SUPPLEMENTAL INFORMATION

Identification of novel Spx regulatory pathways in *Bacillus subtilis* uncovers a close relationship between the CtsR and Spx regulons

Daniel F. Rojas-Tapias[1,2] & John D. Helmann[1]*

[1] Department of Microbiology, Cornell University, Ithaca, NY 14853, US.

[2] Current address: Infectious Diseases and Microbiome Program, The Broad Institute of MIT and Harvard, Cambridge, MA 02142, US.

* Corresponding author: John D. Helmann, Ph.D. Department of Microbiology, Wing Hall, Cornell University, Ithaca, NY 14853-8101. Phone: +1-607-255-6570. Fax: 607-255-3904. e-mail: jdh9@cornell.edu

Running title: Regulation of Spx by ClpCP and McsB.

Supplementary Experimental Methods

Strains construction

Tables

Table S1 Sites of transposon insertions
Table S2 List of strains
Table S3 List of primers
Table S4 Co-occurrence of Spx and related proteins in representative Firmicutes species

Figures

Fig. S1 The phenotype due to *ctsR* inactivation is explained by ClpP proteolysis as well as another unknown pathway.
Fig. S2 *In vivo* ClpX-independent Spx degradation in *Bacillus subtilis* occurs through ClpCP.
Fig. S3 Elevated arginine phosphorylation affects Spx activity
Fig. S4 Spx accumulation negatively affects bacterial growth
Fig. S5 Alignment of Spx proteins in different species

Supplementary Experimental Methods

Strains construction

For overlap PCR, one first round of PCR without primers was performed using the following program: initial denaturation (98ºC x 1'), 5 cycles of assembly (98ºC x 10'', Tm+5ºC x 40'', and 72ºC x 30'' per kb''), 5 cycles of assembly (98ºC x 10'', Tm ºC x 40'', and 72ºC x 30'' per kb), and 5 cycles of assembly (98ºC x 10'', Tm-5ºC x 40'', and 72ºC x 30'' per kb). Equimolar amounts of both DNA fragments were used (~50 ng of the smallest fragment) for assembly. Then 3 μl of the resulting product was used for conventional PCR amplification; the PCR reactions were performed as recommended by the manufacturer. For deletion of the erythromycin or kanamycin cassettes in the strains from the Bacillus Genomic Stock Center (BGSC) the pDR244 plasmid was used (1).

For construction of the *spx* allele under control of the hy-spank promoter [i.e. P_{hs}-*spx (cm)*], the PCR product obtained with primers DR445 and DR446 was used. For construction of the IPTG-inducible *ctsR* allele [i.e. P_{hs}-*ctsR (cm)*], the fragment containing the *ctsR* gene was amplified using the primers DR315 and DR316. To generate the *ctsR*^{R63E} allele under IPTG control [i.e. P_{hs}-*ctsR*^{R63E} *(cm)*], the mutagenic primers DR345 and DR346 were additionally used, and the resulting PCR products were ligated by overlap PCR. The resulting fragments of *spx*, *ctsR*, and *ctsR*^{R63E}, as well as the pPL82 vector (2), were digested with XmaI and XbaI, ligated using the T4 ligase, and transformed into *E. coli* DH5a. The generated vectors were used to transform *B. subtilis*.

For construction of the mutant *spx* alleles, the following mutagenic primers were used *spx*^{R14K}: DR415 and DR416, *spx*^{R91K}: DR393 and DR394, *spx*^{R92K}: DR426 and DR427, *spx*^{R100K}: DR419 and DR420, *spx*^{R112K}: DR397 and DR398. And the primers DR445 and DR446 were used as external primers. The fragments and the pPL82 vector were digested with XmaI and XbaI, ligated using the T4 ligase, and transformed into E. coli DH5a. The generated vectors were used to transform *B. subtilis*.

For construction of the *amyE::ctsR* (cm) complementation cassette, a fragment covering the *ctsR* coding region and its promoter were amplified using primers DR450 and DR451, purified, and digested with EcoRI and BamHI. For construction of the *amyE::ywlE* (cm) complementation cassette, the primers DR362 and DR363 were used. The product was digested with EcoRI and HindIII. The resulting PCR products were cloned into pDG1662 (3), which was digested using the appropriate restriction enzymes. Both insert and vector were ligated using T4 ligase, and transformed into *E. coli*. The generated vector was used to transform *B. subtilis*.

For construction of the pMUTIN-Flag (spec) vector, the pMUTIN-Flag (ery) (4) backbone (without the erythromycin resistance cassette) was amplified using primers DR240 and DR241. The spectinomycin resistance cassette was amplified using the primers DR238 and DR239 using the plasmid pDG1661 (3) as template. The resulting PCR fragments were digested with DpnI, and joined using Gibson Assembly. The integrity of the resulting vector was verified by PCR and sequencing.

For construction of P_{spac}-*clpX*, we amplified the N-terminal region of *clpX* with two sets of primers: first, we used primers DR166 and DR366, and then DR365 and DR342. The goal was to eliminate an internal HindIII restriction site. The resulting PCR products were ligated by overlap PCR. The resulting fragment, as well as the pMUTIN4-Flag (spec) vector, were digested with HindIII and KpnI, ligated using T4 ligase, and transformed into *E. coli* DH5α. The resulting plasmids were transformed into *E. coli* TG1 to generate multimeric plasmids, and then 1 μg of plasmid DNA used to transform *B. subtilis*.

