Additional role for the ccd operon of F-plasmid as a transmissible persistence factor

Arti Tripathi*, Pooja C. Dewan*, Bipasha Barua*, and Raghavan Varadarajan**

*Molecular Biophysics Unit, Indian Institute of Science, Bangalore 560 012, India; and **Chemical Biology Unit, Jawaharlal Nehru Center for Advanced Scientific Research, Bangalore 560 004, India

Edited by Sankar Adhya, National Institutes of Health, National Cancer Institute, Bethesda, MD, and approved June 20, 2012 (received for review December 26, 2011)

Toxin-antitoxin (TA) systems are found on both bacterial plasmids and chromosomes, but in most cases their functional role is unclear. Gene knockouts often yield limited insights into functions of individual TA systems because of their redundancy. The well-characterized F-plasmid-based CcdAB TA system is important for F-plasmid maintenance. We have isolated several point mutants of the toxin CcdB that fail to bind to its cellular target, DNA gyrase, but retain binding to the antitoxin, CcdA. Expression of such mutants is shown to result in release of the WT toxin from a functional preexisting TA complex as well as derepression of the TA operon. One such inactive, active-site mutant of CcdB was used to demonstrate the contribution of CcdB to antibiotic persistence. Transient activation of WT CcdB either by coexpression of the mutant or by antibiotic/heat stress was shown to enhance the generation of drug-tolerant persisters in a process dependent on Lon protease and RecA. An F-plasmid containing a ccd locus can, therefore, function as a transmissible persistence factor.

CcdA-CcdB | conditional regulation | protein–protein interaction

Many bacterial genomes harbor a class of genes referred to as toxin-antitoxin (TA) genes. These genes were initially discovered on low copy-number plasmids of Escherichia coli and appear to be involved in plasmid maintenance in the bacterial population (1). TA systems comprise a pair of genes organized in an operon encoding a stable toxin and a labile antitoxin that antagonizes it (1). Plasmid-based TA systems are also known as addiction modules and selectively eliminate daughter cells that do not inherit a plasmid copy during cell division (1). This mechanism, also called postsegregational killing, occurs in daughter cells devoid of a plasmid copy. The unstable antitoxin counter-part is degraded more rapidly by host proteases than the toxin. The toxin is released from the TA complex and interacts with an essential host target. This interaction often results in cell death but in some cases, as shown below, may also result in growth inhibition (2).

Homologs of plasmid-based TA systems have recently been discovered on the chromosomes of a large number of bacteria, many of which are pathogenic (3). The biological role of these TA systems in bacteria still remains controversial. A number of different models have been proposed to explain their presence on the chromosome (4). TA systems are difficult to study because overexpression of the active toxin component typically leads to cell death. Because 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 (5). In the present work, we describe a methodology to conditionally regulate expression of a toxin gene in a dose-dependent fashion. The method involves the release of the WT toxin from the TA complex by an overproduced mutant toxin that has a high affinity for the antitoxin but a low affinity for the cellular target of WT toxin (Fig. S1). This approach was validated using the well-established plasmid-based CcdAB TA system and used to demonstrate that this system plays a role in bacterial persistence. Bacterial persistence is a phenotype of dormant cells present at a low frequency in a growing population and characterized by tolerance to the presence of a variety of antibiotics, even in the absence of an active, specific resistance mechanism. Persisters are likely to be clinically important (6, 7). In the present work, we show that the F-plasmid derived ccd operon, whether located on a multicopy plasmid or in a single copy on the E. coli chromosome, plays a significant role in the generation of persisters. This finding is in addition to its well-studied role in plasmid maintenance (8). The methodology described here may also be used to probe the role of specific TA systems in other organisms where making knockouts are difficult or which have multiple homologous TA systems.

Results

Inactive, Active-Site Mutants of CcdB. CcdAB is one of the most well-studied plasmid-based TA systems and is involved in maintenance of F-plasmid in E. coli (8). CcdB is a DNA gyrase poison that entraps a cleavage complex between gyrase and DNA (9). In the presence of its antagonist, CcdA, CcdB is sequestered in the form of a CcdAB complex. However, if the cell loses the F-plasmid, the labile CcdA is degraded by the ATP-dependent Lon protease (2), releasing CcdB from the complex to act on its target DNA gyrase, which eventually leads to cell death. The crystal structure of CcdB in complex with a fragment of DNA gyrase has been determined. The active-site residues of CcdB are defined as those that are involved in direct interaction with DNA gyrase, as determined by Ala and Asp scanning mutagenesis (10) and confirmed by X-ray crystallography of the CcdB:GyrA14 fragment complex (11). These comprise residues Ile24, Ile25, Asn95, Phe98, Trp99, and Ile101 (Fig. 1). We have previously reported the construction and phenotypic characterization of a large library comprising of a total of 1,430 (75%) of the 1,900 possible single-site mutants of CcdB (12). The mutants were expressed under control of the arabinose inducible pBAD promoter in pBAD24ccdB (13). In this system, the level of expression of each mutant can be modulated by varying the arabinose concentration in the medium.

No structure was available for the CcdA:CcdB complex when this work was initiated. However, we hypothesized that there should be CcdB mutants that affect binding to DNA gyrase but not CcdA. Such mutants should be nontoxic when overexpressed in a CcdB-sensitive E. coli strain that lacks WT CcdB. However, when overexpressed in F-plasmid containing strains that express WT CcdB and its antitoxin CcdA, such mutants should cause cell death by titrating out CcdA, thereby permitting WT CcdB to bind to its cellular target. To validate this hypothesis, 10 inactive mutants previously isolated (12) at active-site residues involved

Author contributions: A.T., P.C.D., and R.V. designed research; A.T. and P.C.D. performed research; B.B. and R.V. contributed new reagents/analytic tools; A.T., P.C.D., and R.V. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

