A plasmid-encoded *Klebsiella michiganensis* HipBA type II toxin–antitoxin system makes a significant contribution to plasmid maintenance

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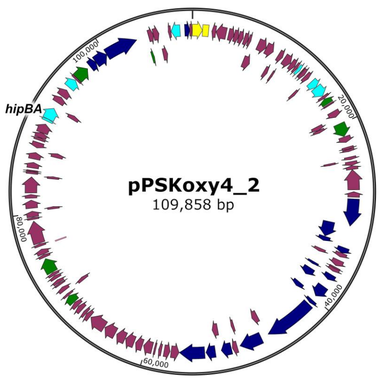
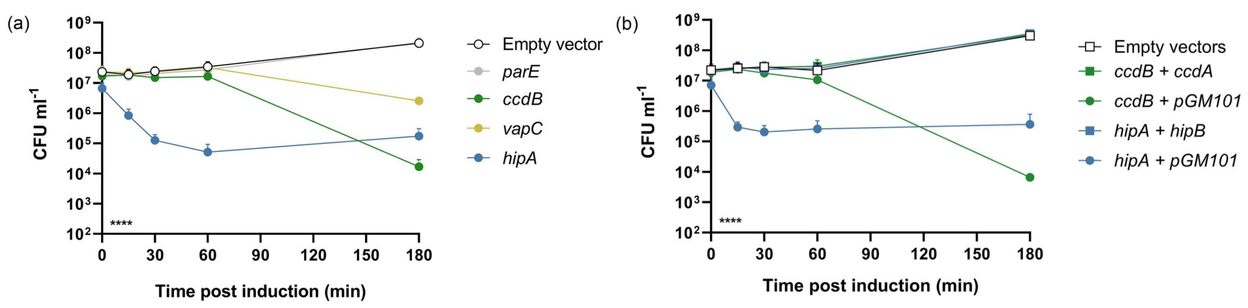
Type II toxin–antitoxin (TA) systems are commonly found encoded on the mobile genetic elements and chromosomes of both Gram-positive and Gram-negative bacteria. They serve numerous roles within the bacterial cell and were originally characterized as post-segregational killing (PSK) or “addiction” systems. Such systems function through the interaction of a stable toxic protein, which causes bacterial cell lysis or cessation of growth, and a labile antitoxin protein that directly sequesters and abrogates the effect of the toxin but is itself actively and specifically degraded by cellular proteases. During PSK, loss of the mobile genetic element encoding a type II TA system allows the antitoxin pool to be rapidly depleted while the toxin remains stable, preventing the growth of any daughter cells that lack the mobile genetic element. To date, TA systems of *Klebsiella* species have received little attention.

The HipBA TA system is typically encoded on bacterial chromosomes where it contributes to antimicrobial tolerance by interfering with translation during cellular stress. Here, we show that plasmid-encoded HipBA from a clinical isolate of *Klebsiella michiganensis* (PS_Koxy4) is responsible for highly effective plasmid addiction; the first such evidence of a HipBA module contributing to plasmid stability. This has important implications for enteric pathogen evolution and horizontal gene transfer in the era of multidrug resistance.

PS_Koxy4 carries a large IncA/C plasmid encoding a highly toxic *hipBA* system

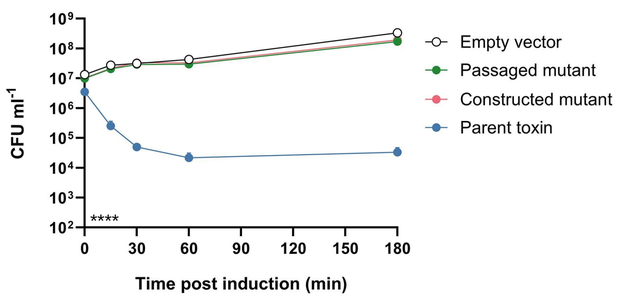


Map of the *K. michiganensis* IncA/C plasmid, pPSKoxy4_2. Yellow: plasmid replication. Dark blue: conjugation/transfer. Cyan: plasmid stability. Green: DNA modification/repair. The *hipBA* locus is indicated.



