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Plant-derived compatible solutes proline betaine and betonicine confer enhanced osmotic and temperature stress tolerance to *Bacillus subtilis*

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L-Proline is a widely used compatible solute and is employed by *Bacillus subtilis*, through both synthesis and uptake, as an osmostress protectant. Here, we assessed the stress-protective potential of the plant-derived L-proline derivatives *N*-methyl-L-proline, L-proline betaine (stachydrine), *trans*-4-L-hydroxproline and *trans*-4-hydroxy-L-proline betaine (betonicine) for cells challenged by high salinity or extremes in growth temperature. L-Proline betaine and betonicine conferred salt stress protection, but *trans*-4-L-hydroxyproline and *N*-methyl-L-proline was unable to do so. Except for L-proline, none of these compounds served as a nutrient for *B. subtilis*. L-Proline betaine was a considerably better osmostress protectant than betonicine, and its import strongly reduced the L-proline pool produced by *B. subtilis* under osmotic stress conditions, whereas a supply of betonicine affected the L-proline pool only modestly. Both compounds downregulated the transcription of the osmotically inducible *opuA* operon, albeit to different extents. Mutant studies revealed that L-proline betaine was taken up via the ATP-binding cassette transporters OpuA and OpuC, and the betaine-choline-carnitine-transporter-type carrier OpuD; betonicine was imported only through OpuA and OpuC. L-Proline betaine and betonicine also served as temperature stress protectants. A striking difference between these chemically closely related compounds was observed: L-proline betaine was an excellent cold stress protectant, but did not provide heat stress protection, whereas the reverse was true for betonicine. Both compounds were primarily imported in temperature-challenged cells via the high-capacity OpuA transporter. We developed an *in silico* model for the OpuAC–betonicine complex based on the crystal structure of the OpuAC solute receptor complexed with L-proline betaine.

INTRODUCTION

When faced with increases in the external osmolarity, many micro-organisms amass compatible solutes to counteract water efflux; they thereby adjust turgor to physiologically appropriate values and promote cell growth under otherwise osmotically unfavourable circumstances (Bremer & Krämer, 2000). L-Proline is a well-known representative of this class of compounds (Csonka, 1989; Kempf & Bremer, 1998). In addition to its role as a water-attracting organic osmolyte, the function-preserving properties of L-proline for macromolecules provide an additional level of cellular protection for bacterial cells challenged by high

Abbreviations: ABC, ATP-binding cassette; AHT, anhydrotetracycline hydrochloride; PDB, Protein Data Bank; PNPG, *p*-nitrophenyl-α-D-glucopyranoside.

Three supplementary figures are available with the online version of this paper.

osmolarity (Fisher, 2006; Ignatova & Gierasch, 2006; Street *et al.*, 2006).

Bacillus subtilis belongs to those micro-organisms that can derive osmoprotection by L-proline (Brill *et al.*, 2011a; von Blohn *et al.*, 1997; Whatmore *et al.*, 1990; Zaprasis *et al.*, 2013). It amasses large amounts of this amino acid under high-osmolarity growth conditions through an osmotically inducible biosynthesis route that is distinct from that employed when L-proline is produced for anabolic purposes (Brill *et al.*, 2011a, b). Cellular L-proline pools exceeding 0.5 M can be found when the osmotic stress is severe (Brill *et al.*, 2011a; Hoffmann *et al.*, 2013; Zaprasis *et al.*, 2013). Attesting to the critical role of compatible solute synthesis by micro-organisms for managing osmotic stress (Csonka, 1989; Kempf & Bremer, 1998), the genetic disruption of the osmotically inducible L-proline biosynthesis route causes an osmotically sensitive growth phenotype (Brill *et al.*, 2011a). Osmostress protection of *B. subtilis* can also be achieved through L-proline uptake and the osmotically inducible OpuE transporter is key to this process (Hoffmann *et al.*, 2012; von Blohn *et al.*, 1997; Zaprasis *et al.*, 2014). However, compared with the metabolically inert compatible solute glycine betaine (Boch *et al.*, 1994), an exogenous supply of L-proline is not a particularly effective osmoprotectant for *B. subtilis* (Zaprasis *et al.*, 2013). This is rooted in (i) the different biophysical properties of glycine betaine and L-proline, and their different effects on the solvation properties of the cytoplasm (Cayley *et al.*, 1992; Street *et al.*, 2006), and (ii) the ability of *B. subtilis* to use exogenously provided L-proline as a nutrient (Moses *et al.*, 2012) – a process that partially diverts it from fulfilling its role as an osmoprotectant (Zaprasis *et al.*, 2013).

