Supplemental Figure Legends

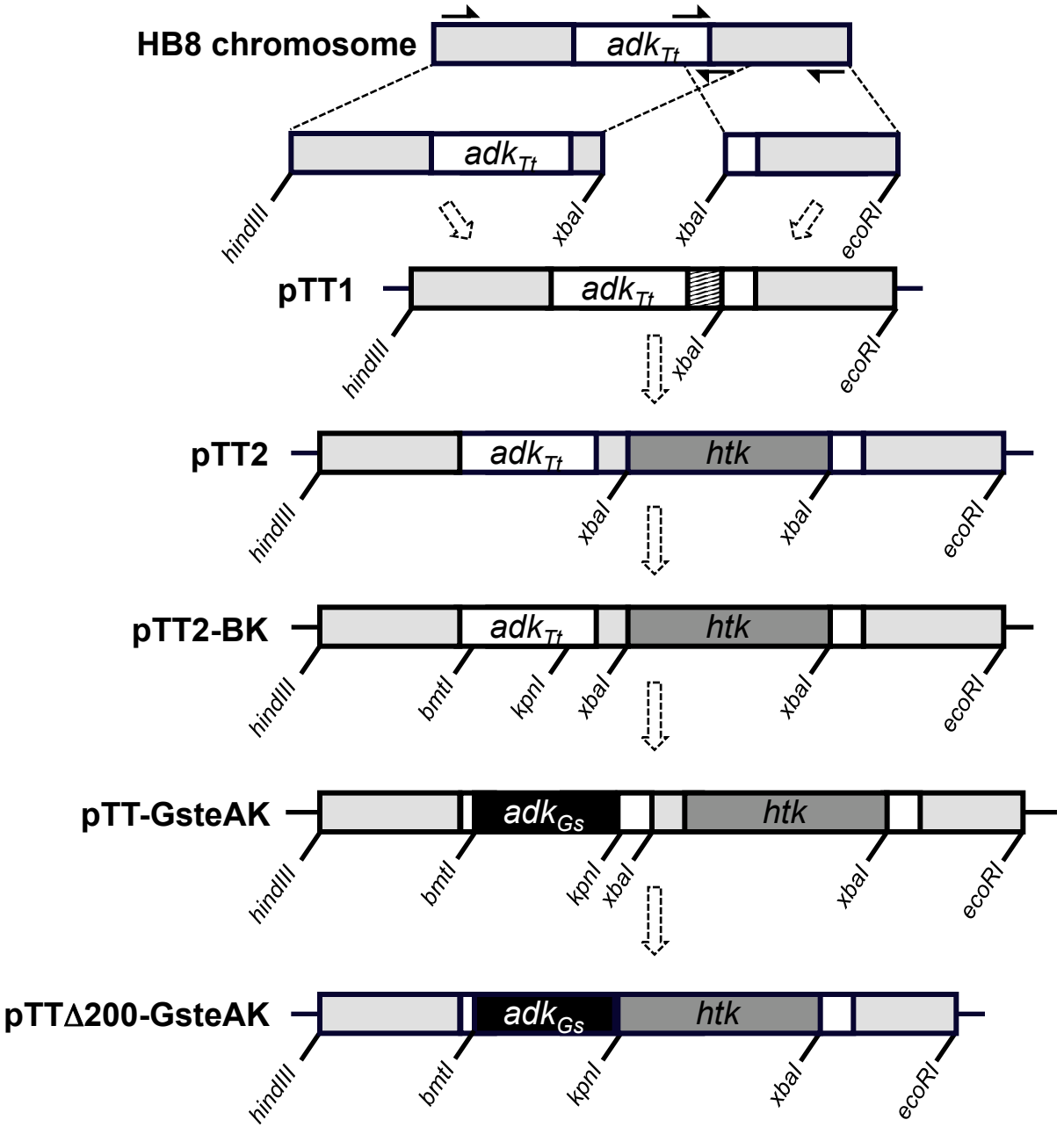
Fig. S1. Creation of an *adk* gene replacement vector. HB8 chromosomal DNA was PCR amplified and cloned into pUC18 to create pTT1, a vector harboring *T. thermophilus adk* (white) and approximately one thousand base pairs of adjacent chromosomal DNA (hatched). The DNA cloned into pTT1 contains a tandem duplication of the last 121 bp of adk_{Tt} and 45 bp of DNA adjacent to the adk_{Tt} stop codon. The gene encoding a thermostable kanamycin nucleotidyltransferase (*htk*) fused to a ribosomal binding site was cloned into the *xbaI* site of pTT1 to create pTT2. Quikchange mutagenesis was used to introduce *bmtI* and *kpnI* sites into pTT2 to create pTT2-BK, and the gene encoding *G. stearothermophilus* adenylate kinase (adk_{Gs}) was cloned into these new restriction sites to create pTT-GsteAK. To prevent chromosomal recombination of *htk* without adk_{Gs}, *htk* was subcloned into pTT-GsteAK using the *kpnI* and *xbaI* sites to create pTTΔ200-GsteAK. This vector contains the pUC18 origin of replication for *E. coli*, but it lacks an origin of replication for *T. thermophilus*.

