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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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Summary

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.

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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 nanomolar or low micromolar 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; 2012; Levine *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-

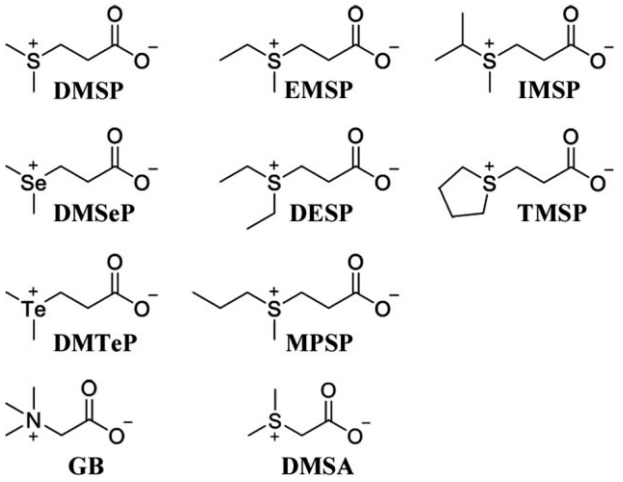

Fig. 1. Chemical structures of DMSP and its natural and synthetic derivatives. GB, glycine betaine.

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*., 2014a,b). 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*., 2014a,b).

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 dimethylseleniopropionate (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 [Spizizen's minimal 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). Because 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, its natural selenium analogue 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 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 with the established heat stress protectant glycine betaine (Holtmann and Bremer, 2004) (Fig. 2C).

Import of DMSP and its derivatives downregulates 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

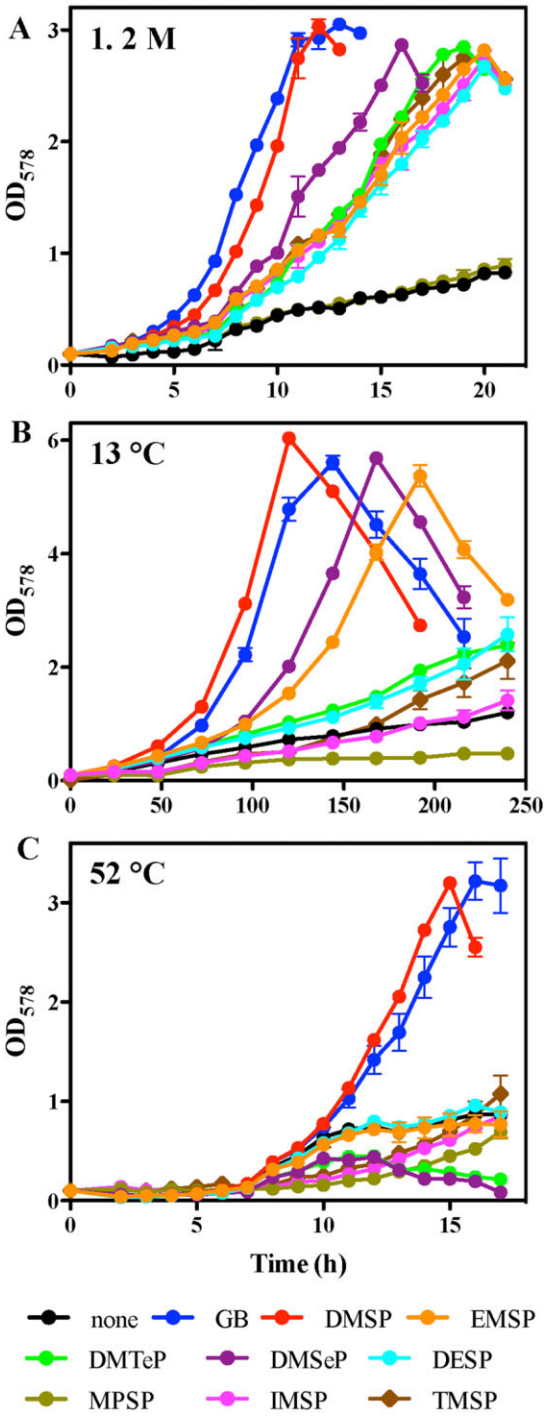

Fig. 2. Protection of *B. subtilis* against salt, cold and heat challenges. (A) Cells of the *B. subtilis* strain JH642 were grown at 37°C in SMM containing 1.2 M NaCl either in the absence or the presence of the indicated compounds. (B) Cultures of the *B. subtilis* strain 168 were propagated at 13°C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

