

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 ecophysiologicaly 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 osmoadaptive 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.

46 **Introduction**

47
48 The tertiary sulfonium compound dimethylsulfoniopropionate (DMSP) (Fig. 1) is an integral
49 constituent of the global sulfur cycle operating on our planet (Charlson et al., 1987; Kiene et al.,
50 2000). It is produced in vast amounts (about 10^9 tons annually) by marine phytoplankton and
51 macroalgae and also by a restricted number of plants that typically populate ecosystems near the sea
52 (Yoch, 2002; Otte et al., 2004; Curson et al., 2011a; Reisch et al., 2011; Moran et al., 2012). These
53 organisms can attain high intracellular concentrations of DMSP through synthesis (up to 400 mM)
54 (Stefels, 2000) and upon cell lysis (e.g., after attack by grazing zooplankton and viruses) or osmotic
55 down-shock, release it into open ocean waters, estuarine ecosystems and sediments. In these natural
56 habitats, DMSP can be found in nM or low μ M concentrations (Kiene et al., 1998; Van Duyl et al.,
57 1998; Vila-Costa et al., 2006). Microorganisms can then take advantage of environmental DMSP
58 either as a stress protectant (Welsh, 2000), or as a nutrient (Curson et al., 2011b; Rinta-Kanto et al.,
59 2011; Levine et al., 2012; Rinta-Kanto et al., 2012; Todd et al., 2012).

60 Evidence for several ecophysiological functions of DMSP has been provided. It is considered
61 to act as an antioxidant, as a cryoprotectant, as a chemical cue in the grazing interactions between
62 zooplankton and phytoplankton and as a chemo-attractant for DMSP-consuming bacteria in their
63 relations with the corresponding DMSP-producing dinoflagellate (Karsten et al., 1992; Wolfe et al.,
64 1997; Bayles and Wilkinson, 2000; Sunda et al., 2002; Miller et al., 2004). It is, however, best known
65 for its role as an osmolyte for the producer organisms (Stefels, 2000), most of which live in high-
66 saline environments (Yoch, 2002; Curson et al., 2011a; Reisch et al., 2011; Moran et al., 2012).
67 Notably, osmostress protection by DMSP can also be conferred through its uptake by microorganisms
68 that do not produce it (Gouesbet et al., 1994; Pichereau et al., 1998; Cosquer et al., 1999; Bayles and
69 Wilkinson, 2000; Murdock et al., 2014).

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

75 their well-established role in osmoregulation, as they also serve as stabilizers of proteins, improve
76 their solubility and preserve the functionality of cell components or even of entire cells (Lippert and
77 Galinski, 1992; Bourot et al., 2000; Manzanera et al., 2002; Ignatova and Gierasch, 2006; Street et al.,
78 2010; Auton et al., 2011). The term chemical chaperone has been coined in the literature to reflect
79 these beneficial traits (Diamant et al., 2001).

80 Bacteria can derive protection against abiotic stress both through synthesis and uptake of
81 compatible solutes (Kempf and Bremer, 1998; Bremer and Krämer, 2000). A microorganism in which
82 these processes are well characterized, both at the physiological and at the molecular level is the
83 ubiquitously distributed Gram-positive bacterium *Bacillus subtilis* (Bremer, 2002). Upon a high
84 osmolarity challenge, *B. subtilis* produces very large amounts of the compatible solute proline as a cell
85 protectant (Whatmore et al., 1990; Brill et al., 2011; Hoffmann et al., 2013). This bacterium has also
86 been shown to attain relief from sustained osmostress through the import of different types of
87 compatible solutes, most of which are chemically related to either glycine betaine or proline (von
88 Blohn et al., 1997; Bremer, 2002; Hoffmann and Bremer, 2011; Bashir et al., 2014b; Bashir et al.,
89 2014a). The uptake of compatible solutes by *B. subtilis* is mediated via a set of osmotically inducible
90 uptake systems, the Opu family of transporters (Bremer, 2002). These transporters also serve for the
91 import of compatible solutes when they are used as protectants against extremes in either low or high
92 growth temperatures (Holtmann and Bremer, 2004; Hoffmann and Bremer, 2011; Bashir et al., 2014b;
93 Bashir et al., 2014a).

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

103 **Results**

104 *DMSP is not a nutrient for B. subtilis*

105 Many microorganisms can catabolize DMSP (Curson et al., 2011a; Reisch et al., 2011; Moran et al.,
106 2012). To test if *B. subtilis* could use it as sole carbon or sulfur source, cultures of the wild-type strain
107 JH642 were grown in a chemically defined medium (SMM or SMM with 0.4 M NaCl) in which
108 glucose (28 mM) was replaced with 33 mM DMSP as the sole carbon source. No growth was
109 observed after 20 h of incubation of the cultures (Fig. S1A). Likewise, no growth was observed when
110 DMSP was offered to the cells as sole sulfur source (15 mM) (Fig. S1B). Since DMSP can sometimes
111 be toxic, we also tested a lower concentration (2 mM) of DMSP in our growth assays; no growth was
112 observed under these conditions either (Fig. S1A and B). We therefore conclude that *B. subtilis*
113 belongs to the group of microorganisms that cannot exploit DMSP as a nutrient.

