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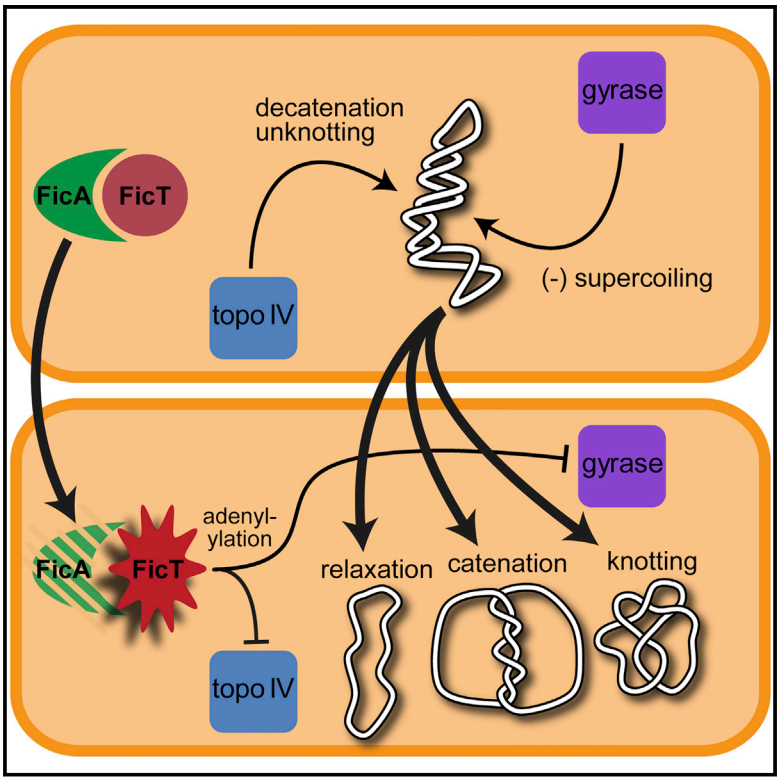
Adenylylation of Gyrase and Topo IV by FicT Toxins Disrupts Bacterial DNA Topology

Graphical Abstract



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In Brief

Harms et al. reveal that the FicTA toxin-antitoxin module acts via adenylylation of DNA gyrase and topoisomerase IV. This modification inactivates both targets by blocking the ATPase activity that is central to their enzymatic functions, and it reversibly inhibits bacterial growth via the knotting, catenation, and relaxation of cellular DNA.

Highlights

- FicT toxins modify DNA gyrase and topoisomerase IV by adenylylation
- Adenylylation inactivates both targets by blocking their ATPase activity
- Target inactivation causes DNA knotting, catenation, and relaxation in vivo
- Suspending control of DNA topology by FicT toxins induces reversible growth arrest

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Adenylylation of Gyrase and Topo IV by FicT Toxins Disrupts Bacterial DNA Topology

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SUMMARY

Toxin-antitoxin (TA) modules are ubiquitous molecular switches controlling bacterial growth via the release of toxins that inhibit cell proliferation. Most of these toxins interfere with protein translation, but a growing variety of other mechanisms hints at a diversity that is not yet fully appreciated. Here, we characterize a group of FIC domain proteins as toxins of the conserved and abundant FicTA family of TA modules, and we reveal that they act by suspending control of cellular DNA topology. We show that FicTs are enzymes that adenylylate DNA gyrase and topoisomerase IV, the essential bacterial type IIA topoisomerases, at their ATP-binding site. This modification inactivates both targets by blocking their ATPase activity, and, consequently, causes reversible growth arrest due to the knotting, catenation, and relaxation of cellular DNA. Our results give insight into the regulation of DNA topology and highlight the remarkable plasticity of FIC domain proteins.

INTRODUCTION

Bacterial toxin-antitoxin (TA) modules comprise a toxin that interferes with bacterial growth via the inactivation of essential cellular processes and an antitoxin that prevents functionality of the toxin. In the more prevalent type II TA modules, the antitoxin is a protein that inhibits the toxin via tight binding until it is degraded in response to cellular signaling. Though they were originally discovered as post-segregational killing systems mediating plasmid addiction (Jensen and Gerdes, 1995), it is now known that many TA modules act via the induction of a transient bacteriostatic condition that converts bacteria into persister cells (Lewis, 2010; Maisonneuve et al., 2013).

The majority of TA module toxins that have been studied reversibly interfere with protein translation somehow, e.g., as mRNA endonucleases or as kinases that phosphorylate proteins involved in ribosome function (Christensen and Gerdes, 2003; Germain et al., 2013; Leplae et al., 2011). However, several other molecular mechanisms, such as an abrogation of the proton-motive force or the impairment of cytoskeletal assembly, have been described (recently reviewed by Unterholzner et al., 2013). Two different plasmid addiction modules, CcdBA and ParDE, act via poisoning of bacterial topoisomerases in a manner similar to quinolone antibiotics (Bernard and Couturier, 1992; Jiang et al., 2002). Topoisomerase poisoning manipulates the catalytic cycle of these enzymes to cause the formation of chromosome breaks and readily overcomes dedicated DNA repair functions, resulting in a bactericidal effect (Couturier et al., 1998; Deghorain et al., 2013). Here, we looked for other TA modules, beyond these two poisons, that exploit their vital role in bacterial cells. We characterize a family of filamentation induced by cAMP (FIC) domain proteins as toxins of the FicTA type II TA module that, instead of poisoning targets by direct binding, acts via the enzymatic inhibition of bacterial topoisomerases by post-translational modification.

Proteins containing FIC domains typically mediate adenylylation (also known as AMPylation), the covalent transfer of an adenosine 5′-monophosphate (AMP) moiety onto target proteins. They contain an HxFx(D/E)GNGRxxR signature motif at their active site that is critical for the catalysis of adenylylation, though a few cases of secondarily diverged motifs promoting different molecular activities have been described (Engel et al., 2012; Garcia-Pino et al., 2014). Previous research on FIC domain proteins has focused on a few representatives that had secondarily evolved into host-targeted virulence factors of bacterial pathogens (Worby et al., 2009; Yarbrough et al., 2009), but the majority are genuine bacterial proteins of unknown function. We recently described the adenylylation activity of these bacterial FIC domain proteins, and we examined their inhibition by a conserved regulatory module that interferes with productive binding of the ATP substrate. Interestingly, the regulatory module of one major group of bacterial FIC domain proteins, categorized as class I, is part of a separate small protein that inhibits the activity of its cognate FIC domain via a tight interaction, thereby alleviating growth inhibition caused by the FIC domain's activity (Engel et al., 2012). Such a regulatory arrangement satisfies the

central definition of type II TA modules, so we named the class I FIC domain proteins FIC domain toxin (FicT) and their cognate antitoxins FIC domain antitoxin (FicA) (Goepfert et al., 2013). However, the enzymatic targets and biological functions of the FicTA module have remained elusive.

In this study, we characterize different representatives of the FicTA type II TA family and show that FicTs inactivate DNA gyrase and topoisomerase IV (topo IV) via adenylylation at their ATP-binding site. Adenylylation abrogates the ATP hydrolysis that is central to the function of these topoisomerases, and, consequently, it blocks all their physiologically relevant activities in vitro. The expression of FicTs causes reversible growth arrest accompanied by strong DNA knotting and catenation combined with a varying degree of DNA relaxation in *Escherichia coli*, demonstrating that both targets are inactivated in vivo. Their functionality as enzymes, as well as their bacteriostatic and not bactericidal effect on bacterial cells, distinguishes FicTs from other TA module toxins that target topoisomerases.

