



20 **Abstract**

21 Despite the advent of new techniques for genetic engineering of bacteria, allelic exchange through  
22 homologous recombination remains an important tool for genetic analysis. Currently, *sacB*-based  
23 vector systems are often used for allelic exchange, but counter-selection escape, which prevents  
24 isolation of cells with the desired mutation, limits its utility. To circumvent this limitation, we  
25 engineered a series of “pTOX” allelic exchange vectors. Each plasmid encodes one of a set of  
26 inducible toxins, chosen for their potential utility in a wide range of medically important  
27 Proteobacteria. A codon-optimized *rhaS* transcriptional activator with a strong synthetic ribosome  
28 binding site enables tight toxin induction even in organisms lacking an endogenous rhamnose  
29 regulon. Expression of the blue *amiICP* or magenta *tsPurple* non-fluorescent chromoproteins  
30 facilitates monitoring of successful single- and double-crossover events using these vectors. The  
31 versatility of these vectors was demonstrated by deleting genes in *Serratia marcescens*,  
32 *Escherichia coli* O157:H7, *Enterobacter cloacae*, and *Shigella flexneri*. Finally, pTOX was used to  
33 characterize the impact of disruption of all combinations of the 3 orthologous *S. marcescens*  
34 peptidoglycan amidohydrolases on chromosomal *ampC* beta-lactamase activity and corresponding  
35 beta-lactam antibiotic resistance. Mutation of multiple amidohydrolases was necessary for high  
36 level *ampC* derepression and beta-lactam resistance. These data suggest why beta-lactam  
37 resistance may emerge during treatment less frequently in *S. marcescens* than in other AmpC-  
38 producing pathogens like *E. cloacae*. Collectively, our findings suggest that the pTOX vectors  
39 should be broadly useful for genetic engineering of Gram-negative bacteria.

## 40 **Importance**

41 Targeted modification of bacterial genomes is critical for genetic analyses of microorganisms.  
42 Allelic exchange is a technique that relies on homologous recombination to substitute native loci for  
43 engineered sequences. However, current allelic exchange vectors often enable only weak  
44 selection for successful homologous recombination. We developed a suite of new allelic exchange  
45 vectors, pTOX, which were validated in several medically important Proteobacteria. They encode  
46 visible non-fluorescent chromoproteins that enable easy identification of colonies bearing  
47 integrated vector, and permit stringent selection for the second step of homologous recombination,  
48 yielding modified loci. We demonstrate the utility of these vectors by using them to investigate the  
49 effect of inactivation of *Serratia marcescens* peptidoglycan amidohydrolases on beta-lactam  
50 antibiotic resistance.

## 51 **Introduction**

52 The ever-increasing availability of bacterial genome sequence data has driven the demand for  
53 widely applicable and facile techniques enabling site-specific targeted mutagenesis. In general,  
54 such techniques can be divided into those that rely on exogenous enzymes versus those that  
55 depend exclusively on endogenous enzymes. Examples of methods in the former category include  
56 those utilizing the Lambda Red recombinase (“recombineering” (1)), those employing clustered  
57 regularly interspaced short palindromic repeat (CRISPR)/Cas9 systems (2), or a combination of the  
58 two (3, 4). These systems can be fast and reliable, but often require organism-specific  
59 modifications, rely on efficient transformation, and can leave genetic scars or result in off-target  
60 mutations.

61

62 In contrast, “allelic exchange” utilizes endogenous homologous recombination enzymes to facilitate  
63 the replacement of a native genomic region with a foreign sequence of interest. This is a versatile  
64 technique that can routinely yield mutations ranging from kilobase-scale deletions or insertions to  
65 the generation of precise point mutations. The early allele exchange vectors resulted in antibiotic-  
66 marked strains (5, 6); subsequent advances using counter-selectable cassettes allowed the  
67 generation of truly scarless, unmarked mutant strains. However, many genes used in counter-  
68 selection strategies (*e.g.* *rpsL*, *pheS*, *thyA*, *ccdB*) require a specific host genotype, limiting their  
69 widespread utility (7). Background-independent counter-selection strategies utilizing *tetAR* (8),  
70 *sacB* (9), or a combination of the two (10) are valuable but often require considerable optimization.  
71 Moreover, counter-selection escape, where the integrated allelic exchange vector remains lodged  
72 in the genome, preventing isolation of the desired mutant, remains common with such schemes  
73 even after optimization. This has been a key technical obstacle limiting wider use of allelic  
74 exchange.

75

76 Recently, a powerful negative selection system using inducible toxins derived from toxin-antitoxin  
77 systems or from Type VI secreted effector-toxins was developed for use with recombineering (11).  
78 Here, we repurpose these toxins for use in allelic exchange and engineer a counter-selection  
79 escape surveillance system using visible chromoproteins derived from the *Acropora millepora*  
80 coral. We demonstrate the utility of these new allele exchange vectors, designated “pTOX,” in  
81 multiple medically important Proteobacteria. These vectors were used to systematically delete all  
82 combinations of the three peptidoglycan hydrolases in *Serratia marcescens* to characterize their  
83 contributions to beta-lactam antibiotic resistance.

84

## 85 **Results**

### 86 **Engineering and testing of pTOX vectors**

87 The motivation for this work arose from our difficulties adapting common genetic tools for use in  
88 *Serratia marcescens*, an Enterobacteria that is a common cause of healthcare-associated urinary  
89 tract infections, pneumonia, and bacteremia (12). While chemical- and electro-transformation is  
90 possible in many *S. marcescens* strains (13, 14), it is often cumbersome and inefficient, which  
91 reduces the utility of Lambda red recombinase- and CRISPR/Cas9-based systems for genetic  
92 manipulation. Because of this, we sought to construct a conjugatable allelic exchange vector for *S.*  
93 *marcescens* that would be widely useful.

94

95 Our set of new vectors (the pTOX vectors) is derived from pDS132, a *sacB*-based suicide plasmid  
96 that contains the conditional ( $\pi$ -dependent) R6K origin of replication (15). The *sacB* cassette was  
97 replaced with a rhamnose-inducible toxin obtained from the pSLC vector series (11) (Fig 1A).

98 Reasoning that a given toxin would be most useful in a strain that did not encode a chromosomal  
99 copy of that same toxin (and presumably the corresponding antitoxin or immunity protein), we  
100 identified a minimal set of three toxins (*yhaV*, *mqsR*, and *tse2*), of which at least one should be  
101 effective in the majority of medically important proteobacteria (Fig S1). Additional steps in the

102 construction of this set of vectors included: 1) Introduction of a codon-optimized *rhaS*  
103 transcriptional activator (16) with a strong synthetic ribosome binding site (17) to enable use of the  
104 well-characterized and stringent rhamnose-inducible system for toxin activation (18) even in strains  
105 that lack a rhamnose regulon; 2) introduction of a strong forward transcriptional terminator  
106 upstream of the multiple cloning site, minimizing read-through into the multiple cloning site and  
107 facilitating the manipulation of toxic genes; and 3) introduction of a greatly expanded polylinker  
108 region (19) (Fig 1A) to facilitate insertion of new sequences into the vectors. Two versions of this  
109 set of plasmids, encoding either chloramphenicol or gentamicin resistance, were created (Supp  
110 Table 2). All molecular cloning was performed in the presence of glucose, which inhibits toxin  
111 production through catabolite repression.

112  
113 The utility of each of the three toxins was validated in *S. marcescens* ATCC 13880, which lacks  
114 *rhaS* and endogenous versions of the 3 toxins. First, a region homologous to the targeted  
115 chromosomal locus was inserted into the pTOX multiple cloning site (see the Methods for more  
116 detail and Fig 1B for a schematic). Next, conjugation was used to introduce pTOX derivatives into  
117 *S. marcescens*. Single cross-over merodiploids were selected on the appropriate antibiotic. To  
118 assess the utility of the heterologous *rhaS*, we then compared the growth of merodiploids to wild-  
119 type *S. marcescens* in either glucose- or rhamnose-containing media. Toxin-containing  
120 merodiploids grown in glucose-containing media grew indistinguishably from wild-type, while  
121 growth in rhamnose-containing media was undetectable (Fig 2). These observations reveal that  
122 *yhaV*, *mqsR*, and *tse2* enable robust growth inhibition in *S. marcescens* and that the exogenous  
123 *rhaS* is sufficient for stringent control of their expression.

124  
125 A limitation of the *sacB* counter-selection system is the occasional outgrowth of merodiploids that  
126 have either mutated the *sacB* gene or acquired resistance to its product (9). Such counter-  
127 selection escape can confound isolation of double-crossover events. To assess whether counter-

128 selection escape also confounds *yhaV*-, *mqsR*-, or *tse2*-based selections, we randomly selected  
129 23 colonies representing putative double-crossovers (based on growth in the presence of  
130 rhamnose) from 3 independent experiments for each of the three toxin-vectors. All 207 colonies  
131 screened were chloramphenicol sensitive and lacked pTOX vector sequences by PCR (Table 1  
132 and Fig S2). These observations suggest that selection mediated by the 3 toxins is potent and that  
133 the frequency of counter selection escape is very low.

134

### 135 **Utility of pTOX vectors in diverse pathogens**

136 To investigate the versatility of the pTOX vectors, we tested their capacity to mediate diverse allele  
137 replacements, beginning with the *S. marcescens hexS* locus. *S. marcescens* ATCC 13880, like  
138 many isolates of this opportunistic pathogen, produces the red prodigiosin pigment; however,  
139 production is only robust at reduced temperatures, due to relief of repression mediated by the  
140 negative regulator *hexS* (20). A pTOX derivative encoding sequences flanking *hexS* was used to  
141 delete this regulator from the *S. marcescens* chromosome, resulting in prodigiosin hyperproduction  
142 even at 37°C (Fig 3A). Subsequently, we have replaced more than 20 loci in *S. marcescens* using  
143 pTOX1, pTOX2, and pTOX3 (Supplemental Table 2). All attempts have been successful, though  
144 like other allelic exchange methods, the ratio of wild-type to mutant double-crossovers can vary  
145 from balanced to skewed.

146

147 We also tested the utility of the pTOX vectors in 3 additional Gram-negative pathogens.

148 *Escherichia coli* O157:H7 (EHEC) is an important cause of foodborne diarrhea as well as a  
149 systemic microangiopathy which can lead to hemolysis and renal failure. A pTOX3 derivative was  
150 used to delete *lacZ*, which produces a beta-galactosidase that enables wild-type EHEC to ferment  
151 lactose. As seen in Fig 3B, deletion of EHEC *lacZ* yielded colonies that are white on agar  
152 containing the chromogenic lactose analog 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside (X-  
153 gal). Derivatives of pTOX3 were also used to replace nearly 20 additional loci in EHEC.

154  
155 *Shigella flexneri* is an increasingly antibiotic-resistant cause of dysentery. In *S. flexneri*, most  
156 secreted virulence proteins (effectors) are encoded by a large, unstable virulence plasmid.  
157 Recombineering is useful in performing single gene deletions on the plasmid, but multiple gene  
158 deletions leave identical scar sequences that can enable undesired recombination within the  
159 plasmid. pTOX3 was used to delete the virulence plasmid *ipgH* locus (Fig 3C) as well as  
160 chromosomal loci.

161  
162 Finally, pTOX was efficacious in *Enterobacter cloacae*, an opportunistic hospital-associated  
163 pathogen associated with urinary tract and bloodstream infections. *E. cloacae*, like *S. marcescens*,  
164 possesses an inducible chromosomal beta-lactamase, AmpC, which hydrolyzes most beta-lactam  
165 antibiotics. A pTOX3 derivative was used to delete *E. cloacae ampC*. Colonies harboring the *ampC*  
166 deletion exhibited no detectable beta-lactamase activity, whereas colonies that reverted to wild-  
167 type (*ampC*<sup>+</sup>) did (Figure 3D). Collectively, these observations suggest that pTOX may be widely  
168 useful in Gram-negative bacteria, particularly for those where other methods are difficult or  
169 unavailable.

