

1 **Title:**

2 Mobile-CRISPRi: Enabling Genetic Analysis of Diverse Bacteria

3

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17

18 **Introductory paragraph:**

19 The vast majority of bacteria, including human pathogens and microbiome species, lack genetic

20 tools needed to systematically associate genes with phenotypes. This is the major impediment

21 to understanding the fundamental contributions of genes and gene networks to bacterial

22 physiology and human health. CRISPRi, a versatile method of blocking gene expression using a

23 catalytically inactive Cas9 protein (dCas9) and programmable single guide RNAs (sgRNAs), has

24 emerged as a powerful genetic tool to dissect the functions of essential and non-essential genes

25 in species ranging from bacteria to human. However, the difficulty of establishing effective

26 CRISPRi systems in non-model bacteria is a major barrier to its widespread use to dissect

27 bacterial gene function. Here, we establish “Mobile-CRISPRi”, a suite of CRISPRi systems that  
28 combine modularity, stable genomic integration and ease of transfer to diverse bacteria by  
29 conjugation. Focusing predominantly on human pathogens associated with antibiotic resistance,  
30 we demonstrate the efficacy of Mobile-CRISPRi in Proteobacteria and Firmicutes at the  
31 individual gene scale by examining drug-gene synergies and at the library scale by  
32 systematically phenotyping conditionally essential genes involved in amino acid biosynthesis.  
33 Mobile-CRISPRi enables genetic dissection of non-model bacteria, facilitating analyses of  
34 microbiome function, antibiotic resistances and sensitivities, and comprehensive screens for  
35 host-microbe interactions.

36

37 **Main text:**

38 CRISPRi (Clustered Regularly Interspaced Short Palindromic Repeats interference) is a  
39 programmable method for controlling gene expression that has enabled systematic interrogation  
40 of gene phenotypes in diverse organisms<sup>1-6</sup>. In bacterial CRISPRi, an sgRNA-dCas9 complex  
41 binds to a target gene by base-pairing and reduces gene expression by sterically blocking  
42 transcription elongation (Fig. 1a)<sup>1,4</sup>. New CRISPRi targets are easily programmed by  
43 substituting the first 20 nt of the sgRNA sequence (spacer) to match the non-template strand of  
44 the target gene, making design and construction of CRISPRi libraries that target specific sets of  
45 genes or the entire genome straightforward<sup>4,7,8</sup>. Genetic screens using CRISPRi libraries have  
46 contributed new insights into fundamental biology and molecular medicine including identifying  
47 functions for uncharacterized essential genes<sup>4,7</sup> and drug modes of action<sup>4,9</sup>.

48 CRISPRi provides several advantages over other methods for genetic manipulation in  
49 bacteria. CRISPRi knockdowns can be induced<sup>1,3-6</sup> and titrated/tuned<sup>4,10</sup>, enabling depletion of  
50 essential gene products without complex strain construction strategies that remove genes from  
51 their native regulation. Dissecting genetic redundancy via multiplexed CRISPRi targeting  
52 several genes in the same cell<sup>4,11</sup> requires markedly less effort than construction of multiple-

53 deletion strains. At the genome scale, CRISPRi expands on prior transposon-based gene  
54 perturbation methods such as Tn-seq<sup>12</sup> by allowing all genes—including essential genes that  
55 cannot be studied through deletion—to be systematically targeted so that a relatively small  
56 strain library provides comprehensive coverage of the genome. Moreover, the DNA sequences  
57 encoding sgRNAs serve as unique barcodes to differentiate CRISPRi strains mixed in a pool,  
58 allowing for competitive fitness measurements using next generation sequencing<sup>8</sup>. CRISPRi  
59 blocks expression of downstream genes in operons<sup>1,4</sup>, but this property can be used to further  
60 simplify libraries by targeting operons instead of genes.

61         Despite these advantages, CRISPRi has been used in only a few bacterial species both  
62 because CRISPRi has been transferred using species-specific<sup>4</sup> or narrow host-range<sup>1,3,5,6,13</sup>  
63 strategies, and because components need to be optimized for function in different species. To  
64 overcome this barrier, we developed “Mobile-CRISPRi”—a suite of modular and transferable  
65 CRISPRi components that can stably integrate into the genomes of diverse bacteria. The  
66 modularity of every component of Mobile-CRISPRi makes it straightforward to clone in  
67 organism-specific sgRNA libraries and other components (e.g. promoters, Fig. 1b). Mobile-  
68 CRISPRi achieves transfer and genomic integration by distinct mechanisms for Proteobacteria  
69 and Firmicutes. For Proteobacteria, Mobile-CRISPRi is transferred from *Escherichia coli* using  
70 the broad host range RP4 plasmid conjugation machinery, and is orientation and site specifically  
71 integrated into the recipient genome downstream of the highly conserved *glmS* gene using the  
72 extensively characterized Tn7 transposition system (Fig. 1c, top)<sup>14,15</sup>. Consistent with previous  
73 reports<sup>15</sup>, this strategy was unsuccessful in Firmicutes such as *Bacillus subtilis* (Supplementary  
74 Fig. 1), leading us to develop a strategy to transfer CRISPRi using the ICE *Bs1* conjugation and  
75 integration machinery. Here, Mobile-CRISPRi is transferred from *B. subtilis* to other Bacillales  
76 Firmicutes (e.g., *Staphylococcus aureus*—although the host range has not been experimentally  
77 defined), and integrated into *trnS-leu2*<sup>16</sup>(Fig. 1c, bottom). Critically, Mobile-CRISPRi integrations  
78 downstream of *glmS* and into *trnS-leu2* do not disrupt those gene’s functions<sup>14,16</sup>, occur in a

79 specified orientation, and are stable in the absence of selection for  $\geq 50$  generations (Fig. 2a),  
80 enabling studies of antibiotic function in which maintaining selection is problematic or  
81 impossible.

82 To assess the efficacy of Mobile-CRISPRi in non-model bacteria, we measured  
83 CRISPRi transfer and knockdown efficiency primarily in species involved in human disease. We  
84 quantified transfer efficiency by counting the number of recipient colonies (i.e., transconjugants)  
85 on selective agar plates (Fig. 2b). We found that most strains showed transfer efficiencies that  
86 were sufficient for genome-scale sgRNA library construction (e.g., *Enterobacter sp.*  $\sim 10^{-2}$ - $10^{-3}$ ,  
87 and *L. monocytogenes*  $\sim 10^{-2}$ ), whereas other strains were more suited for single gene  
88 knockdown approaches (e.g., *Acinetobacter baumannii*  $\sim 10^{-6}$ ).

89 To assess CRISPRi knockdown efficacy across species, we created a “test” version of  
90 Mobile-CRISPRi consisting of *rfp* (encoding Red Fluorescent Protein, or RFP) and either an  
91 sgRNA targeting *rfp*, or a “control” version lacking an sgRNA to normalize *rfp* expression. Our  
92 quantification of *rfp* knockdown in single cells using flow cytometry indicated that knockdown  
93 efficiency ranged from  $\sim 8$ -fold in *Pseudomonas aeruginosa* to  $\sim 130$ -fold in *S. aureus*, with a  
94 median knockdown of  $\sim 35$ -fold across all measured species (Fig. 2c). We confirmed that  
95 CRISPRi was also functional against native genes by targeting *P. aeruginosa* pyocyanin  
96 production (Supplementary Fig. 2). In this visual assay, the culture appears yellow, as  
97 production of the blue pigment pyocyanin is decreased.