For construction of the conditional P_{spac}-*mcsA-mcsB-clpC* and P_{spac}-*clpC* strains using the pMUTIN-Flag (spec) vector, the primers DR452 and DR453, and DR454 and DR455 were used, respectively. The PCR fragments and pMUTIN-Flag (spec) vector were digested with HindIII and KpnI. The digested fragments were then ligated into the digested vector using the T4 ligase. The resulting plasmids were transformed into *E. coli* TG1 to generate multimeric plasmids, and then 1 μg of plasmid DNA used to transform *B. subtilis*.

For construction of the *yjbH::kan* strain the upstream and downstream regions of the *yjbH* region were amplified using the primers DR16 and DR17, and DR18 and DR19 using the genomic DNA of the wild-type strain as template. The kanamycin cassette was amplified using the primers 1295 and 1296 using as template gDNA of the strain HB10216 (5). The three fragments were joined by overlap PCR, and the resulting PCR fragment was used to transform the wild-type strain (HB18501). The kanamycin resistant colonies were verified by PCR and sequencing.

Supplementary Tables

Table S1. Sites of transposon insertions[1]

Gene	Frequency	Function
IolR	8	Transcriptional repressor of the *iol* operon, regulation of myo-inositol catabolism
ctsR	5	Transcription repressor of class III heat shock genes (*clpC* operon, *clpE*, *clpP*)
lacZ Reporter/Spx	4	*spx* gene or P_{trxB}-*lacZ* transcriptional fusion
galK	3	Galactokinase. Galactose utilization
menH	3	Menaquinone biosynthesis methyltransferase
ywlE	1	Protein arginine phosphatase
degV	1	Fatty acid binding protein
gndA	1	NADP-dependent phosphogluconate dehydrogenase. Pentose phosphate pathway
hrcA	1	Transcriptional repressor of class I heat-shock genes
hutP	1	Transcriptional antiterminator
mccB	1	Cystathionine lyase/ homocysteine gamma-lyase
murAB	1	UDP-N-acetylglucosamine 1-carboxyvinyltransferase
pbuG	1	Hypoxanthin/ guanine permease
sigH	1	RNA polymerase sigma factor SigH
yabR	1	Unknown
ybcF	1	Similar to carbonic anhydrase
yckB	1	Similar to amino acid ABC transporter (binding protein)
ydcI	1	Unknown
yfnE	1	Unknown
yhcE	1	Similar to ABC transporter (membrane protein)
ylaJ	1	Efficient spore germination
yqhT	1	Degradation of proline-containing peptides
yuiF	1	Unknown
ywcI	1	Unknown
yybS	1	Unknown

[1] Insertions sites were mapped using inverse PCR as described in Materials and Methods. Linkage tests have not been conducted to determine if each of these insertions affect Spx activity. Experiments with null mutations confirmed the initially observed effects for *ctsR* and *ywlE,* as described in the main text.

Table S2. Strains used in this study

Strains	Genotype	Construction or source
BKE13700	clpE::ery	BGSC strain.
HB18501	168 trpC2	Lab strain
HB18658	thrC::P_{trxB}-lacZ (ery)	(6)
HB18703	yjbH::kan	Lab strain
HB18807	amyE::P_{hs}-spx^{DD} (spec)	(7)
HB18930	clpX::spec	(5)
HB18931	clpC::ery	gDNA of BKE00860 into WT.
HB18961	clpP::tet	(5)
HB18984	ΔctsR	Derivative of BKE00830 from BGSC. The erythromycin cassette was looped out using pDR244.
HB18986	ΔmcsB	Derivative of BKE00850 from BGSC. The erythromycin cassette was looped out using pDR244.
HB23017	ΔctsR thrC::P_{trxB}-lacZ (ery)	This work. gDNA of HB18658 was moved into HB18984.
HB23019	ΔmcsB thrC::P_{trxB}-lacZ (ery)	gDNA of HB18658 was moved into HB18986.
HB23040	ΔctsR amy::P_{hs}-ctsR (cm) thrC::P_{trxB}-lacZ (ery)	A pPL82-based vector containing the ctsR coding region was transformed into HB23017.
HB23041	ΔctsR amy::P_{hs}-$ctsR^{R63E}$ (cm) thrC::P_{trxB}-lacZ (ery)	A pPL82-based vector containing the $ctsR^{R63E}$ coding region was transformed into HB23017.
HB23043	ΔywlE	Derivative of BKE36930 from BGSC. The erythromycin cassette was looped out using pDR244.
HB23053	ΔywlE thrC::P_{trxB}-lacZ (ery)	gDNA of HB18658 was moved into HB23043.
HB23054	ΔywlE amyE::ywlE (cm) thrC::P_{trxB}-lacZ (ery)	The coding region of ywlE was cloned into pPL82 and moved into HB23053.
HB23067	ΔywlE thrC::P_{trxB}-lacZ (ery) spx::kan	gDNA from HB23268 was transformed into HB23043.
HB23199	ΔmcsB ΔywlE	DNA from a ywlE::ery strain was transformed into ΔmcsB, and the erythromycin resistance cassette was looped out with the pDR244 plasmid.

