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Abiotic stress protection by ecologically abundant dimethylsulfoniopropionate and its natural and synthetic derivatives: insights from *Bacillus subtilis*

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Abstract :

Dimethylsulfoniopropionate (DMSP) is an abundant osmolyte and anti-stress compound produced primarily in marine ecosystems. After its release into the environment, microorganisms can exploit DMSP as a source of sulfur and carbon, or accumulate it as an osmoprotectant. However, import systems for this ecophysiologically important compatible solute, and its stress-protective properties for microorganisms that do not produce it are insufficiently understood. Here we address these questions using a well-characterized set of Bacillus subtilis mutants to chemically profile the influence of DMSP import on stress resistance, the osmostress-adaptive proline pool and on osmotically controlled gene expression. We included in this study the naturally occurring selenium analogue of DMSP, dimethylseleniopropionate (DMSeP), as well as a set of synthetic DMSP derivatives. We found that DMSP is not a nutrient for B. subtilis, but it serves as an excellent stress protectant against challenges conferred by sustained high salinity or lasting extremes in both low and high growth temperatures. DMSeP and synthetic DMSP derivatives retain part of these stress protective attributes, but DMSP is clearly the more effective stress protectant. We identified the promiscuous and widely distributed ABC transporter OpuC as a high-affinity uptake system not only for DMSP, but also for its natural and synthetic derivatives.

Introduction

The tertiary sulfonium compound dimethylsulfoniopropionate (DMSP) (Fig. 1) is an integral constituent of the global sulfur cycle operating on our planet (Charlson et al., 1987; Kiene et al., 2000). It is produced in vast amounts (about 10^9 tons annually) by marine phytoplankton and macroalgae and also by a restricted number of plants that typically populate ecosystems near the sea (Yoch, 2002; Otte et al., 2004; Curson et al., 2011a; Reisch et al., 2011; Moran et al., 2012). These organisms can attain high intracellular concentrations of DMSP through synthesis (up to 400 mM) (Stefels, 2000) and upon cell lysis (e.g., after attack by grazing zooplankton and viruses) or osmotic down-shock, release it into open ocean waters, estuarine ecosystems and sediments. In these natural habitats, DMSP can be found in nM or low μM concentrations (Kiene et al., 1998; Van Duyl et al., 1998; Vila-Costa et al., 2006). Microorganisms can then take advantage of environmental DMSP either as a stress protectant (Welsh, 2000), or as a nutrient (Curson et al., 2011b; Rinta-Kanto et al., 2011; Levine et al., 2012; Rinta-Kanto et al., 2012; Todd et al., 2012).

Evidence for several ecophysiological functions of DMSP has been provided. It is considered to act as an antioxidant, as a cryoprotectant, as a chemical cue in the grazing interactions between zooplankton and phytoplankton and as a chemo-attractant for DMSP-consuming bacteria in their relations with the corresponding DMSP-producing dinoflagellate (Karsten et al., 1992; Wolfe et al., 1997; Bayles and Wilkinson, 2000; Sunda et al., 2002; Miller et al., 2004). It is, however, best known for its role as an osmolyte for the producer organisms (Stefels, 2000), most of which live in high-saline environments (Yoch, 2002; Curson et al., 2011a; Reisch et al., 2011; Moran et al., 2012). Notably, osmostress protection by DMSP can also be conferred through its uptake by microorganisms that do not produce it (Gouesbet et al., 1994; Pichereau et al., 1998; Cosquer et al., 1999; Bayles and Wilkinson, 2000; Murdock et al., 2014).

DMSP is a zwitter-ion with no net charge at physiological pH and a member of a selected class of highly water-soluble organic osmolytes, the compatible solutes. Members of all three domains of life exploit these types of compounds to offset the detrimental effects of high salinity and high osmolarity on cellular water content, volume, and physiology (Kempf and Bremer, 1998; Roeßler and Müller, 2001; Yancey, 2005). However, the beneficial effects of compatible solutes extend beyond

their well-established role in osmoregulation, as they also serve as stabilizers of proteins, improve their solubility and preserve the functionality of cell components or even of entire cells (Lippert and Galinski, 1992; Bourot et al., 2000; Manzanera et al., 2002; Ignatova and Gierasch, 2006; Street et al., 2010; Auton et al., 2011). The term chemical chaperone has been coined in the literature to reflect these beneficial traits (Diamant et al., 2001).

Bacteria can derive protection against abiotic stress both through synthesis and uptake of compatible solutes (Kempf and Bremer, 1998; Bremer and Krämer, 2000). A microorganism in which these processes are well characterized, both at the physiological and at the molecular level is the ubiquitously distributed Gram-positive bacterium *Bacillus subtilis* (Bremer, 2002). Upon a high osmolarity challenge, *B. subtilis* produces very large amounts of the compatible solute proline as a cell protectant (Whatmore et al., 1990; Brill et al., 2011; Hoffmann et al., 2013). This bacterium has also been shown to attain relief from sustained osmostress through the import of different types of compatible solutes, most of which are chemically related to either glycine betaine or proline (von Blohn et al., 1997; Bremer, 2002; Hoffmann and Bremer, 2011; Bashir et al., 2014b; Bashir et al., 2014a). The uptake of compatible solutes by *B. subtilis* is mediated via a set of osmotically inducible uptake systems, the Opu family of transporters (Bremer, 2002). These transporters also serve for the import of compatible solutes when they are used as protectants against extremes in either low or high growth temperatures (Holtmann and Bremer, 2004; Hoffmann and Bremer, 2011; Bashir et al., 2014b; Bashir et al., 2014a).

Members of the genus *Bacillus* can colonize a great variety of ecosystems (Earl et al., 2008; Logan and De Vos, 2009), including marine and estuarine habitats and sediments (Siefert et al., 2000; Miranda et al., 2008; Ettoumi et al., 2013). In these ecosystems, *B. subtilis* would certainly have access to the ecologically abundant DMSP but it is unknown whether it catabolizes DMSP and/or can derive stress protection from DMSP after its uptake. Here we address these ecologically important questions through the evaluation of DMSP, its natural selenium analogue dimethlyselenoniopropionate (DMSeP), its synthetic tellurium derivative dimethyltelluriopropionate (DMTeP), and five DMSP-inspired synthetic compounds whose sulfonium head-groups have been extensively chemically modified (Fig. 1).

Results

DMSP is not a nutrient for B. subtilis

Many microorganisms can catabolize DMSP (Curson et al., 2011a; Reisch et al., 2011; Moran et al., 2012). To test if *B. subtilis* could use it as sole carbon or sulfur source, cultures of the wild-type strain JH642 were grown in a chemically defined medium (SMM or SMM with 0.4 M NaCl) in which glucose (28 mM) was replaced with 33 mM DMSP as the sole carbon source. No growth was observed after 20 h of incubation of the cultures (Fig. S1A). Likewise, no growth was observed when DMSP was offered to the cells as sole sulfur source (15 mM) (Fig. S1B). Since DMSP can sometimes be toxic, we also tested a lower concentration (2 mM) of DMSP in our growth assays; no growth was observed under these conditions either (Fig. S1A and B). We therefore conclude that *B. subtilis* belongs to the group of microorganisms that cannot exploit DMSP as a nutrient.

Stress protection by DMSP and its derivatives against high salinity and extremes in growth temperature

We tested the stress-protective properties of DMSP, it natural selenium analogue dimethlyseleniopropionate (DMSeP) (Ansede and Yoch, 1997; Ansede et al., 1999), and six synthetic DMSP derivatives (Dickschat et al., 2010; Brock et al., 2014) (Fig. 1) for *B. subtilis* cells that were continuously challenged either by extremes in salinity (1.2 M NaCl) or growth temperatures (13° C and 52° C). DMSP exerted the same level of osmostress protection as the highly effective compatible solute glycine betaine (Boch et al., 1994) (Fig. 2A). DMSeP was also a good osmostress protectant and was followed in its potency by the synthetic DMSP derivatives dimethyltelluriopropionate (DMTeP), ethylmethylsulfoniopropionate (EMSP), diethylsulfoniopropionate (DESP), isopropylmethylsulfoniopropionate (IMSP), and tetramethylenesulfoniopropionate (TMSP). In contrast, methylpropylsulfoniopropionate (MPSP) did not serve as an osmoprotectant for *B. subtilis* (Fig. 2A).

Many compatible solutes used by *B. subtilis* as osmoprotectants (Bremer, 2002) also protect cells against stress at the cutting edges of the temperature spectrum that *B. subtilis* cells can populate in a defined chemical medium (Holtmann and Bremer, 2004; Hoffmann and Bremer, 2011). DMSP

resembled the established cold protectant glycine betaine in its ability to promote growth at 13° C, a temperature that otherwise severely restricts growth of *B. subtilis* (Hoffmann and Bremer, 2011) (Fig. 2B). The naturally occurring DMSeP and the synthetic EMSP also offered reasonably good cold stress protection, while the remaining synthetic DMSP derivatives afforded either no protection or provided cellular protection at a very low level (Fig. 2B).

