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Dimethylglycine Provides Salt and Temperature Stress Protection to *Bacillus subtilis*

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Glycine betaine is a potent osmotic and thermal stress protectant of many microorganisms. Its synthesis from glycine results in the formation of the intermediates monomethylglycine (sarcosine) and dimethylglycine (DMG), and these compounds are also produced when it is catabolized. *Bacillus subtilis* does not produce sarcosine or DMG, and it cannot metabolize these compounds. Here we have studied the potential of sarcosine and DMG to protect *B. subtilis* against osmotic, heat, and cold stress. Sarcosine, a compatible solute that possesses considerable protein-stabilizing properties, did not serve as a stress protectant of *B. subtilis*. DMG, on the other hand, proved to be only moderately effective as an osmotic stress protectant, but it exhibited good heat stress-relieving and excellent cold stress-relieving properties. DMG is imported into *B. subtilis* cells primarily under osmotic and temperature stress conditions via OpuA, a member of the ABC family of transporters. Ligand-binding studies with the extracellular solute receptor (OpuAC) of the OpuA system showed that OpuAC possesses a moderate affinity for DMG, with a K_d value of approximate 172 μM; its K_d for glycine betaine is about 26 μM. Docking studies using the crystal structures of the OpuAC protein with the sulfur analog of DMG, dimethylsulfonioacetate, as a template suggest a model of how the DMG molecule can be stably accommodated within the aromatic cage of the OpuAC ligand-binding pocket. Collectively, our data show that the ability to acquire DMG from exogenous sources under stressful environmental conditions helps the *B. subtilis* cell to cope with growth-restricting osmotic and temperature challenges.

Glycine betaine (*N*,*N*,*N*-trimethylglycine) (Fig. 1) is, without any doubt, the most widely used compatible solute in nature, as it is employed by members of all three kingdoms of life as an effective osmotic stress protectant (1–3). The physicochemical properties of glycine betaine, through its preferential exclusion from protein surfaces, make it highly compliant with protein function and lead to the stabilization of proteins under otherwise denaturing conditions (4–6). The term "chemical chaperone" has been used in the literature to address this general property of compatible solutes (7, 8). This attribute probably contributes significantly to the ability of glycine betaine to serve as a cytoprotectant of microorganisms against both high and low growth temperature extremes (9, 10). Remarkably, this can even lead to the efficient thermal protection of the hyperthermophilic archaeon *Archaeoglobus fulgidus* at 90°C by an external supply of glycine betaine (11).

Microorganisms can derive strong osmotic stress protection through the uptake of glycine betaine (12–15), and they employ various types of osmotically controlled transport systems for this task (1, 13, 14, 16–18). These uptake systems are often regulated in response to increases in external osmolarity both at the level of the actual transport activity (14, 16, 18, 19) and at the level of transcription of their structural genes (1, 14, 17, 19). As a result of this two-tier level of regulation, microbial cells are provided with an increased capacity to import glycine betaine under both acute and sustained osmotic stress conditions. The accumulation of compatible solutes is sensitively tied to the severity of the environmentally imposed osmotic stress (20, 21) and thereby allows the cell to relieve the harmful effects of high external salinity and osmolarity on turgor, physiology, and growth (14).

Bacteria can also synthesize glycine betaine to achieve osmotic stress protection (22–27). There are two pathways by which to produce glycine betaine under aerobic conditions: (i) oxidation of the precursor choline, a glycine betaine synthesis route that is frequently found not only in microbes but also in plants and animals, and (ii) sequential methylation of glycine, a pathway used primarily in halophilic archaea, sulfur bacteria, and some cyanobacteria. The choline-to-glycine betaine synthesis pathway relies on the import of choline and the subsequent two-step oxidation of this precursor molecule with glycine betaine aldehyde as the intermediate (22–25, 28, 29). The synthesis pathway that rests on the methylation of glycine does not require the uptake of a precursor molecule and yields the intermediates monomethylglycine (sarcosine) and dimethylglycine (DMG) (Fig. 1) (27, 30–33).

Glycine betaine not only functions as a stress protectant but also can serve as a nutrient for a considerable number of microorganisms (25, 27). Catabolism of glycine betaine under aerobic conditions proceeds through its sequential demethylation to glycine and the subsequent enzymatic conversion into serine, which then, in turn, is catabolized to the central metabolite pyruvate (25, 26, 34, 35). Glycine betaine can also be used as a growth substrate under anaerobic conditions (e.g., by the acetogenic Gram-positive bacterium *Eubacterium limosum*) with the concomitant forma-

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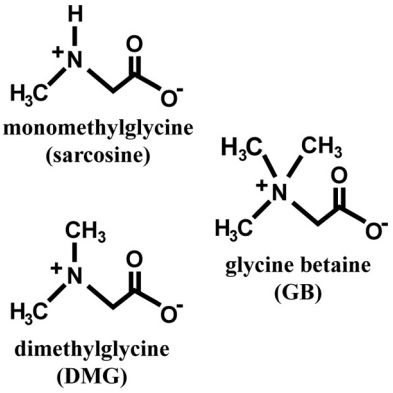

FIG 1 Chemical structures of monomethylglycine (sarcosine), DMG, and glycine betaine.

tion of DMG (36), and as shown recently, methanogens can also demethylate it to DMG (37). Hence, both in the synthesis of glycine betaine from glycine and during its catabolism, sarcosine and DMG are formed. It is thus likely that, in addition to the environmentally widely distributed glycine betaine molecule (15, 38), both sarcosine and DMG should be present in natural ecosystems and could thus potentially be scavenged by bacteria from environmental sources and used by nonproducer cells either as nutrients (25, 35, 39) or as stress protectants (40, 41).

Bacillus subtilis is well studied with respect to the excellent osmotic and temperature stress-relieving properties of glycine betaine (9, 10, 22), the transporters that mediate its high-affinity uptake (42–44), and the importers of the glycine betaine precursor choline and the enzymes that mediate its oxidation to glycine betaine (45). Here we have assessed the antistress protective potential of the glycine betaine metabolism intermediates DMG and sarcosine (Fig. 1) for osmotically and thermally challenged *B. subtilis* cells. We found that DMG possesses very good temperature stress-relieving properties both at the upper (52 to 53°C) and, in particular, at the lower (13°C) growth boundaries of *B. subtilis*. However, it served as an only modestly effective osmotic stress protectant. We identified the osmotically controlled ABC transporter OpuA (21, 43) as the main entry route of DMG into the *B. subtilis* cell under osmotic and temperature stress conditions. Sarcosine, on the other hand, had no noticeable stress-relieving potential for either osmotically or thermally challenged cells. Like glycine betaine (12), neither DMG nor sarcosine was used as a nutrient by this soil bacterium.

MATERIALS AND METHODS

Chemicals. Glycine betaine, DMG, sarcosine, the chromogenic substrate *para*-nitrophenyl-α-D-glucopyranoside (α-PNPG) for the TreA enzyme (46), and the ninhydrin reagent for the quantification of proline by a colorimetric assay (47) were all purchased from Sigma-Aldrich (Steinheim, Germany). [1-^{14}C]glycine betaine (55 mCi mmol^{-1}) was purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO). Anhydrotetracyline hydrochloride, desthiobiotin, and Strep-Tactin Superflow chromatography material were purchased from IBA GmbH (Göttingen, Germany). The antibiotic ampicillin was purchased from Carl Roth GmbH (Karlsruhe, Germany).

