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1 **CreC Sensor Kinase Activation Enhances Growth of *Escherichia coli* in**  
2 **the Presence of Cephalosporins and Carbapenems.**

3

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13

14

15 **Abstract**

16

17 **Mutants with enhanced growth in the presence of an antibiotic are more difficult**  
18 **to identify than mutants where the antibiotic's minimum inhibitory concentration**  
19 **increases, because they are not amenable to lethal selection *in vitro*. We report**  
20 **that activatory mutations in the CreC signal sensor enhance growth of *Escherichia***  
21 ***coli* in the presence of cefoxitin, cefotaxime and meropenem without increasing**  
22 **their minimum inhibitory concentrations. Enhanced growth is dependent on over-**  
23 **production of the inner-membrane cre-regulon protein CreD.**

24

25 **Text**

26 In *Escherichia coli*, CreC is a sensor kinase and CreB is a response regulator and  
27 together they form a two-component regulatory system that controls the expression  
28 of the cre regulon, a group of genes with poorly characterised functions (1-3). We  
29 have previously shown that an activatory mutation in CreC in *E. coli* MG1655 confers  
30 the Cet phenotype: tolerance of the protein antibiotic Colicin E2, through over-  
31 production of the protein Yiel (3). Whilst the mechanism by which Yiel confers colicin  
32 resistance is not clear it may be due to modifications in the outer envelope which  
33 restrict entry of colicin E2 to the cell (3).

34

35 To learn more about the Cet phenotype, we used phenotype microarray analysis to  
36 characterise differences between CTX6, a well-characterised Cet mutant (3), versus  
37 CTX6 $\Delta$ creB, where cre regulon hyper-expression is ablated and the Cet phenotype is  
38 reversed (3). Phenotype microarray analysis was performed by Biolog (Hayward CA,  
39 USA). Of almost 2000 growth conditions tested in the phenotype microarray,  
40 significantly improved growth of CTX6 versus CTX6 $\Delta$ creB was seen in media with 13  
41 different chemical additions. Eight of these chemicals are  $\beta$ -lactam antibiotics (Table  
42 1). MICs of  $\beta$ -lactams were not noticeably higher against CTX6 than CTX6 $\Delta$ creB,  
43 according to E-test and broth microdilution methodologies (data not shown) but the  
44 CreC activatory mutation in CTX6 was reproducibly seen to enhance growth at sub-  
45 MIC concentrations of some  $\beta$ -lactam drugs during growth curve analysis. For these  
46 assays, 500  $\mu$ l of an overnight culture grown in LB medium (Oxoid) were used to  
47 inoculate 10 ml of fresh LB in a sealed universal bottle and the antimicrobial drug of  
48 interest was added. The starting optical density at 600 nm (OD<sub>600</sub>) of each subculture  
49 was approximately 0.1. Cultures were incubated at 37°C with 150 rpm shaking and  
50 OD<sub>600</sub> measured every 60 min using a spectrophotometer by taking 1 ml from the  
51 culture. For example, *E. coli* MG1655 (the parent of CTX6) suffers a temporary  
52 slowing of growth at 120 min following addition of half the MIC of ceftaxime (Fig 1A)  
53 or cefotaxime (Fig 1B) (both from Sigma). Later, a post-antibiotic effect occurs, and  
54 growth of the population increases again (Fig. 1). CTX6 does not suffer such a  
55 prolonged slowing of growth after 120 min and there is a significant enhancement in  
56 OD<sub>600</sub> at 180 or 360 min after the addition of ceftaxime or cefotaxime, respectively,

57 relative to MG1655 ( $p < 0.05$  for both). CTX6 $\Delta creB$  displays the wild-type phenotype  
58 (Fig. 1) confirming that CreBC hyper-activation is responsible for enhanced growth in  
59 the presence of these  $\beta$ -lactam antibiotics.