Fig. S2. Creation of *E. coli–T. thermophilus* shuttle vectors for protein expression. (A) The *E. coli-T. thermophilus* shuttle vector pWUR112 containing a bleomycin selectable marker (*shble*) that has been engineered to function in hyperthmophiles was modified through Quikchange mutagenesis to remove the unique *ecoRI* and *xbaI* restriction sites and create pWUR112-ΔEX. An ampicillin resistance cassette (*bla*) amplified with primers that incorporated flanking *kpnI-ecoRI-xbaI* and *speI-pstI-kpnI* was cloned into the *kpnI* site of pWUR112-ΔEX to create pJJS. A PCR product containing the *T. thermophilus slpA* promoter with flanking *xbaI* and *speI-pstI* sites was cloned into pJJS using *xbaI* and *pstI* to create pJJS-Pro, and adk_{Tn} was PCR amplified with flanking *ecoRI-xbaI*, digested with XbaI and PstI, and clone into the *speI* and *pstI* sites of pJJS-Pro to create pJJS-TnAK. (B) The multicloning site of pJJS-Pro contains a promoter and ribosomal binding site.

Figure S3. The two-step approach used to create vectors that coexpress TnN and TnC^{C156A} protein fusions from a single polycistronic transcript. Plasmids were first created that have all pairwise combinations of *tnN* and tnC^{C156A} fused to *cheA*, *cheX*, and *cheY* adjacent to a ribosomal binding site. In step 1, the genes encoding each

TnC^{C156A} fragment fusion were digested with EcoRI and SpeI, and cloned into the EcoRI and XbaI digested pJJS-Term vectors. In step 2, the vectors created in step 1 were digested with XbaI and PstI and cloned into SpeI and PstI digested plasmids harboring genes encoding each TnN fusion protein. In the resulting plasmids, three genes (shble, tnN fusions, tnC fusions) are transcribed as a single polycistronic transcript that is driven by the *slpA* promoter and terminated by the *slpA* terminator.

