

1 **Activation of programmed cell death and counter-defense functions of phage accessory**  
2 **genes**

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33 **Viruses have been evolving host-modifying factors for billions of years. Genomes**  
34 **of bacterial and archaeal viruses are replete with fast-evolving, uncharacterized accessory**  
35 **genes (AGs), most of which likely antagonize host defenses or other viruses<sup>1,2</sup>. Systematic**  
36 **investigation of AGs could uncover a multitude of biological mechanisms involved in**  
37 **virus-host competition, but AG identification in genomic databases remains a challenge.**  
38 **We developed an integrated computational and high-throughput discovery platform to**  
39 **identify AGs in virus genomes and assay their functions in complementary phage**  
40 **infection-dependent and -independent contexts. Our approach showcases how phages**  
41 **interact with the principal layers of antiviral immunity, including cell surface modifications,**  
42 **restriction systems, and abortive infection (Abi) mechanisms, which operate**  
43 **simultaneously in the same host. We discovered multiple Enterobacteriophage AGs**  
44 **associated with counter-defense functions that activate rather than inhibit antiviral**  
45 **immunity in cells, including the surprising finding that anti-restriction AGs elicit**  
46 **programmed cell death (PCD) activity of some restriction-modification (R-M) systems. We**  
47 **propose that counter-defense AGs that trigger PCD create a conundrum for phages**  
48 **whereby keeping the AGs causes PCD but losing them exposes the phage to restriction**  
49 **by bacteria. Strategies employed by viruses to avoid this double jeopardy could be an**  
50 **important factor in virus evolution that remains to be explored.**

## 51 Introduction

52  
53 To a large extent, the evolution of life is a story of the virus-host arms race<sup>3</sup>. As hosts  
54 evolve elaborate antiviral defense mechanisms, viruses retort with equally versatile counter-  
55 defenses. Signatures of this arms-race are evident in prokaryotic genomes, where diverse  
56 defense systems tend to be encoded within 'defense islands'<sup>4</sup>, and in virus genomes, where  
57 hotspots known as accessory regions (ARs) contain clusters of highly variable anti-defense  
58 genes<sup>1,2</sup> (Figure 1a). These accessory genes (AGs) are at the forefront of virus-host co-  
59 evolution, and many of them are known or predicted to interface with host defenses<sup>2</sup>.  
60 Bacteriophage AGs are typically non-essential, undergo frequent horizontal transfer, and  
61 diversify rapidly under enhanced evolutionary pressure<sup>1</sup>. The characterized AGs are involved in  
62 antagonizing other phages or bacterial defense systems such as restriction-modification (R-M)<sup>5</sup>  
63 and CRISPR-Cas<sup>6</sup>, but a vast pool of AGs in rapidly growing (meta)genomic sequence  
64 databases remains unexplored<sup>7</sup>. Determining the identity and functions of AGs systematically  
65 remains an important and unaddressed problem.

66 Comparative genomic approaches can exploit the highly organized architecture of virus  
67 genomes to identify evolutionary hotspots. However, ARs have so far only been characterized in  
68 isolated phage-families<sup>1,8-12</sup>, and AGs have not been studied at scale. We therefore developed a  
69 scalable, high-throughput platform to identify and functionally test AGs from large datasets of  
70 phage genomes. Here, we focus on AGs that can inhibit, and in some cases, also activate  
71 bacterial immunity. We uncover a multi-layered defense architecture and extensive co-evolution  
72 of defense and counter-defense mechanisms, including an R-M system that can trigger PCD in  
73 addition to its canonical defense functions and another R-M locus providing decoy immunity  
74 previously only known in eukaryotes<sup>13</sup>. On the phage side, we discover proteins that alter host  
75 metabolism and modify the cell surface, or inhibit antiviral restriction. Moreover, we identify  
76 several counter-defense AGs that trigger abortive infection (Abi) responses and propose that  
77 bacteria routinely exploit phage AGs to sense infection.

## 78 Results and Discussion

### 79 **A high throughput screening platform for identifying and testing virus accessory genes**

80  
81 Given that AGs are usually defined within a cluster of closely-related viruses<sup>8-11</sup>, AG  
82 detection is highly sensitive to the genome clustering strategy. We therefore sought to identify  
83 ARs without *a priori* sequence comparison or clustering. All pairwise combinations of all phage  
84 genes were evaluated sequentially (Figure 1b) to determine if any pair of conserved genes  
85 bounds an AR (Supplementary File 1). We also did not rely on any gene ontology to identify  
86 AGs (eg. genes similar to or encoded next to known counter-defense factors). Starting with an  
87 initial set of 1706 Enterobacteriophage genomes, 2014 non-redundant ARs containing 10888  
88 putative AGs were identified in 1217 phages. All ARs were then scored and ranked by diversity  
89 (See Methods). A sample AR is shown in Figure 1c. A typical AR tallied 11-30 unique AGs  
90 cumulatively across multiple related phages (we discarded ARs encountered in <6 genomes).  
91 AGs were mostly of ~50-100 aa in length. A typical phage genome contained <5 ARs and <10  
92 AGs, while some phages encompassed many more AGs (Figure 1d).

93  
94 We manually inspected the 200 highest-scoring ARs and chose multiple (2-5) AGs from  
95 62 of these (see Supplementary File 2 for detailed notes on each AR and rationale for AG  
96 selection). We synthesized 200 AGs, of which 54 were from ARs containing known counter-  
97 defense factors (Supplementary File 3), whereas 135 AGs were from ARs consisting entirely of  
98 genes of unknown function. Eleven phage genes with previously elucidated functions were  
99 included as controls (Abc2, ArdA, Arn, Cor, Dmd, Imm, Ip1, Ocr, Ral, SieA, Stp). To identify AG  
100 function(s), the genes were barcoded and chromosomally integrated under an IPTG-inducible

101 promoter in a subset of the *E. coli* Reference (ECOR) strains<sup>14</sup> (one AG per cell) in pools.  
102 Fitness was measured in triplicate by next-generation sequencing of AG-specific barcodes upon  
103 AG-expression (Figure 1e, Supplementary File 4).

104

### 105 **Phage AGs trigger programmed cell death responses in bacteria**

106 Several AGs produced “conditional lethal” phenotypes, imposing a severe fitness burden  
107 in a minority of strains, suggestive of PCD induction (Figure 2a). Although most known triggers of  
108 abortive defense systems are conserved phage proteins<sup>15-18</sup>, we hypothesized that these strains  
109 harbor systems that sense variable phage proteins encoded by AGs. Surprisingly, several of  
110 these trigger AGs were known anti-restriction factors. Anti-restriction-induced cell death  
111 mechanisms might serve as a backup to thwart phage-encoded counter-defense strategies. PCD  
112 in these wild strains is triggered by some R-M inhibitors but not others, even discriminating  
113 between the two DNA mimics Ocr<sup>19</sup> and ArdA<sup>20</sup> (Supplementary Figure 1a). A novel AG, *orf7*  
114 (hereafter, *aop1* for Activator Of PCD) also triggers PCD in ECOR55 and 66 similarly to Ocr and  
115 ArdA, but does not inhibit Type I R-M in MG1655 (Supplementary Figure 1b). These observations  
116 suggest a diversity of PCD mechanisms.

117 We transformed the respective wild hosts with the trigger AGs and confirmed that they  
118 induce PCD (Figure 2b). We then performed genome-wide transposon suppressor screens to  
119 map the loci causing PCD (Figure 2c). Transposon insertions in the causal system would be  
120 expected to disable PCD and allow survival of the bacteria despite heterologous expression of a  
121 trigger AG. We identified putative PCD systems in all tested hosts, except for ECOR22 with *orf98*  
122 and ECOR66 with *orf1*, *orf7*, or *orf169*, where no suppressor mutants could be obtained  
123 (Supplementary File 5).

124

### 125 **Restriction-modification systems can elicit PCD**

126 Surprisingly, a Type III R-M system in ECOR22 (hereafter, EcoR22I) was required for cell-  
127 death in response to Ocr (Figure 2c), the DNA mimic that blocks both Type I and Type III R-M<sup>21</sup>.  
128 R-M defenses are ubiquitous in bacteria, and so are anti-restriction factors in phages<sup>22</sup>. In  
129 response, bacterial anti-anti-restriction mechanisms such as retrons<sup>23</sup>, PARIS<sup>24</sup>, and PrrC<sup>25</sup>  
130 provide backup defense. Our results suggest that in ECOR22, the frontline (R-M) and backup  
131 (PCD) defense functions coalesced into an integrated defense system. To test this hypothesis  
132 directly, we reconstituted the PCD and R-M activities of EcoR22I in a model *E. coli* strain.

133 In its native host, the *mod* and *res* genes of EcoR22I are associated with an SNF2-like  
134 helicase related to the ancillary DISARM helicase, *drmD*<sup>26</sup>, and all 3 genes are encoded in a P4  
135 satellite prophage hotspot<sup>24</sup>. However, transposon insertions that suppressed Ocr-toxicity in  
136 ECOR22 were found only in *mod* and *res* (Figure 2c, Supplementary Figure 2). We overexpressed  
137 only the *mod* and *res* genes of EcoR22I in a lab strain of *E. coli* and found that EcoR22I caused  
138 PCD upon Ocr induction but not upon induction of ArdA or Aop1, consistent with the behavior of  
139 the wild host (Figure 2d). Ocr was not toxic in the lab strain on its own (see Figure 3a). An F54V  
140 Ocr mutant, previously reported to evade detection by PARIS<sup>24</sup>, also lost the ability to trigger  
141 EcoR22I PCD, as did various other Ocr mutants<sup>19</sup> (Supplementary Figure 1c). Because EcoR22I  
142 is the only R-M system in the lab strain and in ECOR22, EcoR22I likely detects Ocr directly.

143 Next, we tested whether the restriction activity of EcoR22I could block phage infection.  
144 We constructed an active-site mutant of the PD-(D/E)xK restriction nuclease (*res\**: D1024A,  
145 D1043A double mutant) and compared immunity conferred by wild type and *res\** EcoR22I against  
146 a panel of phages. EcoR22I but not the catalytic site mutant blocked  $\lambda$ vir (Figure 2e). Furthermore,  
147  $\lambda$ vir passaged through the *res\** host (and thus modified by the EcoR22I methyltransferase)  
148 regained the ability to infect cells carrying wildtype EcoR22I, indicating that EcoR22I indeed  
149 restricts only unmodified  $\lambda$ vir DNA.

150 We then tested if the immune (R-M) and PCD functions of EcoR22I could be uncoupled.  
151 EcoR22I *mod* and *res* individually did not induce PCD when co-expressed with Ocr, indicating  
152 that PCD induction is a property of the complex (Figure 2f). The *res*\* nuclease-dead mutation of  
153 EcoR22I that completely abrogated  $\lambda$ vir restriction also decreased PCD by 100-fold, although it  
154 did not fully abolish Ocr-induced toxicity (Figure 2f). These results show that the EcoR22I PD-  
155 (D/E)xK nuclease motif is involved in both R-M and PCD but does not appear to be fully  
156 responsible for the latter.

157 Thus, EcoR22I mounts both a targeted antiviral defense and a PCD response in the  
158 presence of certain phage AG products (Figure 2g). Coupling restriction and PCD could allow  
159 cells to respond dynamically depending on the progress of the infection<sup>27</sup>.

160

### 161 **Decoy immunity in bacteria**

162 A second R-M-like system also elicited PCD in the presence of anti-R-M AGs. This locus  
163 in ECOR55 contains *hsdS* and *hsdM* genes typical of Type I R-M but lacks the nuclease (*hsdR*),  
164 and instead has a tightly linked gene that encodes a 61aa protein. This small gene was identified  
165 in our suppressor screens as the PCD-inducing effector activated by Ocr, Aop1, and ArdA (Figure  
166 3a). We named this system Ronin, after the master-less samurai in the 1962 Japanese film,  
167 *Harakiri*. Ronin induces cell suicide in ECOR55 when Ocr, ArdA, or Aop1 are expressed (Figure  
168 2b), but only responds to ArdA and Aop1 when cloned into a lab strain (Figure 3b).

169 Several lines of evidence suggest that the 61aa protein (hereafter, RonA) in Ronin is the  
170 PCD effector. The presence of a predicted transmembrane helix suggests RonA might disrupt  
171 membrane integrity (Figure 3c). Over-expression of *ronA* alone is lethal, but this effect is  
172 suppressed by HsdMS co-expression (Figure 3d). Ronin activity is independent of the  
173 methyltransferase function of HsdMS because PCD was not abrogated by an F287G mutation in  
174 motif IV (NPPF) of the *hsdM* catalytic domain<sup>28</sup> (Figure 3e). However, removal of *ronA* inactivates  
175 Ronin, such that anti-restriction proteins no longer trigger PCD (Figure 3e). The 235-bp non-  
176 coding region upstream of *ronA* also contributes an unknown, essential functionality  
177 (Supplementary Figure 3). Live cell imaging showed that Ronin activation arrested growth without  
178 lysis, consistent with RonA-mediated inner-membrane disruption as a PCD mechanism (Figure  
179 3f). As *ronA* is not lethal when co-expressed with HsdM and HsdS (hereafter, HsdMS), we surmise  
180 that its activity is sequestered or suppressed by HsdMS. This model is reminiscent of PrrC  
181 activation upon blocking of EcoPrrI restriction by T4-encoded peptide Stp. Similarly to Stp binding  
182 EcoPrrI and releasing the Abi anticodon nuclease PrrC<sup>25</sup>, an anti-restriction protein could bind  
183 Ronin HsdMS to release RonA, the PCD effector.

184 Examination of the gene neighborhoods of *ronA* homologs showed that they are always  
185 linked to *hsdS* and *hsdM* genes and occur in two predominant genetic architectures. Ronin is  
186 either linked to an atypical Type I/Type IV composite R-M system or to a BREX system (Figure  
187 3g). The BREX-linked architecture is exclusive to *E. coli*. Most (77%) Ronin-encoding *E. coli*  
188 genomes contain no Type I R-M systems anywhere in the genome, and therefore lack *hsdR* genes  
189 (Supplementary File 6). The predominantly nuclease-lacking configuration in *E. coli* suggests that  
190 PCD is Ronin's primary function. This is reminiscent of decoy immunity (especially common in  
191 plants), where a host factor mimics the target of a pathogen effector. The effector then binds the  
192 decoy instead of its actual target, and the decoy alerts the host to the presence of the pathogen<sup>13</sup>.

193 Thus, Ronin appears to be a Type I R-M derivative that lost the defense nuclease but  
194 gained the ability to sense anti-restriction effectors and trigger PCD. Ronin encodes the canonical  
195 target of Ocr/ArdA, the Type I R-M HsdMS complex, and its activation likely depends on these  
196 anti-restriction proteins binding HsdMS (Figure 3h). Consistent with this, Ronin could still sense  
197 the Ocr F54V mutant that evades PARIS and EcoR22I but retains anti-restriction activity<sup>24</sup>.  
198 Surprisingly, however, Ronin also responded to various Ocr mutants that lost their anti-restriction  
199 activity (Supplementary Figure 1c). Because it almost always appears linked to an R-M or BREX



200 system, both of which can be blocked by *Ocr*<sup>29</sup>, Ronin might drive evolution of *Ocr*-encoding  
201 phages to a BREX/R-M susceptible state through the loss of *Ocr*. Decoy immunity could thus be  
202 a powerful host strategy to constrain virus escape from restriction systems in bacteria.

### 203 204 **Counter-defense-associated AGs trigger Abi mechanisms encoded in prophages**

205 We identified three prophage-encoded Abi loci that induced PCD in response to counter-  
206 defense-associated AGs (Supplementary File 5). First, we found that heterologous *orf116*  
207 expression triggered PCD in ECOR15. Transposon-insertions identified the P4 satellite prophage  
208 (especially the ~100-bp intergenic region marked by a star in Figure 4a) as the Abi locus. *orf116*  
209 is the poorly characterized *abc1* gene of phage P22 that is adjacent to the RecBCD inhibitor *abc2*  
210 (Figure 4a)<sup>30</sup>. We cloned the P4 satellite prophage into a plasmid and reconstituted its PCD effect  
211 in a lab strain. P4 cannot be induced without a co-resident P2 prophage (which was absent in the  
212 lab strain), ruling out prophage-induction as the cause of PCD. *Abc1* contains a helix-turn-helix  
213 (HTH) domain typical of DNA-binding proteins (Supplementary File 7). Thus, *Abc1* might trigger  
214 PCD by interfering with P4 transcriptional regulation, perhaps upregulating the lethal gene *kil*.

215 Second, the *gop-beta-cll* Abi system is a toxin-antitoxin-like locus located in the P4  
216 hotspot<sup>24,31</sup> with apparent anti-phage activity<sup>24</sup>. Our suppressor screens showed that *gop-beta-cll*  
217 toxicity is activated by *orf184*, which is widely distributed among coliphages and is present in the  
218 same AR with the anti-restriction gene *ral* (Figure 4b). We cloned *gop-beta-cll* into a lab strain  
219 and confirmed that *orf184* triggered PCD (Figure 4b).

220 Finally, a distinct prophage-encoded system in ECOR61 inhibited growth upon expression  
221 of *orf24*, which is narrowly distributed and present in the same AR with the anti-restriction gene  
222 *ocr* (Figure 4c). We found suppressor transposon-insertions in three prophage genes, including  
223 an *lcd*-like cell-division-inhibitor with a long N-terminal extension (Superfamily cl41269). However,  
224 expression of all 6 linked genes (including a DNA primase) was necessary to reconstitute PCD in  
225 a lab strain, still yielding a muted effect (Figure 4c) compared to that in the native host (Figure  
226 2b). The *lcd*-like protein is the likely effector because PCD induction was abrogated upon its  
227 removal (Figure 4c). These findings demonstrate that prophages can activate superinfection  
228 exclusion mechanisms in response to highly variable phage AGs.

### 229 230 **Phage AGs disable host defenses**

231 In the previous sections, we show that phage AGs can activate immune pathways that  
232 lead to cell death or dormancy. Next, we wondered how AGs might affect the progress of phage  
233 infections in ECOR strains. We layered phage infection onto our AG screens, measuring fitness  
234 in the presence or absence of infection by 8 model phages (Figure 1e). Forty-five phage-host  
235 combinations were selected where phage replicative titers were highly attenuated compared to  
236 growth on a sensitive lab strain (Supplementary Figure 4a). This phenotype suggests that an  
237 antiviral mechanism was blocking phage replication, such that expressing an AG that inhibits  
238 this defense system could alleviate restriction. Although 8 model phages were used in our  
239 infection-based screens, the AGs were taken from hundreds of diverse phage genomes (see  
240 Figure 1).

241 To identify AGs with counter-defense phenotypes, we compared infected samples with  
242 uninfected controls and identified several host-phage-AG combinations that were depleted from  
243 the infected pools (Figure 5a). Each AG that sensitized any wild strain to infection was then  
244 individually validated in that strain against the entire phage set. In these validation experiments,  
245 some additional phage-host combinations that were omitted in the initial screen also displayed  
246 AG counter-defense phenotypes (Supplementary Figure 4b). In all, increased plaquing of  
247 several phages was observed upon expression of 3 known Type I R-M inhibitors (*Ocr*, *Ral*,  
248 *ArdA*), 6 novel AGs, and the T4 internal protein 2 (*IplI*) in a total of 28 phage-host combinations.  
249 These AGs likely antagonize host factors that inhibit phage reproduction.

250 To identify antiviral mechanisms inhibited by AGs, we employed two unbiased  
251 approaches: (1) transposon-knockout screens to pinpoint gene disruptions in hosts that  
252 phenocopy AG expression (i.e. increased phage infection) (Supplementary File 8) and (2)  
253 affinity-purification and mass spectrometry (AP-MS) of AGs to identify binding partners in the  
254 wild strains (Supplementary File 9). We performed these assays for every host found to be  
255 sensitized to infection by any AG.

