Supporting Information

DNA damage checkpoint activation affects peptidoglycan synthesis and late divisome components in *Bacillus subtilis*

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Running Title: Regulation of the DNA damage checkpoint
Key words: YneA, DNA damage checkpoint, FtsW, cell division.

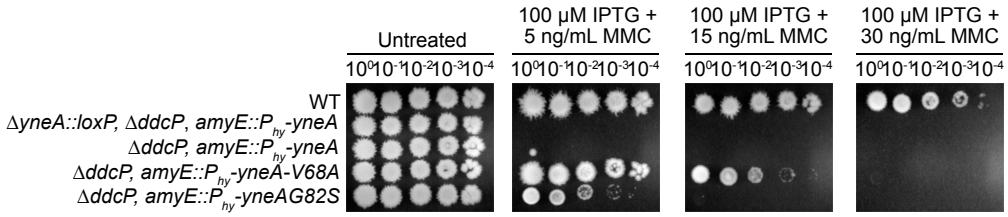

Figure S1. The *yneA* variants are recessive to native *yneA* in the presence of increasing concentrations of DNA damage. Spot titer assay using *B. subtilis* strains WT (PY79), Δ*yneA::loxP* Δ*ddcP amyE::P*$_{hy}$*-yneA* (EAM48), Δ*ddcP amyE::P*$_{hy}$*-yneA* (PEB681), Δ*ddcP amyE::P*$_{hy}$*-yneA-V68A* (EAM57) and Δ*ddcP amyE::P*$_{hy}$*-yneA-G82S* (EAM58) spotted on the indicated media.

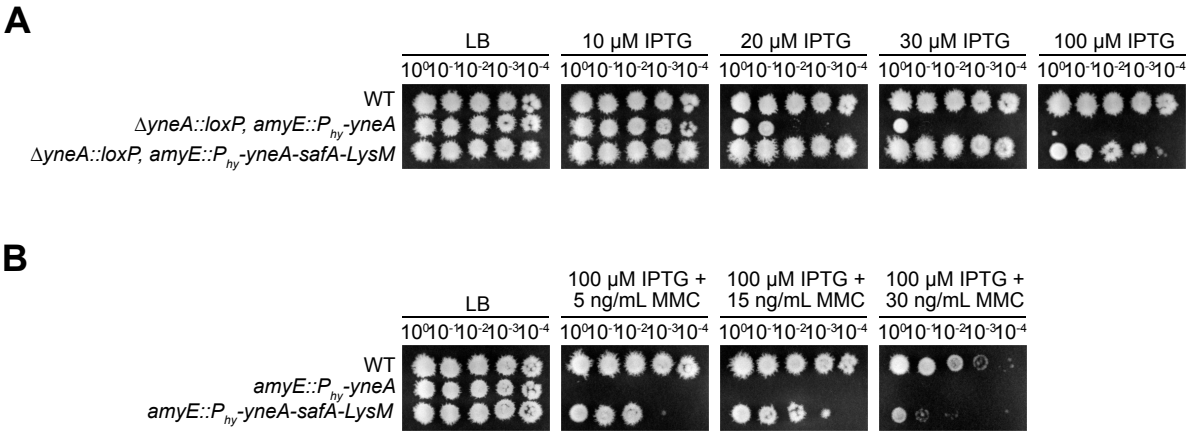

Figure S2. YneA LysM domain swap fails in checkpoint activation. Spot titer assay using *B. subtilis* strains WT (PY79), **(A)** Δ*yneA::loxP amyE::P$_{hy}$-yneA* (EAM46) and Δ*yneA::loxP amyE::P$_{hy}$-yneA-safA-LysM* (EAM80) **(B)** *amyE::P$_{hy}$-yneA* (PEB677) and *amyE::P$_{hy}$-yneA-safA-LysM* (EAM82) spotted on the indicated media.

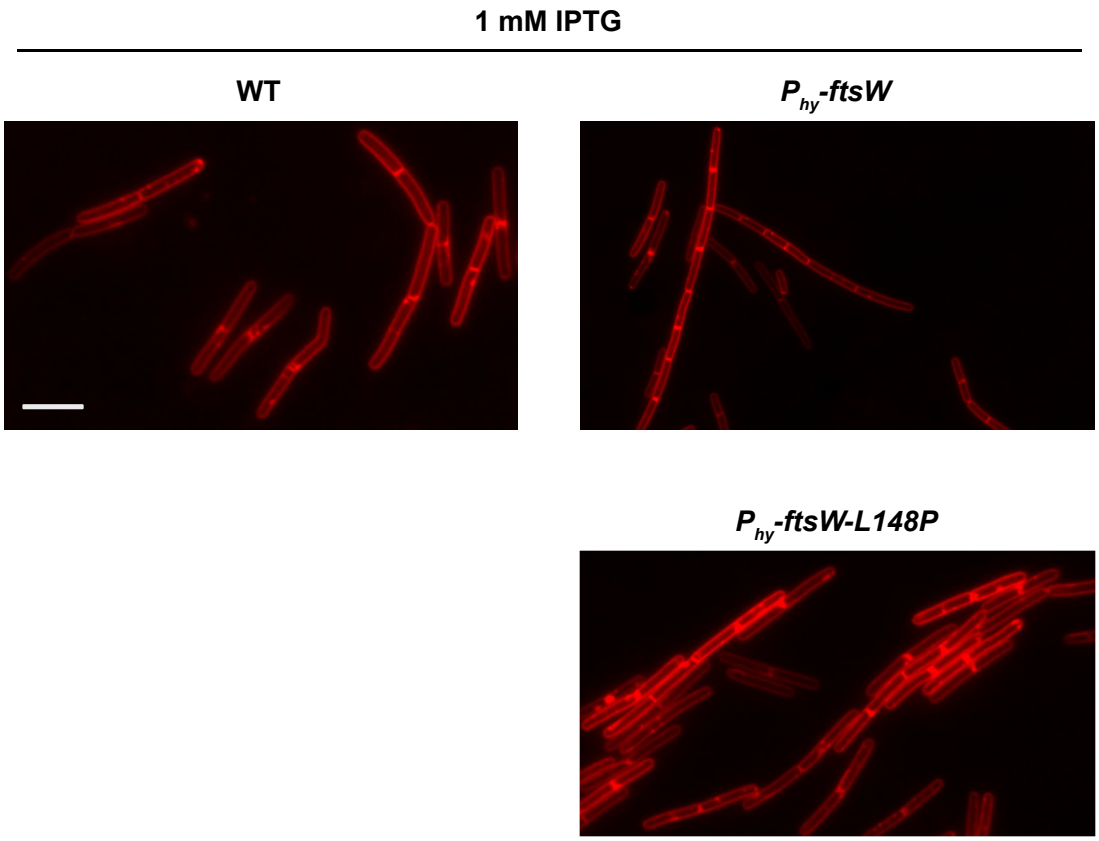

Figure S3. Representative images of WT, cells expressing FtsW or FtsW-L148P. The white bar corresponds to 10 μm.