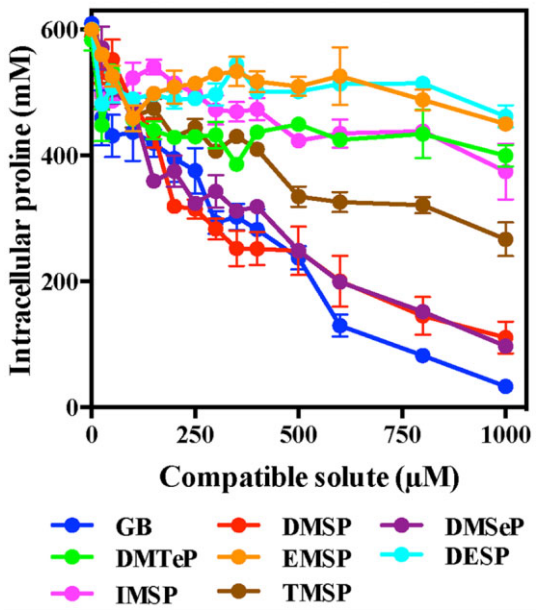

Fig. 3. Influence of DMSP and its derivatives on the cellular proline pool build up via *de novo* synthesis under osmotic stress conditions. Cells of the *B. subtilis* strain JH642 were grown in SMM containing 1.2 M NaCl in the absence or presence of various concentrations (25–1000 µM) of the indicated compounds to mid-exponential phase (OD_{578nm} of about 1.6) and were then used the determination of their proline content by a colourimetric assay. The data shown are the results from two independently grown cultures and two technical replicas of the proline assay.

osmoprotectants downregulates 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 a similar effect (Fig. 3). All other DMSP derivatives affected the proline pools more modestly, with TMSP conferring an intermediate effect (Fig. 3).

DMSP and its derivatives reduce the level of opuA *expression at high salinity*

The import of glycine betaine by osmotically stressed cells not only allows maintenance of a physiologically appropriate level of cellular hydration (Cayley *et al*., 1992), but it also affects gene expression in *B. subtilis* on a global scale (Kohlstedt *et al*., 2014). The activity of the promoter for the *opuA* operon is a good reporter for such effects because it is both strongly induced by high osmolarity and also responsive to cellular pools of various compatible solutes built up through transport processes (Hoffmann *et al*., 2013; Bashir *et al*., 2014a,b).

Strain MBB9 carries a chromosomal copy of an *opuA-treA* operon fusion that expresses this hybrid reporter gene under the control of the *opuA* promoter (Hoffmann *et al*., 2013). The level of the TreA reporter enzyme, a salt-tolerant phospho-α-(1,1)-glucosidase (Gotsche and Dahl, 1995), can thus be used as a read-out for the assessment of the potential influence of DMSP and its derivatives on osmotically controlled gene expression. Cultures of strain MBB9 were grown in the absence or presence (1 mM) of these solutes in SMM or in SMM containing 1.2 M NaCl. In the absence of a compatible solute in the growth medium, transcription of the *opuA-treA* reporter fusion was induced about 8.5-fold when the external salinity was increased (Table 1). The osmoprotectants glycine betaine and carnitine (Boch *et al*., 1994; Kappes and Bremer, 1998) reduced the salt-induced level of *opuA* transcription about fivefold to sixfold, and so did DMSP (Table 1). DMSP, glycine betaine and carnitine also downregulated (between 3.6 and 4-fold) the level of *opuA-treA* expression found in the absence of added NaCl and thereby still permitted an osmotic upregulation (between 4.9 and 6.6-fold) in the expression level of the reporter fusion (Table 1). The reduction in the level of *opuA-treA* transcription by the

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

	TreA activity [U (mg protein^{-1})]	
Compatible solute	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.

