Philipps Universität Marburg

Analysis of *Bacillus subtilis* spore germination and outgrowth in high-salinity environments

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4. **Nagler K, Julius C, Moeller R.** 2016. Germination of spores of astrobiologically relevant *Bacillus* species in high-salinity environments. Astrobiology **16**(7), AST-2015-1419.R2. *Accepted for publication.*

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Des Weiteren wurde an den folgenden Manuskripten mitgewirkt, die nicht Teil der vorliegenden kumulativen Dissertation sind:

De Ory A, Nagler K, Carrasco B, Raguse M, Zafra O, Moeller R, de Vera M. 2016. Identification of a conserved 5'-dRP lyase activity in bacterial DNA repair ligase D and its potential role in base excision repair. Nucleic Acids Res. **44**(4):1833-1844.

Cockell C, Samuels T, Sirks E, Mayer M, Friswell I, Nicholson N, Moeller R, Nagler K, Raguse M, Schroeder A, Berger T, Rettberg P. 2015. A 500-year experiment. Astron. Geophys. **56:**1.28-1.29.

Cockell C, Samuels T, Mayer M, Sirks E, Friswell I, Moeller R, Nagler K, Raguse M, Schroeder A, Berger T, Rettberg P. 2014. The 500-year experiment. Microbiology Today **41**(2):95-96.

Für meine Familie

Contents

Table of figures

I. Summary

Upon nutrient depletion, the soil bacterium *Bacillus subtilis* can form highly resistant, metabolically dormant spores. Spores consist of a dehydrated core (harboring the spore genome) enveloped in an inner spore membrane, a peptidoglycan germ cell wall and cortex, an outer spore membrane, and a proteinaceous coat. When specific nutrients ('germinants') become available again, they can bind to germinant receptors in the inner spore membrane and induce spore revival, consisting of a germination and an outgrowth phase. During germination, spores lose their resistance, release ions and Ca^{2+}-dipicolinate (Ca^{2+}-DPA) from the core in exchange for water ('core rehydration'), and hydrolyze their cortex. When core rehydration is sufficient to allow enzymatic activity, metabolism is re-activated. This hallmarks the beginning of the outgrowth phase, during which spores undergo molecular reorganization and elongate.

The effects of high salt concentrations and osmotic stress on spore revival were previously poorly investigated, although this topic is relevant for basic research, food microbiology, soil ecology, and astrobiology. Therefore, in this doctoral thesis, the impact of high salinity on *Bacillus* spore revival was examined, primarily focusing on *B. subtilis* spore germination in the presence of high NaCl concentrations.

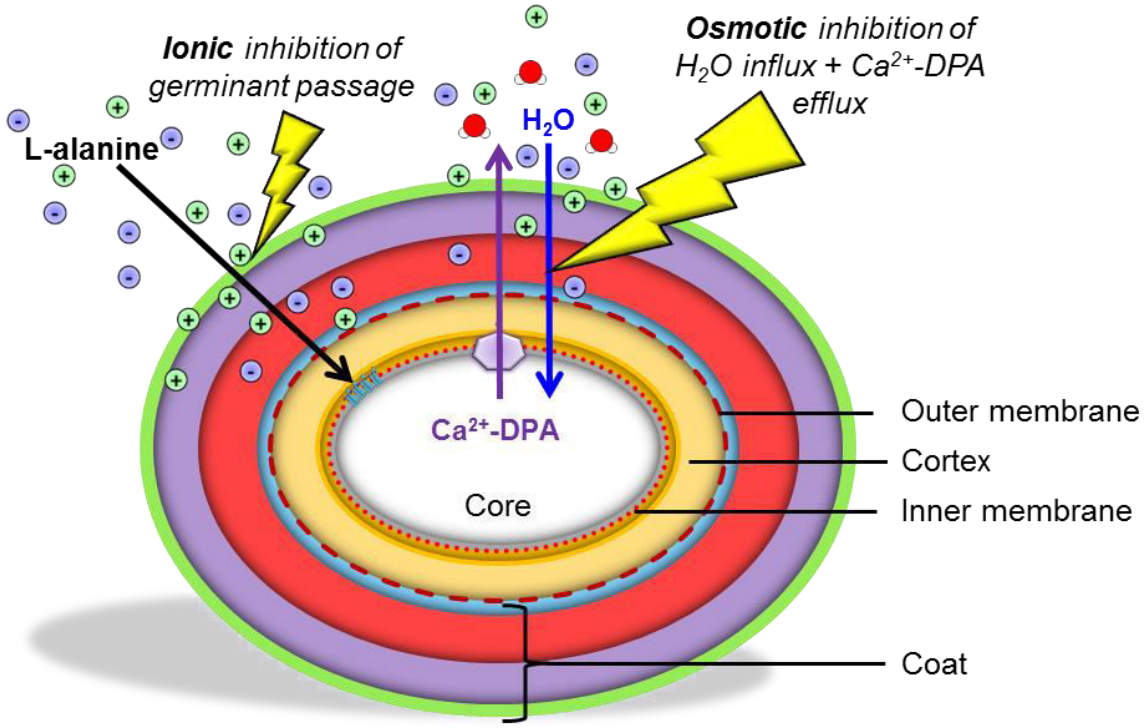
In general, increasing salt concentrations exerted increasingly detrimental effects on germination, although some spores initiated germination despite very high salinities. In the presence of high NaCl concentrations (≥ 1.2 mol/L), *B. subtilis* spore germination was delayed, slower, more heterogeneous, and less efficient. Other salts also inhibited germination, although their inhibitory strength varied depending on ion concentrations, ionic species (and their combination), and the chemical properties of the salt. Although ionic stress was indeed an important factor, high concentrations of non-ionic osmotic solutes had similar inhibitory strengths as iso-osmotic NaCl concentrations, suggesting that osmotic stress plays a decisive role in NaCl-inhibition. Strikingly, spores having strong coat defects showed exacerbated inhibition by NaCl but not by non-ionic solutes, indicating an important role of the spore coat (possibly in combination with the outer spore membrane) in protecting the subjacent inner spore structures (i.e. cortex, germ cell wall, germination apparatus, and inner spore membrane) from ionic stress.

Based on these findings, a first mechanistic model for germination inhibition by high salinity is proposed (see **graphical abstract** below). In this model, ionic interactions with the germinant and/or spore coat slow germinant passage to the germinant receptors, thereby

delaying germination initiation. Subsequently, osmotic inhibition of core rehydration and concomitant Ca^{2+}-DPA release slows germination after its initiation.

While metabolic reactivation was observable at up to 4.8 mol/L NaCl, successful outgrowth in terms of elongation was observable at up to 2.4 mol/L NaCl, but only under nutrient-rich conditions. Transcriptomic analyses of salt-stressed outgrowing spores indicated many similarities to vegetative cells exposed to sustained high salinity, including the induction of *B. subtilis'* complete genetic repertoire of osmoprotectant uptake and compatible solute synthesis.

Taken together, this doctoral thesis yielded the first mechanistic model for inhibitory high-salinity effects on *B. subtilis* spore germination as well as the first comprehensive transcriptomics study of the salt stress response of outgrowing *B. subtilis* spores. These results contribute to the basic understanding of the influence of salt on *B. subtilis'* life cycle, and are valuable for the aforementioned applied research fields as well.



Graphical abstract: Schematic depiction of the proposed mechanistic model for NaCl inhibition of *B. subtilis* spore germination. According to the model, ionic interactions with the nutrient germinant (e.g. L-alanine) and/or the spore coat slow nutrient passage through the spore integuments, thereby delaying germination initiation. After germination is initiated, high external osmolarity impedes core rehydration and concomitant Ca^{2+}-DPA release.

I. Zusammenfassung

In Folge von Nährstoffverknappung kann das Bodenbakterium *Bacillus subtilis* resistente, metabolisch inaktive Sporen bilden. Diese Sporen bestehen aus einem dehydrierten Sporenkern der das Sporengenom enthält, umschlossen von einer inneren Sporenmembran, einer Keimzellwand und einem Cortex aus Peptidoglycan, einer äußeren Sporenmembran und einer mehrlagigen Protein-Schicht (‚Coat'). Wenn bestimmte Nährstoffe (‚Keimungsinduktoren') verfügbar werden, können sie an Keimungsrezeptoren in der inneren Sporenmembran binden. Dies induziert die Spore-Reaktivierung, welche sich aus einer Keimungsphase und einer Auswachsphase (‚Outgrowth') zusammensetzt. Während der Keimung verlieren Sporen ihre Resistenzen, ersetzen die Ionen und das Ca^{2+}-Dipicolinat (Ca^{2+}-DPA) des Sporenkerns mit Wasser (Kern-Rehydrierung) und hydrolysieren ihren Cortex. Wenn der Wassergehalt des Kerns hoch genug für Enzymaktivitäten ist, wird der Metabolismus reaktiviert. Dies kennzeichnet den Beginn der Auswachsphase, während welcher sich die gekeimten Sporen molekular reorganisieren und ihr Elongationswachstum beginnt.

Wie sich hohe Salzkonzentrationen und osmotischer Stress auf die Sporen-Reaktivierung auswirken war bisher kaum erforscht, obwohl dieses Themengebiet für Grundlagenforschung, Lebensmittelmikrobiologie, Bodenökologie und Astrobiologie relevant ist. Daher wurden im Rahmen dieser Doktorarbeit die Auswirkungen von Salzstress auf die *Bacillus* Sporen-Reaktivierung mit Hauptaugenmerk auf die Keimung von *B. subtilis* in Gegenwart hoher NaCl Konzentrationen untersucht.

Im Allgemeinen wirkten sich steigende Salzkonzentrationen zunehmend negativ auf die Keimung aus, obwohl ein Teil der Sporen trotz hoher Salinität die Keimung initiierte. In der Gegenwart von hohen NaCl Konzentrationen (≥ 1.2 mol/L) keimten *B. subtilis* Sporen verspätet, langsamer, heterogener und ineffizienter. Auch andere Salze inhibierten die Keimung, doch ihre inhibitorische Stärke variierte stark in Abhängigkeit von der jeweiligen Ionenkonzentration, der Art und Kombination der Ionen und den chemischen Eigenschaften des Salzes. Obwohl dies unterstreicht, dass ionischer Stress eine Rolle in der Inhibition spielt, hatten hohe Konzentrationen nicht-ionischer, osmotisch wirksamer Solute eine ähnlich inhibitorische Wirkung wie NaCl-Lösungen mit äquivalenter Osmolarität. Somit ist osmotischer Stress eine wesentliche Determinante der Keimungsinhibition durch NaCl. Interessanterweise keimten Sporen mit Coat-Defekten in der Gegenwart von NaCl noch wesentlich schlechter als intakte Sporen, nicht jedoch in der Gegenwart nicht-ionischer Solute. Dies legt nahe, dass der Coat (möglicherweise zusammen mit der äußeren

Sporenmembran) eine wichtige Funktion zum Schutz der unterliegenden Sporenstrukturen (d.h. Cortex, Keimzellwand, Keimungsapparat und innere Sporenmembran) vor ionischem Stress besitzt.

Basierend auf diesen Beobachtungen wurde im Rahmen der vorliegenden Doktorarbeit das erste mechanistische Modell zur Keimungsinhibition durch Salzstress erarbeitet (siehe oben, **Graphical Abstract**). Laut diesem Modell erschweren ionische Interaktionen mit dem Keimungsinduktor und/oder dem Coat die Diffusion des Induktors zu den Rezeptoren in der inneren Sporenmembran, so dass die Keimung verspätet ausgelöst wird. Im Anschluss behindert osmotischer Stress die Kern-Rehydrierung und den damit verknüpften Ca^{2+}-DPA Efflux, was den Gesamtprozess der Keimung verlangsamt.

Obwohl eine metabolische Reaktivierung von Sporen selbst in der Gegenwart von 4.8 mol/L NaCl noch messbar war, konnte Outgrowth im Sinne von Elongation bis zu einer NaCl Konzentration von 2.4 mol/L in nährstoffreichem Medium beobachtet werden. Eine Transkriptom-Analyse Salz-gestresster, auswachsender Sporen zeigte viele Ähnlichkeiten zur Stress-Antwort kontinuierlich gestresster vegetativer Zellen. So wurde unter anderem das gesamte genetische Repertoire zur Synthese und Aufnahme kompatibler Solute induziert.

Zusammenfassend konnte im Rahmen der vorliegenden Doktorarbeit das erste mechanistische Modell zur Keimungsinhibition durch Salzstress erarbeitet und die erste umfassende Transkriptom-Studie zur Salzstress-Antwort auswachsender Sporen durchgeführt werden. Diese Ergebnisse geben neue Einblicke in die Auswirkungen von Salzstress auf den Lebenszyklus von *B. subtilis* und könnten für die obengenannten Forschungsgebiete von angewandtem Nutzen sein.

II. Abbreviations

3PGA	=	3-phosphoglyceric acid
AGFK	=	Germinant mixture of L-asparagine, D-glucose, D-fructose, and K^+
ATP	=	Adenosine triphosphate
a_w	=	Water activity
Ca^{2+}-DPA	=	1:1 complex of calcium and dipicolinate
c-di-AMP	=	Cyclic diadenylate monophosphate
CFU	=	Colony forming units
CLE	=	Cortex-lytic enzyme
DIC	=	Differential interference-contrast
DNA	=	Deoxyribonucleic acid
DPA	=	Dipicolinic acid (pyridine-2,6-dicarboxylic acid)
GB	=	Glycine betaine
GR	=	Germinant receptor
IM	=	Inner spore membrane
M	=	Mole per liter (mol/L)
MPa	=	Mega Pascal
mRNA	=	Messenger RNA
OD	=	Optical density
OM	=	Outer spore membrane
PG	=	Peptidoglycan
RNA	=	Ribonucleic acid
RNA-seq	=	RNA sequencing
SASPs	=	Small, acid-soluble spore proteins
TEM	=	Transmission electron microscopy
T_{lag}	=	Time point in germination when rapid release of Ca^{2+}-DPA starts
$T_{release}$	=	Time point in germination when rapid release of Ca^{2+}-DPA ends
$\Delta T_{release}$	=	Germination-phase of rapid release of Ca^{2+}-DPA ($T_{release}$ - T_{lag})
T_{lys}	=	Time point in germination when cortex hydrolysis ends
ΔT_{lys}	=	Germination-phase of cortex hydrolysis (T_{lys} - $T_{release}$)

CHAPTER 1

Introduction

1.1. *Bacillus subtilis* and its response to changing environments

Bacillus subtilis is a gram positive, rod-shaped bacterium that can be ubiquitously found in soil (Wood *et al.*, 2001; Nicholson, 2004; Earl *et al.*, 2008; Mandic-Mulec *et al.*, 2015). In its preferred habitat, the upper soil layers, it is frequently challenged by changing environmental conditions and has therefore evolved a broad range of survival strategies, including sporulation, development of natural competence, formation of biofilms, cannibalism, as well as a diverse set of responses to specific stresses (Miller and Wood, 1996; Wood *et al.*, 2001; Errington, 2003; Earl *et al.*, 2008; Lopez *et al.*, 2009; Marles-Wright and Lewis, 2010; Young *et al.*, 2013). All stress responses involve altered expression of numerous genes leading to major cellular changes, and are therefore precisely regulated (Marles-Wright and Lewis, 2007; Hecker *et al.*, 2007; Lopez *et al.*, 2009; Schultz *et al.*, 2009; Nicolas *et al.*, 2012). While, for instance, *B. subtilis* reacts to fluctuations in external osmolarity by elaborately adjusting its internal solute pool (**section 1.1.1.**; reviewed e.g. in Hoffmann and Bremer, 2016), it undergoes a complex differentiation process upon nutrient limitation, leading to the formation of an endospore (**section 1.1.2.**; reviewed e.g. in Errington, 2003). The emerging endospores (hereafter referred to as spores) are highly resistant against a broad range of environmental extremes and can stay dormant for many years (**section 1.2.**; reviewed e.g. in Nicholson *et al.*, 2000). However, when nutrients become available again, a spore can revive ('germination' and 'outgrowth') and thereby convert back into a vegetative cell (**section 1.3.**; reviewed e.g. in Setlow, 2013). Within this thesis, spore revival in the presence of high salt concentrations was investigated due to its importance for basic research, soil ecology, food microbiology, and astrobiology (**section 1.4**).

1.1.1. Osmotic stress response of vegetative *B. subtilis* cells

In soil, flooding and desiccation can quickly alter osmotic conditions, thus creating hypo- and hyperosmotic environments, respectively (Miller and Wood, 1996; Wood *et al.*, 2001; Bremer, 2002). Since water moves along its osmotic gradient and bacteria cannot transport water actively, they have to adjust their cytoplasms' osmolarity to maintain their turgor (i.e. the outward directed hydrostatic pressure of the cell) and thus their ability to grow (Kempf and Bremer, 1998; Poolman *et al.*, 2004; Hoffmann and Bremer, 2016). When hyperosmotic conditions arise (i.e. higher external solute concentration), *B. subtilis* cells

rapidly take up large amounts of K^+ ions to increase their internal osmolarity and to avoid plasmolysis due to dehydration of the cytoplasm (Whatmore *et al.*, 1990; Wood *et al.*, 2001). Cells shocked with 0.4 M NaCl can approximately double their internal K^+ concentration from ca. 350 mM to ca. 650 mM (Whatmore *et al.*, 1990). The major K^+ uptake system in *B. subtilis* is the moderate-affinity transporter KtrAB (Holtmann *et al.*, 2003). It is composed of the dimeric membrane protein KtrB and a cytoplasmic octameric KtrA ring that can interact with adenosine triphosphate (ATP) as well as cyclic diadenylate monophosphate (c-di-AMP) and has a regulatory function (**Fig. 1**; Nelson *et al.*, 2013; Vieira-Pires *et al.*, 2013). In the absence of KtrAB, cells cannot cope with a salt shock of 0.6 M NaCl, although KtrAB is not the sole K^+ uptake system of *B. subtilis*: the low-affinity transporter KtrCD also contributes to salt stress adaptation (Holtmann *et al.*, 2003). While rapid accumulation of K^+ allows survival upon sudden increases in osmolarity, very high K^+ concentrations are not compatible with essential cell functions over extended periods of time (Whatmore *et al.*, 1990; Record *et al.*, 1998). Therefore, as a secondary response, *B. subtilis* replaces K^+ by accumulating compatible solutes, i.e. highly soluble organic compounds that do not disturb cell functions (Whatmore *et al.*, 1990; Kempf and Bremer, 1998; Wood *et al.*, 2001). The major compatible solutes used by *B. subtilis* are glycine betaine (GB) and proline, but other compatible solutes (mostly chemically related to GB or proline) can also be used (Hoffmann and Bremer, 2016).

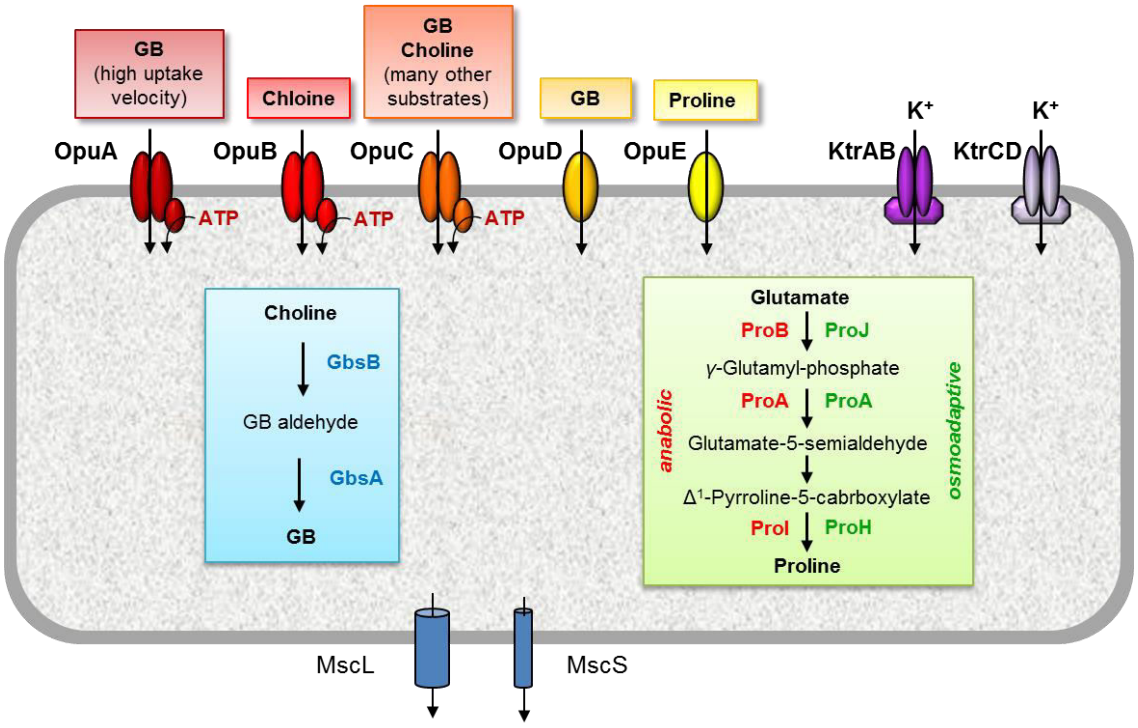


Figure 1: Overview of the specific hyperosmotic stress response in *B. subtilis*. Adapted from Hoffmann and Bremer (2016)

Compatible solutes can either be synthesized or taken up from the surrounding medium (**Fig. 1**; Kempf and Bremer, 1998). The uptake of osmoprotectants, i.e. compatible solutes and their precursors, is mediated by five Opu[1] transporters (OpuA, OpuB, OpuC, OpuD, OpuE) with different affinities and substrate specificities (**Fig. 1**; Kempf and Bremer, 1998; Hoffmann and Bremer, 2016). *B. subtilis* can import GB via OpuA, OpuC, and OpuD, or synthesize it by oxidation of its precursor choline, which needs to be imported via OpuB or OpuC (Boch *et al.*, 1996; Kappes *et al.*, 1996; Kempf and Bremer, 1998). While OpuC has a very broad substrate spectrum, OpuB only imports choline and glycine betaine aldehyde (Kempf and Bremer, 1998; Hoffmann and Bremer, 2011). Glycine betaine aldehyde is derived from choline by the alcohol dehydrogenase GbsB and then converted to GB by the glycine betaine aldehyde dehydrogenase GbsA (**Fig. 1**; Boch *et al.*, 1994; Boch *et al.*, 1996). In contrast to GB, proline can also be synthesized *de novo* by three enzymatic reactions using glutamate as precursor (Whatmore *et al.*, 1990). Depending on the purpose of proline requirement, *B. subtilis* can activate two different interlinked proline-synthesis pathways: an anabolic pathway involving ProB, ProA and ProI, or an osmoadaptive pathway involving ProJ, ProA, and ProH, which represent a γ-glutamyl kinase, a γ-glutamyl-phosphate reductase, and a Δ^1-pyrroline-5-carboxylate reductase, respectively, in both pathways (Brill *et al.*, 2011a; Hoffmann and Bremer, 2016). Compatible solute levels in the cytoplasm are precisely adjusted to the current needs of the cell by various mechanisms involving transcriptional and feedback regulation (Poolman *et al.*, 2004; Gutiérrez-Preciado *et al.*, 2009; Brill *et al.*, 2011b; Nau-Wagner *et al.*, 2012; Hoffmann *et al.*, 2013).

B. subtilis can respond in different ways depending on how osmotic stress is imposed (Spiegelhalter and Bremer, 1998; Young *et al.*, 2013). Upon a sudden salt shock, *B. subtilis* activates the σ^B-mediated general stress response, leading to the differential expression of about 200 genes conferring enhanced, albeit non-specific resistance against various environmental stresses (Spiegelhalter and Bremer, 1998; Steil *et al.*, 2003; Hecker *et al.*, 2007; Nannapaneni *et al.*, 2012; Young *et al.*, 2013). However, when *B. subtilis* is confronted with incremental and sustained salt stress, it induces a specific osmotic stress response under the control of the house-keeping sigma factor σ^A (Spiegelhalter and Bremer, 1998; Steil *et al.*, 2003; Hahne *et al.*, 2010; Young *et al.*, 2013).

The molecular details of global osmotic stress perception and the corresponding signal transduction leading to altered gene expression are still elusive, although the two-component

[1] Opu = **o**smo**p**rotectant **u**ptake

regulatory system DegSU, the sensor kinase KinD, and the stressosome have been implicated in this process (Marles-Wright and Lewis, 2010; Hoffmann and Bremer, 2016). However, individual proteins can also directly perceive and respond to osmotic stress (Poolman *et al.*, 2004). For instance the OpuA transporter in *Lactococcus lactis*, and possibly also in *B. subtilis*, is activated by high intracellular ion concentrations that alter the interaction of a charged cytoplasmic domain with anionic membrane lipids (Poolman *et al.*, 2004; Hoffmann and Bremer, 2016). In contrast, when hypoosmotic conditions arise, *B. subtilis* can release solutes primarily via the mechanosensitive channel MscL, which opens when the tension within the cytoplasmic membrane increases (**Fig. 1**; Poolman *et al.*, 2004; Hoffmann *et al.*, 2008). Thus, electrostatic interactions of proteins and lipids are important in bacterial osmosensing (Poolman *et al.*, 2004).

Taken together, *B. subtilis* has an elaborate osmotic stress response system ensuring survival under hyperosmotic conditions (Hoffmann and Bremer, 2016). Notably, although the formation of a desiccation-resistant endospore may seem to be a suitable strategy for escaping non-growth-permissive high-salinity conditions, sporulation is suppressed at an early stage by osmotic stress, likely to prevent the initiation of a process that cannot be completed under the prevailing environmental and nutritional conditions (Ruzal and Sánchez-Rivas, 1998; Hoffmann and Bremer, 2016; Widderich *et al.*, 2016).

1.1.2. Sporulation as response to nutrient deprivation

An important factor for microbial growth and survival is the availability of sufficient nutrients. To survive conditions under which nutrients are sparse or absent, *B. subtilis* and its close relatives have evolved the ability to form endospores, which are highly stress resistant, metabolically inert endurance states (Nicholson *et al.*, 2000; Higgins and Dworkin, 2012). Due to their dormancy, all spore features required for resistance and revival must be developed during sporulation (Setlow, 2013).

Sporulation is an elaborate, but also energetically costly process: one sporulation cycle lasts about seven hours, involves the transient and highly concerted expression of more than 600 genes, and irreversibly leads to the death of the mother cell (Errington, 2003; Hilbert and Piggot, 2004; Eichenberger, 2012; Nicolas *et al.*, 2012). Therefore, sporulation requires finely tuned genetic and cellular regulation, starting with a phosphorelay signaling cascade that controls sporulation initiation (Hilbert and Piggot, 2004; Schultz *et al.*, 2009; Higgins and

Dworkin, 2012). The phosphorelay integrates intra- and extracellular cues (e.g. cell density, nutrient starvation, cell cycle) to prevent sporulation under inappropriate conditions (Hilbert and Piggot, 2004). It consists of several kinases, phosphotransferases, and phosphatases that determine the phosphorylation state of the master regulator of sporulation, Spo0A (Schultz *et al.*, 2009; Higgins and Dworkin, 2012; Tan and Ramamurthi, 2014). A sufficient amount of phosphorylated Spo0A initiates sporulation (Fujita *et al.*, 2005; Lopez *et al.*, 2009).

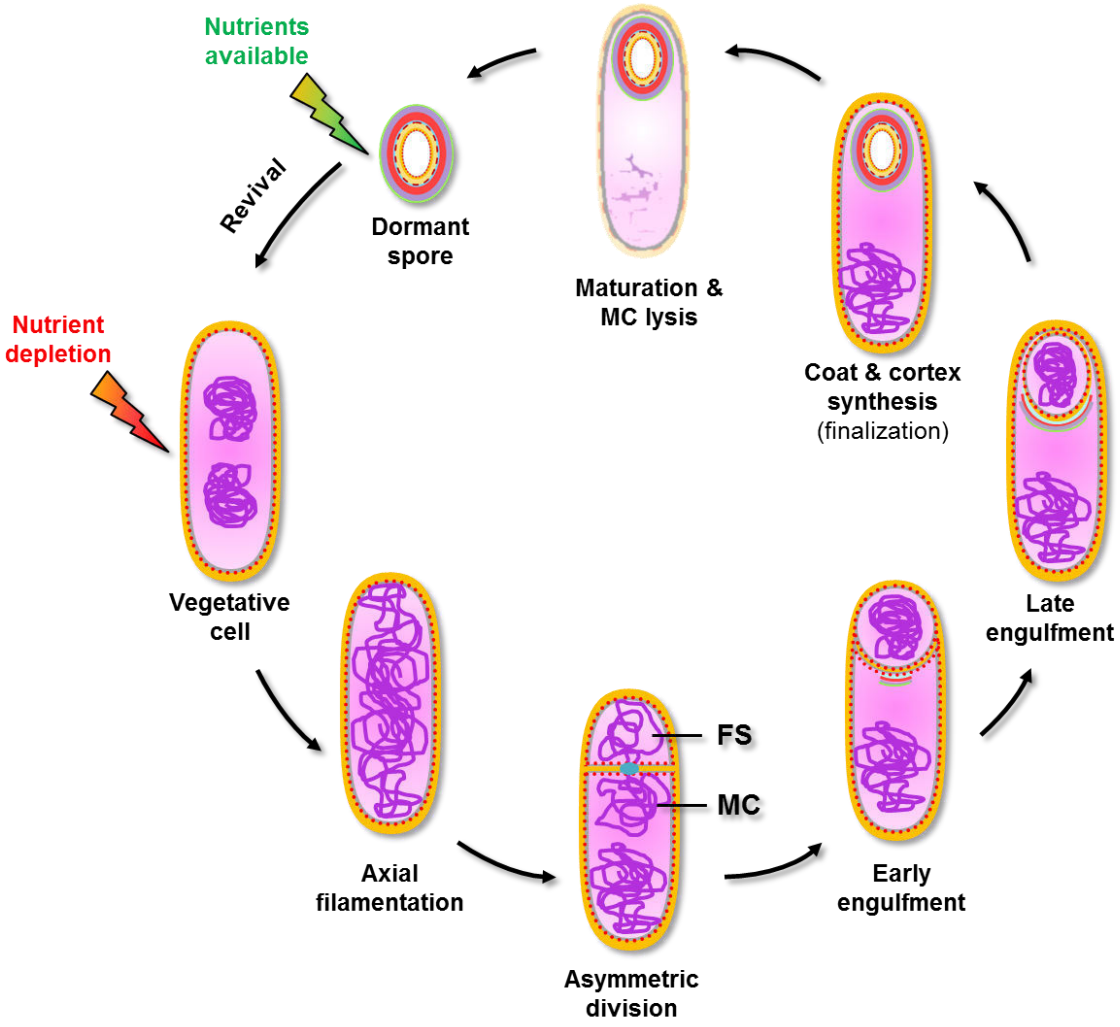


Figure 2: Schematic depiction of *B. subtilis* sporulation (adapted from Errington, 2003). Upon nutrient depletion, a vegetative cell undergoes asymmetric division. The mother cell (MC) engulfs the forespore (FS), builds up the protective coat and cortex, and ultimately lyses to release the spore. The spore remains dormant until nutrients become available and induce spore revival (i.e. germination and outgrowth).

At the beginning of sporulation, two identical, newly-replicated chromosomes are attached to the opposing cell poles, thereby forming the so-called axial filament (**Fig. 2**; Eichenberger, 2012; Tan and Ramamurthi, 2014). Subsequently, the cell forms an asymmetric sporulation septum near one of the poles (Eichenberger, 2012). Importantly, the asymmetric septation is almost completed when only ca. one-third of one chromosome (including its *oriC* region) has been imported into the forespore compartment (Wu and Errington, 1994; Higgins and Dworkin, 2012). This leads to a transient genetic asymmetry required for further differentiation: while the first third of the chromosome can already by transcribed, the residual two-thirds are not expressed until they have been pumped into the forespore (Wu and Errington, 1994; Bath *et al.*, 2000; Higgins and Dworkin, 2012). The genetic asymmetry contributes to the activation of the alternative sigma factor σ^F and thereby to the start of a finely-tuned gene expression series subjected to an elaborate spaciotemporal gene regulation (Hilbert and Piggot, 2004; Higgins and Dworkin, 2012). Alternative sigma factors, i.e. the forespore-specific σ^F and σ^G and the mother cell-specific σ^E and σ^K, are the central regulators of the complex expression network and are themselves precisely regulated (Errington, 2003; Hilbert and Piggot, 2004). Intercompartmental crosstalk ensures the exactly timed series of alternative sigma factor activation ($\sigma^F \rightarrow \sigma^E \rightarrow \sigma^G \rightarrow \sigma^K$) (Hilbert and Piggot, 2004).

After asymmetric division as well as σ^F and σ^E activation, the mother cell engulfs the forespore (**Fig. 2**; Hilbert and Piggot, 2004). Throughout this process, the cell wall between the two compartments is remodeled while the mother cell membrane migrates around the forespore (Higgins and Dworkin, 2012). After engulfment, the forespore is a double-membrane-enclosed compartment within the mother cell's cytosol, which supplies the forespore with nutrients via a 'feeding tube' (Errington, 2003; Higgins and Dworkin, 2012; Tan and Ramamurthi, 2014).

While engulfment is still in progress, the formation of the spore cortex and spore coat begins (**Fig. 2**; McKenney *et al.*, 2013). The cortex is composed of peptidoglycan (PG) and localized between the inner and the outer forespore membrane (**Fig. 3, Fig. 4**; Popham, 2002). Although spore PG has structural modifications (see below), cortex synthesis is generally similar to vegetative cell wall synthesis: disaccharide-pentapeptide precursors are synthesized and modified by Mur proteins within the mother cell, tethered to and flipped across the outer forespore membrane (Popham, 2002; Tan and Ramamurthi, 2014). Within the intermembrane space, transglycosylation and transpeptidation lead to the formation of crosslinked glycan strands (Tan and Ramamurthi, 2014). Cortex synthesis is temporally and structurally linked to

the synthesis of the spore coat, a multilayered proteinaceous structure that is localized outside of the outer forespore membrane (**Fig. 3, Fig. 4**; Popham, 2002; Henriques and Moran, 2007; McKenney *et al.*, 2013; Tan and Ramamurthi, 2014). Coat morphogenesis is a complex, dynamic process that begins with the formation of a protein-scaffold cap on the mother cell proximal pole of the forespore (**Fig. 2**; McKenney and Eichenberger, 2012; McKenney *et al.*, 2013). The scaffold cap is already structured into four layers that can later be distinguished in the coat of a mature spore, i.e. the basement layer, the inner coat, the outer coat, and the crust (**Fig. 4**; McKenney *et al.*, 2010; McKenney and Eichenberger, 2012; McKenney *et al.*, 2013). Assembly of each layer depends on the presence of specific morphogenetic proteins (SpoIVA, SafA, CotE, and CotXYZ, respectively): *spoIVA* mutants exhibit coat material that is not attached to the spore as well as a cortex defect; *safA* mutants lack the inner but not the outer coat; in *cotE* mutants the outer coat and crust are absent; and in *cotX*/*cotY*/*cotZ* mutants no crust is attached (Driks, 1999; McKenney *et al.*, 2010; McKenney *et al.*, 2013; Tan and Ramamurthi, 2014). Starting from the scaffold cap, the forespore is encased by groups of coat proteins in successive waves (McKenney and Eichenberger, 2012). Although the molecular mechanism of encasement is not yet known, physical interaction of SpoIVA with other morphogenetic proteins, SpoVM and SpoIVD, is required (Henriques and Moran, 2007; McKenney *et al.*, 2013; Tan and Ramamurthi, 2014). While protein-protein interactions are important for proper coat protein localization, temporal coordination of coat assembly is based on transcriptomic regulation of coat protein synthesis by σ^E and σ^K, and their modulation by SpoIIID and GerE, respectively (Driks, 1999; Henriques and Moran, 2007; McKenney and Eichenberger, 2012; McKenney *et al.*, 2013). When all coat proteins are assembled, enzymatic crosslinking and modification contributes to spore maturation (Driks, 1999; Henriques and Moran, 2007).

Throughout sporulation, the forespore protoplast (i.e. the later spore core) undergoes various molecular changes towards establishing dormancy and resistance, including dehydration and mineralization (see below; Errington, 2003). When all spore features are in place, the mother cell lyses, thereby releasing the dormant spore into the environment (**Fig. 2**; Higgins and Dworkin, 2012). It should be noted that *B. subtilis* spores can undergo changes (‘maturation’) after release from the mother cell (Hornstra *et al.*, 2009). Changes in coat structure occurring after mother cell lysis can involve enzymatic activity and contribute to spore resistance (Ragkousi and Setlow, 2004; Sanchez-Salas *et al.*, 2011; Setlow, 2014b). Moreover, it was reported that the RNA content of released spores can change depending on environmental conditions, which affects spore revival (Segev *et al.*, 2012; Segev *et al.*, 2013).

However, metabolism of endogenous compounds is undetectable after mother cell lysis so that spores can be considered metabolically dormant (Ghosh *et al.*, 2015).

1.2. Morphology and properties of *Bacillus* spores

In the absence of nutrients, spores can stay dormant for extended periods of time. The exact extent of spore longevity is unknown, with reports ranging from hundreds to thousands or even millions of years (Cano and Borucki, 1995; Vreeland *et al.*, 2000; Nicholson, 2004; McKenney *et al.*, 2013). Outlasting such long phases of quiescence, throughout which damaged biomolecules cannot be repaired or replaced, is only possible due to particular structural, biochemical, and biophysical spore properties conferring metabolic inertness as well as resistance against many harmful influences (Setlow, 1994; Nicholson *et al.*, 2000; Setlow, 2006; Leggett *et al.*, 2012; Setlow, 2014b). In general, dormancy and the remarkable resistance against environmental insults such as heat, desiccation, radiation, and a broad range of chemicals are due to synergistic effects of multiple spore features (Setlow, 1994; McKenney *et al.*, 2013; Setlow, 2014b). Typically, a *Bacillus* spore has a layered structure composed of a proteinaceous spore coat, an outer membrane, a thick PG cortex, a thin PG germ cell wall, an inner membrane, and a central spore core (Setlow, 2013; **Fig. 3; Fig. 4**).

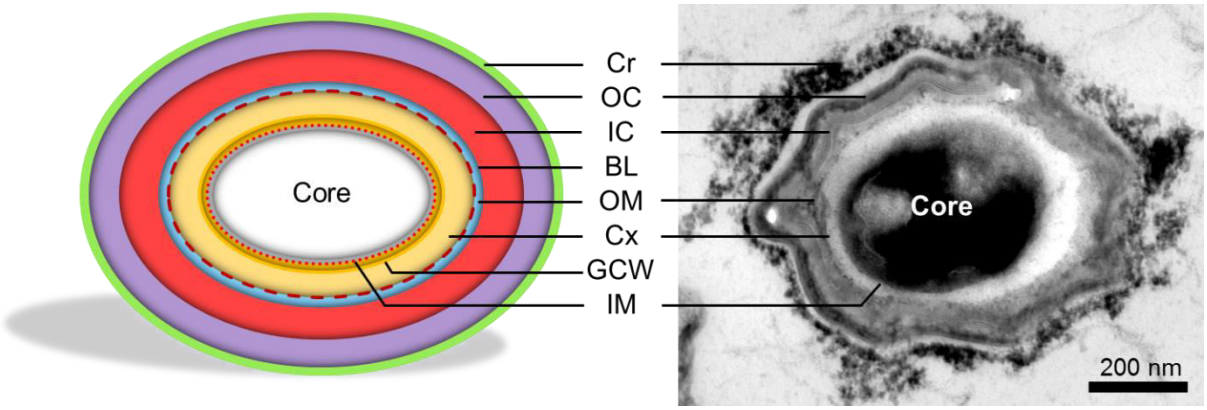


Figure 3: Morphology of a dormant *B. subtilis* spore. The image shows a schematic cross-section (left) and a transmission electron micrograph (right). The spore shown on the right was stained by ruthenium red to visualize the crust layer. Abbreviations: Cr = crust; OC = outer coat; IC = inner coat; BL = basement layer; OM = outer membrane; Cx = cortex; GCW = germ cell wall; IM = inner membrane (adapted from Nagler *et al.*, 2015)

1.2.1. Functional morphology of the multilayered *B. subtilis* spore

The outermost protective feature of *B. subtilis* spores is the **spore coat**. While transmission electron microscopy (TEM) allows clear discrimination of a lamellar inner coat and an electron-dense outer coat (**Fig. 3**), coat morphogenesis studies have established a four-layer coat model, in which the morphogenetic proteins SpoIVA, SafA, CotE, and CotXYZ are required for the assembly of the basement layer, inner coat, outer coat, and crust, respectively (**Fig. 3; Fig. 4**; **section 1.1.2.**; Henriques and Moran, 2007; McKenney *et al.*, 2010; McKenney and Eichenberger, 2012; McKenney *et al.*, 2013). The coat is composed of more than 70 different proteins and enzymes that are partially crosslinked, thus contributing to the spore's rigidity and mechanical resistance (Driks, 1999; Henriques and Moran, 2007). Functionally, the coat acts as a 'molecular sieve' that allows passage of small molecules (e.g. amino acids), but restrains access of larger molecules (> 5 kDa) such as exogenous lytic enzymes (Gerhardt and Black, 1961; Bassi *et al.*, 2012; McKenney *et al.*, 2013; Setlow, 2014b; Knudsen *et al.*, 2016). Moreover, the coat can detoxify various chemicals (e.g. hypochlorite and nitrous acid) by unspecific and enzymatic reactions, thereby protecting the more essential inner spore structures from damage (Ghosh *et al.*, 2008; Bassi *et al.*, 2012; Setlow, 2014b).

Underneath the coat, spores possess a mother-cell-derived **outer spore membrane** (OM; **Fig. 3; Fig. 4**). Although its permeability and function in mature spores are unclear, recent electron cryotomographic images on plunge-frozen samples clearly revealed its presence in mature *B. subtilis* spores (Butzin *et al.*, 2012; Leggett *et al.*, 2012; McKenney *et al.*, 2013; Tocheva *et al.*, 2013; Setlow, 2014a). At least in *B. megaterium* the coat-OM complex behaves dielectrically, forming an insulating layer around the cortex (Carstensen *et al.*, 1979). Yet, the passage of small molecules through the OM is somehow possible as exemplified by the movement of nutrients like L-alanine to the germination receptors localized in the inner spore membrane (see below; Swerdlow *et al.*, 1981; Griffiths *et al.*, 2011; Butzin *et al.*, 2012).

Below the coat and OM lies the **spore cortex**, which is composed of modified PG (**Fig. 3; Fig. 4**; Setlow, 2013). Spore PG differs from vegetative PG by the absence of teichoic acids, a lower degree of peptide crosslinking between the glycan strands, and the presence of muramic-δ-lactam (Popham, 2002). A pivotal function of the cortex is the establishment and maintenance of spore core dehydration, which is crucial for dormancy and resistance to various harmful conditions (Popham, 2002; Bassi *et al.*, 2012; Setlow, 2014b). Although it is

still not entirely clear how core hydration is established and maintained, the concept of an expanded osmoregulatory cortex was proposed (Gould and Dring, 1975; Bassi *et al.*, 2012). In this model, the cortex is the most hydrated spore region, harbors high amounts of mobile cations that interact with the electronegative PG, and has a low water activity (a_w = 0.85) so that it can dehydrate the spore core (also having an a_w of 0.85) by osmosis (Gould and Dring, 1975; Carstensen *et al.*, 1979; Algie, 1984; Bassi *et al.*, 2012). At least in *B. megaterium*, the OM is involved in insulating the cortex and its mobile counter ions from environmental ionic strength (Carstensen *et al.*, 1979). A second, albeit thinner PG layer termed **germ cell wall** is located underneath the cortex (**Fig. 3; Fig. 4**). Germ cell wall PG does not exhibit the cortical PG modifications, which is essential for selective cortex hydrolysis during germination (see below; Setlow, 2013; Tan and Ramamurthi, 2014). A function of the germ cell wall in spore resistance is not known, but after germination it becomes the outgrowing spore's cell wall (Leggett *et al.*, 2012; Setlow, 2013; Setlow, 2014b).

The **inner spore membrane** (IM) is situated beneath the germ cell wall, surrounds the spore core, and has a very low permeability (**Fig. 3; Fig. 4**; Gerhardt and Black, 1961; Black and Gerhardt, 1962; Knudsen *et al.*, 2016). The low permeability of the IM relates to its very high viscosity and lipid immobility, suggesting the lipid bilayer is in a gel state (Cowan *et al.*, 2004; Loison *et al.*, 2013). Low lipid mobility might be due to the 1.3- to 1.6-fold compression of the IM surface, which is relieved during germination without requiring new lipid synthesis (Cowan *et al.*, 2004). The IM becomes the plasma membrane of germinated spores and attains normal permeability and viscosity after germination is completed (Cowan *et al.*, 2004; Loison *et al.*, 2013). Despite previous reports about cardiolipin-enrichment in the IM, the membranes of spores and vegetative cells were later reported to have a similar composition (Kawai *et al.*, 2006; Griffiths and Setlow, 2009). Notably, alterations of the IM composition can influence the germination rate as well as spore resistance to wet heat and oxidizing agents (Griffiths and Setlow, 2009). The IM has a distinctive proteome, as it harbors many proteins required for germination (Griffiths *et al.*, 2011; Zheng *et al.*, 2016).

The central part of the spore is the **spore core**, which is analogous to a vegetative cell's protoplast and contains DNA, RNA, various proteins (**Fig. 3;** Setlow, 2013). An important characteristic of the spore core is its low water content of approximately 28 to 57% of wet weight as opposed to ca. 80 % of wet weight in vegetative cells (Beaman and Gerhardt, 1986; Setlow, 2013). Core dehydration is thought to be an important factor for enzymatic dormancy, heat resistance, and resistance to some chemicals (Beaman and Gerhardt, 1986;

Nicholson *et al*., 2000; Leggett *et al*., 2012). For many years the constitution of the spore core and the physical state of water therein was controversial (Bassi *et al*., 2012). However, recent studies reject the hypothesis of a glassy core with immobile water and strongly support the so-called 'gel scenario', in which spore core water is mobile and thus able to move within a matrix of immobilized macromolecules (Black and Gerhardt, 1962; Sunde *et al*., 2009; Kaieda *et al*., 2013; Knudsen *et al*., 2016). Indeed, the spore core exhibits very low protein mobility, with rotational immobilization possibly preventing irreversible aggregation of heat-denatured proteins, thus contributing to the spore's heat resistance (Cowan *et al*., 2003; Sunde *et al*., 2009). Furthermore, heat resistance is affected by core mineralization: while generally higher amounts of inorganic minerals go along with a lower core water content and consequently higher heat resistance, it should be noted that this resistance also depends on the types of minerals present (Beaman and Gerhardt, 1986; Leggett *et al*., 2012). Similarly, the presence of large amounts of dipicolinic acid (DPA, pyridine-2,6-dicarboxylic acid), which is complexed 1:1 with divalent ions (mostly Ca^{2+} as Ca^{2+}-DPA) and accounts for about 10 % of a spore's dry weight (ca. 20% of spore core dry weight), also supports core dehydration and thereby heat resistance (Paidhungat *et al*., 2000; Setlow, 2013).

Another important characteristic feature of the spore core is the high abundance of small, acid-soluble spore proteins (SASPs), which are only synthesized by the forespore late in sporulation and can be subdivided into major (α/β-type) and minor SASPs (Setlow, 1994; Lee *et al*., 2008; Leggett *et al*., 2012). While both SASP types are degraded to amino acids that can be exploited during early outgrowth, the major SASPs are important for spore resistance against genotoxic chemicals, desiccation, heat, γ- and UV-radiation, as they protect the spore from DNA damage (Lee *et al*., 2008; Setlow, 2014b). Therefore they bind the DNA until saturation, thereby inducing a conformational change ('A-B-DNA conformation') towards a highly ordered, crystalline nucleoid (Lee *et al*., 2008; Dittmann *et al*., 2015). In contrast, the minor SASPs do not bind to the DNA and have no known role in spore resistance (Setlow, 1994; Leggett *et al*., 2012).

The spore core also differs from vegetative cells by minimal to undetectable amounts of ATP and reduced pyridine nucleotides such as NADH and NADPH, which likely contributes to metabolic dormancy (Setlow and Kornberg, 1970a; Setlow, 1994). Instead, developing spores accumulate 3-phosphoglyceric acid (3PGA), which is converted to ATP after germination is completed (Setlow, 1994; Magill *et al*., 1996). 3PGA accumulation is regulated by the low spore core pH (ca. 6.3 - 6.5) that is established after approximately

4 hours of sporulation and decreases the activity of phosphoglycerate mutase (Magill *et al.*, 1996). Taken together, the complex, multilayered *B. subtilis* spores deviate from vegetative cells by a multitude of properties, which enables the spores to passively outlast inhospitable environmental conditions for an extended period of time.

1.2.2. Interspecific morphological variation among spores of *Bacillus* species

Bacillus spores of different species have many similarities such as their morphogenesis and layered structure, but there is also interspecific variation (see also **2.3.2.**; Moir and Smith, 1990; Setlow, 1994; Henriques and Moran, 2007; Paredes-Sabja *et al.*, 2011; Leggett *et al.*, 2012; Setlow, 2013). The largest deviation from the overall *B. subtilis* spore morphology is the presence of an outermost exosporium in various *Bacillus* species, including *B. anthracis*, *B. cereus*, and *B. thuringiensis* (Stewart, 2015). The exosporium is a glycoprotein layer outside of the coat that is involved in spore resistance and plays an important role for interaction with the environment and/or host organism (Henriques and Moran, 2007; Stewart, 2015). Moreover, spores of different *Bacillus* species can exhibit variations in spore size, shape, spore core content (e.g. mineralization, water content), coat structure (i.e. appearance and thickness of coat layers), and protein composition (Plomp *et al.*, 2005; Carrera *et al.*, 2007; Henriques and Moran, 2007; Setlow *et al.*, 2012; Bassi *et al.*, 2012; Checinska *et al.*, 2015). Nevertheless, many *B. subtilis* spore proteins have homologs in other species, as for instance at least 50 *B. subtilis* coat proteins and morphogenetic factors are conserved in *B. anthracis* and *B. cereus* (Jedrzejas and Huang, 2003; Henriques and Moran, 2007; Paredes-Sabja *et al.*, 2011). While interspecific variation of *Bacillus* spores can contribute to different overall spore properties and resistance, it should be noted that intraspecific variation is also common, and the precise sporulation conditions (e.g. medium composition and temperature) have a significant impact on spore constitution and resistance (Leggett *et al.*, 2012; Ramirez-Peralta *et al.*, 2012; Setlow *et al.*, 2012; Setlow, 2013; Checinska *et al.*, 2015).

1.3. *Bacillus* spore germination and outgrowth

Although mature spores are metabolically dormant, they can perceive environmental cues: when nutrients become available, thus signifying a potential improvement of environmental conditions, spores can convert back into growing, vegetative cells (reviewed e.g. in Setlow, 2013). This spore revival consists of two phases: germination and outgrowth. Germination is defined as the time interval between the irreversible commitment of a spore to germinate and the onset of metabolism (Moir and Smith, 1990; Setlow, 2013). Throughout germination, spores lose their resistance and refractivity, release ions and Ca^{2+}-DPA from the spore core and take up water in preparation for the resumption of metabolic activity (Paredes-Sabja *et al.*, 2011; Setlow, 2013). The initiation of metabolism hallmarks the beginning of the outgrowth phase, which ends with the first cell division (Keijser *et al.*, 2007; Setlow, 2013). Throughout the two revival phases, the spore undergoes a broad range of molecular, morphological, chemical, and physiological changes (Santo and Doi, 1974; Keijser *et al.*, 2007; Plomp *et al.*, 2007; Paredes-Sabja *et al.*, 2011; Segev *et al.*, 2013; Setlow, 2013; Luu and Setlow, 2014a).

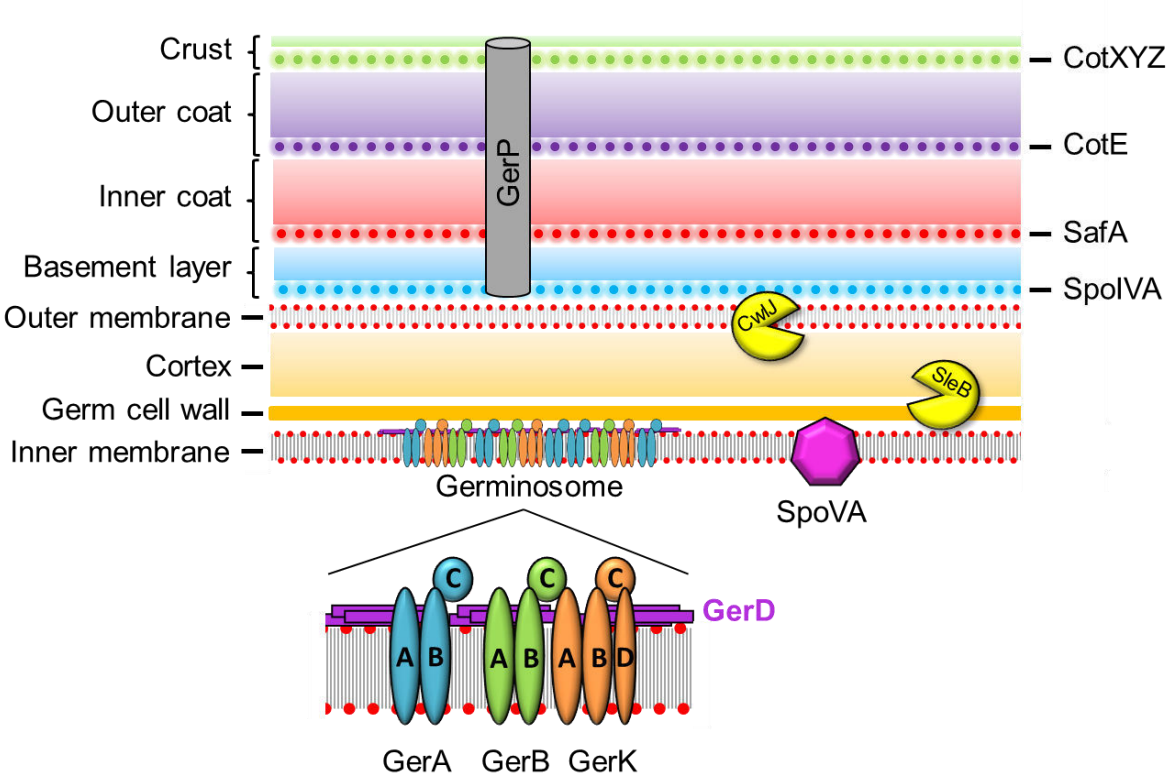


Figure 4: Spore layers surrounding the core and most important components of the germination apparatus. Major morphogenetic coat proteins (required for the proper assembly of their respective layer) are given on the right. Layers and proteins are not shown to scale.

1.3.1. Induction of *B. subtilis* spore germination

Germination can be induced by various means and the agent that triggers germination is called 'germinant' (Setlow, 2013; Setlow, 2014a). Germinants are frequently categorized as either 'nutrient germinants' or 'non-nutrient germinants'. Nutrient germinants could in principle be utilized for metabolism, even though their uptake and metabolism is completely irrelevant for germination initiation (Setlow, 2013). However, as nutrient and non-nutrient germinants trigger germination in different manners, the term is still useful for discrimination (**Fig. 5;** Paredes-Sabja *et al*., 2011; Setlow, 2013).

In general, nutrient germinants include various amino acids, sugars, nucleosides, and salts, but the exact range of active nutrient germinants is species-specific (Ross and Abel-Santos, 2010; Setlow, 2013). The most commonly used nutrient germinants for *B. subtilis* are L-alanine, L-valine, and a mixture of L-asparagine, D-glucose, D-fructose and KCl termed AGFK (Hills, 1950; Wax and Freese, 1968; Atluri *et al*., 2006; Setlow, 2013). In nutrient mixtures, components that can enhance germination but are not capable of inducing germination by themselves (like glucose, fructose, and K^+ in AGFK) are called 'cogerminants' (Atluri *et al*., 2006).

The most commonly used non-nutrient germinants for *B. subtilis* are exogenous Ca^{2+}-DPA, the cationic surfactant dodecylamine, and high pressures (Riemann and Ordal, 1961; Rode and Foster, 1961; Setlow, 2013). These agents can trigger germination in different ways, are less species-specific, and in most cases do not involve GRs, whereas the central germination events (i.e. ion and Ca^{2+}-DPA release, core rehydration, cortex hydrolysis) take place regardless of the germination trigger (Paidhungat and Setlow, 2000; Paredes-Sabja *et al*., 2011; Setlow, 2013).

1.3.2. The nutrient germination pathway in *B. subtilis*

Nutrient germinants are recognized stereospecifically by germinant receptors (GRs) in the IM (Paidhungat and Setlow, 2000; Paidhungat and Setlow, 2001; Griffiths *et al*., 2011). Hence, nutrient germinants first have to pass through the spore integuments (i.e. coat, OM, cortex, and germ cell wall) to induce germination (**Fig. 4**; Butzin *et al*., 2012; Setlow, 2013). Although incompletely understood, this process is facilitated by proteins of the hexacistronic *gerP* operon (Behravan *et al*., 2000; Butzin *et al*., 2012). *B. subtilis* spores possess three

homologous GRs with known substrates: the GerA receptor recognizes L-alanine and L-valine, whereas GerB and GerK act cooperatively to induce germination in response to AGFK (**Fig. 5**; Paidhungat and Setlow, 2000; Atluri *et al*., 2006). Each GR is composed of three different subunits (termed A, B, and C), two of which are transmembrane proteins (A and B subunits), whereas the C-subunit is a lipoprotein on the outer surface of the IM (**Fig. 4**; Setlow, 2013). A fourth D-subunit was recently identified for GerK and seems to modify its activity (Ramirez-Peralta *et al*., 2013). Unfortunately, the exact structure of a complete GR has not yet been resolved and the mechanism of nutrient binding to GRs is not understood, although it likely induces a conformational change (Setlow, 2014a; Wang *et al*., 2015b). All GRs colocalize in a single cluster termed 'germinosome' (**Fig. 4**) that is important for rapid and efficient germination (Pelczar *et al*., 2007; Griffiths *et al*., 2011). Germinosome assembly occurs during sporulation and requires the peripheral IM protein GerD as a scaffold, but it is unclear if further proteins belong to the germinosome and what its precise structure is (Pelczar *et al*., 2007; Griffiths *et al*., 2011; Setlow, 2013; Li *et al*., 2014; Setlow, 2014a).

When sufficient germinants have bound to GRs, a spore irreversibly commits to germinate: even if the germinant is removed by washing, acidification or replacement by a strong competitive inhibitor (e.g. D-alanine), the committed spore will progress through the germination process (Yi and Setlow, 2010; Setlow, 2013). Commitment temporally coincides with the release of large amounts of cations including H^+, K^+, Na^+, and Zn^{2+} (**Fig. 5**; Dring and Gould, 1971; Swerdlow *et al*., 1981; Setlow, 2003; Yi and Setlow, 2010). So far, the function of this ion release, its relation to commitment, by which channels it is mediated, and whether anions are released as well is not clear (Setlow, 2014a). Yet, the H^+ release causes a rise in spore core pH from approximately 6.5 to 7.7, which is important for the resumption of active growth (Setlow, 1994; Setlow, 2003). Subsequent to commitment and ion efflux, Ca^{2+}-DPA is released, most likely via channels encoded by the heptacistronic *spoVA* operon (Vepachedu and Setlow, 2004; Vepachedu and Setlow, 2007a; Li *et al*., 2012). However, the structure of the SpoVA channel is not known (Setlow, 2014a). Concomitant to ion and Ca^{2+}-DPA release, the spore core partially rehydrates, but how water uptake is mediated is - again - not known (Setlow, 2013; Setlow, 2014a). These ion, Ca^{2+}-DPA, and water fluxes are often referred to as 'stage I' of germination (**Fig. 5**; Setlow *et al*., 2001; Setlow, 2003).

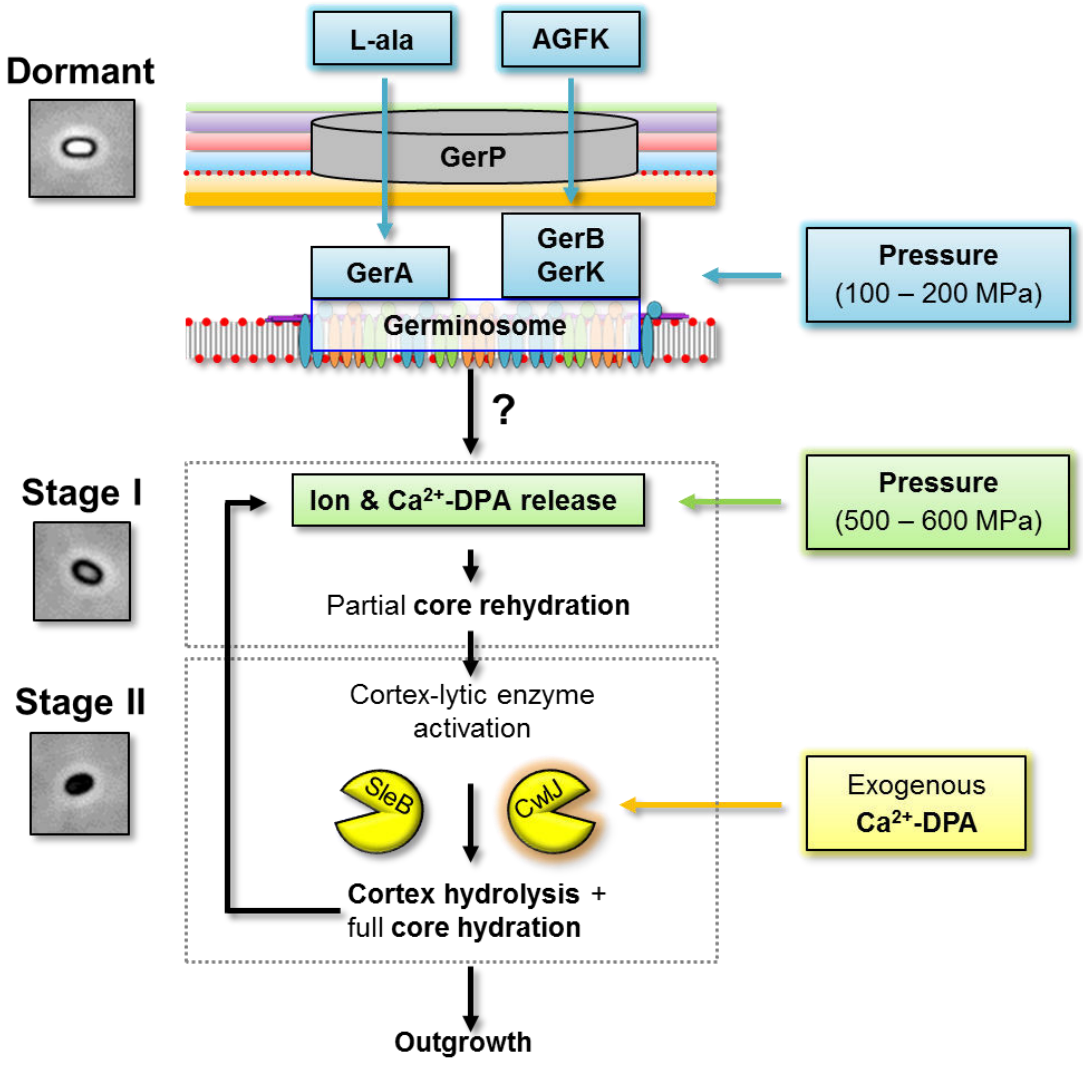


Figure 5: Scheme of *B. subtilis* nutrient and non-nutrient germination pathways. Germinants (i.e. nutrients, pressure and exogenous Ca^{2+}-DPA) and their targets are depicted in the same colors. Phase-contrast images of dormant (phase-bright), stage-I-germinated (phase-gray), and fully germinated (phase-dark) spores are shown on the left. Adapted from Setlow, 2003.

In 'stage II', the cortex is hydrolyzed by the cortex-lytic enzymes (CLEs) CwlJ and SleB (Ishikawa *et al.*, 1998; Setlow, 2003). Either one of these two CLEs is required to successfully progress through stage II and complete germination, as spores of *cwlJ sleB* double mutants get stuck in an intermediate phase-gray state and cannot form colonies (**Fig. 5**; Ishikawa *et al.*, 1998; Setlow *et al.*, 2001; Setlow, 2013). CwlJ is located near the coat-cortex boundary and is activated by Ca^{2+}-DPA efflux, whereas SleB is situated on the outer surface of the IM as well as the coat-cortex boundary and has an unknown activation mechanism (Paidhungat *et al.*, 2001; Chirakkal *et al.*, 2002; Bagyan and Setlow, 2002; Setlow, 2013).

Both CLEs specifically hydrolyze the cortex and not the germ cell wall, as they require the muramic-δ-lactam modification of cortical PG for cleavage (Popham *et al.*, 1996; Tan and Ramamurthi, 2014). Cortex hydrolysis allows further water uptake and an approximately twofold core volume expansion (Setlow, 2003; Cowan *et al.*, 2004). Core expansion goes along with a ca. 1.6-fold surface increase in the absence of lipid and PG synthesis (Cowan *et al.*, 2004; Setlow, 2013). As a consequence of all germination-induced changes, a germinated spore attains normal IM permeability, IM viscosity, IM lipid mobility, water content, and protoplast protein mobility, thus being prepared to reactivate metabolism (Cowan *et al.*, 2003; Cowan *et al.*, 2004; Loison *et al.*, 2013; Setlow, 2013).

1.3.3. Non-nutrient-induced germination of *B. subtilis* spores

The mechanisms of germination induction by non-nutrient germinants are diverse (**Fig. 5**; Setlow, 2003). Exogenous Ca^{2+}-DPA, just as the endogenous Ca^{2+}-DPA released during germination stage I, activates the CLE CwlJ by an unknown mechanism (Riemann and Ordal, 1961; Paidhungat *et al.*, 2001; Setlow, 2014a). CwlJ-dependent cortex hydrolysis then allows spore core expansion, water uptake and the release of ions and (endogenous) Ca^{2+}-DPA without requiring GRs (Paidhungat and Setlow, 2000; Setlow, 2003). Severe coat defects (e.g. due to chemical decoating or knockout of major morphogenetic proteins; see **section 1.1.2**) abolish germination induction by exogenous Ca^{2+}-DPA due to loss of CwlJ (Paidhungat and Setlow, 2000; Bagyan and Setlow, 2002). The cationic surfactant dodecylamine is thought to trigger germination by opening SpoVA channels, thereby inducing Ca^{2+}-DPA release (Rode and Foster, 1961; Setlow *et al.*, 2003; Vepachedu and Setlow, 2007a). It should be noted that decoated spores germinate better with dodecylamine and that dodecylamine-germinated spores cannot progress into outgrowth, because the alkylamine kills the germinated spores (Rode and Foster, 1961; Setlow *et al.*, 2003; Knudsen *et al.*, 2016). High pressure can induce germination by different mechanisms: 100 to 300 MPa pressure activate the GRs in the absence of nutrient germinants, whereas pressures > 400 MPa trigger Ca^{2+}-DPA release by opening SpoVA channels and/or by a phase shift of the IM (Wuytack *et al.*, 2000; Paidhungat *et al.*, 2002; Reineke *et al.*, 2013). While non-nutrient germination induction via exogenous Ca^{2+}-DPA, dodecylamine, and high pressures have been known for decades, it was found quite recently that PG fragments can also trigger spore revival via the protein kinase PrkC (Shah *et al.*, 2008; Setlow, 2013).

Altogether, there are various agents that can induce germination and frequently the same spore components are involved. However, many molecular details of the different germination pathways still remain to be determined (Setlow, 2014a).

1.3.4. Germination kinetics of *B. subtilis* spores

Spore germination can be observed by various methods (Moir and Smith, 1990; Nicholson and Setlow, 1990). The extent of germination at a given time point can for instance be evaluated by determining colony forming ability after a pasteurization treatment that is only survived by heat resistant (i.e. dormant) spores. However, as germination is a rapid process, the aforementioned approach cannot precisely resolve germination kinetics. A well-established method to investigate germination kinetics is the spectrophotometric measurement of the optical density (OD) changes in a germination culture. As germinating spores lose their refractivity, an OD_{600nm} decrease indicates germination (Moir and Smith, 1990; Nicholson and Setlow, 1990). After mixing spores with germinants, there is a short lag time with constant OD_{600nm}, followed by a rapid OD_{600nm} decrease, which ends after approximately 15 min under optimal conditions (Setlow and Kornberg, 1970a; Setlow *et al.*, 2012; Sinai *et al.*, 2015).

As OD measurements reflect the average germination of a spore population, it is important to note that germination of individual spores in isogenic spore populations can be quite heterogeneous (Moir and Smith, 1990; Setlow *et al.*, 2012). Single-spore analyses have shown that especially the lag time between germinant addition and Ca^{2+}-DPA release varies strongly among individual spores (Peng *et al.*, 2009; Zhang *et al.*, 2010; Setlow *et al.*, 2012). The extent of the lag time phase is linked to the number of functional GRs/spore, which is on average ca. 2500 GRs/spore, but can vary within isogenic populations (Griffiths *et al.*, 2011; Zhang *et al.*, 2010; Stewart and Setlow, 2013). Although incompletely understood, GR levels are not the only determinant of germination heterogeneity, but seem to be involved in the rate-limiting step (Zhang *et al.*, 2013). To decrease germination heterogeneity within populations, spores are usually subjected to a sub-lethal heat shock (‘heat activation’, e.g. 70°C for 30 min) prior to germination analyses, which is thought to affect the GRs either directly or indirectly via heat-induced membrane changes, thereby decreasing the lag time (Setlow, 2013; Luu *et al.*, 2015). In general, lag time duration is not only affected by intraspecific variation and heat activation, but also varies with germination temperature and germinant concentration (Setlow, 2013).

