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

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Summary

The ProJ and ProH enzymes of *Bacillus subtilis* catalyse together with ProA (ProJ-ProA-ProH), osmostress-adaptive synthesis of the compatible solute proline. The *proA*-encoded gamma-glutamyl phosphate reductase is also used for anabolic proline synthesis (ProB-ProA-ProI). Transcription of the *proHJ* operon is osmotically inducible whereas that of the *proBA* operon is not. Targeted and quantitative proteome analysis revealed that the amount of ProA is not limiting for the interconnected anabolic and osmostress-responsive proline production routes. A key player for enhanced osmostress-adaptive proline production is the osmotically regulated *proHJ* promoter. We used site-directed mutagenesis to study the salient features of this stress-responsive promoter. Two important features were identified: (i) deviations of the *proHJ* promoter from the consensus sequence of SigA-type promoters serve to keep transcription low under non-inducing growth conditions, while still allowing a finely tuned induction of transcriptional activity when the external osmolarity is increased and (ii) a suboptimal spacer length for SigA-type promoters of either 16-bp (the natural *proHJ* promoter), or 18-bp (a synthetic promoter variant) is strictly required to allow regulation of promoter activity in proportion to the external salinity. Collectively, our data suggest that changes in the local DNA structure at the *proHJ* promoter are important determinants for osmostress-inducibility of transcription.

Introduction

The semi-permeable nature of the cytoplasmic membrane forces essentially all free-living members of the *Bacteria* and *Archaea* to be vigilant about fluctuations in the osmolarity of their surroundings as these will inevitably trigger passive water fluxes in or out of the cell (Bremer and Krämer, 2000; Booth, 2014). Under high osmolarity conditions, water will exit and will thereby elicit the dehydration of the cytoplasm. This can cause a drop in vital turgor to physiologically non-sustainable values, which in turn will severely impair growth (Wood, 2011). Many members of the *Bacteria* and *Archaea* counteract these negative consequences of high osmolarity surroundings either through the accumulation of large quantities of potassium and chloride ions through uptake (the *salt in* strategy) (Galinski and Trüper, 1994), or through the build-up of large pools of physiologically compliant organic osmolytes, the compatible solutes (the *salt out* strategy) (Brown, 1976). The accumulation of compatible solutes can be accomplished through synthesis and uptake (Kempf and Bremer, 1998; Roeßler and Müller, 2001; Sleator and Hill, 2002), and in both processes, the degree of osmotic stress dictates the size of the cellular compatible solute pool (Kuhlmann and Bremer, 2002; Hoffmann *et al*., 2013b). Different types of compatible solutes have been selected as osmostress protectants during microbial evolution (da Costa *et al*., 1998; Kempf and Bremer, 1998; Roeßler and Müller, 2001), and L-proline is one of them (Csonka, 1989; Fichman *et al*., 2014). Proline is highly water-soluble and its accumulation not only serves as a countermeasure for water exit from osmotically stressed cells but its function-preserving and protein anti-aggregating physico-chemical properties also

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helps to optimize the composition of the cytoplasm for biochemical activities (Cayley *et al*., 1992; Ignatova and Gierasch, 2006; Street *et al*., 2006).

One of the microorganisms that make intensive use of proline as an osmostress protectant is *Bacillus subtilis* (Whatmore *et al*., 1990; von Blohn *et al*., 1997; Brill *et al*., 2011a; Zaprasis *et al*., 2015). Although it is mostly associated with soil and plant roots, it can actually live in rather different environments, including sediments and marine ecosystems and even in the gastro-intestinal tract of animals and humans (Earl *et al*., 2008; Cutting, 2011; Ettoumi *et al*., 2013; Mandic-Mulec *et al*., 2015; Rodriguez-Torres *et al*., 2017). In its varied habitats, *B. subtilis* has to cope with fluctuations in the external osmolarity (Hoffmann *et al*., 2008; Hoffmann and Bremer, 2016). It adjusts physiologically to increases in the environmental osmolarity through synthesis of the compatible solutes proline and glycine betaine (from the precursor choline), and the import of a large set of osmostress protectants via the osmotically controlled family of Opu transport systems (Hoffmann and Bremer, 2016; 2017). Among the 15 naturally occurring compounds *B. subtilis* can exploit for osmostress protection (Hoffmann and Bremer, 2017), proline is the only one it can synthesize *de novo* (Whatmore *et al*., 1990; Brill *et al*., 2011a) and is also the only one which it can catabolize (Belitsky, 2011; Moses *et al*., 2012).

As in many other microorganisms (Fichman *et al*., 2014), proline is synthesized by *B. subtilis* from the precursor glutamate (Belitsky *et al*., 2001; Commichau *et al*., 2008). Three enzymes participate in its biosynthesis: γ-glutamyl kinase, γ-glutamyl phosphate reductase and Δ^1-pyrroline-5-carboxylate reductase (Fig. 1A). However, it possesses, with the exception of γ-glutamyl phosphate reductase (ProA), iso-enzymes for the first (ProB/ProJ) and last (ProI/ProH/ProG) steps in proline biosynthesis (Belitsky *et al*., 2001). From this set of enzymes, two physiologically distinct biosynthetic routes are assembled (Fig. 1A) that meet the different production levels of proline required for either anabolic or osmostress protective purposes (Whatmore *et al*., 1990; Brill *et al*., 2011a,b). The anabolic proline biosynthetic route encompasses the sequential activities of the ProB-ProA-ProI enzymes while the corresponding ProJ-ProA-ProH enzymes form the osmostress-adaptive route (Fig. 1A).

Both genetic regulatory circuits and biochemical control mechanisms tie the pool size of proline formed by the anabolic ProB-ProA-ProI route in non-stressed cells to ongoing protein biosynthetic activities. The genes for the γ-glutamyl kinase (ProB) and for the γ-glutamyl phosphate reductase (ProA) are organized as an operon (*proBA*), while that for the Δ^1-pyrroline-5-carboxylate reductase (ProI) is encoded by a separate gene (*proI*) (Fig. 1A) (Belitsky *et al*., 2001). A common denominator of *proBA* and *proI* transcription is their control via a T-box system (Brill *et al*., 2011b), an RNA-based regulatory device (Sherwood and Henkin, 2016) that gauges cellular proline pools through the loading-status of a proline-specific tRNA and its interaction with a particular proline-specific codon in the 5' non-translated regions of the *proBA* and *proI* genes (Brill *et al*., 2011b). The proline-responsive T-box system allows enhanced full-length *proBA* and *proI* transcription only when the proline pool of the cell is insufficient for adequate protein biosynthesis (Brill *et al*., 2011b). A second level of control is afforded through feedback inhibition of the ProB enzyme activity (Fujita *et al*., 2003; Fichman *et al*., 2014), a regulatory step that already sets in at rather low cellular proline concentrations (Chen *et al*., 2007). Through these two levels of genetic and biochemical control, *B. subtilis* maintains cellular proline pools approximately between 10 and 20 mM in vegetative cells (Whatmore *et al*., 1990; Hoffmann *et al*., 2013b; Zaprasis *et al*., 2015), and thereby avoids a wasteful overproduction of an amino acid that is energetically expensive to synthesize (Akashi and Gojobori, 2002).

It is apparent that the regulatory systems controlling the ProB-ProA-ProI anabolic proline biosynthetic route are set to limit proline production (Chen *et al*., 2007; Brill *et al*., 2011b) and are therefore unsuited to deliver the very large quantities of proline required for adequate cellular osmostress protection (Whatmore *et al*., 1990; Hoffmann *et al*., 2013b). Proline pools as high a 500 mM are found in *B. subtilis* cells that are severely osmotically stressed (e.g., with 1.2 M NaCl) (Hoffmann *et al*., 2013b; Zaprasis *et al*., 2015). To ensure osmostress protection via proline synthesis, *B. subtilis* possesses a specialized proline biosynthetic route (ProJ-ProA-ProH) that uses ProB- and ProI-related iso-enzymes for the first (ProJ) and third (ProH) step, while still relying on the *proA*-encoded γ-glutamyl phosphate reductase for the second step (Fig. 1A) (Belitsky *et al*., 2001; Brill *et al*., 2011a). Transcription of the *proHJ* operon is strongly up-regulated in response to high osmolarity (Brill *et al*., 2011a), and there is circumstantial evidence that the enzymatic activity of the *proJ*-encoded γ-glutamyl kinase is not, or at least not strongly, subjected to feedback control (Fujita *et al*., 2003; Fichman *et al*., 2014). Disruption of the osmostress responsive proline biosynthetic route causes osmotic sensitivity (Brill *et al*., 2011a), highlighting the central role played by this compatible solute for cellular osmostress adjustment by *B. subtilis*.

The different regulatory circuits controlling *proBA* and *proHJ* transcription (Fig. 1B) (Brill *et al*., 2011a,b) raise the question of how osmotically stressed *B. subtilis* cells can supply the ProA enzyme in amounts that will not limit situation-conform osmostress adjustment. This conundrum has yet to be addressed experimentally. *Bacillus licheniformis*, which also relies on proline production for its cellular osmostress response (Kuhlmann and Bremer, 2002; Bursy

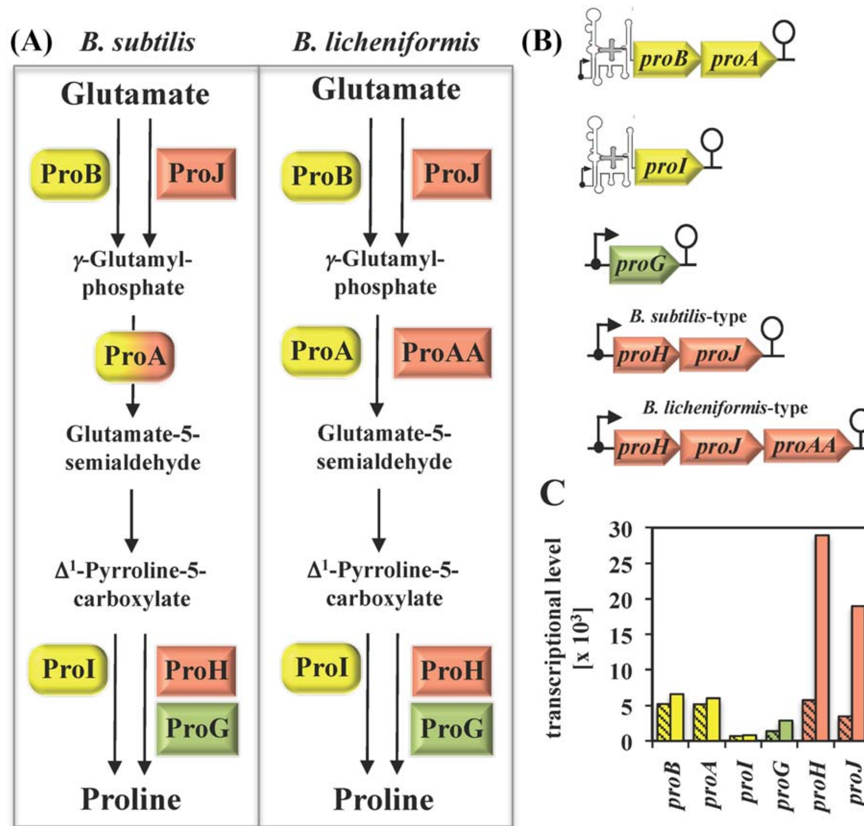

Fig. 1. Proline biosynthesis routes of *B. subtilis* and *B. licheniformis* and their genetic control.
A. The anabolic (ProB-ProA-ProI) and the osmostress responsive (ProJ-ProA-ProH; ProJ-ProAA-ProH) proline biosynthetic pathways of *B. subtilis* and *B. licheniformis* respectively, are shown. ProG has Δ^1-pyrroline-5-carboxylate reductase activity but its true physiological function is unknown (Belitsky *et al*., 2001).
B. Chromosomal organization of the anabolic and osmostress-responsive proline biosynthetic genes from *B. subtilis* and *B. licheniformis*. Full-length transcription of the *proBA* and *proI* genes from both *Bacilli* are regulated via a T-box system that is encoded in the 5′-UTR of these genes (Brill *et al*., 2011b; Sherwood and Henkin, 2016), and this is indicated through the drawn mRNA secondary structure cartoon. The genetic organization of the osmotically regulated *proH-proJ* and *proH-proJ-proAA* genes is shown and indicated as *B. subtilis*- and *B. licheniformis*-type respectively (Brill *et al*., 2011a; Schroeter *et al*., 2013). Transcriptional terminator sequences are indicated by lollipops.
C. The transcriptional levels of the various *pro* genes from *B. subtilis* cells grown in SMM in the absence (hatched bars) or the presence of 1.2 M NaCl (solid bars) are shown. These data were extracted from a tiling-array transcriptional study with cellular and environmentally challenged *B. subtilis* cells (Nicolas *et al*., 2012).

et al., 2007), avoids this potential complication by possessing not only T-box-controlled anabolic *proBA* and *proI* genes but also an additional osmotically controlled gene cluster (*proH-proJ-proAA*) that encodes the full set of proline biosynthetic enzymes (Fig. 1A and B) (Schroeter *et al*., 2013).

