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Table S1: Strains used in this study

***B. subtilis* Strain**	**Relevant Genotype**	**Source/Ref[1]**
BD170	*trpC2 thr5*	Lab strain
BD630	*hisA1 leuA8 metB5*	Lab strain
BD1991	*hisA1 leuA8 metB5, amyE::comK'-lacZ$^+$ cm*	Hahn *et al.*, 1995
BD2594	*hisA1 leuA8 metB5, amyE::comK'-lacZ$^+$ cm::spc*	Turgay *et al.* 1998
BD2741	*hisA1 leuA8 metB5, ΔylbF::spc*	Tortosa *et al.*, 2000
BD3032	*hisA1 leuA8 metB5, ΔymcA::spc*	Lab Strain
BD4319	*hisA1 leuA8 metB5,amyE: P_{xly}-gfp spc*	Lab Strain
BD4498	*hisA1 leuA8 metB5, eps'-lacZ$^+$ tet*	Kearns *et al.*, 2005
BD4576	*hisA1 leuA8 metB5, Δspo0A::kan*	Lab Strain
BD5402	*hisA1 leuA8 metB5, ylbF-yfp cm*	This Study
BD5406	*hisA1 leuA8 metB5, amyE::comK'-lacZ$^+$ spc, ylbF-yfp cm*	This Study
BD5409	*hisA1 leuA8 metB5, amyE::comK'-lacZ$^+$ cm, ΔylbF::spc*	This Study
BD5410	*hisA1 leuA8 metB5, amyE::comK'-lacZ$^+$ cm, ΔymcA::spc*	This Study
BD5494	*hisA1 leuA8 metB5, ymcA-yfp cm*	This Study
BD5495	*hisA1 leuA8 metB5, amyE::comK'-lacZ$^+$ spc, ymcA-yfp cm*	This Study
BD5563	*hisA1 leuA8 metB5, eps'-lacZ$^+$ tet, ΔymcA::spc*	This Study
BD5564	*hisA1 leuA8 metB5, eps'-lacZ$^+$ tet, ΔylbF::spc*	This Study
BD5635	*hisA1 leuA8 metB5, ΔyaaT::ery, P_{yaaT}–lacZ$^+$*	This Study
BD5669	*hisA1 leuA8 metB5, P_{spoIIG}-luc cm, ΔymcA::spc*	This Study
BD5670	*hisA1 leuA8 metB5, P_{spoIIG}-luc cm, ΔylbF::spc*	This Study
BD5671	*hisA1 leuA8 metB5, P_{spoIIG}-luc cm, ΔyaaT::ery*	This Study
BD5807	3610*, ΔymcA::spc*	This Study
BD5808	3610*, ΔylbF::spc*	This Study
BD5809	3610*, ΔyaaT::ery*	This Study
BD5829	*hisA1 leuA8 metB5, P_{abrB}-luc cm*	This Study
BD5860	*hisA1 leuA8 metB5 P_{abrB}-luc cm, Δspo0A::km*	This Study
BD5966	*hisA1 leuA8 metB5, P_{spoIIG}-luc cm*	Prepiak *et al.* 2011
BD6213	*hisA1 leuA8 metB5, P_{spoIIG}-luc cm, ΔrapA-phrA km*	This Study
BD6214	*hisA1 leuA8 metB5, P_{spoIIG}-luc cm, ΔrapA-phrA km, ΔymcA::spc*	This Study
BD6215	*hisA1 leuA8 metB5, P_{spoIIG}-luc cm, ΔrapA-phrA km, ΔylbF::spc*	This Study
BD6216	*hisA1 leuA8 metB5, P_{spoIIG}-luc cm, ΔrapA-phrA km, ΔyaaT::ery*	This Study
BD6219	*hisA1 leuA8 metB5, P_{spoIIG}-luc cm, Δspo0E::tet*	This Study
BD6220	*hisA1 leuA8 metB5, P_{spoIIG}-luc cm, Δspo0E::tet, ΔymcA::spc*	This Study
BD6221	*hisA1 leuA8 metB5, P_{spoIIG}-luc cm, Δspo0E::tet, ΔylbF::spc*	This Study
BD6222	*hisA1 leuA8 metB5, P_{spoIIG}-luc cm, Δspo0E::tet, ΔyaaT::ery*	This Study
BD6371	*hisA1 leuA8 metB5, ΔyaaT::ery*	This Study
BD6379	*hisA1 leuA8 metB5, P_{spoIIG}-luc tet, amyE::P_{spac}-spo0A cm*	This Study
BD6385	*hisA1 leuA8 metB5, P_{spoIIG}-luc tet, amyE::P_{spac}-spo0A cm, ΔymcA::spc*	This Study
BD6386	*hisA1 leuA8 metB5, P_{spoIIG}-luc tet, amyE::P_{spac}-spo0A cm, ΔylbF::spc*	This Study
BD6387	*hisA1 leuA8 metB5, P_{spoIIG}-luc tet, amyE::P_{spac}-spo0A cm, ΔyaaT::ery*	This Study
BD6380	*hisA1 leuA8 metB5, P_{spoIIG}-luc cm, amyE:: P_{spac}-sad-67 D56N tet*	This Study
BD6381	*hisA1 leuA8 metB5, P_{spoIIG}-luc cm, amyE:: P_{spac}-sad-67 D56N tet, ΔymcA::spc*	This Study
BD6382	*hisA1 leuA8 metB5, P_{spoIIG}-luc cm, amyE:: P_{spac}-sad-67 D56N tet, ΔylbF::spc*	This Study
BD6383	*hisA1 leuA8 metB5, P_{spoIIG}-luc cm, amyE:: P_{spac}-sad-67 D56N tet, ΔyaaT::ery*	This Study
BD6410	*hisA1 leuA8 metB5, amyE:: $P_{hyperspank}$-yfp-yaaT spc*	This Study
BD6411	*hisA1 leuA8 metB5, amyE::comK'-lacZ$^+$ cm, ΔyaaT::ery*	This Study
BD6412	*hisA1 leuA8 metB5, eps'-lacZ$^+$ tet, ΔyaaT::ery*	This Study
BD6413	*hisA1 leuA8 metB5, ΔymcA::ery, P_{ymcA}-lacZ$^+$*	This Study
BD6414	*hisA1 leuA8 metB5, ΔylbF::ery, P_{ylbF}-lacZ$^+$*	This Study
BD6530	*hisA1 leu8 metB5, ΔymcA::ery, P_{ymcA}-lacZ$^+$, Δspo0A::kan*	This Study
BD6531	*hisA1 leuA8 metB5, ΔylbF::ery, P_{ylbF}-lacZ$^+$, Δspo0A::kan*	This Study
BD6532	*hisA1 leuA8 metB5, ΔyaaT::ery, P_{yaaT}–lacZ$^+$, Δspo0A::kan*	This Study
BD6600	*hisA1 leuA8 metB5, P_{spoIIG}-luc cm, ΔkinD::tet*	This Study
BD6601	*hisA1 leuA8 metB5, P_{spoIIG}-luc tet, ΔkinD::tet, ΔymcA::spc*	This Study
BD6602	*hisA1 leuA8 metB5, P_{spoIIG}-luc tet, ΔkinD::tet, ΔylbF::spc*	This Study
BD6603	*hisA1leuA8 metB5, P_{spoIIG}-luc tet, ΔkinD::tet, ΔyaaT::ery*	This Study
BD6707	*hisA1 leuA8 metB5, P_{abrB}-luc cm, ΔylbF::spc*	This Study
NCIB 3610	Natural isolate of *Bacillus subtilis*	D. Kearns