When DMSP and its full set of derivatives were assayed for their heat stress protection potential at a growth temperature of 52° C, only DMSP provided thermoprotection to *B. subtilis* at a level comparable to the established heat stress protectant glycine betaine (Holtmann and Bremer, 2004) (Fig. 2C).

Import of DMSP and its derivatives down-regulates the size of the osmostress-adaptive proline pool

The adaptation of *B. subtilis* to sustained high osmolarity growth conditions is afforded through the biosynthesis and accumulation of large amounts of the compatible solute proline (Whatmore et al., 1990; Brill et al., 2011). In this adjustment process, the intracellular proline concentration is sensitively linked to the degree of the osmotic stress imposed by the environment onto the cell (Brill et al., 2011; Hoffmann et al., 2013). Proline pools approaching or exceeding 0.5 M can be found when the osmotic stress is severe (Hoffmann et al., 2013; Zaprasis et al., 2013). In turn, the import of various kinds of osmoprotectants down-regulates the cellular proline content of high osmolarity challenged cells in a finely tuned fashion and thereby allows the saving of precious energy sources and biosynthetic building blocks for proline production (Akashi and Gojobori, 2002; Hoffmann et al., 2013; Bashir et al., 2014b).

To test if DMSP and its various derivatives would exert similar dampening effects on the size of the newly produced proline pool, we grew strain JH642 in SMM containing 1.2 M NaCl in the absence or the presence of various concentrations of these compounds and measured the free proline content of the cells. As observed previously (Hoffmann et al., 2013), the presence of glycine betaine in the growth medium resulted in a significantly reduced intracellular proline pool as the glycine betaine concentration in the medium was increased from 25 μM to 1 mM (Fig. 3). DMSP and DMSeP exerted

158 a similar effect (Fig. 3). All other DMSP derivatives affected the proline pools more modestly, with
159 TMSP conferring an intermediate effect (Fig. 3).
160
161 *DMSP and its derivatives reduce the level of opuA expression at high salinity*
162 The import of glycine betaine by osmotically stressed cells not only allows to maintain a
163 physiologically appropriate level of cellular hydration (Cayley et al., 1992), but it also affects gene
164 expression in *B. subtilis* on a global scale (Kohlstedt et al., 2014). The activity of the promoter for the
165 *opuA* operon is a good reporter for such effects, both because it is strongly induced by high osmolarity
166 but also responsive to cellular pools of various compatible solutes built up through transport processes
167 (Hoffmann et al., 2013; Bashir et al., 2014b; Bashir et al., 2014a).
168 Strain MBB9 carries a chromosomal copy of an *opuA-treA* operon fusion that expresses this
169 hybrid reporter gene under the control of the *opuA* promoter (Hoffmann et al., 2013). The level of the
170 TreA reporter enzyme, a salt-tolerant phospho-α-(1,1)-glucosidase (Gotsche and Dahl, 1995), can thus
171 be used as a read-out for the assessment of the potential influence of DMSP and its derivatives on
172 osmotically controlled gene expression. Cultures of strain MBB9 were grown in the absence or
173 presence (1 mM) of these solutes in SMM or in SMM containing 1.2 M NaCl. In the absence of a
174 compatible solute in the growth medium, transcription of the *opuA-treA* reporter fusion was induced
175 about 8.5-fold when the external salinity was increased (Table 1). The osmoprotectants glycine betaine
176 and carnitine (Boch et al., 1994; Kappes and Bremer, 1998) reduced the salt-induced level of *opuA*
177 transcription about five- to six-fold and so did DMSP (Table 1). DMSP, glycine betaine and carnitine
178 also down-regulated (between 3.6 and 4-fold) the level of *opuA-treA* expression found in the absence
179 of added NaCl and thereby still permitted an osmotic up-regulation (between 4.9 and 6.6-fold) in the
180 expression level of the reporter fusion (Table 1). The reduction in the level of *opuA-treA* transcription
181 by the DMSP selenium analogue DMSeP and the tested synthetic DMSP derivatives in salt-stressed
182 and non-stressed cells was less pronounced, between 1.5- and 2.5-fold under osmotic stress conditions
183 and between 1.7- and 3.5-fold in non-stressed cells (Table 1).
184
185 *Uptake of DMSP and its derivatives relies on the ABC transporters OpuA and OpuC*

The osmoprotective effect of an exogenous supply of compatible solutes depends on their import (Kappes et al., 1996; Hoffmann et al., 2013). *B. subtilis* possesses five osmotically inducible uptake systems for these compounds, the Opu (***o***smo***p***rotectant ***u***ptake) family of transporters which comprises both multi-component ABC transporters (OpuA, OpuB and OpuC) and single component uptake systems that belong either to the MFS (OpuE) or to the BCCT (OpuD) super-families (Bremer, 2002). A comprehensive set of mutant strains is available, each expressing only one of these transport systems, while the genes for the other transporters have been deleted (Table 3) (Hoffmann and Bremer, 2011). These *B. subtilis* mutant strains thus allow a determination which transporter is used by a given osmoprotectants through a simple growth assay. When applied to DMSP and its natural and synthetic derivatives, we found that DMSP can confer osmostress protection in strains that possess either an intact OpuA or OpuC system, whereas all other DMSP derivatives were imported only via OpuC (Fig. 4). Consequently, in a strain with simultaneously defective OpuA and OpuC transporters, osmoprotection by DMSP and its derivatives was lost, while that afforded by glycine betaine remained (Fig. 4) since it can be imported not only via the ABC transporters OpuA and OpuC but also through the BCCT (betaine-carnitine-choline-transporter)-type transporter OpuD (Kappes et al., 1996; Ziegler et al., 2010).

We also tested the role of the individual Opu transporters for the import of DMSP and its derivatives under both cold- and heat-stress conditions. The same transporters used for the uptake of these solutes in salt-stressed cells were also used by cells exposed to either sustained cold (13° C) (Fig. S2A) or sustained heat stress (52° C) (Fig. S2B).

Kinetic parameters of OpuA and OpuC for DMSP and its derivatives

To study the uptake of DMSP and its derivatives by *B. subtilis* in more detail, competition assays with DMSP and radiolabeled [1-^{14}C]glycine betaine were conducted. We first studied the import of DMSP via the OpuA ABC transporter in cells that were grown in the presence of 0.4 M NaCl. Uptake of [1-^{14}C]glycine betaine exhibited Michaelis-Menten kinetics (Fig. 5A) and yielded a K_m value of 3 ± 1 µM, which agrees very well with a previous estimate of 2.4 µM (Kappes et al., 1996). In contrast to

the high-affinity import of glycine betaine by OpuA, uptake of DMSP was a low-affinity process and yielded a K_i value of 912 ± 275 μM (Fig. 5A).

Next, we studied the import of DMSP and its derivatives via the OpuC ABC transporter in osmotically stressed cells (with 0.4 M NaCl). Uptake of [1-^{14}C]glycine betaine proceeded with high affinity and yielded a K_m of 7 ± 1 μM (Table 2), again a value that is in excellent agreement with a previous report (K_m: 6 μM) (Kappes et al., 1996). OpuC-mediated import of DMSP was a high-affinity process as well and yielded a K_i value of 39 ± 7 μM (Fig. 5B). The transport characteristics of OpuC for the uptake of six tested DMSP derivatives yielded similar K_i values (Table 2), thereby identifying this transporter as a high affinity uptake system for DMSP and its natural and synthetic DMSP derivatives (Fig. 1). The details of the uptake characteristics of the studied DMSP derivatives are documented in Fig. S3.

Uptake studies with [1-^{14}C]glycine betaine in the presence of MPSP (Fig. 1) as a potential inhibitor of the OpuC-mediated transport process demonstrated that this synthetic DMSP derivative did not compete with glycine betaine import (Fig. S4). MPSP is the DMSP derivative to which we could not ascribe a biological function in *B. subtilis* (Fig. 2) and this can now be understood. However, it is not immediately evident why this particular DMSP derivative (Fig. 1) is not recognized as a substrate by the OpuC transporter.

In silico docking of DMSP into the ligand-binding sites of the OpuAC and OpuCC solute receptor proteins

The primary substrate recognition component of microbial binding-protein-dependent ABC transporters are the extracellular solute receptor proteins of these systems (Berntsson et al., 2010). OpuAC and OpuCC are the extracellular substrate binding proteins of the OpuA and OpuC ABC transport systems (Kempf and Bremer, 1995; Kappes et al., 1999) and crystal structures of these proteins in complex with various substrates have been reported (Horn et al., 2006; Smits et al., 2008; Du et al., 2011). Since no ligand binding protein associated with an ABC transport system has been crystalized in the presence of DMSP, we carried out *in silico* modeling studies to derive clues on the molecular determinants governing the binding of DMSP by the OpuAC and OpuCC proteins. We

241 relied for these ligand-docking experiments on crystallographic data available for the OpuAC protein
242 in complex with the sulfur analog of glycine betaine, dimethlysulfonioacetate (DMSA; Fig. 1) (PDB
243 code 3CHG) (Smits et al., 2008), and the OpuCC:glycine betaine complex (PDB code 3PPP) (Du et
244 al., 2011) (Fig. 6). Crystallographic data relevant for the properties of the DMSP ligand were extracted
245 from the structure of the DMSP lyase DddQ from *Silicibacter lacuscaerulensis* (PDB database entry
246 4LA2) (Li et al., 2014).