Bacterial strains, media, and growth conditions. The *B. subtilis* strains used in this study have all been described previously (10, 21, 44, 48), and their genetic properties are summarized in Table 1. Osmoprotection and heat stress protection growth assays were conducted with *B. subtilis* wild-type laboratory strain JH642 and mutant derivatives thereof (Table 1). Cold stress protection growth assays, on the other hand, were conducted with *B. subtilis* laboratory strain 168 and mutant derivatives thereof (Table 1), since strain JH642 carries a mutation in the acetolactate synthase gene that confers a cold-sensitive growth phenotype (49). *B. subtilis* strains were routinely cultivated in Spizizen's minimal medium (SMM) with 0.5% (wt/vol) glucose as the carbon source, 15 mM $(NH_4)_2SO_4$ as the nitrogen source, and L-tryptophan (20 mg liter^{-1}) and L-phenylalanine (18 mg liter^{-1}) to satisfy the auxotrophic requirements of *B. subtilis* strains JH642 (*trpC2 pheA1*) and 168 (*trpC2*), respectively (Table 1). A solution of trace elements (50) was added to SMM to improve the growth of *B. subtilis* strains; the osmolarity of the growth medium was adjusted by adding NaCl from a 5 M stock solution to it. The use of glycine betaine, DMG, and sarcosine as sole carbon sources by *B. subtilis* was assessed by replacing glucose (28 mM) as the carbon source in SMM with 33 mM glycine betaine, 42 mM DMG, and 55.5 mM sarcosine. The use of these solutes as sole nitrogen sources by *B. subtilis* was tested by replacing the ammonium source [$(NH_4)_2SO_4$ at 15 mM] present in SMM with 30 mM either glycine betaine, DMG, or sarcosine. Utilization of these compounds as nutrients by *B. subtilis* was assessed after growth for 20 h at 37°C by measuring the optical density at 578 nm (OD_{578}) of the cultures.

For growth experiments, *B. subtilis* cultures were inoculated from exponentially growing precultures into prewarmed (37°C) SMM or SMM containing 1.2 M NaCl to an OD_{578} of about 0.1; the cultures were then subsequently propagated at 37°C in a shaking water bath set to 220 rpm.

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

Strain	Relevant genotype	Origin or reference
JH642[b]	*trpC2 pheA1*	J. Hoch; BGSC[a] 1A96
168[b]	*trpC2*	BGSC (1A1)
RMKB20	JH642 Δ(*opuA*::*erm*)*4 opuC-20*::Tn*10*(*spc*) Δ(*opuD*::*neo*)*2*	44
RMKB22	JH642 Δ(*opuA*::*erm*)*4 opuB-20*::Tn*10*(*spc*) Δ(*opuD*::*neo*)*2*	44
RMKB24	JH642 Δ(*opuA*::*erm*)4 Δ(*opuBD*::*tet*)*23 opuC-20*::Tn*10*(*spc*) Δ(*opuD*::*neo*)*2*	44
RMKB33	JH642 Δ(*opuA*::*erm*)*4* Δ(*opuBD*::*tet*)*23 opuC-20*::Tn*10*(*spc*)	44
RMKB34	JH642 Δ(*opuB*::*tet*)*23 opuC-20*::Tn*10*(*spc*) Δ(*opuD*::*neo*)*2*	44
JGB23	168 Δ(*opuA*::*erm*)*4* Δ(*opuBD*::*tet*)*23 opuC20*::Tn*10* (*spc*)	10
JGB24	168 Δ(*opuA*::*erm*)*4* Δ(*opuBD*::*tet*)*23* Δ(*opuD*::*neo*)*2*	10
JGB25	168 Δ(*opuBD*::*tet*)*23 opuC20*::Tn*10* (*spc*) Δ(*opuD*::*neo*)*2*	10
JGB26	168 Δ(*opuA*::*erm*)*4 opuC20*::Tn*10* (*spc*) Δ(*opuD*::*neo*)*2*	10
JGB27	168 Δ(*opuA*::*erm*)*4* Δ(*opuBD*::*tet*)*23 opuC20*::Tn*10* (*spc*) Δ(*opuD*::*neo*)*2*	10
MBB9	JH642 *amyE*::[Φ(*opuAA-treA*)*1 cat*] (*treA*::*neo*)*1*	21

[a] BGSC, *Bacillus* Genetic Stock Center (Columbus, OH).
[b] *B. subtilis* strains 168 and JH642 are domesticated wild-type laboratory strains (85).

Precultures of *B. subtilis* strains used for temperature stress experiments were incubated at 37°C until they reached the mid-exponential growth phase and then inoculated into fresh SMM to an OD_{578} of about 0.12. For cold stress experiments, the cultures were immediately transferred into a shaking water bath set to 13°C and 220 rpm and their growth was monitored over time. For heat stress experiments, we transferred the inoculated medium to a shaking water bath set to room temperature, followed by a slow increase of the water temperature to 52 or 52.2°C, respectively, within 20 min. The growth curves of *B. subtilis* cultures propagated in 100-ml shake flasks filled with 20 ml medium were recorded.

Measurements of intracellular proline pools. The intracellular proline content of *B. subtilis* cells was determined by a colorimetric assay that detects L-proline as a colored proline-ninhydrin complex that can be quantified by measuring the absorption at 480 nm of the solution in a spectrophotometer (47). For these assays, *B. subtilis* cells were grown in SMM with 1.2 M NaCl in the absence or presence of 1 mM glycine betaine until they reached an OD_{578} of about 1.5; harvesting and processing of the cells, details of the assay conditions, and calculation of the intracellular volume of *B. subtilis* cells have all been described previously (21, 51).

Measurements of TreA enzyme activities in *opuAA-treA* reporter strains. Cultures of strain MBB9 harboring a chromosomal copy of an *opuAA-treA* reporter fusion (Table 1) were grown in SMM or SMM with increased salt concentrations until they reached an OD_{578} of 0.5; subsequently 1 mM glycine betaine, DMG, or sarcosine was added to the cultures and cell growth was allowed to continue for 90 min. Cells were then harvested by centrifugation for assays of the TreA reporter enzyme; TreA is a highly salt-resistant phospho-α-glucosidase whose enzyme activity can readily been quantitated by a colorimetric assay (46). This enzyme assay was conducted with α-PNPG as the substrate and under conditions that were detailed previously (21, 52). One unit of TreA enzyme activity is defined as the conversion of 1 μmol of substrate per minute per milligram of protein. The protein concentrations of the samples were estimated from the OD of the *B. subtilis* cell cultures (53). In the *opuAA-treA* reporter fusion, the promoterless *treA* gene is expressed from the promoter of the *opuA* operon (*opuA-opuAB-opuAC*); it is stably inserted as a single copy into the nonessential *amyE* gene of the *B. subtilis* chromosome via a double recombination event (21, 48). In this strain, the native *treA* gene is disrupted (Table 1) so that the measured TreA enzyme activity of the cells reflects only that produced in response to the transcriptional activity of the *opuAA-treA* reporter gene fusion.

Competition assay of radiolabeled glycine betaine import by DMG and sarcosine. Cells of a set of JH642-derived *B. subtilis* strains expressing only the OpuA, OpuC, or OpuD glycine betaine transporter were grown in SMM with 1.2 M NaCl to an OD_{578} of about 0.3. Samples (2 ml) were withdrawn and mixed with 20 μl of a [1-^{14}C]glycine betaine (55 mCi mmol^{-1}) solution (this mixture contained 1 μM radiolabeled glycine betaine, and the final concentration of glycine betaine added to the cells was 10 μM); glycine betaine uptake was monitored by removing cell samples at 20-s intervals. These transport assays were conducted either in the absence or in the presence of nonradiolabeled competitors (e.g., glycine betaine, DMG, and sarcosine); the competitors used were present at an excess of 10-, 100-, 250-, 500-, or 1,000-fold. Uptake assays, processing of the cells, and the quantification of the imported radiolabeled glycine betaine by scintillation counting followed previously established procedures (21, 42).

