Stress-induced activation of the proline biosynthetic pathway in *Bacillus subtilis*: a population-wide and single-cell study of the osmotically controlled proHJ promoter

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Summary

*Bacillus subtilis*, in its natural habitat, is regularly exposed to rapid changes in the osmolarity of its surrounding. As its primary survival strategy, it accumulates large amounts of the compatible solute proline by activating the de novo proline biosynthesis pathway and exploiting the glutamate pools. This osmotically-induced biosynthesis requires activation of a SigA-type promoter that drives the expression of the *proHJ* operon. Population-wide studies have shown that the activity of the *proHJ* promoter correlates with the increased osmotic pressure of the environment. Therefore, the activation of the *proHJ* transcription should be an adequate measure of the adaptation to osmotic stress through proline synthesis in the absence of other osmoprotectants. In this study, we investigate the kinetics of the *proHJ* promoter activation and the early adaptation to mild osmotic upshift at the single-cell level. Under these conditions, we observed a switching point and heterogeneous proline biosynthesis gene expression, where the subpopulation of cells showing active *proHJ* transcription is able to continuously divide, and those unresponsive to osmotic stress remain dormant. Additionally, we demonstrate that bactericidal antibiotics significantly upregulate *proHJ* transcription in the absence of externally imposed osmotic pressure, suggesting that the osmotically-controlled proline biosynthesis pathway is also involved in the antibiotic-mediated stress response.

Introduction

Microorganisms in their natural habitat are constantly challenged with rapid changes of their surroundings. For instance, soil-inhabiting bacteria frequently encounter interchanging periods of flooding and drying of the soil’s topmost layers, causing osmotic stress (Hoffmann and Bremer, 2016). When the osmolarity of the environment fluctuates, the integrity of the semi-permeable cell membrane and sufficient hydration of the cytoplasm are challenged with passive water fluxes in and out of the cell (Bremer and Krämer, 2019). Therefore, bacteria employ various adaptive mechanisms to maintain cell turgor and minimize cellular damage. As an initial response to high osmolarity conditions, microbes must counteract the outflow of intracellular water by amassing large quantities of potassium and accumulating highly soluble organic osmolytes, known as compatible solutes (Csonka, 1989; Whatmore *et al.*, 1990; Wood *et al.*, 2001; Godard *et al.*, 2020). Importantly, unlike most ions, compatible solutes do not interfere with cellular functions because of their physicochemical properties. These physiologically compliant organic osmolytes can accumulate to high levels, maintain the cell’s turgor and avoid over-crowding of the cytoplasm even in high osmolarity environments (Csonka, 1989; Kempf and Bremer, 1998; van den Berg *et al.*, 2017). In *Bacillus subtilis*, a Gram-positive bacterium typically found in the upper layers of the soil (Earl *et al.*, 2008), the initial uptake of potassium ions is...
followed by accumulation and/or synthesis of proline (from glutamate) and glycine betaine (from the acquired choline pools) (Kempf and Bremer, 1998; Brill et al., 2011a; Hoffmann et al., 2012; Zaprasis et al., 2015). However, in contrast to all other osmoprotectants exploited by B. subtilis, proline is the only one that can be synthesized de novo (Whatmore et al., 1990; Brill et al., 2011a; Hoffmann et al., 2013). Accumulation of large amounts of proline requires the cell to re-route its metabolism to provide precursors for its synthesis (Kohlstedt et al., 2014; Godard et al., 2020). Members of Bacillus sp. convert the precursor glutamate to proline in three enzymatic steps carried out sequentially by \( \gamma \)-glutamyl kinase (ProB/ProJ), \( \gamma \)-glutamyl phosphate reductase (ProA/ProAA) and \( \Delta^1 \)-pyrroline-5-carboxylate reductase (ProL/ProH/ProG/ComER) (Fig. 1B) (Belitsky et al., 2001; Schroeter et al., 2013; Forlani et al., 2017; Godard et al., 2020). Depending on the cellular demand for proline, B. subtilis utilizes two distinct biosynthetic pathways interlinked via the unique ProA to synthesize appropriate amounts of proline (Belitsky et al., 2001). Under non-stress conditions, the cell profits from the anabolic route and activates ProB-ProA-Prol enzymes to support minimal proline levels sustaining protein synthesis (10 mM–20 mM) (Whatmore et al., 1990; Hoffmann et al., 2013; Zaprasis et al., 2015). However, when the cell’s demand for proline increases due to osmotic stress, the cell employs the osmo-adaptive pathway and the ProJ-ProA-ProH enzymes (Belitsky et al., 2001; Brill et al., 2011a). For instance, when B. subtilis suffers from severe osmotic upshift, the upregulation of the osmo-adaptive proline biosynthesis pathway elevates the intracellular proline pools from 20 mM to 500 mM (Whatmore et al., 1990; Brill et al., 2011a; Hoffmann et al., 2013). In B. subtilis, the genes encoding for the osmo-adaptive ProH and Proj enzymes are genetically organized in the proHJ operon. The transcription of the proHJ operon is driven by an osmotically controlled promoter, the sequence of which is recognized by the house-keeping sigma factor of B. subtilis SigA (Helmann, 1995; Brill et al., 2011b). Notably, the activity of the proHJ promoter and the ensuing proline pools have been shown to be linearly dependent on the degree of the osmotic stress imposed onto the cell (Hoffmann et al., 2013; Schroeter et al., 2013; Godard et al., 2020). Hence, monitoring proHJ promoter activity and measuring intracellular proline pools can indicate the physiological state of osmotically stressed cells (Hoffmann et al., 2017). However, previous studies on the adaptation to osmotic stress and dynamics of the proHJ promoter have been based entirely on population-wide analyses (Brill et al., 2011a; Hoffmann et al., 2013). Thus, the activation of proHJ transcription has always been assessed by averaging the activities of all B. subtilis cells in a given culture, thereby missing potential phenotypic variation (heterogeneity) in proHJ promoter activity.