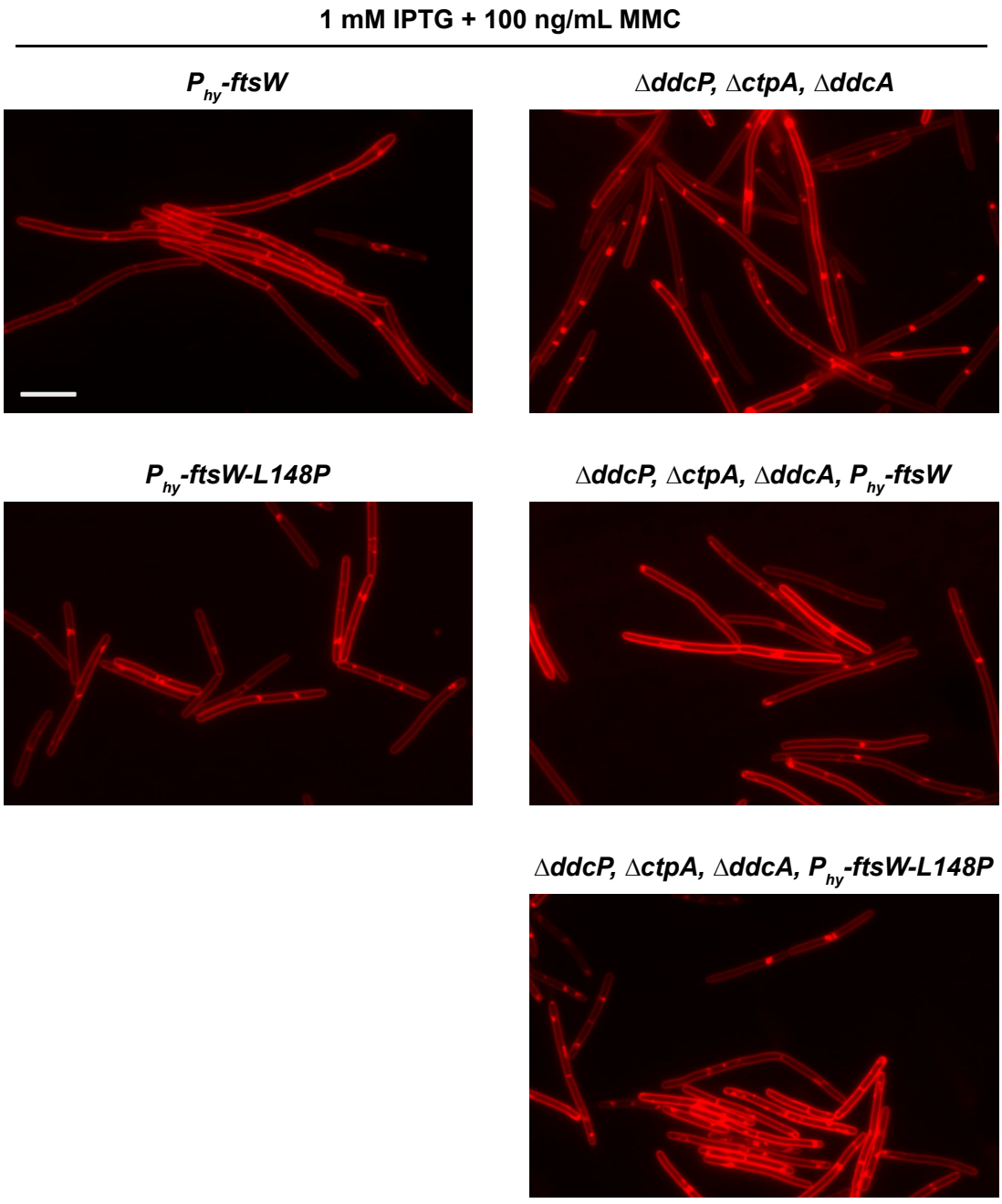

Figure S4. Representative images of cells expressing FtsW or FtsW-L148P under the condition indicated. The white bar corresponds to 10 μm.

Supporting Tables

Table S1. Other mutations identified in the sequenced strains that occurred outside of *ftsW*.

Gene Annotation	Mutation	Count
Putative transporter *ybxG*	I18V (ATC → GTC)	1
Cystathionine beta-lyase *metC*	D192N (GAT → AAT)	1
Putative membrane protein *yerD*	M92I (ATG → ATA)	1
Ribulokinase	M108V (ATG → GTG)	1
Histidyl-tRNA synthetase	G297S (GGC → AGC)	1
Polyketide synthase *pksL*	Q3722R (CAG → CGG)	1

Count refers to the number of occurrences observed in the 25 different sequenced lines.

Table S2. Cell lengths of the strains indicated in Fig. 5A.

Strain	Genotype	Cell length (mean ± sd)
PY79	WT	5.66 ± 1.26 (n = 895)
EAM72	*amyE::P_{hy}-ftsW*	4.69 ± 1.01 (n = 800)
EAM69	*amyE::P_{hy}-ftsW-L148P*	6 ± 1.19 (n = 519)

Strains were measured after growing in the presence of 1 mM IPTG for approximately 1.5 hours. The number of cells scored in each distribution is represented by “n=” and the genotype of each strain is indicated.

Table S3. Cell lengths of the strains indicated in Fig. 5B.

Strain	Genotype	Cell length (mean ± sd)
EAM72	*amyE::P_{hy}-ftsW*	10.15 ± 2.97 (n = 2277)
EAM69	*amyE::P_{hy}-ftsW-L148P*	7.27 ± 1.64 (n = 2549)
PEB639	Δ*ddcP,* Δ*ctpA,* Δ*ddcA*	16.5 ± 5.21 (n = 592)
EAM73	Δ*ddcP,* Δ*ctpA,* Δ*ddcA, amyE::P_{hy}-ftsW*	15.86 ± 4.89 (n = 428)
EAM65	Δ*ddcP,* Δ*ctpA,* Δ*ddcA, amyE::P_{hy}-ftsW-L148P*	8.81 ± 2.43 (n = 839)

Strains were measured after growing in the presence of 1 mM IPTG and 100 ng/mL MMC for approximately 1.5 hours. The number of cells scored in each distribution is indicated as “n=” and the genotype of each strain is also indicated.

Supporting Table S4. Plasmids used in this study.