60

61 Microarray transcriptomics has previously revealed that six genes are differentially  
62 regulated >10-fold in CTX6 in a CreB-dependent manner; none of these are known to  
63 be involve in  $\beta$ -lactam resistance or tolerance, e.g. porin, efflux pump,  $\beta$ -lactamase  
64 or penicillin binding protein genes (3). The use of previously constructed deletion  
65 mutants of the CreB-regulated genes in CTX6 (3) revealed that *creD* is gene  
66 responsible for enhanced growth of CTX6 in the presence of cefoxitin, as illustrated  
67 in figure 2. Deletion of *creD* in the CTX6 background reduced the OD<sub>600</sub> of a culture  
68 at 180 min post addition of cefoxitin to the same extent as deletion of *creB* or *creC*  
69 ( $p < 0.005$  for each).

70

71 It is possible to activate cre regulon gene expression by over-expressing the response  
72 regulator *creB* in an otherwise wild-type background strain. We did this, as  
73 previously, using an arabinose expression system (2) and found that it enhances  
74 growth in the presence of  $\beta$ -lactam antibiotics, even at antibiotic concentrations  
75 greater than the MIC. For example, with cefotaxime, the growth curve of MG1655  
76 carrying a control plasmid shows the drug inhibiting growth, lysing cells and  
77 overwhelming the population, causing the OD<sub>600</sub> of the culture to reduce to basal  
78 levels (Fig. 3). Carriage of a plasmid allowing CreB over-production does not affect  
79 the MIC – the drug still kills the population – but the area under the growth curve is  
80 greater. There is more growth prior to killing, as seen particularly at 120 min post  
81 cefotaxime treatment in figure 3 ( $p < 0.01$ ). Deletion of *creD* blocks the ability of CreB  
82 over-production to improve the growth of MG1655 ( $p < 0.05$  at 120 min post  
83 cefotaxime treatment [Fig 3]) confirming a role for CreD over-production in this  
84 CreBC mediated  $\beta$ -lactam tolerance phenotype.

85

86 To see if the  $\beta$ -lactam tolerant, CreC hyperactive mutant CTX6 gives a fitness  
87 advantage relative to CTX6 $\Delta creB$  over repeated cycles of growth, pairwise  
88 competition experiments were performed over 4 days. In order to mark CTX6 $\Delta creB$

89 so that it could be quantified in a culture when mixed with CTX6, the derivative  
90 CTX6*creB*::Chl<sup>R</sup> was used. Here, *creB* has been disrupted by insertion of a  
91 chloramphenicol resistance gene rather than deleted as used in earlier experiments,  
92 though the effect on CreBC regulated gene expression is known to be identical  
93 whether *creB* is disrupted or deleted (2). CTX6*yjeJ*::Chl<sup>R</sup> (3) was used in parallel as a  
94 control in these experiments to confirm that any change in fitness seen was specific  
95 to disruption of *creB* and not due to Chl<sup>R</sup>; *yjeJ* is not responsible for the growth  
96 enhancement phenotype of CTX6 (Fig. 2). This control also allowed us to be sure that  
97 other, unrelated, mutations affecting growth in  $\beta$ -lactams did not readily accumulate  
98 in the *E. coli* strains used.

99  
100 To perform these pairwise competition experiments, initially, cultures of both strains  
101 to be competed were inoculated separately into LB and incubated for 24 h at 37°C  
102 with shaking at 160 rpm. Next, 5  $\mu$ l of each culture were used to inoculate a separate  
103 flask containing 50 ml of DM25 minimal medium (4), which was prepared from Davis  
104 minimal medium (Difco, Oxford, UK) supplemented with glucose and thymine (25  
105 mg.l<sup>-1</sup> and 2 mg.l<sup>-1</sup> respectively). Cultures were incubated at 37°C with shaking as  
106 above. After overnight incubation, 500  $\mu$ l of each culture was transferred into a fresh  
107 50 ml aliquot of DM25 minimal medium, again separately, and the inoculated  
108 medium was divided into six screw top universal bottles and incubated at 37°C  
109 overnight with shaking, as above. Upon inoculation, these cultures were referred to  
110 as day zero cultures. Day one (mixed) culture started with 250  $\mu$ l each of the two day  
111 zero cultures to be competed being mixed together in the same flask containing 50  
112 ml of fresh DM25 medium plus a  $\beta$ -lactam antibiotic as necessary. There were six  
113 flasks (six biological replicates) for each competition experiment. The day one  
114 (mixed) cultures were incubated for 24 h (approximately 7 generations of growth are  
115 permitted given glucose limitation in the medium) at 37°C with shaking. Each day,  
116 one culture was then sub-cultured by transferring a 500  $\mu$ l of the culture into a  
117 separate flask containing 50 ml of DM25 medium (containing the same  $\beta$ -lactam, if  
118 necessary) to generate day two (mixed) cultures. The last step was repeated daily  
119 until four days of mixed cultures had passed. The competition between the two  
120 strains was measured by performing a serial dilution and counting the number of