RESULTS

Phylogeny of the FicTA Family of TA Modules

We previously established VbhTA of *Bartonella schoenbuchensis* as a model for the FicTA family, and we demonstrated that the antitoxin VbhA inhibits the adenylylation activity of the VbhT toxin and relieves the associated growth arrest in *E. coli* (Engel et al., 2012). Since VbhT is unique among class I FIC domain proteins in that it contains a bona fide type IV secretion signal (called Bep intracellular delivery [BID] domain) at its C terminus, we decided to investigate the molecular and biological functions of VbhT along with a diverse set of other FicTs. Class I FIC domain proteins form a separate branch of this domain family and make up 5%–10% of the more than 2,000 bacterial FIC domain proteins (Engel et al., 2012). A deeper look at their internal phylogeny revealed a substructure of different groups most of which exclusively contain proteins with a regular HxFx(D/E)GNGRxxR signature motif or closely related sequences, suggesting that adenylylation is their original and most abundant molecular activity (Figure 1A).

As a typical single-domain FIC protein with a canonical active site motif, YeFicT of *Yersinia enterocolitica* str. 8081 is distantly related to VbhT but also to EcFicT of *E. coli* K-12, the first FIC domain protein that was described (Figure 1B; Utsumi et al., 1982). EcFicT itself is unsuitable as a model for FicTs in general because it belongs to a cluster of enterobacterial FicT homologs that exhibit a distinctly altered FIC domain signature motif (Figures 1A and 1B; Goepfert et al., 2013). To examine the general significance of our findings among class I FIC domain proteins, we also studied PaFicT of *Pseudomonas aeruginosa* PAO1, which is only remotely related to VbhT or YeFicT and displays a largely divergent protein sequence apart from a few stretches, including its mostly canonical Fic signature motif (Figures 1A and 1B). All FicT homologs investigated in this study were found to be encoded together with bona fide FicAs containing an FIC domain inhibition module, suggesting that they share the same mode of regulation (Figure 1C). To exclude any influence of the resident EcFicTA module on our experiments, we typically used a Δ*ecficAT* derivative of *E. coli* K-12 throughout this work.

Ectopic Expression of Diverse FicTs Results in Reversible Inhibition of Bacterial Growth

The expression of different FicTs from a single-copy vector in the absence of their cognate FicAs resulted in strong inhibition of *E. coli* growth (Figures 2A and 2B). This effect required the FicTs' adenylylation activity, because mutation of the catalytic histidine within the FIC domain signature motif HxFx(D/E)GNGRxxR into alanine (H/A mutants) abolished the growth inhibition (Figure 2A). Similarly, the subsequent expression of cognate antitoxins reversed the growth arrest caused by FicTs (Figure 2C), demonstrating that their activity leads to a classical bacteriostatic condition that is a common feature of many TA systems (Pedersen et al., 2002). We note that the ectopic expression of VbhT appeared to be less potent in growth inhibition than that of YeFicT, PaFicT, or only the FIC domain of VbhT alone (VbhT [fic]; Figure 2B), possibly due to differential expression of the constructs or because the BID domain of VbhT sterically reduces toxin activity. While PaFicT readily caused bacterial growth inhibition in its natural host *P. aeruginosa*, no such effect was obtained with EcFicT in *E. coli* (Figures S2A–S2C).

FicTs Adenylylate GyrB and ParE, the B Subunits of DNA Gyrase and Topo IV

To unravel the molecular mechanism of the reversible growth inhibition caused by the FicTs' adenylylation activity, we next aimed at uncovering the identity of the adenylylated target(s). For this purpose we performed in vitro adenylylation assays with lysates of *E. coli* that had expressed VbhT, and we used a pull-down approach similar to the one published by Grammel et al. (2011) for target identification. Although we failed to achieve significant enrichment of potential targets, we serendipitously discovered an adenylylated peptide that belonged to GyrB of *E. coli* (not shown). The theoretical molecular weight of GyrB (90 kDa) fits well with the apparent molecular weight of the bona fide endogenous target adenylylation detected by autoradiography in adenylylation assays with VbhT (Figure 2D and our previous work [Engel et al., 2012]). Ectopic expression of GyrB both of *E. coli* or *B. schoenbuchensis* unambiguously showed that these proteins are adenylylated by VbhT in bacterial lysates in vitro (Figure 2D). Given the very high similarity in sequence, structure, and function to GyrB, we further suspected that its paralog ParE also might be targeted by VbhT (Sissi and Palumbo, 2010). Indeed, like for GyrB, ectopic expression of ParE of *E. coli* and *B. schoenbuchensis* clearly demonstrated adenylylation by VbhT (Figure 2D). However, unlike for GyrB, no adenylylation of endogenous ParE was detectable by visual inspection of autoradiographs of our adenylylation experiments with cleared lysates of *E. coli* expressing VbhT (Figure 2D). We believe that this discrepancy was caused by largely different target abundances, because *E. coli* cells contain approximately ten times more molecules of GyrB than of ParE (Alexander Schmidt, personal communication). As expected from its peculiar FIC domain signature motif and the lack of bacterial growth inhibition, no adenylylation activity was detected for EcFicT (Figures S4B and S4C).

GyrB and ParE are the B subunits of the two bacterial type IIA topoisomerases DNA gyrase and topo IV that control cellular DNA topology by maintaining negative supercoiling and