Plasmid number	Plasmid name	Reference/Source
pEM1	pPB194-*amyE::P_{hy}-yneA-V68A-spec^R*	This study
pEM2	pPB194-*amyE::P_{hy}-yneA-G10D-spec^R*	This study
pEM4	pPB194-*amyE::P_{hy}-yneA-G82S-spec^R*	This study
pEM5	pPB194-*amyE::P_{hy}-yneA-Δ5-spec^R*	This study
pEM6	pPB194-*amyE::P_{hy}-yneA-Δ10-spec^R*	This study
pEM7	pPB194-*amyE::P_{hy}-yneA-Δ15-spec^R*	This study
pEM13	pPB194-*amyE::P_{hy}-ftsW-spec^R*	This study
pEM26	pPB194-*amyE::P_{hy}-yneA-safA-LysM-spec^R*	This study
pPB47	pPB47	(Burby, Simmons et al. 2018)
pPB192	pPB47-*amyE::P_{xyl}-yneA-cam^R*	(Burby, Simmons et al. 2019)
pPB194	pPB194	(Burby, Simmons et al. 2018)
pPB185	pPB185	(Burby, Simmons et al. 2018)

Supporting Table S5. Oligonucleotides used in this study.

Primer name	Sequence
oEAM20	cttagtcgactaaggaggtatacatatgagtaaagaatctattatttttgtcg
oPEB857	gaattagcttgcatgcggctagcttactatcttacagttgctaattcatatg
oPEB3F	gctagccgcatgcaagctaattcg
oPEB259	atgtatacctccttagtcgactaagcttaattgttatccgctcacaattacacacattatgccacacc ttgtagata
oPEB866	tttatgcagcaatggcaagaac
oPEB867	gccgactcaaacatcaaatcttac
oEAM21	gaattagcttgcatgcggctagcttattcatatgcatcctgatgcttctttttc
oEAM22	gaattagcttgcatgcggctagcttaatgcttctttttcaatgggatcactaac
oEAM23	gaattagcttgcatgcggctagcttatgggatcactaactcatcaccc
oEAM28	cttagtcgactaaggaggtatacatatgcggcagctgtttgctttaattg
oEAM29	gaattagcttgcatgcggctagcttattacagataaacagttttttgagctgtttc
oEAM42	tcccggtgaaggtcaaggacaaggccaaatgaacccgggtcaagaccgaga
oEAM43	gttgtaaaacgacggccgaattcttagttatcaattttcatcttccttttagcagct
oEAM44	tcccggtgaaggtcaaggacaaggccaaatggagtttgtcattggattattaattg
oEAM45	gttgtaaaacgacggccgaattcttagttactaagcggatatgtcagctttgat
oEAM46	tcccggtgaaggtcaaggacaaggccaaatgagtatgaaaaataaactgaaaaactt
oEAM47	gttgtaaaacgacggccgaattcttagttattaccacctctgatgttcgtcttc
oEAM48	tcccggtgaaggtcaaggacaaggccaattgaatttttccagggaacgaacg
oEAM49	gttgtaaaacgacggccgaattcttagttactacttgctcttcttctccacatt
oEAM50	tcccggtgaaggtcaaggacaaggccaaatgagcaatttagcttaccaacca
oEAM51	gttgtaaaacgacggccgaattcttagttatcattcctgtatgtttttcacttttttatc
oEAM52	tcccggtgaaggtcaaggacaaggccaaatgcggcagctgtttgctttaatt
oEAM53	gttgtaaaacgacggccgaattcttagttattacagataaacagttttttgagctg
oEAM54	tcccggtgaaggtcaaggacaaggccaaatgccattaacgccaaatgatattc
oEAM55	gttgtaaaacgacggccgaattcttagttattattccttttcctcaaatacagcg
oEAM56	tcccggtgaaggtcaaggacaaggccaaatgccaaaaaagaataaatttatgaatagag
oEAM57	gttgtaaaacgacggccgaattcttagttattaatcaggatttttaaacttaaccttg
oPEB1021	attatgccgcatctgtccaact
oPEB1022	gcaaggcgattaagttgggtaa
oPEB1024	ttctcgccggatgtactggaaac
oPEB1025	tggcttaactatgcggcatcaga
oPEB1034	ctagagggtgaaggtcaaggacaaggccaaatgagtaaagaatctattatttttgtcggt
oPEB1035	gtactgagagtgcaccatattacttagttactatcttacagttgctaattcatatgca
oPEB1041	atcccggtgaaggtcaaggacaaggccaattgaaacggcaattaaaactgtttttttattg
oPEB1042	gttgtaaaacgacggccgaattcttagttattacatttctttttttcagtgtttcaattgc
oPEB1058	tcccggtgaaggtcaaggacaaggccaaatgagtaaagaatctattatttttgtcggt
oPEB1059	gttgtaaaacgacggccgaattcttagttactatcttacagttgctaattcatatgca
oPEB1070	tctagagggtgaaggtcaaggacaaggccaaatgaacccgggtcaagaccgaga
oPEB1071	actgagagtgcaccatattacttagttatcaattttcatcttccttttagcagct
oPEB1072	tagagggtgaaggtcaaggacaaggccaaatggagtttgtcattggattattaattg
oPEB1073	ttgtactgagagtgcaccatattacttagttactaagcggatatgtcagctttgat
oPEB1074	tagagggtgaaggtcaaggacaaggccaaatgagtatgaaaaataaactgaaaaactt

oPEB1075	ttgtactgagagtgcaccatattacttagttattaccacctctgatgttcgtcttc
oPEB1092	ctagagggtgaaggtcaaggacaaggccaattgaatttttccagggaacgaacg
oPEB1093	actgagagtgcaccatattacttagttactacttgctcttcttctccacatt
oPEB1094	agagggtgaaggtcaaggacaaggccaaatgagcaatttagcttaccaacca
oPEB1095	actgagagtgcaccatattacttagttatcattcctgtatgtttttcacttttttatc
oPEB1096	agagggtgaaggtcaaggacaaggccaaatgcggcagctgtttgctttaatt
oPEB1097	actgagagtgcaccatattacttagttattacagataaacagtttttttgagctg
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oPEB1099	tactgagagtgcaccatattacttagttattattccttttcctcaaatacagcg
oPEB1100	tagagggtgaaggtcaaggacaaggccaaatgccaaaaaagaataaatttatgaatagag
oPEB1101	ttgtactgagagtgcaccatattacttagttattaatcaggatttttaaacttaaccttg
oPEB1102	agagggtgaaggtcaaggacaaggccaaatgtcagatcaatttaacagccgt
oPEB1103	gtactgagagtgcaccatattacttagttattaatttgtttttttcaatggatgatgagtt
oPEB1104	ctagagggtgaaggtcaaggacaaggccaaatgcttgctgataaagtaaagctttc
oPEB1105	ttgtactgagagtgcaccatattacttagttatcaatcataaagcttgctgccaaaaacg
oPEB1108	tagagggtgaaggtcaaggacaaggccaattgaaacggcaattaaaactgtttttttattg
oPEB1109	gtactgagagtgcaccatattacttagttattacatttctttttttcagtgtttcaattgc
oPEB1110	gatcccggtgaaggtcaaggacaaggccaaatgcttgctgataaagtaaagctttc
oPEB1111	tgtaaaacgacggccgaattcttagttatcaatcataaagcttgctgccaaaaacg
oPEB1112	gatcccggtgaaggtcaaggacaaggccaaatgtcagatcaatttaacagccgt
oPEB1113	gttgtaaaacgacggccgaattcttagttattaatttgtttttttcaatggatgatgagtt

Supporting Table S6. Strains used in this study.

