Title

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Journal

Proceedings of the National Academy of Sciences of the United States of America, 114(10)

ISSN

0027-8424

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Publication Date

2017-03-07

DOI

10.1073/pnas.1619273114

Peer reviewed

Activation of contact-dependent antibacterial tRNase toxins by translation elongation factors

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Edited by Joe Lutkenhaus, University of Kansas Medical Center, Kansas City, KS, and approved January 30, 2017 (received for review November 22, 2016)

Contact-dependent growth inhibition (CDI) is a mechanism by which bacteria exchange toxins via direct cell-to-cell contact. CDI systems are distributed widely among Gram-negative pathogens and are thought to mediate interstrain competition. Here, we describe *tsf* mutations that alter the coiled-coil domain of elongation factor Ts (EF-Ts) and confer resistance to the CdiA-CT^{EC869} tRNase toxin from enterohemorrhagic *Escherichia coli* EC869. Although EF-Ts is required for toxicity in vivo, our results indicate that it is dispensable for tRNase activity in vitro. We find that CdiA-CT^{EC869} binds to elongation factor Tu (EF-Tu) with high affinity and this interaction is critical for nuclease activity. Moreover, in vitro tRNase activity is GTP-dependent, suggesting that CdiA-CT^{EC869} only cleaves tRNA in the context of translationally active GTP·EF-Tu·tRNA ternary complexes. We propose that EF-Ts promotes the formation of GTP·EF-Tu·tRNA ternary complexes, thereby accelerating substrate turnover for rapid depletion of target-cell tRNA.

cell communication | protein synthesis | toxin-immunity proteins | ribonuclease | bacterial competition

Bacteria use several strategies to compete and cooperate with neighboring microorganisms in the environment. Contact-dependent growth inhibition (CDI) represents one important form of interbacterial competition that is common among Gram-negative pathogens (1–3). CDI is mediated by the CdiB/CdiA family of two-partner secretion proteins, which assemble as a complex on the surface of CDI$^+$ bacteria. CdiB is an Omp85 β-barrel protein embedded in the outer membrane, where it functions to export long filamentous CdiA effector proteins. CdiA effectors project from the inhibitor-cell surface and bind to receptors on susceptible neighboring bacteria. Upon binding receptor, CdiA transfers its C-terminal toxin domain (CdiA-CT) into the target bacterium through an incompletely understood translocation mechanism (4, 5). Genome and protein database surveys show that CdiA effectors carry a wide variety of distinct toxins (1, 6–8). CDI$^+$ cells protect themselves from self-intoxication by producing CdiI immunity proteins, which bind specifically to cognate CdiA-CT domains and neutralize their toxic activities. Because *cdi* loci encode an elaborate network of toxin/immunity protein pairs, the systems are hypothesized to mediate interstrain competition and self-/non–self-recognition.

Our previous studies have shown that CDI toxins inhibit cell growth using different mechanisms. The CdiA-CT^{EC93} domain deployed by *Escherichia coli* isolate EC93 increases target-cell permeability to protons (9, 10), suggesting that this toxin forms pores in the inner membrane. Many other CdiA-CT toxins are nucleases that must be delivered into the target-cell cytoplasm to inhibit growth. CdiA-CT^{3937} from *Dickeya dadantii* 3937 has potent DNase activity that destroys the target-cell chromosome (1, 11), whereas the CdiA-CT^{ECL} toxin from *Enterobacter cloacae* ATCC 13047 cleaves 16S rRNA to block protein synthesis (12). tRNA molecules are particularly common substrates for CDI nuclease toxins. *Burkholderia pseudomallei* isolates K96243, 1026b, and E479 deploy tRNase toxins with distinct specificities. CdiA-CT^{K96243} has anticodon nuclease activity on tRNA^{His}, tRNA^{Asp}, tRNA^{Asn}, and tRNA^{Tyr} isoacceptors, and CdiA-CT^{E479} cleaves the T-loop of tRNA molecules between conserved residues Ψ54 and T55 (13, 14). CdiA-CT$_{II}^{Bp1026b}$ preferentially cleaves within the aminoacyl acceptor stem of tRNA^{Ala} to block translation (15). Other unrelated CdiA-CT toxins from *E. coli* isolates EC869 and 3006 also cleave tRNA acceptor stems but are specific for tRNA^{Gln} and tRNA^{Ile}, respectively (5, 16). Thus, interbacterial competition has exerted a selective pressure to evolve diverse tRNase toxins with distinct specificities.

Most CDI nuclease domains efficiently cleave their substrates in vitro, but the CdiA-CT^{EC536} toxin deployed by uropathogenic *E. coli* 536 requires an additional factor to promote its tRNA anticodon nuclease activity (17). Using biochemical approaches, we discovered that the biosynthetic enzyme *O*-acetylserine sulfhydrylase A (CysK) binds the toxin with high affinity and stimulates its nuclease activity. This interaction is critical for toxin activity, and target bacteria deleted for *cysK* are fully resistant to CdiA-CT^{EC536} toxin (17). Because *cysK* mutations confer CDI-resistance (CDI^R) to target bacteria, the advantage of an additional toxin-activation step is not clear. Recent work indicates that CysK stabilizes the CdiA-CT^{EC536} fold and promotes toxin interaction with tRNA (18). It is also possible that CdiA-CT^{EC536} modulates CysK activity in immune sibling cells, perhaps serving a role in intercellular signaling. To explore whether other CDI toxins are also subject to extrinsic activation, we used a genetic approach to identify target-cell factors required for growth inhibition by the CdiA-CT^{EC869} tRNase from enterohemorrhagic *E. coli* EC869. We isolated two CDI-resistant (CDI^R) mutants with Ala202Glu and Arg219Pro missense substitutions in *tsf*, which encodes the essential translation factor EF-Ts. Both mutations alter the EF-Ts coiled-coil domain and significantly diminish CdiA-CT^{EC869} tRNase activity in target bacteria. We find that the CdiA-CT^{EC869} toxin binds to EF-Tu with high affinity and only cleaves tRNA in the context of GTP·EF-Tu·tRNA ternary complexes. Although wild-type EF-Ts is required for CdiA-CT^{EC869} toxicity in target bacteria, it appears to be dispensable for in vitro tRNase

Significance

Contact-dependent growth inhibition (CDI) systems enable cells to bind competing bacteria and deliver toxins that cleave nucleic acids or form membrane pores. Here, we characterize a CDI toxin that specifically cleaves transfer RNA (tRNA), thereby blocking protein synthesis and inhibiting bacterial growth. Remarkably, two highly conserved and essential translation factors, EF-Ts and EF-Tu, are critical for this toxic nuclease activity. The toxin binds EF-Tu with high affinity and only cleaves tRNA in complex with the translation factor. EF-Ts appears to increase the rate of tRNA turnover. The activities of two other distinct CDI toxins are also regulated by EF-Ts. We propose that the regulation of toxin activity by the protein synthesis apparatus may play a role in intercellular communication.