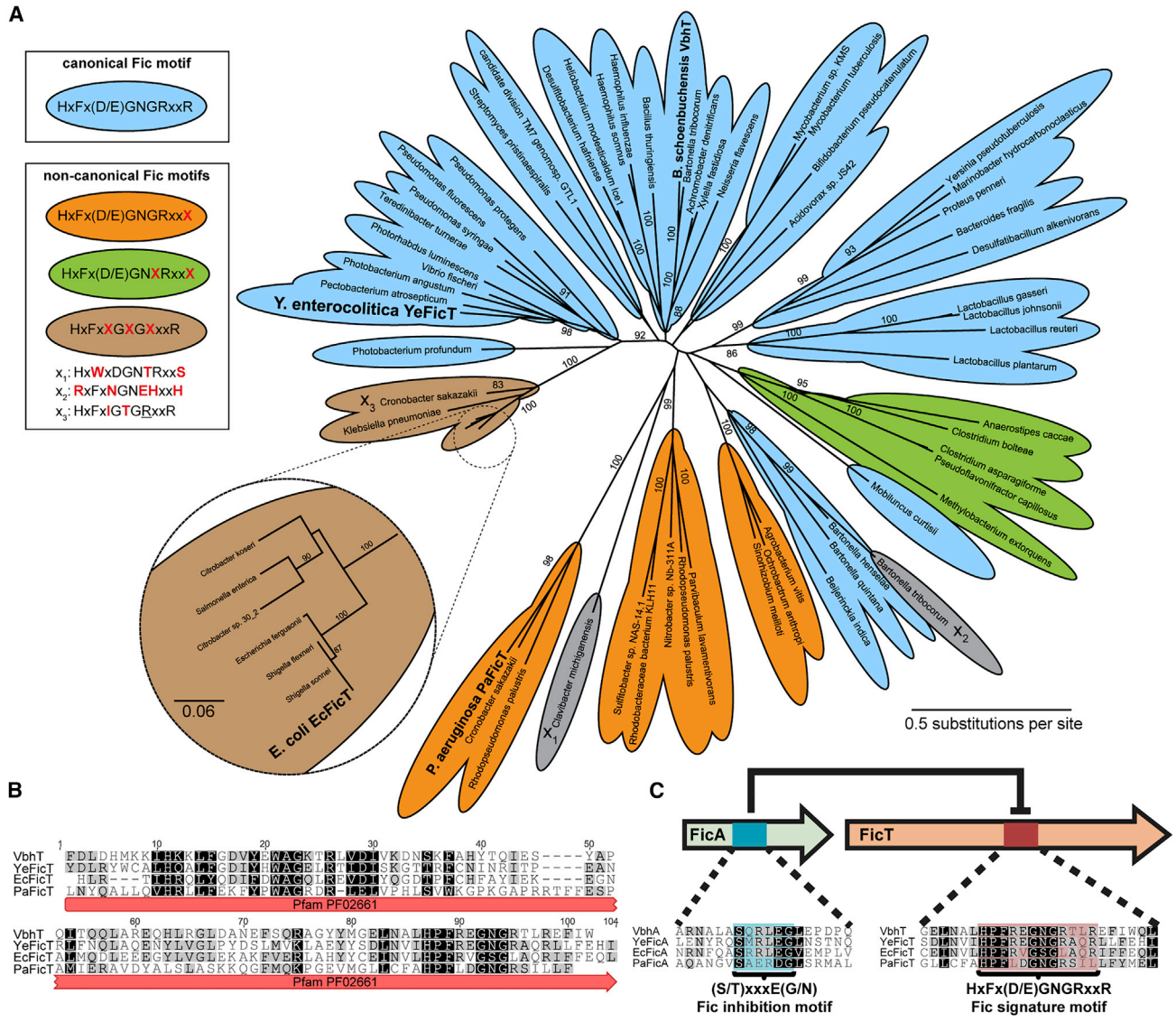

Figure 1. Phylogeny of Class I FIC Domain Proteins and a Comparison of FicA and FicT Homologs Investigated in This Study

(A) A maximum likelihood phylogeny was constructed based on the FIC domains of a representative set of class I FIC proteins (see the Supplemental Experimental Procedures). The positions of VbhT, EcFicT, YeFicT, and PaFicT are highlighted in bold. Different subgroups are color-coded according to the FIC domain signature motif, revealing that most class I proteins display the canonical HxFx(D/E)GNGRxxR sequence (blue) or a close variant of it (orange and green). However, one group of enterobacterial FicT homologs including EcFicT diverges more strongly (brown). Bootstrap values are shown if >75/100, and the protein accession numbers of all sequences are available in Figure S1.

(B) The FIC domain core (Pfam PF02661; release 27.0) of FicT homologs was aligned using ClustalW (implemented in Geneious v.7.1.7) and the alignment was manually curated. Coloring reflects amino acid similarity according to the Blosum62 score matrix with black = 100% identity and white = <60% identity. While VbhT, YeFicT, and EcFicT share >26% identical sites and 40%–50% pairwise sequence identity, PaFicT aligns only poorly to the others outside of a few conserved sequence stretches.

(C) Protein sequences of FicA and FicT homologs were aligned as described in (B) and the signature motifs were extracted for illustration. All four FicA homologs contain an (S/T)xxxE(G/N) motif with the inhibitory glutamate being a chemically similar aspartate in PaFicA, and only EcFicT diverges considerably from the canonical HxFx(D/E)GNGRxxR FIC domain signature motif (see A and B).

removing DNA catenation and knotting, respectively (Sissi and Palumbo, 2010). Therefore, their activities are generally essential for all processes in bacterial cells that involve the manipulation of closed circular DNA, such as chromosome replication, segregation, and transcription (Vos et al., 2011). Due to their remarkable conservation and essential functions, the bacterial type IIA topoisomerases are targets of several groups of antimicrobials, bacteriocins, as well as the CcdBA and ParDE type II TA modules. These toxins, the bacteriocins, as well as quinolone-based antimicrobials, are poisons that interfere with the topoisomerase catalytic cycle to trap the usually only transient cleavage complex, in which the enzyme forms a polypeptide bridge that is

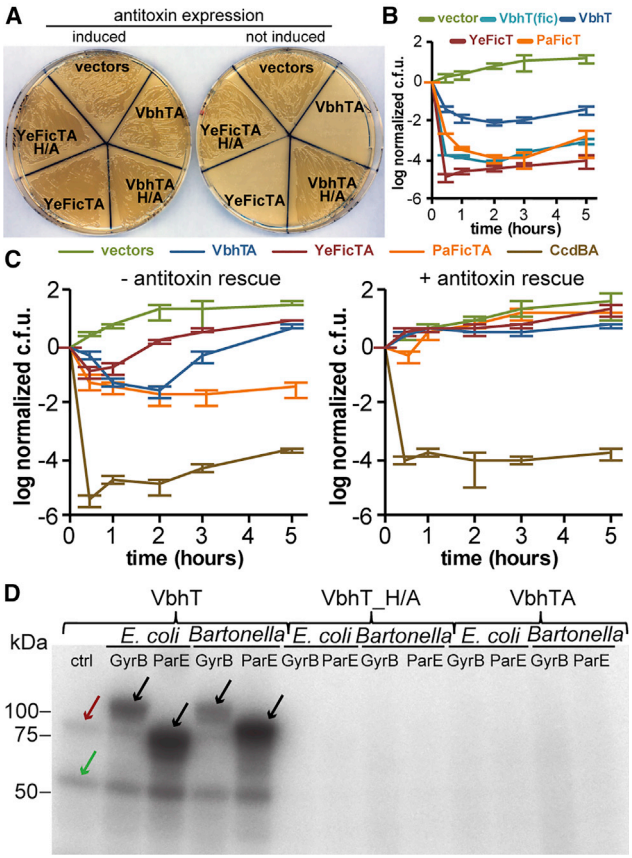

Figure 2. FicTs Reversibly Inhibit Bacterial Growth and Adenylylate Gyrase and Topo IV

(A) FicTs, but not catalytically inactive mutants (H/A with a H136A substitution at the active site), inhibit *E. coli* growth on LB agar plates in the absence of cognate FicAs. Similarly, activation of the genuine PaFicTA module in *P. aeruginosa* caused bacterial growth inhibition (see Figure S2C).

(B) We followed the colony-forming units per milliliter of exponentially growing *E. coli* cultures in which FicT expression had been induced with 2 mM IPTG. The curves show a marked growth inhibition by YeFicT, PaFicT, or only the FIC domain of VbhT (VbhT(fic)) and a lower potency of the VbhT full-length construct. No growth inhibition was observed with EcFicT (Figure S2A). Note that the induction with 2 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) is typically employed for the biological characterization of TA module toxins expressed from the P*lac* single-copy vector that we used (e.g., by Christensen and Gerdes, 2003) and does not constitute an unphysiological overexpression.

(C) The colony-forming units per milliliter of exponentially growing *E. coli* cultures harboring plasmids for the expression of toxins under P*lac* and cognate antitoxins under P*ara* control were monitored over time after the induction of FicT expression by spotting on LB agar plates containing D-glucose (to inhibit toxin expression) or L-arabinose (to induce antitoxin expression). We find that the growth inhibition by FicTs, but not gyrase-poisoning toxin CcdB, is reversed by subsequent antitoxin expression. Weaker growth inhibition by FicTs in (C) compared to (B) is likely the consequence of leaky antitoxin expression.

(D) Cleared lysates of *E. coli* that had expressed different VbhT (full-length) constructs or target candidates were mixed, incubated with [α-^{32}P]-ATP to trace adenylylation, and then analyzed using SDS-PAGE and autoradiography. The autoradiograph shows VbhT auto-adenylylation (58 kDa; green arrow) and adenylylation of endogenous GyrB (90 kDa; red arrow) as well as ectopically expressed glutathione S-transferase (GST) fusions of GyrB and ParE of *E. coli* and *B. schoenbuchensis*, respectively (black arrows). A Coomassie stain of the same SDS-PAGE gel confirmed the presence of all ectopically expressed proteins (Figure S2D). Using mass spectrometry, we mapped the adenylylation sites to be tyrosine 109 in *E. coli* GyrB and tyrosine 105 in *E. coli* ParE (Figure S3). VbhT did not adenylylate GyrA or ParC, the A subunits of gyrase and topo IV (Figure S4A), and no adenylylation activity was detected for EcFicT (Figures S4B and S4C).