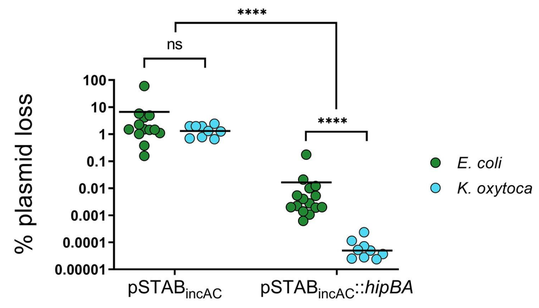
Characterization of plasmid-borne TA systems in PS_Koxy4. Mean bacterial viability after induction of the indicated putative toxin genes from pBAD33. (a) Gene expression induced alone in *E. coli* NEB 5-alpha. (b) Toxin gene expression induced in *E. coli* NEB 5-alpha in the presence (gene indicated) or absence (‘pGM101’; empty vector) of cognate antitoxin genes. Data are mean + SEM ($n \geq 3$ biological replicates). ****$P < .0001$ (two-way ANOVA with Tukey’s post testing); shows the overall effect of strain in each experiment.

Induced expression of *hipA* in *K. oxytoca* generates non-toxic mutants



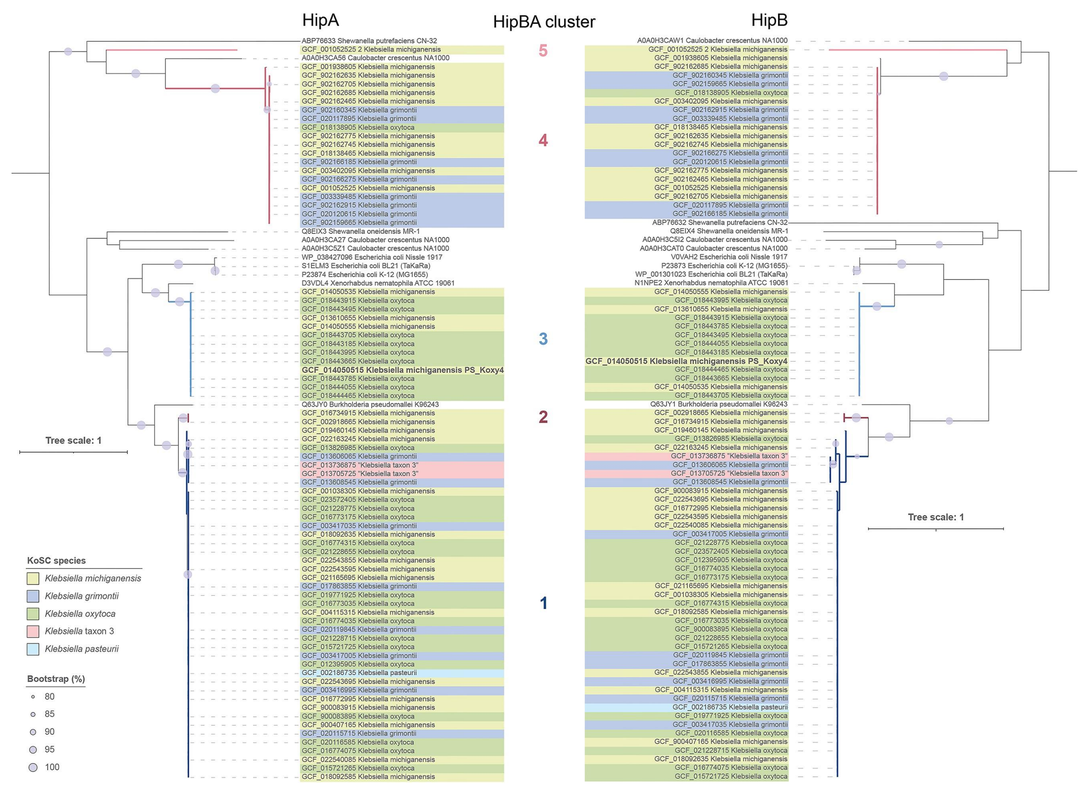
A non-toxic *hipA* mutant was generated by passage of expression vector through *K. oxytoca*. Mean bacterial viability after induction of expression from pBAD33: ‘Passaged mutant’ is a T175P variant extracted from *K. oxytoca* after a toxicity experiment; ‘constructed mutant’ is a T175P variant created through site-directed mutagenesis *in vitro*; ‘parent toxin’ is the wild-type variant. Gene expression induced in *E. coli* NEB 5-alpha. Data are mean + SEM. ****$P < .0001$ (two-way ANOVA with Tukey’s post testing); shows the overall effect of strain in each experiment.