To whom correspondence should be addressed. E-mail: varadar@mbu.iisc.ernet.in.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1121217109/-/DCSupplemental.
in DNA gyrase binding were characterized. In initial experiments, mutants were overexpressed in two different *E. coli* strains, one containing an F-plasmid encoding WT CcdB and the other lacking the F-plasmid. The resulting phenotypes were characterized. The *E. coli* strains used were: Top10, which lacks the F-plasmid (and hence CcdA and CcdB), and either XL1-Blue or Top10F, both of which carry the F-plasmid containing the *ccd* operon. The selected mutants (Table S1), when overexpressed in the Top10 strain with saturating inducer concentration (0.1% arabinose), showed an inactive phenotype. This finding demonstrates that in contrast to WT CcdB, these mutants lack the ability to bind and poison the DNA gyrase, and thus to cause cell death. When the same inactive CcdB mutants were overexpressed in either Top10F or XL1-Blue, 7 of the 10 showed complete absence of colonies (Fig. S2), comparable to the phenotype observed with overexpression of WT CcdB. This finding indicated that most inactive, active-site mutants of CcdB, when overexpressed, were able to titrate out WT CcdA from the WT CcdA-CcdB complex. The released WT CcdB likely caused cell death by binding to its cellular target, DNA gyrase. Although Top10F and XL1-Blue are recA−, similar results were obtained in the recA containing *E. coli* strain AB264. As a negative control, several inactive, buried-site mutants (Table S1) were also overexpressed in both Top10 and Top10F− strains. These buried-site mutants are expected to have a distorted, destabilized, and aggregation-prone structure and thus are not expected to be able to release WT CcdB from the CcdAB complex. As expected, cells expressing these mutants did not show any growth defects and showed growth comparable to cells overexpressing the thioredoxin control protein, in both strains.

WT CcdB Causes Growth Arrest When Expressed at Low Levels. To examine whether lower amounts of free WT CcdB cause reversible growth arrest or cell death in our system, Top10F− and AB264 strains were transformed with inactive, active-site mutants and plated on LB/amp plates containing variable amounts of arabinose. Normal growth was observed in the range 0% to 1 × 10⁻²% arabinose. From 1 × 10⁻² to 2 × 10⁻²% arabinose, colony size became progressively smaller, although the number of colonies was unchanged and at higher arabinose concentrations (1 × 10⁻³% and higher) no colonies were observed. To examine if the growth inhibition at low arabinose concentrations was reversible, the strain carrying F-plasmid was transformed with the inactive, active-site mutant 100TCcdB and grown in liquid culture. Log-phase cells (OD₆₀₀ = 0.2) were induced with varying concentrations of arabinose for 2 h and plated on LB/amp plates containing 0.2% glucose to repress further expression of 100TCcdB from the Pₛᵦᵦ promoter. It was found that cells induced with up to 0.01% arabinose showed reversible growth inhibition, but at higher arabinose concentrations there was significant cell death (Fig. S3). This finding demonstrates that exposure of cells to low levels of WT CcdB causes reversible growth inhibition but higher levels cause cell death.

In Vitro Studies of CcdB Mutants Binding to DNA Gyrase and CcdA. To confirm that the inactive, active-site mutants of CcdB still bind CcdA but have diminished affinity for gyrase, four such mutants (24K, 24M, 95P, 100T) were purified and characterized by surface plasmon resonance (SPR). Purified GyrA14 (residues 363–494) or CcdA was immobilized on the surface of a CM5 chip. WT CcdB and all mutants were passed over the chip surface. Values of the measured parameters are summarized in Table S2. The data show that the mutants bind 5- to 50-fold more weakly to GyrA than WT CcdB, but bind CcdA with similar or higher affinity than WT CcdB.

Expression of Inactive, Active-Site Mutant Leads to Derepression of the *ccd* Promoter. The phenotypic effects observed on overexpression of the inactive, active-site mutants in the presence of WT CcdAB could arise through two different mechanisms. The inactive mutant could displace WT CcdB from its complex with CcdA either directly or by binding to free CcdA. In the latter case, the consequent decrease in free CcdA would lead to dissociation of WT CcdB from its complex with CcdA to maintain equilibrium. In either event, the free WT CcdB could then bind to its cellular target, DNA gyrase, resulting in growth inhibition or cell death. Alternatively (or in addition), an increase in the CcdB:CcdA ratio as a result of mutant expression could lead to derepression of the *ccd* operon. The data show that the mutants bind 5- to 50-fold more weakly to GyrA than WT CcdB, but bind CcdA with similar or higher affinity than WT CcdB. In the presence of excess 100TCcdB, the newly synthesized CcdB could bind to its cellular target, DNA gyrase. To determine whether expression of an inactive, active-site mutant of CcdB results in derepression of the *ccd* operon, two different approaches were used. In the first approach, the ability of excess 100TCcdB to decrease binding of WT CcdAB complex to promoter DNA was studied by SPR (Fig. S4) and EMSA (Fig. 2). In both cases, it was observed that DNA binding of the CcdAB complex was decreased in the presence of excess 100TCcdB, as described previously for WT CcdB (14). It can also be seen that the complex of
Ciprofloxacin-induced for 100TCcdB expression for 1 h were subsequently monitored for cell viability. For this process, AB264/p100TccdB cells showed then washed, plated on 0.2% glucose containing medium to re-

mycin C, cefotaxime, kanamycin, tobramycin) for 4 h. Cells were containing antibiotics or without any antibiotic. Bacterial growth resistance because of mutation during the course of the experi-

sistance because of mutation during the course of the experi-

S6). To rule out the possibility of development of antibiotic re-

Fig. 2. Binding of CcdA-WTcdB and CcdA-100TCcdB complex to ccdPO monitored by EMSA. A radiolabeled DNA fragment containing the ccdPO was incubated with increasing concentrations of CcdA-WTcdB (lanes 1–5) or CcdA-100TCcdB complex (lanes 6–8) at a CcdA:CcdB ratio of 1:5.1. The data show that CcdA-100TCcdB complex binds less well to DNA than CcdA-WTcdB (lanes 5 and 8) and also that addition of 100TCcdB to the preformed CcdA-

ccdP/O-gfp with CcdA. In an alternate approach, a GFP reporter was fused to the ccd operon by reducing ccd expression. These data show that in addition to its well-studied role in plasmid maintenance, the CcdAB system may also be involved in the generation of persisters.

