Activation of an anti-bacterial toxin by the biosynthetic enzyme CysK: mechanism of binding, interaction specificity and competition with cysteine synthase

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Contact-dependent growth inhibition (CDI) is a wide-spread mechanism of inter-bacterial competition. CDI+ bacteria deliver CdiA-CT toxins into neighboring bacteria and produce specific immunity proteins that protect against self-intoxication. The CdiA-CT toxin from uropathogenic Escherichia coli 536 is a latent tRNase that is only active when bound to the cysteine biosynthetic enzyme CysK. Remarkably, the CysK:CdiA-CT binding interaction mimics the ‘cysteine synthase’ complex of CysK:CysE. The C-terminal tails of CysE and CdiA-CT each insert into the CysK active-site cleft to anchor the respective complexes. The dissociation constant for CysK:CdiA-CT (Kd ~ 11 nM) is comparable to that of the E. coli cysteine synthase complex (Kd ~ 6 nM), and both complexes bind through a two-step mechanism with a slow isomerization phase after the initial encounter. However, the second-order rate constant for CysK:CdiA-CT binding is two orders of magnitude slower than that of the cysteine synthase complex, suggesting that CysE should outcompete the toxin for CysK occupancy. However, we find that CdiA-CT can effectively displace CysE from pre-formed cysteine synthase complexes, enabling toxin activation even in the presence of excess competing CysE. This adventitious binding, coupled with the very slow rate of CysK:CdiA-CT dissociation, ensures robust nuclease activity in target bacteria.

Though long considered to be isolated and independent unicellular organisms, bacteria engage in a multitude of cooperative and competitive behaviors. Many bacteria secrete soluble antibiotics and bacteriocins1-4, which diffuse through the environment and kill competing bacteria at a distance. More recently, proximity-dependent inter-bacterial competition systems have been characterized5-8. This phenomenon was first described in Escherichia coli isolate EC93, which inhibits the growth of other E. coli strains in a contact-dependent manner5. Contact-dependent growth inhibition (CDI) is mediated by CdiB/CdiA two-partner secretion proteins, which transfer protein toxins between Gram-negative bacteria8-10. CdiB is an outer-membrane transport protein that exports CdiA onto the cell surface. CdiA forms a long β-helical filament that extends from the inhibitor cell to bind specific receptors on neighboring bacteria. Upon binding receptor, CdiA delivers its C-terminal toxin domain (CdiA-CT) into the target cell to inhibit growth. CDI+ bacteria also express CdiL immunity proteins, which bind to the CdiA-CT domain and neutralize toxin activity to prevent self-intoxication. Analysis of CdiA from many species has revealed that the family carries a wide variety of C-terminal toxin domains, each with a distinct activity11-13. Thus, a given CdiL immunity protein only protects against its cognate toxin and not the

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Toxins deployed by other bacteria. Together, these observations suggest that CDI systems mediate inter-bacterial competition for growth niches and other environmental resources.

We recently discovered that the CDI toxin deployed by uropathogenic *E. coli* 536 is a latent tRNase that is only active when bound to the biosynthetic enzyme CysK16. CysK is a pyridoxal 5′-phosphate (PLP)-dependent O-acetyl-L-ser sulphhydrolase that catalyzes the last step of cysteine biosynthesis in eubacteria, plants and some archaea17–19. *E. coli* and many other bacteria encode an additional isozyme, CysM. Both sulphhydrolases are coordinately regulated with the enzymes responsible for sulfate reduction to bisulfide20, although the functional role of CysM is less well characterized. The structure and catalytic properties of CysK from Gram-negative bacteria and plants have been characterized thoroughly20–33. CysK has long been known to form a high-affinity “cysteine synthase” (CS) complex with CysE, which is a serine O-acetyltransferase responsible for the penultimate reaction in cysteine biosynthesis34–37. The three-dimensional structure of the cysteine synthase complex is unknown, but biochemical studies indicate that each CysE hexamer binds to two CysK homodimers38. Moreover, it is well established that the flexible C-terminal tail of CysE inserts into the CysK active site to anchor the complex36, 37, 39. The C-terminal Ile residue of CysE is particularly critical, and deletion or mutation of this conserved residue consistently interferes with cysteine synthase assembly39, 40. The Ile side-chain mimics substrate to bind the CysK active site, and consequently the cysteine synthase complex is dissociated with micromolar concentrations of O-acetyl-L-Ser1–4. Remarkably, the *E. coli* 536 CdiA-CT toxin mimics CysE and uses its C-terminal peptide motif to bind the CysK active site16, 41. In fact, a number of proteins engage in so-called “moonlighting” binding interactions with CysK homologs32. One intriguing example is the EGL-9 prolyl hydroxylase from *Caenorhabditis elegans*, which uses its C-terminal Ile tail to interact with CYSL-1, a CysK homolog that has lost biosynthetic activity but retains the bisulfide-binding site. Under hypoxic conditions, bisulfide accumulates and promotes EGL-9/CYSL-1 binding. The sequestered EGL-9 is no longer able to hydroxylate HIF-1, which stabilizes the transcription factor and increases the expression of genes required to respond to hypoxia42. Thus, CysK homologs have been co-opted to regulate diverse biological activities.

Here, we explore the thermodynamics and kinetics of the CDI-CS interaction with *E. coli* CysK (EcCysK) to gain insight into toxin activation. We find that the dissociation constant for the EcCysK:CdiA-CT complex is comparable to that of the CS complex, suggesting that EcCysE could attenuate toxicity by competing with CdiA-CT for access to EcCysK. In addition, the second-order rate constant for cysteine synthase complex formation is ~200-fold greater than the rate constant for EcCysK:CdiA-CT binding. Though cysteine synthase assembly is kinetically favored, CdiA-CT toxin is still activated in the presence of competing EcCysE. Robust toxin activation reflects the ability of CdiA-CT to displace EcCysE from pre-formed cysteine synthase complexes. This property, coupled with the very slow rate of EcCysK:CdiA-CT dissociation, ensures toxin activation upon entry into target bacteria. Finally, we show that CysK homologs from different bacterial species support CdiA-CT toxin activity to varying degrees. Although CDI-mediated toxin delivery only occurs between closely related bacteria43, 44, CdiA-CT toxin homologs are found in several species and therefore must interact with different CysK variants. Because CDI genes are acquired through horizontal gene transfer45, 46, we propose that the toxin domain evolved to bind a highly conserved partner that is ubiquitous in bacteria.