170

### 171 **Chromoproteins facilitate visual detection of pTOX transconjugants**

172 Conjugation efficiency can vary between species and strains. For organisms like *S. marcescens*, in  
173 which conjugation can be inefficient, we incorporated an additional module coding for the AmilCP  
174 protein into the pTOX vectors (Fig 4A). AmilCP is a non-fluorescent blue chromoprotein derived  
175 from the *Acropora millepora* coral; we sought to use its blue coloration as an additional method to  
176 discriminate wild-type colonies from transconjugants. To this aim, multiple combinations of  
177 promoters and ribosomal binding sites were tested to identify those which provided coloration  
178 sufficient for discrimination without special equipment.

179



180 The series of *amilCP* modules were first tested in *E. coli* donors, where we found that the tac  
181 promoter (21) or the apFAB46 promoter (22) offered the deepest blue coloration (Fig 4B, Fig S3A).  
182 This level of *amilCP* expression did not incur a detectable fitness cost (Fig S3B); however, several  
183 strategies to increase colony coloration further (e.g increasing *amilCP* copy number) led to toxicity  
184 and were not pursued. pTOX vectors containing *amilCP* driven by the tac promoter and a pTOX  
185 vector expressing the magenta *tsPurple* chromoprotein driven by apFAB46 were created (Fig 4C)  
186 (23). Both AmilCP and TsPurple were visible in re-streaked merodiploid colonies after 24-48 hours  
187 of incubation (Fig 4D), though coloration was not as saturated as when expressed from the pTOX  
188 plasmids (which have medium-copy origins). Therefore, the pTOX chromoprotein modules may  
189 prove useful for monitoring the success of single- and double-crossover, particularly in organisms  
190 with inefficient conjugation.

191

### 192 **Application of the pTOX vectors to study inducible antibiotic resistance**

193 To further interrogate the utility of the pTOX suite, we used these vectors to characterize the role of  
194 the *S. marcescens* peptidoglycan (PG) amidohydrolases in inducible beta-lactam resistance  
195 mediated by the AmpC beta-lactamase. The PG component of the bacterial cell wall consists of a  
196 repeated disaccharide polymer linked through peptide cross-links. The peptidoglycan  
197 amidohydrolases facilitate remodeling of the cell wall by catalyzing hydrolysis of the amide bond  
198 linking the polysaccharide to the peptide component, generating muropeptide breakdown products  
199 that can subsequently be recycled in the cytoplasm (24). When the classical cytoplasmic PG  
200 amidohydrolase, *ampD*, becomes saturated with substrate in the setting of catastrophic remodeling  
201 precipitated by beta-lactam antibiotics such as penicillins and cephalosporins, the accumulation of  
202 muropeptides leads to *ampC* derepression. The associated beta-lactam resistance enables  
203 subsequent restoration of PG homeostasis (25).

204

205 In *E. cloacae* and *Citrobacter freundii*, expression of *ampC* at basal levels is sufficient for clinical  
206 resistance to penicillins and early-generation cephalosporins. After exposure to beta-lactams and  
207 the resulting accumulation of mucopeptide breakdown products, transcriptional upregulation can  
208 lead to transient intermediate resistance to late-generation cephalosporins such as ceftriaxone.  
209 Under conditions where there is selection for high-level cephalosporin resistance (i.e. in individual  
210 patients who are subjected to prolonged cephalosporin treatment), mutation of the *ampD*  
211 amidohydrolase can occur. This leads to a constitutive increase in cytoplasmic mucopeptide that is  
212 sufficient for high level derepression of *ampC* and resistance to ceftriaxone (26, 27). However, it is  
213 unclear whether the insights gained from studies of *E. cloacae* and *C. freundii* can be generalized  
214 to all AmpC-producing organisms, because the pathway to full derepression may be more  
215 complicated in organisms with multiple orthologous amidohydrolases. For example, in  
216 *Pseudomonas aeruginosa*, full derepression of *ampC* requires inactivation of additional *ampD*  
217 orthologues (28), while in *Yersinia enterocolitica*, deletion of all three *ampD* orthologues does not  
218 result in obvious clinical resistance (29).

219  
220 Systematic investigation of the contribution of *S. marcescens* PG amidohydrolases to *ampC*  
221 derepression and resulting beta-lactam resistance has not been performed. We found that *S.*  
222 *marcescens* encodes 3 PG amidohydrolases, which, by sequence homology (30) we denote *ampD*  
223 (WP\_033641266.1), *amiD* (WP\_016928349.1), and *amiD2* (WP\_048796451.1) (Fig 5A, Fig S4).  
224 Creation of pTOX derivatives targeting each of the *S. marcescens* PG amidohydrolases allowed  
225 the rapid generation of all combinations of single, double, and triple mutants (Fig. S5). We found  
226 that, of the single mutants, only  $\Delta amiD2$  had a significant increase in basal AmpC activity (Fig. 5B);  
227 however, this corresponded to only a 2-fold increase in cephalosporin MICs (Table 2). In contrast,  
228 the  $\Delta ampD\Delta amiD2$  double mutant had a more than 50-fold increase in AmpC activity, which  
229 resulted in an 8-fold increase in the ceftriaxone and a 4-fold increase in cefepime MICs. The triple  
230 mutant exhibited no further increase in AmpC activity or in MICs. The Clinical and Laboratory

231 Standards Institute (CLSI) has recently updated their guidelines on MIC breakpoints above which  
232 there is a potential for clinical resistance. Under the new breakpoints, the  $\Delta ampD\Delta amiD2$  double  
233 mutant and triple mutant, with MICs of 2, would be considered to have “intermediate” resistance to  
234 ceftriaxone, but to still be fully sensitive to ceftazidime and cefepime. In comparison, inactivation of  
235 the single *E. cloacae ampD* was reported to result in a ceftriaxone MIC of 32 (from a baseline of  
236 0.5) (26).

237

## 238 Discussion

239 Our findings suggest that the pTOX suite of allele exchange vectors described here should  
240 facilitate the genetic engineering of diverse Proteobacteria. Each of the pTOX vectors includes a  
241 rhamnose inducible toxin that may circumvent escape from counter selection, which can limit *sacB*-  
242 based allele exchange vectors. These toxins have been used to facilitate recombineering (11), and  
243 inducible toxins for allelic exchange promise to be a broadly generalizable approach, as systems  
244 have recently also been described for *Vibrio* and *Aeromonas* species (31) as well as for the  
245 archaeon *Pyrococcus yayanosii* (32).

246

247 The pTOX vectors contain an expanded multiple cloning site, multiple antibiotic resistance  
248 cassettes, and chromoprotein modules that facilitate monitoring of crossover events. The utility of  
249 the pTOX vectors and all 3 of the different toxins they encode was demonstrated through creation  
250 of multiple deletions in 4 different pathogens, including *S. flexneri*, an organism in which allele  
251 exchange has been difficult. All of these vectors have been deposited at Addgene to facilitate their  
252 distribution. Besides their utility for engineering Gram-negative organisms in research labs, these  
253 vectors may also be useful in the context of undergraduate education.

254

255 *S. marcescens*, along with *E. cloacae*, *C. freundii*, *Klebsiella aerogenes* and *Morganella morganii*,  
256 are members of a group of pathogenic *Enterobacteriaceae* with the potential for high level,

257 inducible expression of AmpC, which in some cases has been linked to resistance to almost all  
258 penicillins and cephalosporins (33). Original reports of cephalosporin failure in *E. cloacae* (34)  
259 engendered the practice of using ultra-broad spectrum antibiotics (such as cefepime or  
260 carbapenems, which are resistant to AmpC hydrolysis) in the treatment of serious infections by  
261 pathogens with the potential for AmpC overexpression. However, this approach has untoward  
262 consequences, including increasing infections with carbapenem-resistant Enterobacteriaceae (35).

263  
264 It is not clear if routine use of ultra-broad spectrum antibiotics is warranted for all organisms with  
265 inducible AmpC expression. A recent review (36) emphasized that besides *E. cloacae*, the data on  
266 cephalosporin failure for pathogens with inducible AmpC is sparse. What data do exist emphasize  
267 that true on-treatment emergence of beta-lactam resistance is probably rare, at least in *S.*  
268 *marcescens* and in *Morganella morganii* (37). *In vitro* experiments also hint at important  
269 heterogeneity among these pathogens; in this setting, the development of spontaneous  
270 cephalosporin resistance has been reported to be nearly 100-fold lower in *S. marcescens*  
271 compared to *E. cloacae* and *C. freundii*, and 10-fold lower still in *M. morganii* (38).

272  
273 Our observations suggest that ultra-broad-spectrum antibiotics may be not be necessary for  
274 treatment of *S. marcescens* infections. We used the pTOX vectors to investigate the role of *S.*  
275 *marcescens*' 3 peptidoglycan amidohydrolases on inducible beta-lactam antibiotic resistance. We  
276 found that deletion of a single amidohydrolase locus had a minimal effect on cephalosporin MICs,  
277 and that even the absence of all 3 amidohydrolase loci did not consistently render *S. marcescens*  
278 resistant to this class of antibiotics, although the triple mutant and the  $\Delta ampD\Delta amiD2$  double  
279 mutant did exhibit intermediate resistance to ceftriaxone. Thus, the effects of amidohydrolase  
280 deletion in *S. marcescens* differ from those in *C. freundii* and *E. cloacae*, in which resistance arises  
281 following the loss of a single amidohydrolase. Importantly, though current CLSI breakpoints would  
282 classify the  $\Delta ampD\Delta amiD2$  double mutant as having "Intermediate" resistance to ceftriaxone, there

283 is no evidence of increased clinical failure in this range (39). This is important since ceftriaxone is  
284 less expensive, has more convenient dosing intervals, and is a narrower spectrum agent compared  
285 to cefepime or carbapenems. Further work with additional *S. marcescens* isolates to clarify the  
286 generalizability of our findings is warranted.

287

## 288 **Materials and Methods**

289

### 290 pTOX construction

291 The DNA components for the pTOX series were obtained from pDS132 (15), the pSLC  
292 recombineering series (11) which was a gift from Swaine Chen (Addgene plasmid # 73194),  
293 pON.mCherry (21) which was a gift from Howard Shuman (Addgene plasmid # 84821), strain  
294 TP997 (40) which was a gift from Anthony Poteete (Addgene plasmid # 13055), and direct gene  
295 synthesis (from Integrated DNA Technologies) and were assembled using Gibson or HiFi  
296 Assembly (New England BioLabs) unless otherwise stated. All restriction enzymes were obtained  
297 from New England Biolabs and all PCR was performed with primers from Integrated DNA  
298 Technologies and Q5 polymerase (New England Biolabs). All cloning steps were performed in  $\pi$ -  
299 carrying hosts (either DH5 $\alpha$ pir (41) for propagation or MFD- $\pi$  (42) for conjugation) under catabolite  
300 repression in LB containing the appropriate antibiotic and 2% glucose (w/v).

