targeting the genome at *phzM* (Ind.). An empty vector (E.V.) and a non-induced *phzM*  
645 targeting strain (N.I.) were used as controls. Mean OD values measured at 600 nm are shown for  
646 8 biological replicates each, error bars indicate SD values.

647



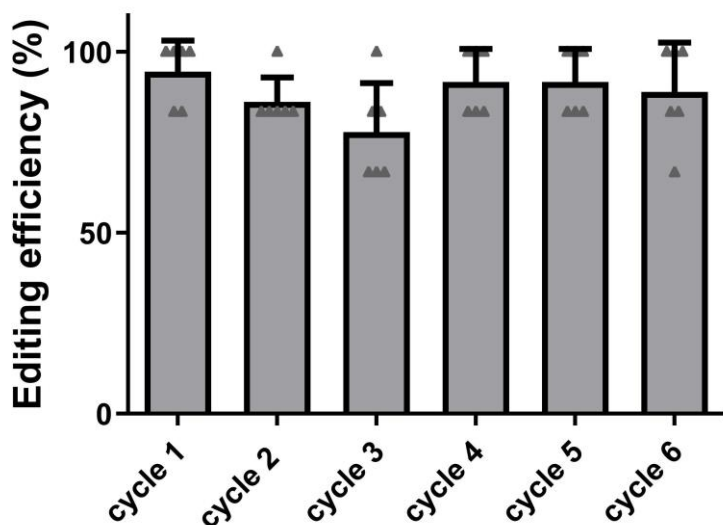


648

649

650 **Supplementary Figure 6.** Schematic overview of the generation of deletions with predetermined  
651 coordinates of various sizes. Sequences with ~400 bp homology to genomic sites (purple and  
652 yellow boxes for the short deletion, red and orange boxes for the long deletion) were cloned into  
653 the vector crRNA vector.