**Results**

Two CdiA-CT toxin domains bind each CysK dimer. Our previous work has shown that the CdiA-CT toxin domain forms a stable complex with EcCysK and that this interaction is required for toxic tRNase activity in *vivo* and in *vitro*16. The initial study suggested that the C-terminal tail of CdiA-CT inserts into the EcCysK active site, and this conclusion was recently confirmed by crystal structures of the EcCysK:CdiA-CT complex42. Therefore, we reasoned that EcCysK:CdiA-CT complex formation could be monitored by measuring changes in pyridoxal 5′-phosphate (PLP) fluorescence. This spectrophotometric approach has been used extensively to track cysteine synthase complex formation38 as well as to identify CysK inhibitors49–54. Indeed, PLP fluorescence increased about five-fold when EcCysK was titrated with increasing concentrations of CdiA-CT (Fig. 1A). This effect is very similar to that observed for the cysteine synthase complexes of *Haemophilus influenzae*38 and *E. coli*16. Moreover, the fluorescence spectrum of EcCysK:CdiA-CT exhibited a blue-shift in the emission maximum from 505 to 498 nm compared to free EcCysK (Fig. 1A). This latter change indicates that the fluorophore is in a less polar environment, consistent with insertion of the toxin's C-terminal Ile residue into the EcCysK active site. Stoichiometric titrations determined a molar ratio of 1:1:1 (Fig. 1B), in agreement with crystal structures showing two CdiA-CT domains bound to each EcCysK dimer42.

CdiA-CT and CysE bind to CysK with comparable affinity. The affinity of the EcCysK:CdiA-CT complex can be estimated through titrations of dilute EcCysK with toxin. We observed the same spectroscopic changes upon CS complex formation38, but the emission spectra evolved over time, stabilizing after about 20 min at the lowest toxin concentrations (Fig. 1C). Fitting of Eq. 2 to binding data collected after 20 min yields an estimated *Kₐ* of about 10 nM (Fig. 1C). The protein concentrations required for fluorescence-based titrations limit the measurable dissociation constants to about 5 nM. Therefore, we used an orthogonal assay to measure binding interactions more accurately. Because CdiA-CT occludes the EcCysK active site, complex formation can be monitored by measuring fractional sulphhydrolase activity as a function of toxin concentration (Fig. 1D). The half maximal inhibitory concentration ([IC₅₀]) of 15.4 ± 0.6 nM was obtained by fitting Eq. 3 to these data. Accounting for substrate concentration and the *Kₐ*, the *IC₅₀* was converted to an inhibition constant (*K*) of 11.0 ± 0.4 nM using Eq. 4. This value is in agreement with the EcCysK:CdiA-CT binding constant calculated from surface plasmon resonance data37. Moreover, EcCysE inhibits EcCysK activity with a *K* of 6.2 ± 0.7 nM35, indicating that the toxin and EcCysE bind to EcCysK with similar affinities.

CdiI immunity protein binds specifically to CdiA-CT toxin, but also forms a ternary complex with CdiA-CT and EcCysK16. 42. A recent report has suggested that the CdiA-CT:CdiI complex has a six-fold higher affinity for
EcCysK than CdiA-CT toxin alone. However, the crystal structure of the EcCysK:CdiA-CT:CdiI ternary complex shows that the immunity protein makes no direct contacts with EcCysK. To monitor the influence of CdiI on EcCysK:CdiA-CT complex formation, we measured EcCysK sulfhydrylase activity in the presence of CdiA-CT and excess CdiI (Fig. 1D). The calculated $K_i$ in the presence of CdiA-CT and CdiI was $6.4 \pm 0.5 \text{ nM}$ under the same conditions in the presence of $2.4 \mu M$ CdiI (open squares).

**Figure 1.** EcCysK:CdiA-CT complex formation. (A) Fluorescence emission spectra of EcCysK excited at 412 nm. Spectra were collected with EcCysK (1.15 $\mu M$) in the presence of the indicated concentrations of CdiA-CT. (B) Stoichiometry of the EcCysK:CdiA-CT complex. 1 $\mu M$ EcCysK was titrated with CdiA-CT to saturation. The intersection of the lines corresponds to a CdiA-CT:EcCysK ratio of 1.1:1 (dashed line). (C) Determination of the EcCysK:CdiA-CT dissociation constant. EcCysK (80 nM) was titrated with increasing concentrations of CdiA-CT, and fluorescence emission at 500 nm monitored at the indicated times. The solid line indicates the Eq. 2 fit to the 20 min data set, with $K_d = 10 \pm 11 \text{ nM}$ and $[\text{CysK}] = 134 \pm 33 \text{ nM}$. (D) CdiA-CT inhibits EcCysK sulfhydrylase activity. EcCysK (6 nM) was titrated with CdiA-CT and sulfhydrylase activity measured as described in the Methods. Eq. 3 was fitted to the dependence of $v/v_0$ on CdiA-CT concentration, yielding an apparent $IC_{50}$ that was used to calculate a $K_i$ of $11.0 \pm 0.4 \text{ nM}$ (closed circles). A $K_i$ of $6.4 \pm 0.5 \text{ nM}$ was calculated under the same conditions in the presence of $2.4 \mu M$ CdiI (open squares).
conformational change. However, given the unusually slow binding kinetics, rapid formation of an encounter complex followed by a slow conformational rearrangement is likely (Fig. 2E). As previously observed for the cysteine synthase (CS) complex the initial fast step was not associated with changes in the fluorescence emission, and therefore only the slow rate-limiting process can be measured by this technique. According to this model, the dependence of $k_{\text{obs}}$ on protein concentration represents the linear portion of the hyperbole and is equal to the second-order rate constant ($k_3/K_d$). Fitting Eq. 6 to the dependence yields $k_3/K_d$ of $2 \times 10^4$ M$^{-1}$s$^{-1}$. By contrast, the second-order rate constant for the H. influenzae cysteine synthase complex is about $10^7$ M$^{-1}$s$^{-1}$ and $4 \times 10^6$ M$^{-1}$s$^{-1}$ for the EcCysK:CdiA-CT complex (Fig. S1). Thus, the EcCysK:CdiA-CT complex forms ~200-fold more slowly than the E. coli cysteine synthase complex.

Figure 2. Pre-steady state kinetics of EcCysK:CdiA-CT assembly. (A, B) Representative time courses for the interaction of CdiA-CT (1 µM and 10 µM) with EcCysK (200 nM) (panel A) and EcCysK (1.7 µM and 16 µM) with CdiA-CT (270 nM) (panel B) as monitored by fluorescence emission intensity upon excitation at 412 nm. Individual traces are presented in grey, and the dashed black lines represent Eq. 5 fits to the time-course binding data. (C) Dependence of the observed kinetic constant ($k_{\text{obs}}$) on EcCysK and CdiA-CT concentrations. The lines represent linear equation fits with slopes of $0.019 \pm 0.007$ µM$^{-1}$s$^{-1}$ and $0.017 \pm 0.007$ µM$^{-1}$s$^{-1}$. (D) Observed kinetic constant as a function of CysK concentration and temperature. Solid lines represent linear equation fits with slopes of $0.099 \pm 0.019$ µM$^{-1}$s$^{-1}$ (37 °C), $0.017 \pm 0.007$ µM$^{-1}$s$^{-1}$ (20 °C) and $0.005 \pm 0.0003$ µM$^{-1}$s$^{-1}$ (5 °C). (E) Two-step model for EcCysK:CdiA-CT complex formation including a slow conformational change. CysK:CdiA-CT is the encounter complex, and CysK:CdiA-CT* corresponds to the isomerized, nucleolytic complex.
CdiA-CT competes with CysE for binding to CysK. The comparable affinities of the cysteine synthase and EcCysK:CdiA-CT complexes suggest that the toxin competes with EcCysE for access to EcCysK. We first used an indirect approach to test whether CdiA-CT interferes with the assembly of cysteine synthase complexes. Because EcCysE activity is stimulated when bound to EcCysK55, we titrated the CS complex with CdiA-CT and measured serine acetyltransferase activity. The maximal rate of serine acetylation was obtained with 28 nM EcCysE and 19 nM EcCysK, conditions in which the two proteins are at stoichiometric amounts based on the 3:2 CysE:CysK stoichiometry of the cysteine synthase complex. Pre-incubation of EcCysK with increasing concentrations of CdiA-CT reduced the stimulatory effect, decreasing acetyltransferase activity down to a plateau equivalent to that of free EcCysE (Fig. 3A).