### 256 **AGs antagonize conserved host pathways and barrier defenses**

257 Six novel AGs – *orf48*, *orf63*, *orf92*, *orf116* (*abc1*, see above), *orf126*, and *orf148* – all  
258 exhibited counter-defense phenotypes in multiple host strains. Because the broad-spectrum  
259 effects of these AGs did not correlate with the variable defense repertoires of these strains  
260 (Supplementary Figure 4c), we sought to determine whether they targeted conserved host  
261 antiviral pathways.

262 Genome-wide knockout screens indicated that disrupting the host O-antigen or capsule  
263 genes phenocopied the phage-sensitization effects of all 6 broad-spectrum counter-defense  
264 AGs with almost all tested phages (Supplementary File 8). Some of the most pronounced  
265 phenotypes we observed were that transposon-mediated O-antigen disruption (Figure 5b) or  
266 expression of *orf48*, *orf63*, or *orf92* (Figure 5c) both converged on the same effect, namely,  
267 enhanced infection by phage T5 (which does not natively encode these AGs). We hypothesized  
268 that T5 adsorption was blocked by the wild-type O-antigen structures in these hosts, but  
269 heterologous AG expression enabled infection by modifying or removing the O-antigen. We  
270 therefore examined LPS preparations from every host where any AG exhibited a counter-  
271 defense phenotype. Hosts expressing *orf48*, *orf63*, or *orf92* exhibited subtle downshifts in the O-  
272 antigen banding pattern (Figure 5d), as observed previously in some seroconverting lysogens  
273 as a means to exclude superinfecting phages<sup>32</sup>. Specifically, the following host-AG  
274 combinations showed visible downshifts in O-antigen bands: *orf63* and *orf92* in ECOR1, *orf48*  
275 and *orf92* in ECOR3, and *orf48* and *orf63* in ECOR22. These downshifts correlate with the host-  
276 AG pairs where T5 infection was especially strongly ( $10^{5-6}$ -fold) enhanced (Figure 5c). We  
277 reasoned that the AGs might interfere with O-antigen biosynthesis, and tentatively named *orf48*,  
278 *orf63* and *orf92*, *gnarl1*, 2, and 3 respectively, after the skin-eating demon in the popular TV  
279 series *Buffy the Vampire Slayer*.

280 All known seroconverting prophages modify O-antigens using enzymes such as  
281 glycosyltransferases and acetyltransferases<sup>32-36</sup>. *Gnarl*-encoding phages in our genomic  
282 dataset do not encode any such seroconverting enzymes. *Gnarl* proteins (40aa – 90aa) are also  
283 much smaller than typical enzymes, hinting that these AGs might inhibit O-antigen-modifying  
284 enzymes, constituting a distinct mode of host seroconversion. *Gnarl* genes are not restricted to  
285 either lytic or lysogenic phages; *gnarl1* and *gnarl3* are present in lytic phages (Supplementary  
286 File 10), whereas *gnarl2* is present in the classical temperate phage Mu (gene E6/Mup07).  
287 Seroconversion could occur in lysogenic infections to exclude superinfecting phages, or in lytic  
288 infections as receptor-masking to prevent newly synthesized virions from unproductively binding  
289 lysed cell fragments<sup>37</sup>.

290 To identify the cellular targets of *gnarl* proteins, we analyzed AP-MS data for all novel  
291 counter-defense AGs. Each accessory gene product bound distinct host proteins, although the  
292 profiles of *orf116* (*abc1*) and *orf126* were somewhat correlated (Figure 5e). Gene Ontology  
293 pathway analysis identified UDP-glucose biosynthesis factors as significantly enriched in the  
294 *gnarl3* set, suggesting that *gnarl3* inhibits a step in this pathway. Among these, GalU catalyzes  
295 the formation of UDP-glucose<sup>38</sup> to build the LPS outer core<sup>39</sup>; GalF regulates the levels of UDP-  
296 glucose<sup>40</sup>; and GalE reversibly converts UDP-glucose to UDP-galactose (also an LPS outer  
297 core component)<sup>41</sup>. Overexpression of *galU*, but not *galE*, *galF*, or *wecB*, completely reversed  
298 the *gnarl3* T5-sensitization phenotype in ECOR1, ECOR3, and ECOR22 (Figure 5f). suggesting  
299

300 a specific interaction between *galU* and *gnarl3*. The same effect was observed with the  
301 unrelated phage T4 (Supplementary Figure 5), indicating the *gnarl3-galU* interaction was phage-  
302 agnostic. Overexpressing *galU* without *gnarl3* did not affect phage sensitivity except in  
303 ECOR22, where it partially potentiated infection for unknown reasons. Coupled with LPS  
304 electrophoresis and AP-MS data, this observation suggests a model where *gnarl3* inhibits GalU,  
305 reducing the availability of UDP-glucose and resulting in a modification of the cell envelope that  
306 potentiates phage adsorption. More broadly, it is notable that the most prevalent and potent  
307 “counter-defense” phenotype for multiple, distinct AGs appears to hinge on removing barrier  
308 defenses through diverse mechanisms.

309

### 310 **Barrier defenses and restriction systems provide layered immunity**

311 The existence of broad-spectrum counter-defense AGs underscores the importance of  
312 barrier defenses. In strain ECOR21, transposon insertions within the O121-specific O-antigen  
313 pathway<sup>42</sup> indeed sensitized the strain to multiple phages, but not T2 (Figure 6a). We wondered  
314 if an additional defense mechanism in ECOR21 complemented barrier defenses by restricting  
315 T2 infection (Figure 6b). In the AG screen, internal-protein IplI (*orf143*) partially sensitized  
316 ECOR21 to T2 (Figure 5a), suggesting that T2 is blocked by an IplI-inhibited system in addition  
317 to O121 O-antigen. Interestingly, although phage T2 itself does not encode IplI, phage T4  
318 encodes IplI as well as the only internal-protein from T-even phages with a known function, Ipl.  
319 Ipl is packaged into the phage capsid and disables canonical Type IV R-M (consisting of two  
320 enzymes, GmrS and GmrD) upon co-injection with phage DNA<sup>43</sup>.

321 To identify the IplI-inhibited system, we eliminated ECOR21 O-antigen by deleting  
322 *wecA*<sup>44</sup> (Figure 6c) and repeated the transposon screen, looking for mutants in the  
323 ECOR21 $\Delta$ *wecA* background that now became sensitive to T2. We obtained several transposon  
324 insertions in an atypical BREX system (Figure 6d) in which a fused Type IV R-M enzyme  
325 GmrSD (also known as BrxU) is embedded<sup>45</sup>. Type IV R-M systems target glucosyl-hmC  
326 modified DNA of T-even phages (T2, T4, T6)<sup>43</sup>. The entire ECOR21 BREX-GmrSD system  
327 cloned into a lab strain and expressed from its native promoter blocked T2, but an active-site  
328 mutant in GmrSD (GmrSD\*) did not (Figure 6e). Inactivation of the BREX system ( $\Delta$ *pgIX*) had  
329 no impact on T2, confirming that GmrSD causes T2 inhibition and is the likely target of IplI.  
330 Consistent with this conclusion, expression of IplI, but neither Ocr (anti-Type I R-M and anti-  
331 BREX<sup>29</sup>) nor Ipl (which inhibits canonical GmrS-GmrD two component Type IV R-M, but not the  
332 fused GmrSD<sup>37,43</sup>), abolished GmrSD defense (Figure 6e).

333 To test sufficiency, we expressed GmrSD or the catalytic mutant GmrSD\* alone.  
334 Overexpressing GmrSD was sufficient to block all T-even phages, while the catalytic mutant had  
335 no effect (Figure 6f). Leaky expression of GmrSD from the uninduced plasmid still inhibited  
336 replication of T2 and T6 (which do not encode IplI), but not the IplI-encoding phage T4. When  
337 IplI was additionally expressed, T2 and T6 plaquing was restored, whereas plaquing of T4 did  
338 not improve further (Figure 6g). Of the many described T-even phage internal proteins<sup>12</sup>, IplI  
339 is only the second (after Ipl) with an identified target. The observed redundancy between barrier  
340 defenses and restriction systems likely obscures phenotypes of other anti-restriction AGs as  
341 well.

342

### 343 **Conclusion**

344

345 Our strategy for identifying AGs and their interactions with host defenses can tease apart  
346 the layers of bacterial anti-phage immunity, providing a holistic view of the most salient  
347 defenses across multiple strains. Deploying this platform in pathogenic bacteria will help tailor  
348 phage therapy approaches to overcome species- and strain-specific antiviral mechanisms. In *E.*  
349 *coli*, we discovered a diverse array of AG functions that interact with multilayered host immunity

350 at every level. AG-encoded proteins modify the cell surface to block viral entry, disable antiviral  
351 restriction systems inside the cell, and trigger PCD mechanisms in the host and its prophages,  
352 while cellular decoy immunity guards the host against phage counter-defense strategies. We  
353 show here how restriction systems work in tandem with barrier defenses on the cell surface, and  
354 with Abi/decoy guardians within the cell to protect the host.

355 We report surprising exaptation of well-studied R-M systems that are triggered by anti-  
356 restriction proteins to induce PCD. Our results further expand the conceptual framework of  
357 layered anti-phage defense in bacteria, whereby anti-anti-restriction mechanisms such as  
358 retrons<sup>23</sup>, PARIS<sup>24</sup>, and PrrC<sup>25</sup> provide backup immunity via Abi. In particular, EcoR22I combines  
359 both R-M and PCD functions, and Ronin is a PCD mechanism that mimics an R-M system to  
360 thwart phage anti-R-M AGs under the decoy immunity strategy previously identified only in  
361 eukaryotes. Thus, experimentally assaying AGs in wild bacterial strains can uncover defense  
362 functions that cannot be identified by comparison with known defense or counter-defense factors.  
363 For instance, EcoR22I does not have any domains currently known to be involved in PCD.  
364 Similarly, *ronA*-like toxins have no previously characterized links to R-M.

365 Assaying AGs individually in diverse hosts also directly identifies otherwise elusive triggers  
366 of defense systems. Anti-phage defense systems have been shown to detect highly conserved  
367 features of phages (nucleic acids, capsids, terminases, or portal proteins)<sup>15-18</sup>. We show that a  
368 variety of poorly conserved phage AGs can also trigger defense mechanisms in the host and its  
369 prophages. Non-essential gene products might seem ill-suited for this role because the virus could  
370 simply lose the gene to escape detection. However, all AGs we found to trigger PCD are  
371 associated with common counter-defense functions, trapping the virus into a Scylla vs Charybdis  
372 catch where a counter-defense AG triggers PCD upon infection, but losing that AG renders the  
373 virus vulnerable to immunity. The strategies viruses employ to navigate this treacherous narrows  
374 are currently unknown and might be an important force in virus evolution. The host counterpoints  
375 to virus AGs were likely overlooked because many viruses encoding inhibitors and triggers of  
376 specialized host defenses can be difficult to procure and culture. Advanced technologies for DNA  
377 synthesis combined with rapidly growing metagenomic sequence databases are starting to paint  
378 a more complete picture of virus-host coevolution.



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380

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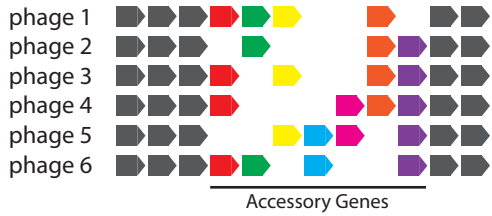
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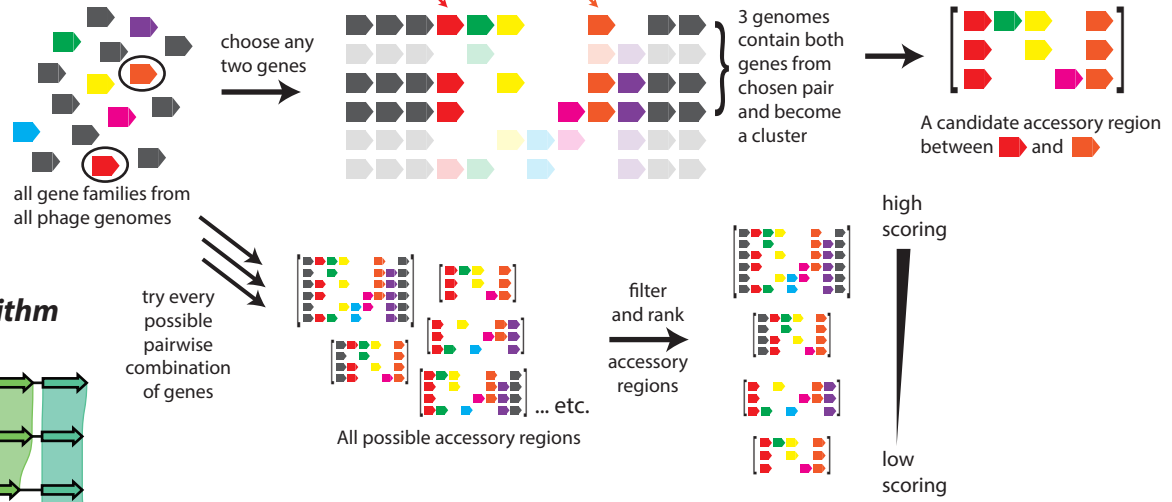
511



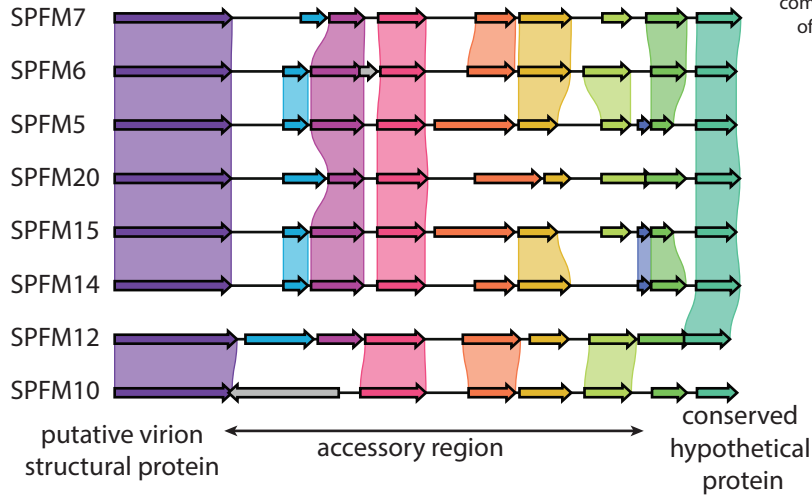
### A. Core vs. Accessory genes in a hypothetical phage cluster



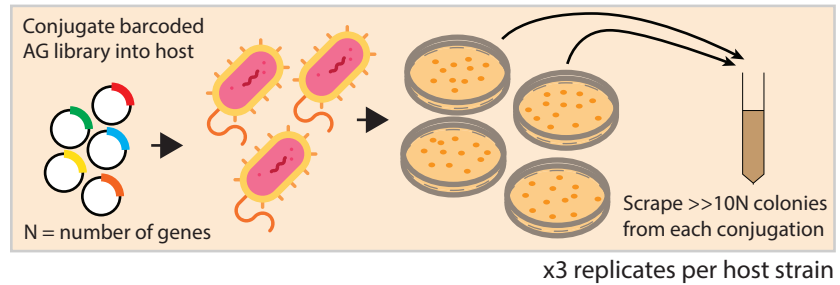
### B. Algorithm to find accessory regions without a priori genome clustering



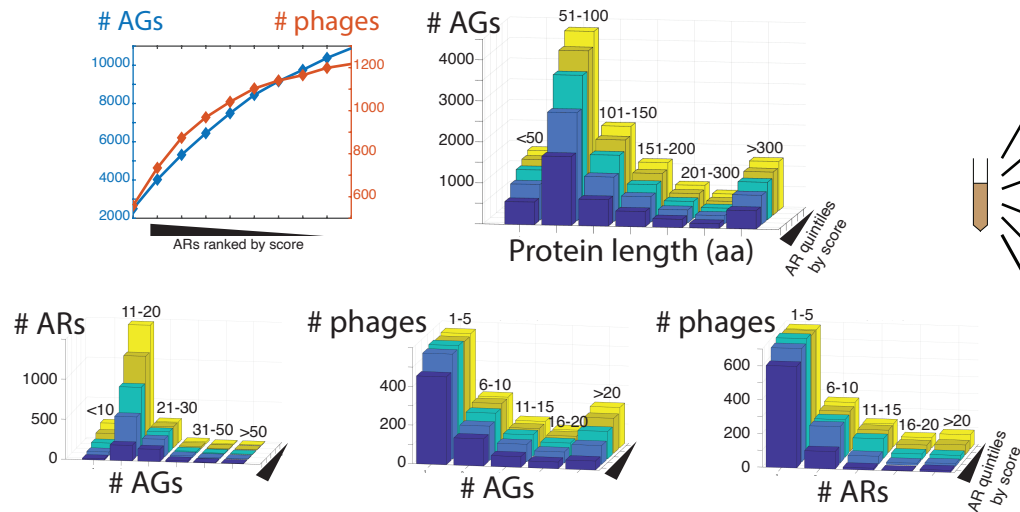
### C. Sample accessory region found by the algorithm



### E. Screen to assay AG phenotypes in parallel

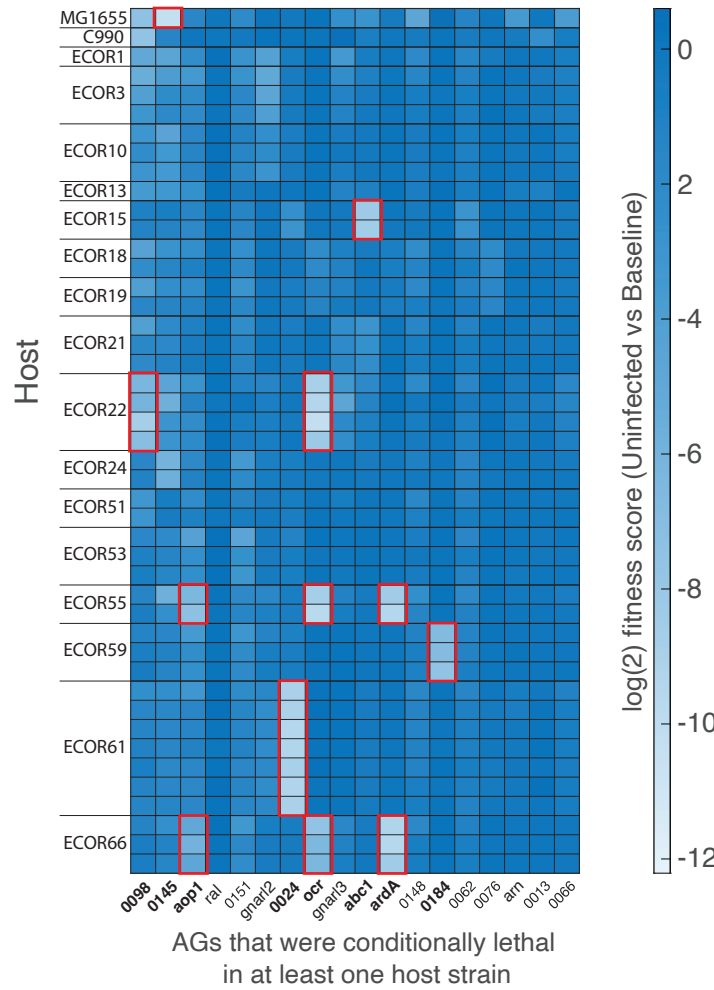


### D. Characteristics of ARs and AGs

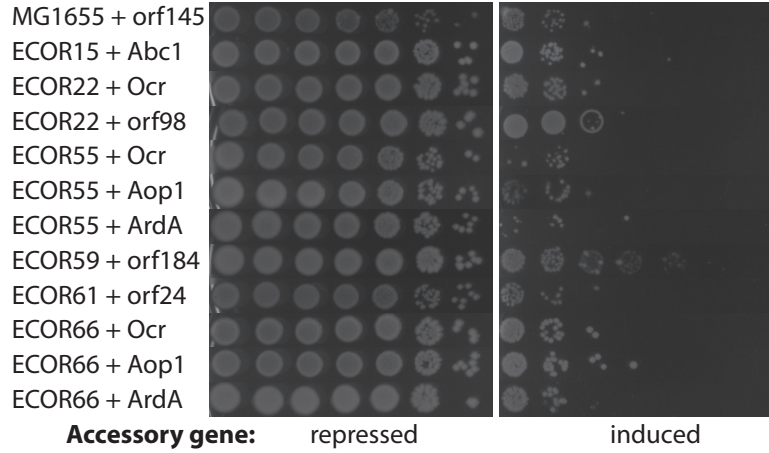


513 **Figure 1. Platform for identifying and testing phage AGs. (A)** Schematic of a genomic region  
514 showing core (shown in grey) and accessory (in various colors) genes in a hypothetical phage  
515 genome cluster. **(B)** Schematic of algorithm to exhaustively identify accessory regions using  
516 pairwise combinations of genes, without *a priori* genome clustering. **(C)** A sample high-scoring  
517 AR in a family of *Salmonella* phages. Purple shading denotes >80% and green denotes >90%  
518 nucleotide identity. **(D)** Statistical features of ARs and AGs. 2D chart depicts saturation curves  
519 (cumulative distribution functions) of unique AGs (left axis) and phage genomes (right axis)  
520 represented in ~2000 non-redundant ARs ranked by score on the X-axis. 3D bar graphs show  
521 various distributions (length of AGs, number of AGs per AR, number of AGs per phage, and  
522 number of ARs per phage). Color-coded histograms in each bar graph are cumulative i.e., the  
523 first histogram (dark blue) shows the top 20% highest-scoring ARs, the next (light blue) represents  
524 top 40% and so on. **(E)** Schematic of the AG screen.  
525

