



# Contribution of the Chromosomal *ccdAB* Operon to Bacterial Drug Tolerance

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**ABSTRACT** One of the first identified and best-studied toxin-antitoxin (TA) systems in *Escherichia coli* is the F-plasmid-based CcdAB system. This system is involved in plasmid maintenance through postsegregational killing. More recently, *ccdAB* homologs have been found on the chromosome, including in pathogenic strains of *E. coli* and other bacteria. However, the functional role of chromosomal *ccdAB* genes, if any, has remained unclear. We show that both the native *ccd* operon of the *E. coli* O157 strain (*ccd*<sub>O157</sub>) and the *ccd* operon from the F plasmid (*ccd*<sub>F</sub>), when inserted on the *E. coli* chromosome, lead to protection from cell death under multiple antibiotic stress conditions through formation of persisters, with the O157 operon showing higher protection. While the plasmid-encoded CcdB toxin is a potent gyrase inhibitor and leads to bacterial cell death even under fully repressed conditions, the chromosomally encoded toxin leads to growth inhibition, except at high expression levels, where some cell death is seen. This was further confirmed by transiently activating the chromosomal *ccd* operon through overexpression of an active-site inactive mutant of F-plasmid-encoded CcdB. Both the *ccd*<sub>F</sub> and *ccd*<sub>O157</sub> operons may share common mechanisms for activation under stress conditions, eventually leading to multidrug-tolerant persister cells. This study clearly demonstrates an important role for chromosomal *ccd* systems in bacterial persistence.

**IMPORTANCE** A large number of free-living and pathogenic bacteria are known to harbor multiple toxin-antitoxin systems, on plasmids as well as on chromosomes. The F-plasmid CcdAB system has been extensively studied and is known to be involved in plasmid maintenance. However, little is known about the function of its chromosomal counterpart, found in several pathogenic *E. coli* strains. We show that the native chromosomal *ccd* operon of the *E. coli* O157 strain is involved in drug tolerance and confers protection from cell death under multiple antibiotic stress conditions. This has implications for generation of potential therapeutics that target these TA systems and has clinical significance because the presence of persisters in an antibiotic-treated population can lead to resuscitation of chronic infection and may contribute to failure of antibiotic treatment.

**KEYWORDS** toxin-antitoxin, persistence

In the early 1940s it was reported that a small fraction of staphylococcal cells, about one in a million, survived prolonged penicillin treatment (1). These cells were termed persisters. Later it was established that persisters are phenotypic variants in a given population that show antibiotic tolerance due to probable differences in their physiology under a given set of conditions. Upon subsequent culturing, they give rise to a population that retains antibiotic sensitivity. They are different from resistant cells in which resistance is genetically encoded and is passed on from one generation to the next. In contrast, persisters are transient and rare, ranging from  $10^{-2}$  to  $10^{-6}$  in frequency. After their initial discovery by Joseph Bigger, various studies have probed the conditions and genes involved in the generation of persisters (2–4). Exposure to

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two different classes of antibiotics, namely, beta-lactams and fluoroquinolones, has been shown to produce a fraction of persister cells as large as  $10^{-1}$  in the case of *hip* mutants (5). HipAB belongs to the family of toxin-antitoxin (TA) systems, with HipB being the antitoxin for the toxin HipA (6).

Many bacterial genomes harbor a class of genes referred to as TA genes. These genes were initially discovered on low-copy-number plasmids such as the F plasmid of *Escherichia coli*, and they are known to be involved in plasmid maintenance in the bacterial population by a process known as postsegregational killing (7). TA systems comprise of a pair of genes organized in an operon encoding a stable toxin and a labile antitoxin that antagonizes it (7). In most cases, the first gene encodes an antitoxin, while the second gene encodes a toxin that targets an essential cellular function. The antitoxin usually acts as a transcriptional repressor by binding to operator sites within its own promoter. Therefore, transcription of both genes is typically autoregulated by their encoded protein products. This negative feedback loop helps in maintaining steady-state levels of both toxin and antitoxin such that the toxin/antitoxin ratio is  $<1$  (8). Under conditions of stress, the labile antitoxin is degraded. The resulting increase in the toxin/antitoxin ratio leads to transient derepression of the operon and fresh synthesis of both antitoxin and toxin (9). In the case of the *ccdAB* system on F-plasmid-containing cells, the toxin CcdB is expressed along with its unstable antidote, CcdA, forming a TA complex. In cells lacking the F plasmid, CcdA is degraded, and in the absence of fresh CcdA synthesis, CcdB kills the cell, ensuring F-plasmid maintenance. Homologs of plasmid-based TA systems have recently been discovered on the chromosomes of a large number of bacteria, many of which are pathogenic (10).

The biological function of TA loci when present on a plasmid is often clear, but the roles of many chromosomal TA loci are unclear. There are different models that have been proposed to explain their presence on the chromosome. For the chromosomal *ccd* systems, these include the antiaddiction model (11), which states that the chromosomally encoded *ccd* system (*ccd<sub>Ech</sub>*) protects the cell against postsegregational killing mediated by its F-plasmid *ccd* (*ccd<sub>F</sub>*) homolog, whereas for the *ccd<sub>O157</sub>* system, it has been suggested that this system is devoid of any biological role in the *E. coli* species (12). Models proposed for other chromosomal TA systems include the programmed cell death (PCD) model, the growth modulation model, the persistence model, and the development model (13). TA loci have been proposed as general stress response elements in prokaryotes (14–17). Despite a plethora of studies on TA systems, in most cases their functions remain to be elucidated. A common difficulty encountered during study of a specific TA system is functional redundancy with other TA systems present in the same organism. Hence, study of the *in vivo* role of a particular TA locus requires the selective activation of that specific locus in the background of other TA loci. Ectopic overexpression of the active, toxin component often leads to cell death, further complicating elucidation of TA system function. Since many bacteria contain multiple homologous TA systems with redundant functions, multiple TA systems may need to be knocked out before there is an observable phenotype (18).

Various chromosomal TA systems as well as stress-responsive pathways in bacteria have been shown to be overexpressed in transcriptomic studies done on isolated persister cells. The pathogenic *E. coli* O157:H7 strain harbors the *ccd* operon in its genome. A global analysis of the transcriptional response to nutritional stress in this strain (19) revealed an increased expression of the *dinJ-yafQ*, *chpBS*, and *ccdAB* TA loci upon entry into stationary phase, indicating that these systems are actively transcribed in the stationary phase (19). However, their contribution to cell survival still remains to be elucidated.