121 colony forming units (cfu) per ml of CTX6 and of CTX6::Chl<sup>R</sup> in each mixture at the  
122 end of each day (including day zero). CTX6::Chl<sup>R</sup> was counted following plating on LB  
123 agar containing 30 mg.l<sup>-1</sup> chloramphenicol. CTX6 was counted by plating on LB agar  
124 with no antibiotic and subtracting the CTX6::Chl<sup>R</sup> count. The selection rate constant  
125 (r) was used as a measure to estimate the fitness cost of Chl<sup>R</sup> insertion after each day  
126 of the competition by comparing M, the Malthusian parameter for each strain in the  
127 competition (5) so that  $M = \ln(N_1/N_0)$ , where  $N_0$  is the density of the strain (cfu/ml)  
128 at the start of the day (density at the end of the previous day divided by 100 to take  
129 account of the dilution factor on subculture) and  $N_1$  is the density of the strain  
130 (cfu/ml) at the end of the day. The selection rate for a competition is therefore  
131 calculated as  $r = M_1 - M_2$ , Where  $M_1$  relates to CTX6 and  $M_2$  relates to CTX6::Chl<sup>R</sup>.  
132 For each competition (one mixed culture) there were four selection rate values, one  
133 for each day, and for each fitness cost experiment, six competitions were run. Hence  
134 for each competition between two strains, 24 r value datapoints are obtained.  
135 Differences in these sets of r value data for different comparisons were assessed  
136 using an unpaired t-test with Welch's correction to assess the statistical significance  
137 of the differences observed.

138

139 In the absence of antibiotics, neither CTX6*creB*::Chl<sup>R</sup> nor CTX6*yieJ*::Chl<sup>R</sup> had reduced  
140 fitness compared with the CreC hyper-active mutant CTX6 (Table 2). However, in the  
141 presence of half the MICs of cefotaxime, ceftazidime or meropenem (AdooQ  
142 Bioscience), an approximately 15% per day reduction in relative fitness (W) was  
143 observed for CTX6*creB*::Chl<sup>R</sup> versus CTX6 ( $p < 0.05$  for all comparisons) though there  
144 was no significant effect of disrupting *yieJ* (Table 2). We also performed competition  
145 experiments using ampicillin or ceftazidime (both from Sigma), but no significant  
146 difference in fitness between CTX6 and CTX6*creB*::Chl<sup>R</sup> was seen (Table 2). The  
147 reason for the specificity of this  $\beta$ -lactam tolerance effect is not clear but may  
148 facilitate future work to understand the mechanism of CreD mediated  $\beta$ -lactam  
149 tolerance in CTX6 and other CreC hyperactive mutants. To put the observed changes  
150 in relative fitness into perspective: starting with a ratio of approximately 1:1 for the  
151 two competing strains, after 4 days of competition the ratio was approximately 10:1  
152 in favour of CTX6 in the presence of half the MIC of ceftazidime, cefotaxime or

153 meropenem. MICs of meropenem were identical against five chloramphenicol  
154 resistant and five susceptible colonies from the mixed culture of CTX6 and CTX6::Chl<sup>R</sup>  
155 at day zero, and after completion of the fitness cost experiment using meropenem.  
156 Accordingly, the observed relative increase in CTX6 population density over time is  
157 not caused by the accumulation of further mutations in CTX6 causing meropenem  
158 MIC to notably rise but instead is a phenotype caused by CreC hyper-active in *E. coli*  
159 MG1655.

