Structural basis for regulation of a tripartite toxin-antitoxin system by dual phosphorylation

René L. Bærentsen¹*, Stine Vang Nielsen²*, Jeppe Lyngsø³, Francesco Bisiak¹, Jan Skov Pedersen³, Kenn Gerdes⁴, Michael A. Sørensen²†, Ditlev. E. Brodersen¹†

¹Department of Molecular Biology and Genetics, Aarhus University, Gustav Wieds Vej 10c, DK-8000 Aarhus C, Denmark, ²Department of Biology, University of Copenhagen, Ole Maaløes Vej 5, DK-2200 København N, ³Department of Chemistry and Interdisciplinary Nanoscience Centre (iNANO), Gustav Wieds Vej 14, DK-8000 Aarhus C, ⁴Voldmestergade 8, DK-2100 København Ø, Denmark.

*These authors contributed equally.

†To whom correspondence should be addressed (mas@bio.ku.dk, phone +45 35323711 or deb@mbg.au.dk, phone +45 21669001).

Keywords: HipBA; STK; Ser/Thr kinase; TrpS; tRNA synthetase

Running title: Regulation of a tripartite TA system
ABSTRACT

Many bacteria encode multiple toxin-antitoxin (TA) systems targeting separate, but closely related, cellular functions. The toxin of the *E. coli* hipBA system, HipA, is a kinase that inhibits translation via phosphorylation of glutamyl-tRNA synthetase. Enteropathogenic *E. coli* (EPEC) O127:H6 encodes an additional, tripartite TA module, hipBST, for which the HipT toxin was shown to specifically target tryptophanyl-tRNA synthetase, TrpS. Surprisingly, the function as antitoxin has been taken over by the third protein, HipS, but the molecular details of how activity of HipT is controlled remain poorly understood. Here, we show that HipBST is markedly different from HipBA and that the unique HipS protein, which is homologous to the N-terminal subdomain of HipA, has evolved to function as antitoxin by breaking the kinase active site. We also show how auto-phosphorylation at two conserved sites in the kinase toxin serve to dually regulate binding of HipS and kinase activity. Finally, we demonstrate that the HipBST complex is dynamic and present a cohesive model for the regulation and activation of this type of three-component system.
INTRODUCTION

Bacteria employ a wide range of mechanisms adapt to changing environments and imminent threats such as antibiotics and phage attack, including activation of toxin-antitoxin (TA) systems that can release intracellular toxins to rapidly alter metabolism or reduce growth (Harms et al., 2018). Canonical (type II) TA systems are small, bicistronic loci that encode a protein toxin and its cognate antidote (antitoxin) that tightly interact to form a higher-order complex in the inactive state, usually capable of controlling transcription via a DNA-binding domain on the antitoxin (Harms et al., 2018). There are also examples of tricistronic TA loci, in which the third component is either a chaperone required for folding of the antitoxin and thus, toxin inhibition (Bordes et al., 2016) or an additional, transcriptional regulator (Hallez et al., 2010; Zielenkiewicz & Ceglowski, 2005). A striking observation is that some microorganisms contain a very large number of similar, paralogous TA systems, such as the human pathogen, *Mycobacterium tuberculosis*, which contains at least 47 paralogous TA systems of the *vapBC*-type, for which the VapC toxin appears to target unique non-coding RNA species (Sharrock et al., 2018; Winther et al., 2016). Likewise, *Salmonella enterica* encodes several paralogous *tacTA* systems in which the TacT toxins function by acetylating the primary amine of amino acids of charged transfer RNA molecules (Grabe et al., 2021). These cases raise important questions about the evolutionary benefit of harbouring multiple, highly similar toxin systems as well as how cross-reactivity is prevented.

In the widespread and diverse *hipBA* (high persister) system, the HipA toxin is a serine-threonine kinase (STKs) (Hanks et al., 1988; Stancik et al., 2018), while the cognate antitoxin, HipB, contains a helix-turn-helix (HTH) motif and inhibits the toxin by a mechanism which is not fully understood (Gerdes et al., 2021; Schumacher et al., 2009). In the most well-studied and canonical *hipBA* system in *Escherichia coli* K-12, the toxin (HipAec) specifically targets glutamyl-tRNA synthetase (GltX)
by phosphorylation of an ATP-binding motif conserved in type I aminoacyl-tRNA synthetases (Eriani et al., 1990; Sekine et al., 2003), thereby inhibiting its activity and blocking translation (Germain et al., 2013). Accumulation of uncharged tRNA\textsuperscript{Glu} subsequently induces the stringent response via RelA-mediated (p)ppGpp synthesis on starved ribosomes (Haseltine & Block, 1973; Pacios et al., 2020; Winther et al., 2018). In contrast to most type II TA systems, the cognate antitoxin, HipB does not directly interact with the HipA active site and inhibition has therefore been proposed to occur by several mechanisms, including blocking conformational changes in the kinase required for catalysis, sequestration of HipA on DNA via the HipB DNA binding domain (Schumacher et al., 2009), or allosterically via placement of a C-terminal Trp residue of HipB into a pocket on HipA (Evdokimov et al., 2009). In an additional layer of complexity in regulation, HipA\textsubscript{Ec} is regulated by \textit{trans} autophosphorylation at a conserved serine (Ser150) situated in a loop (the "Gly-rich loop") near the active site, which is required for ATP binding and functionally similar to the P loop in eukaryotic kinases (Huse & Kuriyan, 2002; Schumacher et al., 2012). Phosphorylation at this site causes a conformational change in which the loop is ejected from its burrowed position inside the active site, rendering the kinase unable to bind ATP (Schumacher et al., 2012).

Enteropathogenic \textit{E. coli} (EPEC) O127:H6, an important diarrheal pathogen of young children, contains, in addition to the canonical \textit{hipBA} locus, a homologous tricistronic TA system, \textit{hipBST}, encoding three separate proteins. HipB, by analogy to HipBA, contains a putative DNA-binding HTH domain while HipT is a kinase like HipA and functions as toxin by phosphorylating and inactivating tryptophanyl-tRNA, TrpS (Gerdes et al., 2021; Vang Nielsen et al., 2019). Surprisingly, the third protein, HipS, which at the sequence level corresponds to the N-terminal subdomain 1 of the larger HipA kinase, was shown to function as antitoxin by an unknown mechanism (Vang Nielsen et al., 2019). Moreover, HipT was shown to be subject to autophosphorylation at two conserved serine
positions in the Gly-rich loop, but the functional consequences of this are also not known. Here, we show that the HipBST complex from pathogenic *E. coli* is markedly different from HipBA and that inhibition of HipT is achieved through direct interaction with a conserved residue in HipS. Two structures of HipT in separate, phosphorylated states further demonstrate that HipT is found in an inactive conformation regardless of phosphorylation state inside the HipBST complex. Finally, we show, using phosphomimetic kinase variants *in vivo* and analysis of phosphorylation patterns and flexibility *in vitro*, that the two phosphorylation positions control HipT activity and binding of antitoxin (HipS), and that the HipBST complex is dynamic. Together, our data provide a mechanistic understanding of how the HipS protein can function as antitoxin and suggests that the corresponding domain in HipA can have important, regulatory roles as well.

**RESULTS**

**Dual autophosphorylation sites are conserved in HipT kinases**

One of the most surprising features of the HipBST system is that HipS shows sequence similarity to the N-terminal subdomain 1 of HipA, the toxin of the *hipBA* system, while simultaneously functioning as antitoxin ([Figure S1](#)) (Vang Nielsen et al., 2019). Moreover, phylogenetic analysis has demonstrated that HipT and HipA toxins are relatively closely related within a very broad superfamily of HipA-homologous kinases (Gerdes et al., 2021). To gain further insight into the basis of this important difference, we initially performed a focused phylogenetic analysis of the 48 HipT orthologues identified so far among bacterial genomes (Gerdes et al., 2021). Alignment and clustering reveals a largely bifurcated, phylogenetic tree for which one large branch (the "SIS group") contains HipT kinases with two serine residues in close proximity in the Gly-rich loop corresponding to the known autophosphorylation sites in HipT of *E. coli* O127:H6 ([Figure 1a](#)). In the remainder of the HipT orthologues, this region contains variable Ser/Thr motifs, including TxT, Sxx, xxT, SxT, and SxS, but the functional consequences of this are also not known.
Figure 1. Phylogenetic analysis of HipT. a. Phylogenetic guide tree of 48 HipT orthologues with sequences motifs (potential phosphorylation sites) indicated on the side. The "SIS group" (red) is by far the largest group followed by the T[IV][TP] group (green), and SIQ group (blue). Sequences used in the alignment in b are shown with red letters. Numbers on the branches indicate bootstrap confidence levels. b. Sequences of the Gly-rich loop (orange background), including the potential phosphorylation sites for selected HipT orthologues compared to HipA from E. coli K-12. Known phosphorylation sites in E. coli O127:H6 HipT (top) and E. coli K-12 HipA (bottom) are indicated with arrows and conserved sequence motifs with bold white text. c. Sequence logo for the Gly-rich loop derived from all 48 HipT sequences. d. Consensus motif for the HipT Gly-rich loop with known phosphorylation sites in red. Φ indicates a hydrophobic residue, while Ψ are aliphatic residues.
Txx. We note that the single position between the two Ser/Thr residues invariably is occupied by a small hydrophobic residue (I, L, or V) and that a small subbranch close to the SIS group represents an interesting variation with a Gln in the second autophosphorylation position (the "SIQ" group). The remainder of the Gly-rich loop across a range of HipT kinases is highly similar to HipA_Ec, which has the sequence ISVAGAQEK (Figure 1b-c). We can thus derive a consensus motif for the HipT Gly-rich loop, which includes several conserved hydrophobic positions in addition to the two Ser/Thr residues (Figure 1d). In summary, phylogenetic analysis reveals that a large fraction of HipT kinases contain a conserved SIS motif with two potential phosphorylation positions in their Gly-rich loops but also that this region displays some sequence diversity, which may point to subtle differences in regulation.

**HipBST forms a hexameric higher-order complex**

Comparison of the HipBST and HipBA sequences reveals that HipS shows similarity to the N-terminal subdomain 1 of HipA (Figure S1 and S2a) and that both HipB and HipT of hipBST contain N-terminal extensions of around 35 residues not found in HipB and HipA of hipBA, respectively (Figure 2a and S1). To understand the molecular basis for the functional and structural differences, we determined the crystal structure of HipBST from enteropathogenic E. coli O127:H6 expressed with inactive HipT (D233Q) to 2.9 Å by molecular replacement using HipA_Ec as search model (Figure 2b). The refined structure (R=19.5% / R_free=22.8%) has two copies of each of the three proteins in the asymmetric unit and is generally well-defined for HipB and HipT, while HipS appears to be more flexible. Analysis by the protein interaction server, PISA (Krissinel & Henrick, 2007), suggests that the biological assembly is comprised of a dimer of HipBST heterotrimers, which are held together through dimerisation of HipB (Figure 2b). This is consistent with the observation that the HTH domain is usually found as a dimer in DNA-binding proteins (Brennan & Matthews, 1989).
Figure 2. Crystal structure of the *E. coli* O127:H6 HipBST complex. a. Schematic representation of *E. coli* O127:H6 HipBST and canonical *E. coli* HipBA showing corresponding proteins and domains. Dashed lines indicate similar domains and grey areas represent regions missing domains in the crystal structures. HipS (beige) is structurally similar to the N-subdomain 1 of HipA (dark purple), HipT has an additional N-terminal mini-domain not found in HipA (light blue) while HipA has a short linker between the two N-subdomains (light purple). b. *E. coli* O127:H6 HipBST forms a hetero-hexameric complex with HipS (beige) on top of HipT (blue) through dimerisation of HipB (green) also generating a helix-turn-helix DNA-binding motif (HTH, dashed box). c. Crystal structure of the *E. coli* K-12 HipBA complex (PDB: 2WIU) shown as cartoon with HipA in blue purple (N-terminal subdomain 1) and purple and the HipB homodimer in two shades of green (Evdokimov et al., 2009). d. The linker between N-subdomain 1 and 2 (dark purple) physically linking the two domains in HipA<sub>Ec</sub>. e. The N-terminal mini-domain of HipT (blue), which is absent from HipA.
In this complex, the two HipB monomers interact strongly (interface area = 1,405 Å²) while the interface between HipB and HipT is relatively small (513 Å²). The biological relevance of the hetero-hexamer is supported by analytical gel filtration (Figure S2b) and small-angle X-ray scattering (SAXS, Figure S6), the latter of which showed a major species with a total mass of 129 ± 8 kDa, in good agreement with the theoretical mass of 125.6 kDa. HipB consists of an α-helical bundle of four helices including the predicted DNA-binding HTH motif (residues 62-83), which forms a canonical HTH DNA-binding domain exposed at the bottom of the hetero-hexamer (Figure 2b). The helical bundle is followed by a small β strand that forms an antiparallel sheet through pairing with the corresponding region in the neighbouring molecule of HipB. The N-terminal extension of HipB (36 residues) is not visible in the electron density, but secondary structure prediction indicates that it forms a long α-helix (Figure S1a). HipS is closely associated with HipT and consists of a solvent-facing, five-stranded, anti-parallel β sheet of which four strands are located in the N terminus (residues 1-52). These are followed by a small domain of three helices (residues 53-94) that forms the interface to HipT followed by the fifth β strand (residues 95-102, Figure 2b and S3a).

The overall architecture of the HipBST complex consists of two HipST toxin:antitoxin complexes separated in space by the dimer of the HipB and in this way differs markedly different from HipBAEc in which two HipA toxins are closely packed head-to-tail around two HipB antitoxins (Figure 2c). This configuration is key to the proposed way in which HipB can function as antitoxin, namely by restricting the movement of the N-terminal subdomain of HipA (Figure 2c, blue) (Schumacher et al., 2009). As suggested from sequence analysis, HipS bears a striking structural resemblance to the N-terminal subdomain 1 of HipA (Schumacher et al., 2012), both with respect to its overall fold and placement in relation to HipT. As a result of this, the HipS C-terminus overlaps with a extended linker that bridges N-subdomain 1 to N-subdomain 2 in HipA (Figure 2d). HipT corresponds structurally
to the core kinase fold of HipA, except for the presence of an additional N-terminal mini-domain (residues 1-41), the role of which is possibly to anchor N-subdomain 2 (residues 42-140) to the core of the kinase in the absence of the physical coupling to HipS (Figure 2a and 2e). Interestingly, the overall structure of the HipBST complex is reminiscent of the structure of a HipBA complex from *Shewanella oneidensis*, which was crystallised in complex with DNA (Figure S2c) (Wen et al., 2014). This suggests that the open conformation is not a specific result the tripartite configuration and superpositioning of the DNA-binding domains (Figure S3b) and analysis of potential interactions with DNA (Figure S3c) suggests HipBST could bind DNA in a similar way.

**HipT has an inactive conformation inside the HipBST complex**

For HipAEc, two discrete active site conformations are observed depending on phosphorylation state and whether substrate (ATP) is bound or not (Schumacher et al., 2015; Schumacher et al., 2012; Schumacher et al., 2009). In its non-phosphorylated form and in the presence of ATP, the flexible Gly-rich loop is found in an inward conformation in which main chain amino groups on Ala154 and Gln155 coordinate the ATP phosphate groups (Figure 3a) (Schumacher et al., 2009). Phosphorylation of Ser150, however, causes ejection of the loop through strong interactions of the phosphate group with active sites residues, which prevents binding of ATP (Figure 3b) (Schumacher et al., 2012). In the HipBST crystal structures, we find the Gly-rich loop of HipT forms a short α-helix and is in a conformation most similar to the ejected form of the HipAEc loop despite HipT not being phosphorylated (Figure 3c). In this conformation, Ser59, which is known to undergo autophosphorylation in HipT, forms interactions to several active site residues, including Asp210 and the Ser57 auto-phosphorylation site (Vang Nielsen et al., 2019). We conclude that inside the HipBST complex, HipT in its non-phosphorylated state is maintained in an inactive conformation that does not support ATP binding.
Figure 3. Trp65 is essential for HipS to function as antitoxin. 