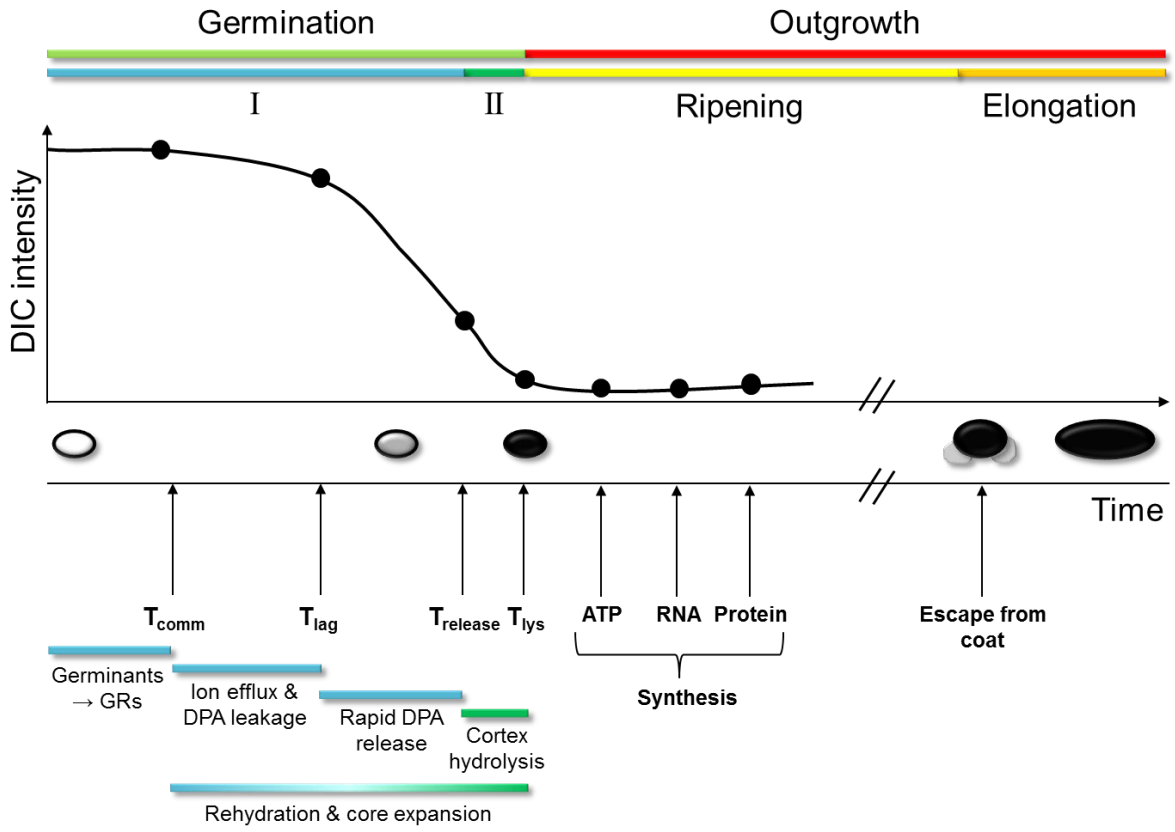


Figure 6: Germination and outgrowth kinetics of a single *B. subtilis* spore. The figure depicts the revival phase (top), the change in observable differential interference-contrast (DIC) intensity during germination (black line), the phenotypic changes (middle), and hallmark events (bottom) of germination and outgrowth. Note that the duration of individual phases can vary in populations. I = stage I of germination; II = stage II of germination; T_{comm} = time point of commitment; T_{lag} = start of rapid Ca^{2+}-DPA release; $T_{release}$ = time point when ≥ 75% is released; T_{lys} = end of cortex hydrolysis

Based on single-spore observations, the following kinetic germination model can be deduced. After mixing spores and germinants, no changes are measureable until the spore commits to germinate (T_{comm}; **Fig. 6**). In this pre-commitment phase, germinants diffuse through the spore integuments and bind to the GRs, thereby likely inducing a conformational change (Wang *et al.*, 2015b). This information is directly or indirectly transduced to SpoVA channels, which in turn alters their conformation from closed (C_0) to a metastable, activated-but-closed (C_1) state (**Fig. 7**; Vepachedu and Setlow, 2007b; Kong *et al.*, 2015; Wang *et al.*, 2015b). The C_1 state stores the germination stimulus, but can decay back to C_0 after some time (Wang *et al.*, 2015b). When, however, a threshold level of SpoVA channels is in C_1 state, the cooperative action among SpoVA proteins (and possibly the germinosome) causes a second conformational switch of SpoVA (O_1 state) and presumably also the opening of

unknown ion channels, so that the spore is now committed to germinate (Griffiths *et al.*, 2011; Setlow, 2013; Setlow, 2014a; Wang *et al.*, 2015a; Wang *et al.*, 2015b). O_1-state SpoVA slowly leaks Ca^{2+}-DPA, and although the duration of the Ca^{2+}-DPA leakage phase varies among individual spores, the total amount of leaked Ca^{2+}-DPA (around 20%) is relatively constant (Peng *et al.*, 2009; Wang *et al.*, 2015a; Wang *et al.*, 2015b). This suggests the existence of some Ca^{2+}-DPA leakage threshold, and when it is reached (at T_{lag}), SpoVA changes to a fully open (O_2) conformation (**Fig.6**; **Fig.7**; Wang *et al.*, 2015a; Wang *et al.*, 2015b). T_{lag} hallmarks the beginning of the main, rapid Ca^{2+}-DPA release phase termed $\Delta T_{release}$ (Peng *et al.*, 2009). $\Delta T_{release}$ is quite constant among individual wild type *B. subtilis* spores, taking approximately 2 to 3 min regardless of heat activation, germinant concentration, and GR levels (Peng *et al.*, 2009; Zhang *et al.*, 2010; Setlow *et al.*, 2012; Wang *et al.*, 2015a). When all Ca^{2+}-DPA is released (at $T_{release}$), a further slow refractivity decrease due to cortex hydrolysis, water uptake, and core expansion (ΔT_{lys}) can be observed (**Fig. 6**; Wang *et al.*, 2015a).

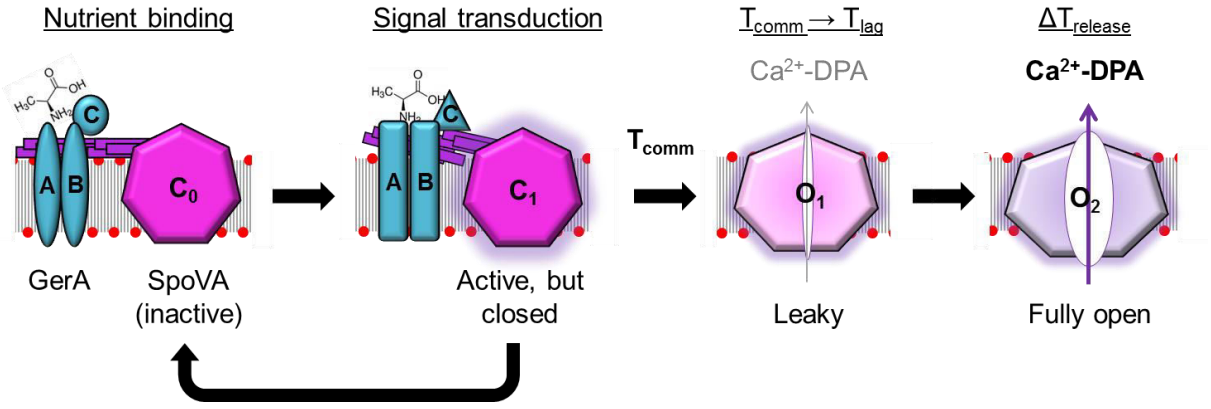


Figure 7: Model for SpoVA activation. The germination signal generated by a GR upon nutrient binding is transduced to an inactive, closed (C_0) SpoVA channel (left), thereby changing it to a metastable, activated-but-closed state that can either decay back to C_0 or upon commitment (T_{comm}) change to a partially open (O_1) state that leaks a fraction of Ca^{2+}-DPA prior to T_{lag}. Upon T_{lag}, SpoVA switches to the fully open O_2 state involved in the rapid Ca^{2+}-DPA release during $\Delta T_{release}$. Adapted from Wang *et al.*, 2015b.

1.3.5. Germination of different *Bacillus* species

The central series of germination events (i.e. ion and Ca^{2+}-DPA release, core rehydration, and cortex hydrolysis) takes place in spores of all *Bacillus* species (Swerdlow *et*

al., 1981; Paredes-Sabja *et al*., 2011; Setlow, 2013). Moreover, single-spore germination kinetics (**Fig. 6**) were shown to apply to germination of *B. cereus* and *B. megaterium* spores as well (Zhang *et al*., 2010). However, an important difference among spores of different *Bacillus* species is the repertoire of GRs and their substrate-specificity (Ross and Abel-Santos, 2010; Paredes-Sabja *et al*., 2011). *B. cereus*, for instance, possesses seven putative GR operons and responds to a broad range of nutrient germinants including L-glutamine, L-cysteine, L-threonine, inosine, nucleosides, and L-alanine (Ross and Abel-Santos, 2010; Giebel *et al*., 2012). Moreover, different bacilli have a variable array of different signal integration strategies, involving combinations of one or several nutrient germinants binding to one or several GRs (Ross and Abel-Santos, 2010). Yet, *Bacillus* species GRs are generally homologous members of the GerA family that exhibit sequence homology among the individual A, B, and C subunits of different species, and although the genetic arrangement of GR genes can vary, approximately 50% of all GRs are organized in tricistronic operons (Ross and Abel-Santos, 2010; Paredes-Sabja *et al*., 2011; Setlow, 2013). Not only are many GRs homologous to *B. subtilis'* GerA; L-alanine can also serve as efficient germinant or cogerminant in many bacilli and even in *Clostridium* species (Ross and Abel-Santos, 2010). Aside of the GRs, other germination proteins are also subject to minor interspecific variation. For instance, spores of several *Bacillus* species have two *spoVA* operons, and *B. anthracis* spores possess an additional *cwlJ* homolog (Paredes-Sabja *et al*., 2011; Giebel *et al*., 2012). While such variations may or may not affect germination in specific molecular aspects, spore germination is generally very similar among different *Bacillus* species (Paredes-Sabja *et al*., 2011; Setlow, 2013).

1.3.6. The outgrowth phase of germinated *Bacillus* spores

Spore outgrowth is defined as the time interval between the onset of metabolic activity and the first vegetative cell division (Keijser *et al*., 2007; Setlow, 2013). Outgrowth consists of a 'ripening' phase, in which a germinated spore undergoes numerous molecular changes including synthesis of essential biomolecules in the absence of morphological change, followed by an elongation phase, in which it escapes from its spore coat remnants and elongates (**Fig. 6; Fig. 8**; Setlow and Kornberg, 1970a; Setlow and Kornberg, 1970b; Santo and Doi, 1974; Setlow, 2003; Keijser *et al*., 2007; Plomp *et al*., 2007; Segev *et al*., 2013; Sinai *et al*., 2015).

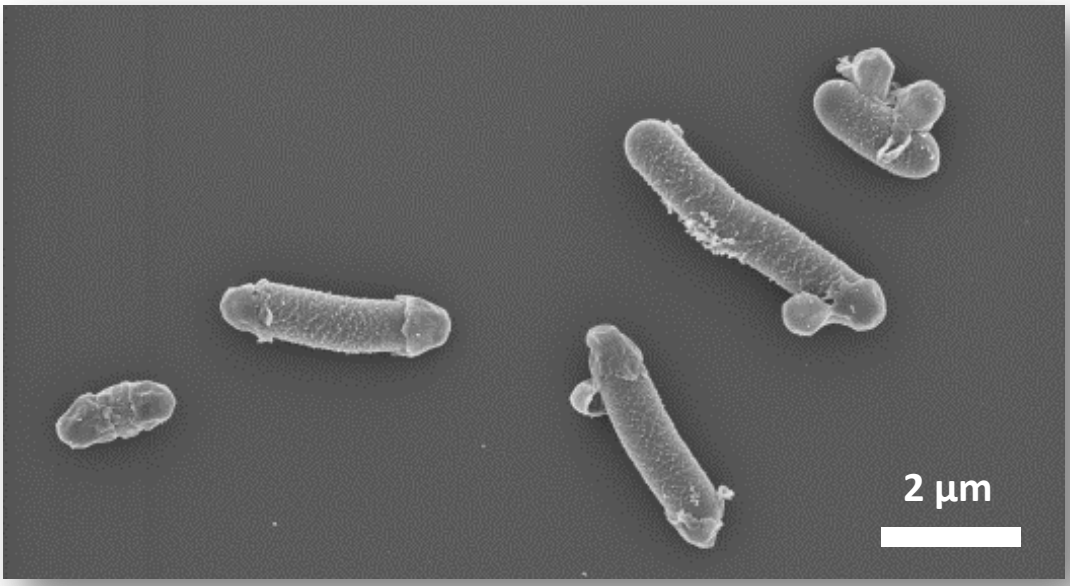


Figure 8: Scanning electron microscopic image of *B. subtilis* outgrowth. The image depicts different progression into the elongation phase among outgrowing spores in an isogenic spore population germinated under optimal conditions. Spore coat remnants are still attached to the outgrowing spores (K. Madela and K. Nagler, unpublished data).

Metabolism is initiated as soon as the spore core has reached a sufficient hydration to allow enzymatic activity (Setlow, 2003). The earliest outgrowth events involve the exploitation of endogenous resources to acquire the energy and building blocks for protein biosynthesis (Setlow and Kornberg, 1970a; Setlow and Kornberg, 1970b). Within few minutes after germination initiation, ATP is generated from the spore's 3PGA depot without requiring exogenous resources (**Fig. 6**; Setlow and Kornberg, 1970a). The enzymes required for ATP synthesis are already present in the dormant spore, and at least phosphoglycerate mutase, which initiates 3PGA catabolism, is activated by rehydration and the pH upshift (Setlow and Kornberg, 1970a; Setlow, 1994; Setlow, 2003). Shortly after ATP production, RNA synthesis is initiated, which is facilitated by the degradation of dormant spore RNA to yield nucleotides (Setlow and Kornberg, 1970a; Setlow and Kornberg, 1970b). Among the early synthesized RNA is mRNA encoding essential metabolic enzymes including enzymes for nucleotide biosynthesis, but also transporters, DNA repair proteins, and regulatory proteins (Setlow and Kornberg, 1970b; Keijser *et al.*, 2007; Sinai *et al.*, 2015). Amino acids for protein biosynthesis are initially generated by SASP degradation, which is initiated by the endoprotease Gpr that is already present (albeit inactive) in dormant spores (Setlow and Kornberg, 1970a; Setlow and Kornberg, 1970b; Setlow, 1994).

Approximately 15 min after germination initiation, the spore switches mainly to the use of extracellular nutrients and begins with *de novo* nucleotide synthesis (Setlow and Kornberg, 1970a; Setlow and Kornberg, 1970b; Keijser *et al.*, 2007). Aside from the activation and biosynthesis of proteins required for transcription, translation and metabolic energy generation, the early phase of ripening also encompasses other important processes including nutrient uptake, ion homeostasis, and DNA repair (Keijser *et al.*, 2007; Sinai *et al.*, 2015). Note that DNA repair, which involves nucleotide excision repair, spore photoproduct lyase, and base excision repair, relies not solely upon newly synthesized proteins, but also on proteins that are already present in the dormant spore (Wang *et al.*, 2006; Keijser *et al.*, 2007; Wells-Bennik *et al.*, 2016). Later in the ripening phase, the outgrowing spore prepares for elongation growth, cellular reshaping, and escape from the spore coat (Santo and Doi, 1974; Keijser *et al.*, 2007; Sinai *et al.*, 2015).

The exact timing of events during ripening is variable. Unsurprisingly, the composition of the germination medium affects the pace of ripening, with higher nutrient availability allowing faster molecular reorganization (Paidhungat and Setlow, 2002; Keijser *et al.*, 2007). Moreover, the molecular cargo of the dormant spore (especially its RNA content) was recently found to affect the duration of ripening (Segev *et al.*, 2013; Sinai *et al.*, 2015). While intact rRNA may allow a rapid start of translation, it was proposed the dormant spore mRNA may not only serve as nucleotide reservoir, but may also be translated, although this has not been proven experimentally (Keijser *et al.*, 2007; Segev *et al.*, 2013).

The disassembly of and escape from the spore coat hallmarks the elongation phase of outgrowth (**Fig. 6; Fig. 8**). The timing of the beginning of coat degradation is variable, but under ideal, nutrient rich conditions, notable signs of coat disintegration become apparent between 30 to 40 min in TEM sections (Santo and Doi, 1974). Atomic force microscopic analyses of *B. atrophaeus* outgrowth indicate that degradation of the coat surface starts with the formation of micro-etch pits that subsequently extend to fissures (Plomp *et al.*, 2007). How coat disassembly is mediated on a molecular level is not known, but an involvement of proteases was proposed (Plomp *et al.*, 2007; Keijser *et al.*, 2007; Setlow, 2013). The actual elongation phase starts (under optimal conditions) approximately 60 to 70 min after germination, leads to the formation of a typical rod shape, and ends with cell division (Santo and Doi, 1974; Plomp *et al.*, 2007; Sinai *et al.*, 2015). Throughout elongation the outgrowing spore possesses a complete metabolic proteome, synthesizes structural cell components such as cell wall material, and prepares for cell division (Keijser *et al.*, 2007; Sinai *et al.*, 2015).

Overall, outgrowth is an elaborate, well-regulated process involving a multitude of transcriptomic and proteomic changes: approximately 30 % of the *B. subtilis* genome is expressed and roughly 650 different proteins are newly synthesized, more than 200 of which in the first 30 min of outgrowth (Keijser *et al.*, 2007; Sinai *et al.*, 2015).

Taken together, sporulation, dormancy, and spore revival represent a sophisticated example of microbial differentiation that is increasingly understood, although much remains to be learned with regard to molecular mechanisms as well as how these processes are affected by environmental conditions.

1.4. Aims and relevance

1.4.1. Aims

The central goal of this doctoral thesis was to develop a comprehensive picture of the effects of high salinity on *Bacillus* spore germination and outgrowth with regard to various different aspects. Although it is long-known that high salt concentrations can exert inhibitory effects on *B. subtilis* spore germination (Fleming and Ordal, 1964), this finding was not analyzed in detail. Hence, as a first step, general effects of the 'model salt' NaCl on *B. subtilis* spore germination were systematically elucidated using various techniques (Nagler *et al.*, 2014; **Chapter 2**). Additionally, the influence of a set of different salts on *B. subtilis* spore germination was characterized to determine, which properties of a salt are relevant for germination inhibition (Nagler and Moeller, 2015; **Chapter 3**). Since the molecular processes in germination were barely understood at the time of the study by Fleming and Ordal (1964), the mechanistic basis for their findings remained elusive. Given the advanced understanding of *B. subtilis* spore germination, a third goal of this thesis was to shed light on the mechanisms and spore features that are detrimentally affected by high NaCl concentrations (Nagler *et al.*, 2015; **Chapter 4**). Moreover, it was investigated to which extent the observations made in *B. subtilis* can be transferred to spore germination of other *Bacillus* species, which required the establishment of an interspecifically comparative germination system (Nagler *et al.*, 2016; **Chapter 5**). Regarding *B. subtilis* outgrowth at high salinity it was previously reported that sufficient energy availability is crucial for adaptation processes, which remained to be investigated (Tovar-Rojo *et al.*, 2003). Thus, a final aim of in this doctoral thesis was to characterize the salt stress response during *B. subtilis* outgrowth using RNA sequencing (Nagler *et al.*, *in preparation for submission*; **Chapter 6**).

1.4.2. Relevance

In the context of different research fields, *Bacillus* spores can be regarded as a model for prokaryotic differentiation, as pathogenic agents (e.g. *B. anthracis*), as contaminants of food (e.g. *B. cereus*) and other products, as bioindicators (e.g. sterilization control), and as pharmaceutical vehicles (e.g. vaccine development), among others (Nicholson, 2004; Abee *et al.*, 2011; Setlow, 2014b; Wells-Bennik *et al.*, 2016). In all cases, profound knowledge on spore germination is important, but unfortunately there are still numerous open questions regarding the mechanisms and principles of germination (Setlow, 2014a). As the use of agents that inhibit germination can provide new insight in the germination process itself (Cortezzo *et al.*, 2004), investigation of high-salinity effects on spore revival is relevant in terms of basic research. Moreover, this topic is of interest for applied research fields, including (i) food microbiology, (ii) soil ecology, and (iii) astrobiology.

(i) Microbial contamination of food can lead to food spoilage and, depending on the bacterial species, can also cause food-borne disease (Setlow, 2014b; Wells-Bennik *et al.*, 2016). Thus, food is often preserved by a combination of an inactivation treatment (such as heat) and growth limiting factors within the food, such as low a_w, which can for instance be achieved by salt (Wells-Bennik *et al.*, 2016). Since dormant spores are resistant against many inactivation treatments as well as desiccation and other adverse conditions, their presence in foods in particularly problematic for the food industry (Setlow, 2014b; Bassi *et al.*, 2016; Wells-Bennik *et al.*, 2016). Spore revival is the prerequisite for food spoilage and toxin production, but also goes along with a loss of the spore resistance properties (Abee *et al.*, 2011; Setlow *et al.*, 2012; Setlow, 2014b; Bassi *et al.*, 2016). Thus, spore germination and outgrowth, and how these processes are affected by surrounding conditions such as low water activity, is of applied interest for the food industry (Setlow, 2014b; Wells-Bennik *et al.*, 2016). In this regard, spores of *B. subtilis* can serve as a non-pathogenic, well-established model for pathogenic species such as *B. cereus*.

(ii) Bacilli are important for soil ecology, as they can for instance improve plant health as well as growth and are involved in biogeochemical cycling (Kloepper *et al.*, 2004; Nicholson, 2004; Mandic-Mulec *et al.*, 2015). Soil osmolarity can fluctuate considerably by natural desiccation, but also due to human influences such as high-efficiency-oriented agricultural activities (Bremer, 2002; Datta and de Jong, 2002; Zalidis *et al.*, 2002; Ju *et al.*, 2007; de Souza Silva and Fay, 2012; Hoffmann and Bremer, 2016). The latter can be problematic, as the consequent soil salinization detrimentally affects soil quality and

represents a growing worldwide ecological issue (Datta and de Jong, 2002; Zalidis *et al.*, 2002; Ju *et al.*, 2007; de Souza Silva and Fay, 2012). Therefore, it is important to understand, how salt affects the life cycle (including spore revival) of ubiquitous soil microbes like *B. subtilis*.

(iii) High-salinity effects on spore germination and outgrowth are also of interest for astrobiology and especially 'planetary protection', which refers to the prevention of contamination of extraterrestrial celestial bodies with terrestrial microbes (Crawford, 2005). Despite their assembly in clean rooms and strict hygienic measures, spacecraft are often contaminated with a certain amount of microbes ('bioload') including spore-forming bacteria such as *Bacillus* species (Crawford, 2005; Nicholson *et al.*, 2009). Due to their multiple resistances as well as their dormancy, spores are able to survive space travel and therefore have the potential to be transferred to another planet or moon (Nicholson *et al.*, 2000; Nicholson *et al.*, 2009). Hence, it is important to assess, whether potentially transferred spores can revive under extraterrestrial conditions. A central prerequisite for spore revival is the availability of liquid water (Stevenson *et al.*, 2015). In our solar system, there are at least three celestial bodies, on which the occurrence of liquid water is assumed: Mars, where so-called recurring slope lineae indicate seasonally occurring liquid brines (Ojha *et al.*, 2015) as well as the moons Europa (Jupiter moon) and Enceladus (Saturn moon), both likely harboring vast salt water oceans under their icy crust (Marion *et al.*, 2003; Hand and Carlson, 2015; Thomas *et al.*, 2015). Hence, in each case, extraterrestrial liquid water would be in close association with salts, thus prompting the question, to which extent spores can revive in high-salinity environments.

CHAPTER 2

High salinity alters the germination behavior of *Bacillus subtilis* spores with nutrient and non-nutrient germinants

Nagler K, Setlow P, Li YQ, Moeller R. 2014. High salinity alters the germination behavior of *Bacillus subtilis* spores with nutrient and non-nutrient germinants. Appl. Environ. Microbiol. **80**(4):1314-1321.

In this publication, the effect of high NaCl concentrations on the germination of wild type *B. subtilis* spores was systematically investigated. Using different spectrophotometric and microscopic approaches it could be shown that high salinity delays germination initiation, prolongs subsequent events, and decreases overall germination efficiency, although a fraction of the spore population initiated germination despite non-growth-permissive salt concentrations. Germination inhibition at the stages of germinant receptor accessibility, germinant binding to the receptors, signal transduction, ion efflux, Ca^{2+}-DPA release, and cortex hydrolysis was considered and discussed. This paper laid the foundation for the following mechanistic studies on the effects of high salinity on *B. subtilis* spore germination and outgrowth.

Author contributions:

K. Nagler designed, performed, and evaluated the experiments (except DIC microscopy), and prepared the text and figures of the manuscript. **P. Setlow** supported DPA release and commitment analyses, gave scientific input, and edited the manuscript text. **Y.Q. Li** performed and evaluated DIC microscopy. **R. Moeller** gave scientific input and edited the manuscript text.

Applied and Environmental Microbiology

High Salinity Alters the Germination Behavior of Bacillus subtilis Spores with Nutrient and Nonnutrient Germinants

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High Salinity Alters the Germination Behavior of *Bacillus subtilis* Spores with Nutrient and Nonnutrient Germinants

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The effect of high NaCl concentrations on nutrient and nonnutrient germination of *Bacillus subtilis* spores was systematically investigated. Under all conditions, increasing NaCl concentrations caused increasing, albeit reversible, inhibition of germination. High salinity delayed and increased the heterogeneity of germination initiation, slowed the germination kinetics of individual spores and the whole spore population, and decreased the overall germination efficiency, as observed by a variety of different analytical techniques. Germination triggered by nutrients which interact with different germinant receptors (GRs) was affected differently by NaCl, suggesting that GRs are targets of NaCl inhibition. However, NaCl also inhibited GR-independent germination, suggesting that there is at least one additional target for NaCl inhibition. Strikingly, a portion of the spore population could initiate germination with L-alanine even at NaCl concentrations near saturation (~5.4 M), suggesting that spores lack a salt-sensing system preventing them from germinating in a hostile high-salinity environment. Spores that initiated germination at very high NaCl concentrations excreted their large depot of Ca^{2+}-pyridine-2,6-dicarboxylic acid and lost their heat resistance, but they remained in a phase-gray state in the phase-contrast microscope, suggesting that there was incomplete germination. However, some metabolic activity could be detected at up to 4.8 M NaCl. Overall, high salinity seems to exert complex effects on spore germination and outgrowth whose detailed elucidation in future investigations could give valuable insights on these processes in general.

When confronted with nutrient depletion, *Bacillus subtilis* can form endospores, which are highly resistant to a broad range of stresses, including heat, radiation, and various chemicals, and it can remain dormant (i.e., metabolically inactive) over long periods of time (1, 2). Vreeland et al. (3), for instance, reported the isolation and revival of *Bacillus* sp. from a brine inclusion in a 250 million-year-old salt crystal, where the bacteria might have survived in the form of spores. Despite their dormancy, spores can perceive improvements in environmental conditions by recognizing the presence of appropriate nutrients, which can cause spores to induce germination, initiating their conversion back into growing cells (4, 5).

The recognition of nutrient germinants is mediated by specific germinant receptors (GRs) (4). Three different types of GRs, each composed of at least three subunits, have been identified in *B. subtilis*: GerA, which responds to L-alanine and L-valine, as well as GerB and GerK, which act together to respond to a nutrient mixture termed AGFK (L-asparagine, D-glucose, D-fructose, and K^+) (6–8). All GRs seem to interact with each other in some fashion and colocalize in a discrete cluster within the spore's inner membrane, likely forming a functional germination unit termed the germinosome that is important for quick and efficient germination (9–11).

The recognition of nutrients by GRs can, in a yet-unknown way, initiate a series of characteristic germination events (2). Initially, a spore commits to germinate, i.e., it continues to progress through germination even when the germinant is removed or germinant binding is reversed and inhibited (12). Commitment is followed by two stages of germination: first, large amounts of monovalent cations and then the spore core's huge depot of pyridine-2,6-dicarboxylic acid (dipicolinic acid, or DPA) in a 1:1 chelate largely with Ca^{2+} (Ca^{2+}-DPA) are excreted, accompanied by some spore core hydration (2, 12, 13). Subsequently, the peptidoglycan spore cortex is hydrolyzed by two redundant cortex-lytic enzymes (CLEs), CwlJ and SleB, leading to further core hydration and core expansion (2, 13). Throughout these two stages, the spore loses its resistance properties and ultimately enters outgrowth, a phase which is characterized by the onset of metabolism and macromolecular synthesis (2, 13).

In addition to nutrient germinants, spore germination can also be triggered by nonnutrients that do not require the action of GRs, such as exogenous Ca^{2+}-DPA, or cationic surfactants, such as dodecylamine (2, 14). Ca^{2+}-DPA is thought to activate CwlJ directly, initiating germination at the stage of cortex hydrolysis, while dodecylamine appears to directly trigger the opening of an inner membrane Ca^{2+}-DPA channel composed of SpoVA proteins (2, 4, 15–17).

The exact environmental conditions during sporulation and germination can have major impacts on the efficiency of these processes and the properties of the resultant spore and cell (18, 19). In 1964, Fleming and Ordal (18) reported that the ionic environment influences not only *B. subtilis* sporulation but also spore germination. In their study, they only investigated the effects of low to moderate salt concentrations on L-alanine-dependent germination, although knowledge of the effects of high salt concentrations on spore germination has significant relevance for

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several applied fields. These include food microbiology with regard to food preservation, because spores can cause food spoilage and food-borne diseases (20). In addition, as *B. subtilis* is an abundant soil inhabitant and soil salinization represents an increasing worldwide ecological problem, this topic is also important with regard to soil ecology (21, 22). Although high-salinity effects on and subsequent responses of growing *B. subtilis* cells are increasingly understood, much less is known about the impact of high salt concentrations on spore germination (23). Here, we present the first systematic study of the impact of high salinity on *B. subtilis* spore germination. We show that NaCl exerts strong inhibitory effects on germination, and that nutrient and nonnutrient germination are affected differently. Based on these observations, we discuss possible mechanisms underlying salt inhibition of spore germination.

MATERIALS AND METHODS

Spore production and purification. All experiments were carried out with *Bacillus subtilis* 168 (*trpC2*) originally obtained from the German Collection of Microorganisms and Cell Cultures GmbH (DSMZ, Braunschweig, Germany) as DSM402. Spores were produced on Schaeffer's sporulation medium plates (SSM) (24) that were incubated for 6 days at 37°C. The biomass was harvested, suspended in water, and centrifuged at 2,990 × *g* and 4°C. The pellet was resuspended in lysozyme solution (1 mg/ml lysozyme, 10 μg/ml DNase I, 2.5 μg/ml $MgSO_4$ in 10 mM Tris-HCl [pH 7]) and incubated for 30 min in a shaking incubator at 37°C. After a subsequent 15-min incubation at 80°C, the spores were washed at least seven times with water by centrifugation. Spores were stored in water at 4°C in screw-cap reaction tubes with glass beads until they were used. The purity of the spore stocks as checked by phase-contrast microscopy was ≥99%.

Spore germination. Spores were heat activated at 70°C for 30 min in order to ensure synchronized germination. For spectrophotometric measurements, germination was carried out on 96-well plates, each containing 200 μl of germination medium composed of 10 mM Tris-HCl (pH 8; with or without NaCl), 50.5 mM D-glucose, 0.5 mM L-tryptophan, and the germinant of interest: 10 mM L-alanine, 30 mM L-valine, or AGFK (10 mM L-asparagine, 100 mM D-glucose [no additional 1% glucose], 100 mM D-fructose, 100 mM KCl). Nonnutrient germination was carried out in 200 μl of 60 mM Ca^{2+}-DPA (pH 8; adjusted with dry Tris base) without further additions (4, 5). Germination media were inoculated with 40 μl heat-activated spores to a starting optical density of ca. 0.5 at 600 nm (OD_{600}), corresponding to a total of ca. 4×10^7 spores per well, and were incubated at 37°C in a multiplate reader (BioTek ELx808IU) that read the OD_{600} of the culture, with 5 s of shaking before all readings. Each germination condition was tested with six replicates. The OD_{600} data were normalized by division of each reading by the first reading (t_0), yielding the relative OD_{600} given in percentages. A 60% decrease in relative OD_{600} corresponds to germination of the whole spore population (8 and data not shown). It should be noted that there was often some decrease in the OD_{600} during incubation of spores without any germination trigger. However, phase-contrast microscopy confirmed that this decreased OD_{600} did not correspond to germination (data not shown), consistent with previous reports about this phenomenon, which was suggested to be due to spore aggregation and adsorption to test tube walls (8).

Analyses of DPA release and germination commitment were performed as described previously using the germination media described above and measuring DPA release by its fluorescence with Tb^{+3} in a Gemini EM multiwell fluorescence plate reader (Molecular Devices, Sunnyvale, CA) as described previously (12). Commitment was always investigated by the addition of D-alanine at various times in germination to a final concentration of 10 mM. D-Alanine serves to inhibit further commitment but allows committed spores to progress through germination (12). DPA release assays with L-alanine and commitment analyses with L-valine were performed in quadruplicate and duplicate, respectively.

To monitor spore germination via plating, spores were germinated in test tubes with 5 ml of the respective germination medium. To determine the amount of heat-resistant CFU (CFU_{Heat}), samples were withdrawn at various times, heat shocked (10 min, 80°C), diluted in water, plated on nutrient broth agar plates, and incubated overnight at 37°C prior to counting colonies; all plating experiments were performed in duplicate.

To view germination by microscopy, spores were germinated in 2-ml tubes in a shaking incubator. At various times, 5-μl samples were withdrawn and fixed by applying them to a microscope slide coated with 1% agar. For analysis of spore recovery after incubation in germination media with very high NaCl concentrations, spores were incubated for 4 h in germination media containing 3.6 M or 5.4 M NaCl, washed once with 10 mM Tris-HCl (no NaCl, no alanine) at room temperature, suspended in NaCl-free germination medium with L-alanine, and incubated at 37°C for 30 min. Micrographs were taken using a Zeiss fluorescence microscope (Axio Imager M2; Carl Zeiss MicroImaging GmbH) equipped with an AxioCam MRm and processed using the AxioVision 4.8.2 software and GIMP 2.6.12. The micrographs were evaluated by manual counting (at least 500 counts per time point) and assigning the individual spores to one of three distinguishable, phase-contrast microscopic phenotypes: bright (i.e., dormant spores), gray (i.e., germination induced, but not completed), or dark (i.e., germination completed).

Analysis of the germination of multiple individual spores by differential interference contrast (DIC) microscopy was as described previously (25) using the germination media described above. This analysis allowed the determination of a number of kinetic parameters for the germination of individual spores, including (i) the time between germinant addition and the initiation of fast DPA release (T_{lag}), (ii) the time for completion of DPA release ($T_{release}$), (iii) the time period for fast DPA release ($\Delta T_{release} = T_{release} - T_{lag}$), and (iv) the time period for the hydrolysis of the great majority of the spore cortex peptidoglycan once fast cortex hydrolysis has been initiated (ΔT_{lys}).

Statistical analyses of the data given in Tables 1 and 2 were performed by *t* tests using a significance level of $P < 0.05$. In Table 2, only T_{lag} and $\Delta T_{release}$ were tested. To analyze the onset of metabolic activity, germinating cultures containing 39.8 mM the redox dye alamarBlue were monitored spectrophotometrically as described above and previously (26, 27), but with OD measurements at both 600 nm and 550 nm. In short, alamarBlue changes its color from blue to pink when it reacts with reduced metabolites (e.g., FADH and NADH) that are newly synthesized after germination (26–28). OD_{600} and OD_{550} changes of parallel control germinations without alamarBlue were subtracted to determine OD changes due to the color changes while excluding the OD drop due to any refractility decrease.

RESULTS

L-Alanine-triggered germination is inhibited by NaCl. When germination was induced by L-alanine in NaCl-free medium, typical changes indicating efficient germination could be detected by all utilized methods. Within 15 to 30 min after the addition of germinant, the relative OD_{600} (Fig. 1A) and differential interference contrast (DIC; see Fig. 3A) intensity decreased rapidly, more than 99% of the spores turned dark in the phase-contrast microscope (Table 1; also see Fig. S1A and B in the supplemental material), large amounts of DPA were released (Fig. 2A), and ≥99.7% of the spores lost their wet heat resistance (see Fig. S1E). However, when NaCl was present in the germination medium, the typical germination-related changes were altered. With ≤1.2 M NaCl, there were moderate effects on germination. The refractility decrease, DPA release, and commitment were slightly delayed and slowed, although the overall efficiencies of these events were similar to those in the absence of NaCl (Fig. 1A, 2, and 3A and Table 1). Analysis of single-spore germination by DIC microscopy

TABLE 1 Phase-contrast microscopy analyses of L-alanine germination at various NaCl concentrations[e]

NaCl concn (M) and time[b] (min)	Difference (%) between phase-contrast microscopy phenotypes[a]		
	Bright	Gray	Dark
0			
0	97.9	0.0	2.1
30	0.9	0.0	99.1
60	0.0	0.2	99.8
120	0.2	0.2	99.6
240	0.1	0.1	99.8
1.2			
30	3.6[c]	1.2[c]	95.2[c]
60	2.3[c]	0.3	97.4[c]
120	2.0[c]	0.6	97.5[c]
240	0.8[c]	0.2	98.9[c]
3.6			
30	71.5[c,d]	26.0[c,d]	2.4[c,d]
60	45.7[c,d]	47.7[c,d]	6.6[c,d]
120	43.1[c,d]	47.9[c,d]	9.0[c,d]
240	26.8[c,d]	67.9[c,d]	5.4[c,d]
R30	0.6	14.2	85.2
4.2			
240	82.8[c,d]	13.3[c,d]	3.9[c,d]
4.8			
240	88.7[c,d]	9.8[c]	1.6[c,d]
5.4			
240	93.2[c,d]	4.7[c]	2.0[c,d]
R30	0.2	0.8	99.0

[a] Spores were classified by their appearance as described in Fig. S1 in the supplemental material.
[b] R30, recovery (counts 30 min after transfer to NaCl-free germination medium).
[c] Significantly different ($P < 0.05$) from the value at the respective time point at 0 M NaCl.
[d] Significantly different ($P < 0.05$) from the value at the respective time point at 1.2 M NaCl.
[e] At various times, samples of L-alanine germinating cultures were examined by phase-contrast microscopy (≥500 spores counted at each time point) as described in Materials and Methods.

also showed that several kinetic germination parameters were significantly different in the presence of 1.2 M NaCl (Table 2). In agreement with the observations on germination of spore population, 1.2 M NaCl delayed germination onset (increased T_{lag}) and increased the time required for the release of the great majority of spore DPA (increased $\Delta T_{release}$).

At NaCl concentrations of >1.2 M, the effects of NaCl on germination became much more pronounced, as the OD_{600} decrease and DPA release were progressively delayed and slowed and their overall magnitudes reduced (Fig. 1A, D, and 2A). The decreased germination efficiency at increasing NaCl concentrations was also observed microscopically, as a significant percentage of spores remained phase bright (Table 1). Overall, the relative OD_{600} changes in the first 30 min after germination initiation exhibited a reverse sigmoid functionality when plotted against the NaCl concentration in the germination medium (Fig. 1D). Using spectrophotometry, OD_{600} decreases could be detected during germination at up to 4.2 M NaCl, and with phase-contrast microscopy some refractility decrease of a small percentage of spores was observed at up to 5.4 M NaCl. However, during germination at very high salinity (≥3.6 M), phase-gray spores accumulated while the percentage of phase-bright spores decreased more or less strongly depending on the NaCl concentration, whereas the amount of phase-dark spores remained essentially constant (Table 1; also see Fig. S1C and D in the supplemental material). Interestingly, during germination with 3.6 M NaCl, the amounts of total CFU and heat-resistant CFU (80°C, 10 min) were quite similar and approximately equal to the amount of phase-bright spores (see Fig. S1E). This suggests (i) that the phase-gray spores were not as heat resistant as dormant spores (note that similar results were obtained using a milder heat treatment of 60°C for 30 min) and (ii) that primarily the heat-resistant spores were viable after plating. At 3.6 M NaCl, several kinetic germination parameters of individual spores were altered much more than at moderate salinity (Table 2). However, even at such a high salinity, the interval between the end of DPA release ($T_{release}$) and the completion of cortex hydrolysis (ΔT_{lys}) was not significantly affected. Most strikingly, increasing NaCl concentrations greatly increased the heterogeneity in germination onset within the spore population (Fig. 3). Fluorometric analyses revealed that high salinity delayed commitment, albeit not as strongly as the onset of DPA release, resulting in an increased time interval between commitment and T_{lag} at NaCl concentrations of ≥2.4 M (Fig. 2B and data not shown). Taken together, NaCl seems to exert inhibitory effects on L-alanine germination, with increasing salinity leading to decreased and slower germination. It is also notable that an excess of L-alanine (100 mM instead of 10 mM) did not significantly influence or counteract the negative effects of NaCl on germination (data not shown).

To test the reversibility of NaCl-dependent germination inhibition, spores that were incubated for 4 h in L-alanine germination medium containing 3.6 M or 5.4 M NaCl were washed, transferred to NaCl-free L-alanine germination medium, and examined by phase-contrast microscopy. The vast majority of spores in both cultures turned from bright or phase-gray to dark within 30 min

TABLE 2 Analysis of L-alanine germination of multiple individual spores by DIC microscopy[a]

NaCl concn (M)	T_{lag} (min)	$T_{release}$ (min)	$\Delta T_{release}$ (min)	ΔT_{lys} (min)	I_{lag} (AU)	$I_{release}$ (AU)
0	3.1 ± 2.2	4.9 ± 2.5	1.8 ± 0.5	7.6 ± 2.1	0.95 ± 0.03	0.21 ± 0.03
1.2	12.7 ± 7.4[b]	18.9 ± 8.8	6.4 ± 3.0[b]	13.0 ± 7.2	0.93 ± 0.06	0.22 ± 0.08
3.6	21.6 ± 16.5[b,c]	30.3 ± 17.3	8.6 ± 4.5[b,c]	9.6 ± 5.6	0.91 ± 0.06	0.16 ± 0.06

[a] Spores were germinated as described in Materials and Methods and reference 25. To determine the given parameters, between 83 and 200 individual spores were investigated. Mean values and standard deviations of T_{lag} (time of onset of rapid DPA release), $T_{release}$ (time at which DPA release was complete), $\Delta T_{release}$ ($T_{release} - T_{lag}$), ΔT_{lys} (time between completion of DPA release and completion of cortex hydrolysis), I_{lag} (DIC intensity at T_{lag}), and $I_{release}$ (DIC intensity at $T_{release}$) are given. AU, arbitrary units.
[b] Significantly different ($P < 0.05$) from T_{lag} or $\Delta T_{release}$ at 0 M NaCl.
[c] Significantly different ($P < 0.05$) from T_{lag} or $\Delta T_{release}$ at 1.2 M NaCl.

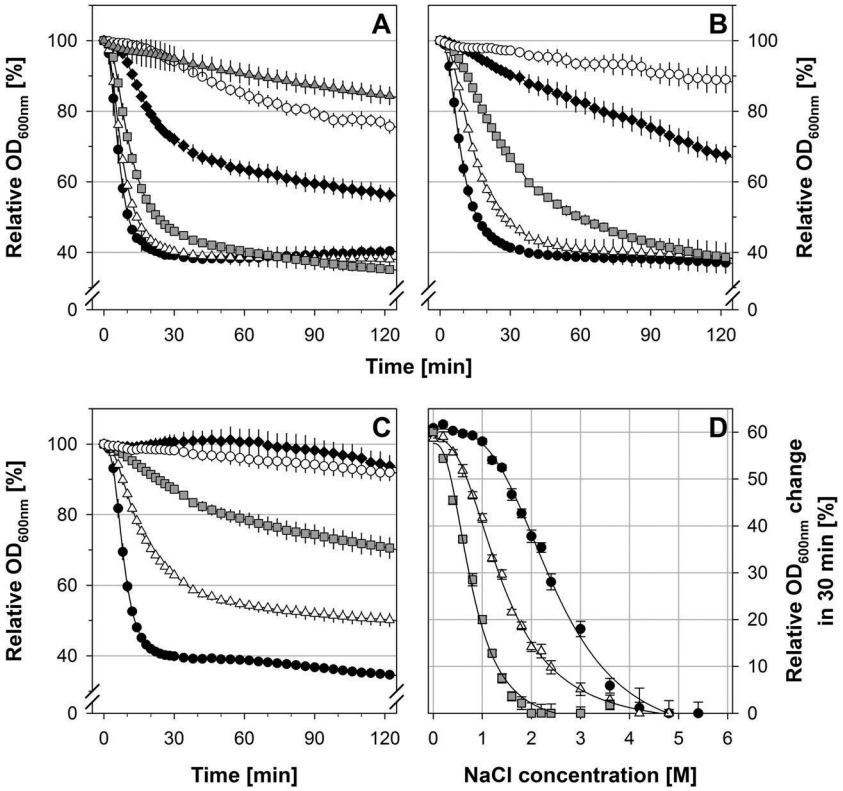

FIG 1 Effects of NaCl on nutrient germination. Germination was triggered by L-alanine (A), L-valine (B), and AGFK (C) in germination media containing no NaCl (black circles) or 0.6 M (white triangles), 1.2 M (gray squares), 2.4 M (black diamonds), or 3.6 M NaCl (white circles) and was measured by the OD_{600} as described in Materials and Methods. It should be noted that a certain OD_{600} decrease was observed in the absence of a germination trigger (A, gray triangles) which does not correspond to germination (8 and data not shown). (D) Relative OD_{600} changes in the first 30 min after germination initiation by L-alanine (black circles), L-valine (white triangles), or AGFK (gray squares) at various NaCl concentrations. Regression curves are shown, with four-parameter logistic curves with $R^2 = 0.997$ (L-alanine), $R^2 = 0.999$ (L-valine), and $R^2 = 0.992$ (AGFK). Error bars represent standard deviations.

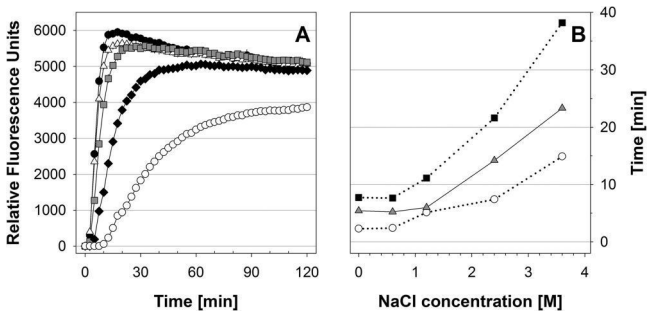

FIG 2 Effects of NaCl on DPA release and commitment. (A) DPA release during L-alanine germination at 0 M (black circles), 0.6 M (white triangles), 1.2 M (gray squares), 2.4 M (black diamonds), or 3.6 M NaCl (white circles) was monitored by changes in fluorescence intensity due to the formation of fluorescent terbium-DPA complexes (see Materials and Methods). Control experiments showed that high NaCl concentrations had no notable effect on terbium-DPA fluorescence (data not shown). (B) Times required for reaching 50% DPA release (black squares; % of maximum) and 50% commitment (white circles; % of maximum) in L-valine germinations in media containing 0 to 3.6 M NaCl. The lag times (Δ_{50}) between 50% DPA release and 50% commitment are given as gray triangles. Note that L-alanine commitment was extremely rapid (>90% commitment within 1 min at 0 M and 0.6 M NaCl), hampering accurate measurement at low salinity. Therefore, only the results for commitment in L-valine germinations are depicted. However, high-salinity effects on germination were similar for these two nutrient triggers.

of transfer, suggesting that the inhibitory effect of NaCl is reversible (Table 1).

The extent of germination inhibition by NaCl varies among nutrient germinants. Besides L-alanine, L-valine and AGFK also can be utilized as germination triggers for *B. subtilis* spores. While L-valine interacts with the same GR as L-alanine (GerA), AGFK activates two other GRs, GerB and GerK (2, 7, 8). Due to these different modes of action, the impact of NaCl on germination triggered by these two germinants was also investigated.

As shown in Fig. 1, the NaCl-dependent germination inhibition varied notably among the tested germinants. Comparing L-alanine and L-valine (Fig. 1A, B, and D), the differences in germination inhibition were relatively small at low NaCl concentrations, as the OD_{600} drop was only slightly more delayed in L-valine germination. However, with increasing NaCl concentrations the differences among the OD_{600} profiles for L-alanine and L-valine germination became more pronounced, with L-valine germination being slower than L-alanine germination, although the overall OD_{600} decrease after 4 h was similar with both germinants. Importantly, applying 10-fold higher L-valine concentrations (300 mM instead of 30 mM) yielded germination curves highly similar to the ones obtained with 10 mM L-alanine, regardless of the NaCl concentration (Fig. 1A and data not shown). Thus, NaCl seems to affect L-alanine- and L-valine-triggered germination in the same manner. This was further substantiated when comparing DPA

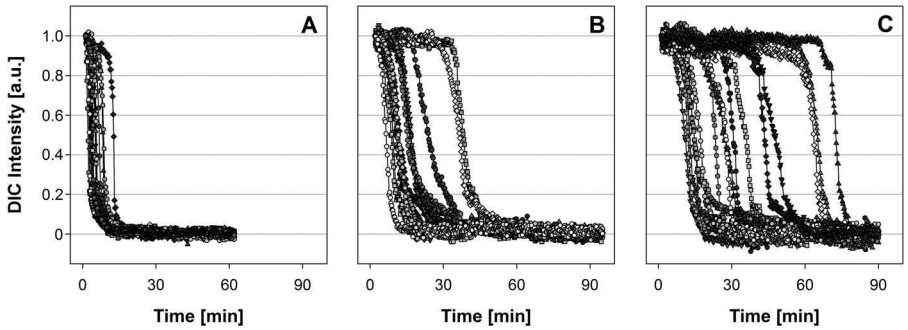

FIG 3 Kinetics of L-alanine germination of multiple individual spores by DIC microscopy. Spores were germinated as described in Materials and Methods and reference 25. Changes in DIC intensities of randomly selected spores that successfully germinated in the presence of 0 M NaCl (A), 1.2 M NaCl (B), and 3.6 M NaCl (C) are given in arbitrary units (a.u.).

release and commitment kinetics between these two germinants (Fig. 2B and data not shown).

Strikingly, the effect of NaCl on AGFK-triggered germination was much greater than that on GerA-dependent germination (Fig. 1C and D), as even 0.2 M NaCl greatly slowed AGFK germination. NaCl concentrations of ≥0.6 M also reduced the total OD_{600} decrease, leaving a considerable percentage of the spore population in a refractile state (data not shown). At 2.4 M NaCl, AGFK-triggered germination was almost completely blocked, as seen with both phase-contrast microscopy (data not shown) and OD_{600} measurements (Fig. 1C). Although plotting the relative OD_{600} change in 30 min after mixing spores and the different germinants against the salinity of the medium gave curves that all exhibited reverse sigmoid functionality, the slopes of the exponential regions differed (Fig. 1D). While the L-alanine and L-valine curves had gradients of −1.9 and −2.1, respectively, the AGFK curve was much steeper (gradient, −3.5). Hence, high NaCl concentrations had a differential impact on GerA- versus GerB/GerK-dependent germination.

Nonnutrient germination is also inhibited by NaCl. Germination at various NaCl concentrations was induced using exogenous Ca^{2+}-DPA, which is thought to trigger germination by direct activation of the cortex-lytic enzyme CwlJ, and with no GR involvement (2, 4, 15). In the absence of NaCl, the onset of the OD_{600} decrease in Ca^{2+}-DPA-dependent germination was significantly later than that for nutrient-dependent germination, and the drop also was not as steep (Fig. 4A). Consistent with the OD_{600} observations, phase-contrast microscopy showed that almost all spores had germinated by 120 min, although a few spores were still refractile or only phase-gray at the end of the experiment (Fig. 4B). NaCl could clearly inhibit Ca^{2+}-DPA germination, as observed by OD_{600} measurements and phase-contrast microscopy. Even 0.6 M NaCl greatly slowed germination, again leaving a large percentage of the spore population refractile until the end of the experiment (Fig. 4A and C). These effects were even more pronounced with 1.2 M NaCl, which allowed only a small fraction of spores to germinate within 4 h (Fig. 4A and D), while 2.4 M NaCl completely blocked Ca^{2+}-DPA germination (Fig. 4A and E). Hence, high NaCl concentrations inhibited both nutrient and nonnutrient spore germination.

Metabolism is initiated during outgrowth even with high NaCl. Using spectrophotometric, microscopic, and fluorometric methods, it is possible to monitor whether or not spores undergo essential steps of germination, such as DPA release, core swelling, and cortex hydrolysis, but this does not indicate if the germinated spores are viable and able to progress into outgrowth. In order to test this, spores were germinated at various salinities in the presence of the redox dye alamarBlue, which is an indicator for metabolic activity. Upon reaction with reducing electron-transfer metabolites, such as NADH or FADH, that must be newly synthesized after germination, alamarBlue changes color from blue to pink (26–28). The color change can be quantified spectrophotometrically at 600 nm (disappearance of the blue form) and 550 nm (appearance of the pink form).

When spores were germinated with L-alanine plus alamarBlue in the absence of NaCl, the OD_{600} and OD_{550} curves both exhibited a short lag phase of about 15 min until they began to decrease and increase, respectively (Fig. 5A and B). Over a long time, the OD changes were linear, although the OD_{550} curve reached a plateau toward the end of the experiment. At NaCl concentrations of ≤1.8 M, the OD profiles were just slightly altered; the OD changes were slowed slightly but had a similar overall magnitude to the changes observed in the absence of NaCl. Hence, spores that germinated under these salt conditions seemed to be viable and could clearly initiate metabolism, which is consistent with the colony-forming ability observed in plating experiments (data not shown). The effects of ≥2.4 M NaCl on metabolic activity in germinating cultures were much more pronounced, with large alterations in

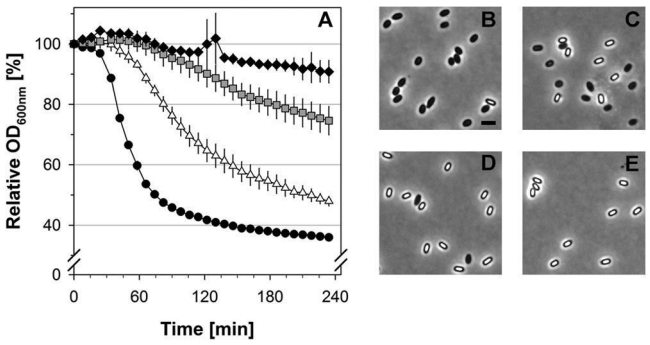

FIG 4 Effects of NaCl on Ca^{2+}-DPA germination. Germination was triggered by 60 mM Ca^{2+}-DPA and was measured as described in Materials and Methods. (A) OD_{600} measurements of germination in media with 0 M (black circles), 0.6 M (white triangles), 1.2 M (gray squares), and 2.4 M NaCl (black diamonds). (B to E) Phase-contrast microscopy 240 min after initiation of Ca^{2+}-DPA germination with 0 M (B), 0.6 M (C), 1.2 M (D), and 2.4 M NaCl (E). Scale bar, 2 μm.

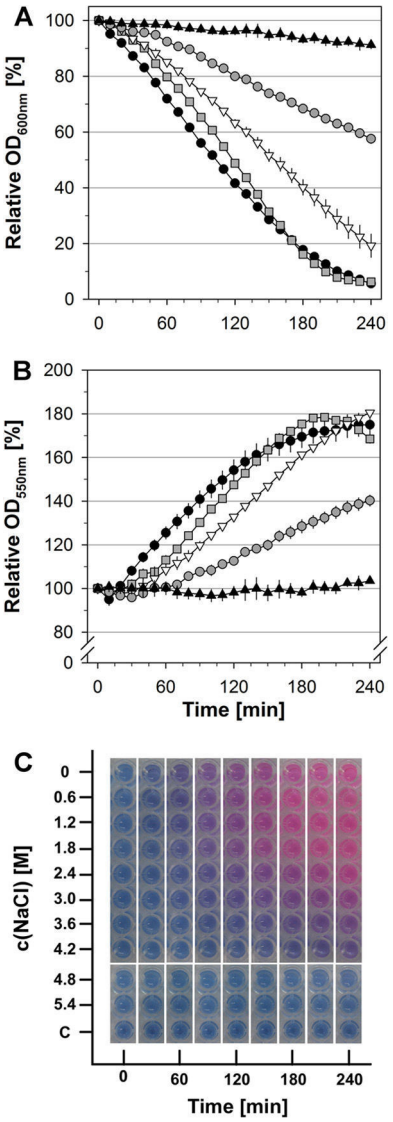

FIG 5 Onset of metabolic activity after L-alanine germination. (A and B) Spores were germinated with L-alanine in the presence of 10 μM alamarBlue at 0 M (black circles), 1.8 M (gray squares), 2.4 M (white triangles), 3.6 M (gray circles), and 4.8 M (black triangles) as described in Materials and Methods. The disappearance of the blue color (A) and the appearance of the pink color (B) were measured at 600 nm and 550 nm, respectively. OD readings from parallel control germinations (same media without alamarBlue) were subtracted from the OD data shown in panels A and B so that the indicated OD changes only represent the color changes. (C) Photographs were taken at 30-min intervals and show the color changes in media containing 0 M to 5.4 M NaCl within the same wells over time. C (control), medium with alamarBlue.

the OD_{600} and OD_{550} profiles, and progressive delays in the onset of color changes, as well as decreases in the magnitude of color changes with increasing NaCl concentrations. Nevertheless, some metabolic activity was detectable at up to 4.8 M NaCl, which is striking, since microscopy suggested that most spores were unable to complete germination normally at such a high NaCl concentration (Table 1).

DISCUSSION

While the overall effects of high salinity on vegetative *B. subtilis* cells are well understood (23), very little is known about the impact of elevated salt concentrations on spore germination. One study on wild-type spore germination in the presence of salts was performed decades ago (18), in which only the impact of relatively low salt concentrations (≤1 M) on L-alanine germination was analyzed using OD_{600} measurements. It was reported that low salt concentrations can enhance germination, whereas higher concentrations can have a detrimental impact (18). Accordingly, within this current study it could be shown that high salinity exerts diverse inhibitory effects on *B. subtilis* spore germination, although L-alanine-responsive germination was surprisingly efficient despite very high NaCl concentrations (see below).

In principle, inhibition by NaCl could occur at many stages within the germination process, including (i) the access of germinants to GRs, (ii) germinant binding to GRs, (iii) signal transduction from the GRs, (iv) ion efflux from the spore core followed by (v) DPA efflux, and ultimately (vi) cortex hydrolysis (2, 29).

Generally, the current work showed that GRs are one likely target for NaCl inhibition, as L-alanine or L-valine germination via the GerA GR was affected much less by NaCl than was AGFK germination triggered by the GerB and GerK GRs (Fig. 1A to D). Furthermore, increasing NaCl concentrations increased T_{lag} (the time of onset of rapid DPA release) and delayed commitment (Table 2 and Fig. 2B, respectively), and both of these kinetic parameters have been shown to be influenced by to the amount of activated GRs (12, 30). Therefore, it could be hypothesized that germinant passage through the spore integuments is hampered by NaCl, which would be consistent with the small germinant L-alanine being the most efficient and the multicomponent germinant AGFK being the least efficient at high salinities. For successful germination with AGFK, sufficient amounts of its individual components must reach the GRs, and the charge of K^+ in particular could hamper its passage considering potential ionic interactions with Na^+ and Cl^-. Although the details of germinant passage through the spore outer layers to the GRs are not clear, there is evidence that this process is facilitated by GerP proteins (31, 32), and these proteins' function might be affected by NaCl. Future research on mutant spores with coat, cortex, and GerP alterations could give new insights into whether or not NaCl influences germinant passage to the GRs. Additionally or alternatively, it could be hypothesized that NaCl hampers germination initiation at the level of germinant binding to the GRs. Inhibition at this stage has previously been reported for several L-alanine analogues, such as D-alanine (29, 33). However, an exclusively competitive inhibition mode for NaCl seems rather unlikely, as a 10-fold increase of L-alanine concentration did not eliminate the inhibitory effects of NaCl on GerA-dependent germination. Unfortunately, little is known about GR structures and particularly their binding sites (34); thus, a potential interference of NaCl at this stage (e.g., blockade of, or ionic interactions at, the binding site) is conceivable but highly speculative. To determine if germinant passage through the spore integuments and/or ligand binding to the GRs are crucial for NaCl-dependent germination inhibition, high-salinity effects on germination triggered by pressures of 100 to 200 MPa that is assumed to activate the GRs without germinant binding (35) could be analyzed in future research. Altogether, the GRs seem to be a likely direct or indirect site of NaCl inhibition, especially so when taking into consideration that many germination inhibitors were previously shown to block the action of GRs and their inhibition was reversible (29), which seems to be the case for NaCl inhibition as well.

(iii) As this study showed that the lag time between commitment and DPA release was increased at very high salinity (≥2.4 M NaCl) (Fig. 2B), signal transduction from the GRs causing subsequent germination is also likely to be affected by NaCl. Unfortunately, this signal transduction process is not understood (34). An important feature early in this process might be the germinosome, a colocalized cluster of all GRs plus GerD and possibly further proteins, most likely forming a functional unit required for quick, cooperative, and efficient germination (10). Within this cluster, protein-protein interactions (among GR subunits of individual GRs, and/or between the different GRs and associated proteins) seem likely to play a fundamental role (10, 36, 37), and some of these interactions might be hampered or even disrupted by high NaCl concentrations. This would be consistent with the finding that AGFK-responsive germination is most strongly affected by NaCl, since AGFK requires the cooperative action of GerB plus GerK (8). Furthermore, GR interactions with, or the action of, a putative signal integrator involved in synergistic effects of multiple germinants might be affected by high salinity (8, 38).

Although effects of NaCl on the above-mentioned processes are likely based on the observations within this study, additional inhibition seems to occur farther downstream in the germination pathway, as high salinity slowed DPA release and also inhibited GR-independent germination with exogenous Ca^{2+}-DPA. Thus, the next potential NaCl inhibition targets would be the yet-unidentified ion channels involved in rapid cation efflux (29, 34, 39) and/or the DPA excretion channels that seem to be at least in part encoded by the *spoVA* operon, although their organization and mode of action is not yet understood (17, 34, 40, 41). In a previous study reporting inhibitory effects of multivalent metal ions on the germination of decoated *B. subtilis* spores, the authors proposed that these ions form complexes with DPA, which could then act as a plug for the DPA channels (42). Whether this would also be possible for monovalent ions is unclear, but ionic interactions would certainly be conceivable.

(vi) The excretion of DPA precedes the full hydrolysis of the spore cortex, which is required for completion of the germination process, as it allows full core hydration and the onset of metabolic activity (13, 43). Cortex degradation is mediated by two redundant cortex-lytic enzymes (CLEs), SleB and CwlJ, with the latter CLE likely activated by Ca^{2+}-DPA (2, 4, 15). As both CLEs are located in the spores' outer layers (44, 45), they would likely be exposed to large amounts of NaCl in high-salinity media, representing further potential targets for NaCl-dependent germination inhibition. This would be supported by the observations that Ca^{2+}-DPA germination is strongly inhibited by NaCl (Fig. 4) and that spores seem to be arrested in some intermediate stage of germination (phase-gray spores; see Fig. S1 in the supplemental material) at very high salinities. However, DIC microscopy suggested that the time between completion of DPA release and completion of cortex hydrolysis (ΔT_{lys}) is not significantly affected by NaCl (Table 2), some metabolic activity was detectable even at high salinity, and inhibition of Ca^{2+}-DPA germination might well be an indirect effect (e.g., by interactions of exogenous Ca^{2+}-DPA with NaCl ions, preventing it from activating CwlJ). Further research on CLE mutant spores, analysis of CLE activity at elevated ionic strength, and/or investigation of cortex integrity would be required to conclude if CLEs are indeed affected by high salinity.

Altogether, there are several possible targets for NaCl inhibition within the germination pathway. Based on the observations within this study, it seems as if high salinity inhibits germination at several stages, with at least one inhibition site being GR related, at least one being subsequent to GR-ligand binding, and possibly another (direct or indirect) leading to decreased germination in response to exogenous Ca^{2+}-DPA in the presence of high NaCl concentrations. Although high salinity clearly exerted inhibitory effects on spore germination, a certain portion of the spore population was able to initiate germination in response to L-alanine despite very high NaCl concentrations. Even at 4.8 M NaCl, a small fraction of the spore population lost some refractility and some metabolic activity was detectable. In this context it is notable that vegetative growth in a nutrient-poor environment is already strongly impaired at 1.2 M NaCl (46). Thus, it seems that dormant spores do not possess a salt-sensing system preventing them from germinating in a non-growth-permissive, high-salinity environment.

Future research will focus on the investigation of factors that are involved in mediating successful L-alanine germination despite elevated salt concentrations, on the salt stress mechanisms and viability during outgrowth, and on the evaluation of the mechanisms of NaCl-dependent germination inhibition discussed above. This could not only help to extend the knowledge of *B. subtilis*' salt stress response but also might lead to a better understanding of the germination process itself.

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The results of this study will be included in the Ph.D. thesis of K.N.

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CHAPTER 3

Systematic investigation of germination responses of *Bacillus subtilis* spores in different high-salinity environments.

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In this publication, the effect of high concentrations of 32 different salts on the germination of wild type *B. subtilis* spores was systematically investigated to analyze the chemical basis for salt inhibition. It was shown that the inhibitory strength of different salts varies considerably. Even though osmotic effects seemed to play an important role for germination inhibition, the ionic composition, ionic concentrations and chemical properties of the salts present during germination were decisive for the extent of inhibition. This paper provided new insights on the salt properties that are involved in germination inhibition and indicated the importance of osmotic effects. It also emphasized that the fluxes of ions, Ca^{2+}-DPA, and water represent likely salt inhibition targets in the germination pathway.

Author contributions:

K. Nagler designed, performed, and evaluated the experiments, and prepared the text and figures of the manuscript. **R. Moeller** gave scientific input and edited the manuscript text.

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RESEARCH ARTICLE

Systematic investigation of germination responses of *Bacillus subtilis* spores in different high-salinity environments

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One sentence summary: The spore germination of the ubiquitous soil bacterium *Bacillus subtilis* is detrimentally affected by the concentration of different salts, where the anionic and cationic salt components inhibit germination at different targets.

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ABSTRACT

High-salinity environments play an increasingly important role in ecology regarding soil salinization due to human-induced processes, but also need to be considered in terms of natural soil desiccation and extreme habitats. It has been shown previously that spore germination of the ubiquitous soil bacterium *Bacillus subtilis* is detrimentally affected by the presence of high NaCl concentrations, but the underlying mechanisms and effects of other salts remained obscure. To address these two points, we performed a systematic analysis with 32 different salts using spectrophotometric and microscopic methods. It could be shown that inhibitory strength varies considerably among different salts. Although osmotic effects seem to play an important role, ionic composition and concentration (especially of the anion) as well as chemical properties seem to be decisive for the extent of germination inhibition. At the current state of knowledge, fluxes of ions, Ca^{2+}-DPA and water are likely affected by all salts, whereas the exact inhibition mechanism of each salt might further depend on the respective properties of the involved ions. Hence, the observed inhibition likely is a result of several phenomena interacting with each other. Altogether this study highlights the complex impact of ionic environments on the life cycle of spore formers.

Keywords: spore germination; salt stress; salt-dependent germination inhibition; ions; metals; osmotic stress

INTRODUCTION

In its natural habitat, the ubiquitous soil bacterium *Bacillus subtilis* is frequently confronted with a variety of environmental stresses, including desiccation and nutrient depletion, and has therefore evolved a broad range of stress responses (Marles-Wright and Lewis 2007; Earl, Losick and Kolter 2008). Upon starvation it can form dormant spores that are highly resistant against harsh environmental conditions (reviewed in Nicholson *et al.* 2000; Setlow 2006, 2007). Spores can remain in this inert state over long periods of time, but when nutrients become available again they germinate and thus turn back into growing cells (Gest and Mandelstam 1987; Priest 1993; Setlow 2003). Nutrients are recognized via germination receptors (GRs) and there are at least three different types with differing specificities: GerA that binds L-alanine, and GerB and GerK that act cooperatively to respond to a nutrient mixture composed of L-asparagine, D-glucose, D-fructose and K^+ (AGFK) (Paidhungat and Setlow 2000; Atluri *et al.* 2006). The GRs are heterotrimeric inner membrane proteins that—together with the scaffold protein GerD and possibly further proteins—colocalize in a cluster

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termed germinosome, which is thought to be a functional unit allowing rapid germination (Hudson *et al.* 2001; Paidhungat and Setlow 2001; Griffiths *et al.* 2011). Nutrient binding then initiates, by a yet-unknown mechanism, a series of germination events that can be subdivided into germination commitment and two subsequent stages (Setlow, Melly and Setlow 2001; Yi and Setlow 2010). In stage one, the spore releases large amounts of cations and subsequently its large depot of Ca^{2+}-DPA [i.e. pyridine-2,6-dicarboxylic acid (dipicolinic acid,) in a 1:1 chelate mainly with Ca^{2+}] from the spore core (Setlow 2003; Paredes-Sabja, Setlow and Sarker 2010). This efflux goes along with a partial core hydration (Setlow 2003). In stage two, the spore's peptidoglycan cortex is degraded by the cortex-lytic enzymes (CLEs) CwlJ and SleB, allowing further core hydration and expansion (Ishikawa, Yamane and Sekiguchi 1998; Setlow 2003). After germination is completed, the spore's metabolism and macromolecular synthesis are initialized (outgrowth phase; Setlow 2003; Keijser *et al.* 2007).

It has previously been shown that high concentrations of NaCl have inhibitory effects on *B. subtilis* spore germination (Fleming and Ordal 1964; Nagler *et al.* 2014). These included delayed and increased heterogeneity of germination initiation, slowed germination kinetics on single-spore and population level and an overall decreased germination efficiency (Nagler *et al.* 2014). Interestingly, some spores within a population still seem to initiate but not complete germination in response to L-alanine at very high NaCl concentrations, leading to the emergence of phase-gray spore states (Nagler *et al.* 2014). Altogether NaCl exerts complex effects on germinating spores, but the underlying mechanisms are not yet understood.

Although NaCl is a model salt on which most work on salt stress response of vegetative *B. subtilis* cells has focused, there are several reasons why an investigation of the impact of different salts on spore germination is important. *Bacillus subtilis*' natural soil habitats usually contain a range of different salts, whose local concentration can greatly increase by desiccation, agricultural fertilization (especially with phosphates, nitrates and sulfates), wastewater irrigation and depositions, and high-efficiency-oriented agricultural practices (Bremer 2002; Datta and de Jong 2002; Lucho-Constantino *et al.* 2005; Ju *et al.* 2007; Rusan, Hinnawi and Rousan 2007; de Souza Silva and Fay 2012). Indeed, soil salinization and consequent decreases in soil quality and productivity are a growing, worldwide ecological issue and monitoring soil health can be challenging (Datta and de Jong 2002; Metternicht and Zinck 2003; Arias *et al.* 2005; de Souza Silva and Fay 2012). Thus, detailed knowledge on the effects of a variable ionic environment on soil microbes as well as on the underlying mechanisms is important with regard to soil ecology. In this context, *B. subtilis* represents a suitable model bacterium, as it is not only globally well characterized but also has ecological functions such as enhancing plant resistance and growth (Kloepper, Ryu and Zhang 2004). Moreover, several spore-forming *Bacillus* species and close relatives have been isolated from high-salinity environments (e.g. soil of the region of potassium salt mining, deep-sea hypersaline sediments) and were previously identified as contaminants on space crafts including Mars landers (Sass *et al.* 2008; Nicholson, Schuerger and Race 2009; Yastrebova *et al.* 2009). On Mars and other celestial bodies, water, as a prerequisite of life, is often expected to be tightly associated with salts (e.g. with hygroscopic minerals or in brines; Zorzano *et al.* 2009; Davila *et al.* 2010). Hence, knowledge on the impact of salts on spore germination is relevant for understanding mechanisms of adaptation to, and survival in, different extreme terrestrial and extraterrestrial environments. Besides it should be noted that a variety of salts such as KNO_3 (E252), $MgCl_2$ (E511), sodium and potassium phosphates (E339, E340) are also commonly used as supplements in food as preservatives, acidity regulators, flavor enhancer, etc., hence rendering this topic of applied interest for the food industry as well (European Commission, DG Health and Consumers, Food and Feed Safety 2013).

Altogether it is crucial to understand which features of a salt determine its inhibitory strength, to get a better insight on the mechanisms of salt-dependent germination inhibition. Therefore, it was our goal within this study to systematically assess *B. subtilis* spore germination behaviors in the presence of 32 different salts with interchanging anions and cations and different chemical properties, including halides, sulfates, phosphates, nitrates, perchlorates and potassium thiocyanate. We could show that within a concentration range from 0.06 to 2.4 mol L^{-1}, the inhibitory strengths vary considerably among the investigated salts.

MATERIALS AND METHODS

Salts

Within this study, the following 32 different salts were analyzed with respect to their impact on *B. subtilis* spore germination: KCl, KBr, KI, KF, NaCl, NaBr, NaI, NaF, LiCl, RbCl, CsCl, $FeCl_2$, $FeCl_3$, $CaCl_2$, $MgCl_2$, $SrCl_2$, KSCN, $MgSO_4$, Na_2SO_4, NH_4Cl, $(NH_4)_2SO_4$, Na-HPO_4, K-HPO_4, KNO_3, NH_4NO_3, $NaClO_4$, $Mg(ClO_4)_2$, $CoCl_2$, $CuCl_2$, $MnCl_2$, $NiCl_2$ and $ZnCl_2$. All salts were ordered from Sigma-Aldrich and had ACS reagent grade (assay $\geq$ 99%). If solubility allowed it, four concentrations (0.06, 0.6, 1.2 and 2.4 mol L^{-1}) were tested for each salt. Salts were solved in 10 mmol L^{-1} Tris-HCl (pH 8) and the pH was adjusted to 8 if necessary. The osmolalities of salt-containing germination media were measured using the automatic digital osmometer OM-815 (Vogel) that utilizes the freezing point depression principle.

Spore production and purification

Experiments were carried out with *B. subtilis* 168 (*trpC2*) originally obtained from the German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) as DSM 402. Spores were produced on Schaeffer's sporulation medium plates (Schaeffer, Millet and Aubert 1965) that were incubated for 6 days at 37°C. The biomass was harvested, suspended in water, centrifuged at 2990 $\times$ g and 4°C. The pellet was resuspended in lysozyme solution [1 mg mL^{-1} lysozyme, 10 μg mL^{-1} DNase I, 2.5 μg mL^{-1} $MgSO_4$ in 10 mmol L^{-1} Tris-HCl (pH 7)] and incubated for 30 min in a shaking incubator at 37°C (Nicholson and Setlow 1990; Nagler *et al.* 2014). After subsequent 15 min incubation at 80°C, the spores were washed at least seven times with water by centrifugation. Spores were stored in water at 4°C in screw-capped reaction tubes with glass beads until usage. The purity of the spore stocks as checked by phase-contrast microscopy was $\geq$99% (Nagler *et al.* 2014).