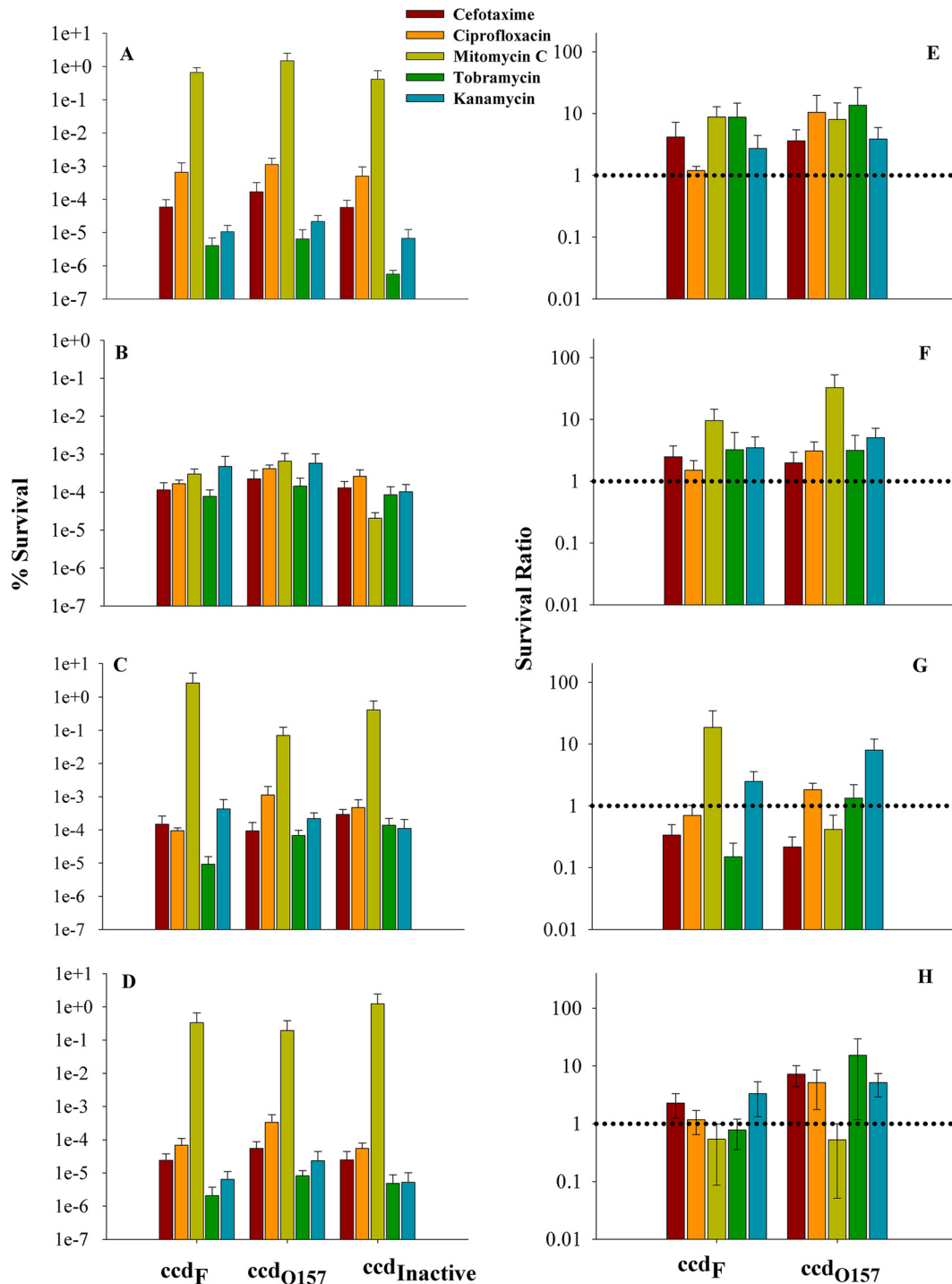
Most chromosomal TA systems are thought to be acquired by horizontal transmission from their plasmidic counterparts, and there is conservation of both target and antitoxin binding residues in the chromosomal CcdB toxin relative to its F-plasmid counterpart. It was recently shown that the *ccd* operon of the F plasmid can also function as a transmissible persistence factor (20). Hence, it becomes important to test

whether the *ccd* operon in the chromosomal context has a role to play in bacterial drug tolerance that is similar to or enhanced compared to that of its F-plasmid counterpart.

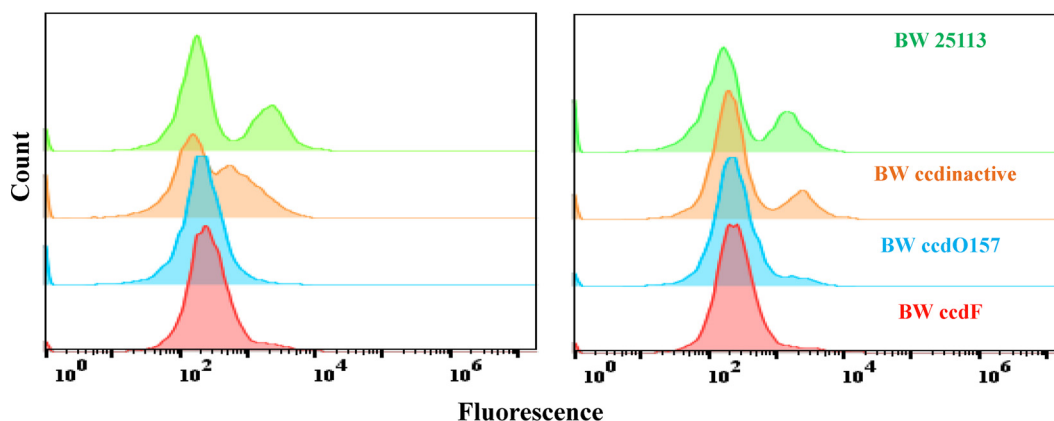
This issue is clinically significant, as the presence of persisters in an antibiotic-treated population can lead to resuscitation of chronic infection and may contribute to failure of antibiotic treatment (2, 21). It has been previously demonstrated that prolonged antimicrobial therapy selects for high-persistence variants. Given that many of these TA systems are found on the chromosomes of pathogenic strains of *E. coli*, it is important to study the contribution of these chromosomally encoded TA systems toward persister generation under antibiotic stress conditions. This can have implications for generation of potential therapeutics that target these TA systems. We have therefore probed the role of the chromosomally encoded CcdAB TA system. We found that both the native *ccd*<sub>O157</sub> and the F-plasmid-carried *ccd* operon, when in a chromosomal context, confer protection from cell death under antibiotic stress, with the *ccd*<sub>O157</sub> chromosomal form showing better protection and weaker DNA gyrase poisoning activity.

## RESULTS

**Activation of *ccd* of *E. coli* O157 by heat or antibiotic stress.** A DNA fragment containing the complete *ccd* operon from pathogenic *E. coli* O157:H7 was synthesized and inserted into the 77-bp intergenic region between the *folA* and *apaH* genes of *E. coli* strain BW25113 using lambda red recombination (22). We chose the *Escherichia coli* BW25113 strain because it is the parent strain of the Keio collection with a known genotype [F<sup>-</sup> Δ(*araD-araB*)567 *lacZ*4787(Δ)::*rrnB3* LAM<sup>-</sup> *rph1* Δ(*rhaD-rhaB*)568 *hsdR514*] and the complete genome is sequenced (23). Bioinformatics analysis has shown that most known chromosomal *ccd* operons are located in this locus in the *E. coli* chromosome. The resulting strains were BW25113 *ccd*<sub>O157</sub> (contains the *ccd* operon from *E. coli* O157:H7 strain), BW25113 *ccd*<sub>F</sub> (contains the F-plasmid-borne *ccd* operon), and BW25113 *ccd*<sub>inactive</sub> (control strain containing the inactive, truncated *ccd* operon from a serogroup O51 *E. coli* O157:H7 strain). All three strains were first exposed to a prestress that involved either heat (48°C for 20 mins) or a sublethal dose of ampicillin (2.5 μg/ml for 1 h) or ciprofloxacin (0.008 μg/ml for 1 h). Multidrug-tolerant persisters are known to increase upon exposure to sublethal levels of antibiotic, at least in case of the clinically relevant bacterium *Staphylococcus aureus* (24). The prestress was followed by exposure of the cells to different antibiotics at lethal doses of 10× the MIC (cefotaxime, 100 μg/ml; ciprofloxacin, 0.4 μg/ml; mitomycin C, 10 μg/ml; kanamycin, 50 μg/ml; and tobramycin, 25 μg/ml) for 4 h. We used these antibiotics because prior studies have shown that persisters isolated under different conditions show various degrees of tolerance to different antibiotics, but typically only 2 or 3 antibiotics were examined (18, 25). In order to carry out a more comprehensive study we used multiple different antibiotics while studying the contribution of the chromosomal *ccdAB* operon to bacterial drug tolerance. We took at least one representative from each major class of antibiotics, based on their mechanism of action: kanamycin and tobramycin as aminoglycoside-type bactericidal antibiotics; cefotaxime, a member of the cephalosporin class of antibiotics that act on actively growing cells by inhibiting cell wall synthesis; mitomycin C, which selectively inhibits DNA synthesis; and ciprofloxacin, which is a fluoroquinolone and inhibits DNA gyrase, similarly to the CcdB toxin. Cells were then washed, plated on LB medium, and grown for 14 to 18 h in order to count the number of viable colonies. Figure 1A to D show the percent survival of the *E. coli* strains BW25113 *ccd*<sub>O157</sub>, BW25113 *ccd*<sub>F</sub>, and BW25113 *ccd*<sub>inactive</sub> in the presence of heat, a sublethal dose of ampicillin or ciprofloxacin, and without prestress, respectively. The survival ratios shown in Fig. 1E to H for the respective prestresses for the *E. coli* strains BW25113 *ccd*<sub>O157</sub> and BW25113 *ccd*<sub>F</sub> were calculated as percent survival of the strain containing active *ccd*/percent survival of the reference strain containing inactive truncated *ccd* (BW25113 *ccd*<sub>inactive</sub>). A survival ratio of greater than one indicates that protection from the antibiotic is due to specific expression of the Ccd toxin. There was a general nonspecific increase in survival for all three strains upon exposure to prestress. When the survivals of strains BW25113 *ccd*<sub>O157</sub> and BW25113 *ccd*<sub>F</sub> were