160

### 161 *Conclusions*

162 Antibiotic resistance is defined in terms of a minimum inhibitory concentration (MIC)  
163 of an antibiotic measured following growth of a very dilute starting culture in the  
164 presence of that antibiotic. After a set period of incubation, the MIC is defined as the  
165 concentration of drug required to completely inhibit growth. If the MIC of a drug  
166 against a bacterial isolate is greater than a consensually defined breakpoint – based  
167 on clinical experience and pharmacokinetic/dynamic properties of the antibiotic –  
168 the isolate is said to be resistant (6). However, in vivo, antibiotic concentrations are  
169 dynamic, and there are periods when they are sub-MIC, so even susceptible bacteria  
170 can multiply before the drug concentration raises to overcome it. Whilst this does  
171 not, of itself, cause treatment failure, it might provide time for resistant mutants to  
172 emerge, or for symptoms to persist in the patient before a cure is established.  
173 Because of this possibility, we are interested in mutations that confer growth  
174 enhancement in the presence of an antibiotic without necessarily altering the  
175 antibiotic's MIC. This phenotype of enhanced growth in the presence of antibiotic is  
176 somewhat different from the much-discussed phenotype of “persistence”, where  
177 bacteria reduce their metabolic activity and/or growth rate to escape from the lethal  
178 actions of antibiotics, which tend to only kill growing cells (7).

179

180 In this paper, we have identified a mechanism – hyper-production of CreD due to  
181 constitutive activation of CreC by mutation – that confers growth enhancement in  
182 the presence of  $\beta$ -lactams in *E. coli*. The clinical relevance of this finding is unclear,  
183 and the mechanism by which CreD confers this phenotype is unknown. Interestingly,  
184 whilst CreC in *E. coli* is not activated by  $\beta$ -lactam challenge (1,2) CreBC shares



185 significant sequence identity with the BlrAB two-component system in *Aeromonas*  
186 spp (8,9), which is activated in response to  $\beta$ -lactam challenge, and controls  $\beta$ -  
187 lactamase production and so confers  $\beta$ -lactam resistance (10). Importantly, BlrAB  
188 also activates transcription of *blrD*, which is a homologue of *E. coli creD* (9). Indeed, a  
189 similar two component system, CreBC/BlrAB in *Pseudomonas aeruginosa* is also  
190 activated during  $\beta$ -lactam challenge (11,12), and whilst it does not control  $\beta$ -  
191 lactamase production, it does control transcription of the *creD* homologue in this  
192 species (11,12). Interestingly, CreD/BlrD hyper-production in *P. aeruginosa* does not  
193 enhance  $\beta$ -lactam MICs unless the chromosomally encoded AmpC cephalosporinase  
194 of this species is over-produced in parallel (12). It is possibility, therefore, that CreC  
195 hyper-activation leading to CreD hyper-production in *E. coli* might enhance  $\beta$ -lactam  
196 MICs in the presence of plasmid mediated  $\beta$ -lactamase(s), something that is worthy  
197 of future investigation.

198

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202

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207

### 208 **Conflict Statement**

209 All authors declare that there is no conflict of interest

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212

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253

254

255 **Tables**

256 **Table 1. Chemicals in Phenotype Microarray analysis where significant changes in**  
257 **growth – measured as an increased area under the growth curve (AUC) – were**  
258 **seen in CTX6 (Cet) versus CTX6 $\Delta$ *creB*.**

259

<b>Moderate Effect (26-75% increase in AUC)</b>	<b>Major Effect (&gt;75% increase in AUC)</b>
Nafcillin	Amoxicillin
Ampicillin	Cefotaxime
Cefuroxime	Aztreonam
Cefoxitin	Phenylarsine oxide
Carbenicillin	
Cetylpyridinium chloride	
Thallium Acetate	
9-Aminoacridine	
Sodium Arsenate	

260

261

262 **Table 2. Effect on fitness of disrupting *creB* in CTX6 measured in pairwise**  
 263 **comparison with CTX6 during growth in the presence of half MIC of various**  
 264 **antibiotics.**

265 Fitness was measured over four days, and is an average of six repetitions, as set out  
 266 in the text. Data are mean percent fitness difference per day. Negative values mean  
 267 that the ChlR derivative is less fit than CTX6 in pairwise competition. Stars represent  
 268 differences where  $p < 0.05$  according to an unpaired t-test with Welch's correction.