Overexpression, purification, and ligand-binding assays with OpuAC. The *B. subtilis* OpuAC ligand-binding protein without its natural lipid anchor (43) was overproduced by using *Escherichia coli* B host strain BL21 harboring plasmid pMH24 (*opuA*$^+$), a derivative of the expression plasmid pASK-IBA6 (IBA GmbH, Göttingen, Germany). In this plasmid, the coding region for the mature OpuAC protein is fused at its 5′ end in frame with the OmpA signal sequence and a short Strep-tag II affinity peptide to allow the purification of the recombinant OpuAC protein by affinity chromatography on Strep-Tactin Superflow material (54). The expression of the hybrid *opuAC* gene in plasmid pMH24 is mediated via the TetR repressor-controlled and anhydrotetracycline-inducible *tet* promoter present on the pASK-IBA6 expression plasmid (IBA GmbH, Göttingen, Germany). Cultures of strain BL21(pMH24) were grown at 37°C in a chemically defined minimal medium (MMA [53]); the conditions for the induction of *opuAC* expression by the addition of anhydrotetracycline to the culture and the growth and harvesting of OpuAC-overproducing cells have been previously described (54). For lysis of the *E. coli* cells harvested from 1 liter of culture, the bacterial pellet was resuspended in 10 ml cold buffer W (50 mM Tris-HCl, 100 mM NaCl, pH 8.0) and the cells were disrupted by passage four times through a French pressure cell (SLM Aminco) at 1,000 lb/in^2. Unbroken cells and cellular debris were removed by ultracentrifugation (35,000 × *g*, for 1 h at 4°C). Purification of OpuAC from the cleared cell extract via Strep-Tactin affinity chromatography, removal of the Strep-tag II affinity peptide from the recombinant protein by factor Xa cleavage, and subsequent anion-exchange chromatography followed previously described procedures (54). The purified OpuAC protein was concentrated by ultrafiltration (Vivaspin 6 concentrator columns; Sartorius Stedim Biotech, Göttingen, Germany) in 10 mM Tris HCl (pH 7.0) and kept at 4°C for subsequent ligand-binding experiments by fluorescence spectroscopy. From a 1-liter culture of strain BL21 (pMH24), we typically obtained approximately 3.5 mg pure OpuAC protein.

Ligand-binding assays. To assess the binding affinity of OpuAC for glycine betaine and DMG, we measured the intrinsic tryptophan fluorescence of the protein at 300 to 400 nm with a Cary Eclipse fluorescence spectrometer (Varian, Surrey, United Kingdom). Michaelis-Menten kinetics were deduced by comparing the maximum emission wavelengths in the absence and presence of various ligand concentrations as previously described (54, 55), with the following modifications. The assay buffer used contained 10 mM Tris-HCl (pH 7.0) supplemented with 10 mM NaCl. Glycine betaine binding was measured with a concentration of 1 μM OpuAC protein; DMG binding analysis was conducted with various concentrations (1, 2, and 3 μM) of OpuAC. Ligand binding was assayed at a temperature of 22.5°C. Analysis and fitting of the spectrophotometric data were performed with GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA).

Docking of DMG into the ligand-binding site of the OpuAC solute receptor. To assess the molecular determinants governing the binding of DMG by the OpuAC protein, we conducted *in silico* modeling experiments by using the OpuAC-DMSA (dimethylsulfonioacetate) crystal structure (Protein Data Bank [PDB] entry 3CHG) as the template; this crystal structure has a resolution of 2.8 Å (54). The OpuAC-DMSA crystal structure was chosen as the template for the modeling study since DMG and the sulfobetaine DMSA are chemically closely related compounds. We first exchanged the DMSA ligand in the OpuAC-DMSA complex (54) with a DMG molecule *in silico*, a process that involved only the replacement of the sulfur atom present in DMSA with the nitrogen atom found in DMG. This new OpuAC-DMG *in silico* model was then refined against the structural factors of the OpuAC-DMSA data set (54) by using the programs *Coot* (56) and REFMAC (57) to define the bond lengths and angle of the *in silico* docked DMG ligand. Interactions of OpuAC with this ligand were manually analyzed within a distance range of 3.2 to 3.8 Å from the DMG molecule. The same procedure was used to obtain the OpuAC-sarcosine *in silico* model.

Preparation of images of crystal structures. Images of the experimentally determined OpuAC-DMSA crystal structure (54) and of the *in silico*-generated OpuAC-DMG and OpuAC-sarcosine complexes were prepared by using the PyMOL software package (http://www.pymol.org).

RESULTS

Sarcosine and DMG cannot be used as nutrients. We focus here on the stress-relieving properties of sarcosine and DMG (Fig. 1), a process that could potentially be affected by the ability of *B. subtilis* to use these compounds as nutrients. This was previously observed for proline (48, 51), the only osmoprotectant that *B. subtilis*

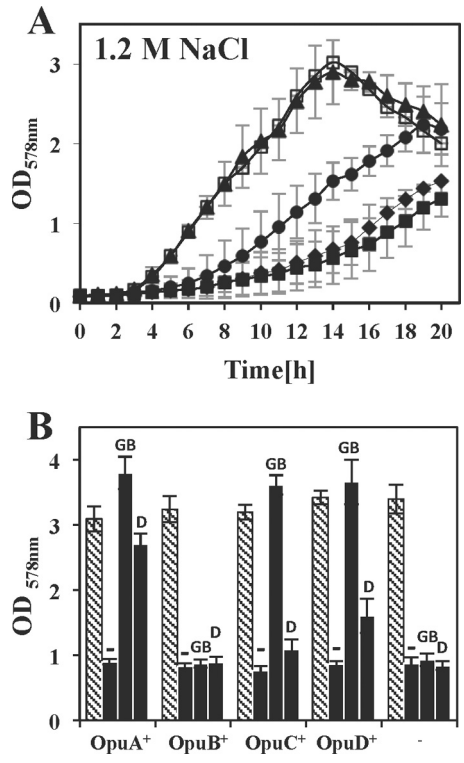

FIG 2 Protection of *B. subtilis* against high-salinity growth conditions by compatible solutes. (A) Growth curves of wild-type strain JH642 cultivated at 37°C in SMM with 1.2 M NaCl in the absence (■) or presence of the solute sarcosine (◆), DMG (●), or glycine betaine (▲) at a final concentration of 1 mM. A culture without 1.2 M NaCl served as an unstressed control (□). The values shown are the means and standard deviations of three independently grown cultures. (B) Growth yields of cultures grown in the absence (hatched bars) or presence (black bars) of 1.2 M NaCl without (−) or with the addition of 1 mM glycine betaine (GB) or DMG (D). A set of mutants derived from *B. subtilis* strain JH642 that each express only one or none (−) of the Opu transporters were grown for 20 h at 37°C; the growth yields of the cultures were determined by OD_{578} measurement. The values shown are the means and standard deviations of two independently grown cultures. The osmotically controlled proline transporter OpuE was present in all of the strains.

can synthesize *de novo* (58, 59), can import from exogenous sources (60, 61), and can catabolize (48). We therefore tested the use of sarcosine and DMG either as sole carbon and energy sources or as sole nitrogen sources. *B. subtilis* was unable to use either sarcosine or DMG as a nutrient either in SMM or in SMM containing an increased concentration of salt (1.2 M NaCl) (see Fig. S1 in the supplemental material). This finding agrees well with the previous report that *B. subtilis* cannot catabolize glycine betaine (12). Hence, the potential stress-relieving properties of the glycine betaine metabolites sarcosine and DMG for *B. subtilis* will not be diminished by their degradation.

DMG provides only a moderate degree of osmotic stress relief, and sarcosine lacks osmoprotective properties altogether. We grew *B. subtilis* laboratory strain JH642 in a chemically defined medium (SMM) with glucose as the sole carbon source in the absence or presence of 1.2 M NaCl and then studied the osmotic-stress-relieving abilities of sarcosine and DMG relative to those of glycine betaine. As expected from previous studies (12), glycine betaine was a strong osmoprotectant when provided at a final concentration of 1 mM (Fig. 2A); on the other hand, DMG at the same concentration exhibited rather modest osmotic-stress-relieving potential, whereas sarcosine conferred a negligible degree of osmotic stress protection (Fig. 2A).

B. subtilis possesses three uptake systems for glycine betaine (OpuA, OpuC, and OpuD) (42–44), and an isogenic set of strains each possessing only one of these transporters has previously been constructed (10, 42). This set of mutant strains is therefore a convenient tool allowing a rapid assessment of which Opu system of *B. subtilis* imports a given compatible solute under osmotic stress conditions. We applied such an experiment to DMG and compared it with the use and import of glycine betaine as an osmotic stress protectant. Glycine betaine afforded good osmotic stress protection, regardless of whether it was imported via the OpuA, the OpuC, or the OpuD system (Fig. 2B). This contrasted with that of the osmotic stress resistance profile afforded by DMG, as it was imported primarily via the OpuA transporter with a very minor contribution of the OpuD system (Fig. 2B). Osmoprotection by both glycine betaine and DMG was completely abolished when the OpuA, OpuC, and OpuD transporters were simultaneously inactivated (Fig. 2B), demonstrating that no other DMG uptake route exists in *B. subtilis* outside the physiologically well-studied Opu transporters (10, 42). As expected from the restricted substrate specificity of the OpuB system for the glycine betaine precursors choline and glycine betaine aldehyde (44, 45, 62), the OpuB transporter played no role in the uptake of DMG (Fig. 2B).