**FIG 1** Survival of *E. coli* strains containing the *ccd* operon upon antibiotic exposure. The BW25113 strains BW25113 *ccd*<sub>O157</sub>, BW25113 *ccd*<sub>F</sub>, and BW25113 *ccd*<sub>Inactive</sub> were first exposed to a prestress that involved either heat or a sublethal dose of ampicillin or ciprofloxacin. Survival of these strains was monitored in the presence of various antibiotics. Percent survival was calculated for each strain as the ratio of CFU after antibiotic exposure to CFU prior to antibiotic exposure. The survival ratio was calculated as the ratio of the percent survival of a strain containing active *ccd* to the percent survival of the reference strain containing inactive truncated *ccd* (BW25113 *ccd*<sub>Inactive</sub>). (A to D) Percent survival of *E. coli* strains BW25113 *ccd*<sub>O157</sub>, BW25113 *ccd*<sub>F</sub>, and BW25113 *ccd*<sub>Inactive</sub> in the presence of heat or a sublethal dose of ampicillin or ciprofloxacin or without prestress, respectively. (E to H) Survival ratios of *E. coli* strains BW25113 *ccd*<sub>O157</sub> and BW25113 *ccd*<sub>F</sub> in the presence of heat or a sublethal dose of ampicillin or ciprofloxacin or without prestress, respectively. The y axis is shown on a log scale, and error bars indicate the standard error from 4 independent experiments. Each experiment was done using three biological replicates. The survival ratio for strain BW25113 *ccd*<sub>O157</sub> in the absence of prestress is higher than that for strain BW25113 *ccd*<sub>F</sub> against cefotaxime, ciprofloxacin, tobramycin, respectively. Survival of both strains BW25113 *ccd*<sub>O157</sub> and BW25113 *ccd*<sub>F</sub> is greater than that of strain BW25113 *ccd*<sub>Inactive</sub> for most antibiotics, as indicated by the dotted lines.



**FIG 2** Monitoring cell survival following antibiotic exposure using flow cytometry. Cells were subjected to antibiotic stress with 25  $\mu\text{g/ml}$  tobramycin (left panel) and 100  $\mu\text{g/ml}$  cefotaxime (right panel) for 4 h. Cells were pelleted, washed in saline, and grown in the presence of 0.01% propidium iodide (PI) solution for 2 h for staining of dead cells. PI fluorescence for a given volume of cells was recorded in a BDAccuri flow cytometer. A rightward shift in the population shows that a larger fraction of cells showed fluorescence due to PI staining in the case of both BW25113 and BW25113 *ccd*<sub>inactive</sub> than in the case of either BW25113 *ccd*<sub>F</sub> or BW25113 *ccd*<sub>O157</sub>.

compared with that of BW25113 *ccd*<sub>inactive</sub>, 2- to 10-fold higher survival was seen for both BW25113 *ccd*<sub>O157</sub> and BW25113 *ccd*<sub>F</sub> (Fig. 1). Survival of BW25113 *ccd*<sub>O157</sub> was higher than that of BW25113 *ccd*<sub>F</sub> against cefotaxime, ciprofloxacin, and tobramycin (Fig. 1), especially under conditions of heat prestress. When ciprofloxacin prestress was given, no enhancement in survival relative to that of BW25113 *ccd*<sub>inactive</sub> was seen, except toward mitomycin C (15-fold) in strain BW25113 *ccd*<sub>F</sub>. Surprisingly a similar protection from antibiotic killing was seen even in the absence of a prestress for the *ccd*<sub>O157</sub> strain (Fig. 1D and H). These data show that persisters surviving treatment with different antibiotics are not identical and may be induced by different mechanisms. The difference in the survival ratios upon different antibiotic stresses could potentially indicate the shutdown of only selected metabolic processes that are targets for different antibiotics, for example, cell wall synthesis for ampicillin and cefotaxime. A similar experiment was carried out in the *E. coli* MG1655 strain. The plasmids with the *ccd* operons cloned in them (see Table S2 in the supplemental material) were individually transformed into the MG1655 strain, and relative survival was monitored under different antibiotic stress conditions. Results similar to those with the BW25113 strain were obtained (see Fig. S1 in the supplemental material).

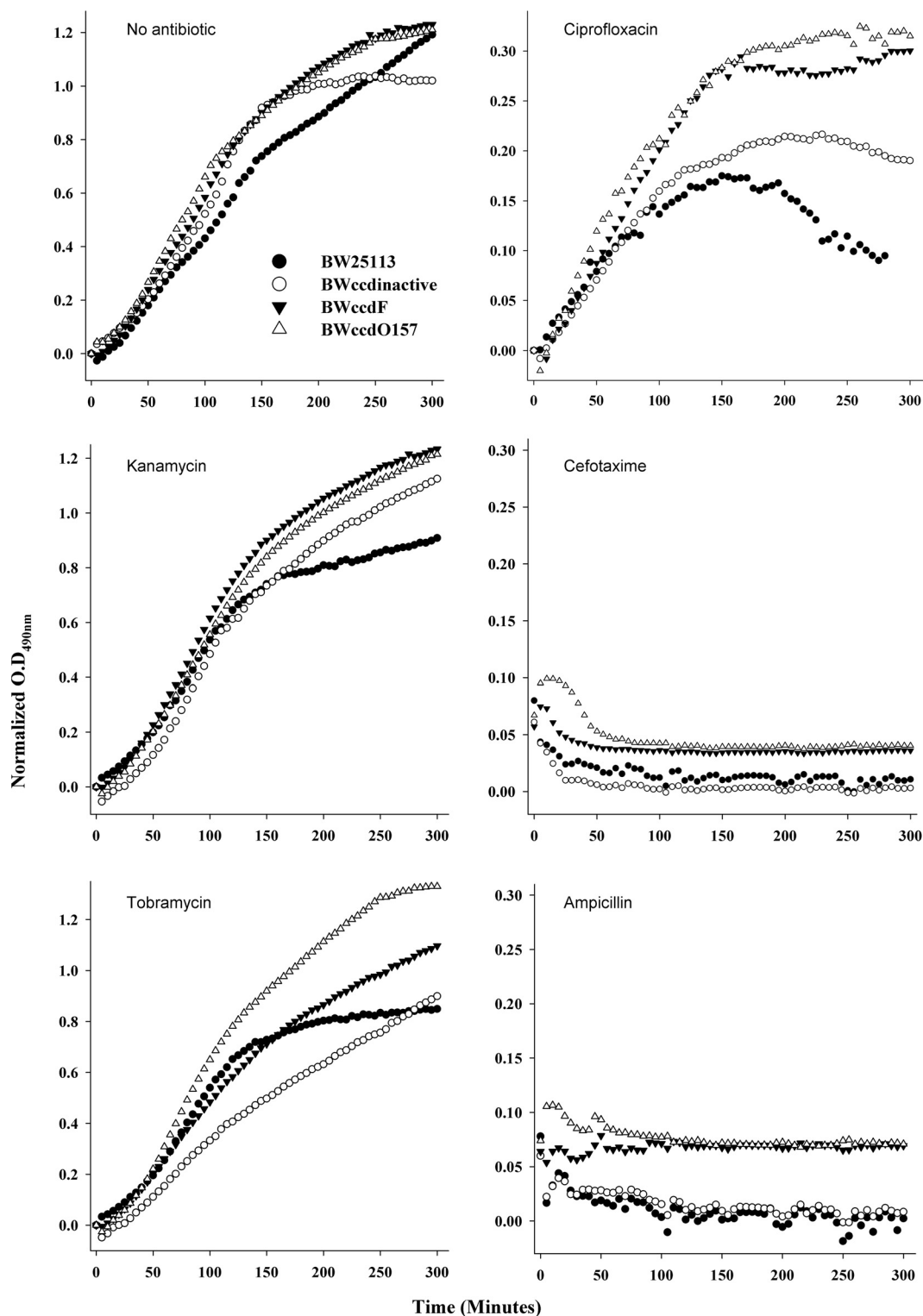
**Estimating cell survival using flow cytometry.** In addition to the viable cell count on plates, we verified our results on cell survival using a flow cytometry-based approach. The forward scatter (FSC)-side scatter (SSC) plot gives a measure of cell size and granularity. Differences in these parameters can be seen between live and dead cells. Log-phase cultures were subjected to antibiotic stress followed by flow cytometric analysis. Further, the cells were stained with propidium iodide (PI) after antibiotic treatment to measure the relative levels of cell survival for different strains. This dye is known to stain dead cells due to increased selective permeability of dead cells but is generally excluded by persisters (26). A larger fraction of cells showed fluorescence due to PI staining in the case of both strains BW25113 and BW25113 *ccd*<sub>inactive</sub> than in the case of either BW25113 *ccd*<sub>F</sub> or BW25113 *ccd*<sub>O157</sub> cells (Fig. 2; see Table S4 in the supplemental material).