The genome sequence of *B. subtilis* carries the hallmarks of a bacterium that lives in association with plants and plant detritus (Belda *et al.*, 2013). Indeed, most of the considerable number of compatible solutes taken up by *B. subtilis* (Bremer, 2002; Hoffmann & Bremer, 2011) are produced by plants (Hanson *et al.*, 1994; Rhodes & Hanson, 1993). In addition to L-proline, plant-derived L-proline derivatives (Hanson *et al.*, 1994; Rhodes & Hanson, 1993; Servillo *et al.*, 2011; Trinchant *et al.*, 2004) have been implicated as osmostress or temperature stress protectants, or as nutrients for micro-organisms. Examples are the betaines of L-proline and hydroxyproline (Alloing *et al.*, 2006; Amin *et al.*, 1995; Bayles & Wilkinson, 2000; Haardt *et al.*, 1995; Kumar *et al.*, 2014; Watanabe *et al.*, 2012; White *et al.*, 2012; Zhao *et al.*, 2013).

Here, we asked whether the L-proline derivatives D-proline, *N*-methyl-L-proline, *N*,*N*-dimethyl-L-proline (L-proline betaine; also known as stachydrine), *trans*-4-hydroxy-L-proline and *trans*-4-hydroxy-L-proline betaine (betonicine) (Fig. 1) could be catabolized by *B. subtilis*, and, more specifically, whether these compounds could be used by this soil bacterium as protectants against osmotic and high/low-temperature challenges. L-Proline betaine and betonicine were identified as metabolically inert cell protectants against extremes in osmolarity and growth temperatures.

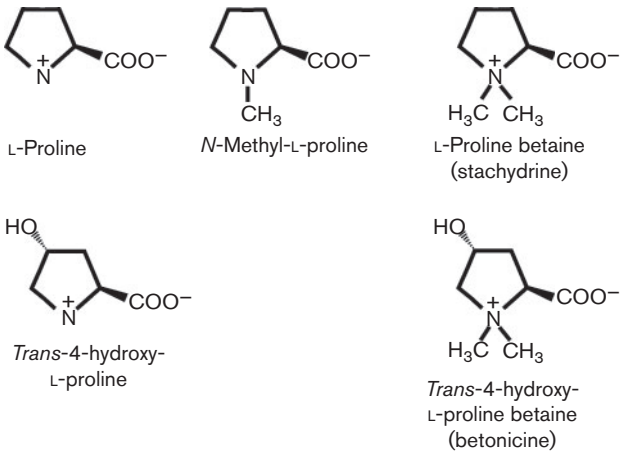

Fig. 1. Chemical structures of L-proline, and its methylated and hydroxylated derivatives.

METHODS

Chemicals. Glycine betaine, L-proline, D-proline, the chromogenic substrate *p*-nitrophenyl-α-D-glucopyanoside (PNPG) for the TreA enzyme (Gotsche & Dahl, 1995), and the ninhydrin reagent for the quantification of proline by a colorimetric assay were purchased from Sigma-Aldrich. L-Proline betaine and betonicine were obtained from Extrasynthese, and *N*-methyl-proline (Alloing *et al.*, 2006) was a gift from D. Le Rudulier (University of Nice, France). *Trans*-4-hydroxy-L-proline, *trans*-4-fluoro-L-proline and *cis*-4-fluoro-L-proline were obtained from Bachem. Anhydrotetracycline hydrochloride (AHT), desthiobiotin and Strep-Tactin Superflow chomatrography material were purchased from IBA, and the antibiotics ampicillin and spectinomycin were obtained from Carl Roth. Anion-exchange chromatography material (HiTrap Q Sepharose FF) was purchased from GE Healthcare Bio-Science and the protease factor Xa was obtained from Merck.

Bacterial strains. The genetic properties of the *B. subtilis* strains used in this study are summarized in Table 1. All strains were described previously, except RMKB27, which was constructed by transforming strain GNB8 [Δ(*opuA*::*erm*)*4*] (Kappes *et al.*, 1999) with chromosomal DNA of strain RMKB20 (Table 1) and selecting for spectinomycin-resistant colonies in order to transfer the *opuC*::Tn*10*(*spc*) mutation. Osmostress and heat stress protection growth assays were conducted with the *B. subtilis* laboratory strain JH642 (*trpC2 pheA1*) and its mutant derivatives (Table 1). Strain JH642 carries a mutation in the acetolactate synthase gene that makes it cold sensitive (Wiegeshoff & Marahiel, 2007); hence, cold stress protection growth assays were conducted with the *B. subtilis* laboratory strain 168 (*trpC2*) and its mutant derivatives. The overproduction of the *B. subtilis* OpuAC ligand-binding protein was carried out in the *Escherichia coli* B strain BL21 carrying plasmid pMH24 (*opuAC*$^{+}$) (Bashir *et al.*, 2014; Smits *et al.*, 2008).

