Bacterial addiction module toxin Doc inhibits translation elongation through its association with the 30S ribosomal subunit

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Bacterial toxin–antitoxin (TA) systems (or ''addiction modules'') typically facilitate cell survival during intervals of stress by inducing a state of reversible growth arrest. However, upon prolonged stress, TA toxin action leads to cell death. TA systems have also been implicated in several clinically important phenomena: biofilm formation, bacterial persistence during antibiotic treatment, and bacterial pathogenesis. TA systems harbored by pathogens also serve as attractive antibiotic targets. To date, the mechanism of action of the majority of known TA toxins has not yet been elucidated. We determined the mode of action of the Doc toxin of the Phd-Doc TA system. Doc expression resulted in rapid cell growth arrest and marked inhibition of translation without significant perturbation of transcription or replication. However, Doc did not cleave mRNA as do other addiction-module toxins whose activities result in translation inhibition. Instead, Doc induction mimicked the effects of treatment with the aminoglycoside antibiotic hygromycin B (HygB): Both Doc and HygB interacted with 30S ribosomal subunits, stabilized polysomes, and resulted in a significant increase in mRNA half-life. HygB also competed with ribosome-bound Doc, whereas HygB-resistant mutants suppressed Doc toxicity, suggesting that the Doc-binding site includes that of HygB (i.e., helix 44 region of 16S rRNA containing the A, P, and E sites). Overall, our results illuminate an intracellular target and mechanism of TA toxin action drawn from aminoglycoside antibiotics: Doc toxicity is the result of inhibition of translation elongation, possibly at the translocation step, through its interaction with the 30S ribosomal subunit.

antitoxin | hygromycin B | bacteriophage P1 | 16S rRNA | postsegregational killing

Toxin–antitoxin (TA) systems, also known as suicide or addiction modules, represent an adaptation used by most free-living bacteria (1, 2). TA modules are found either in bacterial genomes, on extrachromosomal bacterial plasmids or in bacteriophage genomes that lysogenize as low-copy plasmids. The protein sequences of known TA systems (>650 to date) fit into ≈10 conserved families. TA system toxin proteins are distinct from classic bacterial toxin proteins such as anthrax, cholera, or diphtheria (which are actually exotoxins). In contrast, TA toxins function inside the cell, and they enlist a cognate, labile antitoxin to impart reversible control over cell growth by modulating the level of the antitoxin relative to the stable toxin protein.

In general, TA systems appear to have evolved as genetic adaptations that impart distinct functions depending on whether they are carried on mobile elements or integrated into the chromosome. Chromosomal TA systems appear to be responsible for the phenotypic switch to a quasidormant state that enables cell survival during stress (e.g., antibiotic treatment, UV exposure, temperature extremes or nutrient deprivation) (3). This quasidormant state can be rapidly reversed if the stressor is removed within a certain window of time, triggering the production of antitoxin that sequesters free toxin. Once cells pass beyond this reversible window of time into a "point of no return," they can no longer be rescued from their quasidormant state (4–6). Therefore, chromosomal TA toxin action only leads to cell death (proposed to represent a type of programmed cell death distinct from apoptosis in eukaryotic systems) if and when the cell reaches the limit of its capacity to either sustain quasidormancy and/or lose the ability to initiate the synthesis of enough antitoxin to reverse toxin action upon release from stress. This pathway of bacterial programmed cell death has been proposed to facilitate death of a subpopulation of damaged cells to preserve food for the population as a whole, serve as a defense against the spread of bacteriophage infection, and act as a mechanism to eliminate cells carrying deleterious mutations that would otherwise be passed to the following generations (7). In fact, we have recently demonstrated that *Myxococcus xanthus* MazF initiates bacterial programmed cell death that is instrumental for multicellular development of this organism (8). Chromosomal TA systems have recently been linked to medically important phenomena such as biofilm formation (communities of microorganisms that propagate on solid surfaces) and bacterial persistence upon antibiotic exposure (9–13). More importantly, with the increase in antibiotic-resistant pathogens, TA systems would make attractive targets for new antibiotics designed to induce toxin-mediated cell suicide (7).

The only Phd-Doc addiction module studied to date is that derived from the bacteriophage P1, which lysogenizes in *Escherichia coli* cells as a stable low-copy plasmid. In contrast to chromosomal TA systems, the P1-encoded *phd-doc* TA system—as well as other plasmid-encoded TA systems such as *ccdAB*, *kis-kid*, *pemIK*, and *parDE*—ensure their stable maintenance in host cells by a mechanism called postsegregational killing (14). If a bacterial cell is cured of a plasmid that harbors a TA module (e.g., the plasmid fails to segregate to a newly divided cell) the fate of the host cell is sealed by the presence of the existing antitoxin and toxin proteins. In fact, the alternative term "addiction module" for TA systems relates to their role in postsegregational killing. Because the antitoxin is susceptible to protease degradation, the amount of free toxin increases as the antitoxin levels diminish, eventually leading to cell death. Therefore, although toxicity is reversible in chromosomal TA systems, the inability to synthesize new antitoxin (because of the absence of the *phd-doc* TA module DNA after plasmid loss) should preclude reversibility of P1-mediated Doc toxicity. In fact, the TA system harbored by bacteriophage P1 was discovered and named as a consequence of the properties of the antitoxin Phd (whose coexpression with the toxin gene was demonstrated to prevent host

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death) and toxin Doc (which was responsible for host cell death on curing) (15).

Both the regulation of the *phd-doc* TA module, and the biophysical properties of the P1 Phd-Doc TA complex, have been studied in detail. Phd-Doc and their corresponding genes exhibit characteristic properties of TA systems. The genes encoding Phd and Doc reside in an operon (16), and the antitoxin (Phd, 73 aa/8.1 kDa) and toxin (Doc, 126 aa/13.6 kDa) are relatively small proteins. Two Phd antitoxin polypeptides form a 2:1 heterotrimer with one Doc toxin polypeptide (17) to both inhibit the toxin activity of Doc and autoregulate antitoxin–toxin module transcription (18, 19). Phd dimers are subject to cleavage by ClpXP protease (20) and can also autoregulate the module (21, 22). However, the mechanism of the Doc-mediated cell death is not known.