Author contributions: A.M.J., F.G.-S., C.S.H., and D.A.L. designed research; A.M.J., F.G.-S., and J.S. performed research; A.M.J., F.G.-S., C.S.H., and D.A.L. analyzed data; and A.M.J., F.G.-S., C.S.H., and D.A.L. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1619273114/-/DCSupplemental.

activity. We propose that EF-Ts promotes tRNase activity by accelerating the delivery of tRNAs to EF-Tu·toxin complexes, thereby increasing the rate of substrate cleavage.

Results

We used a genetic approach to identify target-cell factor(s) required for CdiA-CT^{EC869}–mediated growth inhibition. Cells that deploy CdiA-CT^{EC869} significantly inhibit the growth of wild-type *E. coli*, but target bacteria are fully protected when they express the CdiI^{EC869} immunity protein (Fig. 1*A*, compare EC869 to mock). We then selected for CDI^{EC869}-resistant (CDI^{R}) target-cell mutants, reasoning that the protective mutations would disrupt genes required for toxin import and/or activation. Independent pools of CDI-sensitive (CDI^{S}) *E. coli* target cells were subjected to mutagenesis with UV light. To avoid isolating mutations that disrupt the CdiA receptor BamA, the mutagenized target cells were also provided with the *bamA* gene on a multicopy plasmid. The target-cell pools were then cocultured with CdiA-CT^{EC869}–expressing inhibitors to enrich for resistant mutants (Fig. S1*A*). Most of the UV-irradiated pools (22/24) failed to yield CDI^{R} mutants, but resistant populations were obtained from pools 3 and 17 after three rounds of selection (Fig. S1*B*). We then used complementation analysis to map the CDI^{R} mutations. The CDI^{R} mutant isolated from pool 3 was first labeled with GFP, then transduced with a cosmid library of *E. coli* chromosomal DNA. The resulting clones were cocultured individually with CDI^{EC869} inhibitors in a microtiter plate and target-cell growth monitored by GFP fluorescence (Fig. S1*A*). We identified one cosmid that rendered the mutant sensitive to CDI. Given the very low frequency of CDI^{R} mutations induced by UV irradiation, we reasoned that the affected gene was likely essential and therefore sequenced candidate genes within 40 kb of one end of the cosmid insert, located at 193,800 bp on the *E. coli* chromosome (19). We identified an Ala202Glu missense mutation in the *tsf* gene, which encodes the essential translation elongation factor EF-Ts. Analysis of the second CDI^{R} mutant from pool 17 revealed an Arg219Pro substitution in *tsf*. Introduction of *tsf(A202E)* and *tsf(R219P)* alleles into wild-type *E. coli* MG1655 conferred CDI^{R} phenotypes, demonstrating that the mutations are sufficient for resistance to the CDI^{EC869} toxin (Fig. 1*A*). This resistance is specific, because *tsf(A202E)* and *tsf(R219P)* cells are sensitive to the CdiA-CT$_{o11}^{EC869}$ DNase toxin from *E. coli* EC869, the CdiA-CT^{ECL} 16S rRNase toxin from *E. cloacae*, the CdiA-CT^{EC536} anticodon nuclease from *E. coli* 536, and the CdiA-CT^{EC93} pore-forming toxin from *E. coli* EC93 (Fig. 1*A*). Both *tsf* mutations alter residues in the coiled-coil domain of EF-Ts (Fig. 1*C*), suggesting that this region of the translation factor is important for toxin activity. The coiled-coil domain is not present in eukaryotic or mitochondrial orthologs and can be deleted from *E. coli* without loss of cell viability (20). Therefore, we generated a *tsf(Δcoil)* target strain and found that these cells are also resistant to CdiA-CT^{EC869} activity (Fig. 1*A*). Together, these results indicate that the coiled-coil domain of EF-Ts plays a critical and specific role in the CDI^{EC869} growth inhibition pathway.

CdiA-CT^{EC869} is an RNase that preferentially cleaves tRNA^{Gln} and tRNA^{Asn}, and this tRNase activity can be detected in CDI competition cocultures (Fig. S2) (16). Therefore, we asked whether CdiA-CT^{EC869} tRNase activity is detectable in the *tsf* mutants. Northern blot analysis revealed cleaved tRNA$_{CUG}^{Gln}$ in total RNA isolated from cocultures with *tsf*$^{+}$ target cells, but no tRNase activity was detected when *tsf*$^{+}$ target cells express *cdiI*^{EC869} from a plasmid (Fig. 1*B*). There was no evidence of tRNase activity in *tsf(A202E)*, *tsf(R219P)*, or *tsf(Δcoil)* target cells (Fig. 1*B*), suggesting that wild-type EF-Ts is required for toxin activity. Alternatively, it is possible that EF-Ts is required for toxin entry into the target bacteria. We addressed this latter possibility using a biochemical approach. We overexpressed His$_6$-tagged CdiA-CT^{EC869} in complex with CdiI^{EC869}, then purified the toxin by Ni^{2+}-affinity chromatography under denaturing conditions to remove the immunity protein. The isolated toxin was refolded and its nuclease activity assayed in S30 extracts prepared from *tsf*$^{+}$, *tsf(A202E)*, and *tsf(Δcoil)* cells.