Media and growth conditions. *B. subtilis* strains were grown in Spizizen's minimal medium (SMM) enriched with a solution of trace elements (Harwood & Archibald, 1990) and 0.5 % (w/v) glucose as the carbon source. This medium was supplemented with

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

Strain	Relevant genotype	Source* or reference
JH642	*trpC2 pheA1*	J. Hoch; BGSC 1A96
168	*trpC2*	BGSC 1A1
RMKB20	JH642 Δ(*opuA*::*erm*)*4 opuC20*::Tn*10*(*spc*) Δ(*opuD*::*neo*)*2*	Kappes *et al.* (1996)
RMKB22	JH642 Δ(*opuA*::*erm*)*4 opuB-20*::Tn*10*(*spc*) Δ(*opuD*::*neo*)*2*	Kappes *et al.* (1996)
RMKB24	JH642 Δ(*opuA*::*erm*)*4* Δ(*opuBD*::*tet*)*23 opuC20*::Tn*10*(*spc*)Δ(*opuD*::*neo*)*2*	Kappes *et al.* (1996)
RMKB27	JH642 Δ(*opuA*::*erm*)*4 opuC20*::Tn*10*(*spc*)	R. Kappes, University of Marburg, Germany
RMKB33	JH642 Δ(*opuA*::*erm*)*4* Δ(*opuBD*::*tet*)*23 opuC20*::Tn*10*(*spc*)	Kappes *et al.* (1996)
RMKB34	JH642 Δ(*opuB*::*tet*)*23 opuC20*::Tn*10*(*spc*) Δ(*opuD*::*neo*)*2*	Kappes *et al.* (1996)
JGB23	168 Δ(*opuA*::*erm*)*4* Δ(*opuBD*::*tet*)*23 opuC20*::Tn*10* (*spc*)	Hoffmann & Bremer (2011)
JGB24	168 Δ(*opuA*::*erm*)*4* Δ(*opuBD*::*tet*)*23* Δ(*opuD*::*neo*)*2*	Hoffmann & Bremer (2011)
JGB25	168 Δ(*opuBD*::*tet*)23 *opuC20*::Tn*10* (*spc*) Δ(*opuD*::*neo*)*2*	Hoffmann & Bremer (2011)
JGB26	168 Δ(*opuA*::*erm*)*4 opuC20*::Tn*10*(*spc*) Δ(*opuD*::*neo*)*2*	Hoffmann & Bremer (2011)
JGB27	168 Δ(*opuA*::*erm*)*4* Δ(*opuBD*::*tet*)*23 opuC20*::Tn*10* (*spc*) Δ(*opuD*::*neo*)*2*	Hoffmann & Bremer (2011)
JSB8	JH642 Δ(*proHJ*::*tet*)*1*	Brill *et al.* (2011b)
MBB9	JH642 *amyE*::[Φ(*opuAA–treA*)*1 cat*] (*treA*::*neo*)*1*	Hoffmann *et al.* (2013)
SMB10	JH642 *amyE*::[Φ(*putB–treA*)*1 cat*] (*treA*::*neo*)1	(Moses *et al.*, 2012)

*BGSC, Bacillus Genetic Stock Center (Columbus, OH, USA).