We next examined the influence of EcCysE on the EcCysK:CdiA-CT complex, first testing whether excess EcCysE blocks toxin activation during CDI. We incubated target bacteria with inhibitor cells that deploy CdiA-CT, then isolated RNA from the mixed culture to detect toxic tRNase activity by Northern blot hybridization56, 63. To facilitate this analysis, we over-expressed tRNA\textsubscript{CCU}Arg in the target-cell population. Because this substrate is present at very low levels in wild-type E. coli62, essentially all of the tRNA\textsubscript{CCU}Arg detected by Northern blot is derived from target bacteria. Most of the tRNA\textsubscript{CCU}Arg substrate was cleaved within 1 h of co-culture (Fig. 3B, Figure 3. EcCysE and CdiA-CT compete for binding to CysK. (A) CdiA-CT blocks formation of the cysteine synthase complex. Increasing concentrations of CdiA-CT were pre-incubated with EcCysK (19 nM) for 20 min prior to the addition of L-Ser (20 mM) and EcCysE (28 nM). Reactions were then initiated by addition of 0.3 mM acetyl-CoA and acetyltransferase activity measured by monitoring the decrease in acetyl-CoA absorption at 232 nm as described in the Methods. The activities of isolated CysE and CysE in the CS complex are shown by horizontal reference lines. (B) EcCysE inhibits CdiA-CT toxin activation \textit{in vivo}. Inhibitor and target cells were co-cultured as described in the Methods. Total RNA and protein were isolated upon initial mixing and after 1 h, and analyzed by Northern blot (top panel) and SDS-PAGE (bottom panel). Where indicated, target cells over-produced EcCysE (+) or truncated EcCysE lacking the C-terminal tail (no tail). Target cells carried a deletion of the cysK gene (Δ) where indicated. Asterisks (*) in the bottom panel indicate over-produced EcCysE proteins. (C) EcCysE inhibits CdiA-CT toxin activation \textit{in vitro}. EcCysK and EcCysE were pre-incubated at the indicated concentrations (µM) prior to addition of CdiA-CT and RNA substrate as described in the Methods. Reactions were quenched after 10 min at 37 °C, then run on 8 M urea-polyacrylamide gels and visualized by ethidium bromide staining. The migration positions of 5 S rRNA and tRNA are indicated.
compare lanes 1 & 5); and this nuclease activity was dependent on EcCysK, because substrate was not degraded when AcysK mutants were used as target bacteria (Fig. 3B, lane 8). We then over-produced EcCysE in target cells and examined the effect on toxin activity. Notably, EcCysE was readily detected by SDS-PAGE analysis of crude lysates prepared from the co-culture (Fig. 3B, bottom panel), indicating that target cells likely contained enough EcCysE to saturate endogenous EcCysK. As predicted, over-produced EcCysE suppressed toxin activity, but substantial RNA degradation was still detected in target cells (Fig. 3B, lane 6). By contrast, an EcCysE variant lacking 11 residues from the C-terminus was less effective in blocking toxin activity (Fig. 3B, lane 7), consistent with the importance of these residues in CS complex stability. We obtained similar results with in vitro TRNase assays. As reported previously, CdIA-CT has no appreciable nuclease activity in vitro, but efficiently cleaves tRNA when reactions are supplemented with EcCysK (Fig. 3C, compare lanes 2 & 3). To examine the effect of EcCysE on nuclease activity, we pre-incubated EcCysK with EcCysE for 20 min to assemble cysteine synthase complexes. CdIA-CT was then added, and the protein mixture incubated for an additional 30 min prior to the addition of tRNA substrate. Even when used in eight-fold excess over EcCysK (with respect to cysteine synthase stoichiometry), EcCysE did not block TRNase activity to the same extent as CdII immunity protein (Fig. 3C, compare lanes 6 & 7). Together, these results demonstrate that CdIA-CT toxin is activated efficiently even in the presence of EcCysE.

The homodimeric structure of EcCysK provides a possible explanation for robust CdIA-CT activation in the presence of competing EcCysE. We reasoned that if only one EcCysK active site per dimer is occupied by EcCysE in the cysteine synthase complex, then the other active site should be available to bind toxin. This model predicts that EcCysK can bind EcCysE and CdIA-CT simultaneously. To explore this hypothesis, we sought to isolate EcCysE:EcCysK:CdIA-CT ternary complexes. We equilibrated His6-tagged CdIA-CT with untagged EcCysK and EcCysE for 1 h, then subjected the mixture to Ni²⁺-affinity chromatography. EcCysE clearly interacted with His₆-CdIA-CT under these conditions, but none of the EcCysE co-purified with the His₆-CdIA-CT:EcCysK complex, even at concentrations up to 15μM (Fig. 4A). Instead, there appeared to be competition for EcCysK occupancy, with much of the EcCysK remaining in the “free” fraction due to its association with EcCysE (Fig. 4A). These results show that high-affinity ternary complexes of EcCysE:EcCysK:CdIA-CT do not form, indicating that the binding of toxin and EcCysE to EcCysK is mutually exclusive.

We then developed a native PAGE approach to monitor the relative proportions of EcCysE:EcCysK and EcCysK: CdIA-CT in complex mixtures. Electrophoresis conditions were optimized to allow unambiguous identification of each complex based on its gel mobility (Fig. 4B, compare lanes 2 & 4). Simultaneous mixing of EcCysE, EcCysK and CdIA-CT resulted in the formation of both complexes, with lower levels (39%) of EcCysK:CdIA-CT with respect to CS (61%) (Fig. 4B, lane 3, Fig. 4C, lane 1). A similar result was obtained when EcCysE was pre-incubated with EcCysK (Fig. 4C, lane 5). By contrast, the proportion of CdIA-CT:EcCysK complex increased significantly (75%) when toxin and EcCysK were pre-incubated before the EcCysE addition (Fig. 4C, lane 6). This latter observation suggests that the CdIA-CT:EcCysK complex reaches equilibrium more slowly than CS. The finding is further supported by a time-driven experiment where EcCysK, EcCysK and CdIA-CT are mixed simultaneously and monitored over time. The proportion of EcCysE: CdIA-CT:EcCysK complex increased from about 40% to 60% after 40 min of incubation (Fig. 4C, lanes 1–4 and Supplemental Fig. S2). This result confirms that equilibrium conditions are reached slowly when EcCysE and CdIA-CT compete for EcCysK occupancy, and further suggests that CdIA-CT might displace EcCysE from the CS complex.