98 Our initial assessment of Mobile-CRISPRi used pathogenic strains possessing at least  
99 rudimentary tools for perturbing gene function. To determine whether Mobile-CRISPRi functions  
100 in an environmental isolate with no existing genetic system, we tested transfer and knockdown  
101 in *Vibrio casei*, a  $\gamma$ -proteobacterium originally isolated from French wash-rind cheeses<sup>17</sup> and  
102 broadly associated with cheese microbiomes<sup>18</sup>. We found that Mobile-CRISPRi transferred to *V.*  
103 *casei* with library scale efficiency ( $\sim 10^{-3}$ , Fig. 2a), and a modest, but useful knockdown efficiency  
104 ( $\sim 8$ -fold, Fig. 2b). The modular nature of Mobile-CRISPRi allows for further optimization of

105 knockdown efficiency; for instance, by using *Vibrio*-specific promoters for *dcas9* and sgRNA  
106 expression. We conclude that Mobile-CRISPRi is an effective genetic tool for gene knockdowns  
107 in diverse, non-model bacteria.

108         The emergence of multi-drug resistant pathogenic bacteria is an urgent threat to human  
109 health that requires both new antibiotics and a better understanding how existing antibiotics  
110 work<sup>19</sup>. Knowledge of the mechanism by which antibiotics kill bacteria—the mode of action  
111 (MOA)—is critical to advance new antibiotics from the laboratory to the clinic<sup>20</sup>. Because the full  
112 complement of genes in a bacterial genome (i.e., genetic background) can affect antibiotic  
113 function<sup>20</sup>, the MOA should ideally be determined directly in clinically relevant strains. However,  
114 most pathogenic bacterial lack genetic tools to systematically perturb the functions of essential  
115 genes that typically encode antibiotic targets. Importantly, we previously showed that the ability  
116 to titrate the knockdown level enables the systematic study of essential genes in *B subtilis*. A  
117 low (~3-fold) level of knockdown allowed sufficient growth to determine the MOA of an  
118 uncharacterized antibiotic by virtue of its synergistic effects on growth<sup>4</sup>.

119         To explore whether Mobile-CRISPRi could be used to explore MOA in pathogenic  
120 Proteobacteria associated with antibiotic resistance (i.e., Gram-negative rods), we examined  
121 synergy between the antibiotic trimethoprim and the essential gene *folA*, which encodes the  
122 trimethoprim target dihydrofolate reductase<sup>21</sup>. We found that targeting *folA* with CRISPRi in  
123 *Enterobacter aerogenes*, *Klebsiella pneumoniae*, and *P. aeruginosa* strongly increased  
124 sensitivity to trimethoprim (Fig. 3) and shifted the minimal inhibitory concentration (MIC) by 2-4-  
125 fold, depending on the species (Fig. 3a). Even though CRISPRi knockdown in *P. aeruginosa* is  
126 at a lower efficiency compared to other strains (Fig. 2b), there was still a clear shift toward  
127 sensitivity (Fig. 3a). Moreover, concentrations of trimethoprim below the MIC for the WT,  
128 completely inhibited growth of the *folA* knockdown strains, clearly demonstrating synergy (Fig.  
129 3b). Thus, Mobile-CRISPRi targeting essential genes can be used to generate sensitized strains  
130 for antibiotic MOA studies.

131 A compelling feature of CRISPRi is the ease of pooled knockdown library construction,  
132 either for defined gene sets or at the genome scale<sup>8</sup>. As a proof of principle, we used Mobile-  
133 CRISPRi to construct a 40-member library of selected genes in the opportunistic pathogen,  
134 *Enterobacter cloacae* (Supplementary Tables 1 and 2). In the pooled context, each sgRNA  
135 functions as a barcode, enabling quantification of each knockdown strain in the pool. To  
136 evaluate our pipeline, we performed two different pooled experiments. In the first, all steps from  
137 initial cloning to analysis were performed in a pool (Fig. 4a). This revealed that all sgRNA strains  
138 were present and had reasonable representation in the pool (31/40 sgRNA counts were within  
139 one standard deviation of the median, with a maximum 50-fold difference in representation). In  
140 the second, each sgRNA plasmid was constructed individually, and an equimolar mixture of  
141 plasmids was used to transform *E. coli* and perform downstream steps (Fig. 4b). This assessed  
142 the variability of all steps downstream of cloning and revealed a maximum 2-fold difference in  
143 representation. Thus, Mobile-CRISPRi transfer and integration is highly uniform, with essentially  
144 all variability derived from the initial cloning step. Further optimization or alternative cloning  
145 strategies<sup>8</sup> may decrease the variability in sgRNA representation.

146 Our library consists of 10 amino acid biosynthesis genes, 4 putative essential genes and  
147 6 well-characterized genes, each targeted by 2 sgRNAs (Supplementary Table 1). For fitness  
148 measurements, we grew our library in glucose minimal medium in competition with a 100-fold  
149 excess wild-type *E. cloaca*. We measured the relative frequency of each strain in the library  
150 after 6 and 12 generations with and without CRISPRi induction to initiate knockdown. Using the  
151 fitness calculation of van Opijnen *et al.*<sup>12</sup>, we found that fitness of strains with sgRNAs targeting  
152 amino acid biosynthesis and those targeting some putative essential genes decreased, whereas  
153 representation of non-essential genes that are unrelated to amino acid biosynthesis remained  
154 constant (Fig. 4c; Supplementary Table 2). Fitness for affected strains was more pronounced at  
155 12 doublings than at 6 doublings, suggesting that a larger number of generations was required  
156 to dilute out existing protein products. Additionally, both guides generally decreased the fitness

157 of the essential and auxotrophic genes, but with more variability than previously observed<sup>4</sup>,  
158 potentially due to cross-feeding of nutrients between strains in the pooled context. Finally, the  
159 fitness measurements from the completely pooled construction (Fig 4a), and those in the equal  
160 representation library (Fig. 4b, Supplementary Table 2) were highly correlated ( $R^2=0.92$ )  
161 indicating that the initial frequency of the strain in the pooled library did not affect the  
162 measurement of the fitness (Fig. 4d).

163 We also screened an arrayed library of individual knockdown strains to confirm the  
164 auxotrophy of amino acid biosynthesis gene knockdown strains, finding that their poor growth in  
165 minimal medium was suppressed by relevant amino acids (Supplementary Fig. 3 and  
166 Supplementary Table 3). Thus, knockdown effects are specific to the targeted gene and do not  
167 represent off-target effects of CRISPRi. We conclude that Mobile-CRISPRi enables both pooled  
168 and arrayed library construction and straightforward assaying of phenotypes in non-model  
169 bacteria.

