Supporting information

Synthesis of the compatible solute proline by *Bacillus subtilis*: point mutations rendering the osmotically controlled *proHJ* promoter hyperactive

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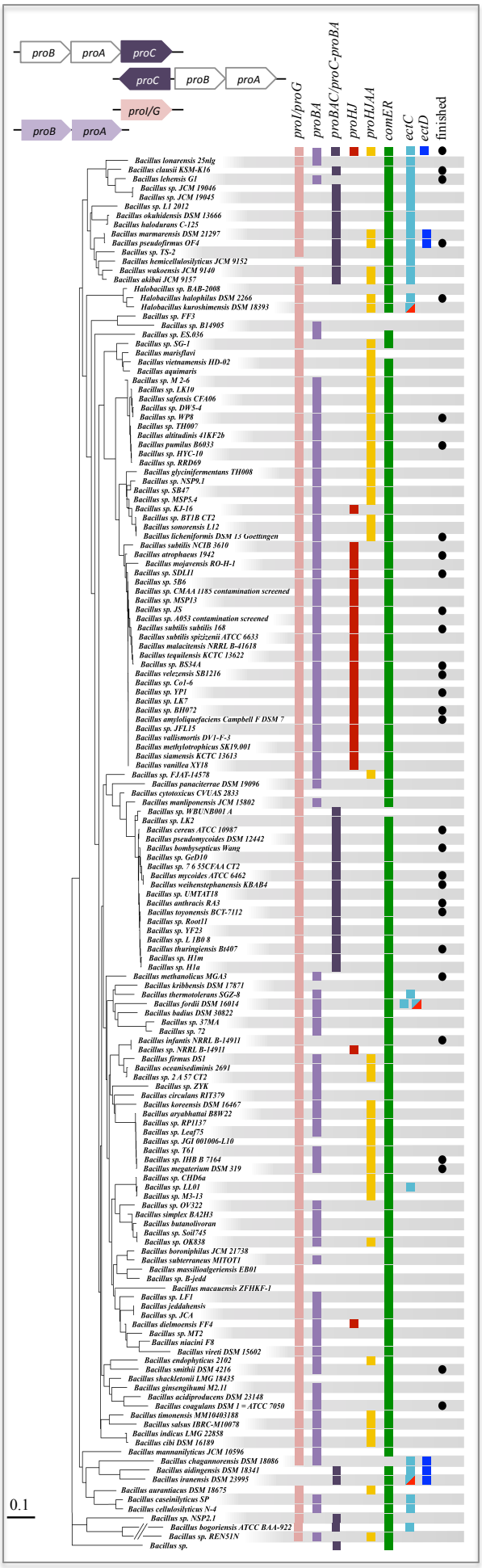

Fig. S1. Phylogenetic tree of *Bacillus* and *Halobacillus* species and their predicted proline biosynthetic genes. We compared a set of 152 genome sequences [both unfinished and finished (●)] from the genera *Bacillus* and *Halobacillus*, whose 16S DNA information were available, to generate a phylogenetic tree at the IMG/M homepage (Integrated microbial genome and metabiome samples database; https://img.jgi.doe.gov/cgi-bin/m/main.cgi). Subsequent BLAST searches against these genome sequences using the *B. subtilis* ProH, ProA and ProB protein sequences as queries were performed. The gene neighborhoods of the resulting hits were analyzed and revealed the following types of L-proline biosynthesis operons: the osmo-adaptive *B. subtilis*-type *proH-proJ-* (■), the osmo-adaptive *B. licheniformis*-type *proH-proJ-proAA-* (■), the anabolic *proBAC-* (■) and *proBA-* operons (■).

Species harboring genes for the synthesis of the compatible solute ectoine [*ectABC* (■) and or hydroxyectoine (*ectD*; ■)] were identified by a BLAST search using the EctC amino acid sequence of *Virgibacillus pantothenticus* (Gene ID: 2655618993) and the EctD amino acid sequence of *Salibacillus salexigens* (Bursy et al., 2007) as search query. Some *Bacilli* possess only the gene for the ectoine synthase [*ectC* (■] but lack the *ectAB* biosynthetic genes; this phenomenon has been observed in many other microbial species (Widderich et al., 2014). It is not clear if these strains can synthesize ectoine by themselves or whether they rely on the import of the EctC substrate from the environment (Kurz et al., 2010).