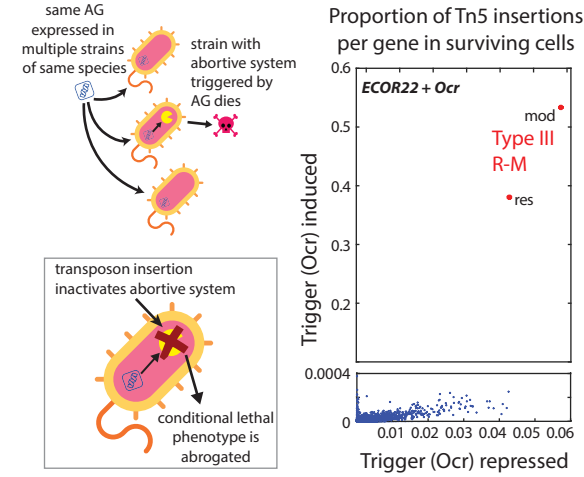
### A. Accessory genes with conditional lethal effects in any host



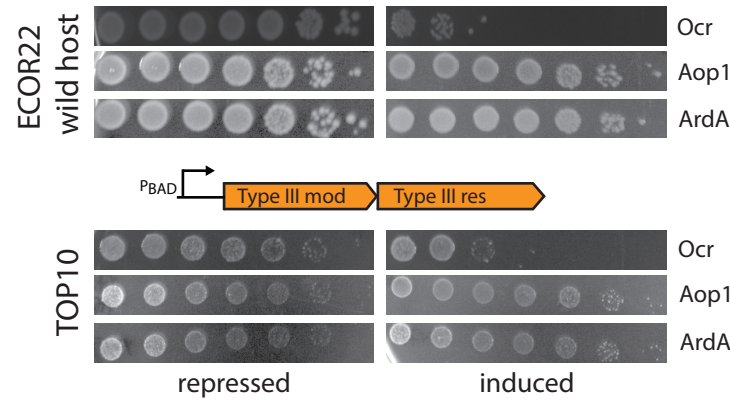
### B. Fitness cost of conditional lethal interactions in native hosts



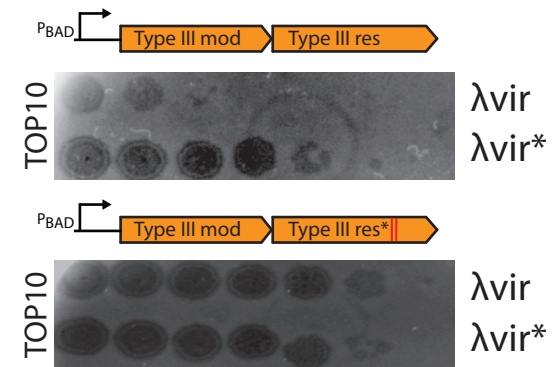
### C. Whole genome Tn5 suppressor screens



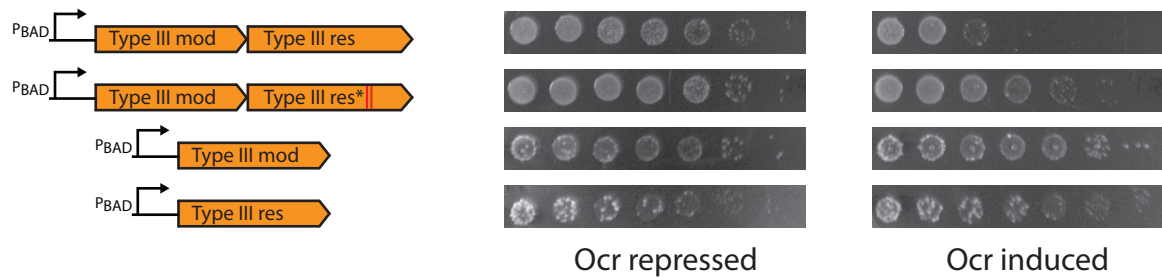
### D. Type III R-M from ECOR22 kills cells upon Ocr expression



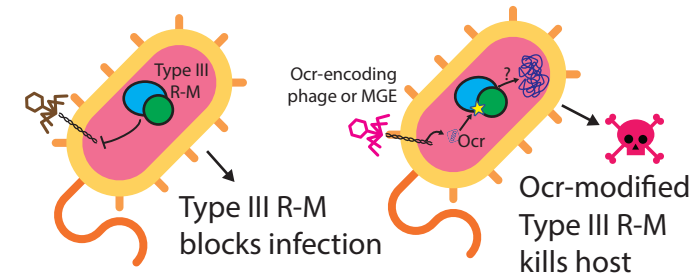
### E. λvir grown on nuclease-dead (res\*) mutant can evade EcoR22III restriction



### F. EcoR22III restriction endonuclease site is partially responsible for Ocr-induced PCD



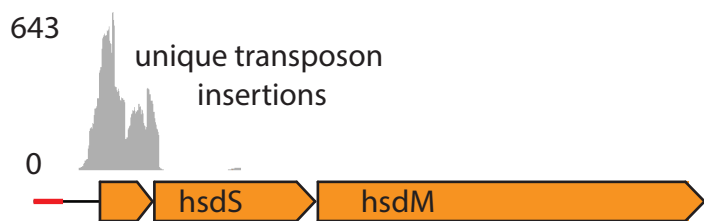
### G. Hypothetical model of the same R-M system mounting both targeted and abortive defenses



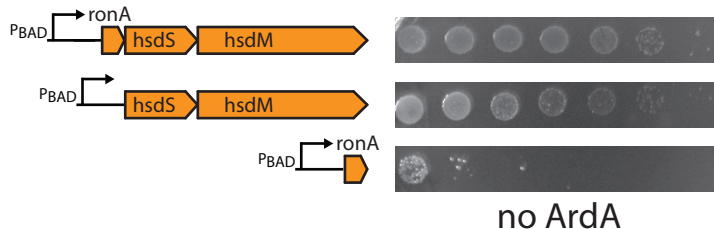
527 **Figure 2. AGs that trigger programmed cell death. (A)** Heatmap of log<sub>2</sub>-transformed fitness  
528 scores comparing induced and uninduced samples without phage infection. Of 200 AGs tested,  
529 only AGs that exhibited lethality in some (but not all) hosts are shown. Red boxes/bold names  
530 highlight conditional-lethal AGs selected for further study. Named AGs are *orf7:aop1*, *orf87:ral*,  
531 *orf63:gnarl2*, *orf1:ocr*, *orf92:gnarl3*, *orf116:abc1*, *orf169:ardA* and *orf2:arn*. **(B)** Host-AG  
532 combinations in red boxes from (C) tested individually for AG-induced lethality (serial tenfold  
533 dilutions of saturated culture; AGs repressed: left, induced: right). **(C)** Schematic depicts  
534 hypothetical strain-specific Abi system being triggered by AGs, and inactivated in follow-up  
535 transposon knockout screens (inset). Data from a representative screen with a Tn5 library  
536 constructed from ECOR22 with chromosomally encoded *ocr*. Proportion of transposon insertions  
537 per gene in surviving cells with *ocr* repressed is plotted on the X-axis, and with *ocr* induced on  
538 the Y-axis. Y-axis interrupted to resolve genes other than Type III R-M *mod*, *res*. **(D)** Abortive  
539 phenotype of EcoR22I in its native host, and reconstituted when cloned onto a plasmid and  
540 expressed in TOP10 (AGs repressed: left, induced: right). **(E)** Plaque assay showing R-M  
541 phenotype of EcoR22I against methylated and unmethylated  $\lambda$ vir in TOP10.  $\lambda$ vir\* were methylated  
542 by passaging in a TOP10 strain with nuclease-dead EcoR22I (*res\** double mutant; D1024A,  
543 D1043A) and tested for their ability to evade restriction by wildtype or *res\** EcoR22I. **(F)** As in (D),  
544 PCD activity of EcoR22I variants in TOP10 (wildtype, nuclease dead (*res\**), and *mod*, *res* genes  
545 expressed individually). **(G)** Hypothetical model of EcoR22I R-M and PCD activities. EcoR22I kills  
546 its host in the presence of *Ocr*, partially relying on its nuclease activity. “pBAD” indicates the  
547 construct was overexpressed from the plasmid in (F-H).  
548



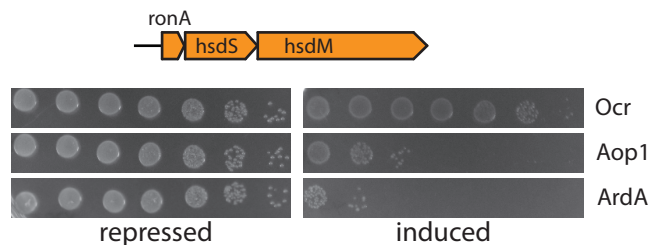
**A. Transposon insertions that inactivate PCD in ECOR55 despite expression of anti-R-M genes**



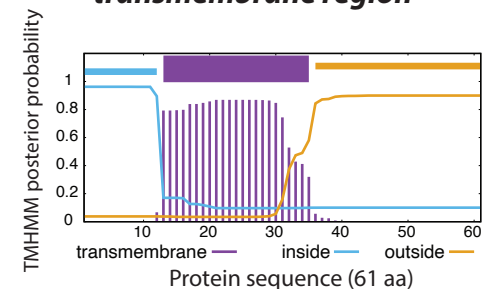
**D. RonA is toxic upon overexpression but not with hsdMS present**



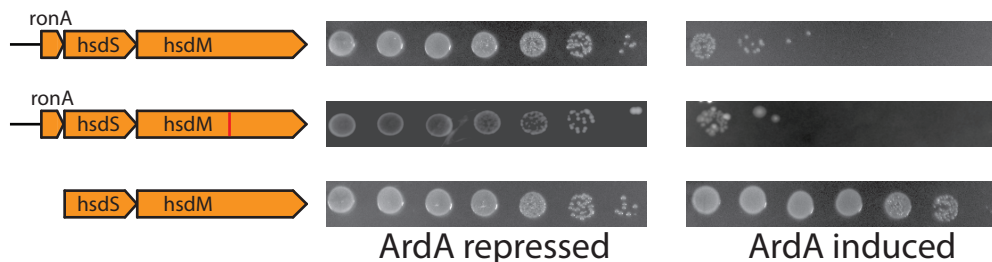
**B. Ronin system from ECOR55 kills cells upon aop1 or ardA induction**



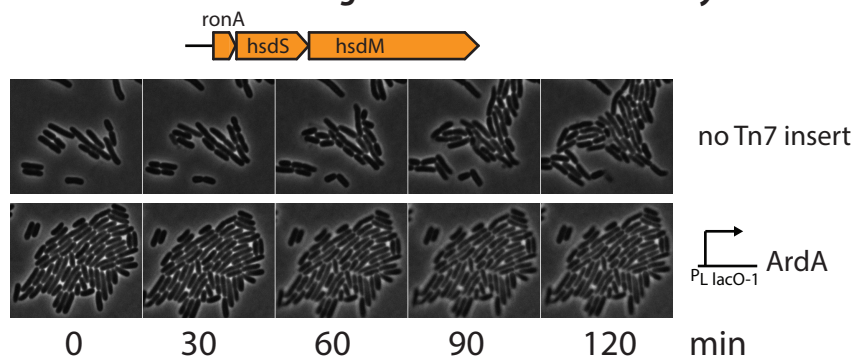
**C. RonA contains a predicted transmembrane region**



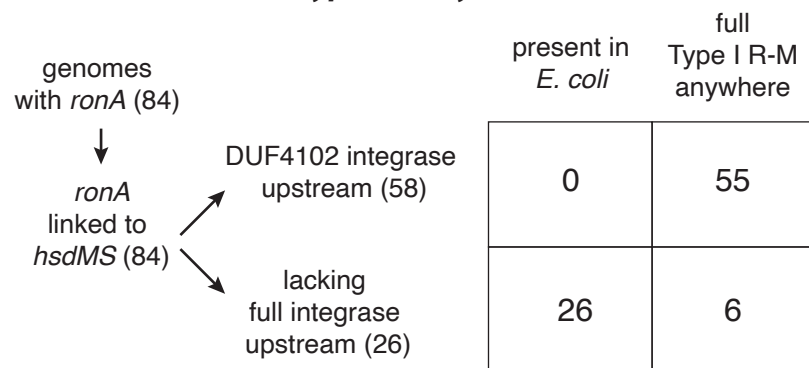
**E. RonA is necessary for ArdA-induced PCD activity**



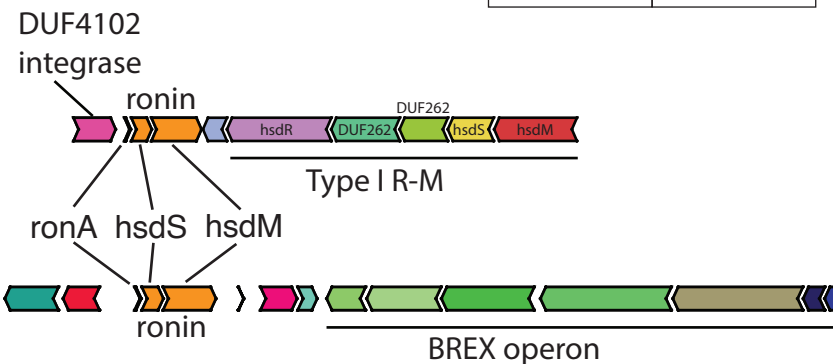
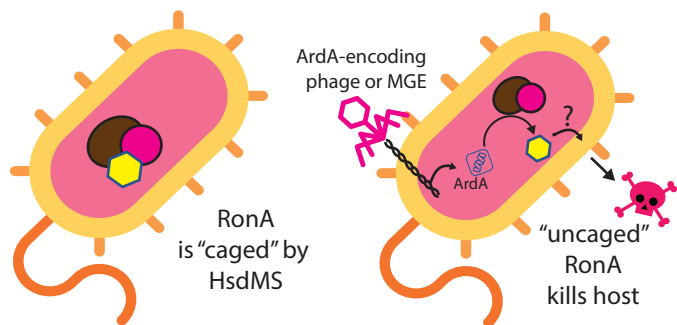
**F. Ronin activation causes growth arrest but not cell lysis**



**G. RonA-associated type I R-M systems (ronin) lack the restriction endonuclease**

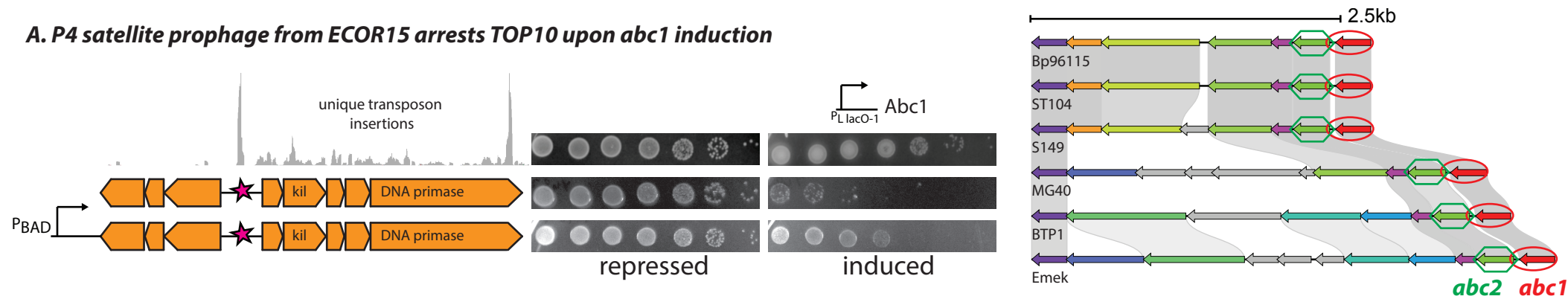


**H. Hypothetical model of RonA "uncaging"**

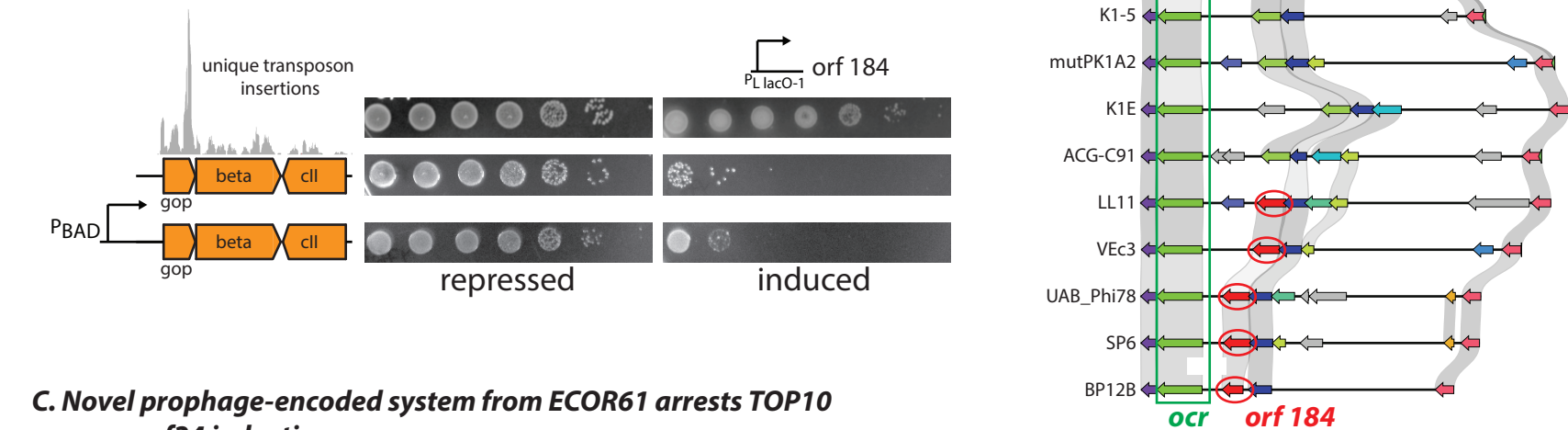


550 **Figure 3. Decoy immunity in bacteria. (A)** Distribution of unique transposon insertions  
551 recovered from survivors in ECOR55 Tn5 libraries upon *aop1* induction, mapped to Ronin. **(B)**  
552 Schematic of Ronin system from ECOR55. Abortive phenotype reconstituted in TOP10 upon  
553 expression of Ronin from a plasmid (serial tenfold dilutions of saturated culture; AGs repressed:  
554 left, induced: right). **(C)** Prediction of transmembrane character along the 61 aa RonA sequence.  
555 **(D)** Lethality of Ronin components tested when overexpressed (indicated as “pBAD”) in TOP10  
556 without AGs. **(E)** As in (B), genetic requirements for the Abi phenotype of Ronin. Red bar in *hsdM*  
557 (second row) denotes the F287G active-site mutation. **(F)** Time-lapse imaging of TOP10 cells  
558 expressing Ronin with (bottom) or without (top) chromosomally-integrated *ArdA*. **(G)** Two  
559 prevalent architectures of *ronA*-linked Type I R-M-like Abi system (*ronin*), based on the presence  
560 of an upstream DUF4102 integrase. Number of (*E. coli* and other) genomes with a full Type I R-  
561 M system (with *hsdR*, *hsdM*, *hsdS* genes) anywhere in the genome is shown for each Ronin  
562 architecture. **(H)** Schematic depicting hypothetical *RonA* “uncaging” when *ArdA* is present.  
563