654

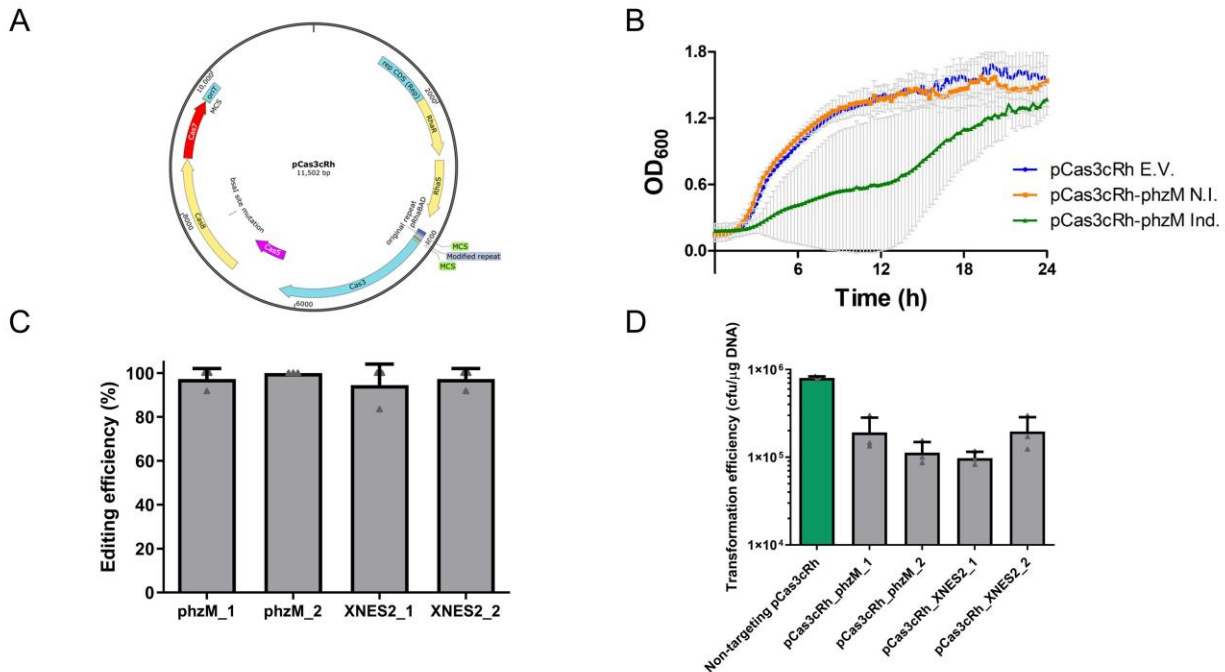


655

656

657 **Supplementary Figure 7.** Deletion efficiencies observed over six cycles of iterative self-targeting.  
658 Six genomic targets were targeted in six different orders. Six survivors were analyzed using site-  
659 specific PCR after each cycle, for a total of 36 analyzed colonies (6\*6) after each cycle.

660

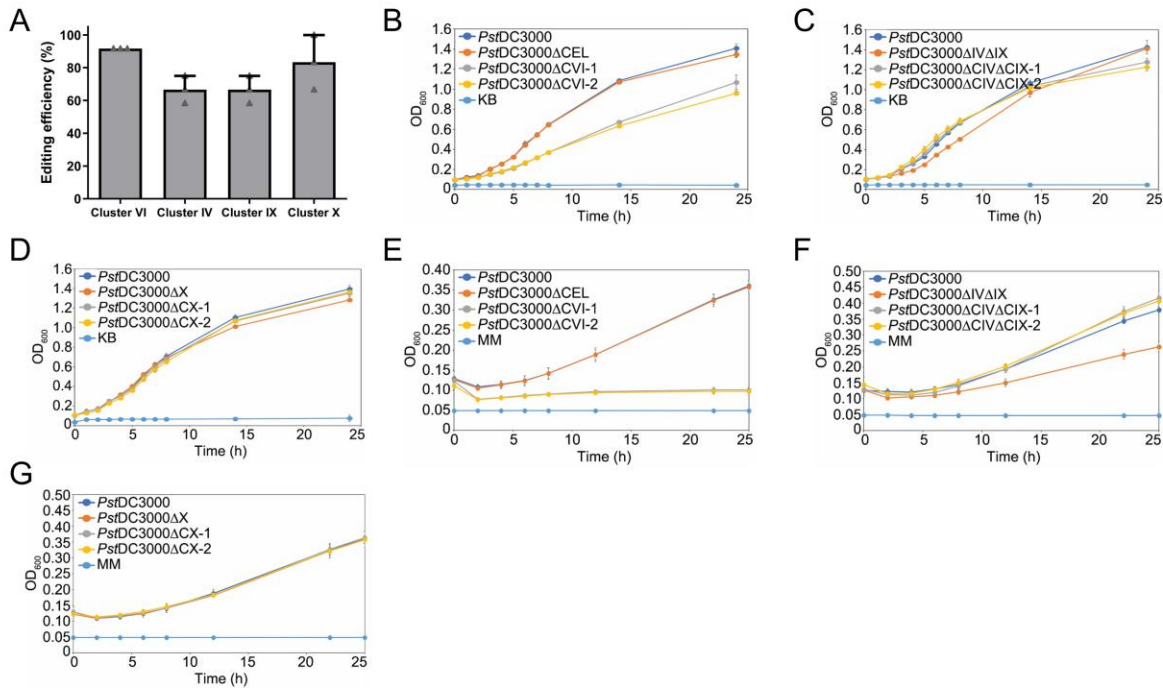


661

662

663 **Supplementary Figure 8. A)** Map of the I-C CRISPR-Cas all-in-one plasmid pCas3cRh carrying  
 664 I-C crRNA and genes *cas3*, *cas5*, *cas8*, and *cas7* under the control of the rhamnose-inducible  
 665 *rhaSR-PrhaBAD* system. **B)** Growth curve of PAO1 transformed with the pCas3cRh vector  
 666 expressing a self-targeting crRNA targeting *phzM* (Ind.). An empty vector (E.V.) and a non-  
 667 induced *phzM* targeting strain (N.I.) were used as controls. Mean OD values measured at 600 nm  
 668 are shown for six biological replicates each. **C)** Deletion efficiencies for WT PAO1 using the all-  
 669 in-one vector pCas3cRh carrying all necessary components of the I-C CRISPR-Cas system.  
 670 Values are averages of three replicates where 12 individual colonies were analyzed using site-  
 671 specific PCR. Error bars show standard deviations. **D)** Transformation efficiencies with self-  
 672 targeting pCas3cRh vectors expressing crRNAs for *phzM* or *XNES2* compared to a non-targeting  
 673 control (green bar) in PAO1. Values are means of 3 replicates each, error bars represent SD  
 674 values.

