environmental microbiology

Environmental Microbiology (2020) **22**(8), 3266–3286 doi:10.1111/1462-2920.15087

Impact of high salinity and the compatible solute glycine betaine on gene expression of *Bacillus subtilis*

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Summary

The Gram-positive bacterium *Bacillus subtilis* is frequently exposed to hyperosmotic conditions. In addition to the induction of genes involved in the accumulation of compatible solutes, high salinity exerts widespread effects on *B. subtilis* physiology, including changes in cell wall metabolism, induction of an iron limitation response, reduced motility and suppression of sporulation. We performed a combined whole-transcriptome and proteome analysis of *B. subtilis* 168 cells continuously cultivated at low or high (1.2 M NaCl) salinity. Our study revealed significant changes in the expression of more than one-fourth of the protein-coding genes and of numerous non-coding RNAs. New aspects in understanding the impact of high salinity on *B. subtilis* include a sustained low-level induction of the SigB-dependent general stress response and strong repression of biofilm formation under high-salinity conditions. The accumulation of compatible solutes such as glycine betaine aids the cells to cope with water stress by maintaining physiologically adequate levels of turgor and also affects multiple cellular processes through interactions with cellular components. Therefore, we additionally analysed the global effects of glycine betaine on the transcriptome and proteome of *B. subtilis* and revealed that it influences gene expression not only under high-salinity, but also under standard growth conditions.

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Introduction

Life depends on the availability of water (Stevenson *et al*., 2015a), a parameter that can rapidly change, in particular in terrestrial habitats where microorganisms perform critical tasks for soil ecology (Stevenson and Hallsworth, 2014; Stevenson *et al*., 2015b). One of the main habitats of the Gram-positive bacterium *Bacillus subtilis* is the upper layer of the soil (Mandic-Mulec *et al*., 2015), where changes in the osmotic conditions occur frequently through flooding and desiccation. An increase in external osmolarity triggers water efflux from the cell, thereby causes dehydration of the cytoplasm and a reduction in turgor pressure, which consequently impairs growth (Wood, 2011; Hoffmann and Bremer, 2017; Bremer and Krämer, 2019).

To counteract these detrimental effects, cells initially take up potassium ions (Whatmore and Reed, 1990), which are subsequently replaced by physiologically compliant organic osmolytes, the compatible solutes (Kempf and Bremer, 1998; Hoffmann and Bremer, 2017; Gunde-Cimerman *et al*., 2018). The import of potassium ions by *B. subtilis* is mediated by two Ktr-type transporters, KtrAB and KtrCD (Holtmann *et al*., 2003; Mikusevic *et al*., 2019), and by KimA (Gundlach *et al*., 2017; Tascon *et al*., 2020). These transporters are controlled by the second messenger c-di-AMP, a signalling molecule essential for potassium homeostasis in *B. subtilis* (Corrigan *et al*., 2013; Gundlach *et al*., 2019). The accumulation of compatible solutes allows the cell to reduce the cytoplasmic potassium concentration through the activities of potassium export systems (Whatmore *et al*., 1990; Fujisawa *et al*., 2007; Hoffmann and Bremer, 2017). Of the at least 15 naturally occurring compatible solutes employed by *B. subtilis* to provide osmotic stress protection (Hoffmann and Bremer, 2017), proline is the only one that it can synthesize *de novo* (Whatmore *et al*., 1990; Brill *et al*., 2011). *Bacillus subtilis* uses five osmotically regulated *o*smostress

*p*rotectant *u*ptake systems, the Opu transporters, to import the various compatible solutes. Particularly effective osmostress protection is conferred by glycine betaine, the probably most widely used compatible solute in nature (Yancey, 2005). *Bacillus subtilis* can either take up glycine betaine from the environment via OpuA, OpuC and OpuD or synthesize it via the GbsAB enzymes from the precursor choline imported via OpuB and OpuC (Boch *et al*., 1994; Kappes *et al*., 1996).

The intracellular accumulation of compatible solutes such as glycine betaine is not only important for maintenance of turgor pressure under hyperosmotic conditions (Cayley and Record Jr, 2003; de Lima Alves *et al*., 2015), but it can also stabilize proteins and other cellular components (Bourot *et al*., 2000; Ignatova and Gierasch, 2006; Street *et al*., 2006; Guinn *et al*., 2011; Stadmiller *et al*., 2017). The function-preserving attributes of compatible solutes in general (Bolen and Baskakov, 2001; Street *et al*., 2006), and that of glycine betaine in particular, led to the description of these organics osmolytes as chemical chaperones (Chattopadhyay *et al*., 2004). The amassing of compatible solutes thus lets the cell to avoid water stress caused by high salinity or high osmolarity surroundings (Kempf and Bremer, 1998; Gunde-Cimerman *et al*., 2018) and allows simultaneously the optimization of the solvent properties of the bacterial cell to maintain the functionality of key biochemical reactions (Wood, 2011; Bremer and Krämer, 2019). It should be noted that water stress can also be induced by hydrophobic substances, compounds that are frequently found as pollutants in soil ecosystems (Bhaganna *et al*., 2010), and by various types of highly osmotically active chaotropes (e.g. NH_4NO_3, $MgCl_2$, $CaCl_2$; de Lima Alves *et al*., 2015). The accumulation of compatible solutes, kosmotropic compounds, thus provides a means to offset physicochemical constrains on cellular physiology and aids pro- and eukaryotic microorganisms to cope with low water-activity (Kempf and Bremer, 1998; Cayley and Record Jr, 2003; Stevenson and Hallsworth, 2014; Stevenson *et al*., 2015a; Gunde-Cimerman *et al*., 2018). In addition to their role as osmostress protectants and their beneficial effects on the functionality of proteins and various cellular components, the amassing of compatible solutes can also influence gene expression, particularly affecting those genes that are involved in the synthesis or uptake of these compounds (Barron *et al*., 1986; Spiegelhalter and Bremer, 1998; Romeo *et al*., 2003; Gunasekera *et al*., 2008; Brill *et al*., 2011; Hoffmann *et al*., 2013).

Salt stress is one of the strongest inducers of the general stress response, a cellular adaptation triggered by a variety of environmental and cellular cues (Völker *et al*., 1995). Activation of the general stress sigma factor, SigB, triggers coordinated expression of more than 200 genes (Helmann *et al*., 2001; Petersohn *et al*., 2001; Price *et al*., 2001; Nicolas *et al*., 2012), thus providing *B. subtilis* with a non-specific and pre-emptive multiple stress resistance (reviewed in Hecker *et al*., 2007; Price, 2011). In a systematic analysis of stress survival rates of *B. subtilis* mutants defective in individual SigB-controlled genes, it was found that roughly 20% of the SigB-regulon members can be associated with cellular protection against severe salt shock (Höper *et al*., 2005). Following a sudden osmotic upshift, SigB contributes to the expression of *opuD* and *opuE* encoding transporters for glycine betaine and proline respectively (Hoffmann and Bremer, 2017). The activity of SigB is regulated by a signal transduction cascade starting with two main signalling pathways that respond to environmental stress and energy depletion respectively. Activation of SigB by environmental stresses, including ethanol, salt and heat, is predominantly described as a transient response (Boylan *et al*., 1993; Völker *et al*., 1995; Young *et al*., 2013; Cabeen *et al*., 2017).

In addition to the detrimental influences of high salinity on hydration of the cytoplasm and magnitude of turgor, salt stress exerts additional effects on the physiology of *B. subtilis*, in particular by alterations of the composition of the cytoplasmic membrane and of the properties of the cell wall (Lopez *et al*., 1998; 2000; 2006; Steil *et al*., 2003 Hahne *et al*., 2010). The impairment of cell wall function might also cause the induction of the regulons controlled by the extracytoplasmic function (ECF) sigma factors SigM and SigW (Helmann, 2016) in response to salt shock as revealed by transcriptional profiling (Petersohn *et al*., 2001; Steil *et al*., 2003; Hahne *et al*., 2010) and functional studies (Horsburgh and Moir, 1999).

The response of *B. subtilis* to high salinity can be divided into two phases: an initial reaction to a sudden increase in salinity and the subsequent cellular adaptation to prolonged growth under high-salinity conditions. It was previously shown that only a small portion of the genes that are immediately induced or repressed by salt shock also display significant differences in their transcriptional profile in *B. subtilis* cells continuously cultivated at high versus low salinity (Steil *et al*., 2003). These findings indicate that salt shock and prolonged growth at high salinity require partially different adaptation reactions.

Previous studies of salt-adapted *B. subtilis* cells had revealed alterations in gene expression that affect multiple aspects of cellular physiology, namely uptake and synthesis of osmoprotectants (Kappes *et al*., 1996; von Blohn *et al*., 1997; Kappes *et al*., 1999; Brill *et al*., 2011; Hoffmann *et al*., 2013), cell wall metabolism and cell division (Dartois *et al*., 1998; Steil *et al*., 2003; Fischer and Bremer, 2012), iron metabolism (Hoffmann *et al*., 2002; Steil *et al*., 2003), degradative enzyme synthesis (Kunst and Rapoport, 1995), endospore formation (Kunst and Rapoport, 1995; Ruzal *et al*., 1998; Widderich *et al*., 2016)

and motility (Steil *et al*., 2003). In one of these studies global gene expression profiling of high-salinity adaptation of *B. subtilis* was performed using a first generation DNA array with PCR products spotted on a nylon membrane (Steil *et al*., 2003). This study was conducted with a SigB-deficient mutant of *B. subtilis* JH642, a widely used laboratory strain derived from the legacy *B. subtilis* strain 168 (Brehm *et al*., 1973).

In order to reveal additional facets of adaptation of *B. subtilis* to growth under sustained high-salinity conditions (generated with 1.2 M NaCl; Boch *et al*., 1994), we performed a whole-transcriptome analysis of the *B. subtilis* 168 wild-type using strand-specific tiling arrays. Additionally, the transcriptome data were complemented by analysing the proteome of cells cultured under the same conditions. Because the accumulation of compatible solutes (e.g. glycine betaine) affects multiple facets of the cell's response to sustained osmotic stress, we also analysed the global effects of glycine betaine on the transcriptome and proteome of *B. subtilis* cells cultured either in the absence or presence of NaCl-induced stress.

