The WalR-WalK signaling pathway modulates the activities of both CwlO and LytE through control of the peptidoglycan deacetylase PdaC in *Bacillus subtilis*

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Supplemental Figures and Tables

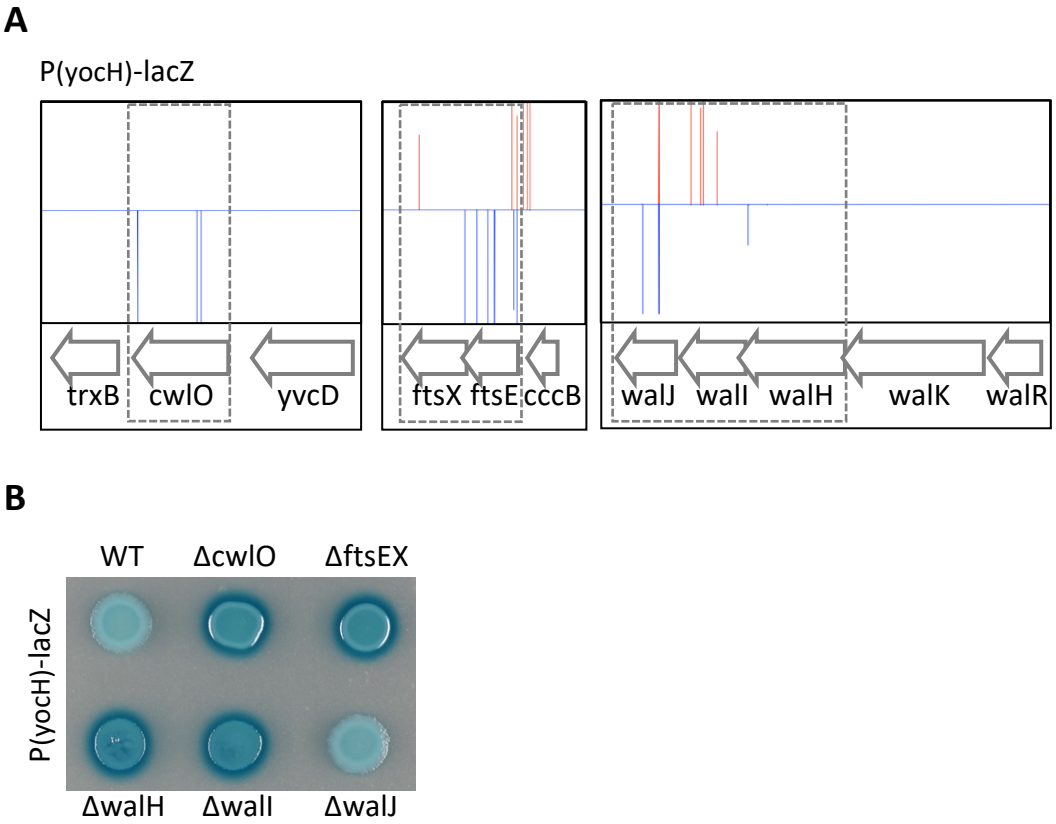

Figure S1: Transposon-sequencing screen for activators of WalR-WalK signaling identifies known regulators. To identify mutants that increase WalRK signaling, a transposon was used to mutagenize a strain harboring the WalR activity reporter P(yocH)-lacZ. **(A)** Transposon insertion profile from three regions of the *B. subtilis* genome. Each line indicates a transposon insert site, its height represents the number of sequencing reads, and its color (red or blue) indicates its orientation. Insertions in *cwlO, ftsEX, walH*, and *walI* that are known to regulate WalRK signaling are shown. **(B)** Strains harboring the indicated deletions in the P(yocH)-lacZ background were confirmed to increase WalRK signaling when strains were spotted on LB agar plates containing 100mg/mL X-gal. Images were taken after overnight incubation at 37˚C. Transposon insertions in walJ were hits in the screen but a deletion mutant did not validate.