Strain	Genotype	Reference/Source
PY79	Prototroph Spβ°	(Youngman, Perkins et al. 1984)
PEB324	Δ*ddcP*	(Burby, Simmons et al. 2018)
PEB439	Δ*yneA::loxP*	(Burby, Simmons et al. 2018)
PEB441	Δ*ddcP,* Δ*yneA::loxP*	(Burby, Simmons et al. 2018)
PEB639	Δ*ddcP,* Δ*ctpA,* Δ*ddcA*	(Burby, Simmons et al. 2019)
PEB643	Δ*ddcP,* Δ*ctpA,* Δ*ddcA,* Δ*yneA::loxP*	(Burby, Simmons et al. 2019)
PEB677	*amyE::P_{hy}-yneA*	(Burby, Simmons et al. 2018)
PEB681	Δ*ddcP, amyE::P_{hy}-yneA*	(Burby, Simmons et al. 2018)
PEB844	Δ*ddcP,* Δ*ctpA,* Δ*ddcA, amyE::P_{hy}-yneA*	This study
PEB852	Δ*ddcP,* Δ*ctpA,* Δ*ddcA, amyE::P_{xyl}-yneA*	This study
EAM46	Δ*yneA::loxP, amyE::P_{hy}-yneA*	This study
EAM48	Δ*ddcP,* Δ*yneA::loxP, amyE::P_{hy}-yneA*	This study
EAM49	Δ*yneA::loxP, amyE::P_{hy}-yneA-V68A*	This study
EAM50	Δ*yneA::loxP, amyE::P_{hy}-yneA-G10D*	This study
EAM52	Δ*yneA::loxP, amyE::P_{hy}-yneA-G82S*	This study
EAM53	Δ*yneA::loxP, amyE::P_{hy}-yneA*Δ*5*	This study
EAM54	Δ*yneA::loxP, amyE::P_{hy}-yneA*Δ*10*	This study
EAM55	Δ*yneA::loxP, amyE::P_{hy}-yneA*Δ*15*	This study
EAM56	Δ*ddcP,* Δ*ctpA,* Δ*ddcA,* Δ*yneA::loxP, amyE::P_{hy}-yneA*	This study
EAM57	Δ*ddcP, amyE::P_{hy}-yneA-V68A*	This study
EAM58	Δ*ddcP, amyE::P_{hy}-yneAG82S*	This study
EAM63	Δ*ddcP,* Δ*yneA::loxP, amyE::P_{hy}-yneA-G10D*	This study
EAM64	Δ*ddcP,* Δ*ctpA,* Δ*ddcA, amyE::P_{hy}-ftsW-A99V*	This study
EAM65	Δ*ddcP,* Δ*ctpA,* Δ*ddcA, amyE::P_{hy}-ftsW-L148P*	This study
EAM66	Δ*ddcP,* Δ*ctpA,* Δ*ddcA, amyE::P_{hy}-ftsW-P158L*	This study
EAM67	Δ*ddcP,* Δ*ctpA,* Δ*ddcA, amyE::P_{hy}-ftsW-P158S*	This study
EAM68	*amyE::P_{hy}-ftsW-A99V*	This study
EAM69	*amyE::P_{hy}-ftsW-L148P*	This study
EAM70	*amyE::P_{hy}-ftsW-P158L*	This study
EAM71	*amyE::P_{hy}-ftsW-P158S*	This study
EAM72	*amyE::P_{hy}-ftsW*	This study
EAM73	Δ*ddcP,* Δ*ctpA,* Δ*ddcA, amyE::P_{hy}-ftsW*	This study
EAM78	Δ*ddcP,* Δ*yneA::loxP, amyE::P_{hy}-yneA-V68A*	This study

EAM79	*∆ddcP, ∆yneA::loxP, amyE::P_{hy}-yneA-G82S*	This study
EAM80	*∆yneA::loxP, amyE::P_{hy}-yneA-safA-LysM*	This study
EAM82	*amyE::P_{hy}-yneA-safA-LysM*	This study
EAM83	*∆ddcP, ∆yneA:loxP, amyE::P_{hy}-yneA∆5*	This study
EAM84	*∆ddcP, ∆yneA:loxP, amyE::P_{hy}-yneA∆10*	This study
EAM85	*∆ddcP, ∆yneA:loxP, amyE::P_{hy}-yneA∆15*	This study

Supporting Table S7. Plasmid list for bacterial two-hybrid assay

Plasmid	Vector	Description	Primers[a]
pPB275	pKT25	YneA	oPEB1058 and 1059
pPB291	pUT18C	DivIB	oPEB1070 and 1071
pPB292	pUT18C	EzrA	oPEB1072 and 1073
pPB293	pUT18C	SepF	oPEB1074 and 1075
pPB296	pUT18C	DivIC	oPEB1092 and 1093
pPB297	pUT18C	FtsL	oPEB1094 and 1095
pPB298	pUT18C	FtsW	oPEB1096 and 1097
pEM25	pUT18C	FtsW-L148P	oPEB1096 and 1097
pPB299	pUT18C	DivIVA	oPEB1098 and 1099
pPB300	pUT18C	Pbp2b (*pbpB*)	oPEB1100 and 1101
pPB301	pUT18C	Pbp1 (*ponA*)	oPEB1102 and 1103
pPB302	pUT18C	GpsB	oPEB1104 and 1105
pPB303	pUT18C	CtpA-S297A	oPEB1108 and 1109
pPB267	pUT18C	YneA	oPEB1034 and 1035
pEM16	pKT25	DivIB	oEAM42 and 43
pEM17	pKT25	EzrA	oEAM44 and 45
pEM18	pKT25	SepF	oEAM46 and 47
pEM19	pKT25	DivIC	oEAM48 and 49
pEM20	pKT25	FtsL	oEAM50 and 51
pEM21	pKT25	FtsW	oEAM52 and 53
pEM22	pKT25	FtsW-L148P	oEAM52 and 53
pEM23	pKT25	DivIVA	oEAM54 and 55
pEM24	pKT25	Pbp2b (*pbpB*)	oEAM56 and 57
pPB305	pKT25	Pbp1 (*ponA*)	oPEB1112 and 1113
pPB304	pKT25	GpsB	oPEB1110 and 1111
pPB271	pKT25	CtpA-S297A	oPEB1041 and 1042

[a]Primer pairs used to amplify the gene fragments are provided (see Supporting Table 2 for primer sequences).