In this work, we pinpoint the molecular mechanism of Doc action derived from bacteriophage P1. Doc expression results in rapid cell-growth arrest accompanied by inhibition of translation without significant perturbation of transcription or replication. However, Doc does not cleave mRNA, as documented with several other TA toxins whose activities result in translation arrest. In fact, mRNA is significantly stabilized upon Doc induction. Doc induction exhibited other significant parallels to treatment with the aminoglycoside antibiotic hygromycin B (HygB), suggesting similarity in their mechanisms of action. Doc toxicity appears to result from a block in translation elongation resulting from Doc interaction with the 30S ribosomal subunit. Furthermore, the ability of HygB to compete for Doc binding to the 30S ribosomal subunit, coupled with the loss of Doc toxicity in a HygB-resistant bacterial strain suggests that the Doc-binding site includes that of HygB—the highly conserved 16S rRNA helix 44 at the 30S–50S interface of the ribosome containing the P and A sites essential for protein translation.

Results

Doc Expression Leads to Translation Arrest. In contrast to classic secreted toxins designed to kill those cells that do not possess immunity, TA system toxins always act inside the cell that synthesizes them, and their toxicity is reversible. Therefore, we predicted that Doc is likely to disrupt one of the essential cellular machineries that carry out RNA transcription, protein translation, or DNA replication instead of targeting the cell wall.

To dissect Doc function in living cells, we used an *E. coli* strain containing an arabinose-inducible low-copy pBAD33 plasmid with a pACYC origin (23) to enable Doc expression. TA toxin expression from this relatively tightly regulated system is a reliable method for mimicking the normal process of the activation of a single toxin *in vivo* (i.e., through protease cleavage of its cognate antitoxin to release free toxin). Although selected stress conditions can be used to activate TA toxins by inducing cleavage of their cognate antitoxins, there is no known trigger for Doc only. In fact, it is likely that at least some stresses can trigger activation of more than one of the *E. coli* TA toxin family members (24).

We first used permeabilized *E. coli* cells to determine whether toxin expression affects DNA replication, transcription, or translation by quantifying incorporation of isotope labeled precursors of DNA, RNA or protein, respectively. We harvested *E. coli* cells that had been grown with or without arabinose for Doc induction, permeabilized their membranes after a brief exposure to toluene (25), and measured ATP-dependent DNA, RNA, or protein synthesis (ATP must be supplied to drive energy, requiring metabolic processes in permeabilized cells). Our results suggested that Doc targets translation, but not transcription or replication, because only [^{35}S]methionine incorporation was inhibited [supporting information (SI) Fig. 7].

We then measured the effect of Doc induction on growth and translation *in vivo*. As expected, Doc expression was toxic; cell growth was arrested within 20 min of Doc induction (Fig. 1*A*), with a corresponding reduction in colony-forming units that paralleled the trend of the growth profile (data not shown). Aliquots of cells

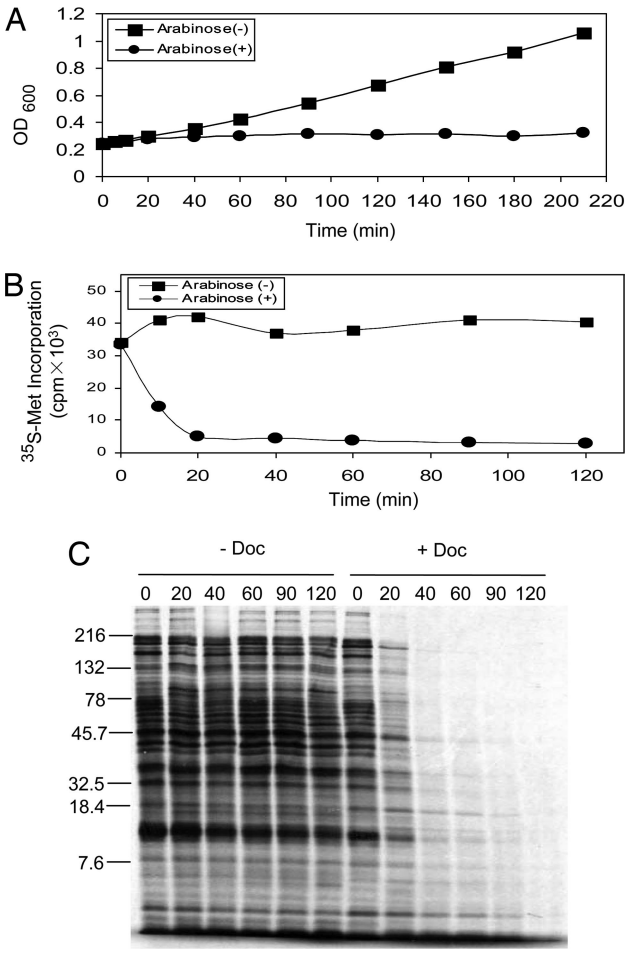


Fig. 1. Doc expression leads to translation arrest. (*A*) Growth profile of Doc-induced (Arabinose+) or uninduced (Arabinose−) BW25113 cells containing pBAD33-Doc grown at 37°C in M9 medium containing 0.2% glycerol as the sole carbon source with 25 μg/ml chloramphenicol. (*B*) Quantification of [^{35}S]methionine incorporation into Doc-induced (Arabinose+) or uninduced (Arabinose−) cells. (*C*) [^{35}S]methionine incorporation of Doc-induced or uninduced cells *in vivo*. Equivalent amounts of cell lysate, derived from equal culture volumes, were subjected to SDS/PAGE, followed by autoradiography. Time points correspond to those in *B*; cell samples for experiments in *B* and *C* were derived from the growth-profile experiment shown in *A*. Molecular mass markers (kDa) are shown on the left.

from early time points from the same growth profile experiment were also labeled with [^{35}S]methionine. Consistent with our results using permeabilized cells, Doc induction inhibited translation because [^{35}S]methionine incorporation rapidly decreased within 20 min of Doc induction and leveled off to <10% of normal incorporation from the 40 min through the last 120-min time point (Fig. 1 *B* and *C*). These results are consistent with an earlier study that documented the effect of Doc on MazE-MazF (24).