- **a.** Overview of the HipA active site, bound to ATP (green, PDB: 3DNT) (Schumacher et al., 2009). Residues interacting with the nucleotide are highlighted as well as the Gly-rich loop (residues 150-156, green) including Ser150. D309Q is a mutated active site residue.

- **b.** The active site of HipA phosphorylated on Ser 150 (green/orange, PDB: 3TPE) with the Gly-rich loop (green) in the outward-

- **c.** HipT (HipS bound)

- **d.** Diagram showing the interactions of HipA with different ligands.

- **e.** Secondary structure representation of HipA.

- **f.** Table showing the effects of different conditions on the expression of HipT and HipS.

**Figure 3** Trp65 is essential for HipS to function as antitoxin. a. Overview of the HipA active site, bound to ATP (green, PDB: 3DNT) (Schumacher et al., 2009). Residues interacting with the nucleotide are highlighted as well as the Gly-rich loop (residues 150-156, green) including Ser150. D309Q is a mutated active site residue. b. The active site of HipA phosphorylated on Ser 150 (green/orange, PDB: 3TPE) with the Gly-rich loop (green) in the outward-

---

**CC-BY 4.0 International license**

The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under a CC-BY 4.0 International license.
facing conformation (Schumacher et al., 2012). c. The active site of HipBS-bound HipTD233Q with the Gly-rich loop (residues 58-63, ruby) in the outward-facing conformation. D233Q is the mutated active site residue. d. Overview and detailed interactions between HipS (beige) and HipT (blue) with its Gly-rich loop (ruby) and interacting residues indicated. e. Top, HipS Trp65 (beige) is placed in a pocket on the surface of HipT (blue); Bottom, the corresponding residue (Pro69) in HipA (purple, PDB: 3TPD). f. *E. coli* MG1655 harbouring empty pBAD33 vector ("pBAD33") or pSVN1 (pBAD33::*hipT*) in combination with empty pNDM220 vector ("pNDM220"), pSVN109 (pNDM220::*hipS*), or pSVN178 (pNDM220::*hipSW65A*) as indicated. Plates contained 0.2% glucose (to repress *hipT*), 0.2% arabinose (to induce *hipT*), or 0.2% arabinose plus 200 µM IPTG (to induce *hipS*, or *hipSW65A*). The plates are representative of at least three independent experiments.
HipS inhibits HipT through insertion of a hydrophobic residue near the active site

To understand how HipS, which is homologous to the N-terminal subdomain 1 of HipA, can function as the antitoxin in the HipBST system, we then focused on the specific interactions found between HipS and HipT. There are three major areas of contact between the two proteins with several strong interactions on both sides of the kinase domain, involving both hydrogen bonds and hydrophobic and charged interactions (Figure 3d). Near the Gly-rich loop, HipS Glu63 points directly into the HipT active site while Trp65 is wedged into a deep cavity formed by the HipT Gly-rich loop in its outward conformation. While Glu63 is conserved in the N-terminal subdomain of HipA Ec (Asp67) and therefore less likely contribute to functional differences between the two systems, the Trp65 motif (Figure 3e, top) is structurally different from the corresponding interaction in HipA Ec, which has the helix (HipS α3, Figure S2a) in a different orientation and a proline residue at this position (Figure 3e, bottom). Moreover, Trp65 is conserved in many HipS orthologues (Figure S2a). We hypothesised that placement of the bulky Trp residue inside HipT might block transition of the Gly-rich loop from the outward to the inward conformation, likely required for ATP binding. To test this, we mutated the Trp65 in HipS to alanine and tested the ability of the antitoxin to antagonise the kinase in E. coli K-12 on both solid media and in liquid culture (Figure 3f and S3d). In both experiments, absence of the bulky Trp65 side chain strongly reduced the antitoxin activity of HipS, confirming that this interaction is critical to inhibition of HipT. We conclude that Trp65 is essential for the ability of HipS to inhibit HipT, presumably either by increasing the affinity of HipS for HipT, or by preventing the Gly-rich loop from adopting the conformation required for ATP binding.

The conformation of HipT in the HipBST complex is independent of phosphorylation

In contrast to HipA Ec, which undergoes autophosphorylation at a single site (Ser150), HipT was found to harbour two sites in its Gly-rich loop (Ser57 and Ser59), which are subject to autophosphorylation
individually but not concomitantly (Vang Nielsen et al., 2019). To understand the individual roles of the two autophosphorylation positions, we next determined crystal structures of two HipBST complexes with HipT serine residues mutated to alanine individually: HipBST\textsuperscript{S57A} (2.4 Å, Table S1) and HipBST\textsuperscript{S59A} (3.4 Å). Inspection of the electron densities near the active site revealed that in both cases, the remaining serine residue (i.e. Ser57 in HipBST\textsuperscript{S59A} and Ser59 in HipBST\textsuperscript{S57A}) had become phosphorylated prior to crystallisation (Figure 4a and S4a-b). For HipBST\textsuperscript{S59A}, phosphorylation of Ser57 was incomplete and could only be modelled in one of the two HipT molecules in the asymmetric unit (Figure S4b). The phosphate group of Ser59 (P-Ser59) forms strong interactions to Lys161, His212, and the catalytic Asp210 inside the active site (Figure 4a, top), while P-Ser57 is located further away from the active site and forms hydrogen bonds to Tyr162 and Asp210 (Figure 4a, bottom). In both structures, however, the Gly-rich loop maintains its outward conformation, incompatible with ATP binding, suggesting that HipT is inactive. The phosphate group on Ser59 occupies a position very similar to phosphorylated Ser150 in HipAE\textsubscript{c}, despite the fact that this residue aligns to Ser57 at the sequence level (Figure S4c, top and 1b). This position structurally overlaps with the γ phosphate of ATP in the structure of ATP-bound HipAE\textsubscript{c}, suggesting that phosphorylation prevents nucleotide binding in the kinase active site (Figure 4b). Intriguingly, the phosphoryl group of P-Ser57 overlaps structurally with that of phosphorylated Ser147 in HipAS\textsubscript{o}, which corresponds to HipAE\textsubscript{c} Ser150 at the sequence level (Figure S4c, bottom). In order to confirm the phosphorylation state of the HipT variants \textit{in vitro}, we analysed purified HipBST complexes on Phos-tag SDS-PAGE gels, which contain functional molecules that cause retention of phosphorylated protein species in the gel (Figure 4c, top). Here, we observed a marked difference in migration between HipT from wildtype HipBST, HipBST\textsuperscript{S59A}, and HipBST\textsuperscript{S57A} confirming that HipT\textsuperscript{S57A} is predominantly phosphorylated while HipT\textsuperscript{S59A} is only partially modified, consistent with the crystal structures. Surprisingly, wildtype HipBST complex is completely non-phosphorylated when purified in this way,
Figure 4. The phosphoserine positions in HipT have distinct functional roles. 

a. Overview of the HipT kinase active site in the D233Q mutant as well as S57A (top) and S59A (bottom) structures. The phosphate groups on Ser57 (in S59A) and Ser59 (in S57A) are shown in orange and relevant nearby residues are highlighted.

b. Close-up of the HipT active site as observed for the S57A mutant with relevant active site residues shown as sticks. The Gly-rich loop is shown in red with pSer59 indicated. Overlaid is ATP and 2 Mg²⁺ ions derived from the structure of HipA (PDB: 3DNT, salmon/green) (Schumacher et al., 2009).

c. HipT bands on Phos-tag gels (stained by Coomassie Blue) of purified HipBST complex before (-) and after (+) a Heparin column step to separate various complex species, as well as before (-) and after (+) treatment with 10 mM ATP, as indicated. Phosphorylated (P-
HipT) and non-phosphorylated ("HipT") protein species are indicated. The gels are representative of two independent experiments. d. Top, growth curves measured by OD$_{600}$ of *E. coli* MG1655 harbouring single autophosphorylation mutants pSVN194 (pBAD33::hip$^{S57D}$, "hipT (DIS)"), pSVN195 (pBAD33::hip$^{S59D}$, "hipT (SID)"), pSVN199 (pBAD33::hip$^{S57A}$, "hipT (AIS)"), pSVN201 (pBAD33::hip$^{S59A}$, "hipT (SIA)"), wildtype HipT pSVN1 (pBAD33::hipT, "hipT (SIS, wt)"), or the empty pBAD33 vector, as indicated, in combination with pSVN109 (pNDM220::hipS). hipT (ara) and hipS (IPTG) expression were induced at the indicated time points. The curves show the mean values of results from at least two independent experiments with error bars indicating standard deviations (hidden when small); Bottom, growth curves of *E. coli* MG1655 harbouring double autophosphorylation mutants pSVN203 (pBAD33::hip$^{S57A+S59A}$, "hipT (AIA)"), pSVN205 (pBAD33::hip$^{S57D+S59A}$, "hipT (DIA)"), pSVN207 (pBAD33::hip$^{S57A+S59D}$, "hipT (AID)"), or pSVN209 (pBAD33::hip$^{S57D+S59D}$, "hipT (DID)"), or the empty pBAD33 vector, as indicated, in combination with pSVN109 (pNDM220::hipS). hipT (ara) and hipS (IPTG) expression were induced at the indicated time points. The curves show the mean values of results from at least two independent experiments with error bars indicating standard deviations (hidden when small). e. Northern blot analysis probing against tRNA$^{\text{trpT}}$ based on RNA extracted from *E. coli* MG1655 harbouring pSVN110 (pNDM220::hipB-S) in combination with pSVN1 (pBAD33::hipT, "hipT (S$^{S7}$S$^{59}$, wt)"), pSVN194 (pBAD33::hip$^{S57D}$, "hipT (D$^{S7}$S$^{59}$)"), pSVN195 (pBAD33::hip$^{S59D}$, "hipT (S$^{S7}$D$^{59}$)"), pSVN209 (pBAD33::hip$^{S57D+S59D}$, "hipT (D$^{S7}$D$^{59}$)") or pSVN205 (pBAD33::hip$^{S57D+S59A}$, "hipT (D$^{S7}$A$^{59}$)") at indicated time points in minutes before (-) and after (+) addition of arabinose (0.2%) to induce wildtype hipT or autophosphorylation mutants. Quantification of the bands (represented as % deacylated tRNA) is shown below the gel. The control ("ctrl") is chemically deacylated tRNA. The percentage of deacylated tRNA (bottom) was based on quantification of total tRNA$^{\text{trpT}}$. Results from strains harbouring pSVN1 (pBAD33::hipT), pSVN194 (pBAD33::hip$^{S57D}$), pSVN209 and (pBAD33::hip$^{S57D+S59D}$) are representative of two independent experiments, whereas results from strains harbouring pSVN195 (pBAD33::hip$^{S59D}$) and pSVN205 (pBAD33::hip$^{S57D+S59A}$) are from single experiments.
but incubation with ATP for 30 minutes leads to a visible increase in phosphorylation of wildtype HipT while no further phosphorylation seemed to take place for the mutants upon incubation with ATP (Figure 4c, bottom). In summary, we conclude that the conformation of the HipT active site loop (Gly-rich loop) inside the HipBST complex is independent of the autophosphorylation state.

The two phosphoserine positions in HipT have separate functional roles

We next sought to understand the functional implications of autophosphorylation of HipT at the two positions. To this end, we designed a set of HipT mutants with either Ala (to prevent autophosphorylation) or Asp (to mimic the phosphorylated state) in one or both of the two Ser positions. Liquid culture assays showed that both wildtype HipT S57IS59 and HipT S57ID59 caused growth inhibition upon overexpression in E. coli K-12 and could be repressed by concomitant expression of HipS (Figure 4d, top) (Vang Nielsen et al., 2019). In contrast to this, growth inhibition by the HipT D57IS59 variant could not be repressed upon induction of HipS. Moreover, mutation to alanine at either position caused very little (S57IA59) or no (A57IS59) growth inhibition, suggesting that the serine residues or their phosphorylated counterparts are important for HipT activity on the target TrpS (Figure 4d, top). To rule out any effects of autophosphorylation of the non-mutated Ser position, we created double mutants for which both serine residues were mutated into either alanine or aspartate (Figure 4d, bottom). Of these, HipT D57ID59 causes immediate growth inhibition while HipT D57IA59 shows a similar but delayed phenotype, and consistent with the observation above, neither could be repressed by HipS induction. And as before, placing alanine at either position either completely abolished (A57IA59 and A57ID59) or strongly delayed (D57IA59) growth inhibition. To rule out any effects due to the fact that HipB is missing in this setup, we finally repeated the experiments with concomitant induction of both HipB and HipS and obtained very similar results (Figure S4d and S4e).
To confirm that the growth inhibition observed was in fact due to phosphorylation and inactivation of TrpS, we took samples from *E. coli* cultures both before and at several time points after induction of wildtype HipT as well as all variants that caused markedly growth inhibition, extracted total RNA, and performed Northern blotting using probes against tRNA<sub>trpT</sub> (Figure 4e) as well as tRNA<sub>gltTUVW</sub> and tRNA<sub>argVYZQ</sub> (Figure S4f) as controls. Here, charging of the tRNA is visible as a shift of the probed RNA band and thus allows direct assessment of the activity of TrpS *in vivo* in the presence of the various HipT variants. Induction of all variants caused accumulation of deacylated tRNA<sub>trpT</sub> (Figure 4e) but no accumulation of the two control tRNAs (Figure S4f), which strongly suggests that the observed growth effects are due to reduction in the cellular pool of acylated tRNA. Taken together, we conclude that HipT autophosphorylation at Ser57 prevents HipS inhibition and does not prevent phosphorylation of the target, TrpS. On the other hand, phosphorylation at Ser59 does not affect HipS inhibition, indicating that phosphorylation at the two positions serve separate, functional roles.