The OpuA and OpuD transporters possess only modest affinities for DMG. The OpuA, OpuC, and OpuD transporters all possess a high affinity for their common substrate glycine betaine, with K_m values in the low micromolar range when tested in the absence of osmotic stress or under conditions of moderately increased salinity (with 0.4 M NaCl) (42). The OpuA system is the dominant glycine betaine importer of *B. subtilis* because of its considerable transport capacity (21). Its V_{max} in cells not osmotically stressed even exceeds that of the OpuC and OpuD systems in cells grown under inducing conditions (in the presence of 0.4 M NaCl), and it outperforms the OpuC and OpuD transporters by about 3- to 4-fold in moderately salt-stressed cells (42).

Since neither DMG nor sarcosine was available to us in radiolabeled form, we qualitatively assessed the affinity of the OpuA, OpuC, and OpuD transporters for these glycine betaine metabolites by performing a series of competition experiments with [^{14}C]glycine betaine. In these experiments, we tested the possible uptake of DMG via the various Opu systems in cells that had been grown in SMM containing 1.2 M NaCl. DMG was a competitor for [^{14}C]glycine betaine uptake regardless of whether it was imported via the OpuA, the OpuC, or the OpuD uptake system (Fig. 3). For each of these glycine betaine transporters, DMG was a weak competitor since an at least 250-fold increase over the glycine betaine concentration used (10 μM) was required to produce any noticeable effect at all (Fig. 3). A 1,000-fold excess of DMG was insufficient to reduce [^{14}C]glycine betaine import via OpuA to a degree similar to that afforded by the addition of just a 10-fold excess of unlabeled glycine betaine to the transport assay mixtures (Fig. 3A); however, such a large excess reduced the [^{14}C]glycine betaine import activity of the OpuC and OpuD systems to a basal level (Fig. 3B and C).

Surprisingly (42), OpuC-mediated [^{14}C]glycine betaine uptake was not very efficient in cells that were grown in the presence of 1.2 M NaCl; it was strongly reduced in comparison with that observed in cells grown in the presence of only moderately in-

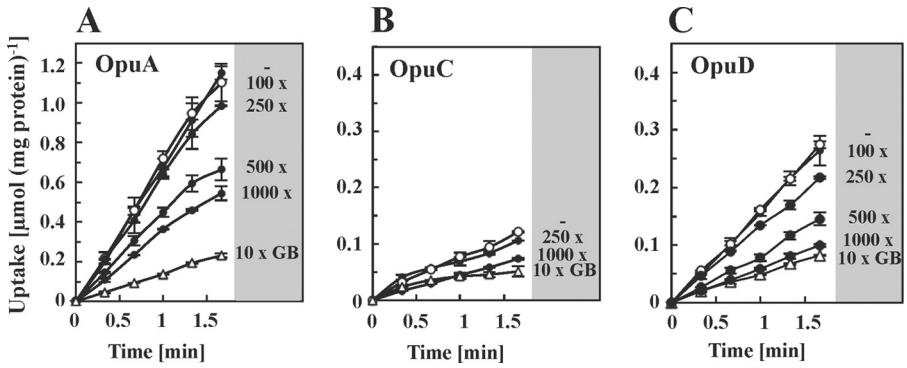

FIG 3 Inhibition of [1-^{14}C]glycine betaine by an excess of DMG. *B. subtilis* mutant strains expressing only the indicated Opu glycine betaine (GB) uptake systems were cultivated in SMM with 1.2 M NaCl. When the cultures reached the early exponential growth phase (OD_{578} of about 0.3), 2-ml aliquots were withdrawn and immediately mixed with a solution containing a mixture of nonlabeled glycine betaine and [1-^{14}C]glycine betaine (the final concentration in the assay was 10 μM), and the uptake of glycine betaine (○) was then monitored over time. Inhibition of glycine betaine uptake was tested in parallel assays with excesses of DMG (100-, 250-, 500-, and 1,000-fold) as indicated (●). As a control, a 10-fold excess of unlabeled glycine betaine was added to the labeled glycine betaine standard mixture (△) to monitor the inhibition of [1-^{14}C]glycine betaine import by glycine betaine itself. The values shown are the means and standard deviations of three independent cultures.

creased salinity (0.4 M NaCl) (see Fig. S2 in the supplemental material). Furthermore, competition between [^{14}C]glycine betaine and DMG in cells grown at high salinity (with 1.2 M NaCl) was very weak (Fig. 3B).

In growth experiments, we did not observe a significant degree of osmotic stress protection by sarcosine (Fig. 2A). This raised the question of whether sarcosine is an ineffective osmoprotectant of *B. subtilis* or whether it simply cannot enter the cell effectively. Competition experiments between sarcosine and [^{14}C]glycine betaine in a set of isogenic strains expressing only the OpuA, OpuC, or OpuD transport system revealed that [^{14}C]glycine betaine uptake (the final substrate concentration in the assays was 10 μM) was not inhibited by a 1,000-fold excess of sarcosine (see Fig. S3 in the supplemental material). This firmly indicates that sarcosine is not a physiologically relevant substrate for any of these transporters.

Glycine betaine, but not DMG, strongly reduces the size of the osmotic-stress-relieving proline pool. The *de novo* synthesis of the compatible solute proline is critical for the ability of *B. subtilis* to cope with high salinity (20, 59). Pool sizes of about 0.5 M in severely osmotically stressed *B. subtilis* cells (with 1.2 M NaCl) can be achieved (21, 51, 58, 61). Uptake of glycine betaine reduces the size of the osmotic-stress-responsive proline pool in a dose-responsive manner (21). We tested whether DMG would exert an effect similar to that of glycine betaine on the proline pool in high-salinity-challenged cells (21). High salinity triggered an increase in the proline content of the cells from about 12 mM to approximately 530 mM. The presence of 1 mM glycine betaine in the high-salinity growth medium reduced the proline pool again to a level found in cultures not osmotically stressed. DMG did not exert such an effect (Table 2).

DMG modulates *opuA* transcription only modestly. We found that there was another important difference between the activities of glycine betaine and DMG in osmotically stressed *B. subtilis* cells. This difference concerns the ability of glycine betaine to efficiently influence the expression of osmotically inducible genes. Both newly synthesized glycine betaine and glycine betaine that is scavenged from external sources strongly downregulate both the osmotically noninduced and, in particular, the osmotically induced levels of *opuA* transcription (21). We tested a possible influence of DMG on *opuA* expression by monitoring the induction of an *opuAA-treA* reporter gene fusion in response to osmotic stress and the presence of either glycine betaine or DMG. Glycine betaine downregulated the expression of the reporter construct very strongly, whereas we observed only modest effects of DMG on the level of *opuAA-treA* transcription (Table 3).

To sum up these data, DMG is only a rather modest osmoprotectant of *B. subtilis* in comparison with glycine betaine (Fig. 2A). It also lacks the ability of glycine betaine to significantly modulate

TABLE 2 Size of intracellular proline pool in response to increases in salinity and the presence of external compatible solutes[a]

Presence of NaCl	Compatible solute	Mean intracellular proline concn (mM) ± SEM
−	None	12 ± 2
+	None	534 ± 39
+	GB	12 ± 2
+	DMG	529 ± 47

[a] Cultures of wild-type *B. subtilis* strain JH642 were grown in SMM in the absence (−) or presence (+) of 1.2 M NaCl with either 1 mM glycine betaine (GB) or 1 mM DMG. Once the cultures had reached an OD_{578} of about 1.5, they were harvested by centrifugation and then processed to determine the proline content of the cells by a colorimetric assay (47).