675



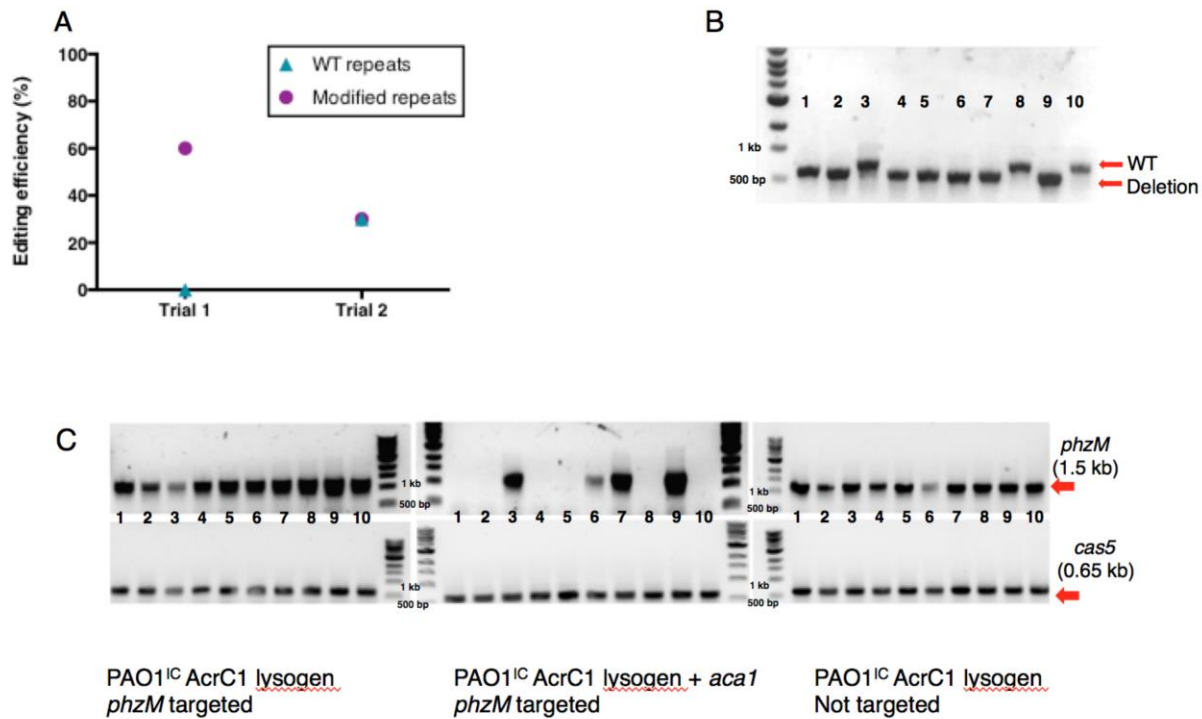
676

677

678 **Supplementary Figure 9. A)** Percentage of survivors with targeted deletions in clusters of non-  
 679 essential virulence effector genes in *P. syringae* pv. *tomato* DC3000. Values are averages of  
 680 three biological replicates where 12 individual colonies were analyzed using site-specific PCR for  
 681 each, error bars show standard deviations. **B)** *In vitro* growth of cluster VI deletion strains in King's  
 682 medium B (KB).  $\Delta$ CEL is the previously published polymutant, while  $\Delta$ CVI-1 and  $\Delta$ CVI-2 are Cas3-  
 683 generated mutants. Error bars represent standard deviation,  $n = 4$ . **C)** *In vitro* growth of cluster  
 684 IV, cluster IX deletion strains in KB.  $\Delta$ CEL is the previously published polymutant, while  
 685  $\Delta$ CIV $\Delta$ CIX-1 and  $\Delta$ CIV $\Delta$ CIX-2 are Cas3-generated mutants. Error bars represent standard  
 686 deviation,  $n = 4$ . **D)** *In vitro* growth of cluster X deletion strains in KB.  $\Delta$ CEL is the previously  
 687 published polymutant, while  $\Delta$ CX-1 and  $\Delta$ CX-2 are Cas3-generated mutants. Error bars represent  
 688 standard deviation,  $n = 4$ . **E)** *In vitro* growth of cluster VI deletion strains in apoplast mimicking  
 689 minimal media (MM).  $\Delta$ CEL is the previously published polymutant, while  $\Delta$ CVI-1 and  $\Delta$ CVI-2 are  
 690 Cas3-generated mutants. Error bars represent standard deviation,  $n = 4$ . **F)** *In vitro* growth of  
 691 cluster IV, cluster IX deletion strains in MM.  $\Delta$ CEL is the previously published polymutant, while  
 692  $\Delta$ CIV $\Delta$ CIX-1 and  $\Delta$ CIV $\Delta$ CIX-2 are Cas3-generated mutants. Error bars represent standard  
 693 deviation,  $n = 4$ . **G)** *In vitro* growth of cluster X deletion strains in MM.  $\Delta$ CEL is the previously  
 694 published polymutant, while  $\Delta$ CX-1 and  $\Delta$ CX-2 are Cas3-generated mutants. Error bars represent  
 695 standard deviation,  $n = 4$ .

696

697



698

699

700 **Supplementary Figure 10. A)** Editing efficiencies for the *Pseudomonas aeruginosa*  