All data points and error bars in (A)–(C) are mean and SD of three independent experiments.

covalently linked to both ends of a double-strand break (Chen et al., 1996; Deghorain et al., 2013; Vos et al., 2011). This break is exposed upon removal of the topoisomerase, e.g., after collision of transcription complexes or replication forks with the trapped cleavage complex, which ultimately results in cell death (Aldred et al., 2014). In contrast, gyramide A and aminocoumarin drugs like novobiocin merely inactivate their topoisomerase targets as competitive inhibitors of their ATP hydrolysis activity, which abrogates the cellular control of DNA topology, but does not directly trigger cell death (Hardy and Cozzarelli, 2003; Rajendram et al., 2014).

Adenylylation at the ATP-Binding Site Inhibits ATP-Dependent Activities of Gyrase and Topo IV In Vitro

Using mass spectrometry, we found that VbhT adenylylates Tyr109 and its homolog Tyr105 in GyrB and ParE of *E. coli*, respectively (Figure S3). This highly conserved residue is part of the ATP lid loop (residues 99–120 of *E. coli* GyrB) and borders the ATP-binding site (Brino et al., 2000; Stanger et al., 2014). We therefore assayed the ATPase activity of GyrB43, a construct that has been used repeatedly to assay the ATPase activity of DNA gyrase (Brino et al., 2000), as well as a corresponding construct of ParE. Adenylylation indeed inhibited the ATPase activity of both targets (Figure 3A).

Given that ATP hydrolysis is crucial for the cellular activities of DNA gyrase and topo IV (Bates et al., 2011), we next used recombinant DNA gyrase and topo IV to assess the effect of adenylylation on different activities of both enzymes in vitro. As expected, adenylylation prevented all ATP-dependent activities of both targets, namely the supercoiling of a relaxed reporter plasmid by DNA gyrase, the relaxation of supercoiled reporter plasmid by topo IV, and the decatenation of kinetoplast DNA (kDNA), a meshwork of catenated DNA rings, by topo IV (Figures 3B and 3C). Unlike these activities, the cleavage complex stabilization (poisoning) of type II topoisomerases with fluoroquinolone drugs is ATP independent (Pierrat and Maxwell, 2003) and was not affected by adenylylation (Figure 3D). These results suggest that a block of the targets' ATPase activity by adenylylation is causal to their observed inhibition in vitro, and they indicate that FicTs pull the plug on gyrase and topo IV to suspend their control of cellular DNA topology in vivo.

FicTs Cause Varying Levels of DNA Gyrase Inhibition In Vivo

Any inhibition of DNA gyrase would decrease the negative supercoiling of cellular DNA. We therefore used DNA of high-copy plasmid pAH160 isolated from *E. coli* expressing FicTs to directly visualize changes in DNA topology via high-resolution agarose gel electrophoresis with chloroquine to resolve negative supercoiling (see the Supplemental Experimental Procedures). As

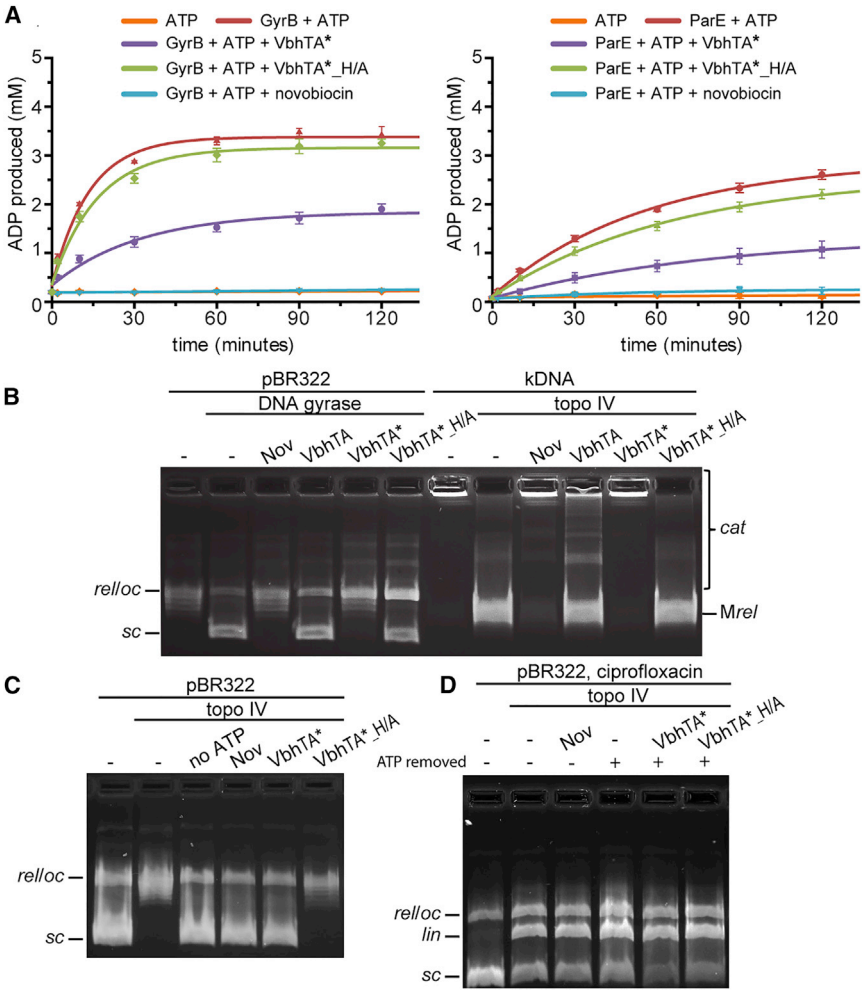

Figure 3. Adenylylation Inhibits the ATPase Activity of Gyrase and Topo IV and Blocks All Their ATP-Dependent Activities In Vitro

(A) The curves represent ATP hydrolysis by GyrB (left) and ParE (right) as monitored by ADP production. Addition of catalytically inactive VbhTA*_H/A had no effect on ATP hydrolysis (compare green and red curves). However, adenylylation by the active VbhTA* construct (lacking antitoxin inhibition) progressively inhibited ATP hydrolysis by both GyrB and ParE (purple curves). As expected, the ATPase inhibitor novobiocin immediately blocked ATP hydrolysis (cyan curve). Data points and error bars are mean and SD of three independent experiments. We verified that the GyrB and ParE constructs used for these experiments are adenylylated by VbhTA* (Figures S4B and S4C).

(B) The supercoiling and decatenation activities of recombinant DNA gyrase and topo IV were probed by monitoring the supercoiling (*sc*) of relaxed pBR322 (*rel*) and the decatenation of highly catenated kDNA (*cat*) into monomers (M*rel*), respectively. The targets were pre-incubated with different VbhT constructs for 30 min prior to the addition of DNA substrates. The agarose gel resolving the reaction products shows that both enzymes are fully inhibited by adenylylation as well as by novobiocin (Nov). *oc*, open circular (nicked) plasmid.