TABLE 3 *opuA* promoter activity in response to salt stress and the presence of the compatible solutes glycine betaine and DMG

Presence of NaCl	Mean TreA activity (U mg protein^{-1}) ± SEM[a]		
	Without compatible solute	With glycine betaine	With DMG
−	51 ± 1	15 ± 1	26 ± 1
+	567 ± 2	85 ± 5	455 ± 28

[a] Cultures of *opuAA-treA* reporter fusion strain MBB9 were grown in the absence (−) or presence (+) of 1.2 M NaCl without or with the addition of 1 mM glycine betaine or DMG. Once the cultures had reached an OD_{578} of about 1.5, cells were harvested by centrifugation and subsequently assayed for the activity of the TreA reporter enzyme (46).

the osmotic-stress-responsive proline biosynthetic activities of the cell (Table 2), and it exerts only a minor influence on the level of the osmotic induction of *opuA* expression (Table 3). This data set therefore insinuates that the uptake of DMG by *B. subtilis* (Fig. 2) is of rather limited physiological relevance. However, this picture changed entirely when we assessed the temperature stress-protective effects of DMG both at the upper and at the lower boundaries of *B. subtilis* culture growth in chemically defined medium (9, 10).

Heat stress protection by DMG. The upper boundary of growth of *B. subtilis* wild-type laboratory strain JH642 in a glucose-based minimal medium is approximately 52°C; at 53°C, growth is no longer possible (9). Both the import of glycine betaine and its synthesis from choline confer heat stress protection at the very cutting edge of growth at high temperatures. However, glycine betaine accumulation is unable to overcome the upper temperature growth boundary of 53°C (9).

We first tested the thermal protection of *B. subtilis* by sarcosine and DMG at 52°C and compared the growth behavior of the cells with that of cultures that had received 1 mM glycine betaine. DMG conferred heat stress protection to a degree similar to that afforded by glycine betaine, whereas sarcosine enhanced growth only marginally (Fig. 4A). Under these temperature conditions (52°C) and in the absence of osmotic stress, glycine betaine entered the *B. subtilis* cells via the OpuA, OpuC, and OpuD transporters (Fig. 4B). DMG was also imported through these uptake systems when it served as a thermoprotectant, with OpuA making the major contribution for DMG uptake and OpuD just playing a very minor role (Fig. 4B). There is a notable difference between the profile of the Opu transporter mediating DMG uptake under sustained heat stress (Fig. 4B) and under continuous osmotic stress conditions (Fig. 2B). The DMG-importing capacity of OpuC is apparently sufficiently high to promote a significant level of stress relief at a high growth temperature (Fig. 4B), but the OpuC system is unable to import enough DMG to protect from osmotic stress (Fig. 2B).

At 52°C, *B. subtilis* is still able to grow in the absence of a compatible solute such as glycine betaine or DMG (Fig. 4A). When we increased the growth temperature by just 0.2°C to 52.2°C, the growth of wild-type laboratory strain JH642 was completely prevented but the presence of 1 mM glycine betaine afforded strong heat stress protection (Fig. 4C). DMG had a stimulating effect on cell growth, but it was a less effective thermoprotectant than glycine betaine (Fig. 4C). Glycine betaine initially strongly promoted cell growth, but the cultures then started to lyse (Fig. 4A and C); the reason for the onset of cell lysis is unknown. Although the growth behavior of the cells propagated at 52.2°C in the presence of 1 mM DMG was unusual, the protective effects of DMG against a continued high-temperature challenge set it apart from sarcosine, which does not possess any heat stress-relieving properties for *B. subtilis* at this growth temperature (Fig. 4C). DMG was unable to promote the growth of heat-stressed *B. subtilis* cells at 53°C (data not shown), as previously shown for other compatible solutes (9).

Cold stress protection by DMG. *B. subtilis* laboratory strain 168 is unable to grow at 13°C in a minimal medium (SMM) with glucose as the carbon source, but the addition of 1 mM glycine betaine effectively enables the growth of cells at a continuous low temperature (10). As the growth temperature is successively lowered from 37°C to 15°C, the cellular glycine betaine pool continuously rises and reaches a pool size (about 400 mM) (10) that

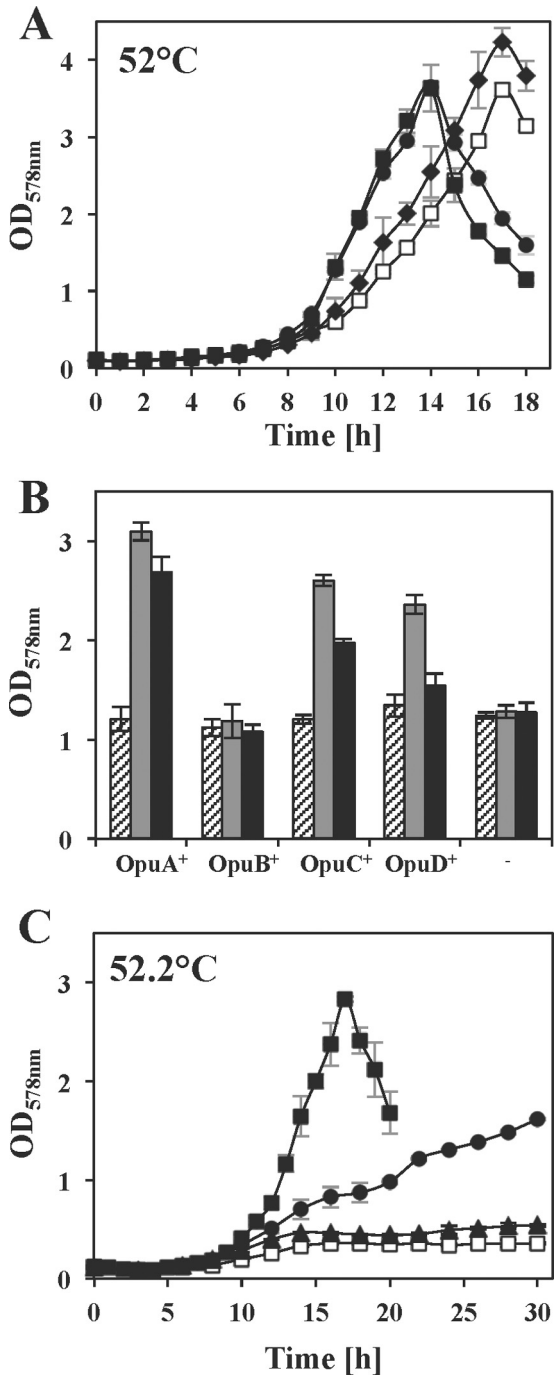

FIG 4 Protection of *B. subtilis* growth against high-temperature challenges. (A, C) Growth curves of wild-type strain JH642 cultivated in SMM at 52°C (A) or at the upper growth limit of 52.2°C (C) in the absence (□) or presence of the solute monomethylglycine (◆), DMG (●), or glycine betaine (■) at a final concentration of 1 mM. The values shown are the means and standard deviations of three independently grown cultures. (B) Growth yields of cultures grown in SMM at 52°C in the absence (hatched bars) or presence of 1 mM DMG (black bars) or glycine betaine (gray bars). A set of *B. subtilis* mutants derived from strain JH642 that each express only one or none (−) of the relevant Opu transporters were grown for 14 h at 52°C; the growth yields of the cultures were determined by OD_{578} measurement. The values shown are the means and standard deviations of two independently grown cultures. The osmotically controlled proline transporter OpuE was present in all of the strains.