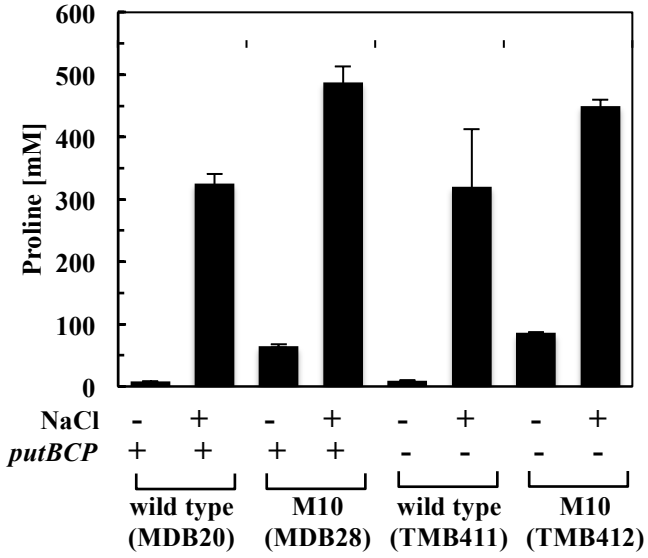

Fig. S2. Proline pools were determined in cells that possess either an intact PutBCP proline import and degradation system (+), or those lacking it (-). *B. subtilis* strains carrying either the wild type *proHJ* operon or its mutant derivative (*proHJ*-M10) were cultivated in SMM without (-) or with (+) 1.2 M NaCl. After the cultures have reached mid-exponential growth phase (OD_{578nm} of about 1.7) cells were harvested and assayed for its proline content using a colorimetric assay (Bates et al., 1973). Shown are the averaged data from three to four biological replicates.

Table S1. Primers and plasmids used for *proH* promoter mutagenesis and strain construction

primer pairs used for site directed mutagenesis			resulting plasmid derived from	
name	sequence (5´→ 3´)	mutation	pJS35 [a]	pMD15 [b]
1-MutHJ fwd	gacatcatttcctcacgaggtaacattttaaacgtgtgaaaatgcg	M1	pMD1	
1-MutHJ rev	cgcattttcacacgtttaaaatgttacctcgtgaggaaatgatgtc			
2-MutHJ fwd	gacatcatttcctcacgtcgtaacattttaaacgtgtgaaaatgcg	M2	pMD2	
2-MutHJ rev	cgcattttcacacgtttaaaatgttacgacgtgaggaaatgatgtc			
7-MutHJ fwd	gacatcatttcctcacgtggtaacagtttaaacgtgtgaaaatgcg	M3	pMD8	
7-MutHJ rev	cgcattttcacacgtttaaactgttaccacgtgaggaaatgatgtc			
4-MutHJ fwd	gacatcatttcctcacgtggtatcattttaaacgtgtgaaaatgcg	M4	pMD5	
4-MutHJ rev	cgcattttcacacgtttaaaatgataccacgtgaggaaatgatgtc			
5-MutHJ fwd	gacatcatttcctcacgtggtaaaattttaaacgtgtgaaaatgcg	M5	pMD4	pMD16
5-MutHJ rev	cgcattttcacacgtttaaaatttaccacgtgaggaaatgatgtc			
6-MutHJ fwd	gacatcatttcctcacgtggtataattttaaacgtgtgaaaatgcg	M6	pMD7	pMD17
6-MutHJ rev	cgcattttcacacgtttaaaattataccacgtgaggaaatgatgtc			
8-MutHJ fwd	ggccttcaaacttgacatttcctcacgtggtaacatttaaacg	M7	pMD6	pMD18
8-MutHJ rev	cgtttaaaatgttaccacgtgaggaaaatgtcaagtttgaaggcc			
9-MutHJ fwd	ggccttcaaacttgacatcatttcctcatcgtggtaacatttaaacg	M8	pMD9	
9-MutHJ rev	cgtttaaaatgttaccacgatgaggaaatgatgtcaagtttgaaggcc			
10-MutHJ fwd	ggccttcaaacttgacatcatttttcctcacgtggtaacattttaaacg	M9	pMD10	pMD19
10-MutHJ rev	cgtttaaaatgttaccacgtgaggaaaaatgatgtcaagtttgaaggcc			
12-MutHJ fwd	ggccttcaaacttgacatcatttttcctcacgtggtataattttaaacg	M10	pMD12	pMD20
12-MutHJ rev	cgtttaaaattataccacgtgaggaaatgatgtcaagttgaaggcc			

[a]Plasmid pJS35 carries the 153 bp *proHJ* promoter fragment fused to a promotre-less *treA* reporter gene.