(C) The relaxation activity of topo IV was assayed analogously to the experiments in (B). Topo IV readily relaxed negatively supercoiled pBR322, but was inhibited by the depletion of ATP by the ATPase inhibitor novobiocin (Nov) and by adenylylation by VbhTA*. ATP had been depleted via the phosphorylation of D-glucose by hexokinase (see also Figure S4D).

(D) The poisoning of topo IV with ciprofloxacin is revealed by the appearance of linear (*lin*) reporter plasmid and not affected by the depletion of ATP or the ATPase inhibitor novobiocin, as expected from Pierrat and Maxwell, (2003). Similarly, adenylylation had no effect on topo IV poisoning.

The asterisk in VbhTA* indicates the presence of mutant VbhA(E24G) that is unable to inhibit VbhT, resulting in an active, adenylylation-competent toxin construct (Engel et al., 2012; see also the Supplemental Experimental Procedures).

expected, the treatment of *E. coli* with a high concentration of novobiocin that completely inactivated DNA gyrase resulted in collapse of the negative supercoiling (Figure 4A). However, a dose of VbhT, VbhT(fic), or YeFicT expression that strongly impaired bacterial growth (2 hr of full induction; compare Figure 2B) caused only a slight stretch of the topoisomer distribution toward DNA relaxation, while the expression of PaFicT resulted in full relaxation of the reporter plasmid (Figure 4A). These divergent findings led us to suspect that the transient nature of local DNA relaxation for the single topological domain of our simple reporter plasmid may have masked more prominent effects of a potential partial inhibition of DNA gyrase upon expression of our VbhT and YeFicT constructs.

We therefore used another reporter plasmid encoding a relaxation-induced P*gyrB::gfpmut2* GFP promoter fusion to record any DNA gyrase inhibition inside single cells by flow cytometry. For validation, we confirmed that *E. coli* harboring this plasmid showed dose-dependent induction of GFP fluorescence upon inhibition of DNA gyrase with different concentrations of novobiocin (Figure 4B). In this system the expression of VbhT or YeFicT resulted in a rather weak but detectable induction of GFP fluorescence, while VbhT(fic) elicited a stronger response and the expression of PaFicT had a similar effect as the highest concentrations of novobiocin (Figure 4B). Furthermore, we constructed a chromosomal P*gyrB::gfpmut2* reporter in order to directly assess the supercoiling of the nucleoid and avoid potential artifacts from analyzing plasmid reporters, which can be misleading (Rovinskiy et al., 2012). Like with the plasmid-based assay (Figure 4B), different concentrations of novobiocin caused a dose-dependent induction of GFP fluorescence, while FicT expression resulted in weak (VbhT and YeFicT), moderate (VbhT(fic)), or strong DNA relaxation (PaFicT; Figure 4C). These findings mirror our results from high-resolution agarose gel electrophoresis, and, thus, they indicate that the expression of our VbhT and YeFicT constructs is unlikely to achieve levels of DNA gyrase inhibition in vivo that would greatly contribute to growth inhibition.

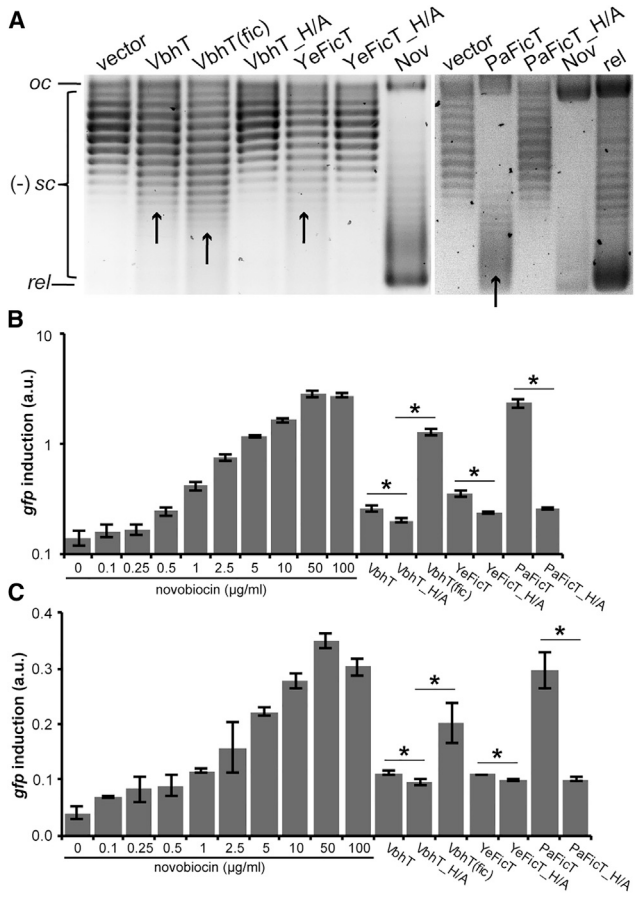

Figure 4. FicTs Cause Divergent Levels of DNA Gyrase Inhibition In Vivo

(A) Reporter plasmid pAH160 was analyzed by high-resolution agarose gel electrophoresis (in the presence of 12.5 μg/ml chloroquine to resolve negative supercoiling) following isolation from *E. coli* that had expressed different FicT constructs for 2 hr. The gel shows only a slight stretching of the pAH160 topoisomer distribution toward DNA relaxation with VbhT or YeFicT (arrows), indicative of weak inhibition of DNA gyrase, while high concentrations of novobiocin (Nov; 100 μg/ml) or the expression of PaFicT fully abrogate the negative supercoiling.

(B) The induction of a relaxation-sensitive P*gyrB::gfpmut2* module on a reporter plasmid was analyzed by flow cytometry in *E. coli* that had expressed different FicT constructs for 2 hr. As expected, the treatment with novobiocin elicited a dose-dependent response (10 μg/ml fully inhibit DNA gyrase in *acrA*-deficient *E. coli* [Khodursky et al., 2000]). Different FicT constructs induced weak (VbhT and YeFicT), intermediate (VbhT(fic)), or strong (PaFicT) expression of GFP ($p < 0.01$, unequal variance t test).

(C) A chromosomal P*gyrB::gfpmut2* module was used to assay nucleoid relaxation as described for the plasmid sensor in (B). Similarly, our set of FicT constructs differentially induced the expression of GFP (all $p < 0.05$, unequal variance t test).

All data points and error bars are mean and SD of biological triplicates.

The more prominent effect with VbhT(fic) compared to VbhT in full length may be due to differences in expression or reflect an evolution of the VbhT FIC domain to stronger activity in order to partially compensate for sterical hindrance by its C-terminal BID domain. Despite that, neither VbhT(fic) nor PaFicT were more potent toxins than YeFicT (Figure 2B), suggesting that