Supporting Experimental Procedures

Individual strain construction

PEB844 (Δ*ddcP,* Δ*ctpA,* Δ*ddcA, amyE::P*$_{hy}$*-yneA*): PEB639 (Δ*ddcP,* Δ*ctpA,* Δ*ddcA*) was transformed with plasmid pPB194.

PEB852 (Δ*ddcP,* Δ*ctpA,* Δ*ddcA, amyE::P*$_{xyl}$*-yneA*): PEB639 (Δ*ddcP,* Δ*ctpA,* Δ*ddcA*) was transformed with plasmid pPB192.

EAM46 (Δ*yneA::loxP, amyE::P*$_{hy}$*-yneA*): PEB439 (Δ*yneA::loxP*) was transformed with genomic DNA isolated from PEB677 (*amyE::P*$_{hy}$*-yneA*).

EAM48 (Δ*ddcP,* Δ*yneA::loxP, amyE::P*$_{hy}$*-yneA*): PEB441 (Δ*ddcP,* Δ*yneA::loxP*) was transformed with genomic DNA isolated from PEB677 (*amyE::P*$_{hy}$*-yneA*).

EAM49 (Δ*yneA::loxP, amyE::P*$_{hy}$*-yneA-V68A)*: PEB439 (Δ*yneA::loxP*) was transformed with plasmid pEM1.

EAM50 (Δ*yneA::loxP, amyE::P*$_{hy}$*-yneA-G10D)*: PEB439 (Δ*yneA::loxP*) was transformed with plasmid pEM2.

EAM52 (Δ*yneA::loxP, amyE::P*$_{hy}$*-yneA-G82S)*: PEB439 (Δ*yneA::loxP*) was transformed with plasmid pEM4.

EAM53 (Δ*yneA::loxP, amyE::P*$_{hy}$*-yneA*Δ*5*): PEB439 (Δ*yneA::loxP*) was transformed with plasmid pEM5.

EAM54 (Δ*yneA::loxP, amyE::P*$_{hy}$*-yneA*Δ*10*): PEB439 (Δ*yneA::loxP*) was transformed with plasmid pEM6.

EAM55 (Δ*yneA::loxP, amyE::P*$_{hy}$*-yneA*Δ*15*): PEB439 (Δ*yneA::loxP*) was transformed with plasmid pEM7.

EAM56 (Δ*ddcP,* Δ*ctpA,* Δ*ddcA,* Δ*yneA::loxP, amyE::P*$_{hy}$*-yneA)*: PEB643 (Δ*ddcP,* Δ*ctpA,* Δ*ddcA,* Δ*yneA::loxP*) was transformed with genomic DNA isolated from PEB677 (*amyE::P*$_{hy}$*-yneA)*.

EAM57 (Δ*ddcP, amyE::P*$_{hy}$*-yneA-V68A*): PEB324 (Δ*ddcP*) was transformed with genomic DNA isolated from EAM49 (Δ*yneA::loxP, amyE::P*$_{hy}$*-yneA-V68A*).

EAM58 (Δ*ddcP, amyE::P*$_{hy}$*-yneA-G82S*): PEB324 (Δ*ddcP*) was transformed with genomic DNA isolated from EAM52 (Δ*yneA::loxP, amyE::P*$_{hy}$*-yneA-G82S*).

EAM63 (Δ*ddcP, ΔyneA::loxP, amyE::P$_{hy}$-yneA-G10D*): PEB324 (Δ*ddcP*) was transformed with genomic DNA isolated from EAM50 (Δ*yneA::loxP, amyE::P$_{hy}$-yneA-G10D*).

EAM64 (Δ*ddcP, ΔctpA, ΔddcA, amyE::P$_{hy}$-ftsW-A99V*): The ORF of *ftsW-A99V* was PCR amplified using oEAM28 and oEAM29 primers. Genomic DNA isolated from WDH25 (*ftsW-A99V*) was used as the template. pPB194 was PCR amplified using oPEB3F and oPEB259 primers. These two PCR products were used in a Gibson assembly reaction to generate a plasmid to integrate *ftsW-A99V* under the control of P$_{hy}$ at the *amyE* locus (*ftsW-A99V*-pPB194). Clones were verified by Sanger sequencing with oPEB866 and oPEB867. PEB639 (Δ*ddcP, ΔctpA, ΔddcA*) was transformed with this plasmid (*ftsW-A99V*-pPB194).

EAM65 (Δ*ddcP, ΔctpA, ΔddcA, amyE::P$_{hy}$-ftsW-L148P)*: The ORF of *ftsW-L148P* was PCR amplified using oEAM28 and oEAM29 primers. Genomic DNA isolated from WDH30 (*ftsW-L148P*) was used as the template. pPB194 was PCR amplified using oPEB3F and oPEB259 primers. These two PCR products were used in a Gibson assembly reaction to generate a plasmid to integrate *ftsW-L148P* under the control of P$_{hy}$ at the *amyE* locus (*ftsW-L148P*-pPB194). Clones were verified by Sanger sequencing with oPEB866 and oPEB867. PEB639 (Δ*ddcP, ΔctpA, ΔddcA*) was transformed with this plasmid (*ftsW-L148P*-pPB194).

EAM66 (Δ*ddcP, ΔctpA, ΔddcA, amyE::P$_{hy}$-ftsW-P158L)*: The ORF of *ftsW-P158L* was PCR amplified using oEAM28 and oEAM29 primers. Genomic DNA isolated from WDH19 (*ftsW-P158L*) was used as the template. pPB194 was PCR amplified using oPEB3F and oPEB259 primers. These two PCR products were used in a Gibson assembly reaction to generate a plasmid to integrate *ftsW-P158L* under the control of P$_{hy}$ at the *amyE* locus (*ftsW-P158L*-pPB194). Clones were verified by Sanger sequencing with oPEB866 and oPEB867. PEB639 (Δ*ddcP, ΔctpA, ΔddcA*) was transformed with this plasmid (*ftsW-P158L*-pPB194).

EAM67 (Δ*ddcP, ΔctpA, ΔddcA, amyE::P$_{hy}$-ftsW-P158S)*: The ORF of *ftsW-P158S* was PCR amplified using oEAM28 and oEAM29 primers. Genomic DNA isolated from WDH32 (*ftsW-P158S*) was used as the template. pPB194 was PCR amplified using oPEB3F and oPEB259 primers. These two PCR products were used in a Gibson assembly reaction to generate a plasmid to integrate *ftsW-P158S* under the control of P$_{hy}$ at the *amyE* locus (*ftsW-P158S*-pPB194). Clones were verified by Sanger sequencing with oPEB866 and oPEB867. PEB639 (Δ*ddcP, ΔctpA, ΔddcA*) was transformed with this plasmid (*ftsW-P158S*-pPB194).

