Molecular mechanism of toxin neutralization in the HipBST toxin-antitoxin system of *Legionella pneumophila*

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Toxin–antitoxin (TA) systems are ubiquitous genetic modules in bacteria and archaea. Here, we perform structural and biochemical characterization of the *Legionella pneumophila* effector Lpg2370, demonstrating that it is a Ser/Thr kinase. Together with two upstream genes, lpg2370 constitutes the tripartite HipBST TA. Notably, the toxin Lpg2370 (HipTLp) and the antitoxin Lpg2369 (HipSLp) correspond to the C-terminus and N-terminus of HipA from HipBA TA, respectively. By determining crystal structures of autophosphorylated HipTLp, its complex with AMP-PNP, and the structure of HipTLp-HipSLp complex, we identify residues in HipTLp critical for ATP binding and those contributing to its interactions with HipSLp. Structural analysis reveals that HipSLp binding induces a loop-to-helix shift in the P-loop of HipTLp, leading to the blockage of ATP binding and inhibition of the kinase activity. These findings establish the *L. pneumophila* effector Lpg2370 as the HipBST TA toxin and elucidate the molecular basis for HipT neutralization in HipBST TA.

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Toxin–antitoxin (TA) systems are bacterial and archaeal genetic modules enriched in mobile genetic elements and chromosomes that comprise two or more closely linked genes encoding a toxin protein and its cognate antitoxin. Since the discovery that the ccdB/ccdA TA system maintains stable inheritance of the mini-F plasmid in *Escherichia coli*, the biological roles of TA systems have been demonstrated to include maintaining stabilization and fitness of mobile genetic elements such as plasmids and protection against phages. Toxins are stable enzymes (e.g., RNases and kinases) or other proteins (e.g., gyrase inhibitors and pore-like toxins) that, in the absence of cognate antitoxin, interfere with vital cellular processes such as DNA replication and protein translation. Antitoxins are unstable proteins or RNAs that counteract toxins. Based on the antitoxin nature and toxin-neutralization mechanism, TA systems can be divided into types I–VIII.

In type II TA systems such as HipBA modules, toxin neutralization depends on direct binding of a proteinaceous antitoxin. HipA from HipBA TA module of the *E. coli* strain K-12 is a 440-amino acid (aa) Ser/Thr kinase that phosphorylates the tRNA-bound glutamate–tRNA ligase GltX at Ser239, thereby inhibiting protein translation. The growth arrest induced by *E. coli* HipA can be counteracted by HipB, a cro/C1-type helix–turn–helix (HTH) domain-containing protein. The structures of HipBA modules from *E. coli* (HipBAEc) and *Shewanella oneidensis* (HipBAso) reveal that both TA modules form a HipA2–HipB2 heterotetramer in which HipB binds far from the kinase catalytic center of HipA. Such neutralization strategy differs from most of other type II TA systems, where antitoxins binding usually occludes the active site or mediate allosteric regulation of the toxin.

Recent studies demonstrated that TA systems containing toxins homologous to the *E. coli* HipA are widely distributed in bacterial...
Legionella pneumophila, the causative agent of Legionnaires’ disease, extensively modifies host signal transduction pathway, especially the post-translational modifications such as ubiquitination and phosphorylation, by translocating hundreds of effectors into the host cell via the Dot/Icm system20. Importantly, the toxin HipT and the antitoxin HipS of the HipBST system were found to correspond to the N-terminal subdomain 1 and the core kinase domain of the E. coli HipA, respectively. The third protein of the HipBST module, HipB, is analogous to HipB of the HipBA system and appears to enhance the neutralization effect of HipS by binding to an already formed HipT–HipS heterodimer26. Recently, a preprint study reported the structure of HipBST heterotrimer from E. coli serotype O127:H6 and concluded that ATP binding in HipT is prevented by comparing its structure in the heterotrimer to the available structures of E. coli HipBA complex27. However, the general mechanism for toxin neutralization in HipBST TA systems is not fully elucidated.

Among these TA systems, a tripartite system designated HipBST was recently identified and experimentally characterized in the enteropathogenic E. coli serotype O127:H628. HipT, which serves as the toxin in the HipBST system, phosphorylates TrpS at Ser197, and its toxicity can be counteracted by the small protein encoded by the adjacent gene hipS29. Importantly, the toxin HipT and the antitoxin HipS of the HipBST system were found to correspond to the N-terminal subdomain 1 and the core kinase domain of the E. coli HipA, respectively. The third protein of the HipBST module, HipB, is analogous to HipB of the HipBA system and appears to enhance the neutralization effect of HipS by binding to an already formed HipT–HipS heterodimer30. Recently, a preprint study reported the structure of HipBST heterotrimer from E. coli serotype O127:H6 and concluded that ATP binding in HipT is prevented by comparing its structure in the heterotrimer to the available structures of E. coli HipBA complex31. However, the general mechanism for toxin neutralization in HipBST TA systems is not fully elucidated.

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N-terminal lobe by 17.3 Å (Fig. 2e, f). Conversely, the six C-terminal α-helices of Lpg2370 and HipAEc, including the catalytic residues and some ATP-binding residues, are almost perfectly aligned (Fig. 2e).