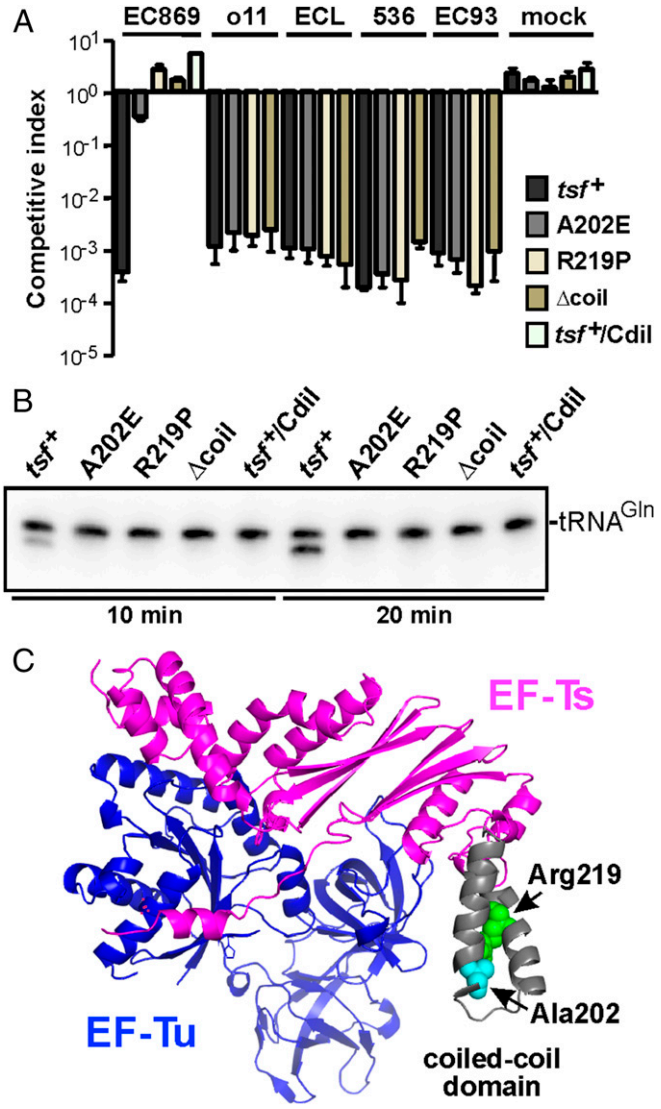

Fig. 1. *E. coli tsf* mutants are resistant to CdiA-CT^{EC869}. (*A*) *E. coli* MG1655 target strains carrying the indicated *tsf* alleles were cocultured with *E. coli* EPI100 inhibitor cells that deploy CdiA-CT^{EC869} (EC869), CdiA-CT$_{o11}^{EC869}$ (o11), CdiA-CT^{ECL} (ECL), CdiA-CT^{EC536} (536), or CdiA-CT^{EC93} (EC93) toxins. After 3 h, viable cell counts for each population were determined and used to calculate the competitive index as described in *Materials and Methods*. Mock inhibitors do not express a CDI system. Data are presented as averages ± SEM for three independent experiments. (*B*) *E. coli* EPI100 inhibitor cells expressing CdiA-CT^{EC869} were cultured at a 1:1 ratio with *E. coli* MG1655 target strains carrying the indicated *tsf* alleles. Total RNA was isolated after 10 and 20 min and subjected to Northern blot hybridization for tRNA$_{CUG}^{Gln}$. Because CDI^{EC869} inhibitor cells are immune to toxin activity, 50% substrate conversion is indicative of complete cleavage in target cells. (*C*) Structure of the *E. coli* EF-Tu·EF-Ts complex [Protein Data Bank (PDB) ID code 1EFU]. The locations of EF-Ts Ala202, Arg219, and the coiled-coil domain are indicated.

tRNA$_{CUG}^{Gln}$ cleavage was observed upon toxin addition to wild-type cell extract, but no activity was detected in *tsf(A202E)* and *tsf(Δcoil)* extracts (Fig. 2*A*). Again, the nuclease activity was specific to the CdiA-CT^{EC869} toxin, because tRNA$_{CUG}^{Gln}$ cleavage was blocked in reactions supplemented with purified CdiI^{EC869}. Together, these results indicate that EF-Ts promotes CdiA-CT^{EC869} tRNase activity.

The critical role of EF-Ts in CdiA-CT^{EC869} toxicity appears to be similar to CdiA-CT^{EC536}, which only exhibits tRNA anticodon nuclease activity when bound to CysK (17, 18). To determine whether EF-Ts activates CdiA-CT^{EC869} in the same manner, we

tested CdiA-CT^{EC869} activity in defined in vitro reactions. No tRNase activity was observed when *E. coli* tRNA was treated with purified CdiA-CT^{EC869} (Fig. 2*B*, lane 5), suggesting that the toxin requires an additional factor for activity. However, the inclusion of purified EF-Ts led to only a modest increase in nuclease activity (Fig. 2*B*, lane 6). This latter result led us to consider whether another factor may be required in conjunction with EF-Ts. Notably, we found that elongation factor EF-Tu copurifies with the CdiA-CT·CdiI^{EC869} complex under nondenaturing conditions (Fig. S3), suggesting that this translation factor may contribute to toxin activity. Markedly, a combination of purified CdiA-CT^{EC869}, EF-Tu, and wild-type EF-Ts was sufficient to cleave most of the tRNA$_{CUG}^{Gln}$ molecules in vitro, provided the reaction was supplemented with GTP (Fig. 2*B*, compare lanes 2 and 3). However, purified EF-Ts carrying the Ala202Glu substitution did not support robust tRNase activity under the same conditions (Fig. 2*B*, compare lanes 3 and 8). Further, we found that the nucleotide requirement is specific, as neither ATP nor GDP supported full nuclease activity in vitro (Fig. 2*C*, compare lanes 3 and 4–5). Because GTP is required for high-affinity binding of tRNA to EF-Tu, this latter result strongly suggests that the toxin cleaves substrate in the context of GTP·EF-Tu·tRNA complexes. To address the structural feasibility of this model, we used S1 nuclease protection to map the cleavage site on tRNA$_{CUG}^{Gln}$ (Fig. 3*A*) and found that CdiA-CT^{EC869} cleaves between C70 and A71, near the 3′-end of the acceptor stem (Fig. 3 *B* and *C*). Crystal structures of GTP·EF-Tu·tRNA ternary complexes show that the scissile phosphodiester is close to EF-Tu but is solvent-exposed to allow nuclease access (21, 22). Taken together, these data demonstrate that efficient toxin activity requires EF-Ts, EF-Tu, and GTP.