247 The OpuAC:DMSA complex (Smits et al., 2008) was chosen as the starting structure for the
248 modeling since DMSP and DMSA are chemically closely related sulfur-containing molecules (Fig. 1).
249 An aromatic ligand-binding cage in the OpuAC protein is formed by the side chains of three Trp
250 residues (Trp^{72}, Trp^{178} and Trp^{225}) that are arranged in form of a prism (Horn et al., 2006). The
251 positively charged dimethylsulfonio head group of DMSA is accommodated within this aromatic
252 micro-environment via cation-π interactions. Its carboxylate interacts via hydrogen bonds with the
253 backbone nitrogens of Gly^{26} and Ile^{27} (Fig. 6). To derive a OpuAC:DMSP model, we first exchanged
254 *in silico* the DMSA ligand in the OpuAC:DMSA complex by a DMSP molecule and then refined the
255 resulting *in silico*-generated complex against the structure factors of the OpuAC:DMSA structure
256 deposited in the PDB file 3CHG (Smits et al., 2008) to ensure the correctness of the bond length and
257 angles of the DMSP ligand. As expected, our *in silico* model envisions that the positively charged
258 dimethylsulfonio head group of the DMSP ligand is also accommodated by the above described
259 aromatic cage via cation-π interactions, but due to the increased length of the main carbon chain of the
260 DMSP molecule by a CH_2 group (Fig. 1), the position of the sulfur atom is slightly shifted (by about
261 0.7 Å) relative to that of the DMSA ligand. As a consequence of this shift, the carboxylate of DMSP is
262 still able to interact with the backbone nitrogens of Gly^{26} and Ile^{27} in the OpuAC protein (Fig. 6),
263 interactions that are also found in the OpuAC:DMSA complex (Smits et al., 2008).

264 The reduced number of cation-π and van der Waals interactions of the OpuAC:DMSA
265 complex in comparison with the OpuAC:glycine betaine crystal structure, decreases the binding of
266 DMSA by OpuAC relative to glycine betaine by five-fold; from a K_d of about 20 μM for glycine
267 betaine to a K_d of about 100 μM for DMSA (Smits et al., 2008). Given the low affinity of the OpuA
268 transporter for DMSP (K_i of about 1 mM) (Fig 5A), one must assume that the predicted shift in the

position of the dimethylsulfonio head group of DMSP within the OpuAC ligand binding site (Fig. 6) is sub-optimal for the stability of the OpuAC:DMSP complex. Ligand binding by OpuAC is sensitive to slight variations. Even conservative amino acid substitutions in the aromatic cage by other aromatic residues that cause an altered geometry in the cation-π interactions can have drastic consequences for the affinity of OpuAC for different ligands (Smits et al., 2008). The covalent radii for the S, Se and Te are 103 pm, 117 pm and 135 pm, respectively (Housecroft and Sharp, 2008). The resulting increasing bulkiness of the positively charged head-groups of DMSP, DMSeP, and TMSTeP (Fig. 1) is likely a contributor why the DMSeP and DMTeP molecules cannot be stably bound by the OpuAC solute receptor protein. As reflected by the high K_i value of the OpuA transporter for DMSP (approximately 1 mM) (Fig. 5A), binding of DMSP by the OpuAC receptor protein is thus a borderline case.

Since no crystal structure of the OpuCC protein with a sulfur-betaine such as DMSA is available, we used the OpuCC:glycine betaine complex (Du et al., 2011) (Fig. 6) as the starting structure for our *in silico* modeling study. Within the OpuCC protein, the positively charged trimethlyammonium head group of glycine betaine is housed and coordinated via four Tyr residues (Tyr^{71}, Tyr^{117}, Tyr^{197} and Tyr^{221}) arranged in form of an aromatic cage. The carboxylate of glycine betaine protrudes out of this aromatic cage and is bound and spatially orientated within the binding site via interactions with Gln^{19} and Thr^{74} (Du et al., 2011). Our *in silico* docking experiment suggests a similar, but not identical, position of the DMSP molecule within the ligand-binding site (Fig. 6). Despite the shift in the overall position of the DMSP molecule, the same stabilizing interactions found for the glycine betaine ligand in the experimentally determined OpuCC:glycine betaine complex are also present in the *in silico* generated OpuCC:DMSP structure (Fig. 6). As expected, the positively charged dimethylsulfonio head group of DMSP is accommodated within the aromatic cage via cation-π interactions. The carboxylate of DMSP, however, interacts differently with the OpuCC protein, a result of the increased chain length of the DMSP molecule. While the interaction of the DMSP ligand with Gln^{19} is retained, the interaction with Thr^{74} is lost and instead a new interaction with the backbone nitrogen of Ser^{51} is established (Fig. 6). Notably, such an interaction of the carboxylate of the carnitine ligand with the backbone of Ser^{51} has also been observed in the crystal structure of the OpuCC:carnitine complex (Du et al., 2011). Hence, the described spatial orientation for ligands with

an increased length in their main carbon chain (e.g., carnitine and DMSP) seems to represent a stable interaction platform with the OpuCC solute receptor protein. This will subsequently allow high-affinity import of these types of substrates via the OpuC transporter as found here for DMSP (Table 2) and as already reported for carnitine (Kappes and Bremer, 1998).

Bioinformatics assessment of the distribution of OpuA- and OpuC-type transporters within the genus Bacillus

Since our growth assays and transport studies revealed the reliance of DMSP import on the OpuA and OpuC transporters, we wondered how widely these compatible solute uptake systems are distributed among members of the genus *Bacillus*. We therefore conducted a BLAST-P analysis of *Bacillus* species with fully sequenced genomes represented in the Integrated Microbial Genomes and Metagenomes database maintained by the DOE Joint Genome Institute (Nordberg et al., 2013). We used for this search the amino acid sequences of the OpuAC and OpuCC solute receptor proteins (Kempf and Bremer, 1995; Kappes et al., 1999) as the query sequences. This search uncovered 88 finished genome sequences that are derived from 18 distinct *Bacillus* species; 84 strains possessed an OpuAC protein and 86 strains possessed OpuCC (Table S1). Hence, OpuA- and OpuC-type transporters are found in essentially every *Bacillus* species whose genome sequence was inspected. The vast majority (82 out of 88) simultaneously possesses both OpuA and OpuC; none of the inspected genomes lacked both of these osmolyte uptake systems (Table S1). An alignment of the amino acid sequences of the retrieved OpuAC and OpuCC proteins revealed that the amino acids forming the characteristic aromatic ligand binding cages (Horn et al., 2006; Smits et al., 2008; Du et al., 2011) are highly conserved and would therefore be able to contribute to DMSP binding via cation-π interactions as suggested by our *in silico* modeling studies (Fig. 6).

Discussion

Members of the genus *Bacillus* are ubiquitous in nature (Earl et al., 2008; Logan and De Vos, 2009) and can be found in marine and estuarine ecosystems and in sediments (Siefert et al., 2000; Miranda et al., 2008; Ettoumi et al., 2013). It is highly likely that *B. subtilis* will have access to DMSP in these

habitats since this compound is produced abundantly in marine environments (Stefels, 2000; Yoch, 2002; Curson et al., 2011a; Reisch et al., 2011; Moran et al., 2012). In contrast to many microorganisms living in marine ecosystems (Curson et al., 2011b; Rinta-Kanto et al., 2011; Levine et al., 2012; Rinta-Kanto et al., 2012; Todd et al., 2012), our data show that *B. subtilis* cannot use DMSP as a nutrient. However, it can exploit DMSP as an excellent stress protectant against challenges conferred by sustained high salinity or lasting extremes in high and low growth temperature. This can be done with a degree of efficiency matching that of the stress-protective effects of glycine betaine, probably the most widely used compatible solute in nature (Yancey, 2005).

By chemical profiling a set of well-defined transporter mutants, we found that DMSP uptake by osmotically and temperature-stressed *B. subtilis* cells are mediated under laboratory conditions by two ABC transport systems, OpuA and OpuC. The *in silico* assessment of the occurrence of these transporters revealed their presence in most Bacilli with a fully sequenced genome. We therefore surmise that the osmotic and temperature stress protection afforded through DMSP import that we describe here in detail for the model organism *B. subtilis* (Barbe et al., 2009; Belda et al., 2013) will be of ecophysiological relevance for most members of the large and diverse *Bacillus* genus (Earl et al., 2008; Logan and De Vos, 2009).