CdIA-CT toxin is activated by CysK from diverse bacterial species. CdIA-CT is a member of the Ntox28 RNase family and closely related toxin domains are found in CysK proteins from Enterobacter cloacae, Yersinia enterocolitica and Pseudomonas syringae. In this assay, separate plasmids that express CdIA-CT or CysK are simultaneously introduced into E. coli AcysK cells, and transformants are selected on antibiotic-supplemented media. Because CdIA-CT is toxic when bound to EcCysK, cells that take up both plasmids are unable to grow, and therefore stable transformants are not obtained even when toxin expression is repressed with D-glucose in the media (Fig. 5A). To control for transformation efficiency, we introduced a catalytically inactive CdIA-CT construct carrying the His178Ala mutation and obtained several transformants (Fig. 5A). We then tested plasmids encoding heterologous enzymes that share between 50% and 96% sequence identity with EcCysK. These CysK homologs share virtually identical active sites, and 8 of the 13 residues that make direct contact with the toxin domain are conserved (Fig. S5). As expected, closely related enzymes from Enterobacter cloacae (ECLCysK, 96% identity) and Dickeya dadantii (DdCysK, 91% identity) promoted CdIA-CT toxicity in the transformation assay (Fig. 5A). More distantly related CysK proteins from Haemophilus influenzae (HiCysK, 68% overall identity, 92% identity in toxin-binding residues) and Bacillus subtilis (BsCysK, 50% overall identity, 92% identity in toxin-binding residues) also activated the toxin in vivo (Fig. 5A). However, NiCysK from Neisseria lactamica (53% identity, 69% identity in toxin-binding residues) only supported toxicity when its expression was fully induced with L-arabinose (Fig. 5A, compare glucose and arabinose plates). This latter result suggests that CdIA-CT has significantly lower affinity for NiCysK. Similar results were obtained when we tested the function of heterologous CysK in CDI competition co-cultures. We provided E. coli AcysK target cells with cysK-his expression plasmids and incubated the resulting strains with inhibitor bacteria that deploy CdIA-CT. Growth inhibition was assessed by enumerating viable target bacteria after three hours of co-culture. Target cells lacking CysK were resistant to growth inhibition, and viable cell counts increased during the incubation (Fig. 5B). By contrast, target cells expressing EcCysK, DdCysK and HiCysK were inhibited, with each population showing ~100-fold losses in viability (Fig. 5B). The other CysK enzymes were less effective at promoting toxicity, particularly NiCysK, which showed less than a ten-fold decrease in viable cell counts (Fig. 5B). To ascertain the levels of heterologous CysK in target cells, we performed immunoblot analysis using antibodies to the His epitope appended to the C-terminus...
of each enzyme. This analysis revealed lower levels of BsCysK and NlCysK (Fig. 5C), perhaps accounting for the resistance of NlCysK expressing cells to growth inhibition.

Finally, we examined toxin binding and activation by heterologous CysK in vitro. We first used affinity co-purification to screen interactions between CdiA-CT and His₈-tagged CysK proteins. This approach showed that DdCysK, ECLCysK and BsCysK all form high-affinity complexes with CdiA-CT (Fig. 6A). Because the toxin failed to co-purify with HiCysK and NlCysK (Fig. 6A), we quantified the binding interactions using fluorimetric titrations and determined dissociation constants of 3.3 ± 0.3 µM for the HiCysK:CdiA-CT complex and 6.4 ± 0.6 µM for NlCysK:CdiA-CT (Fig. 6B). Thus, CdiA-CT binds these latter enzymes with ~1,000-fold lower affinity than EcCysK. Consistent with this low affinity, high concentrations of HiCysK and NlCysK were required to activate the CdiA-CT nuclease in vitro (Fig. 6C). As we found in the in vivo analyses, NlCysK was the least effective at promoting toxin activity. In fact, tRNase reactions supplemented with NlCysK up to 10 µM did not go to completion after 1 h incubation. Together, these results show that CdiA-CT toxin can be activated by a variety of CysK enzymes, but the binding constants span several orders of magnitude.

Discussion

Here, we show that EcCysK and CdiA-CT form a high-affinity complex with two toxin domains bound per EcCysK homodimer. These results are broadly consistent with a prior thermodynamic study by Kaundal et al. 57, though our data indicate that CdiA-CT could displace EcCysK from pre-formed CS complexes and suggest that CdiI has less of an effect on binding affinity. However, both studies show that complex formation is remarkably slow. Kaundal et al. used surface plasmon resonance to measure a $k_{on}$ of 6.2·10³ M⁻¹·s⁻¹, and here we calculate
A second-order rate constant of $2 \times 10^4 \text{M}^{-1}\text{s}^{-1}$ for EcCysK:CdiA-CT binding, consistent with slow conformational rearrangements following the formation of an encounter complex. The cysteine synthase complex also exhibits a two-step binding mechanism, which is perhaps not surprising given that the CdiA-CT toxin mimics CysE by inserting its C-terminus into the CysK active site, anchoring the interaction to allow further conformational changes. For the CS complex, the slow conformational changes entail closure of the CysK active site, but allosteric changes in CysE are also likely because its $O$-acyetyltransferase activity is stimulated in the $E. coli$ complex (see Fig. 3A). We note that EcCysK adopts an open active-site conformation in the EcCysK:CdiA-CT crystal structure, indicating that the toxin does not induce significant structural changes in EcCysK. Together, these observations suggest that conformational changes in CdiA-CT are responsible for the slow phase of complex formation. This model also accounts for toxin activation, whereby EcCysK-induced structural changes organize the nuclease active site for catalysis. CdiA-CT is probably delivered in a partially unfolded state (vide infra) and folding to the final active conformation likely represents the slow, rate limiting step in complex formation. Reversal of this isomerization, which is described by the $k_4$ rate constant is even slower (see Fig. 2E). Direct determination of $k_4$ is hindered by the intrinsically high error in the calculation of the $y$-axis intercept. However, Eq. 8 can be used to estimate the rate constant for the reversal of isomerization at $2.2 \times 10^{-4} \text{s}^{-1}$, which is two orders of magnitude slower than the corresponding value of $0.024 \text{s}^{-1}$ for the CS complex and in good agreement with the overall $k_{off}$ calculated by SPR. Thus, the activated toxin complex dissociates exceptionally slowly, prolonging nuclease activity in target cells. Such long residence times have been observed for antigen-antibody and protease-inhibitor interactions and are important in open biological systems, where ligand concentrations vary over time.