Phenotypic heterogeneity is inherent to many regulatory and physiological processes and may arise from a responsive event to specific environmental conditions or from noisy gene expression (Elowitz et al., 2002; Kusell, 2005). The ability to switch phenotypes allows microorganisms to survive in challenging surroundings, and most importantly, persist during antibiotic treatments (Balaban et al., 2004; Wood et al., 2013). Therefore, it is of high relevance to look more in-depth into B. subtilis’ response to osmotic stress on a single-cell level.

In this study, we elucidate the early response of B. subtilis to mild osmotic upshift at the single-cell level by focusing on the transcriptional up-regulation of proHJ proline synthesis genes. Using time-lapse microscopy, we show heterogeneous proHJ transcriptional activation under 0.6 M NaCl treatment and estimate the time needed for cells to adjust to the osmotic upshift and resume cell division. Data presented here show that only cells exceeding a threshold level of proHJ expression can efficiently grow and divide under osmotically non-favourable conditions, whereas those with low expression of proHJ do not divide and likely remain dormant. Additionally, we demonstrated that bactericidal antibiotics with different molecular targets as well as direct exposure to hydrogen peroxide significantly upregulate the proHJ transcription, in the absence of osmostress, suggesting that the osmotically controlled proline biosynthesis pathway is likely involved in antibiotic ROS-mediated stress response.

**Results**

**Early adaptation to the elevated osmolarity shows increased PproHJ activity in B. subtilis**

During osmotic upshift, B. subtilis induces proline biosynthesis to utilize it as a compatible solute in the absence of exogenous osmoprotectants (Brill et al., 2011a; Hoffmann et al., 2012). To study the adaptation to the hyperosmotic environment, we followed the expression of the proHJ operon that encodes the osmostress-adaptive proline biosynthesis enzymes ProH and Proj. For this purpose, we designed a B. subtilis 168 strain with the sfGFP (Sp) gene sequence transcriptionally fused to the proHJ promoter with its own RBS and the twenty-nine codons of proH. With the use of this reporter strain, we followed the GFP fluorescent signal, and hence the activity of proHJ transcription (Fig. 1A). To ensure that de novo synthesized proline was the only available osmoprotectant for B. subtilis, all experiments were carried out in SMM minimal media, which possess an osmolarity of 356 mosmol kg\(^{-1}\) (Hoffmann et al., 2013).
supplemented with 0.5% glucose as a primary carbon source.