Spore germination

Spores were heat activated at 70°C for 30 min in order to ensure synchronized germination. For spectrophotometric measurements, germination was carried out in 96-well plates, each containing 200 μL of germination media composed of 10 mmol L^{-1} Tris-HCl (pH 8) with the indicated concentration of the respective salt and 10 mmol L^{-1} L-alanine. Germination media were

inoculated with 40 μL heat-activated spores to a starting optical density of ca. 0.5 at 600 nm ($OD600_{nm}$) corresponding to a total of ca. 4×10^7 spores per well, and were incubated at 37°C in a multiplate reader (BioTek ELx808IU) that read the OD_{600nm} of the culture, with five seconds of shaking before all readings. Each germination condition was tested with at least four replicates, and all data are expressed as averages ± standard deviations. The OD_{600nm} data were normalized by division of each reading by the first reading (t_{0min}), yielding the relative OD_{600nm} given in percentage. A 60% decrease in relative OD_{600nm} corresponds to germination of the whole spore population (Atluri *et al.* 2006; Nagler *et al.* 2014; and data not shown). It should be noted that there was often some decrease in the OD_{600nm} during incubation of spores without any germination trigger. However, phase microscopy confirmed that this decreased OD_{600nm} did not correspond to germination (data not shown), consistent with previous reports about this phenomenon, which was suggested to be due to spore aggregation and adsorption to test tube walls (Atluri *et al.* 2006).

DPA release was measured by its fluorescence with Tb^{3+} as described previously (Yi and Setlow 2010) using a Tecan Infinite M200 Pro fluorometer and the germination media described above. Each condition was tested in triplicate and all data are expressed as averages ± standard deviations.

To view germination by microscopy, spores were incubated in 96-well plates, as described above. After four hours, 5 μL samples were withdrawn and fixed by applying to a microscope slide coated with 1% agar. Micrographs were taken using a Zeiss fluorescence microscope (Axio Imager M2, Carl Zeiss MicroImaging GmbH) equipped with an AxioCam MRm and processed using the AxioVision 4.8.2 software and GIMP 2.6.12. The micrographs were evaluated by manual counting using the ImageJ plugin Cell Counter (at least 340 counts per time point) and assigning the individual spores to one of three distinguishable, phase-contrast microscopic phenotypes: bright (i.e. dormant spores), gray (i.e. germination induced, but not completed) or dark (i.e. germination completed). The classification was verified by measuring gray intensities with ImageJ software version 1.46r.

Data evaluation and mathematical analyses

For numerical comparison of different spectrophotometric germination profiles and assessment of different inhibitory strengths, a so-called RI/OD value was calculated as follows: the maximum germination rate υ_{max} (in percentage relative OD_{600nm} per minute) was derived from the linear part of the germination-specific OD_{600nm} decrease for each germination profile (Fig. S2, Supporting Information). For every tested condition, the respective υ_{max} was divided by υ_{max} in the absence of salt to yield a relative inhibition (RI) value:

$$\mathrm{RI} = \frac{\upsilon_{max}\,(\text{without salt})}{\upsilon_{max}\,(\text{with salt})}.$$

Hence, an RI value of 1 indicates normal germination, whereas RI values >1 signify germination inhibition. Yet, the RI value takes neither an increase in lag time prior to germination initiation nor the overall magnitude of the OD_{600nm} decrease into account. Therefore, the RI value for each germination was put into relation with the respective decrease of relative OD_{600nm} within 30 min (ΔOD_{30min}), which in the absence of salt is ca. 60%, i.e. complete germination. Dividing the RI by its respective ΔOD_{30min} value yields the RI/OD value as an indicator of inhibitory strength of a salt at a specific concentration:

$$\mathrm{RI/OD} = \frac{(\upsilon_{max}\,(\text{without salt})/\upsilon_{max}(\text{with salt}))}{(\Delta OD_{30\,min}(\text{with salt}))}.$$

An RI/OD value of 1.67 indicates no salt-dependent inhibition, whereas smaller or greater values imply a positive or negative effect of the salt on germination, respectively. Ranges of RI/OD values were grouped as follows: 1–1.5 = germination enhancement; 1.6–2 = no notable effect; 2.1–6 = weak inhibition; 6.1–35 = intermediate inhibition; >35 = strong inhibition. Regression analyses for correlations between RI/OD values and osmolalities were performed using the analysis tool of SigmaPlot version 12.0.

RESULTS

Sodium halides, potassium halides and monovalent cation chlorides

At very low concentrations (0.06 mol L^{-1}), sodium and potassium halides (except fluorides), and all monovalent cation chlorides similarly enhanced germination, i.e. shorter initial lag times, larger maximum germination rates (υ_{max}) and RI/OD values < 1.5 (Fig. 1A and Table 1). The presence of 0.06 mol L^{-1} NaF and KF did not affect germination behavior. Expectedly, increasing concentrations had detrimental effects, i.e. increased lag times, and decreased υ_{max} and germination efficiencies, but to different extents (Fig. 1, Tables 1 and 4; Table S1 and Fig. S2, Supporting Information).

At up to 1.2 mol L^{-1}, germination profiles of the monovalent cation chlorides were similar, with all salts exerting only weak inhibitory effects (RI/OD ≤ 6; Tables 1 and 4). Yet, at 2.4 mol L^{-1} the germination profiles and thus the inhibitory strengths were much more variable among these chlorides, ranging from intermediate ($RI/OD_{2.4M\ NH4Cl} \approx 15$) to strong inhibition ($RI/OD_{2.4M\ RbCl} \approx 188$). Indeed, phase-contrast microscopy confirmed that with 2.4 mol L^{-1} NH_4Cl, the vast majority (97 ± 2%) of spores turned phase-dark, while the same concentration of RbCl kept about 64% (±7%) in a phase-bright state that is typical for dormant spores (Table S1, Supporting Information). Although spores germinated in the presence of 2.4 mol L^{-1} LiCl turned dark gray instead of phase-dark, major inhibition was not apparent in terms of OD_{600nm} decrease (RI/OD 22.4 ± 3.2), and DPA release was as for NaCl (Fig. S1, Supporting Information).

Germination profiles in the presence of NaBr and KBr resembled the profiles with the respective chlorides at up to 1.2 mol L^{-1}, although the bromides already exhibited intermediate inhibitory strengths (RI/OD > 6) at this concentration (Fig. 1, Table 4). Interestingly, germination cultures with 2.4 mol L^{-1} NaBr and KBr both exhibited a similar lag time prior to OD_{600nm} decrease and DPA release, but the overall magnitude of the OD_{600nm} drop as well as the amount of phase-dark spores after four hours were similar among NaCl and NaBr on the one hand, and KCl and KBr on the other hand (Fig. 1D and Table 1; Table S1 and Fig. S1, Supporting Information).

Potassium and sodium iodide were both similarly more inhibitory than their respective bromides and chlorides These salts had notably longer lag times and intermediate inhibitory strengths at concentrations of 0.6 mol L^{-1} (Fig. 1B and Table 4), were strongly inhibitory at 1.2 mol L^{-1} (Fig. 1C) and almost completely blocked germination at 2.4 mol L^{-1} (Fig. 1D). These

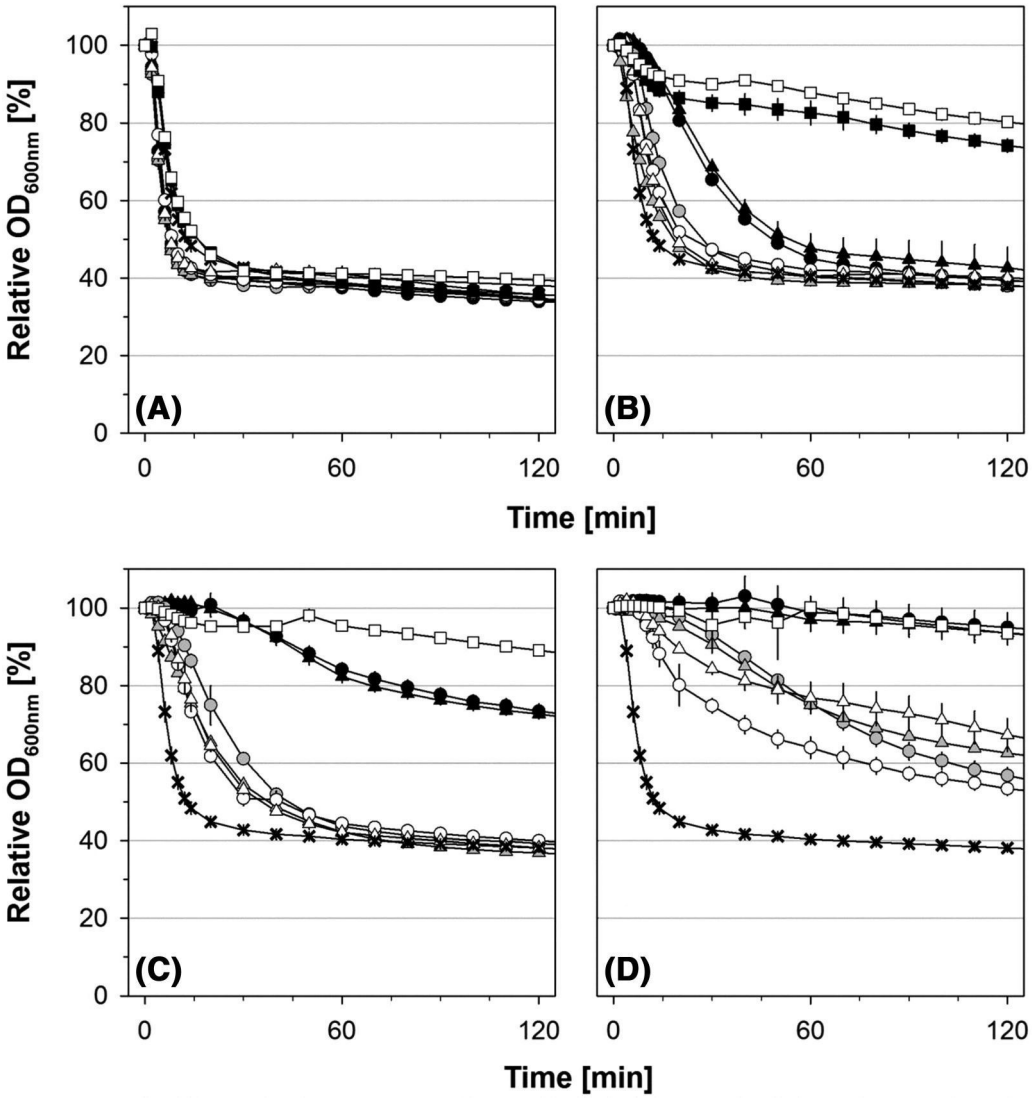

Figure 1. Germination in the presence of sodium and potassium halides at various concentrations. Germination profiles show the changes in relative OD_{600nm} in the presence of (Panel **A**) 0.06 mol L^{-1}, (Panel **B**) 0.6 mol L^{-1}, (Panel **C**) 1.2 mol L^{-1} and (Panel **D**) 2.4 mol L^{-1} of NaCl (white circles), NaBr (gray circles), NaI (black circles), NaF (black squares), KCl (white triangles), KBr (gray triangles), KI (black triangles) and KF (white squares). The germination curve in the absence of salts is indicated by black crosses in each graph.

observations were confirmed by microscopy, which furthermore revealed the emergence of phase-gray spore states at KI and NaI concentrations of 1.2 mol L^{-1} (Table S1, Supporting Information).

Potassium and sodium fluoride were the strongest inhibitors among the tested monovalent cation halides. Even 0.6 mol L^{-1} KF and NaF exerted significant inhibition (RI/OD = 76 and 29, respectively; Fig. 1B, Tables 1 and 4), allowing only ca. 28 or 44% of the spore population to turn phase-dark, respectively (Table S1, Supporting Information). Unlike iodides, emergence of phase-gray spore states was not observable.

Taken together, these results suggest that for the examined monovalent cation halides the anionic components predominantly determined the inhibitory strength at concentrations ≤1.2 mol L^{-1}, whereas the cation seemed to modify the germination profiles distinctly at higher concentrations.

Alkaline earth metal chlorides

At very low concentrations (0.06 mol L^{-1}), none of the three tested alkaline earth metal chlorides, i.e. $MgCl_2$, $CaCl_2$, and $SrCl_2$, had notable positive or negative effects on germination (RI/OD values = 1.6–1.7, Table 2). At higher concentrations (≥0.6 mol L^{-1}), they were much stronger inhibitors than all tested monovalent cation chlorides and bromides, causing a strongly delayed germination initiation and severe inhibition at 1.2 mol L^{-1}, allowing only 2–3% of the spores to turn completely phase-dark (Tables 2 and 4; Table S1, Supporting Information). Yet, a portion of the spore population (31–90%) turned phase-gray and thus still seemed to initiate germination at this concentration (Nagler *et al.* 2014). Among the tested alkaline earth metal chlorides, $MgCl_2$ continuously was the least and $CaCl_2$ the most inhibitory salt (Table 2; Table S1, Supporting Information).

Table 1. Inhibitory strength of monovalent cation chlorides, sodium and potassium halides.

	RI/OD* (Mean ± SD)			
Salt	0.06 mol · L^{-1}	0.6 mol · L^{-1}	1.2 mol · L^{-1}	2.4 mol · L^{-1}
LiCl	1.1 ± 0.0	1.6 ± 0.0	3.1 ± 0.0	22.4 ± 3.2
NaCl	1.1 ± 0.0	2.9 ± 0.0	5.1 ± 0.1	22.7 ± 2.4
KCl	1.2 ± 0.0	2.5 ± 0.1	5.7 ± 0.3	59.2 ± 11.7
RbCl	1.1 ± 0.0	2.6 ± 0.3	4.2 ± 0.3	188.0 ± 47.3
CsCl	1.1 ± 0.0	1.9 ± 0.1	4.0 ± 0.3	92.0 ± 16.8
NH_4Cl	1.1 ± 0.0	2.0 ± 0.0	3.0 ± 0.0	15.1 ± 0.3
NaF	1.8 ± 0.1	29.0 ± 3.6	n.s.	n.s.
KF	1.7 ± 0.1	76.3 ± 12.6	526.9 ± 159.3	N/A
NaBr	1.1 ± 0.0	3.7 ± 0.4	10.2 ± 0.3	184.7 ± 33.6
KBr	1.1 ± 0.0	2.8 ± 0.2	7.9 ± 0.3	136.3 ± 2.8
NaI	1.1 ± 0.0	13.4 ± 0.5	392.4 ± 66.5	N/A
KI	1.1 ± 0.0	16.0 ± 2.3	353.7 ± 33.2	N/A

N/A = no germination detectable via OD_{600nm} measurements. SD = standard deviation.
n.s. = not soluble.
*RI/OD values were calculated as described in the section 'Material and Methods' and are the ratio of the RI value (i.e. the quotient of the maximum germination rate v_{max} in the presence and absence of salt) and the decrease of the relative OD_{600nm} within 30 min (ΔOD_{30min}), with an RI/OD value of 1.67 indicating no salt-dependent inhibition, whereas smaller or greater values imply a positive or negative effect of the salt on germination, respectively.

Table 2. Inhibitory strength of alkaline earth metal and transition-metal chlorides.

	RI/OD (Mean ± SD)			
Salt	0.06 mol · L^{-1}	0.6 mol · L^{-1}	1.2 mol · L^{-1}	2.4 mol · L^{-1}
$MgCl_2$	1.6 ± 0.0	5.1 ± 0.3	75.0 ± 18.8	N/A[a]
$CaCl_2$	1.7 ± 0.1	8.3 ± 0.7	1189.9 ± 196.6	N/A
$SrCl_2$	1.7 ± 0.0	6.7 ± 0.6	673.4 ± 161.9	N/A
$MnCl_2$	3.1 ± 0.1	8.8 ± 0.6	1774.2 ± 1076.7	N/A
$FeCl_2$	4.0 ± 1.0[b,c]	26.8 ± 0.5[c]	N/A	N/A
$FeCl_3$	24.5 ± 4.2	N/A[a]	N/A	N/A
$CoCl_2$	3.2 ± 0.1	8.3 ± 0.4	180.1 ± 52.2	N/A
$NiCl_2$	4.0 ± 0.3	16.9 ± 2.3	583.5 ± 156.2[b]	N/A[a,b]
$CuCl_2$	63.3 ± 7.4[b]	64.9 ± 13.3	N/A[a]	N/A[a]
$ZnCl_2$	7.8 ± 0.5	172.9 ± 50.0	N/A[a]	N/A

N/A = no germination detectable via OD_{600nm} measurements.
[a]Presence of phase-gray spores (>1%) suggests some germination initiation without completion (see also Table S1, Supporting Information).
[b]Flocculation caused spore aggregation (might distort spectrophotometric measurements).
[c]Spores exhibited altered morphology.

Interestingly, $MgCl_2$ had a similar inhibitory effect at 0.6 mol L^{-1} as NaCl had at 1.2 mol L^{-1}, suggesting that the 2-fold chloride concentration in $MgCl_2$ containing germination cultures might play a role for the severity inhibition.

Transition-metal chlorides

All of the seven tested transition-metal chlorides were very strong germination inhibitors, causing weak to intermediate inhibition at concentrations as low as 0.06 mol L^{-1} (Tables 2 and 4). However, the inhibitory strengths among the different chlorides were very variable, suggesting that for transition-metal chlorides, the cationic species has a much stronger impact on germination compared to the other tested chlorides. The variability was also not only apparent by spectrophotometric measurements, but even more so by microscopy. In many cases, accumulation of phase-gray spore states could be observed, although to very different extents, and the shades of gray ranged from very light to dark gray (Table S1, Supporting Information and data not shown). Furthermore, the presence of 0.06 mol L^{-1} of $FeCl_2$ and $CuCl_2$ and of 1.2 mol L^{-1} $NiCl_2$ led to flocculation around the spores, causing spore aggregation and thus disturbing OD_{600nm} measurements. $FeCl_2$ additionally seemed to deform spores, as they vastly exhibited irregular morphologies after four-hour incubation in germination medium containing this salt (data not shown). Notably, neither spore deformation nor aggregation could be observed with $FeCl_3$. Overall, $FeCl_3$ and $ZnCl_2$ were the strongest inhibitors among the transition-metal chlorides (Table 2; Table S1, Supporting Information).

Sulfates, nitrates and phosphates

At a concentration of 0.06 mol L^{-1}, all tested sulfates, nitrates and phosphates improved germination, except for $MgSO_4$ which had no effect (Table 3). Taking all salts within this study into consideration, this suggests that monovalent cation salts have a high potential in enhancing germination at low concentrations tested in this work (i.e. 0.06 mol L^{-1}) regardless of the respective anion, while multivalent cations do not seem to have this effect (Table 4).

At concentrations ≥0.6 mol L^{-1}, inhibitory strengths increased from sulfates over phosphates to nitrates (Table 3;

Table 3. Inhibitory strength of sulfates, nitrates, phosphates and chaotropic salts.

Salt	RI/OD (Mean ± SD)			
	0.06 mol · L^{-1}	0.6 mol · L^{-1}	1.2 mol · L^{-1}	2.4 mol · L^{-1}
$(NH_4)_2SO_4$	1.2 ± 0.0	1.8 ± 0.1	3.0 ± 0.1	12.1 ± 0.3
Na_2SO_4	1.2 ± 0.0	1.8 ± 0.1	3.5 ± 0.1	n.s.
$MgSO_4$	1.7 ± 0.0	1.9 ± 0.0	2.6 ± 0.0	34.7 ± 9.4
NH_4NO_3	1.1 ± 0.0	2.2 ± 0.1	6.2 ± 0.2	41.8 ± 7.0
KNO_3	1.1 ± 0.0	3.6 ± 0.4	9.6 ± 1.1	109.0 ± 6.7
K_2HPO_4/KH_2PO_4	1.1 ± 0.0	2.1 ± 0.0	6.3 ± 0.6	30.8 ± 24.6[a]
Na_2HPO_4/NaH_2PO_4	1.1 ± 0.0	1.8 ± 0.0	4.2 ± 0.1	30.2 ± 9.1[a]
$NaClO_4$	1.1 ± 0.0	245.9 ± 44.8	N/A	n.s.
$Mg(ClO_4)_2$	2.1 ± 0.1	109.0 ± 12.3	N/A	n.s.
KSCN	1.1 ± 0.0	169.3 ± 28.7	N/A	N/A

N/A = No germination detectable via OD_{600nm} measurements.
n.s. = not soluble.
[a]Precipitation after 20–30 min.

Table 4. Classification and relative order of inhibitory strengths (as determined by RI/OD values) of all salts tested in this study.

Effect[a] (RI/OD range)	0.06 mol · l^{-1}	0.6 mol · l^{-1}	1.2 mol · l^{-1}	2.4 mol · l^{-1}
Enhancement (1–1.5)	$CsCl = NH_4Cl = KSCN = KNO_3 = NaBr = NH_4NO_3 = NaCl = KI = KBr = NaI = RbCl = K\text{-}HPO_4 = Na\text{-}HPO_4 = NaClO_4 = LiCl \approx KCl = (NH_4)_2SO_4 = Na_2SO_4$	–	–	–
No notable effect (1.6–2)	$MgCl_2 \approx CaCl_2 = MgSO_4 = SrCl_2 = KF < NaF$	$LiCl < Na\text{-}HPO_4 = (NH_4)_2SO_4 = Na_2SO_4 \approx MgSO_4 = CsCl \approx NH_4Cl$	–	–
Weak inhibition (2.1–6)	$Mg(ClO_4)_2 < MnCl_2 \approx CoCl_2 < NiCl_2 = FeCl_2$[b]	$K\text{-}HPO_4 \approx NH_4NO_3 < KCl \approx RbCl < KBr \approx NaCl < KNO_3 \approx NaBr < MgCl_2$	$MgSO_4 < (NH_4)_2SO_4 = NH_4Cl \approx LiCl < Na_2SO_4 < CsCl < Na\text{-}HPO_4 = RbCl < NaCl < KCl$	–
Intermediate inhibition (6.1–35)	$ZnCl_2 < FeCl_3$	$SrCl_2 < CoCl_2 = CaCl_2 < MnCl_2 < NaI < KI < NiCl_2 < FeCl_2 < NaF$	$NH_4NO_3 \approx K\text{-}HPO_4 < KBr < KNO_3 < NaBr$	$(NH_4)_2SO_4 < NH_4Cl < LiCl < NaCl < Na\text{-}HPO_4 < K\text{-}HPO_4 < MgSO_4$
Strong inhibition (>35)	$CuCl_2$[b]	$CuCl_2 < KF < Mg(ClO_4)_2 < KSCN < ZnCl_2 < NaClO_4$	$MgCl_2 < CoCl_2 < KI < NaI < KF < NiCl_2$[b] $< SrCl_2 < CaCl_2 < MnCl_2$	$NH_4NO_3 < KCl < CsCl < KNO_3 < KBr < NaBr < RbCl$
Complete inhibition	–	$FeCl_3$	KSCN, $Mg(ClO_4)_2$, $NaClO_4$, $CuCl_2$, $FeCl_2$, $FeCl_3$, $ZnCl_2$	KSCN, $CaCl_2$, $MgCl_2$, $SrCl_2$, KI, KF, NaI, Na_2SO_4, $CoCl_2$, $CuCl_2$, $FeCl_2$, $FeCl_3$, $MnCl_2$, $NiCl_2$, $ZnCl_2$

[a]Salts were grouped according to their inhibitory strengths as determined by RI/OD calculation. The respective range is given for each effect.
[b]Flocculation and aggregation likely distorted OD_{600nm} measurements.

Table S1, Supporting Information). Germination profiles within each of these substance groups were very similar at concentrations ≤ 1.2 mol L^{-1}, again pointing out a potentially important role of the anionic component on germination inhibition. At all concentrations, $(NH_4)_2SO_4$ was the least and KNO_3 the most inhibitory. Generally, sulfates, nitrates and phosphates seemed to have weak to intermediate inhibitory strengths when compared to the other tested salts (Table 4).

Chaotropic salts

All three chaotropic salts investigated within this study, i.e. sodium and magnesium perchlorate and KSCN were very strong inhibitors, although $NaClO_4$ and KSCN could still improve germination at concentrations of 0.06 mol L^{-1} (Tables 3 and 4). Nevertheless, already 0.6 mol L^{-1} of each of the three salts caused strong inhibition, keeping ca. 60–76% of the spore populations in a phase-bright state (Table S1, Supporting Information).

At concentrations ≥1.2 mol L^{-1}, germination was no longer observable (Table 3; Table S1, Supporting Information). As chaotropic salts can dissociate protein complexes (Sawyer and Puckridge 1973), their effects on GR clusters of the germinosome were investigated as well. Therefore, spores of the strain KGB04 (PS832 with *gerE*::spc, *cotE*::tet *gerKB-gfp* ermC; from Griffiths *et al.* 2011) expressing the GFP-labeled GerK subunit GerKB were incubated in 1.2 mol L^{-1} KSCN, 1.2 mol L^{-1} $Mg(ClO_4)_2$ and H_2O (as control) for four hours and then analyzed by fluorescence microscopy. Expectedly, the spores incubated in water exhibited fluorescent foci (Fig. S3 A, Supporting Information), which are due to clustering of the GRs as described in Griffiths *et al.* (2011). Spores incubated with chaotropic salts likewise exhibited fluorescent foci (Fig. S3 B and C, Supporting Information), suggesting that at least GerK clusters were not disrupted, as this would be expected to cause a more even fluorescence distribution (Griffiths *et al.* 2011).

Osmotic stress

The presence of high salt concentrations does not only exert ionic stress on organisms, but also causes notable osmotic stress. To elucidate the impact of osmotic stress on germination, spores were germinated in the presence of high concentrations of glycerol (Fig. 2A). The osmolalities of the glycerol-containing germination media were adjusted to correspond to the osmolalities of different standard NaCl concentrations (i.e. 0.6, 1.2, 2.4 and 3.6 mol L^{-1}). Medium with 0.78 mol L^{-1} glycerol with an osmolality of 1.11 osm kg^{-1} (corresponding to 0.6 mol L^{-1} NaCl) slightly enhanced germination (RI/OD = 1.4), while 1.7 mol L^{-1} glycerol (i.e. 2.25 osm kg^{-1}, corresponding to 1.2 mol L^{-1} NaCl) caused very weak inhibition (RI/OD = 2.1). Although at higher glycerol concentrate ions inhibition became more pronounced (RI/OD values of 3.2 and 4.8 mol L^{-1} glycerol were 6.5 and 249.1, respectively), causing a delayed and decreased germination, the inhibitory strength of glycerol was generally weaker compared to NaCl-containing media with corresponding osmolalities (Fig. 1 and Table 1).

To elucidate the impact of osmotic stress on the inhibitory strength of media containing different salts more broadly, the RI/OD values obtained for all germinations within this study were plotted against the respective osmolality of the medium (Fig. 2B). Although increasing osmolalities tended to cause stronger inhibition, regression analysis indicated no linear correlation ($r^2 = 0.08$) between osmolality and RI/OD values. When regarding different types of salts (e.g. monovalent cation chlorides, halides, chaotropic salts, etc.) separately, regression analysis also did not show correlations ($r^2 < 0.5$), with the exception of the nitrates ($r^2 = 0.663$) and phosphates ($r^2 = 0.856$), both of which only included two different salts each.

Taken together, these results suggest that although osmotic stress seems to play a notable role in germination inhibition, it is not the only determinant of inhibitory strength in salt solutions.

DISCUSSION

Although soil salinity represents a growing ecological problem (Metternicht and Zinck 2003; de Souza Silva and Fay 2012), its influence on important stages in the life cycle of soil-dwelling spore formers is not well understood so far. Low salt concentrations were previously reported to enhance *B. subtilis* spore germination (Fleming and Ordal 1964), which was confirmed within this current study. Importantly, increased germination rates at a salt concentration of 60 mmol L^{-1} were observed for all tested monovalent cation salts except fluorides (no notable effect), but for no salt with multivalent cations (Table 4). While K^+ ions are known co-germinants, i.e. agents that stimulate germination, but do not trigger germination alone, other monovalent cations might function in a similar manner, possibly also by interacting with GRs (Atluri *et al.* 2006; Paredes-Sabja, Setlow and Sarker 2010). Yet, further research is required to address germination enhancement at low salt concentrations adequately.

In contrast, high salinity has detrimental effects on germination (Fleming and Ordal 1964; Nagler *et al.* 2014). For elevated NaCl concentrations, delayed and increased heterogeneity of germination initiation, slowed germination kinetics and an overall decreased germination efficiency have been reported (Nagler *et al.* 2014). While the exact inhibition mechanisms remained obscure, likely potential NaCl inhibition sites might be (i) GR accessibility and (ii) germinant binding, (iii) signal transduction from the germinosome leading to (iv) ion and DPA release accompanied by water influx, and ultimately (v) cortex

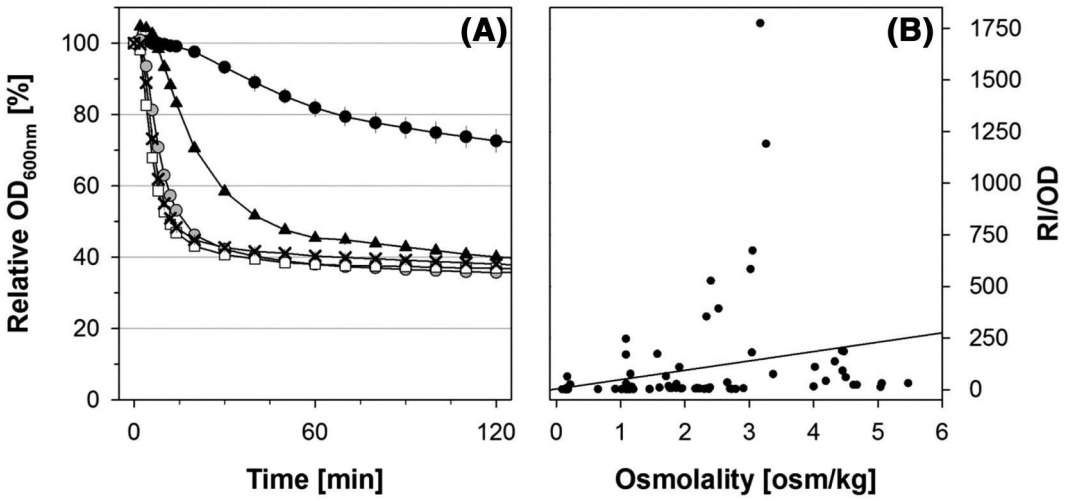

Figure 2. Effects of osmolality on germination. (**A**) Germination in glycerol-containing germination media. Glycerol concentrations were adjusted to have corresponding osmolalities to different standard NaCl concentrations and were 0.78 mol L^{-1} (1.11 osm kg^{-1}; corresponds to germination medium with 0.6 mol L^{-1} NaCl; white squares), 1.7 mol L^{-1} (2.25 osm kg^{-1}; 1.2 mol L^{-1} NaCl; gray circles), 3.2 mol L^{-1} (4.67 osm kg^{-1}; 2.4 mol L^{-1} NaCl; black triangles) and 4.8 mol L^{-1} (6.68 osm kg^{-1}; 3.6 mol L^{-1} NaCl; black circles). (**B**) Regression analysis for correlations between RI/OD values and the respective osmolalities of all tested germination media.

hydrolysis by CLEs (Nagler *et al.* 2014). This current study showed that germination inhibition can also be caused by a range of other salts, with increasing concentrations of all tested salts leading to increased inhibition. Yet, the extent of inhibition was very variable among the different salts and seems to depend on anionic and cationic concentrations, ionic species (and their combination), chemical properties of a salt and osmolality of the medium.

For all salts except the strongly inhibitory transition-metal chlorides, the anionic species seemed to be the major determinant of inhibitory strength at intermediate concentrations. These were $\leq 1.2\,mol\,L^{-1}$ for $MgSO_4$ and salts with monovalent cations, but $\leq 0.6\,mol\,L^{-1}$ for alkaline earth metal chlorides (Fig. S2, Supporting Information), which might relate to the 2-fold absolute amount of chloride at the same molar concentration for the latter salts. At higher concentrations, the cation seemed to play a more prominent role for the extent of inhibition. Possibly, this could hint at different inhibition mechanisms for anions and cations. For transition-metal chlorides, the chemical properties and higher chemical flexibility (e.g. possible oxidation states, potential for complex formation) due to their special electron configuration might be involved in the predomination of the cation and their extraordinary inhibitory strengths. Redox processes on the spore surface as previously detected for marine *Bacillus* sp. spores (He and Tebo 1998) could also contribute to inhibition and might explain the observed flocculation and spore aggregation with $CuCl_2$, $FeCl_2$ and $NiCl_2$ (Table 2).

When comparing the inhibitory strengths of the tested salts to several of their physicochemical and thermodynamic properties (e.g. enthalpy of solvation, electronegativity, molecular weight and ionic radii), no definite correlation could be identified (data not shown). However, there was a striking correlation between the inhibitory strength of the tested salts and the order of their ions in the Hofmeister series: while weak inhibitors tended to be rather kosmotropic (i.e. salts that increase protein stability, e.g. sulfates), chaotropic salts (i.e. salts that favor protein denaturation and complex disintegration, e.g. perchlorates, KSCN and iodides) were strong inhibitors (Sawyer and Puckridge 1973; Zhang and Cremer 2010; Fig. 3). Since a large number of proteins and protein interactions is involved in mediating germination (Paredes-Sabja, Setlow and Sarker 2010), this finding suggests that the outstanding inhibitory strengths of chaotropic salts might be due to detrimental effects on germination proteins and/or their interactions (Zhang and Cremer 2006), although GerK clusters were not disrupted (Fig. S3, Supporting Information). Besides, other spore structures like the coat or inner membrane could be compromised by chaotropic salts as well (Duda *et al.* 2004).

While the extraordinary inhibitory strengths of transition-metal chlorides and chaotropic salts are likely due to their distinctive chemical properties, germination inhibition by the other tested salts is likely related to more general ionic and osmotic effects. Within the first stage of germination, large amounts of cations (especially K^+, Na^+ and H^+) and Ca^{2+}-DPA are released from the spore core and replaced by inflowing water (Swerdlow, Setlow and Setlow 1981; Setlow 2003). Since spores do not contain substantial amounts of ATP (Setlow, Melly and Setlow 2001), it is likely that the release of ions and Ca^{2+}-DPA is driven by a chemical gradient, which might be attenuated by high extracellular salt concentrations, thus hampering germination. Consistently, high salinity can indeed delay Ca^{2+}-DPA efflux (Fig. S1, Supporting Information; Nagler *et al.* 2014). In addition, the transporters for ion and Ca^{2+}-DPA release might directly be affected by ions. While neither the role of cation release nor the transporters mediating it have been identified yet (Paredes-Sabja, Setlow and Sarker 2010), Ca^{2+}-DPA transport is assumed to involve proteins of the heptacistronic *spoVA* operon (Vepachedu and Setlow 2005, 2007; Li *et al.* 2012). With regard to the finding that low amounts (10–5 mmol L^{-1}) of some multivalent cations strongly inhibit decoated spores and in part (e.g. Zn^{2+}) also intact spores, it was proposed previously that DPA might form complexes with other metals than its usual chelate partner Ca^{2+}, thus blocking the Ca^{2+}-DPA transporters (Yi *et al.* 2011). This idea was supported by a correlation of the inhibitory strengths of the examined cations with the stability constants for their complexes with DPA (Chung *et al.* 1971). For the divalent cation chlorides within this study, a trend of inhibitory strength correlating with DPA chelate stabilities was also recognizable, with the moderately inhibitory $MgCl_2$ forming rather unstable and the strong inhibitor $CuCl_2$ forming very strong complexes with DPA^{2-} (stability constant log K_{ML} of 1.66 and 7.97, respectively; Chung *et al.* 1971).

Aside from these ionic effects, it could be shown that osmotic stress also plays an important role for germination inhibition (Fig. 2): high concentrations of osmotically active particles in the surrounding medium might reduce the availability of free water molecules that can be taken up into the spore core, thus interfering with core rehydration that is essential for successful completion of germination and the subsequent onset of metabolic activity during outgrowth (Setlow 2003; Sunde *et al.* 2009). However, the lack of a direct correlation between the osmolality of all tested germination media and inhibitory strength, as well as the lower inhibition by glycerol compared to NaCl at the same osmolality, suggests that germination inhibition is mediated by the combination of ionic and osmotic effects.

In summary, there are several reasonable explanations for the different inhibitory strength of different salts. At the current state of knowledge, fluxes of ions, Ca^{2+}-DPA and water are all likely to be affected by the presence of any type of salt, although the exact inhibition mechanism of each salt might

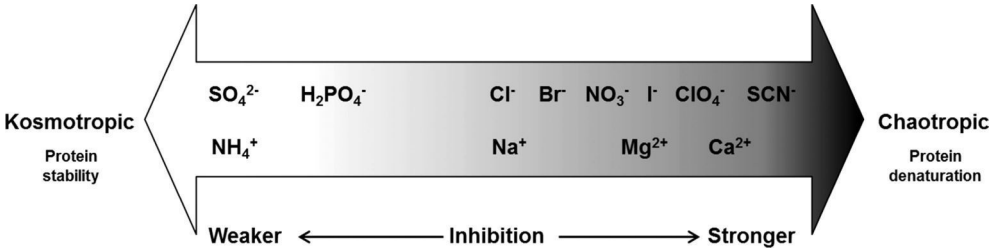

Figure 3. Relevant ions of the Hofmeister series correlated with the inhibitory strengths of their salts. Note that only some cations are shown, as their order varies considerably depending on the reference (Zhang and Cremer 2006, 2010).

further depend on the respective properties of the involved ions (e.g. charge, tendency for complex formation, chaotropicity, etc.). Therefore, the observed inhibition likely is a result of several phenomena interacting with each other, thus leading to very individual germination behaviors and making it difficult to correlate the inhibitory strength of a salt to any of its specific properties, unless one effect is so strong that it superimposes others.

Overall, this study pointed out that different salts can strongly interfere with life cycles and stress responses of soil bacteria such as *B. subtilis*. This could have broad consequences for the microbial community in soil, for biogeochemical cycles and for other organisms such as plants or fungi interacting with soil-dwelling microbes (Miller and Wood 1996; Nicholson 2002; Kloepper, Ryu and Zhang 2004; Falkowski, Fenchel and Delong 2008). It is striking that relatively low concentrations of some salts can have severe effects, hence emphasizing the need for sustainable agricultural practices combined with suitable measures to monitor soil quality, which however is not simple (Schoenholtz *et al.* 2000; Arias *et al.* 2005). Microbial soil quality indicators are already being used (Arias *et al.* 2005) and potentially spore germination could also be utilized as a bioindicator for soil health in the future. This application would greatly benefit from an increased understanding of the molecular mechanisms of germination in general and how biologically harmful conditions, such as pollutants or salinity, interfere with this process (Cabezali *et al.* 1994; Francis and Tebo 1999; Frische and Höper 2003).

With regard to high-salinity-dependent germination inhibition, much remains to be learned about its mechanistic details, which will be addressed in future research including molecular genetic analyses. Moreover, physiological and transcriptomic information on the salt stress response during the subsequent outgrowth phase would be of interest to obtain a more coherent picture of the impact of high salinity on the different life cycle stages of *B. subtilis* and will also be studied in prospective investigations (Zhang, Li and Nie 2010).

SUPPLEMENTARY DATA

Supplementary data is available at FEMSEC online.

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CHAPTER 4

Involvement of coat proteins in *Bacillus subtilis* spore germination in high-salinity environments

Nagler K, Setlow P, Reineke K, Driks A, Moeller R. 2015. Involvement of coat proteins in *Bacillus subtilis* spore germination in high-salinity environments. Appl. Environ. Microbiol. **81**(19):6725-6735.

This publication describes the involvement of coat proteins in *B. subtilis* spore germination at high salinity. Using various mutants, chemically decoated spores, and different experimental approaches it was shown that severe coat defects have exacerbated detrimental effects on spore germination in the presence of high NaCl concentrations. This exacerbated effect seemed to be mainly due to ionic effects, indicating that the spore coat is important for protection of the inner spore layers from ionic stress. Moreover, this study yielded valuable insights on the mechanism of germination inhibition by salt, again pointing at ion, Ca^{2+}-DPA and water fluxes as most likely inhibition targets.

Author contributions:

K. Nagler designed, performed, and evaluated the experiments (except TEM microscopy and HPLC analyses), and prepared the text and figures of the manuscript. **P. Setlow** supported DPA-release and commitment analyses, gave scientific input, and edited the manuscript text. **K. Reineke** supported the pressure experiments, performed HPLC analyses incl. evaluation, and edited the manuscript text. **A. Driks** performed and evaluated TEM microscopy and edited the manuscript text. **R. Moeller** gave scientific input and edited the manuscript text.

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Involvement of Coat Proteins in *Bacillus subtilis* Spore Germination in High-Salinity Environments

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The germination of spore-forming bacteria in high-salinity environments is of applied interest for food microbiology and soil ecology. It has previously been shown that high salt concentrations detrimentally affect *Bacillus subtilis* spore germination, rendering this process slower and less efficient. The mechanistic details of these salt effects, however, remained obscure. Since initiation of nutrient germination first requires germinant passage through the spores' protective integuments, the aim of this study was to elucidate the role of the proteinaceous spore coat in germination in high-salinity environments. Spores lacking major layers of the coat due to chemical decoating or mutation germinated much worse in the presence of NaCl than untreated wild-type spores at comparable salinities. However, the absence of the crust, the absence of some individual nonmorphogenetic proteins, and the absence of either CwlJ or SleB had no or little effect on germination in high-salinity environments. Although the germination of spores lacking GerP (which is assumed to facilitate germinant flow through the coat) was generally less efficient than the germination of wild-type spores, the presence of up to 2.4 M NaCl enhanced the germination of these mutant spores. Interestingly, nutrient-independent germination by high pressure was also inhibited by NaCl. Taken together, these results suggest that (i) the coat has a protective function during germination in high-salinity environments; (ii) germination inhibition by NaCl is probably not exerted at the level of cortex hydrolysis, germinant accessibility, or germinant-receptor binding; and (iii) the most likely germination processes to be inhibited by NaCl are ion, Ca^{2+}-dipicolinic acid, and water fluxes.

The soil bacterium *Bacillus subtilis* possesses a broad range of different stress responses, as it is frequently confronted with changing conditions in its natural habitat (1). Upon nutrient depletion, *B. subtilis* forms endospores that are dormant and highly resistant to harsh environmental conditions (reviewed in references 2 and 3). The resistance properties of the spore strongly depend on its structure and composition: the dehydrated spore interior (the core) is surrounded by a relatively impermeable inner membrane, a germ cell wall, and protective integuments, i.e., the spore cortex and coat (2–4). The cortex is composed of modified peptidoglycan and is an important factor for establishing the low core water content required for wet heat resistance (5). In developing spores, the cortex is separated from the coat by an outer membrane, but its fate and integrity in mature spores are unclear (4). The coat is an elaborate structure composed of more than 70 different proteins that are synthesized by the sporulating mother cell, and it plays a major role in protecting spores from chemicals and exogenous enzymes (2, 4, 6). It consists of at least four layers (the basement layer, inner coat, outer coat, and crust), whose presence depends on specific morphogenetic proteins (SpoIVA, SafA, CotE, and CotXYZ, respectively) (4, 7). Without SpoIVA, coat material is present but not attached to the spore and the cortex is missing; in *safA* mutant spores, the inner coat but not the outer coat is absent, while *cotE* mutant spores lack an outer coat and the crust (4) (for an overview, see Fig. 1). Coat morphogenesis is a complex, concerted, interlinked process that involves an interaction network of many different proteins (4, 8). It begins with the deposition of coat proteins on one pole of the developing spore at a relatively early time in sporulation and progresses with the coordinated encasement of the complete spore by groups of coat proteins in successive waves driven by a temporally controlled program (8). The proteins SpoVID and SpoVM play key roles for encasement, which is blocked in their absence; i.e., other coat proteins cannot properly assemble around the developing spore (4, 8).

Although mature spores are metabolically inert, they can detect the presence of specific small molecules, such as certain amino acids and sugars, triggering the conversion back into growing cells, a process called germination (9, 10). Nutrients are recognized via germinant receptors (GRs) that colocalize in a functional cluster, termed the germinosome, in the inner spore membrane and that have different specificities: GerA recognizes L-alanine, whereas GerB and GerK cooperatively respond to a mixture of L-asparagine, D-glucose, D-fructose, and K^+ (AGFK) (9, 11, 12). Upon recognition of nutrients, spores commit to germinate (13). Germination starts by the release of large amounts of monovalent cations, followed by the release of the spore core's large depot of pyridine-2,6-dicarboxylic acid (dipicolinic acid [DPA]) in complex with Ca^{2+} (Ca^{2+}-DPA) in parallel with partial core hydration (9, 10). Subsequently, the cortex is hydrolyzed by the two redun-

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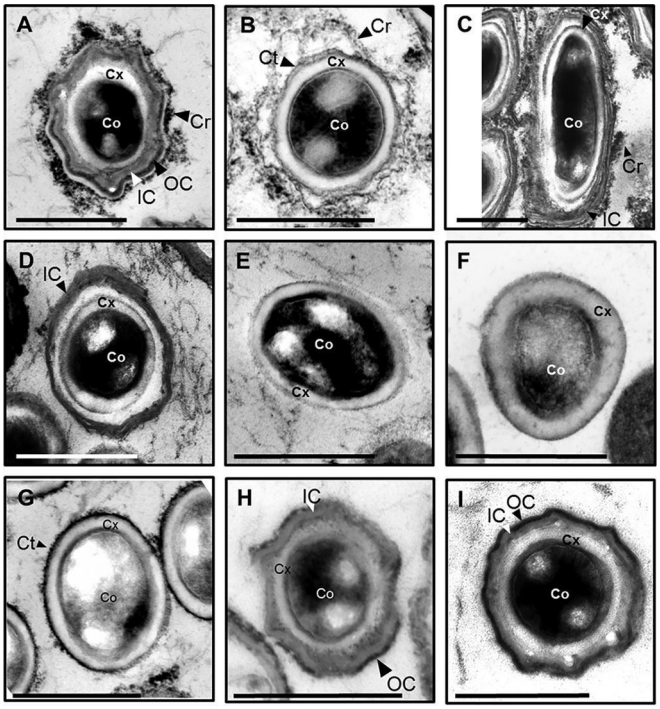

FIG 1 TEM images of wild-type spores (strain 168) (A), chemically decoated wild-type spores (B), and spores lacking *safA* (KN19) (C), *cotE* (KN20) (D), *safA* and *cotE* (KN23) (E), *gerE* and *cotE* (KN32) (F), *spoVID* (KN22) (G), *cotXYZ* (H), or *gerP* (KW05) (I). Co, core; Cx, cortex; IC, inner coat; OC, outer coat; Ct, coat; Cr, crust. Bars = 500 nm.

dant cortex-lytic enzymes (CLEs) CwlJ and SleB, which allows further core hydration, core expansion, and, ultimately, outgrowth (9, 10, 13, 14). Germination can also be induced by nonnutrient factors: exogenous Ca^{2+}-DPA, for instance, can directly activate the CLE CwlJ independently of GRs by a yet unknown mechanism, while high pressure can either activate GRs without germinant binding (100 to 300 MPa) or trigger Ca^{2+}-DPA release directly (>400 MPa) (15–19).

In soil, not only is nutrient availability variable but also other parameters, such as salinity, can change considerably due to desiccation or human influence, leading to high local salt concentrations (20, 21). Previous studies showed that high salinity is detrimental to *B. subtilis* spore germination (22–24). Increasing NaCl concentrations delay germination initiation and increase its heterogeneity, slow down the germination process, and decrease its overall efficiency (23). Germination might be negatively affected by high salt concentrations at several stages (23). These include (i) GR accessibility, (ii) germinant binding to the GRs, (iii) signal processing within and signal transduction from the germinosome, leading to (iv) ion and Ca^{2+}-DPA release with concomitant water influx, and (v) cortex hydrolysis by CLEs. So far, however, the mechanistic details of germination inhibition by NaCl remain obscure.

In *B. subtilis*, the spore coat is the first barrier between the spore and the surrounding medium and, therefore, must be traversed by germinants to induce germination. Consequently, spores with an altered coat structure and/or composition were germinated in the presence of high NaCl concentrations and germination was monitored by spectrophotometry, fluorometry, and microscopy. The investigated spores lacked complete coat layers, individual coat proteins, or CLEs. Spores were also germinated by high pressures of 150 and 550 MPa to study the influence of high NaCl concentrations on germination that does not require germinant passage through the spore's integuments and germinant binding to GRs. This study yielded new, valuable insights into the mechanisms of high-salinity-dependent germination inhibition and evaluated the role of coat proteins in spore germination in high-salinity environments.

(The results of this study are included in the Ph.D. thesis of Katja Nagler.)

MATERIALS AND METHODS

Strains, spore production, spore purification, and electron microscopy. The *Bacillus subtilis* strains used in this study are listed in Table 1 and are isogenic derivatives of the wild-type strain 168 (*trpC2*). For strain construction, the genomic DNA of different mutant strains (original strain names are given in Table 1) was isolated using a peqGOLD bacterial DNA kit (peqlab, VWR International GmbH, Erlangen, Germany). Production of competent strain 168 cells and transformation were performed as described in reference 25, except that a longer DNA incubation time (1 h) and no DNase step were used. Chemical decoating of strain 168 spores was as described previously by a 2-h incubation at 70°C in 1% SDS–0.1 M NaOH–0.1 M NaCl–0.1 M dithiothreitol, followed by extensive washing (26).

Spores were produced in 2× SG (Schaeffer medium with glucose) liquid cultures that were incubated at 37°C for 6 days in a shaking incubator (200 rpm). Spores were harvested and washed by centrifugation with water at least seven times. If necessary, residual cell debris or germinated spores were removed by polyethylene glycol 4000 purification as described previously (27). Spore germination was not affected by this additional purification procedure (data not shown). The purity of the spore stocks, as checked by phase-contrast microscopy, was ≥99%.

For thin-section transmission electron microscopy (TEM), spores were fixed with ruthenium red as described in reference 28.

Spore germination by nutrients. Spores were heat activated at 70°C for 30 min in order to ensure synchronized germination. For spectrophotometric measurements, germination was carried out in 96-well plates, each containing 200 μl of germination medium composed of 10 mM

TABLE 1 *B. subtilis* strains used within this study

Strain	Genotype[a]	Reference or source (original strain)
168	*trpC2*	Laboratory stock
KN19	*trpC2 safA::tet*	48 (PE277)
KN20	*trpC2 cotE::cat*	8 (PE618)
KN21	*trpC2 (cotX cotYZ)::neo*	8 (PE620)
KN22	*trpC2 spoVID::kan*	8 (PE697)
KN23	*trpC2 safA::tet cotE::cat*	P. Eichenberger (PE1720)
KN32	*trpC2 cotE::cat gerE::erm*	This study[b]
KN33	*trpC2 cotB::spc*	49 (RH201)
KN34	*trpC2 cotC::spc*	E. Ricca (RH101)
KN35	*trpC2 cotG::erm*	50 (ER203)
KN36	*trpC2 cotH::spc*	34 (ER220)
KN37	*trpC2 cotS::cat*	51 (AZ541)
KW05	*trpC2 gerP::tet*	36 (PS4228)
KF111	*trpC2 cwlJ::tet*	17 (FB111)
KF112	*trpC2 sleB::spc*	17 (FB112)
KF113	*trpC2 cwlJ::tet sleB::spc*	17 (FB113)

[a] Where appropriate, chloramphenicol (5 μg/ml), neomycin (10 μg/ml), spectinomycin (100 μg/ml), erythromycin (1 μg/ml), kanamycin (10 μg/ml), or tetracycline (10 μg/ml) was added to the medium.
[b] MTB862 (from H. Takamatsu) transformed into KN20.

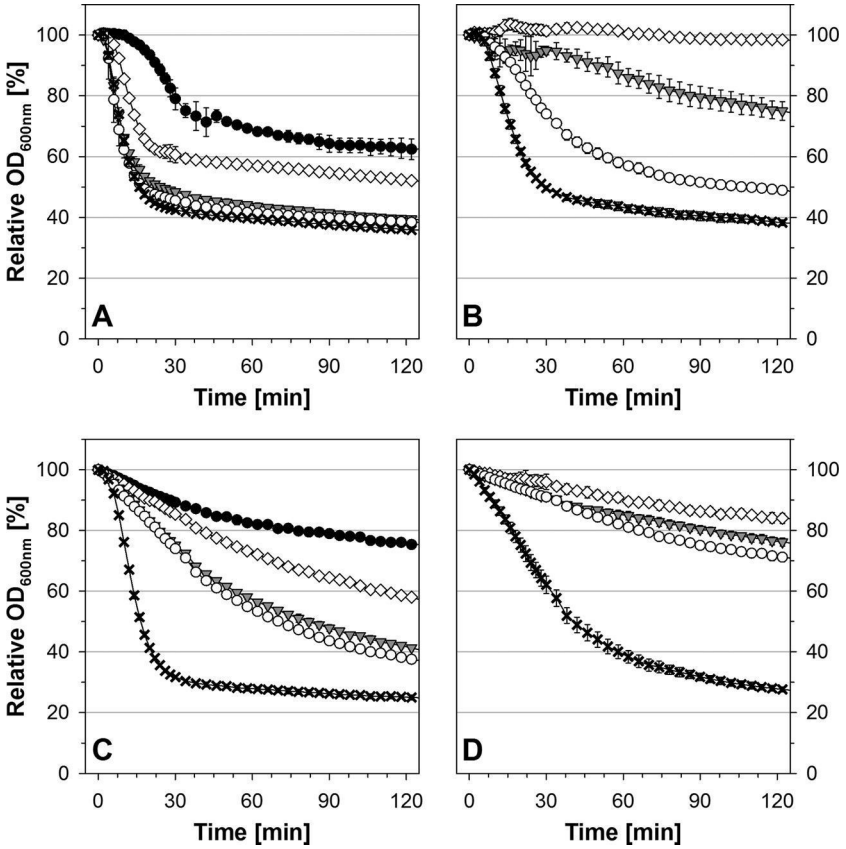

FIG 2 Nutrient germination of untreated (A, B) and decoated (C, D) strain 168 spores in the presence and absence of different NaCl concentrations. Spores were germinated by treatment with L-alanine (A, C) or AGFK (B, D), as described in Materials and Methods. The germination media contained no salt (black crosses), 0.6 M NaCl (white circles), 1.2 M NaCl (gray inverted triangles), 2.4 M NaCl (white diamonds), or 3.6 M NaCl (black circles).

Tris-HCl (pH 8; with or without NaCl), 50.5 mM D-glucose, 0.5 mM L-tryptophan, and the germinant of interest (10 mM L-alanine or AGFK, composed of 10 mM L-asparagine, 100 mM D-glucose {i.e., without the addition of 50.5 mM glucose}, 100 mM D-fructose, 100 mM KCl). The germination media were inoculated with 40 μl heat-activated spores to a starting optical density (OD) at 600 nm (OD_{600}) of ca. 0.5, corresponding to a total of ca. 4×10^7 spores per well, and were incubated at 37°C in a multiplate reader (ELx808IU; BioTek) that read the OD_{600} of the culture. The plates were shaken for 5 s before all readings were taken. Each germination condition was tested with at least four replicates. The OD_{600} data were normalized by division of each reading by the first reading (at time zero), yielding the relative OD_{600} (given in percent). A 60% decrease in the relative OD_{600} corresponds to germination of the whole spore population (11; data not shown). It should be noted that there was often some decrease in the OD_{600} during the incubation of spores without any germination trigger. However, phase-contrast microscopy confirmed that this decreased OD_{600} did not correspond to germination, as shown previously (11; data not shown). To monitor germination by microscopy, spores were germinated in 96-well plates as described above. After 4 h, 5-μl samples were withdrawn and fixed by application to a microscope slide coated with 1% agar. Micrographs were taken using a Zeiss fluorescence microscope (Axio Imager M2; Carl Zeiss MicroImaging GmbH) equipped with an AxioCam MRm camera.

Analyses of DPA release and germination commitment were performed as described previously (13), using the germination media described above and measuring the amount of DPA released by its fluorescence with Tb^{3+} in a Gemini EM multiwell fluorescence plate reader (Molecular Devices, Sunnyvale, CA). Commitment times were determined by the addition of D-alanine (final concentration, 10 mM) at various times to L-alanine germination cultures. D-Alanine serves to inhibit further commitment but allows committed spores to progress through germination (13). DPA release assays and commitment analyses were performed in quadruplicate and duplicate, respectively.

The osmolalities of the germination media were measured using an automatic digital osmometer (OM-815; Vogel) that utilizes the freezing point depression principle.

Spore germination by nonnutrient factors. Germination by exogenous Ca^{2+}-DPA was carried out at 37°C in 200 μl of 60 mM Ca^{2+}-DPA (pH 8, adjusted with dry Tris base) without further additions and measured in a multiplate reader as described above.

Germination by high pressure was performed with wild-type spores. The general procedure was as described previously (19) with the following specifications: shrinkable tubes were filled with 300 μl of 1.6×10^8 spores/ml in 10 mM Tris-HCl containing either 0, 1.2, 2.4, or 3.6 M NaCl and were then hermetically sealed. The samples were exposed to either 150 MPa or 550 MPa for 10 min at 37°C (the exposure was under isothermal and isobaric conditions during the pressure dwell time). Germination was monitored in terms of colony-forming ability by plating and determination of the amount of DPA released by high-pressure liquid chromatography (HPLC). For plating, the spores were incubated for at least 1 h at room temperature (to allow the completion of germination). The samples were split, and one half was heat treated (10 min, 80°C) prior to serial dilution of all samples in phosphate-buffered saline (0.7% $Na_2HPO_4 \cdot 12 H_2O$, 0.4% NaCl, 0.3% KH_2PO_4) and plating on nutrient broth (NB) agar. For HPLC, the samples were sterile filtered directly after pressure treatment and then frozen at −30°C until analysis, which was performed as

TABLE 2 Maximum germination rates (v_{max}) for all tested strains for L-alanine germination at different NaCl concentrations[a]

Strain	Mean ± SD v_{max} with NaCl at the following concn:				
	0 M	0.6 M	1.2 M	2.4 M	3.6 M
168	4.8 ± 0.2	6.3 ± 0.3	4.8 ± 0.1	3.1 ± 0.2	1.3 ± 0.4
D168	4.4 ± 0.1	**0.9 ± 0.0**	**0.8 ± 0.0**	**0.5 ± 0.0**	0.4 ± 0.0
KN19 (*safA*)	**2.1 ± 0.0**	**0.4 ± 0.1**	**0.3 ± 0.0**	**0.2 ± 0.0**	NA
KN20 (*cotE*)	**2.0 ± 0.1**	**0.4 ± 0.0**	**0.3 ± 0.0**	**0.2 ± 0.0**	NA
KN21 (*cotXYZ*)	4.4 ± 0.2	**4.5 ± 0.2**	**3.5 ± 0.4**	**2.1 ± 0.1**	0.6 ± 0.0
KN22 (*spoVID*)	**2.6 ± 0.1**	**0.7 ± 0.0**	**0.6 ± 0.0**	**0.5 ± 0.0**	0.3 ± 0.0
KN23 (*cotE safA*)	**2.2 ± 0.0**	**0.3 ± 0.0**	**0.2 ± 0.0**	**0.2 ± 0.0**	NA
KN32 (*cotE gerE*)	**1.3 ± 0.0**	**0.1 ± 0.0**	**0.1 ± 0.0**	**0.1 ± 0.0**	NA
KF111 (*cwlJ*)	**3.1 ± 0.1**	ND	**3.3 ± 0.1**	ND	1.3 ± 0.2
KF112 (*sleB*)	4.3 ± 0.2	ND	4.3 ± 0.3	ND	1.1 ± 0.0
KF113 (*cwlJ sleB*)	**0.8 ± 0.0**	ND	**0.8 ± 0.0**	ND	**0.1 ± 0.0**
KW05 (*gerP*)	**0.7 ± 0.1**	ND	**1.4 ± 0.1**	ND	0.4 ± 0.0

[a] D168, strain 168 spores were chemically decoated as described in Materials and Methods; NA, no germination detectable via OD_{600} measurements; ND, not determined. Values in bold are significantly ($P < 0.001$) different from the value obtained for strain 168 spores under the respective condition.

described previously (19). DPA concentrations were calculated using a DPA calibration curve. All experiments were performed as independent triplicates.

Data evaluation and mathematical analyses. For convenient comparison of the extent of NaCl-dependent germination inhibition in spores of different *B. subtilis* strains, a relative inhibition (RI)/OD value was calculated as follows (24): the maximum germination rate (v_{max}; in percent relative OD_{600} per minute) was derived from the linear part of the germination-specific OD_{600} decrease for each germination profile (see Fig. S1 in the supplemental material). For every tested condition, the respective v_{max} was divided by v_{max} in the presence of NaCl to yield an RI value, where RI is equal to v_{max} (without NaCl)/v_{max} (with NaCl).

Hence, an RI value of 1 indicates normal germination, whereas RI values of >1 signify germination inhibition. However, the RI values do not take into account either increases in lag time prior to germination initiation or the overall magnitude of the OD_{600} decreases. Consequently, the RI value for each germination condition was also expressed relative to the respective decrease of the relative OD_{600} within 30 min ($\Delta OD_{30\ min}$). Division of the RI by the respective $\Delta OD_{30\ min}$ value yields the RI/OD value as a further indicator of the extent of inhibition at a specific NaCl concentration: RI/OD = (v_{max} [without NaCl]/v_{max} [with NaCl])/$\Delta OD_{30\ min}$ (with NaCl).

An RI/OD value of 1.67 indicates no salt-dependent inhibition, whereas smaller or greater values imply a positive or negative effect of the salt on germination, respectively.

Statistical evaluation of the data given in Tables 2 and 3 was performed by *t* tests, with a *P* value of <0.001 indicating a significant difference.

RESULTS

Germination of chemically decoated wild-type spores. Transmission electron microscopic analysis of ruthenium red-stained, thin-sectioned wild-type spores (in the strain 168 background) revealed the three major layers of the coat: the crust, the outer coat, and the inner coat (Fig. 1A). After chemical decoating, the inner and outer coat layers were reduced in thickness and no longer distinguishable, and the crust appeared to be displaced from the spore surface (Fig. 1B). In the absence of NaCl the decoated spores germinated normally in response to L-alanine and slower than intact spores with AGFK but overall with an efficiency equal to that of intact spores with AGFK (Fig. 2; Tables 2 and 3). It should be noted that the absence or alteration of the coat affects the light absorption properties of the spores, thus leading to a larger decrease in the OD_{600} value during germination, with a decrease of about 70% to 80% (depending on the coat alteration) instead of the usual 60% decrease representing full germination of a population of intact spores (11). Therefore, overall germination efficiency was additionally checked by phase-contrast microscopy.

In the presence of NaCl, the germination of decoated spores with L-alanine was significantly worse than that of wild-type spores: at an NaCl concentration as low as 0.6 M, the OD_{600} decrease was considerably slower and took much longer (Fig. 2A and C), whereas 0.6 M NaCl even increased the maximum germination rate (v_{max}) of untreated wild-type spores (Table 2). Phase-contrast microscopy further revealed that at 0.6 to 2.4 M NaCl, while the vast majority of decoated spores were able to exit the phase-bright (i.e., dormant) state, many turned only phase gray instead of phase dark (data not shown). As reported previously (23), the latter finding might indicate the successful initiation of germination but inhibition of the completion of germination. In

TABLE 3 RI/OD values for all tested strains for L-alanine germination at different NaCl concentrations[a]

Strain	Mean ± SD RI/OD with NaCl at the following concn:			
	0.6 M	1.2 M	2.4 M	3.6 M
168	1.4 ± 0.1	2.0 ± 0.0	4.0 ± 0.4	19.1 ± 7.6
D168	**19.8 ± 0.9**	**22.2 ± 0.7**	**63.1 ± 4.4**	**112.9 ± 19.2**
KN19 (*safA*)	**56.3 ± 17.1**	**92.7 ± 23.2**	**277.7 ± 56.8**	NA
KN20 (*cotE*)	**50.4 ± 7.1**	**86.1 ± 8.0**	**156.1 ± 36.1**	NA
KN21 (*cotXYZ*)	1.7 ± 0.1	2.3 ± 0.2	**5.5 ± 0.1**	**62.1 ± 5.2**
KN22 (*spoVID*)	**21.5 ± 1.5**	**26.2 ± 3.0**	**63.6 ± 2.8**	**124.2 ± 23.2**
KN23 (*cotE safA*)	**92.7 ± 11.3**	**175.7 ± 35.1**	291.9 ± 125.2	NA
KN32 (*cotE gerE*)	242.1 ± 706.1	**136.2 ± 7.6**	**258.9 ± 73.4**	NA
KF111 (*cwlJ*)	ND	2.0 ± 0.1	ND	11.0 ± 2.7
KF112 (*sleB*)	ND	**2.4 ± 0.1**	ND	20.9 ± 1.2
KF113 (*cwlJ sleB*)	ND	**4.9 ± 0.1**	ND	434.9 ± 277.6
KW05 (*gerP*)	ND	1.6 ± 0.2	ND	17.5 ± 2.5

[a] RI/OD values were calculated as described in Materials and Methods and are the ratio of the RI value (i.e., the quotient of v_{max} in the presence and absence of salt) and the decrease of the relative OD_{600} within 30 min ($\Delta OD_{30\ min}$), with an RI/OD value of 1.67 indicating no salt-dependent inhibition, whereas smaller or greater values imply a positive or negative effect of the salt on germination, respectively. D168, strain 168 spores were chemically decoated as described in Materials and Methods; NA, no germination detectable via OD_{600} measurements; ND, not determined. Values in bold are significantly ($P < 0.001$) different from the value obtained for strain 168 spores under the respective condition.

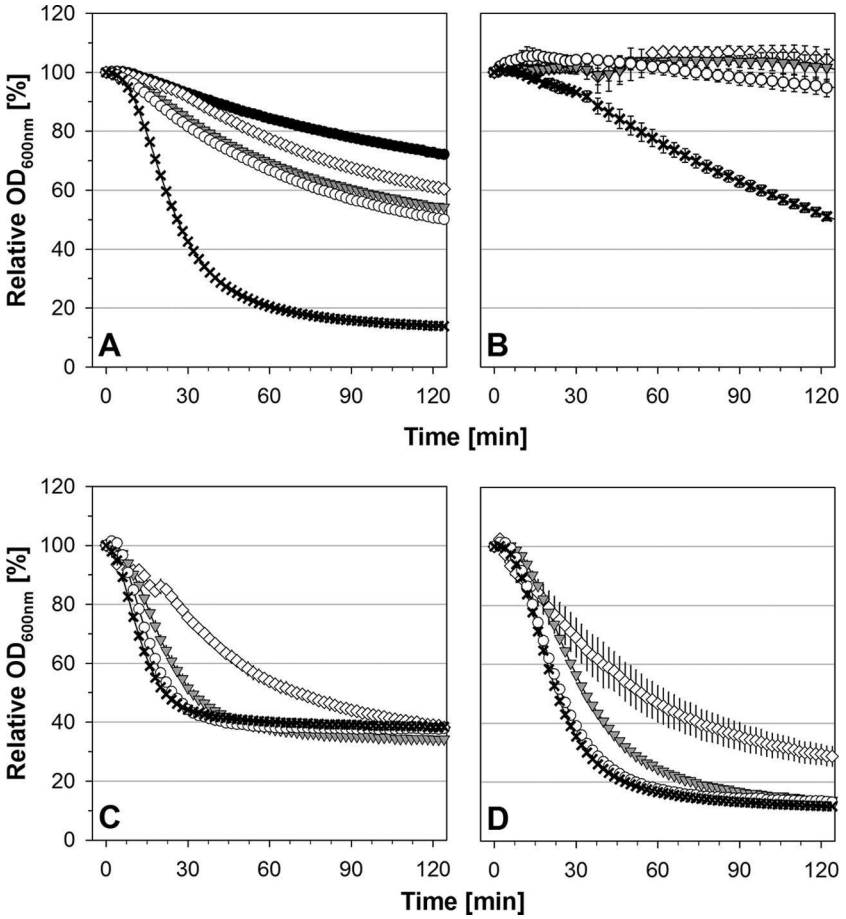

FIG 3 Germination of spores lacking morphogenetic coat proteins. (A, B) Germination of *spoVID* spores was triggered by L-alanine (A) and AGFK (B), and changes in the OD_{600} were recorded as described in Materials and Methods. The germination media contained no salt (black crosses), 0.6 M NaCl (white circles), 1.2 M NaCl (gray inverted triangles), 2.4 M NaCl (white diamonds), or 3.6 M NaCl (black circles). (C, D) L-Alanine germination of wild-type spores (C) and *spoVID* mutant spores (D) in the presence of 0 M (black crosses), 1 M (white circles), 2 M (gray inverted triangles), or 4 M (white diamonds) D-fructose. Germinations with 6 M fructose were monitored by phase-contrast microscopy only.

the presence of 3.6 M NaCl, most decoated spores remained phase bright, although some phase-gray spores were also present.

The AGFK-triggered germination of decoated spores was even more severely affected by salinity, as even 0.6 M NaCl resulted in an incomplete OD_{600} decrease (Fig. 2B and D), with about half of the spore population remaining in the phase-bright state typical for dormant spores.

Germination of spores lacking major coat layers due to mutation. As the chemical decoating procedure is harsh, the possibility cannot be excluded that this procedure also affected spore components other than the coat that are required for germination, hence potentially leading to the observed salt sensitivity, which untreated spores did not exhibit. Therefore, strains lacking the major morphogenetic coat proteins and, thus, complete spore layers were investigated as well.

Spores of a *cotXYZ* mutant (KN21), which lack the outermost coat layer termed the crust (28) (Fig. 1H), generally exhibited wild-type-like germination with L-alanine and AGFK at most NaCl concentrations (Table 3; see also Fig. S1C and S2C in the supplemental material). However, at 3.6 M NaCl, the RI/OD value for *cotXYZ* mutant spores was significantly higher than that for wild-type spores (indicating stronger inhibition) and the v_{max} value was only half that for wild-type spores under this condition (Tables 2 and 3). Hence, the effects of the absence of the crust on nutrient germination became apparent only at a very high salinity.

To address the role of the remainder of the coat (the inner and outer coat layers) in germination in high-salinity environments, we analyzed spores with mutations severely impairing assembly of the inner coat (*safA* mutant strain KN19; Fig. 1C) or the outer coat (*cotE* mutant strain KN20; Fig. 1D), as well as strains preventing coat encasement (*spoVID* mutant strain KN22; Fig. 1G) or most of coat assembly (*cotE safA* mutant strain KN23 and *cotE gerE* mutant strain KN32; Fig. 1E and F, respectively) (4, 6, 29–33). All of these spores germinated very differently from untreated strain 168 spores but had very similar germination profiles among each other (a typical germination profile for these mutant spores is given in Fig. 3A; for additional profiles, see Fig. S1 in the supplemental material and Tables 2 and 3). When germinated with L-alanine in the absence of NaCl, each mutant strain had a longer lag time and a lower v_{max} than the wild type, but almost the complete spore population turned phase dark within 4 h (Table 2). However, as for the chemically decoated spores, even relatively low NaCl concentrations ($\geq$0.6 M) had significant detrimental effects on the germination of spores from each mutant strain, as indicated by

their low v_{max} and high RI/OD values (Tables 2 and 3). Indeed, with increasing NaCl concentrations, an increasing portion of the spore populations remained phase bright, and at 3.6 M NaCl, most appeared not to have germinated. Note that spores of the strains with the most significant impairments in the coat (the *safA cotE* and *gerE cotE* mutant strains; Fig. 1E and F) had the greatest impairments in germination in the presence of NaCl (Tables 2 and 3; see also Fig. S1 in the supplemental material).

When germinated with AGFK, NaCl had even stronger deleterious effects on the germination of *safA*, *cotE*, *spoVID*, *safA cotE*, and *gerE cotE* mutant spores, as at 0.6 M NaCl, germination was no longer detectable by OD_{600} measurements (Fig. 3B; see also Fig. S1 in the supplemental material). Microscopy consistently revealed that in the presence of 0.6 M NaCl, less than ca. 10% of the mutant spores germinated in response to AGFK within 4 h.

Nonnutrient germination initiation by exogenous Ca^{2+}-DPA was possible for the spores with a crust deficiency (*cotXYZ*) but not for spores lacking other coat layers (i.e., *safA*, *cotE*, *spoVID*, *safA cotE*, and *gerE cotE* mutant spores) because severe coat defects caused by mutations or chemical decoating can result in the loss of CwlJ, thus eliminating the ability of Ca^{2+}-DPA to initiate germination (17). Interestingly, in the absence of NaCl, the lag time for *cotXYZ* mutant spores was shorter than that for wild-type spores, whereas in the presence of ≥0.6 M NaCl, *cotXYZ* mutant spores exhibited a notably slower and lower OD_{600} decrease than wild-type spores (see Fig. S4 in the supplemental material).