301

302 pSLC toxin vectors were first linearized with primers prJL1 and prJL2 and joined with the fragment  
303 obtained from pDS132 with primers prJL3 and prJL4 (see Supplemental Table 1 for all primers  
304 used in this study). The mobRP4 from pDS132 was subsequently amplified with primers prJL5 and  
305 prJL6 and assembled with the prior vectors cut with NheI. The chloramphenicol resistance cassette  
306 from pON.mCherry was then amplified with primers prJL7 and prJL8 and inserted into the prior  
307 vectors digested with ClaI and BglIII. The  $\pi$ -dependent origin from pDS132 was next isolated by  
308 SmaI-digestion and inserted into the prior vectors linearized with prJL9 and prJL10. A codon-

309 optimized *rhaS* (with the original primary protein sequence obtained from WP\_000217135.1) and  
310 promoter (see Supplemental Text 1 for the sequence of all directly synthesized DNA fragments  
311 used in this study) was obtained by direct synthesis and assembled into the prior vectors linearized  
312 with prJL11 and prJL12. The expanded polylinker (19) with the forward transcriptional terminator  
313 BBa\_B1002 (IGEM) was obtained by direct synthesis (Sequence 2) and inserted into the prior  
314 vectors linearized with primers prJL13 and prJL14. The artificial ribosome binding site was  
315 generated using the online calculator derived after (17), synthesized as above (Sequence 3) and  
316 assembled into the prior vectors linearized with prJL15 and prJL16 to generate pTOX1 (containing  
317 *yhaV*), pTOX2 (containing *mqsR*) and pTOX3 (containing *tse2*). See Supplemental Table 2 for all  
318 plasmids used in this work. For insertion of *amilCP* or *tsPurple*, the above vectors were cut with  
319 SbfI and Sequence 4 and Sequence 5 inserted. For replacement of the chloramphenicol resistance  
320 cassette with one encoding gentamicin resistance, the appropriate vector was linearized with  
321 prJL17 and prJL18 and assembled with the cassette amplified from strain TP997 (using prJL19  
322 and prJL20). See Supplemental Table 3 for all strains used in this work. Q5 GC enhancer (New  
323 England Biolabs) was used for amplification of *mobRP4* and *tse2*.

324

#### 325 Insertion of homology targeting regions

326 pTOX vectors were cut with SmaI and the relevant homologous regions were assembled after  
327 being amplified with prJL21, prJL22, prJL23, and prJL24 (for *S. marcescens hexS*); prAW1,  
328 prAW2, prAW3, and prAW4 (for EHEC *lacZ*); prCJK1, prCJK2, prCJK3, and prCJK4 (for *S. flexneri*  
329 *ipgH*); prJL25, prJL26, prJL27, and prJL28 (for *E. cloacae ampC*); prJL29, prJL30, prJL31, and  
330 prJL32 (for *S. marcescens ampD*); prJL33, prJL34, prJL35, and prJL36 (for *S. marcescens amiD*);  
331 and prJL37, prJL38, prJL39, and prJL40 (for *S. marcescens amiD2*). Note that some of the overlap  
332 regions in the above primers correspond to a version of pTOX with the original pDS132 polylinker.

333

#### 334 Allelic exchange with pTOX

335 On day 1, the appropriate upstream and downstream sequences from the targeted pathogen are  
336 amplified from gDNA in separate PCR reactions. After column purification of the resulting PCR  
337 product (Denville), the products are assembled with pTOX previously gel-purified after restriction  
338 digestion of the polylinker and electroporated into an *E. coli* strain that could serve as donor in  
339 conjugation. Throughout this work, we routinely used the diaminopimelic acid (DAP) auxotroph  
340 MFD- $\pi$  (42) as the pTOX donor strain. Unless specified, all subsequent steps are performed in the  
341 presence of 2% glucose to avoid premature toxin induction. On day 2, colony PCR was performed  
342 on single MFD- $\pi$  transformant colonies to confirm the appropriate insert size. On day 3,  
343 conjugation was performed between the MFD- $\pi$  bearing pTOX and the pathogen of interest.  
344 Optimizing the conjugation is crucial. For example, we found that conjugation was efficient at 4-8  
345 hours at 37°C with a 3:1 (v/v) ratio of MFD- $\pi$ :pathogen for EHEC, *E. cloacae*, and *S. flexneri*, but  
346 *S. marcescens* had markedly better efficiency when conjugated overnight at 30°C using 50-fold  
347 excess volume of an early logarithmic phase growth culture of MFD- $\pi$ . Exconjugants were isolated  
348 on appropriate antibiotics. On day 4, a single exconjugant colony is resuspended in 2 mL of LB  
349 containing glucose (but no selective antibiotic). This culture is incubated at 37°C with agitation until  
350 OD<sub>600</sub> 0.2, then washed twice with M9 salts (Sigma) with 2% rhamnose (w/v) before plating on the  
351 M9-rhamnose agar described below. A short preliminary outgrowth in broth without selection  
352 minimizes the possibility of the culture becoming dominated with a single double-crossover  
353 rhamnose-resistant clone. On day 5, the desired mutants can be identified with colony PCR on the  
354 resulting double-crossover colonies. The selection is stringent and in this manner, individual  
355 colonies can frequently be isolated from a plate inoculated with the undiluted washed culture from  
356 above, but 10<sup>-1</sup> and 10<sup>-2</sup> dilutions should also be plated.

357

358 For the experiments described in Table 1, primers prJL51 and prJL52 were used; their amplicon  
359 consisted of a small intergenic region that was largely replaced when the expanded polylinker was  
360 inserted.



361

362 *amiICP* coloration optimization

363 pTOX derivatives with different promoters and ribosome binding sites to drive *amiICP* were created  
364 by assembling SbfI-cut pTOX1 with *amiICP* (Sequence 4) amplified with prJL59 and either prJL41  
365 (for J23119-B0030), prJL42 (for J23119-B0034), prJL43 (for CP25-B0030), or prJL44 (for  
366 apFAB46-B0030). The J23119 promoter and B0030 and B0034 ribosome binding sites sequences  
367 were obtained from IGEM. The insulated proD promoter (43) was amplified from pSB3C5-proD-  
368 B0032-E0051 (which was a gift from Joseph Davis and Robert Sauer; Addgene plasmid #107241)  
369 with prJL47 and prJL48, fused by SOE PCR with the *amiICP* coding sequence obtained from PGR-  
370 Blue (44) (which was a gift from Nathan Reyna; Addgene plasmid #68374) using prJL49 and  
371 prJL50, and after XbaI-digestion of this product, it was ligated with XbaI-cut pTOX1. The J23119-  
372 synthetic ribosome binding site (17) was amplified from Sequence 6 with prJL45 and prJL46 and  
373 assembled in SbfI-cut vector and *amiICP* amplified with prJL49 and prJL50 as for proD above.

374

375 *E. coli* DH5 $\alpha$ pir were transformed with the appropriate *amiICP*-containing plasmid. Single colonies  
376 were grown in overnight cultures, diluted 1:100, and then back-diluted once in logarithmic phase  
377 growth so to enable spot-streaking onto solid agar at the identical optical density. Digital images  
378 were taken at 24h and 48h and saturation obtained by splitting the resulting image into an “HSB  
379 Stack” in ImageJ. The peak saturation was subsequently obtained using the “Measure” function,  
380 then normalized by subtracting the peak saturation of the resulting spots from spots of *E. coli*  
381 DH5 $\alpha$ pir carrying pTOX1 without *amiICP*. The resulting values represent the mean  $\pm$  SEM of this  
382 procedure done on 3 different days.

383

384 Beta-lactamase assay

385 Overnight cultures of indicated strains were back-diluted 1:100 (v/v) into fresh media and grown for  
386 an additional 2 hours. Bacteria were then pelleted, washed twice in phosphate-buffered saline, and



387 then flash-frozen in liquid nitrogen. On the day of the assay, pellets were thawed at 37°C and then  
388 subjected to a single round of sonication on ice (Sonic Dismembrator 60, Fisher Scientific,  
389 setting 8, 5 seconds). Lysates were clarified by centrifugation at 20,000 rcf for 60 minutes at 4°C.  
390 Total protein was quantitated by fluorometry using the Qbit Protein Assay Kit (Thermo Fisher).  
391 Beta-lactamase activity was determined by the addition of 80 ng nitrocefin to either 250 ng or 1000  
392 ng of total protein; to facilitate accurate quantitation, 250 ng was used for all cefoxitin-induced *S.*  
393 *marcescens* samples and also for the  $\Delta ampD\Delta amiD2$  double mutant and the triple mutant.  
394 Immediately after addition of nitrocefin with a multi-channel pipettor, absorbance was read  
395 kinetically at 495 nm every 5 minutes in a Synergy HT plate reader (BioTek). For Figure 5, the  
396 slope of the absorbance was normalized to wild-type *S. marcescens* and the amount of total  
397 protein added.

398

#### 399 Minimum inhibitory concentration (MIC) determination

400 Minimum inhibitory concentrations were determined for the indicated *S. marcescens* isolates and  
401 performed by broth microdilution according to CLSI guidelines and after Weigand *et al* (45). Briefly,  
402 overnight cultures were back-diluted in cation-adjusted Mueller-Hinton broth, allowed to grow for 2  
403 hours, and adjusted to a final inoculum of  $5 \times 10^5$  colony-forming units per mL before applying to  
404 wells with the appropriate antibiotic concentration. Results were read after 20 hours of incubation  
405 at 37°C. The results in Table 2 represent the mode of 3 independent experiments.

406

#### 407 Materials and strains

408 Unless otherwise specified, all materials were purchased from Sigma. When appropriate, media  
409 was supplemented with streptomycin 200 µg/mL, gentamicin 5 µg/mL, and chloramphenicol 20  
410 µg/mL for all *E. coli*, *E. cloacae*, and *S. flexneri*. *S. marcescens* exconjugants were isolated at 100  
411 µg/mL chloramphenicol. Diaminopimelic acid (DAP) was used at a final concentration of 0.3 mM, 5-  
412 bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) at 60 µg/mL, glucose at 2% (w/v) in all

413 propagation steps with pTOX vectors. When washing the out-grown single-crossovers, rhamnose  
414 was used at 2% (w/v) in M9 salts. The resulting washed bacteria were plated on M9 agar  
415 supplemented with 0.2% casamino acids (w/v), 0.5 mM MgSO<sub>4</sub>, 0.1 mM CaCl<sub>2</sub>, 25 uM iron chloride  
416 in 50 uM citric acid, the appropriate antibiotic, and rhamnose. Rhamnose at a final concentration of  
417 0.2%-2% facilitated good toxin induction in the organisms we tested; there was no obvious  
418 correlation with the concentration of rhamnose used, but it may be prudent to optimize this in new  
419 organisms. The *S. marcescens* ATCC 13880 isolate used throughout this work is a spontaneous  
420 mutant selected on streptomycin. *E. cloacae* was obtained from ATCC (isolate 13047). EHEC was  
421 isolate EDL933. *S. flexneri* was strain 2457T.

422

#### 423 Miscellaneous analysis

424 All figures and statistical analyses were prepared in Prism 8 (Graphpad). The growth curves in  
425 Supplemental Figure 3 were generated using Bioscreen C (Growth Curves USA). The plasmid  
426 maps were generated with AngularPlasmid and ApE (for the polylinker inset in Figure 1).

427

#### 428 **Acknowledgments**

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430 Investigator Training fellowship; ARW by T32AI132120; MKW by R01 AI-042347 and HHMI. We  
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432 pTOX and for comments on the manuscript.

433

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558 **Tables**

559

560 **TABLE 1** Absence of integrated pTOX in putative double-crossover colonies<sup>a</sup>

	<b>YhaV toxin</b>	<b>MqsR toxin</b>	<b>Tse2 toxin</b>
<b>CAM<sup>R</sup></b>	0/69	0/69	0/69
<b>PCR<sup>+</sup></b>	0/69	0/69	0/69

561 <sup>a</sup> CAM = chloramphenicol resistance was determined using the parental single-  
562 crossover colony as a positive control. An intergenic region of pTOX was used for  
563 colony PCR to avoid false negatives during selection for inactivated open reading  
564 frames.

565

566

567 **TABLE 2** Minimal inhibitory concentrations for amidohydrolase mutants<sup>b</sup>

<b>Strain</b>	<b>MIC (µg/mL)</b>			
	<b>FOX</b>	<b>CTX</b>	<b>CAZ</b>	<b>FEP</b>



<i>S. marcescens</i>	16	0.13	0.25	0.06
ATCC 13880				
SM $\Delta ampD$	16	0.13	0.25	0.03
SM $\Delta amiD$	16	0.13	0.25	0.13
SM $\Delta amiD2$	16	0.25	0.50	0.13
SM $\Delta ampD\Delta amiD$	16	0.50	0.25	0.13
SM $\Delta ampD\Delta amiD2$	16	2.00	0.50	0.25
SM $\Delta amiD\Delta amiD2$	16	0.13	0.25	0.06
SM $\Delta ampD\Delta amiD\Delta amiD2$	16	2.00	0.50	0.25

568 <sup>b</sup> MICs for indicated strain were calculated using broth microdilution according to CLSI  
569 Guidelines. SM, *S. marcescens* ATCC strain 13880. FOX, cefoxitin; CTX, ceftriaxone;  
570 CAZ, ceftazidime; FEP, cefepime.