**Lpg2368-Lpg2369-Lpg2370 constitute the tripartite HipBST TA system of L. pneumophila**

Further analysis of Lpg2370 showed that proteins containing the C-terminal domain of HipA are widespread in bacteria (Fig. 3a). Moreover, the structural similarity between Lpg2370 and E. coli HipA and the fact that E. coli HipA along with HipB from the same genomic locus composes a type II TA system prompted us to examine the locus of lpg2370. Indeed, we found that lpg2370 is preceded by open reading frames (ORFs) of lpg2368 and lpg2369. Analogously to TA systems such as HipBA28, lpg2368, and lpg2369 as well as lpg2369 and lpg2370 overlap by 4 bp. Further analysis suggested that lpg2369 encodes a 102-aa protein similar to the N-terminal region of E. coli HipA and that lpg2368 encodes a 72-aa protein homologous to the helix–turn–helix (HTH) domain of HipB (Fig. 3b and Supplementary Fig. 3a, b). Such locus organization is reminiscent of the HipBST TA module in E. coli O127:H617,18, suggesting that the lpg2368-lpg2369-lpg2370

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**Fig. 1 | L. pneumophila effector Lpg2370 is a Ser/Thr kinase.**

* a TEM-1 β-lactamase translocation assay demonstrates that Lpg2370 is a L. pneumophila effector protein. RAW264.7 cells were challenged with a T4SS-competent wild-type L. pneumophila strain Lp02 or the dotA-mutant deficient strain Lp03 carrying plasmids encoding TEM-1-RalF (positive control), TEM-1-Fabl (negative control), or TEM-1-HipTLp. T4SS-mediated translocation of the fusion proteins into host cells was assessed 2 h after infection by the CCF4-AM-based fluorescence resonance energy transfer assay, scale bars 50 μm.

* b Schematic of Lpg2370 and the Ser/Thr kinase HipA from E. coli K12 (HipAEc). Yellow and blue regions represent the approximate locations of the P-loop and activation motif of HipA, respectively, as well as the locations of corresponding sequences in Lpg2370. Sequence conservation of P-loop and the activation motif is presented as a weblog below the protein schematics.

* c Diagram depicting detection of thiphosphorylated Lpg2370 by thio-phosphate labeling. PNBM: p-nitrobenzylmesylate.

* d Thio-phosphorylation assay with the purified 6×His-tagged Lpg2370. Thio-phosphorylated Lpg2370 was visualized by immunoblotting.

* e Identification of the phosphorylated peptide by LC-MS/MS. The peptide SVQGQVK was observed at charge state 2+ in two forms differing by 70.97 Da in molecular mass.
Table 1 | X-ray data collection and refinement statistics

| Dataset          | pHipTLp | pHipTLp–AMP–PNP | HipTO127–HipSRp |
|------------------|---------|----------------|----------------|
| **Data collection** |         |                |                |
| Wavelength (Å)   | 0.9792  | 0.9792         | 0.9792         |
| Space group      | P2,2,1  | P2,2,1         | P4,2,2         |
| Cell dimensions  |         |                |                |
| a, b, c (Å)      | 52.95, 64.64, 90.42 | 39.81, 86.31, 91.88, 45.22, 45.22, 394.55 |
| α, β, γ (°)      | 90.00, 90.00, 90.00 | 90.00, 90.00, 90.00, 90.00, 90.00, 90.00, 90.00, 90.00 |
| Resolution range (Å) | 27.32–1.46 | 36.52–1.59 | 31.87–1.82 |
| Rmerge (%)       | 0.125 (1.08) | 0.08 (1.22) | 0.084 (1.39) |
| CC1/2            | 0.95 (0.67) | 0.996 (0.870) | 0.999 (0.872) |
| I/σ(I)           | 9.4 (2.00) | 10.3 (2.20) | 22 (2.60) |
| Completeness (%) | 95.32 (90.51) | 99.39 (98.12) | 99.9 (99.70) |
| Multiplicity     | 8.57 (6.44) | 12.3 (9.80) | 24 (24.70) |
| **Refinement**   |         |                |                |
| Resolution (Å)   | 27.32–1.46 | 36.52–1.59 | 31.87–1.82 |
| Rwork (%)        | 17.53 (24.15) | 17.20 (18.60) | 18.59 (27.23) |
| Rfree (%)        | 19.42 (25.06) | 19.26 (21.40) | 19.28 (21.32) |
| Ramachandran plot (%) |         |                |                |
| Favored region   | 98.40 | 98.34 | 98.34 |
| Allowed region   | 1.60  | 1.66  | 0.00  |
| Outliers region  | 0.00  | 0.00  | 0.00  |

One crystal was used for the determination of each structure. Values in parentheses are for the highest-resolution shell.

locus is a potential tricistronic operon encoding component of a HipBST TA system.

