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

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Abstract

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

Text

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

To learn more about the Cet phenotype, we used phenotype microarray analysis to characterise differences between CTX6, a well-characterised Cet mutant (3), versus CTX6Δ*creB*, where cre regulon hyper-expression is ablated and the Cet phenotype is reversed (3). Phenotype microarray analysis was performed by BIolog (Hayward CA, USA). Of almost 2000 growth conditions tested in the phenotype microarray, significantly improved growth of CTX6 versus CTX6Δ*creB* was seen in media with 13 different chemical additions. Eight of these chemicals are β-lactam antibiotics (Table 1). MICs of β-lactams were not noticeably higher against CTX6 than CTX6Δ*creB*, according to E-test and broth microdilution methodologies (data not shown) but the CreC activatory mutation in CTX6 was reproducibly seen to enhance growth at sub-MIC concentrations of some β-lactam drugs during growth curve analysis. For these assays, 500 µl of an overnight culture grown in LB medium (Oxoid) were used to inoculate 10 ml of fresh LB in a sealed universal bottle and the antimicrobial drug of interest was added. The starting optical density at 600 nm (OD_{600}) of each subculture was approximately 0.1. Cultures were incubated at 37°C with 150 rpm shaking and OD_{600} measured every 60 min using a spectrophotometer by taking 1 ml from the culture. For example, *E. coli* MG1655 (the parent of CTX6) suffers a temporary slowing of growth at 120 min following addition of half the MIC of cefoxitin (Fig 1A) or cefotaxime (Fig 1B) (both from Sigma). Later, a post-antibiotic effect occurs, and growth of the population increases again (Fig. 1). CTX6 does not suffer such a prolonged slowing of growth after 120 min and there is a significant enhancement in OD_{600} at 180 or 360 min after the addition of cefoxitin or cefotaxime, respectively,

relative to MG1655 (p <0.05 for both). CTX6Δ*creB* displays the wild-type phenotype (Fig. 1) confirming that CreBC hyper-activation is responsible for enhanced growth in the presence of these β-lactam antibiotics.

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

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

To see if the β-lactam tolerant, CreC hyperactive mutant CTX6 gives a fitness advantage relative to CTX6Δ*creB* over repeated cycles of growth, pairwise competition experiments were performed over 4 days. In order to mark CTX6Δ*creB*

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

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

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

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

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

Conclusions

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

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

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

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Conflict Statement

All authors declare that there is no conflict of interest

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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 CTX6Δ*creB*.**

259

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

260

261

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

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

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

Figure Legends

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

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

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

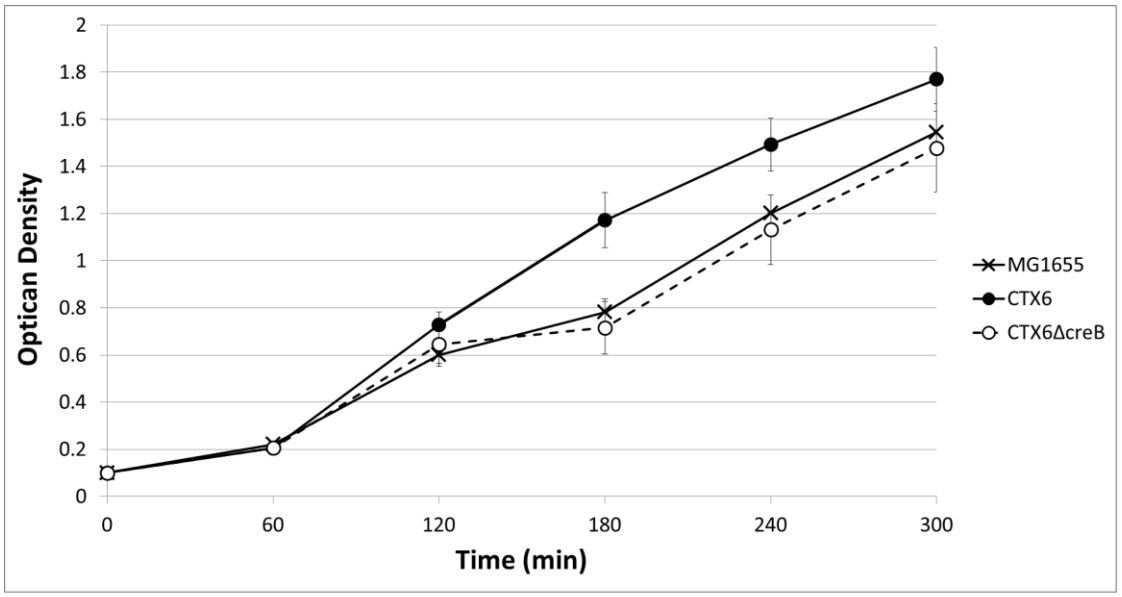
Optical density of an LB culture was measured at 600 nm every hour following subculture and addition of antibiotic (time zero). Data represent means plus/minus standard error of the mean, n=3 after 180 mins post incubation.