571 **Figure Legends**

572 **Fig 1** Allelic exchange with pTOX. A) Plasmid map of pTOX1. R6Kori, the R6K origin of replication;  
573 mobRP4, mobilization region from RP4 conjugative plasmid; *rhaS*, the rhamnose transcriptional  
574 activator; MCS, multiple cloning site; Cam-R, chloramphenicol resistance cassette; pRha,  
575 rhamnose promoter. Vertical black bars of varying width represent terminators. Bottom, expanded  
576 polylinker with restriction sites unique to pTOX1 (*yhaV*) shown. Red arrow, forward transcriptional  
577 terminator. B) pTOX workflow. Step 1: the desired allele is inserted into the MCS using isothermal  
578 assembly and transformed into donor *E. coli*. (yellow bacillus) Step 2: conjugation is performed  
579 between the donor *E. coli* and the organism of interest (red coccobacillus). Step 3: pTOX  
580 integrates into the appropriate chromosomal locus. Step 4: merodiploids are isolated and toxin  
581 induced. Step 5: the desired clone is identified by colony PCR.

582  
583 **Fig 2** Induction of specific bacterial toxins inhibit *S. marcescens* growth. *S. marcescens* wild-type  
584 (Wt) or merodiploid (merodip) harboring the indicated pTOX-carrying toxin were diluted from  
585 exponential phase growth in LB into either 2% (w/v) glucose (gluc) or rhamnose-containing (rham)  
586 LB and incubated with agitation at 37°C. Note that the Wt (gluc) curve is obscured by the Wt  
587 (rham) curve in A and the error bars in C are smaller than the line for all but the merodiploid (gluc).  
588 Means and SEM are depicted from at least 3 independently generated merodiploids.

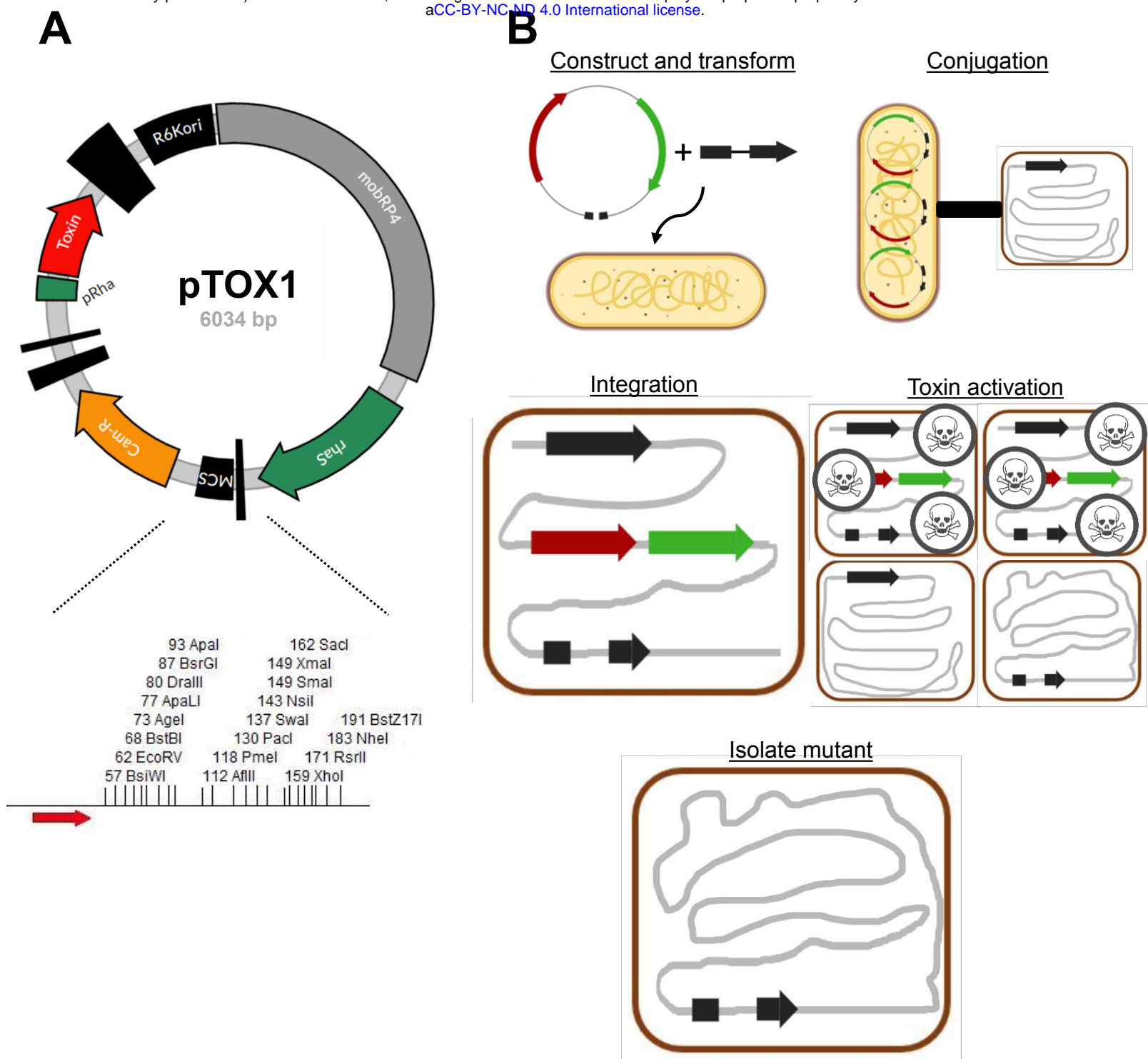
589  
590 **Fig 3** pTOX for genomic modification in multiple pathogens. A) *S. marcescens* colony coloration in  
591 Wt (left) and  $\Delta$ *hexS* (right) grown at 37°C for 1 day. HexS inhibits expression of the red prodigiosin  
592 characteristic of *S. marcescens*. B) *E. coli* O157:H7 colony coloration in Wt (left) and  $\Delta$ *lacZ* (right)  
593 grown on X-gal-containing media. Blue-green colony color indicates lactose fermentation. C) *S.*  
594 *flexneri* colony PCR and results of 1% agarose gel electrophoresis demonstrating deletion of  
595 *ipgH* from *S. flexneri* virulence plasmid. M, marker; Wt, wild-type;  $\Delta$ ,  $\Delta$ *ipgH*. D) *E. cloacae* beta-  
596 lactamase activity in total clarified sonicate from 3 Wt double-crossover colonies and from 3

597 *ΔampC* colonies. Sonicates were incubated with nitrocefin, a chromogenic cephalosporin substrate  
598 which absorbs at 495 nm when hydrolyzed.

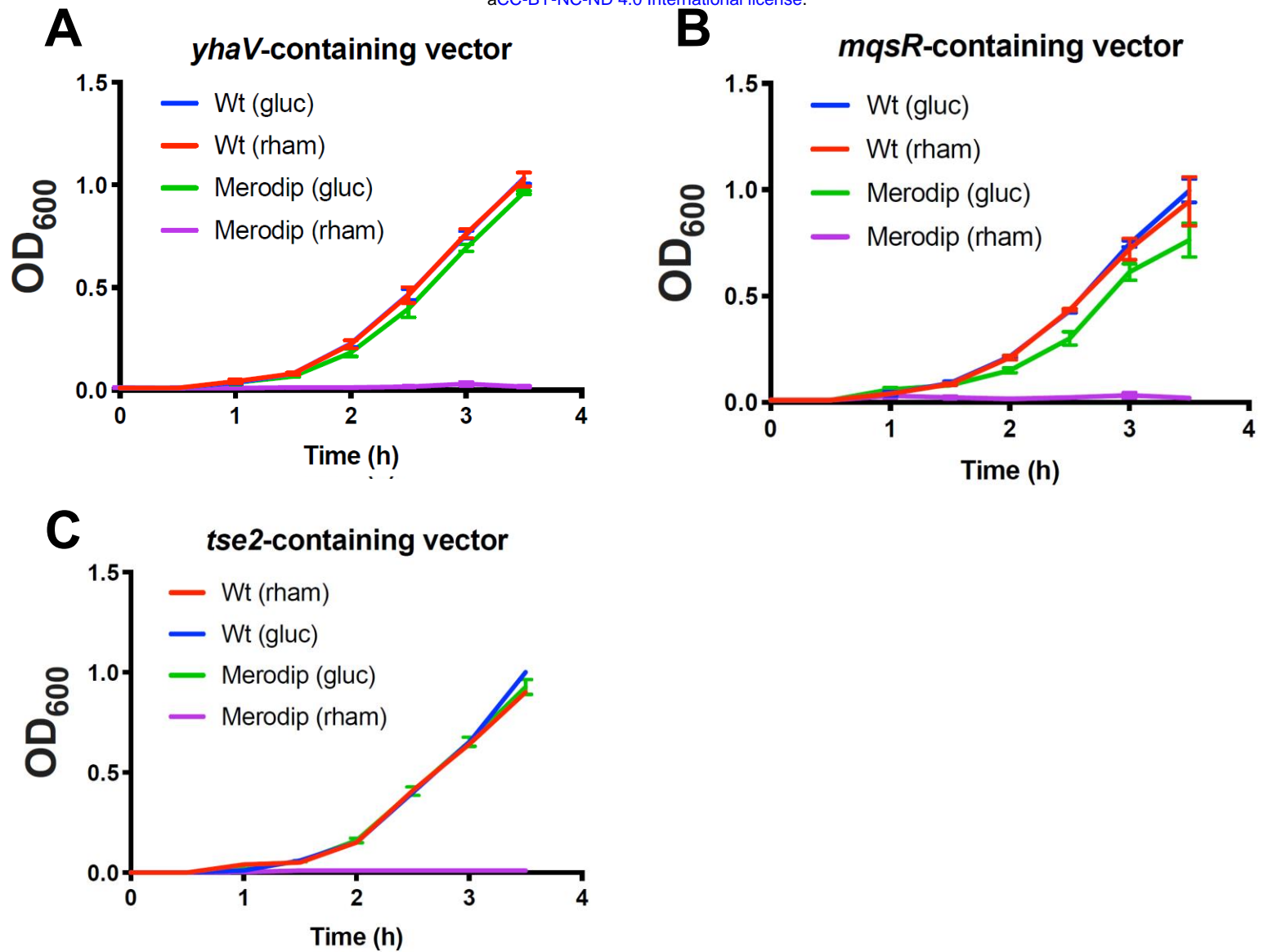
599  
600 **Fig 4** A chromoprotein module facilitates monitoring of conjugation. A) Plasmid map of pTOX4.  
601 R6Kori, the R6K origin of replication; mobRP4, mobilization region from RP4 conjugative  
602 plasmid; *rhaS*, the rhamnose transcriptional activator; *amilCP*, the blue *amilCP* chromoprotein;  
603 MCS, multiple cloning site; Cam-R, chloramphenicol resistance cassette; pRha, rhamnose  
604 promoter. Vertical black bars of varying width represent terminators. B) *tac* and apFAB46-B0030  
605 allow optimal *amilCP* expression. Relative color saturation at 24h and at 48h of pTOX4-containing  
606 colonies with various promoters and ribosome-binding sites (described in more detail in the  
607 Methods). C) Depiction of donor *E. coli* containing (from bottom, clockwise) pTOX without  
608 chromoprotein, with *tac-amilCP*, and with apFAB46-B0030-*tsPurple* after 24h at 37°C. D) *E.*  
609 *cloacae* pTOX merodiploids (from bottom, clockwise) without chromoprotein, with *tac-amilCP* ,  
610 and with apFAB46-B0030-*tsPurple* after 24h at 37°C and an additional 24 hours at 25°C.