The cellular pools of proline in *B. subtilis* cells challenged by high osmolarity are dictated by the severity of the imposed osmotic stress and correlate in essence with it in a linear fashion (Hoffmann *et al*., 2013b). This is largely due to the corresponding transcriptional profile of the *proHJ* promoter (Brill *et al*., 2011a; Nicolas *et al*., 2012) (Fig. 1C) and implies that the *B. subtilis* cell can detect and respond to incremental increases in the external osmolarity in a timely manner. Among all genes of *B. subtilis* exhibiting enhanced transcriptional activity at high salinity, the *proHJ* promoter is one of the most strongly inducible (Steil *et al*., 2003; Hahne *et al*., 2010; Kohlstedt *et al*., 2014). Furthermore, its transcription is truly under osmotic control since it can be induced both by ionic and non-ionic solutes but not by glycerol (Brill *et al*., 2011a), a membrane-permeable solute which cannot establish an osmostress-inducing gradient across the cytoplasmic membrane. The *proHJ* promoter reacts both to suddenly imposed and to sustained increases in environmental osmolarity (Brill *et al*., 2011a), and is in all likelihood recognized by RNA-polymerase complexed with the housekeeping transcription factor SigA (Helmann, 1995; Brill *et al*., 2011a). Its activity is also modulated by the import of compatible solutes such as glycine betaine (Brill *et al*., 2011a; Hoffmann *et al*., 2013b).

Whatever signal perception mechanism(s) is used by *B. subtilis* to detect increases in the external osmolarity (Hoffmann and Bremer, 2016), the ensuing signal transduction process must eventually converge on the *proHJ* promoter. Since its salient features are not understood with respect to osmotic control, we have carried out a detailed site-directed mutagenesis study focusing on key elements of this promoter. We identify here the suboptimal spacing of the *proHJ* −10 and −35 elements as a critical molecular determinant allowing the promoter to direct finely tuned osmotically induced transcription in response to a wide range of environmental osmolarities. Bioinformatics analysis showed that this promoter design is strictly conserved among members of *Bacilli* and *Halobacilli* predicted to synthesize proline as an osmostress protectant. Collectively, our data suggests that the *proHJ* promoter belongs to a group of promoters that are sensitive to changes in DNA topology and might use these alterations to convert environmental changes in osmolarity into changes in transcriptional activity (Higgins *et al.*, 1988; Wang and Syvanen, 1992; Jordi *et al.*, 1995; Dorman and Dorman, 2016). Furthermore, through targeted quantitative proteomics of osmotically non-stressed and stressed cells, we have asked whether the amounts of the ProA protein jointly used by the anabolic and osmostress-adaptive proline synthesis routes of *B. subtilis* (Fig. 1A) are sufficiently high to avoid limiting cellular osmostress adjustment.

Results

Quantification of the anabolic and osmostress-responsive proline biosynthetic enzymes

The anabolic (ProB-ProA-ProI) and osmostress-adaptive (ProJ-ProA-ProH) proline biosynthetic routes of *B. subtilis* are interlinked via the *proA*-encoded γ-glutamyl phosphate reductase (ProA) (Fig. 1A). However, the transcription of the *proBA* and *proHJ* operons are subjected to different genetic regulatory circuits (Brill *et al.*, 2011a,b), thereby raising the question about how enough of the ProA enzyme can be provided by the cell to meet the enhanced proline biosynthetic activity required under high salinity growth conditions. To address this question, we carried out an absolute protein quantification of all proline biosynthetic enzymes via targeted proteomics. For these experiments, we grew wild-type *B. subtilis* JH642 cells in a glucose-based minimal medium (SMM) in the absence or presence of high salt (1.2 M NaCl). The proteins catalysing anabolic proline production (ProB-ProA-ProI) (Belitsky *et al.*, 2001; Brill *et al.*, 2011b) were present in SMM-grown cells at an absolute concentration of 1.7, 4.9 and 1.4 fmol (μg protein extract)$^{-1}$ respectively (Fig. 2). Hence, although the ProB and ProA proteins are encoded in the same operon (Belitsky *et al.*, 2001) (Fig. 1B), the cellular concentration of ProA exceeds that of ProB by 2.9-fold. A similar phenomenon was seen for the *proHJ*-encoded proteins (Fig. 1B), where the amount of the synthesized ProH protein is 5.2-fold higher than that of ProJ (Fig. 2). Indeed, it is frequently observed that proteins synthesized from the same poly-cistronic mRNA are translated with different efficiencies (Quax *et al.*, 2013; Burkhardt *et al.*, 2017). *B. subtilis* possesses three iso-enzymes of Δ^1-pyrroline-5-carboxylate reductase to catalyse the last step in proline biosynthesis (ProI; ProH; ProG) (Belitsky *et al.*, 2001) (Fig. 1A). We found, that levels of ProH [(2.7 fmol (μg protein extract)$^{-1}$] and ProG [(2.5 fmol (μg protein extract)$^{-1}$] were similar to those of ProI [(1.4 fmol (μg protein extract)$^{-1}$] in non-osmotically stressed cells (Fig. 2).

When the same kind of experiment was conducted with salt-stressed cells, we observed a strong increase in the cellular concentration of those enzymes forming the osmostress-adaptive proline biosynthetic route (ProJ-ProA-ProH) (Fig. 2). In comparison to the non-stressed cells, the amount of ProJ was increased by 7.1-fold and a similar value (8.3-fold) was detected for ProH. This increase in ProJ and ProH levels is readily understandable in view of the fact that the transcription of the *proHJ* operon is induced under osmotic stress conditions (Brill *et al.*, 2011a). However, transcription of *proA* is not under osmotic control (Steil *et al.*, 2003; Brill *et al.*, 2011a,b; Nicolas *et al.*, 2012) (Fig. 1C), and the moderate increase (about twofold) of ProA under these growth conditions is somewhat a surprise.

In addition to the anabolic (ProI) and osmostress-adaptive (ProH) Δ^1-pyrroline-5-carboxylate reductases, *B. subtilis* possesses a third representative (ProG) of this type of enzyme (Belitsky *et al.*, 2001) (Fig. 1A). However, the precise physiological function of ProG is not known. Like *proI*, *proG* is transcribed as a mono-cistronic mRNA (Fig. 1B) but unlike *proI* expression, transcription of *proG* is not under control of a T-box regulatory system (Brill *et al.*, 2011b). Judging from a comprehensive tiling array study of *B. subtilis* cells challenged by sustained high salinity (Nicolas *et al.*, 2012), there is an about twofold increase in *proG* mRNA levels (Fig. 1C) and this is reflected by the increase in ProG protein concentrations when stressed and non-stressed cells are compared (Fig. 2). Overall, our proteome analysis shows that no bottleneck in ProA levels seems to exist that would impede adequate osmostress-adaptive proline biosynthesis via the ProJ-ProA-ProH route.

Bioinformatics assessment of the distribution of osmostress-adaptive proline biosynthetic genes among the Bacilli

Osmostress-responsive proline biosynthesis in *Bacilli* is performed by a similar set of enzymes that are encoded either by separate genes clusters (*proH-proJ, proA;* the

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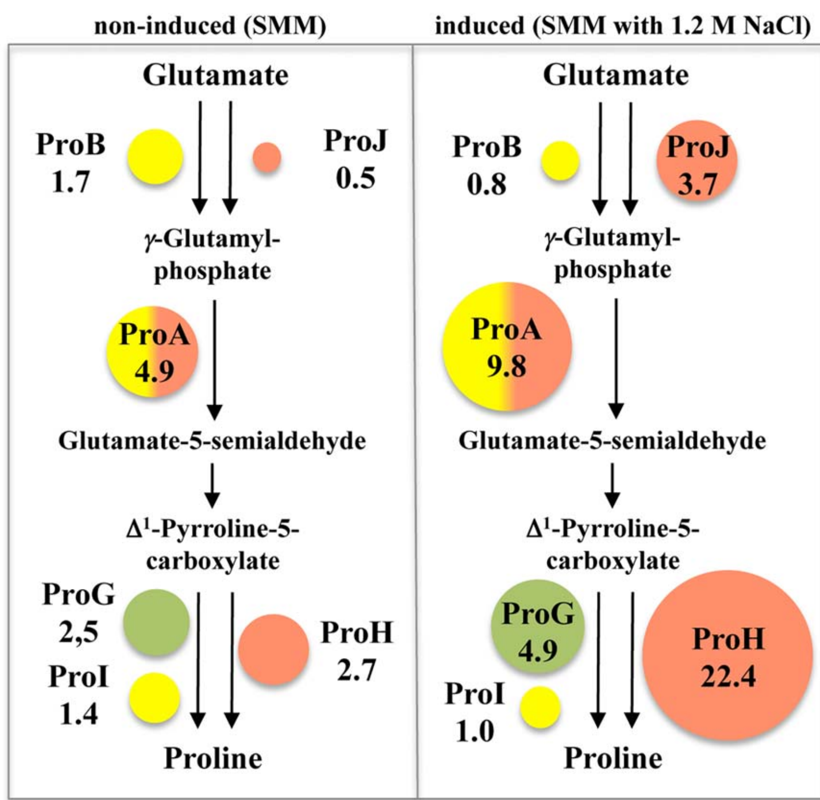


Fig. 2. Absolute protein quantification of the proline biosynthetic enzymes in the *B. subtilis* wild-type strain JH642 by LC-MS/MS analysis. Cultures of strain JH642 were grown in SMM in the absence (non-induced) or the presence of 1.2 M NaCl (induced). Cells were harvested in the mid-exponential growth phase. Proteins were extracted and were used for total protein quantification using synthetic QConCAT proteins comprising peptides from all *B. subtilis* proline biosynthetic proteins. The amounts of the detected proteins are given in fmol (μg total protein)$^{-1}$. The data shown were derived by quantification of proline biosynthetic enzymes from three independently grown *B. subtilis* cultures.