170 We anticipate that Mobile-CRISPRi will be a transformative technology for non-model  
171 bacteria lacking genetic tools and will facilitate cross-species genetic analysis. The existing  
172 Mobile-CRISPRi transfer and knockdown systems are already effective in many organisms, and  
173 its modularity makes it straightforward to expand host range (e.g., combining different transfer  
174 and integration functions, anti-restriction proteins<sup>22</sup>) and increase knockdown efficacy (e.g., use  
175 of “alternative” *dcas9* genes<sup>5,6</sup>). The stability of Mobile-CRISPRi in the absence of selection  
176 suggests that it could be a valuable tool for dissecting the genetics of host-microbe interactions  
177 in both pathogenic and microbiome contexts, and aid in MOA studies in relevant human  
178 pathogens. Interestingly, our approach for transferring CRISPRi mirrors natural transfer of  
179 CRISPR systems by transposons related to Tn7<sup>23</sup>. We will continue to look to nature for novel  
180 approaches to explore the vast landscape of bacterial genetics.

181

182 **Figure legends**



183 **Fig. 1 | Mobile-CRISPRi overview. a**, Mechanism of CRISPRi repression. A dCas9-sgRNA  
184 complex binds to DNA by base-pairing and sterically blocks progression of RNA polymerase  
185 (RNAP), reducing gene expression. **b**, Mobile-CRISPRi modularity. Individual modules are  
186 flanked by unique restriction sites, which can be used for cloning sgRNA libraries or exchanging  
187 other components (e.g. antibiotic resistance marker (AB<sup>R</sup>), or *dcas9* promoter). **c**, Strain  
188 construction using Mobile-CRISPRi. Top: a Tn7 transposon carrying CRISPRi components  
189 (shown in b) and a plasmid containing Tn7 transposition genes are transferred to recipient  
190 bacteria by tri-parental mating. Donor cells contain a chromosomal copy of the RP4 transfer  
191 machinery used to mobilize the Tn7 plasmids. Once inside the recipient cell, Tn7 transposition  
192 proteins integrate the CRISPRi DNA (purple) flanked by left and right Tn7 end sequences  
193 (green) into the recipient genome downstream of the *glmS* gene. Selection on antibiotic plates  
194 lacking DAP eliminates the *E. coli* donors and retains recipients with an integrated CRISPRi  
195 system. Bottom: An ICE element carrying CRISPRi components is transferred to recipient  
196 bacteria by bi-parental mating. Once inside the recipient cell, the ICE integrase inserts ICE into  
197 *trnS-leu2*. Double antibiotic plates that select for ICE and for the intrinsic resistance of the  
198 recipient strain (Streptomycin-resistance in this work) are used to identify recipients with an  
199 integrated CRISPRi system.

200

201 **Fig. 2 | Mobile-CRISPRi stability, transfer, and knockdown efficiency. a**, Mobile-CRISPRi  
202 stability after 50 generations of growth in the absence of antibiotic selection. Stability is the  
203 plating efficiency on kanamycin (the marker associated with Mobile-CRISPRi) vs no antibiotic.  
204 **b**, Mobile-CRISPRi transfer and integration efficiency. ICE or Tn7 containing CRISPRi was  
205 transferred to the species listed. Efficiency was calculated as: %AB<sup>R</sup>/total recipients. **c**,  
206 Efficiency of Mobile-CRISPRi knockdown in various species. Knockdown was tested using a  
207 Mobile-CRISPRi variant containing a constitutively expressed *rfp* reporter and an sgRNA  
208 targeting *rfp*. RFP expression was normalized to a strain lacking either *dcas9* (for *P.*



209 *aeruginosa*) or an sgRNA (all others). Data are represented as mean  $\pm$  s.d. The full species  
210 names for the strains used in panels b and c and their corresponding *rfp* fold knockdowns are:  
211 *Bacillus subtilis* (182-fold), *Listeria monocytogenes* (ND), *Escherichia coli* (65-fold),  
212 *Enterobacter cloacae* (32-fold), *Enterobacter aerogenes* (40-fold), *Shewanella oneidensis* (ND),  
213 *Pseudomonas aeruginosa* (8-fold), *Klebsiella pneumoniae* (34-fold), *Vibrio casei* (8-fold),  
214 *Acinetobacter baumannii* (10-fold), *Salmonella enterica* (54-fold), *Staphylococcus aureus* (132-  
215 fold), and *Proteus mirabilis* (35-fold).

216

217 **Fig. 3 | CRISPRi knockdown of *folA* increases sensitivity to trimethoprim in multiple**  
218 **species. a**, MIC assays for trimethoprim sensitivity in *P. aeruginosa* and *E. aerogenes* with or  
219 without Mobile-CRISPRi targeting *folA*. *K. pneumoniae* clumping increased the measurement  
220 error, although sensitivity is readily apparent. Data are represented as mean  $\pm$  s.d. **b**, Mobile-  
221 CRISPRi targeting *folA* or with a non-targeting control guide in various species were grown with  
222 or without trimethoprim (0.125, 0.5, and 8  $\mu$ g/ml trimethoprim for *E. areogenes*, *K. pneumoniae*,  
223 and *P. aeruginosa*, respectively), and with partial induction of CRISPRi (100  $\mu$ M IPTG for *E.*  
224 *areogenes*, *K. pneumoniae*, 0.1% arabinose for *P. aeruginosa*); growth was monitored by  
225 absorbance at 600 nm. Curves are averages of at least two biological replicates.

226

227 **Fig. 4 | A Mobile-CRISPRi library targeting auxotrophic genes in *E. cloacae*. a**, Tn7 Mobile-  
228 CRISPRi library construction. sgRNAs were cloned as a pool, transformed, and mated into *E.*  
229 *cloacae*, or **b**, sgRNAs were cloned individually, mixed as a pool with equal representation, and  
230 mated into *E. cloacae* as a pool. Representation of individual CRISPRi strains was determined  
231 by Illumina sequencing. **c**, Fitness of CRISPRi strains in glucose minimal media after 6 or 12  
232 doublings with or without CRISPRi induction by IPTG, determined from the library constructed  
233 by pooled cloning. Asterisks indicate strains had no fitness change in pooled screen but

234 significant decrease in arrayed screen. **d**, Comparison of each strain's fitness measure by the  
235 libraries constructed by pooled cloning (a) or individual cloning (b); a linear fit is shown.

236

237 **Fig. S1** | The *B. subtilis* *att*<sub>Tn7</sub> site is not responsible for failure of Tn7 methodology to transfer **a**,

238 Schematic of the transfer experiments. **b**, No transconjugants were obtained from mating a Tn7

239 transposon with a *B. subtilis*-compatible kanamycin resistance marker into *B. subtilis*. **c**, Test to

240 determine if the *B. subtilis* *att*<sub>Tn7</sub> site is responsible for failure of Tn7 methodology to transfer.

241 The *B. subtilis* *att*<sub>Tn7</sub> site with ~1kb flanking DNA was cloned into a replicative plasmid and

242 transformed into an *E. coli* strain in which integration into the chromosomal *att*<sub>Tn7</sub> site is blocked.

243 *E. coli* strains with variants of the *att*<sub>Tn7</sub> plasmid were used as recipients for a Tn7 transposon

244 containing a kanamycin resistance marker. Kan<sup>R</sup> transconjugants were obtained from matings

245 with *E. coli* strains containing the *B. subtilis* *att*<sub>Tn7</sub> site and *B. subtilis* flanking DNA with an *E. coli*

246 *att*<sub>Tn7</sub> site but were not obtained from an empty vector or *B. subtilis* flanking DNA with a precise

247 deletion of *att*<sub>Tn7</sub>, demonstrating that the *B. subtilis* *att*<sub>Tn7</sub> site is compatible with Tn7 integration

248 and suggesting that other factors (such as transfer efficiency or transposon gene expression)

249 are limiting.