In the HipBST TA system of *E. coli* O127:H6, the toxin HipT (denoted HipTO127) can form a heterotrimeric complex with the antitoxin HipS (HipS127) and the HTH domain protein HipB (HipB127). We therefore performed size-exclusion chromatography, pull-down assays and isothermal titration calorimetry (ITC) to analyze interactions between Lpg2370, Lpg2369, and Lpg2368. The co-expressed 6×His-tag recombinant Lpg2370 in isothermal titration calorimetry (ITC) to analyze interactions between Lpg2370, Lpg2369, and Lpg2368-Lpg2369 complex and Lpg2368 are 42 nM by the pull-down assays (Supplementary Fig. 4). These results suggest that Lpg2370 directly interacts with Lpg2369, whereas Lpg2368 binds to Lpg2369 and Lpg2370-Lpg2369 complex and Lpg2368 functions as the antitoxin (Fig. 3e). Co-expression of Lpg2368 and Lpg2369 was also found to counteract Lpg2370-dependent growth inhibition, whereas the expression of Lpg2368 without Lpg2369 could not prevent the growth inhibition (Fig. 3e). Taken together, these results are consistent with the findings on the *E. coli* O127:H6 HipBST module27,28 and demonstrate that Lpg2368, Lpg2369, and Lpg2370 from *L. pneumophila* constitute the tripartite HipBST TA system and will thus hereafter be referred to as Hip1p, HipS1p, and HipP1p, respectively29,30.

The kinase activity of HipT1p is likely independent of P-loop serine autophosphorylation

A comparison of the crystal structure of pHipT1p, and the structures deposited in the PDB revealed that the autophosphorylated P-loop in HipT1p adopts an orientation similar to that of the P-loop in the crystal structure of *E. coli* HipA S50A mutant (Fig. 3f). This observation led us to speculate that pHipT1p can bind ATP. Thermal shift assays performed with the purified wild-type HipT1p revealed a 2.5°C increase in the melting temperature (Tm) in the presence of non-hydrolysable ATP analogue adenylyl-imidodiphosphate (AMP–PNP), suggesting that pHipT1p indeed binds ATP (Supplementary Fig. 7). Likewise, isothermal calorimetry determined that the dissociation constant between pHipT1p and AMP–PNP was about 70 μM (Fig. 4a), which is within the range of ATP-binding affinity expected for other kinases31.

To elucidate how pHipT1p binds ATP, we determined the crystal structure of pHipT1p in complex with AMP–PNP at 1.36 Å resolution (Table 1). The structure of pHipT1p–ATP reveals that AMP–PNP is bound to the P-loop like in other representative kinases (Fig. 4b). The backbone of pHipT1p in the complex is virtually identical to the apo structure (RMSD = 0.35 Å), with the exception of P-loop that bends towards helix α2 to accommodate the AMP–PNP. ATP (AMP–PNP)–interacting residues appear to be conserved among the bacterial HipT toxins, implying a shared mechanism for ATP binding (Fig. 4c). In pHipT1p, the γ-phosphate of AMP–PNP is stabilized by V58, H199, and D219, the β-phosphate forms hydrogen bonds with Q59, K61, and K85, whereas the α-phosphate interacts with K85 and N202. The adenosine moiety interacts with the main chain of K130 and forms π-stacking interactions with the side chain of F132 (Fig. 4b).

A previous study demonstrated that the kinase activity of *E. coli* HipA is essential for the growth arrest of host cells32, and cell growth was inhibited when HipT127 was expressed in *E. coli* BL21 (DE3) cells33. To confirm the role of the residues involved into the ATP binding in the HipT toxins in vivo, HipT127 TA is used to perform the growth inhibition assays due to the easy manipulation of *E. coli* compared to *L. pneumophila*. To investigate whether the above-mentioned residues responsible for ATP binding are essential for the kinase activity of HipT, we performed in vivo toxicity assays with HipT127 variants in which residues corresponding to the SS4 and the highly conserved ATP-binding residues of HipT1p were substituted with alanine. Intriguingly, mutation on the residues corresponding to S57A and S57D HipT127 remain toxic to *E. coli* cells, whereas substitutions of K64 (K61), K86 (K85), H121 (H119), N215 (N202), and D233 (D219) of HipT127 (corresponding residues in HipT1p are indicated in parentheses) eliminated the toxic phenotype (Fig. 4d). Taken together,
these results suggest that unlike in *E. coli* HipA, HipT retains the ATP-binding ability independent of the autophosphorylation on the conserved S54 in the P-loop and that HipT uses a universal mode for ATP recognition.

**Structural basis for the toxin HipT<sub>Lp</sub> recognition by the anti-toxin HipS<sub>Lp</sub>**

Although the toxic activity of HipT in the HipBST TA system has been demonstrated to be counteracted by the anti-toxin HipS<sub>Lp</sub>17,18, the underlying molecular mechanism remains unknown. We therefore sought to determine the structure of the HipT<sub>Lp</sub>–HipS<sub>Lp</sub> complex. To express the HipT<sub>Lp</sub>–HipS<sub>Lp</sub> complex, a ribosomal-binding site (RBS, AGGAGA)<sup>37</sup> was introduced between the stop codon of HipS<sub>Lp</sub> and the start codon of HipT<sub>Lp</sub>. The resultant HipS<sub>Lp</sub>–RBS–HipT<sub>Lp</sub> was cloned into pET21a (+) vector. The crystal structure of the SeMet-labeled HipT<sub>Lp</sub>–HipS<sub>Lp</sub> complex was determined and refined at 1.89 Å resolution (Table 1).