HB23206	*ΔmcsB ΔywlE thrC::P_{trxB}-lacZ (ery)*	*gDNA of HB18658 was moved into HB23199.*
HB23228	*ΔclpC*	*Derivative of BKE00860 from BGSC. The erythromycin cassette was looped out using pDR244. pDR244 was introduced into the clpC::ery strain by SPP1 transduction.*
HB23257	*spx::kan*	*gDNA from HB11373 into HB18501* (8)
HB23258	*ΔctsR spx::kan*	*gDNA of HB23257 was moved into HB18984.*
HB23259	*spx::tet*	*gDNA of HB10339* (5) *in HB18501*
HB23260	*ΔctsR spx::tet*	*gDNA of HB23259 was moved into HB18984.*
HB23261	*spx::kan thrC::P_{trxB}-lacZ (ery)*	*gDNA from spx::kan was transferred into HB18658.*
HB23263	*spx::kan amyE::P_{hs}-spx (cm) thrC::P_{trxB}-lacZ (ery)*	*A pPL82 plasmid with the spx allele was introduced into HB23261.*
HB23264	*spx::kan amyE::P_{hs}-spx^{R14K} (cm) thrC::P_{trxB}-lacZ (ery)*	*A pPL82 plasmid with spx^{R14K} allele was introduced into HB23261.*
HB23265	*spx::kan amyE::P_{hs}-spx^{R92K} (cm) thrC::P_{trxB}-lacZ (ery)*	*A pPL82 plasmid with spx^{R92K} allele was introduced into HB23261.*
HB23266	*spx::kan amyE::P_{hs}-spx^{R112K} (cm) thrC::P_{trxB}-lacZ (ery)*	*A pPL82 plasmid with spx^{R112K} allele was introduced into HB23261.*
HB23268	*spx::kan amyE::P_{hs}-spx (cm)*	*A pPL82-based vector containing the spx coding region was transformed into HB23257.*
HB23272	*ΔctsR spx::kan amyE::P_{hs}-spx (cm)*	*A pPL82-based vector containing the spx coding region was transformed into HB23258.*
HB23278	*spx::kan amyE::P_{hs}-spx^{DD} (spec)*	*DNA from HB18807 was moved into HB23257*
HB23279	*ΔctsR spx::kan amyE::P_{hs}-spx^{DD} (spec)*	*DNA from HB18807 was moved into HB23258*
HB23280	*spx::tet clpX::spec*	*gDNA of HB18930 was moved into HB23259.*
HB23281	*spx::kan amyE::P_{hs}-spx (cm) clpX::spec*	*gDNA of HB18930 was moved into HB23268.*
HB23282	*ΔctsR spx::kan amyE::P_{hs}-spx (cm) clpX::spec*	*gDNA of HB18930 was moved into HB23272.*
HB23285	*spx::tet amyE::P_{hs}-spx (cm)*	*A pPL82-based vector containing the spx coding region was transformed into HB23259.*

HB23286	ΔctsR spx::tet amyE::P_{hs}-spx (cm)	gDNA of HB23285 was moved into HB23260
HB23287	spx::tet amyE::P_{hs}-spx (cm) yjbH::kan	gDNA of HB18703 was moved into HB23285
HB23288	ΔctsR spx::tet amyE::P_{hs}-spx (cm) yjbH::kan	gDNA of HB18703 was moved into HB23286.
HB23291	spx::tet amyE::P_{hs}-spx (cm) clpP::kan	gDNA of BKK34540 was moved into HB23285
HB23292	ΔctsR spx::tet amyE::P_{hs}-spx (cm) clpP::kan	gDNA of BKK34540 was moved into HB23286.
HB23295	spx::kan amyE::P_{hs}-spx (cm) clpX::spec clpC::ery	gDNA of HB18931 was moved into HB23281.
HB23296	spx::kan amyE::P_{hs}-spx (cm) clpX::spec clpE::ery	gDNA of BKE13700 was moved into HB23281.
HB23302	clpX::spec ΔmcsB	DNA from clpX::spec was moved into HB18986
HB23303	clpX::spec ΔywlE	DNA from clpX::spec was moved into HB23043
HB23304	clpX::spec ΔmcsB ΔywlE	DNA from clpX::spec was moved into HB23199
HB23317	spx::kan amyE::P_{hs}-spx^{R91K} (cm) thrC::P_{trxB}-lacZ (ery)	A pPL82 plasmid with spx^{R91K} allele was introduced into HB23261.
HB23319	spx::kan amyE::P_{hs}-spx^{R100K} (cm) thrC::P_{trxB}-lacZ (ery)	A pPL82 plasmid with spx^{R100K} allele was introduced into HB23261.
HB23321	amyE::P_{hs}-mcsA-mcsB(cm)	The coding region of mcsA-mcsB was cloned into pPL82.
HB23332	ΔmcsB ΔctsR	PCR DNA from a ctsR::ery strain was transformed into ΔmcsB, and the erythromycin resistance cassette was looped out with the pDR244 plasmid.
HB23333	amyE::ctsR (cm)	The coding sequence of ctsR as well as its promoter were cloned into pDG1662, and transformed into B. subtilis.
HB23363	ΔmcsB spx::kan amyE::P_{hs}-spx (cm)	gDNA of HB23257 was transformed into HB18986, and then the resulting strains was transformed with gDNA from HB23268.
HB23364	ΔywlE spx::kan amyE::P_{hs}-spx (cm)	gDNA of HB23257 was transformed into HB23043, and then the resulting strains was transformed with gDNA from HB23268.