Phd Can Rescue Doc-Mediated *in Vitro* Translation Arrest and *in Vivo* Growth Arrest. We expressed the *phd-doc* module in a pET expression vector that added a His_6 epitope tag to the carboxyl terminus of the Doc toxin. We then affinity-purified the Phd-Doc complex (Fig. 2*A*, lane 1) using a Ni-NTA column and separated Phd from Doc by denaturation. The stoichiometry of the Coomassie-stained Phd-Doc complex upon affinity purification does not appear to approximate the published 2:1 Phd-Doc ratio. We have noted variability in the ratio of the Coomassie-stained complex from experiment to experiment, suggesting limited accessibility of Phd for His-tagged/column-bound Doc, possibly because of crowding on

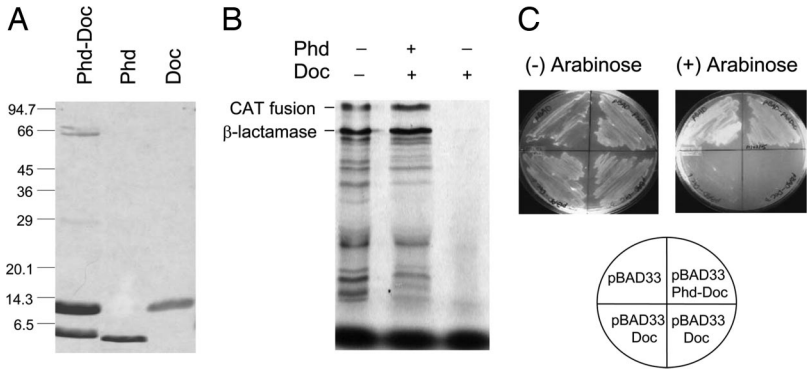


Fig. 2. Phd can rescue Doc-mediated *in vitro* translation arrest and *in vivo* growth arrest. (*A*) Purification of recombinant Phd-Doc–His_6 complex after Ni-NTA-affinity chromatography (lane 1), followed by denaturation and refolding of purified Phd (lane 2), and recapture of Doc over Ni-NTA, followed by denaturation and refolding (lane 3). Molecular mass markers are shown on the left. (*B*) Recombinant Doc inhibits coupled *in vitro* transcription/translation (lane 3), Phd can rescue the inhibition and reconstitute translation (lane 2) to normal levels (as shown in lane 1); the product of the CAT fusion template (39 kDa) comprises one of the two major reaction products, the other β-lactamase (28 kDa) product is present at high levels in T7 S30 extracts because of transcription from the T7 promoter upstream of the CAT fusion that reads through into the ampicillin resistance gene. (*C*) Coexpression of the *phd-doc* module (upper right quadrant), *doc* alone (bottom quadrants), or empty pBAD33 plasmid (top left quadrant). The M9 plate containing 0.2% arabinose, 0.2% glycerol, and 25 μg/ml chloramphenicol was incubated at 37°C overnight.

the affinity column. Also, two proteins migrating at ≈70 and ≈29 kDa that copurified with the Phd-Doc complex were identified by mass spectroscopy as L-glutamine:D-fructose-6-phosphate aminotransferase (GFAT) and the prolyl isomerase SlyD, respectively. Although SlyD has a highly histidine-rich carboxyl-terminal domain and is a well known contaminant with Ni-NTA affinity purifications (26), we have also documented copurification of the histidine-rich protein GFAT upon Ni-NTA purification of other TA systems.

Recombinant Doc was obtained after the denatured TA mixture was again passed through a Ni-NTA column; the Phd flow-through and eluted Doc toxin was renatured by stepwise dialysis (Fig. 2*A*, lanes 2 and 3). Because the recovery of Phd after renaturation was low, we also expressed Phd alone in a pET vector, followed by Ni-NTA purification through its carboxy-His_6 tag as an alternate source of recombinant Phd. Coupled *in vitro* transcription/translation was then performed with or without the addition of Doc alone or both Phd and Doc (Fig. 2*B*). Consistent with our data demonstrating that induction of Doc leads to a block in translation in living and permeabilized cells, recombinant Doc inhibited *in vitro* translation. The effects of Doc can be reversed by addition of antitoxin Phd to the reaction mixture. *In vivo*, we also demonstrated that coexpression of *phd* and *doc* rescues Doc toxicity (Fig. 2*C*). Taken together, our results demonstrate that Doc directly or indirectly inhibits translation, and this effect can be reversed by antitoxin Phd.

Doc Expression Leads to mRNA Stabilization. Some TA toxins lead to translation arrest indirectly by elimination of virtually all of the intracellular mRNA through their sequence-specific endoribonuclease activity [e.g., *E. coli* MazF (27) and ChpBK (28), Kid on plasmid R1 (29), and PemK on plasmid R100 (30)]. We analyzed the effect of Doc expression on the steady-state levels of mRNA *in vivo*. Northern blot analysis of four distinct transcripts revealed that Doc does not degrade mRNA because the levels of each transcript remained constant up to 120 min after Doc induction (SI Fig. 8). This result was in striking contrast to all other TA toxins—MazF, PemK, ChpBK, RelE, and HigB—known to target and rapidly degrade mRNA (27, 28, 30, 31). Therefore, we suspected that the presence of Doc might stabilize mRNA.

To measure mRNA decay rates, we arrested transcription by blocking RNA polymerase activity with rifampicin and determined the effect of Doc expression on *tufA* and *ompA* transcript levels (Fig. 3). Both transcripts were stabilized by Doc and exhibited the same overall trend, with the shorter-half-life transcript *tufA* (32) exhibiting more pronounced stabilization ($t_{1/2}$ increased from 4 min to 48 min) than the longer-half-life *ompA* transcript [which displayed a $t_{1/2}$ increase from 13 min to 48 min (33)]. Therefore, Doc inhibited translation and stabilized mRNA, a combination of phenotypes not exhibited by other TA toxins.