B. subtilis-type) (Brill *et al.*, 2011a), or by a single operon (*proH-proJ-proAA*; the *B. licheniformis*-type) (Schroeter *et al.*, 2013) (Fig. 1B). As a variation of this theme, in *Halobacillus halophilus* osmostress-adaptive proline production depends on a *proH-proJ-proAA* gene cluster as well, while anabolic proline production relies on enzymes that synthesize proline from ornithine (Köcher *et al.*, 2011). To assess the taxonomic distribution of anabolic and osmostress-adaptive proline biosynthetic genes within the families of the *Bacillacea* and *Halobacillaceae*, we searched the Integrated Microbial Genomes & Microbiomes (IMG/M) database of the DOE's Joint Genome Institute (JGI) (https://img.jgi.doe.gov/cgi-bin/m/main.cgi). We extracted from this database 152 genome-sequences from the genera *Bacillus* (149 representatives) and *Halobacillus* (3 representatives), which also had 16S DNA information available for phylogenetic analysis using the 'compare genomes distance tree' tool provided at the IMG/M web site. To identify proline biosynthetic genes within this data set, we used the *B. subtilis* ProH protein sequence (Brill *et al.*, 2011a) as the query for a BLAST analysis (Altschul *et al.*, 1990) since homologs of this protein will occur both in the anabolic and osmostress-responsive pathways (Belitsky *et al.*, 2001; Brill *et al.*, 2011a,b). This search yielded 520 hits to ProH-type proteins. We then extended this analysis further by searching the original 152 genomes in our dataset for homology to either ProB or ProA. In a third step, we inspected the genome neighbourhoods of the ProH, ProB and ProA hits to assign the corresponding genes to either putative anabolic or osmostress-adaptive proline synthetic routes.

With the exception of 18 *Bacilli* with unfinished genome sequences, we found in 133 genomes the full set of anabolic proline biosynthetic genes (Supporting Information Fig. S1). *Bacillus infantis* strain NRRL B-14911 whose genome sequence is completely known, seems to be a proline auxotroph (Massilamany *et al.*, 2016). In the originally inspected dataset of 152 genomes, we found 28 *B. subtilis proH-proJ*-type operons and 50 *B. licheniformis proH-proJ-proAA*-type operons. The strains that featured *B. subtilis proH-proJ*-type operons possess, with a single exception (unfinished genome), also a separate *proA* gene. Hence about half of the *Bacilli* and *Halobacilli* whose genome sequences that we inspected are predicted to physiologically adjust to high-salinity environments through the synthesis of the compatible solute proline (Supporting Information Fig. S1).

The *comER* gene encodes a protein related in its amino acid sequence to Δ^1-pyrroline-5-carboxylate reductases, but ComER is probably not involved in proline biosynthesis

(Inamine and Dubnau, 1995; Belitsky *et al*., 2001). Recent evidence suggests that it participates in the complex genetic control of biofilm and spore formation by *B. subtilis* and *Bacillus cereus* (Yan *et al*., 2016). Through our bioinformatics analysis of the 152 *Bacilli* and *Halobacilli*, we found that almost all of them possess a *comER*-type gene and those few that do not, represent unfinished genome sequences (Supporting Information Fig. S1). This strong evolutionarily conservation suggest an important regulatory function for the ComER protein in cellular development and differentiation of *Bacilli* (Yan *et al*., 2016).

The presumed osmostress-adaptive proline producers possess closely related promoter sequences

The osmotically controlled *B. subtilis proHJ* promoter has been identified through primer extension analysis (Brill *et al*., 2011a), and −10, −35 and −16 elements characteristic for SigA-dependent promoters (Helmann, 1995; Voskuil and Chambliss, 1998) have been found. The −35 element matches perfectly to the consensus sequence (TTGACA) of these types of promoters; the −10 element (TAACAT) deviates at two positions from the consensus sequence (TATAAT), and a TG −16 element frequently found in strong *B. subtilis* promoters (Voskuil and Chambliss, 1998) is present as well. SigA-type promoters typically possess a spacing of 17 bp between the −10 and −35 elements but some *B. subtilis* promoters with spacer length of either 16 bp or 18 bp are known (Helmann, 1995). The *proHJ* promoter has such a sub-optimal spacing (16 bp) (Fig. 3) (Brill *et al*., 2011a). A promoter closely related to that of the *proHJ* operon from *B. subtilis* has been identified for the osmostress-responsive *proH-proJ-proAA* operon from *B. licheniformis* through DNA sequence gazing (Schroeter *et al*., 2013).

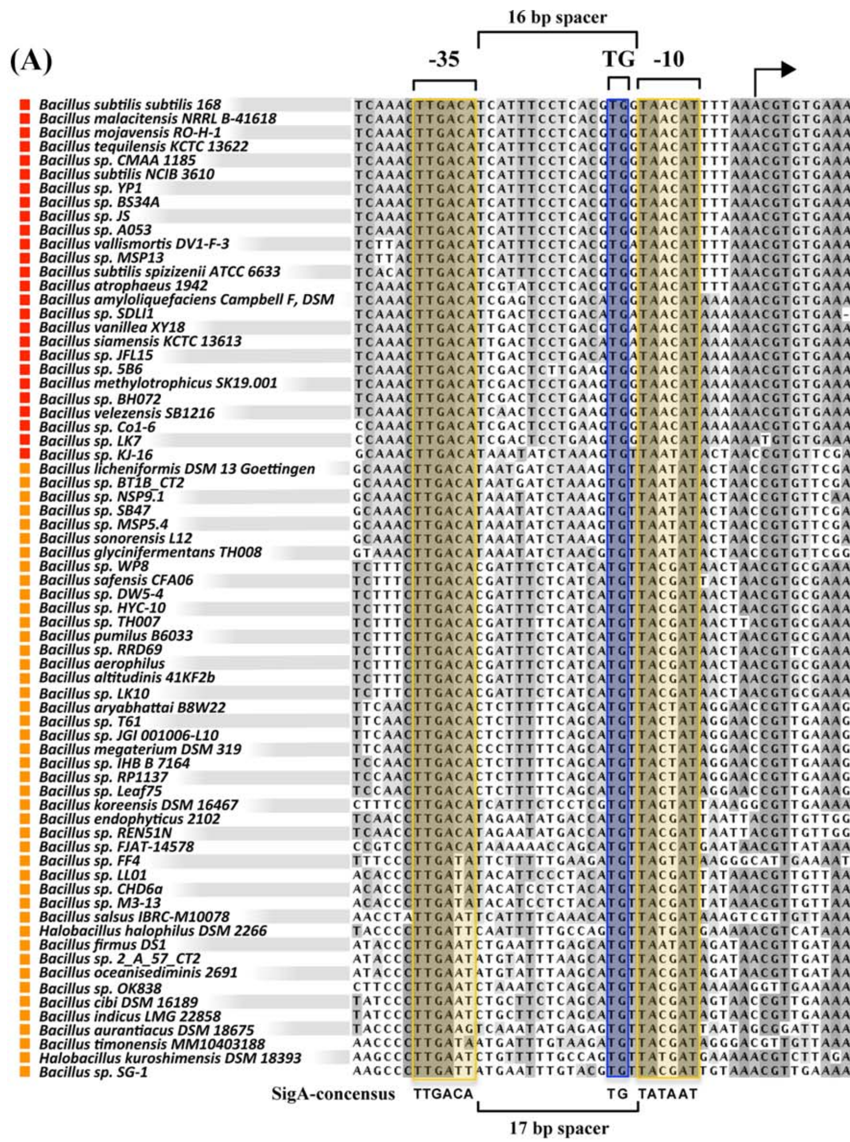
In the next step in our bioinformatics analysis, we inspected the regulatory regions of the *proH-proJ* and *proH-proJ-proAA*-type operons from 70 *Bacillus* and *Halobacillus* species. From the remaining eight gene clusters of bacteria predicted to synthesize proline under osmotic stress conditions (Supporting Information Fig. S1), we could not derive a convincing DNA-sequence alignment. In Fig. 3A, we compiled the predicted 70 promoter sequences, and this alignment highlights the considerable conservation of the −10, −16 and −35 elements. Strikingly, each of the predicted SigA-type promoters has a sub-optimal 16-bp spacer (Fig. 3A). The salient features of these promoters are conserved regardless whether they are present in front of *proH-proJ* or *proH-proJ-proAA* operons from *Bacilli*, or in front of the two *proH-proJ-proAA* gene clusters from *Halobacilli* that we identified in our data base search (Supporting Information Fig. S1).

Site-directed mutagenesis of the B. subtilis *proHJ promoter*

Guided by the compilation of the putative promoter sequences of the *proH-proJ* and *proH-proJ-proAA* operons, we studied the features of the osmoregulated *B. subtilis proHJ* promoter through site-directed mutagenesis. Since its −35 region already corresponds to the consensus sequence of SigA-type promoters (Helmann, 1995; Brill *et al*., 2011a), we focused our analysis on the −10 and the −16 elements, and the sub-optimal spacer (Fig. 4). For this analysis we used a previously described *proHJ-treA* operon fusion construct (Brill *et al*., 2011a) as a read-out for *proHJ* promoter activity. This reporter construct contains a 153-bp *proHJ* promoter DNA fragment transcriptionally fused to a promoter-less *treA* reporter gene encoding a salt-resistant enzyme whose activity can be quantitated with a chromogenic substrate (Schöck *et al*., 1996). The chromosomal *proHJ* fragment carried by the fusion construct extends from 32 bp upstream of the −35 element of the *proHJ* promoter into codon 17 of the *proH* gene. This *proHJ-treA* operon fusion reports an essentially linear increase in promoter activity in response to sustained step-wise increases in the environmental osmolarity, once a threshold-level of added NaCl (100 mM) to a minimal medium (SMM) is exceeded (Fig. 4A) (Brill *et al*., 2011a). As the used *proHJ-treA* operon fusion can be stably inserted as a single copy into the chromosomal *amyE* gene, difficulties in the interpretation of the obtained TreA enzyme activity data due to multi-copy effects are avoided.

In an initial step, we changed the so-called ‘invariant T’ (Helmann, 1995) in the −10 region of the *proHJ* promoter to a G/C base pair. As expected, the transcriptional activity of this promoter variant (M3) was strongly reduced and no longer responsive to an increase in salinity (with 0.4 M NaCl) (Fig. 3B). Many *B. subtilis* SigA-type promoters possess a conserved TG-motif at position −16 that frequently determines promoter strength (Voskuil and Chambliss, 1998). We targeted this −16 motif in the *proHJ* promoter (mutants M1 and M2) and found that these promoter variants exhibited only a very low activity, both in cells that were grown in the absence or in the presence of osmotic stress (Fig. 3B). Taking the data from the M3 mutation in the −10 region and those from the M1 and M2 mutations in the −16 element together, there can be little doubt that the osmostress-responsive *proHJ* promoter is exclusively recognized by the house-keeping transcription factor SigA.