269

270

Competition	Relative Percent Fitness of Chl <sup>R</sup> strain
CTX6 <i>creB</i> :Chl <sup>R</sup> vs CTX6 (No Antibiotic)	+3.0 +/- 3.4
CTX6 <i>yieJ</i> :Chl <sup>R</sup> vs CTX6 (No Antibiotic)	-4.9 +/- 3.9
CTX6 <i>creB</i> :Chl <sup>R</sup> vs CTX6 (Plus Ampicillin)	+4.4 +/- 2.8
CTX6 <i>creB</i> :Chl <sup>R</sup> vs CTX6 (Plus Ceftazidime)	-0.7 +/- 4.8
CTX6 <i>creB</i> :Chl <sup>R</sup> vs CTX6 (Plus Cefoxitin)	-17.7 +/- 3.4*
CTX6 <i>yieJ</i> :Chl <sup>R</sup> vs CTX6 (Plus Cefoxitin)	-2.0 +/- 4.7
CTX6 <i>creB</i> :Chl <sup>R</sup> vs CTX6 (Plus Cefotaxime)	-12.4 +/- 4.9*
CTX6 <i>creB</i> :Chl <sup>R</sup> vs CTX6 (Plus Meropenem)	-15.9 +/- 4.1*

271

272 **Figure Legends**

273

274 **Figure 1 Effect of Cet phenotype on *E. coli* growth curve in the presence of cefoxitin**  
275 **or cefotaxime.**

276 Optical density of an LB culture was measured at 600 nm every hour following  
277 subculture and addition of antibiotic (time zero): top graph, cefoxitin; bottom graph,  
278 cefotaxime, each used at half its MIC against *E. coli* MG1655. Data are mean  
279 plus/minus standard error of the mean, n=6

280

281

282 **Figure 2. Effect of deletion of various Cre regulon genes in the *E. coli* Cet mutant**  
283 **CTX6 on growth in the presence of cefoxitin.**

284 Optical density of an LB culture was measured at 600 nm every hour following  
285 subculture and addition of antibiotic (time zero). Data represent means plus/minus  
286 standard error of the mean, n=3 after 180 mins post incubation.

287

288

289 **Figure 3. Effect of activating the Cre regulon through over-production of CreB on *E.***  
290 ***coli* growth in the presence of cefotaxime.**

291 Optical density of an LB culture was measured at 600 nm every hour following  
292 subculture and addition of cefotaxime (time zero) used at half its MIC against *E. coli*  
293 MG1655. Arabinose (0.2 % w/v) was added in all growth media to stimulate CreB  
294 production from the pUB6073 (pBAD(*creB*)) plasmid as used in (2). Data represent  
295 means plus/minus standard error of the mean, n=3 after 180 mins post incubation.