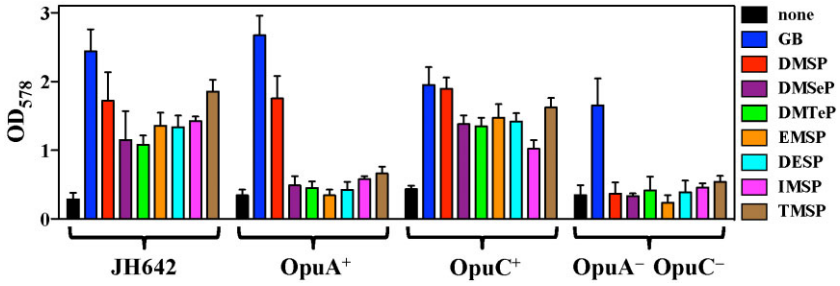

Fig. 4. Import of DMSP and its derivatives via the OpuA and OpuC ABC transport systems under osmotic stress. Cells of the *B. subtilis* strain JH642 and its mutant derivatives SBB1 (OpuA$^+$), SBB2 (OpuC$^+$) and SBB4 (OpuA$^-$ OpuC$^-$) were grown at 37°C in either the absence or the presence of the 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.

DMSP selenium analogue DMSeP and the tested synthetic DMSP derivatives in salt-stressed and non-stressed cells was less pronounced, between 1.5- and 2.5-fold under osmotic stress conditions and between 1.7- and 3.5-fold in non-stressed cells (Table 1).

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). *Bacillus subtilis* possesses five osmotically inducible uptake systems for these compounds, the Opu (***o***smo***p***rotectant ***u***ptake) family of transporters which comprises both multicomponent ABC transporters (OpuA, OpuB and OpuC) and single component uptake systems that belong either to the SSS (OpuE) or to the BCCT (OpuD) superfamilies (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) because 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 radiolabelled [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.

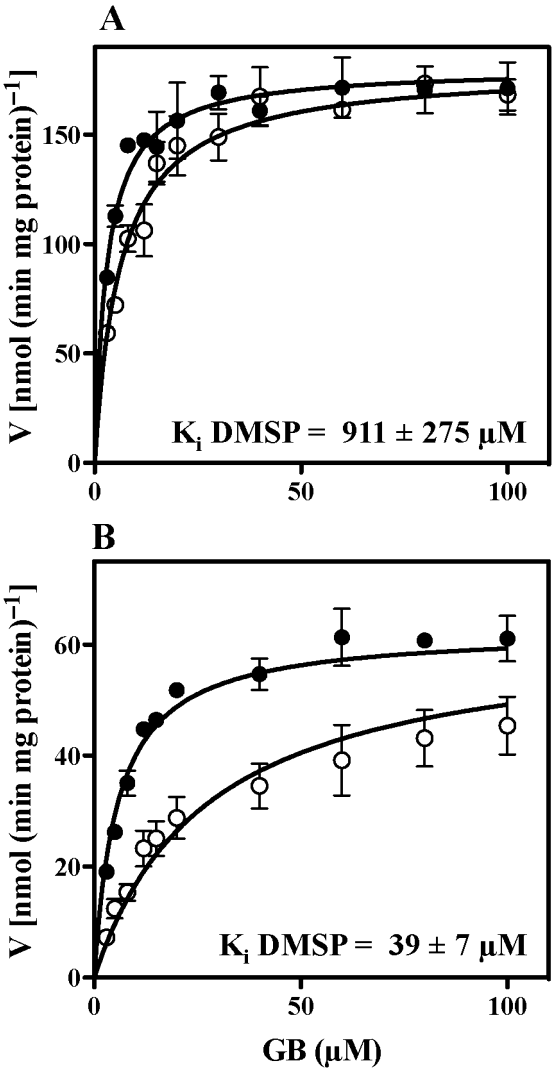

Fig. 5. Kinetic parameters of the OpuA and OpuC transporter system for DMSP. Michaelis–Menten kinetics of the uptake of [1-^{14}C]glycine betaine (closed circles) and determination of the competitive inhibition of glycine betaine import by DMSP (open circles) via the (A) OpuA and (B) OpuC transport systems. The glycine betaine concentration in the uptake assays was varied as indicated, whereas the concentration of DMSP was kept constant; (A) 1000 µM for the transport assays conducted with the OpuA$^+$ strain SBB1 and (B) 150 µM for those conducted with the OpuC$^+$ strain SBB2.

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). Because no ligand-binding protein associated with an ABC transport system has been crystallized in the presence of DMSP, we carried out *in silico* modelling studies to derive clues on the molecular determinants governing the binding of DMSP by the OpuAC and OpuCC proteins. We relied for these ligand-docking experiments on crystallographic data available for the OpuAC protein in complex with the sulfur analogue of glycine betaine, dimethylsulfonioacetate (DMSA; Fig. 1) (PDB code 3CHG) (Smits *et al.*, 2008) and the OpuCC : glycine betaine complex (PDB code 3PPP) (Du *et al.*, 2011) (Fig. 6). Crystallographic data relevant for the properties of the DMSP ligand were extracted from the structure of the DMSP lyase DddQ from *Silicibacter lacuscaerulensis* (PDB database entry 4LA2) (Li *et al.*, 2014).