Further Confirmation of CcdB’s Role in Persistence. The above studies involving overexpression of 100TCcdB suggested a possible role for the ccd operon in the generation of persisters. Use of an-

100TCcdB with CcdA (lanes 6–8, Fig. 2) binds ccdPO DNA, although less well than the WT CcdB:CcdA complex. This finding is consistent with an earlier study (15) that showed that 100R and 100E CcdB mutants led to loss of toxin activity but retained the ability to bind the operator when complexed to CcdA. In an alternate approach, a GFP reporter was fused to the ccd promoter and cloned to yield the plasmid pccdP/O-gfp with a p15A origin. The effect of expression of the inactive mutant, 100TCcdB on GFP expression was monitored in both Top10F’ and Top10 strains. As can be seen in Fig. S5A, the ratio of GFP expression in induced to uninduced cells was appreciably higher for Top10F’ strain. This finding suggests that overexpression of 100TCcdB leads to derepression of the ccd operon by reducing the CcdA:CcdB ratio, resulting in fresh synthesis of WT CcdB.

Overexpression Studies with 100TCcdB Suggest a Possible Role of CcdB in Generation ofPersisters. Plasmids expressing WT CcdB under control of the PBAD promoter are toxic to E. coli, even under highly repressed conditions (0.2% glucose) (12). However, expression of low levels of an inactive, active-site mutant (such as 100TCcdB) in cells containing the WT ccd operon, by the addition of 0.001% arabinose results in growth inhibition. Under these conditions, we examined the effects of the antibiotics ciprofloxacin, mitomycin C, cefotaxime, kanamycin, and tobra-

mycin on cell viability. For this process, AB264/p100TCcdB cells induced for 100TCcdB expression for 1 h were subsequently exposed to lethal doses of different drugs (ciprofloxacin, mito-

mycin C, cefotaxime, kanamycin, tobramycin) for 4 h. Cells were then washed, plated on 0.2% glucose containing medium to repress further expression of 100TCcdB, and the surviving colonies were counted. In comparison with the uninduced cells, induced cells showed ∼850-, 750-, and 350-fold more tolerance to ciprofloxacin, mitomycin C and cefotaxime, respectively, as well as 250- and 150-fold increased tolerance against kanamycin and tobramycin, respectively (Fig. 3). Compared with cells lacking the 100TCcdB plasmid, levels of tolerance were even higher (Fig. S6). To rule out the possibility of development of antibiotic resistance because of mutation during the course of the experiment, persister-derived colonies were replica plated on LB plates containing antibiotics or without any antibiotic. Bacterial growth was found only in the absence of any antibiotic. This finding confirms the generation of true persisters at low levels of CcdB expression. These data show that in addition to its well-studied role in plasmid maintenance, the CcdAB system may also be involved in the generation of persisters.

CcdB-Induced Persister Formation Is Dependent on RecA and Lon Protease. Poisoning of Gyrase:DNA complexes by CcdB in vivo, results in induction of a RecA mediated SOS response (16). The SOS response has previously been linked to persister formation via enhanced expression of TisB (17). To study the role of RecA on CcdB mediated persister formation, 100TCcdB was expressed in AB264 deleted for recA. Relative to the WT strain under identical conditions, the survival was over 100-fold reduced in the presence of ciprofloxacin and mitomycin C, respectively, and smaller reductions in survival were observed for the other antibiotics (Fig. 3). The involvement of RecA in DNA repair (18) explains why recA deletion makes bacteria particularly sensitive toward antibiotics that target DNA either directly (mitomycin C) or indirectly (ciprofloxacin).

Overexpression of inactive 100TCcdB results in an increase in the level of free WTcCdB, because of titration of any CcdA that is present as well as fresh synthesis of WT CcdB. The protease Lon is involved in degradation of free CcdA as well as activation of various genes during stress (19). In the present case, there should be little free CcdA present because of the presence of excess, inactive 100TCcdB. Nevertheless, to investigate the possible role of Lon in CcdB-mediated persister formation, the antibiotic sensitivity was examined in the presence and absence of overexpressed 100TCcdB in E. coli (Fig. S5). The results are consistent with an earlier study (15) that showed that overexpression of inactive 100TCcdB results in an increase in survival ratio relative to the WT strain and 50- to 200-fold decrease in survival ratio relative to the corresponding uninduced control (Fig. 3). This result clearly demonstrates that Lon also plays an important role in CcdB-mediated persister generation, apart from its role in antitoxin degradation (2, 5). Because both Lon and RecA are involved in CcdB-mediated persister generation, the above experiments were repeated in the AB264 lon Δlon double-knockout. In the double-knockout, persisters were further reduced to close to background levels, suggesting that the downstream pathways activated by Lon and RecA to generate persisters are at least partially independent.
active, active-site mutant, such as 100TCcdB, provides a powerful tool to conditionally regulate expression from the WT ccd operon. However, the WT CcdB levels generated using this system may not be physiologically relevant. Expression of the ccd operon is known to be triggered by various stresses, including heat (2, 20). We therefore compared the survival of cells with and without the ccd operon. Cells were first exposed to either heat or a sublethal dose of antibiotic prestress (21) (to derepress the operon) (Fig. S5B) followed by exposure to lethal concentrations of various different antibiotics (Fig. 4). Three different formats were used, namely: (i) Top10 cells containing F-plasmid, present at one to two copies, each molecule of plasmid contains one copy of the ccd operon but numerous other genes as well; (ii) Top10 cells transformed with the small multicopy pcc plasmid (10–12 copies per cell); and (iii) cells in which the ccd operon was integrated in single copy into the chromosome of the E. coli strain BW25113 (see SI Materials and Methods). This process was done not to simulate chromosomal ccd systems, but rather to quantitate the role of the F-plasmidic ccd operon in single copy on persister formation, in the absence of other F-plasmid genes. In each case, the number of persister cells were compared with the relevant control strain lacking the ccd operon (Fig. 4). In all cases, there was a significant increase in survival for cells containing the ccd operon, although the effects were not as large as those seen upon continuous expression of the inactive, active-site mutant (Fig. 3).