701 environmental isolate naturally expressing the Type I-C *cas* genes, transformed with a plasmid  
702 targeting *phzM* with WT repeats or modified repeats. Each data point represents the fraction of  
703 isolates with the deletion out of ten isolates assayed. **B)** Genotyping results for the *Pseudomonas*  
704 *aeruginosa* environmental isolate using the 0.17 kb HDR template. Larger band corresponds to  
705 the WT sequence, smaller band corresponds to a genome reduced by 0.17 kb. **C)** Genotyping  
706 results of PAO1<sup>IC</sup> AcrC1 lysogens after self-targeting induction in the presence or absence of  
707 *aca1* and a non-targeted control. Ten biological replicates per strain were assayed. gDNA was  
708 extracted from each replicate and PCR analysis for the *phzM* gene (targeted gene, top row of  
709 gels) or *cas5* gene (non-targeted gene, bottom row) was conducted. Only cells that co-expressed  
710 *aca1* with the crRNA showed loss of the *phzM* band, indicating genome editing. All replicates had  
711 a *cas5* band, indicating successful gDNA extraction and target specificity for the *phzM* locus.

712

| Region  | Coordinates       | Size   |
|---------|-------------------|--------|
| XNES 1  | 27535 – 142359    | 114 kb |
| XNES 2  | 143267 – 371151   | 228 kb |
| XNES 3  | 491900 – 606160   | 114 kb |
| XNES 4  | 841825 – 986817   | 145 kb |
| XNES 5  | 1147815 – 1249907 | 102 kb |
| XNES 6  | 1260442 – 1491913 | 232 kb |
| XNES 7  | 1974210 – 2150828 | 176 kb |
| XNES 8  | 2216121 – 2375804 | 160 kb |
| XNES 9  | 2376541 – 2923367 | 546 kb |
| XNES 10 | 2972700 – 3079197 | 106 kb |
| XNES 11 | 3155072 – 3309411 | 154 kb |
| XNES 12 | 3587303 – 3802567 | 216 kb |
| XNES 13 | 3897357 – 4062426 | 165 kb |
| XNES 14 | 4294208 – 4457362 | 163 kb |
| XNES 15 | 4576324 – 4753990 | 178 kb |
| XNES 16 | 6025305 – 6180942 | 156 kb |

713

714

715 **Supplementary Table 1.** Extended, non-essential regions (XNES) of *P. aeruginosa* PAO1  
716 genome with contiguous, individually non-essential genes in a complex laboratory medium  
717 exceeding 100 kb. Data based on a transposon sequencing dataset from Turner *et al.*<sup>27</sup>.