114

115 *Stress protection by DMSP and its derivatives against high salinity and extremes in growth* 116 *temperature*

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

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

131 resembled the established cold protectant glycine betaine in its ability to promote growth at 13° C, a
132 temperature that otherwise severely restricts growth of *B. subtilis* (Hoffmann and Bremer, 2011) (Fig.
133 2B). The naturally occurring DMSeP and the synthetic EMSP also offered reasonably good cold stress
134 protection, while the remaining synthetic DMSP derivatives afforded either no protection or provided
135 cellular protection at a very low level (Fig. 2B).

136 When DMSP and its full set of derivatives were assayed for their heat stress protection
137 potential at a growth temperature of 52° C, only DMSP provided thermoprotection to *B. subtilis* at a
138 level comparable to the established heat stress protectant glycine betaine (Holtmann and Bremer,
139 2004) (Fig. 2C).

140

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

142 The adaptation of *B. subtilis* to sustained high osmolarity growth conditions is afforded through the
143 biosynthesis and accumulation of large amounts of the compatible solute proline (Whatmore et al.,
144 1990; Brill et al., 2011). In this adjustment process, the intracellular proline concentration is
145 sensitively linked to the degree of the osmotic stress imposed by the environment onto the cell (Brill et
146 al., 2011; Hoffmann et al., 2013). Proline pools approaching or exceeding 0.5 M can be found when
147 the osmotic stress is severe (Hoffmann et al., 2013; Zaprasis et al., 2013). In turn, the import of
148 various kinds of osmoprotectants down-regulates the cellular proline content of high osmolarity
149 challenged cells in a finely tuned fashion and thereby allows the saving of precious energy sources and
150 biosynthetic building blocks for proline production (Akashi and Gojobori, 2002; Hoffmann et al.,
151 2013; Bashir et al., 2014b).

152 To test if DMSP and its various derivatives would exert similar dampening effects on the size
153 of the newly produced proline pool, we grew strain JH642 in SMM containing 1.2 M NaCl in the
154 absence or the presence of various concentrations of these compounds and measured the free proline
155 content of the cells. As observed previously (Hoffmann et al., 2013), the presence of glycine betaine in
156 the growth medium resulted in a significantly reduced intracellular proline pool as the glycine betaine
157 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 DMS_{Se}P 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*

186 The osmoprotective effect of an exogenous supply of compatible solutes depends on their import
187 (Kappes et al., 1996; Hoffmann et al., 2013). *B. subtilis* possesses five osmotically inducible uptake
188 systems for these compounds, the Opu (*osmoprotectant uptake*) family of transporters which
189 comprises both multi-component ABC transporters (OpuA, OpuB and OpuC) and single component
190 uptake systems that belong either to the MFS (OpuE) or to the BCCT (OpuD) super-families (Bremer,
191 2002). A comprehensive set of mutant strains is available, each expressing only one of these transport
192 systems, while the genes for the other transporters have been deleted (Table 3) (Hoffmann and
193 Bremer, 2011). These *B. subtilis* mutant strains thus allow a determination which transporter is used
194 by a given osmoprotectant through a simple growth assay. When applied to DMSP and its natural and
195 synthetic derivatives, we found that DMSP can confer osmoprotection in strains that possess
196 either an intact OpuA or OpuC system, whereas all other DMSP derivatives were imported only via
197 OpuC (Fig. 4). Consequently, in a strain with simultaneously defective OpuA and OpuC transporters,
198 osmoprotection by DMSP and its derivatives was lost, while that afforded by glycine betaine remained
199 (Fig. 4) since it can be imported not only via the ABC transporters OpuA and OpuC but also through
200 the BCCT (betaine-carnitine-choline-transporter)-type transporter OpuD (Kappes et al., 1996; Ziegler
201 et al., 2010).

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

206

207 *Kinetic parameters of OpuA and OpuC for DMSP and its derivatives*

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

213 the high-affinity import of glycine betaine by OpuA, uptake of DMSP was a low-affinity process and
214 yielded a K_i value of $912 \pm 275 \mu\text{M}$ (Fig. 5A).

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

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

230

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

233 The primary substrate recognition component of microbial binding-protein-dependent ABC
234 transporters are the extracellular solute receptor proteins of these systems (Berntsson et al., 2010).
235 OpuAC and OpuCC are the extracellular substrate binding proteins of the OpuA and OpuC ABC
236 transport systems (Kempf and Bremer, 1995; Kappes et al., 1999) and crystal structures of these
237 proteins in complex with various substrates have been reported (Horn et al., 2006; Smits et al., 2008;
238 Du et al., 2011). Since no ligand binding protein associated with an ABC transport system has been
239 crystalized in the presence of DMSP, we carried out *in silico* modeling studies to derive clues on the
240 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, dimethylsulfonioacetate (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 (Trp⁷², Trp¹⁷⁸ and Trp²²⁵) 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 Gly²⁶ and Ile²⁷ (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 CH₂ 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 Gly²⁶ and Ile²⁷ 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 μ M for glycine
267 betaine to a K_d of about 100 μ M 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