Taken together, these results indicate that the coat is crucial for efficient nutrient germination in the presence of NaCl. Although the crust did not seem to play a major role in nutrient germination, its absence did have an effect on germination kinetics at very high salinity and on nonnutrient germination initiation by Ca^{2+}-DPA.

Germination of *spoVID*-deficient spores under nonionic osmotic stress conditions. It is known that germination can also be inhibited by high concentrations of nonionic solutes, such as glycerol, presumably due to osmotic effects (24). To assess the role of osmotic stress in the enhanced inhibition of high salinity on coat-deficient spores, wild-type and *spoVID* mutant spores were germinated with L-alanine in the presence of high D-fructose concentrations (1, 2, 4, and 6 M); these concentrations have osmolalities corresponding to the germination media with 0.6, 1.2, 2.4, and 3.6 M NaCl, respectively, i.e., 1.1, 2.2, 4.7, and 6.8 osmol/kg, respectively. Fructose concentrations of ≥4 M had inhibitory effects on wild-type spore germination (Fig. 3C), causing an OD_{600} decrease somewhat slower than that achieved with 2.4 M NaCl (Fig. 2A), possibly as a result of the high viscosity of the fructose germination medium. However, germination with high fructose concentrations was more effective, since even at 6 M fructose about 90% of the spore population germinated within 4 h, as determined by phase-contrast microscopy. Surprisingly, in contrast to germination with NaCl, the effects of high fructose concentrations on the germination of *spoVID* spores were very similar to the effects on the germination of wild-type spores (Fig. 3C and D). This difference between NaCl and fructose strongly suggests that the much stronger NaCl inhibition of the germination of coat-deficient spores than that of wild-type spores is primarily due to ionic effects.

Germination of spores with mutations in less pleiotropic coat protein genes. To assess the roles of coat proteins not involved in the recruitment of complete major coat layers in germination in the presence of high NaCl concentrations, we analyzed spores from strains missing the coat protein CotB, CotC, CotG, CotH, or CotS (4). Spores with null alleles of *cotB*, *cotC*, *cotG*, or *cotS* germinated similarly to wild-type spores in the presence and absence of NaCl (see Fig. S1 in the supplemental material). Interestingly, spores bearing a mutation in *cotH*, which results in a partial defect in the outer coat and the lack of a subset of inner and outer coat proteins (34, 35), exhibited a slightly slower germination than wild-type spores in the presence of ≥0.6 M NaCl (see Fig. S1I in the supplemental material).

Spores lacking the *gerP* operon (strain KW05), which encodes proteins thought to facilitate germinant passage through the spore's integuments, had no detectable crust but did not exhibit any further noticeable coat defects (Fig. 1I), as suggested previously (36, 37). However, the germination of *gerP* mutant spores was strikingly affected by the presence of NaCl. While these spores generally germinated more slowly and less efficiently than untreated wild-type spores at all salt concentrations, the presence of up to 1.2 M NaCl clearly enhanced L-alanine germination of *gerP*-deficient spores, as observed by spectrophotometry, microscopy, and DPA release (Fig. 4A and B; see Fig. S1N in the supplemental material). Although DPA release during L-alanine-induced germination of *gerP* mutant spores was slightly delayed by 2.4 M NaCl and much slower than that with 3.6 M NaCl, the total amount of DPA released in 120 min was ca. 1.5-fold higher with 2.4 M NaCl than with 3.6 M NaCl and the level of germination was about equal to the level of germination in the absence of salt (Fig. 4B). In the absence of NaCl, the commitment to germination of *gerP* mutant spores was slower than the commitment of wild-type spores, which is consistent with the longer lag times for the DPA release of *gerP* mutant spores (Fig. 4C and D). Although the presence of 1.2 M NaCl increased the total amount of DPA released from *gerP* mutant spores by about 1.6-fold, the time required for a 50% commitment (C50%) and 50% of the maximum DPA release (G50%) was not decreased by NaCl. However, the time interval between C50% and G50% tended to be larger for *gerP* mutant spores than for wild-type spores.

When *gerP* mutant spores were germinated with AGFK, 0.6 M NaCl slightly increased the amount of DPA released (ca. 1.1-fold), and the release rate was similar to the rate in the absence of NaCl (see Fig. S3 in the supplemental material). At 1.2 M NaCl, the amount of DPA released was slightly decreased (ca. 0.8-fold) and DPA release was delayed, whereas 2.4 M NaCl almost completely inhibited DPA release.

Germination of spores lacking CLEs. It has previously been shown that germination with exogenous Ca^{2+}-DPA, which is GR independent, is also inhibited by NaCl (23). Because Ca^{2+}-DPA triggers germination via CLEs (17) that are localized in the outer spore layers (38, 39), it was hypothesized that they might be one of the targets of NaCl inhibition (23). Since either *cwlJ* or *sleB* needs to be present and functional for complete germination (14), *in vivo* inhibition of the individual enzymes was tested with single mutants of either gene. Interestingly, L-alanine germination of spores of both single mutant strains (KF111 and KF112, bearing mutations in *cwlJ* and *sleB*, respectively) was close to that of wild-type spores in terms of their OD_{600} changes and DPA release, regardless of the NaCl concentration, although DPA release in *cwlJ* spores was slightly slower in general (Fig. 4A and 5A and B; see also Fig. S1 in the supplemental material). Hence, neither of the two CLEs was specifically inhibited by NaCl. However, cortex hydrolysis was important for germination in high-salinity environ-

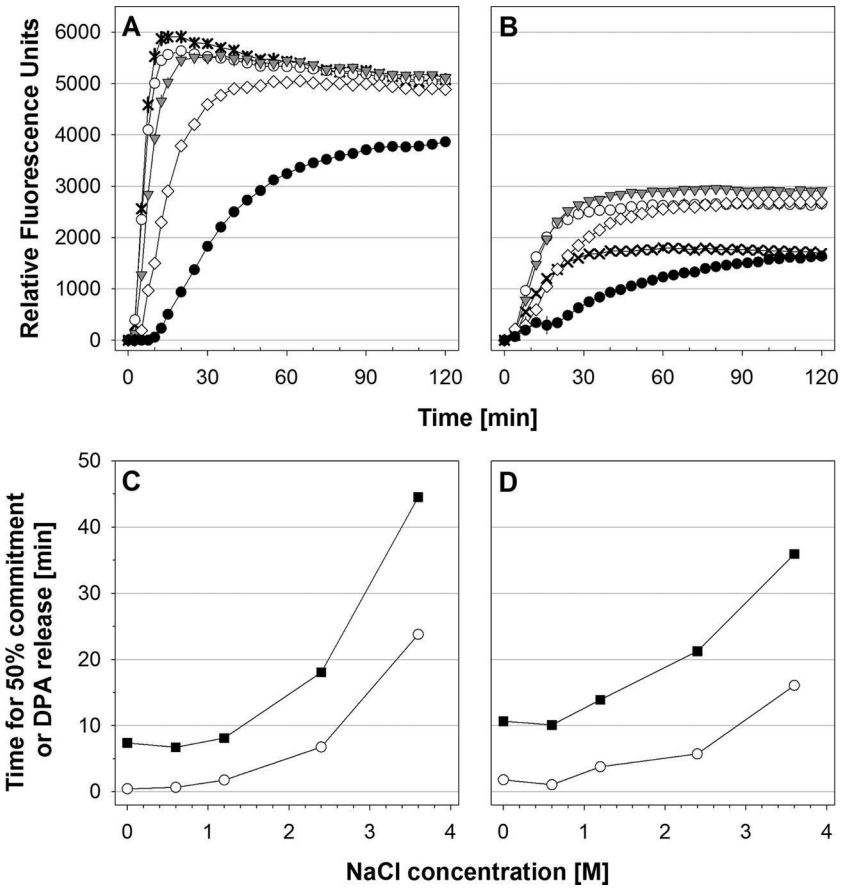

FIG 4 Germination of *gerP*-deficient spores (B, D) compared to that of wild-type spores (A, C). (A, B) Spores were germinated by treatment with L-alanine, and DPA release was measured as described in Materials and Methods. The germination media contained no salt (black crosses), 0.6 M NaCl (white circles), 1.2 M NaCl (gray inverted triangles), 2.4 M NaCl (white diamonds), or 3.6 M NaCl (black circles). (C, D) Time required for 50% commitment (white circles) and 50% of maximum DPA release (black squares) in response to L-alanine at different NaCl concentrations.

ments, since DPA release from *cwlJ sleB* mutant spores (KF113) was inhibited more strongly by high salinity than DPA release from wild-type spores (Fig. 4A and 5). It should be noted, though, that even in the absence of NaCl, DPA release from the *cwlJ sleB* mutant spores was slower and less complete than that from spores of the single CLE mutant strains (Fig. 5C).

Germination by high pressure. To test whether impaired germinant accessibility and/or germinant binding to the GRs is a major reason for NaCl-dependent germination inhibition, wild-type spores were germinated by a 150-MPa-pressure treatment (10 min, 37°C), which does not require germinants to activate GRs (16, 18). With this approach, the requirements for germinant accessibility and/or germinant binding are bypassed, and their role in NaCl-dependent germination inhibition can be assessed. Ger-

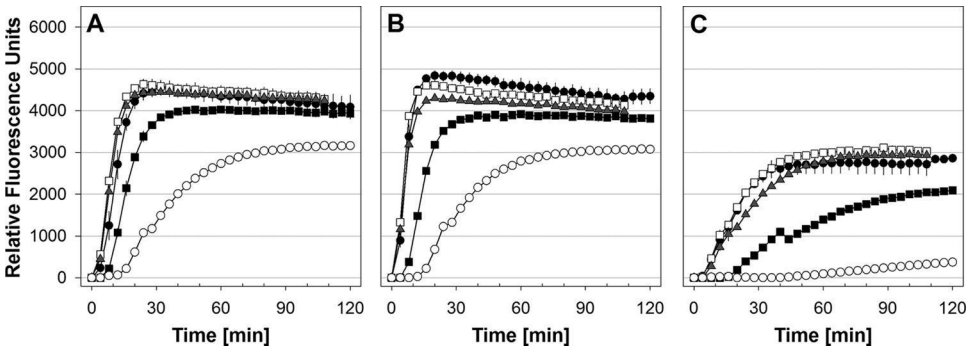

FIG 5 DPA release during germination of CLE mutant spores. Spores lacking *cwlJ* (A), *sleB* (B), or *cwlJ* and *sleB* (C) were germinated by treatment with L-alanine as described in Materials and Methods. Germination media contained 0 M NaCl (black circles), 0.6 M NaCl (white squares), 1.2 M NaCl (gray triangles), 2.4 M NaCl (black squares), or 3.6 M NaCl (white circles).

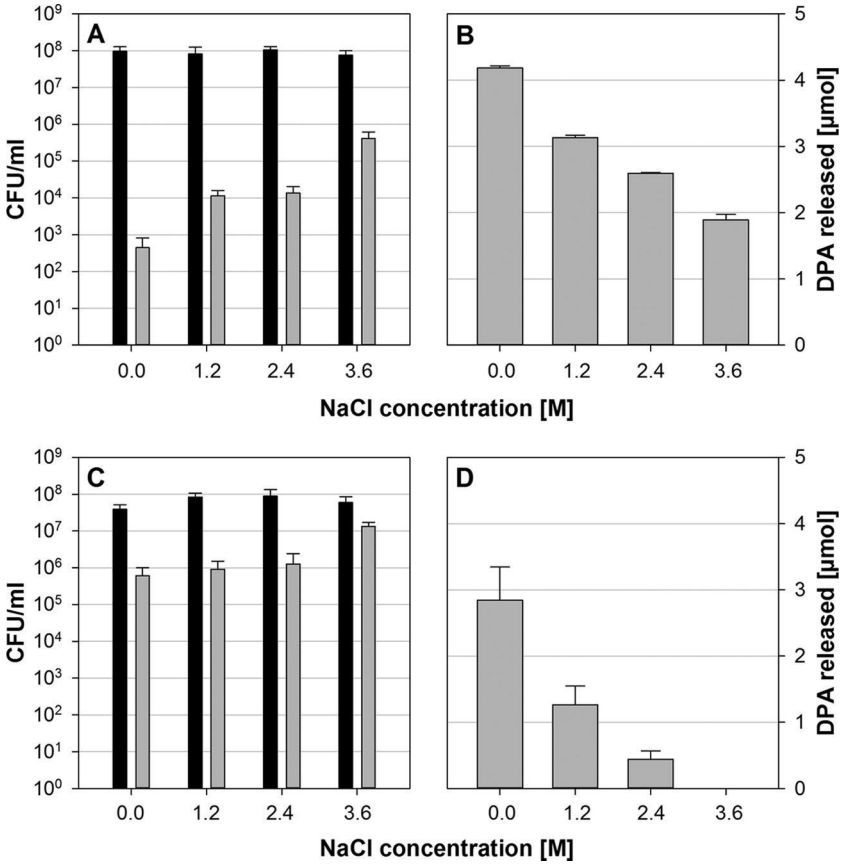

FIG 6 (A to D) Wild-type spore germination at high pressure in the presence of NaCl. Spores were germinated by treatment with pressures of 150 MPa (A, B) and 550 MPa (C, D), and germination was monitored by determination of the amount of DPA released by plating (A, C) and by HPLC (B, D), as described in Materials and Methods. (A, C) Black bars, numbers of CFU per milliliter without heat treatment; gray bars, numbers of CFU per milliliter after 10 min of heat treatment at 80°C. (B, D) The total DPA content of the spores was 4.6 ± 0.1 μmol.

mination was monitored by measurement of DPA release via HPLC analysis and plating with or without a heat treatment to kill any germinated spores. With both methods, it could be shown that germination by 150 MPa is inhibited by NaCl (Fig. 6A and B). As for nutrient-dependent germination, the extent of NaCl inhibition increased with increasing NaCl concentrations (23). Importantly, even at up to 3.6 M NaCl, noticeable germination could be induced by 150 MPa and significant amounts of DPA (ca. 2 μmol) were released.

Spores were also germinated by a higher pressure of 550 MPa. This treatment is thought to directly induce Ca^{2+}-DPA release via Ca^{2+}-DPA channels (16, 18, 19) and/or to induce a phase shift of the inner spore membrane (40–42). Again, it could be shown that germinations induced by 550 MPa were inhibited by NaCl: increasing NaCl concentrations clearly decreased the amount of DPA released, with 3.6 M NaCl no longer allowing any detectable DPA release (Fig. 6D). Germination efficiencies in terms of colony-forming ability were also decreased by NaCl (Fig. 6C), although the effects of 1.2 M and 2.4 M NaCl on the numbers of CFU after heat treatment were much smaller than the effects on DPA release. It should be noted that both the plating efficiencies of heat-treated samples were lower and the amount of DPA released was smaller when germination was induced by treatment of spores with 550 MPa than when it was induced by treatment of spores with 150 MPa. This likely relates to the fact that germination and DPA release by treatment with 550 MPa are possible only during the 10-min pressure treatment, whereas treatment with 150 MPa activates GRs, allowing the process of germination to continue even after decompression. Nevertheless, germination induced by either pressure was clearly inhibited by NaCl.

DISCUSSION

Although inhibition of spore germination by high salinity is very important to both applied and basic research, the underlying mechanistic details have remained obscure. From existing work, the most likely inhibition targets for NaCl include (i) GR accessibility, (ii) germinant binding to the GRs, (iii) signal integration and transduction from the germinosome, leading to (iv) ion and Ca^{2+}-DPA release from the spore core with concomitant water uptake, and (v) cortex hydrolysis via CLEs (23). Previous data suggest that NaCl likely has several inhibition targets: on the one hand, high NaCl concentrations can cause a delay in (and an increased heterogeneity of) germination initiation, as observed by commitment experiments, Ca^{2+}-DPA release, OD_{600} decrease, and single-spore differential interference contrast microscopy (23), suggesting inhibition very early in germination. On the other hand, subsequent events also seem to be inhibited at high salinity, as the time required for the change from the phase-bright (dormant) state to the phase-dark (fully germinated) state is significantly increased, and at very high NaCl concentrations, spores can

get stuck in an intermediate phase-gray state, suggesting incomplete germination (23; K. Nagler, K. Madela, and R. Moeller, unpublished results). Further inhibition targets may also exist, especially when germination occurs in the presence of high concentrations of other salts (24).

With regard to the first proposed inhibition target, GR accessibility, it is conceivable that ions might interact with the complex protein network of the spore coat, thereby affecting its structure and decreasing its permeability for germinants (23). However, the germination of spores lacking major coat layers or the entire coat was much more severely affected by the presence of NaCl than the germination of wild-type spores. This suggests that the coat plays an important role for successful germination in high-salinity environments. It has previously been proposed that there may be an additional permeability barrier outside the inner spore membrane, e.g., the outer membrane or a different coat-associated structure, which might be removed or compromised in severely coat-defective spores (36, 37, 43), thus leading to stronger germination inhibition in high-salinity environments. In contrast, the lack of the outermost coat layer, the crust, did not have notable effects on nutrient germination at NaCl concentrations of $<$3.6 M NaCl. This suggests a novel functional difference between the crust and the other coat layers in the ability to germinate in the presence of environmental stress, such as high salinity.

Analysis of the effects of NaCl on pressure-induced germination further supported the idea that NaCl likely does not inhibit germinant accessibility or germinant binding to GRs: spore germination by a high pressure of 150 MPa was increasingly inhibited by increasing NaCl concentrations, although this treatment led to the direct activation of GRs, thus requiring neither nutrient germinants passing through spore integuments nor germinant-receptor binding (16, 18). Hence, inhibition of 150-MPa-induced germination seems to occur downstream of GR activation. Interestingly, the germination triggered by 550 MPa was also inhibited by NaCl. As treatments with a 550-MPa pressure directly induce Ca^{2+}-DPA release, likely by activation of SpoVA channels and/or a phase shift of the inner spore membrane (16, 18, 19, 40–42), NaCl probably does not inhibit germination at the levels of signal integration and transduction from the germinosome.

Furthermore, because neither *cwlJ* nor *sleB* mutant spores were particularly inhibited by high salinity, it seems that NaCl does not inhibit germination at the level of cortex hydrolysis. However, in the absence of both, the level of CLE inhibition by high NaCl concentrations ($\geq$2.4 M NaCl) was stronger than that in wild-type spores. This inhibition is possibly due to the lack of amplification of germination signaling that is a consequence of the reduced release of Ca^{2+}-DPA in *cwlJ sleB* mutant spores (10, 17). If so, then NaCl might directly interfere with Ca^{2+}-DPA release, consistent with the increase in the delay of germination with increasing salinity.

Nevertheless, assuming that CwlJ itself is not inhibited by high ionic strength, it is not clear why the germination of crust-deficient spores with exogenous Ca^{2+}-DPA is more strongly inhibited by NaCl than the germination of wild-type spores, especially since it is faster in the absence of salt. The crust possibly has a minor protective function for coat proteins apparent only at very high salinities. The faster germination of crust-deficient spores in the absence of NaCl might, in turn, be due to the easier access of exogenous Ca^{2+}-DPA to CwlJ.

Taken together, the results presented in this study suggest that inhibition of wild-type spore germination by NaCl is not mainly at the level of GR accessibility, germinant binding to GRs, signal integration and transduction from the germinosome, or cortex hydrolysis. Therefore, the release of ions and/or Ca^{2+}-DPA and the accompanying water uptake into the spore core seem to be the most likely processes to be detrimentally affected by high salinity. This would be consistent with the assumption of multiple inhibition targets within the germination pathway: while ion release is one of the first measureable responses of germinating spores and may be important for germination initiation, Ca^{2+}-DPA release and especially water uptake occur later during the germination process. Hence, the impairment of the last two processes may be responsible for the slower change from phase bright to phase dark in high-salinity environments.

Possible mechanisms for the inhibition of ion, Ca^{2+}-DPA, and water fluxes include the following: (i) Na^+ and Cl^- ions might directly block channels, (ii) ions and/or Ca^{2+}-DPA might not be able to flow out of the spore core due to the high extracellular ionic strength that eliminates the normal chemical gradient, and/or (iii) water influx might be impaired by a extracellular water activity that is too low. As presented in an earlier study on the effects of a broad range of different salts on *B. subtilis* spore germination (24), salt-dependent germination inhibition seems to result from a combination of ionic and osmotic effects. Thus, all three possibilities mentioned above might concurrently contribute to NaCl inhibition, albeit to different extents.

It is generally assumed that the cortex of dormant spores, as an electronegative structure harboring positive counterions, is in osmotic equilibrium with the core (with both having water activity of ca. 0.85) and, hence, acts as an osmoregulatory structure supporting dehydration of the core (44, 45). This equilibrium might be disturbed in high-salinity environments, consequently causing less effective germination. Moreover, the coat, possibly in complex with the putative outer membrane (if it exists in mature spores) or another coat-associated structure, is assumed to be a dielectrically effective insulating layer around the cortex which may act as an additional permeability barrier that possibly restricts ion movement to some extent (43, 44). In decoated spores, the coat or outer membrane-coat complex is largely removed (43). Thus, the three putatively NaCl-sensitive processes mentioned above, all of which occur in close proximity to the inner spore membrane, are likely exposed more directly to Na^+ and Cl^- ions, thereby explaining the increased inhibition of coat-defective spores by NaCl. This would be consistent with the different inhibitory effects of high fructose concentrations versus high NaCl concentrations on the *spoVID* spores that were used as a proxy for severely coat-deficient spores (Fig. 3): while Na^+ and Cl^- ions might interfere directly with ion and Ca^{2+}-DPA efflux by interaction with channels/transporters or by annihilation of an ionic gradient, fructose can affect only the osmotic equilibrium. In addition, a significant increase in the water permeability of the inner spore membrane, known to occur in coatless spores, might also contribute to these effects (46).

While all these results are consistent with each other, the assumption of NaCl inhibition at the level of ion, Ca^{2+}-DPA, and water fluxes without any involvement of GRs does not explain why germination triggered by L-alanine (and L-valine) is less susceptible to NaCl than AGFK-triggered germination (23). Although ionic interactions of Na^+ and Cl^- with AGFK's K^+ or conformational changes of GR proteins due to high ionic strength

might play a role, further investigations would be required to address this question adequately.

Despite the inhibitory effects that NaCl exerts on spore germination, analysis of spores lacking the *gerP* operon, whose proteins are thought to facilitate germinant passage through the spore integuments (36, 37), revealed that even up to 2.4 M NaCl can also have positive effects on germination, increasing the amount of DPA released. Notably, 1.2 M NaCl, which maximized DPA release in *gerP*-deficient spores, did not accelerate commitment, a process that might involve GR-germinant interactions (13, 47). Therefore, NaCl does not seem to enhance nutrient accessibility to the inner membrane in *gerP*-less spores, e.g., by interaction with spore integuments, but rather affects subsequent processes.

Overall, this study not only showed that the spore coat (possibly in combination with the outer spore membrane or another coat-associated structure) seems to have a protective function during *B. subtilis* germination in high-salinity environments but also provided further evidence that germination inhibition by NaCl likely occurs at the level of ion, Ca^{2+}-DPA, and water fluxes. The importance of spore resistance to high salinity in diverse niches raises the necessity of future work to analyze these fluxes in more detail to provide a precise mechanism for the inhibition of germination in high-salinity environments.

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No conflict of interest is declared.

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CHAPTER 5

Germination of spores of astrobiologically relevant *Bacillus* species in high-salinity environments

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In this publication, spore revival of five different *Bacillus* species (*B. megaterium, B. pumilus* SAFR-032, *B. nealsonii, B. mojavensis,* and *B. vallismortis*) in the presence of high NaCl concentrations was investigated and compared to *B. subtilis*. As spores of different species germinated very differently in rich media and in response to a non-nutrient germinant, a new 'universal' germination trigger termed KAGE (K^+, L-alanine, D-glucose, and ectoine) that allowed comparable germination in the presence and absence of NaCl was developed. Salt-sensitivity differed among the tested species, with *B. subtilis* spores being the least salt-sensitive. This study provided information on interspecific differences in germination capabilities in the presence of NaCl and includes a discussion of the relevance of spore transfer to another celestial body in the context of planetary protection.

Author contributions:

K. Nagler designed, performed (ca. 50%), and evaluated the experiments, and prepared the text and figures of the manuscript. **C. Julius** performed the remaining part of the experiments and was involved in preliminary analyses of the data. **R. Moeller** gave scientific input and edited the manuscript text.

ABSTRACT

In times of increasing space exploration and search for extraterrestrial life, new questions and challenges for planetary protection, aiming to avoid forward contamination of different planets or moons with terrestrial life, are emerging. Spore-forming bacteria such as *Bacillus* species have a high contamination potential due to their spores' extreme resistance, enabling them to withstand space conditions. Spores require liquid water for their conversion into a growing cell (i.e. spore germination and subsequent growth). If present, water on extraterrestrial planets or moons is likely to be closely associated with salts (e.g. in salty oceans or brines), thus constituting high-salinity environments. Spores of *Bacillus subtilis* can germinate despite very high salt concentrations, although salt stress does exert negative effects on this process. In this study, germination and metabolic reactivation ('outgrowth') of spores of five astrobiologically relevant *Bacillus* species (*B. megaterium*, *B. pumilus* SAFR-032, *B. nealsonii*, *B. mojavensis*, and *B. vallismortis*) in high salinity (≤ 3.6 M NaCl) were investigated. Spores of different species exhibited different germination and outgrowth capabilities in high salinity, which strongly depended on germination conditions, especially the exact composition of the medium. In this context, a new 'universal' germination trigger for *Bacillus* spores, named KAGE (KCl, L-alanine, D-glucose, ectoine), was identified, which will be very useful for future comparative germination and outgrowth studies on different *Bacillus* species. Overall, this study yielded interesting new insights on salt stress effects on spore germination and points out the difficulty of predicting the potential of spores to contaminate salty environments on extraterrestrial celestial bodies.

INTRODUCTION

In times of increasing space exploration and the search for extraterrestrial life, there is a growing need for planetary protection considerations, aiming to avoid forward contamination of celestial bodies with terrestrial organisms (Crawford, 2005; Nicholson *et al.*, 2005). Despite countermeasures and assembly in ultra-clean rooms, spacecraft still harbor a notable microbial bioload that could potentially be transferred to extraterrestrial planets or moons (reviewed in Nicholson *et al.*, 2005; Nicholson *et al.*, 2009; Horneck *et al.*, 2010). Spore-forming bacteria are a problematic bioload, because spores are not only metabolically inert and can stay in this dormant state for many years, but are also highly resistant against

many environmental extremes (reviewed in Nicholson *et al.*, 2000; Nicholson *et al.*, 2005; Crawford, 2005; Setlow, 2014). This allows them to withstand certain sterilization measures as well as space conditions for a prolonged period of time (Horneck *et al.*, 2012; Vaishampayan *et al.*, 2012). A prominent bacterium with the ability to form extremely resistant spores is the *Bacillus pumilus* strain SAFR-032, which was isolated from a clean room in the Jet Propulsion Laboratory (JPL) spacecraft assembly facility (Venkateswaran *et al.*, 2003; Link *et al.*, 2004). In general, a downside of the high hygienic standards and the inhospitable environment created in spacecraft assembly facilities is the selective pressure allowing only the most resistant microbes to survive (Crawford, 2005). Such resistant microbes are in turn the most likely to resist extraterrestrial conditions and are therefore a major threat to planetary protection (Nicholson *et al.*, 2000; Crawford, 2005).

Since bacterial spores have a high potential to be transferred to a different planet or moon in a dormant but viable state (Nicholson *et al.*, 2000; Crawford, 2005; Nicholson *et al.*, 2005), it is a major question, whether these spores can convert back into an actively growing cell in the extraterrestrial environment, thus becoming an issue for planetary protection. For this conversion termed spore germination (and generally for bacterial growth) liquid water is essential and would need to be present on the celestial body in question. In recent years, increasing knowledge about our solar system points at the possible presence of liquid water on extraterrestrial celestial bodies ‘nearby’. Recurring slope lineae strongly suggest the presence of liquid brines on Mars and the moons Enceladus and Europa are assumed to have liquid salty oceans underneath their icy crust (Marion *et al.*, 2003; Davila *et al.*, 2010; Sohl *et al.*, 2010; Hand and Carlston, 2014; Martin and Cockell, 2015; Thomas *et al.*, 2015; Ojha *et al.*, 2015). Hence, it is likely that liquid water on potential space mission targets will be in close association with salt. Thus, it is important to elucidate, if spores are able to germinate and subsequently grow in high-salinity environments. For *B. subtilis* it has recently been shown that germination and – in the presence of specific chemicals – metabolic reactivation (‘outgrowth’, see below) can occur despite high salinity, although very high salt concentrations do exert negative, inhibitory effects (Nagler *et al.*, 2014; Nagler and Moeller 2015; Nagler *et al.*, 2015).

An essential factor for successful *Bacillus* spore germination is the presence of specific molecules called germinants. Germinants are required to trigger germination and can be amino acids, sugars, salts, and purine nucleosides (Setlow, 2013). Most germinants (usually referred to as nutrient germinants) bind to specific germinant receptors (GRs) that are located in the inner spore membrane, which surrounds the dehydrated spore core harboring

the DNA, RNA, proteins, and other biomolecules (Paredes-Sabja *et al.*, 2010; Setlow, 2013). To reach the inner membrane, the germinants first have to pass through the spore's integuments, i.e. the proteinaceous spore coat, the peptidoglycan cortex and in some species also an outermost exosporium (Setlow, 2006; Paredes-Sabja *et al.*, 2010). After sufficient germinants have bound to GRs, the spore commits to germinate in a yet unknown fashion (Yi and Setlow, 2010; Setlow, 2013). The germination process has two stages: first, large amounts of cations and the spore's large depot of dipicolinic acid (mostly in complex with Ca^{2+} as 'Ca^{2+}-DPA') are released from the spore core, allowing a partial core hydration (Setlow *et al.*, 2001; Paredes-Sabja *et al.*, 2010; Setlow, 2013). Second, the spore cortex is hydrolyzed (in *B. subtilis* spores mainly by the cortex-lytic enzymes CwlJ and SleB), which permits full core rehydration (Paredes-Sabja *et al.*, 2010; Setlow, 2013). Throughout these two stages, the spore loses its resistance and refractivity, i.e. it changes from a bright to a dark appearance in the phase-contrast microscope (Setlow, 2013). After germination is completed, the spore enters a phase called outgrowth. Outgrowth is defined as the period between the onset of metabolism and the first cell division, throughout which the spore escapes from its spore coat remnants and elongates (Keijser *et al.*, 2007; Setlow, 2013). Aside from nutrient germinants, other factors that do not bind to GRs can induce germination as well: these so-called non-nutrient germinants include high pressure and exogenous Ca^{2+}-DPA among others (Reimann and Ordal, 1961; Paredes-Sabja *et al.*, 2010; Reineke *et al.*, 2013; Setlow, 2013). In *B. subtilis*, exogenous Ca^{2+}-DPA triggers germination by activation of the cortex-lytic enzyme CwlJ (Paidhungat *et al.*, 2001). A similar mechanism can be assumed for other *Bacillus* species as well, as CwlJ orthologues are common among bacilli (Paredes-Sabja *et al.*, 2010). While exogenous Ca^{2+}-DPA can trigger germination in a variety of different *Bacillus* species, suitable nutrient germinants are more species specific (Reimann and Ordal, 1961; Ross and Abel-Santos). In many cases chemically defined nutrient germinants are not yet known, hampering precise spore germination studies (Ross and Abel-Santos, 2010; Nagler, K. and Moeller, R., unpublished results).

In this study, six different (and in part poorly investigated) *Bacillus* species that may be present as bioloads on spacecraft (Nicholson *et al.*, 2005; Nicholson *et al.*, 2009) were analyzed with respect to their ability to germinate and reestablish metabolism in the presence of 0.6 M (3.5 %, similar to Earth oceans), 1.2 M (7.1 %), 2.4 M (14.3 %), and 3.6 M (21.4 %) NaCl. These species included *B. pumilus* SAFR-032, *B. nealsonii*, *B. vallismortis*, *B. mojavensis*, *B. megaterium* and the model species *B. subtilis*. The observed germination and outgrowth responses were highly variable, giving new insights into salt stress effects on

Bacillus spore germination and entailing important implications for astrobiology and planetary protection. Importantly, a common, efficient germinant mixture was identified, allowing defined, systematic, and comparable germination and outgrowth studies.

MATERIALS AND METHODS

Strain selection. In this study, spores of the *Bacillus* species *B. megaterium*, *B. pumilus*, *B. nealsonii*, *B. vallismortis*, and *B. mojavensis* were investigated. With the exception of *B. pumilus* (strain SAFR-032), the type strain of each species was used (**Table 1**). For comparison, the model species *B. subtilis* (laboratory strain 168), which has already been characterized with regard to germination and outgrowth in high salinity, was included (Nagler *et al.*, 2014; Nagler & Moeller., 2015; Nagler *et al.*, 2015).

B. megaterium was chosen, as it is genetically and phenotypically distinct from members of the phylogenetic *Bacillus subtilis* group (**Fig. S1**; Eppinger *et al.*, 2011; Gupta *et al.*, 2013), but represents a fairly well investigated model species for *Bacillus* spore germination (Paredes-Sabja *et al.*, 2010; Setlow, 2013). Thus, spore production techniques and defined germinants are already established and adequate literature resources available (Gupta *et al.*, 2013; Setlow, 2013).

B. pumilus SAFR-032 was isolated from a clean room in the spacecraft assembly facility at NASA's Jet Propulsion Laboratory (JPL-SAF), rendering it a likely bioload for space vehicles (Link *et al.*, 2004). Its spores have an outstandingly high resistance against UV radiation and H_2O_2, enabling this strain to withstand decontamination procedures (Link *et al.*, 2004; Tirumalai *et al.*, 2013). *B. pumilus* SAFR-032 spores have previously been used for astrobiological experiments including prolonged exposure to real space conditions on board the International Space Station (ISS) (Vaishampayan *et al.*, 2012; Horneck *et al.*, 2012; Nicholson *et al.*, 2012). Although germination of *B. pumilus* has been investigated in complex media or when triggered by high pressure (Clousten and Wills, 1969; Nicholson *et al.*, 2012), to our knowledge, no chemically defined nutrient germinant has been identified so far. However, L-alanine was reported to be conductive for germination when used in combination with Spizizen salts and tryptophan (Fajardo-Cavazos *et al.*, 2008).

B. nealsonii was isolated from the JPL-SAF as well, and its spores were found to be resistant against UV, γ-radiation, H_2O_2, and desiccation (Venkateswaran *et al.*, 2003). Hence, *B. nealsonii* spores are also a likely contaminant on spacecraft and of concern for planetary

protection (Nicholson *et al*., 2009). No chemically defined nutrient germinant has been reported for *B. nealsonii* spores so far.

Bacteria thriving in extreme environments need to be taken into consideration in terms of planetary protection as well. Therefore, *B. vallismortis* and *B. mojavensis*, which were isolated from soil in the Death Valley (CA, USA) and the Mojave Desert (CA, USA), respectively, and may thus be well adapted to extreme conditions (especially desiccation), were included in this study. Both species are very closely related to *B. subtilis* (**Fig. S1**; Stackebrandt and Swiderski, 2002), and were shown to be able to grow at 10 % NaCl (Roberts *et al*., 1994; Roberts *et al*., 1996). While *B. vallismortis* has not yet been subjected to detailed germination analyses (i.e. defined nutrient germinants are yet unknown), *B. mojavensis* spores have been shown to germinate in response to the standard *B. subtilis* germinants L-alanine, AGFK (a mixture of L-asparagine, D-glucose, D-fructose, and KCl), and Ca^{2+}-DPA (Alzahrani and Moir, 2014).

Table 1: *Bacillus* sp. strains and respective sporulation conditions

***Bacillus* species (strain)**	**Source**	**Sporulation conditions**		
		Time [h]	**Medium [a]**	**Temp. [°C]**
Bacillus subtilis (DSM402)	DSMZ	48	2 x SG	37
Bacillus megaterium (DSM32; type strain)	DSMZ	65	SSM	30
Bacillus pumilus (SAFR-032)	W. Nicholson (Link *et al*., 2004)	> 80	SSM	30
Bacillus mojavensis (DSM9205; type strain)	DSMZ	65	SSM	30
Bacillus vallismortis (DSM11031; type strain)	DSMZ	79	2 x SG	37
Bacillus nealsonii (DSM15077; type strain)	DSMZ	79	SSM	30

[a] As described in **Table 2**

DSMZ = German Collection of Microorganisms and Cell Cultures GmbH (Braunschweig, Germany)

Spore production and purification. Suitable sporulation conditions (time before harvest, medium, temperature) for proper spore yield of each of the investigated *Bacillus* species were experimentally determined (data not shown) and are listed in **Table 1**. For each species, an exponentially growing pre-culture was used to inoculate 200 ml of the appropriate

liquid sporulation medium in a 2 L flask. The sporulation cultures were then incubated in a shaking incubator (200 rpm) for an appropriate sporulation time (**Table 1**). Spores were sedimented and washed with sterile, distilled water five to ten times by centrifugation ('spore harvest'). Purity was checked by determining the percentage of refractile, phase-bright (i.e. dormant) spores by phase-contrast microscopy. If cell debris and residual vegetative cells were still present after the washing procedure, the spore pellet was resuspended in lysozyme solution (1 mg/ml lysozyme, 10 µg/ml DNase I, 2.5 µg/ml $MgSO_4$ in 10 mM Tris-HCl (pH 7)) and incubated for 30 min in a shaking incubator at 37°C. After a subsequent 15 min incubation at 80°C, the spores were washed at least five times with sterile, distilled water by centrifugation. When the spore suspension, as checked by phase-contrast microscopy, contained ≥ 99% phase-bright spores, it was considered as pure. Spore suspensions were stored in sterile, distilled water at 4°C in screw-capped reaction tubes with glass beads until usage.

Spore germination. While dormant spores are highly refractile when viewed in a phase-contrast microscope ('phase-bright'), they lose their refractivity during germination, thus turning phase-dark (Setlow, 2013). This refractivity loss can be quantified spectrophotometrically by measuring the optical density of the germination culture at 600 nm (OD_{600nm}) or can be observed using a phase-contrast microscope.

For spectrophotometric measurements, germination was carried out in 96-well plates. Each well contained 200 µl of the germination medium stated in the text (**Table 2**). Each germination condition was tested with at least four replicates (i.e. four wells). Mean values and standard deviations of the replicates are given in the figures and tables. Spores were activated by a sub-lethal heat shock (30 min at 70°C) to achieve a higher degree of synchronicity of germination initiation of the spore population (Setlow, 2013). The germination medium in each well was inoculated with 40 µl heat-synchronized spores to a starting OD_{600nm} of about 0.5. Well plates were incubated at an appropriate temperature (according to **Table 1**, if not noted otherwise) in a multi-plate reader (BioTek ELx808IU) that read the OD_{600nm} of each culture every two minutes over four hours, with 5 sec of shaking before all readings. All OD_{600nm} data was normalized to the starting OD_{600nm}: the OD_{600nm} at each measured time point was divided by the first reading (t_{0min}), yielding the relative OD_{600nm} given in percent. An approximately 60% decrease in relative OD_{600nm} indicates that all spores in the germination culture have germinated successfully (Atluri *et al.*, 2006; Nagler *et al.*, 2014). Typically, germination curves as recorded by OD_{600nm} have a reverse sigmoid shape

(e.g. Nagler *et al.*, 2014). For a simplified numerical comparison of the speed of germination under different conditions, maximum germination rates v_{max} were determined (**Table 3; Tables S2, S4, S6**) as follows: individual germination curves (in % relative OD_{600nm}) were recorded as described above (**Fig. 1 – 3, S2**), the linear part of the decrease in relative OD_{600nm} was determined and the slope of that linear part represents the maximum germination rate v_{max} in % relative OD_{600nm} per minute. **Tables S2, S4, S6** additionally include the parameters "lag time" and "ΔOD". Lag time refers to the first time point of the linear part of the decrease in relative OD_{600nm} and indicates the duration of the phase before the majority of the spores loses their refractivity. ΔOD is the difference between starting relative OD_{600nm} (= 100%) and minimum relative OD_{600nm} measured within 4 hours (OD_{Start} - OD_{min}), and describes the magnitude of the germination-dependent OD_{600nm} decrease.

Spectrophotometric measurement of germination can have two drawbacks: on the one hand, there can be some decrease in the OD_{600nm} during incubation of spores that does not correspond to germination, but is rather due to spore adsorption to the well plate (Atluri *et al.*, 2006; Nagler *et al.*, 2014). On the other hand, especially in the presence of high NaCl concentrations, there can be an increase in OD_{600nm} above 100%, which is most likely due to spore clumping. To control for these experimental artifacts, it is advantageous to observe germination additionally by phase-contrast microscopy.

To monitor germination-dependent spore refractivity loss by phase-contrast microscopy**,** spores were germinated in 96-well plates as described above. After four hours, 5 µl samples were withdrawn and fixed by applying to a microscope slide coated with 1% agar. Phase-contrast micrographs were taken using a Zeiss microscope (Axio Imager M2, Carl Zeiss MicroImaging GmbH) equipped with an AxioCam MRm.

Table 2: Sporulation and germination media used in this study

Medium	Composition (per liter) [a]	Comment
Schaeffer's Sporulation Medium (SSM)	8 g nutrient broth, 1 g KCl, 0.2 g $MgSO_4$ * 7 H_2O, 2 ml of each sporulation salt solution [b]	Sporulation medium (Nicholson and Setlow, 1990)
Modified Schaeffer's Sporulation Medium with glucose (2x SG)	16 g nutrient broth, 2 g KCl, 0.5 g $MgSO_4$ * 7 H_2O; 2 ml of each sporulation salt solution [b], 2 ml 50% D-glucose	Sporulation medium (Nicholson and Setlow, 1990)
1 x LB	10 g tryptone, 5 g yeast extract, 10 g NaCl	Complex, nutrient-rich germination medium; no defined germinant

Medium	Composition (per liter) [a]	Comment
Tris medium with exogenous Ca^{2+}-DPA	60 mM Ca^{2+}-DPA pH 8, adjusted with dry Tris base (120 mM $CaCl_2$ and DPA solutions were mixed 1:1 shortly before experiment to avoid precipitation)	Defined germination medium; Ca^{2+}-DPA serves as germinant
Tris-HCl with KAGE	10 mg/ml of each KCl, L-alanine, D-glucose, and ectoine in 10 mM Tris-HCl pH 8	Defined germination medium; KAGE serves as germinant
Modified Spizizen Minimal Medium (SMM)	2 g $(NH_4)_2SO_4$, 14 g K_2HPO_4, 6 g KH_2PO_4, 1 g Na_3-citrate * 2 H_2O, $MgSO_4$ * 7 H_2O, 0.83% D-glucose; pH 7.5	Defined minimal medium (Nicholson and Setlow, 1990); used for outgrowth experiments in this study
SMM with KAGE	10 mg/ml of each KCl, L-alanine, D-glucose, and ectoine in SMM; Additional 0.5 mM L-tryptophan for *B. subtilis* experiments (auxotrophic requirement);	Defined outgrowth medium; KAGE serves as germinant

[a] Media contained additional NaCl as stated in the text, figures and tables

[b] Sporulation salt stock solutions were 1 M $Ca(NO_3)_2$, 0.1 M $MnCl_2$ * 4 H_2O, and 1 mM $Fe(II)SO_4$ * 7 H_2O

Spore outgrowth. To analyze the onset of metabolic activity, spores were germinated in KAGE (SMM) medium (**Table 2**) containing 39.8 mM of the redox dye Alamar Blue. Germination and outgrowth cultures (in well plates) were monitored spectrophotometrically as described above and previously (O'Brien *et al*., 2000; Nicholson *et al*., 2011; Nagler *et al*., 2014) with OD measurements at both 600 nm and 550 nm. Alamar Blue changes its color from blue to pink when it reacts with reduced metabolites (e.g. FADH, NADH) that are newly synthesized after germination (O'Brien *et al*., 2000; Setlow, 2006; Nicholson *et al*., 2011). OD_{600nm} and OD_{550nm} changes of parallel control germinations without Alamar Blue were subtracted to determine OD changes due to the color changes, while excluding the OD drop due to any refractivity decrease (Nagler *et al*., 2014). The maxium rate (v_{max}) of disappearance of blue color at different NaCl concentrations (**Table 4**) was derived from the slopes of the linear parts of the individual Alamar Blue assay curves (**Fig. S3**). Alamar Blue experiments were performed in triplicate for every condition.

RESULTS

Germination in complex, nutrient-rich medium. For the vast majority of spore-forming *Bacillus* species, specific, chemically defined germination triggers (germinants) are unknown. Hence, germination experiments with non-model *Bacillus* species are often performed using a complex, nutrient-rich medium. In our study, the complex, nutrient-rich medium 1 x LB containing an additional 0.6 M, 1.2 M, 2.4 M, and 3.6 M NaCl or no additional NaCl was used to assess the effects of high salinity on *Bacillus* sp. spore germination in nutrient-rich environments. Germination kinetics were monitored by measurement of the OD_{600nm}, which decreases due to loss of spore refractivity during germination (**Fig. 1**). A decrease of relative OD_{600nm} of about 60% indicates that all spores have germinated. Furthermore, germination success was analyzed by phase-contrast microscopy: four hours after mixing of spores with germination media, the phenotypic states of the spores were determined and counted (**Tables S1, S3, S5**): phase-bright spores are dormant (i.e. have not started to germinate), phase-gray spores have initiated, but not completed germination, and phase-dark spores are fully germinated (Nagler *et al.*, 2014).

In nutrient-rich medium without additional NaCl, the laboratory *B. subtilis* strain 168 as well as *B. megaterium* germinated very quickly and efficiently (**Fig. 1A, F; Table S1, S2**). Although nearly the complete spore population of *B. vallismortis* and *B. mojavensis* (99% and 93%, respectively) germinated within four hours under this condition (**Table S1**), the OD_{600nm} decrease was much slower and occurred after a considerable lag time (**Fig. 1B, C; Table S2**). However, germination of *B. pumilus* and especially *B. nealsonii* was much less efficient in nutrient-rich medium without additional NaCl, allowing only 63% and 6% of the spore population to turn phase-dark within four hours, respectively (**Fig. 1D, E; Table S1**).

Strikingly, germination of *B. pumilus* and *B. nealsonii* was strongly enhanced by the presence of additional 0.6 M, 1.2 M and 2.4 M NaCl in the medium (**Fig. 1D, E; Table S1, S2**). In general, elevated salinity affected germination of spores of the six tested species to different extents. *B. subtilis* was affected the least, as even in the presence of 3.6 M NaCl 93% of spores turned phase-dark within four hours (**Fig. 1A; Table S1, S2**). While 0.6 M NaCl only had minor effects on germination of *B. vallismortis*, *B. mojavensis*, and *B. megaterium*, increasing NaCl concentrations slowed OD_{600nm} decreases and germination was increasingly less efficient (**Fig. 1B, C, F; Table S1, S2**). Remarkably, despite the presence of 3.6 M NaCl, 5% (*B. nealsonii*) to 93% (*B. subtilis*) of spores of the investigated species could still germinate (i.e. turn phase-dark) successfully within four hours (**Table S1**).

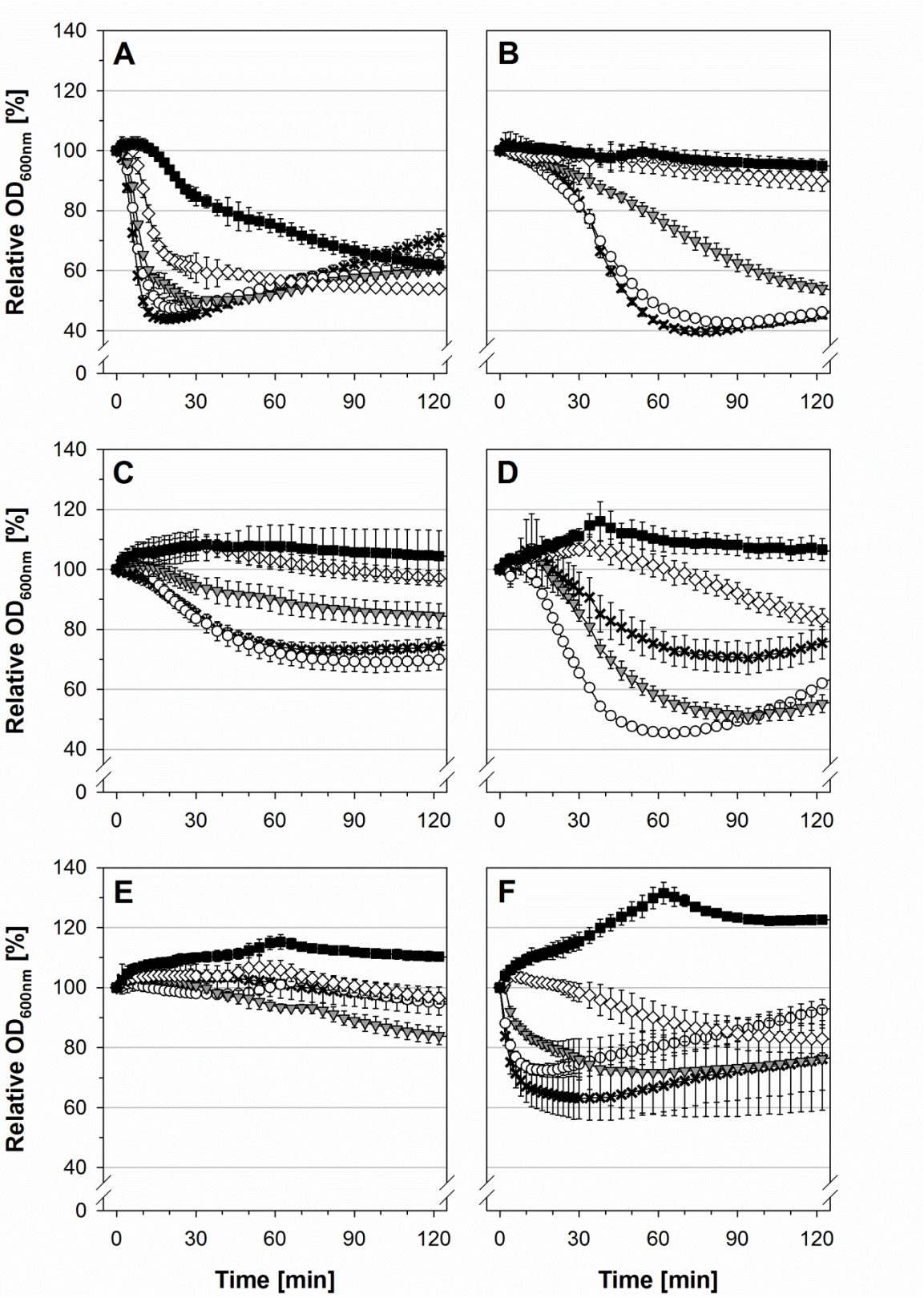

Figure 1: Germination in complex, nutrient-rich medium with and without NaCl. Germination of spores of (A) *B. subtilis* 168, (B) *B. vallismortis*, (C) *B. mojavensis*, (D) *B. pumilus* SAFR-032, (E) *B. nealsonii*, and (F) *B. megaterium* was monitored via the decrease of OD_{600nm} as described in Materials and Methods. Media contained no additional NaCl (black crosses), or an additional 0.6 M NaCl (white circles), 1.2 M NaCl (gray triangles), 2.4 M NaCl (white diamonds), or 3.6 M NaCl (black squares).

Due to the high concentration of nutrients, minerals, and compatible solutes present in the nutrient-rich LB medium, outgrowth (in terms of morphological change) of all species was frequently observed at up to 1.2 M NaCl after four hours by phase-contrast microscopy. However, the extent of outgrowth varied between species and NaCl concentration (**Table S1**). In some cases, spores had just escaped their spore coats, whereas in other cases, cell proliferation was already in progress (data not shown). Again, *B. subtilis* was the most salt-tolerant among the tested species, exhibiting considerable outgrowth (54%) even at 2.4 M NaCl (**Table S1**).

Non-nutrient germination with Ca^{2+}-DPA. Although nutrient-rich medium could induce germination of spores of all tested species, the germination responses (i.e. efficiency and kinetics) were already strongly variable in the absence of additional NaCl, thus hampering the comparison of NaCl effects. Moreover, the exact composition of the nutrient rich LB medium is not chemically defined, so that the substances responsible for germination induction are not known. Therefore, spores were germinated under chemically defined conditions by exogenous Ca^{2+}-DPA, i.e. a non-nutrient germinant, which (in *B. subtilis*) is thought to trigger germination via the cortex-lytic enzyme CwlJ and does not require germinant receptors (GRs) (Paidhungat *et al*., 2001).

In Ca^{2+}-DPA germination medium without NaCl, quick and efficient germination was observable for *B. vallismortis* and *B. pumilus* (**Fig. 2B, C; Table S4**). *B. mojavensis* and *B. subtilis* exhibited a delayed and slower OD_{600nm} decrease, but phase-contrast microscopy revealed that more than 90% of their spores successfully turned phase-dark within four hours (**Fig. 2A, E; Table S3; S4**). Although germination of *B. megaterium* and *B. nealsonii* spores could not unambiguously be observed via OD_{600nm} measurement, ca. 73% and 62% of the spores turned phase-dark within four hours (**Table S3**).

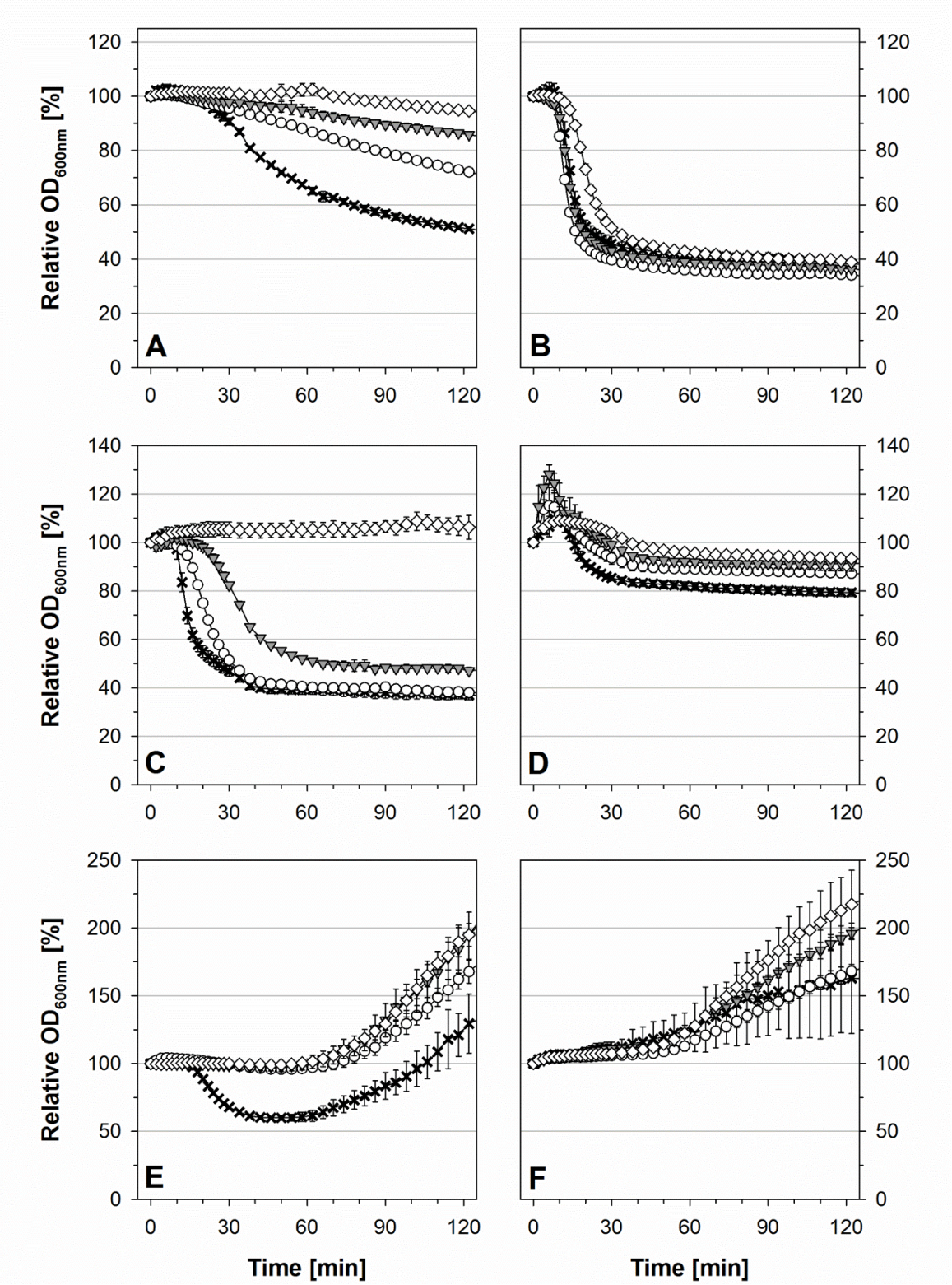

Figure 2: Non-nutrient germination with exogenous Ca^{2+}-DPA. Germination of spores of (A) *B. subtilis* 168, (B) *B. vallismortis*, (C) *B. pumilus* SAFR-032, (D), *B. megaterium* (E) *B. mojavensis*, and (F) *B. nealsonii* was monitored via the decrease of OD_{600nm} as described in Materials and Methods. Media contained no additional NaCl (black crosses), 0.6 M NaCl (white circles), 1.2 M NaCl (gray triangles), or 2.4 M NaCl (white diamonds). Note the different y-axis scales, which were chosen for better readability. OD_{600nm} increases in (E) and (F) are due to precipitation of Ca^{2+}-DPA.

As for germinations in nutrient-rich medium, the effect of NaCl on germination by Ca^{2+}-DPA varied strongly among the tested *Bacillus* species (**Fig. 2; Table S4**). *B. vallismortis* spores were affected the least, with even 2.4 M NaCl merely causing a slightly longer lag-phase prior to the germination-dependent OD_{600nm} drop (**Fig. 2B; Table S4**). Germination of spores of all other tested species was already negatively affected by the presence of 0.6 M NaCl. While the inhibitory effects of NaCl on germination kinetics were relatively small for *B. pumilus* (**Fig. 2C**), they were much more severe for the other species. Especially *B. nealsonii* spores were extremely sensitive to NaCl during Ca^{2+}-DPA germination, with NaCl concentrations ≥ 0.6 M NaCl drastically reducing germination efficiencies to ≤ 7% (**Fig. 2F; Table S3**). Nevertheless, successful germination in the presence of 2.4 M NaCl was possible for spores of all tested species within four hours, albeit to very different extents (4% for *B. nealsonii* and 98% for *B. vallismortis*; **Table S3**). Yet, due to the lack of nutrients in the germination medium, outgrowth was never observed.

Germination with a novel nutrient germinant. Germination with the exogenous Ca^{2+}-DPA gives interesting insights on how NaCl interferes with non-nutrient germination in various *Bacillus* species. However, it is also of major interest to learn about NaCl effects on the more physiologically-relevant nutrient germination, involving germinant binding to GRs. Ideally, nutrient germination is investigated using single nutrients (e.g. amino acids, such as L-alanine or L-valine for *B. subtilis*) or defined nutrient mixtures (e.g. AGFK, i.e. L-asparagine, D-glucose, D-fructose, and KCl for *B. subtilis*). As mentioned above, chemically defined nutrient germinants are unknown for most *Bacillus* species. Hence, a range of substances (sugars, amino acids, and salts) and mixtures thereof were screened (data not shown), aiming to achieve optimal germination responses of the spores tested in this study. Finding a defined nutrient germinant that works equally well in various species would then allow comparable investigation of salt effects on germination.

In this context, a nutrient mixture termed ‘KAGE’, composed of 10 mg/ml of each KCl, L-alanine, D-glucose, and ectoine (in 10 mM Tris-HCl pH 8), was identified to allow efficient germination of spores of all tested species: within four hours, 75% - 100% of the spores turned dark in the absence of NaCl (**Table S5**). In contrast to all other potential germinants that were screened (including L-alanine as a single germinant), KAGE induced rapid OD_{600nm} decreases after short lag times with all tested species in the absence of NaCl, giving the resulting germination profiles the germination-typical reverse sigmoid shape (**Fig. 3; Table S6**). Although the OD_{600nm} decrease in *B. nealsonii* germination was slightly slower

compared to the other five species, it was still remarkably fast (**Fig. 3D; Table S6**), as spores of this species induced germination quite inefficiently in general.

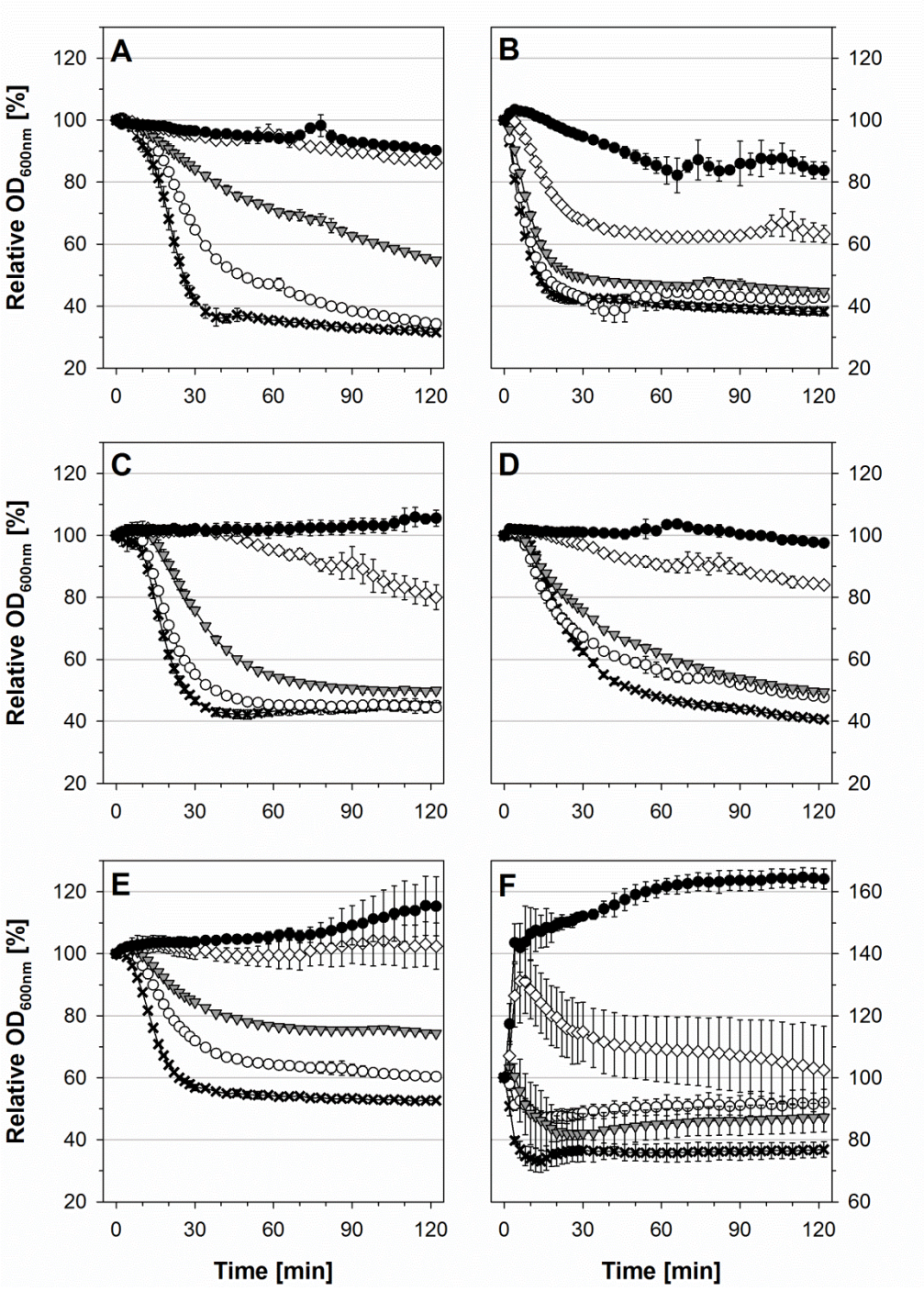

Figure 3: *Bacillus* sp. germination with the novel germinant mixture KAGE (10 mg/ml of each KCl, L-alanine, D-glucose, and ectoine) in 10 mM Tris-HCl pH 8. Media contained no additional NaCl (black crosses), 0.6 M NaCl (white circles), 1.2 M NaCl (gray triangles), 2.4 M NaCl (white diamonds), or 3.6 M NaCl (black circles). (A) *B. vallismortis*, (B) *B. subtilis* 168, (C) *B. pumilus* SAFR-032, (D) *B. nealsonii*, (E) *B. mojavensis*, (F) *B. megaterium*. Note the different y-axis scale in (F), which was chosen for better readability, as spore clumping caused strong OD_{600nm} increases especially at high salinity.

Due to the similar germination responses of spores of the different species with KAGE in the absence of NaCl, salt effects on germination were easily comparable among the species. While 0.6 M NaCl barely affected germination of *B. subtilis* spores (**Fig. 3B**), OD_{600nm} decreases during germination of the other species were already slightly to moderately delayed and slower at this concentration (**Fig. 3; Table S6**). Increasing NaCl concentrations increased this inhibitory effect, albeit to different extents. Overall, *B. subtilis* was affected the least, whereas *B. vallismortis* was most salt sensitive. The OD_{600nm} results were consistent with phase-contrast microscopy (**Table S5**). In the presence of 3.6 M NaCl, significant amounts of spores of *B. subtilis* (86%), *B. pumilus* (35%), and *B. mojavensis* (29%) were still able to turn dark within four hours, whereas *B. vallismortis* (3%), *B. nealsonii* (3%), and *B. megaterium* (4%) germinated poorly at this very high salinity.

Influence of temperature on germination at high salinity. All previous experiments were performed at the optimal growth temperature for each species (i.e. 37°C for *B. subtilis* and *B. vallismortis*, and 30°C for *B. pumilus*, *B. megaterium*, *B. mojavensis*, and *B. nealsonii*). However it is also interesting to find out how deviation from these temperatures affects germination in the presence and absence of NaCl, especially from an astrobiological and ecological point of view. Hence, germination of *B. pumilus* and *B. subtilis* spores at 30°C versus 37°C was compared in response to nutrient-rich medium, exogenous Ca^{2+}-DPA, and KAGE.

In nutrient-rich medium, *B. subtilis* germinated much faster at 37°C than at 30°C as indicated by significantly higher maximum germination rates (v_{max}) at all tested salinities (**Table 3; Fig. S2G, H**). Although *B. pumilus* exhibited higher v_{max} at 37°C in the absence of NaCl and at 0.6 M NaCl, the OD_{600nm} germination profiles suggested that germination was better at 30°C (**Table 3; Fig. S2A, B**). In the presence of ≥ 1.2 M NaCl, there was no unambiguously measurable germination response of *B. pumilus* spores at 37°C. Thus, in nutrient-rich medium, the optimal growth temperatures were also the best germination temperatures.

When germinated with exogenous Ca^{2+}-DPA, *B. subtilis* spores germinated slightly better at 30°C than at 37°C in the presence of 0 – 1.2 M NaCl. At 2.4 M NaCl germination was not clearly measureable at either temperature (**Table 3; Fig. S2I, J**). While the difference of NaCl inhibition of *B. subtilis* germination between 30°C and 37°C was minor, there was a much stronger temperature effect on *B. pumilus* Ca^{2+}-DPA-germination: although *B. pumilus*

had a higher v_{max} at 37°C in the absence of NaCl, inhibitory effects in the presence of 0.6 M NaCl were much more severe than at 30°C (**Table 3; Fig. S2C, D**).

Table 3: Comparison of maximum germination rates (v_{max}) of *B. subtilis* (*B. sub*) and *B. pumilus* (*B. pum*) spores at 30°C vs. 37°C using different germination triggers.

Species	Trigger	°C	v_{max} [% OD_{600nm} decrease pro min][a]				
			0 M NaCl	0.6 M NaCl	1.2 M NaCl	2.4 M NaCl	3.6 M NaCl
B. sub	LB	30	4.2 ± 0.1	3.4 ± 0.1	1.4 ± 0.0	0.5 ± 0.1	0.1 ± 0.0
		37	7.3 ± 0.4	6.6 ± 0.1	5.7 ± 0.1	3.9 ± 0.1	1.1 ± 0.0
	DPA	30	1.4 ± 0.0	0.5 ± 0.0	0.2 ± 0.0	0.0 ± 0.0	x
		37	1.0 ± 0.1	0.3 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	x
	KAGE	30	5.7 ± 0.1	5.5 ± 0.1	5.4 ± 0.1	3.5 ± 0.1	1.4 ± 0.0
		37	5.6 ± 0.3	4.7 ± 0.2	3.7 ± 0.2	1.7 ± 0.1	0.3 ± 0.0
B. pum	LB	30	0.6 ± 0.2	1.8 ± 0.1	1.2 ± 0.1	0.3 ± 0.0	0.1 ± 0.0
		37	1.0 ± 0.5	2.8 ± 0.6	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
	DPA	30	6.9 ± 0.2	3.4 ± 0.0	1.9 ± 0.1	0.0 ± 0.0	x
		37	7.5 ± 0.1	2.0 ± 0.1	0.6 ± 0.0	NA	x
	KAGE	30	3.4 ± 0.1	2.8 ± 0.0	1.4 ± 0.0	0.3 ± 0.0	0.0 ± 0.1
		37	6.9 ± 1.4	8.0 ± 0.1	3.1 ± 0.2	0.4 ± 0.0	0.2 ± 0.0

[a] v_{max} was determined as described in Materials and Methods and is expressed as arithmetic mean values (of at least four replicates) ± standard deviation
LB = complex, nutrient-rich medium (1 x LB medium)
NA = no germination measureable
x = not determined

In contrast to the results obtained in nutrient-rich medium, the optimal germination temperature of the two tested species was not equal to their respective optimal growth temperature when germination was triggered with KAGE. While *B. subtilis* germination in the presence of ≤ 1.2 M NaCl was quite similar at both temperatures (albeit slightly faster at 30°C), germination inhibition by NaCl was clearly stronger at 37°C (**Table 3; Fig. S2K, L**). In contrast, *B. pumilus* clearly germinated faster at 37°C than at 30°C at all salinities (**Table 3; Fig. S2E, F**).

Altogether, these results point out that (i) temperature can affect the degree of germination inhibition by NaCl, (ii) temperature effects on NaCl inhibition vary depending on

the germination trigger, and (iii) temperature effects on NaCl inhibition cannot simply be predicted based on the species' optimal growth temperature.

Outgrowth and metabolic activity after KAGE germination. As *Bacillus* sp. spore germination with KAGE was shown to be efficient and also possible in the presence of NaCl (**Fig. 3; Table S5, S6**), it is important to investigate, whether germinated spores are capable of progressing to the next growth phase called outgrowth as well. Outgrowth is characterized by the onset of metabolic activity, which can be measured with the redox dye Alamar Blue. Alamar Blue changes its color from blue to pink when it reacts with reduced metabolites (e.g. FADH, NADH) that are newly synthesized after germination (O'Brien *et al*., 2000; Setlow, 2006; Nicholson *et al*., 2011). The loss of blue and appearance of pink color can be measured at 600 nm and 550 nm, respectively (germination dependent OD changes are subtracted, see Materials and Methods; Nagler *et al*., 2014). As successful outgrowth also requires the presence of some minerals, outgrowth of the six *Bacillus* species used in this study was analyzed in Spizizen Minimal Medium (SMM; **Table 2**) instead of Tris-HCl buffer.

For all tested strains, significant metabolic activity could be observed over a broad range of salinities, allowing complete color changes from blue to pink within 12 hours (**Table 4**; **Fig. S3,** and data not shown). Strikingly, color changes were frequently slightly to remarkably faster in the presence than in the absence of NaCl, although KAGE germination (in Tris-HCl medium) was always the fastest without NaCl (**Fig. 3; Table S6**). *B. mojavensis* outgrowth cultures, for instance, exhibited the fastest increase in OD_{550nm} and decrease in OD_{600nm} at 1.2 M NaCl, followed by 2.4 M NaCl and 0.6 M NaCl (**Table 4**; **Fig. S3E, F**). The reason for these findings is unclear, but it does not seem to be some mathematical artifact or measurement error, as the measured color changes were also visible by eye (data not shown). Possibly, the increased speed of color changes relates to an upregulated stress response that may go along with an altered metabolic profile of the outgrowing spores in presence of NaCl. Moreover, the presence of additional minerals in the SMM medium as well as the compatible solute ectoine (as a component of KAGE) could contribute to this observation. Besides, NaCl-induced lysis of a portion of the germinated spores could have an effect. Future research will look into this issue with an attention to detail that is beyond the scope of this current study.