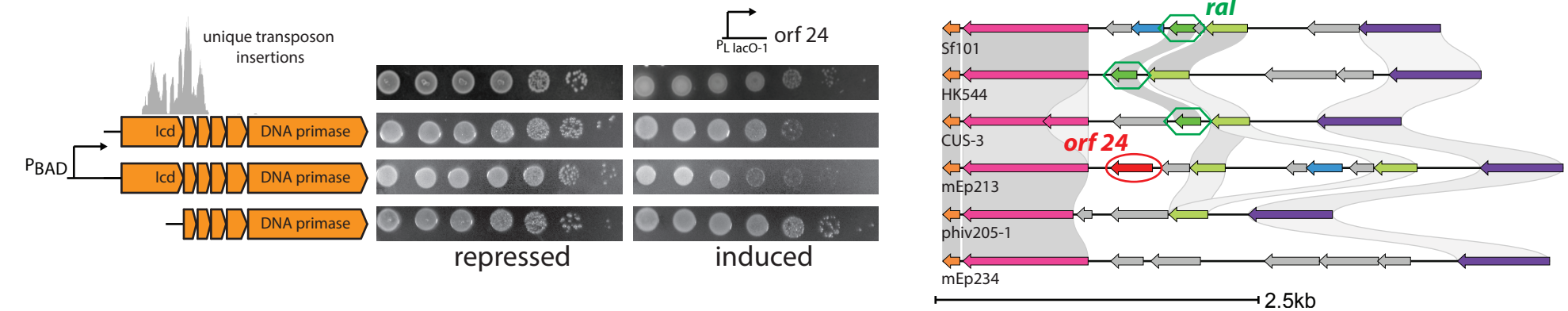
**A. P4 satellite prophage from ECOR15 arrests TOP10 upon *abc1* induction**



**B. *gop-beta-cll* system from ECOR59 arrests TOP10 upon *orf184* induction**



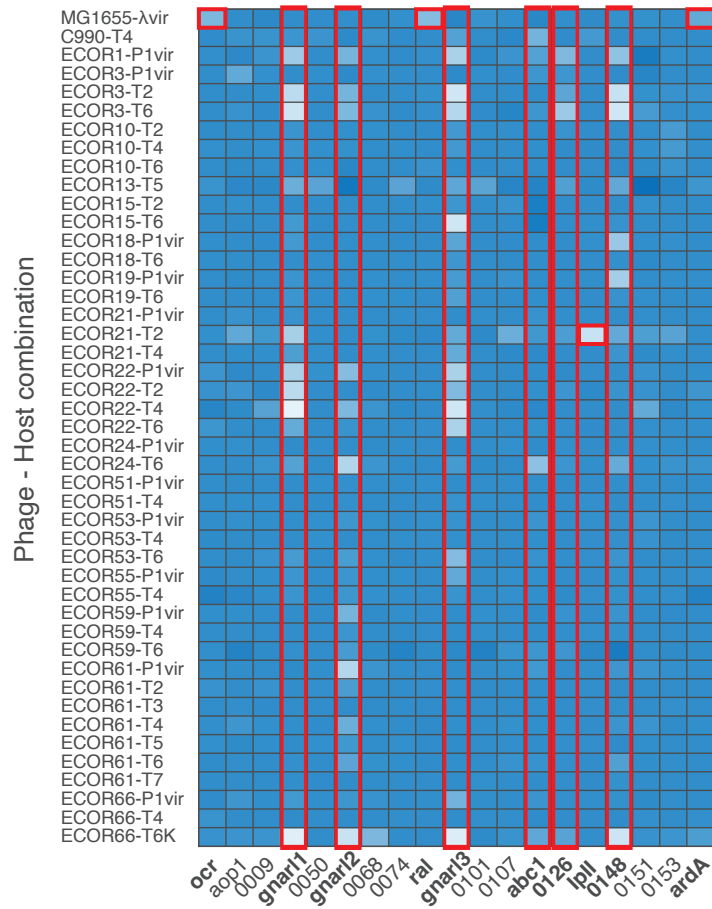
**C. Novel prophage-encoded system from ECOR61 arrests TOP10 upon *orf24* induction**



565 **Figure 4. AGs that trigger prophage-encoded PCD mechanisms. (A)** Abortive phenotype of  
566 P4 satellite prophage from ECOR15 cloned onto a plasmid and expressed in TOP10. *Abc1* is  
567 expressed from a single-copy chromosomal insertion (serial tenfold dilutions of saturated culture;  
568 AGs repressed: left, induced: right). Star marks an intergenic region that was strongly targeted in  
569 transposon suppressor screens. **(B)** As in (A), PCD phenotype of *gop-beta-cll* system from  
570 ECOR59 upon expression of *orf184*. **(C)** As in (A), PCD phenotype of novel prophage-encoded  
571 superinfection exclusion system from ECOR61 upon expression of *orf24*. “*pBAD*” indicates where  
572 the constructs were overexpressed in (A-C). Distribution of unique transposon insertions  
573 recovered from survivors in Tn5 libraries upon trigger AG induction are mapped to each PCD  
574 locus in (A-C). Accessory Regions where trigger AGs in (A-C) were found (*orf116:abc1*, *orf184*,  
575 *orf24*; highlighted with red ovals), with co-occurring counter-defense AGs (*anti-RecBCD abc2*,  
576 *anti-R-M ocr*, *anti-R-M ral*; indicated in green boxes) are shown in the respective panels.  
577

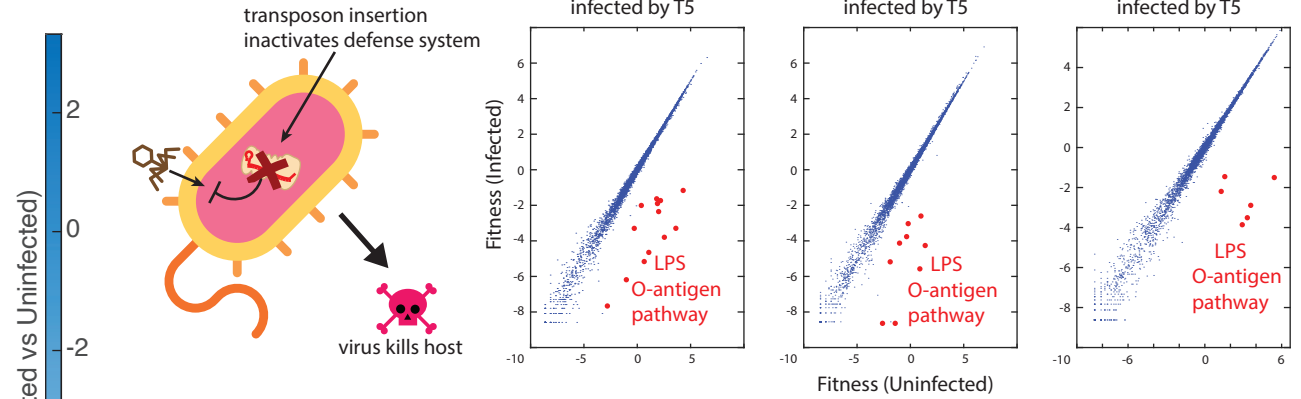


### A. Accessory genes with counter-defense phenotypes in any host

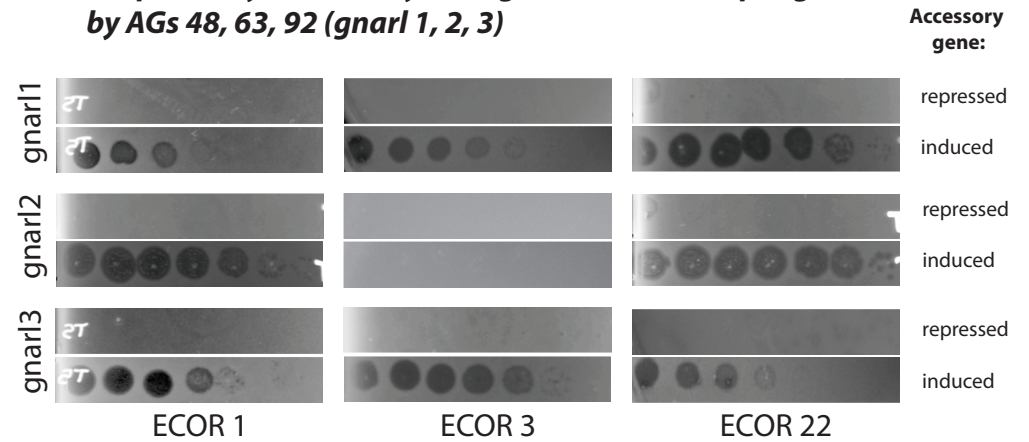


AGs that had a counter-defense effect in at least one host strain

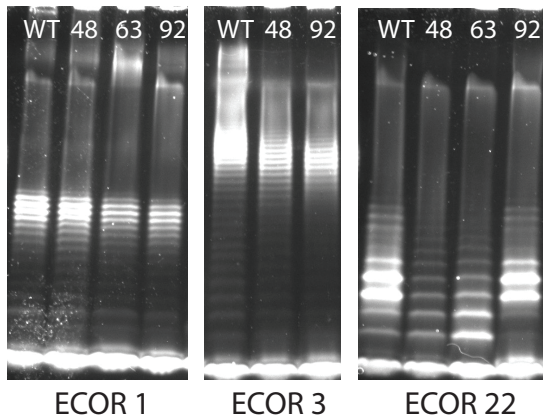
### B. Whole genome Tn5-knockout screening to find phage exclusion mechanisms



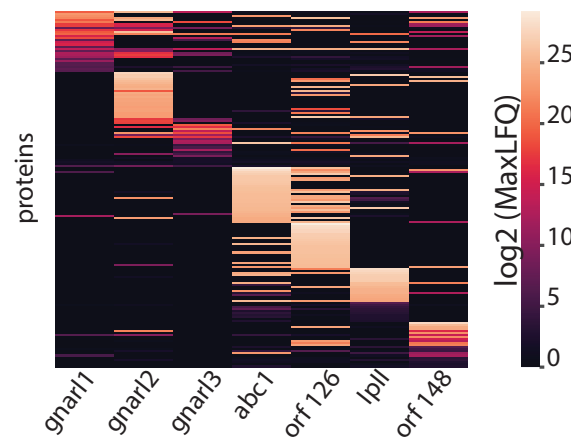
### C. Plaque assays show very strong sensitization to phage T5 by AGs 48, 63, 92 (gnrar1, 2, 3)



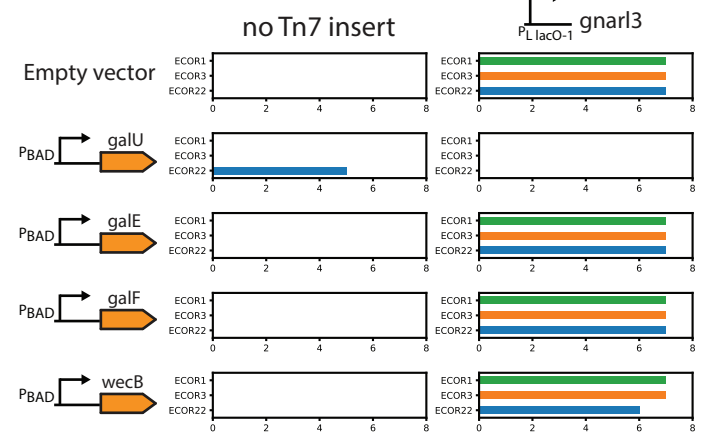
### D. Gel electrophoresis of LPS from wild E. coli expressing AGs 48, 63, 92 (gnrar1, 2, 3)



### E. Heatmap of enriched host-proteins upon AP-MS of broad-spectrum phage AGs

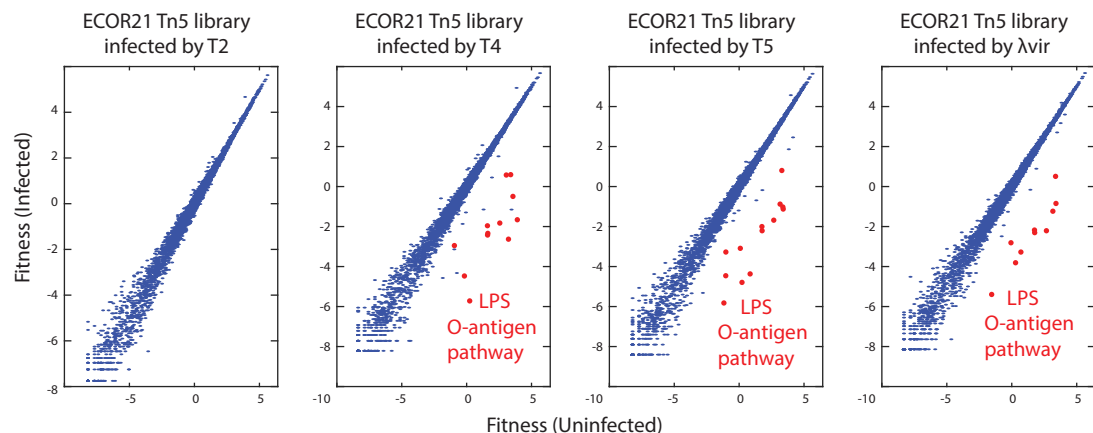


### F. Over-expression of galU reverses T5-sensitization by gnrar3

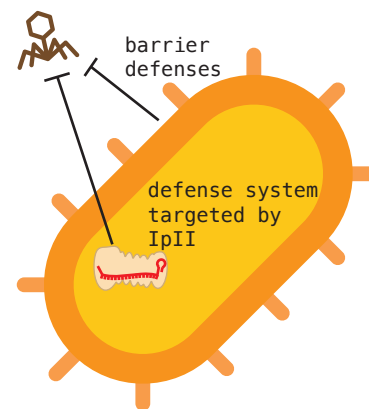


579 **Figure 5. AGs that sensitize hosts to infection by modifying barrier defenses. (A)**  
580 Heatmap of log<sub>2</sub>-transformed fitness scores comparing infected and uninfected samples. Of 200  
581 AGs tested, only AGs with a host-sensitizing phenotype are shown on the X-axis. Red  
582 boxes/bold names highlight counter-defense AGs selected for further study. Named AGs are  
583 *orf1:ocr*, *orf7:aop1*, *orf48:gnarl1*, *orf63:gnarl2*, *orf87:ral*, *orf92:gnarl3*, *orf116:abc1*, *orf143:lpII*  
584 and *orf169:ardA*. **(B)** Schematic of follow-up transposon knockout screens, performed in the  
585 native host without AGs. Graphs show fitness of transposon-mediated knockouts in infected (Y-  
586 axis) and uninfected (X-axis) Tn5-libraries from three representative hosts challenged by T5.  
587 Red dots are gene-disruptions that lower bacterial fitness upon infection (e.g., disruptions in O-  
588 antigen biosynthesis). **(C)** Plaque assays with phage T5 and hosts from **(D)** with *gnarl* genes  
589 repressed (top) or induced (bottom). **(D)** Lipopolysaccharide (LPS) visualized by electrophoresis  
590 from host-AG combinations in **(E)** where AGs produced a phage-sensitizing effect. **(E)** Log<sub>2</sub>-  
591 transformed protein representation scores (MaxLFQ) for the top 30 enriched host proteins upon  
592 expression of AGs. **(F)** log<sub>10</sub>-transformed T5 infection scores in wild hosts ECOR1 (green),  
593 ECOR3 (orange), ECOR22 (blue) with or without UDP-glucose biosynthesis pathway genes  
594 *galU*, *galE*, *galF*, and ECA precursor *wecB* cloned onto a plasmid and overexpressed.  
595

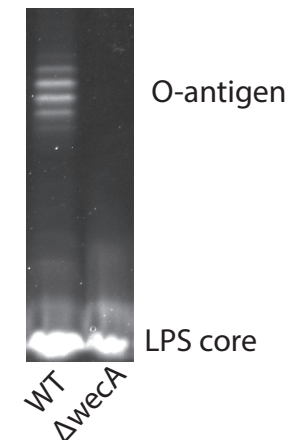
### A. Whole genome Tn5-knockout screening in ECOR21 with various phages



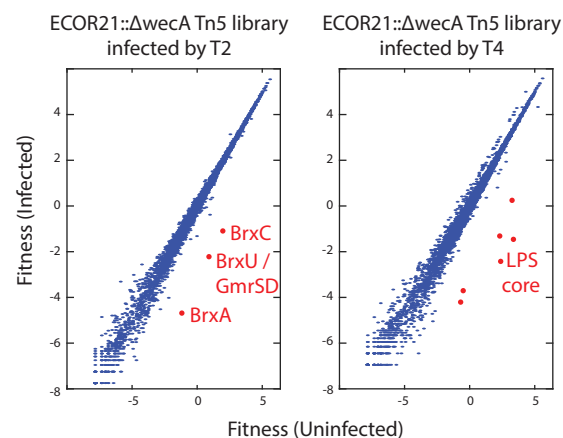
### B. ECOR21 might block T2 at two levels simultaneously



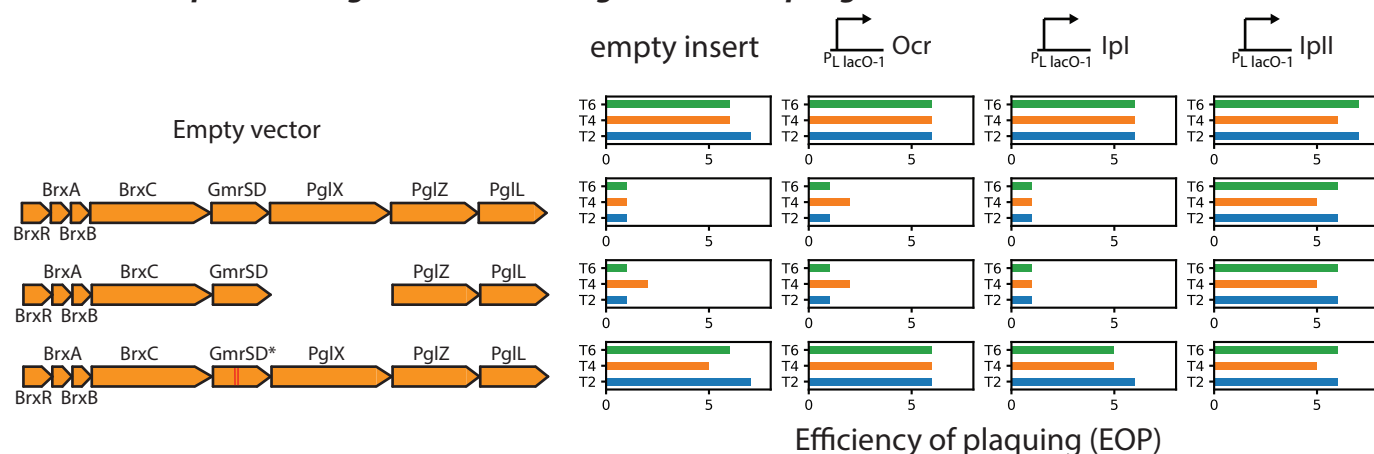
### C. O-antigen removed in ECOR21::ΔwecA