250

251 **Fig. S2** | **Mobile-CRISPRi knockdown of native genes in *P. aeruginosa***. Mobile-CRISPRi

252 was used to target genes involved directly (*phzA1* and *phzM*) or indirectly in pyocyanin

253 biosynthesis (*pqsC*). The loss of blue pigment indicates knockdown of the pyocyanin pathway.

254

255 **Fig. S3** | **Validation of knockdown-induced auxotrophies by arrayed screen. a**,

256 Construction of an ordered CRISPRi library for *E. cloacae*. sgRNAs were cloned individually,

257 transformed into the *E. coli* donor strain, MFD*pir*. Donor strains were arrayed in 96 well plate

258 and then mating and selection were performed on LB agar plate using a Singer ROTOR robot.

259 **b**, Heat map representation of relative fitness (RF) of 40 *E. cloacae* CRISPRi strains (y-axis) in

260 7 glucose minimal media conditions with various supplementation (x-axis). Yellow rectangles  
261 indicate complementation of auxotrophy of strains by relevant amino acids.

262

## 263 **Methods**

### 264 **Construction of Mobile-CRISPRi vectors**

265 A complete list of Mobile-CRISPRi vectors can be found in Supplementary Table 4. All plasmids  
266 were constructed by restriction enzyme digestion of vector DNA followed by either ligation or  
267 NEBuilder HiFi DNA Assembly with insert DNA (all enzymes were purchased from NEB). To  
268 generate the Mobile-CRISPRi vectors, the pUC origin of replication in the Tn7 transposon  
269 plasmid pTJ1<sup>24</sup> was replaced with the R6K  $\gamma$  origin that requires the  $\pi$  protein (encoded by the  
270 *pir* gene) for replication, generating pJMP1050, and ensuring that Mobile-CRISPRi vectors  
271 cannot replicate in recipient cells. Mobile-CRISPRi “backbone” DNA containing unique  
272 restriction sites that flank the cloning modules was synthesized as a gBlock (IDT), and inserted  
273 into a pJMP1050 derivative (pJMP1054) that lacked those restriction sites, generating  
274 pJMP1055. pJMP1055 served as a base for all Tn7-based Mobile-CRISPRi derivatives. New  
275 derivatives were constructed by inserting components into the following modules/restriction  
276 sites: antibiotic markers/*Xho*I, reporter genes (e.g., *rfp*)/*Pme*I, sgRNA promoters and  
277 sgRNAs/*Eco*RI, sgRNA spacers (for creating sgRNA libraries)/*Bsa*I, regulatory genes (e.g.,  
278 *lacI*)/*Sma*I, *dcas9* promoters and ribosome binding sites/*Spe*I, and *dcas9*/*Spe*I-*Asc*I. To create a  
279 Mobile-CRISPRi plasmid that integrates into the ICE *Bs1* element, two ~1kb DNA fragments  
280 flanking the *rapI* gene were amplified from *B. subtilis* 168 gDNA and used to replace the Tn7  
281 transposon ends in a pJMP1055 derivative (pJMP1106), generating pJMP1290. pJMP1290  
282 served as a base for all ICE-based Mobile-CRISPRi derivatives and has the same unique  
283 restriction sites listed for the modules above. New sgRNAs were cloned into the *Bsa*I sites of  
284 Mobile-CRISPRi plasmids by ligating annealed oligos<sup>8</sup>. Oligos were designed to include  
285 overlaps that were complementary to the sticky ends generated by *Bsa*I. Oligos were added to

286 1X NEB buffer 4 at 5  $\mu$ M concentration, denatured for 5 min at 95 °C, and then annealed by  
287 transferring the reactions to room temperature. Annealed oligos were then diluted 1:20, 2 $\mu$ l of  
288 the dilution was ligated to 100ng of Bsal-digested vector for 1hr at room temperature. sgRNAs  
289 were designed as previously described<sup>4</sup>.

290

### 291 **Construction of Mobile-CRISPRi strains and mating assays**

292 A complete list of strains used in the study can be found in Supplementary Table 5. Tn7-based  
293 Mobile-CRISPRi strains were constructed by tri- or quad-parental mating as previously  
294 described in Choi *et al.*<sup>15,25</sup>, with several modifications. All Tn7 matings used MFD*pir*<sup>26</sup> (a *pir*<sup>+</sup>  
295 strain that is dependent on DAP for growth and contains the RP4 transfer machinery)  
296 transformed with either a Tn7 transposase plasmid (pJMP1039—a derivative of pTNS3<sup>27</sup> with a  
297 spontaneous small deletion upstream of the P<sub>c</sub> promoter) or transposon plasmid (various  
298 pJMP1055 derivatives) as mating donors. Matings with *Acinetobacter baumannii* ATCC19606  
299 required the presence of a third donor strain containing the self-mobilizing RP4 transfer plasmid  
300 pRK2013<sup>15</sup> for unknown reasons. Cultures of the two *E. coli* donor strains (transposon and  
301 transposase donors) were grown overnight (~16 hrs) at 37 °C in Lysogeny Broth (LB) + 300  $\mu$ M  
302 DAP (Alfa Aesar B22391) + 100  $\mu$ g/ml ampicillin. Recipient strains assayed here also grew to  
303 saturation in LB after incubation at 37 °C for ~16 hrs. 100  $\mu$ l of each donor and recipient strain  
304 was added to 700  $\mu$ l of LB and mixed by pipetting. Mixes of donor and recipient strains were  
305 pelleted for 2 min at 7000  $\times$  g, washed twice with 1 ml of LB, resuspended in 30  $\mu$ l of LB after  
306 the final wash, pipetted onto a cellulose filter (MF-Millipore HAWG01300) placed on a pre-  
307 warmed LB + 300  $\mu$ M DAP plate, and incubated at 37 °C for 6 hrs. Filters were then transferred  
308 to microcentrifuge tubes containing 200  $\mu$ l of PBS and vortexed to liberate the cells. Cells were  
309 spread onto on media that selects for the Mobile-CRISPRi plasmid and recipient (e.g., LB +  
310 kanamycin) without DAP (the absence of DAP will select against donor *E. coli*). Antibiotic

311 concentrations used for selection were: 6 µg/ml (*B. subtilis*) chloramphenicol, 7.5/50 µg/ml  
312 kanamycin (*B. subtilis*/*S. aureus*), and 100 µg/ml streptomycin.  
313 ICE-based Mobile-CRISPRi strains were constructed by bi-parental mating as previously  
314 described<sup>28,29</sup> with modifications. New ICE donor strains were generated by transformation of *B.*  
315 *subtilis* with Mobile-CRISPRi integration plasmids using natural competence as previously  
316 described<sup>4</sup>. Expression of the ICE anti-repressor, RapI, induces conjugation genes found on the  
317 ICE element and promotes excision<sup>28</sup>. ICE excision and the large insert size of Mobile-CRISPRi  
318 plasmids resulted in very few transformants. To produce a strain with a stable ICE element in  
319 the presence of an IPTG-inducible *rapI* gene that transformed at high efficiency, a *dcas9* gene  
320 linked to a chloramphenicol-resistance marker was integrated into ICE—selection for the  
321 chloramphenicol marker and the extra homology present in the *dcas9* gene improved  
322 transformation efficiency. For mating, one 3 ml LB culture of each donor and recipient strain was  
323 grown from single colonies to exponential phase (~2 hrs at 37 °C); donors were grown in LB +  
324 3.25 µg/ml kanamycin to select for ICE retention. Exponential phase cultures were then back  
325 diluted to an OD600 of 0.02 and grown until OD600 0.2 before inducing *rapI* expression with  
326 1mM IPTG for 1 hr. 2.5 ml of donor and recipient cells adjusted to an OD600 of 0.9 were mixed  
327 with 5 ml of 1X Spizizen salts<sup>30</sup> and vacuum filtered using an analytical CN filter (Nalgene 145-  
328 0020). Filters were transferred to Spizizen agar plates and incubated for 3 hrs at 37 °C.  
329 Transconjugants were selected for plating on kanamycin + streptomycin plates as all recipient  
330 strains were streptomycin resistant.