E. coli strain	Background/Plasmid	Source/Ref
BTH101	Bacterial 2H strain *cya*$^-$ reporter strain	Karimova *et al.*, 2000
PP494	BL21 (DE3)/ p*ET26B-spo0A amp*	Muchová *et al.*, 2004
ED1229	DH5α/ p*UC19-ylbF-yfp amp*	This Study
ED1279	DH5α/ p*UC19-ymcA-yfp amp*	This Study
ED1307	DH5α/ p*T25N-ylbF kan*	This Study
ED1333	Stellar/ p*GEX-6P-1-GST-ymcA amp*	This Study
ED1334	Stellar/ p*GEX-6P-1-GST-ylbF amp*	This Study
ED1350	DH5α/ p*T25N-ymcA kan*	This Study
ED1384	Stellar/ p*T18C-ymcA amp*	This Study
ED1385	Stellar/ p*T25N-yaaT kan*	This Study
ED1427	BL21 (DE3)/ p*ET21B-spo0B-His$_6$ amp*	Fujita and Losick, 2003
ED1428	BL21 (DE3)/ p*ET21B-spo0F-His$_6$ amp*	Fujita and Losick, 2003
ED1444	BL21 (DE3)/ Δ*slrD,* p*ET21B-kinA-His$_6$ amp*	Fujita and Losick, 2003
ED1450	Stellar/ p*TB146-His$_6$-SUMO-yaaT amp*	This Study
ED1459	Stellar/ p*T18C-spo0A amp*	This Study
ED1460	Stellar/ p*T18C-spo0B amp*	This Study
ED1466	Stellar/ p*T18C-kinA amp*	This Study
ED1467	Stellar/ p*T18C-spo0F amp*	This Study
ED1495	Stellar/ p*Mutin4-yaaT-KO amp*	This Study
ED1510	Stellar/ p*DR111-yfp-yaaT amp*	This Study
ED1518	Stellar/p*Mutin4-ymcA amp*	This Study
ED1519	Stellar/p*Mutin4-ylbF amp*	This Study
ED1602	DH5α/p*GEX-6P-1-GST-spo0E amp*	This Study

[1]Knockout mutations and constructs that were not made in this study were obtained from the following: Δ*ymcA* (A. Neyfakh), Δ*spo0A* (M. Fujita), Δ*rapAphrA* (N. Mirouze), Δ*spo0E::tet* (N. Mirouze), Δ*kinD* (R. Losick), P_{spac}-*spo0A* (Fujita *et al.*, 2005), and P_{spac}-*sad-67* (A. Grossman), P_{xyl}-*GFP* (P. Prepiak).

Table S2: Sequences of primers used for plasmid construction

Primer Name	Sequence 5' → 3'
5 ylbF-ecoRI	tggtggGAATTCcgagatgattctgcagtcggagacg
3 ylbF-xhoI	ccaccaCTCGAGggacactttacatccgcagcttcc
5 ymcA-mfeI	tggtggCAATTGggctgaagcgcaaatcaatgag
3 ymcA-xhoI	ccaccaCTCGAGgagagaacagctgttatttgaatgc
5 YFP-YaaT-INF	AAGCTAGCTCCGTCGACTGTACAATGTAATTGGTGTCCGC
3 YFP-YaaT-INF	GAATTAGCTTGCATGCTTAATCTGTGGTTTGTGCGGATA
5 YaaTKO_ecoRI	tggtggGAATTCgaaaagtgatacgtgtggcaga
3 YaaTKO_bamHI	ccaccaGGATCCgccgtctcatactcatcgttc
5 ymcA-GST-INF	GGGGCCCCTGGGATCCACGCTCTACTCAAAAAAAGAC
3 ymcA-GST-INF	GATGCGGCCGCTCGAGTTAGAGAGAACAGCTGTTATTTG
5 ylbF-GST-INF	GGGGCCCCTGGGATCCTATGCGACGATGGAATCCGTG
3 ylbF-GST-INF	GATGCGGCCGCTCGAGTCAGGACACTTTACATCCGCAGC
5 yaaT-SUMO-INF	GAGAACAGATTGGTGGTATGTACAATGTAATT
3 yaaT-SUMO-INF	GTCACCCGGGCTCGAGTTAATCTGTGGTTTGTGCGGATA
5 ymcA-mutin4	tggtggAAGCTTtcaaaaaaagacattgtgcagca
3 ymcA-mutin4	ccaccaGGATCCgaatgcttcacctttgaaccg
5 ylbF-mutin4	tggtggAAGCTTgcttcaaagtgaagctcagcagC
3 ylbF-mutin4	ccaccaGGATCCtccgcctgaaccgcagc
5spo0E-GST-INF	GGGATCCCCGGAATTCATGGGCGGTTCTTCTGAACAAG
3spo0E-GST-INF	GATGCGGCCGCTCGAGTTATTTATTTGCATCATATGCTGGCA
Bacterial 2-Hybrid Primers	**Sequence 5' → 3'**
5 ylbF-2H-bamHI	tggtggGGATCCtatgcgacgatggaatccgtgcg
3 ylbF-N25-kpnI	ccaccaGGTACCggacactttacatccgcagc
5 ymcA-N25-bamHI	tggtggGGATCCatgacgctctactcaaaaaaagac
3 ymcA-N25-kpnI	ccaccaGGTACCgagagaacagctgttat
5 ymcA-C18-INF	CGACTCTAGAGGATCCACGCTCTACTCAAAAAAAGAC
3 ymcA-C18-INF	TTATATCGATGAATTCTTAGAGAGAACACTGTTATTTGAATG
5 yaaT-N25-INF	TGATTACGCCAAGCTTATGTACAATGTAATTGGTGTCCGC
3 yaaT-N25-INF	CCGGGGATCCTCTAGAATCTGTGGTTTGTGCGGA
5 spo0A-C18-INF	CGACTCTAGAGGATCCGAGAAAATTAAAGTTTGTGTTGC
3 spo0A-C18-INF	TTATATCGATGAATTCTTAAGAAGCCTTATGCTCTAACC
5 spo0B-C18-INF	CGACTCTAGAGGATCCAAGGATGTTTCAAAAAATCAAGAA
3 spo0B-C18-INF	TTATATCGATGAATTCCTAGTCCAACCCAATTTCAATCA
5 kinA-C18-INF	CGACTCTAGAGGATCCGAACAGGATACGCAGCATGTTA
3 kinA-C18-INF	TTATATCGATGAATTCTTATTTTTTTGGAAATGAAATTTTAA
5 spo0F-C18-INF	CGACTCTAGAGGATCCATGAATGAAAAAATTTTAATCGTTGAT
3 spo0F-C18-INF	TTATATCGATGAATTCTCAGTTAGACTTCAGGGGCAGATA

Primers listed in lower-case letters were those used for standard cloning, with the restriction sites in capital letters. The primers in all capital letters are those used with the In-Fusion HD cloning kit, and red sequences represented homology to the vector sequence.

Table S3: List of YmcA-YFP, YlbF-YFP and YFP-YaaT co-isolated proteins

				Number of Unique Peptides					
				GFP	YmcA-YFP		YlbF-YFP		YFP-YaaT
Accession #	Gene name	Description	MW (kDa)	Control	#1	#2	#1	#2	#1
P02968	*hag*	Flagellin	33	168	196	12	133	11	52
P42212	***gfp***	**Green fluorescent protein**	**27**	**14**	**52**	**34**	**48**	**25**	**29**
P42175	*narG*	Nitrate reductase alpha chain	139	69	109	26	100	26	83
P37541	***yaaT***	**Stage 0 sporulation protein**	**31**	**0**	**22**	**15**	**56**	**15**	**55**
P37871	*rpoC*	DNA-directed RNA polymerase subunit beta'	134	57	42	16	67	10	56
Q04747	*srfAB*	Surfactin synthase subunit 2	401	46	23	55	35	41	95
P27206	*srfAA*	Surfactin synthase subunit 1	402	46	19	36	31	21	86
O34425	*gapB*	Glyceraldehyde-3-phosphate dehydrogenase	37	23	34	14	44	14	31
P33166	*tuf*	Elongation factor Tu	44	16	12	18	32	10	31
P42921	*rplD*	50S ribosomal protein L4	22	21	19	0	31	0	11
O34412	***ylbF***	**Regulatory protein**	**17**	**0**	**10**	**23**	**50**	**23**	**8**
O31779	***ymcA***	**Uncharacterized protein**	**16**	**0**	**53**	**27**	**48**	**26**	**9**
P37870	*rpoB*	DNA-directed RNA polymerase subunit beta	134	38	29	11	31	7	46
P71079	*fabL*	Enoyl-[acyl-carrier-protein] reductase [NADPH]	27	18	25	3	22	3	13
P71011	*albA*	Antilisterial bacteriocin subtilosin biosynthesis protein	52	22	22	16	11	16	35
P96574	*mtlR*	Transcriptional regulator	79	34	40	0	19	0	26
P80868	*fusA*	Elongation factor G	77	8	4	16	31	8	34
P32397	*hemY*	Protoporphyrinogen oxidase	51	33	29	1	11	0	20
P94391	*ycgN*	1-pyrroline-5-carboxylate dehydrogenase 2	56	9	5	16	30	13	30