The OpuAC : DMSA complex (Smits *et al.*, 2008) was chosen as the starting structure for the modelling because DMSP and DMSA are chemically closely related

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 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 radiolabelled 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}.

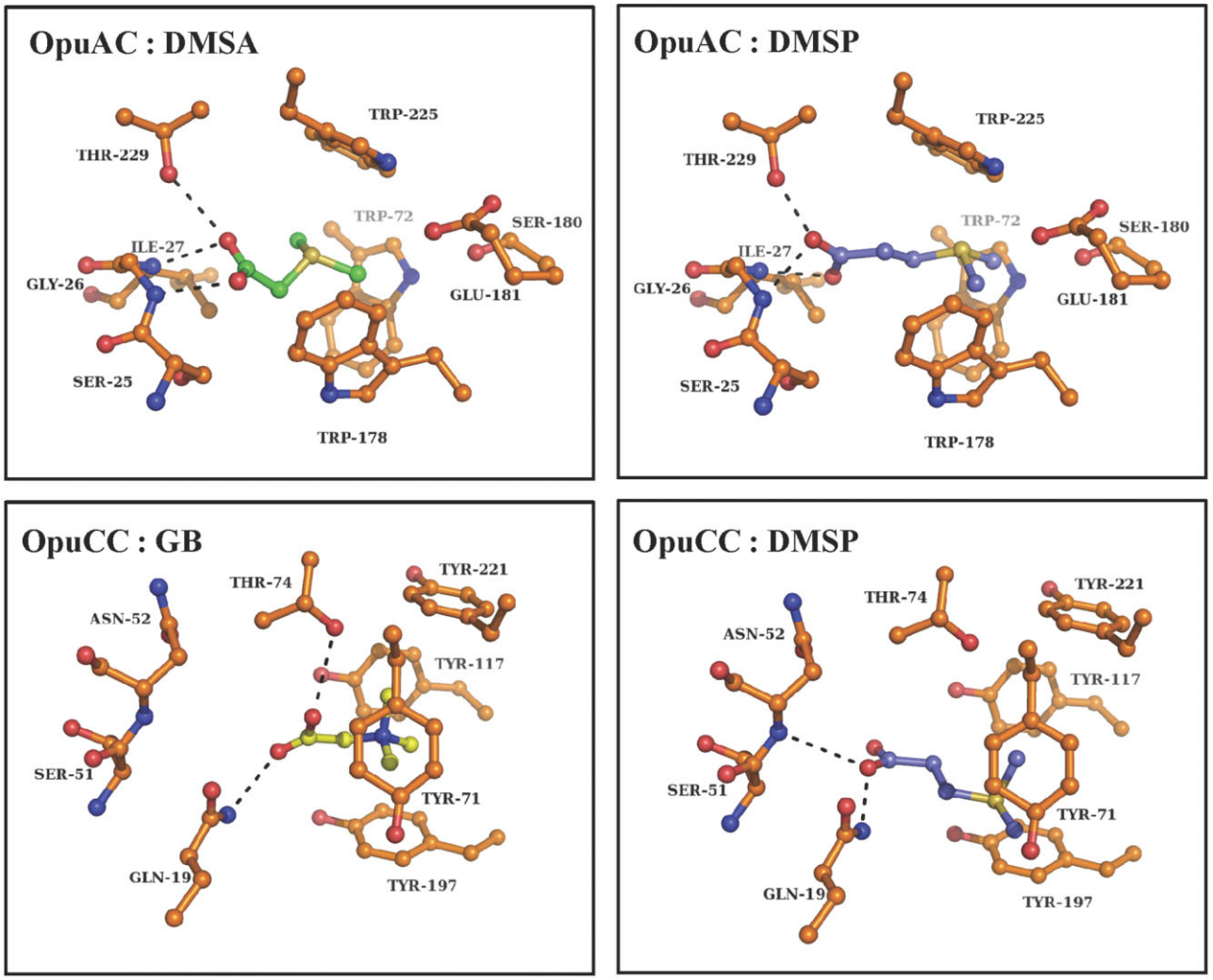

Fig. 6. *In silico* models for the binding of DMSP by the OpuAC and OpuCC solute receptor proteins. Coordination of DMSA within the OpuAC ligand-binding site (OpuAC : DMSA); the structural data were taken from the PDB database (PDB accession code 3CHG). (B) *In silico* model for the OpuAC : DMSP complex.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