[b]Plasmid pMD15 carries the *proHJ* operon under control of its natural promoter and can be used for integration into the *B. subtilis* chromosome at the *amyE* gene.

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

Strain	Relevant genotype	Origin or reference [a]
JH642	*trpC2 pheA1*	J. Hoch; BGSC 1A96
FSB1	(*treA*::*neo*)1	(Spiegelhalter and Bremer, 1998)
JSB8	Δ(*proHJ*::*tet*)1	(Brill et al., 2011a)
JSB36	(*treA*::*neo*)1 (*amyE*::ϕP_{wt} *proH´-treA*, *cml*)	(Brill et al., 2011b)
MDB8	(*treA*::*neo*)1 (*amyE*:: ϕP_{M1} *proH´-treA*, *cml*)	This study
MDB9	(*treA*::*neo*)1 (*amyE*:: ϕP_{M2} *proH´-treA*, *cml*)	This study
MDB13	(*treA*::*neo*)1 (*amyE*:: ϕP_{M3} *proH´-treA*, *cml*)	This study
MDB6	(*treA*::*neo*)1 (*amyE*:: ϕP_{M4} *proH´-treA*, *cml*)	This study
MDB11	(*treA*::*neo*)1 (*amyE*:: ϕP_{M5} *proH´-treA*, *cml*)	This study
MDB12	(*treA*::*neo*)1 (*amyE*:: ϕP_{M6} *proH´-treA*, *cml*)	This study
MDB7	(*treA*::*neo*)1 (*amyE*:: ϕP_{M7} *proH´-treA*, *cml*)	This study
MDB14	(*treA*::*neo*)1 (*amyE*:: ϕP_{M8} *proH´-treA*, *cml*)	This study
MDB15	(*treA*::*neo*)1 (*amyE*:: ϕP_{M9} *proH´-treA*, *cml*)	This study
MDB17	(*treA*::*neo*)1 (*amyE*:: ϕP_{M10} *proH´-treA*, *cml*)	This study
MDB20	Δ(*proHJ*::*tet*)1 (*amyE*::P_{wt} *proHJ, cml*)	This study
MDB24	Δ(*proHJ*::*tet*)1 (*amyE*::P_{M5} *proHJ, cml*)	This study
MDB25	Δ(*proHJ*::*tet*)1 (*amyE*::P_{M6} *proHJ, cml*)	This study
MDB26	Δ(*proHJ*::*tet*)1 (*amyE*::P_{M7} *proHJ, cml*)	This study
MDB27	Δ(*proHJ*::*tet*)1 (*amyE*::P_{M9} *proHJ, cml*)	This study
MDB28	Δ(*proHJ*::*tet*)1 (*amyE*::P_{M10} *proHJ, cml*)	This study
SMB44	Δ(*putBCP*::*spc*)1	Susanne Moses
TMB411	Δ(*proHJ*::*tet*)1 (*amyE*::P_{wt} *proHJ, cml*) Δ*putBCP*::*spc*	This study
TMB412	Δ(*proHJ*::*tet*)1 (*amyE*::P_{M10} *proHJ, cml*) Δ*putBCP*::*spc*	This study

[a] BGSC: Bacillus Genetic Stock Center (Columbus, OH, USA). All strains are genetically derived from the domesticated *B. subtilis* wild-type strain JH642 (Smith et al., 2014).

Table S3. Set of final peptides used for SRM assays in this study

Protein	Peptide sequences
ProA	TVENVQEAVK, ELLDQLENAGVEIR
ProB	VFIGTGSGEQK, QYSLTPGQILLTR
ProG	LLEAETEAGISR, LNELLSVFSR
ProH	SIGAQTLLGAAK, DAENALSSLK, LTELELQYGIK
ProI	ALLETIGDATLVEER, EITSPGGTTEAGLR
ProJ	GIIPIINENDTVTVNR, LEALVDQVVK

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