P18157	*glpK*	Glycerol kinase	55	21	15	24	28	21	30
P21465	*rpsC*	30S ribosomal protein S3	24	15	17	10	17	8	13
P46911	*qcrA*	Menaquinol-cytochrome c reductase iron-sulfur subunit	19	2	35	15	2	7	0
P21464	*rpsB*	30S ribosomal protein S2	28	14	16	5	16	3	12
P42920	*rplC*	50S ribosomal protein L3	23	18	21	0	12	0	6
O31774	*ymdA*	2',3'-cyclic-nucleotide 2'-phosphodiesterase	59	1	1	4	3	4	32
Q03222	*rho*	Transcription termination factor	49	25	26	18	15	18	13
P37809	*atpD*	ATP synthase subunit beta	51	14	6	15	16	13	24
P38021	*rocD*	Ornithine aminotransferase	44	16	14	21	29	17	17
P39634	*rocA*	1-pyrroline-5-carboxylate dehydrogenase	56	14	5	12	21	6	26
P39738	*fliD*	Flagellar hook-associated protein 2	55	23	25	0	12	0	15
P80886	*sucC*	Succinyl-CoA ligase [ADP-forming] subunit beta	41	13	9	19	20	18	29
P50735	*gudB*	NAD-specific glutamate dehydrogenase	47	12	12	12	22	12	21
P42176	*narH*	Nitrate reductase beta chain	55	15	15	7	18	7	25
P09339	*citB*	Aconitate hydratase	99	1	1	21	24	15	42
O07021	*lutB*	Lactate utilization protein B	53	13	12	10	16	9	18
P42435	*nasD*	Nitrite reductase [NAD(P)H]	88	11	11	15	13	14	39
P17820	*dnaK*	Chaperone protein	66	12	7	18	9	16	29
Q08787	*srfAC*	Surfactin synthase subunit 3	144	9	3	8	11	3	25
Q05852	*gtaB*	UTP--glucose-1-phosphate uridylyltransferase	33	13	20	16	17	16	10
P28598	*groL*	60 chaperonin	57	18	18	11	10	10	26
P42919	*rplB*	50S ribosomal protein L2	30	6	9	4	11	2	10
O32006	*yokA*	Resolvase homolog	63	29	23	0	3	0	11
O07603	*yhfE*	Putative aminopeptidase	39	12	12	13	4	12	20
Q01465	*mreB*	Rod shape-determining protein	36	12	11	7	10	6	19
P28366	*secA*	Protein translocase subunit	96	11	3	11	21	10	33
P54716	*glvA*	Maltose-6'-phosphate glucosidase	51	9	13	19	29	17	16
P16263	*odhB*	Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex	46	10	9	19	18	18	21

P08066	*sdhB*	Succinate dehydrogenase iron-sulfur subunit	28	5	5	10	16	9	9
P39633	*rocG*	NAD-specific glutamate dehydrogenase	47	16	7	8	9	6	23
P08065	*sdhA*	Succinate dehydrogenase flavoprotein subunit	65	15	3	16	10	12	29
P25994	*pyrAB*	Carbamoyl-phosphate synthase pyrimidine-specific large chain	118	10	0	29	7	20	30
P21466	*rpsD*	30S ribosomal protein S4	23	9	15	6	14	4	9
P37869	*eno*	Enolase	47	10	6	16	13	15	21
P12425	*glnA*	Glutamine synthetase	50	1	6	15	14	13	20
P54452	*yqeG*	Uncharacterized protein	20	13	10	1	7	0	2
P21880	*pdhD*	Dihydrolipoyl dehydrogenase	50	11	1	4	13	2	21
P37808	*atpA*	ATP synthase subunit alpha	55	5	4	9	15	7	21
P18158	*glpD*	Aerobic glycerol-3-phosphate dehydrogenase	63	10	5	4	19	3	27
P21881	*pdhA*	Pyruvate dehydrogenase E1 component subunit alpha	42	9	6	8	13	7	21
P42412	*iolA*	Methylmalonate semialdehyde dehydrogenase [acylating]	53	5	7	6	17	4	21
P42060	*rplV*	50S ribosomal protein L22	12	9	6	0	7	0	3
P42415	*iolD*	3D-(3,5/4)-trihydroxycyclohexane-1,2-dione hydrolase	70	7	2	8	12	5	25
P14577	*rplP*	50S ribosomal protein L16	16	10	11	5	10	5	5
P21467	*rpsE*	30S ribosomal protein S5	18	10	11	7	13	5	9
P21879	*guaB*	Inosine-5'-monophosphate dehydrogenase	53	14	10	4	14	1	19
O06478	*yfmT*	Putative aldehyde dehydrogenase	53	7	3	5	12	5	24
P09124	*gapA*	Glyceraldehyde-3-phosphate dehydrogenase 1	36	7	6	7	10	5	12
P39793	*ponA*	Penicillin-binding protein 1A/1B	100	19	13	5	6	4	23
P21882	*pdhB*	Pyruvate dehydrogenase E1 component subunit beta	35	9	7	14	15	12	17
P12877	*rplE*	50S ribosomal protein L5	20	11	11	6	12	1	7
P96614	*cshA*	DEAD-box ATP-dependent RNA helicase	55	19	5	2	8	2	13
O32162	*sufB*	FeS cluster assembly protein	53	3	0	6	6	1	17
O31749	*pyrH*	Uridylate kinase	26	9	6	3	6	2	6
P16971	*recA*	Protein recA	38	10	8	4	10	4	12
P21469	*rpsG*	30S ribosomal protein S7	18	6	8	7	5	6	4
O34660	*dhaS*	Putative aldehyde dehydrogenase	54	8	5	15	6	15	17