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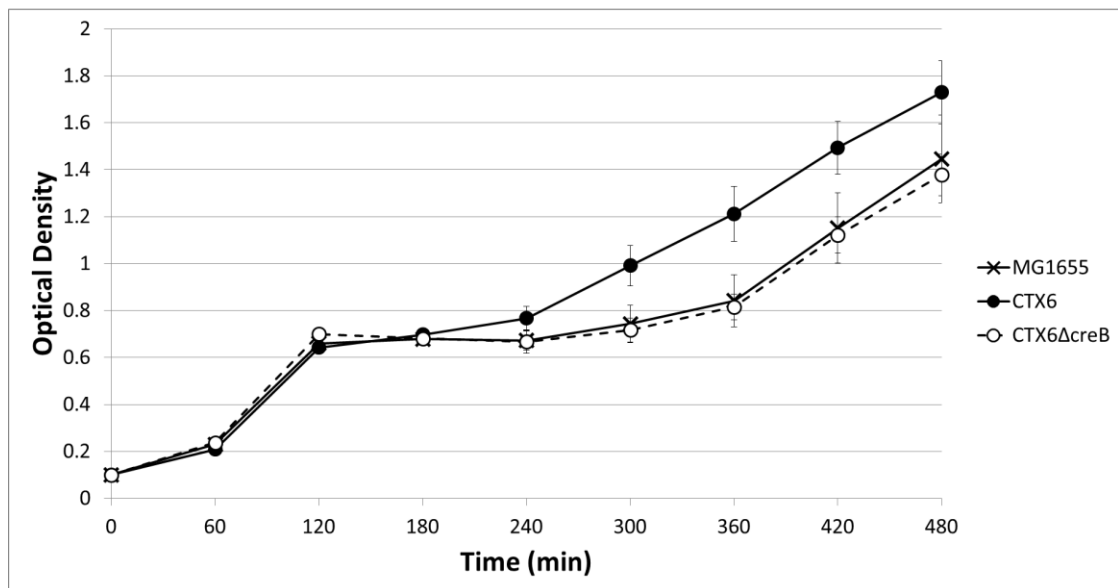
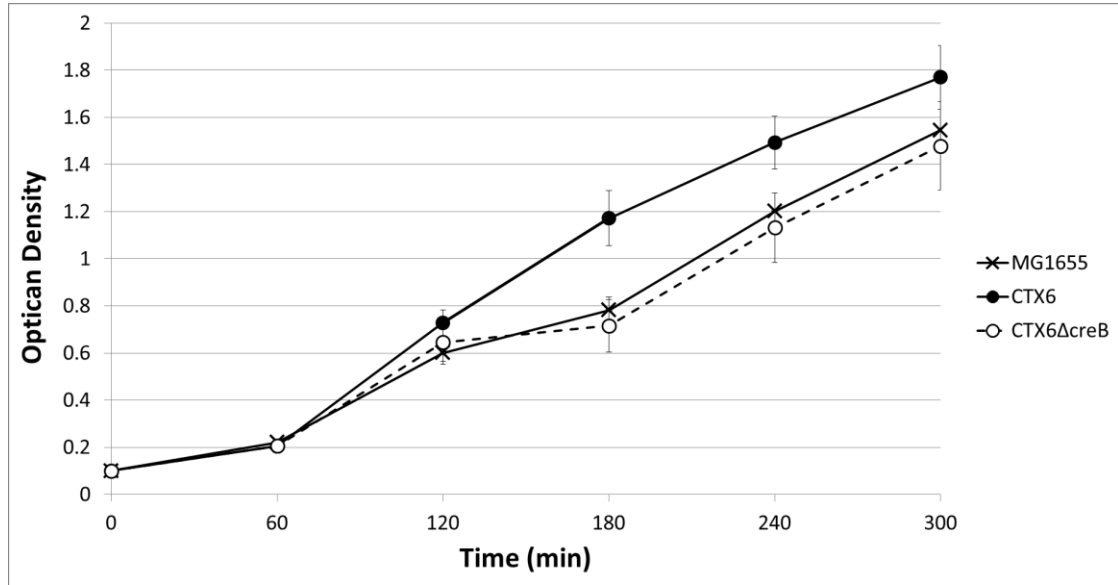
300 **Figures**