269 position of the dimethylsulfonio head group of DMSP within the OpuAC ligand binding site (Fig. 6) is
270 sub-optimal for the stability of the OpuAC:DMSP complex. Ligand binding by OpuAC is sensitive to
271 slight variations. Even conservative amino acid substitutions in the aromatic cage by other aromatic
272 residues that cause an altered geometry in the cation- π interactions can have drastic consequences for
273 the affinity of OpuAC for different ligands (Smits et al., 2008). The covalent radii for the S, Se and Te
274 are 103 pm, 117 pm and 135 pm, respectively (Housecroft and Sharp, 2008). The resulting increasing
275 bulkiness of the positively charged head-groups of DMSP, DMSeP, and TMSTeP (Fig. 1) is likely a
276 contributor why the DMSeP and DMTeP molecules cannot be stably bound by the OpuAC solute
277 receptor protein. As reflected by the high K_i value of the OpuA transporter for DMSP (approximately
278 1 mM) (Fig. 5A), binding of DMSP by the OpuAC receptor protein is thus a borderline case.

279 Since no crystal structure of the OpuCC protein with a sulfur-betaine such as DMSA is
280 available, we used the OpuCC:glycine betaine complex (Du et al., 2011) (Fig. 6) as the starting
281 structure for our *in silico* modeling study. Within the OpuCC protein, the positively charged
282 trimethylammonium head group of glycine betaine is housed and coordinated via four Tyr residues
283 (Tyr⁷¹, Tyr¹¹⁷, Tyr¹⁹⁷ and Tyr²²¹) arranged in form of an aromatic cage. The carboxylate of glycine
284 betaine protrudes out of this aromatic cage and is bound and spatially orientated within the binding
285 site via interactions with Gln¹⁹ and Thr⁷⁴ (Du et al., 2011). Our *in silico* docking experiment suggests a
286 similar, but not identical, position of the DMSP molecule within the ligand-binding site (Fig. 6).
287 Despite the shift in the overall position of the DMSP molecule, the same stabilizing interactions found
288 for the glycine betaine ligand in the experimentally determined OpuCC:glycine betaine complex are
289 also present in the *in silico* generated OpuCC:DMSP structure (Fig. 6). As expected, the positively
290 charged dimethylsulfonio head group of DMSP is accommodated within the aromatic cage via cation-
291 π interactions. The carboxylate of DMSP, however, interacts differently with the OpuCC protein, a
292 result of the increased chain length of the DMSP molecule. While the interaction of the DMSP ligand
293 with Gln¹⁹ is retained, the interaction with Thr⁷⁴ is lost and instead a new interaction with the
294 backbone nitrogen of Ser⁵¹ is established (Fig. 6). Notably, such an interaction of the carboxylate of
295 the carnitine ligand with the backbone of Ser⁵¹ has also been observed in the crystal structure of the
296 OpuCC:carnitine complex (Du et al., 2011). Hence, the described spatial orientation for ligands with

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

301

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

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

320

321 **Discussion**

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

325 habitats since this compound is produced abundantly in marine environments (Stefels, 2000; Yoch,
326 2002; Curson et al., 2011a; Reisch et al., 2011; Moran et al., 2012). In contrast to many
327 microorganisms living in marine ecosystems (Curson et al., 2011b; Rinta-Kanto et al., 2011; Levine et
328 al., 2012; Rinta-Kanto et al., 2012; Todd et al., 2012), our data show that *B. subtilis* cannot use DMSP
329 as a nutrient. However, it can exploit DMSP as an excellent stress protectant against challenges
330 conferred by sustained high salinity or lasting extremes in high and low growth temperature. This can
331 be done with a degree of efficiency matching that of the stress-protective effects of glycine betaine,
332 probably the most widely used compatible solute in nature (Yancey, 2005).

333 By chemical profiling a set of well-defined transporter mutants, we found that DMSP uptake
334 by osmotically and temperature-stressed *B. subtilis* cells are mediated under laboratory conditions by
335 two ABC transport systems, OpuA and OpuC. The *in silico* assessment of the occurrence of these
336 transporters revealed their presence in most Bacilli with a fully sequenced genome. We therefore
337 surmise that the osmotic and temperature stress protection afforded through DMSP import that we
338 describe here in detail for the model organism *B. subtilis* (Barbe et al., 2009; Belda et al., 2013) will
339 be of ecophysiological relevance for most members of the large and diverse *Bacillus* genus (Earl et al.,
340 2008; Logan and De Vos, 2009).

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

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

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

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

380 OpuD transporter, a system that catalyzes glycine betaine import (Kappes et al., 1996) but does not
381 participate in DMSP uptake. OpuD is a member of the BCCT (betaine-carnitine-choline-transporter)
382 family, carriers that are involved in the uptake of various types of compatible solutes (Ziegler et al.,
383 2010). Interestingly, a member (DddT) of the BCCT family was recently identified as a DMSP uptake
384 system in several DMSP-catabolizing species but it was also proficient in glycine betaine import when
385 assessed in a heterologous *E. coli* system (Todd et al., 2010; Sun et al., 2012).