P54420	*asnB*	Asparagine synthetase [glutamine-hydrolyzing] 1 OS	73	6	2	10	8	6	22
P23129	*odhA*	2-oxoglutarate dehydrogenase E1 component	106	6	3	7	13	6	21
P55873	*rplT*	50S ribosomal protein L20	14	3	5	0	8	0	6
Q06797	*rplA*	50S ribosomal protein L1	25	7	7	5	14	2	10
P39126	*icd*	Isocitrate dehydrogenase [NADP]	46	5	1	9	14	6	16
O32129	*lipA*	Lipoyl synthase	34	8	6	0	13	0	12
P26935	*iolg*	Inositol 2-dehydrogenase/D-chiro-inositol 3-dehydrogenase	38	5	3	7	13	2	13
O34909	*yeaR*	Putative adenine deaminase year	67	9	2	0	7	0	17
P54531	*yqiT*	Leucine dehydrogenase	40	2	6	4	13	2	15
O34454	*ykaA*	UPF0111 protein	24	11	10	4	11	2	4
P39754	*glmS*	Glucosamine--fructose-6-phosphate aminotransferase 3	65	5	0	3	5	1	22
P30949	*hemL*	Glutamate-1-semialdehyde 2,1-aminomutase	46	8	4	10	6	10	14
Q45493	*rnjA*	Ribonuclease J 1	62	9	4	3	5	2	17
Q03224	*ywjI*	Uncharacterized protein	34	4	2	2	6	1	13
P39148	*glyA*	Serine hydroxymethyltransferase	45	9	4	8	9	6	17
P54419	*metK*	S-adenosylmethionine synthase	44	10	2	7	15	5	18
P24141	*oppA*	Oligopeptide-binding protein	62	0	2	7	10	5	15
P17631	*dnaJ*	Chaperone protein	41	6	6	3	3	3	18
O31742	*rplS*	50S ribosomal protein L19	13	7	10	2	8	0	5
O34529	*pfkA*	6-phosphofructokinase	34	8	5	4	9	3	8
O31782	*pksN*	Polyketide synthase	610	5	2	4	4	1	15
P05653	*gyrA*	DNA gyrase subunit A	92	10	8	7	3	6	24
P20429	*rpoA*	DNA-directed RNA polymerase subunit alpha	35	10	9	4	8	2	12
P39776	*xerC*	Tyrosine recombinase	35	12	7	0	3	0	0
P28619	*rph*	Ribonuclease PH	27	9	6	0	6	0	6
O32038	*aspS*	Aspartyl-tRNA synthetase	66	5	0	0	15	0	14
P45745	*dhbF*	Dimodular nonribosomal peptide synthase	264	1	4	0	2	0	17
O32267	*tuaH*	Putative teichuronic acid biosynthesis glycosyltransferase	46	15	5	0	5	0	8
P54423	*wprA*	Cell wall-associated protease	96	5	3	4	8	3	9

O32259	*lutC*	Lactate utilization protein C	26	9	5	7	10	7	5
P39120	*citZ*	Citrate synthase 2	42	4	2	9	7	9	13
P36947	*rbsA*	Ribose import ATP-binding protein	55	13	5	6	13	4	11
P37570	*yacI*	Putative ATP:guanido phosphotransferase	41	8	8	2	4	1	15
P45694	*tkt*	Transketolase	72	3	0	6	8	3	19
P94356	*yxkC*	Uncharacterized protein	19	5	5	5	9	5	7
P21470	*rpsI*	30S ribosomal protein S9	14	5	6	0	6	0	3
P25144	*ccpA*	Catabolite control protein A	37	12	3	0	9	0	9
P04969	*rpsK*	30S ribosomal protein S11	14	4	6	0	9	0	5
P14193	*prs*	Ribose-phosphate pyrophosphokinase	35	8	4	10	7	8	9
P42065	*appF*	Oligopeptide transport ATP-binding protein	37	11	9	6	4	5	8
P42414	*iolC*	5-dehydro-2-deoxygluconokinase	36	1	0	4	3	2	16
P39751	*mbl*	MreB-like protein	36	5	5	5	8	5	12
P21883	*pdhC*	Dihydrolipoyllysine-acetyltransferase component of pyruvate dehydrogenase complex	48	3	2	6	3	6	16
P39912	*aroA*	Phospho-2-dehydro-3-deoxyheptonate aldolase	40	8	4	0	6	0	12
P54479	*zur*	Zinc-specific metallo-regulatory protein	17	8	7	0	10	0	5
O31777	*kbl*	Putative 8-amino-7-oxononanoate synthase/2-amino-3-ketobutyrate coenzyme A ligase	43	8	3	3	3	2	15
P94390	*ycgM*	Proline dehydrogenase 2	35	10	7	4	8	3	11
P54377	*gcvPB*	Probable glycine dehydrogenase [decarboxylating] subunit 2	54	10	3	10	3	8	10
P39616	*ywdH*	Probable aldehyde dehydrogenase ywdH	51	11	9	0	3	0	8
O31778	*miaB*	(Dimethylallyl)adenosine tRNA methylthiotransferase	58	3	1	8	11	5	13
P42418	*iolH*	Unknown Protein	34	5	5	9	11	9	10
O31760	*rnjB*	Ribonuclease J 2	61	10	2	5	6	4	13
O34857	*rok*	Repressor	22	12	1	4	0	4	2
P39814	*topA*	DNA topoisomerase 1	79	20	3	9	2	9	15
P37527	*pdxS*	Pyridoxal biosynthesis lyase	32	5	4	6	8	5	7
P08821	*hupA*	DNA-binding protein HU 1	10	7	6	0	7	0	4

P39778	*clpY*	ATP-dependent protease ATPase subunit	53	7	6	6	4	5	11
P39772	*asnS*	Asparaginyl-tRNA synthetase	49	12	4	5	9	4	13
P46337	*iolR*	HTH-type transcriptional regulator	28	10	6	4	5	4	1
P17889	*infB*	Translation initiation factor IF-2	79	5	2	0	6	0	13
P54717	*glvR*	HTH-type transcriptional regulator	29	12	13	6	11	5	3
P24327	*prsA*	Foldase protein	33	2	2	4	4	3	10
P19669	*tal*	Transaldolase	23	7	10	10	11	10	6
P46898	*rplF*	50S ribosomal protein L6	20	7	4	0	5	0	8
P37945	*lon1*	Lon protease 1	87	6	0	2	1	2	17
P29726	*purA*	Adenylosuccinate synthetase	48	3	0	5	6	2	15
P50866	*clpX*	ATP-dependent Clp protease ATP-binding subunit	46	5	1	7	6	5	13
O07573	*nsrR*	HTH-type transcriptional regulator	17	8	5	0	3	0	3
P42923	*rplJ*	50S ribosomal protein L10	18	5	6	0	7	0	7
P02394	*rplL*	50S ribosomal protein L7/L12	13	3	6	0	7	0	6
P20277	*rplQ*	50S ribosomal protein L17	14	3	3	0	4	0	4
P21474	*rpsP*	30S ribosomal protein S16	10	6	4	0	5	0	5
P39142	*pdp*	Pyrimidine-nucleoside phosphorylase	46	3	0	5	3	4	16
P26908	*rplU*	50S ribosomal protein L21	11	3	5	0	3	0	4
O31494	*ydzF*	Uncharacterized HTH-type transcriptional regulator	13	7	7	0	5	0	5
O06714	*sbcC*	Nuclease sbcCD subunit C	129	0	0	0	1	0	17
O31766	*ymfH*	Uncharacterized zinc protease	49	0	0	0	1	0	19
P24281	*yaaK*	UPF0133 protein	12	3	2	4	1	4	4
Q06796	*rplK*	50S ribosomal protein L11	15	3	5	0	7	0	5
P71018	*plsX*	Phosphate acyltransferase	36	6	2	4	1	3	10
P21471	*rpsJ*	30S ribosomal protein S10	12	6	6	4	7	3	5
P12876	*rplX*	50S ribosomal protein L24	11	2	3	0	5	0	4
O06491	*gatB*	Glutamyl-tRNA(Gln) amidotransferase subunit A	53	3	1	5	7	1	11
O06975	*whiA*	Putative sporulation transcription regulator	36	13	10	3	1	3	2
O32117	*yutJ*	NADH dehydrogenase-like protein	40	15	3	3	3	3	5