Results and discussion

Growth of B. subtilis *under high-salinity conditions is associated with profound changes in mRNA and protein levels*

In order to comprehensively characterize the transcriptional adaptation of *B. subtilis* 168 to growth under high-salinity conditions, we performed a whole-transcriptome analysis using a strand-specific DNA tiling array (see Experimental procedures) of cells grown in either Spizizen's minimal medium (SMM) with glucose as the carbon source or in SMM that contained additional 1.2 M NaCl. This level of salt stress puts a considerable constrain on the growth of *B. subtilis* (Boch *et al*., 1994; Fig. 1). Overall, our transcriptional analysis generated gene expression data for 4292 annotated coding sequences (CDSs) and 1445 RNA features (including potential sRNAs, asRNAs and new predicted CDSs) previously identified in a systematic study of the *B. subtilis* transcriptome exposed to a wide range of experimental conditions (Nicolas *et al*., 2012).

Triplicate cultures of the *B. subtilis* wild-type strain BSB1 (168 Trp^+) were grown in SMM and in SMM with 1.2 M NaCl and total RNA was isolated from exponentially growing cells (OD_{600} of 1). After sample processing and quantification of the hybridization signals, an aggregated expression value per gene was computed as the median intensity of probes lying within the respective genomic region (Nicolas *et al*., 2012). Genes were considered as significantly differentially expressed if their mean transcript level ratio between the two conditions was ≤ 0.5 or ≥ 2 and the false discovery rate (FDR) was less than 0.05, a threshold met by more than 95% of all genes with the stringent fold-change cut-off of 2. A group of 832 genes (638 annotated CDSs and 194 RNA features) displayed at least twofold higher expression levels in cultures grown at high salinity, and 995 genes (694 CDSs and 301 RNA features) displayed at least twofold lower expression under high-salinity growth conditions (Table S1).

In order to further characterize the RNA features whose levels were altered in response to increased salinity of the medium, we divided this group into RNA classes defined by the study by Nicolas and colleagues (2012)

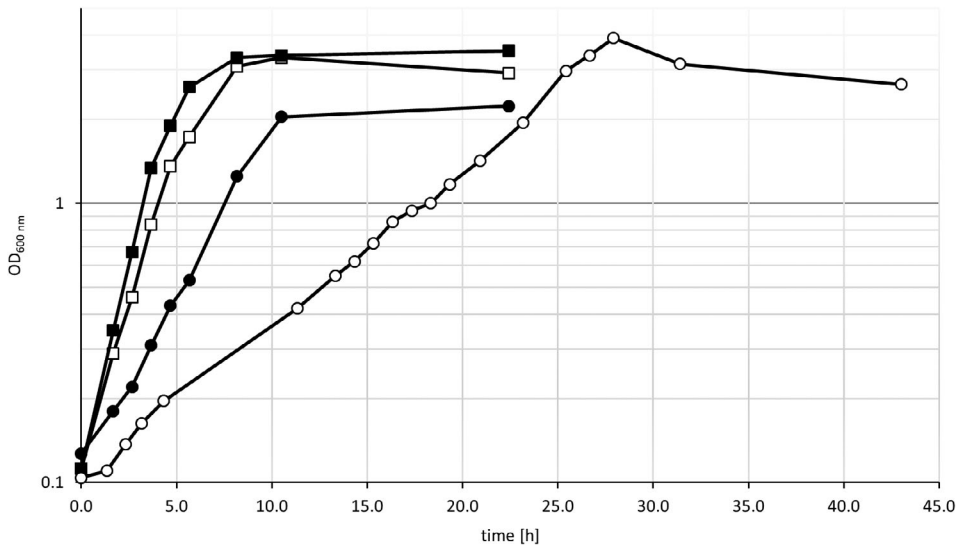

Fig 1. Growth of the *B. subtilis* strain BSB1 in SMM (□) supplemented either with 1 mM glycine betaine (■), 1.2 M NaCl (○) or 1.2 M NaCl and 1 mM GB (●).

Table 1. Summary of RNA features with altered transcript levels under high-salinity conditions (1.2 M NaCl).

	High-salinity-induced			High-salinity-repressed		
Features[a]	*n*	asRNA	CDS	*n*	asRNA	CDS
5′	70	14	3	121	35	7
3′UTR	21	11	–	17	4	4
3′NT	6	6	–	14	12	–
3′PT	14	13	–	15	14	–
Indep	6	2	–	24	14	3
Indep-NT	11	11	–	29	27	–
Inter	33	11	1	59	24	1
Intra	28	8	1	20	1	–
Total	189	76	5	299	131	15

a. RNA-features were categorized according to Nicolas and colleagues (2012); indep/indep-NT RNA-features which were not assigned as asRNA or CDS were classified as potential sRNAs.

(Table 1). Of these, potential regulatory RNAs, i.e. sRNAs and asRNAs, and newly predicted small proteins are of particular interest for further study. Overall, we found altered expression of 7 sRNAs, 207 asRNAs and 20 new potential CDSs (Table S2). In agreement with the repressive effect of high salinity on sporulation-specific gene expression (see below), we noted that 91 of the 131 asRNAs with reduced expression levels are predicted to be controlled by one of the sporulation sigma factors (Nicolas *et al*., 2012; Table S2). In the group of 76 high-salinity-induced asRNAs, we found 29 that are transcribed from predicted SigB-type promoters, which will be further discussed below.

Growth of *B. subtilis* in SMM is strongly impaired by the addition of 1.2 M NaCl (Fig. 1). Surprisingly, growth-rate related effects, e.g. changes in expression of ribosomal protein encoding genes, were only poorly reflected in the transcriptome data (Fig. S1). This might be due to differences in total cellular RNA amount associated with the growth rate, which can cause a systematic bias not eliminated by available data normalization approaches (Borkowski *et al*., 2016). In order to prevent endospore formation during the course of the cultivation, a cultivation procedure was employed that minimized spore formation in SMM-grown cells propagated in the absence of high salinity (see Experimental procedures). Nevertheless, our tiling array data revealed that the expression of sporulation genes was noticeable in the control cultures without additional NaCl, reflecting the fact that a certain, albeit minor fraction of the culture initiates sporulation during growth in defined medium with glucose as the carbon source (Dawes and Thornley, 1970). It is well established that *B. subtilis* cells propagated at high external salinity are blocked at an early stage of the sporulation process (Kunst and Rapoport, 1995; Ruzal *et al*., 1998; Widderich *et al*., 2016). Consistent with this finding, 420 of the 694 CDSs expressed at lower levels in high-salinity-grown compared to control cells, are members of the regulons controlled by the sporulation-specific sigma factors SigF, SigE, SigG and SigK (Arrieta-Ortiz *et al*., 2015; Fig. S2).

In order to complement the transcriptome data, we analysed the proteomes of *B. subtilis* cells cultured under the same conditions. Mass spectrometric analysis resulted in the quantification of 1493 proteins, of which 196 and 254 proteins were found in significantly higher or lower amounts (absolute fold change of ≥ 2), respectively, during growth in high-salinity medium compared to SMM without additional salt (Table S3). Hence, the presence of 1.2 M NaCl had a pronounced effect on cellular protein levels. Similar to what has been reported before (Kohlstedt *et al*., 2014), part of these changes was not reflected in the transcriptome profiles (Fig. S3), as observed, for example, for a number of proteins belonging to the SigD regulon (see below). On the other hand, the effect of high salinity on sporulation was not reflected at the proteome level, because the small fraction of sporulating cells in the cultures resulted in protein levels below the limit of detection of the proteome analysis. Of the proteins with higher amounts under high-salt conditions, more than 72% showed a similar change at the mRNA level (Fig. S3). This proportion was smaller (47%) for the proteins found in lower amounts, whose differential abundance partly reflects the lower growth rate of *B. subtilis* in the presence of 1.2 M NaCl, as observed, e.g. for ribosomal proteins (Fig. S1) and proteins involved in nucleotide biosynthesis.

Cellular functions involved in the adaptation of B. subtilis *to high salinity*

In the following, we focus on the specific adaptation reactions of *B. subtilis* to high-salinity growth conditions, as revealed by our genome-wide transcriptome study. In order to define the underlying transcriptional regulators, we assigned the significantly differentially expressed genes to the known transcription factor regulons of *B. subtilis*. We used a Fisher's exact test to specify the regulons that were significantly enriched (Benjamini–Hochberg adjusted p-value < 0.05) for up- and downregulated genes respectively. This analysis revealed, besides sporulation-specific regulons, six and nine regulons enriched for genes expressed at higher and lower level, respectively, in cultures grown at high versus low salinity (Fig. 2A). The majority of the affected regulons reflect cellular functions previously implicated in the adaptation of *B. subtilis* to high-salinity conditions, namely cell envelope and cell division related functions (SigI, YvrHb, SigV, SigX and SigD), motility and chemotaxis (SigD), high-salinity-induced iron limitation (Fur) and cellular processes controlled by the DegSU two-component regulatory system.

Fig 2. Transcription factor regulons significantly enriched (Benjamini–Hochberg corrected p-value ≤ 0.05) for differentially expressed genes as identified through Fisher's exact test.
(A). For each regulon, the number of differentially expressed genes and the regulon size are shown in parentheses. The general function of the transcription factor is based on the *Subti*Wiki database. (B). Fraction of high-salinity regulated genes whose induction or repression was reversed by addition of 1 mM glycine betaine.

Moreover, the tiling array transcriptome profiles of our study revealed a number of additional regulons enriched for differentially expressed genes that have so far not been implicated in the adaptation of *B. subtilis* to growth under high-salinity conditions (Fig. 2A), namely the SigB regulon, regulons involved in biofilm formation (SinR, RemA) and the CymR regulon controlling cysteine metabolism. These novel findings were further examined.