L-tryptophan (20 mg l^{-1}) and L-phenylalanine (18 mg l^{-1}) to satisfy the auxotrophic requirements of the *B. subtilis* strains JH642 (*trpC2 pheA1*) and 168 (*trpC2*), and their mutant derivatives (Table 1). NaCl from a 5 M stock solution was used to increase the osmolarity of the SMM growth medium. When L-proline, *N*-methyl-L-proline, L-proline betaine, *trans*-4-hydroxy-L-proline and betonicine were used as sole carbon sources, they were added to SMM at concentrations of 33, 28, 24, 33 and 24 mM, respectively. These concentrations were equivalent in carbon content to 28 mM glucose that was used as a control in the growth assays. The ammonium source [$(NH_4)_2SO_4$ (15 mM)] present in SMM was replaced by 30 mM of the various L-proline derivatives to test their use as sole nitrogen source. The use of L-proline and its derivatives as nutrients by *B. subtilis* was assessed by measuring the OD_{578} of the cultures in a spectrophotometer after 20 h of incubation of the cultures at 37 °C in a shaking water bath. For growth experiments assessing the osmostress protective potential of L-proline and its various derivatives, cultures were pre-grown in SMM at 37 °C and then used to inoculate 20 ml SMM (in a 100 ml Erlenmeyer flask) containing 1.2 M NaCl to an OD_{578} ~0.1; the cultures were grown at 37 °C in a shaking water bath (set to 220 r.p.m.). Pre-cultures of strains used for temperature stress experiments were grown at 37 °C until they reached mid-exponential growth phase (OD_{578} 1.5) and the cells were then inoculated into fresh SMM to an OD_{578} ~0.12. For heat stress experiments, the inoculated cultures were transferred to a shaking water bath set to room temperature, which was then followed by a slow increase to either 52 or 52.2 °C over a 20 min time frame. For cold stress experiments, the cultures were transferred immediately into a shaking water bath pre-set to a temperature of 13 °C. The temperature of the water baths used for the heat and cold stress growth experiments was set and controlled with the aid of a calibrated electronic thermometer (Testo).

Measurements of intracellular proline pools. To determine the pool size of *de novo* synthesized L-proline in osmotically stressed cells (Brill *et al.*, 2011a; Hoffmann *et al.*, 2013), we grew the *B. subtilis* cultures in SMM that contained 1.2 M NaCl in the absence or presence of various compatible solutes until they reached an OD_{578} ~1.7. We then used a colorimetric assay that detected proline as a coloured proline–ninhydrin complex, which could be quantified by measuring A_{480} of the solution in a spectrophotometer. Harvesting of the cells, their processing for the L-proline assay and the specifics of the calculation of the intracellular volume of *B. subtilis* cells have all been described previously (Hoffmann *et al.*, 2012, 2013).

Preparation of cell extracts for ^{13}C-NMR spectroscopy. The *B. subtilis* mutant strain JSB8 [Δ(*proHJ*::*tet*)*1*] (Table 1) was grown in SMM (culture volume of 600 ml in a 1 l Erlenmeyer flask) containing 1.2 M NaCl in the absence or presence of 1 mM (final concentration) L-proline betaine or betonicine. After the cultures reached late exponential growth phase (OD_{578} 2.5), the cells were harvested by centrifugation and the solutes were extracted with 20 ml 80 % (v/v) ethanol as described previously (Kuhlmann & Bremer, 2002). Cellular debris was removed by centrifugation at 25 000 ***g*** at 4 °C for 30 min; the supernatant was lyophilized to dryness. For natural abundance NMR measurements, the dried samples were dissolved in 0.6 ml ^2H_2O together with 3 mg D_4-3-(trimethylsilyl) propionate as an internal standard. ^{13}C-NMR spectra (125 MHz) were recorded on a Bruker Avance 500 MHz NMR spectrometer equipped with a 5 mm BBFO probe; the spectra were processed with the program Topspin 3.1 (Bruker). To verify resonance signals for L-proline betaine and betonicine in the cell extracts, we recorded ^{13}C-NMR spectra on authentic samples of L-proline betaine and betonicine under conditions identical to those used to assess the total cell extracts. ^{13}C chemical shifts of individual compounds were referenced with respect to the signal of the internal standard D_4-3-(trimethylsilyl) propionate.

Measurements of TreA enzyme activity in *putB–treA* and *opuAA–treA* reporter fusion strains. In the *putB–treA* and *opuAA–treA* reporter gene fusion strains, a promoterless *treA* gene was fused to the proline-responsive regulatory region of the catabolic *putBCP* operon (Moses *et al.*, 2012) and the osmostress responsive promoter of the *opuA* operon (Hoffmann *et al.*, 2013). These fusions were stably inserted into the *B. subtilis* chromosome as a single copy via a double recombination event in the non-essential *amyE* gene. The details of the growth of the reporter fusion strains, the processing of the cells, the TreA enzyme assay using the chromogenic PNPG as the substrate and the calculation of the TreA enzyme specific activity have all been described previously (Hoffmann *et al.*, 2013; Moses *et al.*, 2012). One unit of TreA enzyme activity is defined as 1 μmol PNPG converted min^{-1}.