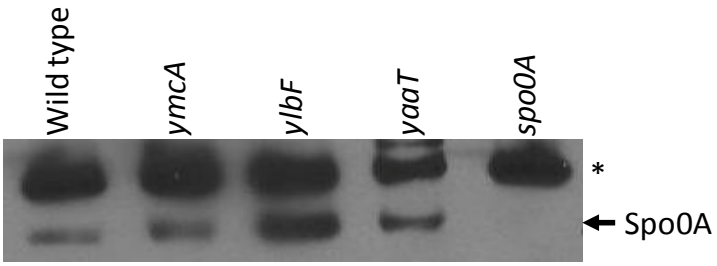
P24137	*oppF*	Oligopeptide transport ATP-binding protein	35	11	2	6	5	4	5
P08838	*ptsI*	Phosphoenolpyruvate-protein phosphotransferase	63	1	0	3	1	1	15
P49814	*mdh*	Malate dehydrogenase	34	2	1	5	9	2	10
P54466	*yqfA*	UPF0365 protein	36	0	1	4	4	2	11
O32165	*sufD*	FeS cluster assembly protein	48	1	0	4	4	1	12
O31728	*sepF*	Cell division protein sepF	17	5	5	3	5	3	3
Q45066	*parC*	DNA topoisomerase 4 subunit A	91	1	0	0	0	0	12
P40924	*pgK*	Phosphoglycerate kinase	42	5	1	2	4	2	7
P70974	*rplM*	50S ribosomal protein L13	16	4	5	4	5	0	3
P94360	*msmX*	Maltodextrin import ATP-binding protein	41	5	3	0	5	0	7
Q03223	*rpmE*	50S ribosomal protein L31	7	2	3	0	6	0	2
P80865	*sucD*	Succinyl-CoA ligase [ADP-forming] subunit alpha	31	3	1	6	10	4	7
P12879	*rpsH*	30S ribosomal protein S8	15	4	2	3	8	3	3
O34338	*mntB*	Manganese transport system ATP-binding protein	28	6	0	5	1	5	2
P37814	*atpF*	ATP synthase subunit b	19	3	0	5	2	3	7
P42437	*nasF*	Uroporphyrinogen-III C-methyltransferase	54	5	0	0	3	0	9
O54408	*relA*	GTP pyrophosphokinase	85	8	0	3	1	3	12
O32201	*liaH*	Similar to phage shock protein, resistance against oxidative stress and cell wall antibiotics	26	2	8	2	4	2	6
P46899	*rplR*	50S ribosomal protein L18	13	2	8	7	5	5	3
P19946	*rplO*	50S ribosomal protein L15	15	4	5	7	5	5	2
P50849	*pnp*	Polyribonucleotide nucleotidyltransferase	77	0	1	1	8	0	10
P71088	*spo0M*	Sporulation-control protein	30	4	4	9	7	9	4
P37486	*yybR*	Uncharacterized HTH-type transcriptional regulator	15	7	7	0	3	0	2
Q45057	*yneB*	Resolvase homolog	25	10	4	0	2	0	1
O31755	*proS*	Prolyl-tRNA synthetase	63	5	0	4	3	0	11
Q45477	*ileS*	Isoleucyl-tRNA synthetase	105	0	0	2	6	2	11
P54482	*ispG*	4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase	41	3	0	3	6	2	10
P54617	*ydjF*	Phage shock protein A homolog	25	0	0	5	0	4	9

O32213	*cysI*	Sulfite reductase [NADPH] hemoprotein beta-component	65	0	0	5	7	2	10
O34949	*ykoM*	Uncharacterized HTH-type transcriptional regulator	18	7	9	3	2	2	3
P05649	*dnaN*	DNA polymerase III subunit beta	42	5	1	8	4	8	11
P21472	*rpsL*	30S ribosomal protein S12	15	2	1	3	6	2	3
P46336	*iolS*	Unknown	35	4	1	7	5	6	9
P35136	*serA*	D-3-phosphoglycerate dehydrogenase	57	0	0	1	5	0	9
P51785	*ilvD*	Dihydroxy-acid dehydratase	60	0	1	4	2	2	12
P20282	*rpsM*	30S ribosomal protein S13	14	3	4	0	3	0	4
P25812	*mnmG*	tRNA uridine 5-carboxymethylaminomethyl modification enzyme	70	3	0	0	2	0	11
Q02113	*lytB*	Amidase enhancer	77	6	2	2	0	0	10
P97032	*yhbD*	Uncharacterized protein	27	10	5	0	0	0	2
O06973	*yvcJ*	UPF0042 nucleotide-binding protein	34	10	6	0	4	0	0
P96683	*ydfF*	Uncharacterized HTH-type transcriptional regulator	26	8	6	0	0	0	0
O32164	*csd*	Probable cysteine desulfurase	45	6	0	3	2	3	7
O34910	*yobT*	Uncharacterized protein	25	4	6	0	4	0	4
Q08352	*ald*	Alanine dehydrogenase	40	4	0	1	3	0	10
P12875	*rplN*	50S ribosomal protein L14	13	4	3	3	5	2	3
O07906	*yraN*	Uncharacterized HTH-type transcriptional regulator	34	10	4	0	4	0	2
P36948	*rbsC*	Ribose transport system permease protein	34	2	1	1	5	1	4
P40871	*dhbE*	2,3-dihydroxybenzoate-AMP ligase	60	4	2	0	0	0	10
O07631	*typA*	GTP-binding protein TypA/BipA homolog	68	1	0	2	2	0	9
O31776	*tdh*	L-threonine 3-dehydrogenase	37	5	1	5	4	5	8
P18255	*thrS*	Threonyl-tRNA synthetase 1	74	5	0	2	5	1	8
P77837	*ureC*	Urease subunit alpha	61	2	0	1	0	1	12
P42924	*rplW*	50S ribosomal protein L23	11	1	4	1	6	1	4
P80861	*yjlD*	NADH dehydrogenase-like protein	42	5	3	2	4	1	4
Q45598	*yydD*	Uncharacterized protein	69	1	0	1	1	0	11
Q795Y4	*yrhE*	Putative formate dehydrogenase	109	3	1	2	0	1	9
O32119	*yutI*	Putative nitrogen fixation protein	12	1	2	0	3	2	4

P46320	*licH*	Probable 6-phospho-beta-glucosidase	49	3	2	8	1	6	7
P21475	*rpsR*	30S ribosomal protein S18	9	5	1	0	4	0	1
P42419	*iolI*	Inosose isomerase	32	3	0	5	8	2	6
O07020	*lutA*	Lactate utilization protein A	26	3	1	15	3	13	4
P40806	*pksJ*	Polyketide synthase	563	3	1	2	2	0	5
P42974	*ahpF*	NADH dehydrogenase	55	2	0	0	2	0	4
P96499	*yvhJ*	Putative transcriptional regulator	43	8	0	0	2	1	6
P24139	*oppC*	Oligopeptide transport system permease protein	34	4	1	0	1	0	2
P28368	*yvyD*	Uncharacterized protein	22	4	2	4	3	4	3
P34957	*qoxa*	Quinol oxidase subunit 2	36	4	0	1	2	1	5
O06008	*adhR*	HTH-type transcriptional regulator	16	3	7	1	2	0	3
Q796K8	*pbpH*	Penicillin-binding protein H	79	0	1	0	2	0	8
O34925	*deoD*	Purine nucleoside phosphorylase deoD-type	25	4	1	1	3	0	5
O06474	*yfmP*	HTH-type transcriptional regulator	17	6	2	4	1	1	1
P17922	*pheT*	Phenylalanyl-tRNA synthetase beta chain	88	0	0	2	0	2	10
P05657	*rpmA*	50S ribosomal protein L27	10	2	3	2	4	2	4
P39765	*pyrR*	Bifunctional protein	20	6	1	12	1	6	3
P80866	*yurY*	Vegetative protein 296	29	0	3	4	2	0	5
P21468	*rpsF*	30S ribosomal protein S6	11	4	4	0	5	0	4
P32395	*hemE*	Uroporphyrinogen decarboxylase	40	4	2	4	5	3	6
O31630	*yjcH*	Uncharacterized protein	28	6	2	7	2	5	0
O34784	*yobI*	Uncharacterized membrane protein	141	1	0	6	0	6	6
A7BJC5 (+1)	*iolB*	5-deoxy-glucuronate isomerase	31	3	0	6	6	4	7
O34384	*yceE*	Uncharacterized protein	21	3	0	3	1	3	4
P39215	*mcpB*	Methyl-accepting chemotaxis protein	72	2	1	2	0	0	6
P42958	*ycsA*	Probable tartrate dehydrogenase/decarboxylase	39	0	1	1	2	0	6
P50740	*fni*	Isopentenyl-diphosphate delta-isomerase	37	9	1	0	0	0	1
O31605	*yjbG*	Oligoendopeptidase F homolog	77	0	1	0	0	0	9