First, the early adaptation to the hyperosmotic environment was analysed on a population-wide scale, and the ProHJ activity was measured in cells exposed to different concentrations of sodium chloride (0 M, 0.5 M, 0.6 M, 0.7 M and 1.2 M NaCl) by flow cytometry. The early response to osmotic upshift was analysed for 10,000 cells in each salt concentration at 30 min intervals for 120 min (Fig. 2). Interestingly, we found that the proHJ expression was moderately induced within the first 60 min after cells were exposed to hyperosmotic conditions in all samples. After 90 to 120 min of treatment with 0.5 M, 0.6 M, 0.7 M and 1.2 M NaCl, B. subtilis cells displayed the highest increase in the average GFP fluorescence in all salt-treated samples compared to the 0 M NaCl control, indicating the approximate time required for B. subtilis to highly induce the proHJ expression and thereby attain osmoprotective proline pools (Fig. 2A) (Whatmore et al., 1990; Hoffmann et al., 2012). These data agree with previous proteomics and transcriptomics studies in B. subtilis under high osmotic stress, revealing the highest mRNA levels of proH and proJ after 60 min of exposure to a high salt concentration (Höper et al., 2006; Hahne et al., 2010).

Previous studies have shown that osmotically challenged B. subtilis increases its intracellular proline concentration to a level that depends on the applied osmotic pressure (Brill et al., 2011a; Hoffmann et al., 2013; Schroeter et al., 2013). Interestingly, the highest variation in the intracellular proline levels was found for cells exposed to mild osmotic stress caused by 0.6 M NaCl (Brill et al., 2011a). The flow cytometry data showed that exposure to different salt concentrations resulted in a population-wide shift in the fluorescence intensity. Although, we did not observe a bimodal distribution of fluorescent signal in any of the cultures pointing on heterogeneous activation of ProHJ (Fig. S1), we detected a small fraction of the osmostressed population with a higher fluorescence signal (Fig. 2B). In the case of the 0.6 M NaCl treatment, the fraction of highly fluorescent cells reached 6.8% of the entire population after 120 min of salt exposure, which likely resulted from the stochastic activation of the promoter (Fig. 2B). These results show that under mild osmostress conditions, a part of the population highly induces proHJ expression in order to synthesize large amounts of the compatible solute proline, whereas the majority of the population maintains moderate levels of proHJ expression (Whatmore et al., 1990; Brill et al., 2011a). The variation in the intracellular proline concentration in the 0.6 M NaCl-treated cells (Brill et al., 2011a; Hoffmann et al., 2017) and the presence of a fraction of highly fluorescent cell variants shown by the flow cytometry data suggests that under
these conditions, the population of genetically identical *B. subtilis* cells diversifies in terms of the activation of the proline biosynthesis pathway.

Upregulation of proHJ transcription on a single-cell level during osmotic upshift – heterogeneous activation of the proline biosynthesis pathway

To further investigate *B. subtilis*’ adaptation to 0.6 M NaCl stress, we performed a time-lapse microscopy experiment on SMM agarose containing 0.6 M NaCl and monitored PproHJ activation at a single-cell level. Interestingly, time-lapse microscopy revealed that under sustained osmotic upshift, *B. subtilis* responds heterogeneously and only part of the isogenic population highly activates proHJ transcription (Fig. 3). After the first 90 min of exposure to the osmotic upshift, cells did not grow nor divide, and initially, only a fraction of the population displayed a low GFP signal, indicating moderate PproHJ activity and upregulation of the proline biosynthesis pathway (Fig. 3). Conversely, GFP-negative cells did not show the PproHJ activation and could not divide. This data suggests that GFP-positive cells were more adapted to elevated osmotic pressure by effectively switching on the proline biosynthesis pathway and reaching for the accumulated proline pools to balance out the adverse effects of the osmotic upshift. Based on these results, we propose that the cell needs to exceed a particular intracellular proline concentration, which allows it to overcome the detrimental effects of water efflux and thereby permits the resumption of growth and division.

Changes in regulation of the osmostress-adaptive proline biosynthesis pathway upon antibiotic stress

We reasoned that a short pretreatment with an osmotic upshift (0.6 M NaCl) can benefit the *B. subtilis* population and helps to increase its survival upon aminoglycoside exposure. Thus, we wondered whether *B. subtilis* activates the osmostress-adaptive proline biosynthesis pathway in response to immediate antibiotic treatment on a population-wide level. To address this question, we followed the activation of the proHJ promoter under antibiotic exposure in cultures with and without the 90 min