**hipB is both required and sufficient for transcriptional repression**

To understand the functional role of HipB, specifically how DNA binding has been separated from the antitoxin function in the tripartite *hipBST* system, we initially constructed a vector-based transcriptional fusion reporter, in which the native *hipBST* promoter region and 5' region of the *hipB* gene was transcriptionally fused to *lacZ* (Figure 5a, right). We then expressed various combinations of *hipB*, *hipS*, and *hipT* in trans in *E. coli* MG1655 ∆lacIZYA cells that do not harbour the *hipBST* operon, utilising two inactivating kinase mutations, *hipT<sup>D210Q</sup>* (Figure 5a, left) and *hipT<sup>D233Q</sup>* (Figure S5a) to avoid toxicity. In both cases, in the absence of expressed *hipBST* components, active transcription could be observed by the blue colour of the colonies (Figure 5a, left, top row, pBAD33),
Figure 5. The HipBST complex is dynamic in vivo. a. Right, transcriptional reporter assay using a plasmid with the hipBST promoter region and a 5' fragment of the hipB gene (including 224 bp upstream of hipB plus the first 73 bp of the hipB gene, shown with a lightening symbol) transcriptionally fused to lacZ. Left, E. coli TB28 harbouring the reporter plasmid (pSVN141, pGH254::P_{hipBST}-'-lacZ') and empty pBAD33 vector or combinations of hipB, hipS, and hipT in the context of the HipT^{D210Q} inactive mutant; pSVN180 (pBAD33::hipB::S::T^{D210Q}, "hipBST^{D210Qw}"), pSVN182 (pBAD33::hipB::S, "hipBS"), pSVN184 (pBAD33::hipB::T^{D210Q}, "hipBT^{D210Qw}"), pSVN187 (pBAD33::hipS::T^{D210Q}, "hipST^{D210Qw}"), pSVN189 (pBAD33::hipB, "hipB"), pSVN190 (pBAD33::hipS, "hipS"), or pSVN192 (pBAD33::hipT^{D210Q}, "hipT^{D210Qw}") were grown, diluted, and spotted onto YT, X-gal agar plates.
containing 0.2% glucose (to repress \(\text{hipB/S/T}^{D210Q}\)), or 0.2% arabinose (to induce \(\text{hipB/S/T}^{D210Q}\)). Results are representative of two independent experiments. b. Left, HipT exchange assay where overexpression of a catalytically inactive kinase (HipT\(^{D210Q}\)) is used to exchange wild type HipT from the HipBST complex \textit{in vivo}. Right, spot assays of \textit{E. coli} O127 harbouring the empty pBAD33 vector, pSVN220 (pBAD33:\:\text{hipT}\(^{D210Q}\), “\text{hipT} (S^{57}I^{59}, \text{wt})”), pSVN221 (pBAD33:\:\text{hipT}\(^{S57D\rightarrow D210Q}\), “\text{hipT} (D^{57}I^{59})”), pSVN222 (pBAD33:\:\text{hipT}\(^{S59D\rightarrow D210Q}\), “\text{hipT} (S^{57}I^{59})”), pSVN223 (pBAD33:\:\text{hipT}\(^{S57A\rightarrow D210Q}\), “\text{hipT} (A^{57}I^{59})”), pSVN224 (pBAD33:\:\text{hipT}\(^{S59A\rightarrow D210Q}\), “\text{hipT} (S^{57}I^{59})”), pSVN225 (pBAD33:\:\text{hipT}\(^{S57A\rightarrow S59A\rightarrow D210Q}\), “\text{hipT} (A^{57}I^{59})”), pSVN226 (pBAD33:\:\text{hipT}\(^{S57D\rightarrow S59A\rightarrow D210Q}\), “\text{hipT} (D^{57}I^{59})”), pSVN227 (pBAD33:\:\text{hipT}\(^{S57A\rightarrow S59D\rightarrow D210Q}\), “\text{hipT} (A^{57}D^{59})”) or pSVN228 (pBAD33:\:\text{hipT}\(^{S57D\rightarrow S59D\rightarrow D210Q}\), “\text{hipT} (D^{57}D^{59})”) on YT agar plates containing 0.2% glucose (to repress \text{hipT} variants), 0.2% arabinose (to induce \text{hipT} variants) or 0.2% arabinose and 500 µM IPTG (to induce \text{hipB}). Results are representative of two independent experiments. c. As b but with additional overexpression of \text{hipB} to avoid renewed transcription. Overnight cultures of \textit{E. coli} O127 harbouring pSVN111 (pNDM220::\text{hipB}) in combination with the empty pBAD33 vector, pSVN211 (pBAD33::\text{hipT}\(^{D210Q}\), “\text{hipT} (S^{57}I^{59}, \text{wt})”), pSVN212 (pBAD33::\text{hipT}\(^{S57D\rightarrow D210Q}\), “\text{hipT} (D^{57}I^{59})”), pSVN213 (pBAD33::\text{hipT}\(^{S59D\rightarrow D210Q}\), “\text{hipT} (S^{57}I^{59})”), pSVN214 (pBAD33::\text{hipT}\(^{S57A\rightarrow D210Q}\), “\text{hipT} (A^{57}I^{59})”), pSVN215 (pBAD33::\text{hipT}\(^{S59A\rightarrow D210Q}\), “\text{hipT} (S^{57}I^{59})”), pSVN216 (pBAD33::\text{hipT}\(^{S57A\rightarrow S59A\rightarrow D210Q}\), “\text{hipT} (A^{57}I^{59})”), pSVN217 (pBAD33::\text{hipT}\(^{S57D\rightarrow S59A\rightarrow D210Q}\), “\text{hipT} (D^{57}I^{59})”), pSVN218 (pBAD33::\text{hipT}\(^{S57A\rightarrow S59D\rightarrow D210Q}\), “\text{hipT} (A^{57}D^{59})”) or pSVN219 (pBAD33::\text{hipT}\(^{S57D\rightarrow S59D\rightarrow D210Q}\), “\text{hipT} (D^{57}D^{59})”) were grown, diluted, and spotted onto YT agar plates containing 0.2% glucose (to repress \text{hipT} variants), 0.2% arabinose (to induce \text{hipT} variants) or 0.2% arabinose and 500 µM IPTG (to induce \text{hipB}). Results are representative of two independent experiments.
while induction of any construct containing hipB efficiently repressed transcription (Figure 5a, left, right plate, hipBST\textsuperscript{D210Q}, hipBS, hipBT\textsuperscript{D210Q}, and hipB). For hipBST and hipBT, leaky expression on repressing glucose plates was sufficient to reduce transcription. We conclude that hipB is both sufficient and required for transcriptional repression of the hipBST locus. Given that the hipBST and hipBT constructs repress transcription even in the absence of inducer, our data are also consistent with HipT (and possibly HipS) stabilising DNA interaction by HipB, as observed for many toxin-antitoxin systems (Harms et al., 2018).

**Phosphorylation of HipT Ser57 prevents HipS binding in vivo**

In principle, the observed lack of HipS antitoxin inhibition of toxicity in the S57D phosphomimetic HipT mutants could be caused by either lack of binding of HipS or hyperactivity of the kinase. To distinguish between these two scenarios, we next tested if inactive HipT can exchange with wildtype HipT inside the HipBST complex in vivo in *E. coli* O127:H6. To achieve this, we constructed a series of HipT autophosphorylation variants on a background of inactive HipTD210Q and asked if expression would release wildtype HipT from endogenous HipBST complexes encoded by hipBST of *E. coli* O127:H6 and thus cause toxicity (Figure 5b, right). Both in the absence and presence of the inducer arabinose, all inactive HipT autophosphorylation variants, except those harbouring the phosphomimetic Asp57 (D\textsuperscript{57IS59}, D\textsuperscript{57IA59}, and D\textsuperscript{57ID59}) were able to induce toxicity in this setup, thus suggesting that exchange can take place when Ser57 is not phosphorylated (Figure 5b, left). Of these, the S\textsuperscript{57IS59}, A\textsuperscript{57IS59}, and S\textsuperscript{57IA59} variants had the strongest ability to release wildtype HipT activity while the S\textsuperscript{57ID59}, A\textsuperscript{57IA59} and A\textsuperscript{57ID59} variants activated HipT at a lower level. To exclude the possibility that expression of inactive HipT would affect the natural transcriptional repression of the endogenous HipBST complex, we repeated the experiment in a setup where excess HipB is expressed from a separate plasmid using IPTG, knowing that this represses transcription (Figure 5c,
right). Due to an uncharacterized growth inhibiting effect observed upon hipBT co-expression for the inactivating kinase mutations, hipTD210Q (Figure 5a, left) and hipTD233Q (Figure S5a), we used genetic constructs with a lower expression level of the HipT mutants for expression in combination with HipB to obtain the data in figure 5c. Here we found similar results even with full transcriptional repression, supporting that the observed toxicity is indeed due to wildtype HipT being exchanged away from preformed endogenous HipBST complexes upon expression of inactive toxin. To confirm that the growth defects observed were in fact dependent on the hipBST system and not a secondary effect in the host, we performed a similar assay in E. coli K-12 with the intact hipBST operon fused to lacZ encoding wildtype HipT (Figure S5b). Growth inhibition was only observed when inactive HipTD210Q/D233Q was expressed in trans confirming that the phenotype is indeed dependent on the hipBST operon. To ensure that the low expression levels of the HipT D210Q autophosphorylation mutants in combination with HipB was not in themselves causing the observed growth inhibitions as seen for high expression levels (Figure 5a, left and S5a), we finally transformed the same vectors used in E. coli O127:H6 into E. coli K-12. None of the vectors caused growth inhibition upon induction (Figure S5f), confirming that the phenotype observed in Figure 5b must be due to the presence of the native hipBST operon. Finally, to rule out any differences in transcription of the hipBST promoter between E. coli K-12 and O127:H6, we confirmed that mRNA expression can be repressed in E. coli K-12 when hipBSTD210Q is present in trans (Figure S5c) and that the transcriptional start sites (TSS) in the two strains are identical (Figure S5d-e). In summary, we conclude that free HipT molecules are able to exchange with those inside the inhibited HipBST complex, except in the S57D mutants, suggesting that phosphorylation at Ser57 controls exchange, likely via control of binding of HipS.

The core HipT kinase domain is flexible in solution
Since the protein exchange experiments suggested that the HipBST complex is dynamic in vivo, we finally performed a careful analysis of the obtained small-angle X-ray scattering (SAXS) data to understand the structure of the complex in solution (Figure S6a-b). We found, that the pair distance distribution function indicates a maximum complex diameter of 170 Å, significantly larger than the value calculated from the crystal structure (140 Å) suggesting some degree of structural flexibility (Figure S6c-d). Moreover, fitting of the crystal structure to the solution data yielded a relatively poor fit, so to accommodate internal flexibility, the HipT structure was split into three parts, roughly corresponding to the N-terminal mini domain (1-59), the N-terminal subdomain 2 (60-169), and the core kinase domain (170-335) (Figure 2a). Following rigid body refinement against the SAXS data with soft restraints between the three parts of HipT, including HipS and HipB, and imposing two-fold symmetry, we obtained a better fit which could be further improved by allowing for partial dimerisation of the hetero-hexamer (see Material and Methods for details). In this model, the core kinase domain of HipT has a looser structure than observed in the crystal structure (root mean square deviation for Ca atoms HipT ~6.5 Å, HipBST ~7 Å) suggesting that the complex in the solution state allows for internal flexibility in the kinase. In summary, in solution structural analysis confirms that the HipBST complex has a flexible structure, likely caused by structural dynamics in the HipT core kinase domain, thus consistent with a model in which HipS (and perhaps HipB) can release and rebind HipT dynamically.

**DISCUSSION**

In this paper, we present a detailed structural and functional analysis of the tripartite HipBST toxin-antitoxin system from enteropathogenic *E. coli* O127:H6. We show that most HipT kinases contain a conserved SIS motif in their Gly-rich loops, allowing for fine-tuned regulation via dual autophosphorylation. Other members of the family contain various combinations of Ser and Thr at these
positions, which may serve similar roles since Hip toxins are Ser/Thr kinases (Pereira et al., 2011).

Crystal structures of the HipBST complex in several, naturally occurring phosphorylation states demonstrate that HipT is found in its inactive state inside the HipBST complex regardless of its autophosphorylation state, consistent with a model whereby HipS, possibly aided by the DNA-binding protein HipB, keeps the kinase in an inactive conformation and thus overrules any effects of autophosphorylation. We show that this inhibition is dependent on a conserved Trp65 in HipS, which couples HipS binding directly to the active site of HipT and the conformation of its Gly-rich loop. Trp65 is located in a motif that differs both structurally and at the sequence level from the corresponding region in the N-terminal subdomain 1 of HipA, which is otherwise very similar to HipS (Gerdes et al., 2021). Together, this suggests that the molecular basis for the role of HipS as antitoxin in the HipBST complex involves prevention of the Gly-rich loop from transitioning from the outward, inactive state, to another conformation compatible with ATP binding (Figure 6).
Figure 6. Model for the action of HipS as antitoxin in the HipBST system. Structural comparison of the positions of the Gly-rich loops in HipT D233Q (blue) and *E. coli* K-12 HipA in its ATP-bound conformation (PDB: 3DNT, green/purple) (Schumacher et al., 2009). Shown is also HipS with Trp65, which is predicted to prevent transition of the Gly-rich loop from an outward-facing (HipT) to an inward-facing (HipA:ATP) conformation (shown with an arrow). The active site residues of HipA as well as ATP:Mg²⁺ are shown with sticks on top of the HipT cartoon. Ser150, which undergoes phosphorylation in HipA, is indicated as well.
The size and topology of the HipT kinase with its lack of the N-terminal subdomain 1 found in HipA is reminiscent of the Cyclin-dependent kinases (Cdks), which are minimal kinases that regulate the eukaryotic cell cycle (Figure S7a) (Lim & Kaldis, 2013). Intriguingly, one of these, Cdk5, also regulates translation through direct phosphorylation of glutamyl-prolyl tRNA synthetase (EPRS) (Arif et al., 2011). Despite differences in tertiary structure (Figure S7b), the residues involved in the active site align well, suggesting common reaction mechanisms. Cyclin-dependent kinases are stabilised by two hydrophobic regions, the R (regulatory) and C (catalytic) spine (Taylor & Kornev, 2011). Of these, the R spine changes conformation upon an activating phosphorylation by flipping the final active site residue into place and stabilising the kinase by forming an interaction to an aspartate residue in the so-called F helix (Baugh et al., 2015). While several of these structural elements are not present in HipT, the bacterial kinase does feature a conserved hydrophobic core similar to the C spine (Figure S7c-e). These regions are more extended than seen in Cdk, in particular due to the presence of two additional, conserved aromatic residues (Y138 and Y232 in HipT, Figure S7d-e). In the structure of ATP-bound HipA, the adenine base intercalates right between these and thereby connects the N and C lobes of the kinase. Interestingly, the invariant small hydrophobic residue found between the phosphor-serine positions in HipT (SIS) would connect the adenine base to the N lobe, suggesting that it is conserved to preserve the ATP binding. In general, it thus appears that the similarities between bacterial and eukaryotic kinases may be larger than hitherto appreciated and consequently, that the vast set of knowledge available for the eukaryotic enzymes might help us understand the Hip kinases as well.

A surprising discovery of this study is that HipT was found with phosphorylation at Ser57 or Ser59 when expressed in the context of the inactive HipBST complex. This suggests that some active (HipS-free) HipT exists in the cell and hence compatible with a model whereby the HipBST complex is...
dynamic and allows HipT to dissociate in vitro. In this context, it is likely that the exposed
conformation of the Gly-rich loop renders it particularly susceptible to phosphorylation by active
HipT kinase. Taken one step further, this could indicate that a secondary effect of HipS binding (apart
from inactivating the kinase) is to expose the kinase to regulation via autophosphorylation in trans.

We still don't understand what controls whether HipT becomes modified at one or the other position
but the structures of HipT^{S57A} and HipT^{S59A} could suggest that subtle changes in the loop can make a
big difference (Figure 4a). Moreover, since Ser57/Ser59 phosphorylation was observed for both
alanine single mutations in vitro, the presence of both serine residues does not appear to be important
for the autophosphorylation activity of HipT. Finally, the observation that the S^{57}I^{59}, A^{57}I^{59}, and
S^{57}I^{59} HipT^{D210Q} variants exhibit a stronger toxicity due to exchange with wildtype HipT than the
A^{57}I^{59} variant in vivo (Figure 5b-c) indicates that the hydroxyl groups of the serine residues play a
role in the binding of HipS.

Intriguingly, although the two autophosphorylation positions in HipT occupy a similar space near the
\( \gamma \)-phosphate of ATP, they serve different roles. HipT is active in both phosphomimetic isoforms (S^{57}D
and S^{59}D) while removal of the hydroxyl group on either serine by mutation to alanine (S^{57}I^{59},
A^{57}I^{59} and A^{57}I^{59}) reduces its ability to cause growth inhibition. In contrast to this, we found that
both S^{57}I^{59} and A^{57}I^{59} remained capable of autophosphorylation, suggesting that the presence of
both of the serine residues or their phosphorylated counterparts could be important for regulating
whether HipT is active on itself or its target (TrpS). Taken together, these results are consistent with
a model in which autophosphorylation at either position is required for target phosphorylation.