**Discussion**

In this study we have used a method to conditionally regulate the expression of TA systems and to obtain new insights into the role of the ccd operon in persistence. Although we have specifically applied this method to a prokaryotic TA system, it can be used for any regulated system, including those within eukaryotic cells wherein an inactive mutant of the protein can titrate a cellular inhibitor. The present studies show that through appropriate point mutations, it is possible to selectively modulate some binding/catalytic functions without affecting others, even in compact, single-domain globular proteins. In many cases it should be possible to set up appropriate genetic screens to isolate such mutants. In a related approach, overexpression of a nontoxic mutant of RelE was used to titrate endogenous RelB and thereby activate endogenous RelE (5). In the relBE, mazEF and many other TA systems the toxin component is enzymatically active. It is therefore relatively straightforward to design mutations that lead to loss of

![Fig. 4](image-url)  
**Fig. 4.** Role of CcdB in persister generation when present in multiple (A–C) or single copy (D–F). (A–C) Top10 strain containing either the multicopy plasmid pcc (Top10pccd) or F-plasmid (Top10F) or lacking any plasmid (Top10) were grown to an OD_{600} of 0.2–0.3. Subsequently, cells were exposed to different prestresses, either a sublethal dose of ampicillin (1 μg/mL) for 1 h (A), heat at 48 °C for 20 min (B), or without any prestress (C). Subsequently, cultures were exposed to different antibiotics at ~10 times the minimal inhibitory concentration for 4 h, washed, and then plated on LB agar media for cfu/mL determination. Survival ratio was calculated and is defined as the ratio of percent survival of the E. coli strain containing the ccd operon (either Top10pccd or Top10F) to that lacking the ccd operon (Top10) after antibiotic exposure. (D–F) A similar experiment was done in strains with the ccd operon integrated into the chromosome. Normalized data for BWccd (containing ccd) E. coli strain with ampicillin (1 μg/mL) prestress (D), heat prestress (E), and without any prestress (F) are shown. Normalization was done with respect to two different reference strains, BWcat (containing insertion of cat marker at the same locus as ccd) and BW25113 (WT E. coli strain, lacking both cat and ccd), respectively. Error bars indicate the SE from three independent experiments.
enzyme activity without significantly affecting antitoxin binding. The CcdAB system is more challenging because CcdB has no enzymatic activity. Furthermore, CcdB has significantly overlapping binding sites for CcdA and DNA gyrase and binds both full-length proteins with $K_D$s that are in the subnanomolar range (22) (Fig. 1B). Despite this challenge, it was possible to isolate CcdB mutants that abolish its Gyrase poisoning activity without affecting CcdA binding (Fig. S2 and Table S1), even at residue positions (such as 247) that are involved in binding both proteins. This is a surprising result, which demonstrates that it should be possible to apply this methodology to many other TA systems, to conditionally activate a single toxin in a background of multiple other TA systems because of the specificity of TA interactions. A recent study has shown that the ccdB$_fi$ system is able to mediate postsegregational killing in an E. coli strain harboring the ccdO157 system on its chromosome. This finding shows that the plasmid ccd$fi$ system is functional even in the presence of its chromosomal counterpart (23). Inhibitors of TA interactions can be important leads for the development of new drugs in case of TA systems that are involved in killing cells. The present study validates the CcdAB system as a potential drug target because complete titration of WT CcdA by an excess of inactive, active-site mutants of CcdB results in cell death.

The factors responsible for generating bacterial persisters are poorly understood. Transcriptional profiling of isolated persisters indicated an overexpression of TA modules (24, 25). Although the ectopic expression of several different toxins, such as RelA, MazF, HipA, and YgiU resulted in increased persistence, though the ectopic expression of several different toxins, such as RelA, MazF, HipA, and YgiU resulted in increased persistence, the chromosomally encoded ccdB$_fi$ operon is involved in plasmid maintenance (8). In the present study, a putative role for the F-plasmid CcdAB TA system in persistence was elucidated. Activation of endogenous CcdB either by CcdA titration or by transient heat or antibiotic stress lead to measurably higher amounts of persisters. Because the ccd operon occurs on the transmissible F-plasmid, it is also an example of a transmissible persistence factor. Homologs of F-plasmid ccd are also present on the chromosome of pathogenic strains of E. coli (E. coli O157: H7 strain EC4206, RefSeq accession no. ZP_03253116.1) and CcdB homologs are present in other human pathogens, such as Shigella dysenteriae (GenBank accession no. EF189125.1) and Vibrio cholera (RefSeq accession no. ZP_01978533). The sequence of the chromosomal ccd systems have diverged significantly from the F-plasmid ccd operon studied here, although in ~70% of cases both components of the chromosomal ccd have retained their toxic activity (26). Molecular evolutionary analysis of 47 isolates of ccdB$_{O157}$ suggested that the chromosomally encoded ccdAB TA systems appear devoid of any biological function and are under neutral evolution (26). Using approaches similar to the ones described herein should clarify whether or not specific chromosomally encoded ccd systems also contribute to persistence.

The present studies also suggest that, in addition to its known role in antitoxin degradation (2, 5), the Lon protease has other roles in the generation of persisters that need to be elucidated. Like ciprofloxacin and other fluoroquinolone antibiotics, CcdB produces DNA lesions by stabilizing a cleaved DNA-gyrase complex. This stabilization leads to induction of the RecA mediated SOS response. It is likely that this induction in turn leads activation of other TA loci, the promoters of which contain a Lex binding site. It has previously been shown that ciprofloxacin can induce formation of persisters by SOS-mediated activation of the risA, as well as other TA systems, which in turn may lead to dormancy (17). A similar mechanism may hold for CcdB also and this is outlined in Fig. 5. A stress that leads to elevated levels of Lon will lead to release of CcdB from its complex with CcdA and the resulting DNA lesion will result in an SOS response, ultimately triggering conversion of cells to a dormant state.

![Schematic representation of CcdB induced persister formation.](image)

**Materials and Methods**

E. coli host strains and plasmids used for this study are listed in Table S3. The Top10 strain, which lacks the F-plasmid-encoded WT CcdA and CcdB, was used to select inactive mutants of CcdB. XL1-Blue, Top10F and AB284 strains have the F-plasmid that carries the ccd operon. All strains except AB284 are recA$^-$. BW25113 is a WT E. coli K12 strain that was used for chromosomal insertions of the ccd operon and cat cassette. The ccdB gene was expressed under control of the arabinosine P$_{BAD}$ promoter in plasmid pBAD24cddb. The CcdB mutants for the study were taken from the library of CcdB mutants described previously (12). The GyrA14 fragment (residues 363–494) (11), CcdA (27), and CcdB (28) were expressed and purified in E. coli. Additional methodological details are given in SI Materials and Methods.