Overexpression, purification and ligand-binding assays with the OpuAC solute receptor protein. Plasmid pMH24 (*opuAC*$^{+}$) was a derivative of the expression plasmid pASK-IBA6 (IBA), and it allowed the expression of the recombinant *opuAC* gene under the control of the TetR-responsive and AHT-inducible *tet* promoter present on the backbone of the expression plasmid. Overproduction and purification of the recombinant OpuAC protein by affinity chromatography were carried out in the *E. coli* B strain BL21 as described previously (Bashir *et al.*, 2014; Smits *et al.*, 2008). The affinities of the OpuAC protein for its ligands glycine betaine, L-proline betaine and betonicine were measured by fluorescence spectroscopy, based on a ligand-binding assay that exploited changes in the intrinsic tryptophan fluorescence of the OpuAC protein upon substrate binding (Horn *et al.*, 2006; Smits *et al.*, 2008). A Cary Eclipse fluorescence spectrometer (Varian) was used for these experiments. The fluorescence spectrum of OpuAC and its changes incurred upon ligand binding were monitored at wavelengths between 300 and 400 nm. Ligand-binding assays were conducted at 22.5 °C in a buffer solution containing 10 mM Tris/HCl (pH 7.0) and 10 mM NaCl. The concentration of the OpuAC protein in the assay was 1 μM, and the concentration of the ligands glycine betaine, L-proline betaine and betonicine was varied between 10 and 1000 μM. Michaelis–Menten kinetics were deduced by comparing the maximum fluorescence intensities in the absence and presence of various ligand concentrations as described previously (Smits *et al.*, 2008). The corresponding fluorescence intensity maxima of the OpuAC protein were at 336–343, 340–346 and 344–348 nm for the glycine betaine, L-proline betaine and betonicine ligands, respectively. Analysis and fitting of the spectrophotometric data were performed using Prism 5 software (GraphPad).

***In silico* docking of betonicine into the ligand-binding site of the OpuAC protein.** The experimentally determined crystal structure of the OpuAC–L-proline betaine complex at 2.8 Å resolution [Protein Database (PDB) ID: 2B4M] (Horn *et al.*, 2006) was chosen as the starting point for *in silico* modelling of the OpuAC–betonicine complex. We first exchanged *in silico* the ligand in the available crystal structure by a betonicine molecule – a process that only involved the substitution of a hydrogen atom at position C-4 in the L-proline ring by a hydroxyl group (Fig. 1). This *in silico* generated OpuAC–betonicine model was then refined against the structure factors of the experimentally determined OpuAC–L-proline betaine complex (Horn *et al.*, 2006) using the programs COOT (Emsley & Cowtan, 2004) and REFMAC (Murshudov *et al.*, 1997) to define the bond length and angle of the placed betonicine ligand with the *in silico* model. Contacts of the betonicine ligand with the OpuAC binding protein were manually analysed and considered with a distance range of 2.8–3.5 Å.

Preparation of figures of crystal structures. Figures of the crystal structure of the OpuAC–L-proline betaine complex (Horn *et al.*, 2006) and of the *in silico* generated model for the OpuAC–betonicine complex generated in this study were prepared using the PyMOL software package (http://www.pymol.org).

RESULTS

Assessing the use of proline derivatives as nutrients and their influence on the expression of proline catabolic genes

L-Proline can be used as a sole carbon, energy and nitrogen source by *B. subtilis* (Moses *et al.*, 2012). We tested whether the L-proline derivatives *N*-methyl-L-proline, L-proline betaine, *trans*-4-hydroxy-L-proline and betonicine (Fig. 1) could be used by *B. subtilis* as nutrients, either as sole carbon or as sole nitrogen sources. We also tested the potential use of these compounds as nutrients under high salinity (0.6 M NaCl) growth conditions as we considered the possibility that their uptake would be stimulated by increased osmolarity of the growth medium (Moses *et al.*, 2012; von Blohn *et al.*, 1997; Zaprasis *et al.*, 2014). Catabolic routes for *trans*-4-hydroxy-L-proline, L-proline betaine and betonicine have been identified in a variety of micro-organisms (Kumar *et al.*, 2014; Watanabe *et al.*, 2012; White *et al.*, 2012; Zhao *et al.*, 2013), but we found that *B. subtilis* cannot use any of the studied L-proline derivatives as nutrients (Fig. 2).