Osmotic-stress specific response

Uptake and synthesis of compatible solutes are central for the cellular adaptation of *B. subtilis* to high-salinity conditions and are mediated by five osmoprotectant uptake systems (OpuA to OpuE; Hoffmann and Bremer, 2017), the GbsB and GbsA enzymes for glycine betaine synthesis from the precursor choline (Boch *et al*., 1996) and the ProJ and ProH enzymes that, together with ProA, catalyse osmostress-adaptive proline biosynthesis (Brill *et al*., 2011; Hoffmann *et al*., 2017). Expression of the encoding genes is generally regulated at the transcriptional level by means of osmotically inducible SigA-type promoters (Hoffmann and Bremer, 2017). Notably, the *opuD* and *opuE* genes each possess a second promoter that is recognized by the general stress sigma factor SigB (Spiegelhalter and Bremer, 1998). In *B. subtilis* cells grown in the presence of 1.2 M NaCl we observed significantly elevated expression of *proHJ*, the *opuA* and *opuB* operons, *opuE*, and *gbsAB* (Table S1).

In the absence of exogenously supplied compatible solutes, *B. subtilis* only synthesizes proline as osmostress protectant; hence, disruption of the osmoregulatory proline biosynthetic *proHJ* operon causes a strong growth defect under hyperosmotic conditions (Brill *et al*., 2011). Accordingly, the *proHJ* genes are strongly induced under high-salinity growth conditions (more than sixfold in the present study) in the absence of externally provided osmoprotectants. Of the five Opu transport systems, three displayed significantly higher expression of their encoding genes during growth in high-salinity medium compared to SMM without additional salt: the *opuA* operon (2.1-fold), the *opuB* operon (twofold) and the *opuE* gene (14-fold). Transcript levels of *opuD* were slightly increased (1.5-fold), while those of the *opuC* operon were unaffected under steady-state high-salinity growth conditions. The OpuE transporter does not only function for the import of exogenously provided proline as an osmoprotectant, but also to recapture proline that is synthesized by salt-stressed *B. subtilis* cells and partly released under high-osmotic steady state growth conditions (Hoffmann *et al*., 2012). As already observed in previous studies (Hahne *et al*., 2010; Kohlstedt *et al*., 2014), proline release can lead to the induction of the catabolic *putBCP* operon needed for the utilization of proline as a nutrient, which encodes the transporter PutP and the proline degrading enzymes PutB and PutC (Moses *et al*., 2012). The *putP* gene was found to be induced more than threefold, the *putB* and *putC* genes more than 15-fold in cells grown at high versus low

salinity. However, it was previously demonstrated that the transport activity of PutP is strongly inhibited in cells grown at high salinity (Zaprasis *et al*., 2014), thus potentially limiting a futile cycle of *de novo* proline synthesis and degradation. The *gbsAB* operon is known to be induced by the addition of choline to the growth medium, a process mediated by the release of the GbsR repressor from its operator site (Nau-Wagner *et al*., 2012). Higher expression of the *gbsAB* operon during growth of *B. subtilis* in SMM with 1.2 M NaCl revealed that high salinity leads to a notable degree of osmotic induction (by about 5.5-fold) of the *gbsAB* operon in the absence of the inducer choline. This finding is in agreement with previous transcriptome data on the response of *B. subtilis* to a sudden osmotic upshift (Hahne *et al*., 2010) and derived from cells grown in a chemostat under sustained high salinity (Kohlstedt *et al*., 2014).

After initially importing large quantities of potassium, osmotically stressed *B. subtilis* cells export part of this ion again, once the synthesis or import of compatible solutes commences (Whatmore *et al*., 1990; Fujisawa *et al*., 2007). In this way a build-up of a long lasting high ionic strength cytoplasm is prevented without impairing the osmotic potential of this cellular compartment. Of the potassium exporter genes of *B. subtilis* (Hoffmann and Bremer, 2017), only the *khtSTU* operon was induced (fourfold) in cells grown at high versus low salinity, whereas *yugO* mRNA levels were unchanged. In the study by Hahne and colleagues (2010), expression of both, *khtSTU* and *yugO*, was upregulated early (10 and 30 min) after the osmotic upshift and *khtSTU* mRNA levels remained high until the last time point (120 min) analysed. Hence, these data collectively support a prominent role of the KhtTU system in the export of potassium ions during the adaptation phase and during continuous growth of *B. subtilis* at high salinity. In addition, we observed an upregulation of the *mrp* operon encoding the major sodium extrusion system of *B. subtilis* as was the case in response to a suddenly imposed salt stress (Hahne *et al*., 2010). The physiologically very important role of extrusion systems for cytotoxic sodium ions in osmostress adaptation of *B. subtilis* is related to the activities of the glycine betaine transporter OpuD [betaine-choline-carnitine transporter (BCCT) family] and the proline transporter OpuE [sodium:solute symporter (SSS) family]. Both transporters import sodium ions together with the compatible solutes (Hoffmann and Bremer, 2017), which therefore need to be timely removed from the cytoplasm in order to prevent the strong growth-inhibiting effects of this ion (Gorecki *et al*., 2014). Finally, expression of the *yrkA* gene encoding a putative magnesium transporter was fivefold higher in cells grown at high versus low salinity. It can be noted in this context that inactivation of the magnesium transporter MgtE of *Staphylococcus aureus* strongly impairs growth of the bacterium under hyperosmotic conditions (Schuster *et al*., 2019).

Cell envelope related functions and motility

Among the regulons enriched for differentially expressed genes were five regulons associated with cell envelope and cell division related functions: SigI, YvrHb, SigV, SigX and SigD (Fig. 2A). Alterations in the envelope composition of *B. subtilis* cells grown under hyperosmotic conditions were first reported based on altered sensitivity towards phage infection or antibiotics targeting the cell wall (Lopez *et al*., 1998). Along with the properties of the cell wall, exposure of *B. subtilis* to high salinity also influences the lipid and fatty acid composition of the cytoplasmic membrane (Lopez *et al*., 1998; 2000; 2006). In the present study, all fatty acid biosynthesis genes regulated by FapR were expressed at lower level under high-salinity conditions, though only half of them (5/10) passed the twofold criterion chosen for significant regulation. Downregulation of mRNAs and proteins involved in fatty acid and lipid biosynthesis was also observed by Hahne and colleagues (2010) in response to a sudden osmotic upshift elicited with NaCl. Their study also revealed expression changes apparently specific for the initial salt stress response, in particular induction of genes affecting membrane fluidity, namely the desaturase gene *des* and the SigW-regulated genes *fabF* and *floT* (Kingston *et al*., 2011; Lee *et al*., 2012). One should note that changes in the lipid profiles of cells can depend on the chaotropic (e.g. $MgCl_2$) or non-chaotropic (e.g. NaCl) nature of the salts used to elicit water stress. This phenomenon is based upon the ability of various salts to destabilize or disorder membranes (Bhaganna *et al*., 2010).

Among the osmotically regulated genes identified in the transcriptome study by Steil and colleagues (2003) were genes involved in peptidoglycan remodelling (*lytF*, *yocH*, *yabE*, *yqiI*), the *dlt* operon involved in teichoic acid biosynthesis, *wapA* encoding a cell wall associated protein, and *gpsB* encoding a key player in the elongation-division cycle of *B. subtilis*, which recruits penicillin-binding protein 1 (PBP1) to the cell division site (Claessen *et al*., 2008). In addition, osmotic induction of the structural gene (*pbpE*) for the peptidoglycan endopeptidase PBP4* was observed (Palomino *et al*., 2009); notably, the individual disruption of the *gpsB* and *pbpE* genes causes osmotic sensitivity (Claessen *et al*., 2008; Palomino *et al*., 2009). We identified changes in the expression of 57 genes involved in cell envelope functions and cell division (Table 2), including most of the genes previously shown to be affected during continuous growth of *B. subtilis* at high salinity (Steil *et al*., 2003) and the early adaptation phase (up to 120 min; Hahne *et al*., 2010) respectively. Of the 35 downregulated genes, 17 possess a promoter recognized by a sporulation-specific

Table 2. High-salinity-regulated genes involved in cell envelope and cell division related functions identified in the present and previous studies.

Gene name[a]	Locus	Product/function	Regulator	Expression ratio[a] (1.2 M NaCl/ no NaCl)	Reference(s)
Cell wall synthesis (19 of 97 genes)[b]					
tagC	BSU35770	Biosynthesis of teichoic acid	LexA	**5.13**	
pbpE	BSU34440	Penicillin-binding protein 4[b]	SigW	1.01	Palomino *et al*., 2009
tagU	BSU35650	Attachment of teichoic acid to peptidoglycan	TagU, SigX	**0.49**	
amj	BSU04230	Lipid II flippase	SigM	**0.46**	Hahne *et al*., 2010
pbpC	BSU04140	Penicillin-binding protein 3		**0.43**	
yfhO	BSU08610	Lipoteichoic acid glycosylation	SigB, SigX	**0.35**	
bcrC	BSU36530	Undecaprenyl pyrophosphate phosphatase	SigI, SigM, SigW, SigX, SigV	**0.32**	Hahne *et al*., 2010
pbpH	BSU13980	Penicillin-binding protein H	AbrB, WalR	**0.32**	
dltABCDE	BSU38500 … BSU38540	Biosynthesis of teichoic acid	SigD, SigM, SigX, Spo0A, stringent resp., YvrHb, SigV	**0.23**	Steil *et al*., 2003
spoVD	BSU15170	Penicillin-binding protein (spore cortex)	SigE, SpoIIID	**0.19**	
pbpI	BSU27310	Penicillin-binding protein 4b	SigE, SigF	**0.18**	
dacB	BSU23190	Penicillin-binding protein 5[b]	SigE	**0.15**	
spoVB	BSU27670	Sporulation-specific MurJ family lipid II flippase	SigE, SpoIIID	**0.14**	
pbpG	BSU37510	Penicillin-binding protein 2d	SigF, SigG	**0.13**	
pbpX	BSU16950	Penicillin-binding protein X	SigM, SigX, SigV	**0.08**	Hahne *et al*., 2010
dacF	BSU23480	Penicillin-binding protein I	AbrB, SigF, SigG	**0.04**	
Cell shape (2 of 10 genes)[b]					
mreBH	BSU14470	Cell shape-determining protein	SigI, WalR	**4.00**	
mbl	BSU36410	MreB-like protein	SigE, stringent resp.	**0.47**	
Autolysis and peptidoglycan hydrolases (17 of 37 genes)[b]					
blyA	BSU21410	N-acetylmuramoyl-L-alanine amidase		**58.04**	
yqiI	BSU24190	Peptidoglycan hydrolase		**23.60**	Steil *et al*., 2003; Hahne *et al*., 2010; Fischer and Bremer, 2012
cwlT	BSU04970	Cell wall hydrolase	ImmR	**21.08**	
xlyA	BSU12810	N-acetylmuramoyl-L-alanine amidase	Xpf	**6.52**	Hahne *et al*., 2010
xepA	BSU12780	PBSX prophage lytic exoenzyme	Xpf	**5.41**	Hahne *et al*., 2010
cwlP	BSU21350	Cell wall hydrolase, wall turnover		**4.62**	
yocH	BSU19210	Peptidoglycan hydrolase	Spo0A, WalR, AbrB	**3.78**	Steil *et al*., 2003; Hahne *et al*., 2010
lytE	BSU09420	Major autolysin, cell elongation and separation	Spo0A, SigI, WalR, SigH	**3.15**	
xlyB	BSU12460	N-acetylmuramoyl-L-alanine amidase		**2.74**	
lytABC	BSU35640 … BSU35620	Major autolysin, cell separation, wall turnover	SinR, SigD, YvrHb, SlrR	**2.04**	
lytF	BSU09370	Major autolysin, cell separation	SinR, SigD, SlrR	0.78	Steil *et al*., 2003; Hahne *et al*., 2010
lytD	BSU35780	Major autolysin, cell separation	SigD	**0.29**	
cwlH	BSU25710	Cell wall hydrolase, N-acetylmuramoyl-L-alanine amidase GerE, SigK		**0.29**	
spoIIP	BSU25530	Cell wall hydrolase	SigF, SpoVT, SigE	**0.24**	
lytH	BSU32340	Peptidoglycan hydrolase	SigG	**0.21**	
iseA	BSU18380	Inhibitor of autolysins	WalR	**0.12**	Steil *et al*., 2003
cwlD	BSU01530	Cell wall hydrolase, N-acetylmuramoyl-L-alanine amidase	LexA, SigE, SigG	**0.11**	
cwlJ	BSU02600	Cell wall hydrolase, spore coat protein	SigE, SpoIIID	**0.02**	