Figure S1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adk_Tt
hindIII
xbaI
xbaI
ecoRI
pTT1
adk_Tt
hindIII
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pTT2
adk_Tt
htk
hindIII
xbaI
xbaI
ecoRI
pTT2-BK
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kpnI
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xbaI
ecoRI
pTT-GsteAK
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bmtI
kpnI
xbaI
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bmtI
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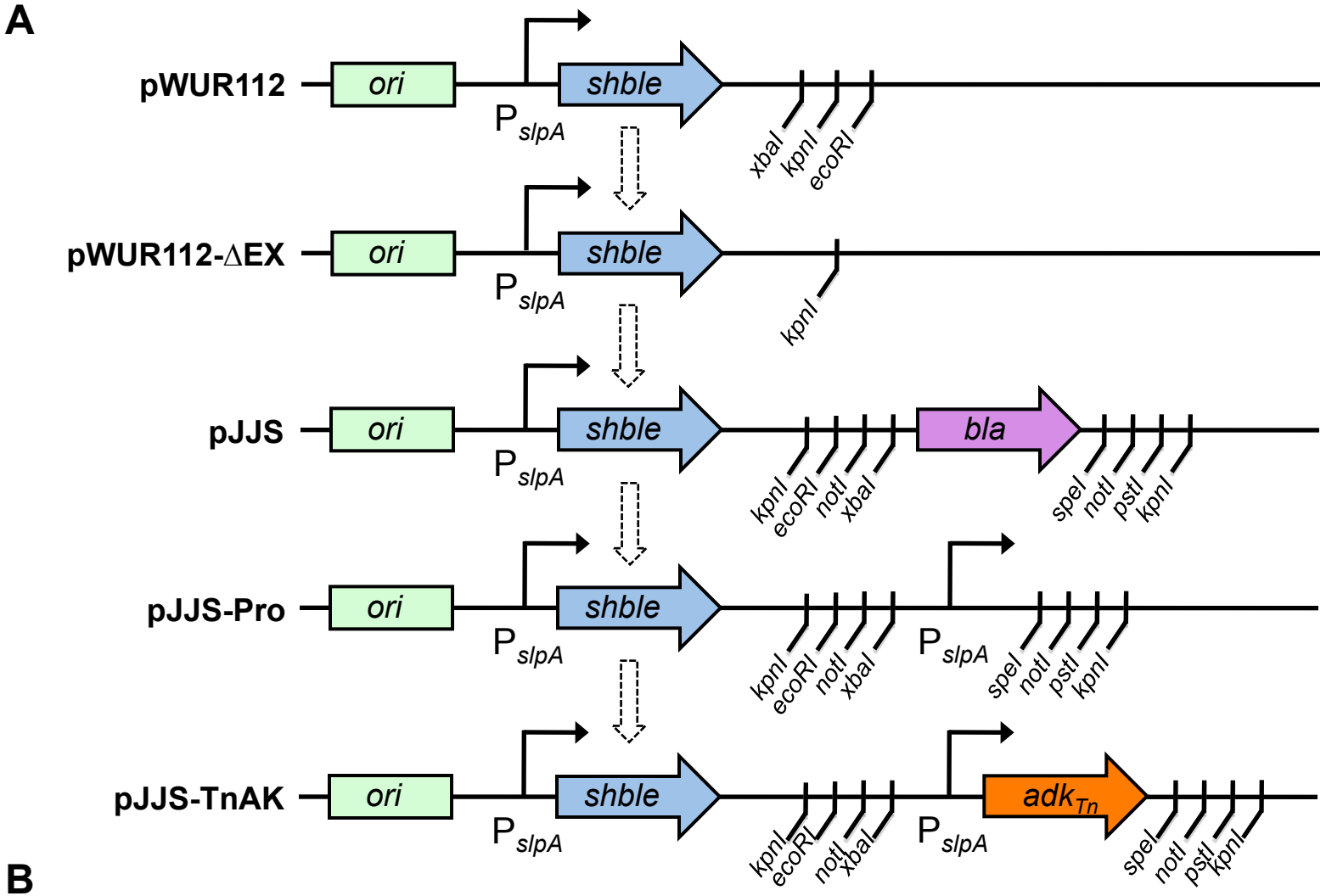
Figure S2



B

kpnI *ecoRI* *notI* *xbaI*

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ACGATAGCGCCGGATGCGGGGAAAAAGGGCTCCTTTTGGGGGGTTTTCCCCGCACCGGGCGGACCTGGGCGGAGAGGAAACGCGGCA