611  
612 **Fig 5** *S. marcescens* peptidoglycan amidohydrolase deletions lead to differential derepression of  
613 *ampC*. A) Phylogenetic analysis performed using the Maximum Likelihood method and JTT matrix-  
614 based model in MEGA X (30)An unrooted tree is shown with the lowest log likelihood (-4913). B)  
615 Clarified sonicates from indicated strains were incubated with equal amounts of nitrocefin, a  
616 chromogenic cephalosporin beta-lactam, and absorbance measured in kinetic mode for 10  
617 minutes. The slope of the line from the first 5 data points were used to calculate beta-lactamase  
618 activity, which was then normalized to Wt. Measurements are shown either without pre-induction,  
619 and those with induction with cefoxitin 4 µg/mL for 2 hours prior to harvesting. Data represent the  
620 mean ± SEM of 4 independent experiments. Comparisons were made between all uninduced  
621 mutants and Wt, and between each induced sample and its uninduced control. \* = p < 0.05 after  
622 performing Bonferroni correction. All induced samples are also significantly different from their

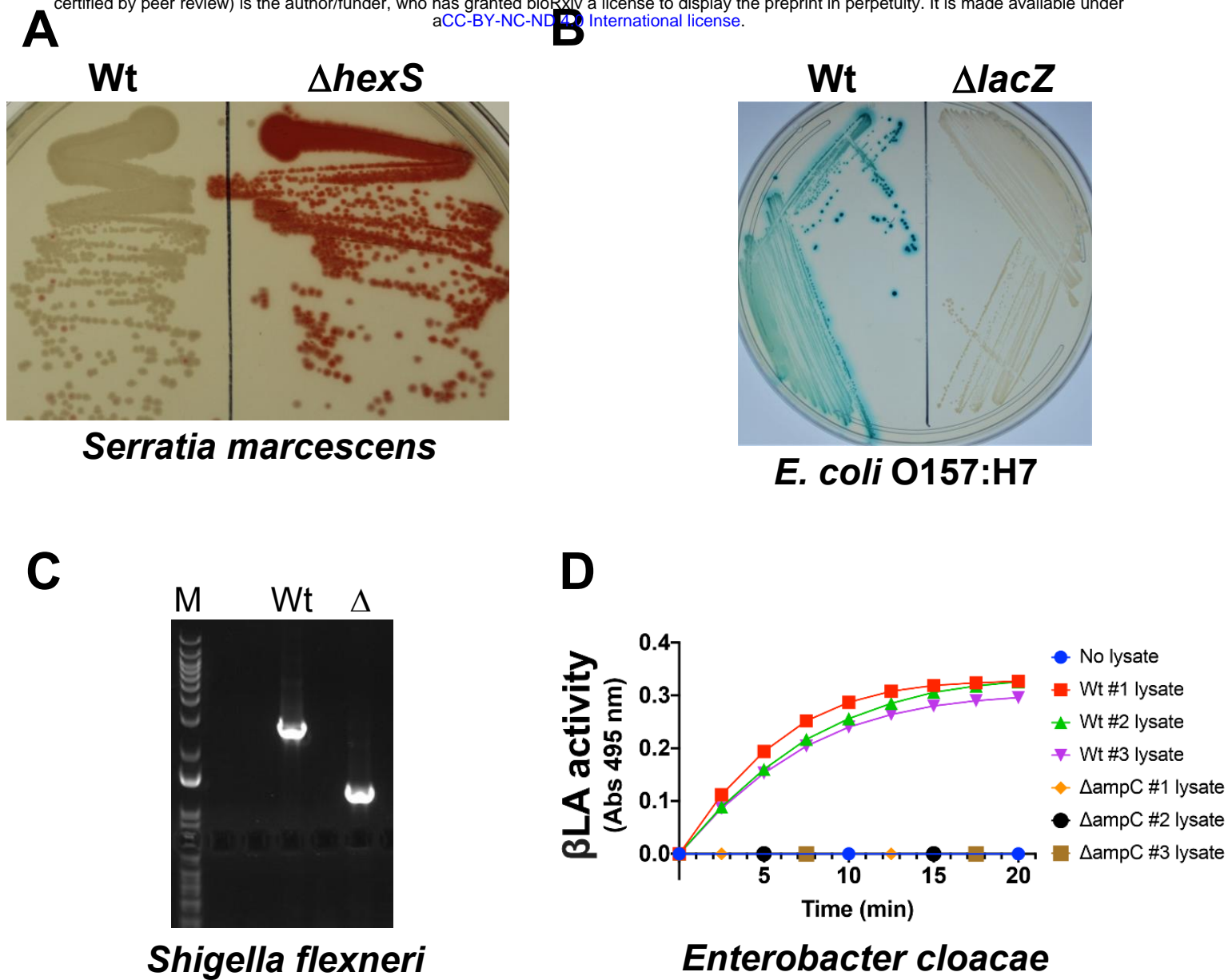
623 uninduced samples, except for  $\Delta ampD\Delta amiD$ ,  $\Delta ampD\Delta amiD2$ , and the triple mutant. These  
624 asterisks are not shown for clarity.



**Fig 1** Allelic exchange with pTOX. A) Plasmid map of pTOX1. R6Kori, the R6K origin of replication; mobRP4, mobilization region from RP4 conjugative plasmid; *rhaS*, the rhamnose transcriptional activator; MCS, multiple cloning site; Cam-R, chloramphenicol resistance cassette; pRha, rhamnose promoter. Vertical black bars of varying width represent terminators. Bottom, expanded polylinker with restriction sites unique to pTOX1 (*yhaV*) shown. Red arrow, forward transcriptional terminator. B) pTOX workflow. Step 1: the desired allele is inserted into the MCS using isothermal assembly and transformed into donor *E. coli*. (yellow bacillus) Step 2: conjugation is performed between the donor *E. coli* and the organism of interest (red coccobacillus). Step 3: pTOX integrates into the appropriate chromosomal locus. Step 4: merodiploids are isolated and toxin induced. Step 5: the desired clone is identified by colony PCR.