(Continues)

Table 2. Continued

Gene name[a]	Locus	Product/function	Regulator	Expression ratio[a] (1.2 M NaCl/ no NaCl)	Reference(s)
Capsule biosynthesis and degradation (7 of 10 genes)[b]					
capBCAE	BSU35900 … BSU35870	Capsule biosynthesis	DegU	**3.31**	Hahne *et al*., 2010
ggt	BSU18410	Gamma-glutamyltransferase		**3.02**	
pghZ	BSU20460	Phage-derived gamma polyglutamic acid hydrolase		**2.19**	
pghL	BSU17820	Phage-derived gamma polyglutamic acid hydrolase	SigK	**0.22**	Hahne *et al*., 2010
Cell division (1 of 35 genes)[b]					
gpsB	BSU22180	Cell division adaptor protein, member of the divisome		1.9	Steil *et al*., 2003
mciZ	BSU23616	FtsZ inhibition peptide	SigE	**0.01**	
Other (11 of 25 genes)[b]					
ybfG	BSU02200	Putative pepdidoglycan binding protein		**7.54**	Steil *et al*., 2003
slp	BSU14620	Small peptidoglycan-associated lipoprotein		**3.89**	Hahne *et al*., 2010
ykfC	BSU12990	D-Glutamyl-L-amino acid peptidase	CodY	**2.38**	
spoIIM	BSU23530	Dissolution of the septal cell wall	SigE	**0.23**	
spoIID	BSU36750	Lytic transglycosylase	SigE, SpoIIID	**0.21**	
ydhD	BSU05710	Spore coat glycosylase	SigE	**0.18**	
gamAP	BSU02360, BSU02350	Glucosamine utilization	GamR	**0.17**	
wprA	BSU10770	Secreted quality control protease	YvrHb, CcpA	**0.14**	Hahne *et al*., 2010
wapA	BSU39230	Cell wall-associated protein precursor	YvrHb, WalR, DegU	**0.02**	Steil *et al*., 2003

a. Significantly differentially expressed genes of the present study are marked in bold (growth in SMM with 1.2 M NaCl compared to SMM without additional salt, expression ratio ≤ 0.5 or ≥ 2 and Benjamini–Hochberg corrected *p*-value ≤ 0.05).
b. Number of significantly differentially expressed genes compared to the total number of genes belonging to a functional category (according to the SubtiWiki database).

sigma factor. Previous studies reported transient induction of the regulons controlled by the ECF sigma factors SigM and SigW following salt shock as well as downregulation of SigX-regulated genes (Steil *et al*., 2003; Hahne *et al*., 2010). In agreement with these data, we observed downregulation of significant portions of the SigV and SigX regulons, whereas the SigM and SigW regulons were not significantly enriched for upregulated genes during prolonged growth under high-salinity conditions. Among others, our study newly identified upregulation of *mreBH* encoding a cell shape-determining protein that is also required for the activity of the cell wall hydrolase LytE. The *mreBH* and *lytE* genes are regulated by the WalKR two-component regulatory system and the alternative sigma factor SigI (Salzberg *et al*., 2013).

SigI, which is mainly involved in cell wall metabolism, also directly promotes transcription of genes subject to regulation by the Fur repressor (Baichoo *et al*., 2002), namely the *dhbACEBF* and *ykuNOP* operons (Ramaniuk *et al*., 2018), which were more than 10-fold induced during growth in high-salinity medium. Under these conditions, cells apparently experience severe iron limitation, leading to the induction of the Fur regulon (Hoffmann *et al*., 2002; Steil *et al*., 2003), of which 30 genes were significantly upregulated in *B. subtilis* BSB1 grown at high versus low salinity (Fig. 2A, Table S1). Restrictions in iron availability to osmotically challenged cells probably result from reduced solubility of iron in high salinity solutions and from the fact that *B. subtilis* strains derived from the domesticated 168 lineage carry a mutation in *sfp* gene leading to insufficient production of the siderophore bacillibactin (May *et al*., 2001; Hoffmann *et al*., 2002).

Expression of the SigD-dependent major autolysin genes (*lytC*, *lytD* and *lytF*) was differently affected: Whereas expression of the *lytABC* operon controlled by SigD and the SlrR-SinR complex (see below) was increased under high-salinity conditions, that of *lytD* was decreased and *lytF* mRNA levels were nearly unaffected. The alternative sigma factor SigD of *B. subtilis* controls genes involved in motility and chemotaxis. Downregulation of the SigD regulon under high-salinity conditions was already observed by Steil and colleagues (2003) and could

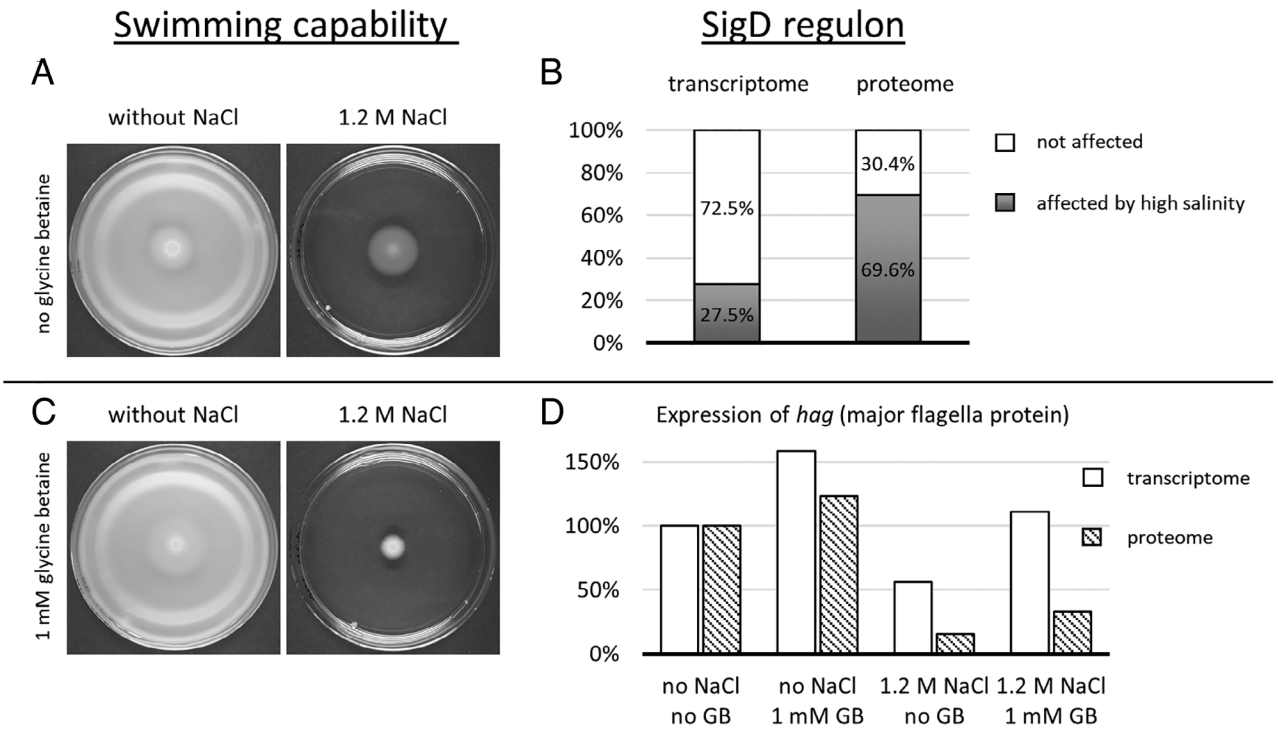

Fig 3. Influence of high salinity on motility of *B. subtilis*.
The *B. subtilis* strain BSB1 was inoculated onto soft agar plates without or with 1.2 M NaCl. (A) or soft agar plates containing 1 mM glycine betaine without or with 1.2 M NaCl. (B) Bar chart displaying the proportion of SigD-regulated genes and proteins that exhibited significantly reduced levels under high-salinity-conditions. In the proteome, 46 of 120 SigD regulon members were identified (set to 100%). (C) Swimming capability was documented after growth at 37 °C overnight. (D) Levels of hag mRNA and Hag protein under the different growth conditions. Values were normalized to growth in SMM without NaCl and glycine betaine.