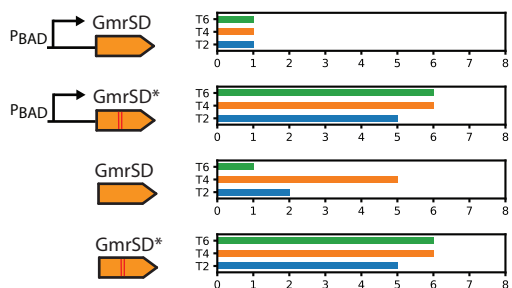
### D. Tn5 screens after removing O-antigen layer



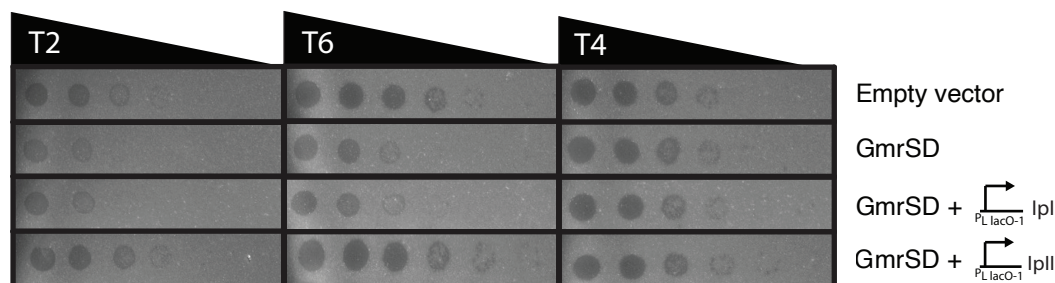
### E. BREX-operon configurations tested against T-even phages with various inhibitors



### F. GmrSD is sufficient to confer resistance against T-even phages



### G. T4 naturally encodes IplI and does not require additional IplI to overcome basal levels of GmrSD restriction



597 **Figure 6. AGs that block canonical antiviral defenses. (A)** Fitness of transposon-mediated  
598 knockouts in infected and uninfected Tn5-libraries from ECOR21 challenged by various phages.  
599 **(B)** Schematic of ECOR21 defense against T2 through both barrier defenses and a defense  
600 system which is putatively blocked by *orf143/lplI*. **(C)** LPS visualized from ECOR21 with the O-  
601 antigen biosynthesis initiator *wecA* deleted by allelic exchange. **(D)** Whole-genome transposon-  
602 knockout screens as in (A) but with the ECOR21:: $\Delta$ *wecA*. **(E)** Variants of the BREX/GmrSD  
603 operon cloned onto a plasmid along with their native promoters and tested in a lab strain of *E.*  
604 *coli* (TOP10) against T-even phages (T2, T4, T6). Removal of the PglX gene inactivates BREX.  
605 Active-site mutations that disable GmrSD are shown as two parallel red bars (GmrSD\* double  
606 mutant; D474A, H475A). *Ocr*, *lpl* and *lplI* are expressed from single-copy chromosomal  
607 insertions. Bar graphs show log<sub>10</sub> infection scores. **(F)** GmrSD and its inactive variant GmrSD\*  
608 cloned onto a plasmid and tested in TOP10 for restriction activity against T-even phages.  
609 “pBAD” indicates the defense construct was overexpressed from the plasmid. **(G)** Plaque  
610 assays with TOP10 cells with uninduced GmrSD (i.e., not overexpressed) challenged by T-even  
611 phages.

## 612 **Methods**

613

### 614 **Algorithm to identify phage accessory regions**

615 1706 non-redundant Enterobacteriophage genomes were obtained (with help from Dr.  
616 Andrew Millard, Leicester) from public databases. These phages were both lytic and lysogenic,  
617 but were sequenced and assembled from cultured isolates (i.e. prophages and metagenomic  
618 samples were not included). All annotated protein records from all the phages were assigned  
619 unique numeric IDs and clustered by MMseqs2<sup>46</sup>. ORF IDs in phage genomes were then  
620 replaced by their MMseqs2 cluster representative IDs in each genome. Thus, each phage  
621 genome can be represented as an ordered list of IDs of clustered proteins. The gene-  
622 neighborhood of every ORF (up to 10 genes upstream and downstream) was extracted from  
623 every virus. Given that phage genomic termini can be difficult to identify, every genome was  
624 considered potentially circularly permuted. Allowing for circularization also ensures all gene-  
625 neighborhoods were the same size and putative ARs towards ends of genomes would not be  
626 missed. A 2D matrix of all possible pairwise combinations of genes was constructed, with every  
627 matrix position containing lists of (a) the phages that each gene appears in, (b) the 10 upstream,  
628 and (c) 10 downstream neighboring ORFs of each gene in every genome. We then traversed  
629 the matrix and assessed every gene pair to identify all possible ARs, defined as the region  
630 between a pair of genes that are both present in at least 6 different phage genomes but are no  
631 more than 10 genes apart in any genome. Hence, every virus genome that contained both  
632 genes of the gene pair was grouped into a possible virus cluster, and genes encoded between  
633 the genes of the query pair were considered members of a possible AR. Regions trivially  
634 containing the same genes in each participating genome were discarded. Genomes involved in  
635 a putative AR were then compared pairwise at the nucleotide level using FastANI<sup>47</sup>, and ARs  
636 with divergent genomes were removed. In practice, this was done by pre-computing an all-vs-all  
637 matrix of genome ANI and then traversing subsets of the matrix containing all the genomes  
638 participating in the putative AR to ensure no cells were empty (FastANI produces no output  
639 when very divergent genomes are compared). Various parameters were extracted from  
640 surviving ARs.  $N$  is the number of unique genes in the AR. The vector  $c$  contains conservation  
641 numbers (the number of different genomes that each gene appears in; also indicated on top of  
642 each gene) for all the unique genes in this AR. The vector  $l$  contains the lengths (in numbers of  
643 genes) of the AR in each genome.  $CV$  denotes coefficient of variation.  $GCdev$  is a vector  
644 containing one minus the absolute deviation in GC content of each accessory gene from its  
645 resident genome. A scaling factor is also included to normalize the score by the number of  
646 phage genomes in the group that have at least one gene in the AR. ARs were scored according  
647 to the formula below.

$$648 \quad ARscore = \frac{N * median(GCdev) * CV(c)}{CV(l) * mean(c)} * \frac{\#non\_empty}{\#genomes}$$

649

650 To filter out ARs that were subsets of other ARs, we successively removed redundant,  
651 low scoring ARs with at least 90% of genes that had already been encountered in higher scoring  
652 ARs. The top 200 ARs were examined manually and 1-15 AGs were selected from 62 ARs  
653 (Supplementary Files 2,3). A worked example of the code is provided in Supplementary File 1.

654

### 655 **Strains and Vectors**

656 The *E. Coli* Reference (ECOR) collection of wild *E. coli* strains was obtained from the  
657 STEC Center (Michigan State University). Lab strains were obtained as follows: MG1655 (from  
658 Dr. Carol Gross, UCSF), C990 (from Dr. Ry Young, Texas A&M), DH5 $\alpha$  (NEB), DH10 $\beta$ /TOP10  
659 (Life Technologies), BW25113 and BW25141 (from Dr. Vivek Mutalik, LBNL), and WM6026  
660 (from Dr. Jason Peters, UW-Madison). All strains were cultured at 37°C with shaking (180-225



661 rpm) in lysogeny broth (LB) (10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl with 15 g/l agar for  
662 plates) supplemented with 100 µg/ml of ampicillin/carbenicillin, 10-20 µg/ml gentamicin (except  
663 WM6026, which yielded more consistent colony sizes with 50 µg/ml), 50 µg/ml kanamycin, and  
664 0.5% w/v glucose, 0.1% w/v L-arabinose, and 1 mM IPTG (isopropyl-b-D-thiogalactopyranoside)  
665 as needed. The WM6026 strain required diaminopimelic acid (DAP) for growth, which was  
666 supplied at 300 µM. All strains were stored in 20% glycerol at -80°C for long term storage.

667 R6K-origin pTn7 plasmids (from Dr. Jason Peters, UW-Madison) – the  
668 pJMP1039/pTn7C1 helper plasmid (carbenicillin) and the pJMP1360/pTn7C185 transposon  
669 vector (gentamicin, carbenicillin) – were used for integration of phage AGs into the *E. coli*  
670 genome. All host defense systems identified in the various screens were placed under the  
671 araBAD promoter (arabinose inducible) in the pBAD Myc/His A vector (Invitrogen). Conjugative  
672 allelic exchange was carried out using R6K-origin pKEK2201 (kanamycin) (from Karl E. Klose,  
673 UT San Antonio). All plasmids were constructed by Gibson Assembly.

674 R6K-origin plasmids were maintained in BW25141 (routine cloning) and WM6026  
675 (conjugative donor strain). All other plasmids were maintained in DH5α or TOP10. All plasmid  
676 sequences were verified by Sanger or whole-plasmid sequencing (Plasmidsaurus, Primordium)  
677 and are available upon request. Frequent plasmid dimerization during the Gibson Assembly  
678 procedure (especially when using the NEBuilder reagent) was ameliorated by using 10-fold less  
679 of one of the insert DNA fragments in a reaction involving at least 2 insert fragments (not  
680 including the backbone).

681

## 682 **AG conjugation into host strains**

683 AGs were delivered to *E. coli* hosts by Tn7 transposition via conjugation performed by  
684 triparental mating as described previously<sup>48</sup>. Briefly, the transposon and helper plasmids were  
685 delivered to the recipient from WM6026 donor strains by overnight incubation on LB agar plates  
686 supplemented with 300 µM DAP and 0.5% glucose to repress AG expression. The next day,  
687 cells were scraped and resuspended in 1 ml LB, and 1000X-20000X dilutions of this conjugation  
688 mixture were plated on LB plates (lacking DAP) supplemented with the appropriate antibiotics  
689 and 0.5% glucose. All strains were maintained under strict glucose repression of the pLacO-1  
690 promoter at every step until screens were performed. Thus, all AGs were maintained as single-  
691 copy chromosomal inserts at the same attTn7 site in the genomic region between *glimS* and  
692 *pstS* genes.

693 Efficient Tn7 transposition is necessary to construct an AG library in any given *E. coli*  
694 host. Tn7 conjugation-transposition efficiencies were determined in 44 ECOR stains containing  
695 an intact *glimS-pstS* region (we note that other strains can support Tn7 integration as well). Tn7  
696 transposons carried only a barcode sequence and the gentamicin-resistance gene to minimize  
697 payload-dependent variation in conjugation efficiency between strains. Efficiency of conjugation  
698 was determined by streaking the conjugation mixture on two different gentamicin concentrations  
699 (25 µg/ml and 10 µg/ml) to account for possible differences in natural antibiotic resistance  
700 between the wild strains. The barcode sequence was verified by Sanger sequencing and both  
701 flanks were checked by PCR to ensure proper integration of the Tn7 transposon at the expected  
702 attTn7 site. Strains that supported Tn7 transposition at high levels and were amenable to agar-  
703 overlay plaque assays were selected for further experiments.

704

## 705 **DNA extraction + Nextera Genomic Sequencing + Analysis**

706 Genomic DNA was extracted using a modified SDS–proteinase K method: Briefly, cells  
707 pelleted from 50 µl of saturated culture (or 5 µl of the cell mixture scraped from selective plates  
708 during AG library construction) were re-suspended in 200 µl of lysis buffer (10 mM tris, 10 mM  
709 EDTA, 400 mg/ml proteinase K, 0.5% SDS) and incubated at 50-55°C for 1 hour. Subsequently,  
710 the temperature was lowered to 37°C and RNase A (Thermo Scientific) was added to a  
711 concentration of 1 mg/ml. After 30-60 min of incubation, the digests subsequently purified using

712 the Genomic DNA Clean & Concentrator Kit (Zymo Research). DNA was prepared for whole-  
713 genome sequencing using the Nextera Flex Library Prep Kit (now, the Illumina DNA Prep Kit)  
714 according to manufacturer's instructions, except that we lowered the volumes of all  
715 tagmentation reactions 5-fold, and used a custom dual-indexed primer set JSW-SS-22:33  
716 (CAAGCAGAAGACGGCATAACGAGAT NNNNNNNN GTCTCGTGGGCTCGG) and JSW-SS-  
717 34:41 (AATGATACGGCGACCACCGAGATCTACAC NNNNNNNN TCGTCGGCAGCGTC) to  
718 amplify libraries for 11-13 cycles using Phusion High Fidelity PCR Master Mix (NEB) instead of  
719 the Illumina-supplied PCR reagents. The N<sub>3</sub> sequences correspond to reverse-complemented  
720 Nextera DNA indexes N701 to N712 and N501 to N508, respectively (Illumina). Libraries were  
721 resolved by agarose gel electrophoresis and DNA was excised in the 300-400 bp range. Gel  
722 slices were purified using the Zymo Gel DNA Recovery Kit and sequenced on the Illumina  
723 MiSeq using 150-cycle v3 or 600-cycle v2 reagent kits.

724 Sequencing adaptors were trimmed from reads using cutadapt, mapped to reference  
725 genomes using bowtie2, and visualized using IGV. Reference genomic contigs of ECOR strains  
726 were obtained from the USDA<sup>49</sup>. SNPs, indels, and large structural variants were analysed  
727 using a mapping-free variant finder<sup>50</sup>.

728

### 729 **Phage propagation and plaque assays**

730 High-titer phage stocks were generated by growth in liquid culture. All phage infections  
731 were performed at 37°C in LB supplemented with 10 mM MgSO<sub>4</sub> and 5 mM CaCl<sub>2</sub>. *E. coli*  
732 BW25113 or C990 were grown to an optical density (at 600 nm; OD<sub>600</sub>) ~ 0.5 (1-5 X 10<sup>8</sup> cfu/ml)  
733 and infected with the desired phage at MOI ~ 5. Infections were allowed to proceed at 37°C with  
734 agitation for 5-6 hours until the cultures were clear. Lysates were clarified by centrifugation  
735 (8000 X g for 2 min) and sterile-filtered using a 0.45 µm SFCA syringe filter (Millipore). Phage  
736 lysates were stored at 4°C.

737 Bacteriophages T2, T3, T4, T5, T6, T7, P1vir were obtained from Dr. Vivek Mutalik  
738 (LBNL), and λvir was obtained from Dr. Ry Young (Texas A&M). To the extent possible, all  
739 phages were grown on *E. coli* C990 which lacks restriction-modification. T4 and P1vir could not  
740 be grown on C990 and were instead grown on *E. coli* BW25113. To ensure that our phage  
741 lysates were free from ancestral Type I R-M methylation marks or other host-passage derived  
742 DNA modifications, we serially passaged all phages in the desired hosts 3 times. All phage  
743 genomes were subsequently verified by Nextera high-throughput sequencing.

744 Phage titers were determined against various hosts by the agar-overlay method with LB  
745 top agar containing 0.7% agar, 10 mM MgSO<sub>4</sub> and 5 mM CaCl<sub>2</sub>. Phage spot-titration assays  
746 were performed with 10-fold serial dilutions of phage lysates (3-5 µl of each dilution pipetted  
747 onto bacteria immobilized in top-agar overlay using a multichannel pipette).

748

### 749 **AG Library Preparation + NGS verification + testing post-conjugation evenness**

750 200 AGs with unique 10 bp DNA barcodes were synthesized (Twist and IDT) and cloned  
751 individually under pLlacO-1 control (IPTG inducible, repressed by glucose) into a modified  
752 pTn7C185 vector (with mobile CRISPRi components sgRNA and dCas9 removed). Plasmid  
753 assembly reactions were performed in 96-well format using the 2X Gibson assembly master mix  
754 (NEB) and transformed into chemically-competent BW25141 cells in 96-well format.

755 Transformations were individually plated on LB agar supplemented with 20 µg/ml gentamicin  
756 and 0.5% glucose and one colony was initially checked by Sanger sequencing from each plate.  
757 Where a successful transformant could not be obtained on the first attempt, more colonies were  
758 sequenced in successive rounds until a plasmid with no mutations in the region spanning the  
759 pLlacO-1 promoter, the AG ORF, and the barcode sequence was obtained. Verified vectors  
760 were miniprep using the ZR Plasmid miniprep – Classic kit (Zymo) and transformed into  
761 chemically-competent WM6026 cells in 96-well format, and each transformation was plated

762 individually on LB agar supplemented with 50 µg/ml gentamicin, 300 µM DAP, and 0.5%  
763 glucose.

764 One colony from each of the 200 WM6026 transformants was grown in 2 ml square-well  
765 96-well plates with shaking overnight, and 100 µl of each culture was then pooled together to  
766 yield ~20 ml of the donor mixture. This mixture was concentrated down to 10 ml by  
767 centrifugation at 6000 g for 10 min, and 5 ml of 60% glycerol (20% final) was added to yield the  
768 donor library. The 15 ml library was divided into single-use 50 µl aliquots (each containing >50M  
769 cells, i.e. >300K of each donor strain) and stored at -80°C.

770 Genomic DNA was extracted from one of the library aliquots and all constructs in the  
771 final pool were verified by Nextera high-throughput sequencing. This allowed us to assess  
772 library evenness as well as verify the fidelity of our cloning, colony picking, and strain pooling  
773 process. Overall, 195 AGs were cloned successfully. One AG (*orf88*) never yielded any colonies  
774 in multiple cloning attempts, possibly due to its cytotoxicity. Three AGs (*orf36*, *orf52*, *orf94*) were  
775 dropouts during the WM6026 growth step, and another two (*orf25*, *orf84*) were discovered to  
776 contain mutations upon high-throughput sequencing that had been missed by Sanger  
777 sequencing. *Orf25* had previously presented difficulties in cloning, and did not yield a mutation-  
778 free construct even after checking >12 colonies (whereas most transformations yielded a  
779 correct construct on the first attempt). The *orf84* construct contained a mixed base at the last  
780 position of the second repeat of the Lac-operator sequence upstream of the ORF, which would  
781 not be expected to affect any downstream assays significantly.

782 The donor library was conjugated into MG1655 *E. coli* according to the Tn7 transposition  
783 procedure (above) and a 1000X dilution of the conjugation mixture was plated on LB agar  
784 supplemented with 20 µg/ml gentamicin and 0.5% glucose. We routinely use multiple 150 mm X  
785 15 mm bacteriological petri dishes (Corning) to obtain sufficient numbers of transconjugant  
786 colonies to assemble libraries. The next day, >>2,000-20,000 transconjugant colonies (10-100X  
787 the number of AGs in the library) were scraped and combined thoroughly, and the thick cell  
788 mixture was directly frozen at -80°C in 20% glycerol. Genomic DNA was extracted from this  
789 mixture and the distribution of DNA barcodes corresponding to individual AGs was measured in  
790 both this post-conjugation strain mixture as well as the genomic DNA extracted from the  
791 WM6026 donor library aliquot.