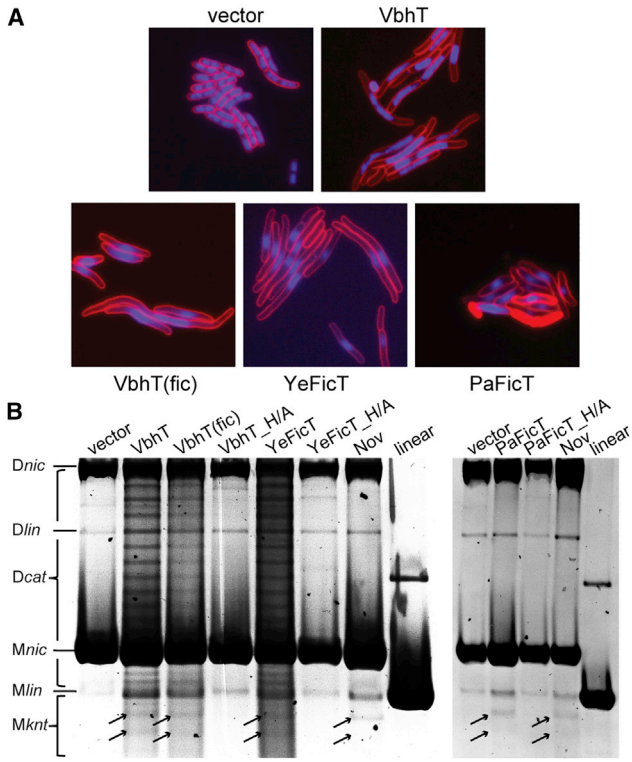

Figure 5. FicT Expression Results in Robust Inhibition of Topo IV In Vivo

(A) *E. coli* cells that had expressed different FicT constructs for 2 hr were stained with FM4-64 (membranes; red) and DAPI (DNA; blue) and analyzed by fluorescence microscopy; representative images are shown. FicT expression induces a *par* phenotype with cell filamentation and unsegregated nucleoids, indicative of strong topo IV inhibition. The nucleoid compaction observed with VbhT(fic), YeFicT, and PaFicT is likely a consequence of DNA gyrase inhibition (Rajendram et al., 2014).

(B) Nicking of the DNA samples used for Figure 4A separates nicked (*nic*)/linear (*lin*) dimers (*D*) and monomers (*M*) and reveals catenation (D*cat*) and knotting (M*knt*, arrows) upon FicT expression. High concentrations of novobiocin (100 μg/ml, *Nov*) or PaFicT expression also inactivate DNA gyrase (note collapsed supercoiling in Figure 4A), leading primarily to DNA knotting in the absence of DNA replication. A lane plot that highlights the bands representing knotted topoisomers is shown in Figure S5.

DNA gyrase inhibition is not the primary driving force of FicT-mediated growth inhibition.

FicTs Robustly Inhibit Topo IV In Vivo

The inactivation of topo IV is known to result in DNA knotting and catenation, which induce a classical phenotype called *par* hallmarked by cell filamentation and the sequestration of unsegregated DNA at the cell center (Kato et al., 1990). We, therefore, used fluorescence microscopy to study cell shape and DNA partitioning in *E. coli* expressing FicTs (Figure 5A). As expected from our biochemical data with VbhT, the expression of any FicT greatly inhibited DNA segregation and bacterial cell division, which were first indications of topo IV inactivation in vivo (Figure 5A). In deviation from a classical *par* phenotype, the cells expressing VbhT(fic), YeFicT, and PaFicT, but not VbhT, displayed

a highly condensed nucleoid morphology. These findings were reminiscent of the phenotype that others have reported for gyramide A treatment, suggesting that the compaction was caused by DNA gyrase inhibition coming on top of the inactivation of topo IV (Rajendram et al., 2014).

As an independent readout of topo IV inhibition in vivo, we reanalyzed the pAH160 samples that we had used for the detection of DNA relaxation by high-resolution agarose gel electrophoresis (see Figure 4A). After nicking to release all supercoiling, the samples were run on plain agarose gels to resolve DNA knotting and catenation. Plasmid isolated from *E. coli* that had expressed any of the FicT constructs showed detectable DNA knots, evidencing robust topo IV inhibition (Figures 5B and S5). DNA knots continuously arise in the tangled nucleoid from random strand passages that occur whenever cellular DNA handling cuts open the chromosome, e.g., during processes of replication, recombination, or repair, and they cannot be removed in the absence of functional topo IV (Deibler et al., 2001). The seemingly low extent of all DNA knotting seen with pAH160 is due to the small size of this reporter plasmid (4,359 bp) and hints at considerable knotting of the nucleoid, since the topological entanglement and size of chromosomal DNA greatly favors knotting and complicates unknotting compared to small plasmids (Witz and Stasiak, 2010). Additionally, a clear ladder of catenanes with various node numbers was detected for VbhT and YeFicT as well as more weakly for VbhT(fic), but not for PaFicT or the novobiocin treatment (dimeric catenanes appear between nicked monomeric and dimeric plasmid; Figure 5B). The absence or reduction of catenation with VbhT(fic) and PaFicT compared to VbhT or YeFicT expression confirms the stronger gyrase inhibition that we detected earlier, because a concomitant inactivation of DNA gyrase and topo IV (but not the latter alone) causes a rapid arrest of DNA replication and, therefore, prevents the formation of catenanes (Adams et al., 1992; Khodursky et al., 2000).

To investigate the molecular mechanism of target inactivation by FicTs in more detail, we then assayed whether the overexpression of GyrB and/or ParE could rescue the growth inhibition caused by FicT expression. Such reversion of the growth arrest is characteristic of a purely inhibitory mechanism like, for example, of the HipA toxin that phosphorylates and concomitantly inactivates GltX (Germain et al., 2013). In contrast, the presence of a susceptible, wild-type target is dominant over the co-expression of even a resistant mutant in case of the gyrase poison CcdB (Bernard and Couturier, 1992). Our results show that the growth inhibition caused by VbhT could be prevented by the overexpression of ParE, implying that FicTs act via an inhibitory mechanism (Figure 6A). No effect was observed for the overexpression of GyrB, confirming previous findings that the inactivation of DNA gyrase has no major contribution to growth inhibition by VbhT (Figure 4).

FicTs Impair Cellular DNA Processing

Our experiments investigating the effects of FicT expression on DNA topology in bacterial cells had strongly suggested that the obstruction of topological control upon topoisomerase inhibition was the cause of the growth arrest observed with FicTs. We therefore explored the consequences of FicT activity inside bacterial cells more deeply, and we genetically confirmed that the expression of FicTs caused major problems with DNA functioning, particularly chromosome replication and segregation (Figure S6A). Furthermore, we investigated the genetic requirements of the observed full reversibility of the growth inhibition caused by FicTs (see Figure 2C). The deletion of *recA*, a factor essential for double-strand break repair (Wigley, 2013), did not abolish the reversibility of bacterial growth inhibition caused by VbhT or YeFicT (Figure 6B). This result confirms that adenylylation does not cause topoisomerase poisoning, which would result in the formation of double-strand breaks. Instead, the viability of bacteria that expressed FicTs was only impaired upon inactivation of both exonuclease V as well as homologous recombination in a *recDA* double mutant or the *recB* knockout (Figure 6B). These two functions constitute parallel pathways of replication fork restart after fork regression and reversal that initiate the processing of collapsed replication forks, thus providing solid evidence that the activity of FicTs interferes with continuous replication fork movement (De Septenville et al., 2012). Others have indeed shown that an inactivation of topo IV results in an inhibition of DNA replication and transcription as well as an impaired topological control of replication forks that is aggravated in different ways by DNA gyrase inhibition (Deibler et al., 2007; Khodursky et al., 2000; Witz et al., 2011).

The single-stranded DNA that is typically exposed at arrested replication forks is the direct molecular inducer of the SOS response, a cascade of genes whose expression is triggered by bacteria in order to respond to DNA damage (Erill et al., 2007). We detected an activation of the SOS response upon FicT expression (Figure 6C) that varied between the different constructs from not significant (VbhT) to similarly strong as with topoisomerase poisons ciprofloxacin or CcdB (VbhT(fic) and PaFicT). Interestingly, the SOS induction roughly correlated with the extent of gyrase inhibition by the different FicT constructs (Figure 6C; compare Figure 4). It is, therefore, likely that the SOS induction is caused by the aggravation of replication fork collapse upon failure of DNA gyrase to continuously remove the positive supercoils forming ahead of the progressing forks. Similarly, it was shown that treatment with gyramide A, an inhibitor of ATP hydrolysis of DNA gyrase, but not topo IV, activated the SOS response in *E. coli* (Rajendram et al., 2014), and we also detected an SOS induction with novobiocin (Figure 6C). Though the SOS response mostly activates DNA repair functions, it can also directly block bacterial cell division upon strong DNA damage and, therefore, could contribute to growth inhibition upon FicT activity (Erill et al., 2007). However, given that the extent of SOS induction did not correlate with the potency of growth inhibition (compare Figures 6C and 2B) and that SOS induction did not considerably contribute to it at relevant levels of FicT expression (Figures S6B and S6C), we conclude that the inhibition of bacterial growth by FicTs in our system was mostly driven by topo IV inhibition.