Surprisingly, in contrast to the A^{57}D^{59} mutant, the D^{57}I^{59} mutant was still active in inhibiting growth
(but with a delayed effect compared to D^{57}I^{59}, S^{57}I^{59}, and D^{57}I^{59}), suggesting that
phosphorylation of Ser57 results in a kinase, which is more potent against its target compared to
phosphorylation of Ser59. Finally, although the D^{57}ID^{59} mutant is fully active in growth inhibition, this may be artificial, as dual phosphorylation has not been observed in vivo (Vang Nielsen et al., 2019). Nevertheless, the D^{57}ID^{59} mutant gives us valuable information, as it shows that growth inhibition does not require the presence of a serine residue and that phosphorylation is likely what determines the active state. The differences in the ability of the S^{57}IA^{59} and D^{57}IA^{59} mutants to cause growth inhibition are also striking. The S^{57}IA^{59} mutant has a very small growth inhibitory effect, whereas D^{57}IA^{59} has a clear effect. One explanation for this observation could be that the S^{57}IA^{59} mutant has a slower rate of autophosphorylation compared to wildtype (S^{57}IS^{59}) and that this would be required for HipT activity. When examining the other autophosphorylation site, Ser59, it is noteworthy, that the S^{57}ID^{59} mutant is fully active, while the A^{57}IS^{59} and A^{57}ID^{59} mutants are inactive.

Another question raised by the structures is how HipS can be bound in the complex with Ser57 in its phosphorylated state if this serves to block HipS binding? In this context, it is important to note, however, that only a minor fraction of the purified complex is phosphorylated (Figure 4c), confirming that if HipT (Ser57-P) binds to HipS at all, it probably binds very poorly. It is also possible that phosphorylation occurred during purification. In either case, we believe that our data is compatible with the model for DNA binding and toxin activation for the HipT kinase toxin shown in Figure 7. Under non-activating conditions, HipBST will be repressing the hipBST operon via binding to promoter DNA. Liberation of HipBST from DNA by an unknown mechanism will result in relaxed promoter repression and increased transcription from the operon. Free HipT could then form three populations, non-phosphorylated HipT, HipT P-Ser57, and HipT P-Ser59. Our results indicate that both phosphorylated forms are active on TrpS and as a result of this, reduce cell growth. Furthermore, HipT P-Ser59 would readily be able to (re-)bind HipS, and therefore form a stable and inactive HipBST complex as well as tightly bind the hipBST promoter, effectively shutting down the kinase.
Figure 7. Proposed model of regulation and activation of the HipBST system. Transcription from the hipBST operon (middle row, left) is repressed by binding of the HipBST hetero-hexamer in which HipT is inactive and the Gly-rich loop (red) is outward-facing when HipS is bound. The dynamic HipBST complex can disassociate to generate free HipT, which can phosphorylate another HipT kinase and perhaps TrpS, using ATP. Autophosphorylation at Ser 57 (top box) severely reduces HipS binding, thereby prolonging the growth inhibitory effect of HipT by keeping the kinase active and preventing full transcriptional repression. On the contrary, autophosphorylation at Ser59 (bottom box) stabilises HipS binding and the HipBST complex, reducing the activity of HipT on TrpS compared to when autophosphorylated at Ser57 and repressing transcription from the hipBST operon.
activity, halting the expression of new protein. On the contrary, HipT P-Ser57 would have reduced HipS binding and therefore stay in its active conformation. The overall effect on growth could then be a balance between these two counter-acting responses.

ACKNOWLEDGEMENTS

The authors are indebted to the beamline staff at P14 in EMBL Hamburg, and BioMAX in MaxIV Lund for help during data collection. This project was funded by grants from the Novo Nordisk Foundation (NNF18OC0030646 to D.E.B.) and Danish Natural Research Foundation’s Centre of Excellence for Bacterial Stress Response and Persistence (grant number DNRF120).

COMPETING INTERESTS

The authors confirm that there are no competing interests.

AUTHOR CONTRIBUTIONS


MATERIALS AND METHODS

Phylogenetic analysis. The previously identified set of 48 HipT orthologues (Gerdes et al., 2021) was used for sequence alignment by Clustal Omega (Sievers & Higgins, 2018) at www.ebi.ac.uk and imported into Jalview (Waterhouse et al., 2009). The phylogenetic tree was visualized using iTOL.
Reconstruction of the phylogenetic tree was accomplished using IQ-TREE that uses the Maximum Likelihood approach and Ultrafast bootstrapping via the CIPRES module in Genious Prime (Minh et al., 2020).

Strains and plasmids. Strains and plasmids are listed in Table S2, and DNA oligonucleotides in Table S3.

Site-directed mutagenesis. Amino acid change HipT<sub>S57A</sub> was constructed by PCR mutagenesis (Table S3) as previously performed (Vang Nielsen et al., 2019). PCR product was digested with DpnI, and resulting plasmid transformed into <i>E. coli</i> strain DH5α.

Protein purification and structure determination. Expression of the <i>E. coli</i> O127:H6 HipBST complex was done using constructs pSVN78 (HipBST<sub>S57A</sub>), pSVN96 (HipBST<sub>D233Q</sub>), and pMME3 (HipBST<sub>S59A</sub>) derived from pSVN78. The constructs contained the genes coding for HipB, HipS, and C-terminal hexa-histidine tagged HipT, all separated, and with optimised Shine-Dalgarno sequences for large scale expression. All constructs contained an IPTG-inducible promoter and ampicillin resistance gene for selection. For each construct, 2 L cultures with a cell density of OD<sub>600</sub> = 0.6 were induced with a final concentration of 1 mM IPTG, and left to express ON at 20°C. Cell pellets were resuspended in lysis buffer (50 mM Tris-HCl, pH 7.5, 300 mM NaCl, 20 mM imidazole, 5% glycerol, 5 mM BME) together with 1 mM PMSF. Cells were lysed by sonication and the cleared lysate was applied to a 5 mL HisTrap HP column (Cytiva) equilibrated in lysis buffer, and washed in wash buffer (50 mM Tris-HCl, pH 7.5, 300 mM NaCl, 40 mM imidazole, 5 mM BME), before eluting with elution buffer (50 mM Tris-HCl, pH 7.5, 300 mM NaCl, 400 mM imidazole, 5 mM BME). The eluate was applied directly to a 5 mL Heparin HF column equilibrated in 70% Buffer A (50 mM Tris-HCl, pH
7.5, 5 mM BME) and 30% Buffer B (50 mM Tris-HCl, pH 7.5, 1M NaCl, 5 mM BME) running on an ÄKTA Pure system (Cytiva). Final separation was achieved after concentrating the Heparin elution to approximately 8 mg/mL using a Vivaspin column with a 30 kDa cutoff (Sartorius), before applying the sample to a Superdex 200 10/300 GL (Cytiva) column equilibrated in gel filtration buffer (20 mM Tris-HCl, pH 7.5, 300 mM NaCl, 5 mM BME). Crystals of HipBST$^{S57A}$ grew as clusters of thin plates in a 2 μL drop with a 1:1 ratio of protein to buffer, which consisted of 0.1 M Bicine, pH 9, 8% MPD and with a protein concentration of 5 mg/mL, and a reservoir of 200 μL. Crystals of all three mutant forms were obtained by a very similar protocol. All crystals were cryoprotected in the mother liquor supplemented with 25% MPD before freezing in liquid N₂.

Data collection for HipBST$^{S57A}$ was performed at P14 in Hamburg and for HipBST$^{S59A}$ and HipBST$^{D233Q}$ at BioMAX in Lund. For HipBST$^{S59A}$ and HipBST$^{D233Q}$, 7,200 images were collected with an oscillation of 0.1° and a transmission of 100%. The HipBST$^{S57A}$ data set was collected with an oscillation of 0.1°, 3,600 images and a transmission of 70%. All data was processed in XDS (Kabsch, 2010), using the CC½ value after scaling as indicator of the data resolution limit (Karplus & Diederichs, 2012). The space group was confirmed using Pointless (Evans, 2006), and all structures were determined using molecular replacement with Phaser (McCoy et al., 2007) from the CCP4 suite (Winn et al., 2011). The data set for HipBST$^{S57A}$ used a heavily truncated HipA to obtain initial phases for HipT, while the data set for HipBST$^{S59A}$ and HipBST$^{D233Q}$ used the solved structure of HipBST as a search model. Refinement was performed using Buster (Smart et al., 2012) using 1 big cycle of 20 small cycles of refinement when building, and 5 big cycles of 100 small cycles of refinement afterwards. Non-crystallographic symmetry (NCS) restraints were used during building since the asymmetric unit (ASU) contained a dimer but some refinement rounds without NCS were also included to allow for differences between the molecules in the ASU. Automatic water placement was...
used and water molecules with no density were removed. Model building was performed in Coot (Emsley et al., 2010). All structures were validated by the MolProbity server and Rama-Z scores.

Final R-work/R-free for HipBST_{D233Q}, HipBST_{S57A}, and HipBST_{S59A}, is 0.19/0.23, 0.21/0.24, and 0.20/0.24, respectively.

**Phos-tag gel of pre- and post-incubation with ATP.** 15% Phos-tag acrylamide gels (Wako) were cast according to the manufacturer's guidelines, except that 100 µM Phos-tag acrylamide was used to ensure proper separation between phosphorylated and non-phosphorylated HipT. The non-phosphorylated HipT control was expressed from pSNN2. The gel was run at 4 °C until the loading dye reached the bottom of the gel and visualized using standard Coomassie Blue staining as for normal SDS-PAGE gels. HipBST samples were run directly on the gel or incubated with ATP for 30 minutes at room temperature in a final mixture of: 2 mM KCl, 3.2 µM ZnSO_{4}, 4 mM MgCl_{2}, 66 µM ATP, 14 µL gel filtration buffer, and 1 mg/mL protein sample.

**Spot assays and growth curves.** Cultures were grown in liquid YT medium or MOPS minimal medium + 0.2% glucose at 37°C shaking at 160 rpm and solid medium used was YT agar plates, which were incubated at 37°C for approximately 16 h. When applicable, media were supplemented with 25 µg/mL chloramphenicol, 30 µg/mL ampicillin, 25 µg/mL kanamycin and/or 40 µg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). For spot assays, cells were grown as overnight cultures, diluted to obtain the same values of OD_{600}, centrifuged at 5,000 rpm for 5 min, washed in phosphate-buffered saline (PBS), and serially diluted before being spotted onto YT agar plates containing the indicated amount of inducer/repressor. Gene expression from plasmids carrying the pBAD promoter was induced by a final concentration of 0.2% arabinose and repressed by 0.2% glucose. Gene expression from plasmids carrying the P_{AI/O4/O3} promoter was induced by a final
concentration of 200 or 500 µM isopropyl-D-1 thio-galactopyranoside (IPTG), as indicated. Growth experiments were done in YT medium with addition of relevant antibiotics, diluted from overnight cultures and grown exponentially for at least 4 h until a seemingly constant doubling time. At $\text{OD}_{600} \approx 0.2$, arabinose (0.2%) was added to induce wildtype $\text{hipT}$ or autophosphorylation mutants. After another 1.5 h, IPTG (200 µM) was added to induce $\text{hipS}$ or $\text{hipBS}$. For each repetition, an independent colony from the strain was used to start separate cultures. No outliers were rejected.

**Northern blot analysis.** Total RNA samples were prepared using hot phenol extractions. Cultures were grown in YT medium (or MOPS minimal medium + 0.2% glucose in the case of the *E. coli* TB28 strain used for primer extension as described below). At indicated time points 10 mL were harvested into 1.25 mL ice-cold stop solution (5% phenol, pH 4.3 in absolute ethanol) and pelleted by centrifugation at 4,000 rpm at 4 °C for 10 min. Cell samples were resuspended in 200 µL ice-cold solution I (0.3 M sucrose; 0.01 M Na-Acetate, pH 4.5) and mixed with 200 µL solution II (2% SDS; 0.01 M Na-Acetate, pH 4.5) and 400 µL phenol (pH 4.3). Samples were vortexed for 10 s. before incubation at 65 °C for 3 min. followed by freezing in liquid nitrogen and centrifugation at 13,000 rpm at room temperature for 5 min. The upper phase was transferred to a new tube with 400 µL phenol (pH 4.3) followed by another round of the vortexing, heating, freezing and centrifugation steps. The RNA was precipitated in 1 mL ice-cold absolute ethanol and 50 µL RNA storage buffer (3 M Na-Acetate, pH 4.7; 10 mM EDTA) at -80 °C for a minimum of 30 min. RNA was then pelleted by centrifugation at 13,000 rpm at 4 °C for 30 min., washed in 70% ice-cold ethanol and air-dried at room temperature. Finally, samples used for northern blotting analysis were resuspended in 20 µL RNA storage buffer, whereas samples used for primer extension were resuspended in 20 µL nuclease-free water. Decylated control sample used for northern blotting analysis was prepared by mixing 4 µL of any of the total RNA samples with 6 µL 1 M Tris-HCl (pH 9) and 50 µL nuclease-free water.
and incubating for 2 hours at 37 ºC. This was followed by the addition of 150 µL ice-cold absolute ethanol and 4 µL 5 M NaCl and the tube was incubated at -20 ºC for 15 min. The sample was then centrifuged at 13,000 rpm at 4 ºC for 30 min. The supernatant was discarded, and RNA pellet was air-dried at room temperature and resuspended in 4 µL RNA storage buffer.

Northern analysis was used for detection and comparison of charged versus uncharged tRNA. 4 µL RNA samples were mixed with 6 µL tRNA loading buffer (0.1 M Na-succinate, pH 5; 8 M urea; 0.05% bromophenol blue and 0.05% xylene cyanol) and separated on 6% polyacrylamide gels with 8 M urea buffered in 0.1 M Na-succinate (pH 5) and run at 35 V. RNA was transferred to a Hybond-N+ membrane (Amersham) at 550 mA constant for 1 hour in blotting buffer (0.8 M Tris; 40 mM EDTA; acetic acid to pH 8.1). Membranes were pre-hybridized at 42 ºC in 6 mL hybridization buffer (5 x SSPE; 5 x Denhardt’s solution; 0.5% SDS; ~0.55 mg/mL herring sperm DNA (Sigma)) for one hour before addition of 10 µM DNA oligonucleotide probe, which had been radioactively labelled with [γ-32P]-ATP, for overnight hybridization. Oligonucleotides complementary to the tRNA anticodons of interest were 5’-end labelled using T4 polynucleotide kinase (Thermo Scientific) and 40 µCi of [γ-32P]-ATP (6000 Ci/mmol) by incubating at 37 ºC for 2 hours. For detection of tRNA

**Primer extension analysis.** Primer extension was used to map mRNA 5’ ends using radioactively labelled primers following extension by reverse transcription. Specific oligonucleotide primers (4

---

Primer extension analysis. Primer extension was used to map mRNA 5’ ends using radioactively labelled primers following extension by reverse transcription. Specific oligonucleotide primers (4
pmol) were radioactively 5’-end labelled with 130 µCi of [γ-32P]-ATP (6000 Ci/mmol) using T4 polynucleotide kinase (Thermo Scientific) and incubated for 30 min at 37 ºC followed by inactivation for 15 min at 75 ºC and cooling on ice. To remove excess unincorporated [γ-32P]-ATP, the reaction was centrifuged through an illustra MicroSpin G-25 Column (Cytiva) at 750 g for 1 min.

Sequencing reactions used as markers of molecular weight were generated using a purified PCR fragment amplified with primers FP43 and RP38 and 0.1 pmol from pKG127 as template DNA. The reactions were incubated with radioactively labelled primer (0.4 pmol) and extensions were performed with DreamTaq DNA polymerase (Thermo Scientific) in the presence of either ddGTP, ddATP, ddTTP or ddCTP termination mix (Roche). PCRs were carried out and reactions were stopped by the addition of an equal volume of 2 x formamide loading buffer (95% formamide; 0.025% bromophenol blue and 0.025% xylene cyanol; 5 mM EDTA; 0.025% SDS).