**ACKNOWLEDGMENTS**

We thank Profs. John E. Cronan, Remy Loris, and Laurence Van Meldenere for the wild GFP, GyrA14, and CcdA plasmids, respectively; Anusmita Sahoo and Bharat Adkar for preparing Fig. 1 and Table S1; and Dr. Pranveer Singh for the construction of the Top10 Escherichia coli strain. A.T. and P.C.D. are Council of Scientific and Industrial Research Fellows.


Supporting Information

Tripathi et al. 10.1073/pnas.1121217109

SI Materials and Methods

Bacterial Growth Monitored as a Function of Arabinose Concentration. Top10, Top10F, XL1-Blue, and AB264 Escherichia coli strains were transformed in parallel with plasmids expressing inactive, active-site mutants of CdcB and WT Trx under control of the arabinose inducible PBAD promoter. Top10 lacks the ccd operon but Top10F (recA'), XL1-Blue (recA'), and AB264 (recA') have the ccd operon present on their resident F-plasmid. Five micro-
liters of the transformation mix was grown overnight in 5 mL of LB/amp (100 μg/mL). Unless indicated otherwise, cultures were grown at 37 °C with shaking at 180 rpm. Fifty microliters of a 10^{-4} dilution was plated on LB-amp plates containing a range of arabinose concentrations (0%, 1 x 10^{-6}, 1 x 10^{-5}, 1 x 10^{-4}, 1 x 10^{-3}, 1 x 10^{-2}, 1 x 10^{-1}, and 2 x 10^{-1}%). Cells transformed with pBADtrx were used as a control.

Measurement of Binding of Inactive, Active- site Mutants to DNA Gyrase or CcdA by Surface Plasmon Resonance. The Biacore 3000 system was used in this study. HBS buffer (10 mM Hepes, 0.15 M NaCl, 3 mM EDTA, 0.005% Surfactant P20, pH 7.4) was used as the binding buffer and 10 mM Glycine-HCl was used as the regeneration buffer. About 800 RU of either the Glycine A14 or CcdA proteins were immobilized on CM5 sensor chips by an amine-coupling reaction. The control channel was treated in the same way as the assay channel but without any immobilized protein. WT CcdB and its inactive, active-site mutants 24K, 24M, 95P, 100T were used as analyte at concentrations ranging from 15.6 nM to 500 nM in HBS buffer. The binding was carried out at 37 °C with a flow rate of 30 μL/min and data were collected for 100 s of association and 200 s of dissociation. To analyze the data, the assay channel was subtracted from the control channel to eliminate nonspecific interaction. Multiple sensorgrams with different concentrations of analyte were overlaid and aligned. Kinetic constants were calculated by the BIAevaluation 3.1 software with nonlinear fitting, the 1:1 (Langmuir) binding model was used, where $K_A = k_a/k_d$ and $K_D = k_D/k_A$.

Kinetics of Bacterial Growth Revival Monitored on Glucose-Containing Medium. Top10, Top10F, and AB264 strains of E. coli transformed with inactive, active-site mutant 100T CcdB were grown overnight in LB/amp and a 1:100 dilution of the overnight culture was grown to an OD_{600} of 0.2. An aliquot of the culture was withdrawn and 10-fold serial dilutions were plated on 2% glucose plates. To each of the remaining cultures, arabi-
nose was added to a final concentration of 0.01 or 0.1%. Sub-
sequently, 500-μL aliquots were withdrawn at different time points and centrifuged at 1,520 × g for 5 min at room temper-
ature. The pellet cells were washed twice with 500 μL of LB. Tenfold serial dilutions were prepared and plated on 0.2% glu-
 cose plates. The survival cfu was calculated at each time point.

Construction of Plasmid (pcddP/O-gfp) Containing Transcriptional Fusion of Promoter-Operator of cdd with WT gfp. A colony PCR was done using E. coli strain XL1-Blue (containing ccd operon on F plasmid) to amplify a 241-bp DNA fragment containing 113-bp ccd promoter using primers cddp1 (ClaI) and cddp2 (SphI) (1). The resulting amplified fragment was digested with ClaI and SphI restriction enzymes and inserted into ClaI and SphI digested pRK6 plasmid (2), thereby replacing the Pbad promoter and the anaC gene with the ccd promoter-operator upstream of a GFP reporter. The clone was confirmed by DNA sequencing.

Construction of pcdd Plasmid. Colony PCR was done to amplify the ccd operon from F plasmid of XL1-Blue strain using primers cddp1 (ClaI) and cddAB (SphI) and cloned as done for plasmid pcddP/O-gfp. The clone was confirmed by DNA sequencing.

Construction of Strain BWcdc and BWcat. The ccd operon and cat gene were amplified separately from the pccdc plasmid by using cddpRK1, cddpRK2 and catpRK1, catpRK2 primers, respectively, and fused by overlap PCR to generate a ccdABcat cassette. Next, 50-bp sequences homologous to the intB gene were added at both ends of the ccdABcat cassette by using primers cat1, cddAB1 and cat2, cddAB2, respectively, in two successive rounds of PCR. The final construct was inserted at the intB locus in E. coli strain BW25113 using λ red recombination (3). Recombinants were screened on chloramphenicol (25 μg/mL) containing LB plates and the presence of the ccd operon was confirmed by both PCR using primers intBP1 and intBP2, as well as DNA sequencing. For the construction of BWcat, cat cassette was amplified from pcdd plasmid by using catpRK1 and catpRK3 primers, respectively, and at both sides of the amplified cat cassette, homologous sequences to intB were added in subsequent PCR steps. A similar procedure was followed for insertion of the cat cassette at the intB locus as described above for BWcdd.

Construction of AB264 Deletion Strains. To construct AB264Δlon, the cat cassette was amplified from pKD3 plasmid by using lon1f and lon1r primers. Sequences homologous to flanking region of lon gene were added in a subsequent PCR by using primers lon2f and lon2r, respectively. The PCR product was electroporated into AB264/pKD46 and the remaining procedure was as previously described (3). Similarly AB264Δarec and AB264Δlonarec strains were constructed by using primers rec1f, rec1r, rec2f, and rec2r, respectively. In all of the constructed strains, the absence of the cat cassette was confirmed by PCR as well by DNA sequencing using primers lon3f and lon3r for the Δlon strain; rec3f and rec3r primers were used for the Δarec strain. All three strains were made markerless by using plasmid pCP20, expressing Flippase recombinase (4). All deletions were verified by PCR.