In the next step, we systematically increased the homology of the *proHJ* −10 sequence (TAACAT) to that of the consensus −10 sequence (TATAAT) (Helmann, 1995). In each of the three constructed mutant variants (M4, M5, M6), transcriptional activity of the promoter was substantially increased both in the absence and in the presence of



(B)

Promoter mutation	Promoter sequence: -35 ⟵ Spacer ⟶ -16 ⟶ -10	TreA activity [U (mg protein)$^{-1}$]: - NaCl	TreA activity [U (mg protein)$^{-1}$]: + 0.4 M NaCl	Salt induction ratio	
SigA	TTGACA $n_{(17\pm1)}$ TG nTATAAT				
wt	TTGACA TCA--TTTCCTCACG- TG GTAACAT	22 ± 1	39 ± 1	1.8	
M 3	TTGACA TCA--TTTCCTCACG- TG GTAACAG	10 ± 1	4 ± 3	0.4	-10 region
M 4	TTGACA TCA--TTTCCTCACG- TG GTATCAT	64 ± 13	146 ± 1	2.3	-10 region
M 5	TTGACA TCA--TTTCCTCACG- TG GTAAAAT	95 ± 6	209 ± 14	2.2	-10 region
M 6	TTGACA TCA--TTTCCTCACG- TG GTATAAT	503 ± 82	738 ± 30	1.5	-10 region
M 1	TTGACA TCA--TTTCCTCACG- AG GTAACAT	9 ± 0	9 ± 1	1.0	TG motif
M 2	TTGACA TCA--TTTCCTCACG- TC GTAACAT	9 ± 1	9 ± 3	1.0	TG motif
M 7	TTGACA TCA-TTTTCCTCACG- TG GTAACAT	418 ± 21	633 ± 53	1.5	spacer
M 8	TTGACA TCA--TTTCCTCACGT TG GTAACAT	485 ± 13	667 ± 42	1.4	spacer
M 9	TTGACA TCATTTTTCCTCACG- TG GTAACAT	123 ± 4	260 ± 20	2.1	spacer
M10	TTGACA TCA-TTTTCCTCACG- TG GTATAAT	507 ± 17	768 ± 48	1.5	

Fig. 3. Alignment of predicted promoters of *B. subtilis*- and *B. licheniformis*-type *proHJ(AA)*-operons and mutational analysis of the *proHJ* promoter region.
A. Based on the BLAST analysis using the *B. subtilis* ProH amino acid sequence as the query, the 5′ untranslated regions of the *proHJ* (red square) and *proHJAA* operons (orange square) were aligned. Putative promoter regions, which are highly similar to the known SigA-dependent *B. subtilis proHJ* promoter, could be identified in all aligned 5′-UTR sequences. The −35 and −10 promoter regions are boxed in yellow, the −16 TG motif is boxed in blue; the degree of the grey shading represents the degree of the conservation of a base-pair at a given position. The transcriptional start site of the *B. subtilis proHJ* operon has been mapped by primer extension analysis (Brill *et al*., 2011a), and is marked by an arrow.
B. Site-directed mutagenesis of the osmotically regulated *proHJ* promoter. *B. subtilis* strains carrying either a chromosomal copy of the *proHJ-treA* wild-type reporter fusion or mutant derivatives of this construct were grown in SMM or in SMM with additional 0.4 M NaCl, and their TreA enzyme activity was measured. The TreA reporter enzyme activity data given are the means and standard deviations of four independent biological replicates. Salt induction ratios were calculated by dividing TreA activities of cells grown in the presence of 0.4 M NaCl by the activities measured in cells grown without NaCl. The mutations introduced into the wild-type *proHJ* promoter sequence are highlighted in red.

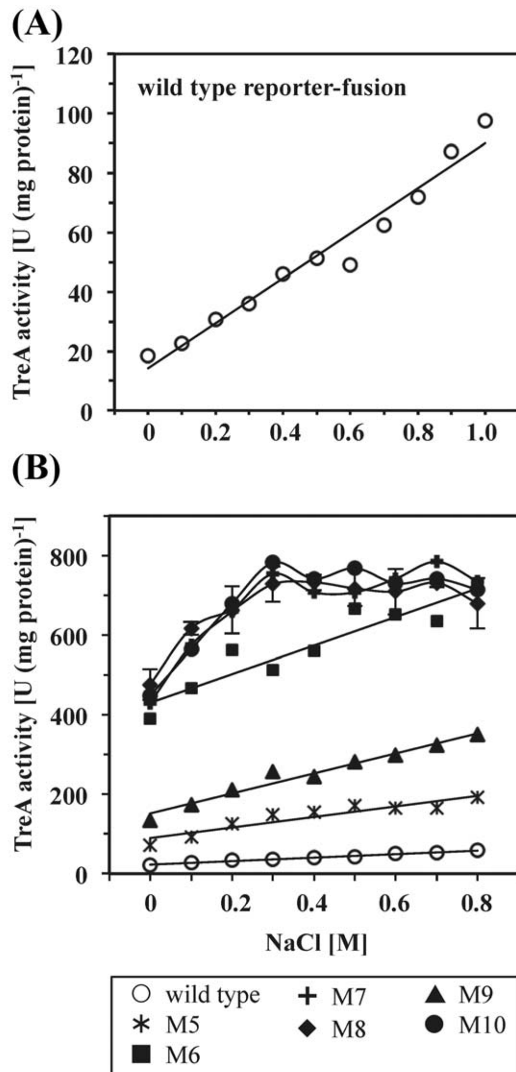


Fig. 4. Transcriptional response of the wild-type and mutant *proHJ* promoters in response to incremental increases in the external salinities. *B. subtilis* strains carrying transcriptional *proHJ-treA* reporter fusions were grown in SMM without or with increasing NaCl concentrations to mid-exponential growth phase (OD_{578} of about 1.5) and were then assayed for their TreA reporter enzyme activity.
A. TreA activities of the *proHJ-treA* wild-type promoter fusion strain JSB36.
B. TreA activities derived from the mutant *proHJ-treA* fusions relative to that exhibited by the wild-type *proHJ-treA* operon fusion strain (open circle). These latter values were re-plotted as a point of reference from the data shown in (A).

osmotic stress (Fig. 3B). Depending on the type of mutation present in the −10 region, promoter activity increased between threefold and 23-fold in cells grown in SMM and between about fourfold and 19-fold in the osmotically stressed cells respectively (Fig. 3B). Particularly striking was the change in promoter activity in the M6 mutant possessing a perfect SigA consensus-like −10 region: in non-induced cells, the basal promoter activity was raised from 22 to 503 units (mg protein)$^{-1}$ of TreA reporter enzyme activity, while still retaining a fold osmostress responsive induction in gene expression similar to that observed for the wild-type *proHJ* promoter when the cells were continuously challenged with 0.4 M NaCl (Fig. 3B).

A strong effect on promoter activity was also afforded when we evaluated two different spacer variants of the *proHJ* promoter in which we had increased the length of the spacer from 16 bp to the more optimal 17-bp spacing of SigA-type promoters (Helmann, 1995). Again, both the non-induced and the osmotically induced levels of promoter activity were strongly enhanced in the corresponding M7 and M8 variants, but maintained only a residual osmotic inducibility in their transcriptional activity (Fig. 3B). Remarkably, an 18-bp spacer variant of the *proHJ* promoter, while typically sub-optimal for SigA-type promoter activity (Helmann, 1995), significantly increased transcriptional activity of the reporter fusion both under non-inducing and inducing growth conditions. As observed for the other promoter variants that were still transcriptionally active, the 18-bp spacer variant remained osmotically inducible (Fig. 3B).

Finally, we constructed a *proHJ* promoter variant with consensus −10, −16 and −35 sequences and an optimal spacing of 17 bp (Helmann, 1995; Voskuil and Chambliss, 1998). These combined changes generated a very strong promoter with levels of transcriptional activity strongly exceeding those of the non-induced and induced levels of the wild-type promoter (by 23- and 20-fold respectively); however, it still was osmotically inducible to some extent (about 1.5-fold) (Fig. 3B). Taken together, our promoter analysis shows that the deviations of the natural *proHJ* promoter from the consensus sequence of SigA-type promoters (Helmann, 1995) serve to keep the transcriptional activity low under non-inducing conditions in order to allow osmotic induction.

Dynamics of the salt-stress response of mutant proHJ promoters

The data summarized in Fig. 3B assess the transcriptional activity of *proHJ* promoter variants under non-inducing conditions and under a single, rather moderate level of continuously imposed salt stress [by adding 0.4 M NaCl to the growth medium; (Boch *et al*., 1994)]. However, *proHJ* promoter activity is highly dynamic and responds in a finely tuned fashion to small increases in the degree of the imposed sustained osmotic stress (Brill *et al*., 2011a). This is shown for the wild-type *proHJ* promoter in Fig. 4A as a point of reference for the promoter variants constructed in this study (Fig. 4B). For representatives of each class of *proHJ* promoter mutants (Fig. 3B), we measured promoter activity in cells grown over a broad range of salinities

(0–0.8 M NaCl) to determine whether the promoters were still able to proportionally respond to step-wise increase in sustained osmotic stress. The chosen level of salinities corresponds to osmolarities ranging between 340 mOsmol (kg H_2O)$^{-1}$ (SMM) and 1 860 mOsmol (kg H_2O)$^{-1}$ (SMM with 0.8 M NaCl) respectively.

The M5 and M6 promoter variants carry mutations that improve the sequence of the −10 region still allow a full proportional response to the salt stress, while operating at differently increased levels of promoter activity relative to the wild-type *proHJ-treA* reporter fusion (Fig. 4B). The M7 and M8 mutants carry different point mutations that increase the spacer length from suboptimal 16-bp to optimal 17-bp for SigA-type promoters (Helmann, 1995) (Fig. 3B). These two spacer mutants possess strongly increased promoter activity (Fig. 3B) but have lost the ability to respond dynamically over the entire spectrum of the imposed osmostress. Instead, there is a linear increase in promoter activity up at a level of 0.3 M NaCl, but after further increases in salinity the promoters do not respond in kind and instead stay at a constant elevated level of activity (Fig. 4B). A promoter mutant (M10) corresponding fully in its −10, −16, −35 elements and spacer length (17-bp) to the consensus sequence of SigA-type promoters (Helmann, 1995) behaved similar to promoter variants possessing a 17-bp spacer in an otherwise *proHJ* wild-type configuration (Fig. 4B). Notably, the mutant (M9) carrying a suboptimal spacer sequence of 18-bp, but harbouring authentic *proHJ* −10 and −35 regions (Fig. 3B), still allowed the *proHJ* promoter to proportionally respond to step-wise increases in the degree of osmotic stress (Fig. 4B).

Influence of proHJ promote variants on osmostress-adaptive proline synthesis

Since most of the *proHJ* promoter variants increased expression of the reporter fusion under both non-inducing and inducing growth conditions (Figs 3B and 4B), we asked if this leads to a corresponding increase in the cellular proline pools. To this end, we introduced representative promoter mutations (M5, M6, M7, M9, M10) (Fig. 3B) into a plasmid carrying the wild-type *proHJ* operon and then inserted these *proHJ* variants via double recombination events in single copy into the chromosomal *amyE* gene of a *B. subtilis* host strain carrying a deletion of the native *proHJ* operon. When such an experiment was carried out with the wild-type *proHJ* locus, the resulting strain (MDB20) produced proline pools similar to that observed in the wild-type strain JH642 grown either in SMM or in SMM with 0.4 M NaCl (Fig. 5). In strain JH642, the corresponding proline pools were 14 mM (in SMM-grown cells) and 130 mM (in cells grown in SMM with 0.4 M NaCl) (Fig. 5). This corresponds to a 9.3-fold increase in proline

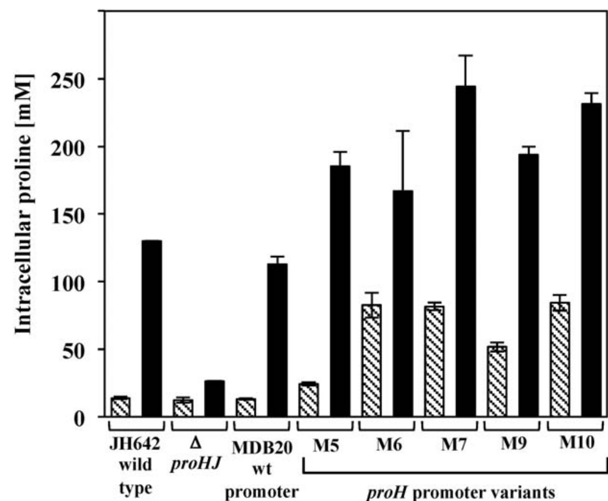


Fig. 5. Intracellular proline pools in strains carrying the *proHJ* operon under control of different promoter variants. The impact of single or multiple mutations introduced into the *proHJ*-promoter sequence was analysed by measuring the intracellular proline pools. For these experiments, we used *B. subtilis* strains harbouring either the wild-type *proHJ* operon or variants carrying mutations in the *proHJ* promoter where the *proHJ* operon had been inserted into the chromosomal *amyE* locus. Cultures were grown in SMM in the absence (hatched bars) or the presence (black bars) of 0.4 M NaCl. Cells were harvested at mid-exponential growth phase (OD_{578} of about 2.0), and their intracellular proline content was determined via a colorimetric assay (Bates *et al.*, 1973; Hoffmann *et al.*, 2012). The data given are the means and standard deviations of four independent biological replicates.

content while the transcriptional activity of the *proHJ-treA* wild-type fusion increased only 1.8-fold under these conditions in response to osmotic stress (Fig. 3B).