HB23365	*spx::kan amyE::P_{hs}-spx (cm) thrC::P_{trxB}-lacZ (ery) clpX::spec*	*gDNA of clpX::spec was transformed into HB23261*
HB23366	*ΔctsR spx::kan amyE::P_{hs}-spx (cm) thrC::P_{trxB}-lacZ (ery) clpX::spec*	*gDNA of clpX::spec was transformed into HB23262*
HB23367	*ΔmcsB spx::kan amyE::P_{hs}-spx (cm) clpX::spec*	*gDNA of HB18930 was moved into HB23363*
HB23368	*ΔywlE spx::kan amyE::P_{hs}-spx (cm) clpX::spec*	*gDNA of HB18930 was moved into HB23364*
HB23371	*ΔctsR ΔmcsB spx::kan amyE::P_{hs}-spx (cm)*	*gDNA of HB23268 was transformed into HB23332*
HB23372	*ΔmcsB ΔywlE spx::kan amyE::P_{hs}-spx (cm)*	*gDNA of HB23268 was transformed into HB23199*
HB23373	*ΔmcsB ΔywlE spx::kan amyE::P_{hs}-spx (cm) clpX::spec*	*gDNA of clpX::spec was introduced into HB23372*
HB23374	*ΔctsR ΔmcsB spx::kan amyE::P_{hs}-spx (cm) clpX::spec*	*gDNA of clpX::spec was introduced into HB23371*
HB23378	*ΔctsR ΔmcsB thrC::P_{trxB}-lacZ (ery)*	*gDNA from HB23363 into HB23332*
HB23380	*ΔctsR ΔmcsB amyE::ctsR (cm) thrC::P_{trxB}-lacZ (ery)*	*gDNA from HB23333 into HB23378*
HB23381	*ΔmcsB ΔctsR amyE::P_{hs}-mcsAB (cm) thrC::P_{trxB}-lacZ (ery)*	*gDNA from HB23321 into HB23378*
HB23400	*P_{spac}-mcsAB-clpC (spec)*	*pMUTIN-Flag (spec) plasmid containing the C-terminal region of ctsR and N-terminal region of mcsB into HB18501.*
HB23401	*P_{spac}-mcsAB-clpC (spec) thrC::P_{trxB}-lacZ (ery)*	*Plasmid DNA into HB18658.*
HB23404	*P_{spac}-clpC (spec) thrC::P_{trxB}-lacZ (ery)*	*Plasmid DNA into HB18658.*
HB23407	*P_{spac}-clpC (spec)*	*pMUTIN-Flag (spec) plasmid containing the C-terminal region of mcsB and N-terminal region of clpC into HB18501.*
HB23414	*ΔywlE spx::kan amyE::P_{hs}-spx (cm) thrC::P_{trxB}-lacZ (ery)*	*A pPL82 plasmid with the spx allele was introduced into HB23067.*

HB23415	ΔywlE spx::kan amyE::P_{hs}-spx^{R14K} (cm) thrC::P_{trxB}-lacZ (ery)	A pPL82 plasmid with spx^{R14K} allele was introduced into HB23067.
HB23416	ΔywlE spx::kan amyE::P_{hs}-spx^{R91K} (cm) thrC::P_{trxB}-lacZ (ery)	A pPL82 plasmid with spx^{R91K} allele was introduced into HB23067.
HB23417	ΔywlE spx::kan amyE::P_{hs}-spx^{R92K} (cm) thrC::P_{trxB}-lacZ (ery)	A pPL82 plasmid with spx^{R92K} allele was introduced into HB23067.
HB23417	ΔywlE spx::kan amyE::P_{hs}-spx^{R100K} (cm) thrC::P_{trxB}-lacZ (ery)	A pPL82 plasmid with spx^{R100K} allele was introduced into HB23067.
HB23419	ΔywlE spx::kan amyE::P_{hs}-spx^{R112K} (cm) thrC::P_{trxB}-lacZ (ery)	A pPL82 plasmid with spx^{R112K} allele was introduced into HB23067.
HB23424	spx::kan amyE::P_{hs}-spx (cm) thrC::P_{trxB}-lacZ (ery) clpP::tet	gDNA of clpP::tet was transformed into HB23261
HB23425	ΔctsR spx::kan amyE::P_{hs}-spx (cm) thrC::P_{trxB}-lacZ (ery) clpP::tet	gDNA of clpP::tet was transformed into HB23262

Table S3. Primers used in this study

Number	Sequence
DR445	ATCACCCGGGGATGTTCATCCTACTAATTAGAGGAG
DR446	ATCGTCTAGACGAGCATTTATTACCAGCAGGTTG
DR315	ATCGACCCGGGGTCAAAGTCAGTAAAGGAGG
DR316	ATCGTCTAGAGACAAATCAATCTTTTCACCC
DR345	TTGTTGAGAGCAAAGAAGGGGGCGGCGGTTACATC
DR346	CCCTTCTTTGCTCTCAACAATATATCCTCTTT
DR450	ATCAGAATTCGTGAGAGTAGGACGCCGCCAAG
DR451	ATCAGGATCCGCACTCTTGACAAATCAATCTTTTCACCC
DR415	TTCATGCAAAAAGGCGAGAGCGTGG
DR416	CTCTCGCCTTTTTGCATGAAGTACAGCTTGGTGATGTG
DR393	CCAGGTTTGCTTAAACGTCCGATCATCATTGACGAAAAACG
DR394	CGGACGTTTAAGCAAACCTGGATGCTCGTTG
DR426	GCTTCGCAAACCGATCATCATTGACGAAAAAC
DR427	GATGATCGGTTTGCGAAGCAAACCTGGATGCTCG
DR419	AAAAAATTGCAGGTCGGATATAACG
DR420	TATCCGACCTGCAATTTTTTTCGTCAATGATGATCGG
DR397	GACGAAATCAGAAAATTCCTGCCAAGAAAAGTTCGCTC
DR398	GGCAGGAATTTTCTGATTTCGTCTTCGTTATATCC
DR166	GGATAACAATTAAGCTTTCGGTAAAAGAAAAAGTGAGAC
DR366	GCTACAAGTTTACGAACCTGATCTTG
DR365	GTTCGTAAACTTGTAGCTGGACCTGGTG
DR342	ATCAGGTACCCAACATCATCAACTTTGCTGTTGG
DR452	ATCGAAGCTTGAGGATATATTGTTGAGAGCAAACG
DR453	ATCAGGTACCGGTGTAATGTTGCTATGAAATGTTTTG