The very low affinity of OpuA for DMSP (K_i of about 1 mM) suggests a limited importance of this transport system for DMSP uptake in natural settings where this compound is typically found in rather low concentrations (Kiene et al., 1998; Van Duyl et al., 1998; Vila-Costa et al., 2006). OpuC, on the other hand, is a high-affinity uptake system and cannot only scavenge DMSP (K_i of about 40 μM), but also its natural selenium analogue DMSeP and several synthetic DMSP derivatives with similar high affinities. To the best of our knowledge, the transport data that we provide here for DMSP uptake in *B. subtilis* via the OpuA and OpuC systems are the first truly quantitative measurements reported for any defined microbial species. Our data also identify the first uptake system (OpuC) for the naturally occurring derivative of DMSP, DMSeP (Ansede and Yoch, 1997; Ansede et al., 1999), in any microorganism and pinpoint OpuC as a flexible transporter through which various synthetic DMSP derivatives (Dickschat et al., 2010; Brock et al., 2014) can be efficiently taken up.

The ABC transporter OpuC is a remarkable osmolyte import system since its substrate specificity is extremely broad (Bremer, 2002; Hoffmann and Bremer, 2011; Bashir et al., 2014b). Most of its ligands possess positively charged and fully methylated head-groups, and these are accommodated via cation-π interactions within an aromatic cage formed by four tyrosine residues present in the extracellular OpuCC substrate-binding protein (Kappes et al., 1999; Du et al., 2011). Given what is known about the molecular determinants for compatible solute binding by substrate-binding proteins of ABC transporters (Bremer, 2011; Tschapek et al., 2011), it is not surprising that OpuCC can accommodate DMSP and its selenium and tellurium analogues within its ligand-binding site with good affinities, as evidenced by the low K_i values of the OpuC transporter for these compounds,

The ligand-binding site present in OpuCC exhibits a considerable degree of structural flexibility (Du et al., 2011) and allows, as suggested by our modeling studies, the capture of ligands with different chain length (e.g., glycine betaine, carnitine and DMSP) through a switch in the binding mode of the carboxylate of its substrates. What is rather surprising, however, is our finding that the sulfur head-group of DMSP can be extensively chemically modified with no significant reduction in the affinity of the OpuC transporter for these synthetic ligands. This is reminiscent of the OpuC-mediated import by *B. subtilis* of a toxic synthetic glycine betaine derivative [2-(dimethyl(4-nitrobenzyl)ammonio) acetate] in which a bulky benzyl group substituted one of its methyl groups (Cosquer et al., 2004). Collectively, the structural plasticity of the OpuCC ligand-binding site (Du et al., 2011) provides the molecular underpinning for the promiscuous nature of the OpuC ABC transport system (Hoffmann et al., 2013; Bashir et al., 2014b).

DMSP import competes with the uptake of glycine betaine in natural marine settings (Kiene et al., 1998; Vila-Costa et al., 2006) and microbial transport systems that mediate glycine betaine uptake are frequently also used for DMSP import (Gouesbet et al., 1994; Pichereau et al., 1998; Cosquer et al., 1999; Murdock et al., 2014). *B. subtilis* is no exception in this regard since both OpuA and OpuC serve for high-affinity glycine betaine import as well (Kempf and Bremer, 1995; Kappes et al., 1996; Kappes et al., 1999). We note in this context, however, that not all microbial glycine betaine import systems can mediate DMSP uptake. This is exemplified by the substrate profile of the *B. subtilis*

OpuD transporter, a system that catalyzes glycine betaine import (Kappes et al., 1996) but does not participate in DMSP uptake. OpuD is a member of the BCCT (betaine-carnitine-choline-transporter) family, carriers that are involved in the uptake of various types of compatible solutes (Ziegler et al., 2010). Interestingly, a member (DddT) of the BCCT family was recently identified as a DMSP uptake system in several DMSP-catabolizing species but it was also proficient in glycine betaine import when assessed in a heterologous *E. coli* system (Todd et al., 2010; Sun et al., 2012).

The growth-enhancing effects of compatible solutes for osmotically stressed bacterial cells probably stem from a combination of their beneficial influence on cellular hydration and turgor, on the ionic strength and solvent properties of the cytoplasm, on the preservation of the solubility of proteins and their functionality, and the maintenance of the integrity of cell components and biosynthetic processes (Cayley et al., 1992; Bourot et al., 2000; Bremer and Krämer, 2000; Diamant et al., 2001; Ignatova and Gierasch, 2006; Street et al., 2010; Auton et al., 2011; Wood, 2011). The physico-chemical attributes of individual compatible solutes (Street et al., 2006; Auton et al., 2011; Diehl et al., 2013; Jackson-Atogi et al., 2013) are, however, also an important determinant for the efficiency and type by which they exert their protective function. For instance, the oxidation of ectoine to 5-hydroxyectoine (Bursy et al., 2007) results in a far better desiccation protection for molecules than that afforded by its precursor ectoine (Tanne et al., 2014), which itself is an excellent stress protectant against various types of challenges (Lippert and Galinski, 1992; Widderich et al., 2014). Similarly, the disparate effects of glycine betaine and proline on the cellular content of potassium, glutamate, and trehalose, and hence on the water activity and osmotic pressure of the cytoplasm, are large enough to make glycine betaine a far more effective osmoprotectant for *E. coli* than proline (Cayley et al., 1992). We probably see all these effects at work when one collectively views the different influence of DMSP and its natural and synthetic derivatives on the growth of salt-challenged *B. subtilis* cells, on the build-up of the osmostress-adaptive proline pool, and on gene expression of the osmotically controlled *opuA* operon.

Cellular protection by compatible solute accumulation against sustained low and high growth temperatures has been reported for a considerable number of microbial species [for a detailed set of references see: (Holtmann and Bremer, 2004; Hoffmann and Bremer, 2011)]. However, the underlying

molecular mechanisms are insufficiently understood. Studies in *B. subtilis* with the cold- and heat-stress protectant glycine betaine have shown that the cellular pools of this compound attained under temperature stress are far lower than those established under osmotic stress conditions (Holtmann and Bremer, 2004; Hoffmann and Bremer, 2011; Hoffmann et al., 2013). This observation indicates that the mechanism(s) for protection by glycine betaine against osmotic and temperature challenges are, at least partially, different. Since cold stress can have significant effects on protein structure (Jaenicke, 1990), the cryoprotective effects of DMSP for *B. subtilis* and other microorganisms (Bayles and Wilkinson, 2000; Angelidis and Smith, 2003; Murdock et al., 2014) might primarily stem from its function as a chemical chaperone. Indeed, DMSP is known to stabilize *in vitro* the enzyme activities of purified phosphofructokinase from rabbit muscle, the cold-labile model enzyme lacate dehydrogenase, and of the malate dehydrogenase from the polar alga *Acrosiphonia arcta* under cold-induced denaturing conditions (Nishiguchi and Somero, 1992; Karsten et al., 1996).

Our work with *B. subtilis* also revealed a new facet of the physiological attributes of DMSP since it conferred effective heat stress protection. As argued above for cold stress protection, the heat-stress protective effects of DMSP might also be ascribed to the chemical chaperone activity of compatible solutes (Caldas et al., 1999; Diamant et al., 2001; Chattopadhyay et al., 2004; Tschapek et al., 2011).

In summary, DMSP not only proved to be a formidable protectant against osmotic stress, but it also effectively rescued growth at the very upper and lower edges of the temperature spectrum that *B. subtilis* cells can populate. Under these conditions, other prominent cellular defense systems of *B. subtilis* (e.g. the cold- and heat-shock response and the SigB-controlled general stress response) fail but DMSP does the job.

Experimental procedures

Chemicals and synthesis of synthetic DMSP derivatives

Glycine betaine, carnitine, the chromogenic substrate [*para*-nitrophenyl-α-D-glucopyranoside; (PNPG)] used for assays of the TreA reporter enzyme, a salt-tolerant phospho-α-(1,1)-glucosidase (Gotsche and Dahl, 1995), and the ninhydrin reagent used for the quantification of proline by a

colorimetric assay (Bates et al., 1973) were purchased from Sigma-Aldrich (Steinheim, Germany). Radiolabeled [1-^{14}C]glycine betaine (55 mCi mmol^{-1}) was obtained from American Radiolabeled Chemicals Inc. (St. Louis, MO; USA). Dimethylsulfoniopropionate (DMSP) was purchased from Carbon Scientific Co. LTD (London, United Kingdom). The antibiotics kanamycin, erythromycin, spectinomycin, and tetracycline were obtained from SERVA Electrophoreses GmbH (Heidelberg, Germany), United States Biochemical Corp. (Cleveland, Ohio; USA) and Sigma-Aldrich (Steinheim; Germany), respectively. Chemicals for the synthesis of DMSP and its derivatives were obtained from Sigma-Aldrich (Steinheim; Germany), or Acros Organics (Thermo Fisher Scientific, Geel; Belgium) and used without further purification. The synthesis of DMSP, dimethylseleniopropionate (DMSeP), dimethyltelluriopropionate (DMTeP), ethylmethylsulfoniopropionate (EMSP), diethylsulfoniopropionate (DESP), methylpropylsulfoniopropionate (MPSP), isopropylmethylsulfoniopropionate (IMSP), and tetramethylenesulfoniopropionate (TMSP) was performed by acid-catalyzed Michael addition of the corresponding dialkyl chalcogenides to acrylic acid as detailed previously (Dickschat et al., 2010; Brock et al., 2014).