**Monitoring growth kinetics and assessing metabolic activity.** Most persister cells that are tolerant to antibiotics are metabolically active, but there are known exceptions (27, 28). Many may exhibit biphasic killing (29). The second phase arises due to killing of the colonies formed from persister cells in cases where the persister cells continue to divide but at a rate lower than normal. We employed the 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) redox dye to monitor growth and assess the metabolic activity of the different strains spectrophotometrically (30). The optical density mea-

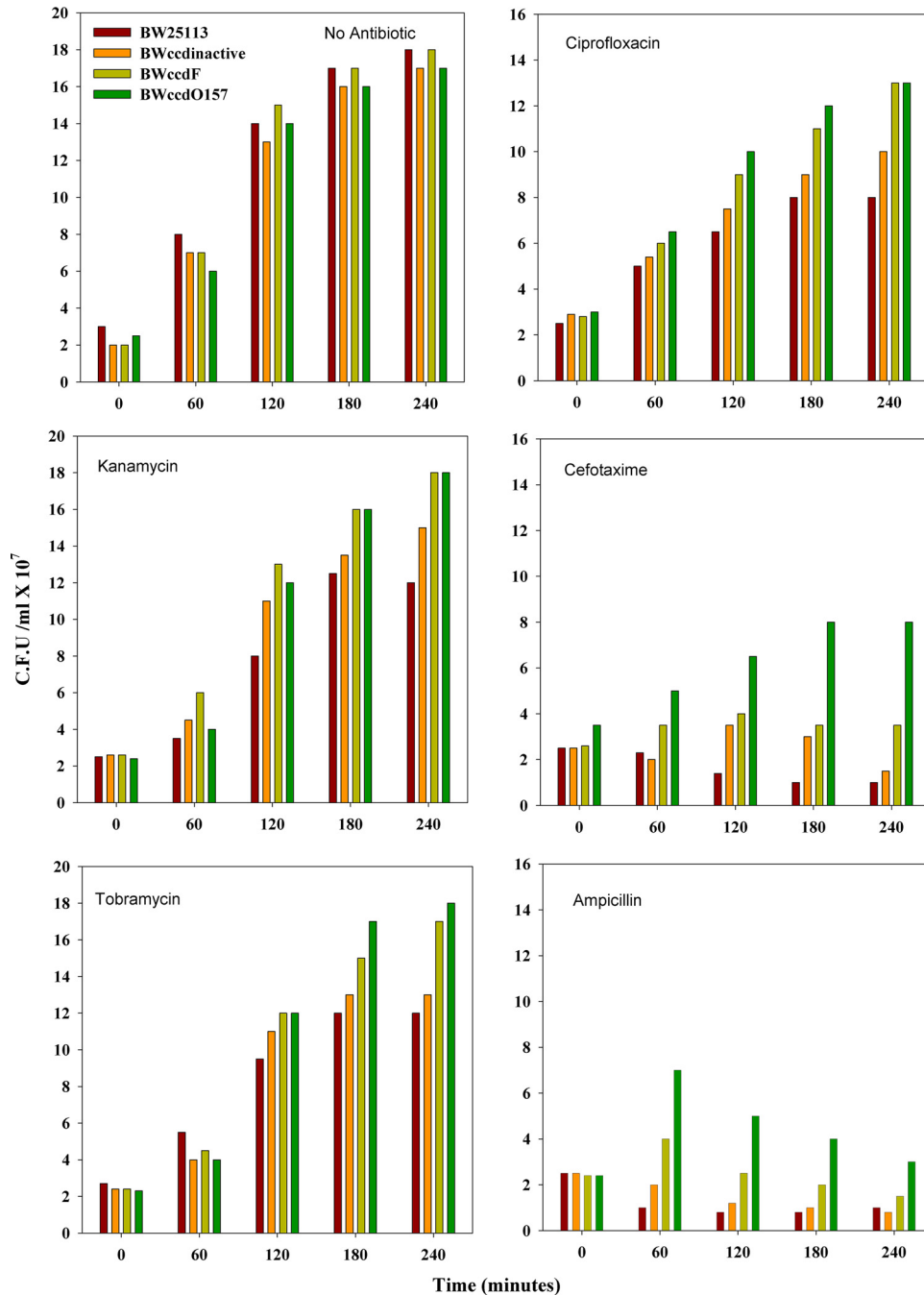
sured at 490 nm ( $OD_{490}$ ) was plotted against time to follow the growth kinetics. In the absence of antibiotic, all the strains showed comparable growth, although the wild-type (WT) BW25113 strain attained the stationary phase earlier than the other strains (Fig. 3). The aminoglycoside-type bactericidal antibiotics kanamycin and tobramycin, which act by inhibiting protein synthesis, did not cause much reduction in growth, although both the BW25113 and BW25113  $ccd_{inactive}$  strains showed lower growth rates than the BW25113  $ccd_F$  and BW25113  $ccd_{O157}$  strains. Lower cell death with these antibiotics may be due to the use of comparatively low concentrations (50  $\mu\text{g/ml}$  and 25  $\mu\text{g/ml}$  for kanamycin and tobramycin, respectively). Cefotaxime, a member of the cephalosporin class of antibiotics, and ampicillin, which is in the penicillin group of beta-lactam antibiotics, both showed typical killing curves for cultures treated with antibiotic. Both act on actively growing cells by inhibiting cell wall synthesis. In both cases, phenotypically sensitive cells showed marked cell death within 1 h of antibiotic treatment, and a plateau of persister cells was observed. Both  $ccd$  operon-containing strains showed similar protection, higher than that for the two control strains. Upon treatment with ciprofloxacin, strains containing the  $ccd$  operon showed better growth than the control strains, and the cells are metabolically more active than after treatment with other antibiotics (Fig. 3). Overall, the  $ccd_{O157}$ -containing cells showed growth in liquid culture similar to or better than that of  $ccd_F$ -containing cells under most antibiotic stress conditions. Similar results were obtained when we monitored growth using measurements of  $OD_{600}$  (see Fig. S2 in the supplemental material) or with viable cell counts (Fig. 4).