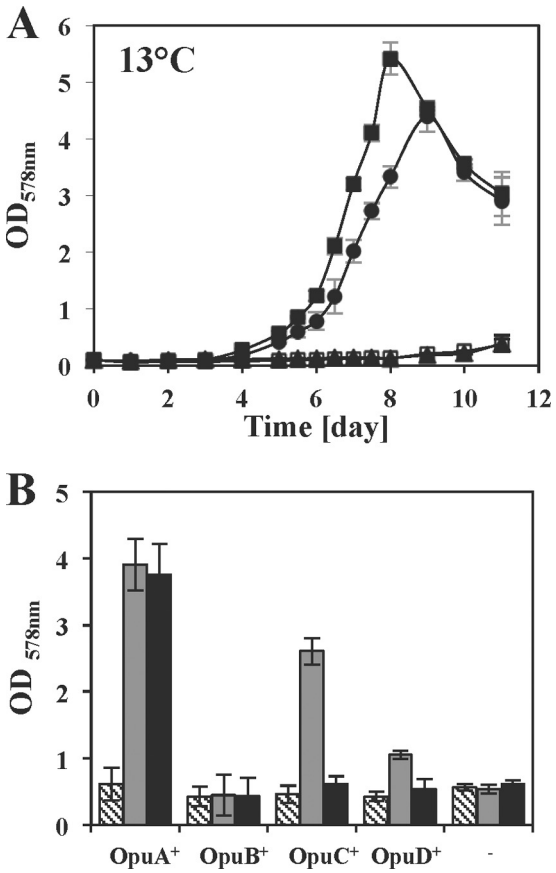

FIG 5 Protection of *B. subtilis* growth against low-temperature challenges. (A) Growth curves of *B. subtilis* wild-type strain 168 cultivated in SMM at 13°C in the absence (□) or presence of the solute sarcosine (◆), DMG (●), or glycine betaine (■) at a final concentration of 1 mM. The values shown are the means and standard deviations of three independently grown cultures. (B) Growth yields of cultures grown in SMM at 13°C in the absence (hatched bars) or presence of 1 mM DMG (black bars) or glycine betaine (gray bars) after 10 days of incubation as determined by OD_{578} measurement. A set of *B. subtilis* strain 168-derived mutants that each express only one or none (−) of the relevant Opu transporters were used for this experiment. The values shown are the means and standard deviations of two independently grown cultures. The osmotically controlled proline transporter OpuE was present in all of the strains.

approximately corresponds to the level found in *B. subtilis* cells continuously stressed with 0.6 M NaCl (21).

DMG is an excellent cold stress protectant of *B. subtilis*, providing strong growth protection at 13°C to a degree similar that of glycine betaine (Fig. 5A), but it did not allow the growth of cells at 11°C (data not shown), a growth pattern in the cold previously established for glycine betaine as well (10). In contrast to DMG, sarcosine was unable to promote the growth of *B. subtilis* at 13°C. An assessment of the contribution of the individual Opu transport system for the acquisition of DMG under cold stress conditions revealed that OpuA was the only transporter of physiological relevance to DMG import (Fig. 5B). Import of glycine betaine under cold stress conditions was also prominently afforded by OpuA, but in contrast to DMG, the OpuC and OpuD transporters also contributed to its uptake (Fig. 5B).

Binding of DMG to the purified OpuAC solute receptor protein. The data presented above show that the OpuA transporter is primarily responsible for the import of DMG under osmotic, heat, and cold stress conditions (Fig. 2A, 4A, and 5A). We therefore studied the binding of DMG by the extracellular solute receptor OpuAC (43, 63), since the affinity of ligand-binding proteins for a given compound typically determines the overall K_m value of the corresponding ABC transporter (64). We overproduced and purified by affinity chromatography a recombinant version of the OpuAC protein (Fig. 6A) that lacks the lipid anchor that naturally tethers it to the outer face of the cytoplasmic membrane (43, 63). To determine the affinity of OpuAC for DMG, we exploited the intrinsic Trp fluorescence exhibited by this protein in ligand-binding assays, a sensitive method that has previously been used to quantify the binding of glycine betaine, its sulfur analog DMSA, and proline betaine by the OpuAC protein (54, 55).

The binding of glycine betaine to the OpuAC protein solution caused a change in the intrinsic Trp fluorescence and also resulted in a blue shift of the maximum of the emission spectrum of 9 nm (Fig. 6B). As detailed previously (54), the change in the intensity of the emission maximum was used to determine the apparent dissociation constant of the OpuAC-glycine betaine complex and a K_d value of 26 ± 2.8 μM was calculated (Fig. 6C). This value is in excellent agreement with previous reports of K_d values of 17 ± 1 μM (55) and 22 ± 4 μM (54), respectively, for glycine betaine binding by OpuAC. Hence, the affinity-purified recombinant OpuAC protein was functional.

The addition of DMG to the OpuAC protein solution reduced the fluorescence intensity, whereas the emission maximum remained at the same wavelength exhibited by OpuAC in the absence of a ligand (Fig. 6B). The DMG concentration-dependent shift in the fluorescence intensity was used to determine the dissociation constant of the OpuAC-DMG complex and a K_d value of 172 ± 24 μM was calculated (Fig. 6D). Hence, the OpuAC-binding protein *in vitro* possesses an approximately 6- to 7-fold lower affinity for DMG than that of glycine betaine.

***In silico* docking of a DMG molecule into the ligand-binding site of the OpuAC solute receptor.** The *B. subtilis* OpuAC protein has been crystalized in complex with its ligands glycine betaine (K_d of about 20 μM), DMSA (K_d of about 100 μM), and proline betaine (K_d of about 270 to 300 μM) (54, 55). In addition, the functional contributions of individual residues to the overall architecture of the substrate-binding pocket in OpuAC have been probed via site-directed mutagenesis, thereby providing a molecular explanation for the observed differences in the binding affinity of OpuAC for these three ligands (54, 55). We built on this knowledge and used a modeling approach to dock the DMG molecule into the OpuAC substrate-binding site *in silico*.

The positive charge of the trimethylammonium head group of glycine betaine is delocalized. This voluminous cation interacts, via cation-π and van der Waals contacts, with the indole moieties of the side chains of Trp-72, Trp-178, and Trp-225 in the OpuAC-glycine betaine crystal structure (55), the so-called aromatic cage (65). Further stabilizing contacts for the carboxylate of the glycine betaine molecule in the ligand-binding pocket of OpuAC are provided through hydrogen bonds with the backbone amide groups of Gly-26 and Ile-27 and the side chain of His-230 (55) (Fig. 7A). The same types of contacts also stabilize the sulfur analog of DMG, DMSA, within the ligand-binding site (54), except that fewer cation-π and van der Waals interactions of the positively charged dimethylsulfonio head group of DMSA can be formed with the residues structuring the aromatic cage (Trp-72, Trp-178, Trp-