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

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

408 molecular mechanisms are insufficiently understood. Studies in *B. subtilis* with the cold- and heat-
409 stress protectant glycine betaine have shown that the cellular pools of this compound attained under
410 temperature stress are far lower than those established under osmotic stress conditions (Holtmann and
411 Bremer, 2004; Hoffmann and Bremer, 2011; Hoffmann et al., 2013). This observation indicates that
412 the mechanism(s) for protection by glycine betaine against osmotic and temperature challenges are, at
413 least partially, different. Since cold stress can have significant effects on protein structure (Jaenicke,
414 1990), the cryoprotective effects of DMSP for *B. subtilis* and other microorganisms (Bayles and
415 Wilkinson, 2000; Angelidis and Smith, 2003; Murdock et al., 2014) might primarily stem from its
416 function as a chemical chaperone. Indeed, DMSP is known to stabilize *in vitro* the enzyme activities
417 of purified phosphofructokinase from rabbit muscle, the cold-labile model enzyme lactate
418 dehydrogenase, and of the malate dehydrogenase from the polar alga *Acrosiphonia arcta* under cold-
419 induced denaturing conditions (Nishiguchi and Somero, 1992; Karsten et al., 1996).

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

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

430

431 **Experimental procedures**

432 *Chemicals and synthesis of synthetic DMSP derivatives*

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

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

450

451 *Media and growth conditions for B. subtilis strains*

452 *B. subtilis* strains were routinely maintained on Luria-Bertani (LB) agar plates or cultured in LB liquid
453 medium (Miller, 1972). The antibiotic concentrations for the selection of *B. subtilis* strains carrying
454 chromosomal mutant alleles marked with an antibiotic resistance cassette were as follows: kanamycin
455 ($5 \mu\text{g ml}^{-1}$), erythromycin ($1 \mu\text{g ml}^{-1}$), spectinomycin ($100 \mu\text{g ml}^{-1}$), and tetracycline ($10 \mu\text{g ml}^{-1}$). For
456 stress protection growth assays by compatible solutes, *B. subtilis* strains were cultivated in Spizizen's
457 minimal medium (SMM) with 0.5% (wt/vol) glucose as the carbon source and a solution of trace
458 elements (Harwood and Archibald, 1990). L-tryptophan and L-phenylalanine were added to growth
459 media at final concentrations of 40 mg ml^{-1} and 36 mg ml^{-1} , respectively, to satisfy the growth
460 requirements of the *B. subtilis* strains JH642 and 168 and their mutant derivatives (Table 3). When the
461 use of DMSP by *B. subtilis* as either sole carbon source was tested, the glucose content (28 mM) in
462 SMM was replaced by 33 mM DMSP; likewise, when the use of DMSP as sole sulfur source was
463 assessed, the sulfur source present in SMM [$(\text{NH}_4)_2\text{SO}_4$; 15 mM] was replaced by 15 mM DMSP in

464 the presence of [(NH₄)₂PO₄; 15 mM] and MgSO₄ was replaced by MgCl₂. Use of DMSP as a nutrient
465 was also tested at a substrate concentration of 2 mM to assess possible toxic effects of higher DMSP
466 concentrations on growth. The osmolarity of the SMM was increased by the addition of NaCl from a 5
467 M stock solution. Compatible solutes were sterilized by filtration (Filtropur S 0.2 μm; Sarstedt,
468 Nürnberg, Germany) and were added to growth media at a finally concentration of 1 mM. Cultures
469 of *B. subtilis* cells were inoculated from exponentially growing pre-cultures in pre-warmed SMM to
470 optical densities (OD_{578nm}) of 0.1. *B. subtilis* cultures were grown in 20-ml culture volumes in 100-ml
471 Erlenmeyer flasks set in a shaking (set to 220 r.p.m.) water bath. Cultures used for heat-stress growth
472 protection assays at 52° C were inoculated from pre-cultures grown at 37° C to an OD₅₇₈ of about 1 to
473 an OD₅₇₈ of 0.1. The cultures were set in a water bath with a temperature of 37° C; the growth
474 temperature was then slowly increased to 52°C over a 20 min time frame. The temperature of the
475 water baths used for the heat and cold stress growth experiments was set and controlled with the aid of
476 a calibrated thermometer (Testo AG, Lenzkirch, Germany).