DISCUSSION

FicTs Inhibit Bacterial Growth by Adenylylation of DNA Gyrase and Topo IV

In this study, we determined the common molecular mechanism of distinct representatives of the conserved and abundant FicTA

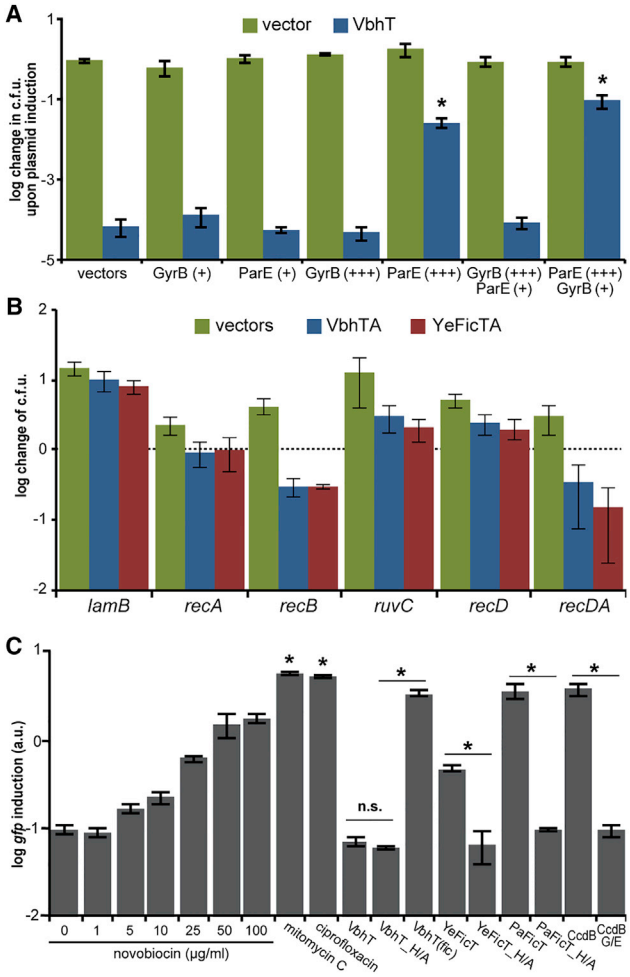

Figure 6. Growth Arrest Caused by FicTs Is Reversible upon Target Overexpression and Accompanied by Impaired DNA Processing

(A) *E. coli* harboring plasmids for the expression of VbhT under P*lac* control as well as target(s) under control of P*ara* (low-copy vectors, denoted by "+" expression level) or P*rha* (high-copy vectors, denoted by "+++" expression level) were spotted in serial dilutions onto different agar plates. The plates contained either no inducer or 2 mM IPTG (for P*lac*) as well as 0.2% L-arabinose and L-rhamnose (for P*ara* and P*rha*). The bar diagram shows the difference in colony-forming units obtained between the conditions of no inducer and all inducers. It is apparent that a strong induction of ParE expression can prevent the growth inhibition caused by VbhT ($p < 0.05$, unequal variance t test against vector control of target expression). Data points are mean values of three independent experiments with error bars denoting SEM.

(B) The reversibility of FicT-mediated growth inhibition was assayed in different *E. coli* mutants by induction of antitoxin expression on LB agar plates subsequent to FicT expression in liquid culture, just as it had been done for the experiment shown in Figure 2C. In this system, a reversible growth inhibition results in no change or an increase in colony-forming units after toxin expression, while a decrease in colony-forming units indicates an irreversible loss of bacterial viability. We detected a fully reversible growth inhibition in the *ruvC* mutant that is highly sensitive to FicT expression (see Figure S6A) as well as in the *lamB* mutant that served as an isogenic control. Similarly, the single inactivation of *recA*, which is essential for double-strand break repair (Wigley, 2013), did not abolish the reversibility of a FicT-mediated growth arrest, confirming that FicTs do not poison their targets. Bacterial viability was only compromised irreversibly upon dual inactivation of exonuclease V (RecBCD) and homologous recombination, which represent the two branches of replication fork restart after fork reversal (*recB* as well as *recDA* mutant; log change of cfu < 0). These results strongly suggest that FicT activity blocks replication fork movement and that fork reversal drives subsequent replication fork restart (De Septenville et al., 2012). Data bars represent mean values of three independent experiments and error bars denote SEM.

(C) We probed an activation of the SOS response upon FicT expression using flow cytometry recording fluorescence from a plasmid-encoded P*sulA::gfpmut2* module with a procedure identical to the detection of DNA relaxation via a P*gyrB::gfpmut2* reporter (Figure 4B). As expected, the gyrase poisons CcdB and ciprofloxacin (1 μg/ml) or the DNA-damaging agent mitomycin C (1 μg/ml) caused massive induction of the reporter, and novobiocin induced a clear, yet weaker, dose-dependent response. FicT expression resulted in a divergent induction of the SOS response that was qualitatively similar to the DNA gyrase inhibition achieved (Figure 4). While SOS induction with VbhT was not significant (n.s.) in this system, VbhT(fic) and PaFicT caused levels of GFP expression similar to those observed with the positive controls (all $p < 0.05$, unequal variance t test). The expression of inactive toxin mutants (FicT_H136A or CcdB_G100E) did not cause any SOS induction. All data points are mean values of three independent experiments with error bars denoting SD. Asterisks indicate $p < 0.05$ (unequal variance t test).

family as the adenylylation and concomitant inactivation of DNA gyrase and topo IV, indicating that this activity is the original and most prevalent function of these proteins. The expression of toxins VbhT, YeFicT, and PaFicT consistently caused a robust inhibition of topo IV activity in *E. coli*, but the extent of DNA gyrase inhibition differed, possibly reflecting divergent histories of host adaptation or a quantitative limitation of our single-copy vector expression system. Though a robust inhibition of topo IV alone is sufficient to inhibit bacterial growth, any additional inhibition of DNA gyrase aggravates this effect because both decatenation and unknotting activities of topo IV are guided by the gyrase-dependent negative supercoiling of the nucleoid (Witz and Stasiak, 2010). As inhibitors of ATP hydrolysis, the FicTs can be compared to drugs like aminocoumarins or gyramides, which have a similar mechanism of type II topoisomerase inhibition (Figure 3A; Hardy and Cozzarelli, 2003; Rajendram et al., 2014). The other known topoisomerase-targeting TA systems CcdBA and ParDE are bactericidal plasmid addiction modules that trigger lethal DNA double-strand breaks by poisoning gyrase activity via non-covalent interaction with the A subunit (Bernard and Couturier, 1992; Jiang et al., 2002).