In the structure of HipT<sub>Lp</sub>–HipS<sub>Lp</sub> complex, a copy of HipT<sub>Lp</sub> and HipS<sub>Lp</sub> each were observed per crystal asymmetric unit. Residues belonging to helices α<sub>1</sub> and α<sub>2</sub> of HipT<sub>Lp</sub> were not visible in the electron density map, whereas the density of the remaining residues was unambiguous (Fig. 5a). All 102 residues of HipS<sub>Lp</sub> were successfully built into the model, showing that HipS<sub>Lp</sub> is a small single-domain protein composed of five β-strands and three α-helices. The overall structure of the HipT<sub>Lp</sub>–HipS<sub>Lp</sub> complex is highly similar to *E. coli* HipA, with HipS<sub>Lp</sub> and HipT<sub>Lp</sub> aligning with the N- and C-terminal portions of *E. coli* HipA, respectively (Supplementary Fig. 8a). HipS<sub>Lp</sub> superimposed with the N-terminus of HipA<sub>Ec</sub> with an overall RMSD of 0.932 Å across 26 Ca. However, a notable difference can be observed on the β4–α2 loop of HipS<sub>Lp</sub>, which is twisted and rotated by ~45° with...
respect to its counterpart in the N-terminus of E. coli HipA (Supplementary Fig. 8b).

In the structure of HipT<sub>Lp</sub>–HipS<sub>Lp</sub> complex, three α-helices of HipS<sub>Lp</sub> form a helix bundle that sits above the cleft formed by the β-sheet in the N-terminal lobe of HipT<sub>Lp</sub>, whereas the β-strands form a flank region in HipS<sub>Lp</sub> (Fig. 5b). HipS<sub>Lp</sub> binds the toxin HipT<sub>Lp</sub> via hydrogen bonding in three main interacting regions, which constitute more than 1100 Å<sup>2</sup> of total buried surface area (Fig. 5c–e). The intermolecular interactions are mainly formed between helices α1, α2 and α1–α2 loop of HipS<sub>Lp</sub> and helix α3 and strand β5 of HipT<sub>Lp</sub>. In the first interacting region, side chains of HipS<sub>Lp</sub> E63 and HipT<sub>Lp</sub> K157 form a salt bridge, side chain of HipS<sub>Lp</sub> E58 engages in polar interactions with the main chain amide and side chain of HipT<sub>Lp</sub> R154, and hydrogen bonds are additionally formed between main chain of HipS<sub>Lp</sub> G59 and
side chain of HipTLp K201 and main chains of HipSLp I65 and HipTLp G57 (Fig. 5c). The second interacting region includes a salt bridge between HipTLp D77 and HipSLp K73 and hydrogen bonds between (i) side chain of HipTLp D133 and HipTLp G94, (ii) side chain of HipTLp R154 and side chain of HipSLp N91 as well as main chain of HipTLp V92, (iii) side chain of HipSLp N91 and main chain of HipTLp V79, and (iv) side chains of HipTLp Q78 and HipSLp Q90 (Fig. 5d). In the third interacting region, the side chain of HipTLp Q145 hydrogen bonds with the main chain of HipSLp S56, whereas the side chain of HipTLp E144 forms hydrogen bonds with the side chain of HipSLp S38 and the main chain of HipTLp L39 (Fig. 5e).

To verify the importance of these interactions for stable binding of HipSLp to HipTLp, we performed pull-down assays with untagged wild-type HipSLp and wild-type or mutant HipTLp carrying a N-terminal 6-His-tag. The HipTLp mutants D133A, R144A, and E144A completely lost their ability to bind HipSLp and the mutants R154A, K157A, K201A exhibited severely reduced HipSLp binding, suggesting that these residues form key interactions with HipSLp (Fig. 5f).

Molecular mechanism for toxin neutralization in the HipBST TA systems

One of the most striking features of the HipBST TA systems is that the role of antitoxin is taken by HipS which corresponds to the N-terminal portion of HipA toxin from the E. coli HipBA system. To better understand how the toxic activity of HipT is neutralized by HipS, we reinspected and compared the structures of apo pHipTLp–pHipTLp–AMP–PNP, and apo pHipTLp–HipSLp complex with ITC and found that HipTLp completely lost the AMP–PNP binding affinity when HipSLp was co-expressed (Supplementary Fig. 11). Considering that HipSLp complex superimposes with RMSD of 0.46 Å over 215 Cα atoms. Notably, Ser54 of HipTLp is phosphorylated in the structure of apo pHipTLp but not in the HipTLp–HipSLp complex (please note that HipTLp–HipSLp was co-expressed in E. coli BL21 (Fig. 2a, b and Supplementary Fig. 8c). Since the residue S54 is phosphorylated when HipTLp is expressed alone, we wondered whether the phosphorylation on S54 influences the interaction between HipTLp–HipsLp and HipBDp. Size-exclusion chromatography revealed that the phosphorylation state of S54 does not appear to have a noticeable effect on interactions between HipTLp–HipSLp and HipBDp. Moreover, structural comparison suggests that the P-loop of HipTLp, which encircles ATP, is critical for catalytic activities in typical Ser/Thr kinases, underwent a conformational change from loop to helix upon HipSLp binding (Fig. 6a, b). Such allosteric regulation induced by the antitoxin binding has not been observed in E. coli HipBA TA system. A conformational change similar to the loop-to-helix change of the HipTLp P-loop in HipTLp–HipSLp complex can also be observed in the recently released structure of HipBSTO127 trimmer (Supplementary Fig. 10), suggesting a common mechanism of toxin neutralization.