718

719 **Supplementary Table 2.** Genomic coordinates and extent of homologous sequences at genomic  
720 deletion junctions of whole-genome sequenced self-targeting strains of *P. aeruginosa*, *P.*  
721 *syringae*, and *E. coli*. See separate Excel File.

722

| Strain | Edited gene | HR edits (%) | Nontemplated edits (%) | No edits (%) | n  | Designed deletion (bp) | HR template length (left + right, bp) |
|--------|-------------|--------------|------------------------|--------------|----|------------------------|---------------------------------------|
| PA14   | <i>psiF</i> | 100          | 0                      | 0            | 12 | 0.5                    | 600 + 600                             |
| PA14   | <i>rebB</i> | 50           | 50                     | 0            | 16 | 4.1                    | 600 + 600                             |
| z8     | <i>ghlO</i> | 100          | 0                      | 0            | 5  | 0.2                    | 600 + 600                             |
| z8     | <i>mexZ</i> | 75           | 25                     | 0            | 12 | 0.6                    | 722 + 600                             |
| z8     | <i>psiF</i> | 100          | 0                      | 0            | 12 | 0.5                    | 600 + 600                             |
| z8     | <i>qsrO</i> | 29(80)*      | 71                     | 0            | 14 | 0.4                    | 751 + 596                             |
| z8     | <i>teg</i>  | 75           | 25                     | 0            | 4  | 6.3                    | 800 + 809                             |

723

724 **Supplementary Table 3.** Summary of HR-mediated genome editing experiments using the Type  
725 I-F CRISPR-Cas3 system. Genes were targeted for deletion in the strains PA14 and z8.  
726 Experiments targeted 4 single genes and 2 gene blocks, *teg* and *rebB*, that comprise X and Y  
727 genes, respectively. Transformants were classified as 1) 'HR edits' that have the HR designed  
728 deletion; 2) 'non-templated edits' that have a non-designed deletion encompassing the targeted  
729 gene, 3) 'no edits' where the targeted gene is intact. (\*) two colony morphologies with different  
730 editing frequencies were obtained in this experiment.

731



732 **Supplementary Table 4.** List of oligonucleotides (including crRNA sequences) used in the study.  
733 See separate Excel File.

734

735 **References**

- 736 1. Makarova, K. S. *et al.* An updated evolutionary classification of CRISPR-Cas systems. *Nat.*  
737 *Rev. Microbiol.* **13**, 722–736 (2015).
- 738 2. Barrangou, R. *et al.* CRISPR provides acquired resistance against viruses in prokaryotes.  
739 *Science* **315**, 1709–1712 (2007).
- 740 3. Garneau, J. E. *et al.* The CRISPR/Cas bacterial immune system cleaves bacteriophage and  
741 plasmid DNA. *Nature* **468**, 67–71 (2010).
- 742 4. Barrangou, R. & Doudna, J. A. Applications of CRISPR technologies in research and beyond.  
743 *Nat. Biotechnol.* 933–941 (2016) doi:10.1038/nbt.3659.
- 744 5. Wiedenheft, B. *et al.* Structures of the RNA-guided surveillance complex from a bacterial  
745 immune system. *Nature* **477**, 486–489 (2011).
- 746 6. Westra, E. R. *et al.* CRISPR immunity relies on the consecutive binding and degradation of  
747 negatively supercoiled invader DNA by Cascade and Cas3. *Mol. Cell* **46**, 595–605 (2012).
- 748 7. Brouns, S. J. J. *et al.* Small CRISPR RNAs Guide Antiviral Defense in Prokaryotes. *Science*  
749 **321**, 960–964 (2008).
- 750 8. Sinkunas, T. *et al.* Cas3 is a single-stranded DNA nuclease and ATP-dependent helicase in  
751 the CRISPR/Cas immune system. *EMBO J.* **30**, 1335–1342 (2011).
- 752 9. Sinkunas, T. *et al.* In vitro reconstitution of Cascade-mediated CRISPR immunity in  
753 *Streptococcus thermophilus*. *EMBO J.* **32**, 385–394 (2013).
- 754 10. Mulepati, S. & Bailey, S. In vitro reconstitution of an *Escherichia coli* RNA-guided  
755 immune system reveals unidirectional, ATP-dependent degradation of DNA target. *J. Biol.*  
756 *Chem.* **288**, 22184–22192 (2013).
- 757 11. Hochstrasser, M. L. *et al.* CasA mediates Cas3-catalyzed target degradation during  
758 CRISPR RNA-guided interference. *Proc. Natl. Acad. Sci. U. S. A.* **111**, 6618–6623 (2014).
- 759 12. Redding, S. *et al.* Surveillance and Processing of Foreign DNA by the *Escherichia coli*  
760 CRISPR-Cas System. *Cell* **163**, 854–865 (2015).