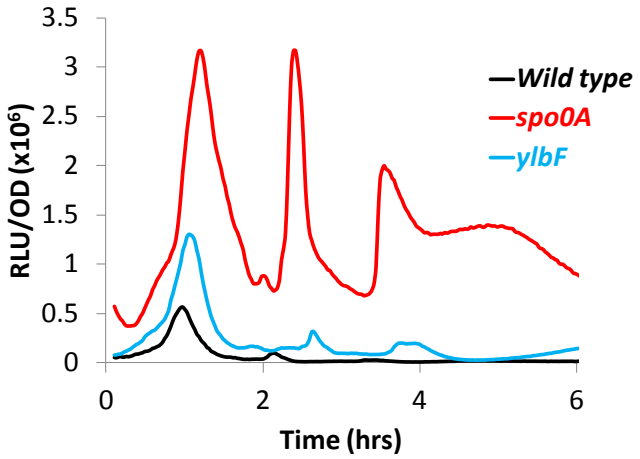
O34774	*yobJ*	Uncharacterized protein	34	3	9	0	3	0	1
O07584	*plsC*	1-acyl-sn-glycerol-3-phosphate acyltransferase	22	4	1	0	1	0	3
O05389	*yrbE*	Uncharacterized oxidoreductase	38	5	0	2	2	0	4
P54510	*yqhL*	Uncharacterized protein	15	7	4	0	2	2	0
P30950	*hemB*	Delta-aminolevulinic acid dehydratase	36	3	0	0	5	0	6
O31501	*swrC*	Swarming motility protein	114	0	0	0	0	0	6
P80698	*tig*	Trigger factor	47	5	1	0	2	0	6
P96583	*topB*	DNA topoisomerase 3	81	0	2	0	1	0	4
O31716	*ykpA*	Uncharacterized ABC transporter ATP-binding protein	61	1	0	8	2	0	6
O07605	*gltT*	Proton/sodium-glutamate symport protein	46	1	0	0	3	0	5
P80700	*tsf*	Elongation factor Ts	32	0	0	0	3	0	6
O32044	*recJ*	Single-stranded-DNA-specific exonuclease	88	0	0	0	1	0	8
P54608	*yhcX*	UPF0012 hydrolase	60	2	0	0	0	0	6
Q02114	*lytC*	N-acetylmuramoyl-L-alanine amidase	53	1	1	2	1	2	4
P94545	*mutSB*	MutS2 protein	87	0	1	0	0	0	4
Q795M6	*yugH*	Putative aminotransferase	42	5	0	5	1	3	3
Q04796	*dapA*	Dihydrodipicolinate synthase	31	0	1	4	2	3	6
O32090	*pcnB*	Nicotinate phosphoribosyltransferase	56	4	0	0	1	0	6
P12013	*gntZ*	6-phosphogluconate dehydrogenase, decarboxylating	52	0	0	0	4	0	4
P38494	*ypfD*	30S ribosomal protein S1 homolog	42	0	0	0	1	0	6
P21476	*rpsS*	30S ribosomal protein S19	11	3	3	2	4	1	2

Figure S1: Spo0A levels are not decreased in *ymcA, ylbF* and *yaaT* mutants.



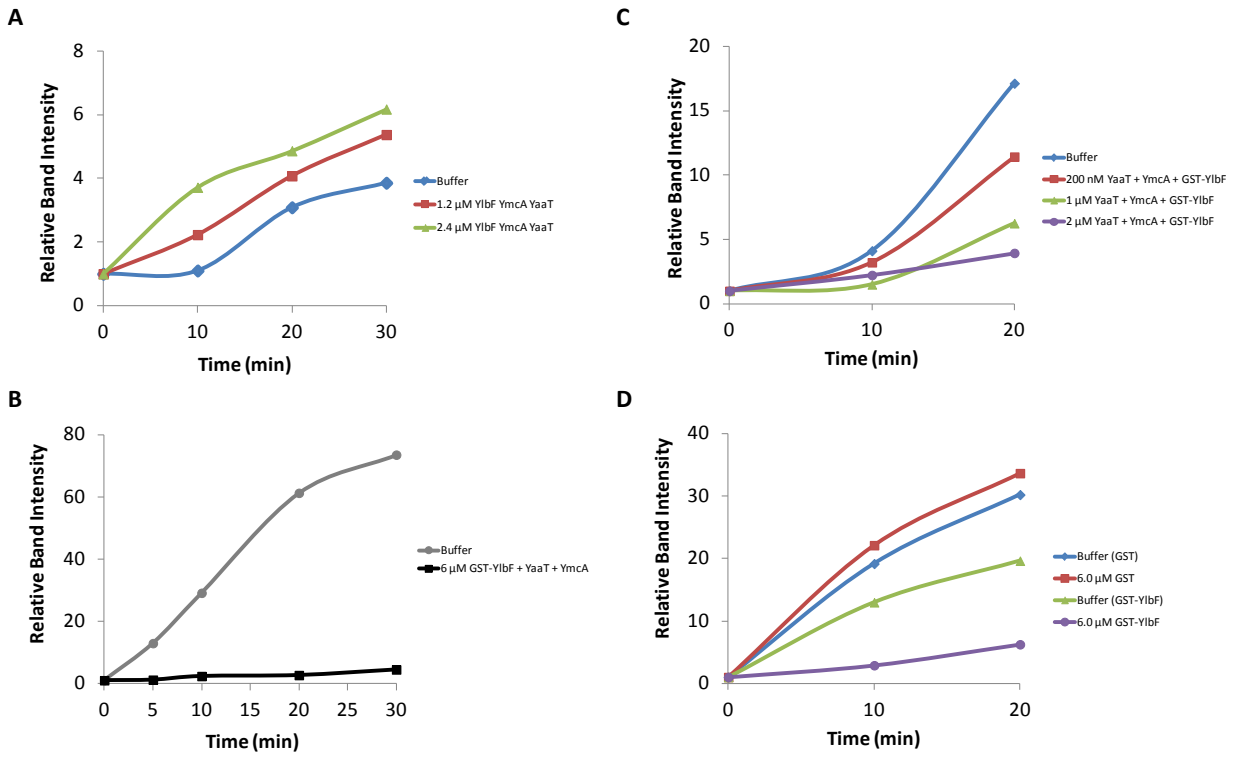
Wild type (BD630), *ymcA* (BD3032), *ylbF* (BD2741), *yaaT* (BD5635) and *spo0A* (BD4576) were grown in DSM until T_0. Whole cell extracts were prepared as described in the Supporting information, and samples were analyzed by immunoblotting with Spo0A antiserum. The asterisk represented a non-specific cross-reacting band that was included in the figure as a loading control.

Figure S2: A *ylbF* mutation has an intermediate effect on transcription of *abrB::luc*.



The *abrB::luc* construct was made as described previously (Mirouze *et al.*, 2011), with the native *abrB* ribosome binding site (RBS) replaced by the *spoVG* RBS. Luciferase assays for expression from the P_{abrB}-*luc* reporter construct were carried out as described in Experimental procedures. Wild type (BD5829), *spo0A* (BD5860), *ylbF* (BD6707).

Figure S3: Concentration-dependent effects of YlbF- and GST-YlbF-containing complexes.



(A) The YmcA-YlbF-YaaT complex exhibits a dose-responsive effect on the rate of the phosphorelay. The phosphorelay proteins were pre-incubated with 1.2 µM or with 2.4 µM YmcA-YlbF-YaaT co-purified complex. The relay was initiated by the addition of ^{32}P-γ-ATP, and samples were collected at the indicated time points. Bands were quantified as described in Experimental procedures, and total phosphate is depicted in each graph. (B-D) GST-YlbF inhibits the phosphorelay. Concentrations of a 1:1:1 mixture of individually purified GST-YlbF, YmcA and YaaT used were as follows: 200 nM, 1 µM, 2 µM (B) or 6 µM (C). To determine if GST-YlbF could inhibit the phosphorelay without its binding partners, 6 µM GST-YlbF was pre-incubated with the relay proteins, and purified GST was used as a control (D). GST and GST-YlbF were purified as described in Experimental procedures for GST-YmcA, with a single exception. GST-YlbF was purified in 20 mM HEPES, pH 7.5, 2 mM $MgCl_2$ instead of phosphate buffer, and thus has a separate buffer control, as indicated. Samples were collected and analyzed as described above.