These observations led us to hypothesize that the loop-to-helix conformational transition induced upon HipSLp binding may obstruct the access of ATP to the kinase active site, resulting in inhibition of the HipTLp kinase activity. Superimposition of the structures of pHipTLp–pHipTLp–AMP–PNP, and the HipTLp–HipSLp further revealed that P-loop in the HipTLp–HipSLp complex overlaps with AMP–PNP in the pHipTLp–AMP–PNP complex (Fig. 6c, d and Supplementary Fig. 11). More specifically, the γ-phosphate and β-phosphate groups of AMP–PNP would clash with the side chains of Q59 and D219, respectively, whereas the α-phosphate would clash with the side chains of K61 and K35 (Fig. 6d). This may account for the unphosphorylated state of the P-loop in the HipTLp–HipSLp complex when they were co-expression (Supplementary Fig. 8c). Such conformational change also occurs in the P-loop of HipBSTO127, suggesting that a similar mechanism is utilized by HipBSTO127 (Fig. 6e, f). To further verify whether ATP binding is abolished, we measured the binding affinity between the HipTLp–HipSLp complex and AMP–PNP with ITC and found that HipTLp completely lost the AMP–PNP binding affinity when binding with HipSLp (Fig. 6g). Consistent with these results, the thermal stability of the HipTLp–HipSLp complex did not change upon the addition of 4 mM AMP–PNP (Supplementary Fig. 11a). Considering that HipSLp, in autophosphorylated form can bind ADP and AMP but not ATP, we also investigated whether the HipTLp–HipSLp complex binds ADP and AMP. Again, the results of ITC experiments suggested that the HipTLp–HipSLp complex has no detectable affinity for ADP or AMP, even at concentrations of 1 mM (Supplementary Fig. 12). The allosteric regulation of the P-loop induced by the antitoxin binding was also observed in the HipBSTO127. Together, these findings suggest that HipSLp binding induces conformational changes in the P-loop of HipTLp, which blocks ATP binding and consequently inhibits the HipTLp kinase activity (Fig. 6h).

Discussion

Although the biological functions of TA systems are often ambiguous and debatable, recent advances in the field and the discovery of numerous novel TA modules increasingly support their roles in viral defense or plasmid stability and interactions between hosts and their mobile genetic elements. Among known TA modules, the type II TA systems are most well understood. Type II TA systems typically consist of two components, though several tripartite type II TA modules have been identified, such as the Rv1955-Rv1956-Rv1957−19. Together, these findings suggest that HipSLp binding induces conformational changes in the P-loop of HipTLp, which blocks ATP binding and consequently inhibits the HipTLp kinase activity.

Most type II antitoxins are composed of an N-terminal DNA-binding domain that regulates transcription from the TA locus through direct interaction with the promoter, and a C-terminal region responsible for the toxin binding and inhibition. HipBA modules are representative type II TA systems and are ubiquitous in bacteria. Based on the similarity searching using E. coli HipA against the sequenced microbial genomes, a three-component widespread HipBST TA was found and experimentally verified. Later bioinformatic analysis has suggested the presence of several other HipBA-like TA in numerous bacterial species. While the toxin HipO127 of HipBSTO127 was found to exert its toxic function by phosphorylating TrpS, and in spite of the similarity between HipBO127 and E. coli HipB,
HipB\textsubscript{O127} however cannot neutralize the HipT\textsubscript{O127} kinase. This task was taken over by HipS, a small protein with homology to the N-terminal part of \textit{E. coli} HipA\textsuperscript{18,19}. Our current study also experimentally identifies a tripartite HipBST\textsubscript{Lp} TA in which the antitoxin HipSL\textsubscript{p} instead of HipBL\textsubscript{p} restores the growth inhibition induced by HipTL\textsubscript{p} in human pathogen \textit{L. pneumophila}, which is in agreement with the HipBST\textsubscript{O127} TA\textsuperscript{18}. Together with the preprint study in ref. \textsuperscript{19}, our study identifies and elucidates the toxin-neutralization mechanism in the tripartite

\textbf{Fig. 4} | Crystal structure of the pHipTL\textsubscript{p}–AMP–PNP complex. a Binding of AMP–PNP to pHipTL\textsubscript{p} monitored by ITC, the presented data is from a single ITC experiment. b Left: cartoon representation of the pHipTL\textsubscript{p}–AMP–PNP complex. The N- and C-termini of pHipTL\textsubscript{p} are labeled. The bound AMP–PNP molecule and pSer54 are shown as sticks and colored red and yellow, respectively. Right: detailed view of the ATP-binding cavity of HipTL\textsubscript{p} and interactions formed with AMP–PNP. The distance between the interacting residues of HipTL\textsubscript{p} and AMP–PNP are in range of 2.7–3.3 Å, which was shown as sticks and hydrogen bonds are indicated with black dashed lines. c Sequence alignment of HipT variants from \textit{L. pneumophila}, \textit{Escherichia coli} O127:H6, \textit{Vibrio halioticoli} and \textit{Haemophilus influenzae} reveals conservation of the residues involved in ATP binding. The ATP-binding residues are encircled with black bold rectangles. d Growth curves of \textit{E. coli} BL21(DE3) cells expressing recombinant wild-type HipT\textsubscript{O127} or its mutant variants S57A, S57D, K64A, K86A, H212A, N215A, and D233A.
HipBST TA systems of *L. pneumophila*. It is clear that the toxin neutralization mechanism in HipBST systems is notably different from the corresponding mechanism in HipBA TA system of *E. coli*. The structural study by Bærentsen et al. on the *E. coli* O127:H6 HipBST found that HipT adopts an inactive conformation in the HipBST complex that prevents ATP binding. However, it was unclear whether the blockage of ATP binding arises from the binding of the antitoxin HipS or autophosphorylation of the conserved P-loop serine. Our structure of the HipTLp–HipSLp complex clearly shows the toxicity of HipTLp is neutralized directly upon HipSLp binding, leading to blocking of the ATP-binding site through steric hindrance. Although this study demonstrates that HipBLp forms a heterotrimer with the HipTLp–HipSLp complex, we failed to obtain crystals of the heterotrimer structure after extensive crystal screening. However, the available structure of *E. coli* HipBST shows that HipB binds to HipT but does not interact with the kinase active site. In addition, overall architecture of HipBStO127 system is reminiscent of the structure of HipBAso system in the HipBAso–DNA complex, implying that HipBST can also bind DNA. Induction of HipB_O127 was found to be required and sufficient for the transcriptional repression of the HipBST TA. Thus, it seems that the toxin neutralization and the autoregulation of the HipBST TA are carried out by the antitoxin HipS and HipB, respectively. Nevertheless, the exact role of HipB in the HipBST system remains to be determined.