Media and growth conditions for B. subtilis strains

B. subtilis strains were routinely maintained on Luria-Bertani (LB) agar plates or cultured in LB liquid medium (Miller, 1972). The antibiotic concentrations for the seletion of *B. subtilis* strains carrying chromosomal mutant alleles marked with an antibiotic resistance cassette were as follows: kanamycin (5 µg ml^{-1}), erythromycin (1 µg ml^{-1}), spectinomycin (100 µg ml^{-1}), and tetracycline (10 µg ml^{-1}). For stress protection growth assays by compatible solutes, *B. subtilis* strains were cultivated in Spizizen´s minimal medium (SMM) with 0.5% (wt/vol) glucose as the carbon source and a solution of trace elements (Harwood and Archibald, 1990). L-tryptophan and L-phenylalanine were added to growth media at final concentrations of 40 mg ml^{-1} and 36 mg ml^{-1}, respectively, to satisfy the growth requirements of the *B. subtilis* strains JH642 and 168 and their mutant derivatives (Table 3). When the use of DMSP by *B. subtilis* as either sole carbon source was tested, the glucose content (28 mM) in SMM was replaced by 33 mM DMSP; likewise, when the use of DMSP as sole sulfur source was assessed, the sulfur source present in SMM [$(NH_4)_2SO_4$; 15 mM] was replaced by 15 mM DMSP in

the presence of [$(NH_4)_2PO_4$; 15 mM] and $MgSO_4$ was replaced by $MgCl_2$. Use of DMSP as a nutrient was also tested at a substrate concentration of 2 mM to assess possible toxic effects of higher DMSP concentrations on growth. The osmolarity of the SMM was increased by the addition of NaCl from a 5 M stock solution. Compatible solutes were sterilized by filtration (Filtropur S 0.2 µm; Sarstedt, Nürnbrecht, Germany) and were added to growth media at a finally concentration of 1 mM. Cultures of *B. subtilis* cells were inoculated from exponentially growing pre-cultures in pre-warmed SMM to optical densities (OD_{578nm}) of 0.1. *B. subtilis* cultures were grown in 20-ml culture volumes in 100-ml Erlenmeyer flasks set in a shaking (set to 220 r.p.m.) water bath. Cultures used for heat-stress growth protection assays at 52° C were inoculated from pre-cultures grown at 37° C to an OD_{578} of about 1 to an OD_{578} of 0.1. The cultures were set in a water bath with a temperature of 37° C; the growth temperature was then slowly increased to 52°C over a 20 min time frame. The temperature of the water baths used for the heat and cold stress growth experiments was set and controlled with the aid of a calibrated thermometer (Testo AG, Lenzkirch, Germany).

Bacterial strains

The *B. subtilis* strains JH642 (*trpC2 pheA1*) (Brehm et al., 1973) (Table 3), a member of the domesticated inage of laboratory strains (Smith et al., 2014), was used for all experiments that addressed the salt- and heat-stress protective potential of DMSP and its derivatives. Since it carries a mutation in the *ilvB* gene that makes it cold sensitive (Wiegeshoff and Marahiel, 2007), the *B. subtilis* laboratory strain 168 (Barbe et al., 2009) (Table 3) was used for studies that probed the potential of these solutes as cold stress protectants. To analyze the transporter activities of individual Opu uptake systems and to avoid a possible cross-talk of components of a given Opu ABC-transporter with another Opu systems, we constructed a set of strains that carry deletions of the complete operons coding for the OpuA-, OpuB- and OpuC ABC transporters. Strain TMB107 [Δ(*opuA*::*tet*)3] was constructed by replacing a 2 700 bp *´opuAA-opuAB-opuAC´* DNA fragment with a 1834 bp DNA fragment carrying a tetracycline resistance cassette which was derived from plasmid pDG1515 (Guerout-Fleury et al., 1995). Strain TMB116 [Δ(*opuB*::*ery*)1] carries a 3 139 bp *´opuBA-opuBB-opuBC-opuBD´* deletion that was replaced with a erythromycin resistance cassette (1 553 bp) derived

492 from plasmid pDG647 (Guerout-Fleury et al., 1995). The [Δ(*opuC*::*spc*)3] mutation was constructed
493 by replacing a 3 419 bp ´*opuCA-opuCB-opuCC-opuCD*´-fragment with a 1 173 bp DNA fragment
494 encoding a spectinomycin resistance cassette which was derived from plasmid pDG1726 (Guerout-
495 Fleury et al., 1995). The formerly described strain RMKB7 carries a gene disruption in the
496 *opuD* gene that encodes a single component glycine betaine uptake system (Kappes et al.,
497 1996). Combinations of single *opu* mutations were constructed by transforming appropriate
498 recipient strains with chromosomal DNA of *B. subtilis* mutants carrying various *opu* alleles marked
499 with antibiotic resistance cassettes (Table 3). Preparation of chromosomal DNA from *B. subtilis*
500 strains, transformation of *B. subtilis* with this DNA, and the selection of transformants via their
501 antibiotic resistance were conducted according to routine procedures (Cutting and Vander Horn, 1990;
502 Harwood and Archibald, 1990). Derivatives of the *B. subtilis strain* 168 carrying gene disruption
503 mutations have been described before (Hoffmann and Bremer, 2011).
504
505 *Determination of cellular proline pools in osmotically stressed cells*
506 The intracellular proline content of osmotically stressed cells of the wild-type JH642 strain was
507 determined by a colorimetric assay detecting proline as a colored prolin-ninhydrine complex, which
508 can be quantified by measuring the absorption of the solution at 480 nm in a spectrophotometer (Bates
509 et al., 1973). Cells of strain JH642 were grown in SMM containing 1. 2M NaCl in the absence or
510 presence of various concentrations (25 µM to 1000 µM) of glycine betaine, DMSP, DMSeP, DMTeP,
511 EMSP, DESP, IMSP and TMSP until they reached an OD_{578nm} of about 1.6. Harvesting of the cells by
512 centrifugation, their processing for the colorimetric proline detection assay, and the details of the
513 calculation of the intracellular volume of *B. subtilis* and of the concentration of proline have all been
514 described previously (Hoffmann and Bremer, 2011; Hoffmann et al., 2013).
515
516 *Transport studies*
517 Cultures of the *B. subtilis* strains SBB1 ($OpuA^+$) and SBB2 ($OpuC^+$) (Table 3) were grown in SMM
518 containing 0.4 M NaCl to an OD_{578nm} of about 0.3. 2-ml aliquots were withdrawn and mixed with a
519 solution of glycine betaine that that been spiked with [1-^{14}C]glycine betaine; the final glycine betaine

concentration in the uptake assays was varied between 3 µM and 100 µM. The transport assays were conducted in the presence of non-radiolabeled DMSP for the $OpuA^+$ strain SBB1 and non-radiolabeled DMSP, DMSeP, DMTeP, EMSP, DESP, IMSP, and TMSP for the $OpuC^+$ strain SBB2. In the transport studies conducted with strain SBB1, DMSP was present as an inhibitor for glycine betaine uptake at a final substrate concentration of 1000 µM. For glycine betaine uptake assays conducted with strain SBB2, the substrate concentration for the inhibitors was set to a final concentration of 150 µM. Uptake assays, processing of the cells, and the quantification of the imported radiolabeled glycine betaine by scintillation counting followed previously established procedures (Kappes et al., 1996). Michaelis-Menten kinetics of [1-^{14}C]glycine betaine uptake and fitting of the competitive inhibition of this transport activity by DMSP and its derivatives were performed with the GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA).