All tested strains carrying mutant *proHJ* promoters possessed elevated proline pools both in cells that were not osmotically stressed and in those subjected to osmotic stress by adding 0.4 M NaCl to the growth media (Fig. 5). For instance, in the M10 mutant strain MDB28, the proline pool was increased to 84 mM in cells grown under non-inducing conditions and to 231 mM in cells propagated under inducing conditions. In comparison with the wild-type strain MDB20, this corresponds to increases in the proline pools of 6.5- and 2.1-fold respectively (Fig. 5).

When we compared the fold-induction in *proHJ-treA* transcription of the M5, M6, M7, M9 and M10 mutant strains to the fold induction in proline content, a disparity between these two parameters became apparent (Table 1). As an example: the proline pool was 11 times lower than that predicted from the increased level of *proHJ-treA* transcription for the M10 promoter mutation, and the same trend was observed for all of the tested *proHJ* promoter variants (Table 1). Hence it is apparent, that factors other than *proHJ* promoter strength also influence the size of the *B. subtilis* proline pools.

Table 1. Ratios of TreA reporter enzyme activity and proline pools derived from the *proHJ* promoter mutants and the corresponding wild type strain.

	TreA activity		Intracellular proline	
	Ratio (mutant/ wild type)		Ratio (mutant/ wild type) [a]	
proHJ promoter mutation	0 M NaCl	0.4 M NaCl	0 M NaCl	0.4 M NaCl
M5	6.8	4.6	1.9	1.6
M6	35.9	16.4	6.4	1.5
M7	41.0	20.1	6.3	2.2
M9	11.6	9.6	4.0	1.7
M10	42.9	23.2	6.5	2.1

a. The TreA reporter enzyme activity of the *proHJ-treA* fusion strains M5, M6, M7, M9 and M10 are expressed as a ratio relative to that of the corresponding *proHJ-treA* wild-type strain JSB36. The ratio of intracellular proline content of strains carrying *proHJ* operon at the *amyE* site is given for the indicated promoter mutants (M5, M6, M7, M9 and M10) relative to the corresponding MDB20 wild-type strain. The values given were extracted and calculated from experimental data reported in Figs 3B and 5.

We investigated this phenomenon further by focusing on the wild-type promoter and its mutant M5 and M10 variants by monitoring *proHJ* mRNA levels. For both the wild-type and the two variants, we observed a strong increase of the corresponding mRNAs under osmostress conditions (SMM with additional 0.4 M NaCl). As expected from the *proHJ-treA* fusion analysis (Figs 3B and 4B), the M5 and M10 mutants possessed elevated mRNA levels both under non-inducing and inducing conditions (Fig. 6). In comparison with the wild-type *proHJ* operon, the induced mRNA levels for mutant M5 are increased 2.6-fold and the corresponding value for the M10 mutant is 22.1-fold (Fig. 6). These values agree reasonably well with those obtained from the *proHJ-treA* fusion analysis that yields values of 4.6-fold for the M5 mutant and 23.2-fold for the M10 mutant (Fig. 3B and Table 1). As a conclusion from this analysis we can state that the transcriptional activity of the *proHJ* promoter and its variants are relatively closely reflected in mRNA levels (Fig. 6) but there is a disconnection with the corresponding proline pools (Fig. 5).

Targeted proteomics of the proHJ M10 mutant variant strain MDB28

In a further step of our analysis, we quantitated the amounts of the proline biosynthetic proteins of the M10 derivative (strain MDB28) through targeted proteomics and compared these values with those of its parent strain MDB20. For this set of experiments we exposed the cells to severe osmotic stress (SMM with 1.2 M NaCl) (Boch *et al*., 1994), growth conditions that have been used in the past for most physiological studies addressing the response of *B. subtilis* to high salinity environments

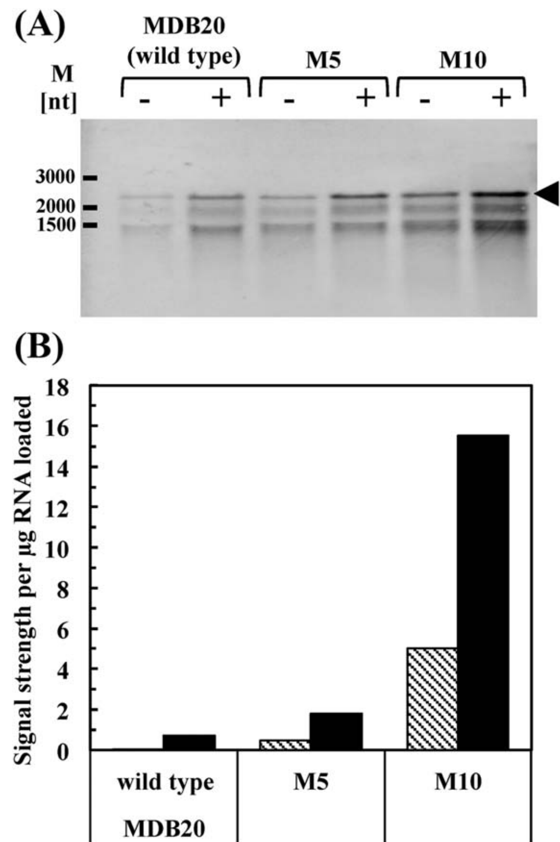


Fig. 6. Northern blot analysis of the *proHJ* mRNA produced under the transcriptional control of either the wild-type *proHJ* promoter or its M5 and M10 mutant derivatives. Total RNA was prepared from cells of the *B. subtilis* strains MDB20 [Δ(*proHJ*) *amyE*::P_{wt}-*proHJ*] and the promoter mutant strains MDB24 [Δ(*proHJ*) *amyE*::P_{M5}-*proHJ*] and MDB28 [Δ(*proHJ*) *amyE*::P_{M10}-*proHJ*]. Cells were grown in SMM without (hatched bars) or with 0.4 M NaCl (black bars) to early exponential growth phase (OD_{578} of about 1.0). The electrophoretically separated RNA was hybridized with a single-stranded anti-sense *proHJ* probe labelled with DIG-UTP.
A. To allow linear quantification of the detected signals, the total RNA amounts that were loaded onto the gel were adjusted as follows: MDB20 (15 µg), MDB24 (9 µg) and MDB28 (3 µg). The arrow indicates the *proHJ* transcript with a length of 2 100 nt; degradation products of the full-length transcript are visible.
B. The strength of the signals of the lull-length *proHJ* mRNA transcript visible in (A) were quantified with the Image Quant software suit (Molecular Dynamics) and are presented as signal strength per µg of total RNA loaded onto the agarose gel.

(Hoffmann and Bremer, 2016). Under these growth conditions, expression of the *proHJ*-reporter fusion was enhanced about 3.9-fold at high salinity, while the transcriptional activity of its M10 promoter mutant derivative was only stimulated about 1.3-fold. However, the basal transcriptional activity of the *proHJ* M10 reporter construct was about 22.8-fold enhanced over that of its wild-type counterpart and its salt-stressed induced levels of TreA reporter enzyme activity greatly exceeded that of the wild-type fusion (by about 7.4-fold) as well (Table 2).

Table 2. TreA reporter enzyme activity and salt induction ratios derived from the *proHJ*-promoter mutant M10 and the corresponding wild type strain.

	TreA activity [U (mg protein)$^{-1}$]		
proHJ-promotermutation	– NaCl	+1.2 M NaCl	Salt induction ratio
wild-type	23	90	3.9
M10	526	670	1.3

a. The TreA reporter enzyme activity of the *proHJ-treA* fusion strains JSB36 (wild type) and MDB17 (M10) grown in minimal medium without or with the addition of 1.2 M NaCl were determined. Shown are the averaged values of two biological replicates.

Reassuringly, through our targeted proteomics approach we found very similar values for the full set of proline biosynthetic proteins under both non-inducing and inducing growth conditions regardless of whether the *proHJ* operon was present at its natural locus in the *B. subtilis* chromosome (strain JH642; Fig. 2) or had been ectopically inserted into the *amyE* gene (strain MDB20; Fig. 7A). For the *proHJ* wild-type strain MDB20, we found a ninefold increase in the amounts of ProJ and a 12-fold increase in the amounts of ProH when non-inducing and osmotic stress conditions are compared (Fig. 7A). The corresponding values for the *proHJ* M10 promoter variant (strain MDB28) were only fourfold and twofold respectively (Fig. 7A). Despite these low induction factors, the cellular amounts of the ProH and ProJ proteins were present at substantially enhanced levels in comparison to the wild-type strain. The ProA protein whose structural gene (*proA*) is not affected by the *proHJ* M10 mutation remained at cellular levels similar to those found in the wild-type strain MDB20 (Fig. 7A). However, in contrast to the situation in the wild-type strain (Fig. 7A), in strain MDB28 (M10 mutation), the amounts of ProA no longer exceeded that of ProJ, suggesting that these ProA levels now become a bottleneck for osmostress-responsive proline biosynthesis in the mutant strain.

Physiological assessment of the proHJ *M10 promoter variant strain MDB28*

We wanted to know whether the elevated ProJ and ProH protein levels in the M10 mutant strain MDB28 would lead to increased proline pools and thereby improve growth under severe osmotic stress conditions. We therefore assessed its growth properties in SMM with 1.2 M NaCl and determined under these growth conditions also the proline pool of the cells. Growth of the wild-type strain MDB20 and that of the MDB28 M10 *proHJ* mutant derivative was identical and the proline pool was only moderately increased from 408 mM in MDB20 to 530 in MDB28 (Fig. 7B and C).

Proline synthesis in *B. subtilis* proceeds from glutamate (Belitsky *et al*., 2001; Commichau *et al*., 2008) and the strongly enhanced proline production successively drains the glutamate pool of the cells as the external osmolarity is step-wise increased on a sustained basis (Brill *et al*., 2011a). To test if the cellular glutamate pool restricts proline biosynthesis and growth, we added 10 mM glutamate to the osmotically stressed cells (SSM with 1.2 M NaCl) as this amino acid can be imported by *B. subtilis* and used to replenish the endogenously synthesized glutamate pool (Zaprasis *et al*., 2015). The addition of glutamate increased the size of the proline pool from 408 to 607 mM in the wild-type strain MDB20 and from 530 to 770 mM in the MDB28 (M10) mutant strain in SMM with 1.2 M NaCl (Fig. 7B). It thus is apparent from these data that under sever osmotic stress, the cellular glutamate pool becomes limiting for realizing the full proline-biosynthetic potential of *B. subtilis*. While the supplementation of the high salinity growth medium with 10 mM glutamate strongly improved growth of strains MDB20 and MDB28 (Fig. 7C), the significant difference in the proline pools of these strains (by 163 mM) was however insufficient to afford a physiologically relevant growth advantage to the M10 mutant strain MDB28 under osmotic stress conditions (Fig. 7B and C).

Two events could impair the cellular proline pools of *B. subtilis*: release and catabolism (Hoffmann *et al*., 2012; Moses *et al*., 2012). In addition to synthesis, maintenance of adequate cellular proline pools also involves the osmotically controlled proline importer OpuE (von Blohn *et al*., 1997), as *B. subtilis* cells excrete, perhaps as a measure to fine-tune turgor, part of the newly synthesized proline into the growth medium that is then subsequently recaptured via OpuE (Hoffmann *et al*., 2012). We found that there is very little proline present in the supernatants of MDB20 (*proHJ* wild-type) and MDB28 (*proHJ* M10 mutant) cultures that were osmotically challenged on a sustained basis (Fig. 7B). Furthermore, the deletion of the catabolic *putBCP* proline import and degradation operon (Moses *et al*., 2012) had no influence on the proline pools of strains MDB20 and MDM28 (Supporting Information Fig. S2). It is apparent from this analysis, that the cellular proline pools observed in the experiments documented in Fig. 7A are skewed neither by excessive excretion, nor by enhanced catabolism.