be linked to a role of the DegSU two-component regulatory system in the control of salt-mediated repression of the *fla*/*che* operon (see below). It comprises 32 genes (including the *sigD* gene), which encode flagella components and chemotaxis constituents. SigD is involved in the control of the *fla*/*che* operon and activates transcription of motility genes outside the *fla*/*che* operon like *hag* (encoding flagellin) and the *motAB* operon (encoding flagellar stator proteins; Estacio *et al*., 1998; Serizawa *et al*., 2004). In the present study, 34 genes of the SigD regulon including 12 genes of the *fla*/*che* operon (Cozy *et al*., 2012) were significantly lower expressed during growth of *B. subtilis* BSB1 at high salinity. For the *B. subtilis* JH642 *sigB* mutant Steil and colleagues (2003) observed a strong downregulation of 50 SigD controlled genes. Since the impact of high salinity on the expression of motility and chemotaxis genes is apparently less pronounced in the *B. subtilis* 168 Trp^+ (BSB1) strain than in JH642, the swimming capability of *B. subtilis* BSB1 under low and high salt conditions was examined on soft agar plates. Indeed, motility of the cells was severely impaired in the presence of 1.2 M NaCl (Fig. 3A). Strikingly, at the proteome level the fraction of the SigD regulon affected by high salinity was significantly higher as compared to the transcriptome (69% versus 27%; Fig. 3B; Table S3). The level of Hag, the structural protein of the flagellum, was more than sixfold reduced in cells grown in the presence of 1.2 M NaCl, whereas the level of the *hag* mRNA decreased only a 1.8-fold (Fig. 3D).

Global regulators involved in osmotic signalling and adaptation

Our data indicate that, in addition to Spo0A, the activity of three global regulators, DegU, AbrB and SigB, was influenced by high-salinity growth conditions (Fig. 2A). The DegSU two-component regulatory system is involved in the complex network that mediates the regulation of cellular processes during the transition from exponential to stationary phase. DegU is crucial for competence development, but is also involved in the regulation of degradative enzyme production, biofilm development and flagellar motility (reviewed in Murray *et al*., 2009). Salt-mediated differential expression of DegU-controlled genes including the *fla*/*che* operon and a role of the DegSU TCS in sensing salt stress were previously reported (Kunst and Rapoport, 1995; Dartois *et al*., 1998;

Ruzal and Sanchez-Rivas, 1998; Steil *et al*., 2003). At low phosphorylation levels, DegU activates transcription of the *fla/che* operon in the presence of the regulator SwrA, whereas high levels of phosphorylated DegU are assumed to inhibit flagellar-based motility in undomesticated strains (Kearns and Losick, 2005; Verhamme *et al*., 2007; Mordini *et al*., 2013). The laboratory strain *B. subtilis* 168 that lacks a functional *swrA* gene (Kearns *et al*., 2004) shows strongly reduced expression of the *fla/che* operon in the presence of high levels of DegU-P (Mäder *et al*., 2002; Amati *et al*., 2004). Expression patterns of other DegU-controlled genes revealed by our study (Table S1) support the notion that *B. subtilis* cells exhibit high DegU-P levels under hyperosmotic growth conditions. In particular, the *aprE* and *capBCEA* operons whose activation requires high DegU-P levels were significantly higher expressed (Verhamme *et al*., 2007; Cairns *et al*., 2013).

The transition state regulator AbrB contributes to the regulation of numerous genes, including those for biofilm formation and the production of antimicrobial compounds (Hamon *et al*., 2004; Strauch *et al*., 2007; Chu *et al*., 2008). Although the majority of AbrB-controlled genes are subject to multiple regulation, 11 of 80 regulon members showing reduced expression at 1.2 M NaCl are supposed to be exclusively regulated by AbrB, including the *ppsABCDE* operon for biosynthesis of the lipopeptide antibiotic plipastatin. The *abrB* gene is under negative control of Spo0A-P (Strauch *et al*., 1990) and the gene encoding the inhibitor of AbrB activity, AbbA, is under positive control of Spo0A-P (Banse *et al*., 2008). It can thus be assumed that low Spo0A-P levels found in *B. subtilis* cells grown at high salinity (Widderich *et al*., 2016) trigger the lower expression of AbrB-regulated genes. However, no differences in *abrB* and *abbA* mRNA and protein levels were detected under the conditions of our study, indicating that AbrB activity, which is presumably modulated by phosphorylation (Kobir *et al*., 2014), rather than its expression level, might be responsible for AbrB-dependent changes in gene expression under high-salinity conditions.

Increased expression of the general stress regulon under high-salinity growth conditions

More than half of the genes assigned to the SigB-controlled general stress regulon (Helmann *et al*., 2001; Petersohn *et al*., 2001; Price *et al*., 2001; Nicolas *et al*., 2012) exhibited significantly higher expression levels in cultures grown at high salinity (Fig. 2A). The alternative sigma factor SigB is known to be activated by osmotic stress, but has been mainly implicated in the short-term response to a sudden osmotic upshift (Völker *et al*., 1995; Young *et al*., 2013). Therefore, we compared induction ratios of SigB-regulated genes during continuous growth at high versus low salinity with those measured 10 min after an osmotic upshift elicited by addition of 0.4 M NaCl using the same tiling array design (Nicolas *et al*., 2012). The data revealed a clear but submaximal induction of the SigB regulon even in cells subjected to prolonged growth under high-salinity conditions (Fig. 4A). The median induction was 18-fold in response to a sudden osmotic upshift but only threefold during continuous growth at high salinity. Using single cell measurements Young and colleagues (2013) recently proposed a single-pulse transient activation of SigB following exposure to environmental insults including salt shock. While we confirm maximal transient activation of SigB following the sudden imposition of salt stress, our data clearly show that SigB activity remains higher than basal level following continuous growth at high salinity during cultivation in shake flasks. This sustained SigB-dependent response of *B. subtilis* is likely caused by the high NaCl concentration used (1.2 M) and in agreement with a previous study by Cabeen and colleagues (2017) showing that high ethanol concentrations also provoke a sustained SigB-dependent stress response.

Bacillus subtilis uses two distinct stress-sensing pathways for activation of SigB, which respond to energy stress (ATP depletion) and to environmental stressors respectively. SigB is kept inactive by its anti-sigma factor RsbW and is released from the SigB/RsbW complex by the unphosphorylated form of the anti-anti-sigma factor RsbV (Alper *et al*., 1996; Yang *et al*., 1996). RsbV is dephosphorylated by either of two phosphatases, RsbU and RsbP. Environmental stress signals are transduced by a large multiprotein complex, the stressosome, that controls the activity of RsbT (reviewed in Marles-Wright and Lewis, 2008), the positive regulator of the phosphatase RsbU (Völker *et al*., 1995; Yang *et al*., 1996), while energy stress signals are conveyed to SigB via RsbQ and the phosphatase RsbP (Vijay *et al*., 2000; Brody *et al*., 2001). In order to confirm that SigB activation during continuous growth of *B. subtilis* at high salinity is mediated by the RsbU-dependent 'stress pathway', we made use of *rsbP* and *rsbU* deletion mutants (Brigulla *et al*., 2003; Table 3). The strains were grown in SMM until OD_{600} of 0.3 and subjected to salt shock by the addition of 0.4 M NaCl or continuously grown in SMM containing 1.2 M NaCl. RNA was isolated from cells harvested before and 10 min after salt shock and from the high-salinity grown culture after reaching an OD_{600} of 1. Northern blot analysis of the transcript levels of the SigB-regulon members *gspA* and *gsiB* revealed that their basal SigB-dependent expression depends on the RsbP phosphatase, whereas induction by salt shock and during continuous growth at high salinity depends on the RsbU phosphatase (Fig. 4B).

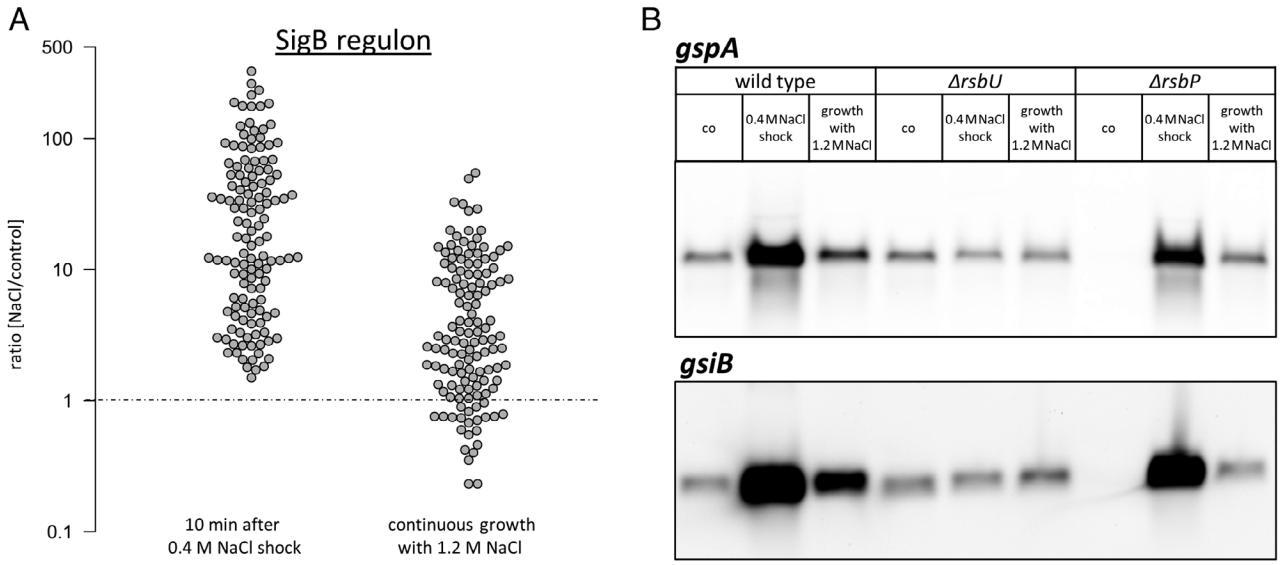

Fig 4. (A) Induction of the general stress regulon in response to a sudden osmotic upshift (Nicolas *et al*., 2012) and during continuous growth of *B. subtilis* BSB1 at high salinity. Genes classified as SigB regulon members by two previously reported independent studies (Nannapaneni *et al*., 2012; Nicolas *et al*., 2012) are shown. (B) Northern blot analysis of the SigB-dependent *gspA* and *gsiB* transcript levels in *B. subtilis* 168 (wild type), BSM21 (168 Δ*rsbU*) and BSM30 (168 Δ*rsbP*), immediately before (co) and 10 min after salt shock (0.4 M NaCl) and during continuous growth at high salinity (1.2 M NaCl). For each sample 3 μg of total RNA per lane were loaded.