Figure 5. Heterologous CysK promotes CdiA-CT toxicity. (A) Activation of internally expressed CdiA-CT toxin. Plasmids encoding CdiA-CT and CysK proteins were introduced into $E. coli$ ΔcysK cells, and transformants isolated on selective media supplemented with D-glucose or L-arabinose as indicated. To control for transformation efficiency, a plasmid encoding catalytically inactive CdiA-CT(H178A) was also tested. (B) CDI competition co-cultures. Inhibitor cells that deploy CdiA-CT were incubated with $E. coli$ ΔcysK target bacteria that express CysK-His$_6$ from the indicated bacterial species. Viable target bacteria were quantified as colony forming units per mL upon mixing and after 3 h of co-culture. Presented data are averages ± standard errors for four independent experiments. (C) Immunoblot analysis of heterologous CysK-His$_6$. Total protein was isolated from the target-cell strains in panel B and analyzed by immunoblotting using antibodies to the His$_6$ epitope. 10 $\mu$g of total protein was loaded in each lane.
many protein-ligand interactions, binding efficacy can be explained entirely by the k_{off} value rather than dissociation constant alone\(^6\).

EcCysE and CdiA-CT bind with comparable affinities to the same site on EcCysK, indicating that the toxin must compete with EcCysE in order to be activated. Further, the toxin is presumably at a disadvantage with respect to endogenous EcCysE, because only a few CdiA-CT domains are delivered into target cells during CDI\(^6\)\(^5\). This is compounded by the fact that the second-order rate constant for CS complex formation is ~200-fold greater than that of the EcCysK:CdiA-CT complex. However, early studies in *Salmonella* Typhimurium suggested that StCysK levels exceed those of StCysE, with only 5–25% of StCysK found in the CS complex\(^6\)\(^6\), \(^6\)\(^7\). On the other hand, *cysE* and *cysK* are regulated by different transcription factors, raising the possibility that their relative proportions are modulated in response to changing growth conditions. For example, high cysteine levels inhibit CysE activity\(^6\)\(^8\), \(^6\)\(^9\), reducing the production of O-acetyl-L-Ser and N-acetyl-L-Ser, which are required as co-activators to induce CysB-dependent transcription of the *cys* regulon\(^1\)\(^1\). Because *cysE* transcription is not regulated by CysB, it is possible that CysE becomes more abundant than CysK when the cell is replete with cysteine. Moreover, recent transcriptomic data show that *cysK* and *cysE* transcript levels are comparable in *S. Typhimurium* cells grown in rich media and other conditions\(^8\)\(^0\). Thus, CdiA-CT activity and CDI could be modulated by environmental conditions, though we have found that target bacteria are still inhibited in cysteine supplemented media (C.M.B. \& C.S.H., unpublished data). These observations indicate that EcCysE levels are no impediment to toxin activation. Moreover, the data presented here show that even supra-physiological EcCysE concentrations are insufficient to block toxin activation. Thus, CdiA-CT competes effectively with EcCysE, and may even displace EcCysK from pre-formed CS complexes. There are no structures available for the CS complex, but biochemical studies indicate that each CysE hexamer engages two CysK dimers (Fig. 7). Further, molecular modeling shows that distance and geometrical constraints prevent CysE from engaging both CysK active sites simultaneously\(^7\)\(^1\). Therefore,
only one active site per CysK dimer is engaged with CysE in the CS complex (Fig. 7). This architecture provides opportunities for CdiA-CT to bind to the unoccupied CysK active site. However, stable EcCysE:EcCysK:CdiA-CT ternary complexes cannot be isolated, suggesting that the binding of CdiA-CT and EcCysE to EcCysK is mutually exclusive. Several studies indicate that the CysK active site undergoes allosteric closure in the CS complex, whereas CdiA-CT binds to EcCysK with an open active-site conformation (Fig. 7). Thus, differential affinities for the open and closed states could account for the observed binding behavior. This model may also explain how CdiA-CT disrupts the CS complex, though we note that because the two complexes have similar affinities, their proportions at equilibrium should reflect the relative concentrations of toxin and EcCysE.

The CdiA-CT toxin from E. coli 536 has evolved a dependence on CysK, but most other CDI toxins do not require additional factors to promote toxicity. One explanation invokes possible physical constraints on CDI toxin delivery, which entails CdiA-CT translocation across the outer and inner membranes of target bacteria. Though the mechanistic basis of CDI toxin transfer is not completely understood, the analogous import of colicins into E. coli requires the unfolding of toxin domains. If CdiA toxins must also unfold during delivery, then there should be a selective pressure for domains with low global stability. This in turn could provide the impetus to evolve binding interactions that compensate for intrinsic instability. Consistent with this hypothesis, CdiA-CT has relatively low thermostability and is significantly stabilized when bound to EcCysK. Thus, EcCysK-binding could ensure that the toxin regains its native fold after delivery into the target-cell cytoplasm. In principle, the CdiA-CT toxin could have evolved binding interactions with any number of cytosolic proteins, but it appears that CysK was selected due to its conservation throughout bacteria. Although uropathogenic E. coli are unable to deliver the CdiA-CT toxin into other bacterial species, there is still a selective pressure for activation in diverse bacteria because CDI systems are encoded on plasmids.

**Methods**

**Bacterial strains and plasmid constructions.** Bacterial strains and plasmids are listed in Table 1. Bacteria were grown in lysogeny broth (LB) or on LB agar unless otherwise noted. Where indicated, media were supplemented with antibiotics at the following concentrations: ampicillin, 150 μg mL⁻¹; kanamycin, 50 μg mL⁻¹; rifampicin, 200 μg mL⁻¹; and tetracycline, 12.5 μg mL⁻¹. The ΔcysK:kan disruption was obtained from the Keio collection and transduced into E. coli strains MG1655 (DE3) and CH10013. Kanamycin-resistance cassettes were subsequently removed with FLP recombinase expressed from plasmid pCP20. Bacterial cysK open-reading frames were amplified by PCR using the following primer pairs: CH2095/CH2102 for D. dadantii 3937, CH2095/ CH2099 for E. cloacae ATCC 13047, CH3466/CH3467 for H. influenzae Rd, CH3345/CH3346 for N. lactamica ATCC 23970, and CH2096/CH2094 for B. subtilis 168 (Table S1). The resulting products were digested with NcoI/SpeI, then ligated to plasmid pCH6505 to generate T7 over-expression constructs, and to plasmid pCH6478 for complementation of E. coli ΔcysK mutants. The N. lactamica cysK fragment was ligated using NcoI/XhoI restriction sites. E. coli cysE was amplified with primers CH3642/CDI235 and ligated to pET21P with KpnI/XhoI restriction sites to generate pCH12028 for the gratuitous over-production of native EcCysE in target cells. This fragment was also ligated to pCH10068 to generate plasmid pCH9764 for the purification of untagged EcCysE. Primers CH3642/CH4125 were used to generate plasmid pCH13299, which over-produces EcCysE lacking 11 residues from the C-terminus. The E. coli cysK gene was amplified with primers CH3865/CH2797 and ligated to pCH10068 for the purification of untagged EcCysK.