331

### 332 **Transfer efficiency assays**

333 Tn7 or ICE mating experiments were carried out in triplicate. Transfer efficiency was calculated  
334 by taking the ratio of transconjugants (antibiotic-resistant Dap<sup>+</sup> colonies for Tn7 matings, and  
335 KanR/StrR colonies for ICE matings) to viable cells (LB colonies for Tn7 matings, and StrR  
336 colonies for ICE matings). For Tn7 transfer to the *B. subtilis* *att*<sub>Tn7</sub> site in *E. coli* (Supplementary

337 Fig. 1a), the native *att*<sub>Tn7</sub> site in *E. coli* K-12 DH10B was occupied by an unmarked Tn7 to  
338 prevent chromosomal transposition, while test *att*<sub>Tn7</sub> sites were cloned onto a chloramphenicol  
339 resistant plasmid.

340

#### 341 **Mobile-CRISPRi stability assays**

342 Four independently generated isolates of *E. coli* K-12 BW25113 and *B. subtilis* 168 containing  
343 Mobile-CRISPRi systems targeting *rfp* were grown to saturation overnight at 37 °C in LB +  
344 kanamycin (30 µg/ml for *E. coli* and 7.5 µg/ml for *B. subtilis*) to select for retention of the of the  
345 Tn7 or ICE element containing CRISPRi. One ml of each culture was centrifuged at 6000 × *g* for  
346 3 min and washed twice with LB to remove any residual kanamycin. The washed cells were  
347 diluted 1:1000 in LB and grown to saturation. The procedure of dilution and growth to saturation  
348 was repeated a total of 5 times for ~50 generations of growth. Cells were then serially diluted  
349 and plated on selective (LB + kanamycin) and non-selective plates (LB). The ratio between  
350 colony counts on LB and LB + kanamycin was used to determine the fraction of cells that  
351 retained the Tn7 or ICE element.

352

#### 353 **RFP knockdown assays**

354 RFP knockdown was measured using flow cytometry or a plate reader (for *A. baumannii* and *V.*  
355 *casei*). Flow cytometry was performed by diluting overnight cultures of Mobile-CRISPRi *rfp*  
356 knockdown strains 1:10,000 into fresh media (LB for all Proteobacteria and *B. subtilis*, Brain  
357 Heart Infusion broth for *S. aureus*) containing CRISPRi inducer (1mM IPTG for all  
358 Proteobacteria except *P. aeruginosa*, 1% arabinose for *P. aeruginosa*, and 0.1 µg/ml  
359 anhydrotetracycline for Firmicutes) and incubating cultures at 37 °C with rotation until the  
360 cultures reached mid-log phase (OD<sub>600</sub> 0.3-0.6). Cultures were then cross-linked with 1%  
361 formaldehyde [final] for 10 min, followed by quenching for 10 min with 0.5 M glycine [final].  
362 Cross-linked cells were then diluted 1:10 in phosphate buffered saline and flowed on a BD

363 LSRII using 610/20 BP filter (PE-Texas-Red fluorochrome). Data for at least 10,000 cells was  
364 collected for four independently constructed strain isolates. For *V. casei*, overnight cultures were  
365 normalized to 2.0 OD600 and then diluted 1:200 in LB with or without 0.5 mM IPTG. After 6  
366 hours growth post-induction the strains were normalized to 0.2 OD600 and washed once in 1X  
367 PBS. The samples were then transferred to a 96well plate (200ul in each well) in triplicate and  
368 measured for ds-Red fluorescence (Ex 557nm Em 592nm) using a bottom-read plate reader  
369 (Tecan). For *A. baumannii*, overnight cultures were diluted 1:10,000 into fresh LB with or without  
370 0.1 mM IPTG. Cells were grown in a 96 well plate with measurements of OD600 and RFP every  
371 10 min. The values reported reflect the RFP knockdown at mid-log growth.

372

### 373 **Pyocyanin knockdown assays**

374 Strains were grown overnight in Kings Medium A Base (HiMedia M1543) to induce pyocyanin  
375 and pyorubin production and 1% arabinose to fully induce *dcas9* expression.

376

### 377 **Antibiotic sensitivity assays**

378 MIC assays were performed using the broth microdilution method as previously described<sup>31</sup>,  
379 except that 0.1% arabinose (for *P. aeruginosa*) or 100  $\mu$ M IPTG (for *E. aerogenes*) was added  
380 to induce *dcas9* expression. Growth curves shown in Fig. 3 were set up in exactly the same  
381 manner as the MIC assays, except that cultures were grown with agitation in a plate reader  
382 (BioTek) for ~16 hrs.

383

### 384 **Construction of Mobile-CRISPRi strains and mating assays**

385 Pooled Tn7-based Mobile-CRISPRi libraries for *E. cloacae* were constructed by following the  
386 procedure for single gene CRISPRi strain construction with several modifications. Equal  
387 concentration of annealed oligonucleotides for each sgRNA (Supplementary Table 1) were  
388 pooled and ligated into a Bsal digested plasmid. Ligation product was transformed into an *E.*



389 *coli pir+* strain. Colonies on selection plates (LB + 100 µg/ml ampicillin) were collected and  
390 resuspended in LB and plasmids were purified from of pooled transformants. Purified pooled  
391 plasmids were transformed into donor strain, MFD*pir*. Transformants were collected and  
392 resuspended in LB + 300 µM DAP + 100 µg/ml ampicillin + 12.5% glycerol and stored at -80 °C.  
393 For comparison, the other donor was prepared by transformation of a pool of individually cloned  
394 plasmids with equal concentration. Tri-parental mating and selection were performed as  
395 described above and selected colonies of *E. cloacae* CRISPRi strains were collected and  
396 resuspended in MOPS salts solution<sup>32</sup> + 12.5% glycerol and stored at -80 °C after measurement  
397 of OD<sub>450</sub> of stock. In order to prepare inoculum of library to screen fitness of library in minimal  
398 media, frozen stock was diluted in glucose minimal medium to OD<sub>450</sub> of 5 and incubated for  
399 recovery for 1 hr. Recovered cell culture was mixed with a 100-fold excess wild-type *E. cloaca*,  
400 then diluted to an OD<sub>450</sub> of 0.01 in 30 ml glucose minimal media with or without IPTG, then  
401 grown in 125 ml flasks at 30 °C with shaking (250 rpm). When the culture reached OD<sub>450</sub> of  
402 0.64, 1 ml of culture was collected for preparation of sequencing library of 6 doubling sample.  
403 For 12 doubling sample, this culture was diluted to an OD<sub>450</sub> of 0.01 in 30 ml and was grown  
404 until the culture reached OD<sub>450</sub> of 0.64. in order to prepare the Illumina sequencing library,  
405 genomic DNA was purified using the Qiagen DNeasy Blood & Tissue kit and sequencing region  
406 was amplified by PCR using the primers harboring indices for different sampling time and  
407 growth conditions. Differentially indexed PCR products were purified by agarose gel  
408 electrophoresis prior Illumina sequencing. Sequences of primers used for preparation of  
409 sequencing libraries are listed in (Supplementary Table 6). Frequencies of strains in each  
410 sample were calculated by dividing the number of reads of sgRNA encoding sequence from  
411 each strain by the number of total read and used for calculation of fitness. Fitness was  
412 calculated as described in van Opijnen *et al.*<sup>12</sup>  $W_i = \ln(N_i(t_2)Xd/N_i(t_1))/\ln((1-N_i(t_2))Xd/(1-N_i(t_1)))$ ,  
413  $N(t)$  is frequency of the mutant in the population at the time points, and  $d$  represents the growth