Measurements of TreA enzyme activity in opuA-treA reporter fusion strains

The *B. subtilis* strain MBB9 carries a *opuA-treA* operon fusion that is expressed from the *opuA* promoter; it is stably inserted via a double-recombination event in the non-essential *amyE* gene (Hoffmann et al., 2013) (Table 3). The expression level of this reporter gene fusion is responsive to both osmotic stress and the intracellular pools of different compatible solutes (Hoffmann et al., 2013; Bashir et al., 2014b; Bashir et al., 2014a). Cells of strain MBB9 were grown in SMM or in SMM containing 1.2 M NaCl in either the absence or the presence of glycine betaine, DMSP, DMSeP, DMTeP, EMSP, DESP, IMSP, and TMSP (the final substrate concentrations of these compounds in the medium was were 1 mM) to mid-exponential growth phase (OD_{578nm} of about 1.5), harvested by centrifugation, and then processed for TreA enzyme activity assays as described previously (Gotsche and Dahl, 1995; Hoffmann et al., 2013). One unit (U) of TreA activity is defined as the enzymatic conversion of 1 µmol of the colorimetric substrate PNPG per min. Protein concentrations of the samples were estimated from the optical density of the *B. subtilis* cell culture (Miller, 1972).

in silico *docking of DMSP into the ligand-binding sites of the OpuAC and OpuCC proteins*

The presumed molecular interaction of DMSP with the OpuAC and OpuCC proteins were assessed by *in silico* docking. The OpuAC:DMSA crystal structure (Smits et al., 2008) was used as the template for the OpuAC:DMSP *in silico* model. The DMSA ligand in the OpuAC:DMSA complex was first exchanged with a DMSP molecule and the generated OpuAC:DMSP model was then refined against the structure factors of the OpuAC:DMSA dataset (Protein database entry 3CHG) (Smits et al., 2008) using the programs COOT and REFMAC (Murshudov et al., 1997; Emsley and Cowtan, 2004) to define the bond lengths and angle of the *in silico* DMSP ligand docked into the OpuAC binding site. The coordinate file for the DMSP ligand was extracted from the crystal structure of the DMSP lyase DddQ (PDB database entry 4LA2) (Li et al., 2014). After refining the *in silico*-generated model, the orientation of DMSP within the ligand-binding site was manually checked by analyzing the interactions of the DMSP molecule with the OpuAC protein within a distance range of 2.8-3.2 Å from the ligand. A similar procedure was used for generating an *in silico* model of the OpuCC:DMSP complex, except that the OpuCC:glycine betaine crystal structure (PDB code 3PPP) (Du et al., 2011) was used as the template. First, the *in silico*-generated OpuAC:DMSP complex was overlaid with the OpuCC:glycine betaine crystal structure. Then, the location of the glycine betaine and DMSP ligands was superimposed and after removing the glycine betaine ligand from the OpuCC:glycine betaine crystal structure, the DMSP coordinates were transferred *in silico* into the OpuCC protein. The thereby generated OpuCC:DMSP complex was than refined and analyzed as described above for the OpuAC:DMSP *in silico* complex.

Preparation of figures of crystal structures of the in silico derived OpuAC:DMSP and OpuCC:DMSP complexes

Figures of the crystal structures of the OpuAC protein in complex with dimethylsulfonioacetate (DMSA) (PDB code 3CHG) (Smits et al., 2008), of the OpuCC protein in complex with glycine betaine (PDB code 3PPP) (Du et al., 2011), and of the *in silico* generated OpuAC:DMSP and OpuCC:DMSP complexes generated in this study were prepared using the PyMOL software package (http://www.pymol.org).

Database searches and phylogenetic analysis of the distribution of OpuA- and OpuC-type transporters in Bacilli

The amino acid sequence of the ligand-binding proteins (OpuAC, OpuCC) of the OpuA and OpuC ABC transporters (Kempf and Bremer, 1995; Kappes et al., 1999) were retrieved from the nucleotide sequence of the *B. subtilis* laboratory strain 168 (Barbe et al., 2009) and used as query sequences for BLAST-P database searches at the Integrated Microbial Genomes and Metagenomes database (IMG; https://img.jgi.doe.gov/cgi-bin/w/main.cgi) maintained by the the Department of Energy (DOE) Joint Genome Institute (Nordberg et al., 2013). We focused our analysis on members of the Bacillus genus with a finished genome sequence. The retrieved OpuAC and OpuCC amino acid sequences were aligned using Clustal W (Thompson et al., 2000) for inspection of conserved residues, in particular for those that from the aromatic cages in the OpuAC and OpuCC proteins (Horn et al., 2006; Du et al., 2011). The genome context of the *opuAC* and *opuCC* genes for the remaining components of the OpuA and OpuC ABC transporters (Kempf and Bremer, 1995; Kappes et al., 1999) was assessed with the bioinformatics tool provided by the IMG platform.

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Table 1. Repression of *opuA* expression by compatible solutes.

Compatible solute	TreA activity [U (mg protein^{-1})]	
	without NaCl	1.2 M NaCl
none	66 ± 2	561 ± 35
Glycine betaine	18 ± 2	88 ± 3
Carnitine	17 ± 2	102 ± 4
DMSP	16 ± 1	107 ± 16
DMSeP	19 ± 1	223 ± 6
DMTeP	30 ± 1	330 ± 19
EMSP	24 ± 1	313 ± 3
DESP	39 ± 3	381 ± 9
IMSP	25 ± 3	297 ± 9
TMSP	19 ± 1	224 ± 10

Cells of the *opuA-treA* reporter fusion strain MBB9 were grown either in SMM or in SMM containing 1.2 M NaCl to mid-exponential growth phase (OD_{578nm} of about 1.5) in the absence or the presence of the indicated compounds and were then assayed for the activity for their TreA reporter enzyme activity. The final concentration of the different compatible solutes added to the growth media was 1mM. The values shown are the averages of two independently grown cultures, where each culture was assayed twice for phospho-α-(1,1)-glucosidase (TreA) activity. The data shown represent the error ranges of the enzyme assays.

Table 2. Kinetic parameters for the uptake of DMSP and its derivatives *via* the OpuC transport system of *B. subtilis*.

Compatible solute	K_i (μM)[a)]
GB	-
DMSP	39 ± 7
DMSeP	28 ± 3
DMTeP	18 ± 2
EMSP	29 ± 4
DESP	24 ± 6
IMSP	48 ± 6
TMSP	18 ± 2

Cells of the *B. subtilis* OpuC$^+$ strain SBB2 were propagated at 37° C in SMM containing 0.4 M NaCl to early-exponential growth phase (OD_{578nm} approximately 0.3) and were then used for uptake studies at 37°C. For the various transport assays, the concentration of glycine betaine (GB) (spiked with [1-^{14}C]glycine betaine) was varied between 3 μM and 100 μM, whereas the concentration of the various inhibitors was kept constant at 150 μM. The data given for the inhibition constant (K_i) for DMSP and its derivatives are the averages of uptake studies conducted with two independently grown *B. subtilis* cultures; the data shown represent the error ranges of the transport assays.

[a)]Transport assays with radiolabeled glycine betaine in the absence of an inhibitor were conducted in parallel with each inhibition experiment. The average and standard deviation of the kinetic data for glycine betaine uptake were K_m 6 ± 1 μM and V_{max} 65 ± 1 nmol min^{-1} mg of protein^{-1}.

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

Strain	Relevant genotype[a)]	Origin/reference
JH642	*trpC2 pheA1*	(Brehm et al., 1973)
RMKB7	JH642 Δ(*opuD::neo*)2	(Kappes et al., 1996)
SBB1	JH642 Δ(*opuC::spc*)3 Δ(*opuD::neo*)2 Δ(*opuB::ery*)1	This study
SBB2	JH642 Δ(*opuA::tet*)3 Δ(*opuD::neo*)2 Δ(*opuB::ery*)1	This study
SBB4	JH642 Δ(*opuC::spc*)3 Δ(*opuA::tet*)3	This study
TMB107	JH642 Δ(*opuA::tet*)3	This study
TMB108	JH642 Δ(*opuC::spc*)3	This study
TMB109	JH642 Δ(*opuA::tet*)3 Δ(*opuD::neo*)2	This study
TMB111	JH642 Δ(*opuC::spc*)3 Δ(*opuD::neo*)2	This study
TMB116	JH642 Δ(*opuB::ery*)1	This study
MBB9[b)]	JH642 *amyE*::[Φ(*opuA-treA*)*1 cat*] (*treA::neo*)	(Hoffmann et al., 2013)
168	*trpC2*	(Barbe et al., 2009)
JGB23	168 Δ(*opuA::erm*)*4* Δ(*opuBD::tet*)*23 opuC20*::Tn*10* (*spc*)	(Hoffmann and Bremer, 2011)
JGB24	168 Δ(*opuA::erm*)*4* Δ(*opuBD::tet*)*23* Δ(*opuD::neo*)*2*	(Hoffmann and Bremer, 2011)
JGB25	168 Δ(*opuBD::tet*)23 *opuC20::Tn10* (*spc*) Δ(*opuD::neo*)*2*	(Hoffmann and Bremer, 2011)

[a)]The OpuA, OpuB and OpuC transport systems are members oft the ABC transporter superfamily family and are multi-component systems. They are encoded by the *opuA* [*opuAA-opuAB-opuAC*], *opuB* [*opuBA-opuBB-opuBC-opuBD*] and *opuC* [*opuCA-opuCB-opuCC-opuCD*] operons (Kempf and Bremer, 1995; Kappes et al., 1999). In the Δ(*opuA::tet*)3, Δ(*opuB::ery*)1 and Δ(*opuC::spc*)3 mutant alleles, the entire coding sequences oft the *opuA*, *opuB* and *opuC* operons has been removed and was replaced by the indicated antibiotic resistance cassettes.

[b)]In the Φ(*opuA-treA*)*1* reporter fusion carried by this strain, a promoterless *treA* gene is placed under the transcriptional control of the osmotically regulated *opuA* promoter; the fusion junction between the truncated *opuA* material and *treA* is present within the *opuAA* gene of the *opuA* operon. The Φ(*opuA-treA*)*1* reporter construct was stably integrated via a double-homologous recombination event as a single copy into the *B. subtilis* genome within the non-essential *amyE* gene that is thereby rendered non-functional.