The presence of low concentrations of L-proline in the growth medium induces the expression of the catabolic *putBCP* operon in a fashion that is dependent on the L-proline-responsive activator protein PutR (Belitsky, 2011; Huang *et al.*, 2011; Moses *et al.*, 2012). To test a possible influence of the various L-proline derivatives on the expression of the *putBCP* catabolic operon, we used a *putB–treA* reporter strain in which the production of the TreA reporter enzyme was under the control of the L-proline-responsive PutR activator protein (Belitsky, 2011; Huang *et al.*, 2011; Moses *et al.*, 2012). Except for L-proline, none of the tested proline derivatives triggered enhanced *putB–treA* expression in cells that had been grown in SMM (Table 2). However, when the salinity of the growth medium was raised with 0.6 M NaCl, L-proline betaine significantly increased *putB–treA* transcription, whereas *N*-methyl-L-proline, *trans*-4-hydroxy-L-proline and betonicine did not cause such an effect (Table 2). Natural abundance ^{13}C-NMR spectroscopy of salt-stressed cells (with 1.2 M NaCl) proved that externally provided L-proline betaine was accumulated by *B. subtilis* in unmodified form (see below). Hence, L-proline betaine served as a gratuitous inducer for the *putBCP* L-proline catabolic operon (Moses *et al.*, 2012); we surmised that this effect was mediated through PutR.

Osmostress protection by proline derivatives

Next, we tested the ability of *N*-methyl-L-proline, *trans*-4-hydroxy-L-proline, L-proline betaine and betonicine to serve as osmostress protectants for *B. subtilis*. We benchmarked their performance against that of exogenously provided L-proline and glycine betaine (Boch *et al.*, 1994; von Blohn *et al.*, 1997; Zaprasis *et al.*, 2013). High salinity severely inhibited the growth of *B. subtilis* in a chemically defined medium (SMM) with 1.2 M NaCl, and both glycine betaine and L-proline exerted osmoprotective effects on cell growth, with glycine betaine being the better osmoprotectant than L-proline (Fig. 3a). L-Proline betaine was about as effective as glycine betaine in relieving osmotic stress, whereas the osmoprotective potential of betonicine resembled that of L-proline (Fig. 3a). In contrast, *N*-methyl-L-proline and *trans*-4-hydroxy-L-proline did not serve as osmoprotectants (Fig. 3a). Hence, small differences in the chemical structure of the L-proline derivatives (Fig. 1) could