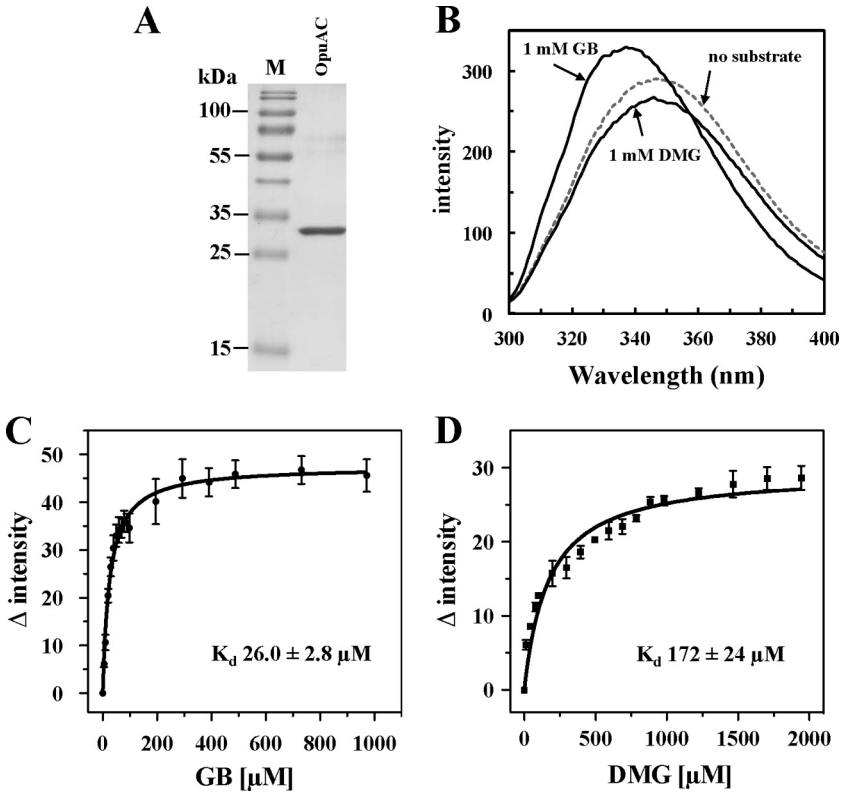

FIG 6 Binding of DMG and glycine betaine by the OpuAC solute receptor protein. (A) SDS-PAGE of the purified OpuAC protein. Lanes: M, molecular mass markers; OpuAC, 2 μg of purified OpuAC protein. (B) Fluorescence spectrum of the purified OpuAC protein (1 μM) in the absence or presence of 1 mM DMG or glycine betaine (GB). (C) Kinetics of glycine betaine binding to purified OpuAC protein (1 μM) assessed by intrinsic-fluorescence spectroscopy in two independent experiments. Changes in fluorescence intensity were determined between 335 and 345 nm since ligand binding caused a shift in the wavelength of the intensity maximum. (D) Kinetics of DMG binding to OpuAC assessed by intrinsic-fluorescence spectroscopy in three independent experiments with 1, 2, and 3 μM OpuAC. Changes in fluorescence intensity at the wavelength of the intensity maximum upon ligand binding were determined from 345 to 347 nm. The values shown are the means and standard deviations of three independent experiments.

225) (see Fig. S4 in the supplemental material). In structural terms, this finding explains the about 5-fold lower affinity of OpuAC for DMSA than for glycine betaine (54).

For our modeling studies, we used the OpuAC-DMSA complex (PDB entry 3CHG) (54) as the template since the DMSA and DMG molecules are closely related in chemical structure and differ only with respect to their fully methylated sulfur or nitrogen head groups. We superimposed the DMG ligand on the DMSA molecule onto the OpuAC-DMSA crystal complex and replaced the sulfur atom present in DMSA with the nitrogen atom found in DMG *in silico*. This new *in silico* structural model was then refined against the structure factors of the OpuAC-DMSA data set (PDB entry 3CHG) (54) by using the programs *Coot* (56) and REFMAC (57). Analysis of this *in silico*-formed OpuAC-DMG complex did not reveal any steric clashes between the DMG ligand and residues protruding into the ligand-binding pocket of OpuAC or its backbone structure (Fig. 7B). In this *in silico* model, the carboxylate of DMG interacts with the backbone amides of Gly-26 and Ile-27 via hydrogen bonds (with distances of 3.4 and 3.0 Å, respectively) and the side chain of His-230 (with a distance of 3.2 Å) in a fashion similar to that found in the OpuAC-DMSA crystal structure (see Fig. S4 in the supplemental material) (55). These interactions fix the position of the carboxylate of DMG within the substrate-binding pocket and orient the dimethylammonium head group of this ligand for cation-π and van der Waals force-driven interactions with residues forming the aromatic cage in the OpuAC protein (Fig. 7B). All distances between the dimethylammonium head group of DMG and the indole moieties of the side chains forming the aromatic cage range from 3.5 to 4.0 Å, perfectly fitting the criterion for van der Waals interactions and fulfilling the requirements of cation-π interactions (66).

Smits et al. (54) have calculated that the trimethylammonium head group of glycine betaine will make 22 cation-π and 6 van der Waals interactions with the side chains forming the aromatic cage in OpuAC (Fig. 7A), whereas 19 cation-π and 6 van der Waals interactions are established between the DMSA molecule and this ligand-binding protein (see Fig. S4 in the supplemental material). Since the structures of the DMSA and DMG molecules can be perfectly overlaid within the OpuAC ligand-binding pocket, the same number of cation-π and van der Waals interactions of the dimethylammonium head group DMG and the side chains of the amino acids forming the aromatic cage will be established. It is thus not surprising that the affinities of the OpuAC solute receptor protein for the DMSA and DMG ligands are on the same order of magnitude, with K_d values of 102 ± 11 μM for DMSA (54) and 172 ± 24 μM for DMG, respectively (Fig. 6D).

We also assessed why sarcosine is not a substrate for the OpuA transport system *in silico* (see Fig. S3A in the supplemental material). The carboxylate of sarcosine (Fig. 1) could potentially make the same hydrogen-bonding interactions established by glycine

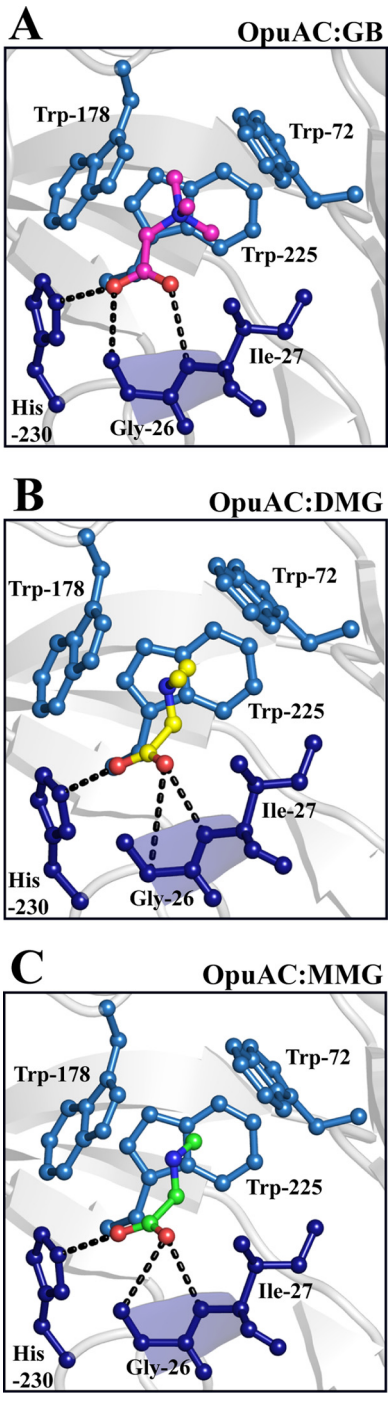

FIG 7 Binding of glycine betaine (A), DMG (B), and sarcosine (C) to the ligand-binding site of the OpuAC solute receptor protein. The experimental data for the OpuAC-glycine betaine (GB) crystal structure shown in panel A were taken from PDB entry 2B4L (55). The structures of the OpuAC-DMG and OpuAC-MMG (sarcosine) complexes were generated *in silico* by using the crystallographic data of the OpuAC-DMSA complex (PDB entry 3CHG) (54) as the template.

betaine, DMSA, and DMG with the OpuAC protein (Fig. 7C). However, the energetics of the accommodation of its monomethyl head group within the aromatic cage are probably insufficient to stably fix sarcosine within the OpuAC-binding pocket, since only nine cation-π and two van der Waals interactions are calculated for the *in silico*-formed OpuAC-sarcosine complex (Fig. 7C). Hence, this significant reduction in the number of cation-π and van der Waals interactions should strongly reduce the affinity of the sarcosine ligand for OpuAC. This suggestion is consistent with data from previous site-directed mutagenesis experiments showing that the replacement of just a single residue from the aromatic ligand-binding cage with a nonaromatic amino acid, and hence the reduction in the maximal possible number of cation-π interactions, is sufficient to abolish DMSA and glycine betaine binding by OpuAC (54).

DISCUSSION

The genome sequence of the soil bacterium *B. subtilis* carries the hallmarks of a microorganism that lives in association with plants and plant detritus (67). Many plants synthesize glycine betaine in response to osmotic stress and will release it into the soil ecosystem through root exudates and decaying tissues (68). The disintegration of glycine betaine-producing microbial cells (15) or rapid osmotic downshifts that trigger the transient opening of mechanosensitive channels (69, 70) will also introduce this compatible solute into the environment. Indeed, glycine betaine can readily be found in soil (71, 72) and other ecosystems (38). This will likely also be the case for the glycine betaine metabolites DMG and sarcosine, although to the best of our knowledge, no detailed studies of their availability in natural habitats have been reported. The presence of sarcosine and DMG in the environment provides the opportunity for microorganisms to use these nitrogen-containing compounds as either nutrients (15, 25) or stress protectants (40). Here we have addressed these processes for *B. subtilis*.