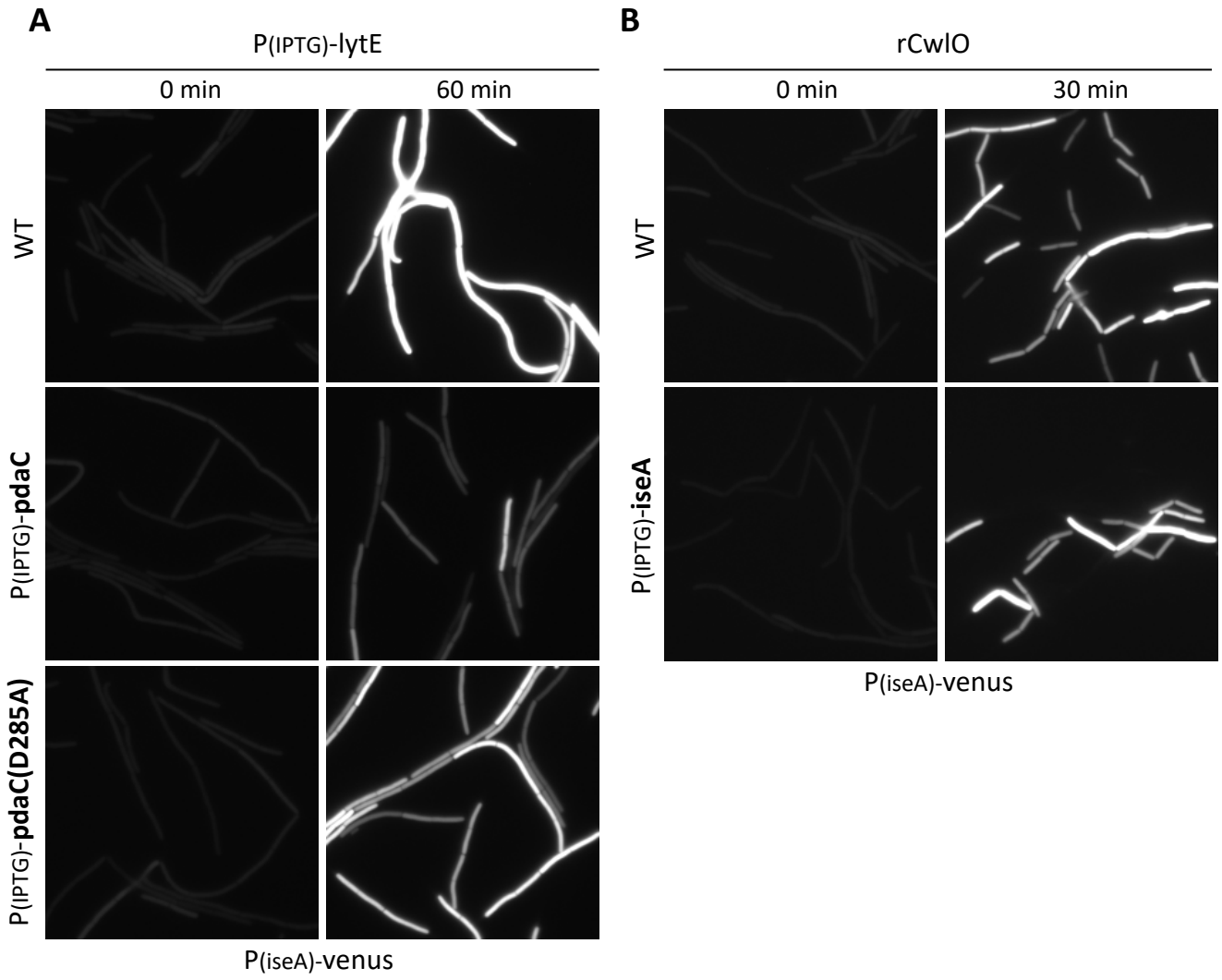

Figure S2: Over-expression of *pdaC* counteracts WalRK inhibition in response to high D,L-endopeptidase activity. (A) Representative fluorescent images of the P(iseA)-venus transcriptional reporter in the indicated strains containing an IPTG-regulated promoter fusion to *lytE*. Cells were grown to OD600 ~0.15 and images were taken before (0 min) and after (60 min) addition of IPTG (50 µM). Over-expression of the catalytic mutant PdaC(D285A) fails to prevent inhibition of WalRK and de-repression of P(iseA)-venus. **(B)** Representative fluorescent images of the P(iseA)-venus transcriptional reporter in the indicated strains before (0 min) and after (30 min) the addition of 70 µg/mL (final) recombinant CwlO (rCwlO). rCwlO inhibited WalRK signaling and de-repressed P(iseA)-venus in wild-type. Over-expression of *iseA* (500 µM IPTG) for 60 min prior to addition of rCwlO did not prevent inhibition of WalRK.