Table 3. *Bacillus subtilis* strains used in this study.

Strain	Genotype	Reference
BSB1	*trp*$^+$	Nicolas *et al*., 2012
168	*trpC2*	Anagnostopoulos and Spizizen, 1961
BSM21	*trpC2 rsbU::kan*	Brigulla *et al*., 2003
BSM30	*trpC2 rsbP::spc*	Brigulla *et al*., 2003

Involvement of the CymR regulon and the oxidative stress response in high-salinity adaptation

Beside the regulons controlled by SigB, Fur and SigI, the CymR regulon was particularly strongly enriched for genes higher expressed during growth of *B. subtilis* at high salinity (Fig. 2A). The global regulator CymR represses the transcription of more than 40 genes involved in cystine uptake and cysteine biosynthesis in response to cysteine availability (Even *et al*., 2006; Tanous *et al*., 2008). Of these, 23 genes were significantly higher expressed during growth of *B. subtilis* 168 (Trp$^+$) in SMM with 1.2 M NaCl compared to medium without additional salt. Among them were the *ssuBACD* operon for aliphatic sulfonate uptake and degradation, *cysK* which encodes cysteine synthase, and *mccAB* which is involved in methionine-to-cysteine conversion. Cysteine and other low molecular weight thiols such as bacillithiol play important roles in protecting cells against reactive oxygen and electrophilic species (reviewed in Chandrangsu *et al*., 2018). In line with this, the CymR regulon has been implicated in oxidative stress adaptation of *B. subtilis* (Hullo *et al*., 2010) and *S. aureus* (Soutourina *et al*., 2010) and was shown to be induced by thiol-reactive electrophiles such as diamide and by superoxide stress (Leichert *et al*., 2003; Mostertz *et al*., 2004; Nguyen *et al*., 2009).

Salt stress was suggested to trigger a secondary oxidative stress response in *B. subtilis* (Höper *et al*., 2006; Reder *et al*., 2012) and other bacteria, for example *Bacillus licheniformis* (Schroeter *et al*., 2013). Moreover, growth of a *B. subtilis* mutant lacking bacillithiol is strongly reduced under high-salinity conditions (Gaballa *et al*., 2010). In light of these findings, the observed induction of the CymR regulon during growth of *B. subtilis* in the presence of 1.2 M NaCl supports a link between hyperosmotic conditions and oxidative stress in *B. subtilis*. Our findings suggest an increased cellular requirement for low molecular weight thiols that function to maintain the protein thiol redox state and to protect active site cysteine residues by S-thiolation (Chi *et al*., 2011). In addition to the CymR regulon, a few other genes involved in the oxidative stress response of *B. subtilis* showed induction by high-salinity, including the SigB-controlled catalase gene *katE*, *mhqNO* (involved in resistance to quinones and diamide) and *ohrA* (peroxiredoxin involved in protection against organic peroxides).

Strong downregulation of the biofilm matrix operons

In addition to different types of cellular differentiation of planctonic cells (e.g. sporulation, development of competence), *B. subtilis* can form biofilms on surfaces. In these morphologically complex structures, cells of different types coexist in a self-produced extracellular matrix (Vlamakis *et al*., 2013; Kalamara *et al*., 2018). Among the genes that displayed the strongest downregulation in the presence of 1.2 M NaCl were those of the *tapA-sipW-tasA* operon (48-, 178- and 157-fold lower expression; Table S1). The encoded proteins are required for polymerization of the amyloid-like protein TasA, a major component of the *B. subtilis* biofilm matrix (reviewed in Cairns *et al*., 2014). The second biofilm matrix operon, *epsA-O* encoding the enzymes for synthesis of the extracellular polysaccharide, was also strongly downregulated (>20-fold). The *tapA* and *eps* operons are repressed by SinR, the master regulator of biofilm formation, when Spo0A-P levels are low. Phosphorylation of Spo0A stimulates transcription of the gene encoding the antagonist protein SinI, which inhibits the DNA-binding activity of SinR. The transcriptional regulator SlrR can also bind to SinR, and the SlrR-SinR complex acts as a repressor of motility and autolysin genes (Chai *et al*., 2010). By concomitant activation of biofilm matrix genes with repression of motility genes, biofilm formation and motility are mutually exclusive cell fates in *B. subtilis* (Vlamakis *et al*., 2013). Under high salinity growth conditions, Spo0A levels and activity are reduced (Widderich *et al*., 2016). Supporting the assumption that the observed expression pattern of the *tapA* and *eps* operons is associated with low Spo0A-P levels, *sinI* expression was 5.5-fold and 8-fold reduced on the mRNA and protein level, respectively, and the *slrR* mRNA amount was 16-fold reduced in cells grown at high versus low salinity (Table S1). This result is in agreement with a regulatory feedback loop that enhances expression of the SinR target gene *slrR*. By forming a complex with SinR, SlrR switches the DNA binding specificity of SinR and prevents binding of SinR to its target promoters. Interestingly, in contrast to other conditions, during growth of *B. subtilis* in the presence of 1.2 M NaCl, strong repression of biofilm genes as well as reduced swimming motility are observed at the same time. As described above, lower expression of SigD-dependent motility and autolysin genes most probably results from high DegU-P levels under high-salinity conditions.

To test if repression of biofilm genes results in a defect in biofilm formation, we tested the ability of *B. subtilis* to form complex colonies as a function of growth medium osmolarity. It was previously shown that domesticated *B. subtilis* 168 strains can develop architecturally complex colonies on different media (Gallegos-Monterrosa *et al*., 2016). *Bacillus subtilis* BSB1 exhibited complex colony architecture when grown on MSgg medium without additional NaCl (Fig. 5A). Strikingly, complex colony formation was already reduced at 0.25 M NaCl and completely abolished in the presence of 0.75 M NaCl. *Bacillus subtilis* was not able to grow on MSgg medium with higher NaCl concentration (Fig. 5A).

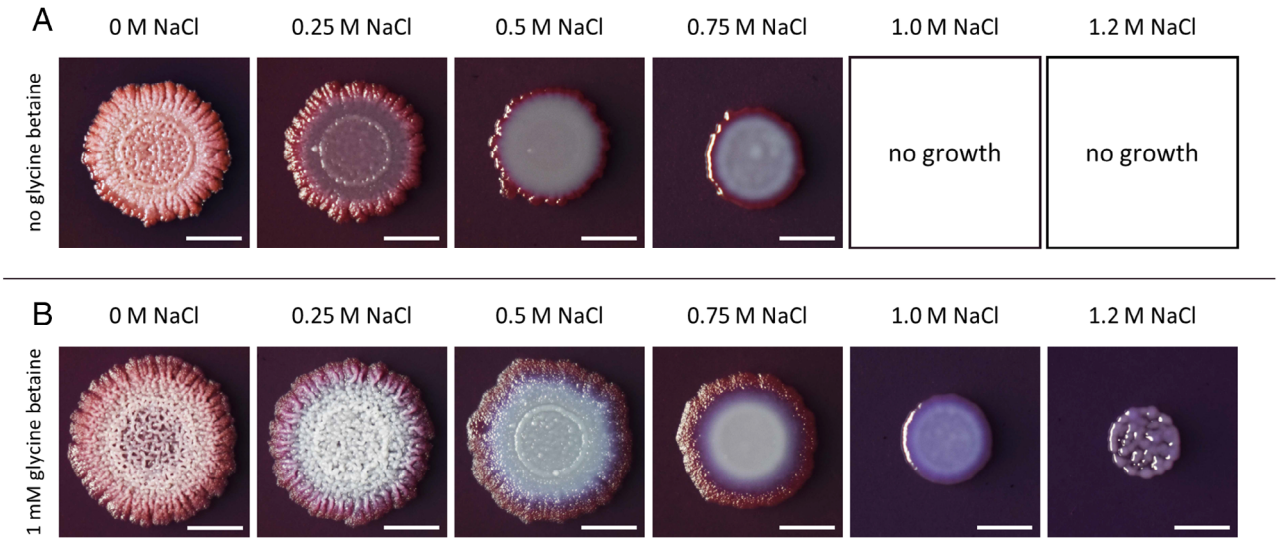

Fig 5. Effects of increasing salinity on biofilm formation in the absence or the presence of glycine betaine. *Bacillus subtilis* BSB1 cells were spotted onto MSgg agar plates containing the indicated concentrations of NaCl in the absence (A) or in the presence (B) of 1 mM glycine betaine. Colonies were imaged after 3 days of incubation at 30 °C. *Bacillus subtilis* BSB1 cells were not able to grow on plates containing 1 M or 1.2 M NaCl, respectively, when no glycine betaine was added. Scale bars: 0.5 cm.

Effects of glycine betaine on gene expression

By accumulating compatible solutes, bacteria are able to maintain turgor at physiologically adequate values, reduce the level of molecular crowding, and optimize the solvent properties of the cytoplasm (Wood, 2011; Stevenson *et al*., 2015a; Bremer and Krämer, 2019). Collectively, this allows the cells to proliferate under unfavourable hyperosmotic conditions. One of the most widely used and particularly effective osmoprotectants in microorganisms is glycine betaine (Kempf and Bremer, 1998; Gunde-Cimerman *et al*., 2018). Previous studies with different microorganisms have shown that its import can influence gene expression. In particular, genes involved in the synthesis or uptake of compatible solutes exhibit reduced induction under osmotic stress conditions (Spiegelhalter and Bremer, 1998; Brill *et al*., 2011; Hoffmann *et al*., 2013). The intracellular glycine betaine pool generated by *B. subtilis* through import is linearly dependent on the salt concentration of the growth medium and reaches 500 mM in the presence of 1 M NaCl (Hoffmann *et al*., 2013). Glycine betaine accumulates to substantial level (about 160 mM) even in *B. subtilis* cells grown in the standard laboratory medium SMM (about 350 mosmol kg^{-1}) without additional salt (Hoffmann *et al*., 2013), indicating that these cells do not truly live stress-free (Hallsworth, 2018). However, to what extent these levels of glycine betaine can affect gene expression, has not been explored so far. Therefore, we included in our study samples of *B. subtilis* BSB1 grown in SMM with 1 mM glycine betaine and in SMM with 1.2 M NaCl/1 mM glycine betaine in order to determine the effects of glycine betaine under both standard and high-salinity growth conditions.