Nevertheless, for all six species, the presence of 3.6 M NaCl had more or less severe inhibitory effects on outgrowth. While for *B. subtilis* cultures the color changes were only delayed by roughly one hour (**Fig. S3A, B**), the delay was much stronger in most other species. *B. pumilus*, *B. mojavensis*, and especially *B. vallismortis* seemed to have the most

difficulties in reestablishing metabolism after germination at 3.6 M NaCl (**Table 4**; **Fig. S3C – H**).

Taken together, outgrowth analyses with Alamar Blue suggest that for the six tested *Bacillus* species not only germination, but also outgrowth is generally possible despite elevated salinities, given the presence of an appropriate nutrient-germinant such as KAGE and appropriate minerals.

Table 4: Onset of metabolic activity after germination with KAGE in SMM, analyzed with Alamar Blue as described in Materials and Methods.

Species[a]	**Rate (v_{max}) of disappearance of blue color at different NaCl concentrations [% OD_{600nm} decrease pro min]** [b]									
	0 M		**0.6 M**		**1.2 M**		**2.4 M**		**3.6 M**	
	MV	SD	MV	SD	MV	SD	MV	SD	MV	SD
B. sub	0.66	0.01	0.82	0.01	1.10	0.03	1.07	0.08	0.46	0.01
B. val	0.44	0.01	0.40	0.01	0.44	0.02	0.28	0.00	0.00	0.01
B. moj	0.24	0.00	0.30	0.00	0.48	0.01	0.43	0.01	0.05	0.00
B. pum	0.91	0.01	1.21	0.02	1.28	0.01	0.65	0.01	0.12	0.04
B. nea	0.68	0.01	0.99	0.01	1.04	0.04	0.82	0.06	0.42	0.03
B. meg	1.13	0.05	1.19	0.06	1.47	0.05	1.42	0.05	0.30	0.03

[a] *B. sub = B. subtilis* 168; *B. val = B. vallismortis*; *B. moj = B. mojavensis*; *B. pum = B. pumilus* SAFR-032; *B. nea = B. nealsonii*; *B. meg = B. megaterium*

[b] As the color change rate from blue to pink correspond to each other, only the rate of disappearance of blue is shown. The rate was determined as described in Materials and Methods For details see **Fig. S3.** v_{max} was determined as described in Materials and Methods and is expressed as arithmetic mean values (of triplicates) ± standard deviation.

DISCUSSION

In order to assess the risk of contaminating celestial bodies with terrestrial microbes during space exploration, it is important to investigate the potential of spore forming bacteria to germinate and outgrow in high-salinity environments (Crawford, 2005; Nicholson *et al.*, 2005). *Bacillus* species represent a good model for such studies, as they are a common bioload on spacecraft (Nicholson *et al.*, 2009). It was shown previously that spores of the

laboratory strain *B. subtilis* 168 are capable of germinating and outgrowing at elevated salinity, although salt does exert inhibitory effects at high concentrations (Nagler *et al.*, 2014; Nagler and Moeller 2015; Nagler *et al.*, 2015). In this current study, it could be shown that spore germination of different *Bacillus* species is variable, but overall similarly affected by NaCl (albeit to different extents), so that these previous findings can be transferred to spores of other *Bacillus* species as well.

For *B. subtilis*, it is currently assumed that NaCl has several inhibition targets: NaCl seems to inhibit at least one early process, thus causing a delay in germination onset, and at least one subsequent process, thereby leading to a slower germination or even causing an arrest in an intermediate, phase-gray state (Setlow *et al.*, 2001; Nagler *et al.*, 2014; Nagler *et al.*, 2015). The most likely processes in *B. subtilis* spore germination to be primarily inhibited by NaCl seem to be ion, Ca^{2+}-DPA, and water fluxes (Nagler *et al.*, 2015). Since these fluxes are almost certainly equally important in *B. subtilis*' close relatives (Paredes-Sabja *et al.*, 2010; Setlow, 2013) and comparable inhibitory effects were observed, NaCl likely acts on the same targets in all tested species. Furthermore, the proteinaceous spore coat of *B. subtilis* is crucial for successful germination at high salinity, as the absence of the coat drastically increases NaCl-dependent germination inhibition (Nagler *et al.*, 2015). Thus, structural differences in the integuments (especially in the coat) of spores of the *Bacillus* species tested here may contribute to the different NaCl sensitivities observed (**Fig. 3**). Additionally, variations in other structural and/or molecular properties of spores of different *Bacillus* species could influence the NaCl-susceptibility of the germination process: the respective numbers of germination-specific proteins (e.g. GRs, ion and Ca^{2+}-DPA transporters), membrane properties, and the ionic composition of the spore core, among others. Indeed, such interspecific variability seems to be relevant, because germination responses as well as NaCl sensitivities of *B. subtilis*, *B. mojavensis*, and *B. vallismortis*, which are only genetically distinguishable, were found to be quite distinct. Possibly, expression levels of germination-specific proteins vary between these very close relatives and/or mutations in key germination proteins may have notable effects. Indeed, the amino acid identity of GR subunits varies from 84% to 90% between *B. subtilis* 168 and *B. mojavensis* (Alzahrani and Moir, 2014). Furthermore, *B. mojavensis* lacks two operons, *yndDEF* and *yfkQRST,* for GRs of unknown function that were identified in *B. subtilis* (Alzahrani and Moir, 2014).

Although NaCl exerted inhibitory effects on germination of spores of all tested *Bacillus* species, germination was still remarkably efficient at high salinity given an appropriate germination medium and trigger. In some cases (e.g. for *B. pumilus* and *B.*

nealsonii germination in nutrient-rich medium) a certain amount of NaCl was even beneficial for germination efficiency. Even outgrowth in terms of initiation of metabolic activity was possible at ≤ 3.6 M NaCl, emphasizing the large flexibility of microbial life. It is unlikely that long-term active growth of non-halophilic bacilli will occur at extreme salinities near 3.6 M NaCl (K. Nagler and R. Moeller, unpublished results). Yet, given appropriate compatible solutes, proliferation in a minimal medium containing 1.2 M NaCl is possible (Boch *et al.*, 1994). Although the salt stress response of vegetative *B. subtilis* cells is increasingly understood (Bremer, 2002; Wood *et al.*, 2001), future research will need to address by which molecular mechanisms outgrowing spores cope with osmotic challenges.

Importantly, this study identified the novel, chemically defined nutrient-germinant 'KAGE' (10 mg/ml of each, KCl, L-alanine, D-glucose, and ectoine) that allowed efficient germination of six tested *Bacillus* species. It has previously been shown that (i) the L-alanine-responsive GR GerA from *B. subtilis* is well-conserved among its close relatives, (ii) GerA orthologues are present in a variety of *Bacillus* species, and (iii) L-alanine is involved in germination induction in many species, albeit usually not as sole germinant as it is the case for *B. subtilis* (Paredes-Sabja *et al.*, 2010; Ross and Abel-Santos, 2010; Alzahrani and Moir, 2014). Moreover, in *B. subtilis*, glucose and K^+ activate the cooperative GRs GerB and GerK, which are also present in *B. mojavensis* and possibly also in the other four species investigated in this study (Atluri *et al.*, 2006; Alzahrani and Moir, 2014). Importantly, in *B. subtilis*, GerK can stimulate germination via GerA (i.e. by L-alanine) in the presence of glucose and K^+ (Atluri *et al.*, 2006). Hence, synergistic effects between GRs might explain the high efficiency of KAGE, even beyond species boundaries (Atluri *et al.*, 2006; Setlow *et al.*, 2013). While the role of ectoine in spore germination is yet obscure, it is a potent osmoprotectant in *Bacillus* species (Bremer, 2002), possibly explaining the efficient onset of metabolic activity despite the presence of NaCl (**Table 4; Fig. S3**). Future research on germination and outgrowth at high salinity will elucidate this finding in more detail. In conclusion, if KAGE turns out to be an efficient 'universal' nutrient germinant for *Bacilli*, it will be very helpful for systematic, comparative spore germination analyses of further *Bacillus* species in the future. This comparative germination system can then also be implemented to analyze germination and outgrowth under more realistic astrobiological and environmental conditions.

Overall, our study showed that germination responses of *Bacillus* spores in general and inhibitory effects of NaCl on germination are variable among the species and strongly depend on the germination medium and the germinant. Interestingly, the availability of large

amounts of nutrients (such as in nutrient-rich LB medium) does not automatically indicate better germination or lesser inhibition. Instead, sufficient amounts of specific substances (e.g. KAGE) seem to determine, whether or not spores of a certain *Bacillus* species can successfully germinate at high (or even low) salinity. Other factors like temperature can also influence both, germination and its inhibition by salt. Surprisingly, the optimal growth temperature is not necessarily the optimal germination temperature. Hence, it is difficult to estimate, whether spores can germinate and outgrow, if the exact environmental conditions are variable or not exactly known, which can have severe implications for planetary protection.

CONCLUSIONS

This study yielded interesting and new insights in *Bacillus* spore germination and how this process is affected by environmental conditions. It could be shown that *Bacillus* spores can revive under harsh environmental circumstances outside of the usual growth spectrum of non-extremophilic bacteria. Especially under sub-optimal conditions, which will likely prevail in much extremer extent on extraterrestrial planets or moons, germination responses and salt sensitivity of germination can be expected to be very variable among different species, hampering forward contamination risk assessment. What is more, extraterrestrial brines or salt oceans are almost certainly composed of a mixture of different salts (Marion *et al.*, 2003; Sohl *et al.*, 2010; Nicholson *et al.*, 2012; Ojha *et al.*, 2015; Stevenson *et al.*, 2015), which will affect spore germination in a different, more complex way than NaCl (Nagler and Moeller, 2015). However, Nicholson *et al.* (2012) showed that germination of *B. subtilis* 168 and *B. pumilus* SAFR-032 spores is, in principle, possible in aqueous extracts of a Mars analogue regolith that mimics the Phoenix landing site.

Although this study clearly suggests that spores can indeed revive under specific inhospitable conditions and that germination success is nearly unpredictable, it has to be considered that circumstances in a real space mission scenario are way more complex and many other factors than salinity and germinant availability need to be taken into consideration with regard to planetary protection. These include radiation (especially UV radiation), vacuum, desiccation, pressure, pH, temperature, gravity, and the exact chemical composition of the environment (Nicholson *et al.*, 2000; Marion *et al.*, 2003; Schuerger *et al.*, 2003; Nicholson *et al.*, 2005; Nicholson and Schuerger, 2005; Horneck *et al.*, 2010; Setlow, 2014). While some of these factors (especially solar radiation) may already kill a major fraction of

the potential spacecraft bioload during space travel in case of insufficient shielding, all of these factors will most likely affect spore germination and outgrowth success on the celestial body in question in a very complex and possibly synergistic fashion (Schuerger *et al.*, 2003; Nicholson *et al.*, 2005; Harrison *et al.*, 2013; Nagler and Moeller, 2015). When looking at isolated environmental extremes, terrestrial organisms have evolutionarily adapted to a very broad range of environmental conditions, but the resistance against - and response to - multiple simultaneous extremes is not well understood so far (reviewed by Harrison *et al.*, 2015, Stevenson *et al.*, 2015). The knowledge that was gained within this study, especially the development of the comparably effective, defined nutrient germinant KAGE, will be advantageous for future investigations of *Bacillus* sp. spore germination under multiple stresses and simulated extraterrestrial conditions.

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ABBREVIATIONS

AGFK	–	Germinant mixture of L-asparagine, D-glucose, D-fructose, and K^+
Ca^{2+}-DPA	–	Calcium dipicolinate
DPA	–	Dipicolinic acid (pyridine-2,6-dicarboxylic acid)
GRs	–	Germinant receptors
JPL-SAF	–	Spacecraft assembly facility at NASA Jet Propulsion Laboratory
KAGE	–	Germinant mixture of K^+, L-alanine, D-glucose, and ectoine
SMM	–	Spizizen Minimal Medium
SSM	–	Schaeffer's Sporulation Medium
OD	–	Optical density

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CHAPTER 6

Analysis of differential gene expression during *Bacillus subtilis* spore outgrowth in high-salinity environments using RNA sequencing

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This manuscript represents the first comprehensive transcriptome analysis of salt-stressed outgrowing spores. Within the first 90 min of outgrowth in the presence of 1.2 M NaCl more than 1000 genes were differentially expressed as determined by RNA sequencing. The salt stress response of outgrowing spores largely resembled the osmospecific response of vegetative cells exposed to sustained high salinity and included strong upregulation of genes involved in osmoprotectant uptake and compatible solute synthesis. The σ^B-dependent general stress response typically involved in the response to salt shocks did not seem to be induced, while the σ^W regulon may play an important role for osmoadaptation of outgrowing spores.

Author contributions:

K. Nagler designed, performed, and evaluated the transcriptomics and spectrophotometric germination experiments, and prepared the text and figures of the manuscript. **A. O. Krawczyk** supported experimental design gave scientific input, and edited the manuscript text. **A. de Jong** supported evaluation of the transcriptomic data. **O. P. Kuipers** arranged RNA sequencing and edited the manuscript text. **K. Madela** and **M. Laue** performed and evaluated the live cell imaging and scanning electron microscopy experiments, and edited the manuscript text. **E. Bremer** and **T. Hoffmann** supported evaluation of transcriptomic data and edited the manuscript text.

ABSTRACT

In its natural habitat, the soil bacterium *Bacillus subtilis* often has to cope with fluctuating osmolality and nutrient availability. Upon nutrient depletion it can form dormant spores, which can revive to form vegetative cells when nutrients become available again. While the effects of salt stress on spore germination have been analyzed previously, detailed knowledge on the salt stress response during the subsequent outgrowth phase is lacking. In this study we investigated the changes in gene expression during *B. subtilis* outgrowth in the presence of 1.2 M NaCl using RNA sequencing. In total, 402 different genes were upregulated and 632 genes were downregulated during 90 min of outgrowth in the presence of salt. The salt stress response of outgrowing spores largely resembled the osmospecific response of vegetative cells exposed to sustained high salinity and included strong upregulation of genes involved in osmoprotectant uptake and compatible solute synthesis. The σ^B-dependent general stress response typically triggered by salt shocks was not induced, whereas the σ^W regulon appears to play an important role for osmoadaptation of outgrowing spores. Furthermore, high salinity induced many changes in the membrane protein and transporter transcriptome. Overall, salt stress seemed to slow down the complex molecular reorganization processes ('ripening') of outgrowing spores by exerting detrimental effects on vegetative functions such as amino acid metabolism.

INTRODUCTION

In its natural habitat, the soil bacterium *Bacillus subtilis* is frequently confronted with fluctuating environmental parameters and has therefore evolved a broad range of elaborate stress responses (Marles-Wright and Lewis, 2007; Lopez *et al*., 2009; Schultz *et al*., 2009; Marles-Wright and Lewis, 2010). Two common environmental stresses in soil are changes in osmolality and limitation of nutrient availability (Wood *et al*., 2001; Bremer, 2002; Nicholson, 2002).

Osmotic stress can be caused by soil flooding and desiccation, creating hypo- and hyperosmotic conditions, respectively (Wood *et al*., 2001). Water passes through semi-permeable cell membranes depending on the osmotic gradient and as *B. subtilis* cells cannot actively transport water, they have to adjust their internal osmolality to avoid cell rupture or plasmolysis (Kempf und Bremer, 1998; Hoffmann and Bremer, 2016). In a first response to high-osmolality environments, *B. subtilis* cells quickly take up large amounts of K^+ to restore

internal osmotic pressure (Whatmore *et al.*, 1990). A key player in K^+ uptake is the moderate-affinity KtrAB transport system, which is supported by the low-affinity KtrCD system (Holtmann *et al.*, 2003). However, prolonged high intracellular K^+ concentrations are not compatible with various cellular functions (Whatmore *et al.*, 1990; Record *et al.*, 1998). Therefore, as a second response, *B. subtilis* replaces K^+ by compatible solutes, highly soluble organic compounds that do not disturb cell physiology, to adjust its intracellular osmotic potential (Whatmore *et al.*, 1990; Kempf und Bremer, 1998). Compatible solutes can either be synthesized or taken up from the environment (Kempf and Bremer, 1998). The most important compatible solutes for *B. subtilis* are glycine betaine (GB) and proline, but while both of these solutes can be imported via specific transporters, only proline can be synthesized *de novo,* using the precursor glutamate (Brill *et al.*, 2011a; Hoffmann and Bremer, 2016). There are two interlinked proline-synthesis pathways (each involving three enzymatic steps) that are activated depending on the purpose of proline requirement: the anabolic pathway involves ProB, ProA and ProI, whereas the osmoadaptive pathway uses ProJ, ProA, and ProH, which represent (in both pathways) a γ-glutamyl kinase, a γ-glutamyl-phosphate reductase, and a Δ^1-pyrroline-5-carboxylate reductase, respectively (Brill *et al.*, 2011a). GB is generated by oxidation of its imported precursor choline in the course of a two-step enzymatic reaction involving the alcohol dehydrogenase GbsB and the glycine betaine aldehyde dehydrogenase GbsA (Boch *et al.*, 1994; Boch *et al.*, 1996). Uptake of compatible solutes (and their precursors) is mediated by five osmotically inducible osmoprotectant uptake transporters (OpuA, OpuB, OpuC, OpuD, OpuE) that differ in their affinities and substrate specificities (Kempf and Bremer, 1998; Hoffmann and Bremer, 2016).

Depending on how salt stress is imposed on *B. subtilis* cells, they can react in distinct manners (Spiegelhalter and Bremer, 1998; Steil *et al.*, 2003; Young *et al.*, 2013). When *B. subtilis* is subjected to a sudden osmotic up-shock, the σ^B-governed general stress response is activated (Spiegelhalter and Bremer, 1998; Nannapaneni *et al.*, 2012; Young *et al.*, 2013). In contrast, upon incremental and sustained salt stress, cells activate a specific osmotic stress response under the regulation of the house-keeping sigma factor σ^A (Spiegelhalter and Bremer, 1998; Steil *et al.*, 2003; Young *et al.*, 2013; Hoffmann and Bremer, 2016). Interestingly, the genes for OpuE (proline uptake) and OpuD (GB uptake) have a dual promoter composed of a σ^A– and a σ^B–specific region (Spiegelhalter and Bremer, 1998; Hoffmann and Bremer, 2016). Nevertheless, it is still not understood how increases in the environmental osmolality are perceived and how this information is processed to adjust gene expression according to the cells' needs (Hoffmann and Bremer, 2016).

A different strategy of *B. subtilis* to cope with environmental (albeit not osmotic) stress is sporulation: upon nutrient depletion *B. subtilis* can form dormant spores that are highly resistant against a broad range of environmental extremes such as heat, desiccation, radiation, and chemicals (Ruzal *et al.*, 1998; Nicholson *et al.*, 2000; Setlow, 2006; Setlow, 2013). A dormant spore consists of a dehydrated spore core (the analogue to a growing cell's protoplast) that is enveloped by a dense inner membrane, a germ cell wall, a cortex made of modified peptidoglycan, and a multilayered proteinaceous spore coat (Setlow, 2006). In *B. subtilis*' natural habitats, its spores can persist in their dormant state for extended periods of time (Nicholson, 2002). However, when nutrients become available again, spores can convert back to vegetative cells via a process called germination. Throughout germination, spores release large amounts of ions and Ca^{2+}-dipicolinate (Ca^{2+}-DPA), hydrolyze their cortex, and rehydrate, which causes the loss of their refractivity and resistance properties (reviewed in Setlow, 2013). After germination is completed, the former spores enter a phase called outgrowth, which is defined as the time period between the onset of metabolic activity and the first cell division (Setlow, 2003; Keijser *et al.*, 2007). Throughout outgrowth the germinated spores undergo molecular reorganization ('ripening'), escape from their spore coats, and elongate (Keijser *et al.*, 2007; Segev *et al.*, 2013; Setlow, 2013; Sinai *et al.*, 2015). Important events in early outgrowth are the generation of ATP, nucleotides, and amino acids from endogenous resources, as well as the onset of macromolecular synthesis (Paidhungat and Setlow, 2002; Setlow, 2003; Keijser *et al.*, 2007; Sinai *et al.*, 2015). On the genomic level, the importance of σ^A as well as the temporal activation of at least 30 % of all *B. subtilis* genes during a well-regulated spore outgrowth program have been reported (Horsburgh *et al.*, 2001; Keijser *et al.*, 2007). These transcriptomic changes are also apparent on the protein level, as outgrowing spores synthesize more than 650 different proteins before entering vegetative growth (Sinai *et al.*, 2015).

While the effects of high salinity on *B. subtilis* spore germination have been analyzed previously (Nagler *et al.*, 2014; Nagler and Moeller, 2015; Nagler *et al.*, 2015), detailed knowledge on the salt stress response during the subsequent outgrowth phase, especially on a transcriptomic level, is lacking. Therefore, we investigated changes in the gene expression profile of outgrowing *B. subtilis* spores in the presence of 1.2 M NaCl by RNA sequencing (RNA-seq). A key result of our study was the observation that the transcriptional profile of salt-stressed outgrowing spores exhibits many similarities to continuously salt-stressed vegetative cells, whereas the σ^B–controlled general stress regulon was not engaged.

MATERIALS AND METHODS

Spore production and purification. Spores of *Bacillus subtilis* 168 (*trpC2*; DSM402) were produced in liquid cultures of modified Schaeffer's Sporulation Medium with glucose (2x SG; as described in Nicholson and Setlow, 1990). All chemicals were ordered from Sigma-Aldrich (St. Louis, MO, USA). The sporulation cultures were incubated at 37 °C for 48 hours in a shaking incubator (200 rpm). Spores were harvested, washed with distilled, sterile water at least seven times, and retrieved by centrifugation. The purity of the spore stocks, as checked by phase-contrast microscopy, was ≥ 99 %. Spores were stored in distilled water in screw-capped glass tubes at 4 °C until use.

Spore germination and outgrowth experiments. Spores were heat activated at 70 °C for 30 min in order to ensure synchronized germination. Germination and outgrowth experiments were performed in germination media composed of Spizizen Minimal Medium (SMM; as described in Nicholson and Setlow, 1990) with or without 1.2 M NaCl, which additionally contained 50.5 mM D-glucose, 0.5 mM L-tryptophan, and 10 mM of the germination trigger L-alanine.

The transcriptomics outgrowth experiments were performed in 45 ml germination medium (500 ml flasks). The medium was inoculated with 1.2 x 10^{10} heat-activated spores (in total) and 15 ml samples were withdrawn at 30 min, 60 min and 90 min after inoculation. The samples were immediately mixed with ice-cold killing buffer (Nicolas *et al*., 2012) and washed with ice cold water by centrifugation (1 min at 10,000 x *g* at 4 °C). The pellet was resuspended in 400 µl ice-cold LETS buffer (0.1 M LiCl, 0.01 M Na_2EDTA, 0.1 M Tris-HCl pH 7.4, 0.2 % SDS), transferred to a pre-cooled Lysing Matrix B tube (MP Biomedicals, Santa Ana, CA, USA) containing 500 µl phenol:chloroform (1:1) and 25 µl 10 % SDS, and used for RNA isolation. For the dormant spore RNA samples, spore suspensions were also heat-treated for consistency. Subsequently they were centrifuged, the pellets were resuspended in LETS buffer, and transferred to Lysing Matrix B tube for RNA isolation. The transcriptomics outgrowth experiments were performed in duplicate using two independent spore batches.

For spectrophotometric measurements, germination was carried out in triplicate in 96-well plates, each containing 200 µl of germination media. Each well was inoculated with 40 µl heat-activated spores to a starting optical density of ca. 0.5 at 600 nm (OD_{600nm})

corresponding to a total of ca. 4 x 10^7 spores per well. The plate was incubated at 37 °C in a multi-plate reader (ELx808IU, BioTek, Bad Friedrichshall, Germany) that read the OD_{600nm} of the culture, with 5 sec of shaking before all readings. The OD_{600nm} data was normalized by division of each reading by the first measured value (t_{0min}), yielding the relative OD_{600nm} given in %. A 60 % decrease in relative OD_{600nm} corresponds to germination of the whole spore population (Atluri *et al*., 2006; Nagler *et al*., 2014).

Microscopy. For scanning electron microscopy (SEM) of outgrowing spores, dormant spores were germinated as described above. Samples were withdrawn 30 min, 60 min, and 90 min after germination initiation, washed with distilled water and fixed in 2.5 % glutaraldehyde. Fixed samples were washed with distilled water, adsorbed to an Alcian blue-coated cover slip, and stored in 2.5 % glutaraldehyde (in 0.05 M HEPES) overnight. Then, samples were washed with distilled water, treated with 1 % osmium, washed again, dehydrated with increasing concentrations of ethanol, and dried by critical-point drying (Emitech K850, United Kingdom). Dried samples were sputter-coated with 3 nm Au/Pd (Polaron E5100) and analyzed by SEM (Gemini 1530, Carl Zeiss Microscopy GmbH, Germany) using an acceleration voltage of 5 kV and the in-lens secondary electron detector.

For live cell imaging of individual spores, dormant spores were dried in a plastic dish (µ-dish, ibidi, Germany). The dried spores were covered with germination medium (as described above) that was solidified with 1.5 % agarose. Germination and outgrowth were observed by phase-contrast with a Nikon TE2000-E Eclipse microscope and a Plan Fluor 100/1.3 Oil objective. Photos were taken every 5 sec and merged into time-lapse videos. Germination parameters (starting time of change from bright to dark and duration of change) were determined using ImageJ (Rasband, 1997).

To monitor germination by phase-contrast microscopy**,** spores were germinated in 96-well plates as described above. At appropriate time points, 5 µl samples were withdrawn and fixed by applying to a microscope slide coated with 1 % agar. Micrographs were taken using a Zeiss fluorescence microscope (Axio Imager M2, Carl Zeiss MicroImaging GmbH, Germany) equipped with an AxioCam MRm.

RNA isolation. RNA isolation was performed with a phenol-chloroform extraction method as follows. The samples (in Lysing Matrix B tubes, see above) were immediately disrupted using a FastPrep device (Eubio, Austria), with four subsequent disruptions (45 s at 6.5 m/s) separated by 1 – 2 min incubation on ice to avoid overheating. After disruption, samples were centrifuged at 4 °C and the supernatant was mixed with chloroform. After centrifugation at 4 °C, the RNA was precipitated by 0.3 M sodium acetate (pH 5.3) in isopropanol for 3 hours on ice. The pellet was washed with 70 % ethanol, dried, resuspended in nuclease-free water, and treated with a RNase-free DNase Set (Qiagen, Hilden, Germany) according to the manufacturer's manual (incubation for 1 h at 37 °C). The treated samples were diluted with nuclease-free water and mixed with the same amount of phenol:chloroform:isoamylalcohol (25:24:1). After centrifugation at 4 °C, the RNA was precipitated by 0.3 M sodium acetate (pH 5.3) in isopropanol overnight at 4 °C. The pellet was washed with 70 % ethanol, dried, and resuspended in 50 µl nuclease-free water.

RNA sequencing and data analyses. RNA concentration of the samples was quantified using a NanoDrop 2000c instrument (Wilmington, DE, USA). Sample quality was determined with an Agilent 2100 Bioanalyzer and an Agilent RNA 6000 Nano Kit (Agilent Technologies, Waldbronn, Germany) according to the manufacturer's manual. The samples were stored at – 80 °C until analysis. RNA-seq was performed by the PrimBio Research Institute (Exton, PA, USA). The obtained raw data containing 4391 genes was then subjected to analyses using the webserver-based RNA-seq analysis pipeline T-Rex as described by de Jong *et al.* (2015). Unless noted otherwise, the transcriptomics data are expressed as the contrast of the RNA that was present in outgrowing spores in the presence of NaCl ('target') against RNA in the absence of NaCl ('control'). T-REx includes two different significance thresholds termed 'TopHits' ($\log_2$ fold change ($\log_2$FC) $\geq$ 2 and *p*-value $\leq$ 0.05) and 'HighFold' ($\log_2$FC $\geq$ 5 and a *p*-value $\leq$ 0.01); unless noted otherwise TopHits values are shown. Hierarchical clustering of transcription profiles was performed with the TIGR Multiexperiment Viewer (MeV, http://www.tm4.org/mev.html). Functional categorization was performed according to the *Subti*Wiki platform (www.subtiwiki.uni-goettingen.de; Mäder *et al.*, 2012; Michna *et al.*, 2016).

RESULTS AND DISCUSSION

Spore germination and outgrowth at high salinity. *B. subtilis* spore germination at high salinity has previously been investigated (Nagler *et al.*, 2014; Nagler and Moeller, 2015; Nagler *et al.*, 2015), but only little is known about the effects of salt stress on the transcriptional profile of outgrowing spores. In this study spores of *B. subtilis* 168 (*trpC2*) were germinated with 10 mM L-alanine in minimal medium (SMM supplemented with L-tryptophan and glucose) containing either no NaCl or 1.2 M NaCl. This salt concentration was chosen in accordance with former studies on the salt stress response in vegetative *B. subtilis* cells and studies on *B. subtilis* spore germination at high salinity (Boch *et al.*, 1994; Steil *et al.*, 2003; Nagler *et al.*, 2014; Nagler *et al.*, 2015; Nagler *et al.*, 2016).

In agreement with previous results (Nagler *et al.*, 2014), the OD_{600nm} decrease of the germination culture that corresponds to germination was slightly slower in the presence of 1.2 M NaCl, but ultimately almost the complete spore population germinated successfully within 30 min (**Fig. 1A**). Due to the low nutrient-content in the minimal medium, no growth could be observed by OD_{600nm} and phase-contrast microscopy within two hours (**Fig. 1A** and data not shown). Single-spore live cell imaging and student's t-test analysis showed that neither the starting time of the change from highly refractive to dark nor the duration of this refractivity change were significantly different in the presence or absence of 1.2 M NaCl (**Fig. 1B**). In contrast, both processes were significantly prolonged at NaCl concentrations ≥ 1.8 M. Furthermore, phase-contrast microscopy and SEM revealed that stressed and non-stressed outgrowing spores had essentially the same morphology at all sample time points (30 min, 60 min, 90 min) of the transcriptomics experiment and were still encased in their spore coats after 90 min (**Fig. 1C, D,** and data not shown). This indicates that the spores germinated under both conditions were in their ripening phase of outgrowth, throughout which the outgrowing spores undergo molecular reorganization, but do not exhibit morphological changes (Segev *et al.*, 2013). Altogether these data show that the transcriptomes of outgrowing spores in the presence and absence of 1.2 M NaCl can be compared to each other and reflect the impact of NaCl on gene expression within the ripening phase of outgrowth.

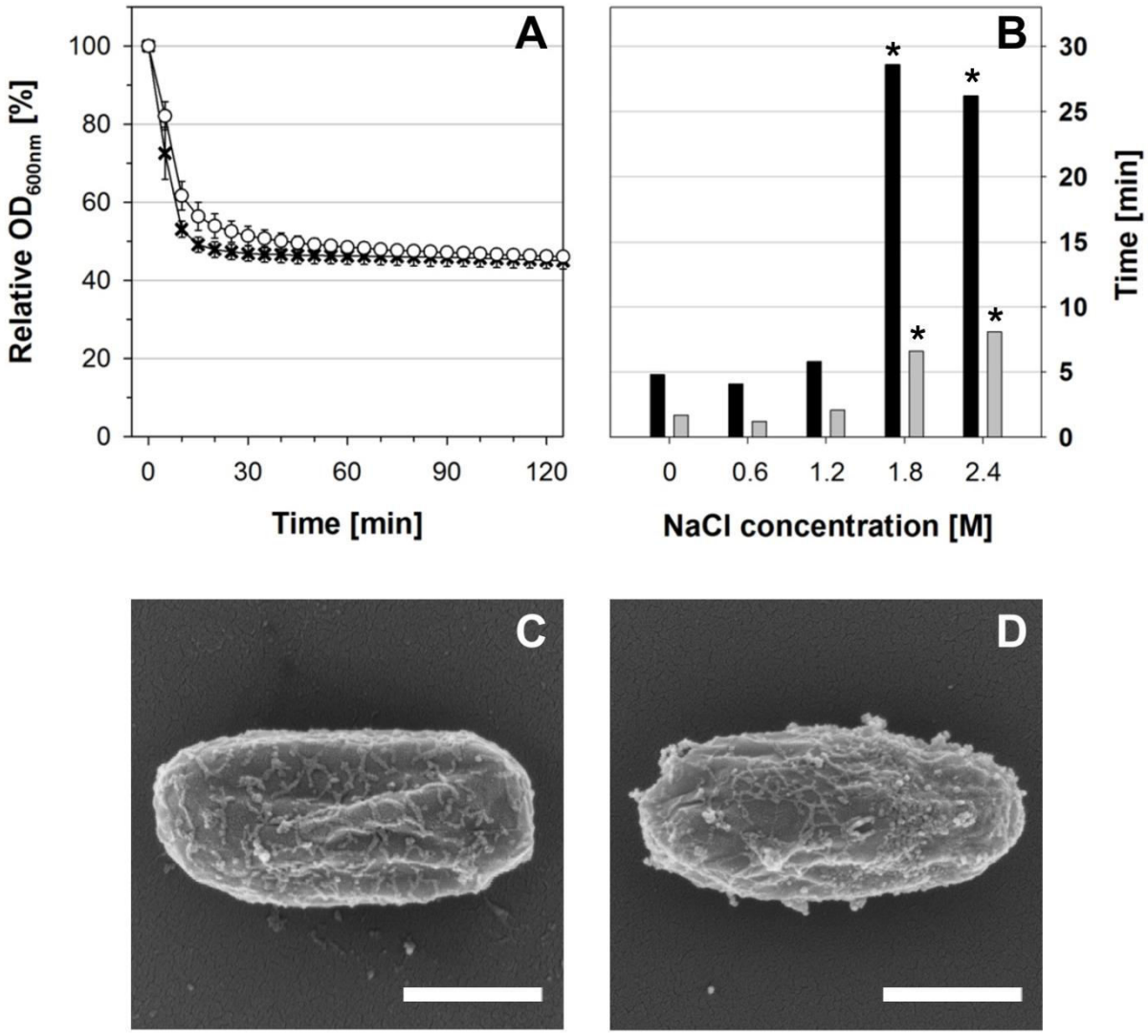


Figure 1: (A) Spore germination and outgrowth profiles in SMM supplemented with glucose, L-tryptophan and L-alanine as measured by OD_{600nm}. The medium contained either 1.2 M NaCl (white circles) or no NaCl (black crosses). (B) Single-spore live cell imaging analyses. The times required for start of refractivity loss (black bars) and duration of refractivity loss (gray bars) at different NaCl concentrations are shown as median values. Asterisks indicate significant ($p \leq 0.01$) differences to the values at 0 M NaCl. (C, D) SEM pictures 90 min after germination initiation (C) in the absence of NaCl or (D) in the presence of 1.2 M NaCl. Scale bars = 500 nm.

Dormant spore RNA. Dormant spores have been shown to contain RNA including sporulation- and spore-related transcripts that are remnants from the spore formation process (e.g. Keijser *et al*. 2007; Segev *et al*., 2012; Bassi *et al*., 2016). RNA can be degraded during early outgrowth, thus serving as a ribonucleotide reservoir for *de novo* RNA synthesis (Setlow and Kornberg, 1970; Keijser *et al*., 2007; Segev *et al*., 2012). Other transcripts may also have

a functional relevance, potentially being rapidly translated at the beginning of spore revival (Keijser *et al*, 2007; Segev *et al*., 2013; Sinai *et al*., 2015). Preliminary microarray data have suggested that degradation of spore-related RNA may be slower during outgrowth under salt stress, thereby falsely indicating these genes as "upregulated" under stress conditions (Nagler, 2012). Hence, to allow for discrimination of RNA that is newly transcribed during outgrowth from the dormant spore transcripts and to investigate the composition of the dormant spore transcriptome, we analyzed the RNA content of dormant spores.

Overall, 955 common transcripts were detected in dormant spore samples of two independent spore batches, albeit partially with low abundance. Yet, a high consistency of transcripts with high abundance among the two samples and a notable overlap with previous studies (Keijser *et al*, 2007; Segev *et al*., 2013) suggest that the RNA content of dormant spores was not random. In total, we found 21 out of the 25 mostly sporulation- or spore-specific transcripts that were detected by Keijser *et al*. (2007), as well as 103 out of 369 different dormant spore transcripts reported by Segev *et al*. (2012). The latter overlap of ca. 35 % can still be considered substantial, regarding the different genetic background (PY79 versus 168) and various differences in the experimental setup.

Out of the 955 detected common dormant spore transcripts, approximately 25 % encode membrane proteins with one third thereof coding for transporters (**Fig. 2**). Another prevalent group of dormant spore RNAs detected is involved in information processing (34 %), especially in proteins synthesis, modification and degradation (216 transcripts). About half of these 216 transcripts were tRNA and tRNA-related genes, which had the overall highest abundances in both replicates. The tRNA reservoir may facilitate a rapid start of translation after germination is completed. Furthermore, 106 mRNAs belong to the "Coping with stress" functional category, one third of which is a part of the σ^B regulon (**Fig. 2**). If indeed translated, they may play an important role during outgrowth under suboptimal conditions. Besides, 157 dormant spore transcripts encode proteins for sporulation and germination, including the highly abundant mRNAs for small acid-soluble spore proteins (SASPs). These mRNAs are most likely residues from sporulation, but might also support outgrowth by providing nucleotides for *de novo* RNA synthesis (Setlow and Kornberg, 1970; Keijser *et al*., 2007). Finally, in agreement with previous reports (Segev *et al*. 2012; Bassi *et al*., 2016), many dormant spore RNAs (partially with very high abundance, e.g. *ytzL*, *yrzQ*, and *ypzG*) code for proteins of unknown function, whose characterization could yield new insights into sporulation and/or spore composition.

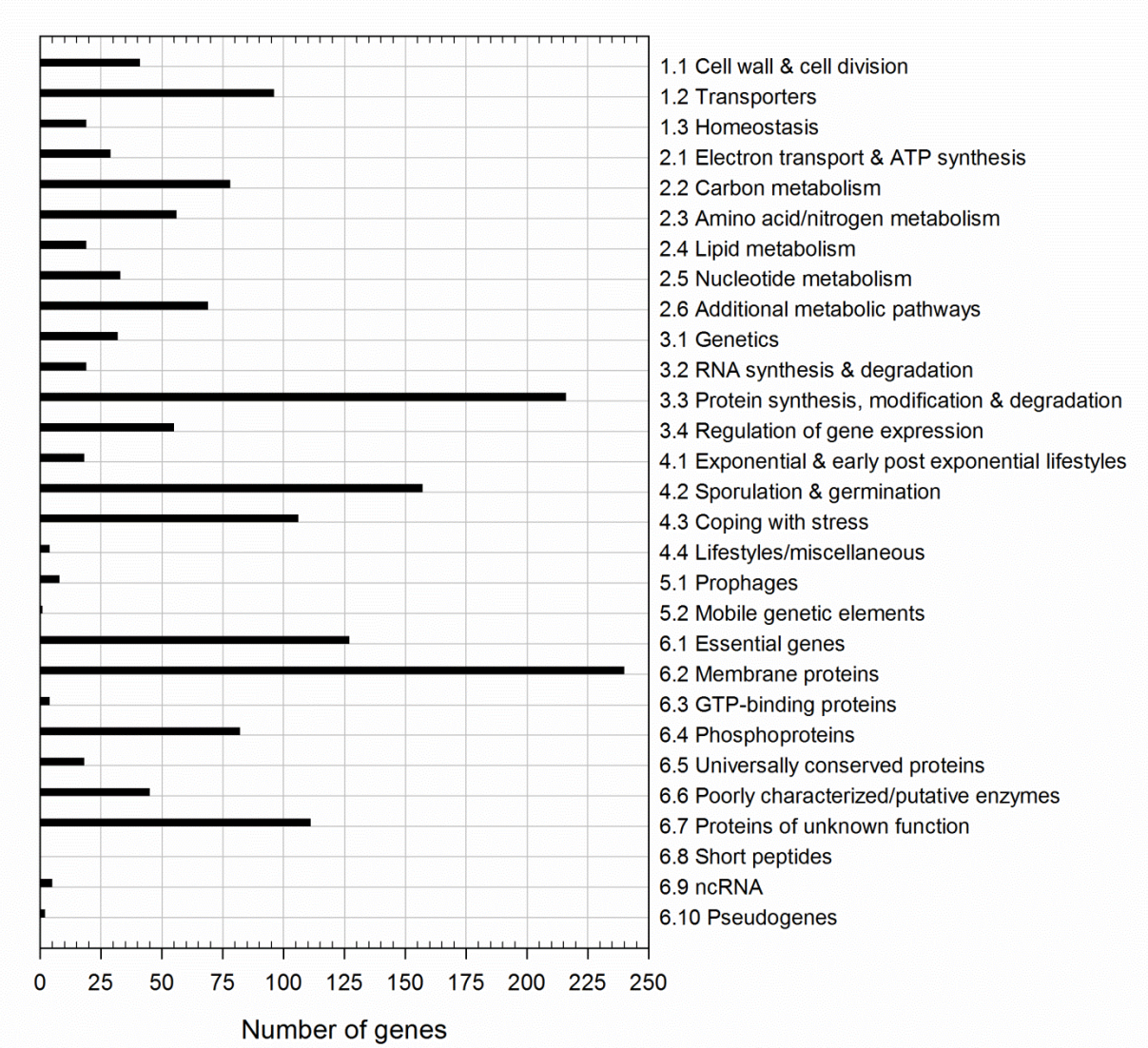


Figure 2: Functional classification of dormant spore transcripts. The 955 transcripts common to both dormant spore replicates were categorized according to *Subti*Wiki (http://subtiwiki.uni-goettingen.de).

Differential gene expression during outgrowth at high salinity. To investigate the impact of salt stress on the ripening phase of outgrowth, RNA from spores germinating in the absence and presence of 1.2 M NaCl was extracted 30 min, 60 min, and 90 min after the initiation of germination. The RNA was subjected to RNA-seq and the results were evaluated using the T-REx analysis pipeline (de Jong *et al.*, 2015). T-REx offers two different significance thresholds termed 'TopHits' ($\log_2$ fold change ($\log_2$FC) ≥ 2 and p-value ≤ 0.05) and 'HighFold' ($\log_2$FC ≥ 5 and a p-value ≤ 0.01); unless noted otherwise TopHits values are shown (in the following paragraph, the respective HighFold values are given in parentheses). In all cases, the RNA-seq data obtained from outgrowth in the presence of NaCl ('target') was contrasted against the data from outgrowth in the absence of NaCl ('control').

In total, 402 (85) genes were upregulated and 632 (190) genes were downregulated during outgrowth in the presence of 1.2 M NaCl compared to outgrowth in the absence of NaCl (**Table 1**). At all investigated time points, the transcriptomes of dormant spores, salt-stressed outgrowing spores, and non-salt-stressed outgrowing spores were clearly distinct from each other according to principal component analysis (PCA) (**Fig. S1).** The strongest alteration of gene expression caused by the presence of NaCl was detected at 30 min of outgrowth (**Table 1; Fig. S1**). This may be due to major salt stress response and adaptation processes that might have taken place at this time point. In addition, high salinity might have postponed the molecular reorganization processes of early outgrowth in a similar manner as it causes a reduced growth rate of vegetative cells (Boch *et al.*, 1994; Hahne *et al.*, 2010). In any case, the transcriptomes of stressed versus non-stressed outgrowing spores became more similar over time, as indicated by the lower number of differentially expressed genes in the 60 min and 90 min samples (**Table 1**). Consistently, the 30 min transcriptome contained much more genes that were exclusively differentially expressed at this time point than the 60 min and 90 min transcriptomes (**Table 2**). Yet, 134 (76) genes were differentially expressed at all three time points of outgrowth under salt stress (**Table 2**).

Table 1: Differentially expressed genes during outgrowth in the presence of 1.2 M NaCl [a]

Time [min][b]	**TopHits** $\log_2FC \geq 2$; $p \leq 0.05$		**HighFold** $\log_2FC \geq 5$; $p \leq 0.01$	
	Upregulated	**Downregulated**	**Upregulated**	**Downregulated**
30	321	523	64	153
60	157	184	41	25
90	118	161	31	38
Total	402	632	85	190

[a] Gene expression in the presence of 1.2 M NaCl was contrasted against gene expression in the absence of NaCl at each respective time point

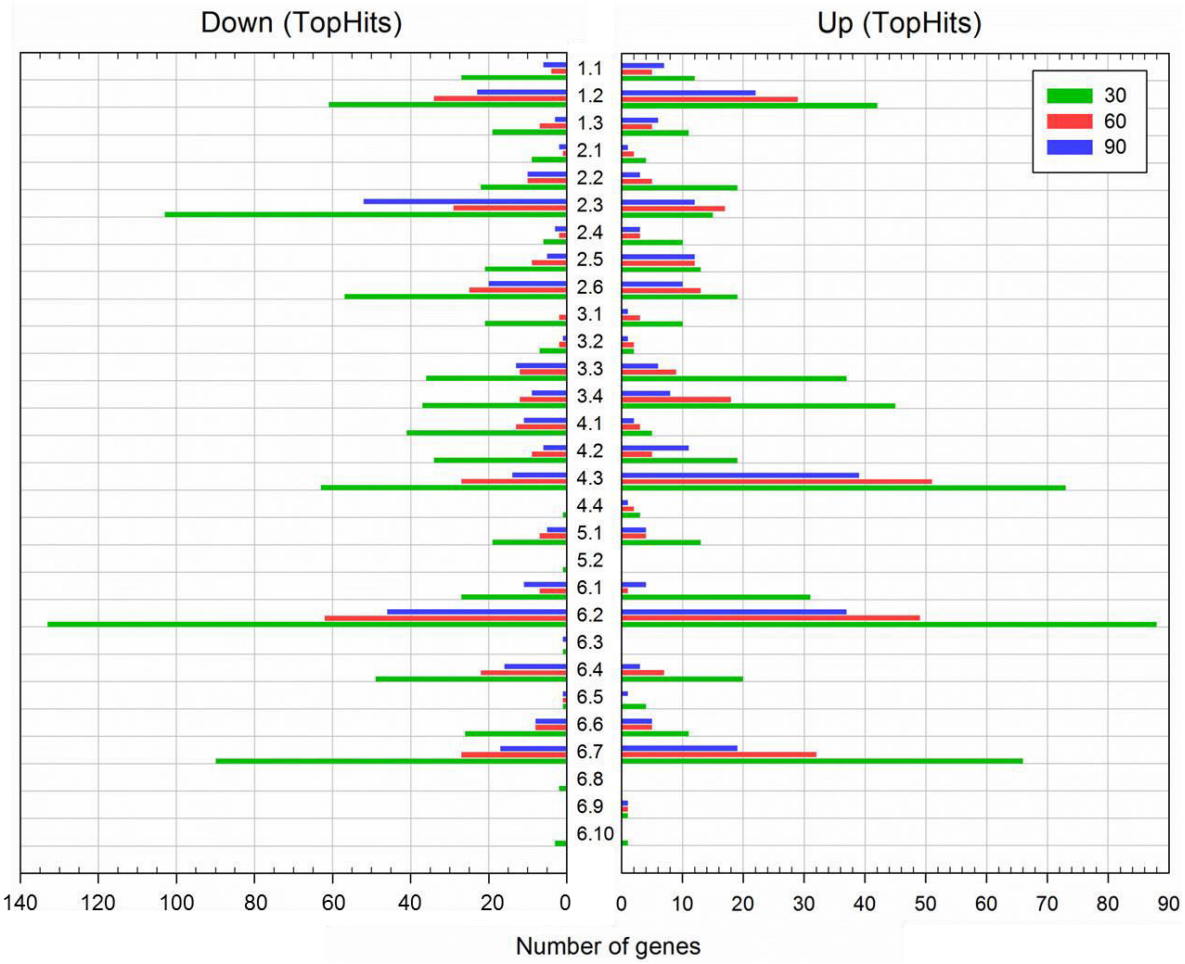
[b] Time of sample withdrawal after mixing spores with germinants

Table 2: Cohesion of contrasts: specific and shared differentially expressed genes among the sample time points

Sample time point(s)			Number of genes [a]	
30 min	**60 min**	**90 min**	**TopHits**	**HighFold**
■			564	329
	■		90	54
		■	68	44
■	■		93	42
	■	■	24	15
■		■	53	30
■	■	■	134	76

[a] The numbers of genes are either specific to the time point (only one gray-filled box), or shared exclusively by the time points indicated by gray fills

For functional interpretation of the changes in the transcriptomic profiles of salt-stressed outgrowing spores, differentially expressed genes were categorized according to the *Subti*Wiki database (Mäder *et al*., 2012; Michna *et al*., 2016). Strong alterations of gene expression – at all time points and in both directions - could be observed in the functional categories "Membrane proteins" (category number 6.2, see **Fig. 3**), "Transporters" (1.2), "Coping with stress" (4.3), and "Proteins of unknown function" (6.7) (**Fig. 3; Tables S1, S2; Database S1**). To a lesser extent and predominantly after 30 min outgrowth at high salinity, genes belonging to the functional categories "Protein synthesis, modification and degradation" (3.3), "Regulation of gene expression" (3.4), and "Essential genes" (6.1) were also notably differentially expressed (**Fig. 3; Tables S1, S2; Database S1**). While most enriched categories exhibited similar extents of up- and downregulation, genes in the categories "Amino acid/nitrogen metabolism" (2.3), "Additional metabolic pathways" (2.6), and "Phosphoproteins" (6.4) were greatly downregulated (**Fig. 3; Tables S1, S2; Database S1**). This might relate to the aforementioned potential high-salinity-dependent retardation of the molecular reorganization processes during the ripening period and/or the reallocation of cellular resources towards salt stress response. The most relevant functional categories and groups are discussed in detail below.



1. Cell wall & cell division	**4. Lifestyles**
1.1 Cell wall & cell division	4.1 Exponential & early post exponential lifestyles
1.2 Transporters	4.2 Sporulation & germination
1.3 Homeostasis	4.3 Coping with stress
2. Metabolism	4.4 Lifestyles (miscellaneous)
2.1 Electron transport & ATP synthesis	**5. Prophages & mobile genetic elements**
2.2 Carbon metabolism	5.1 Prophages
2.3 Amino acid/nitrogen metabolism	5.2 Mobile genetic elements
2.4 Lipid metabolism	**6. Groups of genes**
2.5 Nucleotide metabolism	6.1 Essential genes
2.6 Additional metabolic pathways	6.2 Membrane proteins
3. Information processing	6.3 GTP-binding proteins
3.1 Genetics	6.4 Phosphoproteins
3.2 RNA synthesis & degradation	6.5 Universally conserved proteins
3.3 Protein synthesis, modification & degradation	6.6 Poorly characterized/putative enzymes
3.4 Regulation of gene expression	6.7 Proteins of unknown function
	6.8 Short peptides
	6.9 ncRNA
	6.10 Pseudogenes

Figure 3: Functional classification of differentially expressed genes during spore outgrowth in the presence of 1.2 M NaCl (TopHits). Genes were categorized according to *Subti*Wiki. Sample time points: 30 min (green), 60 min (red) and 90 min (blue).

Hyperosmotic stress response. The functional category "Coping with hyperosmotic stress" includes genes encoding proteins involved in the specific hyperosmotic stress response, i.e. all Opu transporters (OpuA – OpuE); the K^+ uptake systems KtrAB and KtrCD; GbsA, GbsB, and GbsR required for GB synthesis; ProA, ProH, and ProJ responsible for (osmoadaptive) proline synthesis; and the amino-peptidases PapA and PapB that can degrade proline-containing peptides (Boch *et al*., 1996; Kempf and Bremer, 1998; Holtmann *et al*., 2003; Brill *et al*., 2011a; Zaprasis *et al*., 2013). In this current study, 20 of the 25 genes in this category were differentially expressed (**Fig. 4A**). No significant differential expression was detected for *ktrC*, *ktrD*, *papA*, *papB,* and *opuBC* (**Dataset S1**). 17 of the 20 differentially expressed genes were upregulated, including the operons *proHJ*, *gbsAB*, *opuA*, *opuB* (except *opuBC*), *opuC*, and the genes *gbsR*, *opuD*, and *opuE* (**Fig. 4A**). All of these genes encode proteins involved in uptake and synthesis of osmoprotectants, which play a central role in the hyperosmotic stress response of vegetative *B. subtilis* cells (Kempf and Bremer, 1998; Bremer, 2002). Our results indicate that the same genes have an important function in the salt stress response of outgrowing spores as well.

In agreement with previous reports, salt-stressed induced upregulation of *opu* genes was already very strong after 30 min of outgrowth (**Fig. 4A**) and was independent of the transporters' substrate availability (as no substrates were in the medium), reflecting their osmotic control (Hahne *et al*., 2010). It should be noted that *opuBC* from the *opuB* operon was likewise upregulated (around 2.5 $\log_2$FC; **Dataset S1**), but the difference may not have been significant because *opu* genes are reportedly also expressed to some extent in non-stressed outgrowing spores (Keijser *et al*., 2007). Interestingly, the different *opu* operons had variable temporal expression patterns (**Fig. 4A**): *opuD* was only significantly upregulated at 30 min and expression of *opuC* also peaked at 30 min and was only moderately upregulated later. In contrast, *opuA* and *opuE* exhibited continuous high expression at all time points.

During vegetative growth, *opuA* expression is elaborately balanced with the extent of the cell's internal solute pool, as sufficient intracellular amounts of osmoprotectants repress *opuA* transcription (Hoffmann *et al*., 2013). Thus, steady upregulation of the *opuA* operon during outgrowth at high salinity suggests that the outgrowing spores were not able to accumulate sufficient amounts of compatible solutes throughout the whole experiment (**Fig. 4A**).

Moreover, steady expression of *opuE*, which has a σ^A- as well as a σ^B-dependent promoter, is mediated by σ^A during sustained salt stress (Spiegelhalter and Bremer 1998)

suggesting that the osmospecific stress response governed by σ^A is important for the salt stress adaptation during outgrowth. In contrast, *opuD,* which is also controlled by both σ^A and σ^B promoters, exhibited an early, transient upregulation that is rather typical for σ^B–dependent transcription (Spiegelhalter and Bremer 1998; Hoffmann and Bremer, 2011; Young *et al.*, 2013). Nevertheless, the *opuC* operon also showed a transiently high upregulation (followed by moderate upregulation), although it is not a member of the σ^B regulon. While the lower *opuC* upregulation at 60 min and 90 min outgrowth seems to be partly due to an increased expression in the non-stressed outgrowing spores, the *opuC*-repressor *opcR* might also be involved: although the biological function of this *gbsR*-type repressor is still unknown (Lee *et al.*, 2013), its expression pattern was strikingly similar to that of the *opuC* operon (**Fig. 4A**).

Compared to the other *opu* genes, upregulation of the *opuB* genes was more variable, although *opuB* expression in non-stressed outgrowing spores barely changed over time (**Fig. 4A**). Possibly, *opuB* transcription was modulated by the repressor GbsR, which regulates choline uptake (via OpuB) and processing to GB (via GbsA and GbsB), and expression of which was also upregulated during outgrowth at high salinity (**Fig. 4A;** Nau-Wagner *et al.*, 2012). GbsR can directly bind choline, which leads to derepression of the *opuB* and *gbsAB* operons, allowing efficient accumulation of GB (Boch *et al.*, 1996; Nau-Wagner *et al.*, 2012). Strikingly, despite *gbsR* upregulation and the absence of choline, *gbsAB* was upregulated in our experiment. The reason for this *gbsAB* upregulation remains to be determined.

Another evidence for the importance of compatible solutes during outgrowth under salt stress was the strong upregulation of the osmoadaptive proline synthesis genes *proH* and *proJ.* In vegetative cells, osmotic induction of the *proHJ* operon can be observed after a salt shock as well as during sustained high salinity and is mediated by an osmotically controlled σ^A-type promoter (Steil *et al.*, 2003; Hahne *et al.*, 2010; Brill *et al.*, 2011a). Although osmoadaptive proline synthesis requires ProA (encoded in the not osmotically inducible *proBA* operon), *proA* was found to be repressed during outgrowth in high salt conditions (**Fig 4A**), which is in agreement with previous findings (Hahne *et al.*, 2010; Brill *et al.*, 2011a). Transcription of the *proBA* operon is regulated by a tRNA-responsive riboswitch, allowing *proBA* derepression only upon proline starvation (Brill *et al.*, 2011b). Thus, the downregulation of *proA* in salt-stressed outgrowing spores may in fact signify derepression of *proBA* in non-stressed outgrowing spores, as these likely have a higher anabolic proline turnover due to a higher protein biosynthesis rate.

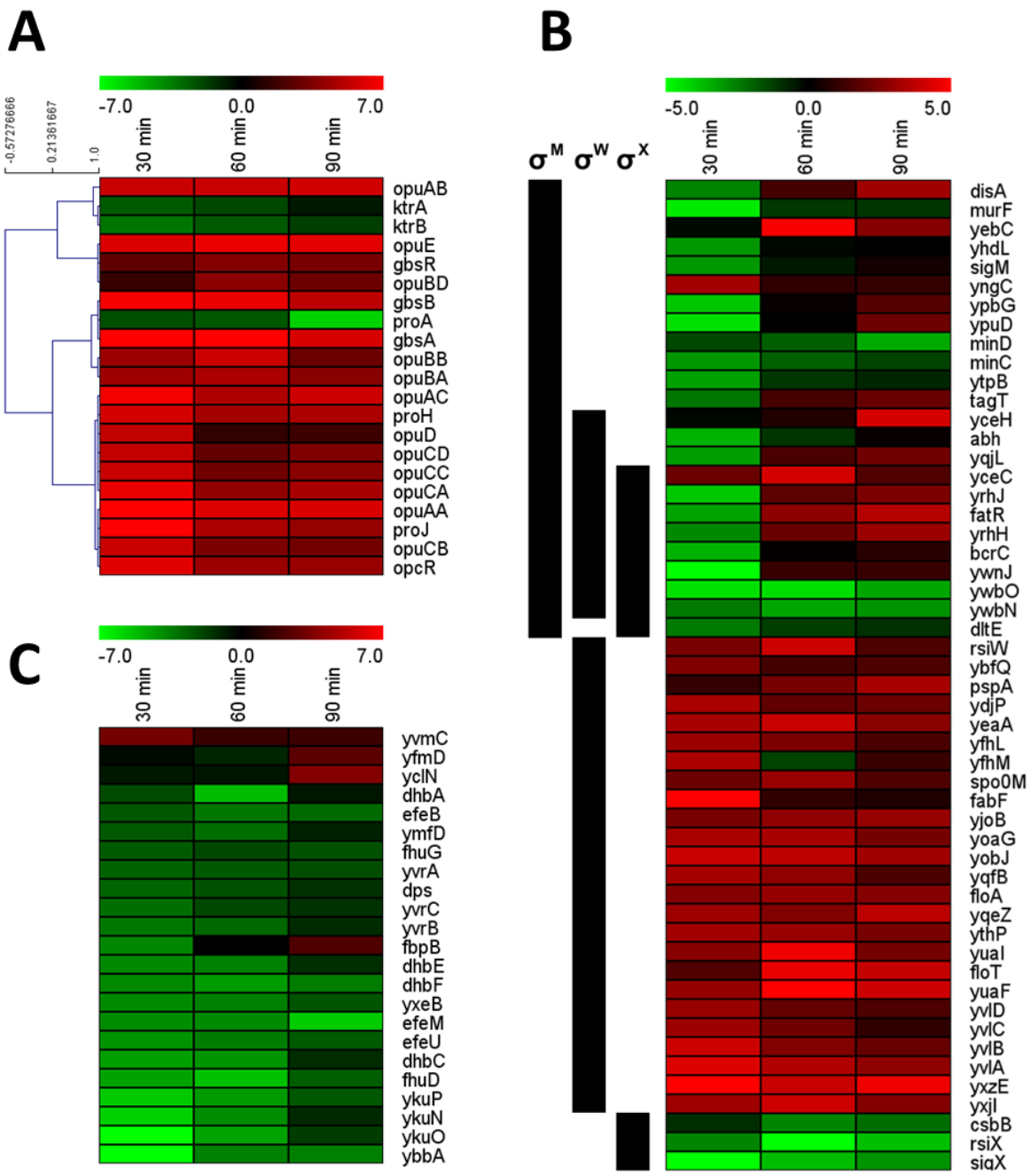


Figure 4: Expression profiles of (A) genes associated with the hyperosmotic stress response, (B) differentially expressed members of the σ^M, σ^W, and σ^X regulons (regulon affiliations are indicated by black bars on the left), and (C) genes associated with iron homeostasis. Only significantly differentially expressed genes are shown. Cutoff values ($\log_2$FC) of the color scale are indicated at the top of each figure.

While most genes involved in the accumulation of osmoprotectants were upregulated during outgrowth at high salinity, the not osmotically inducible *ktrAB* operon was found to be downregulated at all time points (**Fig. 4A**). Since the KtrAB transporter system plays an

important role in K^+ uptake as a first defense against high osmolality (Holtmann *et al.*, 2003) it is possible that the K^+ accumulation phase had already ended within the first 30 min of outgrowth and *ktrAB* expression was downregulated at ≥ 30 min to prevent detrimental effects of further K^+ uptake. As *ktrAB* is regulated by *ydaO*-type riboswitch causing increased transcription termination in the presence of c-di-AMP (Nelson *et al.*, 2013), it would be intriguing to investigate the role of c-di-AMP signaling in salt stress responses and outgrowth in more detail in future studies.

General stress response. Numerous previous studies on osmotically stressed vegetative cells of *B. subtilis* have indicated an induction of the σ^B-dependent general stress response upon sudden osmotic increases (e.g. Spiegelhalter and Bremer, 1998; Petersohn *et al.*, 2001; Höper *et al.*, 2006; Hecker *et al.*, 2007; Nannapaneni *et al.*, 2012; Nicolas *et al.*, 2012; Young *et al.*, 2013). As spores germinated in high-salinity media were also suddenly confronted with salt stress, an involvement of the general stress response would seem plausible. Although the *sigB* gene itself was not differentially expressed at any sample time point (**Dataset S1**), this was not surprising as σ^B is only transiently active after a salt shock (Spiegelhalter and Bremer, 1998; Young *et al.*, 2013). In total, almost one third of the σ^B regulon was differentially expressed at one or several sample time points in our study: 14 genes were significantly upregulated, whereas 31 genes were downregulated (**Table 3; Fig. S2A**). However, all upregulated genes except for one uncharacterized gene (*ydeC*) have additional regulators aside from σ^B (e.g. σ^W), which are likely responsible for the increased expression (see below). Moreover, only one of the 37 general stress response genes whose absence causes a salt-sensitive phenotype (i.e. *yflH;* Höper *et al.*, 2005) was upregulated, whereas 7 others were downregulated (**Fig. S2A**). Indeed, σ^B was previously reported to be dispensable for colony formation from spores on plates containing high salt concentrations (Tovar-Rojo *et al.*, 2003). Altogether, our data suggest that the σ^B-dependent general stress response is not of major significance during outgrowth in the presence of 1.2 M NaCl.

Table 3: Involvement of alternative sigma factors in the salt stress response of outgrowing spores [a]

Regulon	Description	# of genes [b]	Differentially expressed [c]		
			%	#up	#down
sigB	General stress response	151	30	14	31
sigD	Regulation of flagella, motility, chemotaxis & autolysis	24	33	0	27
sigE	Sporulation (early mother cell-specific)	176	9	8	7
sigF	Sporulation (early forespore-specific)	63	11	2	5
sigG	Sporulation (late forespore-specific)	108	10	3	8
sigH	Transcription of early stationary phase genes (sporulation, competence)	37	43	1	14
sigI	Control of a class of heat shock genes	6	50	0	3
sigK	Sporulation (late mother cell-specific)	103	7	2	4
sigL	Utilization of arginine, acetoin & fructose; required for cold adaptation	23	52	0	12
sigM	ECF-type sigma factor responsible for intrinsic resistance against beta-lactam antibiotics	69	35	4	13
sigO-rsoA	Two-subunit sigma factor	5	20	1	0
sigV	ECF-type sigma factor; response to lysozyme	4	0	0	0
sigW	ECF-type sigma factor; activated by alkaline shock, polymyxin B, vancomycin, cephalosporin C, D-cycloserine, & triton X-100	65	54	28	4
sigX	ECF-type sigma factor; cell surface properties	29	45	1	9
sigY	ECF-type sigma factor; maintenance of the SPβ prophage	7	0	0	0
ylaC	ECF-type sigma factor; response to oxidative stress	4	0	0	0
xpf	PBSX phage RNA polymerase sigma factor	10	50	0	5

[a] Classification and description according to *Subti*Wiki and Souza *et al.* (2014).

[b] Number of genes within the regulon

[c] The numbers of up- (#up) and downregulated (#down) genes only include genes that have the same expression direction (up/downregulated) at all three time points. The percentage of differentially expressed genes includes all genes.

Sigma factors and regulons. Aside from σ^B, the alternative sigma factors σ^M, σ^W and σ^X have repeatedly been implicated with cell envelope and salt stress (Horsburgh *et al.*, 2001; Steil *et al.*, 2003; Petersohn *et al.*, 2001; Höper *et al.*, 2006; Hahne *et al.*, 2010). Hence, we

analyzed the role of the different alternative sigma factors and major regulons in the salt stress response of outgrowing spores.

When looking at the expression of the genes encoding the alternative sigma factors, only three significant changes could be observed: downregulation of *sigM* at 30 min outgrowth, upregulation of *sigO* at 60 min outgrowth, and downregulation of *sigX* throughout the whole experiment (**Dataset S1**). While the repression of *sigX* under salt stress has previously been reported (Steil *et al.*, 2003; Hahne *et al.*, 2010; Nicolas *et al.*, 2012), the lack of differential *sigW* expression was in contrast to the study by Hahne *et al.* (2010), in which a significant *sigW* upregulation 30 min and 60 min after the osmotic upshift has been shown. However, as the activity of alternative sigma factors can not only be regulated transcriptionally, but also on the translational and post-translational level (Souza *et al.*, 2014), the involvement of sigma factors can best be estimated by looking at the expression of their regulons (**Table 3**).

In our study, more than half of the genes in the σ^W regulon were differentially expressed, most of them being upregulated (**Table 3; Fig. 4B**), indicating importance of σ^W for the salt stress response during outgrowth. Interestingly, while Hahne *et al.* (2010) reported a maximum induction of the σ^W regulon at 30 min after NaCl addition, we observed three different expression patterns within this regulon: (i) 27 genes were upregulated at all time points, (ii) 4 genes were downregulated at all time points, and (iii) 4 genes were downregulated at 30 min and upregulated subsequently (**Fig. 4B**). Most likely the distinct expression patterns of the latter two gene groups were caused by their simultaneous control by σ^M and σ^X (**Fig. 4B**). The constant upregulation of the σ^W regulon also supports the notion that the salt stress response of outgrowing spores is rather similar to that of cells growing at sustained high salinity and not to that of salt-shocked cells, as salt-shocked cells shut down the σ^W regulon about 20 min after the osmotic upshock (Steil *et al.*, 2003).

In addition to the σ^W regulon, we observed that about one third of the σ^M regulon was differentially expressed, although it should be noted that 10 out of these 24 genes were also members of the σ^W regulon (**Table 3; Fig. 4B**). While Hahne *et al.* (2010) have reported maximum upregulation of the σ^M regulon 60 min after NaCl addition, σ^M-dependent genes tended to be repressed in our study (**Fig. 4B**). Downregulation was predominant at 30 min of outgrowth in the presence of NaCl, which is consistent with the repression of the *sigM* gene at this time point. However, at the later time points, most genes became less repressed or were even upregulated (**Fig. 4B**). Altogether, an involvement of σ^M in salt stress adaptation during

outgrowth is possible, but the tendency for downregulation – in context with the salt-sensitive phenotype of *sigM* mutants (Horsburgh *et al.*, 2001) – suggests that σ^M-dependent genes may be dispensable during the early outgrowth phase under salt stress.

In consistence with previous reports (Steil *et al.*, 2003; Hahne *et al.*, 2010), we found that the σ^X regulon was largely repressed during outgrowth at high salinity (**Table 3**). Notably, all differentially expressed σ^X genes (except *sigX* and the anti-σ^X factor *rsiX*) were also members of the σ^W and/or σ^M regulon (**Fig. 4B**).

Although we detected significant upregulation of *sigO,* which forms a two-subunit sigma factor with RsoA, this finding is almost certainly an artifact, because raw data showed that this gene is barely expressed at all and *rsoA* is not differentially expressed (data not shown).

As summarized in **Table 3**, various genes belonging to the regulons of other alternative sigma factors were differentially expressed during outgrowth at high salinity as well. Most notably, many σ^D–dependent genes involved in motility and chemotaxis were repressed throughout the entire experiment (**Fig. S2B**), which is in excellent agreement with previous findings from salt-stressed vegetative cells (Steil *et al.*, 2003; Höper *et al.*, 2006; Hahne *et al.*, 2010; Nicolas *et al.*, 2012). Furthermore, the differentially expressed genes of the σ^I, σ^L, and Xpf regulons were all downregulated in our experiment (**Table 3; Dataset S1**). Some differential expression was also detected within the sporulation-related σ^E, σ^F, σ^G, σ^K, and σ^H regulons (**Table 3**). However, the portion of differential expression within the σ^E, σ^F, σ^G, and σ^K regulons was relatively low and can be explained by very low expression levels, non-sporulation-related gene functions, and/or different degradation rate of dormant spore transcripts in stressed versus non-stressed outgrowing spores (**Dataset S1**). All differentially expressed σ^H-genes except *spo0M* (which is a member of the σ^W regulon) were downregulated (**Table 3**), consistent with the role of σ^H in sporulation initiation, which is repressed at high salinity, and with the salt-sensitivity of σ^H (Ruzal *et al.*, 1998; Widderich *et al.*, 2016).

Aside from alternative sigma factors, the transcriptional activity of specific regulons can also provide insights into how *B. subtilis* copes with stress. Alignment of all *Subti*Wiki-annotated regulons with our data exhibited various substantial overlaps. While some regulators seemed to be active during outgrowth at high salinity (e.g. BirA, PyrR, LutR), genes in many other regulons behaved exactly opposite to their regulators' functions as

repressors or activators (e.g. AzlB, PucR, RocR, Zur; **Table S3**). The latter observation suggests that these regulons were actively regulated in the control sample, i.e. in non-stressed outgrowing spores, but not in salt-stressed outgrowing spores. Indeed, *azlB* and *zur* have been reported to be overexpressed during outgrowth under non-stress conditions (Keijser *et al.*, 2007).

While the DegS/DegU two-component system has previously been implicated in salt stress sensing and response (Ruzal and Sanchez-Rivas, 1998; Mäder *et al.*, 2002; Steil *et al.*, 2003), only 5 genes (i.e. 19 %) of the DegU regulon were differentially expressed in our study (**Table S3; Dataset S1**). Moreover, there was only a limited overlap (38 %, mostly motility genes) with the DegS/DegU regulated genes that were reported to be differentially expressed in salt-stressed vegetative cells (Steil *et al.*, 2003). Perhaps, the lack of major changes in the DegU regulon can be explained by the upregulation of *rapG*, which encodes a DegU-inhibiting response regulator aspartate phosphatase (Ogura *et al.*, 2003). Nevertheless, the role of DegS/DegU in salt stress adaption during spore outgrowth remains questionable.

Induction of the PerR regulon in vegetative, salt-stressed cells has previously been hypothesized to indicate increased oxidative stress caused by high salinity (Höper *et al.*, 2006). However, in our study, the PerR-dependent catalase gene *katA* was repressed while the other members of the regulon were not differentially expressed (**Table S3; Dataset S1**).