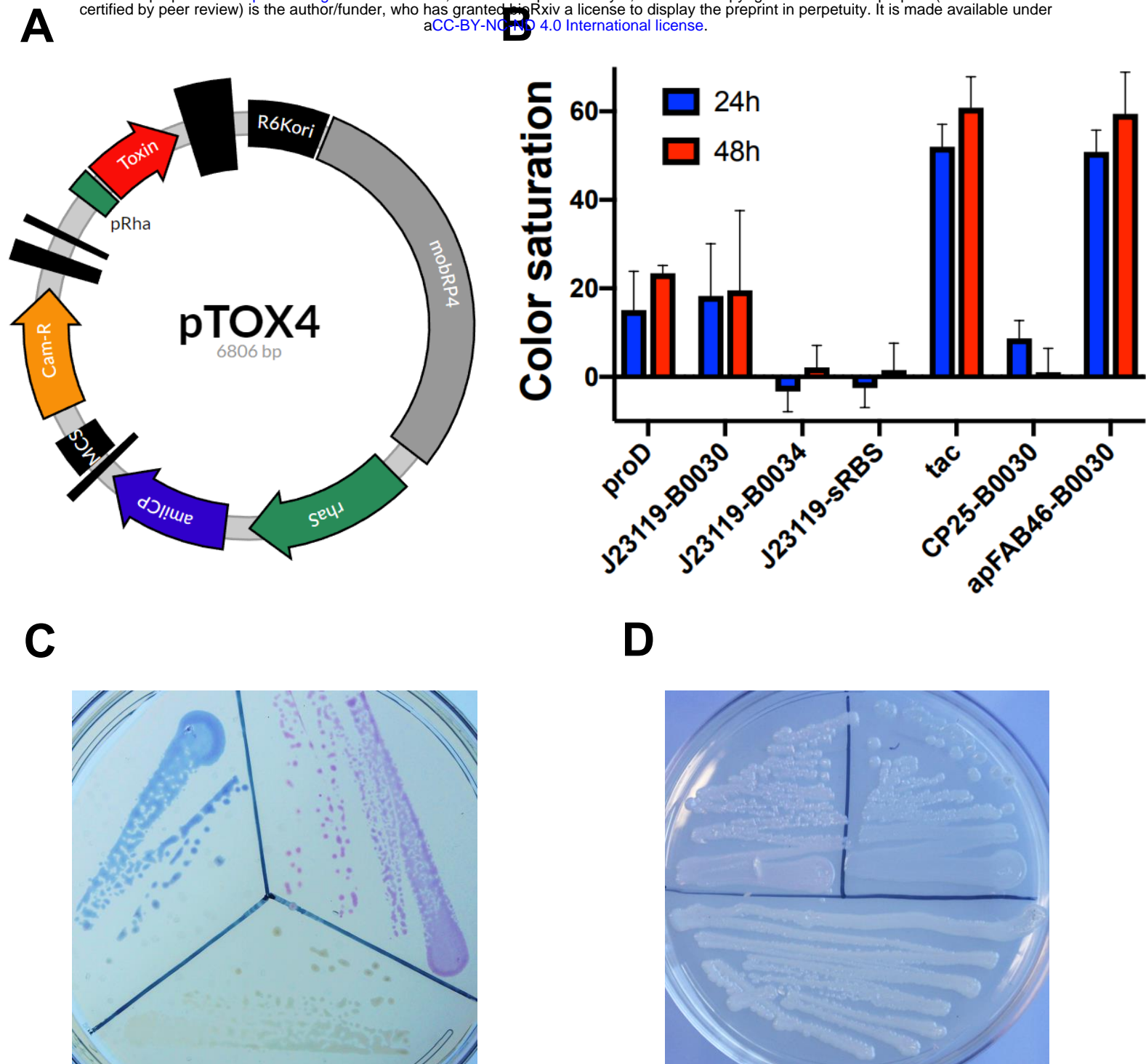


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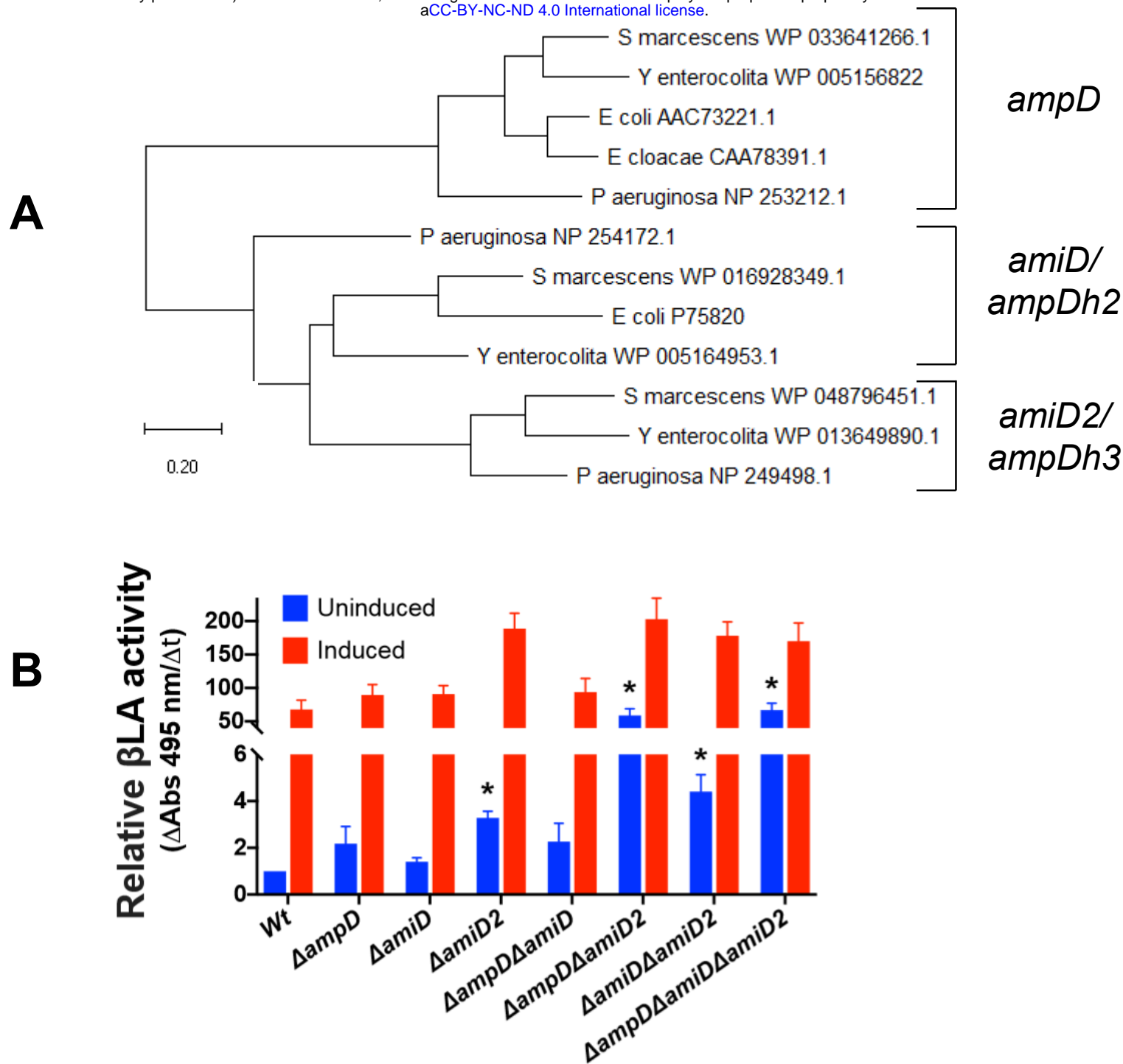
**Fig 3** pTOX for genomic modification in multiple pathogens. A) *S. marcescens* colony coloration in Wt (left) and  $\Delta hexS$  (right) grown at 37°C for 1 day. HexS inhibits expression of the red prodigiosin characteristic of *S. marcescens*. B) *E. coli* O157:H7 colony coloration in Wt (left) and  $\Delta lacZ$  (right) grown on X-gal-containing media. Blue-green colony color indicates lactose fermentation. C) *S. flexneri* colony PCR and results of 1% agarose gel electrophoresis demonstrating deletion of *ipgH* from *S. flexneri* virulence plasmid. M, marker; Wt, wild-type;  $\Delta$ ,  $\Delta ipgH$ . D) *E. cloacae* beta-lactamase activity in total clarified sonicate from 3 Wt double-crossover colonies and from 3  $\Delta ampC$  colonies. Sonicates were incubated with nitrocefin, a chromogenic cephalosporin substrate which absorbs at 495 nm when hydrolyzed.





**Fig 4** A chromoprotein module facilitates monitoring of conjugation. A) Plasmid map of pTOX4. R6Kori, the R6K origin of replication; mobRP4, mobilization region from RP4 conjugative plasmid; *rhaS*, the rhamnose transcriptional activator; *amilCP*, the blue *amilCP* chromoprotein; MCS, multiple cloning site; Cam-R, chloramphenicol resistance cassette; pRha, rhamnose promoter. Vertical black bars of varying width represent terminators. B) *tac* and apFAB46-B0030 allow optimal *amilCP* expression. Relative color saturation at 24h and at 48h of pTOX4-containing colonies with various promoters and ribosome-binding sites (described in more detail in the Methods). C) Depiction of donor *E. coli* containing (from bottom, clockwise) pTOX without chromoprotein, with *tac-amilCP*, and with apFAB46-B0030-*tsPurple* after 24h at 37°C. D) *E. cloacae* pTOX merodiploids (from bottom, clockwise) without chromoprotein, with *tac-amilCP*, and with apFAB46-B0030-*tsPurple* after 24h at 37°C and an additional 24 hours at 25°C.

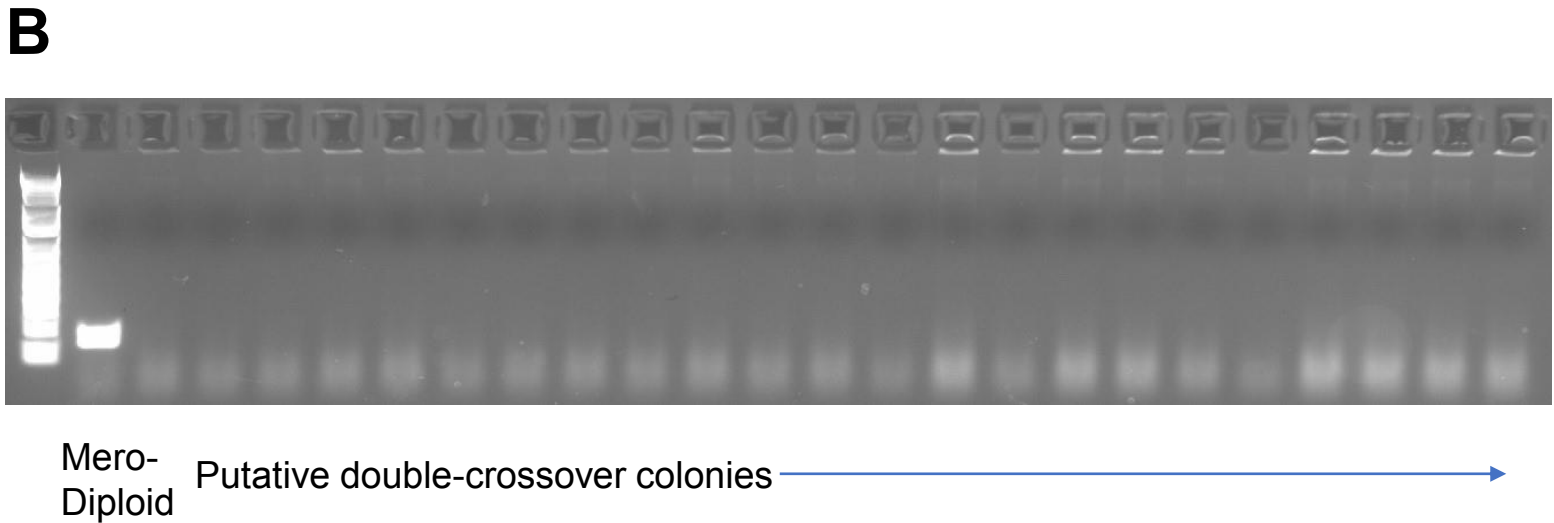
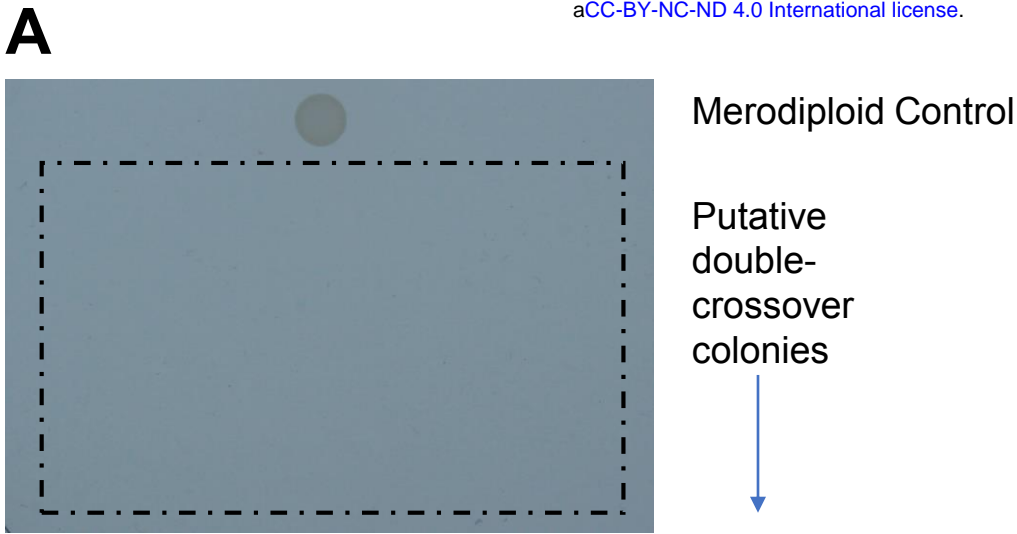




**Fig 5** *S. marcescens* peptidoglycan amidohydrolase deletions lead to differential derepression of *ampC*. A) Phylogenetic analysis performed using the Maximum Likelihood method and JTT matrix-based model in MEGA X. An unrooted tree is shown with the lowest log likelihood (-4913). Scale bar = 0.2 substitutions per site. B) Clarified sonicates from indicated strains were incubated with equal amounts of nitrocefin, a chromogenic cephalosporin beta-lactam, and absorbance measured in kinetic mode for 10 minutes. The slope of the line from the first 5 data points were used to calculate beta-lactamase activity, which was then normalized to Wt. Measurements are shown either without pre-induction, and those with induction with cefoxitin 4  $\mu$ g/mL for 2 hours prior to harvesting. Data represent the mean  $\pm$  SEM of 4 independent experiments. Comparisons were made between all uninduced mutants and Wt, and between each induced sample and its uninduced control. \* =  $p < 0.05$  after performing Bonferroni correction. All induced samples are also significantly different from their uninduced samples, except for  $\Delta ampD\Delta amiD$ ,  $\Delta ampD\Delta amiD2$ , and the triple mutant. These asterisks are not shown for clarity.

		ylbA	mqsR	tse2
<b>Alphaproteobacteria</b>	<i>Ehrlichia</i>	○	○	○
	<i>Anaplasma</i>	○	○	○
	<i>Wolbachia</i>	○	○	○
	<i>Rickettsia</i>	○	○	○
	<i>Brucella</i>	○	○	○
	<i>Bartonella</i>	○	○	○
<b>Betaproteobacteria</b>	<i>Burkholderia</i>	○	✗	○
	<i>Neisseria</i>	✗	○	○
	<i>Bordetella</i>	✗	✗	○
<b>Gammaproteobacteria</b>	<i>Legionella</i>	✗	○	○
	<i>Francisella</i>	○	○	○
	<i>Moraxella</i>	○	○	○
	<i>Acinetobacter</i>	○	○	✗
	<i>Pseudomonas</i>	○	○	✗
	<i>Stenotrophomonas</i>	○	○	○
	<i>Shewanella</i>	○	○	○
	<i>Aeromonas</i>	○	○	○
	<i>Vibrio</i>	○	○	○
	<i>Haemophilus</i>	○	○	○
	<i>Pasteurella</i>	○	○	○
	<i>Hafnia</i>	○	✗	○
	<i>Pantoea</i>	✗	○	○
	<i>Yersinia</i>	○	✗	○
	<i>Serratia</i>	○	○	○
	<i>Klebsiella</i>	✗	✗	○
	<i>Raoultella</i>	○	○	○
	<i>Enterobacter</i>	✗	○	○
	<i>Citrobacter</i>	✗	○	○
	<i>Salmonella</i>	✗	○	○
	<i>Escherichia</i>	✗	✗	○
	<i>Shigella</i>	✗	✗	○
	<i>Proteus</i>	✗	○	○
<i>Morganella</i>	○	○	○	
<i>Providencia</i>	○	○	○	
<b>Epsilonproteobacteria</b>	<i>Helicobacter</i>	○	○	○
	<i>Campylobacter</i>	○	○	○

**Fig S1** Toxin(s) predicted to be useful (green open circle) in diverse pathogens based on absence of toxin homolog by BLASTP in high confidence genomes deposited in NCBI. Red X's denote presence of toxin gene.