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**Fig. 2.** Activation of the proHJ promoter upon NaCl exposure. (A) The graph shows the average fluorescence intensity (per cell) of *B. subtilis* 168 PproHJ-sGFP (Sp) during exposure to different NaCl concentrations. The zero-time point represents the start of NaCl exposure. The dashed line indicates the background GFP signal. The table below (B) shows the population-wide fluorescence distribution of *B. subtilis* PproHJ-sGFP (Sp) after 30, 60, 90 and 120 min of exposure to 0.6 M NaCl. The total sample size was 10,000 individuals (indicated as events).
reach for proline resources to counteract the antibiotic NaCl for 90 min prior to the antibiotic treatment likely. Moreover, we show that cells pre-treated with 0.6 M glutam et al. not only in response to osmotic or cold stress (Brill et al., 2011b; Hoffmann and Bremer, 2011; Hoffmann et al., 2017) but also in response to antibiotic stress. Consequently, we hypothesize that B. subtilis activates the de novo proline biosynthesis pathway as a stress response towards antibiotic-induced oxidative stress. Firstly, to test our hypothesis, we examined the role of oxidative stress on PproHJ kinetics and followed the proHJ activation on a population-wide level in cells challenged with sub-lethal concentrations of hydrogen peroxide. Secondly, prompted by the kanamycin dose-dependent proHJ activation data (Fig. 5), we asked if exposure to other bactericidal antibiotics, which induce ROS formation, activate the de novo proline biosynthesis pathway.

Interestingly, the GFP expression data showed that the activity of the PproHJ promoter decreased when...
cells regained their growth rate, suggesting that the activation of proHJ transcription and proline synthesis contributes to adaptation to oxidative stress.

Next, to compare the levels of PproHJ activation in cells exposed to different bactericidal antibiotics, we decided to use MIC of kanamycin (15,625 µg/ml) and ciprofloxacin (0.47 µg/ml) as study conditions. The analysis of fluorescent data revealed that fluoroquinolones activate the PproHJ significantly higher than aminoglycosides (Fig. 6B). Based on the significant differences in the relative fluorescence between kanamycin and ciprofloxacin-treated cultures, we propose that cells exposed to antibiotics of different classes, which interfere with different primary molecular targets, induce the proHJ transcription to different extents.

Furthermore, to confirm the population-wide fluorescence data and characterize antibiotic-treated cells on a single-cell level, we designed a time-lapse microscope experiment and used a microfluidics device, which allowed us to apply different growth conditions to follow the cell growth and the fluorescent signal of individual cells in real-time. In agreement with the previous fluorescence measurements (Fig. 6B), the analysis of microscopy data confirmed that B. subtilis cells exposed to different bactericidal antibiotics activate the proHJ promoter on different levels (Fig. 6C). Moreover, we observed differences in phenotypes of cells exposed to kanamycin compared to those exposed to ciprofloxacin. In the case of kanamycin treatment, we identified a non-growing population in which only part of the cells exhibited a moderate GFP fluorescence within 8 h of antibiotic exposure. In contrast, the ciprofloxacin-treated population exhibited a high cell-to-cell variation in the GFP expression and severe cell elongation followed by rapid

![Fig. 4.](image-url)
Fig. 5. Oxidative stress activates the proHJ transcription in *B. subtilis* 168. Exponentially growing cultures in SMM were exposed to sub-lethal concentrations of hydrogen peroxide and incubated for 12 h at 37°C. (A) shows the differences in optical densities, whereas (B) represents the relative fluorescent GFP signal measured from the corresponding cultures. The orange curve represents the outgrowth and relative fluorescent signal of control samples without a stressor. The black curve represents the outgrowth and relative fluorescent signal of control samples with MIC of kanamycin. All measurements were performed in biological duplicates, and the mean values were plotted on the graphs. The relative fluorescent signal was estimated by dividing the GFP signal by the cell density. The value is represented in arbitrary units.

Fig. 6. Exposure to bactericides highly activates the proHJ transcription in *B. subtilis* 168. Exponentially growing cultures in SMM were treated with the MIC of kanamycin (green curve) and ciprofloxacin (red curve) and incubated for 16 h at 37°C. (A) shows the differences in optical densities of tested cultures, whereas (B) represents the relative fluorescent GFP signal measured from the corresponding cultures. The grey-shaded area indicates the time frame when micrographs were taken (C). The blue curve represents the outgrowth of control samples without antibiotic treatment. All measurements were performed in biological duplicates, and the mean values were plotted on the graphs. The relative fluorescent signal was estimated by dividing the GFP signal by the cell density. The value is represented in arbitrary units.