Doc Interaction With Ribosomes and the 30S Ribosomal Subunit Can Be Competed by Hygromycin B *in Vivo*. To better understand how Doc exerts toxicity, we next investigated the effect of Doc on free and translating ribosomes. Because Northern blot analysis indicated that Doc was clearly not cleaving mRNA (SI Fig. 8), we suspected that it might bind to ribosomes because TA toxins RelE (34–36) and YoeB (ref. 37 and Y.Z. and M.I., unpublished work) are also known to act through an association with ribosomes. We obtained a ribosomal profile from cells overexpressing Doc only (but not treated with chloramphenicol or HygB) and compared it with those from wild-type untreated cells or HygB-treated cells (Fig. 4 *A–C*). Although chloramphenicol is typically used to immobilize poly-

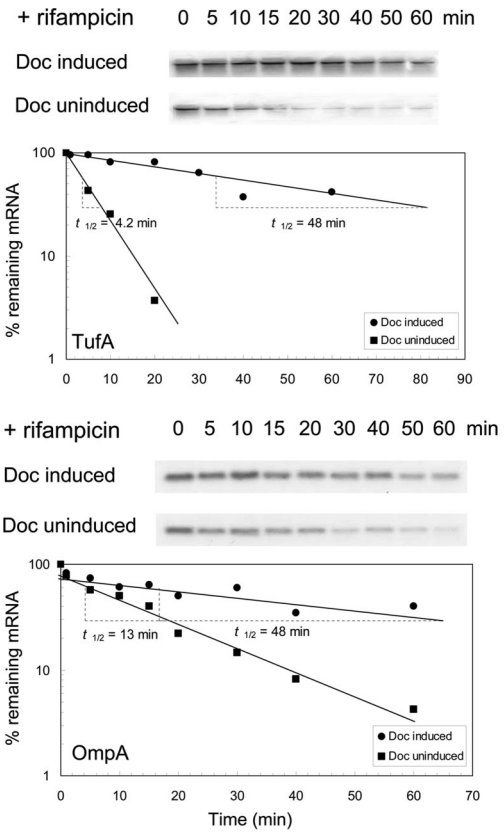


Fig. 3. Doc expression increases mRNA stability. RNA samples were prepared from rifampicin-treated$^{+/-}$ Doc cells; after Northern blot analysis of TufA and OmpA mRNAs, mRNA levels were then quantified and normalized to each uninduced time point.

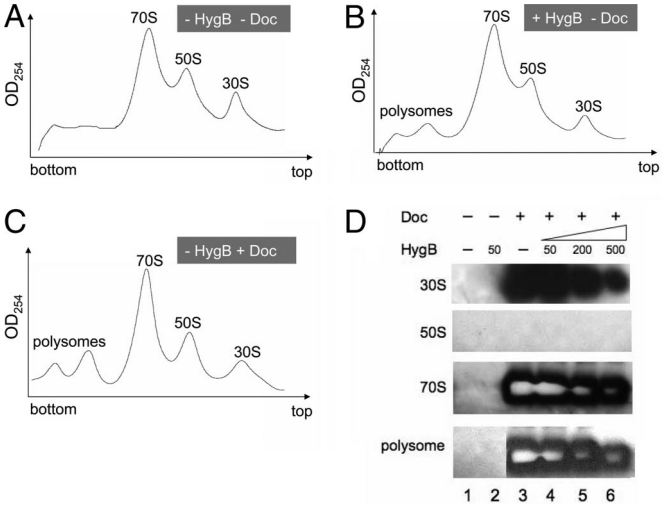
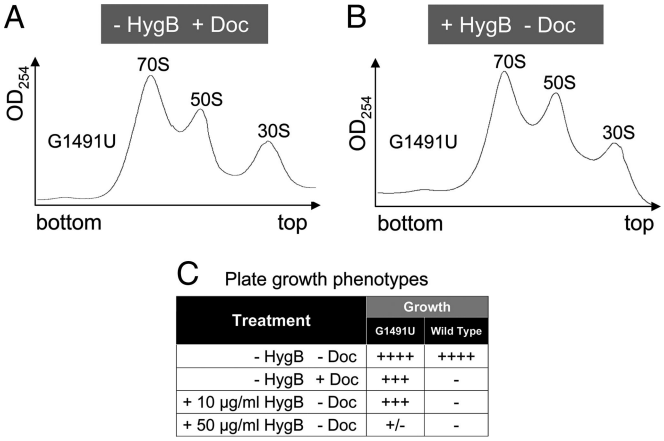


Fig. 4. Doc stabilizes polysomes, associates with 30S ribosomal subunits, and can be competed by HygB. (*A*) −HygB −Doc ribosomes were prepared from BW25113 cells containing pBAD33-Doc under noninducing conditions with no HygB added. (*B*) +HygB −Doc, BW25113 cells containing pBAD33-Doc were treated with 50 μg/ml HygB only for 10 min. (*C*) −HygB +Doc, BW25113 cells containing pBAD33-Doc were induced with 0.2% arabinose only for 20 min. (*D*) Fractions corresponding to free 30S and 50S ribosomal subunits, 70S monosomes, and polysomes from samples treated as labeled above the graphs were collected and subjected to Western blot analysis with a Doc polyclonal antibody. Samples for the HygB competition experiments in lanes 4–6 were exposed to 50 μg/ml, 200 μg/ml, or 500 μg/ml HygB before cell harvesting and ribosome fractionation. We observed strong chemiluminescence with very short exposure times; high-intensity bands appear with varying degrees of white centers that diminish with increasing concentration of HygB exposure. Because the two −Doc polysome samples were not loaded adjacent to the four +Doc polysome samples, they were digitally placed in the same order as the other frames in *D*.



C Plate growth phenotypes

Treatment	Growth	
	G1491U	Wild Type
- HygB - Doc	++++	++++
- HygB + Doc	+++	-
+ 10 μg/ml HygB - Doc	+++	-
+ 50 μg/ml HygB - Doc	+/-	-

Fig. 5. Doc is not toxic in a HygB-resistant mutant. (*A* and *B*) S30 extracts were prepared from *E. coli* cells containing pKK1491U and pBAD33-Doc. Results shown were confirmed in two independent experiments. (*C*) Comparison of plate growth phenotypes of BW25113 cells containing pBAD33-Doc and pKK1491U to BW25113 wild-type cells containing only pBAD33-Doc subjected to the conditions shown. Relative growth rates (strong/wild type (++++), intermediate (+++) weak (+/−), or no (−) growth) were scored after viewing plates grown for the same amount of time overnight in LB plus relevant selective antibiotic at 37°C.

somes on mRNA, we used HygB instead because the pBAD33 plasmid we used to induce Doc expression possessed the chloramphenicol resistance gene as its selectable marker. Interestingly, Doc induction led to stabilization of the polysome peak just as in cells treated with HygB (Fig. 4 *B* and *C*).