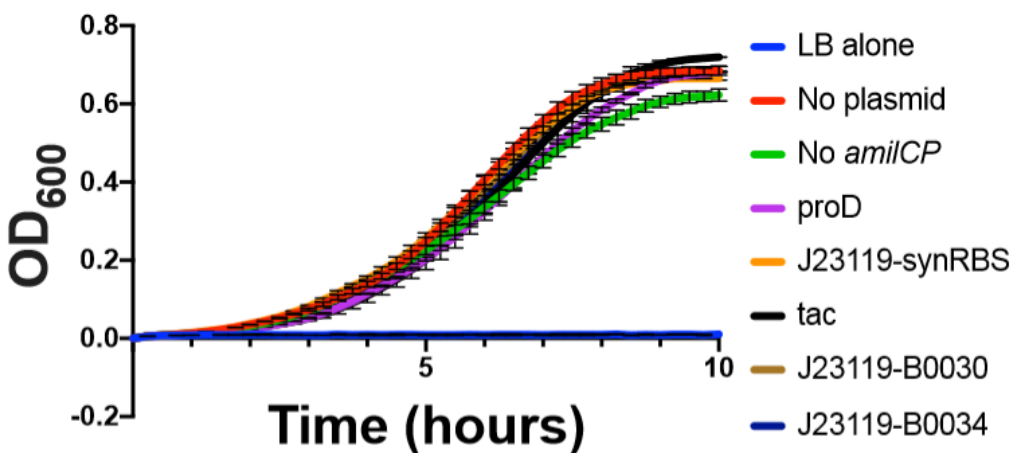


**Fig S2** Putative double-crossover *S. marcescens* colonies (for each toxin, 23 colonies from 3 different crossovers) were randomly selected for characterization. Summary results are in Table 1. A) A colony was resuspended in non-selective LB and then spotted onto LB+chloramphenicol. The top colony is the merodiploid positive control. There is no growth where the putative double-crossover colonies were spotted (hatched box). B) Colony PCR was performed for a small intergenic amplicon (between *rhaS* and the chloramphenicol resistance promoter) to test for presence of retained pTOX. The first lane is the merodiploid positive control.

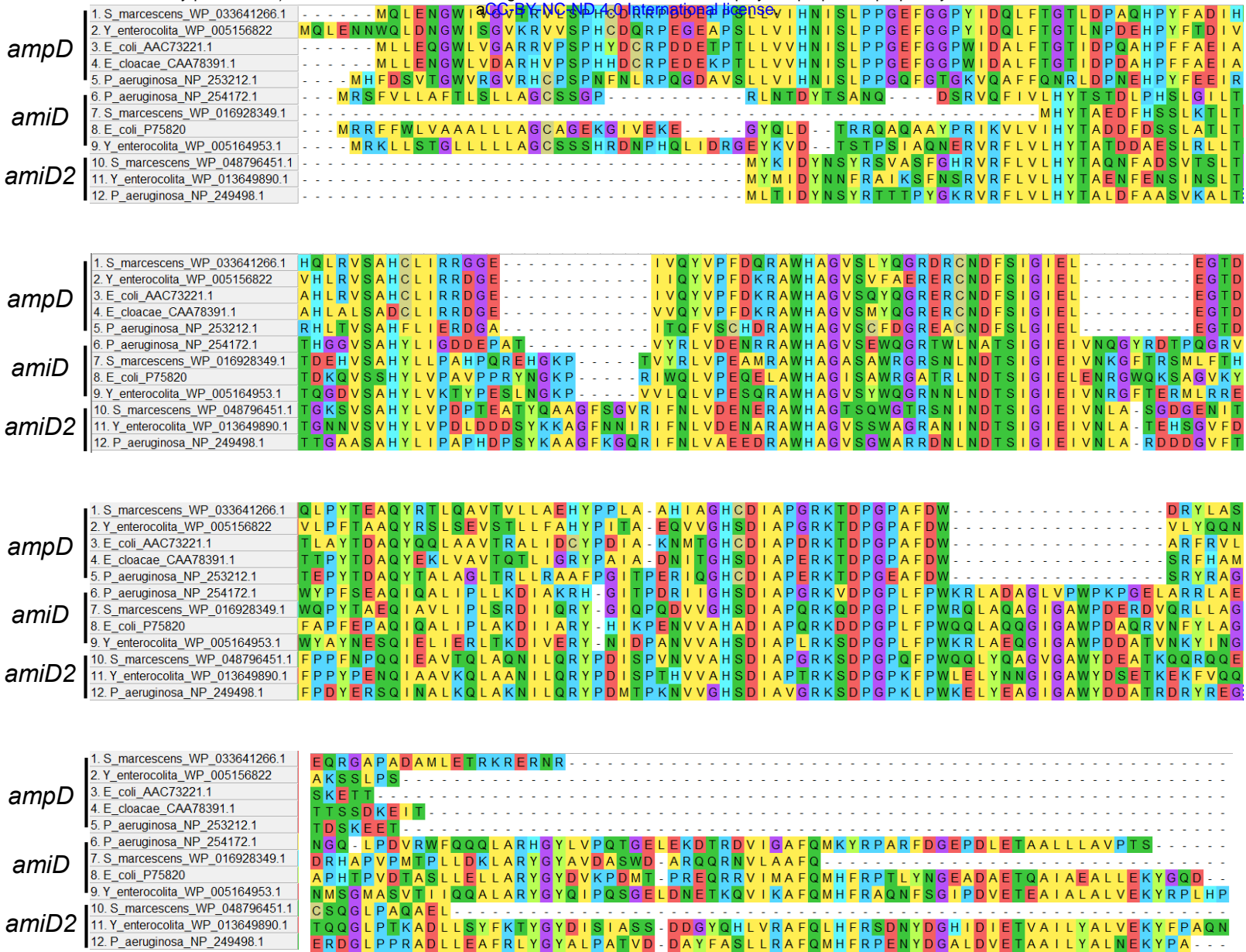
**A**



**B**



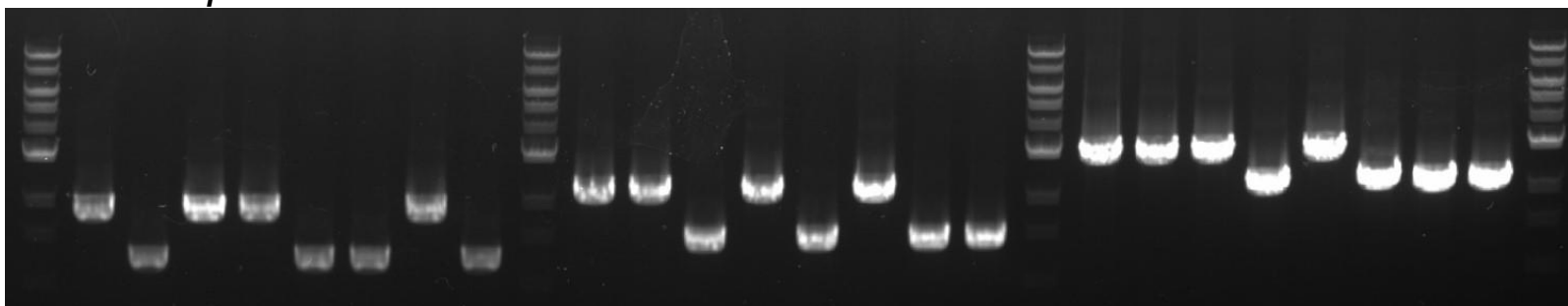
**Fig S3** A) *E. coli* donors containing pTOX vectors with *amilCP* and various promoters and ribosome binding sites (RBS) at 24h at 37°C. From top, clockwise: 1) pTOX with no *amilCP*; 2) pTOX-*amilCP* with proD promoter; 3) pTOX-*amilCP* with J23119 promoter and synthetic RBS; 4) pTOX-*amilCP* with tac promoter; 5) and 6) pTOX-*amilCP* with J23119 promoter with the B0030 or B0034 RBS, respectively. B) Growth curves for *E. coli* donor strains (or LB alone) containing pTOX with *amilCP* driven by indicated promoter/RBS.



*ampD* locus

*amiD* locus

*amiD2* locus



**Fig S5** PCR-based genotyping of *S. marcescens* amidohydrolase mutations. Lanes from left to right: marker, Wt,  $\Delta ampD$ ,  $\Delta amiD$ ,  $\Delta amiD2$ ,  $\Delta ampD\Delta amiD$ ,  $\Delta ampD\Delta amiD2$ ,  $\Delta amiD\Delta amiD2$ ,  $\Delta ampD\Delta amiD\Delta amiD2$ . The *ampD* locus was amplified with prJL53 and prJL54; *amiD* with prJL55 and prJL56; and *amiD2* with prJL57 and prJL58



## **Supplemental Text 1**

### **Sequence 1 – Codon-optimized *rhaS* with promoter**

cgcggaaccctattgtttatcttaataacattcaaatatgtatccgctcatgagacaataaccctgataaatgcttcaataatattgaa  
aaaggaagagtATGACGGTGTGCTGCACTCGGTTGACTTCTTCCCTAGCGGCAATGCCAGCGTTG  
CCATTGAGCCGCGCCTGCCTCAAGCCGACTTCCCGGAGCACCACCACGACTTCCACGAGA  
TCGTTATCGTGGAGCACGGTACCGGCATCCACGTTTTCAACGGCCAACCGTACACGATTAC  
GGCGGTACCGTGTGCTTCGTTTCGTGATCACGACCGCCACTTATACGAGCACACGGACAA  
CTTATGCTTAACCAACGTTTTATACCGTAGCCCTGACCGCTTCCAATTCTGGCGGGCTTA  
AACCAACTGTTACCGCAGGAATTAGACGGCCAATACCCTAGCCATTGGCGTGTGAATCATT  
CGGTGCTGCAACAAGTTCGCCAATTAGTGGCGCAAATGGAGCAACAAGAGGGCGAGAACG  
ACCTGCCGAGCACGGCGAGCCGTGAAATTCTGTTTCATGCAGCTGTTACTGCTGTTACGCA  
AGTCGAGCCTGCAAGAAAATTTAGAGAATTCGGCGAGCCGCCTGAATCTGCTGTTAGCGT  
GGTTAGAAGATCACTTCGCGGACGAAGTTAACTGGGATGCGGTTGCCGACCAGTTCAGCC  
TGAGCTTACGCACCCTGCACCGCCAATGAAACAACAGACCGGCTTAACCCCGCAACGCT  
ATTTAAACCGTTTACGCTTAATGAAGGCGCGCCACTTACTGCGCCATTTCGGAAGCGTCGGT  
GACCGATATTGCGTACCACTGTGGCTTTTTCGGATAGCAATCATTTTCAGCACCCCTGTTCCGT  
CGCGAATTCAATTGGAGCCCTCGCGACATCCGCCAAGGCCGCGACGGTTTTCTTACAGTGA

### **Sequence 2 – Forward terminator and expanded polylinker**

AGCTTCGAGCTAATCGcgcaaaaaaccccgcttcggcggggtttttcgcTGATCACGTACGATATCTTCGA  
ACCGGTGCACATGGTGTACAGGGCCCTAGGATAGGACGTCTTAAGGTTTAAACCAGGTTA  
ATTAATTTAAATGCATCCCGGGACGTCTCGAGCTCGATCGGACCGCGGCCGCTAGCACGT  
ATACCAAGTGTCTGT

### **Sequence 3 – Synthetic strong ribosome binding site**

ttatcttaataacattcaaatatgtatccgctcatgagacaataaccctgataaatgcttcaataatattgaaaaaggaGCTTATC  
ACCGATAAGGAGGTTTTTTAATGACGGTGTGCTGCACTCGGTTGACTTCTTCCCTAGCGGCAA  
TGCCAGCGTTGCCATTGAGCCGCGCCTGCCTCAAGCCGACTTCCCGGAGCAC