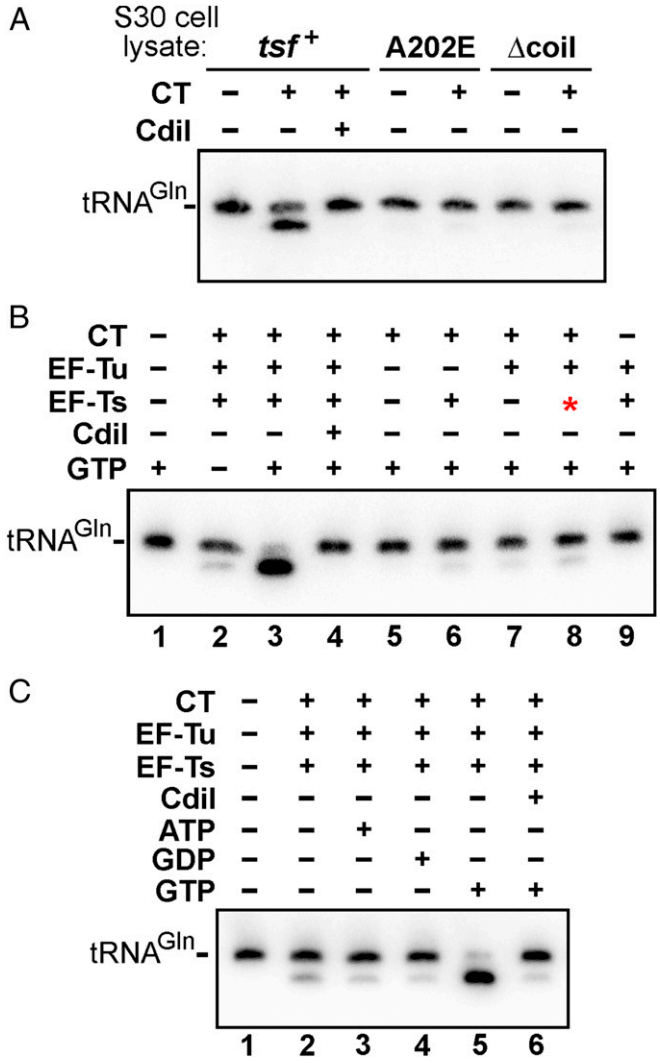

Fig. 2. Efficient CdiA-CT^{EC869} tRNase activity requires EF-Tu, EF-Ts, and GTP. (*A*) *E. coli* S30 cell lysates were supplemented with purified CdiA-CT^{EC869} (0.1 μM) and incubated at ambient temperature for 1 h. Total RNA was isolated and analyzed by Northern blot hybridization for *E. coli* tRNA$_{CUG}^{Gln}$. (*B*) Total *E. coli* RNA (0.1 mg/mL) was treated with purified CdiA-CT^{EC869} (0.1 μM), EF-Tu (0.25 μM), and EF-Ts (0.25 μM) and incubated at ambient temperature for 1 h. Where indicated, CdiA-CT^{EC869} was preincubated with CdiI^{EC869}-His$_6$ (0.3 μM) for 30 min before addition to the reaction. The red asterisk in lane 8 indicates addition of EF-Ts (Ala202Glu). Reactions were supplemented with 1 mM GTP where indicated and analyzed by Northern blot hybridization for *E. coli* tRNA$_{CUG}^{Gln}$. (*C*) Purified CdiA-CT^{EC869} (0.1 μM), EF-Tu (0.25 μM), and EF-Ts (0.25 μM) were incubated with total *E. coli* RNA (0.1 mg/mL) for 1 h at room temperature. Where indicated, CdiA-CT^{EC869} was preincubated with CdiI-His$_6$ (0.3 μM) for 30 min before RNA addition. Reactions were supplemented with ATP, GDP, or GTP (1 μM) where indicated. Reactions were analyzed by denaturing urea polyacrylamide gel electrophoresis and Northern blot hybridization for *E. coli* tRNA$_{CUG}^{Gln}$.

The collaboration between CdiA-CT^{EC869}, EF-Tu, and EF-Ts is reminiscent of the bacteriophage Qβ replicase complex (23, 24). Qβ is a member of the *Leviviridae* family of small RNA phages, which use RNA-dependent RNA polymerases to replicate their genomes. The phage-encoded β-subunit requires host-cell factors EF-Ts, EF-Tu, and ribosomal protein S1 to form the functional replicase complex. Intriguingly, the coiled-coil domain of EF-Ts interacts with the phage β-subunit (Fig. S4*A*) and is critical for replicase activity (25–27). These observations raise the possibility that the *tsf* mutations disrupt formation of a higher order complex with EF-Tu and CdiA-CT^{EC869}. We explored this hypothesis by first examining the plating efficiency of phage R17, which is another levivirus that requires EF-Ts and EF-Tu for replication, on the *tsf* mutants. We found that *tsf(R219P)* and *tsf(Δcoil)* mutants are completely resistant to phage R17, whereas *tsf(A202E)* cells are partially resistant (Fig. S4*B*). To determine if the toxin forms a stable ternary complex with the elongation factors, we tested whether EF-Tu and EF-Ts copurify with His$_6$-tagged CdiA-CT^{EC869} during Ni^{2+} affinity chromatography. EF-Tu bound to the toxin, but substantially less EF-Ts was copurified, even when the binding reactions included EF-Tu and GTP (Fig. S5, lanes 2 and 6). Thus, CdiA-CT^{EC869} binds to EF-Tu with high affinity, but EF-Ts does not appear to associate stably with the complex.

We next considered the possibility that the guanine nucleotide exchange (GEF) function of EF-Ts contributes to toxin activity. If EF-Tu GTPase activity is stimulated by CdiA-CT^{EC869}, then EF-Ts could be required to displace GDP from EF-Tu, thereby accelerating the formation of new GTP·EF-Tu·tRNA substrate complexes. To probe whether GTP hydrolysis accompanies toxin activity, we first tested nuclease activity in reactions supplemented with nonhydrolyzable GDPNP, which supports the same level of tRNA$_{CUG}^{Gln}$ cleavage as GTP (Fig. 4*A*, lanes 5 and 10). We next used TLC to monitor the hydrolysis of radiolabeled GTP in tRNase reactions. In vitro-transcribed tRNA$_{CUG}^{Gln}$ (rather than total *E. coli* tRNA) was used in these experiments to ensure the availability of sufficient substrate to drive detectable GTPase activity. Notably, we did not observe GDP production even in the presence of 0.5 μM wild-type EF-Ts, which supported efficient cleavage of 10 μM tRNA$_{CUG}^{Gln}$ substrate (Fig. 4*B*, lane 5). Because GTPase activity was not detected, we performed a positive-control reaction using polynucleotide kinase and radiolabeled GTP to phosphorylate 5 μM of an oligonucleotide substrate. We observed GDP production in the latter reaction (Fig. 4*B*, lane 1), indicating that GTPase activity is detectable under these assay conditions. Together, these data show that GTP hydrolysis is not concomitant with tRNA cleavage, suggesting that GEF function per se does not promote toxin activity.