RBS *speI* *notI* *pstI* *kpnI*

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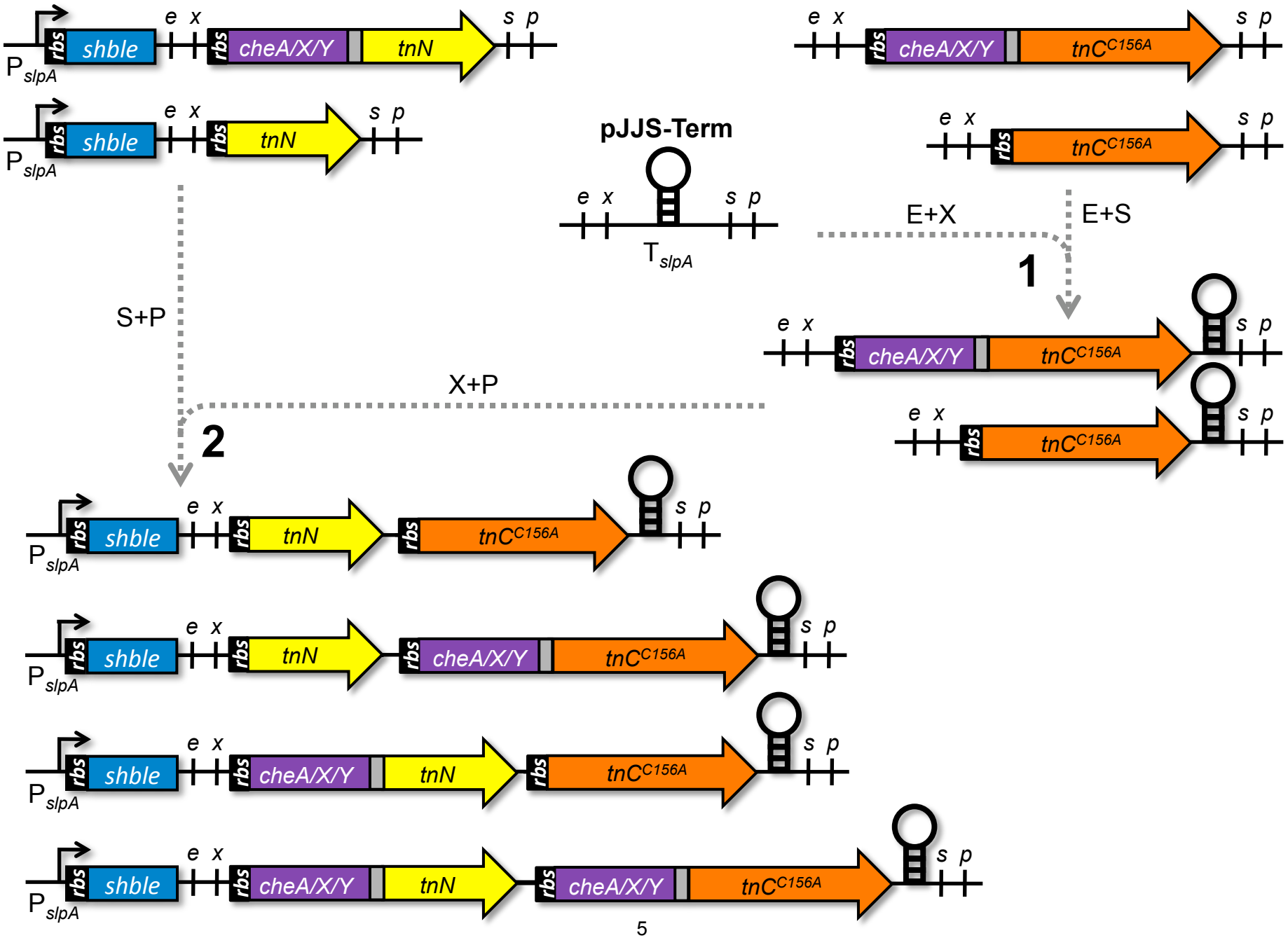
Figure S3


P slpA
rbs
shble
e x
cheA/X/Y
tnN
s p
tnC C156A
pJJS-Term
T slpA
E+X
E+S
S+P
X+P
1
2

Supplemental Tables

Table S1. Plasmids derived from pJJS that were used to construct protein expression vectors. The protein coding region cloned into pJJS is indicated as well as the regulatory elements cloned adjacent to that region.