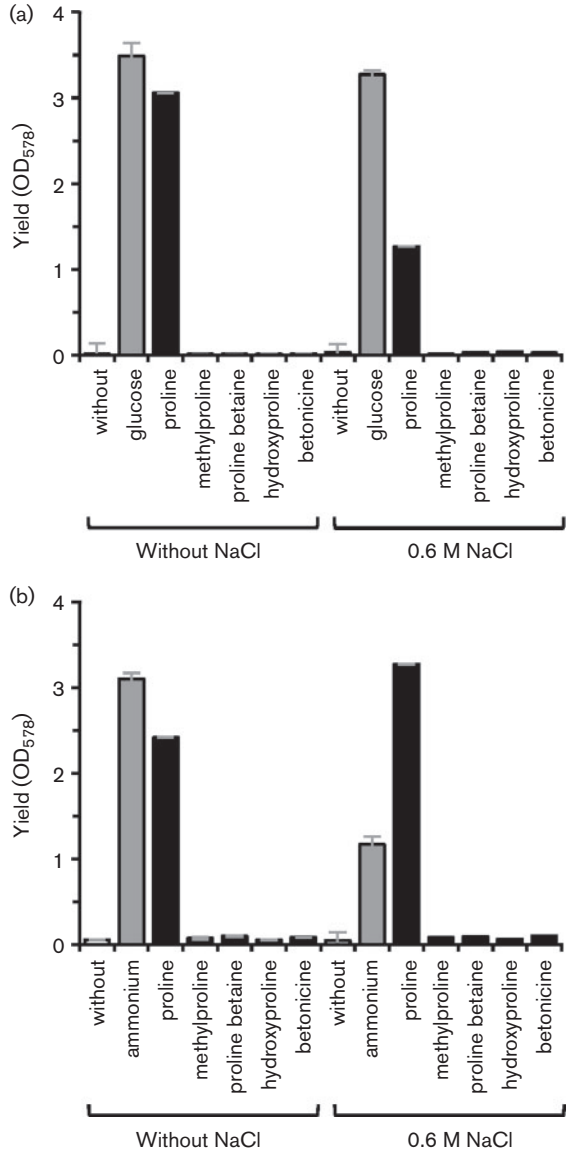

Fig. 2. Use of L-proline and its derivatives as (a) sole carbon and energy source or (b) sole nitrogen source by *B. subtilis*. Growth yields (OD_{578}) of cultures of *B. subtilis* strain JH642 were determined after 20 h of incubation at 37 °C in a shaking water bath. (a) Cells were cultivated in shake flasks containing SMM without a carbon source, with 28 mM glucose as the positive control (grey bars), or 33 mM L-proline, 28 mM *N*-methyl-L-proline, 24 mM L-proline betaine, 33 mM *trans*-4-hydroxy-L-proline or 24 mM betonicine as indicated (black bars). (b) Cells were cultivated in shake flasks containing SMM without a nitrogen source or with 30 mM of the indicated compounds, except for $(NH_4)_2SO_4$, which was present at a final concentration of 15 mM. The influence of high osmolarity on growth yield was assessed by growing each culture in the absence or presence of 0.6 M NaCl. The data shown represent the mean ± SD of two independent cultures.

make a big difference with respect to their stress-protective function for high-salinity-challenged *B. subtilis* cells. We also tested the osmoprotective potential of the D-stereoisomer of proline, but D-proline was not osmoprotective for *B. subtilis*, and neither were the synthetic L-proline derivatives *trans*-4-fluoro-L-proline and *cis*-4-fluoro-L-proline (Fig. S1, available in the online Supplementary Material).

Detection of intracellular L-proline betaine and betonicine in osmotically stressed cells by ^{13}C-NMR spectroscopy

Natural abundance ^{13}C-NMR spectroscopy can be used to detect the dominant compatible solutes accumulated by osmotically stressed cells (Kuhlmann & Bremer, 2002). We used this technique to assess the presence of L-proline betaine and betonicine in *B. subtilis* cells grown in SMM with 1.2 M NaCl. We used a mutant strain that was unable to synthesize osmoprotective levels of L-proline for these experiments (Brill *et al.*, 2011a) in order to reduce the complexity of the NMR signals from the cell extracts. Both L-proline betaine and betonicine were readily detected in the cell extracts, and the NMR traces showed that they were present in an unmodified form (Fig. 4). Although these experiments could not be interpreted quantitatively, they showed that L-proline betaine and betonicine were accumulated from the medium by osmotically stressed *B. subtilis* cells as main organic osmolytes.

Genetic identification of the uptake systems mediating L-proline betaine and betonicine import

As both L-proline betaine and betonicine conferred osmostress protection (Fig. 3a), we asked which compatible solute uptake systems of *B. subtilis* (Bremer, 2002) were used for their import. We used a genetically well-characterized set of mutant strains for this experiment in which only one of the known compatible solute uptake systems (Opu) of *B. subtilis* (Table 1) was functional (Hoffmann & Bremer, 2011). Growth of these strains in high-salinity medium (with 1.2 M NaCl) in the absence or presence (1 mM) of L-proline betaine and betonicine revealed that L-proline betaine was imported via the two ATP-binding cassette (ABC) transporters OpuA and OpuC, and through the betaine-choline-carnitine-transporter-type import system OpuD (Fig. S2). However, betonicine was only taken up via the OpuA and OpuC systems (Fig. S2).

Influence of L-proline betaine and betonicine on the osmostress-adaptive L-proline pool

The size of the L-proline pool formed by *B. subtilis* through *de novo* synthesis is sensitively tied to the severity of the imposed osmotic stress (Brill *et al.*, 2011a; Hoffmann *et al.*, 2013; Whatmore *et al.*, 1990). Notably, an exogenous supply of the potent osmostress protectant glycine betaine (Boch *et al.*, 1994) strongly downregulates the pool size

Table 2. Induction of *putB*–treA expression by L-proline and proline derivatives (mean ± SD of three independent replicates)

The *B. subtilis putB–treA* fusion strain SMB10 (Table 1) was grown in SMM with the indicated salinity. When the cultures reached the early exponential growth phase (OD_{578} 0.3–0.5), various compatible solutes (final concentration 1 mM) were added, growth of the cells was allowed for an additional 1 h and the cells were than processed for TreA enzyme activity assays.

Compatible solute	TreA activity [U (mg protein)$^{-1}$]	
	Without NaCl	0.6 M NaCl
Without	7 ± 1	13 ± 1
L-Proline	120 ± 3	133 ± 4
N-Methyl-L-proline	13 ± 2	12 ± 1
L-Proline betaine	3 ± 1	93 ± 4
Trans-4-hydroxy-L-proline	5 ± 1	11 ± 2
Betonicine	5 ± 1	19 ± 4

of L-proline in osmotically stressed cells (Hoffmann *et al.*, 2013). We therefore wondered whether L-proline betaine and betonicine would confer a similar effect. *B. subtilis* cells grown in SMM with 1.2 M NaCl contained an intracellular L-proline pool of ~560 mM (Fig. 3b). Titration of the concentrations of either glycine betaine or L-proline betaine in the growth medium successively decreased the L-proline pool (Fig. 3b). When either one of these compounds was present in the medium at a concentration of 1 mM, the L-proline pool was reduced to a value (18 mM) found in osmotically non-stressed *B. subtilis* cells (Hoffmann *et al.*, 2013; Whatmore *et al.*, 1990). However, betonicine influenced the L-proline content of the osmotically stressed cells only modestly (Fig. 3b).