We noted that EF-Tu alone supported significant nuclease activity at the high tRNA$_{CUG}^{Gln}$ concentrations (10 μM) used in the GTPase assays (Fig. 4*B*, lane 4). Because CdiA-CT^{EC869} and EF-Tu were purified from *tsf(Δcoil)* cells for this experiment,

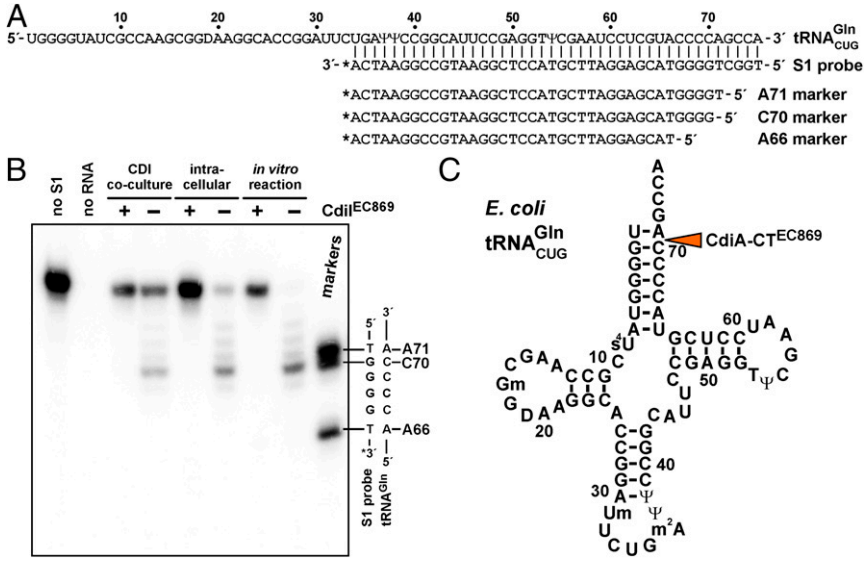

Fig. 3. CdiA-CT^{EC869} cleaves at the tRNA^{Gln} acceptor stem. (*A*) tRNA$_{CUG}^{Gln}$ sequence showing the hybridized S1 probe and oligonucleotide standards used to map the toxin cleavage site. (*B*) S1 nuclease protection assays. RNA was isolated from CDI^{EC869} competition cocultures and cells intoxicated by intracellular CdiA-CT^{EC869} expression. Samples from in vitro nuclease reactions were also analyzed. Where indicated, the neutralizing effect of CdiI^{EC869} immunity protein was examined. RNA samples were incubated with the 3′-radiolabeled S1 probe and treated with S1 nuclease as described in *Materials and Methods*. A portion of the S1 probe-tRNA^{Gln} heteroduplex sequence is shown to the right of the autoradiogram. (*C*) Secondary structure diagram of tRNA$_{CUG}^{Gln}$. The orange arrow indicates cleavage site within the acceptor stem.

and additional EF-Ts(Δcoil) did not enhance activity (Fig. 4*B*, lane 6), we reasoned that EF-Tu alone could be sufficient for toxin activity. Indeed, we obtained efficient GTP-dependent nuclease activity in reactions containing 1 μM tRNA$_{CUG}^{Gln}$ transcript and 0.5 μM EF-Tu (Fig. 5*A*, lanes 4 and 7). However, similar activity was observed in reactions containing toxin and EF-Ts (Fig. 5*A*, lanes 3 and 6). Because EF-Ts does not bind GTP, this latter observation suggests that trace EF-Tu contamination in toxin preparations (Fig. S5, lane 1) may support tRNase activity in conjunction with EF-Ts. To test this possibility, we examined the effect of the antibiotic aurodox on in vitro nuclease reactions. Aurodox binds specifically to EF-Tu and significantly reduces its affinity for tRNA (28). We found that aurodox blocks EF-Tu–dependent tRNase activity (Fig. 5*B*, lanes 2 and 3), consistent with the hypothesis that the toxin acts on GTP·EF-Tu·tRNA complexes. Aurodox also abrogated nuclease activity in the EF-Ts reactions (Fig. 5*B*, lanes 4 and 5), indicating that contaminating EF-Tu contributes to this activity. Together, these results indicate that EF-Tu is required for CdiA-CT^{EC869} nuclease activity, whereas EF-Ts promotes activity at low substrate concentrations.

Finally, we identified two additional CDI toxins that require wild-type EF-Ts to promote their toxicity. Competition cocultures showed that *E. coli tsf(*Δ*coil)* target cells are resistant to growth inhibition by the CDI toxins from *E. coli* isolates NC101 and 96.154 (Fig. S6*A*). In addition, the *E. coli tsf(A202E)* and *tsf(R219P)* mutants exhibited partial resistance to these toxins (Fig. S6*A*). Remarkably, CdiA-CT^{NC101} and CdiA-CT$^{96.154}$ do not share significant sequence identity with each other, nor with the CdiA-CT^{EC869} toxin (Fig. S6*B*). Thus, EF-Ts is critical for the in vivo activities of at least three nonhomologous CDI toxins.