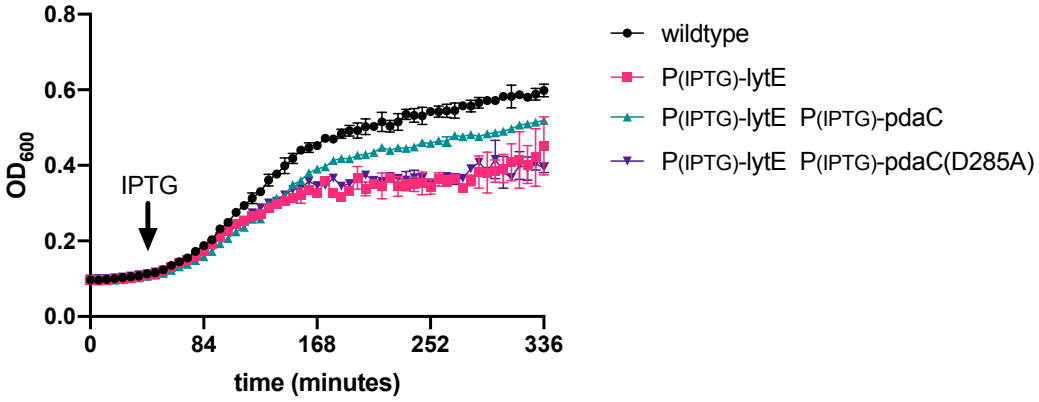

Figure S3: Impaired growth due to *lytE* over-expression can be suppressed by over-expression of *pdaC* but not a catalytic mutant. The indicated strains were grown in LB medium to mid-log, normalized to OD600 = 0.02 in LB, and grown at 37˚C. After 1 hour, IPTG (50 μM) was added to the culture, and growth was resumed. OD600 measurements were taken every 6 minutes for 5 hours. Representative growth curves are from one of three biological replicates.