![Figure 7](image-url)
Protein expression and purification. Proteins were over-produced in *E. coli* BL21(DE3) Tuner™ or CH2016 cells grown in LB media supplemented with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Cells were resuspended in buffer A [20 mM sodium phosphate (pH 7.0), 85 mM sodium chloride, 10 mM 2-mercaptoethanol, 2 mM EDTA] and broken by sonication or French pressure cell. His₆-tagged proteins were purified by Ni²⁺ or Co²⁺-affinity chromatography according to 85 with minor modifications. The His₆ epitope tag was removed from HiCysK using thrombin 36. CysK concentrations were determined by PLP absorbance, calculated by the alkali denaturation method 86. Extinction coefficients at 412 nm are 7,600 M⁻¹·cm⁻¹ for HiCysK.

### Table 1. Bacterial strains and plasmids.

| Strain or plasmid | Description* | Reference |
|-------------------|---------------|-----------|
| **Bacterial strains** | | |
| BL21 Tuner™ (DE3) | *E. coli* B, F-ompT hsdS(rK- mK-) dcm- gal λ(DE3) endA ΔlacZYI Hse, Tet² | Novagen |
| EPI100 | F- merA Δ(mry-hudRMS-merC) p80lacZAM15 ΔlacXZAM15 ΔlacX recA1 endA1 araD139 Nara, leuA7697 gallU gallK λ rpsL nupG, Str² | Epicentre |
| CH2016 | X90 (DE3) Δmna ΔlysD:kan, Rif² Kan² | 81 |
| CH7076 | MG1655 (DE3) | This study |
| CH7718 | X90 (DE3) Δmna ΔcysE:kan, Rif² Kan² | This study |
| CH8804 | X90 (DE3) Δmna ΔlysD ΔcysK:kan, Rif² Kan² | 42 |
| CH10028 | JCM158 ΔcysK:kan, Rif² Kan² | This study |
| CH10801 | JCM158 ΔcysK, Rif² | This study |
| CH13316 | MG1655 (DE3) ΔcysK:kan, Kan² | This study |
| **Plasmids** | | |
| pTrc99a | IPTG-inducible expression plasmid, Amp² | GE Healthcare |
| pET21F | T7 RNA polymerase expression plasmid, Amp² | 11 |
| pCP20 | Heat-inducible expression of FLP recombinase, Cm² Amp² | 79 |
| pDAL866 | Arabinose-inducible expression of the *E. coli cdiBAI* gene cluster, Cm² Amp² | 65 |
| pCH450 | pACYC184 derivative with *E. coli araBAD* promoter for arabinose-inducible expression, Tet² | 82 |
| pCH1043 | pCH405 ∆::argW, over-expresses tRNA CCU Arg, Tet² | 83 |
| pCH6190 | pET21P::cdiA-CT/cdiI, over-produces CdiA-CT and CdiI-His₆, Amp² | 11 |
| pCH6478 | pTrc99A::cdiA-CT3/cdiI₃Dd3937-his₆, Amp² | 47 |
| pCH6505 | pET15c::cdiA-CT/cdiI₃Dd3937, Amp² | 11 |
| pCH7086 | pCH450::cdiA-CT(H178A), Tet² | 16 |
| pCH8215 | pET215::Ec-cysK, Amp² | 16 |
| pCH8936 | pET215::Dd-cysK, Amp² | This study |
| pCH8937 | pET215::ECL-cysK, Amp² | This study |
| pCH8639 | pET215::Bs-cysK, Amp² | This study |
| pCH9280 | pTrc99A::Ec-cysK-his₆, Amp² | 16 |
| pCH9320 | pCH450::cdiA-CT, Tet² | 16 |
| pCH9764 | pSH21::trxA-TEV-cysE, Amp² | This study |
| pCH10068 | pSH21::trxA-TEV-rhs-CT(H208A)-rhsI, Amp² | 80 |
| pCH11846 | pET28a(+):Hi-cysK, over-produces CysK-His₆ from *Haemophilus influenzae*, Kan² | 56 |
| pCH11860 | pTrc99A::Ni-cysK-his₆, Amp² | This study |
| pCH12028 | pET21P::Ec-cysE, Amp² | This study |
| pCH12113 | pTrc99A::Hi-cysK-his₆, Amp² | This study |
| pCH12114 | pET215::Ni-cysK, Amp² | This study |
| pCH12116 | pET215::Hi-cysK, Amp² | This study |
| pCH12286 | pTrc99A::Dd-cysK-his₆, Amp² | This study |
| pCH12287 | pTrc99A::ECL-cysK-his₆, Amp² | This study |
| pCH12288 | pTrc99A::Bs-cysK-his₆, Amp² | This study |
| pCH12673 | Expresses chimeric CdiA EC93-CT EC536, Cm² | 84 |
| pCH12678 | pSH21::trxA-TEV-cysK, Amp² | This study |
| pCH13129 | pET21::his-cdiA-CT(H178A), Amp² | 42 |
| pCH13299 | pET21P::Ec-cysE[Δ(1263-1273)], Amp² | This study |

*Abbreviations: Amp², ampicillin-resistant; Cm², chloramphenicol-resistant; Kan², kanamycin-resistant; Rif², rifampicin-resistant; Str², streptomycin-resistant; Tet², tetracycline-resistant.*
9,370 M$^{-1}$·cm$^{-1}$ for EcCysK, and 8,280 M$^{-1}$·cm$^{-1}$ for NiCysK. Purity was assessed by SDS-PAGE and demonstrated to be greater than 97% (Fig. S4A). All enzymes showed the typical absorption spectrum of fold-type II PLP-dependent enzymes with peaks at 278 and 412 nm and a specific activity of 0.025 U/mg, 0.013 U/mg and 0.016 U/mg for EcCysK, HiCysK and NiCysK (respectively) in agreement with previously reported kinetic data.

EcCysE was over-produced as a fusion with His$_{6}$-thioredoxin (His$_{6}$-TrxA) linked by a TEV protease recognition sequence. Affinity resins were washed with buffer containing 10 mM O-acetyl-L-Ser to dissociate contaminating EcCysK, His$_{6}$-TrxA-EcCysE was eluted with 1 M imidazole and dialyzed against 20 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1% glycerol, 1 mM dithiothreitol, 1 mM EDTA. The fusion was digested with His$_{6}$-tagged TEV protease for 4 h at 25 °C, and the His$_{6}$-TrxA and His$_{6}$-TEV proteins were removed by metal-affinity chromatography. EcCysE was loaded on a FPLC column packed with Ultrogel AcA44 resin (exclusion limit 200 kDa, operating range 17–175 kDa, column volume 63 mL and void volume 20.4 mL) and run at 0.2 mL/min in buffer A. EcCysE eluted at 28 mL with an apparent molecular mass of 167,200 Da, consistent with the expected hexamer quaternary structure. Protein concentration was calculated using an extinction coefficient at 278 nm of 26,900 M$^{-1}$·cm$^{-1}$. Purified EcCysE was ~96% pure (Fig. S4A), with a specific activity of 290 U/mg in agreement with previously published reports.