To the best of our knowledge, HipTLp is the only identified toxin of TA systems secreted into host cells via the type IV secretion system Dot/Icm. Nevertheless, the employment of TA toxins by the pathogenic bacteria during host cell infection is not unprecedented. For instance, the type III secretion system effector AvrRxo1-ORF1 from *Xanthomonas oryzae* pv. *oryzae* constitutes a type II TA system with adjacent AvrRxo1-ORF2. AvrRxo1 can phosphorylate NAD in planta, leading to the suppression of the Nlr22-triggered ROS burst. Moreover, previous investigation of fourteen effectors from *Burkholderia gladioli* revealed that the restriction endonuclease Tox-Rease-5 domain-containing effector TaseTBg can be employed to inhibit the growth of co-habiting bacterial species, and its activity is counteracted by the associated immunity protein TsiTBg. Apart from the effectors LegK1–K5 and LegK7, HipTLp is the sixth effector known to function as a Ser/Thr kinase in *L. pneumophila*. LegK1 phosphorylates NF-κB to activate the genes expression involved in inflammation during...
infection. Phosphorylation of Hsp70 at T495 by LegK445, leading to the inhibition of protein synthesis. LegK7 phosphorylates a conserved scaffold protein MoB1, hijacking the hippo pathway to promote its survival. The exact substrate of HipT in its host remains to be identified in future studies. In summary, these results suggest that TA systems could serve as a reservoir for additional secreted effectors, which sheds light on the evolutionary links between the TA system and the effectors secreted by the pathogenic microorganisms.

![Fig. 6 Toxin-neutralization mechanism in the HipBST TA systems. a Overlay of apo pHipT and structure of HipT-HipS in cartoon representation. Apo pHipT is colored gray and its P-loop is colored yellow, whereas HipT-HipS from the binary complex is colored cyan and its P-loop is colored purple. HipS is colored green. b Close-up view of the overlay showing that HipT-HipS binding induces conformational change of the P-loop. c Overlay of the pHipT–AMP–PNP and the structure of HipT-HipS in cartoon representation. pHipT from the pHipT–AMP–PNP is colored white. The HipT–HipS complex is color-coded as in panel a. d Close-up view of the overlapping between HipT residues and AMP–PNP. AMP–PNP is shown as dotted surface mode and overlapping HipT residues are shown as sticks. e Crystal structure of HipBST (PDB:7ABS) HipT and HipS were colored cyan, green and yellow orange, respectively. f Structural comparison between the HipT-HipS and HipBST. The P-loops of HipT and HipS are colored red and blue, respectively. g ITC thermogram and binding curve demonstrated that the HipT–AMP–PNP complex does not display detectable affinity for AMP–PNP. h Proposed model for toxin-neutralization mechanism in the HipBST TA systems. The toxin HipT is a Ser/Thr kinase in which the P-loop motif is vital for ATP binding and subsequent substrate phosphorylation. Binding of the antitoxin HipS causes conformational changes in the P-loop, which blocks ATP binding and ultimately inhibits the kinase activity of HipT.
Methods

Bacterial strains and growth conditions

The *L. pneumophila* strain Philadelphia-1 derivative Lp02 was used as the progenitor of all derivative strains used in this study. To construct Δ*lp2368-lp2369-lp2370* triple-deletion mutant strain of *L. pneumophila* (termed Δ3), we first constructed deletion plasmids by cloning the upstream and downstream flanking regions into pSR475. Briefly, the 1.2-kb fragment located upstream of *lp2368* and the 1.2-kb fragment located downstream of *lp2370* were obtained by PCR using Δ3 A1/A3 A2 and Δ3 B1/A3 B2 primer pairs and high-fidelity FastPfu DNA polymerase (TransGen) (Supplementary Table 2), respectively. The amplified PCR products were used as templates to produce a DNA fragment containing the flanking regions by fusion PCR with primers Δ3 A1/A3 B2 and high-fidelity FastPfu DNA polymerase (TransGen). After digestion with BamHI and SalI, the DNA fragment was inserted into BamHI/Sall-digested pSR475. The deletion plasmids were then introduced into Lp02 by triparental mating, and conjugants were selected on CYET plates containing kanamycin (20 μg/mL) and streptomycin (50 μg/mL). Deletion mutants were verified by standard colony PCR techniques using primers Δ3 A1/A3 B2 and 2×Taq master mix (Novoprotein) from colonies grown on CYET plates containing 5% sucrose. Moreover, the *lp2370-lp2368-lp2369* gene cassettes were inserted into pZL507 ΔS1, pZL507ΔS2, and pZL507ΔS3, which were then introduced into Δ*lp2368-lp2369-lp2370* strain. For effector translocation, fresh single colonies of Lp02 or Lp03 harboring expression plasmid for TEM-1/pZL2370, TEM-1/Ralf, or TEM-1/Fab fusion proteins were streaked onto BCYE plates 2 days before infection.