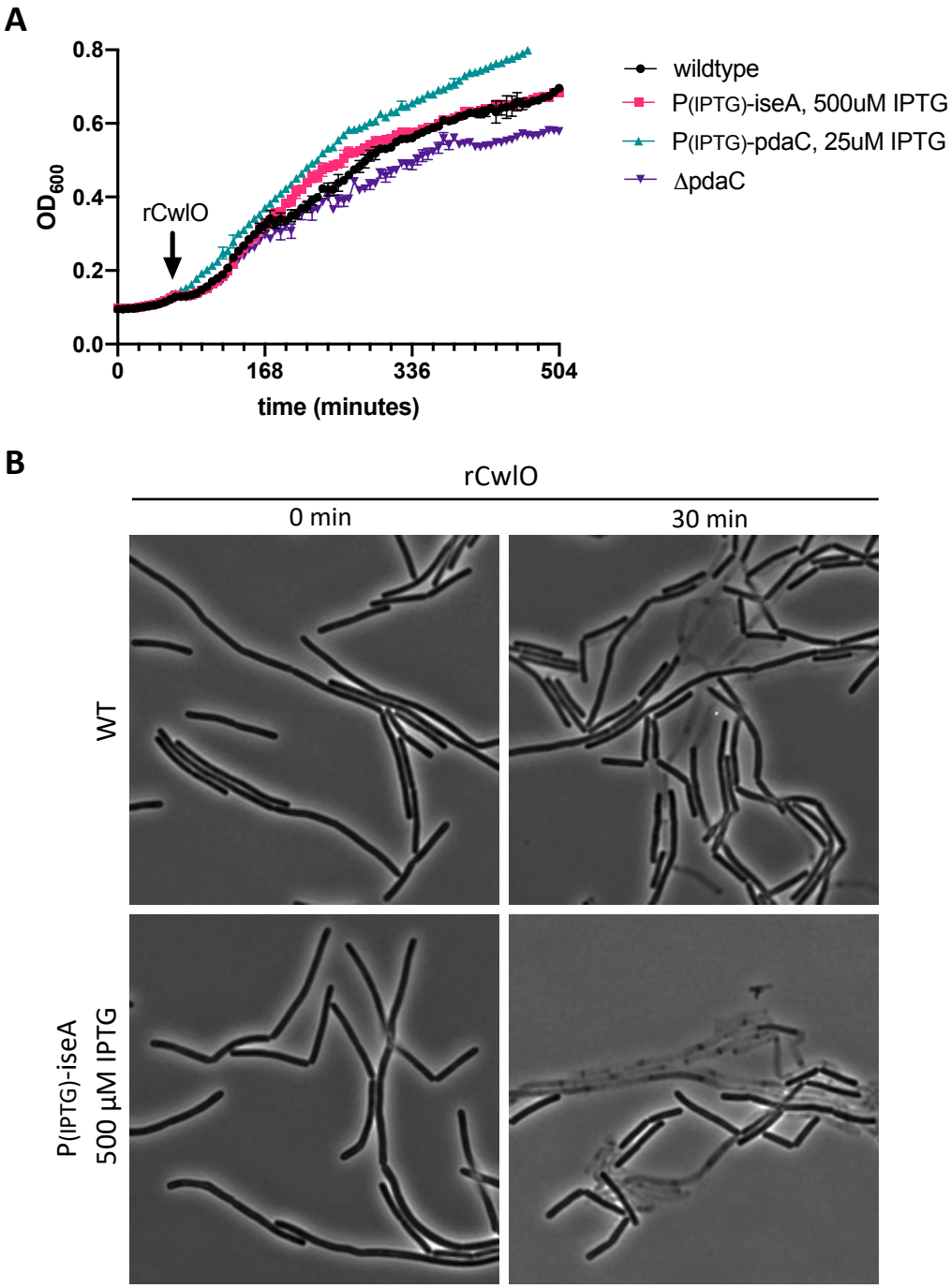

Figure S4: Over-expression of *iseA* does not protect from exogenously-added CwlO . (A) Growth curves of the indicated strains. Wild-type, Δ*pdaC*, and strains harboring an IPTG-regulated promoter fused to *iseA* or *pdaC* were grown for 1 hour in LB medium (with the indicated amount of IPTG) to OD600 of ~0.15. Recombinant CwlO was then added (70 µg/mL final) and growth at 37˚C was resumed. OD600 measurements were taken every 6 minutes for 8 hours. **(B)** Representative phase-contrast images of the indicated strains before (0 min) and after (30 min) addition of rCwlO.