Legends to Figures

Fig. 1. Chemical structures of DMSP and its natural and synthetic derivatives. DMSP: dimethylsulfoniopropionate; DMSeP: dimethylseleniopropionate; DMTeP: dimethyltelluriopropionate; EMSP: ethylmethylsulfoniopropionate; DESP: diethylsulfoniopropionate; MPSP: methylpropylsulfoniopropionate; IMSP: isopropylmethylsulfoniopropionate; TMSP: tetramethylenesulfoniopropionate; GB: glycine betaine; DMSA: dimethlysulfonioacetate.

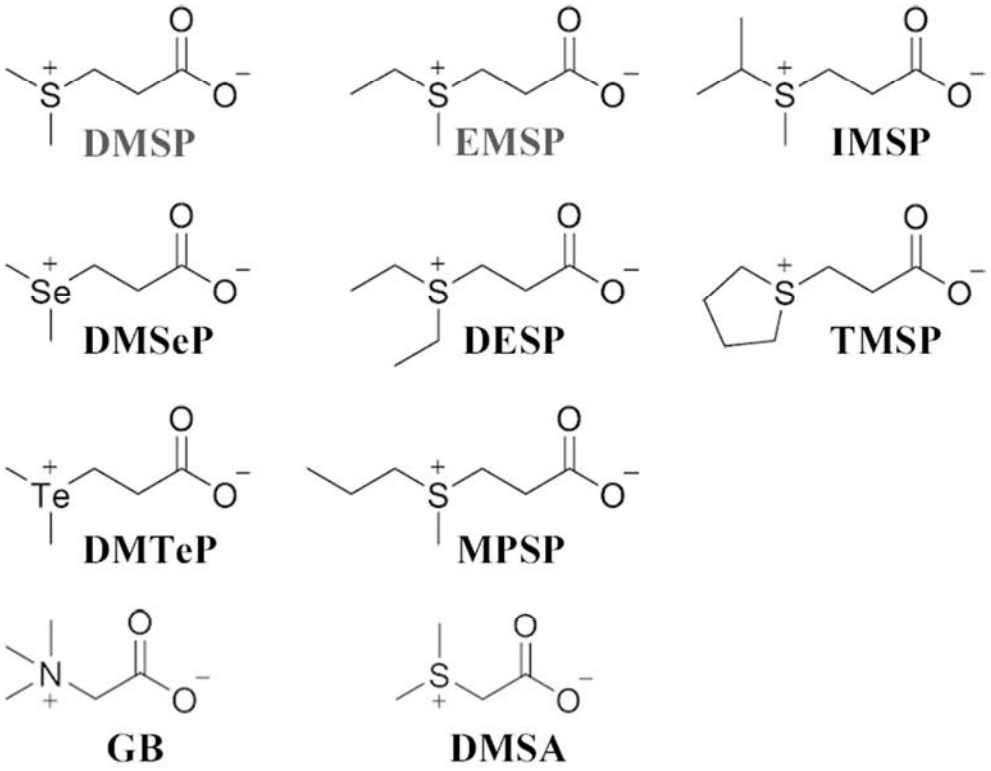
Fig. 2. Protection of *B. subtilis* against salt, cold and heat challenges. (A) Cells of the *B. subtilis* strain JH642 were grown at 37° C in SMM containing 1.2 M NaCl either in the absence or the presence of the indicated compounds. (B) Cultures of the *B. subtilis* strain 168 were propagated at 13° C in SMM in the presence of the indicated compounds. (C) Cells of the *B. subtilis* strain JH642 strain were grown at 52° C in SMM in the presence or absence of the indicated compounds.

Fig. 3. Influence of DMSP and its derivatives on the cellular proline pool build up via *de novo* synthesis under osmotic stress conditions. Cells of the *B. subtilis* strain JH642 were grown in SMM containing 1. 2 M NaCl in the absence or presence of various concentrations (25 µM – 1000 µM) of the indicated compounds to mid-exponential phase (OD_{578nm} of about 1.6) and were then used the determination of their proline content by a colorimetric assay. The data shown are the results from two independently grown cultures and two technical replicas of the proline assay.

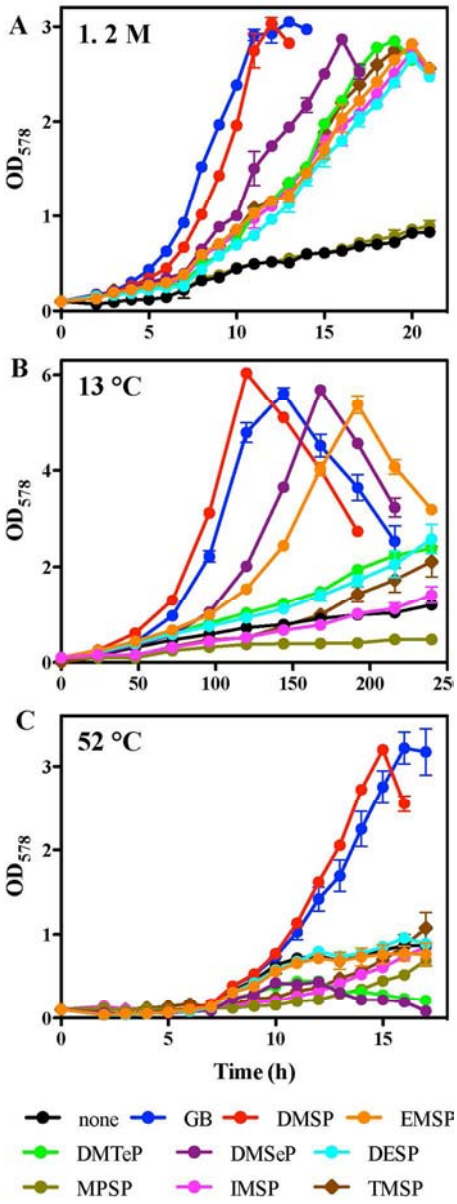
Fig. 4. Import of DMSP and its derivatives via the OpuA and OpuC ABC transport systems under osmotic stress. Cells of the *B. subtilis* strain JH642 and its mutant derivatives SBB1 ($OpuA^+$), SBB2 ($OpuC^+$) and SBB4 ($OpuA^-$ $OpuC^-$) were grown at 37° C in either the absence or the presence of the indicated compounds in SMM containing 1.2 M NaCl; the growth-yield of the cultures was determined by measuring their OD_{578nm} after 13 h of incubation. The values shown represent data from three independent biological experiments with two technical replicas for each experiment.

Fig. 5. Kinetic parameters of the OpuA and OpuC transporter system for DMSP. Michaelis-Menten kinetics of the uptake of [1-^{14}C]glycine betaine (closed circles) and determination of the competitive inhibition of glycine betaine import by DMSP (open circles) via the OpuA (A) and OpuC (B) transport systems. The glycine betaine concentration in the uptake assays was varied as indicated, whereas the concentration of DMSP was kept constant; 1 000 µM for the transport assays conducted with the $OpuA^+$ strain SBB1 (A) and 150 µM for those conducted with the $OpuC^+$ strain SBB2 (B).