- 761 13. Xiao, Y., Luo, M., Dolan, A. E., Liao, M. & Ke, A. Structure basis for RNA-guided DNA  
762 degradation by Cascade and Cas3. *Science* **361**, eaat0839 (2018).
- 763 14. Esvelt, K. M. & Wang, H. H. Genome-scale engineering for systems and synthetic  
764 biology. *Mol. Syst. Biol.* **9**, (2013).
- 765 15. Montalbano, A., Canver, M. C. & Sanjana, N. E. High-Throughput Approaches to  
766 Pinpoint Function within the Noncoding Genome. *Mol. Cell* **68**, 44–59 (2017).
- 767 16. Vercoe, R. B. *et al.* Cytotoxic Chromosomal Targeting by CRISPR/Cas Systems Can  
768 Reshape Bacterial Genomes and Expel or Remodel Pathogenicity Islands. *PLOS Genet* **9**,  
769 e1003454 (2013).
- 770 17. Li, Y. *et al.* Harnessing Type I and Type III CRISPR-Cas systems for genome editing.  
771 *Nucleic Acids Res.* **44**, e34–e34 (2016).
- 772 18. Pyne, M. E., Bruder, M. R., Moo-Young, M., Chung, D. A. & Chou, C. P. Harnessing  
773 heterologous and endogenous CRISPR-Cas machineries for efficient markerless genome  
774 editing in *Clostridium*. *Sci. Rep.* **6**, 25666 (2016).
- 775 19. Zhang, J., Zong, W., Hong, W., Zhang, Z.-T. & Wang, Y. Exploiting endogenous  
776 CRISPR-Cas system for multiplex genome editing in *Clostridium tyrobutyricum* and engineer  
777 the strain for high-level butanol production. *Metab. Eng.* doi:10.1016/j.ymben.2018.03.007.
- 778 20. Maikova, A., Kreis, V., Boutserin, A., Severinov, K. & Soutourina, O. Using endogenous  
779 CRISPR-Cas system for genome editing in the human pathogen *Clostridium difficile*. *Appl.*  
780 *Environ. Microbiol.* AEM.01416-19 (2019) doi:10.1128/AEM.01416-19.
- 781 21. Hidalgo-Cantabrana, C., Goh, Y. J., Pan, M., Sanozky-Dawes, R. & Barrangou, R.  
782 Genome editing using the endogenous type I CRISPR-Cas system in *Lactobacillus crispatus*.  
783 *Proc. Natl. Acad. Sci. U. S. A.* **116**, 15774–15783 (2019).
- 784 22. Dolan, A. E. *et al.* Introducing a Spectrum of Long-Range Genomic Deletions in Human  
785 Embryonic Stem Cells Using Type I CRISPR-Cas. *Mol. Cell* **74**, 936-950.e5 (2019).