HygB is an aminoglycoside antibiotic that predominantly functions as an inhibitor of translation elongation (38–40). HygB treatment stabilizes mRNA in *E. coli* (data not shown); we demonstrated the Doc expression results in mRNA stabilization as well. Structural analysis of HygB interaction with the 30S ribosomal subunit (41) is in agreement with its biochemical properties—it binds the 30S subunit at a single site that includes the A, P, and E sites. Because the phenotypes we observed with Doc expression were consistent with a translation-elongation defect, we tested whether Doc and HygB might target the same region of the 30S subunit. First, we performed Western blot analysis on ribosome profile fractions and found an association of Doc toxin with polysome, 70S, and 30S fractions but not the 50S fractions (Fig. 4*D*, lanes 1–3). We then used the same approach to determine whether addition of increasing amounts of HygB (50, 200, or 500 μg/ml) to Doc-induced cells affected Doc binding. We observed that HygB could compete with Doc binding to its ribosomal targets—as we increased the concentration of HygB, we observed a concomitant decrease in the amount of Doc associated with the 30S, 70S, and polysome fractions (Fig. 4, lanes 4–6).

Doc Is Not Toxic in a Hygromycin B-Resistant Mutant. Because HygB could effectively compete for Doc binding to the 30S ribosomal subunit, it is possible that the region of the 30S subunit bound by Doc includes the HygB-binding site. We used a well characterized HygB-resistant strain (that also exhibits resistance to other aminoglycosides) containing a plasmid that expresses a mutated 16S rRNA at position 1491 (42, 43) to test whether Doc toxicity and interaction with the 30S ribosomal subunit is reduced when Doc expression is induced. The G1491U mutation disrupts the highly conserved C1409-G1491 Watson–Crick base pair in the A site of the decoding region in helix 44 near the 3′ end of 16S rRNA in the 30S subunit (42, 43). In fact, although another 16S rRNA mutation at G1409U also results in HygB resistance, we did not use this strain because the growth rate is slower and the resistance weaker than the G1491U mutant.

We performed ribosome profile experiments and plate-growth assays to assess whether the HygB mutation lessened the severity of the two phenotypes linked to Doc overexpression—the formation of polysome stabilization and toxicity (growth arrest). Remarkably, the severity of both phenotypes was dramatically reduced in the G1491U HygB mutant (Fig. 5). First, polysomes did not accumulate when Doc was induced in the mutant, as was the case after mutant cells were exposed to 50 μg/ml HygB (Fig. 5 *A* and *B*). Growth phenotypes (plate, Fig. 5*C* and liquid culture, SI Fig. 9) of G1491U mutant and wild-type cells subjected to Doc induction or hygromycin treatment were consistent with the ribosome profile phenotypes. More specifically, the G1491U mutant and wild-type cells had comparable growth rates (42). However, Doc induction was no longer toxic and did not cause cell-growth arrest in G1491U mutant cells. These experiments further support a model in which the Doc-binding site includes that bound by HygB.

Discussion

TA systems represent a recently discovered addition to the defense mechanisms enlisted by free-living bacteria to either protect them from stress by initiating signals to pull cells from an active growth mode into a quasidormant phase (chromosomally encoded TA toxins) or facilitate postsegregational killing to maintain an extrachromosomal element that imparts a survival advantage to the cell (P1 bacteriophage or plasmid-encoded toxins). Although the primary goal of chromosomal TA toxins is not to kill but to instead arrest cell growth, their action does eventually lead to bacterial cell death if the stress that triggers TA toxin action is sustained.

The molecular mechanisms by which TA toxins initiate quasidormancy and cell death are gradually being elucidated. To date,

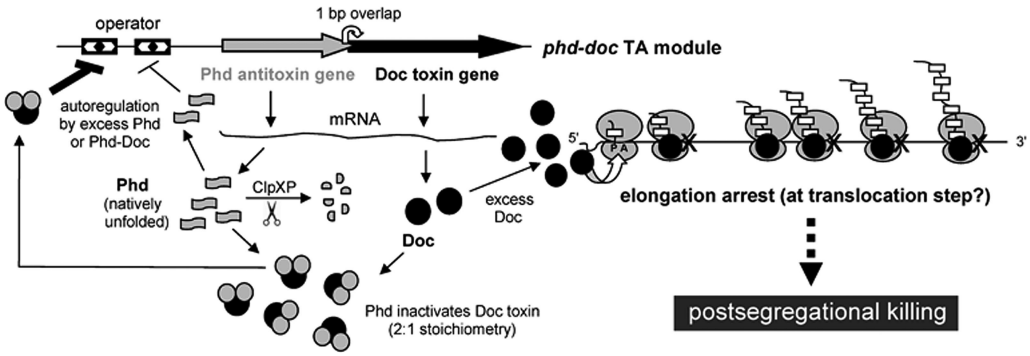


Fig. 6. Features of Phd-Doc function in *E. coli*. Toxicity occurs when free Doc toxin is able to arrest translation elongation by binding to the 30S subunit (denoted by the X); unchecked toxin action leads to bacterial cell death (i.e. postsegregational killing for P1 bacteriophage infected cells). The mechanism of mRNA stabilization by Doc can also be envisioned because stalled ribosomes protect mRNA from degradation. Note that all 30S subunits do not necessarily have to be bound by Doc for both mRNA stabilization and translation arrest to occur because stalled ribosomes block read-through of ribosomes before them.