792 To amplify barcodes for high-throughput sequencing, genomic DNA was subjected to  
793 two successive rounds of PCR. The DNA barcodes were amplified from genomic DNA using  
794 universal primers JSW-SS-170 (CGACGCTCTCCGATCTNNNNN  
795 TGATGTCGTTGTTGCCATCG) and JSW-SS-171 (ACTGACGCTAGTGATCA  
796 CTTTCTGAGCCAGTGTGCT) and the Q5 Hot Start High-Fidelity 2X PCR master mix (NEB)  
797 according to manufacturer's instructions. Sequencing adaptors were attached in a second round  
798 of PCR using amplicons from the first PCR round as the templates, with dual-indexed primer  
799 sets JSW-SS-42:53 (CAAGCAGAAGACGGCATAACGAGAT NNNNNNNN  
800 GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT ACTGACGCTAGTGATCA) and JSW-  
801 SS-54:61 (AATGATACGGCGACCACCGAGATCTACAC NNNNNNNN  
802 ACACTCTTCCCTACACGACGCTCTTCCGATCT), where the N<sub>8</sub> sequences correspond to  
803 reverse-complemented TruSeq HT indexes D701 to D712 and D501 to D508, respectively  
804 (Illumina). Template-matching regions in the primers are underlined. Cycling conditions for  
805 round 1 were as follows: one cycle at 98°C for 1 min; two cycles at 98°C for 10s, 66°C for 30s,  
806 and 72°C for 10s; 22 cycles at 98°C for 10s, and 72°C for 20s; and one cycle at 72°C for 2 min.  
807 Conditions for round 2 were one cycle at 98°C for 1 min; two cycles at 98°C for 10 s, 64°C for  
808 30 s, and 72°C for 10 s; 4 cycles at 98°C for 10 s, and 72°C for 20 s; and one cycle at 72°C for  
809 2 min. 0.5-1 µl of unpurified 1<sup>st</sup> round reaction product was used as template for the 2<sup>nd</sup> round of  
810 PCR. Amplicons from the 2<sup>nd</sup> round were gel-purified by electrophoresis (3% agarose gel, 4.2  
811 V/cm, 2 hours) and quantified using the 1X dsDNA HS kit with the Qubit 4.0 Fluorometer  
812 (Invitrogen). Amplicons were sequenced on a MiSeq using the 150-cycle v3 reagent kit

813 (Illumina) in single-end format (100 bp) with two (8 bp) index reads. DNA barcodes were  
814 trimmed from the reads and the proportions of various AGs in the mixtures were determined  
815 using the prevalence of the corresponding barcode sequence.

816 Concordance between the Nextera whole-genome sequencing and the barcode  
817 sequencing of the WM6026 donor mixture showed that barcode representation was a suitable  
818 proxy for the true distribution of AGs in the mixture and that amplification biases during library  
819 preparation were not a significant source of noise. Concordance between barcode sequencing  
820 in the WM6026 donor and MG1655 post-conjugation recipient mixtures indicated that the Tn7  
821 transposition and colony scraping process does not meaningfully bottleneck AG distribution.

822 The AG library was then conjugated (in triplicate) into ECOR strains that were previously  
823 confirmed to support robust Tn7 transposition. Strain mixtures were prepared in a procedure  
824 analogous to library construction in MG1655, except higher dilutions of the ECOR+AG  
825 conjugation mixtures (10,000-20,000X) were plated and the gentamicin concentration on  
826 selective plates was lowered to 10 µg/ml to account for higher steady state cell densities and  
827 higher gentamicin sensitivity of the wild *E. coli* strains. As with the MG1655 library, thick cell  
828 mixtures comprised of >>2,000-20,000 colonies scraped from each replicate conjugation were  
829 directly stored at -80°C in 20% glycerol and used to seed cultures for the AG screens (below).  
830 Genomic DNA was extracted from each mixture and barcode sequencing was performed to  
831 measure the AG distribution in every replicate of all ECOR strain derived libraries. The  
832 replicates were very highly correlated, and AG distributions in all libraries were confirmed to be  
833 free of significant bottlenecks. These datasets are the reference libraries that serve as the  
834 baseline for AG induction and phage infection experiments.

835

### 836 **MOI calculation for liquid killing assays**

837 Phage adsorption to host cells may be inefficient in very dilute cultures ( $10^{6-7}$  cfu/ml).  
838 Therefore, the apparent multiplicity of infection (MOI-a) might be lower than the expected MOI  
839 (MOI-e). We added 2x serial dilutions of a λvir lysate to a 1:100 dilution of a saturated culture of  
840 *E. coli* BW25113 that had been allowed to acclimate at 37°C with shaking for 15 min. The  
841 proportion of surviving cells was determined (relative to uninfected controls) after a 60-75 min  
842 incubation (one phage replication cycle) by plating on LB agar. An apparent MOI value was  
843 calculated using the Poisson distribution function with zero occurrences  $MOI_a = -\ln(MOI_e)$  for  
844 each phage dilution, assuming that all infected cells would be killed by the virus (i.e. the  
845 proportion of surviving cells would be the zero class in the Poisson distribution). The apparent  
846 and expected MOI values were plotted against each other and the inverse of the slope (1/m) of  
847 the line ( $MOI_a = m \cdot MOI_e + 0$ ) fit through the data was determined as the fold-change  
848 reduction in MOI-a as compared to MOI-e for these experimental conditions. This fold-change  
849 reduction was determined to be ~26x. Hence, all phage infection screens were targeted to an  
850 MOI-a value of ~100 to ensure efficient phage adsorption and infection within the timeframe of a  
851 single phage replication cycle.

852

### 853 **AG Screen + Plaque assay confirmation + Data analysis**

854 Thick cell mixtures from each of the three replicates of the various ECOR+AG libraries  
855 were thawed on ice and diluted into LB supplemented with  $Mg^{2+}$ ,  $Ca^{2+}$ , and 10 µg/ml gentamicin.  
856 Typically, 1-5 µl of the library mixture was diluted in 2 ml broth to yield an initial  $OD_{600}$  of 0.1-  
857 0.15. The cultures were immediately split x2, with one tube used to monitor  $OD_{600}$  values as the  
858 experiments progressed, and the other left untouched for the screen. AG expression was  
859 induced by the addition of 1 mM IPTG after acclimatization for 15 min at 37°C in a shaking  
860 incubator and  $OD_{600}$  values were measured again for each ECOR+AG library at time of  
861 induction. Libraries were then grown for ~80-120 min depending on the relative growth rates of  
862 the various ECOR strains, to  $OD_{600} \sim 1.2-1.5$ . A  $10^{-5}$  dilution of each late-log phase culture was  
863 then plated on LB agar (with 10 µg/ml gentamicin and 0.5% glucose to repress AGs and avoid



864 fitness costs) to obtain a count of viable colony forming units (cfu). Cultures were immediately  
865 diluted ~1:20 in fresh LB (with IPTG, Mg<sup>2+</sup>, Ca<sup>2+</sup>, and 10 µg/ml gentamicin) and 1 ml aliquots  
866 were gently mixed with phage lysates at an MOI-e of ~30-100 (the actual MOI-e values were  
867 recorded the next day when cfu counts were obtained). Parallel uninfected controls were  
868 maintained for each experiment, even when the same ECOR+AG library was being tested  
869 against different phages and the uninfected controls would be interchangeable. Infections were  
870 allowed to proceed for 30-35 min for T-phages and 60-70 min for P1vir and λvir, i.e. one  
871 replication cycle. AG induction was limited to 1-2 hrs and subsequent infections were only  
872 allowed to proceed for ~1 phage replication cycle to prevent prolonged induction of various AGs  
873 from biasing the results. This setup also avoids biases resulting from epigenetic modification of  
874 phages by widespread restriction-modification (R-M) defenses in *E. coli*. However, we note that  
875 allowing sufficient time for the phages to complete cell lysis was necessary as fast-growing cells  
876 were sometimes able to form colonies on LB agar despite having been infected. These colonies  
877 had an uneven size distribution and a “splotchy” appearance, perhaps due to ongoing infection  
878 on the plate that was nevertheless not able to outpace colony formation. After the infection  
879 period was complete, the cultures were vortexed, diluted 1:100, vortexed again, and 50 µl of the  
880 dilution was plated on 2 large (150 mm X 15 mm) LB agar plates (with 10 µg/ml gentamicin and  
881 0.5% glucose to repress AGs and avoid fitness costs). The next day >>2,000-20,000 colonies  
882 were scraped from each infected and uninfected set of plates. 5 µl of the thick resulting cell  
883 mixtures was reserved for genomic DNA extraction, and the rest was frozen at -80°C in 20%  
884 glycerol as a backup. A full technical record of our screens is provided in Supplementary File 4.

885 AG barcodes were amplified and sequenced as with the WM6026 donor library, with a  
886 slight modification. The three biological replicate sets of ECOR+AG experiments were amplified  
887 using JSW-SS-171 and different versions of primer JSW-SS-170 containing extra N's at the  
888 start of the sequencing read. The first replicate was amplified with JSW-SS-170 (containing 5  
889 N's), the second replicate with JBD-SS-254 (CGACGCTCTTCCGATCTNNNNNN  
890 TGATGTCGTTGTTGCCATCG) with 6 N's, and the third replicate with JBD-SS-255  
891 (CGACGCTCTTCCGATCTNNNNNNN TGATGTCGTTGTTGCCATCG) with 7 N's. This ensured  
892 that reads from the three sets would not cross contaminate analyses of replicate experiments.  
893 Amplicons were sequenced to the same depth as the baseline libraries on Illumina MiSeq and  
894 NextSeq instruments in single-end format (100 bp) with two (8 bp) index reads.

895 We required exact matches for the 20 bp primer-derived invariant sequences flanking  
896 the barcodes. These sequences were trimmed and the proportions of various AG-encoding  
897 strains in the mixtures were determined using the prevalence of the corresponding 10 bp  
898 barcode sequences. Barcode counts per AG were normalized by dividing each count by the  
899 median of each experiment, which would represent neutral AG fitness. A log<sub>2</sub> transformation  
900 was subsequently applied to obtain fitness scores. Care was taken to sequence each library to  
901 similar depth such that fitness scores across experiments were distributed in the same range.  
902 Poorly represented AGs were identified in the baseline libraries as well as the infected and  
903 uninfected experimental samples using a low read cutoff of 100, or if the log<sub>2</sub> normalized  
904 average read counts across the three replicates for any AG were >3x the median absolute  
905 deviation below the median for that experiment. Reproducibility was determined using T-tests  
906 across the three replicates, and we enforced a P-value cutoff of 0.2. The magnitude of the AG  
907 fitness effect was determined by (a) subtracting the AG fitness scores in the uninfected library  
908 from the infected libraries to assess whether AGs changed host sensitivity to phage infection,  
909 and (b) subtracting the AG fitness scores in the baseline library from the uninfected libraries to  
910 assess whether AGs change host fitness directly. AGs were selected for further study if they  
911 were not poorly represented to begin with, produced reproducible effects across replicates, and  
912 changed host fitness by at least 4-fold. Although we had targeted our experiments to phage-  
913 host combinations where the host was mostly resistant to the phage in order to find counter-  
914 defense AGs, a few hosts turned out to be more sensitive to certain phages in liquid infection



915 than suggested by agar-overlay plaque assays (perhaps due to abortive antiviral defenses). In  
916 these cases, our platform was also able to robustly identify AGs that conferred a protective  
917 advantage to the host (e.g. Imm and Cor, which both block phage DNA entry <sup>51,52</sup>). In practice  
918 however, these effects can be very variable and the reproducibility filters typically needed to be  
919 relaxed in order to find such superinfection exclusion AGs.

920 All counter-defense AGs were subsequently conjugated into the wild ECOR hosts where  
921 they demonstrated an effect, and tested against all 8 phages (not just the ones that were used  
922 in the screen with that host). In several hosts, the AG was found to additionally sensitize the  
923 host to phages that were omitted in the screen. Not all the screen phenotypes could be  
924 confirmed in agar-overlay plaque assays, possibly due to variations in cell growth between liquid  
925 and solid media, or due to the requirement for successive infection cycles for plaque formation  
926 that would render plaque assays less sensitive than single-infection cycle dynamics in our  
927 screen. Nevertheless, further experiments were performed only in phage-host-AG combinations  
928 that could be confirmed by plaque assay due to ease of follow up testing.

929 Conditional lethal AGs were identified directly from the heatmap of fitness scores, with a  
930 subset selected for further experimentation using an arbitrary fitness cutoff of ~6-7 (~100-fold).

931

### 932 **Tn5 Dropout Screen + Data analysis to find phage exclusion systems**

933 Tn5 whole-genome knockout strain libraries were constructed using the RB-TnSeq  
934 donor library (pKMW7-derived strain APA766 from Dr. Adam Deutschbauer) conjugated with the  
935 recipient ECOR strain in a biparental mating on LB plates supplements with 300  $\mu$ M DAP. The  
936 next day, cell mixtures were scraped and resuspended in 1 ml LB and 1000X dilutions of this  
937 conjugation mixture were plated on 10 large (150 mm X 15 mm) LB plates (lacking DAP)  
938 supplemented with 50  $\mu$ g/ml kanamycin. Approximately 0.5-1 million colonies were scraped  
939 taking care to break colonies apart on the surface of the plates, mixed very thoroughly,  
940 aliquoted and stored at -80°C with 20% glycerol, with ~5-10  $\mu$ l reserved for genomic DNA  
941 extraction and reference library preparation.

942 RB-TnSeq allows the same mutant library to be challenged under various conditions  
943 such as challenge by various phages, and simplifies the downstream sequencing-library  
944 preparation by substituting barcode sequencing for all experimental conditions after initial  
945 characterization of the reference library. To characterize the Tn5-knockout reference libraries in  
946 the various ECOR strains, genomic DNA was extracted from 5-10  $\mu$ l of the cell scrape and  
947 prepared for sequencing using the NEBNext Ultra II FS DNA Library Prep Kit (NEB) with the  
948 following modifications. 500 ng DNA was fragmented for 25 min according to manufacturer's  
949 instructions. The adaptor ligation step was performed as directed albeit with a custom pre-  
950 annealed oligo adaptor comprising of YL001 (/5Phos/GATCGGAAGAG/3ddC/) annealed to one  
951 of YL002:005

952 (AGCGGCAATTTACACAGGACAAGCAGAAGACGGCATAACGAGATNNNNNNNNAGTGGTG  
953 ACTGGAGTTCAGACGTGTGCTCTTCCGATC\*T) where the 4-base underlined sequence

954 serves as a variable barcode unique to each oligo. The USER Enzyme steps post-ligation were  
955 skipped and the bead-cleanup was performed using 40  $\mu$ l and 20  $\mu$ l of Sample Purification  
956 Beads successively as instructed for library size selection in the 150-250 bp range. The PCR  
957 amplification of transposon-genome junctions was performed using the cycling parameters  
958 described in the kit for 11 cycles with Q5 Ultra II FS Master Mix (NEB) using primers YL006  
959 (AGCGGCAATTTACACAGGA) and JBD-SS-272/oAD493

960 (ACACTGGCAGAGCATTACGCCCT) instead of the kit-supplied primers. A second nested-PCR  
961 enrichment step was performed for 11 cycles using the same cycling parameters as before,  
962 using the entire bead-purified reaction product from the first step as a template with primers  
963 YL009 (CAAGCAGAAGACGGCATAACGAG) and JBD-SS-273:280

964 (AATGATACGGCGACCACCGAGATCTACAC TATAGCCT  
965 ACACTCTTCCCTACACGACGCTCTTCCGATCT NNNNNNNN

966 GCAGGGATGTCCACGAGGTCTCT) where N<sub>8</sub> barcodes correspond to reverse-complemented  
967 Illumina TruSeq HT D501-508 indexes. Bead-purified amplicons were quantified using the 1X  
968 dsDNA HS kit with the Qubit 4.0 Fluorometer (Invitrogen) and sequenced on Illumina MiSeq and  
969 NovaSeq instruments in single-end format (150 bp) with two (8 bp) index reads.

970 The first 100 bp of these reads contain transposon derived sequences and a DNA  
971 barcode that uniquely identifies a particular Tn5 insertion. The last 50 bp of these reads contain  
972 host-genome derived sequence that help determine the location of the transposon insertion and  
973 hence construct a map between the Tn5 barcode and the insertion site (IS) in the genome.  
974 Briefly, 20 bp barcodes and 16-50 bp IS sequences were trimmed from the sequencing reads.  
975 To isolate the barcode and IS, we allowed 3 mismatches in the 23 bp invariant sequence  
976 upstream and up to 6 mismatches in the 50 bp downstream of the barcode using a Hamming  
977 distance function to compare the expected and actual sequences. Any reads failing these  
978 sequence-fidelity requirements were discarded. Reads containing an IS that matched the Tn5  
979 transposon vector in the donor strain were also discarded. The 4 Ns adjacent to the underlined  
980 index sequences in YL002:005 were also sequenced as part of one 8 bp index read and served  
981 as a tag to remove PCR duplicates. This allowed us to distinguish between sequencing “reads”  
982 and DNA “fragments” present in the sample at the adaptor ligation step. Thus, reads containing  
983 unique combinations of barcode, PCR tag, and IS were entered into the barcode/IS map for  
984 each Tn5 library, each representing a distinct DNA fragment (but also machine errors in  
985 sequencing, chimeric PCR amplicons, 3’ truncations etc.). The filtered ISs were then mapped to  
986 genes within their respective host genomes. As described previously<sup>53</sup>, it is possible for  
987 transposon barcodes to map to the genomes ambiguously. Barcodes associated with multiple  
988 distinct ISs were retained only if their most frequently represented IS accounted for over 90% of  
989 the fragments containing that barcode. We then counted the number of DNA fragments  
990 recovered per gene and used this as a reference library for subsequent BarSeq experiments to  
991 assay depletion of strains with mutations in specific genes upon phage infection.

992 Tn5 mutant strain libraries of the various ECOR strains were then challenged by various  
993 phages in a procedure analogous to the ECOR+AG screens. Cultures of Tn5 libraries were  
994 initiated with 100 µl of thick cell scrape mixture in 100 ml LB supplemented with 50 µg/ml  
995 kanamycin, Mg<sup>2+</sup>, and Ca<sup>2+</sup>, to an initial OD<sub>600</sub> value of 0.1-0.15. Cultures were grown at 37°C  
996 with shaking for 80-90 min to an OD<sub>600</sub> of 0.7-1. A 10<sup>-5</sup> dilution of each late-log phase culture  
997 was then plated on LB agar (with 50 µg/ml kanamycin) to obtain a count of viable colony forming  
998 units (cfu). Cultures were immediately diluted ~1:10 in fresh LB (with Mg<sup>2+</sup>, Ca<sup>2+</sup>, and 50 µg/ml  
999 kanamycin) and 1 ml aliquots were gently mixed with lysates of the appropriate phages at an  
1000 MOI-e of ~30-100 (the actual MOI-e values were recorded the next day when cfu counts were  
1001 obtained). Since the parental ECOR strains were typically quite resistant to phage infection to  
1002 begin with, only mutants with transposon-inactivated defense systems would be expected to  
1003 drop out upon infection. In practice however, some strains exhibited more pronounced mortality  
1004 upon prolonged phage infection in the Tn5 screens. In particular, in experiments with T2 and the  
1005 Tn5 library constructed in the  $\Delta wecA$  strain of ECOR21 (which was generally more susceptible  
1006 to infection by T2 and other phages), MOI was titrated in three parallel infection screens (with  
1007 varying amounts of phage lysate added: 350 µl, 100 µl, and 25 µl) to empirically ensure specific  
1008 depletion of only the phage-sensitive mutants. Parallel uninfected controls were maintained for  
1009 each experiment, and each set of experiments was performed in biological duplicates. Infections  
1010 were allowed to proceed for 5 min at the benchtop without shaking, and 75 min at 37°C with  
1011 shaking. Since an interaction between the host and phage had already been identified in the  
1012 earlier AG screen, infections were not restriction to a single phage replication cycle, but allowed  
1013 to proceed further to achieve full depletion of the phage-sensitive Tn5-knockout mutants in the  
1014 pool. After the infection period was complete, the cultures were vortexed, diluted 1:10, vortexed  
1015 again, and 100 µl of the dilution was plated on 2 large (150 mm X 15 mm) LB agar plates (with  
1016 50 µg/ml kanamycin). We note that this dilution yields bacterial lawns and in our experience this

1017 preserves diversity of surviving colonies without degrading the signal of dropouts by phage-  
1018 killing in the experiment. The next day, bacterial lawns were scraped from each infected and  
1019 uninfected set of plates. 5  $\mu$ l of the thick resulting cell mixtures was reserved for genomic DNA  
1020 extraction, and the rest was frozen at -80°C in 20% glycerol as a backup. A full technical record  
1021 of our screens is provided in Supplementary File 8.