DR454	ATCGAAGCTTGCGAAGCAGTAGGGAATATCTTTC
DR455	ATCAGGTACCGCTGATGATCCTGTTTCATTACTTCC
DR362	ATCACCCGGGGAATTCGTTTGGATTAAAGCTGCTTTTGC
DR363	ATCGTCTAGAAAGCTTGACCTGTTTGCAGATTTTCTGAC
DR309	CCACCGCGTTTCTATCACTG
DR310	ATCGATAATACGACTCACTATAGGGAGAGCCGCATCCTTCTCTTTACG
DR302	GTGGAAGCTGAGCACATTCA
DR303	ATCGATAATACGACTCACTATAGGGAGAGCTTCGTCTTACCGCTTTTG
6299	GCTTGTAAATTCTATCATAATTG
6300	AGGGAATCATTTGAAGGTTGG
6301	GCATTTAATACTAGCGACGCC
DR16	GCTGTCAGCCGAAATGAGTC
DR17	CCTCAAATGGTTCGCTGTCAAGATGATCGATCCTCCGCTTC
DR18	CCTACGAGGAATTTGTATCGCCGCAGGCGTGCATATG
DR19	GAAATGGAAGAGCTGCTGATG
1295	CAGCGAACCATTTGAGGTGATAGG
1296	CGATACAAATTCCTCGTAGGCGCTCGG
DR238	GATAAGACGGTTCGTGTTCGCCTAGGTCTAGAGGATCGATC
DR239	TACAAAAGCGACTCATAGAACTAATTGAGAGAAGTTTCTATAG
DR240	TTCTATGAGTCGCTTTTGTAAATTTGG
DR241	CGAACACGAACCGTCTTATCTC

Table S4. Co-occurrence of Spx and related proteins in representative Firmicutes species

The co-occurrence of *spx*, *mcsB, mecA*, *yjbH*, and *clpC* was explored in representative strains of *Bacillus subtilis [168]*, *Listeria monocytogenes [ATCC 19117]*, *Staphylococcus aureus [ATCC BAA 39]*, *Enterococcus faecalis [ATCC 29212]*, *Lactobacillus rhamnosus [GG ATCC 53103]*, *Lactobacillus acidophilus [ATCC 4796]*, *Streptococcus pneumoniae [ATCC 49619]*, *Streptococcus pyogenes [NCTC13739]*, and *Lactococcus lactis [IL6288]*, as well as in Firmicutes that do not possess Spx homologs including *Veillonella parvula [DSM 2008]*, and *Blautia producta [PMF1]*, *Ruminococcus albus [DSM 20455]*, *Roseburia hominis [A2 183]*, and *Coprococcus catus [GD 7]*. The analysis was performed using the SyntTax software (9). Only proteins with a score > 15 are displayed in the table below.

	spx	***mcsB***	***yjbH***	***mecA***	***clpC***
Bacillus subtilis	Yes (100)	Yes (100)	Yes (100)	Yes (100)	Yes (100)
Listeria monocytogenes	Yes (86.94)	Yes (47.78)	Yes (30.11)	Yes (38.10)	Yes (76.80)
Staphylococcus aureus	Yes (85.45)	Yes (37.42)	Yes (20.77)	Yes (25.85)	Yes (68.74)
Enterococcus faecalis	Yes (80.60)	No	No	Yes (15.80)	Yes (56.90)
Lactobacillus rhamnosus	Yes (72.76)	No	No	Yes (19.73)	Yes (55.13)
Lactobacillus acidophilus	Yes (71.27)	No	No	No	Yes (47.50)
Streptococcus pneumoniae	Yes (67.16)	No	No	No	Yes (39.62)
Streptococcus pyogenes	Yes (63.06)	No	No	No	Yes (38.22)
Lactococcus lactis	Yes (60.45)	No	No	No	Yes (45.73)
Veillonella parvula	No	Yes (37.82)	No	No	Yes (57.57)
Blautia producta	Yes (16.83)	No	No	No	Yes (50.43)
Ruminococcus albus	Yes (16.53)	No	No	No	Yes (42.80)
Roseburia hominis	No	No	No	No	Yes (48.90)
Coprococcus catus	No	Yes (21.40)	No	No	Yes (51.53)

Supplementary Figures

Fig. S1 The phenotype due to *ctsR* inactivation is explained by ClpP proteolysis as well as another unknown pathway

P_{trxB} activity was assessed in liquid LB on *spx*^{cond} *ΔclpX* (*=clpX::spec*) and *spx*^{cond} *ΔclpP* (*=clpP::tet*) cells grown in presence of 100 μM IPTG to induce *spx* expression. Multiple t-test were performed to compare the mean value of each pair of strains. *, **, *** indicate significant differences with *p<0.05, p<0.01*, and *p<0.001*, respectively. Error bars represent SEM of at least three independent replicates.