TEM-1 β-lactamase translocation assays

To test Dot/Icm-dependent transfer of the fusion proteins into host cells, *L. pneumophila* cells expressing the fusion proteins were grown in the presence of 0.5 mM IPTG to post-exponential phase and used to infect monolayers of RAW264.7 cells that were seeded in 96-well plates at an MOI of 20. The CCF4-AM substrates (Invitrogen, Carlsbad, CA) were mixed with medium in the wells two hours after infection. After further incubation for 1 hour at room temperature, infected cells were inspected under a Nikon IX-80 fluorescence microscope equipped with an integrated HPLC/ESI-MS system (1260 Infinity, Agilent Technologies). Each MYC column was blocked with 5% milk for 1 h at room temperature. The membrane was washed three times with TBST before being incubated with a secondary antibody (HRP-conjugated Affiload goat anti-rabbit (Cat No. SA00001-2)). Western blots were probed with the following primary antibodies: HipSLp (1:2000 dilution), HipTLp (1:2000 dilution), HipBLp (1:2000 dilution). Liquid chromatography-mass spectrometry (LC-MS) analysis

LC-MS was used to analyze autophosphorylation of purified recombinant Lpg2370. After staining of gels with Coomassie blue, excised gel segments were subjected to in-gel trypsin digestion and dried. Electrospray ionization mass spectrometry (ESI-MS) was performed using an integrated HPLC/ESI-MS system (1260 Infinity, Agilent Technologies) equipped with a Luna 3 μm C18 column (100 Å, 250 × 4.60 mm, 5 μm). Peptides were dissolved in 10 μl 0.1% formic acid and were auto-sampled directly onto a homemade C18 column (35 cm × 75 μm i.d., 1.9 μm 3B piped, and 4 °C for 30 min. The supernatant was loaded onto Ni²⁺-NTA column (Qiagen) for purification of target recombinant proteins. After washing with 100 μL of buffer A supplemented with 50 mM imidazole, the target proteins were eluted with buffer B supplemented with 250 mM imidazole. Fractions containing the target protein were pooled, concentrated to 0.5 mL and then purified with Superdex 75 increase column (GE Healthcare) equilibrated with buffer B (20 mM Tris-HCl, pH 8.0, 150 mM NaCl). Protein crystallization and collection of crystallographic data

The purified target protein was concentrated to 0.5 mL and loaded onto a Superdex 75 increase column (GE Healthcare) Fractions
containing purified proteins were then concentrated at 4000×g, 4 °C to -15 mg/mL using an Amicon Ultra 30 K centrifugal filter. To obtain HipTLP-AMP–PNP complex, the purified HipTLP was incubated with AMP–PNP at a 1:1.2 molar ratio at 4 °C for 30 min and concentrated to about 12 mg/mL.

For crystallization of SeMet-labeled HipTLP, HipTLP–AMP–PNP complex, and SeMet-labeled HipTLP–HipSLp complex, the purified and concentrated protein samples were mixed with the reservoir solution at equal volumes and crystallized using the sitting drop vapor diffusion method at 16 °C. Initial crystals of SeMet-labeled HipTLP and HipTLP–AMP–PNP complex were obtained within three days in condition containing 8% Tacsimate (pH 6.0) and 20% v/w PEG 3350. Initial crystals of the SeMet-labeled HipTLP–HipSLp complex were obtained in the condition containing 0.1 M sodium acetate (pH 7.0) and 12% v/w PEG 3350. After extensive optimization, diffraction-quality crystals of SeMet-labeled HipTLP and the HipTLP–AMP–PNP complex were grown in the presence of 10% Tacsimate (pH 6.2) and 20% v/w PEG 3350. Diffraction-quality crystals of the HipTLP–HipSLp complex were grown in the presence of 0.1 M sodium acetate (pH 7.2) and 15% w/v PEG 3350. Harvested crystals were preserved in the respective reservoir solutions supplemented with cryoprotectant and flash-frozen in liquid nitrogen.

**Structure determination and refinement**

All X-ray diffraction data were collected at the BL-02U1 station of the Shanghai Synchrotron Radiation Facility (SSRF). Single-wavelength anomalous diffraction (SAD) datasets of SeMet-labeled HipTLP and SeMet-labeled HipTLP–HipSLp complex were obtained at high resolution and the data were processed with the HKL-2000 package. Autosol program of PHENIX package was used for SAD phasing of SeMet-labeled HipTLP and HipTLP–AMP–PNP complex with initial model building in Coot. Structure re

whereas the residues 1–71 residues were built by iterative manual

mentation was carried out with

the structure of HipTLP as the search model. Structure quality was analyzed during PHENIX refinements and later validated in the PDB validation server. Detailed crystallographic and structure refinement data are listed in Table 1. Structural images were generated using PyMol (Schrödinger, LLC).