Discussion

For those microorganisms that use the *salt out* strategy (Galinski and Trüper, 1994; Kempf and Bremer, 1998; Roeßler and Müller, 2001; Sleator and Hill, 2002), the amassing of compatible solutes is a central aspect in their attempts to ensure proper hydration of the cytoplasm and magnitude of turgor to sustain growth under osmotically unfavourable environmental conditions (Bremer and

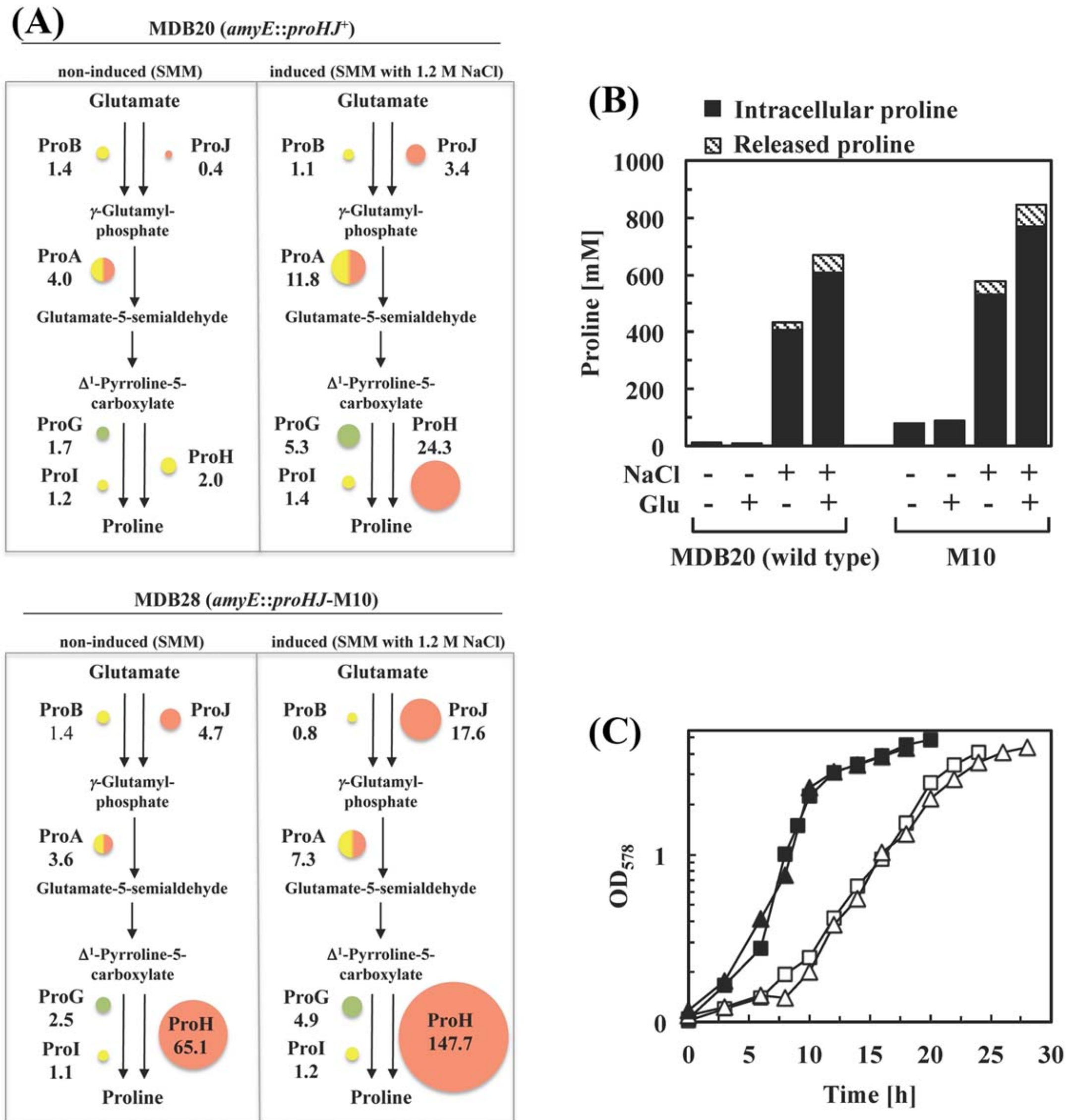


Fig. 7. Absolute quantification of the proline biosynthetic proteins in the *B. subtilis* strain MDB20 and its mutant (M10) derivative MDB28, intracellular proline pools and growth of these strains under osmotic stress conditions in the absence or presence of glutamate.

A. Cultures of strain MDB20 harboring the *proHJ* operon under control of the wild-type *proHJ* promoter and of strain MDB28 harbouring the *proHJ* operon under control of the mutated (M10) *proHJ* promoter were grown in SMM either in the absence or the presence of 1.2 M NaCl. Cells were harvested in mid-exponential growth phase (OD_{578} of about 1.5); proteins were extracted and were used for total protein quantification. LC-MS/MS analysis of the trypsin-digested samples, that had been spiked with QConCAT proteins representing peptides of all *B. subtilis* proteins involved in proline synthesis allowed quantification of all proline biosynthetic enzymes (amounts given in fmol per μg total protein). The reported data are the means of the quantification of proline biosynthetic enzymes from three independently grown *B. subtilis* cultures.

B. Strain MDB20 [Δ(*proHJ*) *amyE*::P_{wt}-*proHJ*] and the promoter-mutant strain MDB28 [Δ(*proHJ*) *amyE*::P_{M10}-*proHJ*] were grown in SMM with the indicated supplements (final NaCl concentration 1.2 M; final glutamate concentration 10 mM) to mid-exponential growth phase (OD_{578} of about 2). Cells were harvested by centrifugation and both the supernatant and the cell pellet were analysed for their proline content by a colorimetric assay (Bates *et al*., 1973). The intracellular proline concentration is shown as black bar. The amount of extracellular proline is given as proline concentration (the amount of the supernatant in relation to the cytoplasmic volume of the whole culture) (hatched bar). The sum of both bars is thought to give the total amount of proline synthesized by the culture. The data given are the means and standard deviations of four independently grown cultures.

C. The *B. subtilis* wild-type strain MDB20 (open and closed squares) and its M10 mutant derivative (open and closed triangles) were grown in SMM of increased salinity (1.2 M NaCl) either in the absence (open symbols) or in the presence (closed symbols) of 10 mM glutamate. The data of each shown growth curve are the means and standard deviations of three independently grown cultures.

Krämer, 2000; Wood, 2011). In the absence of the import of these types of osmostress protectants (Hoffmann and Bremer, 2016; 2017), *de novo* synthesis and high-level accumulation of the compatible solute proline provides a solid base for the osmostress defense of *B. subtilis* (Whatmore *et al*., 1990; Brill *et al*., 2011a). We have focused here on this cellular adjustment process through a combination of targeted proteomics, reporter gene studies and bioinformatics.

The anabolic (ProB-ProA-ProI) and osmostress-adaptive (ProJ-ProA-ProH) proline biosynthetic routes are interconnected in *B. subtilis* via the enzyme activity of the γ-glutamyl phosphate reductase (ProA) (Fig. 1A). Yet these two pathways for proline production are genetically and biochemically controlled through opposing regulatory mechanisms (Fig. 1B and C) that prevent overproduction of proline in osmotically non-stressed cells and allow high-level synthesis upon imposition of osmotic stress (Brill *et al*., 2011a,b). Our targeted and quantitative proteomic analysis now shows that the amounts of ProA under both non-induced and osmotically induced conditions exceed that of its potential interaction partners ProB and ProJ (Fig. 2) and will thus not limit osmostress-responsive production of the compatible solute proline in a wild-type strain.

Osmostress adaptive proline biosynthesis occurs widely among members of the *Bacilli* (Kuhlmann and Bremer, 2002; Bursy *et al*., 2007). Through our bioinformatics analysis of 149 *Bacilli* with available genome sequences that could be taxonomically compared through their 16S DNA sequence, we infer that 51% of these microorganisms will produce proline under osmotic stress conditions (Supporting Information Fig. S1). Twenty-eight of these strains possess the set of genes for the interconnected anabolic and osmostress responsive proline biosynthetic routes originally detected in *B. subtilis* (Brill *et al*., 2011a) (Fig. 1A), and 48 representatives have separate proline production routes for these tasks, as initially found in *B. licheniformis* (Schroeter *et al*., 2013) (Fig. 1A). In a previous study, Bursy *et al*. (2007) experimentally analysed a rather eclectically chosen group of 26 *Bacilli* and related species for their dominantly synthesized compatible solute and found three groups: (i) those that produce only glutamate, (ii) those that produce glutamate and proline and (iii) those that produce glutamate and ectoine (Kuhlmann and Bremer, 2002; Bursy *et al*., 2007). In our bioinformatics analysis of a substantially larger (149 *Bacilli)* and phylogenetically more consistently chosen group of species, we find the same classes with respect to their predicted compatible solute profile (Supporting Information Fig. S1). Species that produce proline dominate (48%), followed by exclusive glutamate producers (38%) and finally by ectoine producers (14%). From growth experiments reported by Bursy *et al.* (2007), it appears that the degree of osmostress tolerance of the studied *Bacilli* increases in the order of the production of glutamate < proline < ectoine (Bursy *et al*., 2007). This agrees rather well with the *in vitro* function-preserving and physico-chemical characteristics of these three compatible solutes (Street *et al*., 2006; Held *et al*., 2010; Cheng *et al*., 2016).

Guided by the compilation of 70 presumptive osmotically regulated promoter sequences driving the expression of osmostress responsive proline biosynthetic genes in *Bacilli* (Fig. 3A), we have asked which sequence features contribute to osmotic inducibility of transcription. We have used the *B. subtilis proHJ* promoter sequence as the model system for this analysis. Reporter strains carrying mutations that optimize the *proHJ* −10 region, in a promoter context that already possesses a perfect consensus −35 element, exhibit increased TreA reporter enzyme activity but they do not lose osmotic inducibility entirely (Figs 3B and 4B). This is even true for a *proHJ* promoter variant (M6) which possesses consensus-type −10, −16 and −35 regions when the spacer is kept at its natural length of 16 bp (Figs 3B and 4B). This promoter mutant directs strongly increased expression levels of the *proHJ-treA* reporter fusion under both non-induced and osmotically induced (with 0.4 M NaCl) conditions but still allows a proportional response to incremental increases in the external salinity (Figs 3B and 4B).

Remarkably, all of the compiled *proHJ*- and *proH-proJ-proAA*-type promoter sequences (Fig. 3A) possess a sub-optimal 16-bp spacing, and we found that this conserved feature is critical for the full dynamic transcriptional response of the *B. subtilis proHJ* promoter to incremental increases in the external salinity. An increase to the consensus 17-bp spacer length of SigA-type promoters not only strongly de-represses promoter activity (Fig. 3B) but also results in the loss of the exquisite sensitivity of the *proHJ* promoter to step-wise increases in the external salinity (Fig. 4). Instead, the M7 and M8 promoter variants that possess such 17-bp spacers allow proportional induction of transcription only to a rather restricted range of salinities (up to 0.3 M NaCl), where upon the promoter activity levels off (Fig. 4B). In striking contrast, lengthening of the spacer to sub-optimal 18-bp restores osmotic inducibility over the entire range of tested external salinities (Fig. 4B). Hence a sub-optimal positioning of the −10 and −35 contacting regions for RNA-polymerase binding is central to enable the *proHJ* promoter to finely tune its transcriptional activity in proportion to the degree of osmotic stress perceived by the *B. subtilis* cell.