Additional Experimental Procedures

Plasmid constructions

Construction of YFP fusion proteins

All oligonucleotides were synthesized by Integrated DNA Technologies. For construction of *ymcA-yfp*, its C-terminal 341 bp without its stop codon was amplified from BD630 genomic DNA, using the primers 5ymcA-mfeI and 5ymcA-xhoI (Table S2, restriction sites indicated by capital letters). The resulting amplicon was digested with *Mfe*I and *Xho*I and ligated into the *EcoR*I and *Xho*I sites of the plasmid *pKL184* (a gift from K. Lemon and A. Grossman). This resulted in a fusion of *yfp* to the C-terminus of the *'ymcA* C-terminal fragment (p*ED1279*). To make the *ylbF-yfp* fusion a similar strategy was used. A C-terminal 394 bp fragment of *ylbF* was amplified using the primers 5ylbF-ecoRI and 3ylbF-xhoI (Table S2), digested with *EcoR*I and *Xho*I and ligated into the same sites of *pKL184* (p*ED1229*). The resulting plasmids were transformed into chemically competent DH5α *E. coli* cells, and the constructs were verified by DNA sequence analysis by Macrogen, Inc. (New York, NY). Once confirmed, the plasmids were transformed into BD630 for Campbell-type integration into the native locus, selecting for chloramphenicol resistance. This created *ymcA-yfp* and *ylbF-yfp* fusions, each under the control of its native promoter, inactivating the native copy of *ymcA* and *ylbF.*

For construction of *yfp-yaaT*, the entire *yaaT* open reading frame (ORF) was amplified from BD630 genomic DNA, using the primers 5YFP-yaaT-INF and 3YFP-yaaT-INF (Table S2, red letters indicate homology to the vector sequence). The vector p*DR111-YFP* (Kramer *et al.*, 2007) was digested overnight with *Sal*I and *Sph*I. For cloning, the In-Fusion HD cloning kit (Clontech) was used, as per manufacturer's instructions. The resulting plasmid was transformed into

Stellar (Clontech) competent cells, and the plasmid sequence was verified as described above. The plasmid (p*ED1510*) was transformed into BD630 selecting for spectinomycin resistance, placing the *yfp-yaaT* fusion under the control of the 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG)-inducible $P_{hyperspank}$ promoter at the ectopic *amyE* locus.

Generation of the yaaT *knockout and promoter fusions*

The *yaaT* gene lies in the middle of a large operon, with genes that are important for DNA replication. To not disrupt the downstream genes, *yaaT* was cloned into *pMutin4* (Vagner *et al.*, 1998). A 465 bp internal fragment of *yaaT* was amplified from BD630 genomic DNA using the primers 5yaaTKO-ecoRI and 3yaaTKO-bamHI (Table S2). Following digestion, the fragment was ligated into the *EcoR*I and *BamH*I sites of *pMutin4*. The plasmid (p*Mutin4-yaaTKO,* p*ED1495*) was transformed into DH5α cells and verified by sequencing. It was then used to transform BD630 by Campbell integration, with selection for erythromycin resistance, creating a knockout of the chromosomal *yaaT* gene, and a transcriptional promoter fusion to *lacZ*, with the downstream genes under control of the IPTG-inducible promoter P_{spac}. To enable β-galactosidase assays with *lacZ* reporter fusions, it was necessary to create a variant of this *yaaT* knockout with the *lacZ* gene contained within *pMutin4* inactivated. For this, *pMutin4-yaaTKO* was digested with *Swa*I and *Fsp*I, which generate blunt ends, removing the *lacZ* coding sequence. After ligation, the new plasmid was verified by restriction digestion and then used to transform BD630, selecting for erythromycin resistance. For all experiments, these strains were grown in the presence of IPTG.

To create *lacZ* transcriptional fusions to the *ylbF* and *ymcA* genes, *pMutin4* was used. The primers 5ylbF-mutin4 and 3ylbF-mutin4 (Table S2) were used to amplify a 400 bp internal fragment of *ylbF* from BD630 genomic DNA. An internal 398 bp fragment was similarly obtained for *ymcA* using the primers 5ymcA-mutin4 and 3 ymcA-mutin4 (Table S2). Both fragments were digested with *Hind*III and *BamH*I and ligated into the *Hind*III and *BamH*I sites of *pMutin4*. The resulting products (p*ED1519* and p*ED1518*) were verified by sequencing, and then transformed into competent BD630, selecting for erythromycin resistance. These constructs generated chromosomal knockouts of the native gene, either *ymcA* or *ylbF*, as well as promoter fusions to *lacZ*. These strains were grown in the presence of 1 mM IPTG for all experiments, although no difference in reporter gene expression was noted when IPTG was omitted (not shown).

Construction of expression plasmids for protein purification

To construct *GST-ymcA* (p*ED1333*), the entire *ymcA* ORF, including its stop codon, was amplified from the BD630 chromosome using the primers 5ymcA-GST-INF and 3ymcA-GST-INF. To construct *GST-ylbF* (p*ED1334*) the entire *ylbF* ORF was amplified using the primers 5ylbF-GST-INF and 3ylbF-GST-INF (Table S2). To create the *GST-spo0E* (p*ED1602*) construct, the entire ORF, including the stop codon, was amplified using the primers 5spo0E-GST-INF and 3spo0E-GST-INF (Table S2). The plasmid p*GEX-6P-1* (GE Healthcare) was linearized overnight using *EcoR*I and *Xba*I for *ymcA* and *ylbF* cloning, and with *EcoR*I and *Xho*I for *spo0E* cloning. The cloning was performed using the In-Fusion HD kit, as per manufacturer's instructions. DNA sequencing confirmed the correct inserts, in-frame with the tags. These resulting constructs express in-

frame N-terminally GST-tagged proteins, with a PreScission Protease (GE Healthcare) site for removal of the tag, all under control of the IPTG-inducible P_{tac} promoter.

To create a *His_6-SUMO-yaaT* expressing plasmid (p*ED1450*), the entire *yaaT* ORF, including its stop codon, was amplified from BD630 genomic DNA using the primers 5yaaT-SUMO-INF and 3yaaT-SUMO-INF (Table S2). The vector *pTB146* (Bendezu *et al.*, 2009) was digested overnight with *Sap*I and *Xho*I, and the In-Fusion HD kit was used for cloning. The resulting plasmid places the *His_6-SUMO-yaaT* construct under T7-polymerase control, and contains a SUMO Protease (Invitrogen) site for removal of the tag. After confirmation by sequencing, the plasmid was transformed into chemically competent BL21 (DE3) cells for expression.

Construction of plasmids for bacterial 2-hybrid assays

The plasmids p*T18C-amp* and p*T25N-kan* (Karimova *et al.*, 1998) were used for cloning. Cloning into p*T18C* fuses a test protein to the C-terminus of the T18 fragment of *cya*, while the p*T25N* vector fuses a test protein to the N-terminus of the T25 fragment of *cya*. Both plasmids result in protein fusions, under the control of the IPTG-inducible *lac UV5* promoter, which is cAMP-CAP independent. The primers to generate the *ymcA* (p*ED1350,* and p*ED1384*), *ylbF* (pED1307) and *yaaT* (pED1385) fusions, as well as fusions to the members of the phosphorelay; *spo0A* (pED1459), spo*0B* (pED1460), *spo0F* (pED1467), and *kinA* (pED1466), are listed in Table S2. For all constructs, PCR was performed on BD630 genomic DNA. The *ylbF* and *ymcA* PCR products were ligated into the *BamH*I and *Kpn*I sites of p*T25N*. For all other constructs, the In-Fusion HD cloning kit was used, and the vectors were linearized overnight using *BamH*I and *EcoR*I for

p*T18C,* and *HinD*III and *Xba*I for p*T25N*. Plasmids were transformed into Stellar or DH5α competent cells for storage.