EAM68 (*amyE::P$_{hy}$-ftsW-A99V)*: The ORF of *ftsW-A99V* was PCR amplified using oEAM28 and oEAM29 primers. Genomic DNA isolated from WDH25 (*ftsW-A99V*) was used as the template. pPB194 was PCR amplified using oPEB3F and oPEB259 primers. These two PCR products were used in a Gibson assembly reaction to generate a plasmid to integrate *ftsW-A99V* under the control of P$_{hy}$ at the *amyE* locus (*ftsW-*

A99V-pPB194). Clones were verified by Sanger sequencing with oPEB866 and oPEB867. PY79 was transformed with this plasmid (*ftsW-A99V*-pPB194).

EAM69 (*amyE::P$_{hy}$-ftsW-L148P)*: The ORF of *ftsW-L148P* was PCR amplified using oEAM28 and oEAM29 primers. Genomic DNA isolated from WDH30 (*ftsW-L148P*) was used as the template. pPB194 was PCR amplified using oPEB3F and oPEB259 primers. These two PCR products were used in a Gibson assembly reaction to generate a plasmid to integrate *ftsW-L148P* under the control of P$_{hy}$ at the *amyE* locus (*ftsW-L148P*-pPB194). Clones were verified by Sanger sequencing with oPEB866 and oPEB867. PY79 was transformed with this plasmid (*ftsW-L148P*-pPB194).

EAM70 (*amyE::P$_{hy}$-ftsW-P158L)*: The ORF of *ftsW-P158L* was PCR amplified using oEAM28 and oEAM29 primers. Genomic DNA isolated from WDH19 (*ftsW-P158L*) was used as the template. pPB194 was PCR amplified using oPEB3F and oPEB259 primers. These two PCR products were used in a Gibson assembly reaction to generate a plasmid to integrate *ftsW-P158L* under the control of P$_{hy}$ at the *amyE* locus (*ftsW-P158L*-pPB194). Clones were verified by Sanger sequencing with oPEB866 and oPEB867. PY79 was transformed with this plasmid (*ftsW-P158L*-pPB194).

EAM71 (*amyE::P$_{hy}$-ftsW-P158S)*: The ORF of *ftsW-P158S* was PCR amplified using oEAM28 and oEAM29 primers. Genomic DNA isolated from WDH32 (*ftsW-P158S*) was used as the template. pPB194 was PCR amplified using oPEB3F and oPEB259 primers. These two PCR products were used in a Gibson assembly reaction to generate a plasmid to integrate *ftsW-P158S* under the control of P$_{hy}$ at the *amyE* locus (*ftsW-P158S*-pPB194). Clones were verified by Sanger sequencing with oPEB866 and oPEB867. PY79 was transformed with this plasmid (*ftsW-P158S*-pPB194).

EAM72 (*amyE::P$_{hy}$-ftsW)*: PY79 was transformed with plasmid pEM13.

EAM73 (Δ*ddcP,* Δ*ctpA,* Δ*ddcA, amyE::P$_{hy}$-ftsW)*: PEB639 (Δ*ddcP,* Δ*ctpA,* Δ*ddcA*) was transformed with plasmid pEM13.

EAM78 (Δ*ddcP,* Δ*yneA::loxP, amyE::P$_{hy}$-yneA-V68A*): PEB441 (Δ*ddcP,* Δ*yneA::loxP*) was transformed with genomic DNA isolated from EAM49 (Δ*yneA::loxP, amyE::P$_{hy}$-yneA-V68A*).

EAM79 (Δ*ddcP,* Δ*yneA::loxP, amyE::P$_{hy}$-yneA-G82S*): PEB441 (Δ*ddcP,* Δ*yneA::loxP*) was transformed with genomic DNA isolated from EAM52 (Δ*yneA::loxP, amyE::P$_{hy}$-yneA-G82S*).

EAM80 (Δ*yneA::loxP, amyE::P$_{hy}$-yneA-safALysM*): PEB439 (Δ*yneA::loxP*) was transformed with plasmid pEM26.

EAM82 (*amyE::P$_{hy}$-yneA-safALysM*): PY79 was transformed with plasmid pEM26.

EAM83 (Δ*ddcP, ΔyneA::loxP, amyE::P$_{hy}$-yneAΔ5*): PEB441 (Δ*ddcP, ΔyneA::loxP*) was transformed with genomic DNA isolated from EAM53 (Δ*yneA::loxP, amyE::P$_{hy}$-yneAΔ5*).

EAM84 (Δ*ddcP, ΔyneA::loxP, amyE::P$_{hy}$-yneAΔ10*): PEB441 (Δ*ddcP, ΔyneA::loxP*) was transformed with genomic DNA isolated from EAM54 (Δ*yneA::loxP, amyE::P$_{hy}$-yneAΔ10*).

EAM85 (Δ*ddcP, ΔyneA::loxP, amyE::P$_{hy}$-yneAΔ15*): PEB441 (Δ*ddcP, ΔyneA::loxP*) was transformed with genomic DNA isolated from EAM55 (Δ*yneA::loxP, amyE::P$_{hy}$-yneAΔ15*).

Individual plasmid construction

pEM1: The ORF of *yneA* containing the *V68A* mutation was PCR amplified using oEAC20 and oPEB857 primers. pPB194 was PCR amplified using oPEB3F and oPEB259 primers. These two PCR products were used in a Gibson assembly reaction to generate a plasmid to integrate *yneA-V68A* under the control of P_{hy} at the *amyE* locus. Clones were verified by Sanger sequencing with oPEB866 and oPEB867.

pEM2: The ORF of *yneA* containing the *G10D* mutation was PCR amplified using oEAC20 and oPEB857 primers. pPB194 was PCR amplified using oPEB3F and oPEB259 primers. These two PCR products were used in a Gibson assembly reaction to generate a plasmid to integrate *yneA-G10D* under the control of P_{hy} at the *amyE* locus. Clones were verified by Sanger sequencing with oPEB866 and oPEB867.

pEM4: The ORF of *yneA* containing the *G82S* mutation was PCR amplified using oEAC20 and oPEB857 primers. pPB194 was PCR amplified using oPEB3F and oPEB259 primers. These two PCR products were used in a Gibson assembly reaction to generate a plasmid to integrate *yneA-G82S* under the control of P_{hy} at the *amyE* locus. Clones were verified by Sanger sequencing with oPEB866 and oPEB867.

pEM5: The ORF of *yneA* with a premature stop codon was PCR amplified using oEAC20 and oEAC21. pPB194 was PCR amplified using oPEB3F and oPEB259 primers. These two PCR products were used in a Gibson assembly reaction to generate a plasmid to integrate *yneAΔ5* C-terminal truncation under the control of P_{hy} at the *amyE* locus. Clones were verified by Sanger sequencing with oPEB866 and oPEB867.