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

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

Figures

Figure 1



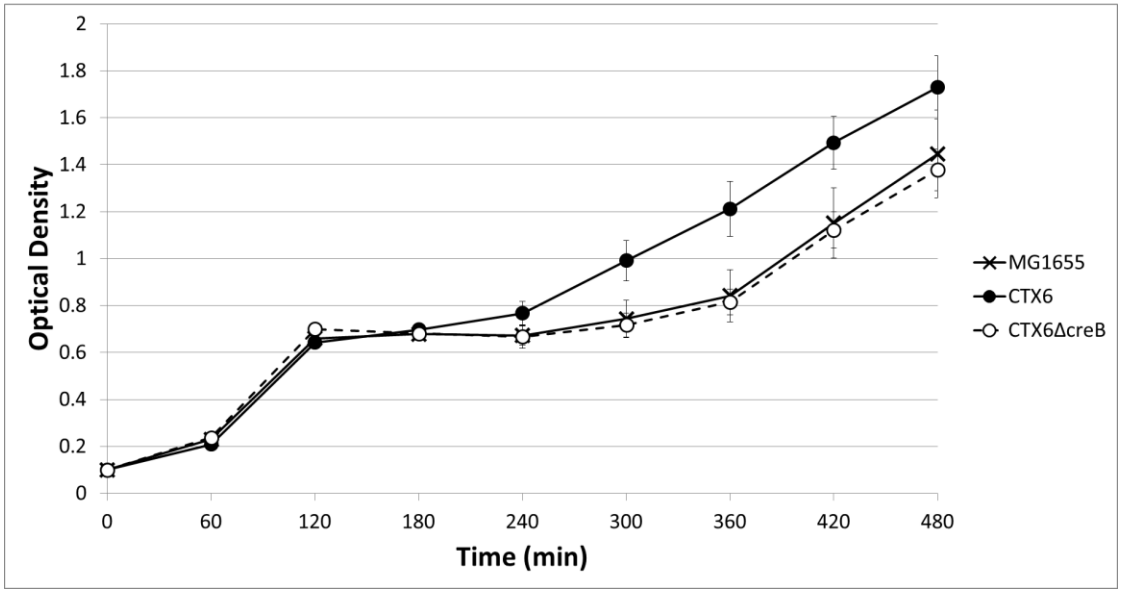


Figure 2

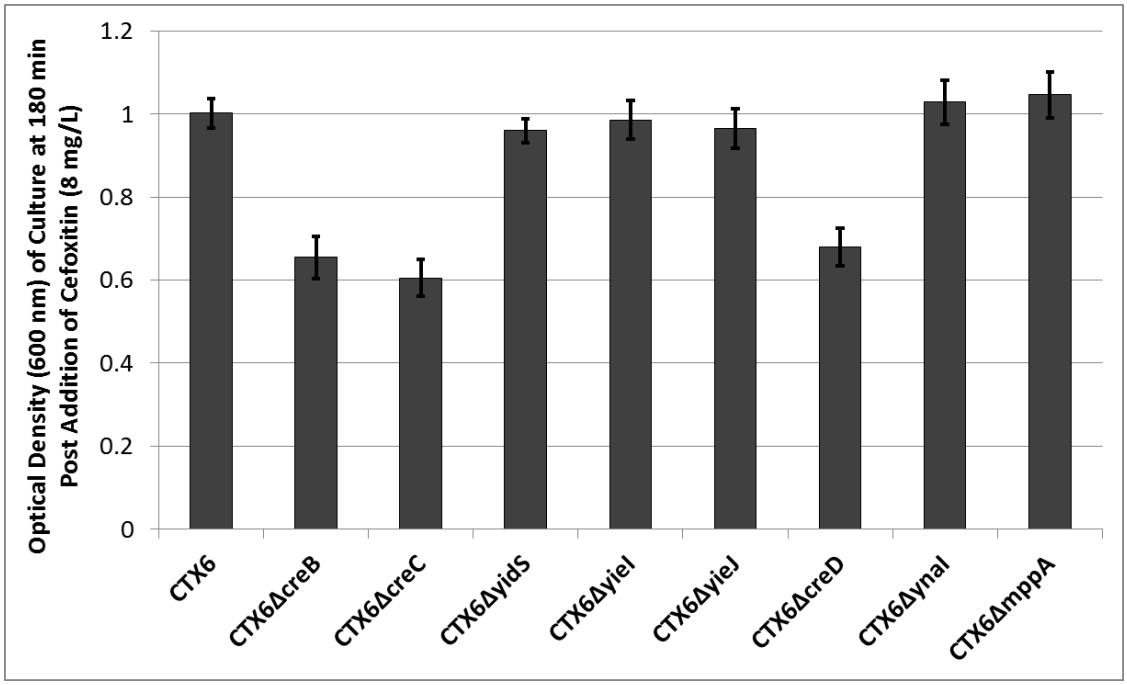
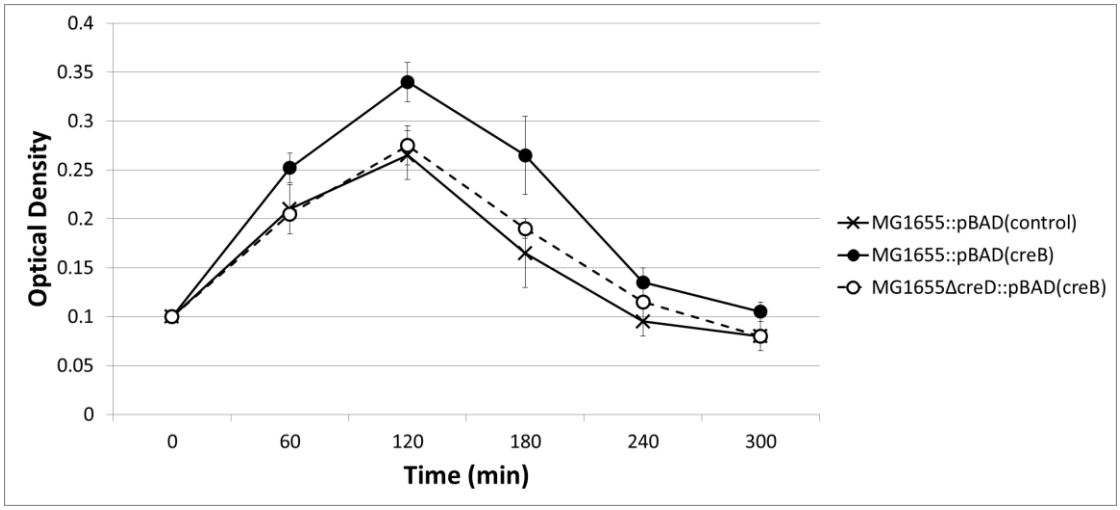

Optical Density (600 nm) of Culture at 180 min Post Addition of Cefoxitin (8 mg/L)
1.2
1
0.8
0.6
0.4
0.2
0
CTX6
CTX6ΔcreB
CTX6ΔcreC
CTX6ΔyidS
CTX6ΔyieI
CTX6ΔyieJ
CTX6ΔcreD
CTX6ΔynaI
CTX6ΔmppA

Figure 3


Optical Density
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0
60
120
180
240
300
Time (min)
MG1655::pBAD(control)
MG1655::pBAD(creB)
MG1655ΔcreD::pBAD(creB)