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lysis. Because the exposure to ciprofloxacin resulted in elongated cells, we suggest that the observed differences in the proHJ activity between kanamycin and ciprofloxacin treatments might not only result from ROS formation but also from changes in the cell’s turgor.

The antibiotic-mediated proHJ induction is independent of surfactant-induced membrane perturbations

Kanamycin and ciprofloxacin belong to the bactericidal group of antibiotics known to indirectly induce cell death by ROS-mediated lipid peroxidation and, in consequence, membrane damage (Dwyer et al., 2009, 2014). It could be argued that the induction of proHJ transcription with antibiotics could be a consequence of antibiotic-induced leakage of the cell membrane and loss of turgor. Hence, we tested if direct perturbation of the cell’s envelope by membrane-acting agents can activate the proHJ transcription. Rhamnolipids are commonly used glycolipid biosurfactants that sensitize Gram-positive and Gram-negative bacteria to aminoglycosides by forming membrane pores and facilitating antibiotic’s uptake (Radlinski et al., 2019). Therefore, we used a sub-lethal concentration of rhamnolipids (15 µg ml⁻¹) to induce ROS-independent membrane damage and follow the PproHJ activation.

Accordingly, we examined the level of the proHJ upregulation in cells with and without kanamycin treatment in the presence of a sub-lethal concentration of rhamnolipids (Fig. 7). The fluorescent data revealed that cells exposed solely to rhamnolipids did not activate the proHJ transcription. Conversely, cells treated with a mix of rhamnolipids and kanamycin displayed strong upregulation of the proHJ promoter (Fig. 7B). This evidence demonstrates that the induction of the proline biosynthetic pathway in antibiotic-stressed B. subtilis is independent of directly induced membrane damage.

Exogenous proline decreases the intracellular ROS levels and benefits the survival under antibiotic stress

B. subtilis imports proline directly from the media via two transporter systems, OpuE and PutP (von Blohn et al., 1997; Moses et al., 2012). While PutP is primarily responsible for the uptake of proline when it is used as a nutrient (Moses et al., 2012), OpuE primarily serves for proline import when it is used as an osmotic stress protectant (von Blohn et al., 1997). Here, we tested if the addition of proline to the media would increase the survival of B. subtilis when exposed to lethal doses of kanamycin. Moreover, to examine the role of proline in oxidative stress response and ROS scavenging, we estimated intracellular ROS levels in antibiotic-stressed B. subtilis with and without proline addition. To follow the changes in the ROS pools, we used 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA), a chemically reduced form of fluorescein. Inside the cell, H₂DCFDA is cleaved by intracellular esterases and oxidized in the presence of hydroxyl radicals to the highly fluorescent 2',7' dichlorofluorescein (DCF). Thus, the conversion of H₂DCFDA to DCF directly corresponds to the ROS levels.

Interestingly, the presence of exogenous 40 mM proline positively affected the growth of kanamycin-stressed cells above the MIC values (Fig. 8A and C). Compared to the control, the cells supplemented with proline displayed significantly lower activation ROS levels when exposed to kanamycin (Fig. 8C and D). Moreover, we...
were able to simultaneously follow the PproHJ activation under tested conditions, and the PproHJ activity elegantly correlates with the ROS levels (Fig. 8E and F). These evidences show that proline has antioxidative potential and the proHJ promoter is activated by ROS. The decrease in the PproHJ in cells with additional proline suggests that the demand for the ProH- and ProJ-dependent proline synthesis decreased when the proline was repurposed from the media.

Discussion

In the absence of external sources of compatible solutes, de novo synthesis and accumulation of proline play a pivotal role in B. subtilis’ adaptation to an adverse hyperosmotic pressure (Whatmore et al., 1990; Brill et al., 2011a). In this study, we followed for the first time the response of B. subtilis to the osmotic upshift and activation of the proHJ transcription on a single-cell level, using PproHJ-tagRFP (Sp) as reporter strain.