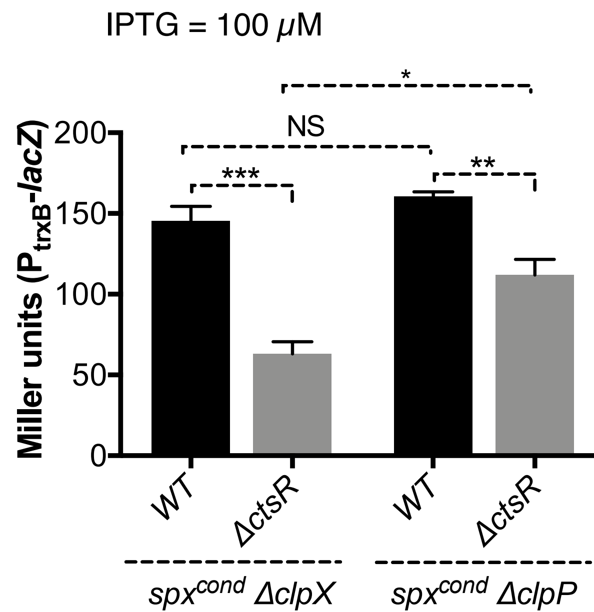


Fig. S2 *In vivo* ClpX-independent Spx degradation in *Bacillus subtilis* occurs through ClpCP

A protein chase was performed in *spx*^{cond} cells lacking *clpX*, *clpX clpE*, *clpX clpC*, or *clpP* to determine Spx stability. For this, cells were grown up to OD_{600}~0.1 and treated with IPTG (100 μM) to induce *spx* expression. When OD_{600} reached 0.4-0.6 cells were subjected to analysis. Samples were taken before and after treatment with 100 μg/ml of chloramphenicol to stop protein synthesis. *ΔclpX* was used to identify *clpX::spec, ΔclpE and ΔclpC* to identify *clpE::ery* and *clpC::ery*, respectively, and *Δclp*P to identify *clpP::tet*. As observed, the Spx levels were lower in *ΔclpXC* cells compared with *ΔclpP*, yet the molecular bases remain unclear.

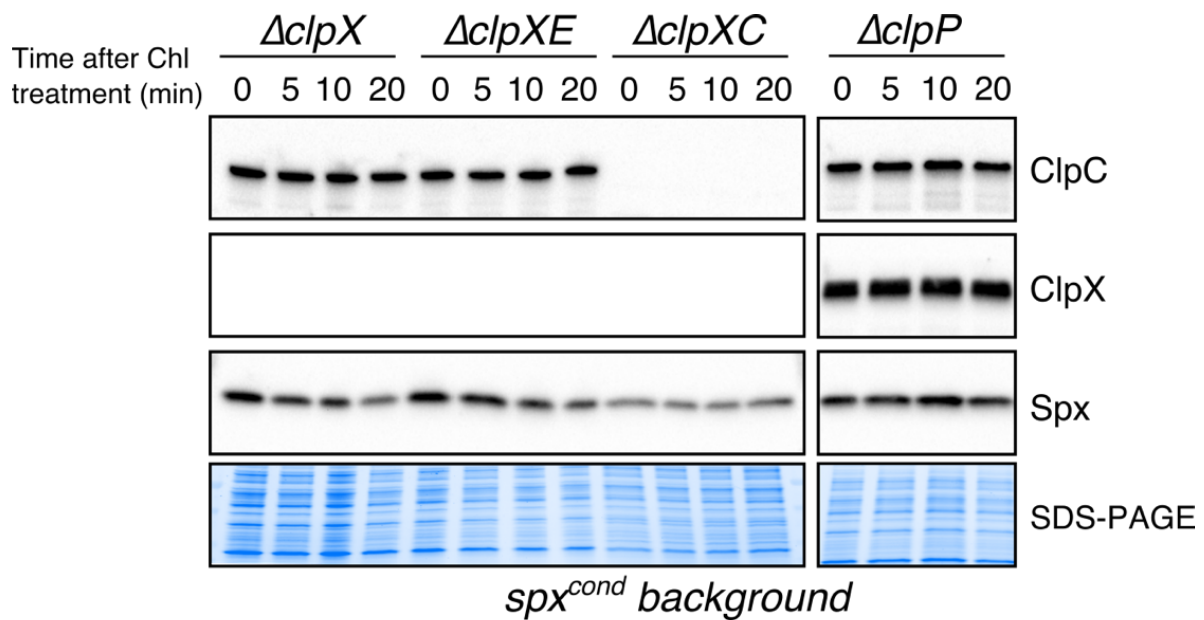
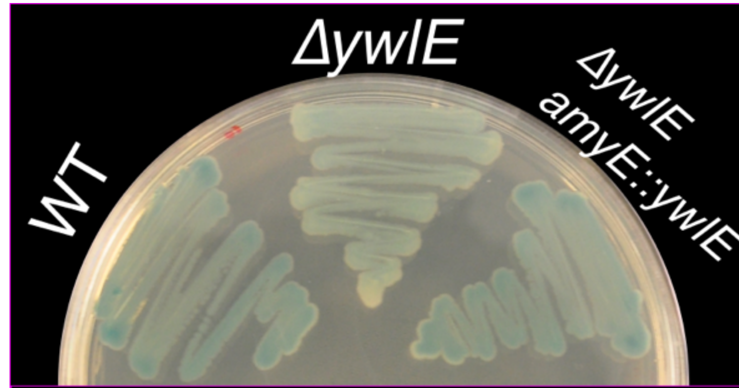


Fig. S3 Elevated arginine phosphorylation affects Spx activity

A) Ectopic complementation of the *ΔywlE* strain also restored the WT phenotype. B) The differences between WT and *ΔywlE* are independent of *spx* transcription and ClpXP-dependent proteolysis.