For extension reactions, radioactively labelled oligonucleotides (0.2 pmol) were hybridized to 10 µg of total RNA by incubating at 80 ºC for 5 min followed by quick cooling on ice for 5 min. Reverse transcriptions were carried out using Superscript III reverse transcriptase (Invitrogen) in 1 x First-Strand buffer plus 10 mM DTT and 1 mM dNTP for 1 hour at 52 ºC and 47 ºC for RP35 and RP37, respectively. Reactions were stopped by the addition of an equal volume of 2 x formamide loading buffer. Sequencing and extension reactions were fractionated on 6% polyacrylamide gels with 8 M urea, buffered in 1 x TBE and run at 45 W. Resulting gels were fixed in fixing solution (50% ethanol; 10% acetic acid), dried and visualized by phosphor imaging. The promoter of hipBST was analysed by primer extension using RP35 and RP37 for mapping of the transcriptional start site from pSVN141 (pGH254: hipBST-lacZ) in E. coli TB28 and the native hipBST operon in E. coli O127, respectively.
Small-angle X-ray scattering measurements and analysis. HipBST\textsubscript{S57A} purified as specified in “Protein purification and structure determination” was used for the SAXS analysis. The SAXS measurements were performed on the optimized NanoSTAR instrument (Bruker AXS) at the iNANO center at Aarhus University (Lyngsø & Pedersen, 2021). It uses a high brilliance Ga metal-jet X-ray source (Excillum), special long optics, a two-pinhole collimation with a home-built ‘scatterless’ slit pinhole, and a VÅNTEC-2000 (Bruker AXS) microgap 2D gas proportional detector. The sample and buffer were measured in the same flow-through quartz capillary, the scattering from the buffer was subtracted from that of the sample, and the data were converted to absolute scale using the scattering from water. The intensity data, $I(q)$, are displayed as a function of the modulus of the scattering vector $q = 4\pi \sin(\theta)/\lambda$, where $2\theta$ is the scattering angle and $\lambda$ is the wavelength of the X-rays. The fit to the data in a Guinier plot gave the radius of gyration $R_g = 52.2 \pm 0.2 \text{ Å}$ and the forward scattering $I(0) = 0.0868 \pm 0.005 \text{ cm}^{-1}$. The molar mass was calculated as: $M = I(0)N_A/c(\Delta \rho_m^2)$, where $N_A$ is Avogadro’s number, $c = 1.01 \text{ mg mL}^{-1}$ is the mass concentration, and $\Delta \rho_m = 2.00 \times 10^{10} \text{ cm g}^{-1}$ is the typical excess scattering length per unit mass for proteins. The radius of gyration and forward scattering can also be obtained from the pair distance distribution function $p(r)$ determined by indirect Fourier transformation (Glatter, 1977; Pedersen et al., 1994) and the values are quite close to those from the Guinier fit: $R_g = 53.8 \pm 0.3 \text{ Å}$ and $I(0) = 0.08651 \pm 0.0005 \text{ cm}^{-1}$, where the latter corresponds to a mass of $M = 129 \text{ kDa}$. The maximum in a normalized Kratky plot using the values of $R_g$ and $I(0)$ from the Guinier fit for normalisation agrees with a relatively compact structure, however, the plateau behaviour for $qR_g > 3$ suggest some openness and potential flexibility of the structure of the complexes. The scattering of the complex from the crystal structure was directly compared to the SAXS data and a poor agreement was observed. To improve the agreement, rigid body refinement (RBR) of the structure was performed using previously described methods (Steiner et al., 2018). For the RBR of HipBST in three connected domains, the

37
first runs showed that there was some degree of oligomerisation present in the sample, since the model
curve is below the measured data at low $q$. Therefore, a dimer factor $S_{agg}(q) = 1 + \frac{A \sin(qD)}{qD}$ (Larsen
et al., 2020) was multiplied with the intensity calculated for the structure. In the expression, $A$ is the
mass fraction of dimers of the HipBST complex and $D$ is the distance between the two copies in the
dimer. This approach gave good fits to the data.

DATA AND SOFTWARE AVAILABILITY

The accession numbers for the structures reported in this paper are PDB: 7AB3 (HipBST HipT
S57A), 7AB4 (HipBST HipT S59A), and 7AB5 (HipBST HipT D233Q).
REFERENCES


site of ribosomes. *Proceedings of the National Academy of Sciences of the U S A*, 70(5), 1564-1568. https://doi.org/10.1073/pnas.70.5.1564


### TABLE S1. Crystallographic data statistics

<table>
<thead>
<tr>
<th></th>
<th>HipBST&lt;sup&gt;D233Q&lt;/sup&gt;</th>
<th>HipBST&lt;sup&gt;S57A&lt;/sup&gt;</th>
<th>HipBST&lt;sup&gt;S59A&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Data Collection</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wavelength (Å)</td>
<td>1.54180</td>
<td>0.97625</td>
<td>1.54180</td>
</tr>
<tr>
<td>Resolution range (Å)</td>
<td>49.9 – 2.9 (3.0 – 2.9)*</td>
<td>46.9 – 2.4 (2.49 – 2.40)*</td>
<td>50.4 – 3.34 (3.5 – 3.4)*</td>
</tr>
<tr>
<td>Space group</td>
<td>C121</td>
<td>C121</td>
<td>C121</td>
</tr>
<tr>
<td>Unit cell dimensions</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a, b, c, (Å)</td>
<td>281.66, 106.47, 57.75</td>
<td>281.68, 106.07, 57.56</td>
<td>285.26, 107.15, 58.45</td>
</tr>
<tr>
<td>α, β, γ (°)</td>
<td>90, 90.75, 90</td>
<td>90, 90.65, 90</td>
<td>90, 90.71, 90</td>
</tr>
<tr>
<td>Total reflections</td>
<td>74,587 (7,335)</td>
<td>625,868 (25,597)</td>
<td>41,777 (4,068)</td>
</tr>
<tr>
<td>Unique reflections</td>
<td>37,716 (3,719)</td>
<td>66,117 (6,503)</td>
<td>22,580 (2,193)</td>
</tr>
<tr>
<td>Multiplicity</td>
<td>2.0 (2.0)</td>
<td>9.5 (3.9)</td>
<td>1.9 (1.9)</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>99.8 (99.3)</td>
<td>99.9 (99.4)</td>
<td>87.71 (84.84)</td>
</tr>
<tr>
<td>R&lt;sub&gt;meas&lt;/sub&gt; (%)</td>
<td>0.13 (1.105)</td>
<td>0.27 (0.08)</td>
<td>0.17 (1.01)</td>
</tr>
<tr>
<td>I/σ(I)</td>
<td>6.62 (0.86)</td>
<td>10.3 (0.72)</td>
<td>7.10 (0.93)</td>
</tr>
<tr>
<td>CC&lt;sub&gt;1/2&lt;/sub&gt;</td>
<td>0.99 (0.37)</td>
<td>0.99 (0.37)</td>
<td>0.97 (0.43)</td>
</tr>
<tr>
<td><strong>Refinement</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average B-factor (Å&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>98.5</td>
<td>86.1</td>
<td>119.4</td>
</tr>
<tr>
<td>protein</td>
<td>98.9</td>
<td>86.5</td>
<td>119.9</td>
</tr>
<tr>
<td>ligands</td>
<td>-</td>
<td>166.7</td>
<td>118.6</td>
</tr>
<tr>
<td>solvent</td>
<td>80.0</td>
<td>76.7</td>
<td>78.3</td>
</tr>
<tr>
<td>No. of reflections</td>
<td>37,706 (3,719)</td>
<td>66,112 (6,504)</td>
<td>22,570 (2,193)</td>
</tr>
<tr>
<td>No. of reflections (free)</td>
<td>1,914 (175)</td>
<td>3,364 (326)</td>
<td>1086 (86)</td>
</tr>
<tr>
<td>R-work (%)</td>
<td>19.5 (31.8)</td>
<td>21.0 (42.8)</td>
<td>20.2 (29.2)</td>
</tr>
<tr>
<td>R-free (%)</td>
<td>22.8 (34.5)</td>
<td>23.8 (46.2)</td>
<td>24.3 (35.4)</td>
</tr>
<tr>
<td>Number of</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>protein (residues)</td>
<td>1,018</td>
<td>1,017</td>
<td>1,023</td>
</tr>
<tr>
<td>solvent (atoms)</td>
<td>203</td>
<td>402</td>
<td>110</td>
</tr>
<tr>
<td>ligand (atoms)</td>
<td>-</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>rmsd (bonds, Å)</td>
<td>0.011</td>
<td>0.013</td>
<td>0.013</td>
</tr>
<tr>
<td>rmsd (angles, degrees)</td>
<td>1.49</td>
<td>1.75</td>
<td>1.66</td>
</tr>
<tr>
<td>Rotamer outliers (%)</td>
<td>9.8</td>
<td>6.0</td>
<td>10.07</td>
</tr>
<tr>
<td>Clashscore</td>
<td>4.4</td>
<td>2.7</td>
<td>5.8</td>
</tr>
<tr>
<td>Ramachandran statistics</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>favoured (%)</td>
<td>95.1</td>
<td>96.0</td>
<td>95.6</td>
</tr>
<tr>
<td>allowed (%)</td>
<td>4.7</td>
<td>3.6</td>
<td>3.9</td>
</tr>
<tr>
<td>outliers (%)</td>
<td>0.2</td>
<td>0.4</td>
<td>0.5</td>
</tr>
<tr>
<td>Rama-Z score</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>whole</td>
<td>-1.63</td>
<td>-0.44</td>
<td>-0.90</td>
</tr>
<tr>
<td>helix</td>
<td>-1.27</td>
<td>-0.40</td>
<td>-0.48</td>
</tr>
<tr>
<td>sheet</td>
<td>-1.36</td>
<td>0.21</td>
<td>0.07</td>
</tr>
<tr>
<td>loop</td>
<td>-0.75</td>
<td>-0.16</td>
<td>-0.73</td>
</tr>
</tbody>
</table>