Name	5' DNA	Protein coding region	3' DNA
pJJS‡	---	---	---
pJJS-Pro‡	P_{slpA}–RBS	---	---
pJJS-Term‡	---	---	T_{slpA}
pJJS-TnN	P_{slpA}–RBS	TnN	---
pJJS-TnC	P_{slpA}–RBS	TnC	---
pJJS-TnC133	P_{slpA}–RBS	TnC^{C133A}	---
pJJS-TnC156	P_{slpA}–RBS	TnC^{C156A}	---
pJJS-AN	P_{slpA}–RBS	$CheA^{P1P2}$–GASGGGSSGGHM–TnN	---
pJJS-XN	P_{slpA}–RBS	CheX–GASGGGSSGGHM–TnN	---
pJJS-YN	P_{slpA}–RBS	CheY–GASGGGSSGGHM–TnN	---
pJJS-AC156	P_{slpA}–RBS	$CheA^{P1P2}$–GASGGGSSGGHM-TnC^{C156A}	---
pJJS-XC156	P_{slpA}–RBS	CheX–GASGGGSSGGHM-TnC^{C156A}	---
pJJS-YC156	P_{slpA}–RBS	CheY–GASGGGSSGGHM–TnC^{C156A}	---
pJJS-rbsTnC156-T_{slpA}	RBS	TnC^{C156A}	T_{slpA}
pJJS-rbsAC156-T_{slpA}	RBS	$CheA^{P1P2}$–GASGGGSSGGHM–TnC^{C156A}	T_{slpA}
pJJS-rbsXC156-T_{slpA}	RBS	CheX–GASGGGSSGGHM–TnC^{C156A}	T_{slpA}
pJJS-rbsYC156-T_{slpA}	RBS	CheY–GASGGGSSGGHM–TnC^{C156A}	T_{slpA}
pJJS-rbsTnN	RBS	TnN	---
pJJS-rbsAN	RBS	$CheA^{P1P2}$–GASGGGSSGGHM–TnN	---
pJJS-rbsXN	RBS	CheX–GASGGGSSGGHM–TnN	---
pJJS-rbsYN	RBS	CheY–GASGGGSSGGHM–TnN	---

‡ Full sequence for vector is provided in supplement.

Table S2. Vectors used for complementation studies in *T. thermophilus* PQN1.

Name	Proteins expressed	Expression
pJJS-TnAK‡	AK_{Tn}	monocistronic
pJJS-N+C‡	TnN & TnC	monocistronic
pJJS-N+C133	TnN & TnC^{C133A}	monocistronic
pJJS-N+C156	TnN & TnC^{C156A}	monocistronic
pJJS-N+C156-poly	TnN & TnC^{C156A}	polycistronic
pJJS-YN+AC156	CheY–TnN & $CheA^{P1P2}$–TnC^{C156A}	polycistronic
pJJS-YN+XC156	CheY–TnN & CheX–TnC^{C156A}	polycistronic
pJJS-YN+YC156	CheY–TnN & CheY–TnC^{C156A}	polycistronic
pJJS-YN+C156	CheY–TnN & TnC^{C156A}	polycistronic
pJJS-N+AC156	TnN & $CheA^{P1P2}$–TnC^{C156A}	polycistronic
pJJS-XN+XC156	CheX–TnN & CheX–TnC^{C156A}	polycistronic
pJJS-N+XC156	TnN & CheX–TnC^{C156A}	polycistronic
pJJS-XN+C156	CheX–TnN & TnC^{C156A}	polycistronic
pJJS-XN+AC156	CheX–TnN & $CheA^{P1P2}$–TnC^{C156A}	polycistronic

‡Full sequence for vector is provided in supplement.

Supplemental DNA Sequences

DNA sequences are provided for the vector used for gene replacement (pTTΔ200-GsteAK), vectors frequently used in the construction of *T. thermophilus* expression vectors (pJJS, pJJS-Pro, and pJJS-Term), as well as representative examples of the expression vectors used for complementation studies (pJJS-TnAK and pJJS-N+C).

pTTΔ200-GsteAK (6,251 bp)

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pJJS (5,395 bp)

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pJJS-Pro (4,646 bp)

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pJJS-Term (4,574 bp)

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pJJS-TnAK (5,315 bp)

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GTC

pJJS-N+C (5,529 bp)

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