Examining the early response to osmotic upshift with flow cytometry revealed that 60–90 min of NaCl exposure is sufficient to induce proHJ transcription and activate the osmotically induced proline biosynthesis in B. subtilis, which is in agreement with the results of related studies (Höper et al., 2006; Hahne et al., 2010; Brill et al., 2011a) (Fig. 2). Additionally, we have shown that the proHJ promoter is upregulated in the majority of
the population and confirmed that the level of its activation depends on the severity of the osmotic upshift (Brill et al., 2011a; Schroeter et al., 2013; Hoffmann et al., 2017). In the heterogeneous model system, where proHJ transcription can be either on or off, a bimodal fluorescence distribution amongst the cell population may be expected. However, the flow cytometry data showed no clear bimodal distribution, but only the presence of a small fraction of highly fluorescent cells (Fig. S1). To complement the flow cytometry data, we were able to show substantial heterogeneity in the activation of the proHJ operon at 0.6 M NaCl using time-lapse microscopy. We demonstrated that under mild osmotic upshift, the clonal population of B. subtilis diversifies into two distinct subpopulations: the proHJ inducers and non-inducers. We showed that cells that activated the proHJ transcription and consequently increased intracellular proline pools were able to resume their pre-shock growth rates, whereas those that did not show the significant upregulation of the proHJ promoter remained dormant (Fig. 3).

It should be noted that the growth conditions within the microcolonies on the agarose pad are different compared to the conditions in liquid media. When growing on solid agarose-based media, the availability of nutrients in the nearest surroundings decreases over time but also, the concentration of secreted compounds increases. Therefore, we hypothesize that cells that highly induce the proHJ expression and boost proline biosynthesis might aid the neighbouring proHJ non-inducers to withstand the osmotic upshift. Since proline can be imported via OpuE (von Blohn et al., 1997) and PutP (Moses et al., 2012) transporter systems, it would be interesting to examine if the proHJ non-inducers recapture proline excreted to the media (Hoffmann et al., 2012). Release of proline by cells that strongly upregulate the proHJ promoter and its recapture by the proHJ non-inducers might reflect the cooperative behaviours, similar to those reported for the glycine betaine transport in Vibrio cholerae (Kapfhammer et al., 2005). The preliminary experiments with opuE and putBCP mutants showed that cells strongly induce the proHJ operon compared to the wild-type strain after 90 min of the osmotic upshift, and the fraction of cells with high GFP signal substantially increased compared to the wild-type strain (82% for ΔopuE and 63% for ΔputBCP) (Fig. S3). This suggests that limited proline recapture (mainly due to the opuE deletion) forces cells to increase the proHJ transcription in most of the cells in the population (Fig. S3).

So far, it has been reported that in B. subtilis, the proHJ promoter is induced in response to ionic and non-
Bacterial strains, culture conditions and media

All tested strains used in this study are derivatives of the *B. subtilis* 168 *trpC2* laboratory strain (Table S1). First transformation steps were performed in the initial hosts, *E. coli* MC1061. Molecular cloning was performed as described by Sambrook *et al.* (1989). Used strains were cultured at 37°C with aeration, initially in liquiduria Bertani media (LB) or agar LB, with 5 μg ml⁻¹ chloramphenicol for engineered *B. subtilis* strains or 100 μg ml⁻¹ ampicillin for *E. coli* strains. Further on, *B. subtilis* strains were grown in Spizizen’s Minimal Medium (Harwood and Cutting, 1990) (SMM) supplemented with 0.5% (w/v) glucose, 1% (v/v) trace elements and, for tryptophan-autotroph strains, 0.5% (w/v) tryptophan. To maintain the same culture conditions, 3-day cultivation was performed as follows: *B. subtilis* was streaked on selective LB agar plates and incubated overnight (O/N) at 37°C. The next morning, 3 ml of liquid LB was inoculated with a single colony and incubated for 8 h at 37°C and 220 rpm in a shaking incubator. After the incubation period, the culture was diluted 1:1000 in supplemented SMM medium and grown O/N at 37°C and 220 rpm. The overnight culture was diluted to OD600 of 0.08 in fresh SMM medium and grown till the OD600 reached 0.3. The exponentially growing culture was immediately
subjected to osmotic stress, and the following measurements were taken.

**Construction of a B. subtilis 168 PproHJ-sfGFP (Sp) reporter fusion**

A promoter PproHJ-sfGFP fusion was made to follow the dynamics of proHJ genes expression at the single-cell level during osmotic stress. A 934 bp region with the proHJ promoter and first 29 codons of proHJ was transcriptionally fused with superfolder GFP (Sp) and the plasmid was integrated into B. subtilis 168 strain via Campbell-type single crossover integration.