Discussion

These results show that CDI^{EC869}-mediated growth inhibition requires two essential and core components of the translational apparatus. There are at least two models to explain how EF-Tu and EF-Ts promote CdiA-CT^{EC869} nuclease activity. The first mechanism entails the formation of a EF-Ts·EF-Tu·CdiA-CT^{EC869} ternary complex, in which the two translation factors form a scaffold that activates the toxin. In this model, the coiled-coil domain of EF-Ts would interact directly with CdiA-CT^{EC869}, analogous to its structural role in the phage Qβ RNA replicase complex (20, 25). However, unlike the Qβ replicase, EF-Ts does not form a stable stoichiometric complex with EF-Tu and CdiA-CT^{EC869}. Together with data showing that in vitro tRNase activity is GTP-dependent and blocked by aurodox, it appears that CdiA-CT^{EC869} only cleaves tRNA in the context of translationally active GTP·EF-Tu·tRNA complexes. We note that although deacylated tRNA can be cleaved in vitro, aminoacylated tRNA has greater affinity for EF-Tu and is almost certainly the physiologically relevant toxin substrate. We propose that EF-Ts acts as a critical accessory factor to load tRNA onto EF-Tu, accelerating the production of GTP·EF-Tu·tRNA substrate for cleavage. This model is supported by recent work from Blanchard and colleagues, who have shown that EF-Ts promotes both the association and dissociation of tRNA from EF-Tu (29). This activity is concomitant to yet distinct from the well-described GEF function of EF-Ts (30). Knudsen and colleagues recently suggested that the coiled-coil domain of EF-Ts, and in particular its conserved basic patch (including Arg219), could promote tRNA loading onto EF-Tu (31). This model is also supported by the observation that the EF-Ts coiled-coil resembles the tRNA-binding region of some aminoacyl tRNA synthetases (32).

Given that most characterized CDI nuclease toxins are autonomous, it is unclear why some systems have evolved to exploit target-cell factors for toxin activation. Because CdiA-CT^{EC869} cleaves near the 3′-end of tRNA$_{CUG}^{Gln}$, we hypothesize that EF-Tu positions the toxin active site near the scissile bond. However, EF-Tu is not necessary for such activity, because other CDI toxins cleave tRNA acceptor stems independently of the translation factor (5, 15). One intriguing explanation is that toxin·EF-Tu interactions serve additional roles in intercellular signaling. In this model, toxins delivered into immune sibling cells would form EF-Tu·CdiA-CT·CdiI ternary complexes that modulate physiology in response to cell density. EF-Tu function could be modulated directly by CdiA-CT·CdiI^{EC869}, analogous to the posttranslational modifications that regulate the translation factor in response to environmental cues (33, 34). Alternatively, it is possible that tRNA fragments produced from sublethal nuclease activity could regulate gene expression in a manner similar to that described for eukaryotes (35). This general model is supported by work from Cotter and colleagues showing that

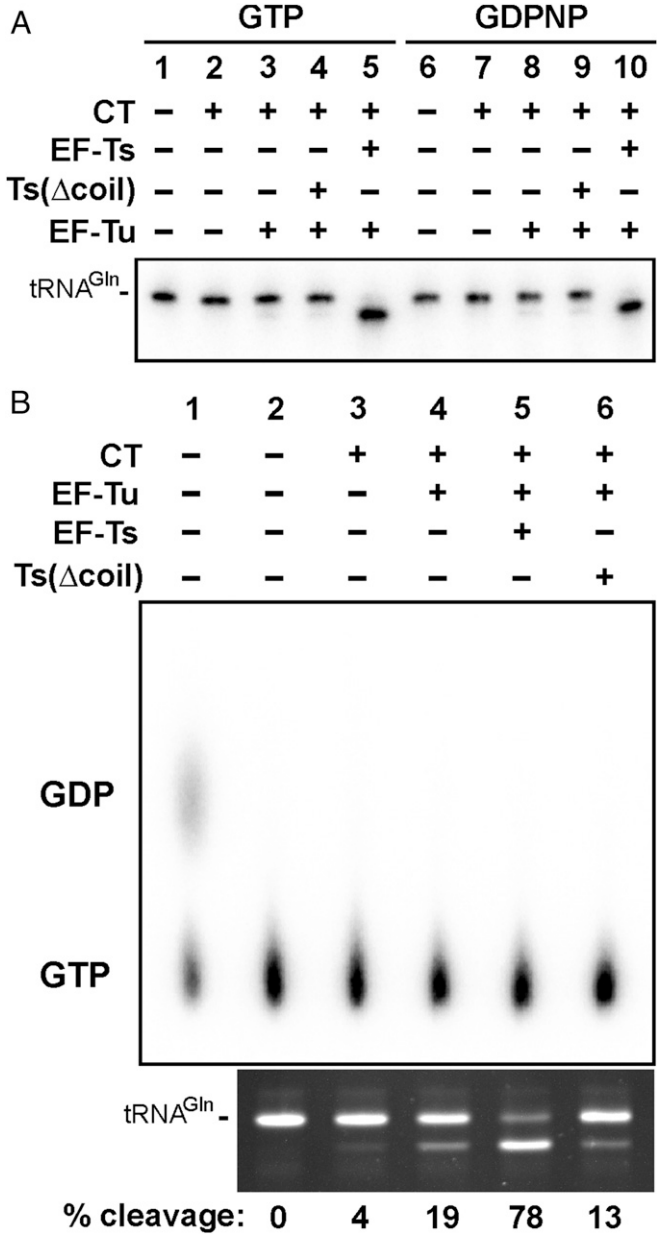

Fig. 4. GTPase activity is not stimulated by CdiA-CT^{EC869}. (*A*) Purified CdiA-CT^{EC869} (0.1 μM), EF-Tu (0.25 μM), and EF-Ts (0.25 μM) were added to total *E. coli* RNA (0.1 mg/mL) as indicated. Reactions were supplemented with GTP or nonhydrolyzable GDPNP (1 mM) and analyzed by Northern blot hybridization for *E. coli* tRNA$_{CUG}^{Gln}$. (*B*) GTPase assays. tRNA$_{CUG}^{Gln}$ transcript (10 μM), radiolabeled GTP (15 μM), CdiA-CT^{EC869} (0.5 μM), EF-Tu, and/or EF-Ts (0.5 μM) were incubated at ambient temperature for 10 min. Lane 1 corresponds to a polynucleotide kinase reaction using radiolabeled GTP as the phosphoryl donor. Reactions were analyzed by TLC (*Upper*) and denaturing polyacrylamide gel analysis (*Lower*) as described in *Materials and Methods*. The migration positions of GDP and GTP are indicated.

intersibling CDI toxin exchange influences biofilm gene expression in *Burkholderia thailandensis* (36).