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

The reduced number of cation-π and van der Waals interactions of the OpuAC : DMSA complex in comparison with the OpuAC : glycine betaine crystal structure decreases the binding of DMSA by OpuAC relative to glycine betaine by fivefold; from a K_d of about 20 μM for

glycine betaine to a K_d of about 100 μM for DMSA (Smits *et al*., 2008). Given the low affinity of the OpuA 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 suboptimal 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 atoms are 103, 117 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) likely contributes 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.

Because 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* modelling 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 relative to that of glycine betaine) 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

Because 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 (IMG) database maintained by the Department of Energy (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* modelling 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 because 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; 2012; Levine *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 scavenge not only 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 because 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 modelling 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). *Bacillus subtilis* is no exception in this regard because both OpuA and OpuC serve for high-affinity glycine betaine import as well (Kempf and Bremer, 1995; Kappes *et al*., 1996; 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 catalyses glycine betaine import (Kappes *et al*., 1996) but does not participate in DMSP uptake. OpuD is a member of the BCCT 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 *Escherichia 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 on 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) and 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. Because 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, of 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 because 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 defence 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 colourimetric assay (Bates *et al*., 1973) were purchased from Sigma-Aldrich (Steinheim, Germany). Radiolabelled [1-^{14}C]glycine betaine (55 mCi mmol^{-1}) was obtained from American Radiolabeled Chemicals (St Louis, MO, USA). DMSP was purchased from Carbon Scientific (London, UK). The antibiotics kanamycin, erythromycin, spectinomycin and tetracycline were obtained from SERVA Electrophoreses GmbH (Heidelberg, Germany), United States Biochemical Corp. (Cleveland, OH, USA) and Sigma-Aldrich respectively. Chemicals for the synthesis of DMSP and its derivatives were obtained from Sigma-Aldrich or Acros Organics (Thermo Fisher Scientific, Geel, Belgium), and used without further purification. The synthesis of DMSP, DMSeP, DMTeP, EMSP, DESP, MPSP, IMSP and TMSP was performed by acid-catalysed 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*

Bacillus subtilis strains were routinely maintained on Luria–Bertani (LB) agar plates or cultured in LB liquid medium (Miller, 1972). The antibiotic concentrations for the selection 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 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,

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

Strain	Relevant genotype[a]	Origin/reference
JH642	*trpC2 pheA1*	Brehm and colleagues (1973)
RMKB7	JH642 Δ(*opuD::neo*)2	Kappes and colleagues (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 and colleagues (2013)
168	*trpC2*	Barbe and colleagues (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 of the ABC transporter superfamily family and are multicomponent 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 of 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.

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. *Bacillus 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 lineage 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. Because 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 analyse 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 that 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 from plasmid pDG647 (Guerout-Fleury *et al*., 1995). The [Δ(*opuC*::*spc*)3] mutation was constructed by replacing a 3 419 bp '*opuCA-opuCB-opuCC-opuCD*'-fragment with a 1 173 bp DNA fragment encoding a spectinomycin resistance cassette that was derived from plasmid pDG1726 (Guerout-Fleury *et al*., 1995). The formerly described strain RMKB7 carries a gene disruption in the *opuD* gene that encodes a single component glycine betaine uptake system (Kappes *et al*., 1996). Combinations of single *opu* mutations were constructed by transforming appropriate recipient strains with chromosomal DNA of *B. subtilis* mutants carrying various *opu* alleles marked with antibiotic resistance cassettes (Table 3). Preparation of chromosomal DNA from *B. subtilis* strains, transformation of *B. subtilis* with this DNA and the selection of transformants via their antibiotic resistance were conducted according to routine procedures (Cutting and Vander Horn, 1990; Harwood and Archibald, 1990). Derivatives of the *B. subtilis strain* 168 carrying gene disruption mutations have been described before (Hoffmann and Bremer, 2011).