EMSA. A 241-bp DNA fragment containing the 113-bp promoter-operator of the ccd operon was used for the DNA-binding assay (1). This fragment was radiolabeled by PCR using (α-32P) ATP, primers cddp1 (ClaI) and cddp2 (SphI) and E. coli F plasmid as a template. Binding reactions were carried out in a 15-μL volume at 30 °C for 30 min. The reaction contained 1 nM of probe, 1 μg of sonicated salmon sperm DNA in 30 mM Tris-HCl, pH 7.5, 200 mM NaCl, 15 mM MgCl2, 3 mM EDTA, 150 μg/mL BSA, 0.3 mM DTT, and varying concentrations of purified proteins. Electrophoresis was performed in Tris borate-EDTA buffer at 50 V in a 5% (wt/vol) polyacrylamide gel at 4 °C. Gel was dried, exposed overnight, and scanned (Typhoon 9210, GE Healthcare).

Surface Plasmon Resonance Analysis. For DNA binding studies, surface plasmon resonance (SPR) analysis was carried out on a Biacore 3000 instrument with streptavidin-coated SA sensor chips. Next, 800 RU of a 5’ biotin-labeled 241-bp double-stranded DNA containing the ccd promoter-operator was immobilized on flow cell 2 by injecting running buffer (15 mM MgCl2, 0.3 mM DTT, 16 mM EDTA, 200 mM NaCl, 20 mM Hepes, 0.005% p20 surfactant, pH 7.5) containing 2 μM DNA,
whereas flow cell 1 was left blank for reference. Fifty microliters of CcdA, CcdA-WTCcdB, CcdA-100TCcdB, or CcdA-WTCCdB complex with additional 100TCcdB, was diluted to the desired concentration in running buffer and injected over both flow cells at a flow rate of 30 µL·min⁻¹. Regeneration was done with 100 mM NaCl. The binding data were fitted using BIAevaluation 3.1 software to obtain on and off rate constants for DNA binding.

**Flow Cytometry Studies.** *E. coli* strains Top10 and Top10F were cotransformed with pccdp/O-gfp and pBAD100TCcdB plasmids and grown overnight in LB containing chloramphenicol (34 µg/mL), ampicillin (100 µg/mL). Overnight cultures of both strains were diluted 100-fold in LB and grown at 37 °C till OD₆₀₀ of 0.2 and the expression of 100TCcdB protein was induced with 0.1% arabinose. After an additional 3 h of growth, cells were washed, resuspended in 0.9% saline, and expression of the GFP reporter was assayed by flow cytometry.

**Isolation of Persisters After Expression of 100TCcdB in E. coli Strain AB264.** *E. coli* strain AB264 transformed with p100TCcdB were grown overnight in LB + amp (100 µg/mL). Following 1:100 dilution, cells were grown to an OD₆₀₀ of 0.2 and induced with 0.001% arabinose for 1 h. An aliquot was withdrawn after 1 h, washed, appropriately diluted, and plated on LB containing 0.2% glucose plates to determine the starting cfu. Each of the remaining cultures was treated with different drugs at 10x 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. The cultures were washed twice, appropriately diluted and plated on 0.2% glucose plates to determine the viable counts after antibiotic treatment. The percent survival was calculated as the ratio (cfu after antibiotic exposure/cfu before antibiotic exposure) × 100. Survival ratio is defined as the ratio of percent survival of the *E. coli* strain containing the *ccd* operon to that lacking the *ccd* operon or to the same strain but with uninduced 100TCcdB.

**Isolation of Persisters in Strains Deleted for lon and recA.** *E. coli* strains AB264 (WT), AB264Δlon, AB264Δrec, and AB264ΔlonΔrec transformed with p100TCcdB were grown overnight in LB + amp (100 µg/mL). Following 1:100 dilution, cells were grown to an OD₆₀₀ of 0.2 and induced with 0.001% arabinose for 1 h. Cells were exposed to antibiotics as in the previous section and plated on glucose containing plates. The percent survival was calculated as the ratio (cfu after antibiotic exposure/cfu before antibiotic exposure) × 100. Survival ratio is defined as the percent survival of the *E. coli* strain expressing 100TCcdB to the same strain with uninduced 100TCcdB.

**Activation of CcdB by Heat or Antibiotic Stress.** Overnight cultures of *E. coli* strains Top10 (lacks ccd operon), Top10pccd (contains ccd operon on multicopy plasmid, pccdp, a pRK5 derivative), Top10F (contains F plasmid borne ccd operon), BWcd (BW25113 with chromosomal insertion of ccd operon and cat cassette), BWcat (BW25113 with chromosomal insertion of only the cat cassette), and BW25113 *E. coli* strain (control strain lacking ccd operon) were diluted 100-fold and grown to OD₆₀₀ of 0.3 (~10⁷ cells/mL). Subsequently, cells were subjected to two different types of sublethal prestresses, namely exposure of cells to high temperature of 48 °C for 20 min or a sublethal dose of ampicillin (1 µg/mL for 1 h) (5). This process was followed by exposure to different antibiotics at lethal doses (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. The cultures were washed twice, appropriately diluted and plated on LB-agar plates to determine the viable counts after antibiotic treatment. The percent survival for each *E. coli* strain was calculated. Percent survival was defined as the ratio (cfu after antibiotic exposure/cfu before antibiotic exposure) × 100. For the calculation of survival ratio, percent survival of BWcd strain was normalized to percent survival of two different reference strains. One was WT *E. coli* strain BW25113 (without any insertion) and another was *E. coli* strain, BWcat, having cat cassette insertion at the same locus as *ccd*. Survival ratio is defined as the ratio of percent survival of the *E. coli* strain containing the *ccd* operon to that for the same strain lacking the *ccd* operon (either BW25113 or BWcat).