- 786 23. Pickar-Oliver, A. *et al.* Targeted transcriptional modulation with type I CRISPR–Cas  
787 systems in human cells. *Nat. Biotechnol.* 1–9 (2019) doi:10.1038/s41587-019-0235-7.
- 788 24. Nam, K. H. *et al.* Cas5d Protein Processes Pre-crRNA and Assembles into a Cascade-  
789 like Interference Complex in Subtype I-C/Dvulg CRISPR-Cas System. *Structure* **20**, 1574–  
790 1584 (2012).
- 791 25. Hochstrasser, M. L., Taylor, D. W., Kornfeld, J. E., Nogales, E. & Doudna, J. A. DNA  
792 Targeting by a Minimal CRISPR RNA-Guided Cascade. *Mol. Cell* **63**, 840–851 (2016).
- 793 26. Marino, N. D. *et al.* Discovery of widespread type I and type V CRISPR-Cas inhibitors.  
794 *Science* **362**, 240–242 (2018).
- 795 27. Turner, K. H., Wessel, A. K., Palmer, G. C., Murray, J. L. & Whiteley, M. Essential  
796 genome of *Pseudomonas aeruginosa* in cystic fibrosis sputum. *Proc. Natl. Acad. Sci.* **112**,  
797 4110–4115 (2015).
- 798 28. Meek, D. W. & Hayward, R. S. Nucleotide sequence of the rpoA-rpiQ DNA of  
799 *Escherichia coli*: a second regulatory binding site for protein S4? *Nucleic Acids Res.* **12**,  
800 5813–5821 (1984).
- 801 29. Chayot, R., Montagne, B., Mazel, D. & Ricchetti, M. An end-joining repair mechanism in  
802 *Escherichia coli*. *Proc. Natl. Acad. Sci.* **107**, 2141–2146 (2010).
- 803 30. Buell, C. R. *et al.* The complete genome sequence of the *Arabidopsis* and tomato  
804 pathogen *Pseudomonas syringae* pv. tomato DC3000. *Proc. Natl. Acad. Sci. U. S. A.* **100**,  
805 10181–10186 (2003).
- 806 31. Lindeberg, M., Cunnac, S. & Collmer, A. *Pseudomonas syringae* type III effector  
807 repertoires: last words in endless arguments. *Trends Microbiol.* **20**, 199–208 (2012).
- 808 32. Kvitko, B. H. *et al.* Deletions in the repertoire of *Pseudomonas syringae* pv. tomato  
809 DC3000 type III secretion effector genes reveal functional overlap among effectors. *PLoS*  
810 *Pathog.* **5**, e1000388 (2009).

- 811 33. Cady, K. C., Bondy-Denomy, J., Heussler, G. E., Davidson, A. R. & O'Toole, G. A. The  
812 CRISPR/Cas adaptive immune system of *Pseudomonas aeruginosa* mediates resistance to  
813 naturally occurring and engineered phages. *J. Bacteriol.* **194**, 5728–5738 (2012).
- 814 34. Bondy-Denomy, J., Pawluk, A., Maxwell, K. L. & Davidson, A. R. Bacteriophage genes  
815 that inactivate the CRISPR/Cas bacterial immune system. *Nature* **493**, 429–432 (2013).
- 816 35. Rauch, B. J. *et al.* Inhibition of CRISPR-Cas9 with Bacteriophage Proteins. *Cell* **168**,  
817 150-158.e10 (2017).
- 818 36. Richter, C. *et al.* Priming in the Type I-F CRISPR-Cas system triggers strand-  
819 independent spacer acquisition, bi-directionally from the primed protospacer. *Nucleic Acids*  
820 *Res.* **42**, 8516–8526 (2014).
- 821 37. Rollins, M. F. *et al.* Cas1 and the Csy complex are opposing regulators of Cas2/3  
822 nuclease activity. *Proc. Natl. Acad. Sci. U. S. A.* **114**, E5113–E5121 (2017).
- 823 38. Pósfai, G. *et al.* Emergent properties of reduced-genome *Escherichia coli*. *Science* **312**,  
824 1044–1046 (2006).
- 825 39. Fehér, T., Papp, B., Pál, C. & Pósfai, G. Systematic Genome Reductions: Theoretical  
826 and Experimental Approaches. *Chem. Rev.* **107**, 3498–3513 (2007).
- 827 40. Csörgő, B., Nyerges, Á., Pósfai, G. & Fehér, T. System-level genome editing in  
828 microbes. *Curr. Opin. Microbiol.* **33**, 113–122 (2016).
- 829 41. Képès, F. *et al.* The layout of a bacterial genome. *FEBS Lett.* **586**, 2043–2048 (2012).
- 830 42. Ghosh, S. & O'Connor, T. J. Beyond Paralogs: The Multiple Layers of Redundancy in  
831 Bacterial Pathogenesis. *Front. Cell. Infect. Microbiol.* **7**, (2017).
- 832 43. Kowalczykowski, S. C. & Eggleston, A. K. Homologous Pairing and Dna Strand-  
833 Exchange Proteins. *Annu. Rev. Biochem.* **63**, 991–1043 (1994).
- 834 44. Bowater, R. & Doherty, A. J. Making Ends Meet: Repairing Breaks in Bacterial DNA by  
835 Non-Homologous End-Joining. *PLOS Genet* **2**, e8 (2006).