**Low-level expression of CcdB<sub>O157</sub> leads to reversible growth inhibition.** We have previously shown (31) that even very low levels of CcdB<sub>F</sub> expression, for example, under highly repressed conditions (0.2% glucose) under the control of the P<sub>BAD</sub> promoter, lead to cell death in sensitive *E. coli* strains such as BW25113. In contrast, we show here that low levels of CcdB<sub>O157</sub> expression under identical conditions do not cause significant cell death (Fig. 5A). A previous study has shown that the  $ccd_F$  system is able to mediate postsegregational killing in an *E. coli* strain harboring the  $ccd_{O157}$  system on its chromosome. This shows that the plasmid  $ccd_F$  system is functional even in the presence of its chromosomal counterpart (32). This requires that the CcdA<sub>O157</sub> antitoxin either binds weakly or does not bind to the CcdB<sub>F</sub> toxin. To explore this further, we employed a previously described methodology (20) to specifically activate a given TA system in the background of others using an active-site inactive mutant toxin. We observed that overexpression of the active-site inactive mutant toxin, CcdB<sub>F</sub> G100T, is nontoxic in cells lacking WT CcdB but leads to cell death at very low levels of induction (0.002%) when transformed in cells having the  $ccd_F$  operon inserted in the chromosome (Fig. 5). This is expected, as the binding of G100T to the cognate antitoxin CcdA<sub>F</sub> releases WT CcdB<sub>F</sub>, which then binds to gyrase, causing cell death. Interestingly, we also saw reduced cell survival at higher levels of induction of the mutant when transformed in cells having the  $ccd_{O157}$  operon inserted in the chromosome (Fig. 5B). This shows that there is weak cross activity between CcdB<sub>F</sub> and CcdA<sub>O157</sub>. CcdB<sub>F</sub> can bind to CcdA<sub>O157</sub> but likely with reduced affinity relative to CcdA<sub>F</sub>, as inferred from the inducer levels at which cells start to die. This is in contrast to a previous study where CcdB<sub>F</sub> and CcdA<sub>O157</sub> were shown to be noninteracting when overexpressed from two different heterologous promoters (32). This is possibly because in the latter case, the CcdA<sub>O157</sub> antitoxin is unable to neutralize overexpressed CcdB<sub>F</sub> completely, and even a small amount of CcdB<sub>F</sub> can bind to gyrase and cause cell death. In contrast, in our experiments, we scored the expression and binding of CcdB<sub>O157</sub> toxin in its native context. Also, we controlled the expression of the active-site inactive mutant by varying the inducer and repressor levels. This finding also demonstrates that exposure of cells to low levels of CcdB<sub>O157</sub> causes growth inhibition rather than cell death.

**Estimates of the relative activities of CcdB<sub>F</sub> and CcdB<sub>O157</sub>.** CcdB<sub>F</sub>, when expressed from the F plasmid, and CcdB<sub>O157</sub>, when expressed from the *E. coli* chromosome, bind to the same target, gyrase A. However, they have been implicated in



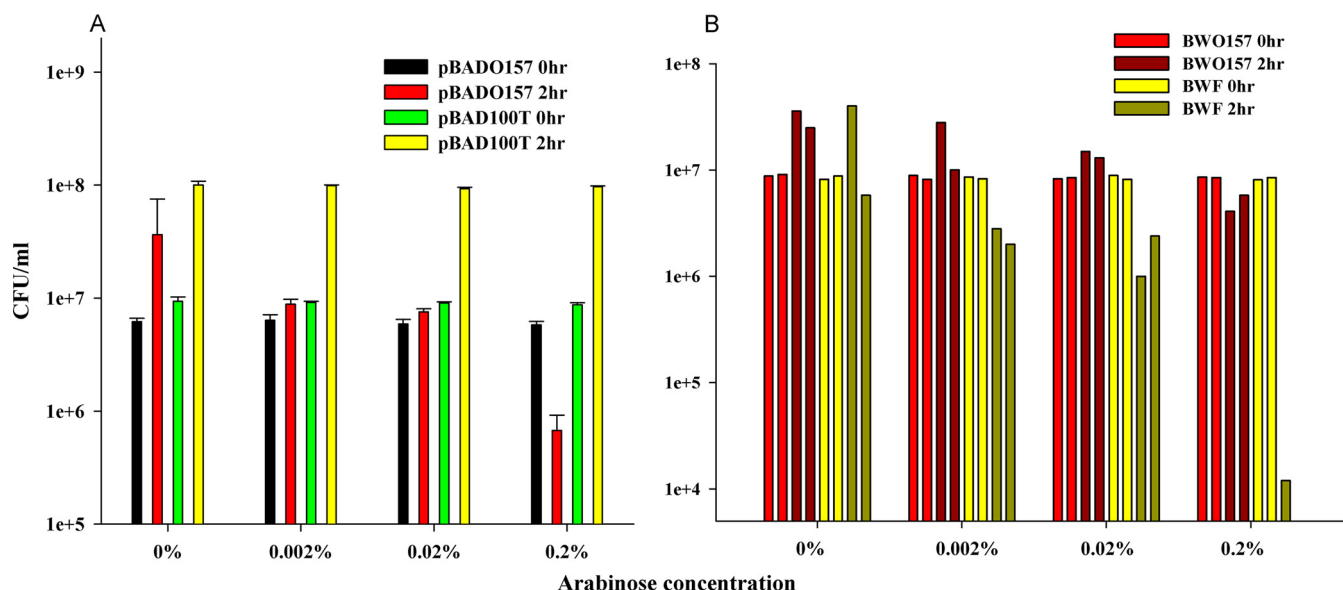
**FIG 3** Growth kinetics and metabolic activity of different strains under antibiotic stress. Mid-log-phase cells were grown in either the absence or presence of various antibiotics (cefotaxime, 100  $\mu\text{g/ml}$ ; ciprofloxacin, 0.4  $\mu\text{g/ml}$ ; ampicillin, 10  $\mu\text{g/ml}$ ; kanamycin, 50  $\mu\text{g/ml}$ ; and tobramycin, 25  $\mu\text{g/ml}$ ) in a 96-well microtiter plate, and growth was monitored using the absorbance of tetrazolium dye (0.02%) at 490 nm. Growth kinetics were monitored for 5 h, and the OD<sub>490</sub> was normalized to zero with respect to the first reading. The average readings from three independent replicates were plotted. Both strains BW25113 *ccd<sub>F</sub>* and BW25113 *ccd<sub>O157</sub>* show protection from cell death upon antibiotic exposure compared to the control strains, BW25113 *ccd<sub>inactive</sub>* and BW25113.



**FIG 4** Viable cell counts for different strains under antibiotic stress. Mid-log-phase cells were grown in either the absence or presence of various antibiotics (cefotaxime, 100  $\mu\text{g/ml}$ ; ciprofloxacin, 0.4  $\mu\text{g/ml}$ ; ampicillin, 100  $\mu\text{g/ml}$ ; kanamycin, 50  $\mu\text{g/ml}$ ; and tobramycin, 25  $\mu\text{g/ml}$ ) in a 96-well microtiter plate. Viable cell counts were measured at intervals of 30 min for 5 h and plotted against time. The average readings from three independent replicates are shown. For clarity, only data at hourly intervals are presented.

different functional roles (12, 32, 33). While the F-plasmid-carried *ccd* operon is involved in plasmid maintenance by postsegregational killing, no clear role has been attributed to the chromosomal *ccd* counterpart. To explore the molecular mechanisms involved in the possibly differential functions of CcdB<sub>F</sub> and CcdB<sub>O157</sub>, we monitored their activities *in vivo* as well as *in vitro*. The *in vivo* results showed that CcdB<sub>O157</sub> is much less efficient than CcdB<sub>F</sub> in causing cell death, as can be seen from significant growth under inducing conditions for CcdB<sub>O157</sub> and no growth even under highly repressed conditions for