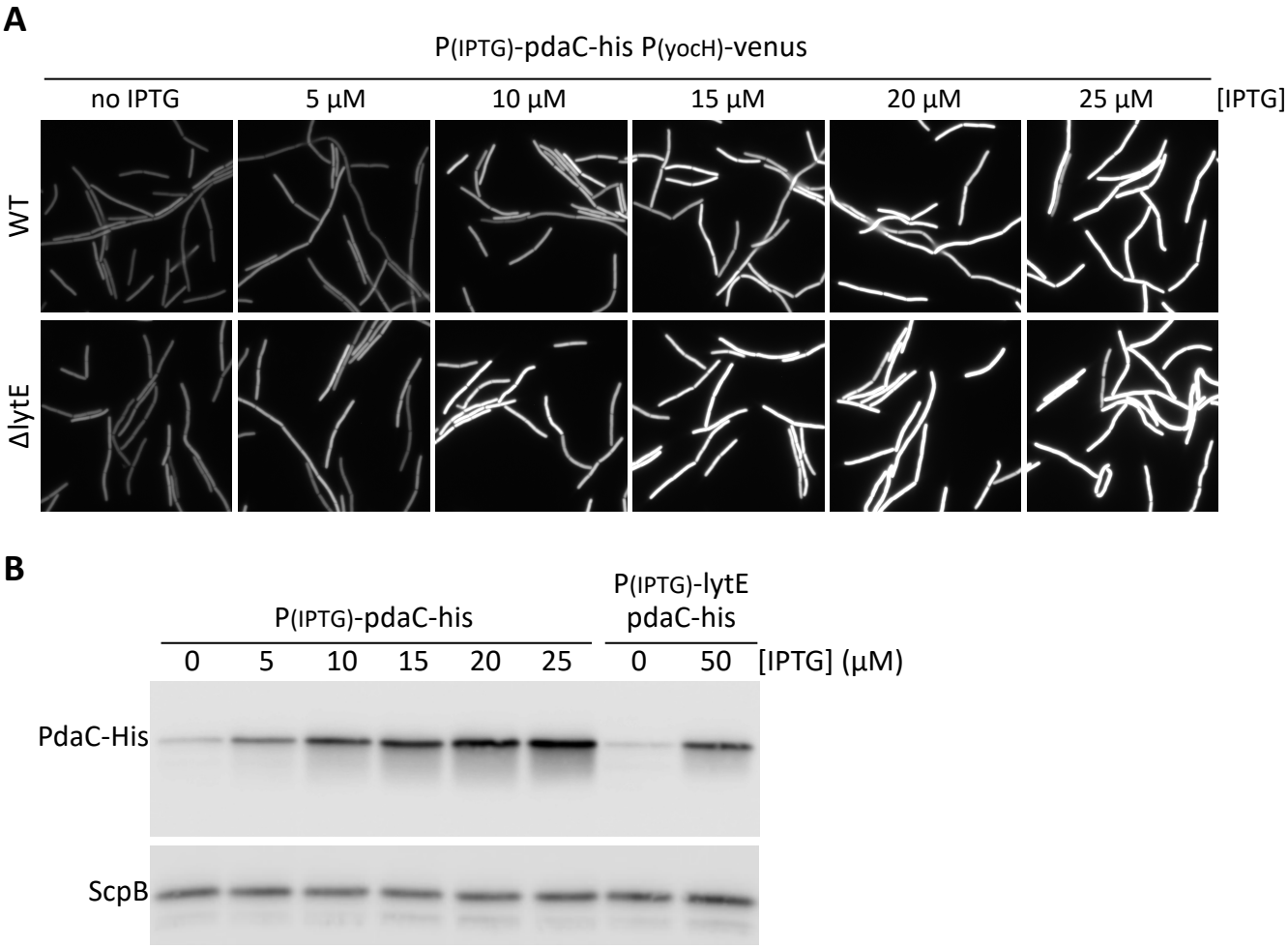

Figure S5: Comparing IPTG-regulated expression of *pdaC* and induction of native *pdaC* in response to increased levels of the D,L-endopeptidase LytE. (A) Representative images of P(yocH)-venus fluorescence in the indicated strains harboring an IPTG-regulated promoter fusion to *pdaC.* Increased WalR-dependent expression of P(yocH)-venus can be detected in the presence of 10 µM IPTG for wild-type, and 5 µM for the strain in which CwlO is the only elongation-specific D,L-endopeptidase present (Δ*lytE*). Strains were grown to OD600 ~0.15 in LB medium, induced with IPTG, and imaged 60 min later. **(B)** Immunoblot analysis of His-tagged PdaC protein levels. Strains contained *pdaC-his* fused to an IPTG-regulated promoter, or under native control at its native locus with an IPTG-regulated promoter fusion to *lytE*. Cells were grown to OD600 ~0.15 in LB medium and samples were taken 60 minutes after induction with the indicated concentration of IPTG. The levels of PdaC-His that result from increased levels of LytE are similar to the levels of PdaC-His produced with 10 µM IPTG. ScpB was used to control for loading.

Table S1. Primary hits from Tn-seq screen for high WalRK activity

Gene	Hit type	Validation?
yycIJ	inactivation	weak
ydhE	inactivation	no
yvrGH	inactivation	weak
cwlO	inactivation	yes
ftsEX	inactivation	yes
walHI	inactivation	yes
dacA	inactivation	weak
ponA	inactivation	weak
tagVUEC	inactivation	no
tagFGH	over-expression	intermediate
ggaAB	inactivation	no
pdaC	over-expression	yes
ltaS	inactivation	intermediate
pgcA	inactivation	no
gtaB	inactivation	weak
manA	inactivation	no
galE	inactivation	yes
fliDST	inactivation	weak
degSU	inactivation	no
lytA	inactivation	no
ybfF	inactivation	no