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303 **Figure 1**

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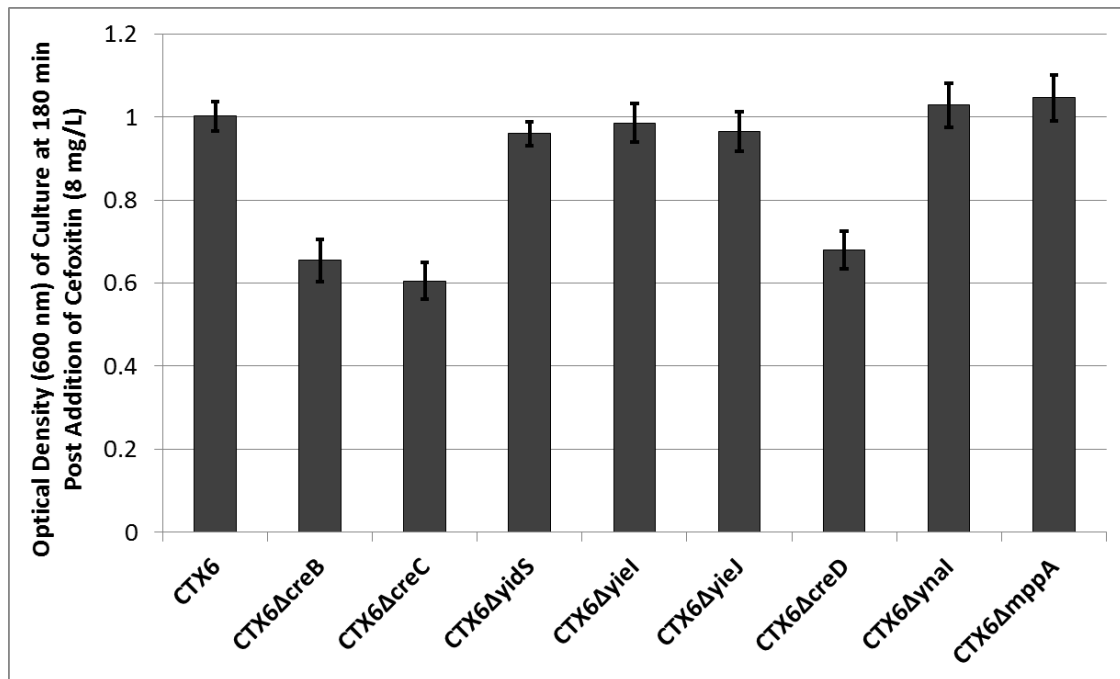
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310 **Figure 2**  
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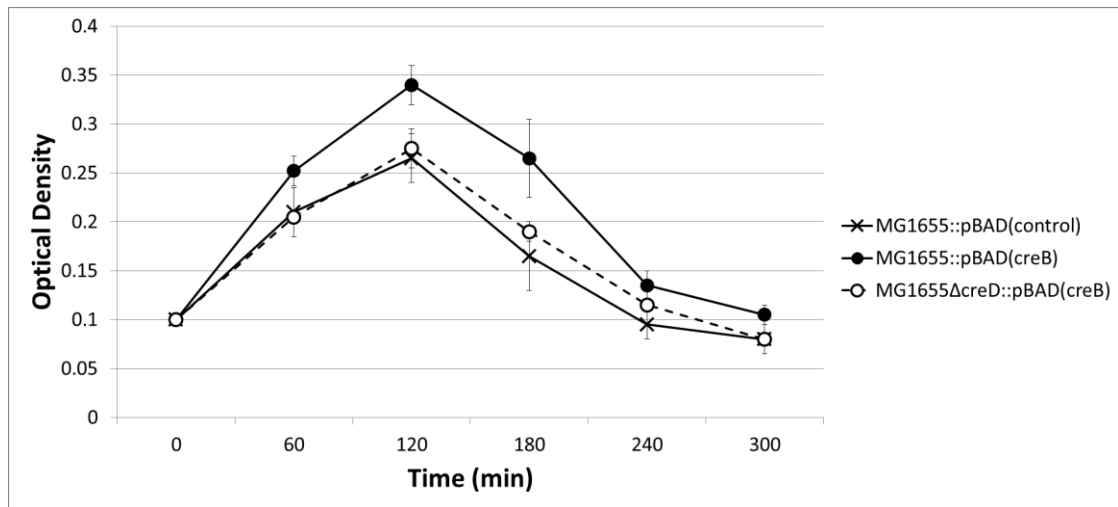


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**Figure 3**



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