A



B

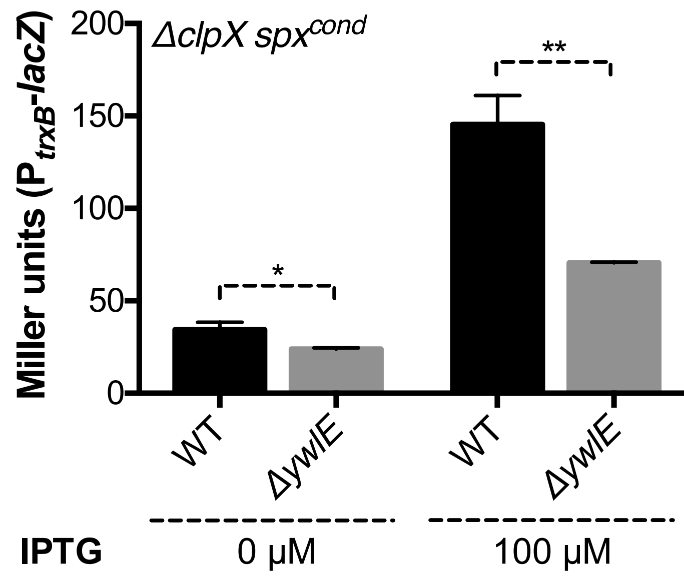


Fig. S4 Spx accumulation negatively affects bacterial growth

Platting efficiency of cells harboring a *spx*^{DD} or *spx* allele under control of an IPTG-inducible promoter. The *spx* alleles were studied in WT and *ΔctsR* cells. Bacteria were platted on LB supplemented or not with 100 μM IPTG.

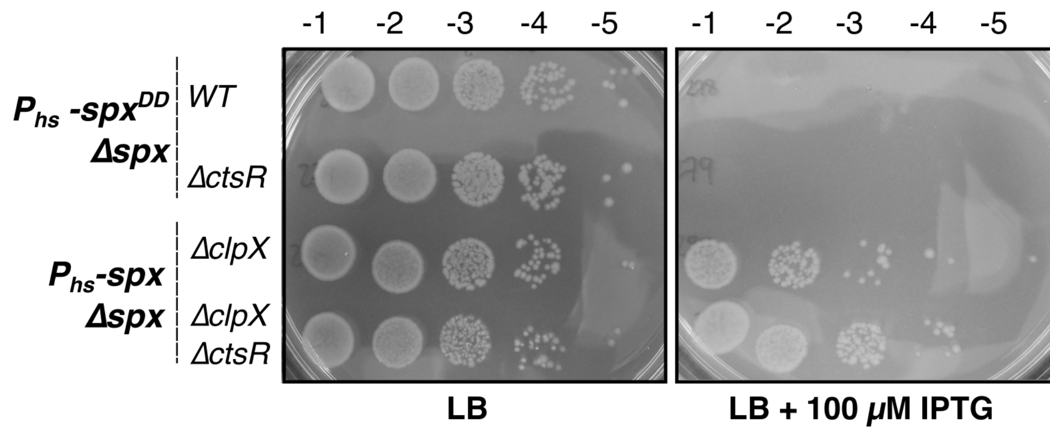
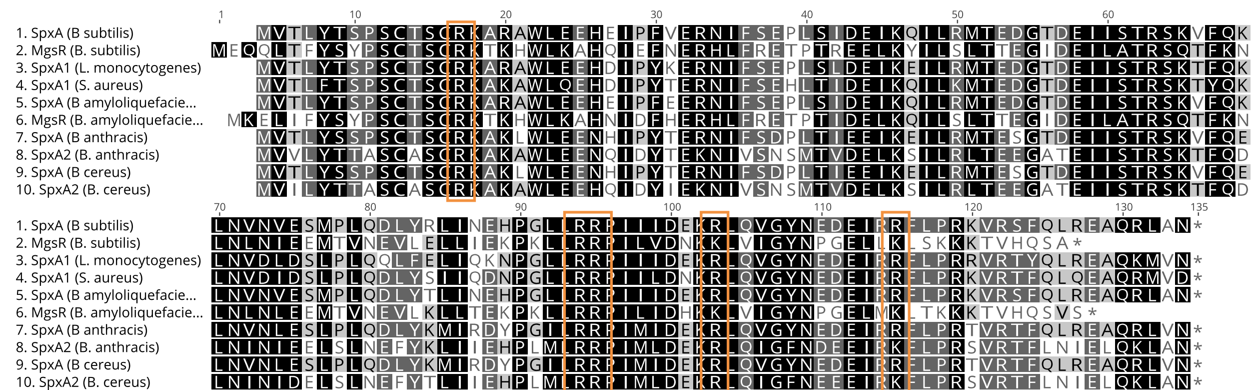


Fig. S5 Alignment of Spx proteins in different species

Spx proteins from representative Firmicutes were aligned using the Geneious alignment algorithm in the Geneious 11.1.5 software. The Blosum62 cost matrix was used with the following parameters: gap open penalty = 12, gap extension penalty = 3, and refinement iterations = 2. A) Alignment of Spx proteins (which display the G52 residue) in species containing McsB. In all Spx proteins the arginine residues are conserved at positions 14, 91, and 92, whereas the positions 100 and 112 are conserved in the closest Spx homologs (see Fig. S5C) but not in the MgsR paralog (where the Arg is replaced by Lys). B) Alignment of Spx proteins in species lacking *mcsB*. The Spx homologs were searched using tblastn, using as template the reference genomes shown in Table S4. In these species the arginine residues at positions 14 and 91 were conserved, but some lysine residues were also observed at these positions. At positions 92 and 112 the arginine residues were highly conserved except for the less similar Spx homologs in *L. lactis* and *L. acidophilus*, respectively. The arginine at position 100 was much less conserved in all these species, and His and Cys residues were found instead. C) Phylogenetic tree of Spx proteins in McsB-containing species shows that the MgsR proteins in both *B. subtilis* and *B. amyloliquefaciens* are more distantly related to other Spx proteins. *Bacillus subtilis* YusI was used as outgroup. The alignment and phylogenetic tree were constructed in the Geneious 11.1.5 software using the default parameters. Additional sequences were obtained from the following RefSeq genomes: *Bacillus amyloliquefaciens DSM7, Bacillus cereus ATCC 14579, Bacillus anthracis str. Ames.*