Overlap with other stress responses. As many survival strategies of *B. subtilis* are closely interlinked (Höper *et al.*, 2005; Lopez *et al.*, 2009; Schultz *et al.*, 2009), the transcriptional profile of genes known to be involved in other stress responses was analyzed as well (summarized in **Table S4**). Our data indicate a large overlap (about 40 %) of differentially expressed genes during outgrowth at high salinity and genes involved in cell envelope stress, which largely reflects the observed changes in the σ^M, σ^W, and σ^X regulons described above (**Table S4; Fig. 4B**). Notably, more than half of the 35 cell envelope stress-related genes that exhibited significant upregulation at one or more of the sample time points encoded hypothetical and poorly characterized proteins, whose role may be interesting to investigate in the future (**Dataset S1**). In addition, several genes encoding heat shock proteins, chaperones (e.g. *groEL*, *groES*, *dnaK*), and proteases (e.g. *clpE*, *clpX*) were found to be upregulated in our experiment (**Dataset S1**). Protein quality control is important during outgrowth (Sinai *et al.*, 2015) and likely even more so during outgrowth at high salinity, since

osmotic upshifts have previously been proposed to cause protein denaturation and misfolding (Hahne *et al.*, 2010). There was also some overlap between genes involved in other stress responses and genes found to be differentially expressed in our study, but most of these correlations rather seemed to be based on the non-specific function of the gene in question (**Table S4; Dataset S1**). Nevertheless, although the functional relevance is not clear, noticeable overlaps between genes differentially expressed in our study and genes in the categories "Resistance against toxins/antibiotics" (27 % overlap, 17 upregulated, 11 downregulated), "Resistance against oxidative and electrophile stress" (22 % overlap, 4 upregulated, 9 downregulated), and "Biosynthesis of antibacterial compounds" (27 % overlap, 4 upregulated, 11 downregulated) were observed (**Table S4; Dataset S1**).

Cell envelope. Vegetative *B. subtilis* cells growing under hyperosmotic conditions exhibit alterations of their cell envelope, i.e. changes in cell wall structure and membrane composition (López *et al.*, 1998; López *et al.*, 2006; Palomino *et al.*, 2009; Hahne *et al.*, 2010). During outgrowth at high salinity, cell envelope stress also seems to be apparent (see above). In total, 44 genes (ca. 23 %) of the category "Cell wall" were differentially expressed in our study (**Dataset S1**). Among these, especially the genes involved in cell wall turnover (8 genes) and cell wall synthesis (10 genes) were repressed, although *tagA* and *tagD* involved in early steps of teichoic acid biosynthesis were upregulated (**Fig. 5A**). PBP4* (encoded by *pbpE*) has previously been proposed to play a role in peptidoglycan modification at high salinity (Palomino *et al.*, 2009; Hahne *et al.*, 2010), but the gene was not differentially expressed in our study (**Dataset S1**). Since most cell wall-related genes did not exhibit differential expression, the extent and relevance of cell wall remodeling during ripening (i.e. in the absence of morphological changes), as well as its susceptibility towards salt stress throughout this phase, remain to be determined.

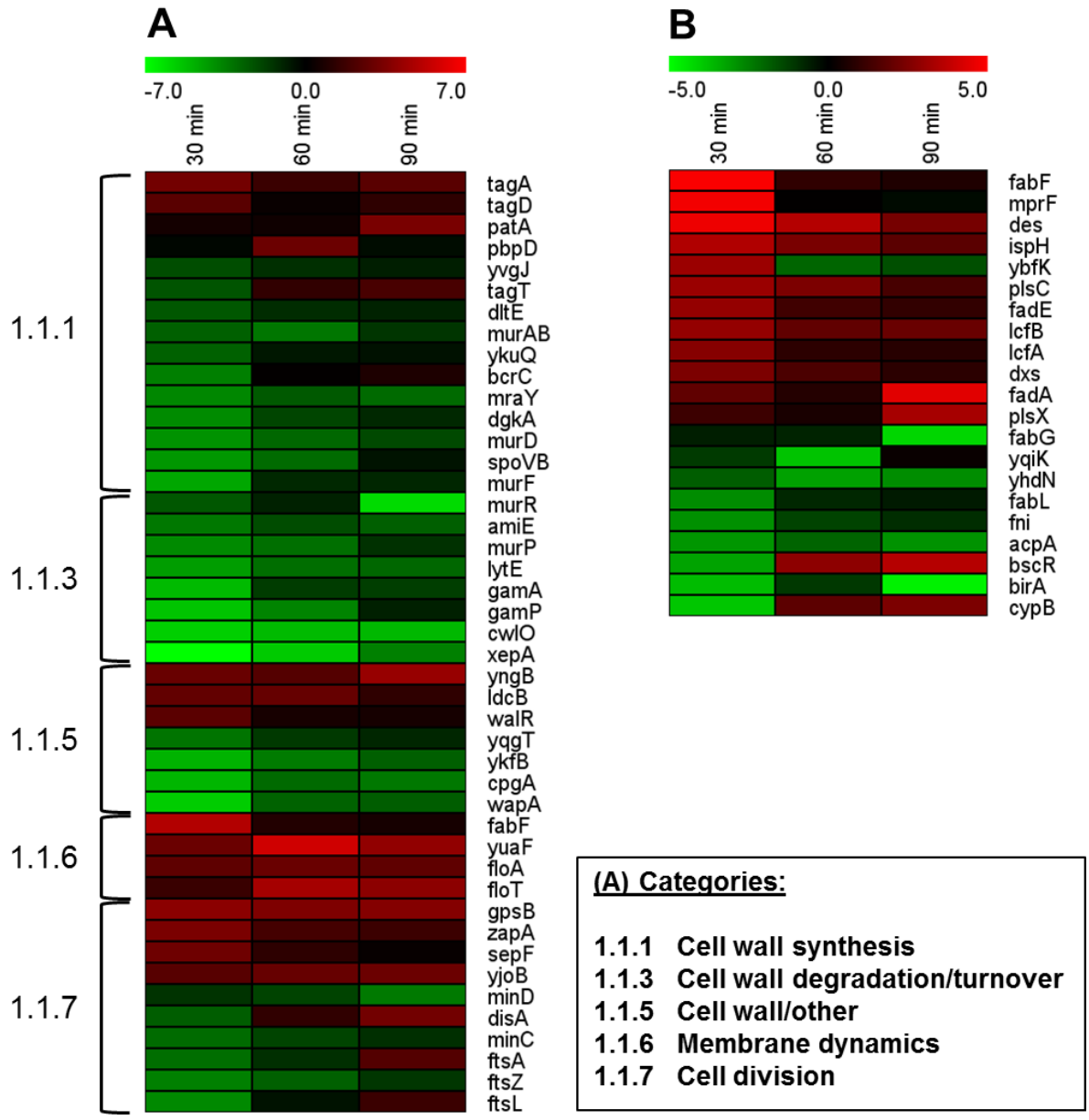


Figure 5: Expression profiles of (A) genes associated with cell wall and cell division and (B) genes involved in lipid metabolism. Only significantly differentially expressed genes are shown. Cutoff values (log_2FC) of the color scale are indicated at the top of each figure.

High-salinity-induced changes in the membrane composition of vegetative cells include an increase in saturated straight-chain fatty acids, unsaturated fatty acids, and cardiolipin, and a decrease in branched fatty acids (López *et al.*, 1998; López *et al.*, 2006). In our study, 21 out of 109 genes involved in lipid metabolism were differentially expressed during outgrowth at high salinity (**Dataset S1**). Genes encoding enzymes for fatty acid utilization tended to be upregulated (**Fig. 5B**), which is in agreement with a previously reported global upregulation of genes involved in degradation of free fatty acids via β-

oxidation (Hahne *et al.*, 2010). In contrast, genes important for fatty acid and lipid biosynthesis exhibited expression changes in both directions. However, since only little is known about membrane remodeling during spore outgrowth, the relevance of the observed transcriptomic differences and their actual impact on membrane composition is unclear. Nevertheless, in agreement with a possible increase in unsaturated fatty acids (López *et al.*, 1998), the *des* gene encoding a fatty acid desaturase was upregulated in our study (**Fig. 5B**). Interestingly, although anionic phospholipids (in particular cardiolipin) play a role in osmoadaptation, possibly by changing biophysical membrane properties such as fluidity (Poolman *et al.*, 2004; Lopez *et al.*, 2006; Romantsov *et al.*, 2009; Unsay *et al.*, 2013) neither the genes encoding cardiolipin synthases (*clsA*, *ywiE*, *ywjE*) nor *pgsA* (encoding a phosphatidylglycerophosphate synthase) were differentially expressed (**Dataset S1**). However, σ^W-dependent upregulation of *fabF* as well as *yuaF* and the flotillin-homologs *floT* and *floA* (**Fig. 4B, Dataset S1**) suggests that changes of the cytoplasmic membrane (e.g. *fabF*-induced fluidity decrease) may also play a role in osmoadaptation of outgrowing spores (Kingston *et al.*, 2011). Hence, it seems worthwhile to investigate membrane remodeling during outgrowth in the presence and absence of high salinity in more detail.

Membrane proteins and transporters. In our study, the membrane protein and transporter transcriptome of outgrowing spores was severely altered by the presence of 1.2 M NaCl (**Fig. 3**). In total, 54 ABC transporter genes (i.e. 26 % of the genes in this category), 6 phosphotransferase system genes (21 %), and 65 other transporter genes (33 %) were differentially expressed in our study (**Dataset S1**). With regard to the importance of solute pool adjustments during osmotic stress adaptation on the one hand, and major molecular reorganization during outgrowth on the other hand, this is not surprising and in agreement with previous transcriptomic and proteomic studies (Steil *et al.*, 2003; Keijser *et al.*, 2007; Hahne *et al.*, 2010; Segev *et al.*, 2013; Hoffmann and Bremer, 2016).

Overall, 44 % of transporter genes exhibited upregulation and 56 % downregulation during outgrowth at high salinity (**Dataset S1**). Among the upregulated ones were (aside from the aforementioned *opu* transporter genes) many genes encoding cation efflux transporters: the *khtSTU* operon involved in K^+ efflux, *mrpABC* of the *mrpABCDEFG* operon encoding *B. subtilis*' major Na^+ extrusion system, as well as *nhaC* and *nhaK* involved in Na^+ and monovalent cation efflux, respectively (Fujisawa *et al.*, 2004; Fujisawa *et al.*, 2005; Gorecki *et al.*, 2014). Upregulation of K^+ efflux systems in salt-stressed cells, which has been reported

previously (Steil *et al.*, 2003; Hahne *et al.*, 2010), is in agreement with the observed downregulation of the KtrAB K^+ importer (see above; **Fig. 4A**). Both transcriptional changes might constitute consequences of the initial K^+ uptake phase of osmoadaptation (Whatmore *et al.*, 1990) that may have ended before 30 min into outgrowth.

Transcriptomic changes towards increased Na^+ efflux most likely represent countermeasures against high intracellular Na^+ concentrations that are toxic for *B. subtilis*, but may for instance build up due to the Na^+-coupled activity of the OpuD and OpuE transporters (**Fig. 4A;** Gorecki *et al.*, 2014; Hoffmann and Bremer, 2016). This would not only be in agreement with observations from vegetative cells (Hahne *et al.*, 2010), but also with the downregulation of Na^+ symporter genes (*yocR, yrbD, yocS, putP, yodF*) in our study (**Dataset S1**). Other upregulated transporter genes are functionally involved (i) in the uptake of zinc, phosphate, sulfonate, sulfate, glucose, gluconate, uracil, and cysteine; (ii) in branched-chain amino acid transport; and (iii) in the export of toxic peptides and antibiotics (**Dataset S1**). Notably, several poorly characterized MFS (Major Facilitator Superfamily) transporter similar to multidrug-efflux transporters and antibiotic resistance proteins were upregulated as well (**Dataset S1**), but the relevance of the export of toxic peptides and antibiotics for the salt stress response of outgrowing spores is unclear.

Another upregulated transporter was the di- and tripeptide importer *dtpT*, which can contribute to osmoprotection by taking up proline-containing peptides (Zaprasis *et al.*, 2013). However, genes encoding two out of three other peptide uptake systems implicated in the same osmoprotective function, Dpp and Opp (Zaprasis *et al.*, 2013), were downregulated throughout our experiment (**Dataset S1**). As it is possible that non-stressed outgrowing spores utilize these systems in general to gain access to a broader nutrient spectrum, the relevance of extracellular peptides in outgrowth and salt-stress adaptation of outgrowing spores remains to be determined. Generally it is plausible that downregulation of many transporter genes in our study was in fact a consequence of higher metabolic and/or biosynthetic activity in non-stressed cells, as these genes are involved in the uptake of common metabolites including purines, nitrate, lactate, and various amino acids (arginine, leucine, branched-chain amino acids, methionine, and proline) (**Dataset S1**).

Although our data showed upregulation of two genes for iron uptake (only at 90 min) and *sufC* involved in the synthesis of Fe-S clusters that were implicated in the salt stress response of vegetative cells (Höper *et al.*, 2006), unexpectedly many iron and iron siderophore uptake systems were downregulated (see below; **Fig. 4C**). With regard to the

differentially expressed phosphotransferase system genes, it should be noted that their transcripts (except for *murP*) were highly abundant in dormant spores (**Fig. 2** and data not shown) so that it is possible that these transcripts represent remnants from dormancy and differential expression during outgrowth may be an artifact.

In total, transporter genes constituted 42 % of the differentially expressed membrane protein genes, emphasizing the importance of transport processes during outgrowth and osmoadaptation within this phase (**Fig. 3; Table S1**). The functions of the membrane proteins encoded by the residual 58 % of differentially expressed genes in this category were very diverse and included genes for regulatory proteins, flagellum and chemotaxis, kinases, dehydrogenases, cell division proteins, and many poorly characterized proteins (**Dataset S1**). Moreover, about 30 % of the differentially expressed non-transporter membrane protein genes encoded hypothetical proteins, some of which may be interesting to investigate in more detail with regard to their role in outgrowth and osmoadaptation.

Iron homeostasis. Iron homeostasis is governed by the central iron regulatory protein Fur, which upon binding of excess iron becomes an active repressor (Hoffmann *et al.*, 2002; Helmann, 2014). Previous proteomics and transcriptomics studies showed that vegetative *B. subtilis* cells grown in SMM containing 1.2 M NaCl experience iron limitation (Hoffmann *et al.*, 2002; Steil *et al.*, 2003). Although the underlying mechanism of this iron limitation is not clear, it was reported that genes involved in the synthesis of the iron siderophore bacillibactin (i.e. organized in the *dhbACEBF* operon) and other members of the Fur regulon were derepressed under high-salinity conditions (Hoffmann *et al.*, 2002; Steil *et al.*, 2003). In contrast, in our study, *dhbA*, *dhbC*, *dhbE*, and *dhbF* and 15 other genes of the category "Acquisition of iron" (1.3.3) were repressed in the presence of 1.2 M NaCl (**Fig. 4C; Dataset S1**). Although the downregulation of only 17 genes (34 %) of the Fur regulon was significant, all other genes in this regulon were also downregulated but below the significance threshold ($\log_2$FC ≥ 2; $p \leq 0.05$). It was previously reported that the *fur* gene itself is overexpressed during outgrowth under non-stress conditions (Keijser *et al.*, 2007), which might explain why differential *fur* expression among the tested conditions was not observed in our study (**Dataset S1**). Thus, the downregulation of the Fur regulon in salt-stressed outgrowing spores may indicate a larger amount of excess iron that would keep the Fur repressor active thereby leading to even lower iron acquisition. Possibly, the iron depot of spores of around 40 µg Fe/g [dry weight] (Granger *et al.*, 2011) is sufficient for spore ripening processes so that excess

iron keeps Fur active and its regulon repressed (Sinai *et al.*, 2015). The increased repression of the Fur regulon during outgrowth at high salinity may indicate lower iron utilization in salt-stressed outgrowing spores, but further in-depth research is needed to address this issue adequately. Additionally, it would be important to assess the influence of the genetic background on high-salinity-dependent iron limitation, because the *B. subtilis* strain used in the study by Steil *et al.* (JH642) carries a mutation (sfp^0) that negatively affects bacillibactin synthesis (Steil *et al.*, 2003).

Metabolism. As outgrowth represents the phase of metabolic reactivation after a dormant phase of indeterminate duration, outgrowing spores in high salinity environments have to simultaneously cope with molecular rearrangements for metabolic initiation and osmoadaptation (Keijser *et al.*, 2007; Sinai *et al.*, 2015). Consistent with these two major challenges, a total of 372 genes involved in metabolism were differentially expressed during 90 min of outgrowth in the presence of 1.2 M NaCl. High salinity affected all aspects of metabolism, however various processes were influenced to different extents: genes in the categories “Electron transport and ATP synthesis” (2.1) and “Lipid metabolism” (2.4) were affected the least; genes of “Carbon metabolism” (2.2) and “Nucleotide metabolism” (2.5) exhibited intermediate alterations; and genes involved in “Amino acid/nitrogen metabolism” (2.3) and “Additional metabolic pathways” (2.6) were affected the most (**Fig. 3; Tables S1 and S2**).

In total, 70 % of the differentially expressed metabolic genes were downregulated, reflecting the detrimental effects of salt stress and suggesting a metabolic decline comparable to the reduced growth rate or growth arrest that can be observed in vegetative salt-stressed cells (Boch *et al.*, 1994; Hahne *et al.*, 2010). Especially the genes related to amino acid metabolism (i.e. amino acid biosynthesis, acquisition and utilization) experienced strong repression (**Fig. 3; Dataset S1**), which is likely to result in generally slower adaptation and ripening processes, both of which require protein biosynthesis (Segev *et al.*, 2013; Sinai *et al.*, 2015; Hoffmann and Bremer, 2016). Moreover, global downregulation of the amino acid metabolism may further impede synthesis of osmoprotective proline from other amino acid precursors (Zaprasis *et al.*, 2015). Although the acquisition of adequate osmoprotectant pools seems *per se* unlikely given the extremely nutrient-poor conditions of our experiment, amino acids that the outgrowing spores may acquire from SASP degradation or from peptides

liberated from the spore coat seem to have low chances to be converted to proline based on our transcriptomic data (Tovar-Rojo *et al.*, 2003; Zaprasis *et al.*, 2013).

Despite the apparently restrained metabolism during outgrowth at high salinity, only 12 genes involved in carbon core metabolism were downregulated in our study (**Dataset S1**). Previous studies on salt-stressed vegetative cells indicated that the enzymes of the tricarboxylic acid (TCA) cycle canalized towards 2-oxoglutarate synthesis and thus ultimately towards glutamate and subsequent proline synthesis (Höper *et al.*, 2006; Hahne *et al.*, 2010). However, we could not detect such an adaptation in our experiment, as most of the TCA cycle genes were either not differentially expressed or even downregulated (**Dataset S1**).

Next to numerous downregulated metabolic routes, the only pathway that was uniformly, significantly upregulated was the uridine-5-phosphate synthesis pathway, with all 8 *pyr* genes (*pyrABCDEFK*) involved in conversion of hydrogen carbonate to UMP showing strong upregulation (average $\log_2$FC = 7.6) at all investigated time points (**Dataset S1**). In contrast, 6 out of 7 differentially expressed purine biosynthesis and acquisition genes as well as all 12 nucleotide utilization genes were downregulated. Unfortunately, the relevance of this difference in pyrimidine and purine assimilation is not clear.

Taken together, our data indicate that high salinity exerted manifold detrimental effects on the metabolism of outgrowing spores, which are especially grave given the low nutrient availability in our outgrowth medium.

Conclusions. In its natural habitats, *B. subtilis* is frequently exposed to increases in environmental salinity, which has profound influences on cellular physiology and growth and triggers adaptive responses (Bremer, 2002; Hoffmann and Bremer, 2016). High salinity exerts detrimental effects on *B. subtilis* spore formation (Ruzal *et al.*, 1998; Widderich *et al.*, 2016), spore germination (Nagler *et al.*, 2014; Nagler and Moeller, 2015; Nagler *et al.*, 2015; Nagler *et al.*, 2016), and, as shown here and previously, spore outgrowth (Tovar-Rojo *et al.*, 2003; Nagler *et al.*, 2014; Nagler *et al.*, 2016). Although it seems counter-intuitive that high salinity inhibits the formation of desiccation resistant spores, blocking this costly cellular differentiation program most likely reflects the inability of starving cells to gather sufficient resources (e.g. for the massive production of osmoprotective proline) required for sporulation during simultaneous salt stress (Brill *et al.*, 2011a; Widderich *et al.*, 2016). In contrast, spore germination and outgrowth can be initiated under salt conditions that do not allow growth of

vegetative cells (Boch *et al.*, 1994; Nagler *et al.*, 2014; Nagler *et al.*, 2016). This behavior indicates that outgrowing spores lack a sensory and regulatory response system to prevent them from revival in non-growth-permissive osmotic conditions, likely resulting in a survival disadvantage (Nagler *et al.*, 2014).

Salt shocks are one of the strongest inducers of the σ^B-controlled general stress regulon of *B. subtilis* (Spiegelhalter and Bremer, 1998; Hecker *et al.*, 2007; Hahne *et al.*, 2010; Nannapaneni *et al.*, 2012; Young *et al.*, 2013). Surprisingly, although we exposed spores to a severe salt shock simultaneously to the germination stimulus, the σ^B-directed stress response system was in its core not turned on by this treatment. This suggests that (i) the stressosome controlling σ^B activity subsequent to a salt shock is either not (sufficiently) present in outgrowing spores, or (ii) that the cellular signal(s) controlling the release of the alternative transcription factor σ^B from its anti-sigma factor RsbW cannot be generated in outgrowing spores (Hecker *et al.*, 2007; Marles-Wright and Lewis, 2010; Young *et al.*, 2013; Hoffmann and Bremer, 2016).

The transcriptional profile of salt-stressed outgrowing spores resembled that of *B. subtilis* cells actively growing under continuous high-salinity conditions in many aspects (Steil *et al.*, 2003; Hahne *et al.*, 2010). Hence, the signals that trigger adaptive responses of *B. subtilis* to counteract sustained high salinity can apparently be perceived by outgrowing spores as well. At all investigated time points, salt-stressed outgrowing spores induced their complete genetic repertoire of osmoprotectant uptake and compatible solute synthesis, emphasizing the pivotal role of these substances (especially of the compatible solutes proline and GB) also during outgrowth (**Fig. 4A**). Unfortunately, the nature of the signal allowing osmotic induction of compatible solute uptake and biosynthesis systems in *B. subtilis* remains to be determined (Bremer, 2002; Hoffmann and Bremer, 2016).

In outgrowing spores, the σ^D regulon was strongly downregulated in response to high salt concentrations (**Fig. S2**), indicating that flagellar biosynthesis, assembly and swimming will be impaired in the emerging vegetative cells. This is surprising as one may have predicted that chemotaxis and the ability to swim would be useful traits to escape from osmotically unfavorable to nutritionally favorable conditions (Wong *et al.*, 1995). Perhaps, the requirement to synthesize 20,000 flagellin subunits (Hag) for the production of a single filament is too resource-consuming for salt-stressed outgrowing spores (Mukherjee and Kearns, 2014). However, the strong down-regulation of flagellar genes and the concomitant

abrogation of swimming have also been observed in *B. subtilis* cells exposed to prolonged high salinity (Steil *et al.*, 2003).

In conclusion, our study provided new insights on the transcriptomic adaptations of outgrowing spores to the presence of high salt concentrations and points out another facet of the perturbing effects that osmotic stress can exert on the life cycle of spore-forming soil bacteria.

ABBREVIATIONS

2x SG	=	Modified Schaeffer's Sporulation Medium with glucose
DPA	=	Dipicolinic acid (pyridine-2,6-dicarboxylic acid)
FC	=	Fold change
GB	=	Glycine betaine
OD_{600nm}	=	Optical density at 600 nm
PCA	=	Principal component analysis
RNA-seq	=	RNA sequencing
SASP	=	Small, acid-soluble spore protein
SEM	=	Scanning electron microscopy
SMM	=	Spizizen Minimal Medium
TCA	=	Tricarboxylic acid

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CHAPTER 7

Discussion

Despite its relevance for basic research, food microbiology, soil ecology, and astrobiology, not much was previously known about how spore revival is affected by high salt concentrations and osmotic stress. Inhibitory effects of high salt concentrations on *B. subtilis* spore germination have been reported (Fleming and Ordal, 1964), but the molecular mechanism of this observation remained elusive. Likewise, sufficient energy availability during outgrowth at high salinity was found to be required for adaptation processes, which were, however, not investigated previously (Tovar-Rojo *et al.*, 2003). The work presented in this doctoral thesis provided many new insights on the effects of high salinity on *Bacillus* spore revival, allowing the development of a mechanistic model for inhibitory salt effects on germination and shedding light on the salt stress adaptation processes during outgrowth.

7.1. Effects of high salinity on *Bacillus* spore germination

7.1.1. High NaCl concentrations negatively affect *B. subtilis* spore germination

In general, increasing NaCl concentrations exerted increasingly detrimental effects on *B. subtilis* spore germination (Nagler *et al.*, 2014; Nagler *et al.*, 2015). At high salinity, spore populations germinated more slowly, more heterogeneously, and less efficiently. Nevertheless, a fraction of the spore population initiated germination in response to nutrients despite extreme NaCl concentrations near saturation (Nagler *et al.*, 2014). This suggests that *B. subtilis* spores do not possess a salt-sensing system to prevent them from germinating in a non-growth-permissive high-salinity environment (Boch *et al.*, 1994), although osmosensing systems are known to exist in various bacteria (Poolman *et al.*, 2004; Nagler *et al.*, 2014). However, most of the spores that initiated germination at ≥ 3.6 M NaCl were arrested in a phase-gray state, suggesting incomplete germination (Setlow *et al.*, 2001; Nagler *et al.*, 2014). Yet, NaCl inhibition was reversible when the spores were washed and transferred to germination medium without salt, indicating that NaCl does not cause permanent damage to the germination apparatus (Nagler *et al.*, 2014). The investigation of germination kinetics supported by single-spore analyses revealed that high NaCl concentrations delayed commitment (T_{comm}), postponed the start of rapid Ca^{2+}-DPA release (T_{lag}), increased the time required for rapid Ca^{2+}-DPA efflux ($\Delta T_{release}$), and also increased the time between commitment and T_{lag} at ≥ 2.4 M NaCl (**Fig. 9**; Nagler *et al.*, 2014; Nagler *et al.*, *in preparation for submission*). This composite inhibition of very early germination events (at the level of initiation) and subsequent events (at the level of refractivity loss) suggests that

NaCl inhibits more than one process (Nagler *et al.*, 2014; Nagler and Moeller, 2015; Nagler *et al.*, *in preparation for submission*).

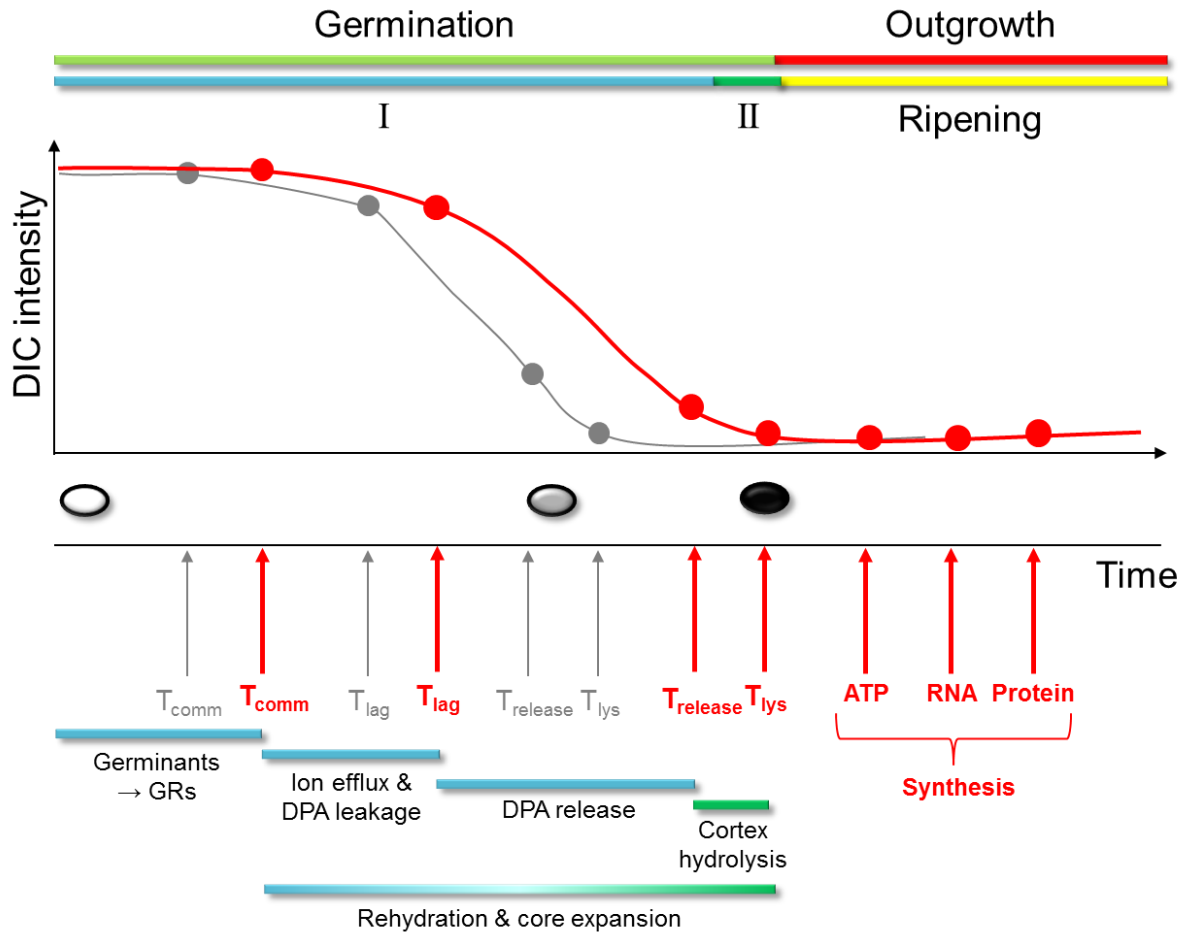

Figure 9: High-salinity effects on the germination kinetics of a single *B. subtilis* spore. A scheme of the change in observable DIC intensity during germination in the absence (gray) or presence (red) of high NaCl concentrations is shown. The time bars and morphological stages refer to germination with NaCl.

Although numerous molecular details of the germination process are still elusive (Setlow, 2014a) interference of high salinity with several known events can be envisioned. Thus, high NaCl concentrations may inhibit (i) GR accessibility and nutrient binding (**section 7.1.2.**), (ii) signal integration and transduction from the GRs (**section 7.1.3.**), (iii) ion, Ca^{2+}-DPA and water fluxes (**section 7.1.4.**), and (iv) cortex hydrolysis (**section 7.1.5.;** Nagler *et al.*, 2014; Nagler *et al.*, 2015). Furthermore, it could be shown that NaCl also detrimentally affects spore germination of other *Bacillus* species (**section 7.1.7.**), and high concentrations of

various salts (**section 7.1.6.**) as well as non-ionic solutes (fructose and glycerol[1]) also exerted inhibitory effects on *B. subtilis* spore germination (Nagler and Moeller, 2015; Nagler *et al.*, 2015; Nagler *et al.*, 2016). Taking all results obtained throughout this doctoral thesis into consideration (**chapters 2 to 6; Supplemental material IV.F** and data not shown), the different possibilities of how high salinity may inhibit nutrient germination will be discussed in the following sections and ultimately merged to a mechanistic model for high-salinity-inhibition of *B. subtilis* spore germination (**section 7.1.8.**).

7.1.2. Inhibition of GR accessibility and nutrient binding

The first germination events that could be detrimentally affected by high NaCl concentrations are the passage of nutrient germinants through the spore integuments to the GRs in the IM and the subsequent binding of the germinants to GRs (**Fig. 5**; Nagler *et al.*, 2014; Nagler *et al.*, 2015). As spores have a negative surface charge that can attract cations while repulsing anions, already the entry of charged germinants (such as zwitterionic amino acids or AGFK's K^+ component) into the spore coat could be slowed by NaCl (Pesce *et al.*, 2014). The germinants' coat passage is facilitated by GerP proteins, as severe coat defects eliminate the Δ*gerP* germination defect (Behravan *et al.*, 2000; Butzin *et al.*, 2012; Setlow, 2014a). In high-salinity germination media, Na^+ and Cl^- ions may interact with the carboxyl and amino groups of the spore coat proteins, thereby possibly decreasing coat permeability to germinants (Kazakov *et al.*, 2008; Nagler *et al.*, 2014; Pesce *et al.*, 2014; Nagler *et al.*, 2015). Compared to the coat, ionic interaction with the cortex should have much more drastic effects (i.e. cortical contraction) due to PG electronegativity and the presence of positive counter ions as described in the concept of an expanded osmoregulatory cortex (Gould and Dring, 1975; Nakatani *et al.*, 1985; Bassi *et al.*, 2012). However, this interaction may normally be prevented by the OM (or another unknown structure) in complex with the coat, acting as a diffusion barrier for ions, which may be traversable for germinants by passage facilitator proteins like GerP or remnants of the sporulation feeding tube (Gerhardt and Black 1961; Carstensen *et al.*, 1979; Ferguson *et al.*, 2007; Butzin *et al.*, 2012; Leggett *et al.*, 2012; Tocheva *et al.*, 2013; Setlow, 2014a; Tan and Ramamurthi, 2014; Knudsen *et al.*, 2016). If ion mobility is not restricted, ionic interaction with the GRs or associated spore components (e.g. other germinosome proteins, IM) may decrease the affinity of GRs for their ligands

[1] It should be noted that although glycerol can pass vegetative cell membranes, the permeability of the dense IM of dormant *Bacillus* spores for glycerol is similar as for glucose and sucrose that cannot pass the membrane (Gerhardt and Black, 1961; Knudsen *et al.*, 2016). Thus, glycerol can induce osmotic stress on spores that are not yet fully germinated.

(Nagler *et al.*, 2014; Nagler *et al.*, 2015). Yet, how exactly germinants bind to their GRs is still incompletely understood (Atluri *et al.*, 2006; Li *et al.*, 2010; Setlow, 2014a).

Several observations would support NaCl-dependent germination inhibition at the level of GRs. First, germination via GerA (by L-alanine or L-valine) was less salt-sensitive than GerB-GerK-dependent germination by AGFK (Nagler *et al.*, 2014; Nagler *et al.*, 2015). This could be due to ionic interactions of Na^+ and Cl^- with AGFK's K^+ during germinant passage and/or GR-binding, or to interaction of Na^+ with GerB/GerK's putative K^+-binding site (Atluri *et al.*, 2006; Nagler *et al.*, 2014). Second, NaCl interfered with germination initiation, i.e. it delayed commitment (T_{comm}) and T_{lag}, and increased the heterogeneity of germination initiation in spore populations (**Fig. 9**; Nagler *et al.*, 2014; Nagler *et al.*, 2015). Importantly, the timing of T_{comm} and T_{lag} as well as the heterogeneity of germination initiation are influenced by the number of GRs (Zhang *et al.*, 2010; Setlow *et al.*, 2012). Hence, if NaCl would hinder germinant passage and/or binding to GRs, GR activation should be slower, thus resulting in delayed and more heterogeneous germination initiation as observed (Nagler *et al.*, 2014). Third, high-salinity inhibition was weaker at low pH (e.g. pH 5) than at high pH (e.g. pH 10; K. Nagler, unpublished results, **Supplemental material IV.F Fig. S1**). At low pH, more carboxyl groups (as the major ionizable groups of the spore integuments) are protonated, hence the surface charge should be smaller (i.e. less negative), thereby possibly decreasing the negative salt effects on germinant diffusion into the coat (Kazakov *et al.*, 2008; Pesce *et al.*, 2014). Fourth, high concentrations of non-ionic osmolytes also inhibited germination, and although they are not capable of ionic interactions with spore integuments, fructose- and glycerol-containing germination media are very viscose at very high (≥ 4 M) concentrations (Nagler and Moeller, 2015; Nagler *et al.*, 2015). Thus, it would be possible that germinant diffusion towards the GRs is slowed, although this does not apply to high-salinity germination media (Nagler *et al.*, 2015).

Nevertheless, various other observations suggest that NaCl does not primarily inhibit germinant passage and binding to GRs. First, although a suboptimal germinant concentration went along with a larger NaCl-sensitivity of germination, increasing germinant concentration beyond the point of saturation (e.g. for L-alanine from 10 mM to 100 mM) did not relieve inhibitory NaCl effects any further. This suggests that Na^+ and/or Cl^- do not mainly compete with the germinants for GR-binding (Nagler *et al.*, 2014). Second, overexpression of GerA or GerB plus GerK did not improve germination via L-alanine or AGFK, respectively, in the presence of NaCl (K. Nagler and P. Setlow, unpublished, **Supplemental material IV.F Fig.**

S2). Thus, the amount of GRs does not seem to be the limiting factor during germination at high salinity. Third, Δ*gerP* spores germinated better (albeit still significantly worse than wild type spores) in the presence of up to 2.4 M NaCl than in the absence of NaCl, which would rather suggest that ionic interaction with the germinant and/or spore integuments somehow improves germinant passage in the absence of an appropriate facilitator system (Nagler *et al.*, 2015). The fact that Δ*gerP* spores are (albeit to a lesser extent) able to germinate in response to nutrients implies that the coat-OM complex does not become completely impermeable by the absence of GerP proteins, e.g. due to the presence of functionally similar proteins, such as GerT or feeding tube remnants (Ferguson *et al.*, 2007; Buzin *et al.*, 2012; Tan and Ramamurthi, 2014; Nagler *et al.*, 2015). Fourth, severely coat-defective spores, in which germinants theoretically should more readily have access to GRs, did not germinate better, but much worse in the presence of ≥ 0.6 M NaCl (Nagler *et al.*, 2015). However, a severe coat defect (which reportedly also compromises the OM) presumably facilitates salt ion access to the cortex, IM and its germination apparatus (Setlow, 2014a; Nagler *et al.*, 2015). Importantly, this exacerbated salt inhibition of coat-deficient spores was clearly an ionic effect, as these spores (e.g. encasement-deficient Δ*spoIVD* spores) germinated wild type-like with high concentrations of non-ionic solutes (Nagler *et al.*, 2015). Hence, with regard to the concept of an expanded osmoregulatory cortex (Gould and Dring, 1975) it seems likely (i) that the coat-OM complex usually restricts ion passage to the cortex, IM, and germination apparatus so that inhibition in intact spores is not mainly due to direct ionic interaction with these structures; and (ii) that the coat-OM complex cannot restrict ion access in spores with severe coat defects so that ionic interaction with the cortex, IM, and germination apparatus strongly disturbs germination of coat-defective spores. Fifth, non-nutrient germination by exogenous Ca^{2+}-DPA (which activates CwlJ without requiring GRs) and by a pressure of 150 MPa (which activates GRs in the absence of nutrients) were both significantly inhibited by elevated NaCl concentrations (Wuytack *et al.*, 2000; Paidhungat *et al.*, 2001; Paidhungat *et al.*, 2002; Nagler *et al.*, 2014; Nagler *et al.*, 2015). Hence, NaCl can unambiguously inhibit germination even if germinant passage and GR binding are bypassed (Nagler *et al.*, 2015).

Taken together, it seems possible that ionic interaction with the germinants and/or coat slows germinant passage towards the GRs, which may therefore contribute to delayed germination initiation (**Fig. 10**). In contrast, in intact wild type spores, the coat-OM complex seems to restrict ion passage to the cortex, IM, and germination apparatus, so that notable inhibition by direct interaction of Na^+ and/or Cl^- with GRs seems rather unlikely. In any case,

significant germination inhibition by high concentrations of NaCl and non-ionic solutes apparently occurs after germinant passage and GR binding.

7.1.3. Inhibition of GR-induced signal transduction

When sufficient germinants have bound to GRs, the spore irreversibly commits to germinate, although many details of this process remain incompletely understood (Yi and Setlow, 2010; Setlow, 2013; Setlow, 2014a). According to a recently proposed model, a GR undergoes a conformational change upon nutrient binding and this information is transduced to a SpoVA Ca^{2+}-DPA release channel, which thereby also changes its conformation from inactive closed (C_0) to a metastable closed (C_1) state (**Fig. 7**; Kong *et al.*, 2015; Wang *et al.*, 2015b). How the information of the GR conformational change is transduced to SpoVA is unclear, but it could be via protein-protein interaction (Vepachedu and Setlow, 2007b; Wang *et al.*, 2015b). This protein-protein interaction may be direct (i.e. between a GR and SpoVA protein) or indirect, e.g. via the germinosome scaffold GerD that may also serve as signal integrator (Vepachedu and Setlow, 2007b; Griffiths *et al.*, 2011; Li *et al.*, 2014; Perez-Valdespino *et al.*, 2014; Setlow, 2014a; Wang *et al.*, 2015b). Thus, if ion movement is not restricted e.g. by the coat-OM complex (see **section 7.1.2**), high ionic concentrations could interfere with numerous non-covalent protein-protein interactions known to prevail in the germination apparatus, such as salt bridges that are important for GerD multimerization, thereby inhibiting signal transduction (Vepachedu and Setlow, 2007b; Li *et al.*, 2014; Nagler *et al.*, 2014; Nagler and Moeller, 2015; Troiano *et al.*, 2015). Besides, protein-lipid interactions could be modified by ions as well, which occurs for example during osmosensory modulation of protein activity as in the case of OpuA of *L. lactis* (Poolman *et al.*, 2004). In addition or alternatively, the IM itself may be involved in signal transduction, and ions could disturb this process for instance by interacting with charged IM lipids (Poolman *et al.*, 2004). Indeed, alterations in spore membrane composition can decrease germination efficiency significantly, although the reason is not yet known (Griffiths and Setlow, 2009).

Unfortunately, the gaps in mechanistic understanding of GR-induced signal transduction prevent direct investigation of how NaCl affects this process. However, disturbance of protein-protein interactions by high salt concentrations would be consistent with the observed delay of commitment and higher salt-sensitivity of AGFK germination that requires GerB-GerK cooperativity (Setlow, 2013; Nagler *et al.*, 2014). Especially chaotropic salts (i.e. salts that promote protein denaturation and protein complex disintegration) such as

perchlorates or KSCN, which were shown to be strong germination inhibitors, may act detrimentally on protein-protein interactions of the germination apparatus (Sawyer and Puckridge, 1973; Zhang and Cremer, 2010; Nagler and Moeller, 2015). Although this does not indicate functionality, at least GR clustering in dormant spores was not destructed by the presence of KSCN or $Mg(ClO_4)_2$ (Nagler and Moeller, 2015).

While detrimental high-salinity effects on the transduction of the germination signal generated by the GRs cannot be ruled out based on available data and literature resources, this process does not seem to be a major NaCl-inhibition target during germination for the following reasons. First, as mentioned above, the coat-OM complex presumably restricts ion passage towards the IM, where signal transduction most likely occurs, so that this process would only be a potential salt inhibition target in coat-deficient spores. If it would be inhibited in intact spores as well, this would not be consistent with the observed germination inhibition by non-ionic osmotic stress. Second, with regard to the possible NaCl-interference with GerD, it seems that this protein is normally not an important site of salt inhibition, as germination of Δ*gerD* spores exhibited a similar NaCl sensitivity as wild type spores, although germination of Δ*gerD* spores is generally less efficient (K. Nagler and P. Setlow, unpublished, **Supplemental material IV.F Fig. S3**). Third, significant germination inhibition in the presence of high NaCl concentrations was also observed when spores were germinated by high pressure of 550 MPa, which directly induces Ca^{2+}-DPA release by opening SpoVA channels and/or by a phase shift of the IM (Wuytack *et al.*, 2000; Paidhungat *et al.*, 2002; Reineke *et al.*, 2013; Nagler *et al.*, 2015). Thus, NaCl can inhibit germination processes that are completely GR-independent (Nagler *et al.*, 2015).

7.1.4. Inhibition of ion, Ca^{2+}-DPA, and water fluxes

Transduction of the germination signal is followed by commitment, which temporally coincides with the efflux of large amounts of cations via unknown channels (**Fig. 9**; Yi and Setlow, 2010; Setlow, 2013; Setlow, 2014a; Wang *et al.*, 2015a). The function of this cation release is not known, but it was proposed to initiate subsequent germination events and may be important for commitment or even essential for germination, as several ion channel blockers can prohibit spore revival (Dring and Gould, 1971; Mitchell *et al.*, 1986; Bassi *et al.*, 2012; Setlow, 2013). Besides, commitment presumably involves a SpoVA conformational change to a loose O_1 conformation allowing slow Ca^{2+}-DPA leakage prior to T_{lag} (**Fig. 7**; Setlow, 2014a; Wang *et al.*, 2015a; Wang *et al.*, 2015b). Although the duration of the leakage

phase is heterogeneous among individual isogenic spores, the total amount of Ca^{2+}-DPA leaking until T_{lag} is relatively constant (ca. 20% of spore Ca^{2+}-DPA), which possibly relates to the attainment of a threshold before the rapid Ca^{2+}-DPA release phase ($\Delta T_{release}$) is initiated by another conformational change creating a fully open (O_2) SpoVA channel (**Fig. 7**; **Fig. 9;** Wang *et al.*, 2015a; Wang *et al.*, 2015b). Ion and Ca^{2+}-DPA efflux are accompanied by water influx into the spore core, with core rehydration being required reactivation of metabolism when entering outgrowth (Setlow, 2003; Setlow, 2013).

Regarding the large amounts of ions present in high-salinity germination media and the consequently high external osmolality, an interference with ion and water movement seems very plausible (Nagler *et al.*, 2014; Nagler and Moeller, 2015; Nagler *et al.*, 2015). This interference may occur in different ways: (i) Na^+ and Cl^- may directly interact with (or block) efflux channels; (ii) passive ion and/or Ca^{2+}-DPA efflux may be impeded because the high extracellular ionic strength eliminate a chemical gradient, and/or (iii) water influx may be impaired osmotically due to the high extracellular osmolarity (Yi *et al.*, 2011; Nagler *et al.*, 2014; Nagler *et al.*, 2015). Moreover, ion and Ca^{2+}-DPA release may be inhibited by charge repulsion or alterations of an electrical gradient by high external ionic strength, but little is known about electrical properties and their changes in germinating *B. subtilis* spores, although dormant spores were reported to lack a membrane potential (Magge *et al.*, 2008; Setlow, 2013).

Numerous observations would be in agreement with high-salinity inhibition at the level of ion, Ca^{2+}-DPA, and water fluxes. First, increasing NaCl concentrations increasingly delayed commitment, which – as noted above – coincides with ion efflux (Setlow, 2013; Nagler *et al.*, 2014). Thus, ion efflux may indeed be a characteristic event in commitment, but it may not occur as readily in the presence of high extracellular ionic strength (Setlow, 2013; Nagler *et al.*, 2014; Nagler *et al.*, 2015). In the presence of an OM, high external osmolarity could impede ion efflux by detracting water from the hydrated cortex, whereas in the absence of a permeability barrier, external ions would likely annihilate the usual chemical gradient across the IM, which serves as the driver of ion release from the core (Bassi *et al.*, 2012; Nagler *et al.*, 2014; Nagler *et al.*, 2015). Second, at $\geq$ 2.4 M NaCl, the time between commitment and T_{lag} was increased, which may indicate that Ca^{2+}-DPA leakage is slowed, so that it takes longer until the aforementioned threshold of leaked Ca^{2+}-DPA is reached (Nagler *et al.*, 2015; Wang *et al.*, 2015a; Wang *et al.*, 2015b). It could be hypothesized that this threshold relates to changes in core expansion (due to rehydration) and concomitant IM

tension increase: upon reaching a critical IM tension, the SpoVA channels may fully open up via SpoVAC, which acts as a mechanosensitive channel (Gould and Dring, 1975; Poolman *et al.,* 2004; Velásquez *et al*., 2014). Third, several different methods indicated a decreased rate of Ca^{2+}-DPA efflux during $\Delta T_{release}$ (**Fig. 9;** Nagler *et al*., 2014; Nagler *et al*., *in preparation for submission*). Although this could be due to ionic interference with SpoVA channels (in case inward ion movement is not restricted), spores having approximately 4-fold increased SpoVA levels did not germinate any better at high salinity than wild type spores, suggesting that the amount of SpoVA is not rate limiting for Ca^{2+}-DPA release at high salinity (K. Nagler and P. Setlow, unpublished results, **Supplemental material IV.F Fig. S4**; Yi *et al*., 2011). Instead, it seems more likely that the large amount on insoluble Ca^{2+}-DPA in the spore core cannot be solubilized (and thus be released) efficiently, as the high external osmolarity almost certainly decreases the rate of water influx (Gould and Dring, 1975; Cowan *et al*., 2003; Nagler *et al*., 2014; Nagler *et al*., 2015). This would furthermore be supported by the significant amount of phase-gray spores emerging during germination at very high NaCl concentrations: just as CLE double mutants that cannot degrade their cortex, therefore cannot fully rehydrate and stay phase-gray, extremely salt-stressed spores may not be able to fully replace Ca^{2+}-DPA with water (Setlow *et al*., 2001; Nagler *et al*., 2014). Fourth, high concentrations of non-ionic solutes significantly inhibited germination, which most likely is mainly due to osmotic effects (Nagler and Moeller, 2015; Nagler *et al*., 2015; K. Nagler, unpublished results). Strikingly, the extent of germination inhibition by iso-osmotic high-salinity and non-ionic high-osmolarity media was relatively similar (Nagler and Moeller, 2015; Nagler *et al*., 2015). This indicates that osmotic stress plays a crucial role in high-salinity inhibition of intact spores and that especially Ca^{2+}-DPA release coupled with water uptake are major inhibition targets (Nagler *et al*., 2014; Nagler and Moeller, 2015; Nagler *et al*., 2015). Yet, ionic stress also plays a notable role during high-salinity inhibition, but ionic effects are much more pronounced (i) during germination of severely coat-defective spores in the presence of high NaCl concentrations (see above; Nagler *et al*., 2015) and (ii) during germination of intact spores in the presence of salts other than NaCl (see **section 7.1.6.**; Nagler and Moeller, 2015).

Taken together, the fluxes of ions, Ca^{2+}-DPA and water seem to be important targets of NaCl inhibition of germination, with osmotic effects likely impeding Ca^{2+}-DPA replacement with water, thereby increasing $\Delta T_{release}$ significantly (**Fig. 9; Fig. 11**).

7.1.5. Inhibition of cortex hydrolysis

The final step in germination, i.e. cortex hydrolysis, may be inhibited by NaCl due to negative effects of high ionic strength on the activity of the two CLEs CwlJ and SleB. While both CLEs are at least in part localized near the coat-cortex boundary, it is not clear whether the coat-OM complex would protect them from ions, because the OM integrity may be breached during stage I of germination, e.g. due to Ca^{2+}-DPA release and consequent cortex contraction (Gould and Dring, 1975; Setlow, 2013). Furthermore, if the coat-OM complex would not (or not efficiently) restrict ion movement into the cortex, the resulting cortex deformation may alter the ability of the CLEs to degrade the cortex (Popham *et al.*, 2012).

Several findings would support an inhibition of germination at the level of cortex hydrolysis: (i) germination triggered by exogenous Ca^{2+}-DPA (i.e. via CwlJ) was strongly inhibited by NaCl (Paidhungat *et al.*, 2001; Nagler *et al.*, 2014); (ii) the emergence of phase-gray spores (beginning at around 3.6 M), which also occur during germination of *cwlJ sleB* mutants that cannot degrade their cortex (Setlow *et al.*, 2001; Nagler *et al.*, 2014); (iii) $\Delta T_{release}$ was significantly increased, which was previously reported for Δ*cwlJ* spores (Peng *et al.*, 2009; Nagler *et al.*, 2014); and (iv) the time required for cortex hydrolysis (ΔT_{lys}) tended to be longer with salt, albeit not significantly (Nagler *et al.*, 2014).

However, other observations argue against cortex hydrolysis being a major salt inhibition target. First, neither CwlJ nor SleB seemed to be particularly inhibited by NaCl as single-knockout mutants of either CLE resulted in wild type-like spore germination[2] (Nagler *et al.*, 2015). Second, germination inhibition by high concentrations of non-ionic solutes cannot be explained by ionic interference with enzyme activity (Nagler and Moeller, 2015; Nagler *et al.*, 2015). Furthermore, NaCl inhibition of germination by exogenous Ca^{2+}-DPA may in fact be an indirect effect, as Na^+ and Cl^- could interact with Ca^{2+}-DPA and/or the coat, thereby restricting its passage to CwlJ (Nagler *et al.*, 2014). Moreover, the emergence of phase-gray spores seems likely to be due to insufficient core rehydration as a consequence of high external osmolarity (see above), which would be consistent with germination inhibition by both, high salinity and non-ionic osmotic stress. Likewise, this osmotic effect seems like a plausible explanation for a prolonged $\Delta T_{release}$ phase.

[2] It should be noted, however, that previous studies (e.g. Peng *et al.*, 2009; Kong *et al.*, 2015) reported Δ*cwlJ* spores would germinate much slower than wild type spores (in the absence of salt) than was observed in the study by Nagler *et al.* (2015). Although this discrepancy may well be due to the different genetic backgrounds (PS832 versus 168, respectively) it may be helpful to reinvestigate high-salinity effects on cortex hydrolysis using direct quantitative measurements of cortex fragment generation.

Taken together, although cortex hydrolysis may to some extent be affected by high salinity (as indicated by slightly increased ΔT_{lys}), it does not seem to be a major inhibition site.

7.1.6. Inhibitory effects of different salts

The effect of high concentrations of different salts on *B. subtilis* spore germination varied considerably, although high salinity always exerted negative effects (Nagler and Moeller, 2015). It could be shown that the inhibitory strength of a salt depends on the exact ionic composition, ionic concentration (especially of the anion), and chemical properties. As mentioned previously, the importance of ionic (as opposed to purely osmotic) effects was for instance very obvious during germination in the presence of different potassium and sodium halides: despite identical osmolarities, the anion was the major determinant of inhibitory strength, although the germination profiles were modified by the cation at concentrations ≥ 2.4 M. Unfortunately, the definite reason why anions play a more prominent role in germination inhibition was not precisely identified, but it may relate to ionic interaction with one or several spore component, i.e. by attraction towards some positively charged structure and/or repulsion from some negatively charged structure (Nagler and Moeller, 2015). As spores have a negative surface charge, it could be speculated that the cationic salt component largely interacts with the spore crust, whereas the anion diffuses further into the spore, where it may interact with free carboxyl and amino groups, thereby altering spore and consequently germination properties (Kazakov *et al.*, 2008; Pesce *et al.*, 2014). Alternatively, the negative charge of the coat may repulse anions, thereby altering the physicochemical conditions around the spore and in the coat (Pesce *et al.*, 2014). Further research may answer this question. The different salts tested by Nagler and Moeller (2015) also had very diverse chemical properties, with chaotropic salts (supporting protein denaturation and complex disintegration) and transition metal chlorides being the most inhibitory salts analyzed (Sawyer and Puckridge, 1973; Zhang and Cremer, 2010; Nagler and Moeller, 2015). Thus, chemical reactions of salts altering spore structures (e.g. the OM and all underlying structures) and thereby germination behavior appear conceivable (Nagler and Moeller, 2015). However, no structural data is available to support such hypotheses, so that the nature of the aforementioned observations would have to be addressed in further studies.

The analysis of effects of different salt on *B. subtilis* spore germination also indicated positive, enhancing effects of some salts when these were present at low concentrations

(60 mM; Nagler and Moeller, 2015). This finding is consistent with several older studies (e.g. Fleming and Ordal, 1964; Foerster and Foster, 1966; Preston and Douthit, 1988). Although the mechanistic basis for this effect remains unclear, it was suggested earlier that low salt concentrations enhance the triggering of germination (Preston and Douthit, 1988). As only salts having monovalent cations (albeit not all) improved germination, the enhancement may mechanistically be similar to the cogerminative effect of K^+, especially as K^+ was proposed to support germination without specific GR-binding (Atluri *et al.*, 2006; Nagler and Moeller, 2015). However, additional data would be required to elucidate such a mechanism.

7.1.7. Germination of spores of other *Bacillus* species at high salinity

As with *B. subtilis*, spore germination of other *Bacillus* species (*B. megaterium, B. pumilus* SAFR-032*, B. nealsonii, B. mojavensis,* and *B. vallismortis*) was found to be increasingly detrimentally affected by increasing NaCl concentrations (Nagler *et al.*, 2016). However, the severity of germination inhibition was found to vary between species, *B. subtilis* (strain 168) spores being the least salt-sensitive among the tested species. The most likely reasons for variable salt-sensitivity seem to be interspecific structural variations, including variations in coat composition and structure, properties and constitution of the OM and IM, composition and properties of the spore core (e.g. mineralization, water content, and a_w), differences in germination proteins (GRs, ion, and Ca^{2+}-DPA channels), and structure of the germination apparatus (Henriques and Moran, 2007; Paredes-Sabja *et al.*, 2011; Bassi *et al.*, 2012; Setlow, 2013; Nagler *et al.*, 2016). Even the spores of the extremely closely related *B. subtilis, B. vallismortis,* and *B. mojavensis* differed notably in salt sensitivity during germination (Nagler *et al.*, 2016). A systematic study using phenotype-genotype linkage may provide powerful new insights on which factors contribute to salt inhibition, even if these factors may be masked by stronger effects.

7.1.8. Mechanistic model of high-salinity inhibition of *B. subtilis* spore germination

Based on the data generated within this doctoral thesis, the following model for *B. subtilis* spore germination in the presence of high NaCl concentrations can be proposed[3]. After mixing nutrient germinants and spores, the germinants diffuse into the spore coat.

[3] References (if not noted otherwise): Nagler *et al.*, 2014; Nagler and Moeller, 2015; Nagler *et al.*, 2015; Nagler *et al.*, 2016; Nagler *et al.*, *in preparation for submission*; K. Nagler, unpublished results; additional references are noted in the text

Germinant diffusion into and inside of the coat can be slowed by ionic interactions of Na^+ and Cl^- (i) with the germinant and/or (ii) with free carboxyl and amino groups of the coat proteins, which may alter the coat structure and permeability (**Fig. 10**; Kazakov *et al.*, 2008; Pesce *et al.*, 2014). GerP and possibly other proteins facilitate the passage of germinants through the coat and OM (Ferguson *et al.*, 2007; Butzin *et al.*, 2012; Setlow, 2014a). Although some Na^+ and Cl^- may be able to trespass the coat-OM complex as well, the majority of ions remains in the coat, which may even lead to the buildup of a chemical and osmotic gradient depending on the extent of OM permeability (Carstensen *et al.*, 1979).

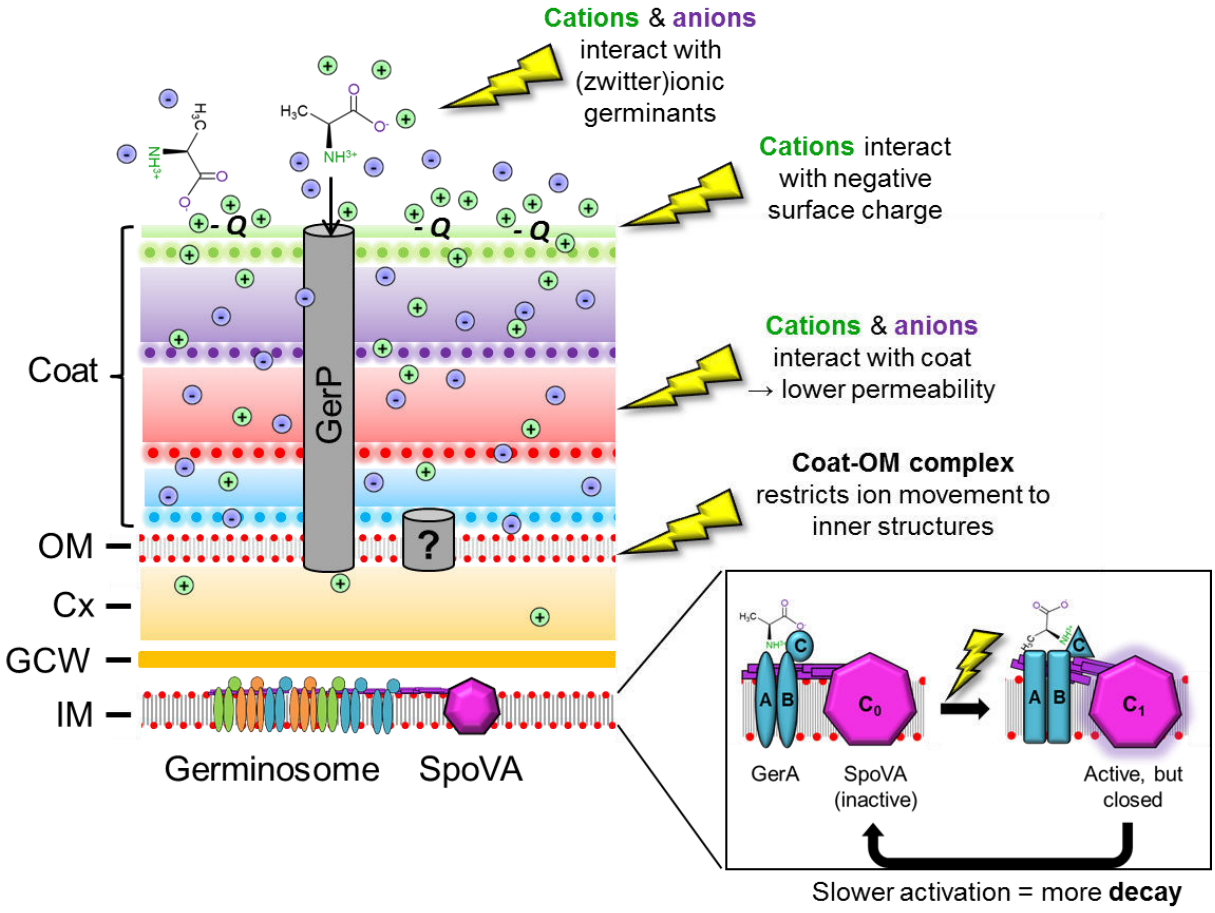

Figure 10: Mechanistic inhibition model: Inhibition at the level of germinant passage and GR activation. L-alanine is shown as germinant. The depicted structures are not drawn to scale. '–Q' = negative surface charge, OM = outer spore membrane, Cx = cortex, GCW = germ cell wall, IM = inner spore membrane, + = Na^+, - = Cl^-, '?' = putative unknown passage facilitator protein

In the absence of the coat-OM permeability barrier (e.g. due to chemical decoating or mutation), Na^+ and Cl^- can interact with the cortex (causing cortex contraction), germ cell wall, and/or the components germination apparatus, thereby causing exacerbated inhibition (Gould and Dring, 1975; Carstensen *et al.*, 1979; Nakatani *et al.*, 1985). In intact spores, however, germinants can bind to GRs without notable ionic disturbance, although high external salinity may also indirectly affect the biophysical conditions inside the OM in some fashion, e.g. by osmotically decreasing cortex hydration (Gould and Dring, 1975; Carstensen *et al.*, 1979; Poolman *et al.*, 2004). Germinant binding induces conformational changes in the respective GRs, which directly or indirectly transduce this information to SpoVA (**Fig. 10;** Vepachedu and Setlow, 2007b; Griffiths *et al.*, 2011; Li *et al.*, 2014; Perez-Valdespino *et al.*, 2014; Setlow, 2014a; Wang *et al.*, 2015b). The SpoVA channels store the germination stimuli in their metastable C_1 state until a threshold amount of C_1-state SpoVA channels is attained (Wang *et al.*, 2015b). Importantly, when high NaCl concentrations hamper germinant access to the GRs, this will increase the time until the threshold level of C_1-state SpoVA is reached. Hence, some of the metastable C_1-state SpoVA may decay back into the inactive C_0 state, resulting in further delay of commitment (**Fig. 7**; **Fig. 10;** Wang *et al.*, 2015b). Yet, when sufficient SpoVA channels are C_1-activated, cooperative action among SpoVA proteins (and possibly the germinosome) causes the conformational switch to the leaky O_1 state of SpoVA and the opening of unknown ion channels, so that the spore is now committed to germinate (Griffiths *et al.*, 2011; Setlow, 2013; Setlow, 2014a; Wang *et al.*, 2015a; Wang *et al.*, 2015b).

Nevertheless, major NaCl interference with germination occurs after commitment. The released ions and some of the initially leaked Ca^{2+}-DPA can interact with the electronegative cortical PG, thereby causing cortex contraction (**Fig. 11;** Gould and Dring, 1975; Nakatani *et al.*, 1985; De Vries, 2004). As a consequence of the decreased cortical pressure, the core starts to expand and take up more water (Gould and Dring, 1975; Bassi *et al.*, 2012). This core rehydration, however, is slowed osmotically by high external salinity (and equivalently by the high osmolarity caused by non-ionic solutes). As the Ca^{2+}-DPA in the core is mainly in its insoluble form, it cannot be released until the core becomes sufficiently hydrated, so that Ca^{2+}-DPA leakage prior to T_{lag} is slower[4] (**Fig. 9; Fig. 11;** Gould and Dring, 1975; Cowan *et al.*, 2003). Ultimately, when a threshold level of Ca^{2+}-DPA has leaked out of the core and was replaced by water, the tension in the IM becomes high enough to fully open the SpoVA channels (O_2 state) in mechanosensitive manner via SpoVAC at T_{lag}

[4] This effect is apparently more pronounced at ≥ 2.4 M NaCl, as the time between commitment and T_{lag} notably increases at such high salt concentrations.

(Poolman *et al.,* 2004; Velásquez *et al.*, 2014; Wang *et al.*, 2015a; Wang *et al.*, 2015b). Just as during the leakage phase prior to T_{lag}, Ca^{2+}-DPA efflux and water influx are coupled during $\Delta T_{release}$, and since the high external osmolarity still impedes core rehydration, the Ca^{2+}-DPA release rate is lower and $\Delta T_{release}$ is prolonged (**Fig. 9**; **Fig. 11**; Gould and Dring, 1975; Cowan *et al.*, 2003).

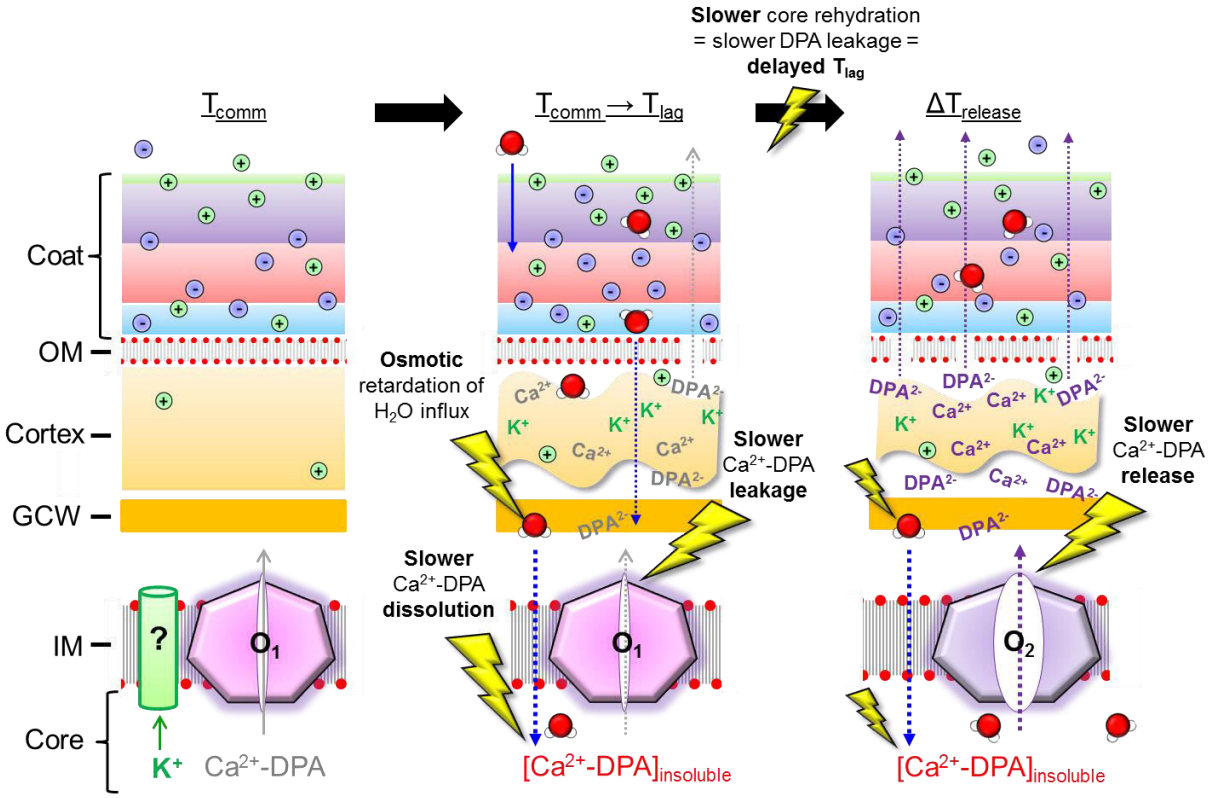

Figure 11: Mechanistic inhibition model: Inhibition at the level core rehydration and Ca^{2+}-DPA efflux. The ions released (and Ca^{2+}-DPA leaked) upon commitment (T_{comm}; left) induce cortex contraction, thereby allowing water influx (red/white molecules & blue arrows). Water influx is osmotically inhibited, leading to slower Ca^{2+}-DPA dissolution and thereby slower Ca^{2+}-DPA leakage, which delays T_{lag} (middle). Inhibition of core rehydration and concomitant Ca^{2+}-DPA continues in (and prolongs) $\Delta T_{release}$ (right). OM = outer spore membrane, GCW = germ cell wall, IM = inner spore membrane

Ca^{2+}-DPA release directly or indirectly leads to the activation of CwlJ and SleB (Popham *et al.*, 2012). Although cortex hydrolysis remains functional despite high salinity, the spores that initiate germination at ≥ 3.6 M NaCl never reach full core hydration for (osmotic) equilibrium reasons and mostly stay phase-gray. However, the water content can nevertheless become high enough to allow the onset of metabolic reactions, although prolonged survival seems rather unlikely.

While this model would explain the observations made for single spores, it may also prompt the question, why NaCl increases the heterogeneity of germination initiation in spore populations. Unfortunately, the exact reasons for heterogeneity in spore populations are not known, although many explanations including stochastic variations in gene expression during sporulation and physicochemical properties can be envisioned (Setlow *et al.*, 2012). Additionally, high salinity could interfere with spore-to-spore communication, which was proposed to be mediated by molecules released during germination (Zhang *et al.*, 2011). While Ca^{2+}-DPA and L-alanine did not act as signal molecules, maybe K^+ efflux could be involved, as K^+ also plays a role in biofilm signaling and no function has been ascribed to the ion efflux in germination so far (Zhang *et al.*, 2011; Beagle and Lockless, 2015; Prindle *et al.*, 2015).

Although the proposed model for *B. subtilis* spore germination in the presence of high NaCl concentrations requires several assumptions, most notably the validity of the osmoregulatory cortex concept (Gould and Dring, 1975) and the presence of an effective diffusion barrier for ions such as the OM, it would be consistent with the observations made within this doctoral thesis. Importantly, it does not only explain inhibition by high salinity, but also by non-ionic osmotic stress. Hence, germinant diffusion towards the GRs may be slowed due to viscosity (instead of ionic interactions), whereas Ca^{2+}-DPA release and core rehydration are hampered by high external osmolarity irrespective of whether the solutes are ionic or not. As osmotic inhibition of core rehydration (and concomitant inhibition of Ca^{2+}-DPA efflux) is a major factor in salt inhibition, the proposed model would also be in agreement with the observation of severe NaCl inhibition of non-nutrient germination that bypasses GRs. Moreover, as the proposed model is based on properties that are common to all *Bacillus* spores (i.e. dehydrated spore core with large amounts of Ca^{2+}-DPA that is enclosed in more or less conserved integuments), it should generally be transferrable to other *Bacillus* species as well, although interspecific variations may be important (Carstensen *et al.*, 1979; Henriques and Moran, 2007; Paredes-Sabja *et al.*, 2011; Bassi *et al.*, 2012; Setlow, 2013; Nagler *et al.*, 2016). Furthermore, the model may also represent a useful basis when analyzing inhibition mechanisms of other salts, as osmotic effects will always play a role, whereas additional effects (depending on the chemical properties of the salt) are expectable (Nagler and Moeller, 2015).

The model may also explain why *B. subtilis* spores apparently do not possess a salt-sensing system capable of prohibiting germination in non-growth permissive high-salinity

environments. Such a system would likely be localized in the same membrane as the germination apparatus, i.e. the IM. However, the presence of the coat-OM complex (or an alternative structure restricting ion movement into the inner spore integuments) as well as a cortex containing mobile ions would blind a salt-sensing system for the external ionic or osmotic stimulus, at least if the system would function in a similar manner as known osmosensory proteins (Poolman *et al*., 2004).

7.1.9. Outlook

In conclusion, the proposed model for *B. subtilis* spore germination in the presence of high NaCl concentrations would be consistent with the experimental results obtained in this doctoral thesis as well as with a broad range of published literature. However, it should be kept in mind that (i) many molecular details of germination are incompletely understood and that (ii) a variety of physical, chemical, and biological factors simultaneously (and often synergistically) contribute to the experimental observations, thus hampering the dissection of the effect in question (Poolman *et al*., 2004; Setlow, 2013; Setlow, 2014a). Hence, to obtain a better understanding of high-salinity effects on *Bacillus* spore germination, a more comprehensive understanding of molecular processes as well as physicochemical changes during germination under ideal conditions would be extremely important. As very much remains to be learned about germination itself (Setlow, 2014a), major questions concerning germination in general as well as germination at high salinity are summarized in **Table S1** (**Supplemental material IV.F**). Answers to at least some of these questions would provide important insights in the molecular germination mechanism and should therefore be addressed in future research.