Neither DMG nor sarcosine is naturally formed by *B. subtilis*, since it uses the choline oxidation pathway to synthesize glycine betaine (22, 45) and cannot catabolize it (12). Not surprisingly, neither sarcosine nor DMG can be used as a nutrient by *B. subtilis* (see Fig. S1 in the supplemental material). However, our data show that *B. subtilis* can exploit exogenously provided DMG as a stress protectant (Fig. 2, 4, and 5). In contrast, it is unable to use sarcosine for this purpose, a finding that can probably be explained by the inability of *B. subtilis* to import this compound effectively. However, our data do not strictly rule out a scenario where sarcosine might be imported through transporters other than the Opu systems studied (see Fig. S3 in the supplemental material) and the *B. subtilis* cell is then unable to use the imported sarcosine as a stress protectant or as a nutrient. We consider this unlikely. Our *in silico* modeling study suggests that the placement of the monomethyl head group of sarcosine within the aromatic cage of the OpuAC protein (Fig. 7C), in contrast to DMG (Fig. 7B), probably does not provide enough interactions to stably capture sarcosine through the "venus flytrap" movements (64) of the two lobes of OpuAC toward each other when it encounters a ligand (54, 55). We therefore surmise that the common design principles (65) that allow the high-affinity recognition of glycine betaine (42) by the OpuA, OpuC, and OpuD transporters preclude the recognition and stable binding of sarcosine by these transporters.

We found that DMG affords moderate osmotic stress protection, notable heat stress protection, and excellent cold stress protection of *B. subtilis* (Fig. 2A, 4C and 5A). It is not uncommon that intermediates in the synthesis or degradation of compatible solutes possess stress-relieving properties for microorganisms. For

instance, an exogenous supply of glycine betaine aldehyde, the intermediate in glycine betaine synthesis from choline, confers osmoprotection on *B. subtilis* cells, provided that this toxic compound is oxidized to the innocuous glycine betaine molecule (22). Conversely, crotonobetaine and γ-butyrobetaine, products of the synthesis and degradation route of L-carnitine in microorganisms, possess excellent osmotic and thermal stress-relieving properties *per se* for *B. subtilis*; they are taken up via the *B. subtilis* OpuC system as metabolically inert compatible solutes (9, 10, 73). In a similar vein, *N*-γ-acetyldiaminobutyrate, an intermediate in the synthesis of the compatible solute ectoine, possesses stress-protective properties and enzyme-stabilizing features by itself and can be imported into bacteria via transport systems that mediate ectoine uptake (74, 75).

DMG is imported under stress conditions primarily via the OpuA ABC system, the major glycine betaine transporter of *B. subtilis* (42, 43). Studies of ligand binding with the primary substrate recognition subunit of the OpuA transporter, the extracellular solute receptor protein OpuAC (54, 55, 63), revealed that it possesses an about 6- to 7-fold reduced affinity for DMG (K_d value of 172 ± 24 μM) in comparison with the main substrate of OpuAC, glycine betaine (K_d value of 26 ± 2.8 μM) (Fig. 6). This is on the same order of magnitude as the binding affinity of OpuAC for the sulfur analog (DMSA) of DMG (K_d value of 102 ± 11 μM) (54). Since DMSA and DMG are chemically closely related (Fig. 7B; see Fig. S4 in the supplemental material), the crystal structure of the OpuAC-DMSA complex (54) provided us with the opportunity to model and visualize the DMG ligand within the substrate-binding pocket of the OpuAC protein (Fig. 7B). This revealed a reduction in the number of possible cation-π and van der Waals interactions between the positively charged dimethylammonium head group of DMG and residues of the aromatic cage relative to those formed by glycine betaine (Fig. 7A and B). Our *in silico* model thus provides the likely molecular underpinning of the reduced binding affinity of OpuAC for DMG that we observed in our ligand-binding assays (Fig. 6C and D). The binding affinity of the purified OpuAC protein for DMG is about 7-fold lower than that for glycine betaine (Fig. 6C and D). However, we noted that in cells grown in SMM with 1.2 M NaCl, effective competition of glycine betaine uptake by DMG via the OpuA system required a DMG concentration considerably higher than a 7-fold excess (Fig. 3A). An understanding of this notable difference in the substrate affinities of the OpuAC ligand-binding protein (*in vitro*) and the overall OpuA transporter (*in vivo*) requires further experimentation.

As studied in detail for glycine betaine and proline, efficient osmotic stress protection of *B. subtilis* is achieved only when a physiologically appropriate pool size of these compatible solutes can be built up to balance the osmotic gradient across the cytoplasmic membrane (21, 58, 59). In comparison with glycine betaine, DMG is not a particularly effective osmotic stress protectant of *B. subtilis* (Fig. 2A). This could be due to the inability of *B. subtilis* to form a pool large enough to accomplish this task effectively. If this were indeed the case, it would also explain the missing effect of DMG on the proline pool buildup under osmotic stress conditions (Table 2) and its very modest influence on the strength of *opuA* expression (Table 3). These two processes are strongly influenced by an exogenous supply of glycine betaine (Table 2 and 3) and its intracellular pool size in osmotically stressed cells (21).

On the other hand, the physicochemical properties of DMG could be sufficiently different from those of glycine betaine that its physiological function is negatively affected so that it cannot sufficiently optimize the solvent properties of the cytoplasm (76). For instance, the effects of the compatible solutes glycine betaine and proline on the cytoplasmic amounts of water and of the potassium, glutamate, and trehalose contents of osmotically stressed *E. coli* cells on the water activity and osmotic pressure of the cytoplasm are large enough to make glycine betaine a significantly better osmoprotectant than proline (77). Furthermore, there are important differences between various types of compatible solutes with respect to their solvation and water-structuring properties (78) and their abilities to preserve the functionality of macromolecules (4). A sizeable number of glycine betaine- and proline-related compounds serve as effective osmotic stress protectants of *B. subtilis* (10, 79). Distinct effects of these osmolytes on the solvation properties of the cytoplasm and the functionality of various classes of biomolecules might underlie the individual capabilities of these solutes to serve as temperature stress protectants of *B. subtilis* (9, 10).

The most notable physiological effects of DMG are related to its ability to serve as a temperature stress protectant at both the upper and lower boundaries of *B. subtilis* growth (Fig. 4 and 5). Its ability to protect cells from the detrimental effects of low (13°C) temperature is impressive and rivals that of glycine betaine (Fig. 5A). We note in this context that the pool size of glycine betaine required for temperature stress protection is much lower than that needed for protection against strong osmotic stress (9, 10). Thus, even if the pool size of DMG attained by transport were insufficient to provide effective osmotic stress protection (Fig. 2A), the considerable transport capacity of the OpuA system (42) would certainly be sufficient to attain the more limited intracellular concentration of DMG needed for cold stress protection.

The ability of compatible solutes to serve as protectants against sustained cold stress is well known (10, 80, 81), but the molecular and physiological foundations of this beneficial effect are not clear. Whatever they might be, we emphasize here that well-studied microbial cold stress responses such as the induction of cold shock proteins, the adjustment in the fluidity of the cytoplasmic membrane, and changes in DNA topology and in the transcriptional and translational profile (82–84) are inadequate to allow cellular adaptation of *B. subtilis* to sustained low temperatures. In its soil and other varied habitats, *B. subtilis* not only faces osmotic stress (79) but also frequently encounters temperatures such as 13°C, a temperature that already causes a severe growth restriction that can be reversed through the import of DMG (Fig. 5A).

Collectively, our data show that the ability to acquire DMG from exogenous sources under stressful environmental conditions will aid the *B. subtilis* cell in its defense against temperature challenges at the very cutting edges of its upper and lower growth limits (9, 10). It will also contribute to the cell's overall physiological efforts to cope with high-osmolarity surroundings (79). Hence, the ability of *B. subtilis* to scavenge the glycine betaine metabolite DMG will certainly increase its competitiveness in the taxing soil ecosystem.

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