Recently Mougous and colleagues reported an interaction between EF-Tu and the Tse6 toxin of *Pseudomonas aeruginosa* PAO1 (37). Tse6 is a type VI secretion system (T6SS) effector with novel NADase activity. The crystal structure of the EF-Tu·Tse6 complex reveals that the toxin makes contact with nucleotide-binding domain 1 of EF-Tu, exploiting a surface that binds directly to EF-Ts. Unlike the CdiA-CT^{EC869} toxin, EF-Tu is not required for the enzymatic activity of Tse6. However, Tse6 mutations that block EF-Tu binding also abrogate the ability to intoxicate target bacteria, suggesting that the interaction with EF-Tu could be required for T6SS-mediated secretion of Tse6 or entry of the effector into the target-cell cytoplasm (37). These observations, together with the data presented herein, show that diverse bacterial toxin domains exploit EF-Tu to promote interbacterial competition.

Materials and Methods

Bacteria and CDI Competition Cocultures. Bacterial strains are listed in Table S1. Bacteria were grown at 37 °C in LB medium or on LB agar unless otherwise noted. Media were supplemented with antibiotics at the following concentrations: ampicillin (Amp) 150 μg·mL^{-1}, kanamycin (Kan) 40 μg·mL^{-1}, chloramphenicol (Cm) 12.5 or 34 μg·mL^{-1}, and spectinomycin (Spc) 50 μg·mL^{-1}. For competition cocultures, inhibitor and target cells were first grown to midlog phase, then mixed at a 10:1 ratio in medium without antibiotics. Cocultures were incubated with shaking at 225 rpm in baffled flasks. After 3 h, viable inhibitor and target cells were enumerated as colony-forming units on selective LB agar. The competitive index was calculated as the ratio of target to inhibitor cells at 3 h divided by the initial ratio.

Plasmid Constructions. Plasmids and oligonucleotides are listed in Tables S2 and S3, respectively. The *tufA*, *tsf*, and *cdiA-CT/cdiI*^{EC869} genes were amplified with primer pairs CH3671/CH367, CH3673/CH2188, and 3875/3834 (respectively) and ligated to plasmid pCH10068 using BamHI/XhoI restriction sites. The *cdiI*^{EC869} gene was amplified with primers 3890/3781 and CH220/CH221 and ligated to pET21b (NdeI/XhoI) and pTrc99KX (KpnI/XhoI), respectively. The *duf-cdiA-CT/cdiI*^{EC869} fragment was amplified from pCH10525 with CH2324/CH3834 and ligated to plasmid pCH6243 using SpeI/XhoI sites.

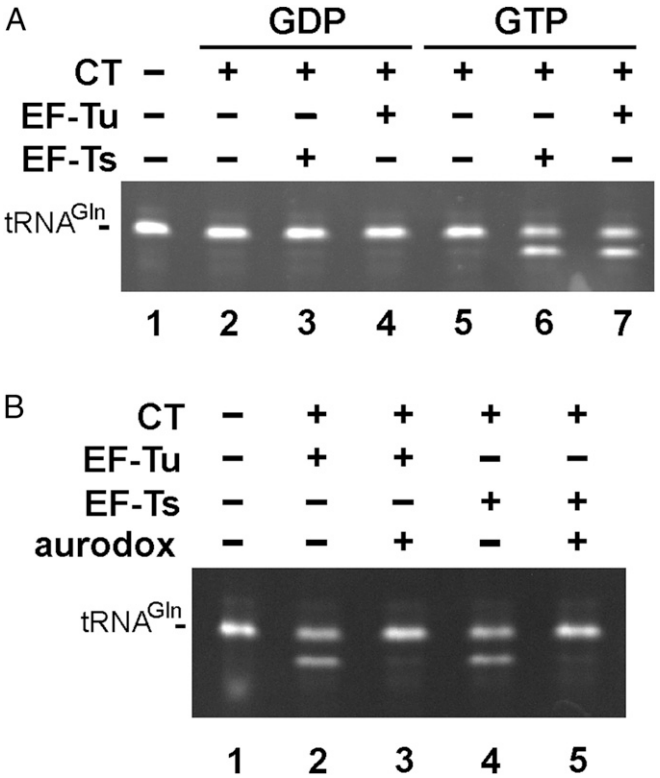

Fig. 5. Roles of EF-Ts and EF-Tu in toxin-dependent tRNA$_{CUG}^{Gln}$ cleavage. (*A*) tRNA$_{CUG}^{Gln}$ transcript (1 μM) was incubated with CdiA-CT^{EC869} (0.5 μM), EF-Tu, and/or EF-Ts (0.5 μM) and GDP or GTP (1 mM) for 10 min at ambient temperature. Reactions were resolved on urea-polyacrylamide gels and visualized by ethidium bromide staining. (*B*) tRNA$_{CUG}^{Gln}$ transcripts (1 μM) were treated with purified CdiA-CT^{EC869} (0.5 μM), EF-Tu, and/or EF-Ts (0.5 μM) and GTP (1 mM) for 10 min at ambient temperature. Aurodox (4 μM) was added to reactions where indicated. Reactions were analyzed as in *A*.

The *cdiA-CT/cdiI* modules from *E. coli* 96.154 and O32:H37 were amplified with primers CH3170/CH3171 and CH3567/CH3568 (respectively) and combined with $cdiA^{EC93}$ fragments amplified with 1527/2470 and 1663/2368 by overlapping-end PCR (15). The final products were electroporated together with plasmid pCH10163 into *E. coli* strain DY378 and recombinant plasmids selected as described (15).

UV Mutagenesis and Complementation Analysis. *E. coli* MG1655 cells carrying pZS21-$bamA^{+}$ were grown to midlog phase, harvested by centrifugation, and resuspended in 0.1 M $MgSO_4$ at an optical density at 600 nm (OD_{600}) of 0.4. The suspension was irradiated at 32 J/m^2 in a Stratalinker 1800. Irradiated cells were collected by centrifugation and resuspended in LB medium and maintained in the dark for all subsequent steps. Irradiated cells were cultured at a 1:1 ratio with *E. coli* EPI100 inhibitors carrying pCH10525 to select for CDI^R mutants. Surviving target cells were isolated on Kan-supplemented LB agar, pooled, and subjected to two additional rounds of CDI^{EC869} selection. Individual colonies were isolated from the final selection and CDI^R phenotypes confirmed in competition cocultures. CDI^R mutations were mapped by complementation analysis. Mutants were transformed with plasmid pRK6, then transduced with a low-copy pREG153 cosmid library of *E. coli* genomic DNA (38). Transductants were individually cocultured with CDI^{EC869} inhibitors in microtiter plates and target-cell growth monitored by fluorimetry. Wells showing low GFP fluorescence indicate growth inhibition, suggesting complementation to the CDI^S phenotype. Results were compared with control cocultures containing mock ($CDI^{—}$) inhibitor cells. The complementing cosmid was sequenced using primer 3513.