Table S1: Validation of hits from the Tn-seq screen. Genes, or gene clusters, that were enriched for transposon insertions are listed. Hits listed as inactivation contained insertions within the coding region. Over-expression hits contained insertions upstream of the coding region, oriented in a direction that the P_{pen} promoter was co-directional with gene transcription. Hits were validated by spotting insertion-deletion mutants harboring P_{yocH}-*lacZ* on LB agar plates containing 100 µg/mL X-Gal. Mutants that generated a subtle or modest increase in blue color compared to wild-type after >24 hours of incubation were designated "weak" or "intermediate", respectively. We suspect the impact on WalRK signaling in these mutants is indirect and/or results from defects in envelope permeability that increases the ability of X-Gal to access ß-galactosidase in the cell cytoplasm as is likely to be the case for the *galE* mutant. Importantly, the increase in blue color was substantially stronger for insertions in *cwlO*, *ftsEX*, *walHI*, and when *pdaC* was over-expressed.

Table S2. *Bacillus subtilis* strains used in this study

Strain	Genotype	Source	Figure
PY79	wildtype	Youngman et al., 1983 [1]	Source of all strains
bGD729	*ycgO*::PyocH-*optRBS-lacZ* (kan)	This study	1, S1
bGD780	*ycgO*::PyocH-*optRBS-lacZ* (kan) Δ*pdaC*::*erm*	This study	1
bGD819	*ycgO*::PyocH-*optRBS-lacZ* (kan) *yvbJ*::Phyperspank-*optRBS-pdaC* (spec)	This study	1
bGD910	*ycgO*::PyocH-*optRBS-lacZ* (kan) Δ*ctaO*::*erm*	This study	1
bGD911	*ycgO*::PyocH-*optRBS-lacZ* (kan) Δ*cotT*::*erm*	This study	1
bGD731	*ycgO*::PyocH-*optRBS-lacZ* (kan) Δ*cwlO*::*cat*	This study	S1
bGD902	*ycgO*::PyocH-*optRBS-lacZ* (kan) Δ*ftsEX*::*cat*	This study	S1
bGD781	*ycgO*::PyocH-*optRBS-lacZ* (kan) Δ*walH*::*erm*	This study	S1
bGD895	*ycgO*::PyocH-*optRBS-lacZ* (kan) Δ*walI*::*erm*	This study	S1
bGD901	*ycgO*::PyocH-*optRBS-lacZ* (kan) Δ*walJ*::*erm*	This study	S1
bGD300	*amyE*::PyocH-*optRBS-venus* (cat)	Dobihal et al., 2019 [2]	2
bGD818	*amyE*::PyocH-*optRBS-venus* (cat) *yvbJ*::Phyperspank-*optRBS-pdaC* (spec)	This study	2, S5
bGD709	*amyE*::PyocH-*optRBS-venus* (cat) *ycgO*::Phyperspank-*optRBS-iseA* (erm)	This study	2
bGD857	*yvbJ*::Phyperspank-*optRBS*-pdaC (spec) Δ*cwlO*::*kan* *amyE*::PyocH-*optRBS-venus* (cat)	This study	2
bGD853	*ycgO*::Phyperspank-*optRBS-iseA* (erm) Δ*cwlO*::*kan* *amyE*::PyocH-*optRBS-venus* (cat)	This study	2
bGD855	*yvbJ*::Phyperspank-*optRBS-pdaC* (spec) Δ*lytE*::*kan* *amyE*::PyocH-*optRBS-venus* (cat)	This study	2
bGD851	*ycgO*::Phyperspank-*optRBS-iseA* (erm) Δ*lytE*::*kan* *amyE*::PyocH-*optRBS-venus* (cat)	This study	2