The CdiA-CT:CdiII-His$_{6}$ complex was expressed in either E. coli BL21(DE3) Tuner™ or CH2016 as described. CdiA-CT and CdiI-His$_{6}$ were separated by metal-affinity chromatography in 8 M urea and the proteins refolded by dialysis into buffer A. The isolated proteins were greater than 95% pure (Fig. S4A), and circular dichroism spectroscopy showed that each protein regained native structure under these conditions (Fig. S4B). CdiA-CT was further purified using size-exclusion chromatography as described for EcCysE above. Protein concentration was estimated using an extinction coefficient at 278 nm of 13,300 M$^{-1}$·cm$^{-1}$ and 8,480 M$^{-1}$·cm$^{-1}$ for CdiA-CT and CdiI, respectively.

**Spectroscopy.** Absorption spectra were collected at 20.0 ± 0.5 °C using a Varian Cary40 spectrophotometer. All spectra were corrected for buffer contributions. Circular dichroism measurements were carried out using a JASCO J-715 spectropolarimeter. Each spectrum is the average of three measurements and is subtracted of the buffer contribution. EcCysE/CdiA-CT binding to CysK was monitored by measuring PLP fluorescence emission at 500 nm following excitation at 412 nm with a JASCO J-715 spectropolarimeter. Each spectrum is the average of three measurements and is subtracted of the buffer contribution. EcCysE/CdiA-CT binding to CysK was monitored by measuring PLP fluorescence emission at 500 nm following excitation at 412 nm with a JASCO J-715 spectropolarimeter. Each spectrum is the average of three measurements and is subtracted of the buffer contribution. EcCysE/CdiA-CT binding to CysK was monitored by measuring PLP fluorescence emission at 500 nm following excitation at 412 nm. CysK emission spectra were collected using a FluoroMax-3 fluorometer (HORIBA) at 20 ± 0.5 °C. Unless otherwise specified, titration samples were equilibrated for 5 min prior to spectra acquisition. All spectra were corrected for buffer contribution, and the slit width set to optimize the signal to noise ratio. For equilibrium binding experiments, the dependence of emission intensity on ligand concentration was determined using the binding isotherm:

$$I = I_0 + \frac{I_{\text{max}} \cdot [L]}{K_d + [L]}$$

(1)

or a quadratic equation that describes tight binding:

$$I = I_0 + I_{\text{max}} \frac{[\text{CysK}] + [\text{CdiA-CT}] + K_d - \sqrt{([\text{CysK}] + [\text{CdiA-CT}] + K_d)^2 - 4 \cdot [\text{CysK}] \cdot [\text{CdiA-CT}]}}{2}$$

(2)

where $I$ is the fluorescence intensity at 500 nm, $I_0$ is a horizontal offset, $I_{\text{max}}$ is the maximum change in fluorescence at saturating [CdiA-CT] and $K_d$ is the dissociation constant for the CysK:CdiA-CT complex.

**Enzyme activity assays.** CysK specific activities were quantified by a continuous spectrophotometric assay using 2-thio-5-nitrobenzoate (TNB) as a nucleophilic substrate. EcCysE specific activity was determined indirectly with 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) as described. An extinction coefficient of 14,150 M$^{-1}$·cm$^{-1}$ at 412 nm was used to quantify TNB in both enzyme assays. EcCysE steady-state kinetics were measured by adaptation of a published method in buffer A without 2-mercaptoethanol at 20 °C. Brieﬂy, O-acetylation of 20 mM L-Ser was monitored by measuring the absorption at 232 nm of the thioester bond (ε$_{232}$ = 4,440 M$^{-1}$·cm$^{-1}$), while varying acetyl-CoA concentrations. At a fixed 0.25 mM acetyl-CoA concentration, EcCysE activity increases as a function of EcCysK concentration. Displacement of EcCysE from the cysteine synthase complex by CdiA-CT was monitored using 28 nM CysE (4.7 nM hexamer) in the presence of 19 nM EcCysK steady-state kinetics were measured by quantifying L-Cys using the discontinuous method of Gaitonde in a 96-well plate format. The sulphydrylase reaction was initiated by addition of 0.6 mM Na$_2$S to a solution containing 6 nM EcCysK, 60 nM bovine serum albumin and variable concentrations of O-acetyl-L-Ser in buffer A. Aliquots (60 µL) were removed at intervals and quenched with 60 µL of acetic acid in a PCR tube strip. Ninhydrin (60 µL) was added with a multichannel pipette and the mixture heated at 100 °C for 10 min in a thermal cycler. The solution was cooled down and 46 µL were added to the wells of a 96-well plate containing 154 µL of cold ethanol. The absorbance at 550 nm was measured using a plate reader and blanks subtracted. The amount of L-Cys produced at each time point was calculated from a calibration curve and a linear equation was fitted to the data to determine initial rate ($v_i$) of production. All kinetic data sets were collected from at least two independent experiments. The kinetic parameters were calculated as follows: $k_{cat} = 241 ± 5$ s$^{-1}$, $K_{M,\text{CysK}} = 5.1 ± 0.3$ mM and $K_{M,\text{CdiA-CT}} = 0.006 ± 0.003$ mM. The dependence of the initial velocity on either EcCysE or CdiA-CT concentration was measured in buffer A containing 2 mM O-acetyl-L-Ser. Morrison's equation (3) was used to calculate $IC_{50}$ and hence the $K_d$ for tight-binding inhibitors.
\[
\frac{v_t}{v_0} = 1 - \frac{([E]_T + [I]_T + IC_{50}) - \sqrt{([E]_T + [I]_T + IC_{50})^2 - 4 \cdot [E]_T \cdot [I]_T}}{2 \cdot [E]_T}
\]

where \([E]_T\) is the total enzyme concentration, and \([I]_T\) is the total inhibitor concentration (EcCysE or CdiA-CT). For competitive inhibitors of a ping-pong reaction\(^6^3, 9^3, 9^4\):

\[
IC_{50} = K_i \left[1 + \frac{[OAS]}{K_{M, OAS}} \cdot \left(1 + \frac{K_{M, HS}}{[HS]}\right)\right]
\]

**Pre-steady state binding kinetics.** Pre-steady state kinetic traces were collected under similar conditions to those reported in ref. 56. Experiments were carried out in buffer A under pseudo-first order conditions with 200 nM EcCysK, 270 nM CdiA-CT or 400 nM EcCysE. The temperature of the loading syringes and the stopped-flow cell compartment was maintained constant with a circulating water bath. Kinetic traces were collected upon direct excitation of PLP at 412 nm using an SX-18MV apparatus (Applied Photophysics) equipped with a 75-watt xenon lamp as a light source and a photomultiplier as a detector. The emission signal was collected at 90° with respect to the excitation source and filtered below 440 nm by a cut-off filter. A single exponential equation

\[
I_t = I_0 + I \cdot e^{-\frac{t}{\tau}}
\]

was fitted to data averaged from three to five kinetic traces. \(I_t\) and \(I_0\) are the emission values at a given time and at zero time, respectively; \(I\) is the total fluorescence change, and \(\tau\) is the relaxation time, such that \(k_{obs}\) is \(1/\tau\). The dependence of \(k_{obs}\) on protein concentrations was obtained from the linear equation:

\[
k_{obs} = k_4 + \left(\frac{k_3}{K_d}\right) \cdot [P]
\]