TA toxins are known to perturb one or more vital processes—DNA replication, RNA transcription, and protein translation—with DNA gyrase, mRNA, and ribosomes serving as toxin targets. We demonstrated that the mechanism of action of Doc is distinct from all of the other characterized TA toxins (summarized in Fig. 6). The majority of the effects of Doc expression mirror those associated with characteristic translation-elongation defects—inhibition of protein synthesis, ribosome binding, mRNA stabilization, and polysome accumulation—there are no other TA toxins known to directly target translation elongation. A Doc-meditated elongation block at the translocation step would lead to an accumulation of stalled ribosomes along the length of actively translating mRNAs during logarithmic growth, consistent with the mRNA stabilization we observed. Note that this mechanism of toxicity would not require that all of the 30S subunits assembled into actively translating 70S ribosomes need be bound by Doc. Translating ribosomes lacking Doc would encounter Doc-arrested ribosomes downstream that physically block their ability to continue elongating. Finally, although we characterized Doc activity derived from the Phd-Doc addiction module in bacteriophage P1, orthologs of this module exist as chromosomal TA systems in several free-living organisms and pathogens. Therefore, the chromosomal counterparts of Phd-Doc are expected to impart stress survival in lieu of postsegregational killing through Doc interaction with the 30S ribosomal subunit.

We have obtained valuable clues to Doc function based on similarity to some features of HygB. The illustration in Fig. 6 summarizes the important features of the *phd-doc* module and the proposed role of Doc in elongation translocation. HygB primarily functions as an inhibitor of translation elongation; it acts at the translocation step by preventing movement of the peptidyl tRNA from the A site to the P site. An elongation block at the translocation step would lead to an accumulation of stalled ribosomes along the length of actively translating mRNAs during logarithmic growth, consistent with the mRNA stabilization we observed. HygB also binds the 30S subunit at a single site that includes the A, P, and E sites (41). Our data suggest that the site on the 30S ribosomal subunit bound by Doc includes the HygB-binding site. The HygB-30S structure revealed that HygB binds to the 30S subunit (composed of 21 ribosome proteins and 16S rRNA) at the top of helix 44. Helix 44 is highly conserved, resides at the 30S–50S interface, and contains the P, A, and E sites; HygB resistance mutations have also been localized to helix 44 (44). HygB primarily inhibits translation elongation but also somewhat decreases mRNA decoding fidelity (38, 40, 45–47). Doc may also act, as does HygB, at the translocation step by preventing movement of the peptidyl tRNA from the A site to the P site.

As the mechanisms of action of more TA toxins come to light, the similarities between certain TA toxin family members (proteins typically ≈10 kDa) and classic small-molecule antibiotics that target the ribosome are striking. In fact, much of our insight into Doc function was illuminated based on the parallels between the effects of Doc and HygB on *E. coli* cells. Ribosomes appear to be particularly effective targets for irreversible or reversible toxicity. In fact, nearly half of all well validated antibiotic targets involve the 30S subunit, 50S subunit, or 70S ribosome (48). Like antibiotics, TA systems Doc, YoeB, and RelE have exploited the same ribosome targets to impart translation arrest. However, these TA systems have also adapted to enable reversible toxicity through the regulatable association of the antitoxin with its cognate toxin. Ongoing structural characterization of the Phd-Doc complex, detailed analysis of the Doc-ribosome interaction will further enhance our understanding of how Doc family members impart toxicity through their association with ribosomes.

Materials and Methods

Strains and Plasmids. We used *E. coli* strains BL21(DE3), BW25113 (*lacI*^{q} *rrnB*$_{T14}$ Δ*lacZ*$_{WJ16}$ *hsdR514* Δ*araBAD*$_{AH33}$ Δ*rhaBAD*$_{LD78}$), or strains containing 16S rRNA plasmids pKK3535 [containing the entire *rrnB* operon) or pKK1491U (the G1491U mutant; (42)] kindly provided by Steven Gregory in the Albert Dahlberg laboratory (Brown University, Providence, RI). The *phd-doc* operon was PCR amplified from bacteriophage P1 with $^{5'}$NdeI–XhoI$^{3'}$ ends and cloned into the corresponding sites of pET21c (Novagen) to create pET21c/Phd-Doc (pNW318) used to coexpress the Phd-Doc–His$_6$ complex. The Phd ORF was PCR amplified with $^{5'}$NdeI–XhoI$^{3'}$ ends and cloned into the corresponding sites of pET21c to create pET21c-Phd (pNW319) and used to produce Phd–His$_6$. The Doc ORF was PCR amplified as a $^{5'}$BamH I–Hind III$^{3'}$ fragment and cloned into the corresponding sites of pET21c to create pET21c–Doc. Plasmid pET21c–Doc was digested with $^{5'}$Hind III–XbaI$^{3'}$ and subcloned into pBAD33 to create pBAD33–Doc (pNW320) (23).

Transcription, Replication, and Translation Competence. [^{35}S]Met (25, 49), [α-^{32}P]dTTP (50), and [α-^{32}P]UTP (51) incorporation into toluene-treated cells was performed as described. *In vivo* [^{35}S]Met incorporation was also performed as described (52).

Purification of Recombinant Phd and Doc. Phd-Doc–His$_6$ was expressed in pNW318, purified by Ni-NTA affinity chromatography, denatured with 6 M guanidine-HCl overnight at 4°C, and again passed through a Ni-NTA column to recover free, denatured Phd antitoxin. The Doc–His$_6$ toxin was then eluted by using buffer B [100 mM NaH_2PO_4, 10 mM Tris-Cl, 250 mM imidazole, 6 M guanidine-HCl (pH 8.0)], dialyzed overnight in buffer B with 3 M guanidine-HCl, passed over a final Ni-NTA affinity column, eluted with buffer A [100 mM NaH_2PO_4, 10 mM Tris-Cl, 250 mM imidazole, 3M guanidine-HCl (pH 8.0)], and dialyzed overnight in 100 mM NaH_2PO_4, 10 mM Tris-Cl (pH 8.0). Phd–His$_6$ was also and purified directly by Ni-NTA chromatography.

***In Vitro* Protein Synthesis.** Prokaryotic cell-free protein synthesis was carried out with the *E. coli* T7 S30 Extract System for Circular DNA (Promega); the +Doc and Phd/Doc samples contained 36.5 μg of Doc–His_6 or 36.5 μg of Doc–His_6 plus 146 μg of Phd–His_6, respectively.