477

478 *Bacterial strains*

479 The *B. subtilis* strains JH642 (*trpC2 pheA1*) (Brehm et al., 1973) (Table 3), a member of the
480 domesticated inage of laboratory strains (Smith et al., 2014), was used for all experiments that
481 addressed the salt- and heat-stress protective potential of DMSP and its derivatives. Since it carries a
482 mutation in the *ilvB* gene that makes it cold sensitive (Wiegeshoff and Marahiel, 2007), the *B. subtilis*
483 laboratory strain 168 (Barbe et al., 2009) (Table 3) was used for studies that probed the potential of
484 these solutes as cold stress protectants. To analyze the transporter activities of individual Opu uptake
485 systems and to avoid a possible cross-talk of components of a given Opu ABC-transporter with
486 another Opu systems, we constructed a set of strains that carry deletions of the complete operons
487 coding for the OpuA-, OpuB- and OpuC ABC transporters. Strain TMB107 [Δ (*opuA::tet*)3] was
488 constructed by replacing a 2 700 bp '*opuAA-opuAB-opuAC*' DNA fragment with a 1834 bp DNA
489 fragment carrying a tetracycline resistance cassette which was derived from plasmid pDG1515
490 (Guerout-Fleury et al., 1995). Strain TMB116 [Δ (*opuB::ery*)1] carries a 3 139 bp '*opuBA-opuBB-*
491 *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 [$\Delta(opuC::spc)3$] 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 μ M to 1000 μ M) 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 [1-¹⁴C]glycine betaine; the final glycine betaine

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

531

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

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

545

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

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

566

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

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

574

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

577 The amino acid sequence of the ligand-binding proteins (OpuAC, OpuCC) of the OpuA and OpuC
578 ABC transporters (Kempf and Bremer, 1995; Kappes et al., 1999) were retrieved from the nucleotide
579 sequence of the *B. subtilis* laboratory strain 168 (Barbe et al., 2009) and used as query sequences for
580 BLAST-P database searches at the Integrated Microbial Genomes and Metagenomes database (IMG;
581 <https://img.jgi.doe.gov/cgi-bin/w/main.cgi>) maintained by the the Department of Energy (DOE) Joint
582 Genome Institute (Nordberg et al., 2013). We focused our analysis on members of the Bacillus genus
583 with a finished genome sequence. The retrieved OpuAC and OpuCC amino acid sequences were
584 aligned using Clustal W (Thompson et al., 2000) for inspection of conserved residues, in particular for
585 those that from the aromatic cages in the OpuAC and OpuCC proteins (Horn et al., 2006; Du et al.,
586 2011). The genome context of the *opuAC* and *opuCC* genes for the remaining components of the
587 OpuA and OpuC ABC transporters (Kempf and Bremer, 1995; Kappes et al., 1999) was assessed with
588 the bioinformatics tool provided by the IMG platform.

589

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602

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- 868

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

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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
DMS _e P	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

871

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

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886 **Table 2.** Kinetic parameters for the uptake of DMSP and its derivatives
 887 *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

888

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

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

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

Strain	Relevant genotype ^{a)}	Origin/reference
JH642	<i>trpC2 pheA1</i>	(Brehm et al., 1973)
RMKB7	JH642 $\Delta(\textit{opuD}::\textit{neo})2$	(Kappes et al., 1996)
SBB1	JH642 $\Delta(\textit{opuC}::\textit{spc})3 \Delta(\textit{opuD}::\textit{neo})2 \Delta(\textit{opuB}::\textit{ery})1$	This study
SBB2	JH642 $\Delta(\textit{opuA}::\textit{tet})3 \Delta(\textit{opuD}::\textit{neo})2 \Delta(\textit{opuB}::\textit{ery})1$	This study
SBB4	JH642 $\Delta(\textit{opuC}::\textit{spc})3 \Delta(\textit{opuA}::\textit{tet})3$	This study
TMB107	JH642 $\Delta(\textit{opuA}::\textit{tet})3$	This study
TMB108	JH642 $\Delta(\textit{opuC}::\textit{spc})3$	This study
TMB109	JH642 $\Delta(\textit{opuA}::\textit{tet})3 \Delta(\textit{opuD}::\textit{neo})2$	This study
TMB111	JH642 $\Delta(\textit{opuC}::\textit{spc})3 \Delta(\textit{opuD}::\textit{neo})2$	This study
TMB116	JH642 $\Delta(\textit{opuB}::\textit{ery})1$	This study
MBB9 ^{b)}	JH642 <i>amyE::</i> [$\Phi(\textit{opuA-treA})1 \textit{cat}$] (<i>treA::neo</i>)	(Hoffmann et al., 2013)
168	<i>trpC2</i>	(Barbe et al., 2009)
JGB23	168 $\Delta(\textit{opuA}::\textit{erm})4 \Delta(\textit{opuBD}::\textit{tet})23 \textit{opuC}20::\textit{Tn}10 (\textit{spc})$	(Hoffmann and Bremer, 2011)
JGB24	168 $\Delta(\textit{opuA}::\textit{erm})4 \Delta(\textit{opuBD}::\textit{tet})23 \Delta(\textit{opuD}::\textit{neo})2$	(Hoffmann and Bremer, 2011)
JGB25	168 $\Delta(\textit{opuBD}::\textit{tet})23 \textit{opuC}20::\textit{Tn}10 (\textit{spc}) \Delta(\textit{opuD}::\textit{neo})2$	(Hoffmann and Bremer, 2011)

904

905 ^{a)}The OpuA, OpuB and OpuC transport systems are members of the ABC transporter
906 superfamily family and are multi-component systems. They are encoded by the *opuA* [*opuAA-opuAB-*
907 *opuAC*], *opuB* [*opuBA-opuBB-opuBC-opuBD*] and *opuC* [*opuCA-opuCB-opuCC-opuCD*] operons
908 (Kempf and Bremer, 1995; Kappes et al., 1999). In the $\Delta(\textit{opuA}::\textit{tet})3$, $\Delta(\textit{opuB}::\textit{ery})1$ and
909 $\Delta(\textit{opuC}::\textit{spc})3$ mutant alleles, the entire coding sequences of the *opuA*, *opuB* and *opuC* operons has
910 been removed and was replaced by the indicated antibiotic resistance cassettes.