pEM6: The ORF of *yneA* with a premature stop codon was PCR amplified using oEAC20 and oEAC22. pPB194 was PCR amplified using oPEB3F and oPEB259 primers. These two PCR products were used in a Gibson assembly reaction to generate a plasmid to integrate *yneAΔ10* C-terminal truncation under the control of P_{hy} at the *amyE* locus. Clones were verified by Sanger sequencing with oPEB866 and oPEB867.

pEM7: The ORF of *yneA* with a premature stop codon was PCR amplified using oEAC20 and oEAC23. pPB194 was PCR amplified using oPEB3F and oPEB259

primers. These two PCR products were used in a Gibson assembly reaction to generate a plasmid to integrate *yneA∆15* C-terminal truncation under the control of P_{hy} at the *amyE* locus. Clones were verified by Sanger sequencing with oPEB866 and oPEB867.

pEM13: The ORF of *ftsW* was PCR amplified using oEAM28 and oEAM29 primers. pPB194 was PCR amplified using oPEB3F and oPEB259 primers. These two PCR products were used in a Gibson assembly reaction to generate a plasmid to integrate *ftsW* under the control of P_{hy} at the *amyE* locus. Clones were verified by Sanger sequencing with oPEB866 and oPEB867.

pEM16: DivIB was amplified with primers oEAM42/43 and the plasmid pKT25 was amplified with primers oPEB1014/1015. These two PCR products were used in a Gibson assembly reaction to generate T25-DivIB fusion for expression in BTH101 cells in a bacterial two-hybrid assay. Clones were selected on LB agar containing 50 μg/mL kanamycin and 0.2% glucose. Clones were verified via Sanger sequencing using oPEB1021 and 1022.

pEM17: EzrA was amplified with primers oEAM44/45 and the plasmid pKT25 was amplified with primers oPEB1014/1015. These two PCR products were used in a Gibson assembly reaction to generate T25-EzrA fusion for expression in BTH101 cells in a bacterial two-hybrid assay. Clones were selected on LB agar containing 50 μg/mL kanamycin and 0.2% glucose. Clones were verified via Sanger sequencing using oPEB1021 and 1022.

pEM18: SepF was amplified with primers oEAM46/47 and the plasmid pKT25 was amplified with primers oPEB1014/1015. These two PCR products were used in a Gibson assembly reaction to generate T25-SepF fusion for expression in BTH101 cells in a bacterial two-hybrid assay. Clones were selected on LB agar containing 50 μg/mL kanamycin and 0.2% glucose. Clones were verified via Sanger sequencing using oPEB1021 and 1022.

pEM19: DivIC was amplified with primers oEAM48/49 and the plasmid pKT25 was amplified with primers oPEB1014/1015. These two PCR products were used in a Gibson assembly reaction to generate T25-DivIC fusion for expression in BTH101 cells in a bacterial two-hybrid assay. Clones were selected on LB agar containing 50 μg/mL kanamycin and 0.2% glucose. Clones were verified via Sanger sequencing using oPEB1021 and 1022.

pEM20: FtsL was amplified with primers oEAM50/51 and the plasmid pKT25 was amplified with primers oPEB1014/1015. These two PCR products were used in a Gibson assembly reaction to generate T25-FtsL fusion for expression in BTH101 cells in a bacterial two-hybrid assay. Clones were selected on LB agar containing 50 μg/mL kanamycin and 0.2% glucose. Clones were verified via Sanger sequencing using oPEB1021 and 1022.

pEM21: FtsW was amplified with primers oEAM52/53 and the plasmid pKT25 was amplified with primers oPEB1014/1015. These two PCR products were used in a Gibson assembly reaction to generate T25-FtsW fusion for expression in BTH101 cells in a bacterial two-hybrid assay. Clones were selected on LB agar containing 50 μg/mL kanamycin and 0.2% glucose. Clones were verified via Sanger sequencing using oPEB1021 and 1022.

pEM22: FtsW-L148P was amplified with primers oEAM52/53 and the plasmid pKT25 was amplified with primers oPEB1014/1015. These two PCR products were used in a Gibson assembly reaction to generate T25-FtsW-L148P fusion for expression in BTH101 cells in a bacterial two-hybrid assay. Clones were selected on LB agar containing 50 μg/mL kanamycin and 0.2% glucose. Clones were verified via Sanger sequencing using oPEB1021 and 1022.

pEM23: DivIVA was amplified with primers oEAM54/55 and the plasmid pKT25 was amplified with primers oPEB1014/1015. These two PCR products were used in a Gibson assembly reaction to generate T25-DivIVA fusion for expression in BTH101 cells in a bacterial two-hybrid assay. Clones were selected on LB agar containing 50 μg/mL kanamycin and 0.2% glucose. Clones were verified via Sanger sequencing using oPEB1021 and 1022.

pEM24: Pbp2b (*pbpB*) was amplified with primers oEAM56/57 and the plasmid pKT25 was amplified with primers oPEB1014/1015. These two PCR products were used in a Gibson assembly reaction to generate T25-PBP2B fusion for expression in BTH101 cells in a bacterial two-hybrid assay. Clones were selected on LB agar containing 50 μg/mL kanamycin and 0.2% glucose. Clones were verified via Sanger sequencing using oPEB1021 and 1022.

pEM25: FtsW-L148P was amplified with primers oPEB1096/1097 and the plasmid pUT18C was amplified using primers oPEB1017/1018. These two PCR products were used in a Gibson assembly reaction to generate a T18-FtsW-L148P fusion for expression in BTH101 cells in a bacterial two-hybrid assay. Clones were selected on LB agar containing 100 μg/mL ampicillin and 0.2% glucose. Clones were verified via Sanger sequencing using oPEB1024 and 1025.

pEM26: Gblock (IDT) was generated with the ORF of *yneA* with the *safA* LysM domain in place of the *yneA* LysM domain while retaining the YneA C-terminal tail. This Gblock was PCR amplified with oPEB856 and oPEB857 primers. pPB194 was PCR amplified using oPEB3F and oPEB259 primers. These two PCR products were used in a Gibson assembly reaction to generate a plasmid to integrate *yneA-safA-LysM* under the control of P_{hy} at the *amyE* locus. Clones were verified by Sanger sequencing with oPEB866 and oPEB867.

pPB267: YneA was amplified with primers oPEB1034/1035 and the plasmid pUT18C was amplified using primers oPEB1017/1018. These two PCR products were used in a Gibson assembly reaction to generate a T18-YneA fusion for expression in BTH101

cells in a bacterial two-hybrid assay. Clones were selected on LB agar containing 100 μg/mL ampicillin and 0.2% glucose. Clones were verified via Sanger sequencing using oPEB1024 and 1025.