1022 To amplify barcodes from the post-selection infected and uninfected Tn5 libraries, we  
1023 followed a modified BarSeq98 method<sup>53</sup>, with two successive rounds of PCR. The transposon  
1024 barcode sequence was amplified by PCR using Q5 Ultra II FS Master Mix (NEB) with primers  
1025 JBD-SS-310 (CGACGCTCTTCCGATCTNNNNNNN GATGTCCACGAGGTCTCT) and JBD-SS-  
1026 311 (ACTGACGCTAGTGCATCA GTCGACCTGCAGCGTACG). Template-matching regions in  
1027 the primers are underlined. Sequencing adaptors were attached in a second round of PCR  
1028 using amplicons from the first PCR round as the templates, with dual-indexed primer sets JSW-  
1029 SS-42:53 and JSW-SS-54:61 (sequences already provided in section describing AG screening).  
1030 Cycling conditions for round 1 were as follows: one cycle at 98°C for 4 min; two cycles at 98°C  
1031 for 30s, 63°C for 30s, and 72°C for 30s; 22 cycles at 98°C for 30s, and 72°C for 30s; and one  
1032 cycle at 72°C for 4 min. Conditions for round 2 were one cycle at 98°C for 1 min; two cycles at  
1033 98°C for 10 s, 64°C for 30 s, and 72°C for 10 s; 4 cycles at 98°C for 10 s, and 72°C for 20 s;  
1034 and one cycle at 72°C for 2 min. 0.5-1  $\mu$ l of unpurified 1<sup>st</sup> round reaction product was used as  
1035 template for the 2<sup>nd</sup> round of PCR. Amplicons from the 2<sup>nd</sup> round were gel-purified by  
1036 electrophoresis (3% agarose gel, 4.2 V/cm, 2 hours) and quantified using the 1X dsDNA HS kit  
1037 with the Qubit 4.0 Fluorometer (Invitrogen). Amplicons were sequenced on Illumina MiSeq and  
1038 NovaSeq platforms in single-end format (150 bp) with two (8 bp) index reads.

1039 DNA barcodes were trimmed from the reads and the proportions of various Tn5-  
1040 knockout mutants in the mixtures were determined using the prevalence of the corresponding  
1041 barcode sequence. Briefly, we allowed 3 mismatches in the 18 bp invariant sequence upstream,  
1042 and 4 mismatches in the 18 bp downstream of the transposon barcodes using a Hamming  
1043 distance function to compare the expected and actual sequences. The frequency of occurrence  
1044 of each barcode (representation number) was counted. Transposon barcodes were matched to  
1045 those found in the reference libraries, inferred to represent knockouts in the genes containing  
1046 the corresponding ISs, and the various barcode representation numbers were added together  
1047 for each gene. Cumulative transposon insertions per gene were then compared between the  
1048 infected and uninfected samples (with the reference library as a baseline to filter out poorly  
1049 represented, presumably essential genes) using the same data analysis procedure as for the  
1050 AG screen, albeit with two biological replicates instead of three.

1051

## 1052 **Tn5 Suppressor Screen + Data analysis to find abortive loci**

1053 Tn5 whole-genome knockout strain libraries were constructed using the RB-TnSeq  
1054 donor conjugated with the recipient ECOR strain as before, with modifications. No phages are  
1055 involved. The recipient ECOR strain was equipped with the AG (as a single copy chromosomal  
1056 Tn7 insertion as usual) which triggers PCD, presumably by activating an Abi system. The trigger  
1057 AG was inserted prior to Tn5 library construction in order to minimize passages of the library  
1058 prior to screening and avoid potential bottlenecks due to fitness effects of the various Tn5  
1059 knockouts. Libraries were constructed as above, except that conjugation was carried out under  
1060 strict glucose repression to prevent PCD activation at this stage, and the conjugation mixture  
1061 scraped from the LB+DAP+glucose plates was then plated directly on LB plates containing 10  
1062  $\mu$ g/ml gentamicin and 1 mM IPTG in addition to 50  $\mu$ g/ml kanamycin. We typically needed to  
1063 plate the entire conjugation mixture scraped from five 100 mm LB+DAP+glucose plate onto five  
1064 150 mm LB+IPTG+Kan+Gm plate in order to obtain sufficient numbers of suppressor colonies  
1065 for characterization by high-throughput sequencing. Control samples were obtained by plating  
1066 1000-2000X dilutions on LB+glucose+Kan+Gm plates. Suitable dilutions were empirically  
1067 determined for the various ECOR hosts. The next day, suppressor colonies were scraped from

1068 the large plates and stored at -80°C with 20% glycerol, with ~5-10 µl reserved for genomic DNA  
1069 extraction and sequencing. Genomic DNA was extracted and libraries were prepared and  
1070 sequenced akin to the treatment of the RB-TnSeq/NEBNext reference libraries detailed above.  
1071 There was no need for barcode sequencing since the screen identifies survivors instead of  
1072 dropouts, and each library was only characterized once after IPTG induction/selection for  
1073 suppressor mutants. Tn5 reference libraries were constructed from the data using the same  
1074 procedures for the baseline libraries constructed in the various naïve (without AGs) ECOR  
1075 strains above. The number of transposon insertions (i.e. the number of uniquely mapping ISs  
1076 from distinct DNA fragments) recovered per gene were used directly as a measure of survival of  
1077 mutants despite expression of PCD-triggering AGs. In practice, no statistical analysis was  
1078 necessary to identify the genetic loci responsible for PCD, as the signal-to-noise ratio was  
1079 sufficiently high to allow visual identification of hits when transposon insertion frequency was  
1080 plotted in control vs experimental samples (i.e. when the trigger AG was repressed by plating on  
1081 glucose-containing media, vs induced with IPTG).

### 1082 1083 **LPS gel electrophoresis**

1084 Lipopolysaccharide was prepared from liquid overnight cultures of wild *E. coli* strains  
1085 expressing the relevant AGs (LB + IPTG). Briefly, 50-100 µl of saturated culture was spun down  
1086 and cells were thoroughly resuspended in 100-200 µl of lysing buffer (62.5 mM Tris-HCl, pH6.8,  
1087 10% glycerol, 2% SDS, 4% β-mercaptoethanol). We routinely supplement 1X Laemmli Buffer  
1088 (BioRad) with SDS and βME to make lysing buffer. Samples were boiled at 100°C for 15 min,  
1089 and allowed to rest at room temperature for 15 min. 100 µg of Protease K were added to each  
1090 sample and protein was digested at 60°C for at least 1 hr. 15 µl of each sample were run onto a  
1091 12% polyacrylamide TGX Mini-Protean Precast gel (BioRad) at 200V for 35 min, and visualized  
1092 using the Pro-Q Emerald 300 LPS stain kit (Thermo Fisher) precisely according to  
1093 manufacturer's instructions.

### 1094 1095 **Allelic Exchange for *wecA* deletion**

1096 The R6K-origin pKEK2201 vector (from Prof. Karl E. Klose) carrying the desired genetic  
1097 modification (eg. a *wecA* deletion) flanked by 1000 bp homology arms was prepared by Gibson  
1098 assembly, and delivered as a suicide vector into wild *E. coli* strains via conjugation from the  
1099 WM6026 auxotrophic donor. Conjugation was performed using the same method used to deliver  
1100 AGs, albeit with biparental instead of triparental mating. Successful transconjugants (containing  
1101 a chromosomally integrated plasmid after the first homologous recombination event HR1) were  
1102 selected by incubation on LB plates supplemented with kanamycin. The second recombination  
1103 event HR2 to complete the allelic exchange was selected via *sacB*-mediated counterselection  
1104 on LB plates supplemented with 300 mM sucrose after overnight growth of HR1  
1105 transconjugants. Successful allele exchange was verified by PCR, and by LPS electrophoresis  
1106 to assay for the absence of O-antigen in the case of the *wecA* deletion.

### 1107 1108 **Live cell Microscopy**

1109 Cells were prepared for microscopy, immobilized under an LB agarose pad, and imaged  
1110 as described previously<sup>24</sup>. Where necessary, AGs were pre-induced with 1 mM IPTG for 30 min  
1111 immediately before harvesting for microscopy.

### 1112 1113 **Verification of abortive loci activity**

1114 Abortive/PCD systems identified by transposon screening of conditionally lethal AGs  
1115 were cloned by Gibson assembly using the NEBuilder HiFi DNA assembly master mix (NEB)  
1116 onto pBAD Myc/His A vectors along with their native promoters wherever possible. AGs that  
1117 trigger PCD systems (or appropriate controls) were conjugated into DH10β/TOP10 strains  
1118 carrying pBAD-PCD plasmids using the Tn7 transposition method (above). Where the



1119 restriction/PCD systems prevented conjugation of AGs, the order of transformations was  
1120 reversed – the AG was conjugated into naïve DH10 $\beta$ /TOP10 cells first, and subsequently these  
1121 strains were transformed with the pBAD-PCD vectors. Successful transconjugants were grown  
1122 overnight with glucose to repress trigger AGs, and viability was tested the next day by spotting  
1123 10-fold serial dilutions of saturated cultures on LB agar plates with four additives: 1) Glucose  
1124 repression to assess background toxicity, (2) Arabinose induction of PCD systems alone to  
1125 assess fitness costs of over-expression of abortive components without the trigger AGs, (3)  
1126 IPTG to assess ability of AGs to trigger PCD without over-expression of abortive components  
1127 (i.e. under leaky expression conditions, or when expressed from the native promoters where  
1128 present), and (4) IPTG and Arabinose to assess ability of AGs to trigger PCD when abortive  
1129 components were also over-expressed.

1130

### 1131 **Affinity purification of AGs from wild *E. coli* hosts**

1132 FLAG-tagged AGs were conjugated into ECOR strains by Tn7-transposition (above)  
1133 according to Supplementary File 9. Each strain was grown overnight at 37°C in 2 mL of LB  
1134 supplemented with .5% glucose and 10 mg/mL gentamicin, then diluted 1:100 into 100 mL LB  
1135 supplemented with 10 mg/mL gentamicin and grown at 37°C for 30 minutes. The media was  
1136 then supplemented with 1 mM IPTG and grown for another 2.5 hours. “Popcorn” pellets were  
1137 collected by centrifugation for 30 minutes at 4,000 X g, resuspension in 100  $\mu$ L of ice-cooled  
1138 lysis buffer [50 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM MgCl<sub>2</sub>, 0.5% NP40  
1139 (Research Products International), 125 U/mL Benzonase (Millipore), 1x protease inhibitor  
1140 cocktail (Roche, cOMplete ULTRA tablets, mini, EDTA-free), 0.5 mg/ml lysozyme (Fisher  
1141 Scientific)], followed by dropwise addition into liquid nitrogen and stored at -80°C. Popcorn  
1142 pellets were lysed by 10 cycles of cryomilling for 2 minutes at 12 counts per second in a SPEX  
1143 SamplePrep 6870D Freezer/Mill and stored at -80°C.

1144 For each phage-host combination, 3 replicate lysates were reconstituted using 200 mg  
1145 of the cryomilled powder resuspended in 750  $\mu$ L of lysis buffer. Lysates were cleared by  
1146 centrifugation at 16,000 X g at 4°C for 15 minutes. Anti-FLAG magnetic agarose beads (Pierce)  
1147 were washed on a magnet with 1 mL of ice-cooled IP buffer (50 mM Tris pH 7.4, 150 mM NaCl,  
1148 1 mM EDTA, 1mM MgCl<sub>2</sub>) twice and resuspended to their original volume in IP buffer. Cleared  
1149 lysates were incubated with 30  $\mu$ L of FLAG beads on an end-over-end tube rotator at 10 rpm at  
1150 4°C overnight. The supernatant was separated from the beads on a magnet. Beads were  
1151 washed once with 700  $\mu$ L of .05% NP-40 and 1x protease inhibitor cocktail in IP, once with 700  
1152  $\mu$ L of .05% NP-40 in IP, and 3 times with 700  $\mu$ L of IP. Proteins were eluted by incubation with  
1153 25  $\mu$ L of 100  $\mu$ g/ml 3xFLAG peptide (Sigma) in 0.05% RapiGest (Waters Corp) in IP buffer on  
1154 an end-over-end tube rotator at 10 rpm at 4°C overnight. The eluate was separated from the  
1155 beads on a magnet. The elution step was then repeated with the same beads and fresh elution  
1156 buffer at room temperature for 1 hour. The eluates were combined and stored at -20°C.

1157

### 1158 **Mass spectrometry sample preparation and data acquisition**

1159 Samples were prepared for Mass Spectrometry (MS) as described previously in the  
1160 Filter-Aided Sample Preparation Protocol<sup>55</sup> with the following modifications. Before transferring  
1161 to an AcroPrep Advance 10k Omega plate, each eluate was resuspended in 100  $\mu$ L of 8M Urea  
1162 (Thermo Scientific) dissolved in 50 mM ammonium bicarbonate (MP Biomedicals) and 5 mM  
1163 TCEP (Aldrich), followed by centrifugation to dryness. During alkylation of cysteine residues, 10  
1164 mM chloroacetamide (Thermo Scientific) was used instead of 10 mM iodoacetamide. Wash  
1165 steps prior to proteolysis were carried out with 200  $\mu$ L of 20 mM ammonium bicarbonate  
1166 (instead of 100  $\mu$ L). Proteolysis was performed without the addition of lysyl endopeptidase.  
1167 Proteolysis was quenched by addition of 10% trifluoroacetic acid (Thermo Scientific) to reach  $\approx$   
1168 pH 2. Peptides were then purified over a BioPureSPE Mini 96-Well Plate (Nest Group). Briefly,  
1169 C18 columns were activated by washing once with 400  $\mu$ L of acetonitrile (Fisher Scientific) and



1170 twice with 400  $\mu$ L of 0.1% formic acid (Thermo Scientific) in water solution, LC–MS grade  
1171 (Thermo Scientific). Samples were loaded and centrifuged at 1500 X g for 2 minutes. The bound  
1172 peptides were washed 3 times with 400  $\mu$ L of 1% formic acid before elution with 150  $\mu$ L of 50%  
1173 acetonitrile in 0.1% trifluoroacetic acid. Peptides were vacuum dried and stored at -80°C.

1174 Samples were resuspended in 15  $\mu$ l of 0.1% formic acid prior to loading for MS. The MS  
1175 acquisition was performed as described<sup>56</sup> with same MS parameters and LC configuration.

1176

### 1177 **Mass Spectrometry data analysis and statistical analysis**

1178 Raw files were processed in FragPipe<sup>57</sup> using the LFQ-MBR workflow with minor  
1179 modifications. The files were searched against a combined database of protein sequences from  
1180 ECOR1, ECOR3, ECOR15, ECOR21, ECOR22, ECOR66<sup>49</sup>, and MG1655 with duplicate entries  
1181 removed and common contaminants added. Decoys were generated by pseudo-inversion.  
1182 Protein and peptide FDR was fixed to 1% and MBR was disabled. Cysteine carbamylation was  
1183 set as fixed modification while N-term acetylation, methionine oxidation and pyro-glu formation  
1184 were set as variable modification with a maximum of 3 modifications per peptide.

1185 MaxLFQ intensities were used for statistical analysis<sup>58</sup>. Each triplicate set of host-AG  
1186 samples was quantile normalized against a triplicate set of samples from the same host  
1187 expressing *orf74*. For quantile normalization, all proteins in each sample were ranked according  
1188 to their MaxLFQ intensity. Next, the mean value of the intensities of proteins occupying a given  
1189 rank was calculated, then each protein's value was replaced with the mean value according to  
1190 its rank. Fold changes were calculated by taking the log<sub>2</sub> ratio of average normalized values  
1191 between the test set and the control set. For each host-AG sample, all positively enriched  
1192 proteins were ranked according to their fold change. For each AG, proteins were assigned  
1193 "summed rank" values by calculating the sum of the ranks of each protein across all hosts  
1194 where that AG was tested. P-values were calculated by student's t-test on the normalized sets  
1195 of replicates from the test and control samples. Top enriched candidates for each AG were  
1196 selected based on their summed ranks and p-values.

1197

### 1198 **Ronin systems phylogenetic analysis**

1199 The *ronA* nucleotide sequence in the ECOR55 Ronin system was used to identify similar  
1200 genes by BLASTN. *RonA*-encoding bacterial genomes were downloaded using the entrez direct  
1201 e-utilities command line tools. Of 100 complete genomes retrieved, *ronA* homologs had been  
1202 annotated in 84. *hsdR* genes and other Type I R-M components were identified using phmmer  
1203 and Defense Finder<sup>59</sup> respectively. Ronin-adjacent genetic architectures were visualized using  
1204 Clinker<sup>60</sup>. All ronin-adjacent architectures are shown in HTML files with mouse-over annotations  
1205 for genes.

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1253

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1263 Conceptualization, S.S., J.B.D.; Methodology, S.S., E.L.; Resources, A.F., D.S.; Software, S.S.;  
1264 Investigation, S.S., H.C., D.S.G.; Formal Analysis, S.S., H.C., M.J., K.S.M., E.V.K.; Visualization,  
1265 S.S., H.C., M.J., K.S.M.; Writing – Original Draft, S.S., H.C.; Writing – Reviewing & Editing, S.S.,  
1266 H.C., M.B., E.V.K., J.B.D.; Supervision, S.S., J.B.D.; Funding Acquisition, J.B.D.

1267

1268 **Competing Interests**

1269 S.S. is co-founder and equity holder in BillionToOne, Inc. J.B.D. is a scientific advisory board  
1270 member of SNIPR Biome, Excision Biotherapeutics, and LeapFrog Bio, consults for BiomX, and  
1271 is a scientific advisory board member and co-founder of Acrigen Biosciences. The Bondy-Denomy  
1272 lab received research support from Felix Biotechnology.

1273

1274 **Additional Information**

1275 Supplementary Information is available for this paper. Mass Spectrometry data available at  
1276 PRIDE <https://www.ebi.ac.uk/pride/login> with Username: [reviewer\\_pxd038604@ebi.ac.uk](mailto:reviewer_pxd038604@ebi.ac.uk) and  
1277 Password: 83OmeYpD. High-throughput sequencing data available at SRA with accession  
1278 PRJNA952709. Correspondence and requests for materials should be addressed  
1279 to [Sukrit.Silas@ucsf.edu](mailto:Sukrit.Silas@ucsf.edu), [Joseph.Bondy-Denomy@ucsf.edu](mailto:Joseph.Bondy-Denomy@ucsf.edu). Reprints and permissions  
1280 information is available at [www.nature.com/reprints](http://www.nature.com/reprints).