In the specific context of the proposed salt inhibition model of spore germination, it would be of major importance to investigate (i) the capability of the coat-OM complex to restrict ion movement towards the inner spore integuments and (ii) the validity of the concept of an expanded osmoregulatory cortex. If these two paradigms can be verified or at least substantiated, it should subsequently be elucidated how exactly germinants gain access to their GRs (involvement of GerP and other proteins?) and how ionic interaction with the coat and germinants slows germinant passage to the GRs. The surprisingly enhanced germination of Δ*gerP* spores in the presence of up to 2.4 M NaCl might serve as a fruitful starting point (Nagler *et al*., 2015). Furthermore, single-spore germination analyses techniques could be utilized to study the reasons for increasing germination heterogeneity of spore populations at

increasing salt concentrations. Testing the aforementioned hypothesis that K^+ efflux upon commitment serves as a signal could also shed more light on the nature and properties of the ion efflux *per se*. Moreover, it would be interesting to identify the additional inhibition sites of salts other than NaCl (e.g. what additional damage is caused by perchlorates). Likewise, using a larger set of *Bacillus* species for a systematic, comparative analysis of spore revival at high salinity (and osmotic stress) may yield new insights on which spore properties determine salt sensitivity during germination and outgrowth.

7.2. Effects of high salinity on *B. subtilis* spore outgrowth

7.1.1. *B. subtilis* outgrowth capability at high salinity

The conversion of a dormant spore to a vegetative cell does not only require successful spore germination, but also accomplishment of the subsequent outgrowth phase (Keijser *et al.*, 2007; Setlow, 2013; Sinai *et al.*, 2015). Despite the detrimental high-salinity effects on *B. subtilis* spore germination, metabolic reactivation (as a hallmark of outgrowth initiation) could be observed at up to 4.8 M NaCl – a salinity at which active growth of a non-halophilic organism should not be possible (Boch *et al.*, 1994; Nagler *et al.*, 2014). Most likely the core water content in the few spores that initiated germination at such extreme salt concentrations got just high enough to breach enzymatic dormancy. In general, outgrowth success at high salinity strongly depends on the germination medium: while 0.6 M NaCl prohibited elongation and proliferation within 24 hours in minimal medium[5], nutrient-rich medium[6] allowed proliferation at up to 2.4 M NaCl (K. Nagler, unpublished data, **Supplemental material IV.F Fig. S5**). This is consistent with the observations made by Tovar-Rojo *et al.* (2003), who reported that sufficient energy was required for some unknown adaptation processes during outgrowth at high salinity.

7.1.2. Transcriptomic salt-stress response of outgrowing *B. subtilis* spores

To investigate, which salt stress adaptation processes are important during outgrowth in high-salinity environments, spores of *B. subtilis* were revived in the absence and presence of 1.2 M NaCl, and their transcriptome was subjected to RNA sequencing (RNA-seq) (Nagler

[5] Spizizen minimal medium supplemented with L-alanine, L-tryptophan, and D-glucose (Nicholson and Setlow, 1990)
[6] 1 x LB medium

et al., *in preparation for submission*). To minimize growth rate effects, the study was performed in minimal medium and investigated the transcriptomes 30 min, 60 min, and 90 min after germination initiation. Thus, the study focused only on the ripening phase that is dedicated to molecular reorganization in the absence of morphological changes (Segev *et al.*, 2013). In total, 402 different genes were upregulated and 632 genes were downregulated during 90 min of outgrowth in the presence of 1.2 M NaCl, indicating a strong impact of salt on the outgrowing spore (Nagler *et al.*, *in preparation for submission*). The effect of high salinity on the outgrowth transcriptome was the strongest after 30 min, which seems to be due to a combination of (i) salt stress-specific adaptations (e.g. upregulation of *opu* genes) and (ii) global retardation of molecular reorganization processes (reflected for instance by downregulation of amino acid biosynthesis genes; Nagler *et al.*, *in preparation for submission*) similar to reduced growth rates of vegetative cells at high salinity (Boch *et al.*, 1994; Hahne *et al.*, 2010). 30 to 90 min after germination, salt-stressed outgrowing spores induced their complete genetic repertoire of osmoprotectant uptake and compatible solute synthesis, emphasizing the pivotal role of these substances also during outgrowth (Nagler *et al.*, *in preparation for submission*). However, as suggested by continuous high *opuA* upregulation, the buildup of a sufficient intracellular compatible solute pool was apparently not possible, which seems plausible regarding the low nutrient availability (and absence of osmoprotectants) in the outgrowth medium used in the study (Hoffmann *et al.*, 2013; Nagler *et al.*, *in preparation for submission*). In this situation, sustained high intracellular concentrations of K^+ might have been the only possibility for turgor maintenance, even though this likely had detrimental effects on cellular functions and may have contributed to the delay of ripening processes (Record *et al.*, 1998; Segev *et al.*, 2013; Hoffmann and Bremer, 2016). Indeed, the outgrowing spores seemed to be challenged with high intracellular ion concentrations as suggested by an extensive upregulation of monovalent cation efflux transporter genes (Hoffmann and Bremer, 2016; Nagler *et al.*, *in preparation for submission*). In general, the expression of transporter and membrane protein genes was strongly altered by salt, pointing at a strong interaction with the inhospitable environment (Nagler *et al.*, *in preparation for submission*).

Although the spores in the RNA-seq study were suddenly confronted with high-salinity upon germination, the observed salt stress response differed from the typical response of salt-shocked vegetative cells, especially as the σ^B-dependent general stress response did not seem to play an important role and may not have been activated at all (Steil *et al.*, 2003; Nannapaneni *et al.*, 2012; Hoffman and Bremer, 2016; Nagler *et al.*, *in preparation for*

submission). Instead, osmospecific adaptations that are typically active in cells grown at sustained high salt concentrations seemed to play a pivotal role in the salt stress response of outgrowing spores (Steil *et al.*, 2003; Hahne *et al.*, 2010; Hoffman and Bremer, 2016; Nagler *et al.*, *in preparation for submission*). Consistent with previous studies on vegetative cells, the σ^W regulon (implicated in cell envelope stress) was found to be important in salt-stressed outgrowing spores (Steil *et al.*, 2003; Petersohn *et al.*, 2001; Höper *et al.*, 2006; Hahne *et al.*, 2010; Nagler *et al.*, *in preparation for submission*). Moreover, as in vegetative cells, motility and chemotaxis genes were repressed (Nagler *et al.*, *in preparation for submission*). While this observation seems quite tragic for the emerging cell at first sight, it might simply relate to the fact that the production of flagella is energetically very costly, because about 20,000 flagellin subunits have to be synthesized for one single filament (Mukherjee and Kearns, 2014). Aside from many similarities, there were also several transcriptomic differences between salt-stressed outgrowing spores and continuously salt-stressed cells, including the downregulation of σ^M and the lack of iron limitation (Hoffmann *et al.*, 2002; Steil *et al.*, 2003; Hahne *et al.*, 2010; Nagler *et al.*, *in preparation for submission*). These differences may be the consequence of two simultaneous challenges, i.e. ripening and osmoadaptation.

Taken together, the RNA-seq study conducted in the framework of this doctoral thesis (Nagler *et al.*, *in preparation for submission*) suggests that (i) high salinity severely changes the outgrowth transcriptome of *B. subtilis*, (ii) outgrowing spores utilize the same salt stress-specific response repertoire as continuously salt-stressed vegetative cells, (iii) outgrowing spores do not seem to employ the general stress response at 30 to 90 min after germination initiation at high salinity, and (iv) many genes of unknown function were differentially expressed, whose characterization may provide interesting new insights on *B. subtilis* salt stress adaptation and outgrowth.

7.2.3. Outlook

While the RNA-seq study (Nagler *et al.*, *in preparation for submission*) provided interesting insights on salt stress-induced transcriptomic changes in outgrowing spores, it also prompts many questions. To which extent do the transcriptomic alterations lead to actual proteomic changes? How is the cytoplasmic membrane remodeled during outgrowth in the presence and absence of osmotic stress? What happens in the time between germination and the 30 min outgrowth sample? What is the function of the σ^W regulon? Is σ^B completely dispensable? What is the function of the strongly up- and downregulated hypothetical and

poorly characterized genes? To which extent do internal spore resources as well as cortex and coat degradation products contribute to osmoprotection? How do outgrowing spores respond to different types and concentrations of osmoprotectants and compatible solutes in the surrounding medium? How is outgrowth affected by different salt concentrations and non-ionic stress? These and many other questions could be subjected in future research to gain new insights into spore ripening and outgrowth (especially under suboptimal conditions) as well as into how osmotic stress is sensed, transduced, and managed. Such knowledge is not only important for basic research, but can also be useful in applied research fields including soil ecology, food microbiology and astrobiology.

7.3. Conclusions

7.3.1. Implications for basic research

The results obtained throughout this doctoral thesis have provided new insights on spore germination and outgrowth under stress conditions. With regard to germination, it could be shown that the spore coat, presumably in complex with the OM, has an important protective function as a shield from ionic stress (Nagler *et al.*, 2015). Moreover, the impact of ionic stress significantly depends on the type of salt in question (Nagler and Moeller, 2015). Yet, the overall osmotic conditions are a major factor during germination: the obtained data suggest that the replacement of Ca^{2+}-DPA with water in the spore core may be important to reach a threshold pressure against the IM required for the induction of rapid Ca^{2+}-DPA release, possibly by opening SpoVA channels in mechanosensitive manner via SpoVAC (Nagler *et al.*, 2014; Velásquez *et al.*, 2014; Nagler *et al.*, 2015; Wang *et al.*, 2015b). Overall, the observations within this study were used to generate a first mechanistic model of high-salinity effects on *Bacillus* spore germination (**section 7.1.8**). Moreover, this doctoral thesis provides the first transcriptomic study on salt stress adaptation during *B. subtilis* outgrowth, which indicated many similarities, but also several interesting difference to the salt stress response of vegetative cells (Nagler *et al.*, *in preparation for submission*).

7.3.2. Implications for food microbiology

As repeatedly demonstrated, *Bacillus* spores generally have the capability to germinate and even initiate outgrowth despite very high NaCl concentrations (Nagler *et al.*, 2014; Nagler *et al.*, 2016; K. Nagler, unpublished, **Supplemental material IV.F Fig. S5**). Thus, salt

in food products will most likely not prevent spore germination at edible salt concentrations. However, salt can delay and slow germination and render germination initiation more heterogeneous, which could be problematic during the food's inactivation treatment (Setlow *et al.*, 2012; Reineke *et al.*, 2013; Nagler *et al.*, 2014; Sevenich *et al.*, 2015; Wells-Bennik *et al.*, 2016). The significantly slower Ca^{2+}-DPA efflux at elevated osmolarity may be of particular importance for high-pressure sterilization treatments during which Ca^{2+}-DPA release is the rate limiting step (Reineke *et al.*, 2013; Nagler *et al.*, 2014; Nagler *et al.*, 2015; Sevenich *et al.*, 2015). Importantly, the effects observed in *B. subtilis* are apparently transferable to other species as well (Nagler *et al.*, 2016) and might be helpful in guiding future research on *Bacillus* species that are more relevant for food spoilage and food-borne disease, such as *B. cereus*. Besides, as some salts were shown to have a high inhibitory potency whereas others enhanced germination at low concentrations, it may be of interest to analyze a food's ion composition to assess the effectiveness of an inactivation treatment based on the 'germinate to exterminate' strategy (Fleming and Ordal, 1964; Setlow *et al.*, 2012; Nagler and Moeller, 2015).

7.3.3. Implications for soil ecology

The results obtained in this doctoral thesis suggest that hypersalinization of soils can detrimentally interfere with the life cycles of soil bacteria such as *B. subtilis*. Hence, salt does not only suppress sporulation (Ruzal and Sánchez-Rivas; 1998; Widderich *et al.*, 2016) so that *B. subtilis* may eventually starve in nutrient-poor high-salinity environments, but it also inhibits spore revival. If soil salinity is still in a permissive range (equivalent to $\leq$ 1.2 M NaCl), *B. subtilis* spores can successfully germinate and initiate outgrowth (Nagler *et al.*, 2014; Nagler and Moeller, 2015). Yet, the emerging cell has a growth disadvantage and may not be able to escape the saline area, as (at least 90 min after germination) motility genes are strongly repressed (Nagler *et al.*, 2014; Nagler *et al.*, *in preparation for submission*; K. Nagler, unpublished data, **Supplemental material IV.F Fig. S5**). Moreover, if specific salts (e.g. transition metal chlorides like $ZnCl_2$) are present in relatively low concentrations (~ 60 mM) germination is severely inhibited, which could be important with regard to heavy metal polluted soils and bioremediation (Nagler and Moeller, 2015). If soil salinity is above a growth-permissive threshold, the transient availability of nutrients (e.g. due to application of organic fertilizers) may have drastic effects on the composition of the spore-forming soil bacteria population: due to the absence of a salt-sensing system, spores can initiate

germination, but have very low survival chances (Nagler *et al.*, 2014; Nagler and Moeller, 2015). Overall, salt can exert notable negative effects on the microbial soil community, which can in turn have a negative impact on soil quality, biogeochemical cycles and other associated organisms that interact with soil-dwelling microorganisms (Miller and Wood 1996; Kloepper *et al.*, 2004; Nicholson, 2004; Mandic-Mulec *et al.*, 2015; Nagler and Moeller, 2015).

7.3.4. Implications for astrobiology

As the results obtained within this doctoral thesis suggest that *Bacillus* spores can in principle revive under harsh environmental conditions beyond the usual growth spectrum of non-extremophilic bacteria, *Bacillus* spores are indeed an issue for planetary protection (Nagler *et al.*, 2014; Nagler *et al.*, 2016). Although the revival experiments conducted here were performed in the NaCl and a few other salts, Nicholson *et al.* (2012) showed that *B. subtilis* and the spacecraft assembly facility clean room isolate *B. pumilus* SAFR-032 are capable of germinating in aqueous extracts of a Mars analogue regolith mimicking the Phoenix landing site (Nagler and Moeller, 2015; Nagler *et al.*, 2016). However, even though *Bacillus* spores exhibited a remarkable potential to germinate despite high salinity, this is not directly transferrable to a real space mission scenario, in which the environmental influences (radiation, temperature, gravity, etc.) are much more complex (Nicholson *et al.*, 2000; Nicholson, 2004; Nicholson *et al.*, 2012; Nagler *et al.*, 2016). Thus, further research on *Bacillus* spore germination in the presence of high salinity and one or more additional stressors could provide more applicable insights with regard to astrobiology. Nevertheless, as *Bacillus* spore revival is strongly influenced by a multitude of factors, it is nearly impossible to predict, whether the spore bioload on a spacecraft can contaminate its destination, thus emphasizing the importance of rigorous spacecraft decontamination (Nagler *et al.*, 2016).

7.3.5. Concluding remarks

In conclusion, this doctoral thesis provides novel insight into the effects inhibiting of high salinity on *Bacillus* spore revival, thereby contributing to basic and applied research. Most notably, a first comprehensive mechanistic model of detrimental salt effects on spore germination could be developed. Growing mechanistic knowledge on the germination process itself will further enhance our understanding on the interference of ionic and osmotic stress with molecular germination events. Moreover, the first comprehensive transcriptomic analysis

of *B. subtilis* spore outgrowth at high salinity provides a rich foundation for future research on salt stress adaptation and on how stress affects the ripening phase of outgrowth. Nevertheless, much remains to be learned about *Bacillus subtilis* spore revival and how it is affected by adverse environmental conditions.

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receptors determines the average rate but not the heterogeneity of spore germination. J. Bacteriol. **195:**1735-1740.

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Zheng L, Abhyankar W, Ouwerling N, Dekker HL, van Veen H, van der Wel NN, Roseboom W, de Koning LJ, Brul S, de Koster CG. 2016. *Bacillus subtilis* spore inner membrane proteome. J. Proteome Res. **15**:585-594.

IV.A Supplemental material: Chapter 2

Nagler K, Setlow P, Li YQ, Moeller R. 2014. High salinity alters the germination behavior of *Bacillus subtilis* spores with nutrient and non-nutrient germinants. Appl. Environ. Microbiol. 80(4):1314-1321.

Figure S1 (Nagler *et al.*, 2014)

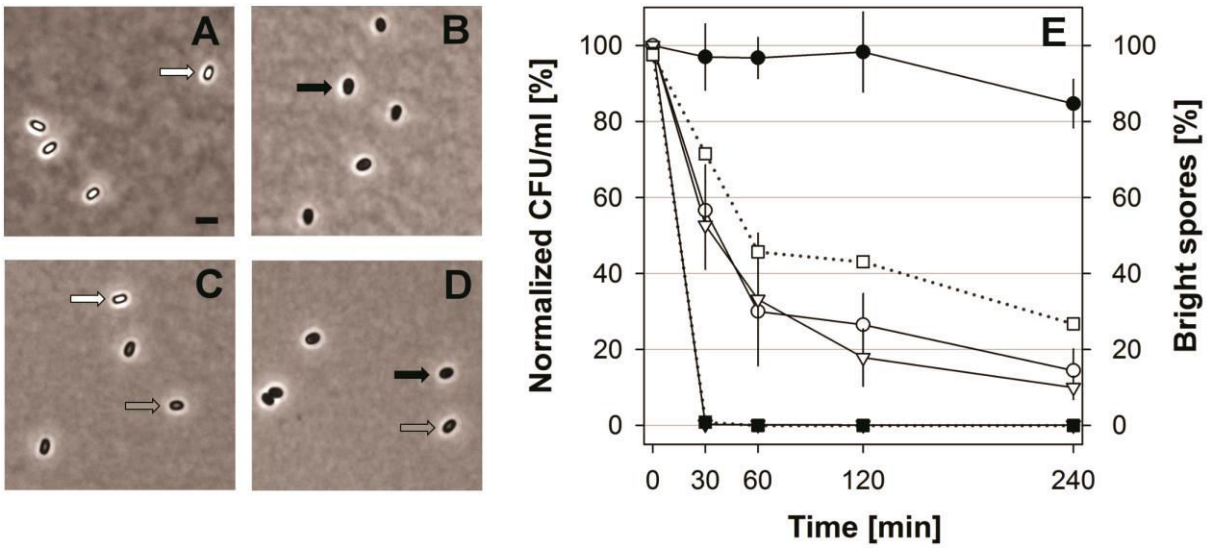


Figure S1. Phase-contrast microscopy, colony forming ability and heat resistance during L-alanine germination at various NaCl concentrations.

Spores were germinated with L-alanine and examined by phase-contrast microscopy and plating as described in Material and Methods. For microscopic analyses, the NaCl concentrations and time points of these representative pictures were (Panel **A**) 0 M, 0 min, (Panel **B**) 0 M, 30 min, (Panel **C**) 3.6 M, 240 min and (Panel **D**) 3.6 M after 30 min recovery as described in Methods. Spores were classified by their appearance as either bright (i.e. dormant; white arrows), phase-gray (gray arrows), and dark (i.e. germination completed; black arrows). Scale bar = 2 µm. (Panel **E**) Changes in CFU during L-alanine germination without NaCl (black symbols) and with 3.6 M NaCl (white symbols). Samples were plated directly (circles) or heat-treated at 80°C for 10 min before plating (triangles). The percentage of bright spores as counted by phase-contrast microscopy is indicated as well (squares).

IV.B Supplemental material: Chapter 3

Nagler K, Moeller R. 2015. Systematic investigation of germination responses of *Bacillus subtilis* spores in different high-salinity environments. FEMS Microbiol Ecol. 91(5). Pii:fiv023. Doi: 10.1093/femsec/fiv023. Epub 2015 Mar 10.

Table S1 (Nagler and Moeller, 2015). Phase-microscopic counts and classification according to spore refractivity after four hours of incubation in germination medium containing the given salt at the given concentration.

Salt	Concentration [M]	% Bright	% Gray	% Dark
		Mean ± SD	Mean ± SD	Mean ± SD
$(NH_4)_2SO_4$	2.4	10 ± 5	4 ± 3	86 ± 5
$CaCl_2$	1.2	67 ± 8	31 ± 6	3 ± 3
	2.4	97 ± 1	0 ± 1	2 ± 1
$CoCl_2$	1.2	21 ± 2	79 ± 2	0 ± 0
	2.4	99 ± 2	0 ± 0	1 ± 2
CsCl	2.4	47 ± 3	1 ± 1	52 ± 3
$CuCl_2$	0.06	7 ± 9	93 ± 8	0 ± 1
	0.6	34 ± 9	65 ± 9	0 ± 1
	1.2	83 ± 11	17 ± 11	0 ± 1
	2.4	97 ± 1	2 ± 1	1 ± 1
$FeCl_2$	0.06	1 ± 1	99 ± 1	0 ± 0
	0.6	14 ± 5	85 ± 5	1 ± 2
$FeCl_3$	0.06	31 ± 3	68 ± 3	1 ± 1
	0.6	79 ± 3	21 ± 3	0 ± 1
KBr	2.4	43 ± 6	1 ± 2	56 ± 6
KCl	2.4	33 ± 4	0 ± 0	67 ± 4
KF	0.06	0 ± 0	1 ± 2	99 ± 2
	0.6	71 ± 7	1 ± 2	28 ± 6
	1.2	80 ± 7	0 ± 0	20 ± 7
	2.4	86 ± 4	0 ± 0	14 ± 4
KI	0.6	3 ± 2	3 ± 2	94 ± 2
	1.2	65 ± 3	20 ± 2	15 ± 3
	2.4	99 ± 1	0 ± 1	1 ± 1
KNO_3	2.4	42 ± 4	5 ± 2	53 ± 4
K-HPO_4	2.4	67 ± 9	16 ± 11	18 ± 19
KSCN	0.6	71 ± 3	0 ± 1	28 ± 3
	1.2	99 ± 1	1 ± 1	0 ± 1

Salt	Concentration [M]	% Bright	% Gray	% Dark
LiCl	2.4	2 ± 1	98 ± 1	0 ± 1
$Mg(ClO_4)_2$	0.6	60 ± 9	40 ± 9	0 ± 1
	1.2	98 ± 2	1 ± 1	1 ± 2
$MgCl_2$	1.2	8 ± 4	90 ± 5	2 ± 2
	2.4	71 ± 6	28 ± 5	1 ± 2
$MgSO_4$	2.4	7 ± 3	2 ± 1	91 ± 3
$MnCl_2$	1.2	39 ± 7	60 ± 7	1 ± 1
	2.4	100 ± 0	0 ± 0	0 ± 0
Na_2SO_4	2.4	11 ± 8	2 ± 2	86 ± 9
NaBr	2.4	16 ± 7	1 ± 1	83 ± 7
NaCl	2.4	12 ± 3	0 ± 0	88 ± 3
$NaClO_4$	0.6	76 ± 9	0 ± 0	24 ± 9
	1.2	98 ± 2	1 ± 1	1 ± 1
NaF	0.06	0 ± 0	0 ± 0	100 ± 0
	0.6	56 ± 5	1 ± 1	44 ± 6
NaI	0.6	1 ± 1	0 ± 1	98 ± 1
	1.2	62 ± 3	36 ± 3	1 ± 1
	2.4	97 ± 1	1 ± 1	1 ± 1
$Na\text{-}HPO_4$	2.4	16 ± 4	14 ± 15	69 ± 19
NH_4Cl	2.4	3 ± 1	0 ± 1	97 ± 2
NH_4NO_3	2.4	47 ± 7	52 ± 7	2 ± 2
$NiCl_2$	1.2	55 ± 12	44 ± 11	1 ± 1
	2.4	96 ± 4	4 ± 4	0 ± 1
RbCl	2.4	64 ± 7	0 ± 0	36 ± 7
$SrCl_2$	1.2	57 ± 4	42 ± 2	2 ± 2
	2.4	99 ± 1	0 ± 0	1 ± 1
$ZnCl_2$	0.06	1 ± 1	98 ± 2	1 ± 2
	0.6	91 ± 4	7 ± 4	1 ± 2
	1.2	94 ± 3	5 ± 2	1 ± 1
	2.4	99 ± 2	1 ± 1	1 ± 1

SD = Standard Deviation

Figure S1 (Nagler and Moeller, 2015)

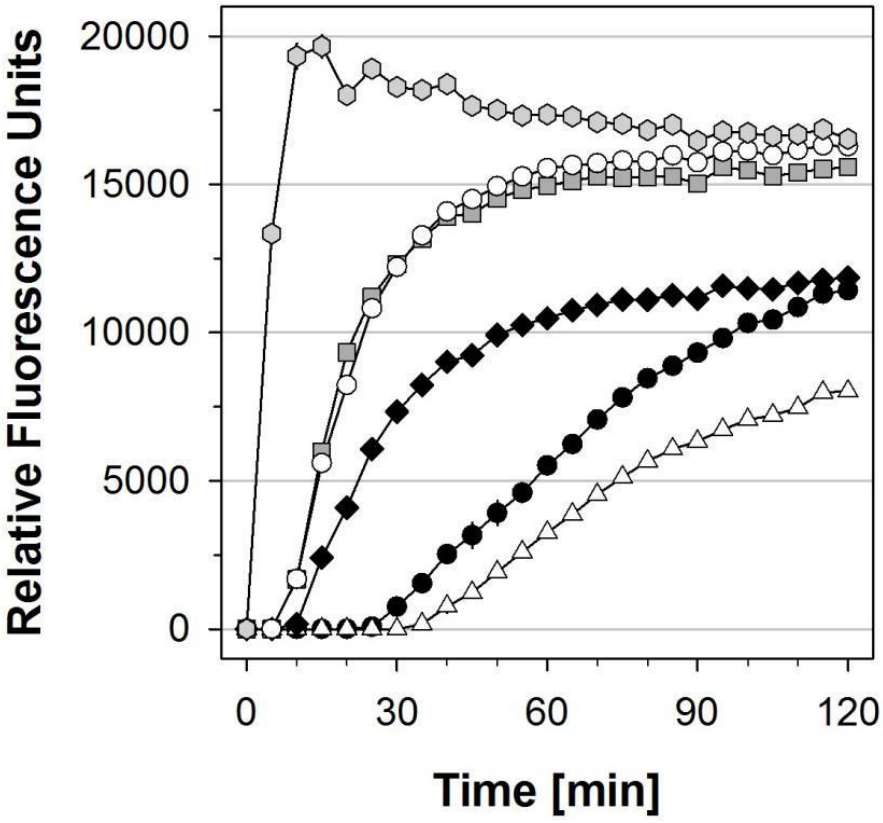
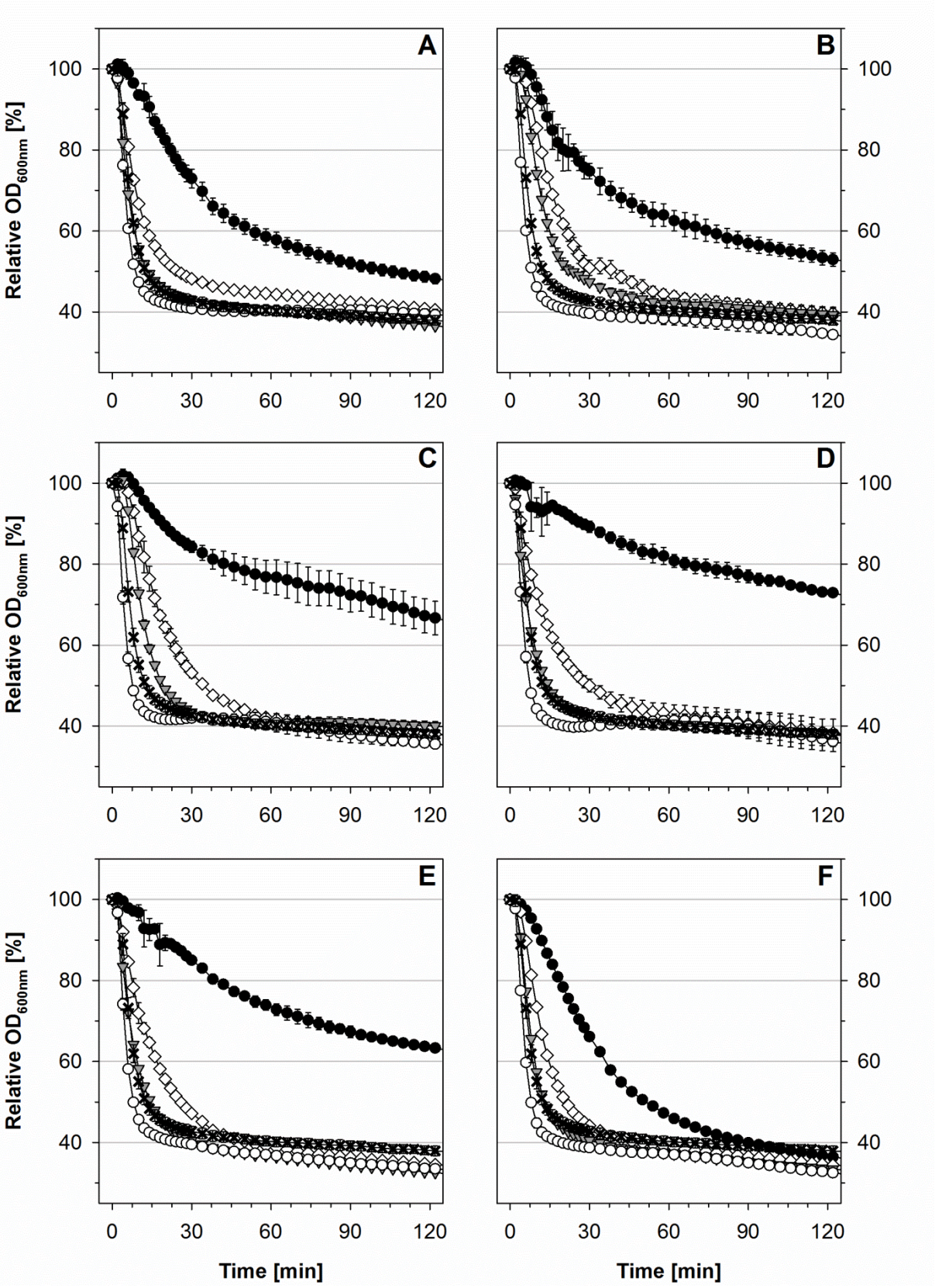
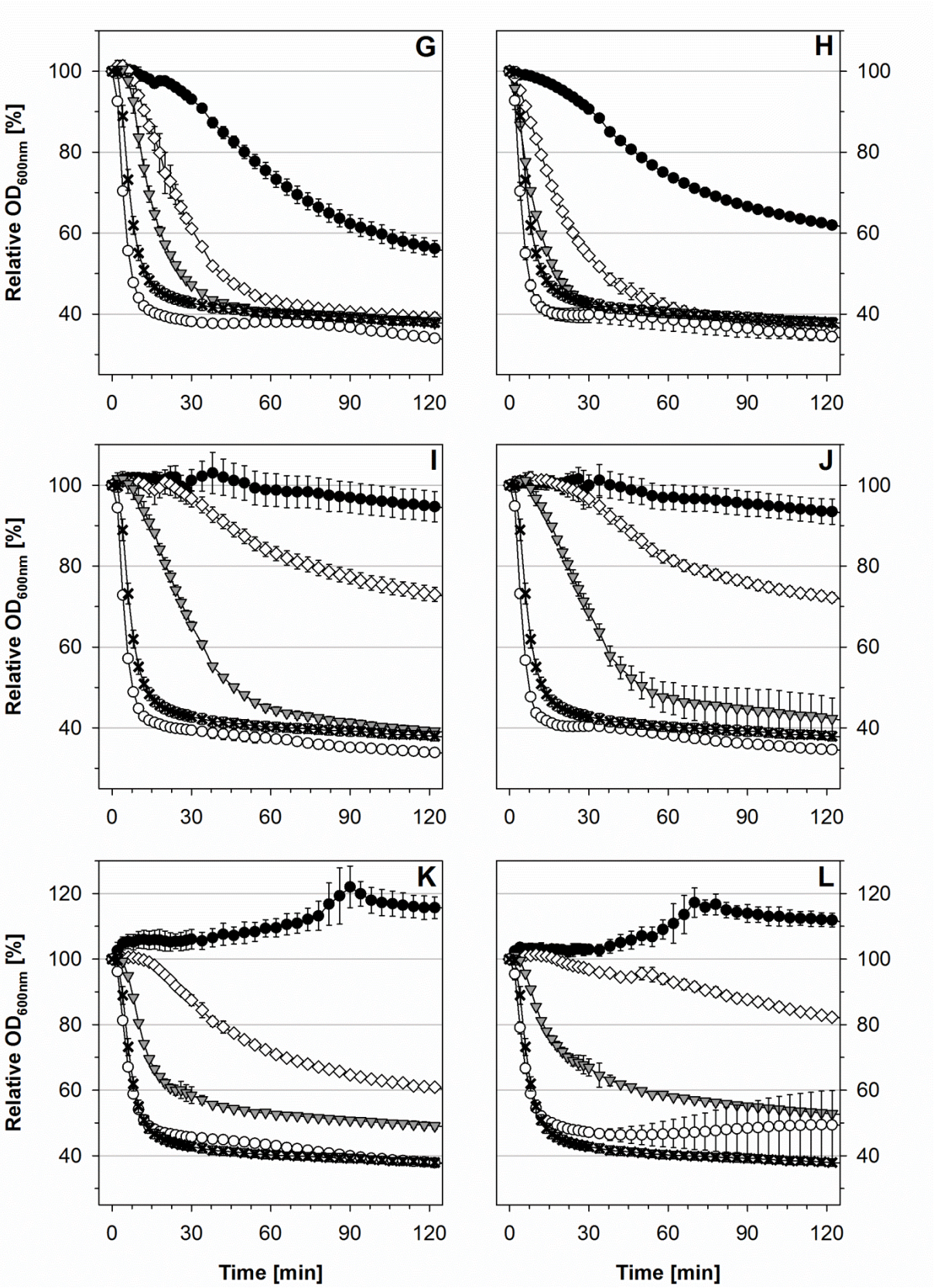


Figure S1. DPA release during germination in the presence of 2.4 M KBr (white triangles), NaBr (black circles), KCl (black diamonds), NaCl (gray squares), LiCl (white circles), and in the absence of salt (gray hexagons). DPA release was measured by the formation of fluorescent Tb-DPA complexes using a fluorometer as described in Material and Methods and in Yi & Setlow, 2010.

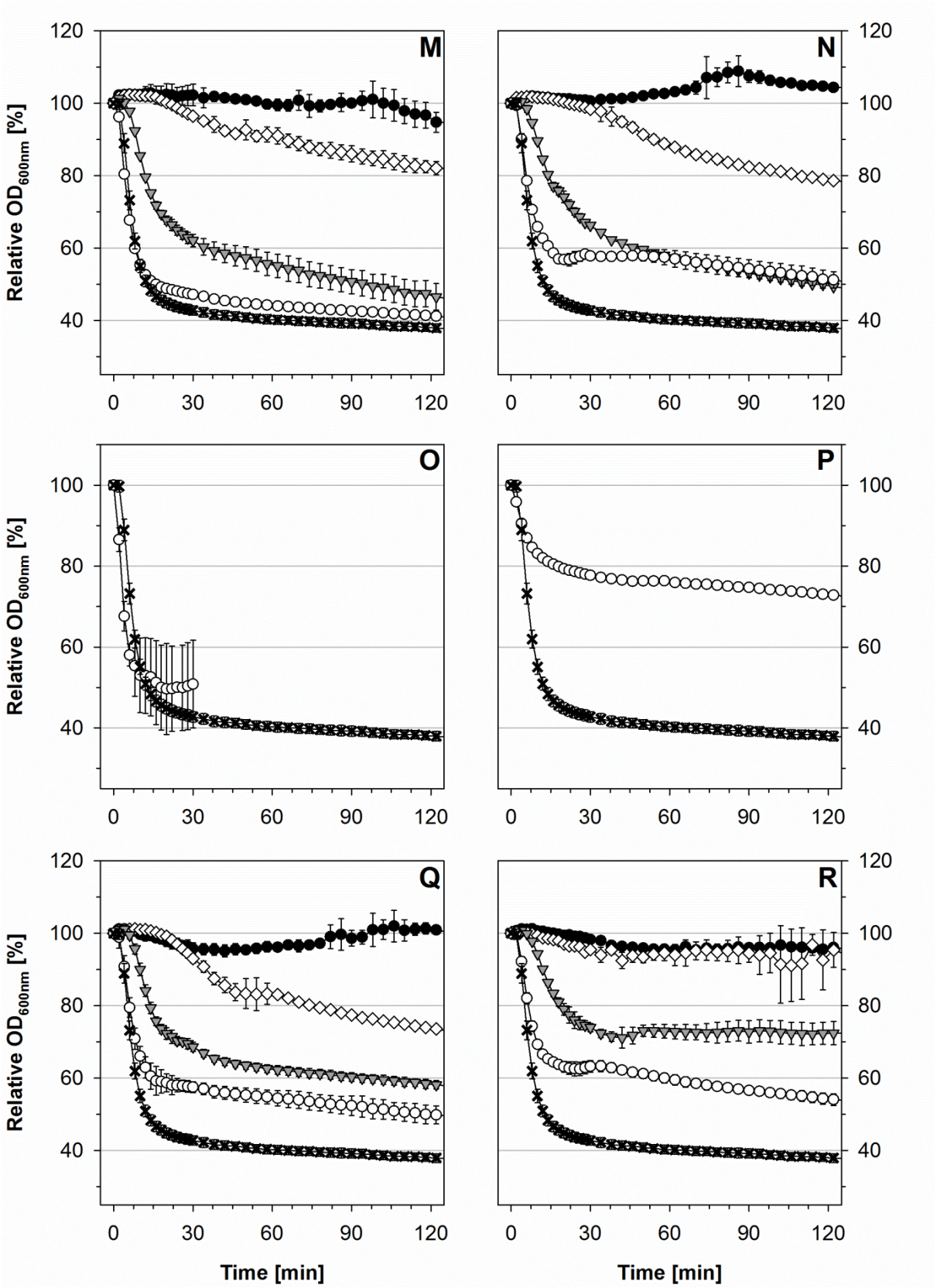
Figure S2 (Nagler and Moeller, 2015):



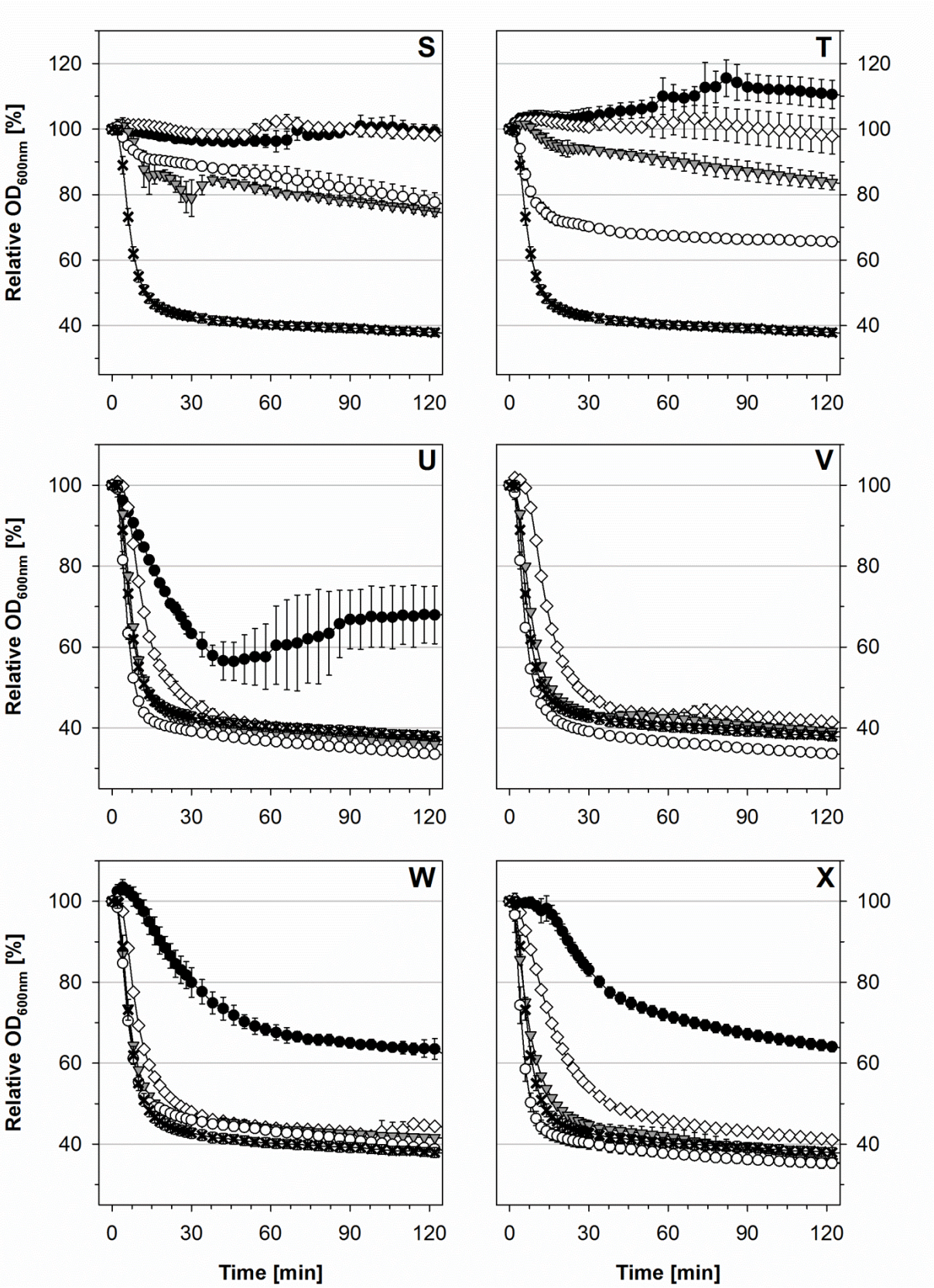
A = LiCl; **B** = NaCl; **C** = KCl; **D** = RbCl; **E** = CsCl; **F** = NH_4Cl



G = NaBr; **H** = KBr; **I** = NaI; **J** = KI; **K** = $MgCl_2$; **L** = $CaCl_2$



M = $SrCl_2$; **N** = $MnCl_2$; **O** = $FeCl_2$; **P** = $FeCl_3$; **Q** = $CoCl_2$; **R** = $NiCl_2$



S = $CuCl_2$; **T** = $ZnCl_2$; **U** = $(NH_4)_2SO_4$; **V** = Na_2SO_4; **W** = $MgSO_4$; **X** = NH_4NO_3

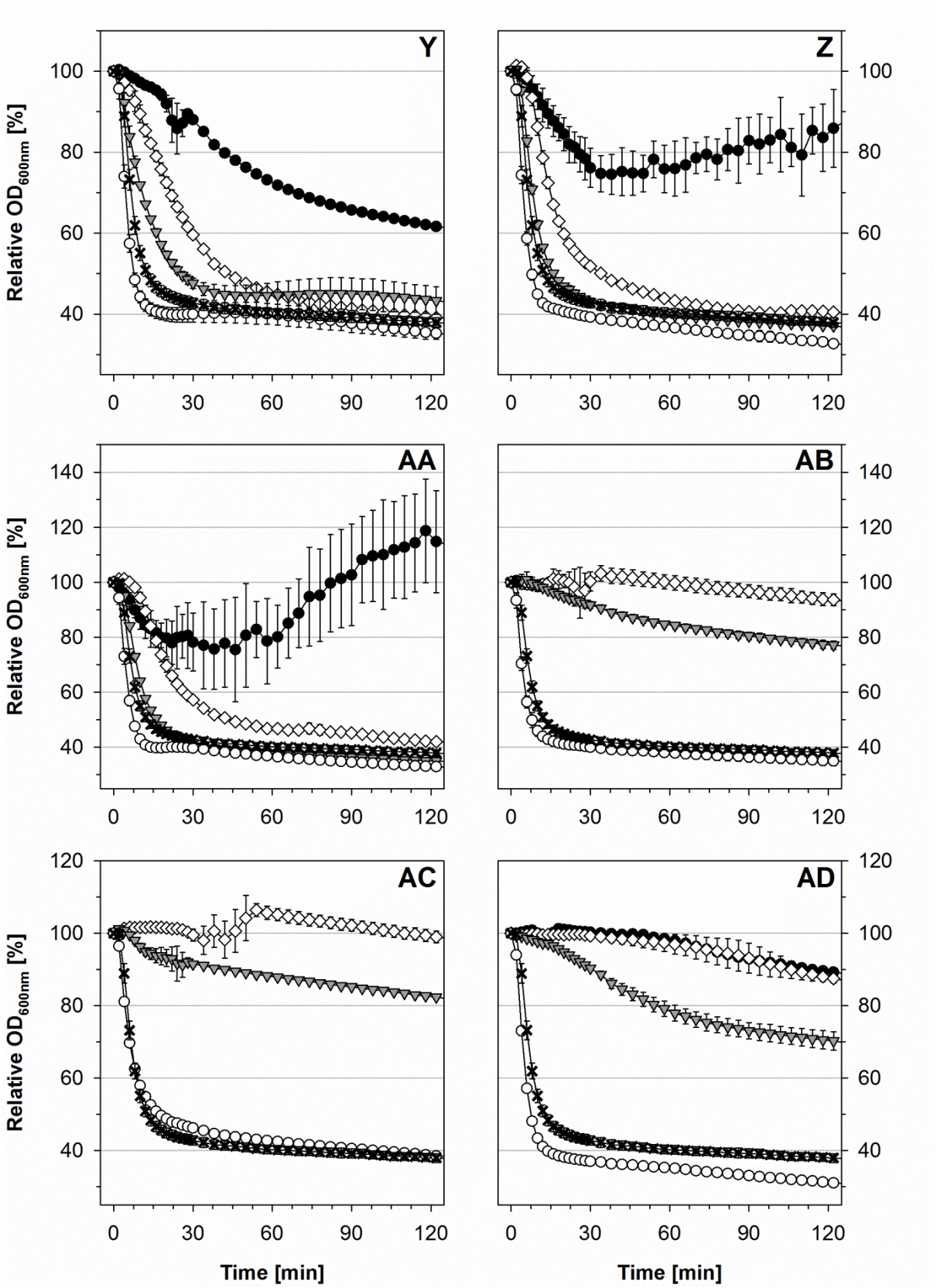


Figure S2: Spectrophotometric germination profiles. Shown are the changes in relative OD_{600nm} of the germination cultures over time in the presence of 0.06 M (white circles), 0.6 M (gray triangles), 1.2 M (white diamonds), and 2.4 M (black circles) of all tested salts. The germination profile in the absence of salts is indicated as black crosses in each diagram. The

profiles are denoted as follows: **A** = LiCl; **B** = NaCl; **C** = KCl; **D** = RbCl; **E** = CsCl; **F** = NH_4Cl; **G** = NaBr; **H** = KBr; **I** = NaI; **J** = KI; **K** = $MgCl_2$; **L** = $CaCl_2$; **M** = $SrCl_2$; **N** = $MnCl_2$; **O** = $FeCl_2$; **P** = $FeCl_3$; **Q** = $CoCl_2$; **R** = $NiCl_2$; **S** = $CuCl_2$; **T** = $ZnCl_2$; **U** = $(NH_4)_2SO_4$; **V** = Na_2SO_4; **W** = $MgSO_4$; **X** = NH_4NO_3; **Y** = KNO_3; **Z** = Na-HPO_4; **AA** = K-HPO_4; **AB** = $NaClO_4$; **AC** = $Mg(ClO_4)_2$; **AD** = KSCN.

Figure S3 (Nagler and Moeller, 2015)

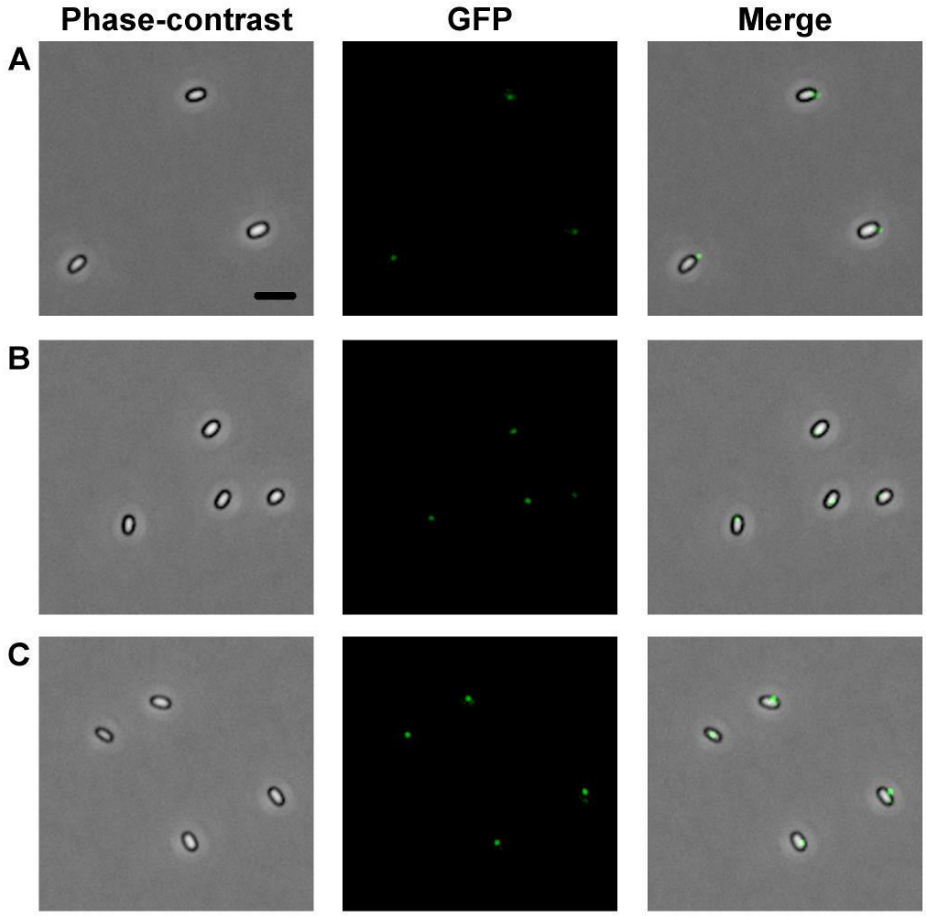
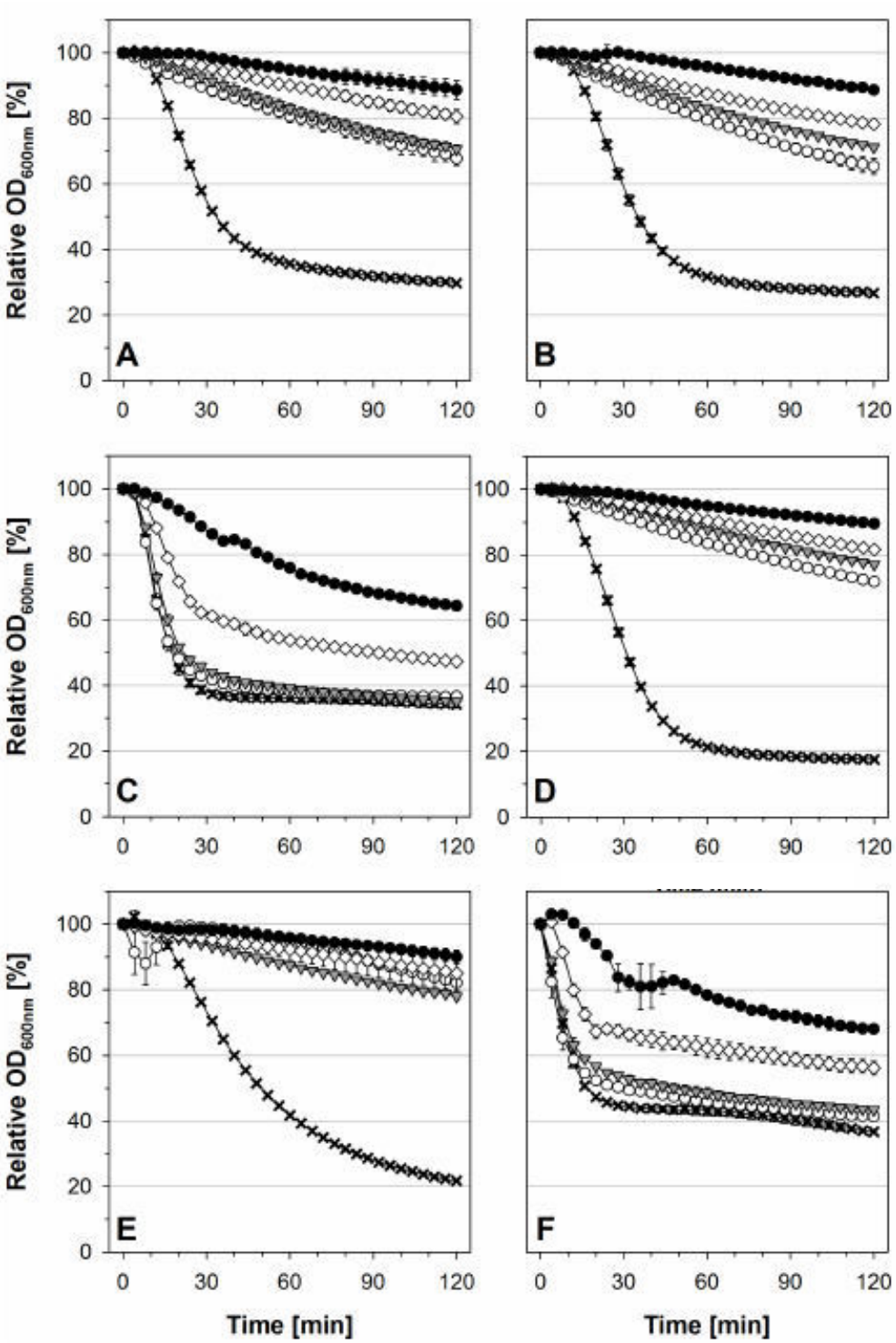


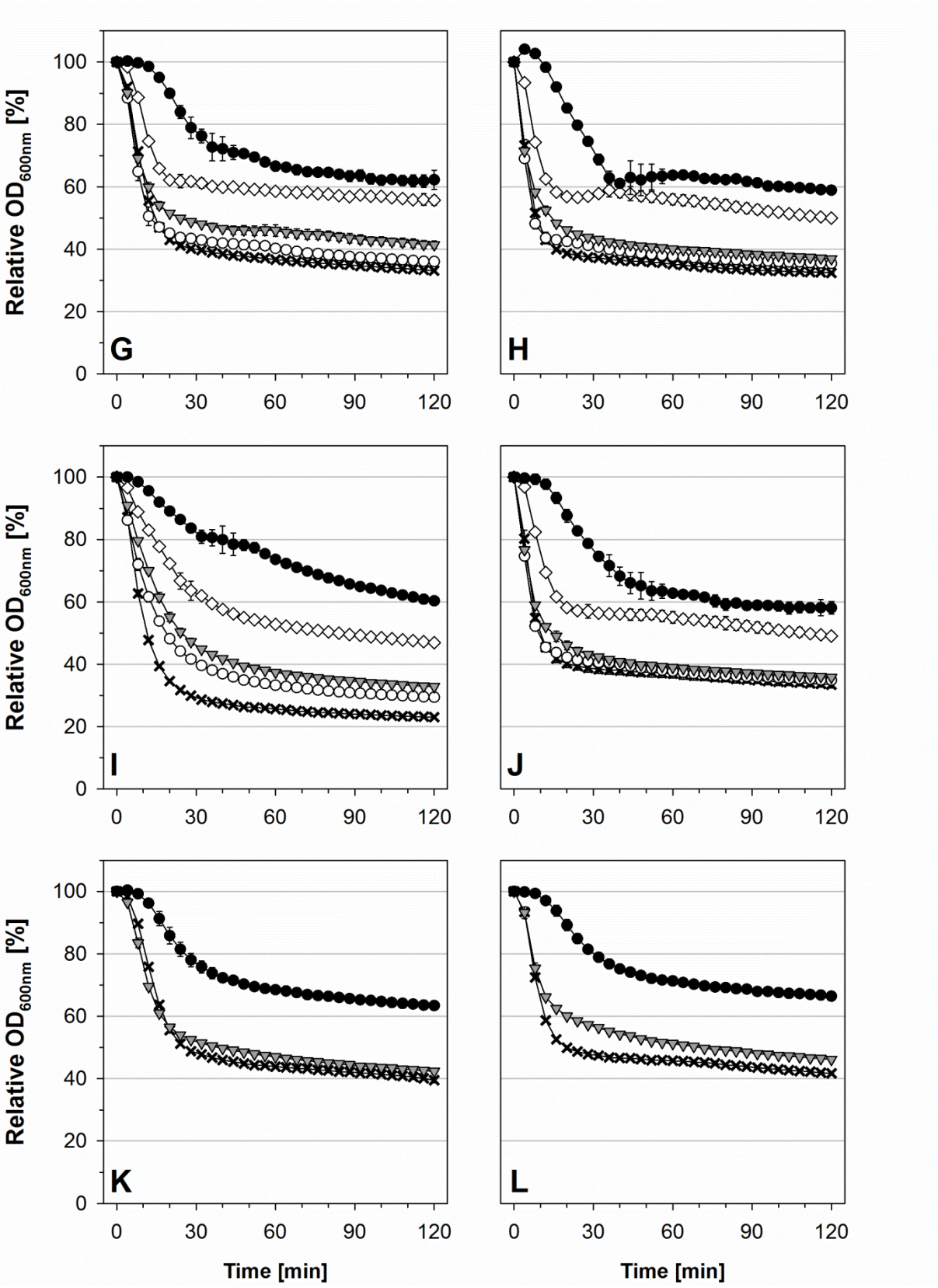
Figure S3: Effect of chaotropic salts on GerK-cluster integrity. Spores of the strain KGB04 (PS832 with *gerE*::spc, *cotE*::tet *gerKB-gfp* ermC; from Griffiths *et al.*, 2011) expressing the GFP-labeled GerK subunit GerKN were incubated in (A) H_2O, (B) 1.2 M KSCN, and (C) 1.2 M $Mg(ClO_4)_2$ for four hours and analyzed by fluorescence microscopy. GerK clusters (green foci) were detectable under all conditions. Scale bar = 2 µm

IV.C Supplemental material: Chapter 4

Nagler K, Setlow P, Reineke K, Driks A, Moeller R. 2015. Involvement of coat proteins in *Bacillus subtilis* spore germination in high-salinity environments. Appl. Environ. Microbiol. 81(19):6725-6735.

Figure S1 (Nagler *et al.*, 2015):




Relative OD600nm [%]
100
80
60
40
20
0
G
H
I
J
K
L
0
30
60
90
120
Time [min]

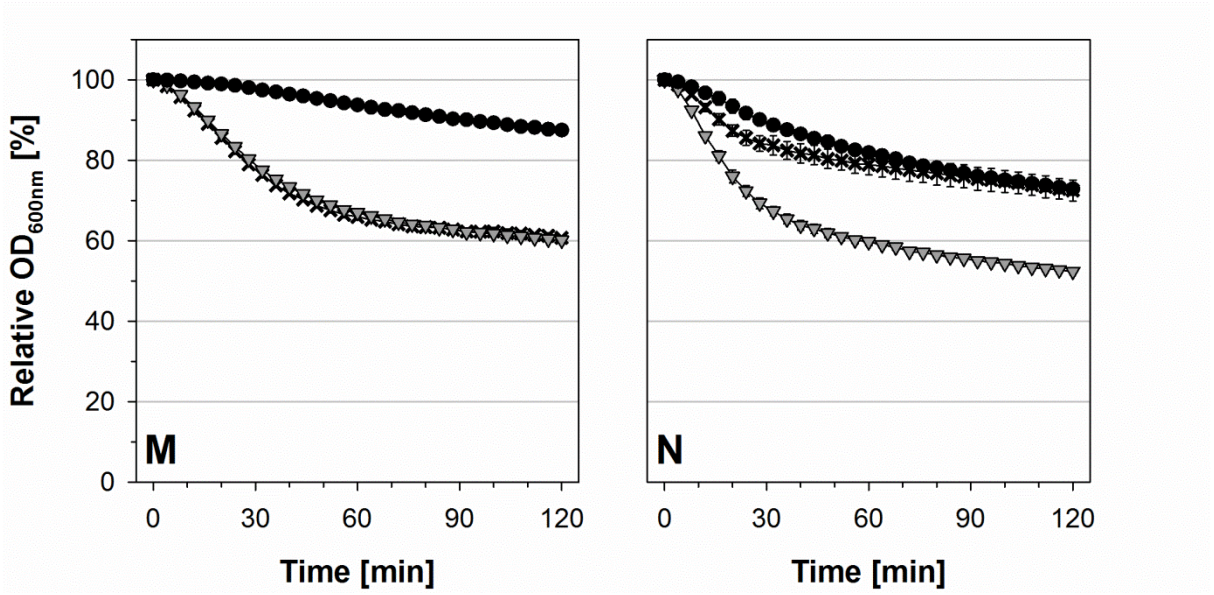


Figure S1: Germination profiles of spores of the different strains. Spores were germinated with L-alanine and OD_{600nm} was measured as described in Materials and Methods. Germination media contained no salt (black crosses), 0.6 M NaCl (white circles), 1.2 M NaCl (gray triangles), 2.4 M NaCl (white diamonds), or 3.6 M NaCl (black circles). (A) KN19 (*safA*), (B) KN20 (*cotE*), (C) KN21 (*cotXYZ*), (D) KN23 (*safA cotE*), (E) KN32 (*cotE gerE*), (F) KN33 (*cotB*), (G) KN34 (*cotC*), (H) KN35 (*cotG*), (I) KN36 (*cotH*), (J) KN37 (*cotS*), (K) KF111 (*cwlJ*), (L) KF112 (*sleB*), (M) KF113 (*cwlJ sleB*), (N) KW05 (*gerP*).

Figure S2:

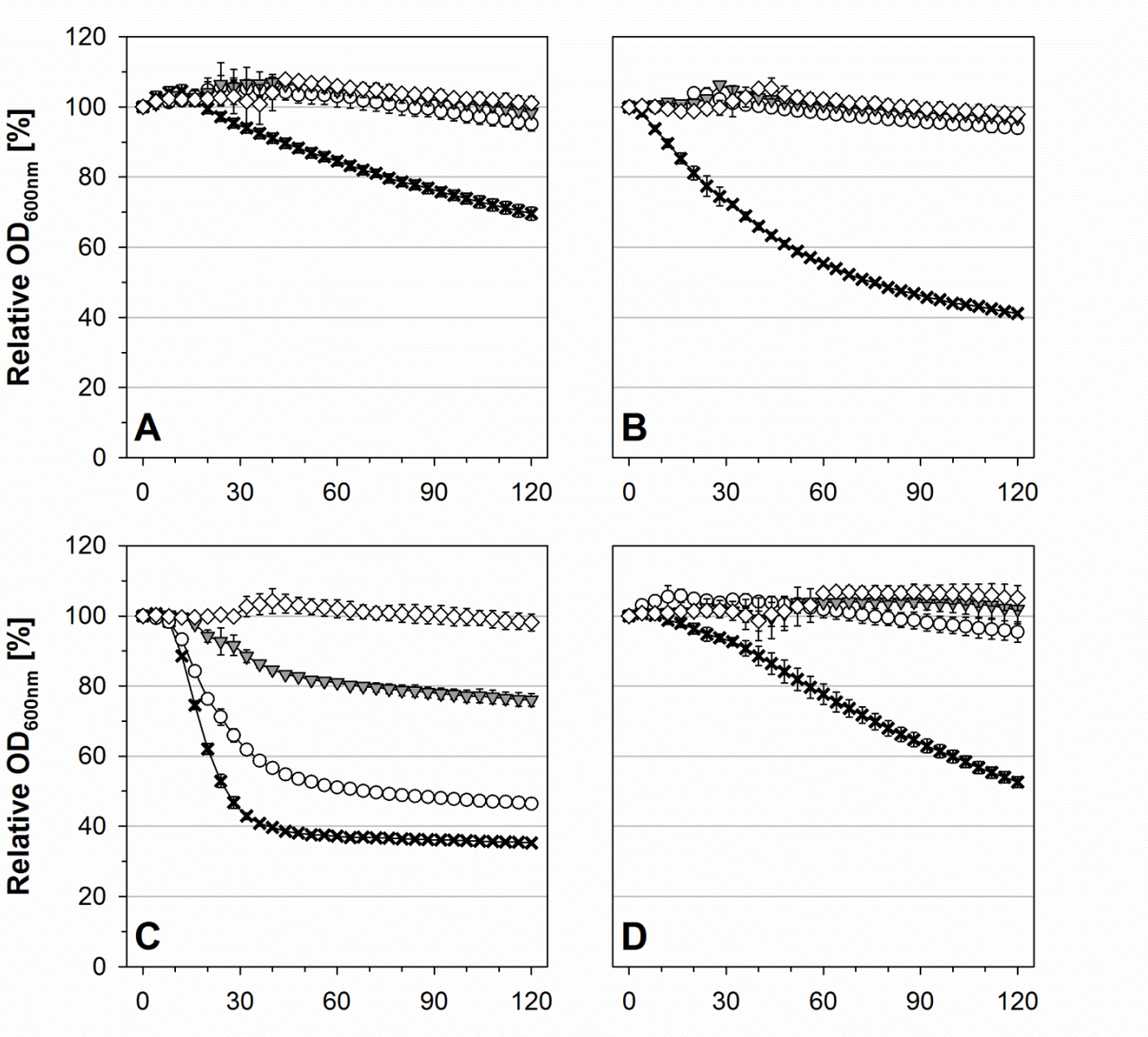


Figure S2: Germination profiles of spores of the different strains. Spores were germinated with AGFK and OD_{600nm} was measured as described in Materials and Methods. Germination media contained no salt (black crosses), 0.6 M NaCl (white circles), 1.2 M NaCl (gray triangles), and 2.4 M NaCl (white diamonds). (A) KN19 (*safA*), (B), KN20 (*cotE*), (C) KN21 (*cotXYZ*), (D) KN23 (*safA cotE*).

Figure S3 (Nagler *et al.*, 2015):

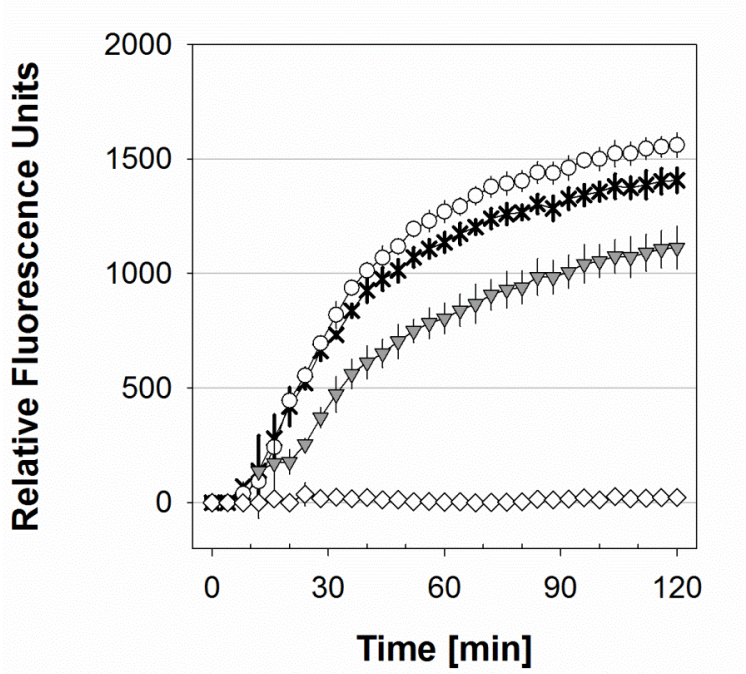


Figure S3: DPA release during AGFK germination of KW05 (*gerP*) spores. Germination media contained 0 M NaCl (black crosses), 0.6 M NaCl (white circles), 1.2 M NaCl (gray triangles), and 2.4 M NaCl (white diamonds).

Figure S4 (Nagler *et al.*, 2015):

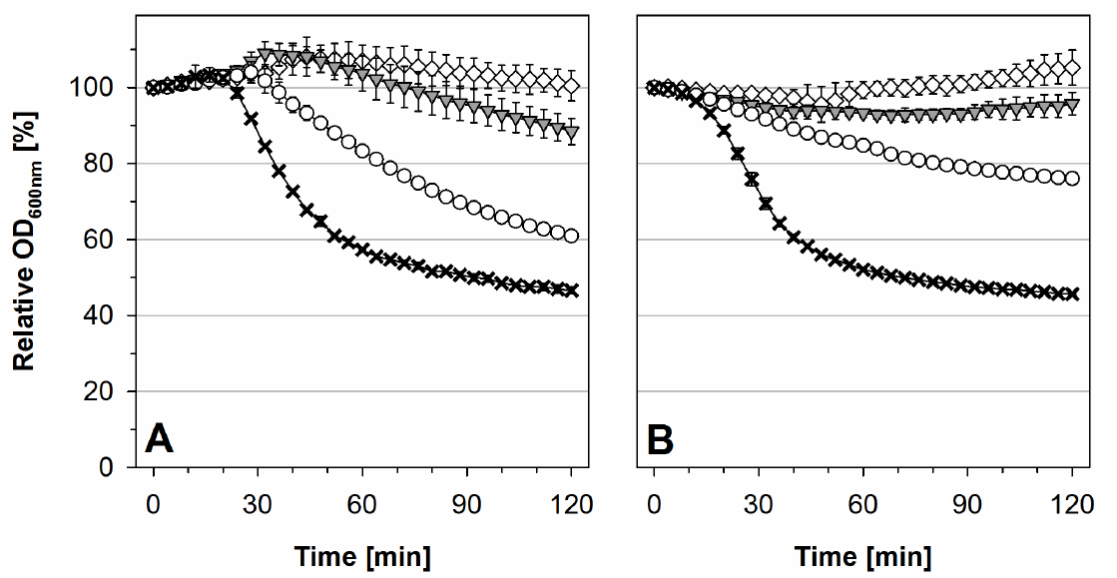


Figure S4: Germination of wild type (A) and *cotXYZ* (B) spores in response to exogenous Ca^{2+}-DPA. Germination media contained no salt (black crosses), 0.6 M NaCl (white circles), 1.2 M NaCl (gray triangles), or 2.4 M NaCl (white diamonds).

IV.D Supplemental material: Chapter 5

Nagler K, Julius C, Moeller R. 2016. Germination of spores of astrobiologically relevant *Bacillus* species in high-salinity environments. Astrobiology 16 (7), AST-2015-1419.R2. *Accepted for publication.*

Table S1 (Nagler *et al.*, 2016): Phase-contrast microscopy analyses of spore germination of various *Bacillus* species in 1x LB medium with different salinities*

Percentages of different phase-contrast microscopy phenotypes [a]											
Species [b]	NaCl [M]	% Bright		% Gray		% Dark		% Outgrowth		% Germ. [c]	
		MV	SD	MV	SD	MV	SD	MV	SD	MV	SD
B. sub	3.6	5	2	2	2	93	3	0	0	93	3
	2.4	0	1	4	5	42	22	54	26	96	5
	1.2	0	0	1	3	11	7	88	8	99	3
	0.6	1	1	0	1	3	3	97	3	99	1
	0	0	0	1	2	3	2	96	3	99	2
B. val	3.6	92	5	2	2	6	4	0	0	6	4
	2.4	89	3	1	1	9	3	2	1	10	3
	1.2	19	8	0	1	4	3	77	7	80	8
	0.6	13	12	0	0	3	2	84	13	87	12
	0	1	1	0	0	2	2	97	2	99	1
B. moj	3.6	68	10	6	4	26	9	0	0	26	9
	2.4	30	15	8	7	62	10	0	0	62	10
	1.2	10	4	4	3	23	4	63	4	86	4
	0.6	9	5	6	6	13	3	72	11	85	10
	0	4	5	3	3	19	11	74	16	93	7
B. pum	3.6	79	8	3	4	18	7	0	0	18	7
	2.4	30	4	2	1	68	4	0	0	68	4
	1.2	4	3	0	0	24	13	72	14	96	3
	0.6	4	4	0	0	11	10	86	14	96	4
	0	37	18	0	0	23	15	41	32	63	18
B. nea	3.6	95	2	0	0	5	2	0	0	5	2
	2.4	73	6	1	1	25	5	1	1	26	6
	1.2	27	10	0	1	13	4	60	10	73	10
	0.6	48	16	1	1	5	2	46	16	52	16
	0	93	5	1	1	4	3	3	3	6	4
B. meg	3.6	54	13	29	17	16	16	0	0	16	16
	2.4	13	12	21	17	66	24	0	0	66	24
	1.2	6	8	3	5	35	18	55	20	90	7
	0.6	3	3	8	6	28	19	61	23	89	7
	0	5	6	4	7	1	3	90	11	91	10

* Four hours after germination initiation, germination cultures were examined by phase-contrast microscopy as described in Materials and Methods.