bGD170	Δ*pdaC*::*erm*	This study	2
bGD919	Δ*pdaC*::*erm* *yhdG*::PpdaC-*pdaC-6xHis* (kan)	This study	2
bGD950	*yvbJ*::Phyperspank-*optRBS-pdaC-6xHis* (spec)	This study	2, S5
bGD975	*amyE*::PyocH-*optRBS-venus* (cat) *yvbJ*::Phyperspank-*optRBS-pdaC-6xHis* (spec)	This study	2
bGD983	*amyE*::PyocH-*optRBS-venus* (cat) *yvbJ*::Phyperspank-*optRBS-pdaC*(D285A)-6xHis (spec)	This study	2
bGD984	*amyE*::PyocH-*optRBS-venus* (cat) *yvbJ*::Phyperspank-*optRBS-pdaC*(H427A)-6xHis (spec)	This study	2
bGD110	*amyE*::PiseA-*optRBS-venus* (cat)	Dobihal et al. 2019 [2]	3, S2
bGD294	*amyE*::PiseA-*optRBS-venus (cat)* *yvbJ*::Phyperspank-*optRBS-lytE* (spec)	Dobihal et al. 2019 [2]	3, S2
bGD708	*amyE*::PiseA-*optRBS-venus* (cat) *yvbJ*::Phyperspank-*optRBS-lytE* (spec) *ycgO*::Phyperspank-*optRBS-iseA* (erm)	This study	3
bGD870	*amyE*::PiseA-*optRBS-venus* (cat) *yvbJ*::Phyperspank-*optRBS-lytE* (spec) *ycgO*::Phyperspank-*optRBS-pdaC* (erm)	This study	3, S2
bGD871	*amyE*::PiseA-*optRBS-venus* (cat) Δ*pdaC*::*erm*	This study	3
bGD869	*amyE*::PiseA-*optRBS-venus* (cat) *ycgO*::Phyperspank-*optRBS-pdaC* (erm)	This study	3
bGD707	*amyE*::PiseA-*optRBS-venus* (cat) *ycgO*::Phyperspank-*optRBS-iseA* (erm)	This study	S2, S4
bGD1018	*yvbJ*::Phyperspank-*optRBS-lytE* (spec) *amyE*::PiseA-*optRBS-venus* (cat) *ycgO*::Phyperspank-*optRBS-pdaC*(D285A) (erm)	This study	S2, S3
bGD810	*yvbJ*::Phyperspank-*optRBS-pdaC* (spec)	This study	4
bGD847	*yvbJ*::Phyperspank-*optRBS-pdaC* (spec) Δ*lytE*::*kan*	This study	4
bGD848	*yvbJ*::Phyperspank-*optRBS-pdaC* (spec) Δ*cwlO*::*kan*	This study	4
bGD997	*amyE*::PiseA-*optRBS-venus* (cat) *ytoI*::Pveg-*mTagBFP* (kan)	This study	4
bGD998	*amyE*::PiseA-*optRBS-venus* (cat) Δ*pdaC*::*erm* *ytoI*::Pveg-*mTagBFP* (kan)	This study	4
bGD999	*amyE*::PiseA-*optRBS-venus* (cat) *ycgO*::Phyperspank-*optRBS-pdaC* (erm) *ytoI*::Pveg-*mTagBFP* (kan)	This study	4

bGD965	*pdaC*::*pdaC-6xHis* (kan)	This study	S5
bGD976	*pdaC*::*pdaC-6xHis* (kan) *yvbJ*::Phyperspank-*optRBS-lytE* (spec)	This study	S5

Table S3. Plasmids used in this study

Plasmid	Description	Source
pGD179	*yvbJ*::Phyperspank-*optRBS-pdaC* (spec, amp)	This study
pGD187	*ycgO*::Phyperspank-*optRBS-pdaC* (erm, amp)	This study
pGD196	*ycgO*::Phyperspank-*optRBS-pdaC*(D285A) (erm, amp)	This study
pGD197	*ycgO*::Phyperspank-*optRBS-pdaC*(H427A) (erm, amp)	This study
pGD203	*yvbJ*::Phyperspank-*optRBS-pdaC*(D285A)-6xHis (spec, amp)	This study
pGD204	*yvbJ*::Phyperspank-*optRBS-pdaC*(H427A)-6xHis (spec, amp)	This study
pIR242	Himar1C9 IR-spec Ppen-IR terminators (amp, erm)	This study
pJM63	P_{T7}-His_6-SUMO-cwlOΔcc	Dobihal et al., 2019 [2]
pYB190	*ycgO*::Phyperspank-*optRBS-iseA* (erm)	Dobihal et al., 2019 [2]
pWX294	empty vector with pACYC origin and MCS (amp)	This study
pWX634	MmeI-TnKRM (spec, amp)	This study
pWX638	pACYC HiMar repG(ts) (amp)	This study
pWX642	pACYC MmeI-TnKRM (spec, erm, amp)	This study