Northern Blot Analysis. Total RNA was isolated by the hot-phenol method (53). *tufA* (elongation factor EF-Tu), *ompA* (outer membrane protein A), *ompF* (outer membrane porin protein), and *lpp* (major outer membrane lipoprotein) mRNAs, were hybridized with radioisotope-labeled PCR fragments corresponding to the ORF only. For $t_{½}$ measurement, Doc was induced for 20 min, 200 μg/ml rifampicin added to −/+ Doc cultures, and RNA prepared at intervals as indicated.

Ribosome Profile Analysis. Approximately seven A_{260} units of each S30 extract was layered onto each 5–40% (wt/vol) continuous sucrose gradient in 10 mM Tris·HCl (pH 7.5), 50 mM NH_4Cl, 10 mM $MgCl_2$, 1 mM DTT and centrifuged at 35,000 rpm in a Beckman SW41 rotor for 3.5 h at 4°C. Gradients were fractionated and analyzed at 254 nm with a FPLC detector to follow rRNA levels. Fractions corresponding to polysomes, 70S, 50S, and 30S were subjected to Western blot analysis using Doc polyclonal antibody. This antibody was generated in rabbits by using pure Phd-Doc complex as the antigen (PRF&L); the specificity of the antibody for Doc (and not Phd) in the ribosome profile fractions was verified by comigration with pure Doc control lanes. Doc-induced samples with HygB treatment were prepared from cells induced with arabinose for 20 min, followed by exposure to 50 μg/ml, 200 μg/ml, or 500 μg/ml HygB for 10 min. Ribosome profile analysis for *E. coli* cells containing pKK1491U and pBAD33-Doc (Fig. 5 *A* and *B*) was performed as described above except that, when HygB was added, the incubation time was increased to 20 min.