Determination of cellular proline pools in osmotically stressed cells

The intracellular proline content of osmotically stressed cells of the wild-type JH642 strain was determined by a colourimetric assay detecting proline as a coloured prolin–ninhydrine complex, which can be quantified by measuring the absorption of the solution at 480 nm in a spectrophotometer (Bates *et al*., 1973). Cells of strain JH642 were grown in SMM containing 1.2 M NaCl in the absence or presence of various concentrations (25 μM to 1000 μM) of glycine betaine, DMSP, DMSeP, DMTeP, EMSP, DESP, IMSP and TMSP until they reached an OD_{578nm} of about 1.6. Harvesting of the cells by centrifugation, their processing for the colourimetric proline detection assay, and the details of the calculation of the intracellular volume of *B. subtilis* and of the concentration of proline have all been described previously (Hoffmann and Bremer, 2011; Hoffmann *et al*., 2013).

Transport studies

Cultures of the *B. subtilis* strains SBB1 ($OpuA^+$) and SBB2 ($OpuC^+$) (Table 3) were grown in SMM containing 0.4 M NaCl to an OD_{578nm} of about 0.3. Two millilitre aliquots were withdrawn and mixed with a solution of glycine betaine 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-radiolabelled DMSP for the $OpuA^+$ strain SBB1 and non-radiolabelled 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 radiolabelled 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, 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*., 2014a,b). 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 colourimetric substrate PNPG per minute. 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 (PDB) 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 analysing 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 analysed 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 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 IMG database (https://img.jgi.doe.gov/cgi-bin/w/main.cgi) maintained by the 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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Supporting information

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

Fig. S1. Use of DMSP as sole carbon and nitrogen source by *B. subtilis*. (A) The use of DMSP as sole carbon source was tested in SMM growth media by replacing glucose (28 mM) with 33 mM DMSP; the growth yield of the cultures was assessed after 20 h of incubation at 37°C. (B) The use of DMSP as sole sulfur source was assessed by replacing the sulfur source [$(NH_4)_2SO_4$; 15 mM] present in SMM with either 2 mM or 15 mM DMSP in the presence of [$(NH_4)_2PO_4$; 15 mM] as the nitrogen source. The $MgSO_4$ (200 μM) present in SMM was simultaneously replaced by 200 μM $MgCl_2$. The growth yield of the cultures was measured after 20 h of incubation at 37°C.

Fig. S2. Uptake of DMSP and its derivatives via the OpuA and OpuC ABC transporters under sustained cold or heat stress. (A) Cells of the *B. subtilis* strain 168 (wild type), JGB25 (OpuA$^+$), JGB24 (OpuC$^+$) and JGB23 (OpuA$^-$ OpuC$^-$) were grown at 13°C in SMM either in the absence or the presence of the indicated compounds. (B) Cultures of the *B. subtilis* strain JH642 (wild type), SBB1 (OpuA$^+$), SBB2 (OpuC$^+$) and SBB4 (OpuA$^-$ OpuC$^-$) were propagated at 52°C in SMM in the presence of the indicated compounds. The growth yield of the cold or heat stressed cultures was assessed by measuring their OD_{578} after 168 h and 13 h respectively.

Fig. S3. Kinetics of the inhibition of [1-^{14}C]glycine betaine uptake through the OpuC transporter in the absence and presence of competitors. Cultures of the *B. subtilis* strain SBB2 (OpuC^{+}) were grown in SMM containing 0.4 M NaCl and were used for transport experiments when they had reached an OD_{578} of about 0.3. Uptake kinetics of [1-^{14}C]glycine betaine was determined at various substrate concentrations either in the absence (closed circle) or the presence of a competitor (open circles). The various competitors were present in the uptake mixtures at a final substrate concentration of 150 μM. The data shown represent the averages of transport assays conducted with two independently grown cultures.

Fig. S4. Competition of DMSP and its derivatives with uptake of radiolabeled [1-^{14}C] glycine betaine. [1-^{14}C] glycine betaine uptake (final substrate concentration in the individual assays: 3 μM) was followed in cells of the the *B. subtilis* strain SBB2 (OpuC^{+}) that had been grown to early log-phase (OD_{578} of about 0.3) in SMM containing 0.4 M NaCl either in the absence or the presence of a potential competitor. The competitor compounds were present in the uptake assays at a concentration of 150 μM. [1-^{14}C] glycine betaine uptake in the absence (black circle) or the presence of unlabelled glycine betaine (blue circle), MPSP (green circle), DMSP (red circles) or DMSeP (purple circle).

Table S1. Bioinformatics' assessment of the distribution of OpuA- and OpuC-type transporters in the genus *Bacillus*.