*Numbers in parentheses refer to the outermost resolution shell.
<table>
<thead>
<tr>
<th><strong>E. coli strains</strong></th>
<th><strong>Description</strong></th>
<th><strong>Reference or source</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>MG1655</td>
<td>Wild-type K12</td>
<td>(Blattner et al., 1997)</td>
</tr>
<tr>
<td>O127</td>
<td>O127:H6 strain E2348/69</td>
<td>(Iguchi et al., 2009)</td>
</tr>
<tr>
<td>TB28</td>
<td>MG1655ΔlacIZYA</td>
<td>Laboratory collection</td>
</tr>
<tr>
<td>pBAD33</td>
<td>p15 araC P&lt;sub&gt;BAD&lt;/sub&gt;, Cm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>(Guzman et al., 1995)</td>
</tr>
<tr>
<td>pBAD322T</td>
<td>pMB1 rop araC P&lt;sub&gt;BAD&lt;/sub&gt;, Tet&lt;sup&gt;r&lt;/sup&gt;</td>
<td>(Cronan, 2006)</td>
</tr>
<tr>
<td>pNDM220</td>
<td>Mini-R1 lac&lt;sup&gt;r&lt;/sup&gt; P&lt;sub&gt;Al/04/03&lt;/sub&gt;, Amp&lt;sup&gt;r&lt;/sup&gt;</td>
<td>(Gotfredsen &amp; Gerdes, 1998)</td>
</tr>
<tr>
<td>pGH254</td>
<td>Mini-R1, lacZYA transcriptional fusion vector, Kan&lt;sup&gt;r&lt;/sup&gt;</td>
<td>Laboratory collection</td>
</tr>
<tr>
<td>pET-15b</td>
<td>pBR322 lacI P&lt;sub&gt;T7&lt;/sub&gt;, Amp&lt;sup&gt;r&lt;/sup&gt;</td>
<td>Novagen</td>
</tr>
<tr>
<td>pKG127</td>
<td>pUC57::hipBST&lt;sub&gt;O127&lt;/sub&gt;</td>
<td>Novagen</td>
</tr>
<tr>
<td>pSVN1</td>
<td>pBAD33::hipT, start codon GTG</td>
<td>(Vang Nielsen et al., 2019)</td>
</tr>
<tr>
<td>pSVN68</td>
<td>pUC57::hipB-S&lt;sup&gt;557A&lt;/sup&gt;H&lt;sub&gt;is6&lt;/sub&gt;, optimized SDs for all genes</td>
<td>This work</td>
</tr>
<tr>
<td>pSVN78</td>
<td>pET-15b::hipB-S&lt;sup&gt;557A&lt;/sup&gt;H&lt;sub&gt;is6&lt;/sub&gt;, optimized SDs for all genes</td>
<td>This work</td>
</tr>
<tr>
<td>pSVN87</td>
<td>pUC57::hipB-S&lt;sup&gt;D210Q&lt;/sup&gt;H&lt;sub&gt;is6&lt;/sub&gt;, optimized SDs for all genes</td>
<td>This work</td>
</tr>
<tr>
<td>pSVN</td>
<td>Description</td>
<td>Source</td>
</tr>
<tr>
<td>--------</td>
<td>------------------------------------------------------------------------------</td>
<td>-------------------------</td>
</tr>
<tr>
<td>pSVN88</td>
<td>pUC57::hipB-S-T&lt;sup&gt;D233Q&lt;/sup&gt;&lt;sub&gt;His6&lt;/sub&gt;, optimized SDs for all genes</td>
<td>This work</td>
</tr>
<tr>
<td>pSVN96</td>
<td>pET-15b::hipB-S-T&lt;sup&gt;D233Q&lt;/sup&gt;&lt;sub&gt;His6&lt;/sub&gt;, optimized SDs for all genes</td>
<td>This work</td>
</tr>
<tr>
<td>pSVN109</td>
<td>pNDM220::hipS, optimized SD</td>
<td>(Vang Nielsen et al., 2019)</td>
</tr>
<tr>
<td>pSVN110</td>
<td>pNDM220::hipB-S, optimized SDs for both genes</td>
<td>(Vang Nielsen et al., 2019)</td>
</tr>
<tr>
<td>pSVN111</td>
<td>pNDM220::hipB, optimized SD</td>
<td>(Vang Nielsen et al., 2019)</td>
</tr>
<tr>
<td>pSVN141</td>
<td>pGH254::P&lt;sub&gt;hipBST&lt;/sub&gt;-hipB&lt;sup&gt;S&lt;/sup&gt;-laZ, transcriptional P&lt;sub&gt;hipBST&lt;/sub&gt;-hipB' lacZ fusion</td>
<td>This work</td>
</tr>
<tr>
<td>pSVN154</td>
<td>pBAD322T:: hipB-S-T&lt;sup&gt;D210Q&lt;/sup&gt;, optimized SDs for all genes</td>
<td>This work</td>
</tr>
<tr>
<td>pSVN178</td>
<td>pNDM220::hipS&lt;sup&gt;W65A&lt;/sup&gt;, optimized SD</td>
<td>This work</td>
</tr>
<tr>
<td>pSVN180</td>
<td>pBAD33::hipB-S-T&lt;sup&gt;D210Q&lt;/sup&gt;, optimized SDs for all genes</td>
<td>This work</td>
</tr>
<tr>
<td>pSVN181</td>
<td>pBAD33::hipB-S-T&lt;sup&gt;D233Q&lt;/sup&gt;, optimized SDs for all genes</td>
<td>This work</td>
</tr>
<tr>
<td>pSVN182</td>
<td>pBAD33::hipB-S, optimized SDs for both genes</td>
<td>This work</td>
</tr>
<tr>
<td>pSVN184</td>
<td>pBAD33::hipB-T&lt;sup&gt;D210Q&lt;/sup&gt;, optimized SDs for both genes</td>
<td>This work</td>
</tr>
<tr>
<td>pSVN185</td>
<td>pBAD33::hipB-T&lt;sup&gt;D233Q&lt;/sup&gt;, optimized SDs for both genes</td>
<td>This work</td>
</tr>
<tr>
<td>pSVN187</td>
<td>pBAD33::hipS-T&lt;sup&gt;D210Q&lt;/sup&gt;, optimized SDs for both genes</td>
<td>This work</td>
</tr>
<tr>
<td>pSVN188</td>
<td>pBAD33::hipS-T&lt;sup&gt;D233Q&lt;/sup&gt;, optimized SDs for both genes</td>
<td>This work</td>
</tr>
<tr>
<td>pSVN189</td>
<td>pBAD33::hipB, optimized SD</td>
<td>This work</td>
</tr>
<tr>
<td>pSVN190</td>
<td>pBAD33::hipS, optimized SD</td>
<td>This work</td>
</tr>
<tr>
<td>pSVN192</td>
<td>pBAD33::hipT&lt;sup&gt;D210Q&lt;/sup&gt;, optimized SD</td>
<td>This work</td>
</tr>
<tr>
<td>--------</td>
<td>------------------------------------------</td>
<td>----------</td>
</tr>
<tr>
<td>pSVN193</td>
<td>pBAD33::hipT&lt;sup&gt;D233Q&lt;/sup&gt;, optimized SD</td>
<td>This work</td>
</tr>
<tr>
<td>pSVN194</td>
<td>pBAD33::hipT&lt;sup&gt;S57D&lt;/sup&gt;, start codon GTG</td>
<td>This work</td>
</tr>
<tr>
<td>pSVN195</td>
<td>pBAD33::hipT&lt;sup&gt;S59D&lt;/sup&gt;, start codon GTG</td>
<td>This work</td>
</tr>
<tr>
<td>pSVN198</td>
<td>pGH254:: P&lt;sub&gt;hipBST&lt;/sub&gt;-hipBST-lacZ, transcriptional P&lt;sub&gt;hipBST&lt;/sub&gt;-hipBST-lacZ fusion</td>
<td>This work</td>
</tr>
<tr>
<td>pSVN199</td>
<td>pBAD33::hipT&lt;sup&gt;S57A&lt;/sup&gt;, start codon GTG</td>
<td>This work</td>
</tr>
<tr>
<td>pSVN201</td>
<td>pBAD33::hipT&lt;sup&gt;S59A&lt;/sup&gt;, start codon GTG</td>
<td>This work</td>
</tr>
<tr>
<td>pSVN203</td>
<td>pBAD33::hipT&lt;sup&gt;S57A+S59A&lt;/sup&gt;, start codon GTG</td>
<td>This work</td>
</tr>
<tr>
<td>pSVN205</td>
<td>pBAD33::hipT&lt;sup&gt;S57D+S59A&lt;/sup&gt;, start codon GTG</td>
<td>This work</td>
</tr>
<tr>
<td>pSVN207</td>
<td>pBAD33::hipT&lt;sup&gt;S57A+S59D&lt;/sup&gt;, start codon GTG</td>
<td>This work</td>
</tr>
<tr>
<td>pSVN209</td>
<td>pBAD33::hipT&lt;sup&gt;S57D+S59D&lt;/sup&gt;, start codon GTG</td>
<td>This work</td>
</tr>
<tr>
<td>pSVN211</td>
<td>pBAD33::hipT&lt;sup&gt;D210Q&lt;/sup&gt;, start codon GTG</td>
<td>This work</td>
</tr>
<tr>
<td>pSVN212</td>
<td>pBAD33::hipT&lt;sup&gt;S57D+D210Q&lt;/sup&gt;, start codon GTG</td>
<td>This work</td>
</tr>
<tr>
<td>pSVN213</td>
<td>pBAD33::hipT&lt;sup&gt;S59D+D210Q&lt;/sup&gt;, start codon GTG</td>
<td>This work</td>
</tr>
<tr>
<td>pSVN214</td>
<td>pBAD33::hipT&lt;sup&gt;S57A+D210Q&lt;/sup&gt;, start codon GTG</td>
<td>This work</td>
</tr>
<tr>
<td>pSVN215</td>
<td>pBAD33::hipT&lt;sup&gt;S59A+D210Q&lt;/sup&gt;, start codon GTG</td>
<td>This work</td>
</tr>
<tr>
<td>pSVN216</td>
<td>pBAD33::hipT&lt;sup&gt;S57A+S59A+D210Q&lt;/sup&gt;, start codon GTG</td>
<td>This work</td>
</tr>
<tr>
<td>Vector</td>
<td>Expression in E. coli, <strong>optimized SD</strong></td>
<td></td>
</tr>
<tr>
<td>----------</td>
<td>----------------------------------------</td>
<td></td>
</tr>
<tr>
<td>pSVN217</td>
<td>pBAD33::\textit{hipT}_{S57D+S59A+D210Q}, start codon GTG</td>
<td></td>
</tr>
<tr>
<td>pSVN218</td>
<td>pBAD33::\textit{hipT}_{S57A+S59D+D210Q}, start codon GTG</td>
<td></td>
</tr>
<tr>
<td>pSVN219</td>
<td>pBAD33::\textit{hipT}_{S57D+S59D+D210Q}, start codon GTG</td>
<td></td>
</tr>
<tr>
<td>pSVN220</td>
<td>pBAD33::\textit{hipT}_{D210Q}, optimized SD</td>
<td></td>
</tr>
<tr>
<td>pSVN221</td>
<td>pBAD33::\textit{hipT}_{S57D+D210Q}, optimized SD</td>
<td></td>
</tr>
<tr>
<td>pSVN222</td>
<td>pBAD33::\textit{hipT}_{S59D+D210Q}, optimized SD</td>
<td></td>
</tr>
<tr>
<td>pSVN223</td>
<td>pBAD33::\textit{hipT}_{S57A+D210Q}, optimized SD</td>
<td></td>
</tr>
<tr>
<td>pSVN224</td>
<td>pBAD33::\textit{hipT}_{S59A+D210Q}, optimized SD</td>
<td></td>
</tr>
<tr>
<td>pSVN225</td>
<td>pBAD33::\textit{hipT}_{S57A+S59A+D210Q}, optimized SD</td>
<td></td>
</tr>
<tr>
<td>pSVN226</td>
<td>pBAD33::\textit{hipT}_{S57D+S59A+D210Q}, optimized SD</td>
<td></td>
</tr>
<tr>
<td>pSVN227</td>
<td>pBAD33::\textit{hipT}_{S57A+S59D+D210Q}, optimized SD</td>
<td></td>
</tr>
<tr>
<td>pSVN228</td>
<td>pBAD33::\textit{hipT}_{S57D+S59D+D210Q}, optimized SD</td>
<td></td>
</tr>
<tr>
<td>pSNN1</td>
<td>pET-15b::\textit{hipT}<em>{S57A}</em>{\text{His6}}, optimized SD</td>
<td></td>
</tr>
<tr>
<td>pSNN2</td>
<td>pET-15b::\textit{hipT}<em>{S57A+D210A}</em>{\text{His6}}, optimized SD</td>
<td></td>
</tr>
<tr>
<td>pMME</td>
<td>pET-15b::\textit{hipB-S}<em>{T</em>{S59A}}_{\text{His6}}, optimized SDs for all genes</td>
<td></td>
</tr>
</tbody>
</table>

*SD, Shine-Dalgarno sequence.*
### TABLE S3. Oligonucleotides and primers.

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>FP1(GTG)</td>
<td>CCCCCGTGCAGGATCCAAAGGAGTTTATAAATGTCGAATTGTGATTCTG</td>
</tr>
<tr>
<td>FP21</td>
<td>GGGGGGGTACCGGATCCAAAATAAGGAGGAAAAAAAAATGATCTGCTGACGGACCCAC</td>
</tr>
<tr>
<td>FP22</td>
<td>CCCCCCTCGAGGATCCAAAATAAGGAGGAAAAAAAAATGATCGGCGAGTGAAAG</td>
</tr>
<tr>
<td>FP43</td>
<td>CCCCCGAATTCCTCTCCCGATGAGATCAGC</td>
</tr>
<tr>
<td>FP46</td>
<td>GGGGGGTACCGGATCCAAAATAAGGAGGAAAAAAAAATGATCGGCGAGTGAAAG</td>
</tr>
<tr>
<td>FP47</td>
<td>GGGGGGTACCGGATCCAAAATAAGGAGGAAAAAAAAATGCGAATTGTCGTATTCTG</td>
</tr>
<tr>
<td>FP48</td>
<td>GGGGGGTACCGGATCCAAAATAAGGAGGAAAAAAAAATGCGAATTGTCGTATTCTG</td>
</tr>
<tr>
<td>FP49</td>
<td>CCCCCGAATTCCTCTCCCGATGAGATCAGC</td>
</tr>
<tr>
<td>RP1</td>
<td>CCCCCCGCATGCGAATTCGCTACAGCGAGCCCCAGACG</td>
</tr>
<tr>
<td>RP11</td>
<td>CCCCCCTCGAGGAAGCTTTTCACAGCAGCCCCAGACG</td>
</tr>
<tr>
<td>RP14</td>
<td>GGGGGAAATCGCTACAGCAGCCCCAGACG</td>
</tr>
<tr>
<td>RP15</td>
<td>GGGGGAAATCGCTACAGCAGCCCCAGACG</td>
</tr>
<tr>
<td>RP32</td>
<td>CCCCCGGATCTCTGCCCATCCTGGAGTTG</td>
</tr>
<tr>
<td>RP35</td>
<td>GTCTGAGGCTCAGATCATTAAAGAACC</td>
</tr>
<tr>
<td>Primer Name</td>
<td>Sequence</td>
</tr>
<tr>
<td>---------------------------------</td>
<td>---------------------------------</td>
</tr>
<tr>
<td>RP37</td>
<td>CGATTGATAAAAAGACAAAAAACAAGATC</td>
</tr>
<tr>
<td>RP38</td>
<td>GTTGGTCGCAGCAGAGATCGCTTTTC</td>
</tr>
<tr>
<td>RP42</td>
<td>GGGGGTACGCTGGAGACTCGCCGATGCAATAG</td>
</tr>
<tr>
<td>RP43</td>
<td>GGGGGGTACCTCACAGCA GCCCAGACG</td>
</tr>
<tr>
<td>hipS W65A Fw</td>
<td>CAGAAGGAGCTCTGCGTCAACGCTA</td>
</tr>
<tr>
<td>hipS W65A Rv</td>
<td>TGACGCAGAGCTCTTCTGGGCG</td>
</tr>
<tr>
<td>HipT S57D Fw</td>
<td>GCGTCAACAAAAAGGGATGGATATTCCGGTT</td>
</tr>
<tr>
<td>HipT S57D Rv</td>
<td>GGGCTGTAACCGGAAATATCCATCCCTTTT</td>
</tr>
<tr>
<td>HipT S59D Fw</td>
<td>GTCAACACAAAAAGGGATGGATATTGACGTTAC</td>
</tr>
<tr>
<td>HipT S59D Rv</td>
<td>TTGGGCTGGTAACCGGCAATACTCATCCCTTT</td>
</tr>
<tr>
<td>HipT S59A Fw</td>
<td>GTCAACACAAAAAGGGATGGATATTGCCCGTTAC</td>
</tr>
<tr>
<td>HipT S59A Rv</td>
<td>TTGGGCTGGTAACCGGCAATACTCATCCCTT</td>
</tr>
<tr>
<td>HipT S57A + S59A Fw</td>
<td>CGTCAACACAAAAAGGGATGGCTATTGCCGTTAC</td>
</tr>
<tr>
<td>HipT S57A + S59A Rv</td>
<td>TTGGGCTGGTAACCGGCAATAGCCATCCCTTT</td>
</tr>
<tr>
<td>HipT S57D + S59A Fw</td>
<td>CGTCAACACAAAAAGGGATGGATATTGCCGTTAC</td>
</tr>
<tr>
<td>HipT S57D + S59A Rv</td>
<td>TTGGGCTGGTAACCGGCAATATCCATCCCTTT</td>
</tr>
<tr>
<td>Offspring</td>
<td>Sequence</td>
</tr>
<tr>
<td>-------------------------</td>
<td>------------------------------------</td>
</tr>
<tr>
<td>HipT S57A + S59D Fw</td>
<td>CGTCAACAAAAAGGGATGGCTATTGACGGTTAC</td>
</tr>
<tr>
<td>HipT S57A + S59D Rv</td>
<td>TTGGGCTGGTAACCGTCAATAGCCATCCCTTTT</td>
</tr>
<tr>
<td>HipT S57D + S59D Fw</td>
<td>CGTCAACAAAAAGGGATGGATATTGACGGTTAC</td>
</tr>
<tr>
<td>HipT S57D + S59D Rv</td>
<td>TTGGGCTGGTAACCGTCAATATCCATCCCTTTT</td>
</tr>
<tr>
<td>hipX S57A Fw</td>
<td>AAGGGATGGCTATTTCGCGTTACCAGCC</td>
</tr>
<tr>
<td>hipX S57A Rv</td>
<td>CGGAAATAGCCATCCTTTTGTGACG</td>
</tr>
<tr>
<td>hipX D210Q Fw</td>
<td>GTAATAACCAGATGCATTACGAAACTTTGG</td>
</tr>
<tr>
<td>hipX D210Q Rv</td>
<td>AAATGCATCTGTTATTACCAGCAACCAGG</td>
</tr>
<tr>
<td>hipX D233Q Fw</td>
<td>CGGATGTATCATGTCTGTTCTGCGCTCCC</td>
</tr>
<tr>
<td>hipX D233Q Rv</td>
<td>GAAACAAACTGATACCGCGCTAACG</td>
</tr>
<tr>
<td>hipBS del Fw</td>
<td>ACGACAAATTCGCCATTTTTTTCTCCTTTTTTCTAGAGGG</td>
</tr>
<tr>
<td>hipBS del Rv</td>
<td>TTCCCCCTCTAGAAAAAAAAATAAGGAGGAAAAAAATGGCGAAT</td>
</tr>
<tr>
<td>hipT D210A Fw</td>
<td>TAAATGCATCGCGTTATTACCAGCAACAA</td>
</tr>
<tr>
<td>hipT D210A Rv</td>
<td>CTGGGTAATAACCGCGATGCATTACGAAACTTTT</td>
</tr>
<tr>
<td>hipT S57S59A Fw</td>
<td>GGGATGAGTATTCGGCGTTACCAGCCCAATTGCAA</td>
</tr>
<tr>
<td>hipT S57S59A Rv</td>
<td>GTAACCGGCAATACTCATCCCTTTTGACCGCGG</td>
</tr>
</tbody>
</table>
SUPPLEMENTARY METHODS

Construction of plasmids. Construction of plasmids is summarised below.

**pSVN68.** The mutation in $\text{hipB-S-T}^{S57A}_{\text{His6}}$ with optimized SDs for all three genes was created using pSVN61 (Vang Nielsen et al., 2019) and primers hipX S57A Fw and hipX S57A Rv in a site-directed plasmid mutagenesis PCR. Eight reactions were carried out at different temperatures with a gradient PCR. The samples were pooled and digested with DpnI to digest the parental plasmid before being transformed into *E. coli* DH5α.

**pSVN78.** $\text{hipB-S-T}^{S57A}_{\text{His6}}$ with optimized SDs for all three genes was sub-cloned from pSVN68 by digesting with XbaI and XhoI, purifying the DNA fragment and ligating into pET-15b.