Under high-salinity conditions, the transcript levels of 427 and 546 genes were up- and downregulated, respectively, when 1 mM glycine betaine was present in the growth medium (Fig. 6; Table S1). Generally, two groups of high-salinity responsive genes can be distinguished according to the effect of glycine betaine. The expression pattern of group 1 genes is not influenced by the presence of glycine betaine. For group 2, the high-salt-mediated regulatory pattern is at least partially reversed by glycine betaine, e.g. Figure 2B: genes belonging to the SigB, CymR, DegU, SigD, SinR and AbrB regulons (Fig. S4). Interestingly, a transcriptome study of *Escherichia coli* exposed to continuous osmotic stress revealed only minor effects of glycine betaine on the expression of osmotically induced genes (Gunasekera *et al*., 2008).

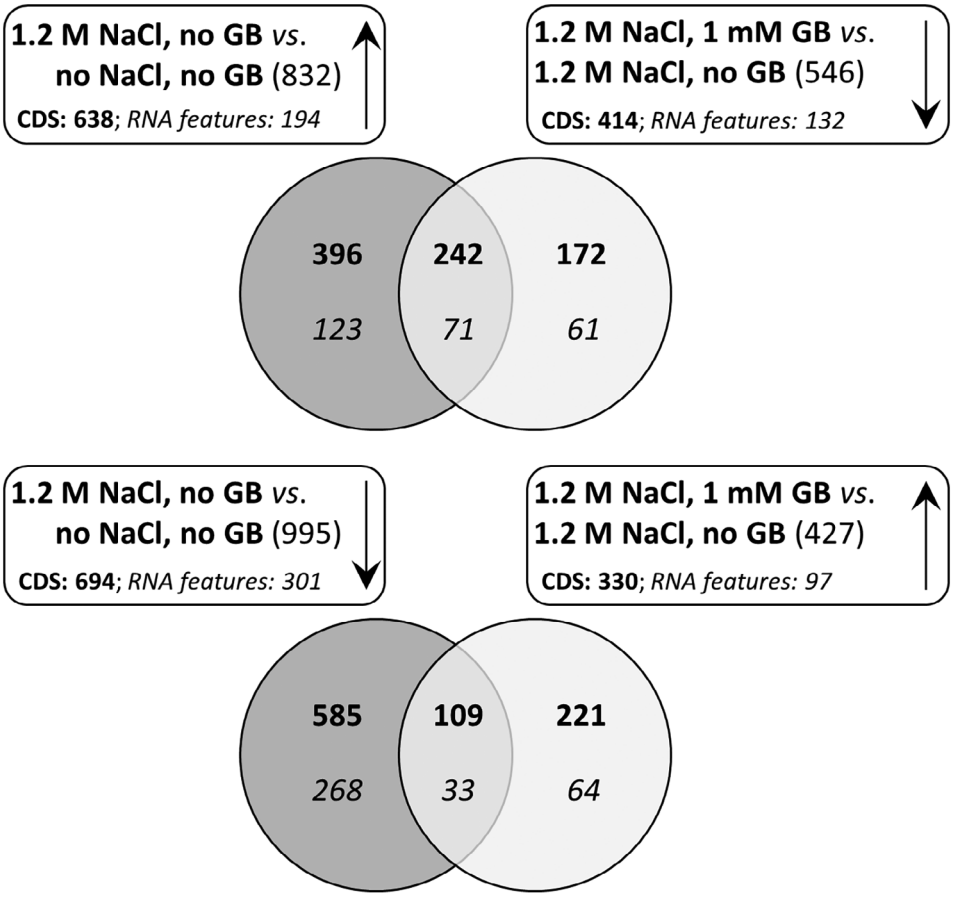

Fig 6. Effects of glycine betaine on high-salinity regulated genes. Venn diagrams show the overlap of genes differentially expressed between SMM with 1.2 M NaCl and SMM (left) and between SMM with 1.2 M NaCl and 1 mM glycine betaine and SMM with 1.2 M NaCl (right). Each group is subdivided into coding sequences (CDS; bold) and RNA features according to Nicolas and colleagues (2012) (italic).

Of the genes expressed at higher level during growth of *B. subtilis* at high versus low salinity, almost 40% (313 of 832; 242 CDS and 71 RNA features) displayed a diminishing effect of the glycine betaine addition (Fig. 6). All high-salinity induced *opu* transporter genes (*opuE* gene, *opuA* and *opuB* operons) belonged to this group of 313 genes. Expression of the *gbsAB* operon showed the same regulatory pattern, but its mRNA level was only 1.9-fold reduced in the presence of glycine betaine.

For the genes that were lower expressed at high versus low salinity, the proportion of genes for which glycine betaine caused the diminishing effect was significantly smaller (142/995, 14%; 109 CDS and 33 RNA features; Fig. 6), because the group of sporulation genes was largely not influenced by glycine betaine. Of the 420 CDSs controlled by SigE, SigF, SigG and SigK, respectively, that displayed lower expression levels under high-salinity conditions, only 14 were positively influenced by glycine betaine (Fig. 2B; Table S1). Thus, our data show that the repressive effects of high salinity on sporulation gene expression are largely independent of the availability of glycine betaine in the growth medium (Fig. S2). In contrast, transcript levels of biofilm genes (*tapA* and *eps* operons) were significantly higher when glycine betaine was present in the high-salt medium, albeit not reaching the levels found in cultures grown in SMM without additional NaCl (Fig. 2B; Table S1). These data are in excellent agreement with the biofilm formation assays. Under high salinity conditions, glycine betaine (1 mM) rescued the ability of *B. subtilis* to form complex colonies on MSgg medium to a considerable degree (Fig. 5B).

We also analysed the effect of glycine betaine on motility under high-salinity conditions. Although for almost half of the salt-affected SigD-regulated genes, glycine betaine had a diminishing effect on the salt-mediated repression of their expression (Fig. 2B), swimming of *B. subtilis* BSB1 was still severely impaired on soft agar plates containing 1.2 M NaCl and 1 mM glycine betaine (Fig. 3C). Perhaps, this still reduced motility might be caused by failure of glycine betaine to restore proper levels of *hag* encoded flagellin. According to our proteome data, Hag exhibited fourfold reduced levels under these conditions compared to medium with 1 mM glycine betaine and no additional salt (Fig. 3D).

Next, we analysed the extent of gene expression changes caused by the addition of 1 mM glycine betaine under low salinity conditions (SMM without additional salt). Overall, we observed only small effects of glycine betaine under this condition (Table S1). Seventy-three genes were significantly higher expressed in the presence of 1 mM glycine betaine. The number of downregulated genes was substantially higher (627); however, most of these genes (538/627) were sporulation genes under the control by SigE, SigF, SigG and SigK. The effect of glycine betaine on the expression of sporulation genes might be due to the slightly higher growth rate under this condition (Dawes and Mandelstam, 1970). In order to reveal the effects of glycine betaine that are independent of the medium salinity, the overlap of differentially expressed genes between both low and high salinity conditions was determined (Fig. S5). Forty-three genes (29 annotated CDSs and 14 RNA features) displayed higher transcript levels and 103 genes (81 annotated CDSs and 22 RNA features) displayed lower transcript levels during growth of *B. subtilis* in glycine betaine containing medium. One-third (9/29) of the upregulated genes are known to be under of the control of ComK, the master regulator for entry into the competence state, including the auto-regulated *comK* gene, which displayed 2.4-fold (SMM) and 5.5-fold (SMM with 1.2 M NaCl) higher expression levels in the presence of glycine betaine. Among the 81 downregulated CDSs were the *opuA*, *opuB* and *opuC* operons involved in osmoprotectant uptake. The *proHJ* operon required for osmo-stress-adaptive proline synthesis displayed the same expression pattern, but its mRNA level was only 1.9-fold reduced by glycine betaine under high-salinity conditions. The data support previous observations that these genes are already transcriptional active at the relatively low osmolarity of the widely used and chemically defined SMM (about 350 mosmol kg^{-1}) in the absence of additional NaCl (Hoffmann *et al*., 2013), an effect that can be reduced by the presence of glycine betaine in the medium. At the proteome level, comparatively few changes triggered by glycine betaine were observed, both under low and high salinity conditions (Fig. S6). This is likely due to the fact that the effects of glycine betaine on gene expression were generally smaller in magnitude than those caused by the addition of 1.2 M NaCl to the growth medium.

Conclusions

In this study, we performed combined transcriptome and proteome analysis of *B. subtilis* BSB1 cells continuously cultivated at low (SMM) or high salinity (SMM with additional 1.2 M NaCl). Increased salinity affected the transcriptional profile of the osmotically stressed cells on a genome-wide scale. More than one-fourth of the protein-coding genes and numerous non-coding RNAs were significantly differentially expressed between high- and low-salinity-grown cells. The data confirmed previous findings regarding (i) the induction of genes involved in uptake and synthesis of osmoprotectants, (ii) high-salinity-induced iron limitation, (iii) suppression of sporulation and (iv) the effects of high salinity on cell envelope functions and motility. Furthermore, our study revealed additional cellular responses to the osmotic challenge such

as a sustained low-level induction of the SigB-dependent general stress response and the induction of the CymR regulon presumably indicating that cells experience increased oxidative stress under high-salinity growth conditions. A regulon-based analysis identified transcriptional regulators particularly involved in the adaptation of *B. subtilis* to high-salinity growth conditions, revealing, among others, changes in the activity of four global regulatory proteins, namely Spo0A, DegU, AbrB and SigB. Strikingly, during growth in high-salinity surroundings *B. subtilis* cultures do not only display reduced swarming motility, but also a strong repression of biofilm matrix genes, most probably resulting from low Spo0A-P levels. This observation provides a new facet in understanding the impact of high salinity on the developmental pathways of *B. subtilis*. A link between biofilm matrix gene expression and the osmostress response has been established previously. In addition to their repression by SinR, the *tapA* and *eps* operons are activated by the biofilm regulator RemA that also functions as an activator for transcription of the *opuA*, *opuB* and *opuC* operons (Winkelman *et al*., 2013).