414 of the bacterial population during library selection. We calculated  $d$  using  $OD_{450}$  change.  
415 Average fitness from two biological replicates is presented in Supplementary Table 2.  
416  
417 Ordered Tn7-based Mobile-CRISPRi libraries for *E. cloacae* were constructed by following the  
418 procedure for single gene CRISPRi strain construction with modifications for automation (Fig.  
419 S4). Each donor Tn7::CRISPRi strains were prepared by transformation of individually cloned  
420 plasmids into MFDpir strain and arrayed in 96 well plate. Equal amount of transposase strain  
421 was added to each well and pinned to LB + 300  $\mu$ M DAP + 2% agar plate using a Singer  
422 ROTOR robot. Wild-type *E. cloacae* cells arrayed in 96 colony format were pinned to the same  
423 plate, which was incubated for 6 hrs. Kanamycin resistant *E. cloacae* CRISPRi strains were  
424 selected on LB supplemented with kanamycin two times and stored at -80 °C as a glycerol  
425 stock. To screen growth phenotype of each strain, cells were pinned from glycerol stocks onto  
426 rectangular LB agar plates in 384-format using a Singer ROTOR robot (four technical replicates  
427 on one plate in this screen). For each screen, exponentially growing cells in 384-format were  
428 then pinned to defined media plates and incubated for 16 hrs at room temperature to avoid  
429 mucoid colony formation. Plates were imaged using a Powershot G10 camera (Canon) when at  
430 a time point at which fitness differences were apparent but growth had not saturated. The  
431 calculation of RF was carried out as described in Koo *et al.*<sup>32</sup> with minor modifications. Relative  
432 fitness (RF) was measured by the colony opacity of each mutant determined with Iris colony  
433 sizing software<sup>33</sup>. The RF of each mutant was calculated as:  $RF = (\text{average colony opacity of}$   
434  $\text{CRISPRi strain})/(\text{average colony opacity of CRISPRi with no sgRNA strain})$ . The average RF  
435 calculated from two same media plates is presented in Supplementary Table 3.  
436

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513

#### 514 **Acknowledgements**

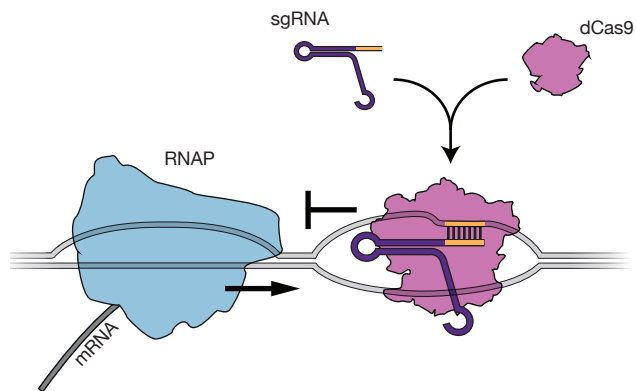
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522

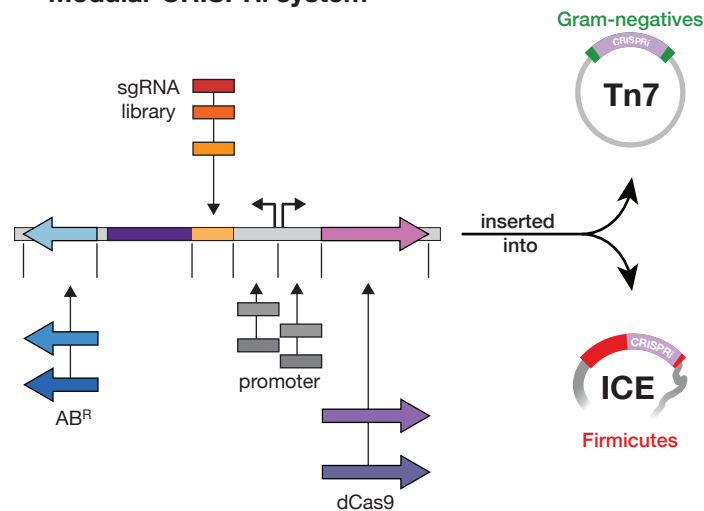
#### 523 **Author contributions**

524 J.M.P., B.M.K., M.M.H., A.D.G, J.E.P., J.N.E., R.J.D., C.A.G., O.S.R. designed the study,  
525 J.M.P., B.M.K., R.P., G.E.H., C.C.H., Y.F.I., C.H.S.L. performed experiments, J.M.P., B.M.K.,  
526 R.P., G.E.H., Y.F.I., J.S.H. analyzed data, and J.M.P., B.M.K., H.O., C.A.G., O.S.R. wrote the  
527 manuscript.