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

917 **Legends to Figures**

918

919 **Fig. 1.** Chemical structures of DMSP and its natural and synthetic derivatives. DMSP:
920 dimethylsulfoniopropionate; DMS_{Se}P: dimethylseleniopropionate; DMT_{Te}P:
921 dimethyltelluriopropionate; EMSP: ethylmethylsulfoniopropionate; DESP: diethylsulfoniopropionate;
922 MPSP: methylpropylsulfoniopropionate; IMSP: isopropylmethylsulfoniopropionate; TMSP:
923 tetramethylenesulfoniopropionate; GB: glycine betaine; DMSA: dimethylsulfonioacetate.

924

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

930

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

937

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

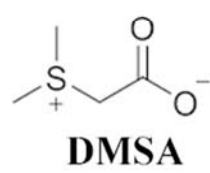
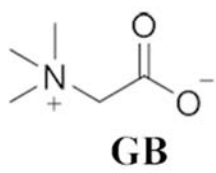
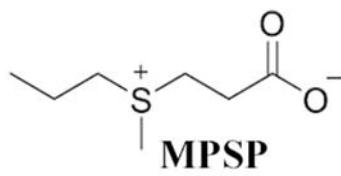
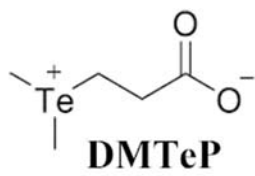
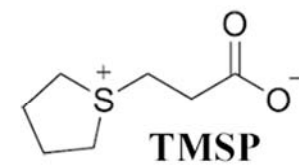
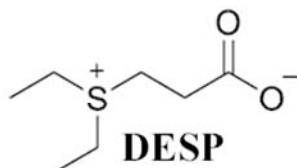
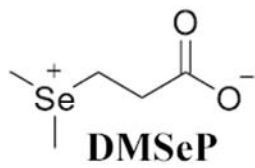
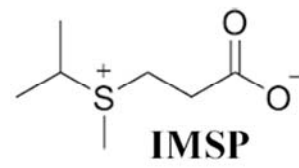
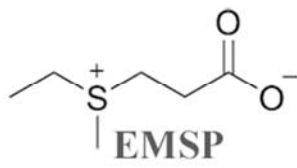
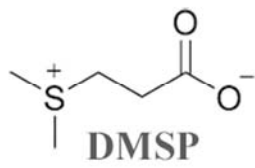
944

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

951

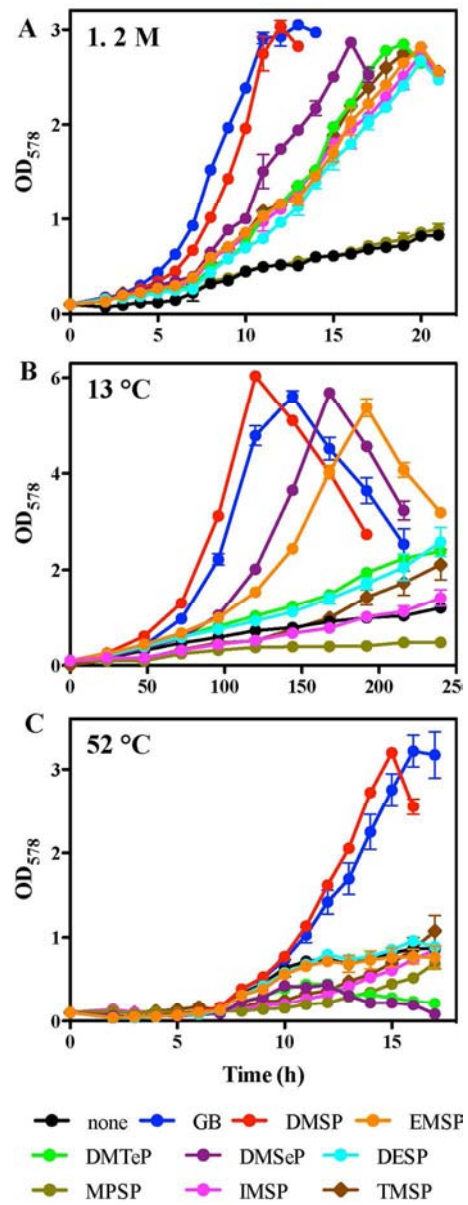
952 **Fig. 6.** *in silico* models for the binding of DMSP by the OpuAC and OpuCC solute receptor proteins.
953 Coordination of DMSA (dimethylsulfonioacetate) within the OpuAC ligand-binding site
954 (OpuAC:DMSA); the structural data were taken from the PDB database (PDB accession code 3CHG).
955 (B)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