**pSVN87.** The mutation in $\text{hipB-S-T}^{D210Q}_{\text{His6}}$ with optimized SDs for all three genes was created using pSVN61 (Vang Nielsen et al., 2019) and primers hipX D210Q Fw and hipX D210Q Rv in a site-directed plasmid mutagenesis PCR. Eight reactions were carried out at different temperatures with a gradient PCR. The samples were pooled and digested with DpnI to digest the parental plasmid before being transformed into *E. coli* DH5α.

**pSVN88.** The mutation in $\text{hipB-S-T}^{D233Q}_{\text{His6}}$ with optimized SDs for all three genes was created using pSVN61 (Vang Nielsen et al., 2019) and primers hipX D233Q Fw and hipX D233Q Rv in a site-directed plasmid mutagenesis PCR. Eight reactions were carried out at different temperatures with a gradient PCR. The samples were pooled and digested with DpnI to digest the parental plasmid before being transformed into *E. coli* DH5α.

**pSVN96.** $\text{hipB-S-T}^{D233Q}_{\text{His6}}$ with optimized SDs for all three genes was sub-cloned from pSVN88 by digesting with XbaI and XhoI, purifying the DNA fragment and ligating into pET-15b.
pSVN141. \( P_{\text{hipBST-hipB}} \), a fragment containing 224 bp upstream of the \( \text{hipB} \) gene plus the first 73 bp of the \( \text{hipB} \) gene was amplified from pKG127 using primers FP43 and RP32. The resulting PCR product was digested with EcoRI and BamHI and ligated into pGH254.

pSVN154. \( \text{hipB-S-T}_{D210Q} \) with optimized SDs for all three genes was amplified from pSVN87 using primers FP21 and RP11. The resulting PCR product was digested with KpnI and HindIII and ligated into pBAD322T.

pSVN178. \( \text{hipS}^{W65A} \) was created by a two-step PCR reaction. Two fragments were amplified from pSVN109 using primers FP22 and hipS W65A Rv in one reaction and hipS W65A Fw and RP14 in the other. The resulting two PCR products were joined by a second round of PCR using both fragments as template DNA and primers FP22 and RP14. The final PCR product was digested with XhoI and EcoRI and ligated into pNDM220.

pSVN180. \( \text{hipB-S-T}_{D210Q} \) with optimized SDs for all three genes was amplified from pSVN87 using primers FP21 and RP11. The resulting PCR product was digested with KpnI and HindIII and ligated into pBAD33.

pSVN181. \( \text{hipB-S-T}_{D233Q} \) with optimized SDs for all three genes was amplified from pSVN88 using primers FP21 and RP11. The resulting PCR product was digested with KpnI and HindIII and ligated into pBAD33.

pSVN182. \( \text{hipB-S} \) with optimized SDs for both genes was amplified from pSVN87 using primers FP21 and RP14. The resulting PCR product was digested with KpnI and HindIII and ligated into pBAD33.

pSVN184. \( \text{hipB-T}_{D210Q} \) with optimized SDs for both genes was created by amplifying two PCR products from pSVN87: \( \text{hipB} \) using primers FP21 and RP42 and \( \text{hipT}_{D210Q} \) using primers FP48 and RP11. The \( \text{hipB} \) fragment was digested with KpnI and PstI, while the \( \text{hipT}_{D233Q} \) fragment was digested with PstI and HindIII. The two digested fragments were then ligated into pBAD33 using the KpnI and HindIII restriction sites.
pSVN185. *hipB-T^{D233Q}* with optimized SDs for both genes was created by amplifying two PCR products from pSVN88: *hipB* using primers FP21 and RP42 and *hipT^{D233Q}* using primers FP48 and RP11. The *hipB* fragment was digested with KpnI and PstI, while the *hipT^{D233Q}* fragment was digested with PstI and HindIII. The two digested fragments were then ligated into pBAD33 using the KpnI and HindIII restriction sites.

pSVN187. *hipS-T^{D210Q}* with optimized SDs for both genes was amplified from pSVN88 using primers FP46 and RP11. The resulting PCR product was digested with KpnI and HindIII and ligated into pBAD33.

pSVN188. *hipS-T^{D233Q}* with optimized SDs for both genes was amplified from pSVN87 using primers FP46 and RP11. The resulting PCR product was digested with KpnI and HindIII and ligated into pBAD33.

pSVN189. *hipB* with optimized SD was amplified from pSVN87 using primers FP21 and RP15. The resulting PCR product was digested with KpnI and HindIII and ligated into pBAD33.

pSVN190. *hipS* with optimized SD was amplified from pSVN87 using primers FP46 and RP14. The resulting PCR product was digested with KpnI and HindIII and ligated into pBAD33.

pSVN192. *hipT^{D210Q}* with optimized SD was amplified from pSVN87 using primers FP47 and RP11. The resulting PCR product was digested with KpnI and HindIII and ligated into pBAD33.

pSVN193. *hipT^{D233Q}* with optimized SD was amplified from pSVN88 using primers FP47 and RP11. The resulting PCR product was digested with KpnI and HindIII and ligated into pBAD33.

pSVN194. *hipT^{S57D}* with start codon GTG was created by a two-step PCR reaction. Two fragments were amplified from pKG127 using primers FP1(GTG) and HipT S57D Rv in one
reaction and HipT S57D Fw and RP1 in the other. The resulting two PCR products were joined by a second round of PCR using both fragments as template DNA and primers FP1(GTG) and RP1. The final PCR product was digested with SalI and SphI and ligated into pBAD33.

**pSVN195.** *hipT*<sup>S59D</sup> with start codon GTG was created by a two-step PCR reaction. Two fragments were amplified from pKG127 using primers FP1(GTG) and HipT S59D Rv in one reaction and HipT S59D Fw and RP1 in the other. The resulting two PCR products were joined by a second round of PCR using both fragments as template DNA and primers FP1(GTG) and RP1. The final PCR product was digested with SalI and SphI and ligated into pBAD33.

**pSVN198.** *HipBST-hipBST*, a fragment containing 224 bp upstream of the *hipB* gene plus the native *hipBST* operon was amplified from pKG127 using primers FP49 and RP43. The resulting PCR product was digested with EcoRI and KpnI and ligated into pGH254.

**pSVN199.** *hipT*<sup>S57A</sup> with start codon GTG was amplified from pSVN78 using primers FP1(GTG) and RP1. The resulting PCR product was digested with SalI and SphI and ligated into pBAD33.

**pSVN201.** *hipT*<sup>S59A</sup> with start codon GTG was created by a two-step PCR reaction. Two fragments were amplified from pKG127 using primers FP1(GTG) and HipT S59A Rv in one reaction and HipT S59A Fw and RP1 in the other. The resulting two PCR products were joined by a second round of PCR using both fragments as template DNA and primers FP1(GTG) and RP1. The final PCR product was digested with SalI and SphI and ligated into pBAD33.

**pSVN203.** *hipT*<sup>S57A+S59A</sup> with start codon GTG was created by a two-step PCR reaction. Two fragments were amplified from pKG127 using primers FP1(GTG) and HipT S57A + S59A Rv in one reaction and HipT S57A + S59A Fw and RP1 in the other. The resulting two PCR products were joined by a second round of PCR using both fragments as template DNA and primers FP1(GTG) and RP1. The final PCR product was digested with SalI and SphI and ligated into pBAD33.
**pSVN205.** hipT<sup>S57D+S59A</sup> with start codon GTG was created by a two-step PCR reaction. Two fragments were amplified from pKG127 using primers FP1(GTG) and HipT S57D + S59A Rv in one reaction and HipT S57D + S59A Fw and RP1 in the other. The resulting two PCR products were joined by a second round of PCR using both fragments as template DNA and primers FP1(GTG) and RP1. The final PCR product was digested with SalI and SphI and ligated into pBAD33.

**pSVN207.** hipT<sup>S57A+S59D</sup> with start codon GTG was created by a two-step PCR reaction. Two fragments were amplified from pKG127 using primers FP1(GTG) and HipT S57A + S59D Rv in one reaction and HipT S57A + S59D Fw and RP1 in the other. The resulting two PCR products were joined by a second round of PCR using both fragments as template DNA and primers FP1(GTG) and RP1. The final PCR product was digested with SalI and SphI and ligated into pBAD33.

**pSVN209.** hipT<sup>S57D+S59D</sup> with start codon GTG was created by a two-step PCR reaction. Two fragments were amplified from pKG127 using primers FP1(GTG) and HipT S57D + S59D Rv in one reaction and HipT S57D + S59D Fw and RP1 in the other. The resulting two PCR products were joined by a second round of PCR using both fragments as template DNA and primers FP1(GTG) and RP1. The final PCR product was digested with SalI and SphI and ligated into pBAD33.

**pSVN211.** hipT<sup>D210Q</sup> with start codon GTG was created by a two-step PCR reaction. Two fragments were amplified from pSVN1 using primers FP1(GTG) and hipX D210Q Rv in one reaction and hipX D210Q Fw and RP1 in the other. The resulting two PCR products were joined by a second round of PCR using both fragments as template DNA and primers FP1(GTG) and RP1. The final PCR product was digested with SalI and SphI and ligated into pBAD33.
pSVN212. *hipT*<sub>S57D+D210Q</sub> with start codon GTG was created by a two-step PCR reaction. Two fragments were amplified from pSVN194 using primers FP1(GTG) and hipX D210Q Rv in one reaction and hipX D210Q Fw and RP1 in the other. The resulting two PCR products were joined by a second round of PCR using both fragments as template DNA and primers FP1(GTG) and RP1. The final PCR product was digested with SalI and SphI and ligated into pBAD33.

pSVN213. *hipT*<sub>S59D+D210Q</sub> with start codon GTG was created by a two-step PCR reaction. Two fragments were amplified from pSVN195 using primers FP1(GTG) and hipX D210Q Rv in one reaction and hipX D210Q Fw and RP1 in the other. The resulting two PCR products were joined by a second round of PCR using both fragments as template DNA and primers FP1(GTG) and RP1. The final PCR product was digested with SalI and SphI and ligated into pBAD33.

pSVN214. *hipT*<sub>S57A+D210Q</sub> with start codon GTG was created by a two-step PCR reaction. Two fragments were amplified from pSVN199 using primers FP1(GTG) and hipX D210Q Rv in one reaction and hipX D210Q Fw and RP1 in the other. The resulting two PCR products were joined by a second round of PCR using both fragments as template DNA and primers FP1(GTG) and RP1. The final PCR product was digested with SalI and SphI and ligated into pBAD33.

pSVN215. *hipT*<sub>S59A+D210Q</sub> with start codon GTG was created by a two-step PCR reaction. Two fragments were amplified from pSVN201 using primers FP1(GTG) and hipX D210Q Rv in one reaction and hipX D210Q Fw and RP1 in the other. The resulting two PCR products were joined by a second round of PCR using both fragments as template DNA and primers FP1(GTG) and RP1. The final PCR product was digested with SalI and SphI and ligated into pBAD33.
pSVN216. *hipT*<sup>S57A+S59A+D210Q</sup> with start codon GTG was created by a two-step PCR reaction. Two fragments were amplified from pSVN203 using primers FP1(GTG) and hipX D210Q Rv in one reaction and hipX D210Q Fw and RP1 in the other. The resulting two PCR products were joined by a second round of PCR using both fragments as template DNA and primers FP1(GTG) and RP1. The final PCR product was digested with Sall and SphI and ligated into pBAD33.

pSVN217. *hipT*<sup>S57D+S59A+D210Q</sup> with start codon GTG was created by a two-step PCR reaction. Two fragments were amplified from pSVN205 using primers FP1(GTG) and hipX D210Q Rv in one reaction and hipX D210Q Fw and RP1 in the other. The resulting two PCR products were joined by a second round of PCR using both fragments as template DNA and primers FP1(GTG) and RP1. The final PCR product was digested with Sall and SphI and ligated into pBAD33.

pSVN218. *hipT*<sup>S57A+S59D+D210Q</sup> with start codon GTG was created by a two-step PCR reaction. Two fragments were amplified from pSVN207 using primers FP1(GTG) and hipX D210Q Rv in one reaction and hipX D210Q Fw and RP1 in the other. The resulting two PCR products were joined by a second round of PCR using both fragments as template DNA and primers FP1(GTG) and RP1. The final PCR product was digested with Sall and SphI and ligated into pBAD33.

pSVN219. *hipT*<sup>S57D+S59D+D210Q</sup> with start codon GTG was created by a two-step PCR reaction. Two fragments were amplified from pSVN209 using primers FP1(GTG) and hipX D210Q Rv in one reaction and hipX D210Q Fw and RP1 in the other. The resulting two PCR products were joined by a second round of PCR using both fragments as template DNA and primers FP1(GTG) and RP1. The final PCR product was digested with Sall and SphI and ligated into pBAD33.
**pSVN220.** hipT\(^{D210Q}\) with optimized SD was amplified from pSVN211 using primers FP48 and RP11. The resulting PCR product was digested with PstI and HindIII and ligated into pBAD33.

**pSVN221.** hipT\(^{S57D+D210Q}\) with optimized SD was amplified from pSVN212 using primers FP48 and RP11. The resulting PCR product was digested with PstI and HindIII and ligated into pBAD33.

**pSVN222.** hipT\(^{S59D+D210Q}\) with optimized SD was amplified from pSVN213 using primers FP48 and RP11. The resulting PCR product was digested with PstI and HindIII and ligated into pBAD33.

**pSVN223.** hipT\(^{S57A+D210Q}\) with optimized SD was amplified from pSVN214 using primers FP48 and RP11. The resulting PCR product was digested with PstI and HindIII and ligated into pBAD33.

**pSVN224.** hipT\(^{S59A+D210Q}\) with optimized SD was amplified from pSVN215 using primers FP48 and RP11. The resulting PCR product was digested with PstI and HindIII and ligated into pBAD33.

**pSVN225.** hipT\(^{S57A+S59A+D210Q}\) with optimized SD was amplified from pSVN216 using primers FP48 and RP11. The resulting PCR product was digested with PstI and HindIII and ligated into pBAD33.

**pSVN226.** hipT\(^{S57D+S59A+D210Q}\) with optimized SD was amplified from pSVN217 using primers FP48 and RP11. The resulting PCR product was digested with PstI and HindIII and ligated into pBAD33.

**pSVN227.** hipT\(^{S57A+S59D+D210Q}\) with optimized SD was amplified from pSVN218 using primers FP48 and RP11. The resulting PCR product was digested with PstI and HindIII and ligated into pBAD33.
pSVN228. $hipT^{S57D+S59D+D210Q}$ with optimized SD was amplified from pSVN219 using primers FP48 and RP11. The resulting PCR product was digested with PstI and HindIII and ligated into pBAD33.

pSNN1. $hipT^{S57A}$ with optimized SD was amplified from pSVN78 using primers $hipB$ del Fw and $hipB$ del Rv. This resulted in the deletion of HipB and HipS.

pSNN2. $hipT^{S57A+D210A}$ with optimized SD was amplified from pSNN1 using primers $hipT$ D210A Fw and $hipT$ D210A Rv.

pMME3. $hipB^{S59A}$ with optimized SD was amplified from pSVN78 using primers $hipT$ S57S59A Fw and $hipT$ S57S59A Rv to introduce the mutations A57S and S59A.
SUPPLEMENTARY FIGURE LEGENDS

Figure S1. Sequence alignment with consensus structural elements of HipBST, HipBA\textsubscript{Ec} and HipBA\textsubscript{So}. a. Alignment of \textit{E. coli} O127:H6 HipB with HipB from \textit{E. coli} K-12 HipBA and HipB from \textit{S. oneidensis} HipBA. Secondary structure observed in \textit{E. coli} O127:H6 HipB is shown above the sequences, except for \(\alpha 1^*\), which is missing from the crystal structures but predicted by JPred4 (Drozdetskiy et al., 2015). b. Alignment of \textit{E. coli} O127:H6 HipS with the N-terminal subdomain 1 from \textit{E. coli} K-12 HipA and \textit{S. oneidensis} HipA. The position of the Gly-rich interacting helix (\(\alpha 3\)) of HipS is indicated. Secondary structure observed in \textit{E. coli} O127:H6 HipS is shown above the sequences c. Alignment of \textit{E. coli} O127:H6 HipT with the main kinase domain of \textit{E. coli} K-12 HipA and \textit{S. oneidensis} HipA. The position of the Gly-rich loop (residue 58-63) and phosphorylation sites (Ser57 and Ser59), catalytic motif (Asp210), and Mg\(^{2+}\)-binding motif (Asp233), are indicated. Secondary structure observed in \textit{E. coli} O127:H6 HipT is shown above the sequences.