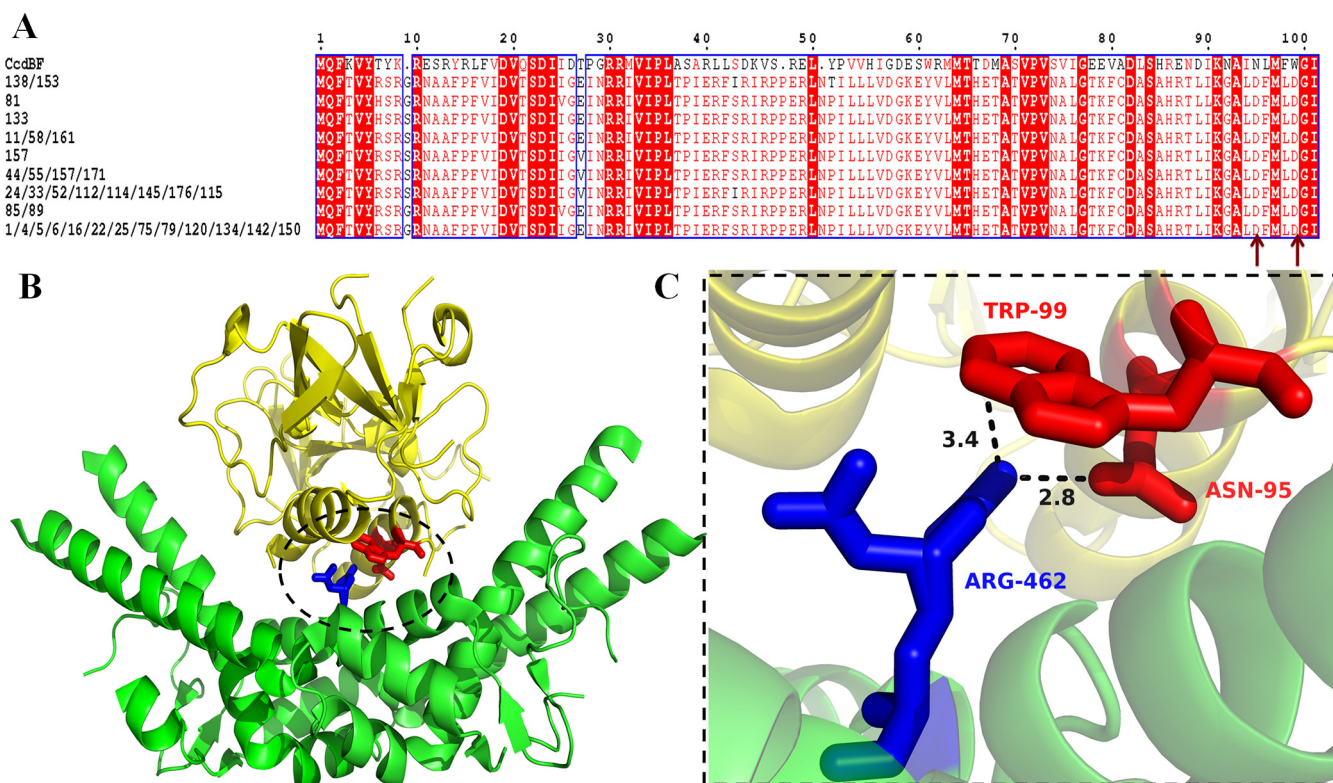


**FIG 5** Low-level expression of  $CcdB_{O157}$  leads to reversible growth inhibition. (A) Plasmids pBAD24 $ccdB_F$ , pBAD24 $ccdB_{O157}$ , and pBAD24 $ccdB100T$  were individually transformed in *E. coli* strain BW25113 and grown in LB medium containing 0.2% glucose. Cells were induced with various concentrations of arabinose at an  $OD_{600}$  of 0.2. The x axis indicates various concentrations of arabinose used for transient expression of the construct. Induced cells were grown for 2 h, washed twice with LB medium, diluted, and plated on 0.2% glucose-containing medium to repress further expression.  $CcdB_F$  expression leads to complete cell death, even under highly repressed conditions.  $CcdB_{O157}$  leads to cell death at very high expression levels (0.2% arabinose) but leads to growth inhibition when the expression is low (0.002% to 0.02% arabinose). The y axis is shown on a log scale. Results from two independent experiments are plotted as two different bars. Overexpression of the G100T mutant is nontoxic to *E. coli* Top10 cells. WT  $CcdB_F$  is toxic to cells even under highly repressed conditions, and no transformants are obtained under any condition. (B) To score the expression of  $ccdB_{O157}$  from its own promoter, the active-site inactive mutant of  $CcdB_F$ , G100T, was transformed into the *E. coli* BW25113  $ccd_F$  (having chromosomal  $Ccd_F$ ) and BW25113  $ccd_{O157}$  (having chromosomal  $Ccd_F$ ) strains individually. Expression of G100T was induced with various concentrations of arabinose (as described above) at an  $OD_{600}$  of 0.2. Cells were grown for 2 h, washed twice, diluted, and plated on 0.2% glucose-containing medium to repress further expression. Overexpressed G100T will titrate out cellular  $CcdA$ , leading to release of WT  $CcdB$  as well as derepression of the  $ccdAB$  operon. The reduction in cell survival at higher levels of induction (0.2% arabinose) of the mutant in BW25113  $ccd_{O157}$  cells shows that there is cross activity between the  $CcdB_F$  and  $CcdA_{O157}$  TA systems. Low expression levels of the mutant (0.002% to 0.02% arabinose) result in reduced toxicity and cause growth inhibition.

$CcdB_F$  (Fig. 5A). *In vitro* gyrase binding of  $CcdB_F$  and  $CcdB_{O157}$  using surface plasmon resonance (SPR) showed that  $CcdB_{O157}$  has 5-fold-lower affinity for its target, GyrA (see Fig. S3 and Table S3 in the supplemental material). Although the dissociation rates for the two proteins were somewhat similar, there was a significant decrease in the association rates. These results are consistent with the experimentally observed lower toxicity of  $CcdB_{O157}$  *in vivo*. Sequence comparison of the two proteins showed that though most of the residues known to be involved in gyrase binding are conserved, two important interacting residues are not conserved. Analysis of the  $CcdB_F$ -gyrase bound structure (PDB no. 1X75) indicated that substitutions N95D and W99D observed in  $CcdB_{O157}$  are likely to disrupt gyrase binding (Fig. 6). Our previous study involving saturation mutagenesis on the  $CcdB_F$  protein also indicates that both the N95D and W99D mutations have an inactive phenotype (34).

## DISCUSSION

One of the first identified and best-studied toxin-antitoxin systems in *E. coli* is the F-plasmid-based  $CcdAB$  system. This is involved in plasmid maintenance through postsegregational killing. More recently,  $ccdAB$  homologs have been found on chromosomes, including those in pathogenic strains of *E. coli* and other bacteria. However, their functional role, if any, has remained unclear. We show that both the native  $ccd$  operon of the *E. coli* O157 strain and the  $ccd$  operon from the F plasmid, when inserted on the *E. coli* chromosome, lead to protection from cell death under multiple antibiotic stress conditions through formation of persisters. The frequency of these persisters ranges from  $10^{-1}$  (for antibiotics such as mitomycin) to  $<10^{-5}$  (for tobramycin). Under many antibiotic stress conditions, the frequency of persisters is increased up to 10-fold compared to that for the isogenic strain having an inactive  $ccd$  operon at the same



**FIG 6** Sequence and structural basis for differential activity of CcdB<sub>F</sub> and CcdB<sub>O157</sub>. (A) Sequence alignment of CcdB<sub>F</sub> and CcdB<sub>O157</sub> serotypes. Sequenced variants of CcdB<sub>O157</sub> from 32 different isolates, which tested positive for toxicity (12), were aligned with the CcdB<sub>F</sub> sequence using the Clustal Omega multiple-sequence alignment tool. Residues that are fully conserved either are active-site residues or form a part of the hydrophobic core (34). (B) CcdB<sub>F</sub>-gyrase A14 bound crystal structure (PDB no. 1X75) (40). The key region of the interaction between CcdB<sub>F</sub> and gyrase A14 is circled. (C) Residues in the interaction between CcdB (in red) and gyrase A14 (in blue) that are not identical in the CcdB<sub>F</sub> and CcdB<sub>O157</sub> sequences (marked with arrows in the multiple-sequence alignment in panel A). CcdB<sub>O157</sub> contains the mutations N95D and W99D relative to CcdB<sub>F</sub>.

locus in the BW25113 genome, showing that the protection from antibiotics is due to specific expression of the Ccd toxin (Fig. 1). Strains with chromosomal *ccd*<sub>O157</sub> show greater drug tolerance, particularly in the presence of heat stress and even in the absence of prestress, conditions that are probably more physiologically relevant. Both the *ccd*<sub>F</sub> and *ccd*<sub>O157</sub> operons may share common mechanisms for activation under stress conditions and also display weak cross activation. CcdB<sub>O157</sub> has weaker affinity for GyrA than the plasmidic counterpart but can still cause some cell death. This suggests that inhibitors of the Ccd<sub>O157</sub> toxin-antitoxin interaction can cause cell death and therefore that the system might represent a drug target. Alternatively, inhibitors of Ccd<sub>O157</sub> might also decrease the fraction of persisters, which could have clinical significance.