Glycine betaine is one of the most effective osmoprotectants for *B. subtilis* (Hoffmann and Bremer, 2017) and supplementation of the medium with 1 mM glycine betaine significantly reduced the expression of 40% of the high-salinity-induced genes. Interestingly, the data also revealed that the repressive effect of high salinity on sporulation gene expression is largely not affected by the availability of glycine betaine in the growth medium. Importantly, glycine betaine can influence gene expression not only under high-salinity conditions, but also during growth of *B. subtilis* in SMM without additional salt.

Experimental procedures

Media and growth conditions

Cultures of *B. subtilis* were inoculated in lysogeny broth (LB) medium and grown to an optical density at 540 nm (OD_{540}) of 0.3 at 37 °C under vigorous shaking. Prewarmed SMM (Harwood and Cutting, 1990) containing 0.5% (wt/vol) glucose as carbon source was inoculated to an OD_{600} of 0.035 by centrifuging the required volume of culture, discarding the supernatant and resuspending the bacterial pellet in SMM. For Northern blot analyses, *B. subtilis* 168 (wild type; Trp^-), BSM21 (168 Δ*rsbU*) and BSM30 (168 Δ*rsbP*) were used and L-tryptophan (20 μg/ml) was added to satisfy tryptophan auxotrophy. When the pre-cultures reached an OD_{600} of 1.0, they were diluted 1:10 in fresh, pre-warmed SMM or S.MM supplemented with 1 mM glycine betaine, 1.2 M NaCl or 1 mM glycine betaine and 1.2 M NaCl. Samples for RNA preparation were collected at OD_{600} of 1.0 by adding 0.5 volume of frozen killing buffer [20 mM Tris/HCl (pH 7.5), 5 mM $MgCl_2$, 20 mM NaN_3] and subsequent centrifugation for 3 min at 8000*g* and 4 °C. After discarding the supernatant, cell pellets were frozen in liquid nitrogen and stored at −80 °C. For proteome analysis the samples were centrifuged for 10 min at 8000*g* and 4 °C and the cell pellets were stored at −80 °C.

For salt shock experiments, *B. subtilis* cultures were inoculated from exponentially growing pre-cultures in SMM as described above. To induce hyperosmotic stress, the osmolarity of the growth medium was increased by addition of 0.4 M NaCl (pre-warmed 4 M stock solution prepared in SMM) at OD_{600} of 0.3. Samples for RNA preparation were collected immediately before and 10 min after the osmotic upshift.

Proteome analysis

Cell disruption, preparation of protein samples and peptides analyses were performed as described previously (Maass *et al*., 2011). Three biological replicates for each condition were analysed. A Q Exactive™ Plus mass spectrometer (Thermo Fisher Scientific, MA, USA) coupled with an UltiMate™ 3000 UHPLC system (Thermo Fisher Scientific, MA, USA) was used for LC-ESI MS/MS analysis of the tryptic peptide mixtures with the parameters given in Table S4 (Palma Medina *et al*., 2019). Data are available at MassiVE (ftp://massive.ucsd.edu/MSV000084766/).

MaxQuant software (Cox and Mann, 2008) version 1.5.3.8 was used to analyse the resulting raw data, analysis details are given in Table S5. The generated LFQ intensities were median scaled and imported in GeneSpring GX 7.3.1 software (Agilent Technologies, Santa Clara, CA, USA) and R 3.5.2 (R CoreTeam, 2018) for further analysis. A Student's *t*-test analysis was performed to identify significantly differentially abundant proteins, defined as those which were identified in all replicates of each condition, with a Benjamini–Hochberg corrected *p*-value (FDR) less than 0.05 and a mean ratio of ≤ 0.5 or ≥ 2. On/off proteins, which were exclusively identified in only one condition in three biological replicates are also considered as significantly differentially present.

RNA preparation and Northern blot analysis

Total RNA was prepared by acid-phenol extraction following mechanical cell disruption as described previously (Nicolas *et al*., 2012). The quality of the RNA was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's instructions. Northern blot analysis was performed as described by

Table 4. Oligonucleotides for Northern blot probes used in this study.

Name	Sequence (5′–3′)
gspA_for	TTGAGGAAAGATGAAATCAT
gspA_rev.T7[a]	CTAATACGACTCACTATAGGGAGAATTCAGTGCATCTTGGTCAT
gsiB_for	ATGGCAGACAATAACAAAATG
gsiB_rev.T7[a]	CTAATACGACTCACTATAGGGAGTTAGTCGTTGTTGCGGGCGTTTCC

a. T7 sequences are underlined.

Homuth and colleagues (1997). Digoxigenin-labelled RNA probes were synthesized by *in vitro* transcription with T7 RNA polymerase and gene-specific PCR products as template. Primer sequences are listed in Table 4. Five micrograms of total RNA per lane was separated on denaturing 1.2% agarose gels. Chemiluminescence signals were detected using a ChemoCam Imager (Intas Science Image Instruments, Göttingen, Germany).

Transcriptome analysis

Thirty-five micrograms of total RNA per sample was DNase-treated using the RNase-Free DNase Set (Qiagen, Hilden, Germany) and purified using the RNA Clean-Up and Concentration Kit (Norgen, Biotek Corp., Thorold, Canada). After quality control (Agilent 2100 Bioanalyzer), 10 μg of purified RNA was subjected to microarray analysis. Synthesis and fluorescence labelling of cDNA followed a strand-specific method using the FairPlay III Microarray Labeling Kit (Agilent Technologies, Santa Clara, CA, USA) and Actinomycin D (Calbiochem, Merck KGaA, Darmstadt, Germany; Nicolas *et al*., 2012). One thousand and two hundred nanograms of Cy3-labelled cDNA was hybridized to a tiling array (containing 383 238 probes covering both strands of the *B. subtilis* 168 genome with a tiling step of 22 nucleotides) following Agilent's hybridization, washing and scanning protocol (One-Color Microarray-Based Gene Expression Analysis, version 5.5). Data were extracted and processed using the Feature Extraction software (version 11.5.1.1). For each gene, the median intensity of probes lying entirely within the corresponding genomic region was calculated, and further data analysis was performed using GeneSpring GX 7.3.1 software (Agilent Technologies, Santa Clara, CA, USA). A *t*-test analysis was performed to identify significantly differentially expressed genes, defined as those with a Benjamini–Hochberg corrected *p*-value (FDR) less than 0.05 and a mean expression ratio of ≤ 0.5 or ≥ 2. Further data visualization and analysis was done in R 3.5.2 (R Core Team, 2018). The tiling array data set is available from NCBI's Gene Expression Omnibus (GEO) database (accession number GSE145124).

Fisher's exact test

A Fisher's exact test was performed comparing the proportion of differentially expressed genes associated with a given transcription factor regulon to the proportion of genes belonging to this regulon within all genes. To this end, the Fisher's test function in R with a Benjamini–Hochberg corrected significance threshold of 0.05 was used. Regulons were defined according to the *Subti*Wiki database (Zhu and Stülke, 2018), except the sporulation-specific regulons (SigFEGK) (Arrieta-Ortiz *et al*., 2015) and the SigB-regulon (Nannapaneni *et al*., 2012; Nicolas *et al*., 2012).

Cell motility assay

Bacillus subtilis BSB1 cells were grown in 3 ml LB medium for 3 h at 37 °C. Five hundred microlitres of this culture was used to inoculate SMM medium and SMM with 1.2 M NaCl with or without 1 mM glycine betaine respectively. When the cultures reached OD_{600} of 1.0–2.0, they were diluted into the corresponding media to an OD_{600} of 0.2. Five microlitres of dilutions was carefully spotted onto the centre of swimming plates containing 30 ml medium (1% tryptone with 0.25% agar; Steil *et al*., 2003) supplemented with the indicated concentrations of NaCl and glycine betaine. Plates were incubated for 18 h at 37 °C.

Biofilm assay

To follow biofilm development, we grew the *B. subtilis* strain BSB1 on MSgg agar plates [5 mM potassium phosphate (pH 7), 100 mM MOPS (pH 7), 2 mM $MgCl_2$, 700 μM $CaCl_2$, 50 μM $MnCl_2$, 50 μM $FeCl_3$, 1 μM $ZnCl_2$, 2 μM thiamine, 0.5% glycerol, 0.5% glutamate and 1.5% agar; Branda *et al*., 2006] with increasing NaCl concentrations (0 M; 0.25 M; 0.5 M; 0.75 M; 1.0 M and 1.2 M; final concentration) without or with 1 mM glycine betaine (final concentration). Matrix components of the biofilm colonies were stained by adding 40 μg/ml Congo red to the MSgg medium. Twenty micrograms per millilitre of Coomassie brilliant blue was added to shade the agar background. For biofilm formation, *B. subtilis* BSB1 cells

were inoculated into LB medium and allowed to grow at 37 °C for 18 h. Two microlitres of a 20-fold dilution of this culture (in fresh LB medium) were spotted onto MSgg plates. After incubation at 30 °C for 3 days biofilms were imaged.

Acknowledgement

We thank Anja Wiechert, Marc Schaffer and Jutta Gade for excellent technical assistance. English and German language versions are available (please select whichever is appropriate for this journal): (approved text for English language journal): Open access funding enabled and organized by Projekt DEAL. (approved text for German language journal): Open Access Veröffentlichung ermöglicht und organisiert durch Projekt DEAL. [Correction added on 10 August 2020, after first online publication: Projekt DEAL funding statement has been added.]

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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:

Data S1 Supporting information Figures S1–S6.

Data S2 Supporting information Table S1.

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