PSKoxy_4 HipBA is a functional plasmid maintenance system



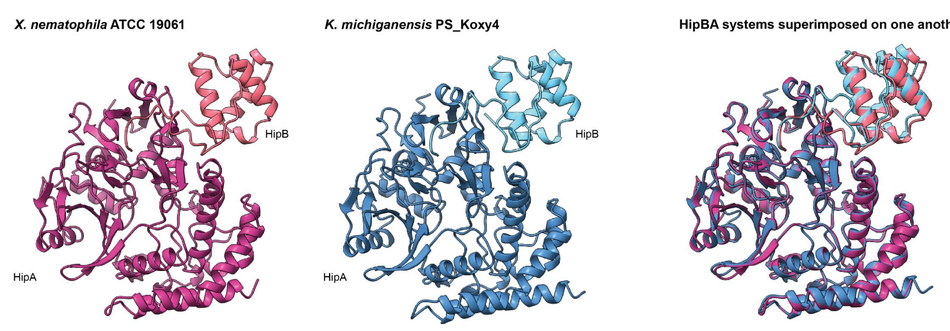
Plasmid loss of pSTAB$_{incAC}$ constructs in *E. coli* and *K. oxytoca* was determined by plating onto neomycin (plasmid$^+$) or sucrose (plasmid$^-$). Each point shows plasmid loss as a percentage of the total viable cell population within a single biological replicate (one independent colony grown on LA for 20–25 generations at 37 °C). Solid lines show the mean. Data were combined from at least two transformant clones per strain/plasmid combination and multiple independent experiments. ****$P < 0.0001$ (two-way ANOVA with Sidak’s post test); ns, not significant.

In silico analyses of HipBA in members of the *K. oxytoca* species complex



Phylogenetic relationships among plasmid-encoded HipA and HipB sequences of the *K. oxytoca* species complex. The trees (both rooted at the mid-point) were created using RAxML from MAFFT-generated multiple-sequence alignments and visualized using iToL. Bootstrap values are presented as a percentage of 100 replications

Plasmid-encoded HipBA is structurally similar to chromosomal HipBA



Generated using AlphaFold-multimer/ChimeraX

Structurally, the plasmid-encoded HipBA system of *K. michiganensis* PS_Koxy4 was almost identical to its closest experimentally validated TA system [sequence alignment score (matchmaker) of 1709.9 between the two HipA sequences; root mean square deviation between 421 pruned atom pairs was 0.488 Å, and across all 432 pairs was 0.816 Å].

We have provided the first experimental evidence that a plasmid-borne HipBA TA system can provide a formidable plasmid-stabilizing effect in both *E. coli* and *K. oxytoca*, most likely through the action of PSK. We have also identified similar plasmid-borne *hipBA* loci across the entirety of the *K. oxytoca* species complex. This adds substantially both to our knowledge of multiple medically-relevant *Klebsiella* species and to the mechanistic understanding of an important and widespread TA system in enteric bacteria.

Read the peer-reviewed, published manuscript

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