Grinding and lysis of cells for mass spectrometry

Bacillus subtilis strains carrying YFP fusions were inoculated into a 100 ml overnight culture in LB containing appropriate antibiotics. The overnight culture was diluted 1:100 (v/v) into 4L fresh DSM and grown for 5-6 hrs to T_1. Cells were harvested by centrifugation, with two 15 min spins at 4500 x *g* in a Beckman Coulter centrifuge (Avanti J-25, rotor JLA 8.1000), followed by two additional 15 min spins at 5000 x *g* (rotor JA-12). The resulting cell pellets were weighed, and 100 μL of 20 mM HEPES, pH 7.5, 1.2% (w/v) polyvinyl-pyrrolidone (Sigma), 1/100 (v/v) EDTA-free protease inhibitor cocktail (Roche Diagnostics) were added per gram of cells. Cells were frozen as small pellets in liquid nitrogen as described previously (Cristea *et al.*, 2005, Carabetta *et al.*, 2010).

Cryogenic cell lysis was performed as described previously (Cristea *et al.*, 2005) by grinding in a Retsch MM 301 Mixer Mill (Retsch, Newtown, PA) for 20 cycles, 3 min per cycle at a frequency of 30 Hz. The lysis buffer contained 20 mM HEPES, pH 7.4, 100 mM potassium acetate, 2 mM $MgCl_2$, 0.1% tween-20 (v/v), 1 μM $ZnCl_2$, 1 μM $CaCl_2$, 0.2% Triton-X, 150 mM NaCl, 10 μg/μl DNaseI, 1:100 protease inhibitor cocktail (Sigma) and 0.1 mg/ml phenylmethylsulphonyl fluoride (PMSF). Three grams of frozen cell powder was immediately added to 21 ml of lysis buffer. When the powder had thawed it was homogenized for 20 s using a PT 10-35 GT Polytron (Kinematica). The clarified lysate was centrifuged for 10 min, 8000 x *g* at 4°C, and the supernatant was used for the affinity purification experiments.

Conjugation of magnetic beads and Immunopurifications

Polyclonal anti-GFP antibody was prepared, purified, and conjugated to M270 Epoxy Dynabeads (Dynal, Invitrogen) as described previously (Cristea *et al.*, 2005). For each immunopurification, 7 mg of the conjugated magnetic beads were incubated with 21 ml cell lysates, prepared as described above, at 4°C for 1 hr (Cristea *et al.*, 2005). The magnetic beads were recovered by passing the mixture over a magnet (Magcraft) for 10 minutes. The beads were then washed six times with lysis buffer, without inhibitors or enzymes, and one time with water. Proteins were eluted from the beads directly into either lithium dodecyl sulfate (LDS)-PAGE sample buffer (Invitrogen) if in-gel digestion was to be performed, or into TEL buffer (26 mM Tris-HCl, 35 mM Tris-base, 127 μM EDTA, 0.5% LDS, pH 8.5) for in-solution digestion. All samples were alkylated with 100 mM iodoacetamide for 30 minutes at room temperature.

In-solution and in-gel protein digestion

For in-gel digestion, samples were resolved on a 4–12% NuPAGE Novex Bis-Tris gel (Invitrogen) according to the manufacturer's instructions. The gels were stained with SimplyBlue Coomassie stain (Invitrogen). Each gel lane was cut up into 1 mm slices, and further cut into small pieces. Gel pieces were destained, dehydrated and rehydrated as previously described (Tsai *et al.*, 2012). After a final dehydration, the gel pieces were incubated with 12.5 ng/μl of sequencing grade trypsin (Promega, Madison, WI) overnight at 37°C. The resulting peptides were extracted from the gel pieces by incubation in 0.5% formic acid for 4 hrs at room temperature (RT), followed by a second incubation in 0.5% formic acid/50% acetonitrile (ACN) for an additional 2

hrs. The extracted peptides were pooled into 4 equal fractions, and were concentrated by vacuum centrifugation to ~12 μl.

For in-solution digestion, eluted protein samples were reduced with 1 mM dithiothreitol (DTT), and digested on a filter (Vivacon 500 centrifugal filters (10K cutoff), Sartorius Stedim Biotech, Goettingen, Germany) with 100 μl of 5 ng/μl trypsin in 50 mM ammonium bicarbonate by a filter-aided sample preparation method (FASP) as described (Wisniewski *et al.*, 2009). The eluted peptide fraction was concentrated by vacuum centrifugation to a final volume of ~12 μl, as before.

Mass Spectrometry

The mass spectrometry procedure was carried out as previously described (Tsai *et al.*, 2012, Grego *et al.*, 2012). Briefly, half of the elution sample was analyzed by nLC-MS/MS on a Dionex Ultimate 3000 RSLC coupled directly to an LTQ-Orbitrap Velos ETD mass spectrometer (ThermoFisher Scientific, San Jose, CA). Peptides were separated by reverse phase chromatography over a 3 hr gradient for in-solution, or a 90 min gradient for in-gel digested samples, from 4 to 35% B, where solvent A is 0.1% formic acid/0.1% acetic acid in water, and solvent B is 0.1% formic acid/0.1% acetic acid in 97% ACN. Details on the mass spectrometer settings can be found in Tsai *et al.*, 2012 and Greco *et al.*, 2012. The MS/MS spectra were acquired by collision-induced dissociation (CID) fragmentation. All immunopurifications and mass spectrometric data analysis were repeated.

Mass spectrometry data processing

Data was analyzed as described previously (Tsai *et al.*, 2012, Greco *et al.*, 2012). Briefly, MS/MS spectra were extracted by Proteome Discoverer (ver. 1.3, Thermo Fisher Scientific, San Jose, CA) and then further analyzed by SEQUEST (ver. 1.3.0.339, Thermo Fisher Scientific, San Jose, CA) and X! Tandem (ver. CYCLONE (2010.12.01.1), The GPM, thegpm.org) for peptide database searching against the UniProt SwissProt sequence database (downloaded 11/2010). The search was performed against the *Bacillus subtilis* database, including some common contaminant sequences (4374 entries). Parameters of the search were as followed as described previously (Tsai *et al.*, 2012), including the following modifications: static modification of carbamidomethylcysteine (+57 Da), variable modifications of methionine oxidation (+16 Da), phosphoserine, threonine, and tyrosine (+80 Da). Peptide spectrum matches (PSMs) were analyzed and validated by Scaffold (ver. 3.2; Proteome Software, Inc.), as described previously (Tsai *et al.*, 2012).

Western blot analysis

Bacillus strains were grown in DSM until T_0 and 1 ml samples taken. Extracts were prepared by pelleting the cells, and washing in an equal volume of STM (50 mM NaCl, 25% sucrose, 50 mM Tris-HCl, pH 8.5, 5 mM $MgCl_2$). Cells were lysed by addition of STM supplemented with 350 μg/μl lysozyme, normalized to Klett value and incubated at 37°C for 5 minutes. Samples were mixed with cracking buffer (0.225 M Tris-HCl, pH 6.8, 50% glycerol, 5% SDS, 0.05% bromophenol blue, 1 % β-mercaptoethanol), boiled for 10 minutes, and equal volumes were loaded on a 12.5% tris-tricine gel. Following SDS-PAGE, proteins were transferred to a Protran nitrocellulose membrane (Whatman), and probed using a 1:5000 dilution of anti-Spo0A

antibody then a 1:5000 dilution of purified goat anti-rabbit antibodies conjugated to peroxidase (Kirkeegard & Perry Laboratories). Bands were visualized using the enhanced chemiluminescence ECL prime kit (Amersham) and Hyblot CL autoradiography film (Denville Scientific Inc).

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