The *ccdAB* system present on the F plasmid has a role in plasmid maintenance. In F-plasmid-containing cells, CcdB is expressed along with an excess of its unstable antidote, CcdA. Hence, CcdB is present almost entirely as a complex with CcdA. In cells lacking the F plasmid, CcdA is degraded, and in the absence of fresh CcdA synthesis, CcdB kills the cell. This process is known as postsegregational killing. There are other reports which suggest activation of the *ccd* operon during nutritional stress (35). Homologs of the *ccd* operon have been discovered on the chromosomes of a variety of bacterial strains, including many pathogenic strains such as those of *Shigella dysenteriae*, *E. coli* O157:H7, and *Vibrio cholerae*. The chromosomal *ccd* operon cloned on a plasmid shows a lower degree of postsegregational killing than *ccdAB*<sub>F</sub> (32). When the *ccd* operon present on the F plasmid is activated by stress, such as heat or a sublethal dose of antibiotics, an increased frequency of drug-tolerant persisters is observed (20). A previous study of *E. coli* O157:H7 suggests that CcdB<sub>O157</sub> is also active and that when

it is expressed alone in the absence of its cognate antidote, CcdA, it causes cell death by poisoning DNA gyrase (32). The *ccd* operon in this bacterium is located in an intergenic region between two metabolic genes, *folA* and *apaH*, which encode dihydrofolate reductase and diadenosine tetraphosphatase, respectively. This operon is absent from the *E. coli* K-12 strains, and a 77-bp intergenic region is present at the same locus. When a bioinformatics analysis was carried out on various serogroups of this strain, it was suggested that the intergenic region between the *folA* and *apaH* genes is a site for transposon insertion. The promoter activity of the Ccd<sub>O157</sub> system was found to be lower than that of the CcdF system, although at the protein level, some of the crucial active-site residues involved in target binding were found to be conserved (32).

One model that was proposed to explain the presence of TA modules on the genomes of bacteria is the stabilization module; i.e., chromosomally encoded TA modules could act against large-scale deletion of neighboring genomic regions. Given that many of these *ccd* TA modules have been consistently found to be integrated between the repeat regions spanned by the *folA* and *apaH* metabolic genes, this could also be one explanation for the role of the *ccdAB* operon on the chromosome.

The antiaddiction model suggests that the presence of TA modules on the chromosome which can cross-react with a plasmidic or phage TA module could potentially give selective advantage to the plasmid-free cells by inhibiting postsegregational killing of cells that lose the plasmid (11). Mutations that are able to evade the antiaddiction will be selected by the plasmidic TA system, and vice versa for the chromosomally encoded TA system. Analysis of the CcdAB complex structure (PDB no. 3HPW) (36) indicates that the presence of an N69Y mutation in CcdA<sub>O157</sub> with respect to CcdA<sub>F</sub> and of a Y8R mutation in CcdB<sub>O157</sub> with respect to CcdB<sub>F</sub> (see Fig. S4 in the supplemental material) will likely hamper the binding of the noncognate Ccd toxin and antitoxin. This implies that the chromosomal *ccd* operon would be unable to cause a strong antiaddiction phenotype in the presence of the plasmidic F plasmid, consistent with previous experimental data (32).

In the present study, we found that there is weak cross talk between the plasmidic toxin and chromosomal antitoxin, since overexpression of the active-site inactive Ccd<sub>F</sub> toxin mutant causes cell death in Ccd<sub>O157</sub>-containing cells. A previous study indicated that chromosomally carried *ccd* operons undergo a decay process within the *E. coli* species and therefore are likely to lose their antiaddictive property, with about 29% of the strains harboring an inactive toxin (12). The present work suggests a probable explanation for the presence of an active *ccd* operon in more than 69% of the *E. coli* strains studied, namely, that they functionally integrate into the host regulatory network, leading to enhanced tolerance to multiple antibiotics through the formation of persisters. It is probable that the chromosomal TA systems, such as the *ccd* operon, are acquired from the plasmidic counterparts and that mutations that lead to lower toxicity but increased drug tolerance have been selected for in the course of evolution.

The *ccd* operon of the F plasmid can function as a transmissible persistence factor (20). Under stress conditions, the antitoxins of multiple TA systems undergo degradation by cellular proteases such as Lon, leading to activation of multiple TA systems, which eventually leads to multidrug tolerance, as previously suggested (20). CcdB poisons the gyrase-DNA complex, blocking the passage of polymerases and leading to double-strand breaks. Additionally, the GyrA subunit has also been found as an inactive binary complex with CcdB when overexpressed (37). Although lethal effects of CcdB are probably due to poisoning of the gyrase-DNA complex, the inactivation pathway may lead to growth inhibition and prevent cell death, as is possible in the case of CcdB<sub>O157</sub>, which shows lower toxicity *in vivo*. Under conditions where there is specific activation of the CcdAB system, the SOS response to DNA damage caused by the CcdB toxin may be the specific trigger for activation of other TA systems. This response is mediated through the DNA repair enzyme RecA and Lon protease. It is very likely that similar pathways operate when the *ccd* operon is placed in a chromosomal context, leading to enhanced persister generation, as previously shown (20) for the plasmidic Ccd<sub>F</sub> TA system. Future experiments aimed at exploring the molecular mechanisms underlying the drug-tolerant phenotype of *E. coli* cells carrying the *ccd* operon in the chromosome

would further aid in designing targeted therapeutics against such antibiotic-tolerant persisters.

## MATERIALS AND METHODS

**Construction of the BW25113  $ccd_{O157}$ , BW25113  $ccd_{inactive}$  and BW25113  $ccd_F$  strains.** The  $ccd$  operons of *E. coli* strain O157:H7 (based on reference strain EDL933) and the F plasmid were both synthesized at GenScript. To enable insertion of the operon at a specific locus in the *E. coli* genome, these constructs had homology at the 5' end with sequences downstream of the *folA* gene of the *E. coli* K-12 chromosome and at the 3' end with a *cat* gene from a pKD3 plasmid. The synthesized fragments were named  $ccd_{O157}$  and  $ccd_F$ , respectively. As a control, the  $ccd$  operon from serogroup O51 of strain O157:H7 was also synthesized at GenScript and named  $ccd_{inactive}$ . The  $ccd_{inactive}$  operon has a truncated  $ccdB$  gene, resulting in an inactive CcdB protein, although the antitoxin sequence and the regulatory regions are intact. To construct  $ccd$ - $cat$  fragments for all synthesized  $ccd$  operons for the purpose of selection, a *cat* gene containing FLP recombination target (FRT) sites was amplified separately from a pKD3 plasmid by using the *cat* (*for2*) and *apaH* (*rev3*) primers, respectively. This fragment has homology at the 3' end with upstream sequences of the *apaH* gene of the *E. coli* K-12 chromosome. All three  $ccd$  operons were fused with the PCR-amplified *cat* gene by overlap PCR. Each  $ccd$ - $cat$  cassette has sequences at the 5' and 3' ends that are similar to the downstream and upstream sequences of the *folA* and *apaH* genes, respectively. The three final constructs,  $ccd_{O157}$ - $cat$ ,  $ccd_F$ - $cat$ , and  $ccd_{inactive}$ - $cat$ , were inserted at a 77-bp intergenic region between the *folA* and *apaH* genes of an *E. coli* strain K-12 locus in *E. coli* strain BW25113 using lambda red recombination (38). A list of all strains and plasmids used in this study is provided in Table S1 in the supplemental material. Sequences for all of these constructs are listed in Table S2 in the supplemental material. Recombinants were screened on chloramphenicol (25  $\mu$ g/ml)-containing LB plates, and the presence of the  $ccd$  operon was confirmed by both PCR and DNA sequencing. The *cat* gene was later removed by transforming each strain individually with plasmid pCP20 expressing the FRT-FLP recombinase, as described previously (38).