Construction and Genetic Manipulation of MG1655 Target Cells. The Δ*wzb* mutation was introduced into *E. coli* MG1655 by allelic exchange. Sequences flanking *wzb* were amplified with primers 3900/3901 and 3902/3903. The upstream fragment was digested with SacI/BamHI and the downstream fragment with BamHI/XbaI. The two fragments were then ligated to pRE112 using SacI/XbaI restriction sites. The resulting plasmid was introduced into *E. coli* DL8705 by conjugation, and exconjugants were selected on LB agar supplemented with Cm. Counterselection was performed on LB agar supplemented with 5% (wt/vol) sucrose (39). Deletion of *wzb* in DL8698 was confirmed by PCR using 3918/3919. The Δ*araBAD::spec* allele from DL5850 was introduced into DL8698 by phage P1 transduction. The *tsf(A202E)* and *tsf(R219P)* mutations were introduced into *E. coli* MG1655 using allelic exchange. The alleles were amplified with 3677/3678 and ligated to pRE112 using SacI/XbaI restrictions sites. The *tsf*(Δ*coil*) allele (in which Val186–Gly225 is replaced by the EPGGEA peptide) (20) was constructed from fragments amplified with 3818/3819 and 3820/3821. The products were combined by OE-PCR with 3818/3821, then ligated to plasmid pRE112 using SacI/XbaI restriction sites. Recombinants were screened by PCR using 3836/3837, and all alleles were confirmed by DNA sequencing. The F′::Tn*10* episome from *E. coli* XL-1 was mated into *E. coli* MG1655 derivatives to generate strains for phage-plating assays.

Protein Purification and Analysis. All proteins were overproduced in *E. coli* CH2016 by addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to 1 mM. After incubation for 2 h, cells were harvested and frozen at –80 °C. Cell pellets were resuspended in buffer A [50 mM Tris·HCl (pH 7.5), 150 mM NaCl, 10 mM β-mercaptoethanol (β-ME), 0.05% Triton X-100, 20 mM imidazole] and broken by two passages through a French press at 20,000 psi. Cell debris was removed by two rounds of centrifugation at 16,000 × *g* at 4 °C. His_6-tagged proteins were purified by Ni^{2+} affinity chromatography in buffer A. For the purification of CdiA-CT^{EC869} fusions, affinity resins were washed with 20 mM Tris·HCl (pH 7.5) and 6 M guanidine–HCl to release $CdiI^{EC869}$ before elution. His_6-tagged proteins were eluted with 20 mM Tris·HCl (pH 7.5) and 250 mM imidazole and dialyzed against buffer B [20 mM sodium phosphate (pH 7.8), 150 mM NaCl, 10 mM β-ME]. After dialysis, His_6-TrxA was cleaved using TEV protease and removed by Ni^{2+} affinity chromatography. Purified proteins were stored at –20 °C in buffer B plus 50% glycerol. Protein–protein interactions were assessed in buffer C [10 mM sodium phosphate (pH 7.5), 150 mM NaCl, 20 mM $MgCl_2$, 1 mM DTT] using 5 μM of each protein in 500 μL reaction at 4 °C as previously described (1).

RNA Isolation and Analysis. Competition cocultures were harvested into an equal volume of ice-cold methanol, and the cells were collected by centrifugation and frozen at –80 °C. Total RNA was extracted with guanidinium isothiocyanate (GITC)-phenol as described (40, 41) and resuspended in 10 mM sodium acetate (pH 5.2), which stabilizes the aminoacyl linkage (42). Total RNA (5 μg) was analyzed by Northern blot hybridization using [^{32}P]-labeled oligonucleotides 3894, CH452, CH800, CH801, and CH1417 as probes (16). S30 extracts were prepared from *E. coli* CH2016, DL8530, and DL8546 cells grown to $OD_{600} \sim 0.5$. Cells were resuspended in buffer B and broken by passage through a French press at 20,000 psi. Lysates were clarified by two cycles of centrifugation at 14,000 × *g* for 10 min. Purified CdiA-CT^{EC869} (0.1 μM) was added to cell supernatants and incubated ambient temperature for 1 h. RNA was then isolated by GITC-phenol extraction and analyzed by Northern blot hybridization. For analysis of toxin activity using purified components, total *E. coli* RNA was incubated with purified CdiA-CT^{EC869}, EF-Tu, EF-Ts, and/or $CdiI^{EC869}$ in buffer C or buffer D [20 mM Tris·HCl (pH 7.5), 150 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT]. $tRNA_{CUG}^{Gln}$ was produced by in vitro transcription as described previously (43). S1 nuclease protection analysis was performed as described (41) using oligonucleotide CH3850 to map the 3′-end of $tRNA_{CUG}^{Gln}$. Migration standard oligonucleotides (CH3851, CH3852, and CH3853) were 3′-labeled with [α-^{32}P]-cordycepin triphosphate and terminal transferase and desalted using a G-25 spin column and 5′-phosphorylated with polynucleotide kinase and unlabeled ATP.

GTPase Assays. In vitro-transcribed $tRNA_{CUG}^{Gln}$ (10 μM) was incubated with CdiA-CT^{EC869}, EF-Tu, and EF-Ts variants (0.5 μM each) in buffer D. Reactions were supplemented with 15 μM GTP (0.033 μM [α-^{32}P]-GTP) and incubated at ambient temperature for 10 min. Positive control reactions were conducted under the same conditions with oligonucleotide 3894 (5 μM) and polynucleotide kinase. Reactions were resolved by TLC on polyethyleneimine cellulose (Polygram cel 300 PEI/UV254) using 0.3 M sodium phosphate (pH 3.5) as the mobile phase. Cellulose sheets were exposed to phosphorimager screens and chromatograms visualized on a Bio-Rad phosphorimager using Quantity One software.

ACKNOWLEDGMENTS. We thank Kurt Fredrick, John Perona, and Zachary Ruhe for technical insight and helpful discussions and Julia Willett for plasmid constructions. This work was supported by NIH Grant R01 GM117373 (to C.S.H. and D.A.L.) and National Science Foundation Grant MCB1545720 (to D.A.L. and C.S.H.). A.M.J. received support from the Ralph M. Parsons Fellowship.

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