Conversely, the growth arrest caused by FicTs is largely reversible (Figure 2C) and relies on a covalent modification of the B subunit of both DNA gyrase and topo IV (Figures 2D and 3), resulting in mere target inactivation and not poisoning. Therefore, target overexpression could prevent the growth arrest caused by VbhT (Figure 6A), and we did not observe an impaired viability of *recA* mutants or the appearance of diffuse nucleoids upon FicT expression, which would have indicated DNA double-strand breaks (Figures 5A and 6B; Rajendram et al., 2014). In distinction from the gyrase-poisoning addiction modules, the mode of target inhibition by FicTs is rather reminiscent of a variety of small proteins that inhibit gyrase by tight interaction that blocks the DNA binding of the topoisomerase and, thus, prevents DNA damage caused by poisoning (Sengupta and Nagaraja, 2008; Tran et al., 2005). However, the ability of FicTs to easily abrogate bacterial growth as well as their action as enzymes constitute major conceptual differences and rather suggest a different biological function, possibly in bacterial persister

formation, that may be revealed by futures studies. Additionally, we anticipate the use of different FicTs as tools in bacterial cell biology to deliberately inactivate topo IV and/or gyrase in vivo in studies investigating the biological roles of these enzymes and the functionalities of bacterial DNA topology.

Evolutionary Connections of FicTA Modules

FicT proteins are related both mechanistically and phylogenetically to the Doc toxin that blocks translation as a kinase that targets elongation factor EF-Tu and that is thought to have evolved from adenylylating ancestors via an inversion of ATP substrate binding (Castro-Roa et al., 2013). The example of Doc highlights the biochemical and functional plasticity that is generally characteristic of FIC domain proteins (Garcia-Pino et al., 2014). Furthermore, a recent computational study revealed the emergence of variant active sites among different clades of FIC domain proteins that indicate repeated functional diversification (Khater and Mohanty, 2015). Similarly, the EcFicT protein and a tight cluster of enterobacterial homologs are hallmarked by a non-canonical FIC domain active site motif (Figure 1; Goepfert et al., 2013) and appear not to display adenylylation activity or bacterial growth inhibition (Figures S2A, S4B, and S4C). It is, therefore, likely that these proteins have secondarily evolved another molecular activity and biological function that may be uncovered by future work.

Unlike EcFicT, YeFicT, and the other FicT homologs, VbhT of *B. schoenbuchensis* uniquely harbors a discernible type IV secretion signal at its C terminus, a BID domain. BID domains are found in relaxases of a number of bacterial conjugation systems, as well as the host-targeted effectors of the genus *Bartonella* (Schulein et al., 2005), suggesting that these bacteria may secrete VbhT into target cells (prokaryotic or eukaryotic). We are currently investigating the biological function of VbhT as a secreted FicT that would constitute another example of the remarkable functional plasticity of FIC domain proteins.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids

Bacterial strains and plasmids used and constructed over the course of this study are described in the Supplemental Experimental Procedures. All oligonucleotide primers are listed in Table S1. All vectors and details of their construction are listed in Table S2.

Toxicity Tests

Unless stated differently, the effect of FicT expression on bacterial viability was investigated in *E. coli* K-12 MG1655 Δ*ecficAT* (AHE573) and derivatives using a two-plasmid system with toxin genes under P*lac* control (derivatives of pNDM220 single-copy vector) and antitoxin genes under P*ara* control (pBAD33 derivatives), as well as occasional other plasmids to co-express enzymatic targets or mutants of FicTs. The PaFicTA module of *P. aeruginosa* PAO1 was investigated via experimental activation by antitoxin sequestration upon expression of catalytically inactive PaFicT_H136Y from a P*ara* vector (pHERD30T derivative; see Figure S2B). Detailed procedures are described in the Supplemental Experimental Procedures.

Protein Expression and Purification

Different VbhT constructs and GyrB43, an N-terminal fragment of GyrB containing the ATPase domain (Ali et al., 1993), or an analogous construct of ParE were expressed and purified as described previously (Engel et al., 2012; Stanger et al., 2014). For reasons of protein solubility, VbhT always was purified in complex with its antitoxin VbhA either in the inhibition-competent wild-type form (represented as VbhTA) or as a VbhA(E24G) mutant, which is unable to inhibit the adenylylation activity of the toxin (indicated as VbhTA*; see also our previous work [Engel et al., 2012]). Further details on the expression and purification of VbhT constructs are given in the Supplemental Experimental Procedures.

ATP Hydrolysis Assay

The ATPase activity of GyrB43 and a corresponding construct of ParE was monitored by fast protein liquid chromatography (FPLC)-based nucleotide quantification using conditions described in the Supplemental Experimental Procedures.

In Vitro Adenylylation Assays

The adenylylation activity of FicT constructs was assessed using cleared lysates of ectopically expressing *E. coli* (Figures 2D and S4A) or purified proteins (Figures S4B and S4C) and [α-^{32}P]-ATP (Hartmann Analytic), as described previously (Engel et al., 2012). In short, the AMP transfer was specifically labeled using the radioactive substrate, reactions were resolved by SDS-PAGE, and adenylylated proteins were visualized by autoradiography.

In Vitro Topoisomerase Assays

In vitro supercoiling, relaxation, decatenation, and poisoning assays with recombinant DNA gyrase or topo IV of *E. coli* were performed using suitable DNA substrates according to the supplier's recommendations (TopoGEN and Inspiralis). Details of the experimental procedures are described in the Supplemental Experimental Procedures.

Flow Cytometry with *gfp* Promoter Fusions

The responses of P*gyrB* (for DNA relaxation [Menzel and Gellert, 1983]) and P*sulA* (for SOS induction) promoters to FicT expression were probed using plasmid-encoded *gfpmut2* promoter fusions of the collection created by Zaslaver et al. (2006) and pUA139, the parental plasmid without promoter, or chromosomal derivatives thereof (AHE1156 and AHE1158; see Strain Construction in the Supplemental Experimental Procedures). Experimental details are given in the Supplemental Experimental Procedures.

High-Resolution Agarose Gel Electrophoresis

Changes in cellular DNA topology upon FicT expression were assessed using the pAH160 reporter plasmid isolated from snap-frozen samples of *E. coli* AHE938 cultures with the Wizard Plus SV Minipreps DNA Purification kit (QIAGEN). Details of the experimental procedure for high-resolution agarose gel electrophoresis to resolve DNA supercoiling, knotting, and catenation are described in the Supplemental Experimental Procedures.

Fluorescence Microscopy

E. coli AHE573 that had expressed FicTs for 2 hr were stained with membrane dye FM4-64 (2.5 μg/ml, Molecular Probes) and DNA dye DAPI (5 μg/ml, Roche) for 30 min in Luria broth (LB) medium in the dark and then transferred onto microscopy slides coated with 1% agarose. Images were acquired using an Olympus IX71 microscope equipped with a CoolSnap HQ2 camera, LED illumination, and a 100× phase contrast objective (all from Applied Precision). Filter sets were as follows: (1) excitation, 390/18; emission, 435/48 (DAPI); and (2) excitation, 542/27; emission, 594/45 (TRITC). Snapshots were taken using SoftWorx 5.5 software (GE Healthcare), and the pictures were adjusted for publication using ImageJ (http://rsbweb.nih.gov/ij/download.html) and Photoshop CS5 extended 12.0.4×64 (Adobe). The microscopy experiment with *P. aeruginosa* (Figure S2C) was performed analogously and details are given in the Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.07.056.

AUTHOR CONTRIBUTIONS

All authors participated in experimental design and data analysis. A.H., F.V.S., P.D.S., and A.G. cloned recombinant plasmids and constructed bacterial strains. F.V.S. and A.G. expressed and purified protein constructs. A.H., F.V.S., P.D.S., A.G., and I.G.d.J. performed experiments. T.G. conducted mass spectrometry analysis. The manuscript was written by A.H., F.V.S., P.D.S., I.G.d.J., K.G., T.S., and C.D.

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