### **Sequence 4 – *amiCP* with *tac* promoter**

ttgacaattaatcatcggtcgtataatgtgtggaaaggcggttcaccgcccgtttcacacaggaaacagaattctttaagaaggagata  
tacatATGTCAGTGATAGCAAAGCAGATGACATACAAAGTATATATGAGCGGTTACTGTAAATG  
GTCACTACTTCGAAGTAGAAGGTGATGGCAAAGGGAAGCCGTATGAGGGTGAACAAACAG  
TGAAGCTTACAGTTACGAAGGGTGGCCCTTTGCCGTTTCGCGTGGGACATTTTGTGCCAC  
AGTGCCAGTACGGGAGCATAACCATCAAGTATCCAGAGGACATAACGACTACGTGA  
AACAGTCTTTCCCGAAGGCTATACCTGGGAGCGCATAATGAACTTTGAAGACGGTGCCG  
TTTTGTACGGTATCGAACGATTCATCAATCCAAGGAAATTGCTTTATTTATCATGTTAAATTT  
CGGGGCTTAACTTTCCGCCAATGGCCCCGTGATGCAGAAGAAAACACTCAGGGCTGGGAAC  
CAAATACCGAGCGCCTTTTCGCTCGGGACGGAATGCTGTTGGGAACAATTTTATGGCGTT  
GAAGCTGGAAGGGGGTGGCCACTATCTCTGTGAATTCAAGACAACATACAAAGCCAAGAA  
GCCTGTCAAGATGCCCGGTTATCACTATGTGGACCGGAAGCTCGATGTCACTAATCACAAC  
AAGGATTATACTTCAGTTGAACAGTGCGAAATTTTCGATCGCTCGCAAACCTGTTCGTAGCAT  
AA

### **Sequence 5 – *tsPurple* with apFAB46 promoter**

AGGCCTtctagagtcgacctgcaaaaaagagtattgacttcgcatctttgtacctataatagattcattactagagaaagaggaga  
aatactagATGGCGTCCCTTGTAAGAAGGATATGTGTGTTAAGATGACAATGGAGGGAACCG  
TCAACGGGTATCACTTTAAGTGTGTTGGCGAGGGAGAAGGCAAACCGTTCGAGGGTACAC  
AGAATATGCGCATTTCGTGTCACCGAAGGGGGCCCTTTGCCCTTTGCTTTTCGATATTTTGGC  
CCCGTGCTGTATGTATGGTTCGAAGACTTTTATCAAACACGTAAGCGGAATCCCTGACTAC  
TTCAAGGAGAGCTTTCCCGAAGGCTTTACTTGGGAACGTACGCAGATTTTTGAAGACGGAG  
GAGTTTTGACTGCCCATCAAGATACATCACTTGAGGGAACTGTTTAATCTACAAAGTTAAG  
GTGTTAGGGACAAATTTTCCTGCCAATGGTCCGGTTATGCAGAAAAGACAGCAGGCTGG  
GAACCGTGTGTAGAGATGTTGTACCCCGCGACGGGGTTCTTTGCGGGCAATCCCTTATG  
GCCTTGAAATGTAAGTACTGATGGAAATCACTTGACATCCCATTTACGCACCACTTATCGCTCGC  
GTAAGCCAAGTAATGCGGTGAACATGCCGGAATTTCACTTCGGAGACCATCGTATTGAAAT  
CTTGAAAGCCGAACAAGGAAAATTTTATGAACAATACGAATCCGCAGTGGCACGTTATTCA  
GATGTTCCAGAAAAGGCGACTTGATAAggcatgcaagcttgctgtt

### **Sequence 6 – J23119 promoter with synthetic ribosome binding site**

TTGACAGCTAGCTCAGTCCTAGGTATAATGCTAGCTGAGAAAAGAAAGGGAAACTAAGGAG  
GTATTTT



## **SUPPLEMENTAL TABLE 1 Primers used in this study**

prAW1	atgcgatatcgagctctcccATGGTGAACATGATGCCGAC
prAW2	TCACACAGGATACAGCTATGTAATAATAACCGGGCAGGCC
prAW3	GGCCTGCCCGGTTATTATTACATAGCTGTATCCTGTGTGA
prAW4	taacaattgtggaattcccTGCCAACGATCAGATGGCGC
prCJK1	GAGAGGGTACCGCATGCGATATCGAGCTCTCCCGGTTTTACCCGAAGTCGGGGCG
prCJK2	GCGGATAACAATTTGTGGAATTCCTCGATGTATACCCGAATGGCAGCC
prCJK3	TTACTCTTTTTCGAACTCCAGTGAGCGCATATTTAATCCTTCTGTAATAC
prCJK4	GTATTACAGAAGGATTAATATGCGCTCACTGGAGTTCGAAAAAGAGTAA
prJL1	agactgggcggtttatgga
prJL2	caagatccgcagttcaacct
prJL3	gcttagtactgactatcaacaggttgaactgcggtcttgcggcaggtatgtgtg
prJL4	caattccggttgcgtgtgtccataaaaccgcccagctctacatgtggaattgtgagcgg
prJL5	tgccaataaccagtagaaacagacgaagaagTCGTGGCCGGATCCAGCCGA
prJL6	gatcgagctccccatccagtgcaagctagattcccggtcatggctg
prJL7	TAAGCAAGATCTctgtgataccgggaagcc
prJL8	tgcttaatcgatgcaacgggaattgaagacia
prJL9	gggtgtcggggcgcagccatgaccccgccgacatcataacggttc
prJL10	gcgataacaattgtggaattccccacgacttctctgctgtt
prJL11	tctagatcgacctgcaggc
prJL12	ttaccttactacggcatccgcttacagacia
prJL13	ttcttgcgccaaggatct
prJL14	catgcgGTACCctctcatcc
prJL15	GCCTCAAGCCGACTTCCCGGAGCACCACCAGACTTCCACGAGAT
prJL16	tcatgagcggatacatatttgaatgtatttagaaaaataaacaataggggttccgcgAG
prJL17	acagtactgcatgagtg
prJL18	agatccttggcggaagaaa
prJL19	gccctgccactcatgcagctactgtCGAATCCATGTGGGAGTTTATTCTTG
prJL20	CAAGTGTCTCTGTTTTcttgcgccaaggatctTAGGTGGCGGTACTTGGGT
prJL21	accgcatgcatatcgagctctcccCGGTTAGCGCACCACTAA
prJL22	CGCCGCGCGGTTATTCTTCTTCTCGTCCGGACGATTTGCAGTTGTCA
prJL23	CAGTATGACAACGCAAACTCGTCCGGACGAAGAAGAATAACGCCG
prJL24	tgagcggataacaattgtggaattcccAGTTAGTGCGCCACATCGAT
prJL25	accgcatgcatatcgagctctcccAATGGTGTAAATCAAGCCCT
prJL26	GCCACCCGGCAAAGGTTTACTGTAGCAGTTATCTTCCGTAATAGCGAG
prJL27	GACTCGCTATTACGGAAGATAACTGCTACAGTAAACCTTTGCCGG
prJL28	tgagcggataacaattgtggaattcccTCGAGGGCGATGACATTGTA
prJL29	accgcatgcatatcgagctctcccTTTGCGGGTATCGAGCAGGC
prJL30	AACAGCGTAAACAGCGTCATTAGCGCAGACACCTCTCTGCGGTGG
prJL31	AAGTACCACCGCAGAGAGGTGTCTGCGCTAATGACGCTGTTTACG
prJL32	gcgataacaattgtggaattcccGGTTTGATAGGCGCGCAGAA
prJL33	accgcatgcatatcgagctctcccGGCGCTGATTTGGTCAGGAT
prJL34	GCAAACGACGGCCTGTAACACCAGAAAGCGAATGCGCC
prJL35	GGCGCATTTCGCTTTCTGGTGTACAGGCCGTGCAGTTTGC
prJL36	tgagcggataacaattgtggaattcccACCCATTTACCATTCTGCG
prJL37	accgcatgcatatcgagctctcccTGCTCGTCTGGTACTCTTC
prJL38	CAGAACGCGCGGGTTTCGGCATCGTAAAGTCCCTCTCTCGCT
prJL39	AATCAAGCGAGAGAGGGACTTTACGATGCCGAAACCGCCGCGTT
prJL40	tgagcggataacaattgtggaattcccCACGATCAGGCTGCGCAGCT
prJL41	GGCTTTCTGCAATAAatcgacctgacagctagctcagcttaggtataatgctagctactagagattaagagga gaaatactagATGTCAGTGATAGCAAAGCAGATG
prJL42	GGCTTTCTGCAATAAatcgacctgacagctagctcagcttaggtataatgctagctactagagaaagaggaga aatactagATGTCAGTGATAGCAAAGCAGATG
prJL43	GGCTTTCTGCAATAAatcgacctgacatttggcagtttattcttgacatgtagtgagggggctggataatcac atagactgtttactagagattaagaggagaaatactagATGTCAGTGATAGCAAAGCAGATG

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prJL44 | GGCTTTCTGCAATAAtcgacctgcaaaaaagagtattgacttcgcatcttttgacctataatagattcattactagagatt
aaagaggagaaatactagATGTCAGTGATAGCAAAGCAGATG
prJL45 | acagcttgctgtaagcggatgccgtaagtaagtaaTTGACAGCTAGCTCAGTC
prJL46 | TCACTTCTTCGCCTTTTGACACCATAAAATACCTCCTTAGTTTCCCT
prJL47 | TCTGTCTAGAttctagagcacagctaacac
prJL48 | tcattgtttagcgtacacctcatctagtactttcctgtgtgac
prJL49 | ctagagtcacacaggaaagtactagatgagtgatcgctaaaca
prJL50 | TCTGTCTAGAttattaggcgaccacaggtt
prJL51 | agactgggcggtttatgga
prJL52 | ggcttcccggatcaacagA
prJL53 | ATCAGGAAGGCATCGGACAG
prJL54 | CTCCAGCGGCGTATTGTG
prJL55 | GCCATTTGATCGAGCACGTC
prJL56 | TCTCTTCCCCGGCGATCTAT
prJL57 | GCTCTGCTACCAGGACGAAG
prJL58 | GATCCCCCAACTCTTCCAGC
prJL59 | gccaaaacagccaagcttgcctgcatgctTATGCTACGACAGGTTTGCG
```

## **SUPPLEMENTAL TABLE 2** Plasmids used in this study

pDS132	From Phillippe <i>et al.</i>
pON.mCherry	From Gebhardt <i>et al.</i>
PGR-Blue	From Bradshaw <i>et al.</i>
pSB3C5- proD-B0032- E0051	From Davis <i>et al.</i>
pSLC-239	From Khetrupal <i>et al.</i>
pSLC-241	"
pSLC-246	"
pTOX1	This work. Encodes the YhaV toxin. CAM <sup>R</sup>
pTOX2	This work. Encodes the MqsR toxin. CAM <sup>R</sup>
pTOX3	This work. Encodes the Tse2 toxin. CAM <sup>R</sup>
pTOX4	This work. Encodes the YhaV toxin and <i>amilCP</i> . CAM <sup>R</sup>
pTOX5	This work. Encodes the MqsR toxin and <i>amilCP</i> . CAM <sup>R</sup>
pTOX6	This work. Encodes the Tse2 toxin and <i>amilCP</i> . CAM <sup>R</sup>
pTOX7	This work. Encodes the YhaV toxin and <i>tsPurple</i> . CAM <sup>R</sup>
pTOX8	This work. Encodes the MqsR toxin and <i>tsPurple</i> . CAM <sup>R</sup>
pTOX9	This work. Encodes the Tse2 toxin and <i>tsPurple</i> . CAM <sup>R</sup>
pTOX10	This work. Encodes the YhaV toxin. Gent <sup>R</sup>
pTOX11	This work. Encodes the MqsR toxin. Gent <sup>R</sup>
pTOX12	This work. Encodes the Tse2 toxin. Gent <sup>R</sup>