Figure S2. Biophysical analysis of the HipBST complex. a. Sequence alignment of \textit{E. coli} O127:H6 and selected HipS orthologues with the N-terminal subdomain 1 of \textit{E. coli} K-12 HipA (bottom). Secondary structure as observed for \textit{E. coli} O127:H6 HipS is indicated above the alignment. Fully conserved residues are shown on a dark orange background, partially conserved residues in orange text, and regions that interact with HipT are shown on a light grey background. Trp65 that intercalates in HipT is shown on a blue background. b. Size exclusion chromatography profile as observed for HipBST\textsuperscript{D233Q}. A similar elution profile was observed for the other HipBST variants used for structural studies. Absorption at 280 (A\textsubscript{280}, blue) and 260 (A\textsubscript{260}, red) nm are shown along with elution volumes of a range of standard proteins (6.5-440 kDa, as indicated). c. The structure of \textit{S. oneidensis} HipBA (PDB: 4PU3, HipA purple, HipB green) bound to DNA (orange backbone, blue bases) (Wen et al., 2014).
Figure S3. Interactions of HipT with HipS and HipB. a. Overall fold of HipS with secondary structure elements labelled (α helices in red and β sheets in yellow). b. Structural comparison between *E. coli* O127:H6 HipB (cartoon, two shades of green) and the corresponding homodimer of *S. oneidensis* HipB (PDB: 4PU3, ribbons, cyan) showing their conserved structure with similar HTH folds (Wen et al., 2014). c. Top, structural alignment of the *E. coli* O127:H6 HipBST complex (HipT, blue; HipS, beige; HipB, green) with *S. oneidensis* HipBA bound to DNA (PDB: 4PU3; HipA, purple; HipB, green) (Wen et al., 2014); Below, close-up views of conserved, basic residues on HipA that contact the DNA backbone (left) and the corresponding region in HipT (right). d. Growth curves of *E. coli* MG1655 grown in YT medium and harbouring empty pBAD33 vector ("pBAD33") or pSVN1 (pBAD33::*hipT*, "*hipT*"), in combination with empty pNDM220 vector ("pNDM220"), pSVN109 (pNDM220::*hipS*, "*hipS*"), or pSVN178 (pNDM220::*hipS*W65A, "*hipS*W65A") as indicated. At the indicated times, 0.2% arabinose was added to induce *hipT* (long arrow) and 200 µM IPTG was added to induce *hipS* or *hipS*W65A (short arrow). The data points represent mean values of results from at least three independent experiments, and error bars show standard deviations (hidden when small).

Figure S4. HipT autophosphorylation at Ser57 and Ser59 affects function differentially. a. Active site region and Gly-rich loop (ruby) of *E. coli* O127:H6 HipT from the HipBSTS57A structure showing the 2mFo-DFc difference electron density covering Ser59, contoured at 1.2σ. The phosphate group was refined to an occupancy of 0.89. b. Similar view from the HipBSTS59A structure showing the 2mFo-DFc difference electron density covering Ser57, contoured at 1.0σ. The phosphate group was refined to an occupancy of 0.72. c. Top, structural alignment of the active sites of *E. coli* O127:H6 HipT S57A (blue) with *E. coli* K-12 HipA.
(HipAEc, purple) highlighting the positions of their respective phosphoserine (P-Ser) residues, as indicated; Bottom, equivalent structural alignment of the active sites of E. coli O127:H6 HipT S59A (blue) with S. oneidensis HipA (HipASo, pale blue). d. Growth curves of E. coli MG1655 strains grown in YT medium and harbouring empty pBAD33 vector, pSVN1 (pBAD33::hipT, "hipT (SIS, wt)"), or single autophosphorylation mutants pSVN194 (pBAD33::hipTS57D, "hipT (DIS)"), pSVN195 (pBAD33::hipTS59D, "hipT (SID)"), pSVN199 (pBAD33::hipTS57A, "hipT (AIS)"), or pSVN201 (pBAD33::hipTS59A, "hipT (SIA)"") in combination with pSVN110 (pNDM220::hipB-S, "hipBS")]. At the indicated times, 0.2% arabinose was added to induce hipT (long arrow) and 200 µM was added to induce hipBS (short arrow). The data points represent mean values of results from two independent experiments, and error bars show standard deviations (hidden when small). e. As in d but for double autophosphorylation mutants pSVN203 (pBAD33::hipTS57A+S59A, "hipT (AIA)"), pSVN205 (pBAD33::hipTS57D+S59A, "hipT (DIA)"), pSVN207 (pBAD33::hipTS57A+S59D, "hipT (AID)"), and pSVN209 (pBAD33::hipTS57D+S59D, "hipT (DID)""). Data points represent mean values of results from two independent experiments, and error bars designate standard deviations. f. Northern blot analysis using probe directed against tRNA_gltTUVW (top) and tRNA_argVYZQ (bottom) based on RNA extracted from E. coli MG1655 harbouring pSVN110 (pNDM220::hipB-S) in combination with pSVN1 (pBAD33::hipT, "hipT (S57S59, wt)"), pSVN194 (pBAD33::hipTS57D, "hipT (D57S59)"), pSVN195 (pBAD33::hipTS59D, "hipT (S57D59)"), pSVN209 (pBAD33::hipTS57D+S59D, "hipT (D57D59)"") or pSVN205 (pBAD33::hipTS57D+S59A, "hipT (D57A59)"") at indicated time points in minutes before (-) and after (+) addition of 0.2% arabinose to induce wildtype hipT or autophosphorylation mutants. Results from strains harbouring pSVN1 (pBAD33::hipT), pSVN194 (pBAD33::hipTS57D), pSVN209 and (pBAD33::hipTS57D+S59D) are representative of two independent experiments,
whereas results from strains harbouring pSVN195 (pBAD33::hipTS59D) and pSVN205 (pBAD33::hipTS57D+S59A) are from single experiments.

Figure S5. Transcriptional regulation of the HipBST system of E. coli O127:H6. a. Transcriptional reporter assay using a plasmid with the hipBST promoter region and a 5' fragment of the hipB gene (including 224 bp upstream of hipB plus the first 73 bp of the hipB gene, shown with a lightening symbol) transcriptionally fused to lacZ. E. coli TB28 harbouring the reporter plasmid (pSVN141, pGH254::PhipBST-hipB'-lacZ) and empty pBAD33 vector or combinations of hipB, hipS, and hipT in the context of the HipT^{D233Q} inactive mutant; pSVN180 (pBAD33::hipB-S-T^{D233Q}, "hipBST^{D233Q}"), pSVN182 (pBAD33::hipB-S, "hipBS"), pSVN184 (pBAD33::hipB-T^{D233Q}, "hipBT^{D233Q}"), pSVN187 (pBAD33::hipS-T^{D233Q}, "hipST^{D233Q}"), pSVN189 (pBAD33::hipB, "hipB"), pSVN190 (pBAD33::hipS, "hipS"), or pSVN192 (pBAD33::hipT^{D233Q}, "hipT^{D233Q}") were grown, diluted, and spotted onto YT agar plates containing 40 µg/ml X-gal and 0.2% glucose (to repress hipB/S/T^{D233Q}) or 0.2% arabinose (to induce hipB/S/T^{D233Q}). Results are representative of two independent experiments. b. Overexpression of inactive HipT^{D210Q} and HipT^{D233Q} increases the level of active wildtype HipT from the hipBST operon transcriptionally fused to lacZ in E. coli TB28. The strain harbouring empty pGH254 vector or pSVN198 (pGH254::PhipBST-hipBST-lacZ, "hipBST") in combination with the empty pBAD33 vector, pSVN192 (pBAD33::hipT^{D210Q}, "hipT^{D210Q}"), or pSVN193 (pBAD33::hipT^{D233Q}, "hipT^{D233Q}") as indicated, were grown, diluted, and spotted onto YT agar plates containing 40 µg/ml X-gal without 0.2% arabinose (to obtain only a leaky expression of hipT^{D210Q/D233Q}), or with 0.2% arabinose (to induce hipT^{D210Q/D233Q}). Results are from a single experiment. c. Primer extension analysis mapping the transcriptional start site in E. coli TB28 harbouring pSVN141 (pGH254::PhipBST-hipB'-lacZ), in combination with the empty pBAD322T vector, or pSVN154 (pBAD322T::hipB-S-T^{D210Q}), as indicated were grown
and harvested at OD$_{600}$≈0.4. d. Primer extension analysis mapping the transcriptional start site in E. coli O127:H6 harbouring pSVN192 (pBAD33::hipT$^{D210Q}$). At OD$_{600}$≈0.4, 0.2% arabinose was added (to induce hipT$^{D210Q}$), samples were taken at indicated time points and primer extension performed. e. The hipBST promoter region showing the transcriptional start site (arrow) and corresponding -10 and -35 sequences (red). The start codon of the hipB gene ("Met") and putative Shine-Dalgarno sequence ("SD") are indicated as well. f. Spot assays using E. coli MG1655 harbouring pSVN111 (pNDM220::hipB) in combination with the empty pBAD33 vector, pSVN211 (pBAD33::hipT$^{D210Q}$, "hipT (S$^{57}$IS$^{59}$, wt)"), pSVN212 (pBAD33::hipT$^{S57D+D210Q}$, "hipT (D$^{57}$IS$^{59}$)"), pSVN213 (pBAD33::hipT$^{S59D+D210Q}$, "hipT (S$^{57}$ID$^{59}$)"), pSVN214 (pBAD33::hipT$^{S57A+D210Q}$, "hipT (A$^{57}$IS$^{59}$)"), pSVN215 (pBAD33::hipT$^{S59A+D210Q}$, "hipT (S$^{57}$IA$^{59}$)")), pSVN216 (pBAD33::hipT$^{S57A+S59A+D210Q}$, "hipT (A$^{57}$IA$^{59}$)"), pSVN217 (pBAD33::hipT$^{S57D+S59A+D210Q}$, "hipT (D$^{57}$IA$^{59}$)"), pSVN218 (pBAD33::hipT$^{S57A+S59D+D210Q}$, "hipT (S$^{57}$ID$^{59}$)"), or pSVN219 (pBAD33::hipT$^{S57D+S59D+D210Q}$, "hipT (D$^{57}$ID$^{59}$)") spotted onto YT agar plates containing 0.2% glucose (to repress hipT$^{D210Q}$ autophosphorylation mutants), 0.2% arabinose (to induce hipT$^{D210Q}$ autophosphorylation mutants) or 0.2% arabinose plus 500 µM IPTG (to induce hipB). Results are from a single experiment.

Figure S6. Analysis of HipBST using small-angle x-ray scattering. a. Background-subtracted and normalized SAXS data measured for HipBST$^{S57A}$ with indirect Fourier transform fit. b. Guinier plot. c. $p(r)$ from indirect Fourier transform. d. Normalized Kratky plot using $I(0)$ and $R_g$ from the Guinier fit.

Figure S7. HipT is structurally similar to eukaryotic cyclin-dependent kinases (Cdks). a. Domain overview (top) and secondary structure comparison (bottom) between E. coli O127:H6
HipT and human CDK5 showing the unique N-terminal mini-domain found in HipT (dashed) and that the N-terminal subdomain 2 is similar to the N-terminal lobe of CDK5 while the core kinase domain is similar to the C-terminal lobe. Conserved features and active site residues are marked with numbers and listed below. 

b. Structural comparison of *E. coli* O127:H6 HipT (top) and human CDK5 (PDB: 4AU8, bottom) (Malmstrom et al., 2012) shown in rainbow colours from N (blue) to C (red) terminus. α-helices are shown as cylinders and for CDK5 named in accordance with classical Cdk nomenclature. The two proteins differ in tertiary fold despite similar topology. c. Top, overview structure of Human CDK9 (salmon, PDB: 3BLT) with the C-spine region shown as surface (cyan) (Baumli et al., 2008); below, details of the active site bound to ATP with relevant residues indicated. d. Top, overview of the *E. coli* O127:H6 HipT structure with the C/R spine region shown as surface (green); below, details of the active site. e. Top, overview of the *E. coli* K-12 HipA structure (PDB: 3DNT) with the C/R spine region shown as surface (orange) (Schumacher et al., 2009); below, details of the active site bound to ATP.
REFERENCES


Figure S1

**a**

E. coli O127:H6 HipB (HipBST)

E. coli O127:H6 HipB (HipBST)

E. coli K-12 HipB (HipBa)

S. oneidensis HipB (HipBa)

**b**

E. coli O127:H6 HipS (HipBST)

E. coli O127:H6 HipS (HipBST)

E. coli K-12 HipA (HipBa)

S. oneidensis HipA (HipBa)

**c**

E. coli O127:H6 HipT (HipBST)

E. coli O127:H6 HipT (HipBST)

E. coli K-12 HipA (HipBa)

S. oneidensis HipA (HipBa)

S. oneidensis HipA (HipBa)
Figure S2  

bioRxiv preprint doi: https://doi.org/10.1101/2022.01.28.478185; this version posted January 31, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under a CC-BY 4.0 International license.
Figure S4

- **Figure S4**

  A graph showing the OD600 over time for different samples. The graph includes data for samples labeled as pBAD33/hipBS, hipT (SIS, wt)/hipBS, hipT (DIS)/hipBS, hipT (SIS)/hipBS, hipT (DIS)/hipBS, and hipT (SIS)/hipBS. The times post induction are -15, 15, 30, 60, and the OD600 values are plotted against time.

  - **Legend:**
    - pBAD33/hipBS
    - hipT (SIS, wt)/hipBS
    - hipT (DIS)/hipBS
    - hipT (SIS)/hipBS
    - hipT (DIS)/hipBS
    - hipT (SIS)/hipBS

  - **Table:**
    | Time post induction | -15 | +15 | +30 | -15 | +15 | +30 | -15 | +30 | +60 |
    |---------------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|
    | control             |     |     |     |     |     |     |     |     |     |
    | Glu-tRNA<sup>GTUW</sup> |     |     |     |     |     |     |     |     |     |
    | tRNA<sup>GTUW</sup>  |     |     |     |     |     |     |     |     |     |
    | Arg-tRNA<sup>GTUQ</sup> |     |     |     |     |     |     |     |     |     |
    | tRNA<sup>GTUQ</sup>  |     |     |     |     |     |     |     |     |     |
glucose (represses hipBST)
arabinose (induces hipBST)
PBAD33
hipBST D233Q
hipBS
hipBT D233Q
hipST D233Q
hipB
hipS
hipT D233Q

Figure S5

A

Figure S5 bioRxiv preprint doi: https://doi.org/10.1101/2022.01.28.478185; this version posted January 31, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under a CC-BY 4.0 International license.
Figure S6
Figure S7

1: Gly-rich motif  3: Glu99/Glu51  5: Asn215/Asn131
2: Lys86/Lys33  4: Asp210/Asp126  6: Asp233/Asp144

bioRxiv preprint doi: https://doi.org/10.1101/2022.01.28.478185; this version posted January 31, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under a CC-BY 4.0 International license.