(6)

to account for a two-step mechanism with a slow conformational change where the plateau cannot be attained under the experimental conditions\(^5^6, 5^9\). The \(K_j\) in Eq. 6 accounts for the first step (i.e. encounter complex formation) of a two-step binding reaction. The dissociation constant as measured under equilibrium conditions accounts for the contributions of both binding and isomerization steps and is usually indicated as \(K_j^{overall}\), indeed for a slow binding mechanism, where \(k_4 < k_3\)\(^5^6, 5^9\):

\[
K_j^{overall} = K_d \cdot \left(\frac{k_3}{k_4}\right)
\]

(7)

If \(k_d/K_d\) and \(K_j^{overall}\) are known, then \(k_4\) can be calculated as follows:

\[
k_4 = K_d^{overall} \cdot \left(\frac{k_3}{k_d}\right)
\]

(8)

**Complex co-purification and native gel electrophoresis.** Purified EcCysK (5 μM) and His\(_6\)-tagged CdiA-CT (5 μM) were incubated with EcCysE (5 or 15 μM) in 20 mM sodium phosphate (pH 7.5), 140 mM NaCl for one h at room temperature. A sample of the mixture was removed (for subsequent SDS-PAGE analysis) and the remainder subjected to Ni\(_{2+}\)-affinity chromatography as described\(^1^6\). Samples of the original mixture (input), the column void (free) and imidazole elution (bound) were analyzed by SDS-PAGE and proteins detected with Coomassie blue stain. Native gel electrophoresis was used to detect cysteine synthase and activated toxin complexes in mixtures. Purified EcCysK (12 μM monomer), EcCysE (18 μM monomer) and CdiA-CT (12 μM) were mixed in various combinations and the resulting complexes resolved on 8% polyacrylamide gels run at 10 mA constant current and 4 °C. The gel running buffer was 5 mM sodium phosphate (pH 7.0) and proteins were detected with Coomassie blue stain. Native-PAGE gels were analyzed using Image Lab™ software (version 5.2.1, Bio-Rad). Software auto analysis procedure was applied to detect lanes and bands with manual adjustments. The exposure time was set to 0.074 s. The relative intensity of each band was calculated using the band % parameter, which calculates band volume as percentage of the total band volume for each sample lane.

**Competition co-cultures and in vivo toxin activity.** *E. coli* EP1100 cells that deploy CdiA-CT from plasmid pCH10673 were used as inhibitors in experiments to determine the effect of EcCysE over-production on toxin activation in target bacteria. Inhibitors were mixed at a 1:1 ratio with *E. coli* CH7076 (cysK\(^+\)) or CH13316 (ΔcysK) target cells that overexpress tRNA\(_{cys}\)\(^{EPI}^6\). Target cells also harbored plasmids pET21P, pCH12028 or pCH13299 (where indicated), and were induced with 1 mM IPTG to allow EcCysE accumulation for 30 min prior to mixing with inhibitor cells. Samples were harvested into an equal volume of ice-cold methanol upon initial cell mixing and after 1 h of co-culture. Cells were collected by centrifugation at 4 °C and frozen at –80 °C. RNA was extracted from frozen cell pellets with guanidinium isothiocyanate-phenol as described previously\(^8^3\). RNA was resolved on 50% urea – 10% polyacrylamide gels and electro-blotted to nylon membrane and hybridized to
5′-radiolabeled oligonucleotide (5′- CTC GCA ATT AGC CCT TAG G)33. Protein was isolated from co-culture samples with two freeze-thaw cycles in urea lysis buffer [8 M urea, 50 mM Tris-HCl (pH 8.0), 150 mM NaCl]. Urea-soluble protein was quantified by Bradford assay and 10 μg resolved on SDS-polyacrylamide gels. Proteins were detected with Coomassie blue stain.

Plasmid co-transformation was used to assess CdiA-CT toxicity in combination with heterologous CysK enzymes. Arabinose-inducible CdiA-CT expression plasmids (100 ng) were introduced into E. coli ΔcysK target cells together with plasmids pTrc99A (no CysK), pCH19280 (EcCysK), pCH11860 (NiCysK), pCH12113 (HiCysK), pCH112286 (DdCysK), pCH112287 (ECLCysK) or pCH12288 (BcCysK). After recovery for 1 h at 37 °C in LB media supplemented with 0.4% D-glucose, cells were plated onto LB-agar supplemented with Tet, Amp and 0.4% D-glucose or L-arabinose to select for transformants carrying both plasmids.

E. coli EPI1001 inhibitors that express the cdiBA system23,36 gene cluster from pDAL866 were used in competition co-cultures to test complementation with heterologous cysK. E. coli CH10801 (ΔcysK) target cells harboring the various cysK expression plasmids were grown to mid-log phase in LB media supplemented with ampicillin, then mixed at a 1:10 ratio with inhibitor cells in LB medium supplemented with 0.2% (w/v) L-arabinose and incubated for 3 h at 37 °C with vigorous shaking in baffled flasks. Viable target-cell counts were enumerated as colony forming units (cfu) ml−1 on LB-agar supplemented with rifampicin. Data are presented as averages ± standard errors for four independent experiments. Heterologous CysK levels were monitored by immunoblot analysis. Total protein was isolated from target-cell strains using urea lysis as described above. Proteins were resolved by SDS-PAGE, electro-blotted onto nitrocellulose, and CysK detected with polyclonal antibodies to the C-terminal His epitope. Immunoblots were visualized using IRDye® 680 (LI-COR) labeled anti-rabbit secondary antibodies and an Odyssey® infrared imager as described previously36.

**In vitro nuclease assays.** CdiA-CT rNase activity was assayed in 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5 mM MgCl2, at 37 °C. To determine the influence of EcCysE on nuclease activity, EcCysK (0.5 μM) was pre-incubated with EcCysE (1.5 to 6 μM) for 25 min prior to the addition of CdiA-CT (0.5 μM). After further incubation for 25 min, reactions were initiated by addition of total E. coli RNA to a final concentration of 2 μg μl−1. Reactions were quenched with SDS-formamide gel-loading buffer after 10 min. CysK (0.1 to 10 μM) enzymes from other bacteria species were assayed in the same manner, except that CdiA-CT was used at 1 μM final concentration and the reactions were quenched after 1 h. All reactions were run on 8 M urea, Tris-borate-EDTA polyacrylamide gels, and RNA visualized by ethidium bromide staining.

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Author Contributions

R.B.: investigation; C.M.B.: investigation; F.G.S.: investigation; S.B.: original idea and planning, funding acquisition, supervision and manuscript reviewing and editing; A.M.: original idea and planning, funding acquisition and manuscript reviewing and editing; C.S.H.: original idea and planning, funding acquisition, supervision, original draft preparation and manuscript reviewing and editing; B.C.: original idea and planning, funding acquisition, supervision, formal analysis, original draft preparation and manuscript reviewing and editing.

Additional Information

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