958

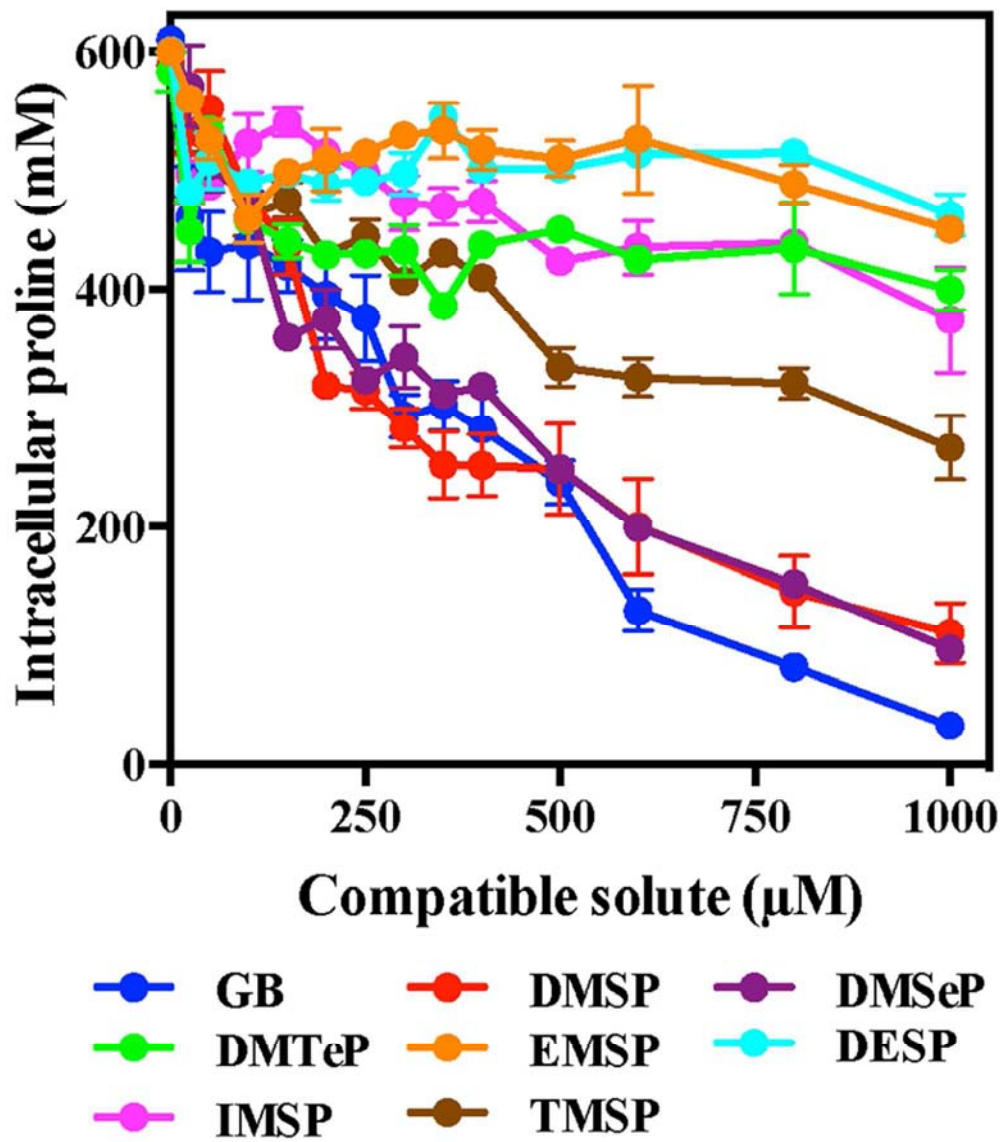


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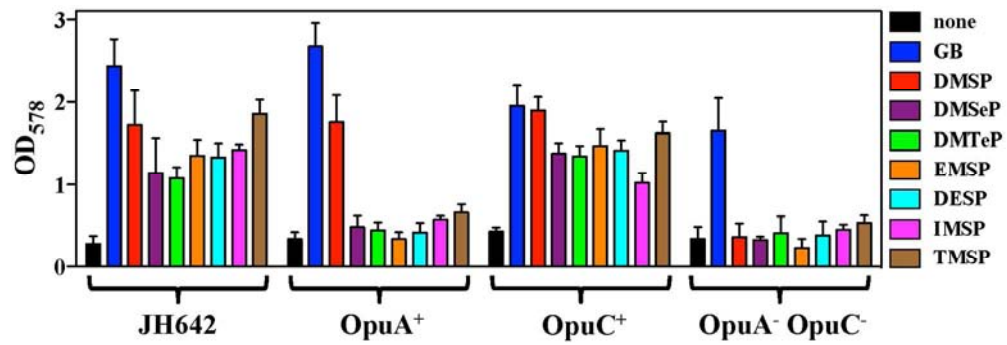
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