**Activation of the  $ccd$  operon by heat or antibiotic stress.** Overnight cultures of *E. coli* strains BW25113  $ccd_{O157}$ , BW25113  $ccd_F$ , and BW25113  $ccd_{inactive}$  were diluted 100-fold and grown to an OD<sub>600</sub> of 0.3. Subsequently, cells were subjected to three different types of sublethal prestresses, namely, exposure of cells to a high temperature (48°C for 20 min) or to a sublethal dose of either ampicillin (2.5  $\mu$ g/ml for 1 h) or ciprofloxacin (0.008  $\mu$ g/ml for 1 h). This was followed by exposure to lethal doses of various antibiotics with different modes of action (cefotaxime, 100  $\mu$ g/ml; ciprofloxacin, 0.4  $\mu$ g/ml; mitomycin C, 10  $\mu$ g/ml; kanamycin, 50  $\mu$ g/ml; and tobramycin, 25  $\mu$ g/ml) for 4 h with growth under shaking conditions (180 rpm) at 37°C. Similar antibiotic concentrations were used for all experiments unless otherwise specified. The cultures were washed twice, appropriately diluted, and plated on LB agar plates at 37°C to determine the viable counts after antibiotic treatment, and the percent survival for each *E. coli* strain was calculated. Percent survival was defined as the ratio of CFU after antibiotic exposure to CFU prior to antibiotic exposure. For the calculation of the survival ratio with respect to the control strain, the percent survival of the  $ccd$  strain (containing the  $ccd$  operon from either the *E. coli* O157:H7 strain or the F plasmid) was normalized to the percent survival of a reference strain containing the truncated  $ccd$  operon,  $ccd_{inactive}$ .

**Estimating cell survival using flow cytometry.** Primary cultures were inoculated in LB medium and grown overnight. Thirty microliters of the overnight culture was inoculated in 3 ml of LB and grown for 2 h to an OD<sub>600</sub> of 0.4. Cells were subjected to antibiotic stresses, namely, 25  $\mu$ g/ml tobramycin and 100  $\mu$ g/ml cefotaxime individually for 4 h. Three milliliters of cell culture was pelleted, washed in saline, and grown in the presence of 0.01% propidium iodide (PI) solution for 2 h for staining of dead cells. One milliliter of cell culture was again pelleted and washed with saline, and PI fluorescence for a 100- $\mu$ l volume of cells was recorded in a BDAccuri flow cytometer using the FL-4 channel.

**Monitoring growth kinetics and assessing metabolic activity.** Two hundred microliters of mid-log-phase cells (OD<sub>600</sub> of 0.4) was grown in the presence of various antibiotics (as mentioned above) in a microtiter plate, and growth was monitored for 5 h by conventional OD<sub>600</sub> measurements as well as by using 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) redox dye (0.02%), which upon formation of formazan reduces to give a purple color having an absorption maximum at 490 nm (30). OD measurements at both 490 nm and 600 nm were made at an interval of every 5 min using a SpectraMax Plus microplate spectrophotometer. In addition, this OD<sub>490</sub> is indicative of the survival of the bacterial population as a function of time of exposure to a given antibiotic. Five microliters of sample was taken from each of the wells every 30 min and plated onto LB medium at three different dilutions to obtain the viable cell count.

**Cloning and expression of CcdB<sub>F</sub> and CcdB<sub>O157</sub> to monitor their relative activities. (i) Cloning, expression, and purification.** The  $ccdB$  genes (both  $ccdB_F$  and  $ccdB_{O157}$ ) were cloned under the control of the P<sub>BAD</sub> promoter in plasmid pBAD24 as described previously (39). pBADCCdB<sub>F</sub> and pBADCCdB<sub>O157</sub> clones were individually transformed into the *E. coli* CSH501 strain, which is resistant to the action of CcdB. This strain harbors the *gyrA462* mutation in its chromosomal DNA, which prevents gyrase from binding to CcdB (33). A 200-ml cell culture in LB was induced with 0.2% (wt/vol) arabinose at an OD<sub>600</sub> of 0.6 and grown for 8 h at 25°C. Cells were harvested by centrifugation at 1,800  $\times$  g for 10 min at 4°C. The pellet was resuspended in HEG buffer (10 mM HEPES, 50 mM EDTA, 10% glycerol), pH 7.4, and sonicated, followed by centrifugation at 11,000  $\times$  g for 30 min at 4°C. The supernatant was loaded onto a GyrA14 affinity column (3-ml bed volume) and incubated at 4°C for 4 h, followed by removal of unbound proteins by washing the column with coupling buffer (5 times the bed volume). Elution of CcdB was carried out with 0.2 M glycine (pH 2.5) into a tube containing an equal volume of 400 mM HEPES

(pH 8.5) at 4°C. The eluted fractions were subjected to 15% SDS-PAGE, and the concentration was determined.

(ii) **In vitro gyrase binding by SPR.** Binding studies were performed with a ProteOn XPR 36 system (Bio-Rad). GyrA14 was immobilized onto the ProteOn GLC chip surface by standard amine coupling. A sensor surface (without GyrA14) that had been activated and deactivated served as a negative control for each binding interaction. Three different concentrations of CcdB were passed across each sensor surface in running buffer (phosphate-buffered saline [PBS; pH 7.4] containing 0.01% P20 surfactant). Protein concentrations ranged from 50 nM to 500 nM. Both binding and dissociation were measured at a flow rate of 60  $\mu$ l/min. In all cases, the sensor surface was regenerated between binding reactions with 4 M MgCl<sub>2</sub>. Each binding curve was corrected for nonspecific binding by subtraction of the signal obtained with the channel where only buffer instead of the analyte was passed. The kinetic parameters were obtained by fitting the data to a simple 1:1 Langmuir interaction model.

(iii) **In vivo activity estimation.** *E. coli* strain BW25113 was individually transformed with the pBADcCdB<sub>F</sub>, pBADcCdB<sub>O157</sub>, and pBADcCdB100T (control) plasmids. Cells were transiently induced with various concentrations of arabinose (0%, 0.002%, 0.02%, and 0.2%) at an OD<sub>600</sub> of 0.2. The induced cells were grown for 2 h, washed twice, diluted, and plated on 0.2% glucose-containing medium to repress further expression. Activity was assayed by plating the transformation mix on LB-ampicillin plates at 37°C in the presence of the following concentrations of glucose (repressor) or arabinose (inducer): 0.2% glucose, 0.02% glucose, 0.002% glucose, 0% glucose/arabinose, 0.002% arabinose, 0.02% arabinose, and 0.2% arabinose.

**Overexpression of a CcdB active-site inactive mutant to probe the specificity and cross-reactivity between the Ccd<sub>F</sub> and Ccd<sub>O157</sub> gene products.** An active-site inactive mutant of CcdB<sub>F</sub> (G100T) was cloned under control of a heterologous arabinose-inducible promoter as described previously (31). This mutant cannot bind to the toxin target (gyrase) and is therefore inactive, but it retains binding to the cognate antitoxin, CcdA. Therefore, overexpression of G100T will lead to cell death only if it binds to the chromosomally expressed CcdA antitoxin, thus releasing the WT CcdB toxin, which is capable of binding to the target. To probe the specificity and cross-reactivity between Ccd<sub>F</sub> and Ccd<sub>O157</sub>, the G100T mutant of CcdB<sub>F</sub> was transformed individually into the BW25113 *ccd<sub>F</sub>* and BW25113 *ccd<sub>O157</sub>* strains. A single colony was picked, inoculated in LB-ampicillin medium containing 0.2% glucose, and grown at 37°C overnight. Expression of G100T was induced with various concentrations of arabinose (0%, 0.002%, 0.02%, and 0.2%) at an OD of 0.2 after washing the cells twice with LB medium. Cells were grown for 2 h, washed twice, diluted, and plated on 0.2% glucose-containing medium to repress further expression. Surviving colonies were counted for each strain.

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JB.00397-17>.

**SUPPLEMENTAL FILE 1**, PDF file, 0.5 MB.

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We declare that no competing interests exist.

K.G., A.T., and R.V. designed the experiments. K.G., A.T., A.S., and R.V. performed the experiments. K.G., A.T., and R.V. analyzed the results and wrote the manuscript.

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