**Mutagenesis**

Base substitutions in this study were introduced using two pairs of complementary primers (sense and antisense strand primers) containing the desired mutation. The primers used in this study were listed in Supplementary Table 2. All constructs were verified by DNA sequencing.

**Isothermal titration calorimetry (ITC)**

ITC experiments were performed in Nano ITC Low Volume (TA instruments). All samples were prepared in the buffer containing 20 mM HEPES (pH 8.0) and 150 mM NaCl. Typically, the titrant concentration in the syringe was 200–500 μM, and the titrant concentration in the reaction cell was 10–20 μM. Titration was conducted at 25 °C using multiple injection method with 150 s intervals. Obtained data were integrated, corrected, and analyzed using the NanoAnalyze software (TA Instruments) with a single-site binding model.

**Pull-down assays**

To perform pull-down assays, wild-type or mutant HipTLP carrying C-terminal 6×His-tag were incubated with Ni-agarose beads for 30 min and then washed twice with buffer containing 20 mM Tris-HCl (pH 8.0) and 150 mM NaCl. The beads were then incubated with untagged wild-type HipSLp (6×His-tag was previously cleaved with TEV protease) for 1 h and then washed twice. The proteins were eluted from beads using a buffer containing 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 250 mM imidazole. Eluted samples were analyzed using SDS-PAGE analysis.

**In vivo toxicity assays**

For in vivo toxicity assays of HipT from E. coli serotype O127:H6 (denoted HipT0127) to E. coli, which was performed by expressing the HipT0127 gene or its mutant variants in E. coli BL21(DE3). The gene encoding HipT0127 was synthesized and cloned into PET2a (+) vector. Plasmids encoding HipT0127–single-point mutants S57A, S57D, K64A, K64D, H212A, N215A, and D233A were prepared using site-specific mutagenesis, and the primers are listed in Supplementary Table 2. Transformed E. coli BL21 (DE3) cells were plated on agar and a single bacterial colony was transferred to 10 mL LB medium for culturing. Expression of wild-type HipT0127 and its mutant variants was induced with IPTG at 0.2 mM concentration when the bacterial cultures reached OD600 = 0.5, after which the bacterial growth curve was measured every 30 min for 6 h.

For the Lpg2370 in vivo toxicity assays in L. pneumoniae Lpg2370, Lpg2370-Lpg2369, Lpg2370-Lpg2368, Lpg2370-Lpg2369-Lpg2368 were overexpressed in L. pneumoniae LP02 or the Δlpg2368Δlpg2369Δlpg2370 deletion strain. Overnight cultures of the tested bacterial strains were diluted in fresh AYE broth to OD600 = 0.1 and split into 2 mL subcultures into which different concentrations of arabinose or IPTG were added. The subcultures were then grown at 37 °C with constant rotation at 180 rpm. Cell viability was assessed by readout of OD600 value every 3 h and plotting the values on a log scale.

**Thermal shift assays**

Thermal shift assays were performed using 1 mg/mL phosphorylated HipTLP, or its mutants incubated with ATP/AMP–PNP and varied concentrations of nucleotides (0–4 mM) in 50 mM Tris-HCl (pH 8.0) and 150 mM NaCl. Then the mixture was loaded in 96-well PCR plates, the fluorescence signals were recorded as a function of temperature using Prometheus NT.48 (NanoTemper Technologies) in FRET mode. Fluorescence intensity was measured at Ex/Em of 350/330 nm. The temperature gradient range was set as 20–95 °C with a 0.5 °C ramp over the course of 30 s. Control assays were conducted in the same buffer without ATP/AMP–PNP. The thermal unfolding value (Tm) for HipTLP was calculated using the curve fitting software PR.Therm-Control (NanoTemper Technologies).

**Phylogenetic analysis**

The sequence of Lpg2370 was blasted in the Uniprot (https://www.uniprot.org/) and all the hit sequences were used for sequence alignment in ClustalW, which was used to build the phylogenetic tree using MEGA software. The phylogenetic tree was visualized using ITOL.

**Statistics and reproducibility**

The TEM-1 β-lactamase translocation assays experiments in Fig. 1a, the western blotting experiment in Fig. 1d, the pull-down assays in Figs. 3c, 5f, Supplementary Fig. 4, and Supplementary Fig. 9 were performed at least triplicate at two independent times.

**Data availability**

The atomic coordinates and structure factors of the autophosphorylated toxin HipTLP, the complex of HipTLP with AMP–PNP, and HipTLP–HipSLp binary complex have been deposited in the Protein Data Bank under the accession codes 7VKC, 7WCF, and 7VKB. Source data are provided with this paper.

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Author contributions
X.K.Z., S.O., and J.Z.Q. conceived the project and designed the experiments, X.K.Z. and Y.Y.W. performed crystallization and resolved the structures, J.L.G. performed the in vivo toxicity assays, J.Q.F., L.Y., N.N.L., Z.J.H., and Z.H.L. contributed to the protein expression and purification. X.K.Z. analyzed the structures, X.K.Z., S.O., Z.Q.L., and J.Z.Q. designed the biochemical experiments and wrote the manuscript.

Competing interests
The authors declare no competing interests.

Additional information
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