Table S4. Oligonucleotide primers used in this study

Oligonucleotide	Sequence
oJM36	agaagcggccgcttattctg
oJM37	ctgagcgagggagcagaactcacttttatatcctccctttac
oJM38	gttgaccagtgctccctgtaataaatatgacaagggccttct
oJM39	tcatccgtctgaagcacac
oJM54	tgctatcggagagcattgg
oJM55	ctgagcgagggagcagaaatcatgaaatcacctaatcttttatatc
oJM56	gttgaccagtgctccctgtaaagtgaaaaagccgttccag
oJM57	taatgtctctgcagtgcgag
oJM40	agttgcaatcacaagtgtatg
oJM41	ctgagcgagggagcagaattcatattttcctccccaaatgtt
oJM42	gttgaccagtgctccctgtaatttttagagaaaacccgttcattgg
oJM53	tcacctgtgagcatataatagtag
oJM3	gattaacgaaaggttgagatgttatgGAGGGAGGAAAGGCAGGA
oJM4	caatggatgatgagtttgtttgtgtCGCCGTATCTGTGCTCTC
oJM28	TTCTGCTCCCTCGCTCAG
oJM29	CAGGGAGCACTGGTCAAC
oGD509	GTGAGCGGATAACAATTAAGCTTacaTAAGGAGGaactactttgTTGGCAAAAAGAATCAAATGGTTTCA
oGD510	GCTAGCatCTGCAGttACTAGTttaTTTCGCTTCTCTTTGTTTTTTAACCTC
oGD517	CGTTGACCAAGAGCATAC
oGD521	gaggcggcctgtatggccGAATTCTTTTATGATGAAATTCCTTAAAAAGGATTGAC
oGD525	gaagAATTgGATCCatGCTAGCatCTCGAGTttaGTGATGATGATGATGATGTTTCGCTTCTCTTTGTTTTT
oGD540	GCTTACTTTCGCCGACGGCCCGAATC
oGD541	GATTCGGGCCGTCGGCGAAAGTAAGC
oGD542	CCATTTTGATTGCCGATATTTACCG
oGD543	CGGTAAATATCGGCAATCAAAATGG
oGD486	cctcaaatggttcgctgGgatccttatttttgacaccagaccaactggtaatggtagcg
oGD487	gtcacaagcagctgggaagGAATTCGAAATCCTTCATGTAAAGGAAC

oGD565	GCTAGCatCTGCAGttACTAGTttaGTGATGATGATGATGATGTTTCGCTTCTCTTTGTTTTTTAACCTCTT
oGD571	ccttttgataaagagagcgtcGAc
oGD572	CgacgctctctttatcaaaaggATTAGAAAAGGCTGTCCGTACG
oGD573	GCAAGTCTTCATGATCAAAACG
oIR541	gccactagttCGAAAAAACGG
oIR542	cggCTGCAgCAACGTTCTTG
oML78	CCATTAGAACATAGGGAGAG
oWX1154	GGCCGGTCGACCAGACCGGGGACTTATCATCCAACCTGTTAGCGGCCGCA
oWX1155	AGCTTGCGGCCGCTAACAGGTTGGATGATAAGTCCCCGGTCTGGTCGACC
oWX1156	TAGTCCACTCTCAACTCCTGATCC
oWX1157	GTCGACCTGCAGGCATGCAAGCTTGAGGGAAACCGTTGTGGTCTCCC
oWX1158	AATAACTAGCATAACCCCTTGGGG
oWX1159	GAGGCCCCAAGGGGTTATGCTAGTTATTGAATTCGTCCAGAAGGTCGATAG
oWX1160	GTTTCCCTCAAGCTTGCATGCCTGCAGGTCGACTCTAGAGGATCCCCGGG

Supplemental References:

1. Youngman, P.J., J.B. Perkins, and R. Losick, *Genetic transposition and insertional mutagenesis in Bacillus subtilis with Streptococcus faecalis transposon Tn917.* Proc Natl Acad Sci U S A, 1983. **80**(8): p. 2305-9.

2. Dobihal, G.S., et al., *Homeostatic control of cell wall hydrolysis by the WalRK two-component signaling pathway in Bacillus subtilis.* Elife, 2019. **8**.