1281 **Supplementary Figure 1. Conditional-lethal phenotypes of known R-M inhibitors in wild *E.***  
1282 ***coli*. (A)** Schematic representation of conditional-lethality data from Figure 1d, showing only  
1283 known R-M inhibitors and an uncharacterized AG, *orf7:aop1*. **(B)** AGs from (A) tested for their  
1284 ability to block EcoKI restriction of phage  $\lambda$ vir in plaque assays with restriction-competent *E. coli*  
1285 MG1655. **(C)** Various mutants of Ocr tested in MG1655 for their ability to block EcoKI restriction  
1286 of  $\lambda$ vir, and in ECOR22 and ECOR55 for their ability to trigger PCD activity of EcoR22I and Ronin  
1287 respectively. F54V mutant of Ocr (known to evade a previously described ARIA, PARIS) still  
1288 triggers ARIA activity in ECOR55 but not ECOR22. Numbered Ocr mutants were described  
1289 elsewhere<sup>19</sup>. Notably, Ral, which operates by allosterically hyperactivating the  
1290 methyltransferase<sup>61</sup> (unlike Ocr and ArdA) does not trigger PCD in any strains. Conversely, Aop1  
1291 elicits PCD from the same systems triggered by Ocr and ArdA in (A) but does not inhibit Type I  
1292 R-M activity in (B). The predicted structure of Aop1 does not resemble a DNA mimic  
1293 (Supplementary File 7), and the protein is not negatively charged (pI ~4 for Ocr/ArdA, but ~8 for  
1294 *aop1*), suggesting a distinct mechanism of R-M inhibition. Thus, depending on their mechanism,  
1295 some R-M inhibitors trigger PCD in these strains, whereas others do not.

1296  
1297 **Supplementary Figure 2. Transposon insertions that suppress AG-induced conditional-**  
1298 **lethal phenotype in ECOR22.** Distribution of unique transposon insertions recovered from  
1299 ECOR22 survivors from Tn5 libraries upon *ocr* expression, mapped to the EcoR22I locus.

1300  
1301 **Supplementary Figure 3. Upstream non-coding region is required for Ronin PCD activity.**  
1302 **(A)** The first 135-bp of the 235-bp upstream non-coding region (marked by a thick red line) are  
1303 required for activity as truncation down to 100-bp causes inactivation. ArdA is chromosomally  
1304 integrated under pLacO-1 control and strains in the left (ArdA repressed) and right (ArdA induced)  
1305 panels are isogenic. **(B)** Over-expression of 235-bp upstream region is not toxic in TOP10  
1306 indicating that it is not the PCD effector. **(C)** As in (A), PCD phenotype of truncated Ronin system  
1307 when overexpressed (indicated as “pBAD”). Over-expression of truncated Ronin did not rescue  
1308 the PCD defect indicating that the upstream region contributes something in addition to the  
1309 promoter.

1310  
1311 **Supplementary Figure 4. Phage-host combinations included in AG screens (A)** Phage  
1312 susceptibility of select wild ECOR strains. Numbers in boxes are log<sub>10</sub>-transformed infection  
1313 scores (7 = 10<sup>7</sup>-fold higher plaquing than 0). Borders indicate that individual plaques were  
1314 visible in that phage-host combination. Combinations with attenuated phage infection that were  
1315 selected for the AG screen are in yellow. Combinations with attenuated infection that were  
1316 attempted but yielded no survivors in liquid infection experiments (perhaps due to Abi  
1317 phenotypes) are in red. **(B)** Phage-host combinations where counter-defense phenotypes were  
1318 confirmed for any AGs. Red denotes combinations where the screen could not be completed  
1319 (as in (A)). Yellow: combinations that were tested where none of the 200 AGs produced  
1320 counter-defense effects. Green: combinations where phage-sensitizing effects could not be  
1321 verified by plaque assays. Blue: combinations where counter-defense phenotypes were  
1322 confirmed by plaque assay (at least 100X higher EOP upon AG expression). **(C)** Defense  
1323 repertoire of selected ECOR strains. Computational prediction of the presence or absence of  
1324 various defense systems in ECOR strains used in the AG screen. Predictions were generated  
1325 using the ISLAND software suite<sup>62</sup>.

1326  
1327 **Supplementary Figure 5. Over-expression of *galU* reverses T4 sensitization by *gnar13*.**  
1328 log<sub>10</sub>-transformed T4 infection scores in wild hosts ECOR1 (green), ECOR3 (orange), ECOR22  
1329 (blue) with or without UDP-glucose biosynthesis pathway genes *galU*, *galE*, *galF*, and ECA  
1330 precursor *wecB* cloned onto a plasmid and overexpressed.



1331  
1332 **Supplementary File 1. Worked example of AG-finding algorithm.** Readme.txt describes how  
1333 to run the code. Sample database of Salmonella phage genomes is included. All ARs from this  
1334 phage dataset are reported in the output file SPFMphages\_accRegions\_nr\_all.txt. Any AR can  
1335 be extracted using the supplied code and then visualized using Clinker<sup>60</sup>. The AR shown in  
1336 Figure 1d is extracted by default.

1337  
1338 **Supplementary File 2. Manually inspected accessory regions from Enterobacteriophage**  
1339 **genomes.** Visualizations of all ~200 accessory regions (ARs) that were manually inspected.  
1340 The first slide in the file shows the layout for the rest, with the AR score calculated according to  
1341 the formula in Methods, followed by a unique identifier for each hypothetical phage group  
1342 (“phamily”). Each AR was also given a short, memorable name (top right). Slides with green  
1343 backgrounds indicate ARs from which genes were selected for the AG screen. Authors’  
1344 impressions upon AR inspection are included in the Notes section.

1345  
1346 **Supplementary File 3. Phage accessory genes selected for AG screens.** 200 accessory  
1347 genes (AGs) selected for screening. Genomic locations, protein and phage genome identifiers,  
1348 and protein descriptions are indicated. IDs denoted by “UDP” correspond to the orf/AG numbers  
1349 used throughout the manuscript.

1350  
1351 **Supplementary File 4. Experimental record of AG screens.** A detailed record of all AG  
1352 screens. The first sheet contains phage-host combinations, dates of the experiment, and  
1353 preliminary results. The next three sheets each correspond to one replicate each of AG  
1354 screening. Empirically measured multiplicity at time of infection, and dilutions used before  
1355 plating are also recorded. Screens that had to be repeated for technical reasons are color-  
1356 coded.

1357  
1358 **Supplementary File 5. Hits from transposon-mediated PCD suppressor screens.** Follow-up  
1359 screens to find inactivating transposon insertions in putative Abi mechanisms that might trigger  
1360 PCD in response to conditional-lethal AGs. These screens are performed with RB-TnSeq libraries  
1361 prepared with ECOR hosts already containing IPTG-inducible trigger-AGs (no phages present).  
1362 Genes inactivated by transposon insertions that were overrepresented in the AG-on condition  
1363 (IPTG-induced) relative to the AG-off condition (Glucose-repressed) are reported as hits.

1364  
1365 **Supplementary File 6. Genomic architectures of all ECOR55-like Ronin systems.**  
1366 Spreadsheet contains accession numbers and genomic locations of all *ronA* homologs. Grouping  
1367 variables indicate whether a Type I R-M system (containing *hsdR*, *hsdM*, and *hsdS*) was found  
1368 anywhere in the genome, and also whether a full (>400 aa) DUF4102 integrase was present  
1369 immediately upstream of Ronin. HTML files split Ronin systems into two groups based on the  
1370 presence of the integrase, and gene annotations can be viewed by mouse-over.

1371  
1372 **Supplementary File 7. AlphaFold predictions for all AGs that produced phenotypes in AG**  
1373 **screens.** PDB files containing predicted structures of *orf7* (*aop1*; triggers PCD by Ronin), *orf24*  
1374 (triggers PCD in ECOR61), *orf48*, *orf63*, *orf92*, (*gnarl1-3*; putative O-antigen modifiers), *orf98*  
1375 (triggers PCD in ECOR22), *orf116* (*abc1*; triggers PCD by P4 prophage), *orf126* (broad-  
1376 spectrum counter-defense), *orf143* (IplI; inhibitor of GmrSD), *orf145* (triggers PCD in MG1655),  
1377 *orf148* (broad-spectrum counter-defense), and *orf184* (triggers PCD by gop-beta-clI).

1378  
1379 **Supplementary File 8. Experimental record of follow-up transposon whole-genome**  
1380 **knockout screens.** The first sheet contains a detailed record of all follow-up transposon  
1381 screens designed to phenocopy counter-defense phenotypes of AGs. These screens are

1382 performed with RB-TnSeq libraries prepared with naïve ECOR hosts (no AGs present). Each  
1383 library is challenged by various phages one at a time. The timeline of each experiment indicates  
1384 library growth characteristics. Empirically measured multiplicity at time of infection, and dilutions  
1385 used before plating are also recorded. Screens were performed in two stages: first, a pilot with  
1386 one phage for each RB-TnSeq library, and then a larger experiment with all other phages that  
1387 showed enhanced infection upon expression of a counter-defense AG. The second sheet lists  
1388 all hits recovered from the screens.

1389  
1390 **Supplementary File 9. Host-AG combinations for affinity-purification/mass-spectrometry.**

1391 Matrix of all host-AG combinations where potential binding partners of AGs were affinity-purified  
1392 from the native host expressing counter-defense AGs, and identified by mass-spectrometry.

1393 Experiments were performed in triplicate. *Orf74* produced no phenotypes during AG screening

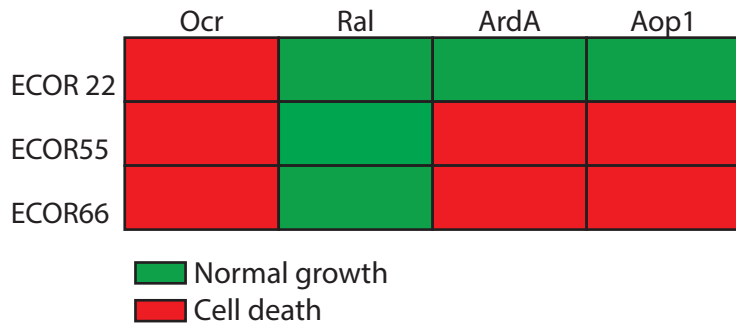
1394 and was used as a control for all experiments in the relevant hosts.

1395

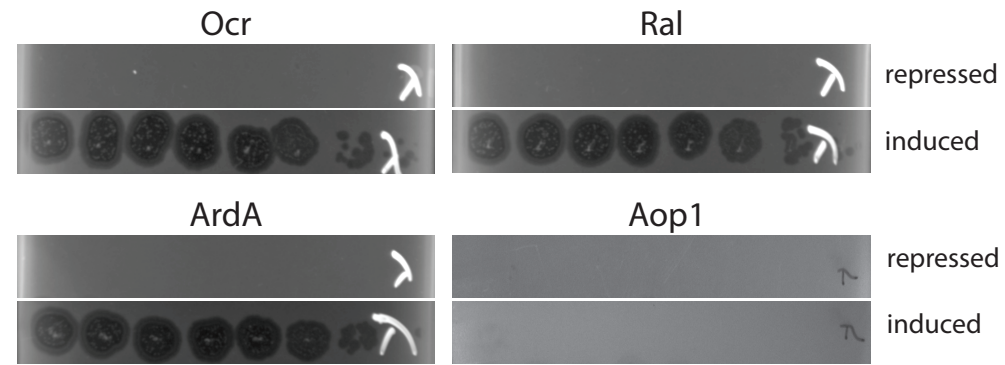
1396 **Supplementary File 10. Accessory regions of AGs selected for further study.**

1397 Visualizations of ARs which contain AGs that produced counter-defense or conditional-lethal  
1398 phenotypes in AG screens.

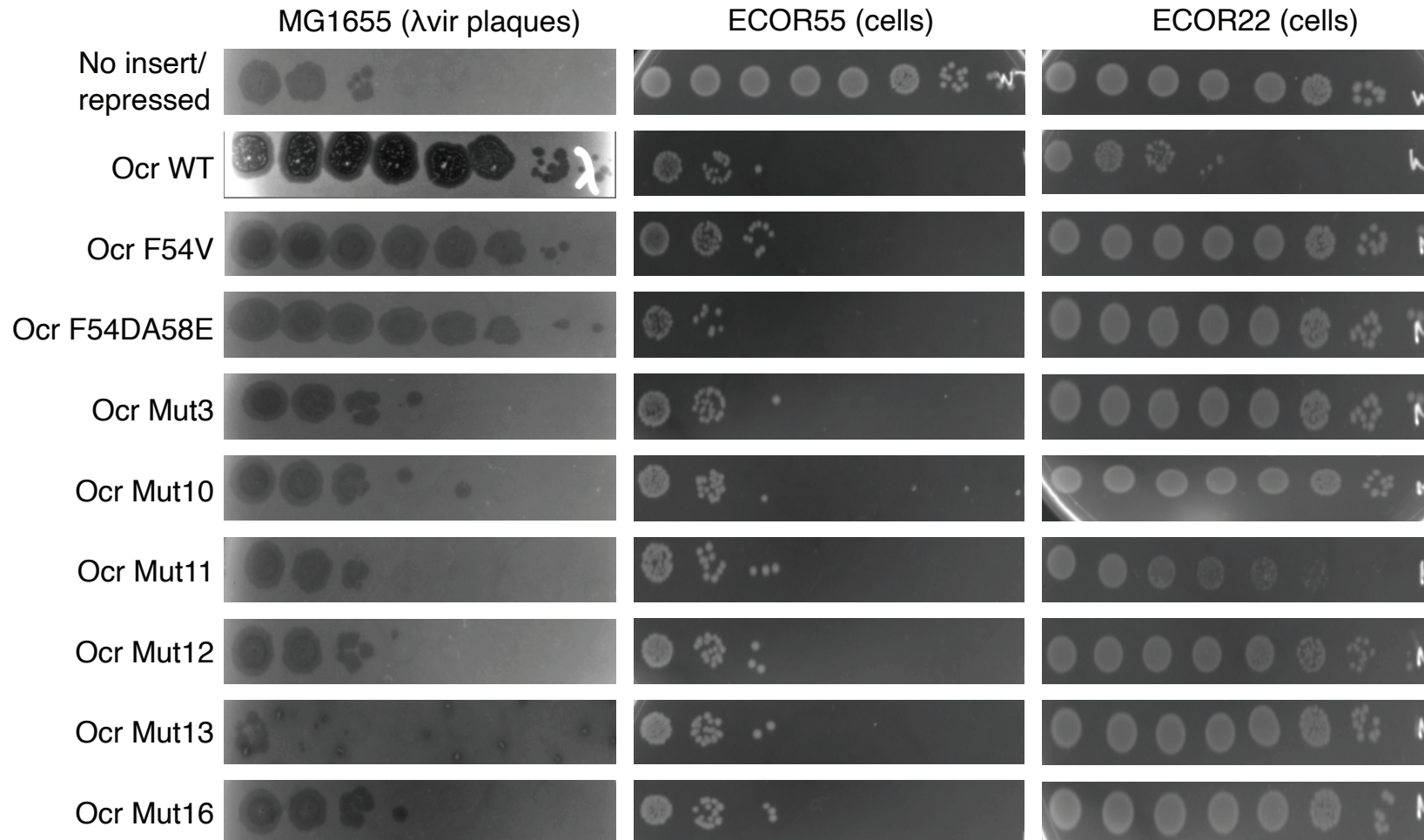
### A. R-M inhibitors trigger abortive defenses in wild *E. coli*



### B. R-M inhibition by AGs in MG1655



### C. Effect of *Ocr* mutations on R-M (plaquing on MG1655) and PCD activity (ECOR55, 22)



unique transposon insertions

5088

0

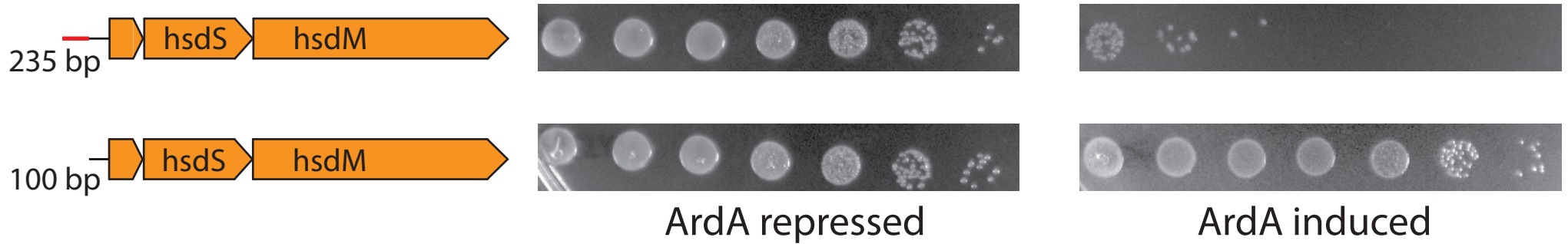
**ECOR22**

**+ *ocr***

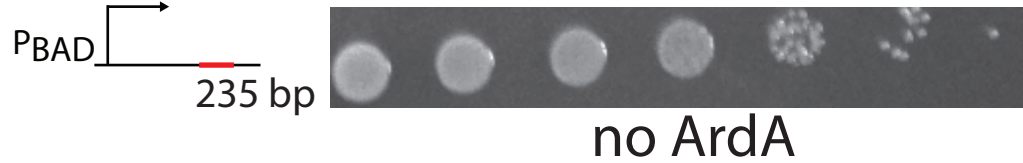




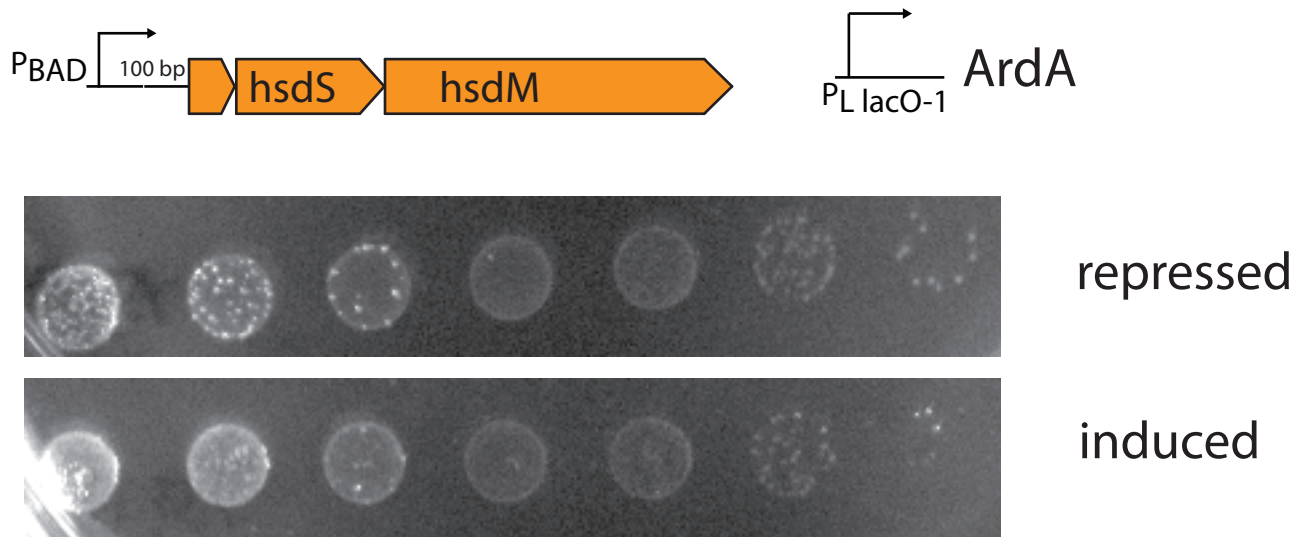
### A. Ronin upstream region is required for PCD activity



### B. Ronin upstream region is not toxic when overexpressed on its own

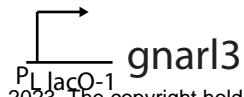


### C. Over-expression of Ronin with truncated upstream region





# no Tn7 insert



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## Empty vector