Whatever the precise signal perception and transduction mechanism might be that is used by *B. subtilis* to communicate changes in the external osmolarity to SigA-type promoters (Spiegelhalter and Bremer, 1998; Fischer and Bremer, 2012; Hoffmann *et al*., 2013b), these processes must eventually target the *proHJ* regulatory region to achieve osmostress-relieving cellular proline pools (Brill

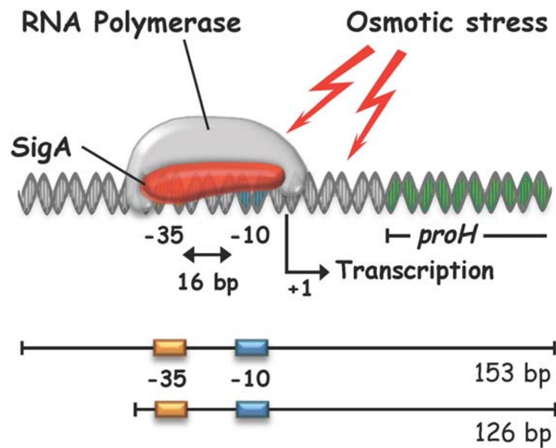


Fig. 8. Genetic organization of the *proHJ* regulatory region mediating osmotically induced transcription. Two DNA fragments encompassing the *proHJ* promoter region have been used for the construction of osmostress-responsive *proHJ-treA* operon fusions, and both respond dynamically to incremental sustained increases in the external salinity. The 153-bp DNA fragment has been used to introduce various mutations into the −10, −16 and spacer regions (this study), and the 126-bp DNA fragment has previously been constructed by deletion analysis of the 5′ region (Brill *et al.*, 2011a). These two *proHJ-treA* reporter fusions differ only in the length of the 5′ region; 32 bp are present in front of the −35 region of the 153-bp DNA fragment, and only six bp are present in front of the −35 region of the 126 bp DNA fragment. Both reporter constructs harbour the same fusion junction to the promoter-less *treA* gene; it lies in codon 17 of the *proH* gene.

et al., 2011a; Hoffmann *et al.*, 2013b). All information required in *cis* for osmotic regulation of *proHJ* promoter activity is present in a 126-bp DNA fragment (Fig. 8); no *trans*-acting regulatory protein or regulatory RNA is currently known that would be required for its osmotic control. Our mutational study of the *proHJ* promoter has shown that even a promoter variant (M10) with a consensus-like SigA-type promoter still retains osmotic inducibility to a restricted extent (Fig. 4B and Table 2). As already observed for the osmotically inducible promoters for the *B. subtilis opuA, opuE* and *yqiHIK* genes (Spiegelhalter and Bremer, 1998; Fischer and Bremer, 2012; Hoffmann *et al.*, 2013b), the core function of deviations from the SigA consensus is to keep *proHJ* expression low under non-stressed conditions (Figs 3B and 4B). This permits facile osmostress induction of transcription but the particular sequences of the −35 and −10 elements by themselves are apparently not responsible for osmotic control (Fig. 3A). The DNA sequences of the spacer region between the *proHJ* −35 and −10 elements are not particular well conserved (Fig. 3A), an observation suggesting that the particular DNA sequence in this region is not key for osmotic control. DNA sequences upstream of the *proHJ* −35 element are of no significant importance either for osmoregulation, since a *proHJ-treA* reporter fusion carrying just six bp 5′ to the −35 region (Fig. 8) retains finely tuned osmotic inducibility (Brill *et al.*, 2011a). We noted however that the region around the transcriptional start site is evolutionarily conserved to a certain degree (Fig. 3A). Notably, the region downstream of the −10 element has not yet been probed for a possible impact on osmoregulation of the *proHJ* promoter.

In addition to RNA-based or transcription-factor-based gene regulation in bacteria, environmentally triggered changes in DNA-topology can play a decisive role in setting the transcriptional activity of promoters (Wang and Syvanen, 1992; Dorman, 1996; Travers and Muskhelishvili, 2005; Dorman and Corcoran, 2009; Dorman and Dorman, 2016). Such changes in DNA topology trigger genome-wide changes in transcriptional profile (Cheung *et al.*, 2003; Lal *et al.*, 2016) and both increases and decreases in supercoiling seem to act as a kind of second messenger transmitting information about fluctuations in the environment to regulatory networks in the cell (Hatfield and Benham, 2002; Peter *et al.*, 2004; Dorman and Dorman, 2016; Lal *et al.*, 2016). Rises in the environmental osmolarity are one of the well known cues that lead to increases in negative superhelicity, both in Gram-negative and Gram-positive bacteria (Higgins *et al.*, 1988; Krispin and Allmansberger, 1995; Alice and Sanchez-Rivas, 1997). Indeed, promoters of a substantial number of osmotically regulated genes are affected by changes in DNA topology (Higgins *et al.*, 1988; Graeme-Cook *et al.*, 1989; Wang and Syvanen, 1992; Jordi *et al.*, 1995; Cheung *et al.*, 2003; Zhang and Baseman, 2011; Keane *et al.*, 2016).

Particularly relevant to the interpretation of the data we present here on the osmostress inducible *proHJ* promoter from *B. subtilis* are reports on the osmotically controlled promoters of the BetL glycine betaine importer from solute *Listeria monocytogenes* and the ProU compatible uptake systems from *Escherichia coli* and *Salmonella typhimurium* (Lucht and Bremer, 1991; Mellies *et al.*, 1994; Jordi *et al.*, 1995; Zhang *et al.*, 1996; Keane *et al.*, 2016). A sub-optimal spacer length of 16- or 18-bp will affect promoter strength due to less efficient RNA-polymerase binding (Auble and Dehaseth, 1988; Browning and Busby, 2016). Negative supercoiling induced changes in DNA-twist have been suggested to function as a transcriptional sensor for environmental changes by promoting a better alignment of the −10 and −35 elements in promoters with suboptimal spacers (Wang and Syvanen, 1992; Dorman, 1996). Such a DNA-twist-dependent mechanism of osmotic control has been proposed to operate at the *betL* promoter, a promoter that possesses a suboptimal spacing of 18-bp (Hoffmann *et al.*, 2013a; Keane *et al.*, 2016). However, a DNA-twist-based mechanism for osmotic control of *proHJ* expression is unlikely the full explanation for our data since *proHJ* promoters with spacer length of 16- or 18-bp responded equally well to the same finely tuned increases in environmental salinity (Fig. 4B). It thus seems improbable that

common osmotically induced supercoiling changes in *B. subtilis* (Krispin and Allmansberger, 1995; Alice and Sanchez-Rivas, 1997) can twist the DNA configuration of these two *proHJ* promoter variants in the same fashion to optimize the alignment of the −10 and −35 regions for productive interactions with RNA-polymerase (Aoyama and Takanami, 1988; Ahmed *et al.*, 2016; Browning and Busby, 2016) and thereby promote a well-graded answer in transcriptional activity in response to increases in the external osmolarity (Fig. 4).

Our experimental data on the salient features of the *B. subtilis proHJ* promoter are most comparable to those reported for the osmotically controlled *proU* promoter. As observed for *proHJ* (Figs 3B and 4B), variants of the *proU* promoter with increased sequence identity to the consensus, feature increased promoter activity without abolishing osmotic control (Lucht and Bremer, 1991; Mellies *et al.*, 1994; Jordi *et al.*, 1995; Zhang *et al.*, 1996). Like *proHJ*, the *proU* promoter possesses a 16-bp spacer. Although tested only for one particular osmotic stress condition, a 17-bp spacer variant of *proU* became in essence constitutive, while the 18-bp spacer mutant showed a degree of osmoregulation comparable to that of the wild-type promoter (Jordi *et al.*, 1995). These and other *in vitro* data led Jordi *et al.* (1995) to suggest that the osmotically induced increase in negative supercoiling increase local DNA flexibility [or writhing (Ahmed *et al.*, 2016)] at the *proU* promoter and that this property is then transformed into osmotic control of transcription (Jordi *et al.*, 1995). According to this model, osmotic regulation is an intrinsic property of the non-standard structure of the *proU* promoter (Jordi *et al.*, 1995; Dorman, 1996). Changes in the DNA-binding behaviour and activity of RNA-polymerase are likely to contribute as well when the intracellular ion and solute pools change upon osmotic stress (Xu and Johnson, 1997; Gralla and Vargas, 2006; Romeo *et al.*, 2007; Gralla and Huo, 2008; Kontur *et al.*, 2010; Cagliero and Jin, 2012; Shikuma *et al.*, 2013). In the absence of knowledge on any *proHJ*-specific regulatory protein, ionic strength- and osmolyte-dependent interactions of RNA-polymerase with promoters combined with high-osmolarity triggered changes in global and local DNA topology is an attractive working hypothesis to possibly explain the osmotic control of *proHJ* transcription (Fig. 8). However, it remains to be seen whether such a model can adequately account for the finely graded increase in *proHJ* transcriptional activity in response to incremental increases in environmental osmolarity (Brill *et al.*, 2011a; Hoffmann *et al.*, 2013b) (Fig. 4A), a process that eventually allows *B. subtilis* to withstand osmotic stress over a broad range of environmental osmolarities through the synthesis of the compatible solute proline (Boch *et al.*, 1994; Brill *et al.*, 2011a).

Experimental procedures

Chemicals

All antibiotics used in this study, the chromogenic substrate (para-nitrophenyl-α-glycopyranoside; pNPG) for TreA enzyme activity assays, and chemicals for buffers used for protein sample preparation were obtained either from Sigma-Aldrich (Steinheim, Germany) or Roth (Karlsruhe, Germany). Chemicals for the preparation of buffers for LC-MS/MS measurements were obtained from J.T. Baker (Avantor, USA).

Media and growth conditions

Selective Luria-Bertani (LB) agar plates used for recombinant DNA work and bacterial strain construction contained the antibiotics ampicillin for *E. coli* cells (final concentration 100 μg ml^{-1}), or chloramphenicol, kanamycin, tetracycline or spectinomycin for *B. subtilis* strains (with final concentrations of 5 μg ml^{-1}, 10 μg ml^{-1}, 15 μg ml^{-1}, 100 μg ml^{-1} respectively). To verify chromosomal integration of DNA constructs at the *amyE* locus of *B. subtilis*, cells were tested for production of the extracellular α-amylase AmyE by flooding colonies grown on LB plates containing 1% starch with Gram's iodine stain [0.5% (w/v) iodine, 1% potassium iodide] for 1 min and scoring for zones of clearing around the colonies after decanting the strain. For growth experiments involving *B. subtilis* strain JH642 (*pheA1 trpC2*) (Smith *et al.*, 2014) and its derivatives, we used Spizizen's minimal medium (SMM) with 0.5% (w/v) glucose as the carbon source, L-tryptophan (20 mg l^{-1}), L-phenylalanine (18 mg l^{-1}) and a solution of trace elements (Harwood and Archibald, 1990). The osmolarity of the individual media was adjusted by the addition of NaCl from 5 M NaCl stock solutions. The osmolality of growth media was measured with an osmometer (model 5500; Wescor, Logan, UT, USA) and is expressed as mOsmol (kg $H_2O)^{-1}$. Bacterial growth was monitored photometrically as the optical density of cultures at 578 nm (OD_{578}). Liquid cultures of *B. subtilis* were grown in 100-ml Erlenmeyer flasks containing 20-ml of medium at 37°C in a water bath with vigorous shaking (220 rpm) for strict aerobic cultivation. Pre-cultures were propagated in SMM without additional NaCl to mid-exponential growth phase (OD_{578} 1.5) and were then used to inoculate main cultures with the indicated NaCl concentrations to an OD_{578} of 0.1.