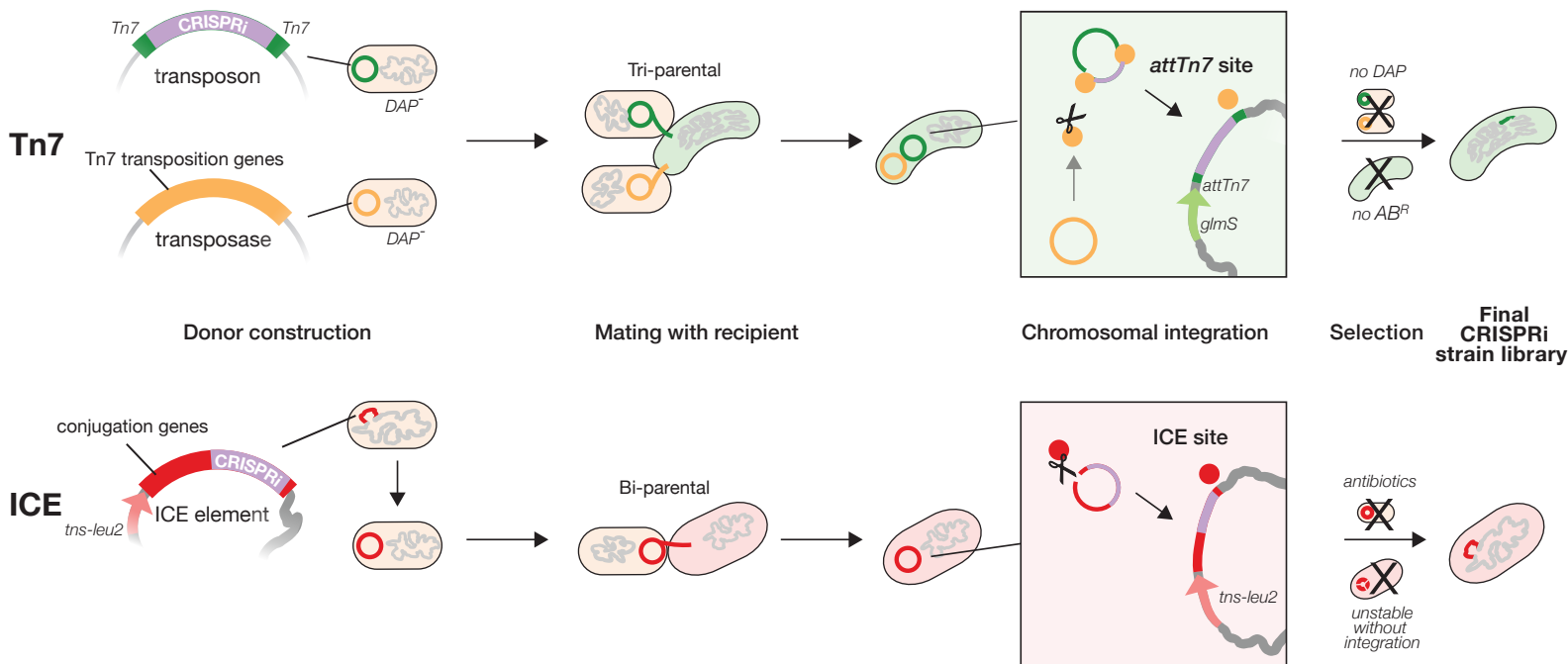
**a** CRISPR interference (CRISPRi)