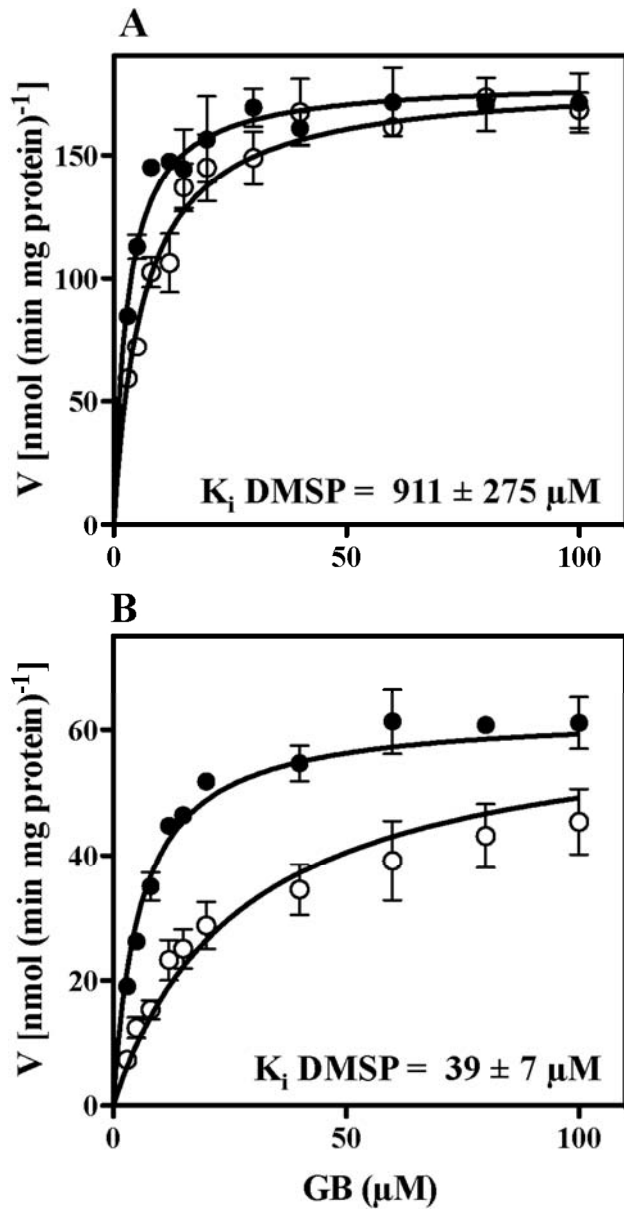
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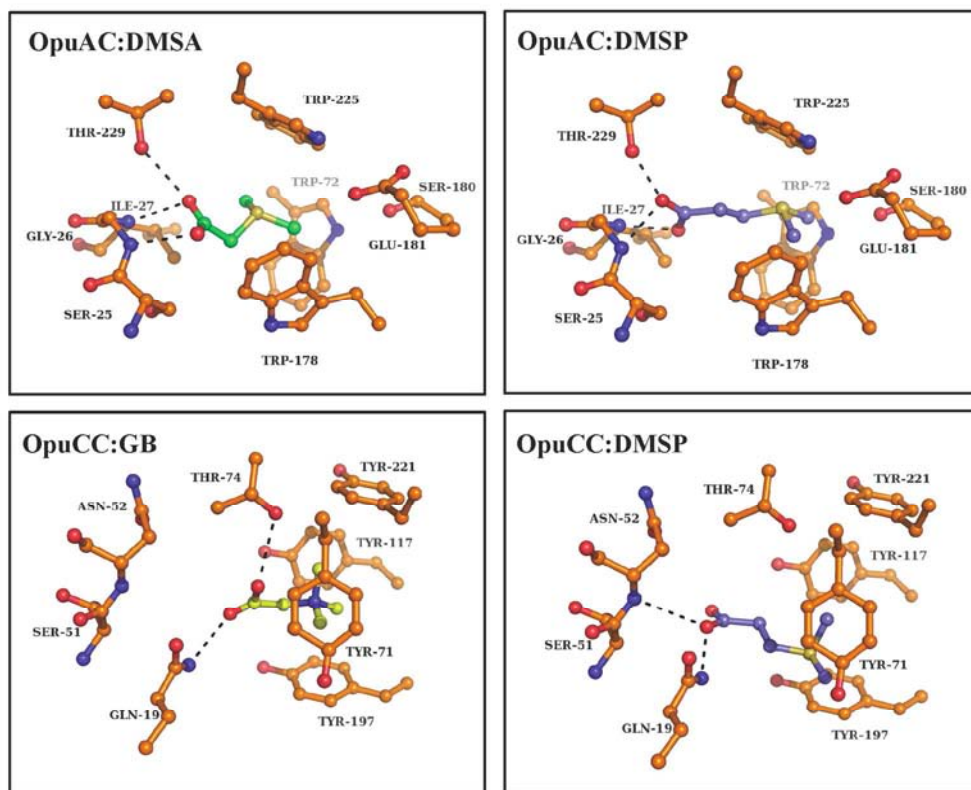
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