Express the WT toxin

Express inactive mutant of the toxin

Increased concentration of WT toxin due to

a) Displacement from TA complex by mutant

b) Derepression of TA operon and fresh synthesis

Active WT toxin binds to target

Inactive toxin fails to bind to target

Express an inactive, active-site mutant of the toxin

Inactive toxin is not intrinsically toxic. Hence, expression of the inactive, active-site mutant in the presence of the WT TA complex offers a more sensitive method of tuning the level of WT toxin (A) than overexpression of WT toxin (B).

Inactive, active-site mutants of CcdB inhibit cell growth only in the presence of the WT ccd operon. Top10 (Δccd) and XL1-Blue (ccd) strains were transformed in parallel with plasmids expressing inactive, active-site mutants of CcdB. Following transformation and overnight growth to correct for differences in transformation efficiency, 50 μL of 10^{-4} dilution was plated on LB/amp plates containing 0% or 0.1% arabinose. Cells transformed with pBADtrx were used as a control to show that overexpression of a nontoxic protein does not affect cell growth. The inactive mutants at the active-site of CcdB (24M, 24K, 95P, and 100T) inhibit cell growth in XL1-Blue strain at 0.1% arabinose in comparison with the growth observed under identical conditions in Top10 strain.
Fig. S3. Effect of toxin expression on cell viability. Viability as a function of time of XL1-Blue and Top10 strains expressing plasmid borne inactive, active-site 100TCcdB mutant. Cells were induced at an OD_{600} of 0.2 with 0.01% or 0.1% arabinose, incubated for various time points, appropriately diluted, and plated on LB/amp plates containing 0.2% glucose to repress 100TCcdB expression. Surviving colonies were counted for each strain. 100TCcdB inhibits growth only in XL1-Blue but not in Top10 strain and the extent of inhibition depends on the level of expression of 100TCcdB. Low expression levels (0.01% arabinose) result in reduced toxicity and cause reversible growth inhibition.

Fig. S4.Binding of CcdA-WTCcdB and CcdA-100TCcdB complex to ccdP/O DNA monitored by SPR. (A) Binding of CcdA-WTCcdB complex, (B) binding of CcdA-100TCcdB complex, (C) binding of CcdA-WTCcdB complex in the presence of varying concentrations of 100TCcdB, where constant concentrations of CcdA (400 nM) and WTCcdB (260 nM) were used. (A and B) Increasing concentrations of CcdA, from bottom to top, are 200, 400, 500, 600, and 800 nM, are used while keeping a constant ratio of 1.5:1 for CcdA to CcdB. (C) Binding is observed in the absence (dotted line) and presence of increasing concentrations of 100TCcdB (solid lines). From top to bottom, 100TCcdB concentrations are 200, 400, 800, and 1,000 nM, respectively. Surface density 800 RU; buffer, pH 7.5 (15 mM MgCl₂, 0.3 mM DTT, 16 mM EDTA, 200 mM NaCl, 20 mM Heps, 0.005% P20 surfactant; flow rate, 30 μL/min. CcdA-100TCcdB binds weaker to the ccdP/O compared with the CcdA-WTCcdB complex. The CcdA-WTCcdB complex is able to bind to ccdP/O in the presence of excess 100TCcdB, but with reduced affinity.
**Fig. S5.** ccd promoter activity is enhanced upon overexpression of an inactive, active-site mutant of CcdB and upon exposure to sublethal prestresses. (A) Both Top10F' (contains F plasmid borne ccd operon) and Top10 (lacks ccd operon) E. coli strains were cotransformed with plasmids pccdP/O-gfp and pBAD100TccdB. Overnight cultures of both strains were diluted 100-fold in LB and grown at 37 °C till OD_{600} of 0.2. The expression of 100TccdB protein was induced with 0.1% arabinose. After 3 h of induction, GFP expression was monitored in both uninduced and induced cells by flow cytometry. Mean fluorescence intensity (MFI) ratio in Top10F' and Top10 strains are shown. MFI ratio is MFI (induced 100TccdB)/MFI (uninduced 100TccdB). This ratio is higher in Top10F' strain relative to Top10 strain, suggesting that titration of CcdA by 100TccdB in the former strain leads to derepression of the ccd operon and additional synthesis of the GFP reporter. (B) Top10 and Top10F' E. coli strains were transformed with plasmid, pccdP/O-gfp and grown to an OD_{600} of 0.2–0.3. Subsequently, cells were exposed to two different prestresses: either a sublethal dose of ampicillin (1 μg/mL) for 1 h or heat at 48 °C for 20 min. GFP expression was subsequently monitored by flow cytometry. The MFI ratio is MFI (with prestress)/MFI (without prestress), shown for both strains. The MFI ratio is higher in strain Top10F' for both types of stress, indicating activation of the ccd operon by both types of stress. Error bars are not visible in plots as similar MFI values were obtained in the two independent experiments.

**Fig. S6.** Viability of various E. coli strains in the presence of different antibiotics. All strains were grown until an OD_{600} of 0.2 and subsequently exposed to lethal doses of different antibiotics for 4 h. The percent survival was calculated as the ratio (cfu after antibiotic exposure/cfu before antibiotic exposure) × 100. (A) The percent survival of individual strains lacking the plasmid pBAD100TccdB. (B) The percent survival of the same strains with uninduced plasmid pBAD100TccdB. The percent survival of strains without plasmid is typically lower than the corresponding strain containing the uninduced plasmid. The y axis is shown in log scale and error bars indicate the SE from two independent experiments.
Table S1. Phenotypes of inactive mutants at both active-site and buried residues in Top10 and XL1-Blue strains