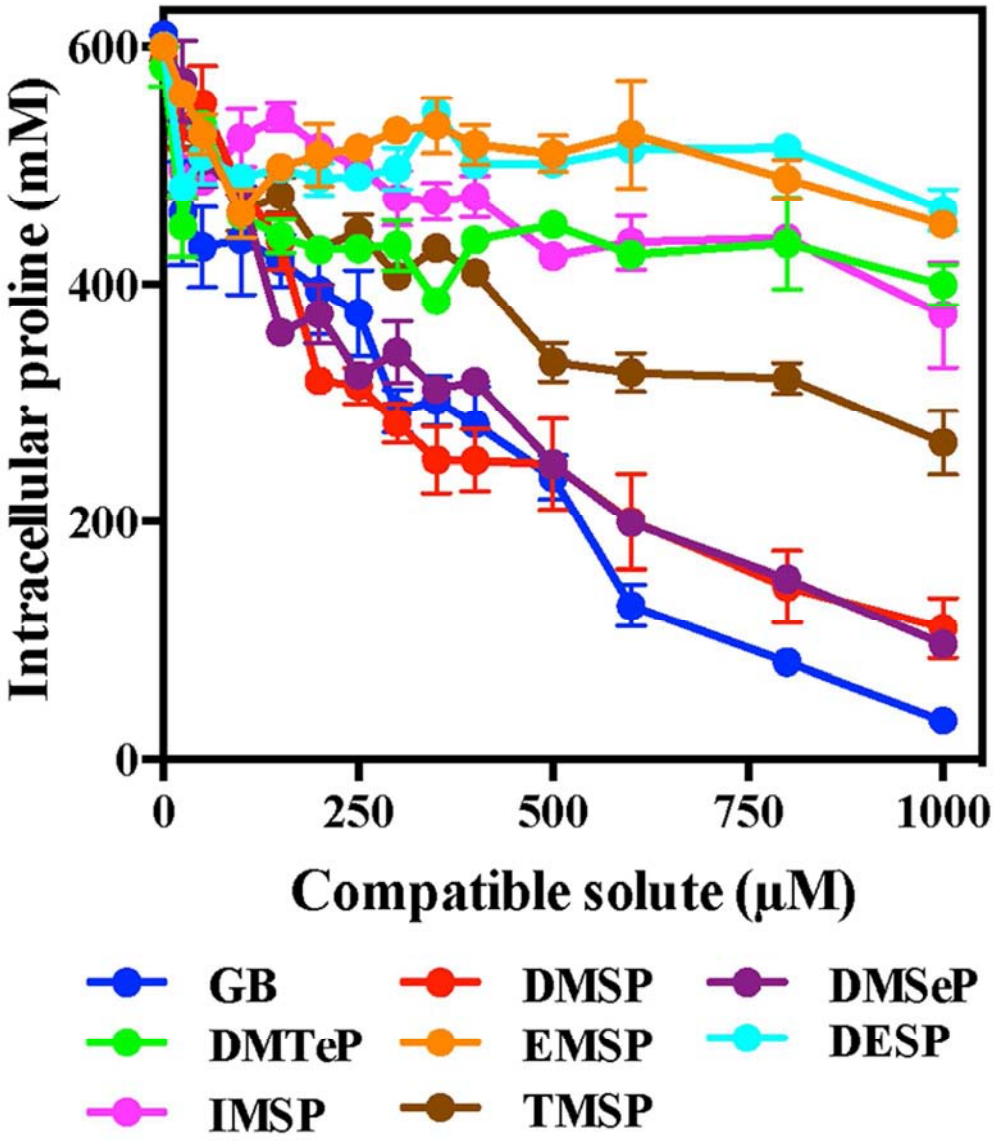
Fig. 6. *in silico* models for the binding of DMSP by the OpuAC and OpuCC solute receptor proteins. Coordination of DMSA (dimethylsulfonioacetate) within the OpuAC ligand-binding site (OpuAC:DMSA); the structural data were taken from the PDB database (PDB accession code 3CHG). (B) *in silico* model for the OpuAC:DMSP complex. (C) Coordination of glycine betaine within the OpuCC ligand-binding site (OpuCC:GB); the structural data were taken from the PDB database (PDB accession code 3PPP). (D) *in silico* model for the OpuCC:DMSP complex.



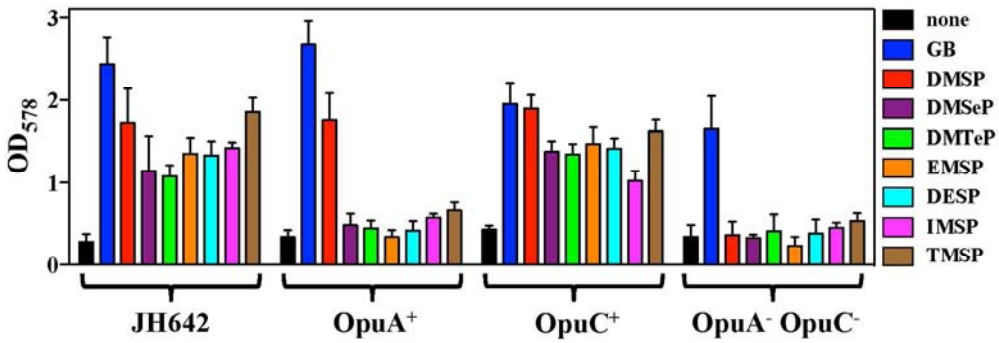
70x60mm (300 x 300 DPI)



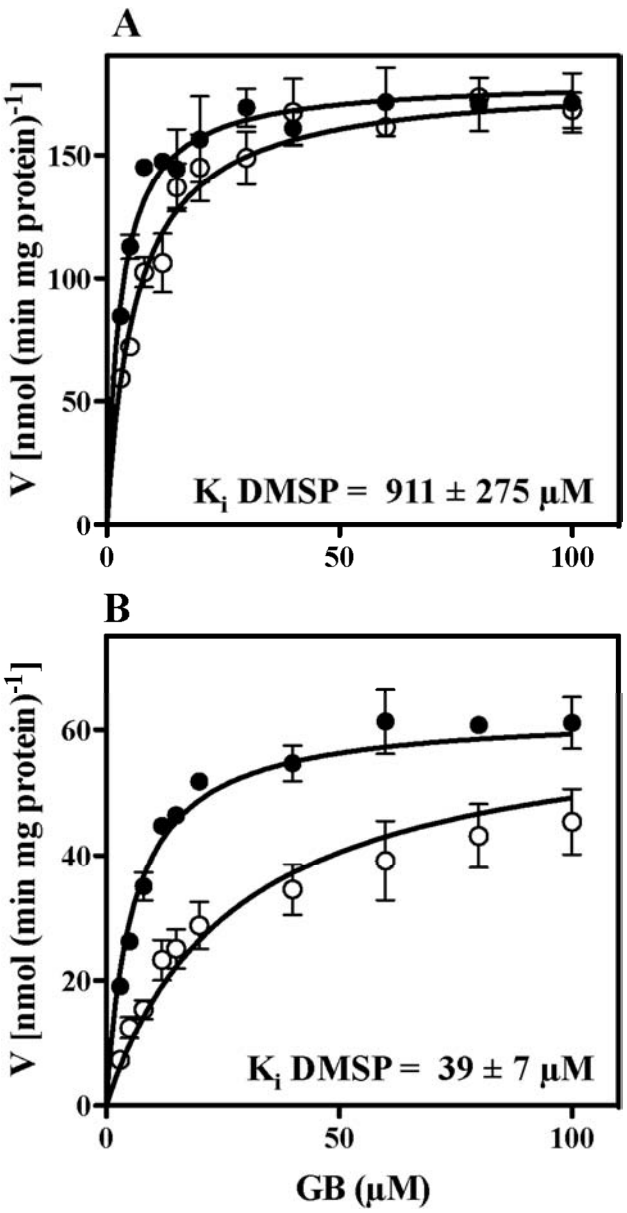
70x187mm (300 x 300 DPI)



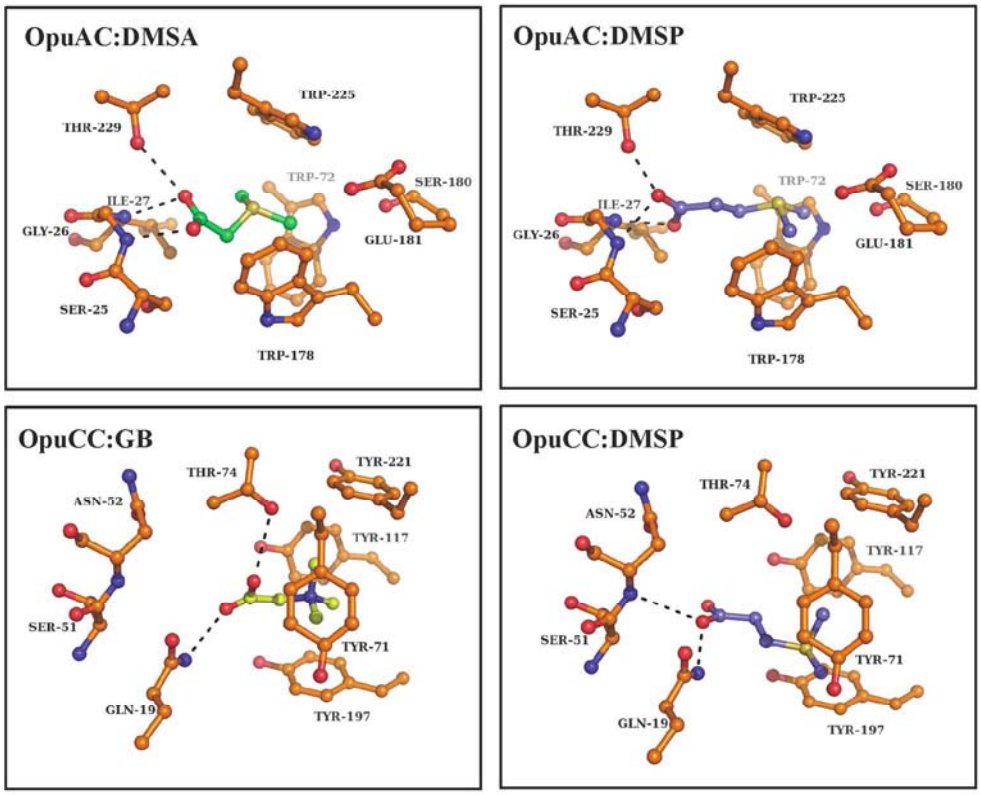
70x80mm (300 x 300 DPI)



148x50mm (300 x 300 DPI)



72x140mm (300 x 300 DPI)



160x131mm (300 x 300 DPI)