**b** Modular CRISPRi system



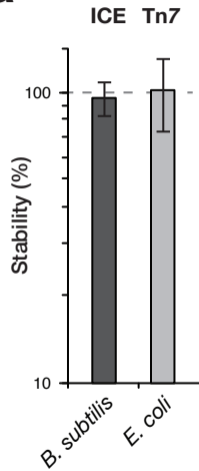
**c** Strain library construction



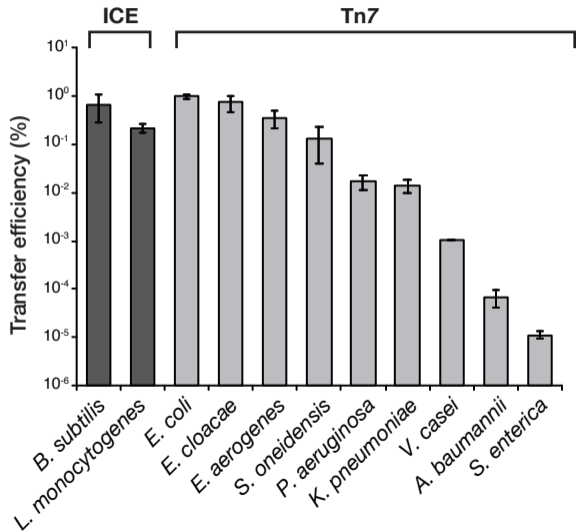


**Figure 2**

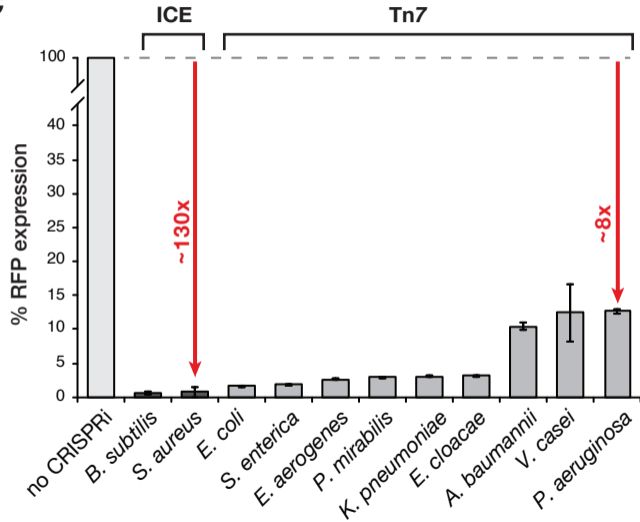
**a**



**b**

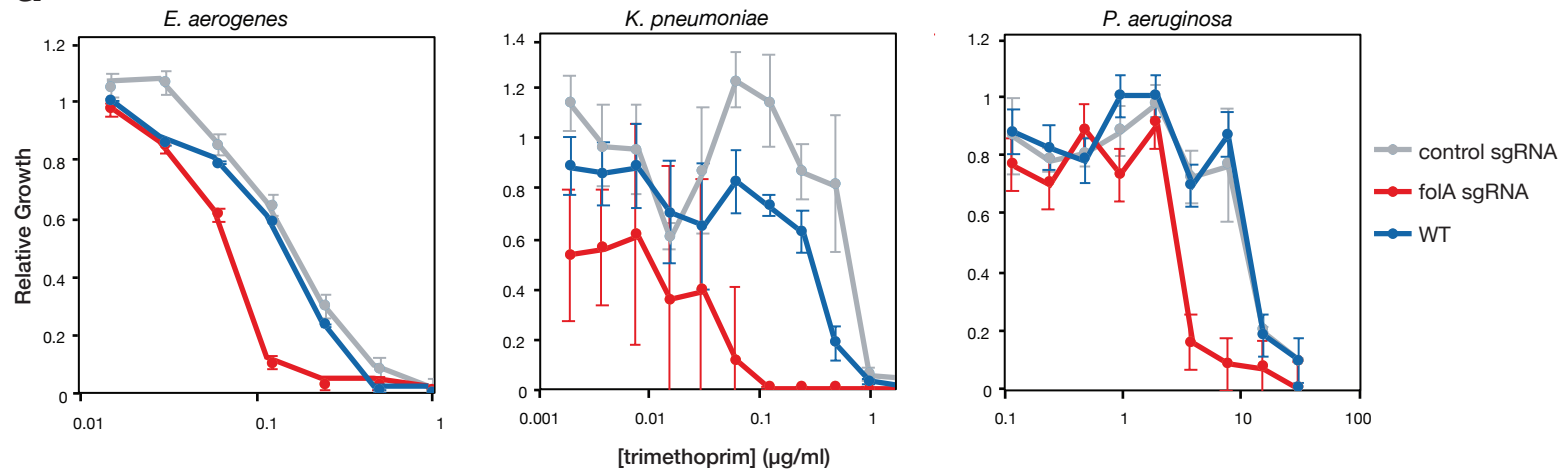


**c**

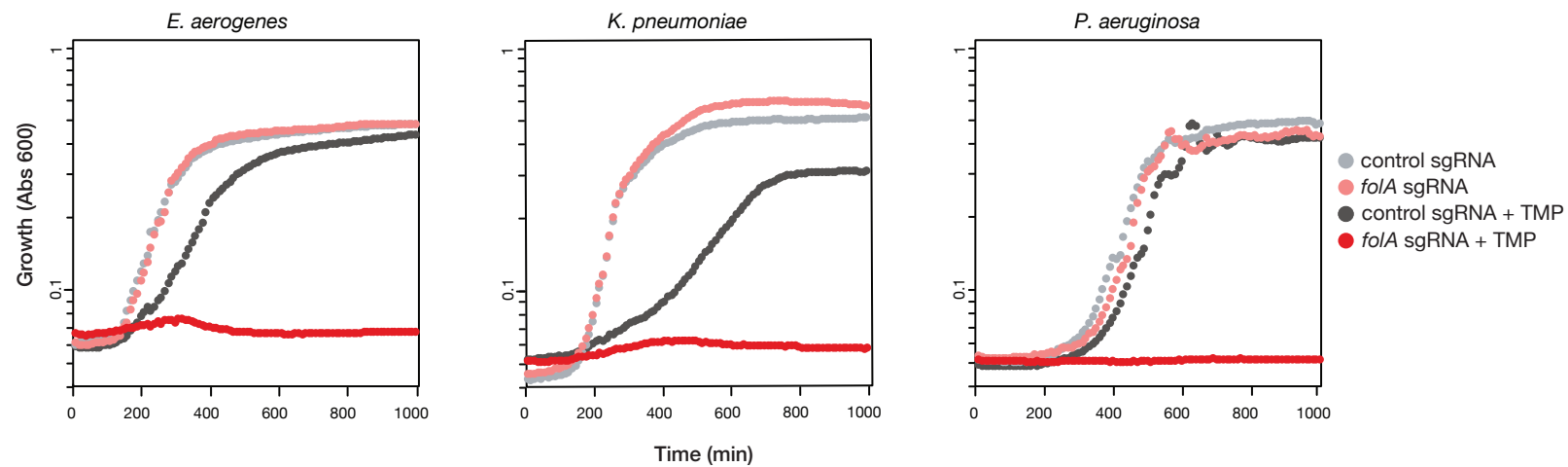


**Figure 3**

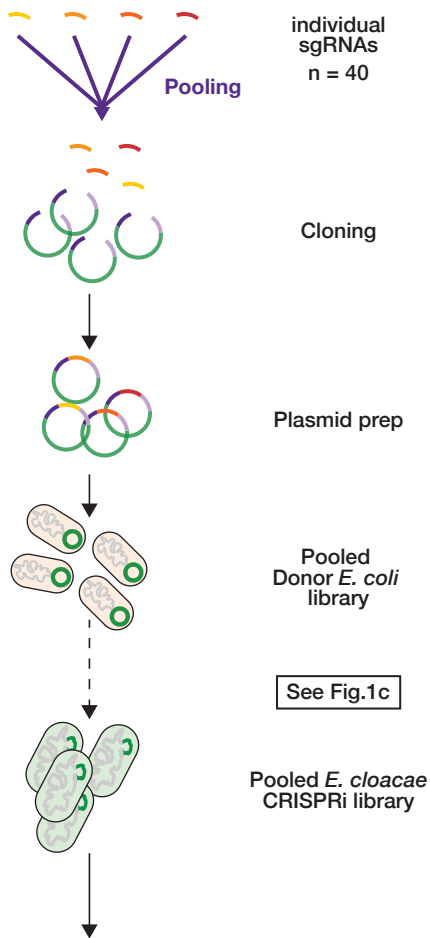
**a**



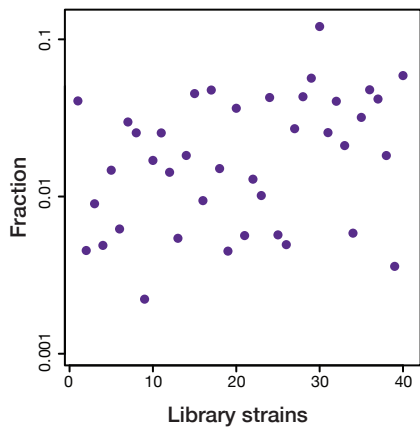
**b**



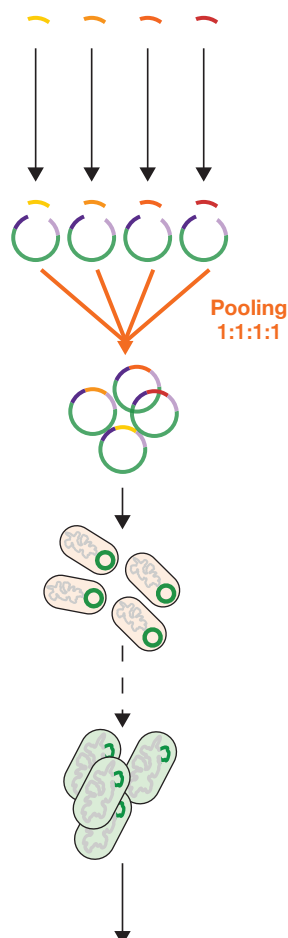
**a** Pooled sgRNA cloning



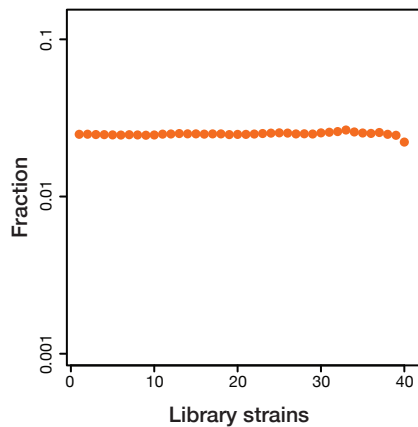
Representation of individual strains



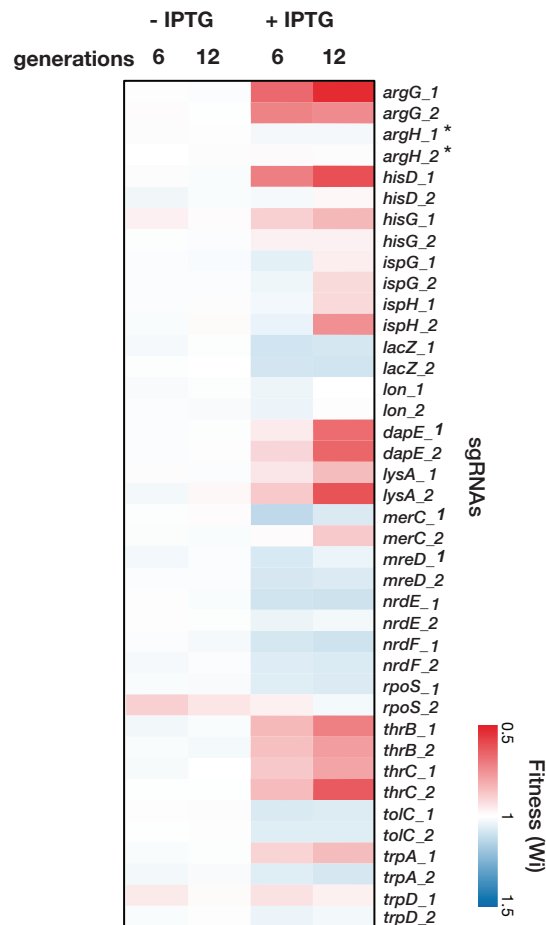
**b** Individual sgRNA cloning



Representation of individual strains



**c** Growth enrichment phenotypes



**d** Correlation of fitness