<table>
<thead>
<tr>
<th>Residue position</th>
<th>WT residue</th>
<th>Mutant residue</th>
<th>ACC (%)</th>
<th>Phenotype</th>
<th>Top10 ASA (CcdA)* (Å²)</th>
<th>X1-Blue ASA (GyrA14)† (Å²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>18‡</td>
<td>V</td>
<td>D</td>
<td>0</td>
<td>I</td>
<td>I</td>
<td>0</td>
</tr>
<tr>
<td>33‡</td>
<td>V</td>
<td>D</td>
<td>1</td>
<td>I</td>
<td>I</td>
<td>4</td>
</tr>
<tr>
<td>34‡</td>
<td>I</td>
<td>D</td>
<td>0</td>
<td>I</td>
<td>I</td>
<td>0</td>
</tr>
<tr>
<td>36‡</td>
<td>L</td>
<td>D</td>
<td>0</td>
<td>I</td>
<td>I</td>
<td>0</td>
</tr>
<tr>
<td>90‡</td>
<td>I</td>
<td>D</td>
<td>0</td>
<td>I</td>
<td>I</td>
<td>0</td>
</tr>
<tr>
<td>93‡</td>
<td>A</td>
<td>D</td>
<td>0</td>
<td>I</td>
<td>I</td>
<td>0</td>
</tr>
<tr>
<td>24§</td>
<td>I</td>
<td>K</td>
<td>66</td>
<td>I</td>
<td>A</td>
<td>98</td>
</tr>
<tr>
<td>24§</td>
<td>I</td>
<td>M</td>
<td>66</td>
<td>I</td>
<td>A</td>
<td>98</td>
</tr>
<tr>
<td>95§</td>
<td>N</td>
<td>D</td>
<td>39</td>
<td>I</td>
<td>A</td>
<td>0</td>
</tr>
<tr>
<td>95§</td>
<td>N</td>
<td>P</td>
<td>39</td>
<td>I</td>
<td>A</td>
<td>0</td>
</tr>
<tr>
<td>98§</td>
<td>F</td>
<td>D</td>
<td>1</td>
<td>I</td>
<td>A</td>
<td>0</td>
</tr>
<tr>
<td>99§</td>
<td>W</td>
<td>K</td>
<td>19</td>
<td>I</td>
<td>A</td>
<td>0</td>
</tr>
<tr>
<td>100§</td>
<td>G</td>
<td>T</td>
<td>4</td>
<td>I</td>
<td>A</td>
<td>1</td>
</tr>
<tr>
<td>101§</td>
<td>I</td>
<td>K</td>
<td>125</td>
<td>I</td>
<td>I</td>
<td>94</td>
</tr>
</tbody>
</table>

I, A, and PA refer to inactive, active, and partially active phenotypes, respectively. Mutants at active-site residues show an active phenotype only in XL1-Blue strain, whereas mutants at buried residues show an inactive phenotype in both strains.

*Average accessible surface area change for residue on CcdB upon interaction with CcdA (PDB ID 3G7Z) (ref. 1).
†Average accessible surface area change for residue on CcdB upon interaction with GyrA (PDB ID 1X75) (ref. 2).
‡Buried-site inactive mutants.
§Active-site inactive mutants.
{Partially active mutant.

Table S2. Rate constants ($k_{on}$, $k_{off}$) and dissociation constants ($K_D$) for binding of WT and inactive, active-site CcdB mutants to GyrA (residues 363–494) and CcdA monitored by SPR at pH 7, 37 °C

<table>
<thead>
<tr>
<th>Immobilized protein</th>
<th>GyrA14</th>
<th>CcdA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$k_{on}$ (M$^{-1}$s$^{-1}$)</td>
<td>$k_{off}$ (s$^{-1}$)</td>
</tr>
<tr>
<td>WT CcdB</td>
<td>2.4 × 10$^6$</td>
<td>2.9 × 10$^{-3}$</td>
</tr>
<tr>
<td>24K</td>
<td>5.8 × 10$^4$</td>
<td>3.9 × 10$^{-3}$</td>
</tr>
<tr>
<td>24M</td>
<td>6.2 × 10$^3$</td>
<td>2.6 × 10$^{-3}$</td>
</tr>
<tr>
<td>95P</td>
<td>3.1 × 10$^4$</td>
<td>4.3 × 10$^{-3}$</td>
</tr>
<tr>
<td>100T</td>
<td>4.2 × 10$^3$</td>
<td>3.5 × 10$^{-3}$</td>
</tr>
</tbody>
</table>

Relative to WT CcdB, inactive, active-site mutants show decreased binding to GyrA but similar or tighter binding to CcdA.

Table S3. *E. coli* strains and plasmid used in this study

<table>
<thead>
<tr>
<th>Strains and plasmids</th>
<th>Genotype/relevant characteristics</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AB264</td>
<td>recA*, F* (ccd operon)</td>
<td>CGSC at Yale</td>
</tr>
<tr>
<td>AB264Δlon recA</td>
<td>recA*, F* (ccd operon), lon⁻</td>
<td>Present work</td>
</tr>
<tr>
<td>AB264Δlon ΔrecA</td>
<td>recA⁻, F* (ccd operon), lon⁻</td>
<td>Present work</td>
</tr>
<tr>
<td>BW25113</td>
<td>recA*</td>
<td>CGSC at Yale</td>
</tr>
<tr>
<td>BWccd</td>
<td>Cm⁹, BW25113 with ccd operon and cat marker inserted at intB locus</td>
<td>Present work</td>
</tr>
<tr>
<td>BWcat</td>
<td>Cm⁹, BW25113 with cat marker inserted at intB locus</td>
<td>Present work</td>
</tr>
<tr>
<td>Top10</td>
<td>recA⁻, Sm⁹</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Top10F</td>
<td>recA⁻, Tet⁰, Sm⁹, F* (ccd operon)</td>
<td>Present work</td>
</tr>
<tr>
<td>XL1-Blue</td>
<td>recA⁻, Tet⁰, F* (ccd operon)</td>
<td>Stratagene</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pBAD24ccdB</td>
<td>Amp⁸, P₉BAD promoter, ccdB</td>
<td>Refs. 1 and 2</td>
</tr>
<tr>
<td>pRK6</td>
<td>Cm⁹, P₉BAD promoter, WT gfp gene</td>
<td>Ref.3</td>
</tr>
<tr>
<td>pccdP/O-gfp</td>
<td>Cm⁹, ccd promoter, WT gfp gene</td>
<td>Present work</td>
</tr>
<tr>
<td>pccd</td>
<td>Cm⁹, ccd operon</td>
<td>Present work</td>
</tr>
</tbody>
</table>

Tet⁰, Sm⁹, Amp⁸ and Cm⁹, are tetracycline, streptomycin, ampicillin and chloramphenicol resistance, respectively. CGSC is the *E. coli* genetic resource, Coli Genetic Stock Center at Yale.