**Flow cytometry**

FACS measurements were performed using a BD Biosciences FACS Canto Flow Cytometer. FACS was used to determine the fluorescence of exponentially growing B. subtilis PproHJ-sfGFP (Sp) after 30, 60, 90 or 120 min of exposure to 0.5 M, 0.6 M, 0.7 M or 1.2 M NaCl. FACS samples were diluted 1000-fold in phosphate-buffered saline (PBS, Boom (Oxoid, Meppel, The Netherlands)) before measurement.

**Microtitre-plate experiments**

Bacterial antibiotic resistance and fluorescent measurements were carried in either Varioskan™ LUX (Thermo Fisher, Waltham, MA, USA) or Tecan T200 (Tecan, Männedorf, Switzerland) multimode microplate reader. An exponentially growing culture was exposed to 0.6 M NaCl for 90 min and applied to an agarose slide or directly applied to an agarose slide containing 0.6 M NaCl. Cells were tracked by imaging phase-contrast and fluorescence every 12 min over 10 h. Image processing was done with ImageJ version 1.50 b software.

**Reactive oxygen species measurements**

ROS levels were measured using 10 μM of 2',7'-dichlorofluorescein diacetate (H2DCFDA) fluorescent dye. All measurements were performed in 96-well black microtitre plates using Varioskan™ LUX multimode microplate reader (Thermo Fisher). An exponentially growing culture was diluted to an OD600 of 0.1 in SMM and exposed to antibiotics. Final microtitre plate volume was 135 μl per well. Cells were grown for a total of 16 h, unless noted otherwise.

**Fluorescence time-lapse microscopy**

Fluorescence time-lapse microscopy was performed using an Olympus IX71 DeltaVision microscope. SMM-agarose microscope slides were prepared with 1.5% (w/v) Low Melting Point Agarose (Sigma-Aldrich, St. Louis, MO, USA) and optionally kanamycin or NaCl. Exponentially growing cells were either exposed to 0.6 M NaCl for 90 min and applied to an agarose slide or directly applied to an agarose slide containing 0.6 M NaCl. Cells were tracked by imaging phase-contrast and fluorescence every 12 min over 10 h.

**Microfluidics experiments**

The experiments were performed with the CellASIC ONIX microfluidic device (Merck Millipore, Darmstadt, Germany) in a B04A plate. The plate was primed with the medium before the experiment, using the manufacturer’s specifications. A pump pressure of 0.25 psi drove the flow rate of the supplied medium. Exponentially growing cells were diluted in the complete SMM to OD600 0.05 and subsequently loaded on the microfluidics plate. The microfluidic plate was mounted on an Olympus IX71 DeltaVision microscope, and fluorescence time-lapse microscopy was performed. The changes of growing conditions in each chamber were performed accordingly: 90 min regrowth in SMM and 8 h of antibiotic treatment. Cells were tracked by imaging phase-contrast and fluorescence every 10 min over 12 h. All experiments were performed at 37°C.

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**Conflict of interest**

None declared.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Fig. S1.** Activation of the proHJ promoter in response to the osmotic upshift. Fluorescence distribution of *B. subtilis* 168 PproHJ::sfGFP (Sp) strain in response to the different osmolarity of the environment after 90 min. The total sample size was 10,000 individual cells.

**Fig. S2.** Differences in the proHJ expression from dividing and non-dividing cells. The mean GFP fluorescent signal of dividing and non-dividing *B. subtilis* cells during 12 h of exposure to the sustained osmotic upshift. 1.5 h time-point, indicated with a dotted line, shows the mean FL value for the early response to the osmotic upshift. The 6 h time-point marks the beginning of the GFP-positive cells division. The mean FL was measured with the MicrobeJ plugin for ImageJ, and the error bars show the SD of the measured signal dividing and non-dividing cells from a single frame.

**Fig. S3.** Activation of the proHJ promoter in osmotically stressed *B. subtilis* mutants. (A) Estimation of the average intensity of GFP fluorescent signal and (B) the distribution of the GFP fluorescent signal in studied populations. The percentages in the brackets indicate the exact percentages

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of cells exhibiting high fluorescent signal. (C) Micrographs of time-lapse microscopy showing adaptation of different mutants of *B. subtilis* to 0.6 M NaCl exposure at 90 min. The GFP fluorescent signal represents the activity of the *proHJ* promoter. The bar scale indicates 10 µm.

Table S1. Strains used in this study.
Table S2. Plasmids used in this study.
Table S3. Oligonucleotides used in this study.
Table S4. Media and buffers.