pPB271: CtpA-S297A was amplified with primers oPEB1041/1042 and the plasmid pKT25 was amplified with primers oPEB1014/1015. These two PCR products were used in a Gibson assembly reaction to generate T25-CtpA-S297A fusion for expression in BTH101 cells in a bacterial two-hybrid assay. Clones were selected on LB agar containing 50 μg/mL kanamycin and 0.2% glucose. Clones were verified via Sanger sequencing using oPEB1021 and 1022.

pPB304: GpsB was amplified with primers oPEB1110/1111 and the plasmid pKT25 was amplified with primers oPEB1014/1015. These two PCR products were used in a Gibson assembly reaction to generate T25-GpsB fusion for expression in BTH101 cells in a bacterial two-hybrid assay. Clones were selected on LB agar containing 50 μg/mL kanamycin and 0.2% glucose. Clones were verified via Sanger sequencing using oPEB1021 and 1022.

pPB305: Pbp1 (*ponA*) was amplified with primers oPEB1112/1113 and the plasmid pKT25 was amplified with primers oPEB1014/1015. These two PCR products were used in a Gibson assembly reaction to generate T25-PBP1 fusion for expression in BTH101 cells in a bacterial two-hybrid assay. Clones were selected on LB agar containing 50 μg/mL kanamycin and 0.2% glucose. Clones were verified via Sanger sequencing using oPEB1021 and 1022.

pPB275: YneA was amplified with primers oPEB1058/1059 and the plasmid pKT25 was amplified with primers oPEB1014/1015. These two PCR products were used in a Gibson assembly reaction to generate T25-YneA fusion for expression in BTH101 cells in a bacterial two-hybrid assay. Clones were selected on LB agar containing 50 μg/mL kanamycin and 0.2% glucose. Clones were verified via Sanger sequencing using oPEB1021 and 1022.

pPB291: DivIB was amplified with primers oPEB1070/1071 and the plasmid pUT18C was amplified using primers oPEB1017/1018. These two PCR products were used in a Gibson assembly reaction to generate a T18-DivIB fusion for expression in BTH101 cells in a bacterial two-hybrid assay. Clones were selected on LB agar containing 100 μg/mL ampicillin and 0.2% glucose. Clones were verified via Sanger sequencing using oPEB1024 and 1025.

pPB292: EzrA was amplified with primers oPEB1072/1073 and the plasmid pUT18C was amplified using primers oPEB1017/1018. These two PCR products were used in a Gibson assembly reaction to generate a T18-EzrA fusion for expression in BTH101 cells in a bacterial two-hybrid assay. Clones were selected on LB agar containing 100 μg/mL ampicillin and 0.2% glucose. Clones were verified via Sanger sequencing using oPEB1024 and 1025.

pPB293: SepF was amplified with primers oPEB1074/1075 and the plasmid pUT18C was amplified using primers oPEB1017/1018. These two PCR products were used in a Gibson assembly reaction to generate a T18-SepF fusion for expression in BTH101 cells in a bacterial two-hybrid assay. Clones were selected on LB agar containing 100 μg/mL ampicillin and 0.2% glucose. Clones were verified via Sanger sequencing using oPEB1024 and 1025.

pPB296: DivIC was amplified with primers oPEB1092/1093 and the plasmid pUT18C was amplified using primers oPEB1017/1018. These two PCR products were used in a Gibson assembly reaction to generate a T18-DivIC fusion for expression in BTH101 cells in a bacterial two-hybrid assay. Clones were selected on LB agar containing 100 μg/mL ampicillin and 0.2% glucose. Clones were verified via Sanger sequencing using oPEB1024 and 1025.

pPB297: FtsL was amplified with primers oPEB1094/1095 and the plasmid pUT18C was amplified using primers oPEB1017/1018. These two PCR products were used in a Gibson assembly reaction to generate a T18-FtsL fusion for expression in BTH101 cells in a bacterial two-hybrid assay. Clones were selected on LB agar containing 100 μg/mL ampicillin and 0.2% glucose. Clones were verified via Sanger sequencing using oPEB1024 and 1025.

pPB298: FtsW was amplified with primers oPEB1096/1097 and the plasmid pUT18C was amplified using primers oPEB1017/1018. These two PCR products were used in a Gibson assembly reaction to generate a T18-FtsW fusion for expression in BTH101 cells in a bacterial two-hybrid assay. Clones were selected on LB agar containing 100 μg/mL ampicillin and 0.2% glucose. Clones were verified via Sanger sequencing using oPEB1024 and 1025.

pPB299: DivIVA was amplified with primers oPEB1098/1099 and the plasmid pUT18C was amplified using primers oPEB1017/1018. These two PCR products were used in a Gibson assembly reaction to generate a T18-DivIVA fusion for expression in BTH101 cells in a bacterial two-hybrid assay. Clones were selected on LB agar containing 100 μg/mL ampicillin and 0.2% glucose. Clones were verified via Sanger sequencing using oPEB1024 and 1025.

pPB300: Pbp2b (*pbpB*) was amplified with primers oPEB1100/1101 and the plasmid pUT18C was amplified using primers oPEB1017/1018. These two PCR products were used in a Gibson assembly reaction to generate a T18-PBP2B fusion for expression in BTH101 cells in a bacterial two-hybrid assay. Clones were selected on LB agar containing 100 μg/mL ampicillin and 0.2% glucose. Clones were verified via Sanger sequencing using oPEB1024 and 1025.

pPB301: Pbp1 (*ponA*) was amplified with primers oPEB1102/1103 and the plasmid pUT18C was amplified using primers oPEB1017/1018. These two PCR products were used in a Gibson assembly reaction to generate a T18-PBP1 fusion for expression in BTH101 cells in a bacterial two-hybrid assay. Clones were selected on LB agar

containing 100 μg/mL ampicillin and 0.2% glucose. Clones were verified via Sanger sequencing using oPEB1024 and 1025.

pPB302: GpsB was amplified with primers oPEB1104/1105 and the plasmid pUT18C was amplified using primers oPEB1017/1018. These two PCR products were used in a Gibson assembly reaction to generate a T18-GpsB fusion for expression in BTH101 cells in a bacterial two-hybrid assay. Clones were selected on LB agar containing 100 μg/mL ampicillin and 0.2% glucose. Clones were verified via Sanger sequencing using oPEB1024 and 1025.

pPB303: CtpA-S297A was amplified with primers oPEB1108/1109 and using pPB185 as a template. The plasmid pUT18C was amplified using primers oPEB1017/1018. These two PCR products were used in a Gibson assembly reaction to generate a T18-CtpA-S297A fusion for expression in BTH101 cells in a bacterial two-hybrid assay. Clones were selected on LB agar containing 100 μg/mL ampicillin and 0.2% glucose. Clones were verified via Sanger sequencing using oPEB833, 1024 and 1025.

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