[a] Spores were classified by their appearance as described in Nagler *et al.*, 2014

[b] *B. sub* = *B. subtilis* 168; *B. val* = *B. vallismortis*; *B. moj* = *B. mojavensis*; *B. pum* = *B. pumilus* SAFR-032; *B. nea* = *B. nealsonii*; *B. meg* = *B. megaterium*

[c] % Germinated = % Dark + % outgrowth

MV = Arithmetic mean value (on average ca. 400 spore counts per condition)

SD = Standard deviation

Table S2 (Nagler *et al.*, 2016): Germination in complex, nutrient rich medium with and without NaCl

Species[a]	NaCl [M]	Lag time [min][b]	v_{max} [% OD_{600nm} decrease pro min][b]	ΔOD (OD_{Start}-OD_{min}) [%][b]	Fig.[c]
B. sub	0	4 ± 0	7.3 ± 0.4	56 ± 1	1A
	0.6	4 ± 0	6.6 ± 0.1	53 ± 2	
	1.2	6 ± 0	5.7 ± 0.1	51 ± 2	
	2.4	8 ± 0	3.8 ± 0.1	47 ± 1	
	3.6	15 ± 1.2	1.1 ± 0	45 ± 2	
B. val	0	31 ± 1.2	1.7 ± 0.1	60 ± 1	1B
	0.6	30.5 ± 1	1.2 ± 0	58 ± 0	
	1.2	50.5 ± 1	0.5 ± 0	48 ± 2	
	2.4	xxx	xxx	xxx	
	3.6	xxx	xxx	xxx	
B. moj	0	13.5 ± 1	0.6 ± 0.1	27 ± 3	1C
	0.6	12 ± 0	0.9 ± 0.1	31 ± 4	
	1.2	15.5 ± 1	0.2 ± 0.1	17 ± 4	
	2.4	xxx	xxx	xxx	
	3.6	xxx	xxx	xxx	
B. pum	0	12.5 ± 7.7	0.7 ± 0.1	30 ± 5	1D
	0.6	12.5 ± 1	1.9 ± 0.1	55 ± 1	
	1.2	16 ± 8	1.2 ± 0.1	49 ± 3	
	2.4	36 ± 5.4	0.3 ± 0	32 ± 3	
	3.6	xxx	xxx	xxx	
B. nea	0	xxx	xxx	xxx	1E
	0.6	xxx	xxx	xxx	
	1.2	25 ± 1.2	0.2 ± 2	24 ± 4	
	2.4	xxx	xxx	xxx	
	3.6	xxx	xxx	xxx	
B. meg	0	0 ± 0	7.2 ± 1	37 ± 7	1F
	0.6	0 ± 0	4.8 ± 0.5	28 ± 2	
	1.2	2 ± 0	3.0 ± 0.2	30 ± 13	
	2.4	xxx	xxx	xxx	
	3.6	xxx	xxx	xxx	

[a] *B. sub = B. subtilis* 168; *B. val = B. vallismortis*; *B. moj = B. mojavensis*; *B. pum = B. pumilus* SAFR-032; *B. nea = B. nealsonii*; *B. meg = B. megaterium*

[b] The parameters lag time, v_{max}, and ΔOD were determined as described in Materials and Methods and are expressed as arithmetic mean values (of at least four replicates) ± standard deviation.

[c] Reference to corresponding germination profile in the main manuscript

xxx = germination was not unambiguously measurable via OD_{600nm} or parameters could not be determined based on the germination profile.

Table S3 (Nagler *et al.*, 2016): Phase-contrast microscopy analyses of spore germination of various *Bacillus* species with exogenous Ca^{2+}-DPA at different salinities*

Percentages of different phase-contrast microscopy phenotypes [a]											
Species [b]	**NaCl [M]**	**% Bright**		**% Gray**		**% Dark**		**% Outgrowth**		**% Germ.** [c]	
		MV	**SD**	**MV**	**SD**	**MV**	**SD**	**MV**	**SD**	**MV**	**SD**
B. sub	2.4	89	8	1	2	9	6	0	0	9	6
	1.2	72	8	1	1	27	7	0	0	27	7
	0.6	44	4	2	0	54	4	0	0	54	4
	0	4	3	2	2	94	4	0	0	94	4
B. val	2.4	2	1	1	1	98	2	0	0	98	2
	1.2	0	0	0	0	99	0	0	0	99	0
	0.6	0	0	1	1	99	1	0	0	99	1
	0	0	0	0	1	100	1	0	0	100	1
B. moj	2.4	61	8	3	3	36	7	0	0	36	7
	1.2	71	7	2	2	27	6	0	0	27	6
	0.6	48	6	3	2	49	6	0	0	49	6
	0	1	1	7	3	92	2	0	0	92	2
B. pum	2.4	56	7	1	2	43	8	0	0	43	8
	1.2	32	3	2	0	66	4	0	0	66	4
	0.6	27	5	0	1	72	5	0	0	72	5
	0	0	1	1	1	99	2	0	0	99	2
B. nea	2.4	96	2	1	1	4	2	0	0	4	2
	1.2	93	4	1	1	7	4	0	0	7	4
	0.6	93	2	0	0	7	2	0	0	7	2
	0	37	4	0	1	62	4	0	0	62	4
B. meg	2.4	23	16	18	23	58	16	0	0	58	16
	1.2	20	3	11	7	69	8	0	0	69	8
	0.6	12	8	8	5	78	10	2	6	80	9
	0	16	6	11	4	72	7	2	3	73	6

* Four hours after germination initiation, germination cultures were examined by phase-contrast microscopy as described in Materials and Methods.

[a] Spores were classified by their appearance as described in Nagler *et al.*, 2014

[b] *B. sub* = *B. subtilis* 168; *B. val* = *B. vallismortis*; *B. moj* = *B. mojavensis*; *B. pum* = *B. pumilus* SAFR-032; *B. nea* = *B. nealsonii*; *B. meg* = *B. megaterium*

[c] % Germinated = % Dark + % outgrowth

MV = Arithmetic mean value (on average ca. 400 spore counts per condition)

SD = Standard Deviation

Table S4 (Nagler *et al.*, 2016): Non-nutrient germination with exogenous Ca^{2+}-DPA

Species[a]	NaCl [M]	Lag time [min][b]	v_{max} [% OD_{600nm} decrease pro min][b]	ΔOD (OD_{Start}-OD_{min}) [%][b]	Fig.[c]
B. sub	0	22.5 ± 1	0.9 ± 0.1	56 ± 2	2A
	0.6	30 ± 1.6	0.3 ± 0	44 ± 1	
	1.2	57.5 ± 1.9	0.1 ± 0	26 ± 1	
	2.4	xxx	xxx	xxx	
B. val	0	10 ± 0	6.3 ± 0.3	64 ± 2	2B
	0.6	10 ± 0	7.0 ± 0.2	68 ± 1	
	1.2	10 ± 0	6.4 ± 0.1	66 ± 1	
	2.4	15.6 ± 0.9	3.9 ± 0.2	64 ± 1	
B. moj	0	16.4 ± 0.9	2.3 ± 0.1	41 ± 2	2E
	0.6	xxx	xxx	xxx	
	1.2	xxx	xxx	xxx	
	2.4	xxx	xxx	xxx	
B. pum	0	10 ± 0	6.9 ± 0.2	66 ± 2	2C
	0.6	16 ± 0	3.4 ± 0	64 ± 1	
	1.2	26.6 ± 0.9	1.8 ± 0.1	56 ± 2	
	2.4	xxx	xxx	xxx	
B. nea	0	xxx	xxx	xxx	2F
	0.6	xxx	xxx	xxx	
	1.2	xxx	xxx	xxx	
	2.4	xxx	xxx	xxx	
B. meg	0	xxx	xxx	xxx	2D
	0.6	xxx	xxx	xxx	
	1.2	xxx	xxx	xxx	
	2.4	xxx	xxx	xxx	

[a] *B. sub* = *B. subtilis* 168; *B. val* = *B. vallismortis*; *B. moj* = *B. mojavensis*; *B. pum* = *B. pumilus* SAFR-032; *B. nea* = *B. nealsonii*; *B. meg* = *B. megaterium*

[b] The parameters lag time, v_{max}, and ΔOD were determined as described in Materials and Methods and are expressed as arithmetic mean values (of at least four replicates) ± standard deviation.

[c] Reference to corresponding germination profile in the main manuscript

xxx = germination was not unambiguously measurable via OD_{600nm} or parameters could not be determined based on the germination profile.

Table S5 (Nagler *et al.*, 2016): Phase-contrast microscopy analyses of spore germination of various *Bacillus* species with KAGE at different salinities*

Percentages of different phase-contrast microscopy phenotypes [a]											
Species [b]	NaCl [M]	% Bright		% Gray		% Dark		% Outgrowth		% Germ. [c]	
		MV	SD	MV	SD	MV	SD	MV	SD	MV	SD
B. sub	3.6	11	3	3	3	86	3	0	0	86	3
	2.4	3	4	2	2	95	4	0	0	95	4
	1.2	1	2	1	1	98	2	0	0	98	2
	0.6	0	0	1	1	99	1	0	0	99	1
	0	0	0	0	1	99	1	1	1	100	1
B. val	3.6	95	6	2	2	3	5	0	0	3	5
	2.4	81	22	0	1	19	22	0	0	19	22
	1.2	55	13	1	2	44	14	0	0	44	14
	0.6	19	9	1	1	80	9	0	0	80	9
	0	8	7	0	1	90	8	1	3	92	7
B. moj	3.6	66	8	5	3	29	7	0	0	29	7
	2.4	23	8	5	4	72	8	0	0	72	8
	1.2	6	2	9	6	85	8	0	0	85	8
	0.6	3	3	5	4	91	6	0	0	91	6
	0	1	1	3	3	94	3	1	2	96	3
B. pum	3.6	49	5	16	4	35	2	0	0	35	2
	2.4	0	0	0	0	100	0	0	0	100	0
	1.2	0	0	0	0	100	0	0	0	100	0
	0.6	0	0	0	0	100	1	0	1	100	0
	0	0	0	0	0	100	0	0	0	100	0
B. nea	3.6	85	5	12	4	3	2	0	0	3	2
	2.4	34	4	1	2	65	5	0	0	65	5
	1.2	7	2	1	0	93	2	0	0	93	2
	0.6	12	6	1	1	83	11	4	5	87	7
	0	5	5	12	27	24	6	59	30	83	24
B. meg	3.6	72	8	24	6	4	5	0	0	4	5
	2.4	11	13	27	21	62	24	0	0	62	24
	1.2	11	8	16	6	73	10	0	0	73	10
	0.6	10	3	13	5	77	5	0	0	77	5
	0	11	3	14	3	69	4	7	2	75	4

* Four hours after germination initiation, germination cultures were examined by phase-contrast microscopy as described in Materials and Methods.

[a] Spores were classified by their appearance as described in Nagler *et al.*, 2014

[b] *B. sub* = *B. subtilis* 168; *B. val* = *B. vallismortis*; *B. moj* = *B. mojavensis*; *B. pum* = *B. pumilus* SAFR-032; *B. nea* = *B. nealsonii*; *B. meg* = *B. megaterium*

[c] % Germinated = % Dark + % outgrowth

MV = Arithmetic mean value (on average ca. 400 spore counts per condition)

SD = Standard Deviation

Table S6 (Nagler *et al.*, 2016): Germination with the novel germinant mixture KAGE (10 mg/ml of each KCl, L-alanine, D-glucose, and ectoine) in 10 mM Tris-HCl pH 8

Species[a]	NaCl [M]	Lag time [min][b]	v_{max} [% OD_{600nm} decrease pro min][b]	ΔOD * (OD_{Start}-OD_{min}) [%][b]	Fig.[c]
B. sub	0	2 ± 0	5.6 ± 0.3	64 ± 3	3B
	0.6	2 ± 0	4.7 ± 0.2	65 ± 2	
	1.2	2 ± 0	3.4 ± 0.1	60 ± 1	
	2.4	6.5 ± 1	1.7 ± 0.1	44 ± 3	
	3.6	xxx	xxx	xxx	
B. val	0	17.5 ± 1	3.6 ± 0.1	72 ± 1	3A
	0.6	17.5 ± 1	1.8 ± 0	70 ± 0	
	1.2	9 ± 1.2	0.6 ± 0	59 ± 1	
	2.4	xxx	xxx	xxx	
	3.6	xxx	xxx	xxx	
B. moj	0	6 ± 0	2.5 ± 0.1	48 ± 0	3E
	0.6	8.5 ± 1	1.5 ± 0	42 ± 0	
	1.2	10 ± 0	0.8 ± 0	30 ± 1	
	2.4	xxx	xxx	xxx	
	3.6	xxx	xxx	xxx	
B. pum	0	10 ± 0	3.3 ± 0.1	58 ± 2	3C
	0.6	10 ± 0	2.7 ± 0.1	57 ± 2	
	1.2	13.5 ± 1	1.4 ± 0.1	53 ± 1	
	2.4	45.5 ± 1	0.3 ± 0	27 ± 6	
	3.6	xxx	xxx	xxx	
B. nea	0	12 ± 0	2.1 ± 0	66 ± 0	3D
	0.6	8 ± 0	2.2 ± 0.2	60 ± 1	
	1.2	8 ± 0	1.2 ± 0	59 ± 1	
	2.4	19.5 ± 1.9	0.2 ± 0	34 ± 2	
	3.6	xxx	xxx	xxx	
B. meg	0	0 ± 0	4.7 ± 0.6	27 ± 4	3F
	0.6	1.5 ± 1	1.0 ± 0.2	13 ± 2	
	1.2	3 ± 2	2.2 ± 0.6	18 ± 6	
	2.4	xxx	xxx	xxx	
	3.6	xxx	xxx	xxx	

[a] *B. sub* = *B. subtilis* 168; *B. val* = *B. vallismortis*; *B. moj* = *B. mojavensis*; *B. pum* = *B. pumilus* SAFR-032; *B. nea* = *B. nealsonii*; *B. meg* = *B. megaterium*

[b] The parameters lag time, v_{max}, and ΔOD were determined as described in Materials and Methods and are expressed as arithmetic mean values (of at least four replicates) ± standard deviation.

[c] Reference to corresponding germination profile in the main manuscript

xxx = germination was not unambiguously measurable via OD_{600nm} or parameters could not be determined based on the germination profile.

Figure S1 (Nagler *et al.*, 2016):

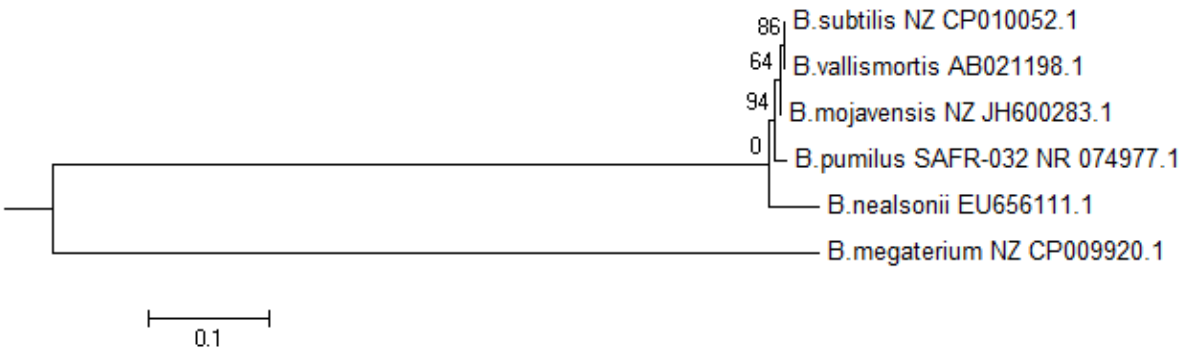
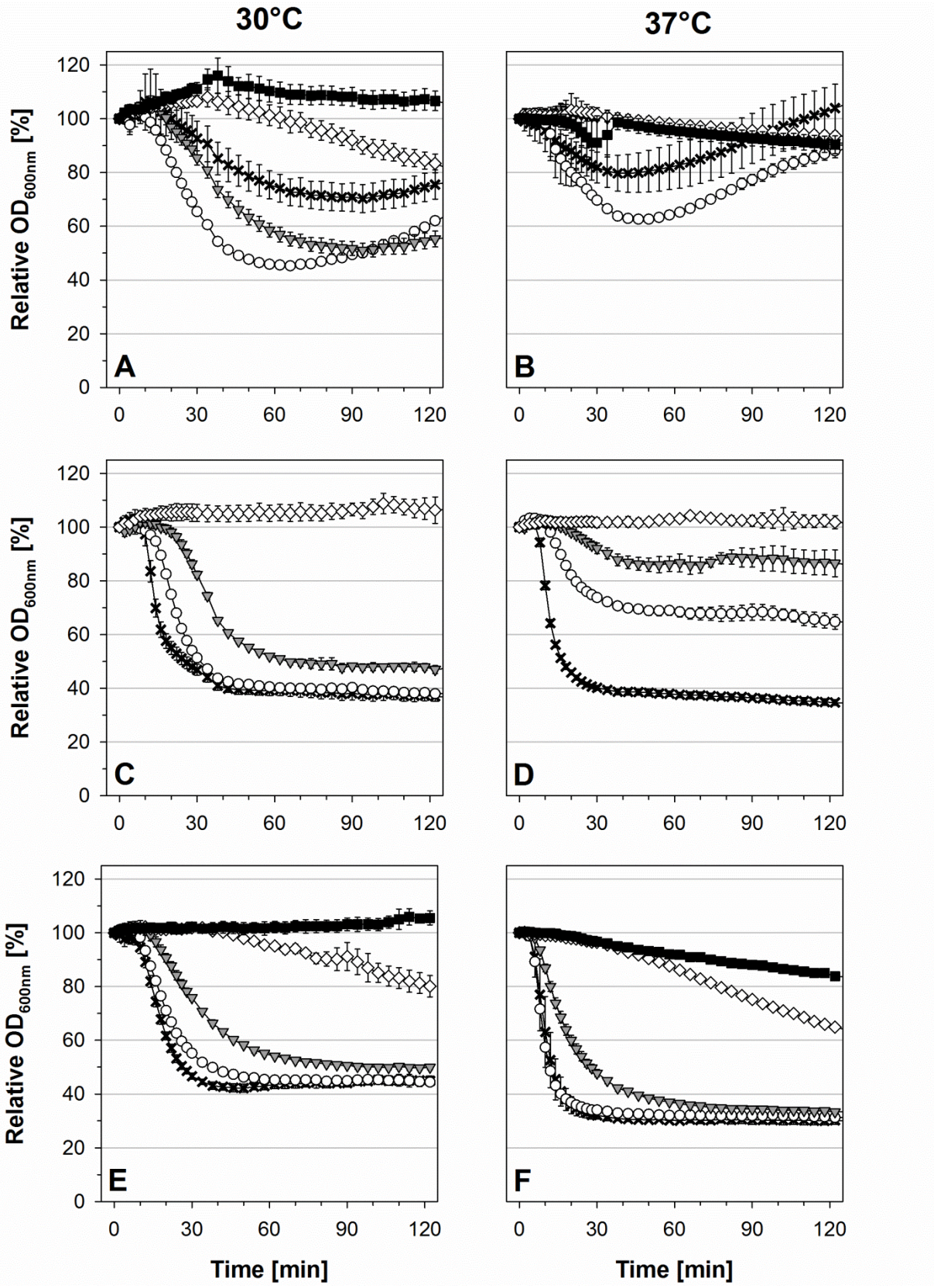


Figure S1: Molecular phylogenetic analysis based on 16S rRNA gene sequences (obtained from the GenBank database; http://www.ncbi.nlm.nih.gov/genbank) of the *Bacillus* species used in this study. The phylogenetic tree was generated with the software MEGA 5.2.2 (Tamura *et al.*, 2011) with the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993) using default settings. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Accession numbers of the sequences used are indicated.

Figure S2 (Nagler *et al.*, 2016):



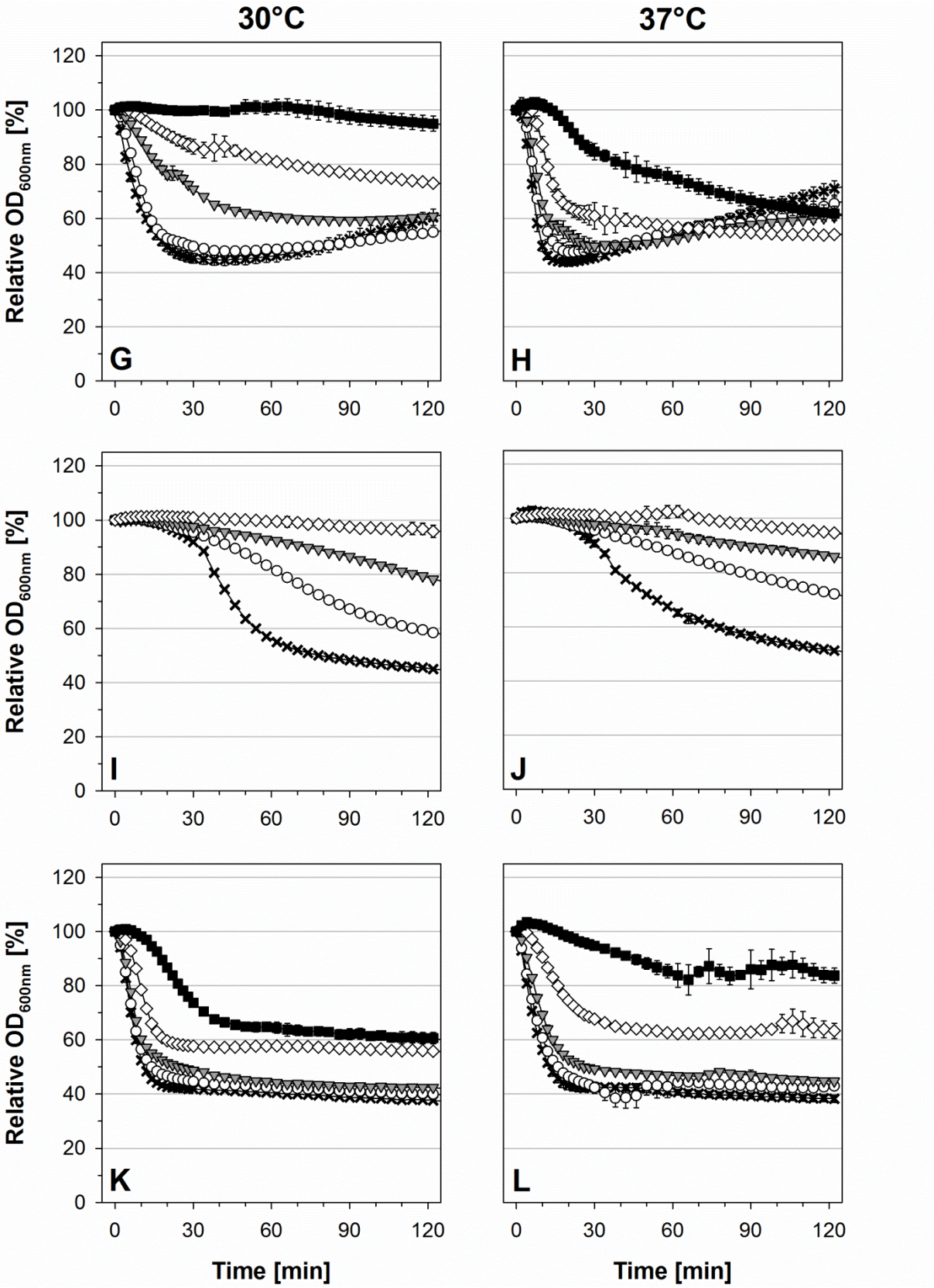
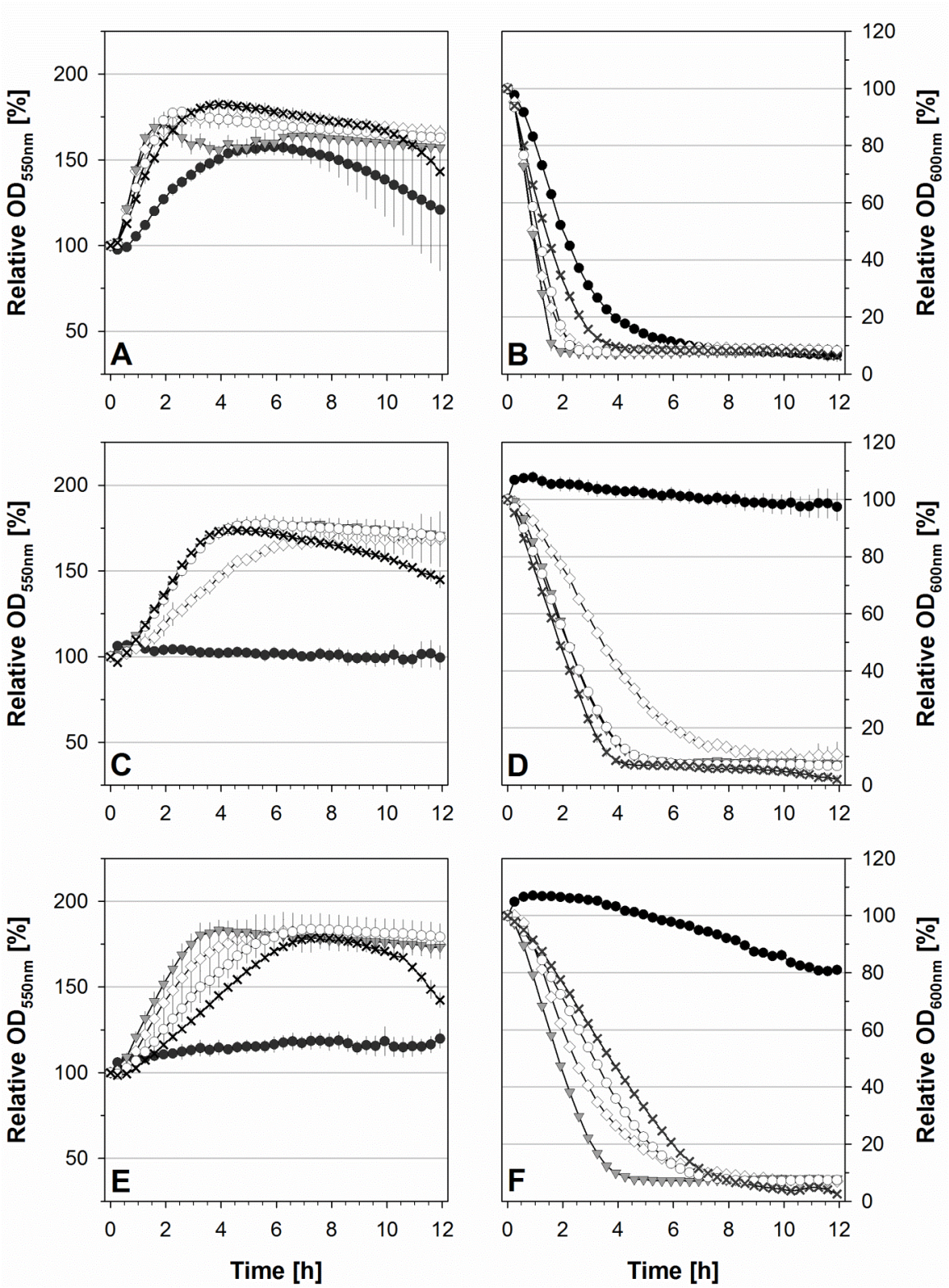


Figure S2: Germination of *B. pumilus* (A – F) and *B. subtilis* (G – L) spores at 30°C (left panel) and 37°C (right panel) with 1 x LB (A, B, G, H), exogenous Ca^{2+}-DPA (C, D, I, J), and KAGE (E, F, K, L). Relative OD_{600nm} was measured as described in Materials and Methods.

Figure S3 (Nagler *et al.*, 2016):



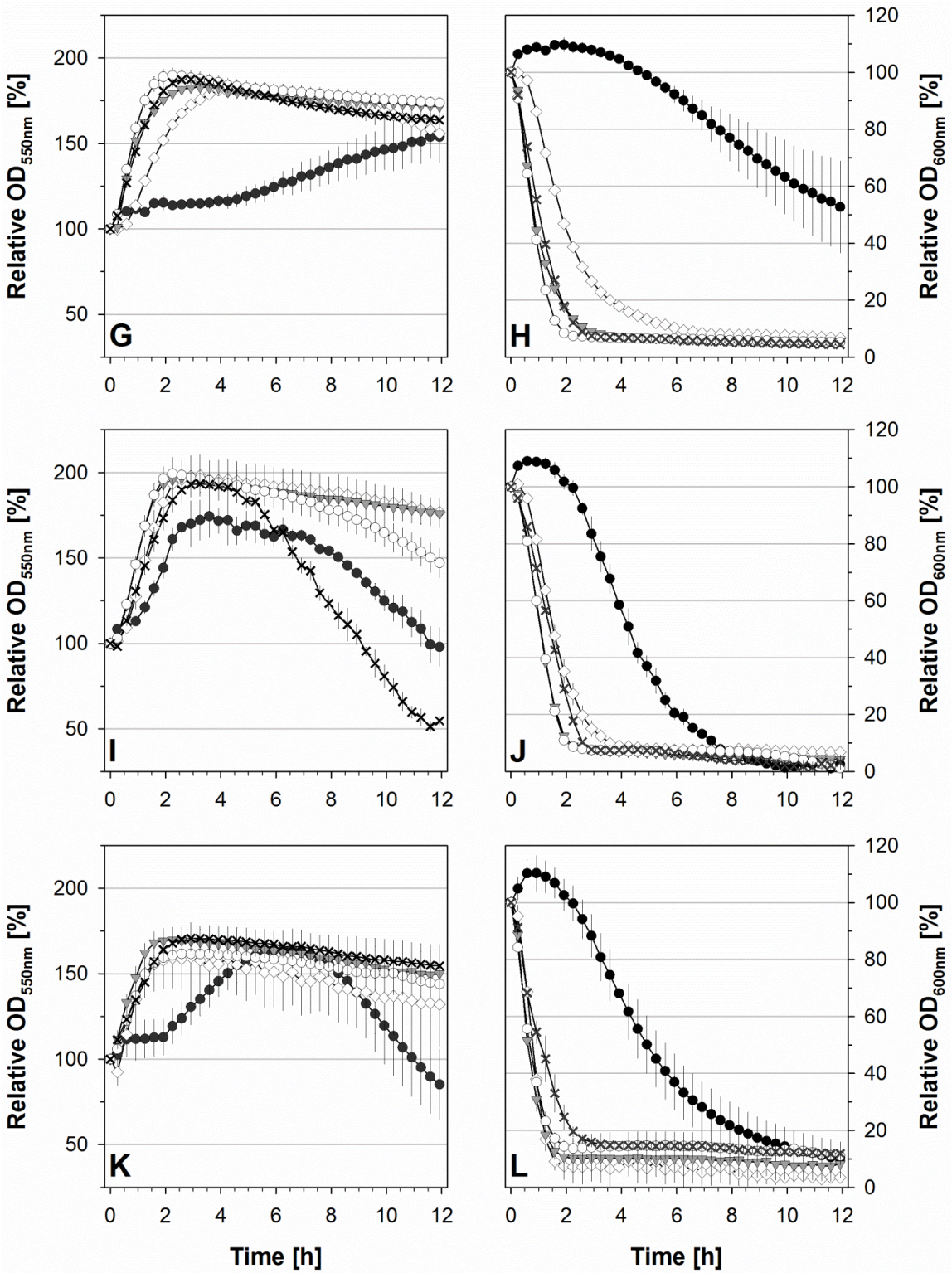


Figure S3: Onset of metabolic activity after germination with KAGE in SMM, analyzed with Alamar Blue as described in Material and Methods. Media contained no additional NaCl (black crosses), 0.6 M NaCl (white circles), 1.2 M NaCl (gray triangles), 2.4 M NaCl (white diamonds), or 3.6 M NaCl (black circles). (A, B) *B. subtilis* 168 [contained additional L-tryptophan due to auxotrophy as described in **Table 2**], (C, D) *B. vallismortis,* (E, F) *B. mojavensis*, (G, H) *B. pumilus* SAFR-032, (I, J) *B. nealsonii*, (K, L) *B. megaterium*. The left data shown in the left and right panel were obtained from OD measurements at 550 nm and 600 nm respectively.

IV.E Supplemental material: Chapter 6

Nagler K, Krawczyk AO, De Jong A, Madela K, Hoffmann T, Laue M, Kuipers OP, Bremer E, Moeller R. 2016. Analysis of differential gene expression during *Bacillus subtilis* spore outgrowth in high-salinity environments using RNA sequencing. Appl. Environ. Microbiol. *In preparation for submission*

Figure S1

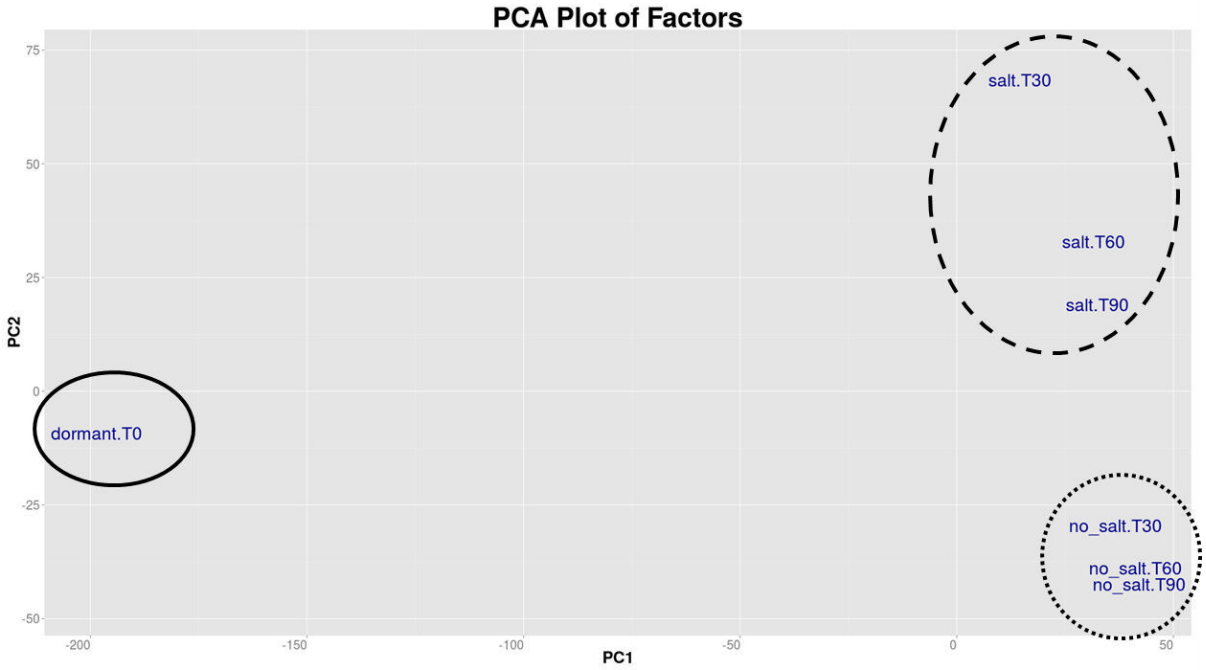


Fig. S1: Principal component analysis (PCA) plot comparing the transcriptome of the tested sample time points (30, 60, and 90 min) consisting of two biological replicates each. Time points of the different conditions are circled: dormant spores (solid line), outgrowth in the presence of NaCl (dashed line), and outgrowth in the absence of NaCl (dotted line).

Figure S2

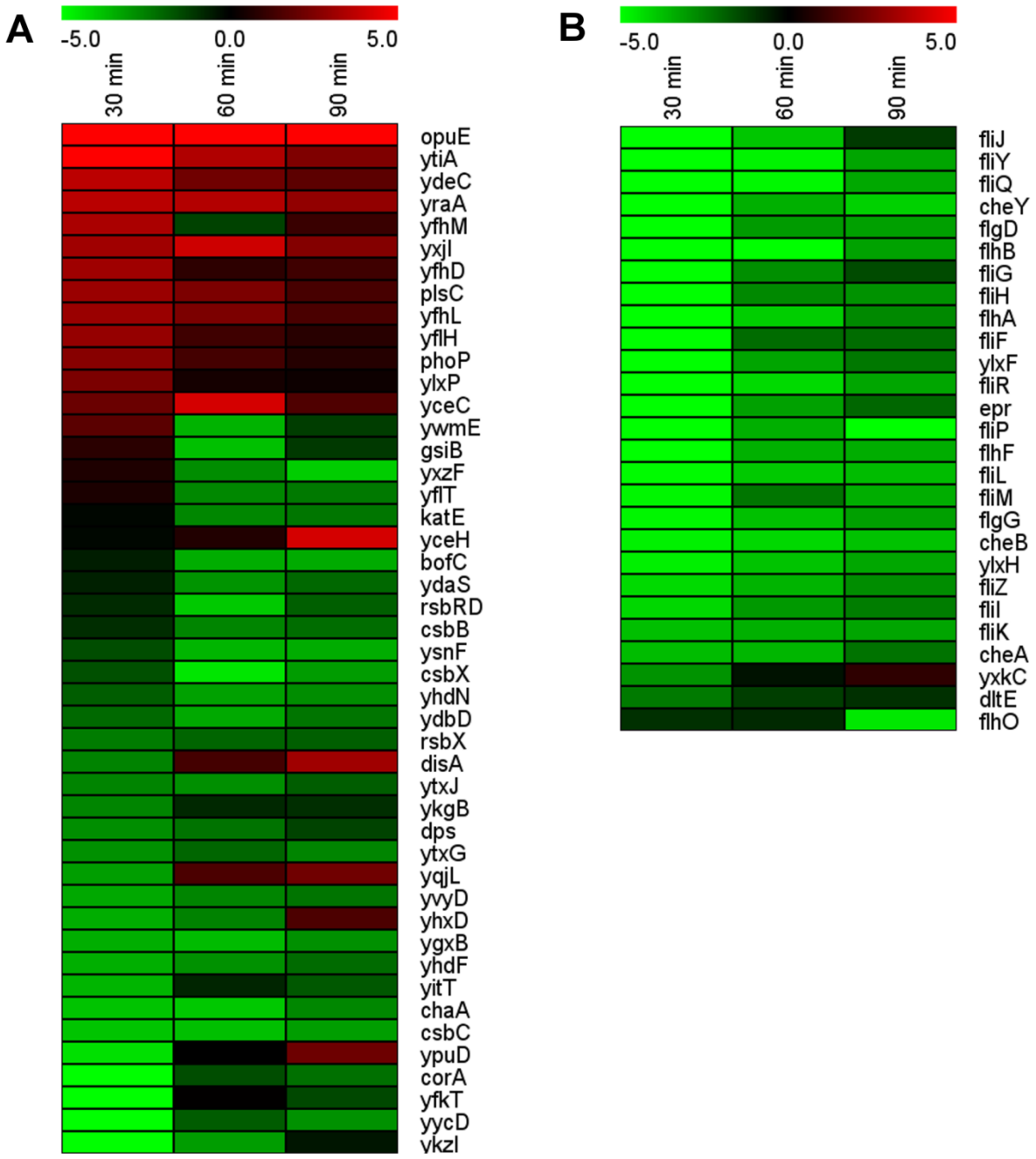


Fig. S2: Expression profiles of differentially expressed genes in (A) the σ^B regulon that governs the general stress response and (B) the σ^D regulon involved in motility and chemotaxis. Only significantly differentially expressed genes are shown. Cutoff values (log_2FC) of the color scale are indicated at the top of the figure.

Table S1: Functional categorization of differentially expressed genes (TopHits)

Category [a]	30 min		60 min		90 min	
	Up	Down	Up	Down	Up	Down
1.1 Cell wall & cell division	12	27	5	4	7	6
1.2 Transporters	42	61	29	34	22	23
1.3 Homeostasis	11	19	5	7	6	3
2.1 Electron transport & ATP synthesis	4	9	2	1	1	2
2.2 Carbon metabolism	19	22	5	10	3	10
2.3 Amino acid/nitrogen metabolism	15	103	17	29	12	52
2.4 Lipid metabolism	10	6	3	2	3	3
2.5 Nucleotide metabolism	13	21	12	9	12	5
2.6 Additional metabolic pathways	19	57	13	25	10	20
3.1 Genetics	10	21	3	2	1	0
3.2 RNA synthesis & degradation	2	7	2	2	1	1
3.3 Protein synthesis, modification & degradation	37	36	9	12	6	13
3.4 Regulation of gene expression	45	37	18	12	8	9
4.1 Exponential & early post exponential lifestyles	5	41	3	13	2	11
4.2 Sporulation & germination	19	34	5	9	11	6
4.3 Coping with stress	73	63	51	27	39	14
4.4 Lifestyles/miscellaneous	3	1	2	0	1	0
5.1 Prophages	13	19	4	7	4	5
5.2 Mobile genetic elements	0	1	0	0	0	0
6.1 Essential genes	31	27	1	7	4	11
6.2 Membrane proteins	88	133	49	62	37	46
6.3 GTP-binding proteins	0	1	0	0	0	1
6.4 Phosphoproteins	20	49	7	22	3	16
6.5 Universally conserved proteins	4	1	0	1	1	1
6.6 Poorly characterized/putative enzymes	11	26	5	8	5	8
6.7 Proteins of unknown function	66	90	32	27	19	17
6.8 Short peptides	0	2	0	0	0	0
6.9 ncRNA	1	0	1	0	1	0
6.10 Pseudogenes	1	3	0	0	0	0

[a] Transcriptomic data was categorized according to the *Subti*Wiki database (www.subtiwiki.uni-goettingen.de).

Table S2: Functional categorization of differentially expressed genes (HighFold)

Category [a]	30 min		60 min		90 min	
	Up	Down	Up	Down	Up	Down
1.1 Cell wall & cell division	0	5	1	1	0	2
1.2 Transporters	16	16	9	3	7	6
1.3 Homeostasis	2	1	3	2	2	1
2.1 Electron transport & ATP synthesis	1	0	0	0	0	0
2.2 Carbon metabolism	4	4	2	0	2	3
2.3 Amino acid/nitrogen metabolism	5	45	4	4	1	20
2.4 Lipid metabolism	0	0	0	0	0	0
2.5 Nucleotide metabolism	10	5	10	2	9	0
2.6 Additional metabolic pathways	2	7	4	4	2	2
3.1 Genetics	0	7	0	0	0	0
3.2 RNA synthesis & degradation	0	1	1	0	0	0
3.3 Protein synthesis, modification & degradation	3	6	1	4	1	0
3.4 Regulation of gene expression	8	7	4	1	0	1
4.1 Exponential & early post exponential lifestyles	1	17	0	1	0	1
4.2 Sporulation & germination	2	11	0	2	4	1
4.3 Coping with stress	22	11	13	1	7	2
4.4 Lifestyles/miscellaneous	1	0	1	0	1	0
5.1 Prophages	1	7	1	1	0	2
5.2 Mobile genetic elements	0	0	0	0	0	0
6.1 Essential genes	1	0	0	3	0	0
6.2 Membrane proteins	24	37	13	4	9	13
6.3 GTP-binding proteins	0	1	0	0	0	0
6.4 Phosphoproteins	5	12	2	1	1	3
6.5 Universally conserved proteins	0	0	0	0	0	0
6.6 Poorly characterized/putative enzymes	1	6	0	2	0	2
6.7 Proteins of unknown function	12	32	5	3	4	5
6.8 Short peptides	0	0	0	0	0	0
6.9 ncRNA	0	0	1	0	1	0
6.10 Pseudogenes	0	1	0	0	0	0

[a] Transcriptomic data was categorized according to the *Subti*Wiki database (www.subtiwiki.uni-goettingen.de).

Table S3: Selected *B. subtilis* regulons and their behavior during outgrowth at high salinity

Regulator	Regulator action	# of genes	Differentially expressed[a]		
			%	# up	#down
AhrC	Activates *roc* genes & represses rest of the regulon (arginine synthesis genes)	16	94	0	15
AzlB	Represses the regulon (branched-chain amino acid transport)	5	100	5	0
BirA	Represses the regulon (biotin synthesis)	7	71	0	5
BkdR	Activates the regulon	7	71	0	5
CcpN	Represses the regulon	4	100	4	0
CodY	Mainly (96 %) repression of the regulon	142	54	1	75
DegU	Mainly (85 %) activation of the regulon	26	19	1	4
GbsR	Represses the regulon	6	83	5	0
FatR	Represses the regulon	2	100	x	x
LutR	Represses the regulon (lactate utilization)	3	100	0	3
Fur	Represses the regulon (iron homeostasis)	50	34	0	17
GltC	Activates the regulon (glutamate synthase)	2	100	0	2
Gly-box	Termination (glycine utilization)	3	100	3	0
GntR	Represses the regulon (gluconate utilization)	4	100	4	0
HxlR	Activates the regulon	2	100	0	2
OhrR	Represses *ohrA* (organic hydroperoxide resistance protein)	1	100	1	0
PadR	Represses the regulon	3	100	3	0
preQ1 riboswitch	Termination (tRNA modification)	4	75	0	3
PucR	Mainly (94%) activation of the regulon	18	78	0	14
PutR	Activates the regulon (proline utilization)	3	100	0	3
PyrR	Antitermination (pyrimidine biosynthesis)	10	100	10	0
RocR	Activates the regulon (amino acid utilization)	7	100	0	7
S-Box	Termination	28	82	4	19
Stringent response	Mainly (96 %) repression of the regulon	121	31	24	13
T-Box	Antitermination	37	43	1	15
YxdJ	Activates the regulon	3	100	3	0
Zur	Represses the regulon	8	75	6	0
AdeR	Activation of *ald* (alanine dehydrogenase)	1	100	0	1
CitR	Represses *citA* (citrate synthase)	1	100	0	1
DesR	Activates *des* (fatty acid desaturase)	1	100	1	0
Thi-box	Termination	14	64	0	9
YdaO riboswitch	Termination in the presence of c-di-AMP	3	100	0	3
YfmP	Represses the regulon	2	100	2	0

[a] The numbers of up and downregulated genes only include genes that have the same expression direction (up/downregulated) at all three time points; x = differential behavior (up- and downregulation) at different sample time points

Table S4: Overlap of differentially expressed genes during outgrowth at high salinity and genes involved in other stress responses

Category [a]	# of genes[b]	Differentially expressed[c]		
		%	# up	#down
Cell envelope stress proteins	133	39	29	16
Acid stress proteins	5	20	1	0
Heat shock proteins	27	30	6	1
Cold stress proteins	16	19	3	0
Coping with hypo-osmotic stress	4	0	0	0
Resistance against oxidative & electrophile stress	63	22	4	9
Resistance against other toxic compounds (nitric oxide, phenolic acids, flavonoids, oxalate)	16	25	4	0
Resistance against toxic metals	22	9	1	1
Resistance against toxins/antibiotics	104	27	17	11
Biosynthesis of antibacterial compounds	55	27	4	11
Toxins, antitoxins & immunity against toxins	41	10	3	1

[a] Transcriptomic data was categorized according to the *Subti*Wiki database (www.subtiwiki.uni-goettingen.de).

[b] Number of genes within the regulon

[c] The numbers of up- (#up) and downregulated (#down) genes only include genes that have the same expression direction (up/downregulated) at all three time points. The percentage of differentially expressed genes includes all genes.

Dataset S1: Overview of differential gene expression. The dataset includes significant differential gene expression information for each sample time point, an overview of all significantly differentially expressed genes (all time points), an overview of all genes including the ones that were not significantly changed, and the list of functional categories obtained from the *Subti*Wiki database (www.subtiwiki.uni-goettingen.de).[Separate .xls file]

IV.F Supplemental material: Chapter 7

Figure S1 [K. Nagler, unpublished data]

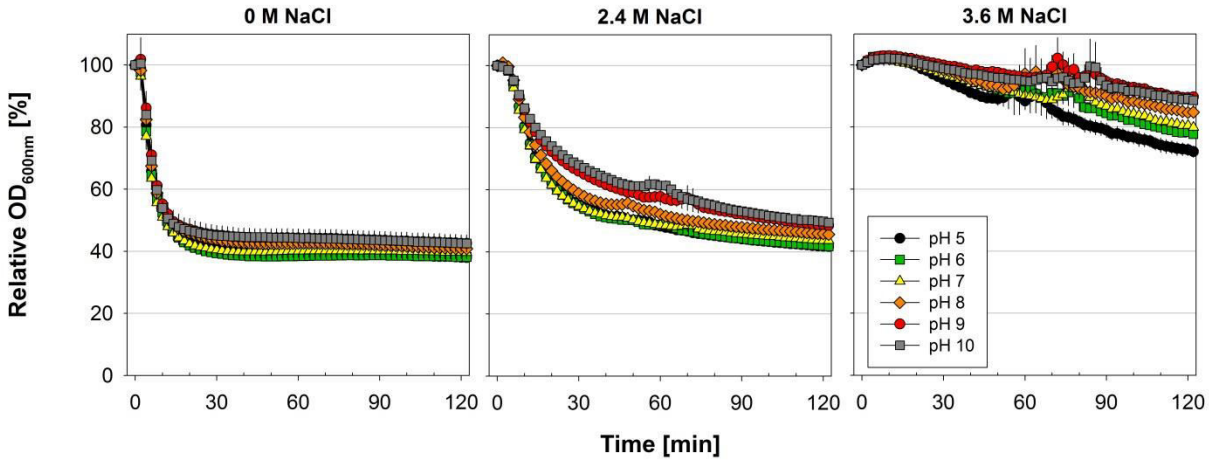


Figure S1: Influence of pH on nutrient germination at high salinity. Spores of *B. subtilis* 168 (*trpC2*) were germinated in Tris-HCl with 10 mM L-alanine and germination was measured spectrophotometrically as described in Nagler *et al.*, 2014. At low pH, germination in the presence of high NaCl concentrations was more efficient and quicker than at high pH.

Figure S2 [K. Nagler and P. Setlow, unpublished data]

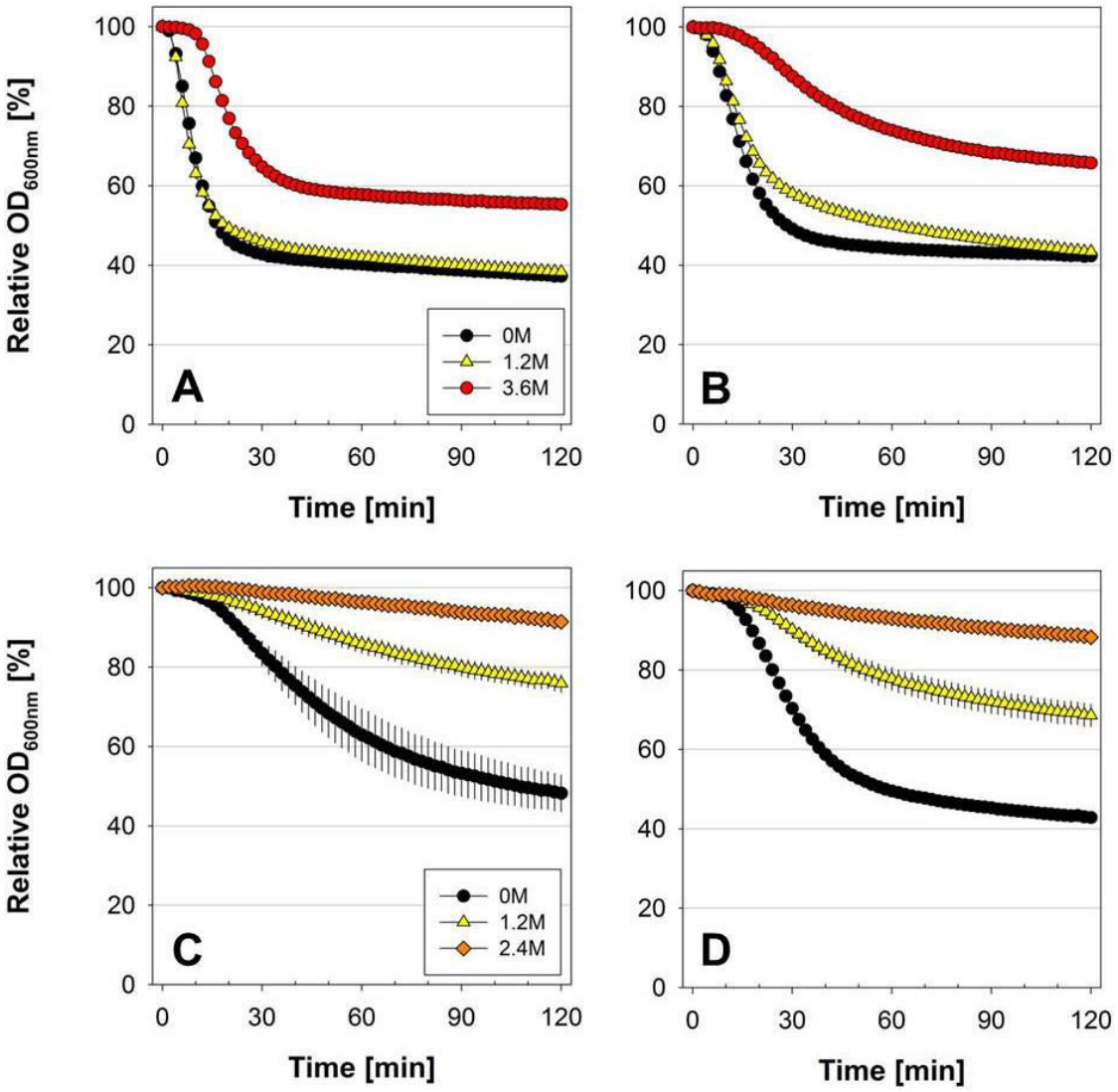


Figure S2: Germination of spores with higher levels of GerA (A, C) or GerB plus GerK (B, D). (A) Germination of GerA overexpression spores (*trpC2* P_{sspD}::*gerA*) with L-alanine. (B) Germination of GerB and GerK overexpression spores (*trpC2* P_{sspD}::*gerB* P_{sspD}::*gerK*) with L-alanine. (C) Germination of GerA overexpression spores with AGFK. (D) Germination of GerB and GerK overexpression spores with AGFK. Spores were prepared, purified and germinated in Tris-HCl supplemented with the appropriate germinant as described in Nagler *et al.*, 2014.

Spores overexpressing the appropriate GRs for the applied germinant (e.g. GerA for L-alanine germination; **A**) germinated better than spores overexpressing the uninvolved GRs (e.g. GerBK for L-alanine germination; **B**). However, inhibitory effects of NaCl were wild type-like, indicating that the number of GRs is not the limiting factor during germination at high salinity.

Figure S3 [K. Nagler and P. Setlow, unpublished data]

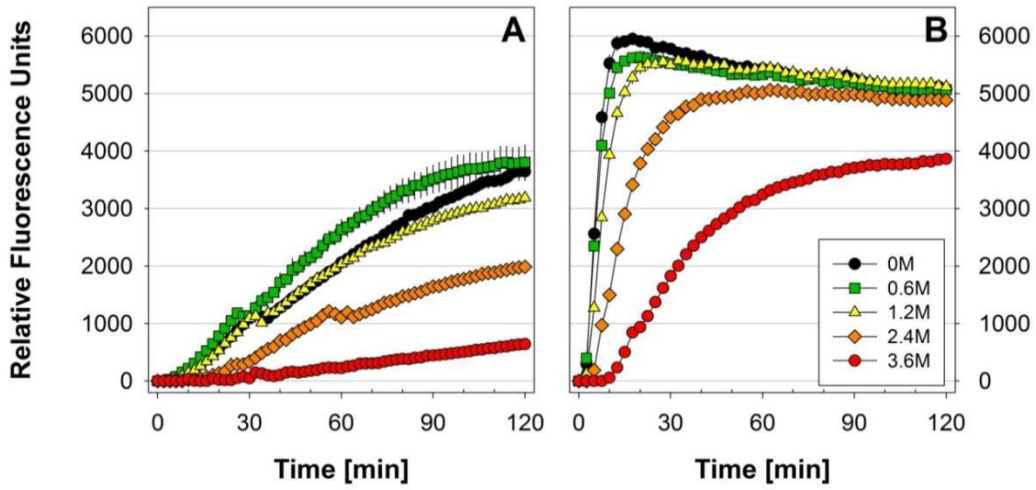


Figure S3: DPA release during L-alanine-induced germination of (A) Δ*gerD* and (B) wild type spores. Spores were germinated in Tris-HCl supplemented with 10 mM L-alanine and DPA release was measured as described in Nagler *et al.*, 2015.

In general, Δ*gerD* spores germinated (i.e. released DPA) more slowly and less efficiently than wild type spores as previously described (Pelczar *et al.*, 2007). Germination of Δ*gerD* spores was slightly enhanced in the presence of 0.6 M NaCl, which is consistent with the report a positive effect of low concentrations of monovalent cations on Δ*gerD* spore germination (Warburg *et al.*, 1985). Other than that, NaCl did not seem to exert any extraordinary effects on L-alanine germination of Δ*gerD* spores compared to wild type spores.

Figure S4 [K. Nagler and P. Setlow, unpublished data]

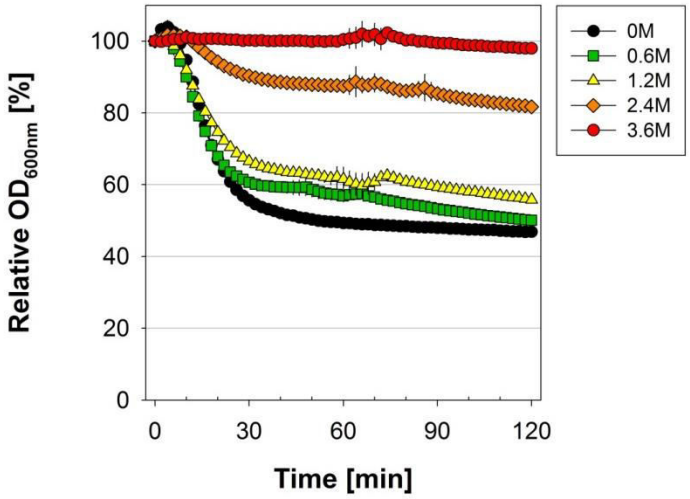


Figure S4: Germination of spores with ca. 4-fold elevated SpoVA levels. Spores of the strain PS3411 (PS832 P_{sspB}*::spoVA;* Vepachedu and Setlow, 2005; Wang *et al.*, 2011) were produced, purified, germinated in Tris-HCl supplemented with 10 mM L-alanine and the OD_{600nm} was measured as described in Nagler *et al.*, 2015.

Figure S5: [K. Nagler, unpublished data]

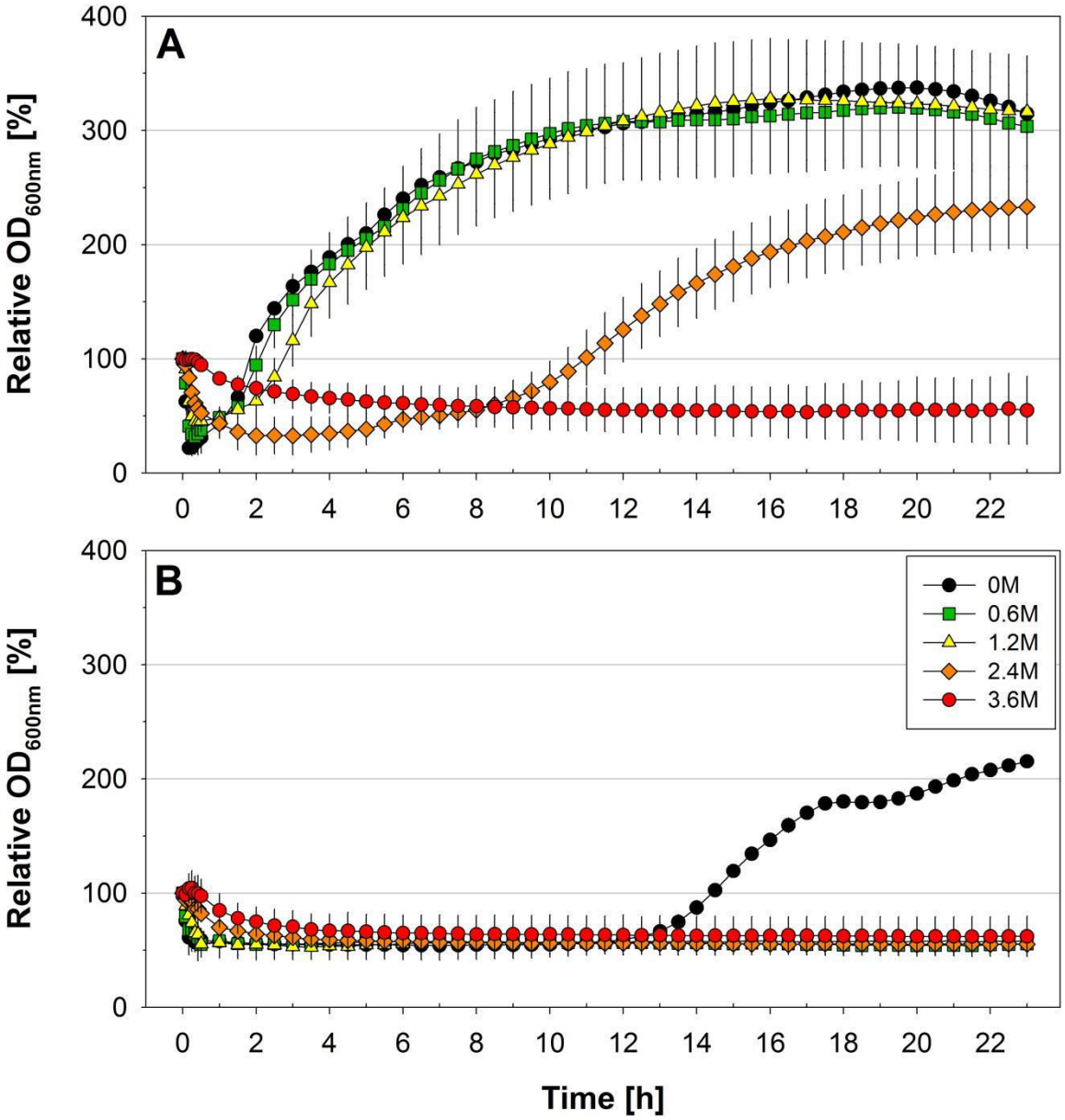


Figure S5: Outgrowth capability of *B. subtilis* spores. *B. subtilis* 168 spore germination and outgrowth in (A) nutrient-rich 1x LB medium and (B) nutrient-poor SMM medium (with L-alanine, D-glucose, and L-tryptophan) were monitored by OD_{600nm} measurement as described in Nagler *et al.* (2016), except that a 24-well plate and 1 ml medium were used.

Table S1: Major open questions concerning molecular germination mechanisms. Filling these knowledge gaps will not only be required for a comprehensive understanding of the germination mechanism, but will also be indispensable for complete understanding of high-salinity interference with this process.

Open questions	References
How do GerP proteins mediate germinant passage on a molecular level?	Butzin *et al.*, 2012; Setlow, 2014a
Does GerT facilitate germinant passage through the spore integuments? If so, how?	Ferguson *et al.*, 2007
To which extent is the OM an intact permeability barrier that restricts ion movement? What are its composition and properties? How can it be traversed by nutrients?	Carstensen *et al.*, 1979; Nagler *et al.*, 2015
If the OM does not restrict ion movement: which other coat-associated structure may have such a function?	Nagler *et al.*, 2015
What is the structure of individual GRs and the whole germinosome?	Griffiths and Setlow, 2011;
How do GRs bind nutrients and generate a germination signal?	Setlow, 2014a
What are the precise functions of the germinosome and how does it mediate these functions?	Griffiths and Setlow, 2011; Setlow, 2014a
How exactly is a germination signal transduced from GR to SpoVA?	Wang *et al.*, 2015b
What is the structure of SpoVA channels and how are they gated? Is the hypothesized four-stage-gating mechanism for SpoVA valid?	Setlow, 2014a; Wang *et al.*, 2015b
What are the thresholds for commitment and initiation of the rapid Ca^{2+}-DPA release?	Wang *et al.*, 2015a; Wang *et al.*, 2015b
What is the function of cation efflux and how is it mediated? Are anions released as well?	Setlow, 2013; Setlow, 2014a
What transporters and channels are involved in molecular movements during germination (ion release, water influx)?	Setlow, 2013
How exactly are SleB and CwlJ activated?	Popham *et al.*, 2012;
How do electrical, physicochemical and general properties of a spore (including volume, pressure, membrane tension and fluidity, pH, and charges) change during germination?	Poolman *et al.*, 2004
Why is the membrane composition important for germination and what are the molecular consequences of an altered membrane composition for germination in the absence and presence of high salinity?	Griffiths and Setlow, 2009

V. Acknowledgements

I am very grateful for every bit of support that I received throughout my time as a PhD student and therefore I would like to sincerely thank everyone who has helped me.

First of all, I would like to thank Prof. Dr. Erhard Bremer for his support, his kind willingness to be my university supervisor (thereby making this project possible) and his valuable scientific opinion and input throughout the past years.

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One of the persons that I want to thank the most is my DLR supervisor Dr. Ralf Möller. I cannot express how grateful I am, but in short I would like to say: Ralf, thank you very much for your never-ending support, your faith in me, giving me a lot of freedom to realize my own ideas and numerous opportunities to do the science that I wanted to do, your kindness, your enthusiasm, and continuous help in every respect of my PhD time. Many thanks for everything.

I would like to thank my dear colleagues of the Space Microbiology Research Group for the wonderful time, I think we were an awesome team! Thank you, Marina Raguse (OMG we made it!), Andrea Schröder, Felix Fuchs, and (again) Ralf - you guys are awesome and made my PhD time become a great (and frequently funny) experience!

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I am particularly grateful for the wonderful collaboration with Prof. Dr. Peter Setlow, for helping me in so many aspects of my scientific work and giving me the opportunity to come to his laboratory for two month, which was a memorable and inspiring experience. Many thanks also to Dr. Barbara Setlow and the rest of the Setlow lab.

In the end, my PhD thesis would also not have been possible without the incredible support in my private life. Therefore, I would like to thank all the wonderful people in my life: my friends, my family including my husband's family, and of course my beloved husband Daniel Nagler. My dearest thanks go out to Mama, Papa and Daniel – I cannot possibly put into words how grateful I am for everything you have done for me, but I owe you billions of thanks for your love and continuous support.

Finally, I also thank *Bacillus subtilis* for his selfless sacrifice, giving countless microscopic lives for my research. For science!

Thank all of you!

VI. Erklärung

Ich versichere, dass ich meine Dissertation

„Analysis of *Bacillus subtilis* spore germination and outgrowth in high-salinity environments“

selbstständig, ohne unerlaubte Hilfe angefertigt und mich dabei keiner anderen als der von mir ausdrücklich bezeichneten Quellen und Hilfen bedient habe.

Die Dissertation wurde in der jetzigen oder einer ähnlichen Form noch bei keiner anderen Hochschule eingereicht und hat noch keinen sonstigen Prüfungszwecken gedient.

_____ _____

Ort, Datum Katja Nagler

Erklärung gemäß der Promotionsordnung der naturwissenschaftlichen Fachbereiche der Philipps-Universität Marburg vom 12. April 2000.

VII. Curriculum Vitae

Personal information

Name	Katja Nagler
Birth date	14.12.1987
Birth place	Leverkusen, Germany

Scientific education

2013 – 2016	Doctoral candidate in the Space Microbiology Research Group at the German Aerospace Center (DLR e.V.), Cologne, Germany
2010 – 2013	Master studies in Biological Sciences at the University of Cologne, Cologne, Germany **Thesis**: „Investigation of *Bacillus subtilis* spore germination at high salinity"
2007 – 2010	Bachelor studies in Biological Sciences at the University of Cologne, Cologne, Germany **Thesis**: „Characterization of the protease RasP and its substrate FtsL in *Bacillus subtilis*"

School education

2005 – 2007	Landrat-Lucas-Gymnasium, Leverkusen, Germany
2004 – 2005	Syracuse High School, Syracuse, KS, USA
1998 – 2004	Freiherr-von-Stein Gymnasium, Leverkusen, Germany

Professional experience

2013	2-month research stay at UConn Health Center, Department of Molecular, Microbial and Structural Biology, Farmington, CT, USA
2010	4-week internship at the Institute of Biochemistry at the University of Cologne, Germany

Publications not included in this thesis

de Ory, Nagler K, Carrasco B, Raguse M, Zafra A, Moeller R, de Vega M. 2016. Identification of a conserved 5'-dRP lyase activity in bacterial DNA repair ligase D and its potential role in base excision repair. Nucleic Acids Res. 44(4):1833-1844.

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Cockell C, Samuels T, Mayer M, Sirks E, Friswell I, Moeller R, Nagler K, Raguse M, Schroeder A, Berger T, Rettberg P. 2014. The 500-year experiment. Microbiology Today 41(2):95-96.

Conference talks

Nagler K, Setlow P, Krawczyk AO, de Jong A, Kuipers OP, Hoffmann T, Bremer E, Madela K, Laue M, Moeller R. **2016**. Mechanistic and transcriptomic salt stress effects on *Bacillus subtilis* spore germination and outgrowth. **European Spores Conference, April 18th – 20th,** London, GB

Nagler K, Setlow P, Reineke K, Rettberg P, Reitz G, Moeller R. **2015**. Towards the identification of mechanisms of high-salinity effects on *Bacillus subtilis* spore germination and outgrowth. **Bacell conference, April 14th – 15th,** Amsterdam, Netherlands

Nagler K, Moeller R, Setlow P, Rettberg P, Reitz G. **2014**. Analysis of *Bacillus subtilis* spore germination in high-salinity environments. **EANA 2014 conference, October 13th - 16th**, Edinburgh, GB

Nagler K, Setlow P, Li YQ, Reitz G, Moeller R. **2014**. Detrimental effects of high salt concentrations on *Bacillus subtilis* spore germination. **European Spores Conference 2014, April 9th – 11th,** London, GB

Nagler K, Moeller R. **2013**. High salinity has negative effects on *Bacillus subtilis* spore germination and outgrowth. **Bacell 2013, April 10th - 11th,** Newcastle upon Tyne, GB

Conference posters

Nagler K, Setlow P, Reineke K, Moeller R, Rettberg P, Reitz G. **2015**. *Bacillus subtilis* spore germination in high-salinity environments: analysis of involved mechanisms and spore components. **VAAM Annual Meeting, March 1st – 4th**, Marburg, Germany

Nagler K, Moeller R, Setlow P, Rettberg P, Reitz G. **2014**. Analysis of *Bacillus subtilis* spore germination in high-salinity environments. **EANA 2014 conference, October 13th - 16th 2014**, Edinburgh, Scotland

Nagler K, Moeller R, Matsumoto K, Reitz G. **2012**. *Bacillus subtilis* spore germination in the

presence of high NaCl concentrations. **Microbial Stress: From Genes to Systems, May 10th - 13th,** Belgirate, Italy

Awards

2013 – 2016	3-year SpaceLife Graduate School Scholarship of the Helmholtz Association of German Research Centers and the German Aerospace Center (DLR e.V.)
2016	FEMS Young Scientist Meeting Grant (YSMG) for participation in the European Spores Conference, April 18th – 20th, London, Great Britain
2015	Student travel grant for participation in the VAAM Annual Conference 2015, March 1st – 4th 2015, Marburg, Germany
2015	2nd prize of the Best Presentation Award at the SpaceLife Students' Workshop, January 20th – 22nd, Rothenburg ob der Tauber, Germany
2014	2nd prize of the EANA 2014 Students' Contest at the EANA 2014 conference, October 13th - 16th 2014, Edinburgh, Scotland
2014	EANA student travel grant for participation in the EANA 2014 conference, October 13th - 16th 2014, Edinburgh, Scotland
2014	FEMS Young Scientist Meeting Grant (YSMG) for participation in the European Spores Conference, April 9th – 11th, London, Great Britain

Additional skills

Languages:	▪ German	- mother tongue
	▪ English	- fluent
	▪ Spanish	- basic skills
	▪ French	- basic skills
IT skills:	▪ MS Office	- advanced skills
	▪ SigmaPlot	- advanced skills
	▪ GIMP	- basic skills
	▪ ImageJ	- basic skills