- 836 45. Hnisz, D. *et al.* Super-enhancers in the control of cell identity and disease. *Cell* **155**,  
837 934–947 (2013).
- 838 46. Tuladhar, R. *et al.* CRISPR-Cas9-based mutagenesis frequently provokes on-target  
839 mRNA misregulation. *Nat. Commun.* **10**, 1–10 (2019).
- 840 47. Smits, A. H. *et al.* Biological plasticity rescues target activity in CRISPR knock outs. *Nat.*  
841 *Methods* 1–7 (2019) doi:10.1038/s41592-019-0614-5.
- 842 48. Choi, K.-H. *et al.* A Tn7-based broad-range bacterial cloning and expression system.  
843 *Nat. Methods* **2**, 443–448 (2005).
- 844 49. Stover, C. K. *et al.* Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an  
845 opportunistic pathogen. *Nature* **406**, 959 (2000).
- 846 50. Choi, K.-H. & Schweizer, H. P. mini-Tn7 insertion in bacteria with single attTn7 sites:  
847 example *Pseudomonas aeruginosa*. *Nat. Protoc.* **1**, 153–161 (2006).
- 848 51. Blattner, F. R. *et al.* The complete genome sequence of *Escherichia coli* K-12. *Science*  
849 **277**, 1453–1462 (1997).
- 850 52. Qiu, D., Damron, F. H., Mima, T., Schweizer, H. P. & Yu, H. D. PBAD-Based Shuttle  
851 Vectors for Functional Analysis of Toxic and Highly Regulated Genes in *Pseudomonas* and  
852 *Burkholderia* spp. and Other Bacteria. *Appl. Environ. Microbiol.* **74**, 7422–7426 (2008).
- 853 53. Gibson, D. G. *et al.* Enzymatic assembly of DNA molecules up to several hundred  
854 kilobases. *Nat. Methods* **6**, 343–345 (2009).
- 855 54. Meisner, J. & Goldberg, J. B. The *Escherichia coli* rhaSR-PrhaBAD Inducible Promoter  
856 System Allows Tightly Controlled Gene Expression over a Wide Range in *Pseudomonas*  
857 *aeruginosa*. *Appl. Environ. Microbiol.* **82**, 6715–6727 (2016).
- 858 55. Borges, A. L. *et al.* Bacteriophage Cooperation Suppresses CRISPR-Cas3 and Cas9  
859 Immunity. *Cell* **174**, 917-925.e10 (2018).
- 860 56. Nyerges, Á. *et al.* Directed evolution of multiple genomic loci allows the prediction of  
861 antibiotic resistance. *Proc. Natl. Acad. Sci.* **115**, E5726–E5735 (2018).

- 862 57. Huynh, T. V., Dahlbeck, D. & Staskawicz, B. J. Bacterial blight of soybean: regulation of  
863 a pathogen gene determining host cultivar specificity. *Science* **245**, 1374–1377 (1989).
- 864 58. Kropinski, A. M. Sequence of the Genome of the Temperate, Serotype-Converting,  
865 *Pseudomonas aeruginosa* Bacteriophage D3. *J. Bacteriol.* **182**, 6066–6074 (2000).
- 866 59. Budzik, J. M., Rosche, W. A., Rietsch, A. & O'Toole, G. A. Isolation and Characterization  
867 of a Generalized Transducing Phage for *Pseudomonas aeruginosa* Strains PAO1 and PA14.  
868 *J. Bacteriol.* **186**, 3270–3273 (2004).
- 869 60. Alikhan, N.-F., Petty, N. K., Ben Zakour, N. L. & Beatson, S. A. BLAST Ring Image  
870 Generator (BRIG): simple prokaryote genome comparisons. *BMC Genomics* **12**, 402 (2011).
- 871
- 872