Construction of plasmids

Reporter gene fusion constructs with mutated *proHJ* promoter variants were constructed using by *proHJ-treA* operon fusion plasmid pJS35 as the DNA template; it contains a 153-bp DNA fragment carrying the *proHJ* wild-type promoter transcriptionally fused to a promoterless *treA* reporter gene (Supporting Information Table S1) (Brill *et al.*, 2011a). Site-specific mutations were introduced into the *proHJ* promoter carried by pJS35 using the Quick change site-directed mutagenesis Kit (Agilent Technologies, Walsbronn, Germany) with primer pairs carrying the individual mutation listed in Supporting Information Table S1. To construct a plasmid carrying the entire *proHJ* operon (along with its authentic promoter region), we amplified the *proHJ* locus from chromosomal DNA of the

B. subtilis strain JH642 using the primers PvuII-proHJ fwd (5′-ggaattccagctggaaacaatacaaatcaataatggcc-3′) and PvuII-proHJ rev (5′-ggaattccagctggcttgcgccttccgcg-3′), yielding a 2225-bp DNA fragment. The *Pvu*II-digested DNA-fragment was ligated into the vector pX (Kim *et al*., 1996) linearized with *Pvu*II as well; this yielded plasmid pMD15. Plasmid pMD15 carries the *proHJ* operon under control of its natural promoter arranged anti-linear to the xylose-inducible promoter present on the pX vector (Kim *et al*., 1996). pX-derived plasmids can be stably integrated into the *B. subtilis* chromosome at *amyE* in single-copy via double homologous recombination events between identical 5′- and 3′ segments of the *amyE* gene present both on the plasmid and the chromosome (Kim *et al*., 1996). The plasmids pMD16, pMD17, pMD18, pMD19 and pMD20 are derivatives of pMD15 and were constructed via site-directed mutagenesis of the *proHJ* promoter using a set of custom-synthesized mutagenesis primer pairs (Supporting Information Table S1). The DNA sequences of all *proHJ-treA* and *proHJ* promoter mutants were verified by DNA sequence analysis and in the case of the wild-type and mutant *proHJ* operons, the entire gene cluster, was sequenced to ensure the presence of the desired mutations and the absence of unwanted alterations.

Bacterial strains and construction of B. subtilis *mutants*

We used the *Escherichia coli* strain DH5α (Invitrogen, Carlsbad, CA, USA) for routine propagation of bacterial strains containing plasmids. All *B. subtilis* strains used in this study are derivatives of the domesticated wild type strain JH642 [(*trpC*2 *pheA*1) BGSC 1A96] (Smith *et al*., 2014). The bacterial strains used and their corresponding genotypes are listed in Supporting Information Table S2. *proHJ-treA* reporter fusion strains were constructed by transforming the *B. subtilis* strain FSB1 [JH642 (*treA*::*neo*)1] (Supporting Information Table S2) with linearized versions of the *proHJ-treA* reporter fusion plasmids pMD1, pMD2, pMD8, pMD5, pMD4, pMD7, pMD6, pMD9, pMD10 and pMD12 (Supporting Information Table S1) and subsequent selection for chloramphenicol-resistant transformants. This selected for the integration for the *proHJ-treA* reporter gene fusion genetically linked to a chloramphenicol resistance gene into the *amyE* locus via homologous recombination events between plasmid-encoded and chromosomal *amyE* DNA sequences. Correct integration of the reporter fusion constructs into the chromosomal *amyE* locus was verified by testing α-amylase activity of the cells. This yielded strains MDB8, MDB9, MDB13, MDB6, MDB11, MDB12, MDB7, MDB14, MDB15 and MDB17 (Supporting Information Table S2). Linearized plasmids pMD16, pMD17, pMD18, pMD19 and pMD20 (Supporting Information Table S1) carrying either the wild-type *proHJ* operon under its osmotically regulated promoter, or promoter variants derived from it, were transformed into competent cells of the *B. subtilis proHJ* mutant strain JSB8 [(Δ*proHJ*::*tet*)1] (Supporting Information Table S2). Integration of the various *proHJ* genes into the *amyE* locus was selected on LB agar plates containing chloramphenicol and then scoring their AmyE phenotype on starch plates. This yielded strains MDB20, MDB24, MDB25, MDB26, MDB27 and MDB28 respectively (Supporting Information Table S2). To analyse the influence of the L-proline degradation and transport genes *putBCP* (Moses *et al*., 2012) on L-proline accumulation, we constructed the strains TMB411 and TMB412 by transforming strains MDB20 and MDB28 with chromosomal DNA of strain SMB44 [Δ(*putBCP*::*spc*)1] (Supporting Information Table S2) and subsequent selection of spectinomycin-resistant transformants.

TreA reporter enzyme activity assays

The *proHJ-treA* operon fusions used in this study rely on the activity of the salt-tolerant TreA [phospho-α-(1,1)-glucosidase] enzyme, whose activity can be quantitated using the chromogenic substrate α-PNPG (Schöck *et al*., 1996). Transcription of the promoterless *treA* reporter gene in these fusion constructs relies on the fused *proHJ* promoter; its translation is dependent on its own ribosome-binding site. These *proHJ-treA* constructs are present in strains in which the authentic *treA* gene is genetically inactivated (Supporting Information Table S2); hence the measured TreA enzyme activity depends exclusively on the transcription of the *proHJ-treA* reporter gene. Growth of the cells for TreA enzyme activity and the details of the assay itself have been described before (Schöck *et al*., 1996; Brill *et al*., 2011a,b; Hoffmann *et al*., 2013b).

Northern blot analysis

To detect and quantitate the *proHJ* mRNA in strains MDB20 and its M5 and M10 mutant derivatives (Supporting Information Table S2), we grew the cells either in SMM or in SMM with additional 0.4 M NaCl to an OD_{587} of about 0.8–1.0. Total RNA from these cells was extracted with phenol as described (Steil *et al*., 2003), electrophoretically separated on a 1.4% agarose gel, transferred to a NY13N nylon membrane (Schleicher & Schuell) and reacted with a DIG-labelled single-stranded anti-sense RNA probe. Signal detection was carried out as described by the manufacturer (Roche Diagnostics, Germany) and quantified on a Storm 860 Imager (Amersham Biosciences) using the Image Quant software from Molecular Dynamics. The DNA fragment used to prepare the *proHJ* probe was generated by a set of DNA primers (proHJ-M-forward: 5′-GAGCGGCGGCGGAGAAGCG-3′; proHJ-M-reverse: 5′-TAATACGACTCACTATAGGGAGGGCTGCGAGCGTATCATTGTCG-3′ – the T7 promoter sequence is underlined) in which one of the primers carried a T7 RNA-polymerase-specific promoter. The single-stranded *proHJ* anti-sense RNA probe (580 nucleotides) was then prepared by *in vitro* transcription using T7 RNA polymerase with the incorporation of DIG-UTP nucleotides (Brill *et al*., 2011a).

Measurement of intracellular and extracellular L-proline pools

To measure the intracellular and extracellular proline content of *B. subtilis* cells, 20-ml cultures were grown either in SMM, or in SMM with either 0.4 M or 1.2 M NaCl until they reached an OD_{578} of about 1.8. In some experiments we added 10 mM glutamate, the precursor of proline, to these cultures. Harvesting and processing the cells for colorimetric proline assays (Bates *et al*., 1973) was done as described (Hoffmann *et al*., 2012; 2013b). Intracellular proline concentrations were calculated by using a cytoplasmic volume of one OD_{578} unit of cell

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culture. Extracellular proline amounts were normalized to an OD_{578} of one of the cell culture (Holtmann *et al*., 2003; Hoffmann *et al*., 2012).

Absolute protein quantification using quantification concatemers

Quantification concatemers (QconCat) (Beynon *et al*., 2005) are artificial proteins labelled with stable heavy isotopes that are assembled from unique proteotypic peptides (Qpeptides) that are chosen from multiple proteins of interest. The QconCat-proteins used in this study covered enzymes of the central carbon metabolism including the glutamate and proline biosynthetic pathways of *B. subtilis*. These QconCat standards were designed adhering to commonly accepted guidelines (Pratt *et al*., 2006). Peptides with the potential post-translational modifications were excluded from the design. Synthesis of the synthetic gene and labelling (^{13}C and ^{15}N) of the QconCat-protein with heavy arginine and lysine and the subsequent purification were done by PolyQuant GmbH (Regensburg, Germany). For each protein, three Qpeptides were designed, which at least two peptides were used for quantification (Supporting Information Table S3).

Protein extracts from frozen pellets were prepared as described previously (Starcher, 2001; Maass *et al*., 2011). Known amounts of QconCAT-protein were spiked into the bacterial protein lysate prior to overnight digestion with trypsin in an enzyme to substrate ratio of 1:25 at 37°C. The reaction was stopped by 1% acetic acid (v/v). The resulting peptide mixtures were resuspended in 100 μl of buffer A (0.1% acetic acid, 2% acetonitrile) and purified using C18-ZipTip columns (Millipore, Bedford). Four hundred nanograms of the purified peptide mixture was used for subsequent MS analysis. Peptide separation was carried out using an Acelaim PepMap 100 column (C18, 3 mm, 100 A°, 15 cm bed length, Dionex) and applying a binary gradient from 0% to 40% buffer B (100% acetonitrile [v/v], 0.1% acetic acid [v/v]) in 70 min and to 100% B in additional 15 min at a flow rate of 300 nl min^{-1}. LC-MS experiments were performed on a triple quadrupole mass spectrometer (TSQ Vantage, Thermo Scientific) operated in nano-electrospray mode. For ionization 1800 V of spray voltage and 250°C capillary temperature were used. The resolution for both quadrupoles, i.e., for Q1 and Q3 was set to 0.7 Da (FWHM). The collision gas pressure of Q2 was set at 1.5 mTorr. Three transitions (product ions) per peptide (precursor) with highly ranked intensities were chosen for targeted analysis. Collision energy was optimized for each of the product ions belonging to the corresponding peptide using the Skyline software suit (MacCoss Lab, University of Washington, USA) (MacLean *et al*., 2010a,b). By combining the optimized collision energies and the recorded retention time for each target peptide a scheduled method was developed to measure all the peptides of interest in a single MS/MS analysis. TSQ Vantage was operated in SRM (single reaction monitoring) mode and data acquisition was done in scheduled SRM manner. Two LC-MS/MS measurements were performed for each sample and the raw files were analysed with the software Skyline 1.0. Ratios of peak areas for endogenous light peptides to heavy Qpeptides were exported from Skyline. Based on the known amount of the spiked Qpeptides the endogenous light peptide concentrations were calculated. Peptides that exhibit missed cleavage and bad chromatographic properties were excluded from quantification of the corresponding protein.

Bioinformatic analysis

From the microbial database of IMG/M (https://img.jgi.doe.gov/m/main.cgi), we identified 152 finished and unfinished genome sequences from the genera *Bacillus* and *Halobacillus* whose 16S DNA information was available through the 'compare genomes distance tree' tool. A BLAST analysis (Altschul *et al*., 1990) of these genome sequences was then carried out using protein sequences of enzymes involved in L-proline (Belitsky *et al*., 2001; Brill *et al*., 2011a,b) and ectoine (Widderich *et al*., 2014) biosynthesis by using the 'find genes by BLAST' tool provided by the IMG/M web-site (https://img.jgi.doe.gov/m/main.cgi). The 'gene neighbourhood' function was subsequently used to identify putative operons involved either in proline or ectoine biosynthesis. Alignments of protein and DNA sequences were performed with the Clustal Omega tool provided at the EMBL-EBI homepage (http://www.ebi.ac.uk/Tools/msa/clustalo/). To identify putative *proHJ* promoters we downloaded and aligned the −100 to −1 DNA sequences of the *proHJ* genes originally found by BLAST analysis. A phylogenetic tree based on the 16S DNA information was built using the 'compare genomes distance tree' tool at IMG/M.

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Author contributions

E.B. conceived and directed this study. T.H., M.B. and P.K.S. performed experiments. L.S., U.M. and U.V. supervised the quantitative proteome analysis and provided help with the analysis and interpretation of the data. E.B. and T.H. wrote the manuscript with input from U.M. and U.V.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

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 neighbourhoods of the resulting hits were analysed 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-proJproAA*-(■), the anabolic *proBAC-* (■) and *proBA*operons (■). Species harbouring 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.

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

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