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1. Gerdes K, Christensen SK, Lobner-Olesen A (2005) Prokaryotic toxin–antitoxin stress response loci. *Nat Rev Microbiol* 3:371–382.
2. Pandey DP, Gerdes K (2005) Toxin–antitoxin loci are highly abundant in free-living but lost from host-associated prokaryotes. *Nucleic Acids Res* 33:966–976.
3. Suzuki M, Zhang J, Liu M, Woychik NA, Inouye M (2005) Single protein production in living cells facilitated by an mRNA interferase. *Mol Cell* 18:253–261.
4. Amitai S, Yassin Y, Engelberg-Kulka H (2004) MazF-mediated cell death in *Escherichia coli:* A point of no return. *J Bacteriol* 186:8295–8300.
5. Engelberg-Kulka H, Hazan R, Amitai S (2005) mazEF: A chromosomal toxin–antitoxin module that triggers programmed cell death in bacteria. *J Cell Sci* 118:4327–4332.
6. Pedersen K, Christensen SK, Gerdes K (2002) Rapid induction and reversal of a bacteriostatic condition by controlled expression of toxins and antitoxins. *Mol Microbiol* 45:501–510.
7. Engelberg-Kulka H, Sat B, Reches M, Amitai S, Hazan R (2004) Bacterial programmed cell death systems as targets for antibiotics. *Trends Microbiol* 12:66–71.
8. Nariya H, Inouye M (2008) MazF, an mRNA interferase, mediates programmed cell death during multicellular *Myxococcus* development. *Cell* 132:55–66.
9. Brooun A, Liu S, Lewis K (2000) A dose–response study of antibiotic resistance in *Pseudomonas aeruginosa* biofilms. *Antimicrob Agents Chemother* 44:640–646.
10. Keren I, Shah D, Spoering A, Kaldalu N, Lewis K (2004) Specialized persister cells and the mechanism of multidrug tolerance in *Escherichia coli. J Bacteriol* 186:8172–8180.
11. Lewis K (2005) Persister cells and the riddle of biofilm survival. *Biochemistry (Moscow)* 70:267–274.
12. Moyed HS, Bertrand KP (1983) hipA, a newly recognized gene of *Escherichia coli* K-12 that affects frequency of persistence after inhibition of murein synthesis. *J Bacteriol* 155:768–775.
13. Spoering AL, Lewis K (2001) Biofilms and planktonic cells of *Pseudomonas aeruginosa* have similar resistance to killing by antimicrobials. *J Bacteriol* 183:6746–6751.
14. Jensen RB, Gerdes K (1995) Programmed cell death in bacteria: Proteic plasmid stabilization systems. *Mol Microbiol* 17:205–210.
15. Lehnherr H, Maguin E, Jafri S, Yarmolinsky MB (1993) Plasmid addiction genes of bacteriophage P1: Doc, which causes cell death on curing of prophage, and phd, which prevents host death when prophage is retained. *J Mol Biol* 233:414–428.
16. Magnuson R, Lehnherr H, Mukhopadhyay G, Yarmolinsky MB (1996) Autoregulation of the plasmid addiction operon of bacteriophage P1. *J Biol Chem* 271:18705–18710.
17. Gazit E, Sauer RT (1999) The Doc toxin and Phd antidote proteins of the bacteriophage P1 plasmid addiction system form a heterotrimeric complex. *J Biol Chem* 274:16813–16818.
18. Gazit E, Sauer RT (1999) Stability and DNA binding of the phd protein of the phage P1 plasmid addiction system. *J Biol Chem* 274:2652–2657.
19. Magnuson R, Yarmolinsky MB (1998) Corepression of the P1 addiction operon by Phd and Doc. *J Bacteriol* 180:6342–6351.
20. Lehnherr H, Yarmolinsky MB (1995) Addiction protein Phd of plasmid prophage P1 is a substrate of the ClpXP serine protease of *Escherichia coli*. *Proc Natl Acad Sci USA* 92:3274–3277.
21. McKinley JE, Magnuson RD (2005) Characterization of the Phd repressor-antitoxin boundary. *J Bacteriol* 187:765–770.
22. Smith JA, Magnuson RD (2004) Modular organization of the Phd repressor/antitoxin protein. *J Bacteriol* 186:2692–2698.
23. Guzman LM, Belin D, Carson MJ, Beckwith J (1995) Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter. *J Bacteriol* 177:4121–4130.
24. Hazan R, Sat B, Reches M, Engelberg-Kulka H (2001) Postsegregational killing mediated by the P1 phage ''addiction module'' phd-doc requires the *Escherichia coli* programmed cell death system mazEF. *J Bacteriol* 183:2046–2050.
25. Halegoua S, Hirashima A, Sekizawa J, Inouye M (1976) Protein synthesis in toluene-treated *Escherichia coli*. Exclusive synthesis of membrane proteins. *Eur J Biochem* 69:163–167.
26. Mukherjee S, Shukla A, Guptasarma P (2003) Single-step purification of a protein-folding catalyst, the SlyD peptidyl prolyl isomerase (PPI), from cytoplasmic extracts of *Escherichia coli*. *Biotechnol Appl Biochem* 37:183–186.
27. Zhang Y, Zhang J, Hoeflich KP, Ikura M, Qing G, Inouye M (2003) MazF cleaves cellular mRNAs specifically at ACA to block protein synthesis in *Escherichia coli*. *Mol Cell* 12:913–923.
28. Zhang Y, Zhu L, Zhang J, Inouye M (2005) Characterization of ChpBK, an mRNA interferase from *Escherichia coli*. *J Biol Chem* 280:26080–26088.
29. Pimentel B, Madine MA, de la Cueva-Mendez G (2005) Kid cleaves specific mRNAs at UUACU sites to rescue the copy number of plasmid R1. *EMBO J* 24:3459–3469.
30. Zhang J, Zhang Y, Zhu L, Suzuki M, Inouye M (2004) Interference of mRNA function by sequence-specific endoribonuclease PemK. *J Biol Chem* 279:20678–20684.
31. Christensen-Dalsgaard M, Gerdes K (2006) Two higBA loci in the *Vibrio cholerae* superintegron encode mRNA cleaving enzymes and can stabilize plasmids. *Mol Microbiol* 62:397–411.
32. Pedersen S, Reeh S (1978) Functional mRNA half lives in *E. coli. Mol Gen Genet* 166:329–336.
33. Deana A, Ehrlich R, Reiss C (1998) Silent mutations in the *Escherichia coli* ompA leader peptide region strongly affect transcription and translation *in vivo*. *Nucleic Acids Res* 26:4778–4782.
34. Christensen SK, Gerdes K (2003) RelE toxins from Bacteria and Archaea cleave mRNAs on translating ribosomes, which are rescued by tmRNA. *Mol Microbiol* 48:1389–1400.
35. Hayes CS, Sauer RT (2003) Cleavage of the A site mRNA codon during ribosome pausing provides a mechanism for translational quality control. *Mol Cell* 12:903–911.
36. Pedersen K, *et al.* (2003) The bacterial toxin RelE displays codon-specific cleavage of mRNAs in the ribosomal A site. *Cell* 112:131–140.
37. Christensen SK, *et al.* (2004) Overproduction of the Lon protease triggers inhibition of translation in *Escherichia coli*: Involvement of the yefM-yoeB toxin-antitoxin system. *Mol Microbiol* 51:1705–1717.
38. Cabanas MJ, Vazquez D, Modolell J (1978) Dual interference of hygromycin B with ribosomal translocation and with aminoacyl-tRNA recognition. *Eur J Biochem* 87:21–27.
39. Cabanas MJ, Vazquez D, Modolell J (1978) Inhibition of ribosomal translocation by aminoglycoside antibiotics. *Biochem Biophys Res Commun* 83:991–997.
40. Eustice DC, Wilhelm JM (1984) Fidelity of the eukaryotic codon–anticodon interaction: Interference by aminoglycoside antibiotics. *Biochemistry* 23:1462–1467.
41. Brodersen DE, *et al.* (2000) The structural basis for the action of the antibiotics tetracycline, pactamycin, and hygromycin B on the 30S ribosomal subunit. *Cell* 103:1143–1154.
42. De Stasio EA, Dahlberg AE (1990) Effects of mutagenesis of a conserved base-paired site near the decoding region of *Escherichia coli* 16 S ribosomal RNA *J Mol Biol* 212:127–133.
43. De Stasio EA, Moazed D, Noller HF, Dahlberg AE (1989) Mutations in 16S ribosomal RNA disrupt antibiotic–RNA interactions. *EMBO J* 8:1213–1216.
44. Pfister P, Risch M, Brodersen DE, Bottger EC (2003) Role of 16S rRNA helix 44 in ribosomal resistance to hygromycin B. *Antimicrob Agents Chemother* 47:1496–1502.
45. Eustice DC, Wilhelm JM (1984) Mechanisms of action of aminoglycoside antibiotics in eukaryotic protein synthesis. *Antimicrob Agents Chemother* 26:53–60.
46. Ganoza MC, Kiel MC (2001) A ribosomal ATPase is a target for hygromycin B inhibition on *Escherichia coli* ribosomes. *Antimicrob Agents Chemother* 45:2813–2819.
47. Gonzalez A, Jimenez A, Vazquez D, Davies JE, Schindler D (1978) Studies on the mode of action of hygromycin B, an inhibitor of translocation in eukaryotes. *Biochim Biophys Acta* 521:459–469.
48. Franceschi F, Duffy EM (2006) Structure-based drug design meets the ribosome. *Biochem Pharmacol* 71:1016–1025.
49. Halegoua S, Hirashima A, Inouye M (1976) Puromycin-resistant biosynthesis of a specific outer-membrane lipoprotein of *Escherichia coli*. *J Bacteriol* 126:183–191.
50. Moses RE, Richardson CC (1970) Replication and repair of DNA in cells of *Escherichia coli* treated with toluene. *Proc Natl Acad Sci USA* 67:674–681.
51. Peterson RL, Radcliffe CW, Pace NR (1971) Ribonucleic acid synthesis in bacteria treated with toluene. *J Bacteriol* 107:585–588.
52. Hirashima A, Inouye M (1973) Specific biosynthesis of an envelope protein of *Escherichia coli*. *Nature* 242:405–407.
53. Sarmientos P, Sylvester JE, Contente S, Cashel M (1983) Differential stringent control of the tandem *E. coli* ribosomal RNA promoters from the rrnA operon expressed *in vivo* in multicopy plasmids. *Cell* 32:1337–1346.