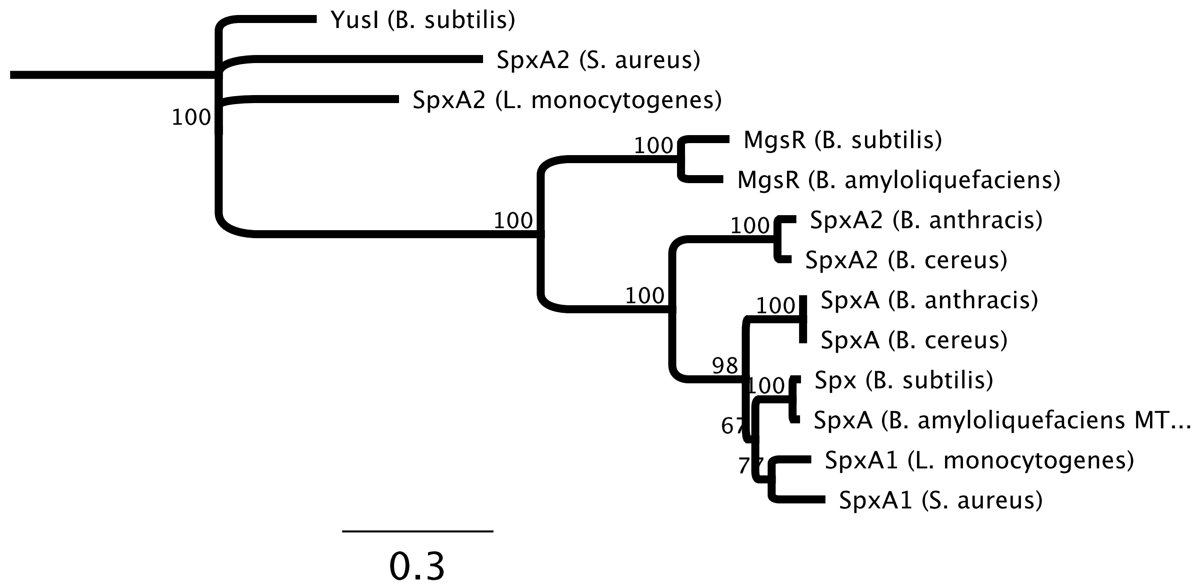
A



B

1. SpxA1 (E. faecalis)
2. SpxA1 (L. acidophilus)
3. SpxA2 (L. acidophilus)
4. SpxA1 (L. rhamnosus)
5. SpxA2 (L. rhamnosus)
6. SpxA1 (L. lactis)
7. SpxA2 (L. lactis)
8. SpxA3 (L. lactis)
9. SpxA4 (L. lactis)
10. SpxA5 (L. lactis)
11. SpxA6 (L. lactis)

C



References

1. Koo B-M, Kritikos G, Farelli JD, Todor H, Tong K, Kimsey H, Wapinski I, Galardini M, Cabal A, Peters JM, Hachmann A-B, Rudner DZ, Allen KN, Typas A, Gross CA. 2017. Construction and Analysis of Two Genome-Scale Deletion Libraries for *Bacillus subtilis*. Cell Syst 4:291–305.e7.
2. Quisel JD, Burkholder WF, Grossman AD. 2001. In vivo effects of sporulation kinases on mutant Spo0A proteins in *Bacillus subtilis*. J. Bacteriol. 183:6573–6578.
3. Guérout-Fleury AM, Frandsen N, Stragier P. 1996. Plasmids for ectopic integration in *Bacillus subtilis*. Gene. 180:57-61.
4. Vagner V, Dervyn E, Ehrlich SD. 1998. A vector for systematic gene inactivation in *Bacillus subtilis*. Microbiology. 144:3097-3104.
5. Luo Y, Helmann JD. 2012. Analysis of the role of *Bacillus subtilis* σ^M in β-lactam resistance reveals an essential role for c-di-AMP in peptidoglycan homeostasis. Mol Microbiol. 83:623-639.
6. Rojas-Tapias DF, Helmann JD. 2018. Stabilization of *Bacillus subtilis* Spx under cell wall stress requires the anti-adaptor protein YirB. PLoS Genet 14:e1007531.
7. Nakano S, Küster-Schöck E, Grossman AD, Zuber P. 2003. Spx-dependent global transcriptional control is induced by thiol-specific oxidative stress in *Bacillus subtilis*. Proc Natl Acad Sci U S A. 100:13603-13608.
8. Gaballa A, Antelmann H, Hamilton CJ, Helmann JD. 2013. Regulation of Bacillus subtilis bacillithiol biosynthesis operons by Spx. Microbiology. 159:2025-2035.
9. Oberto J. 2013 SyntTax: a web server linking synteny to prokaryotic taxonomy. BMC Bioinformatics. Jan 16;14:4. doi: 10.1186/1471-2105-14-4.

.