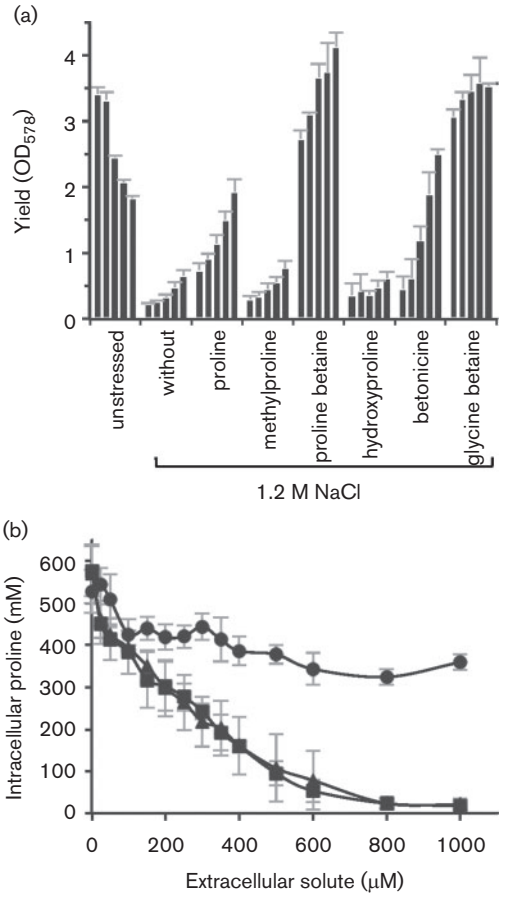

Fig. 3. Protection of *B. subtilis* against high-salinity growth conditions by L-proline and its derivatives, and the influence of L-proline betaine and betonicine on the intracellular L-proline pool. (a) Cultures of *B. subtilis* strain JH642 were grown in SMM without NaCl (unstressed control) or in the presence of high salinity (1.2 M NaCl). The different bars represent the growth yields of the cultures measured after 12, 14, 16, 18 and 20 h in the absence or presence of the indicated compounds (final concentration: 1 mM). (b) Cultures of *B. subtilis* strain JH642 were grown in SMM with 1.2 M NaCl in the presence of the indicated concentrations of the compatible solutes glycine betaine (■), L-proline betaine (▲) or betonicine (●). Cells were harvested after the cultures reached mid-exponential growth phase (OD_{578} 1.7) and the intracellular L-proline pools were determined. The data shown are the mean ± SD of three independent replicates.

Modulation of *opuA* gene expression by L-proline betaine and betonicine

The uptake of glycine betaine downregulates the expression of osmotically induced genes in *B. subtilis* on a genome-wide scale (Kohlstedt *et al.*, 2014). We therefore asked if this would also be the case for L-proline betaine and betonicine, and tested this by using an *opuA–treA* promoter fusion as a read-out – a reporter system that responds to both osmotic stress and the presence of various types of compatible solutes (Bashir *et al.*, 2014; Hoffmann *et al.*, 2013; Kempf & Bremer, 1995). The sustained increase in *opuA* promoter activity in response to continued increases in salinity was reduced strongly by glycine betaine, carnitine and L-proline betaine, whereas betonicine downregulated the expression of the reporter fusion to a much lower extent (Fig. 5). The presence of compatible solutes in the growth medium also affected the non-induced level of *opuA–treA* expression; *opuA* transcription remained salt-inducible, albeit at a much lower level, even in the presence of the tested compatible solutes (Fig. 5). There was an approximately ninefold osmotic induction in the expression level of the reporter fusion in cells grown in the absence of a compatible solute, and similar values of induction were found in cells grown in the presence of glycine betaine (fivefold), L-proline betaine (sevenfold), betonicine (12-fold) and carnitine (eightfold) (Fig. 5). Hence, these data support the previous conclusion that the activity of the *opuA* promoter was responsive to both an increase in the external salinity and the intracellular compatible solute pool (Hoffmann *et al.*, 2013).

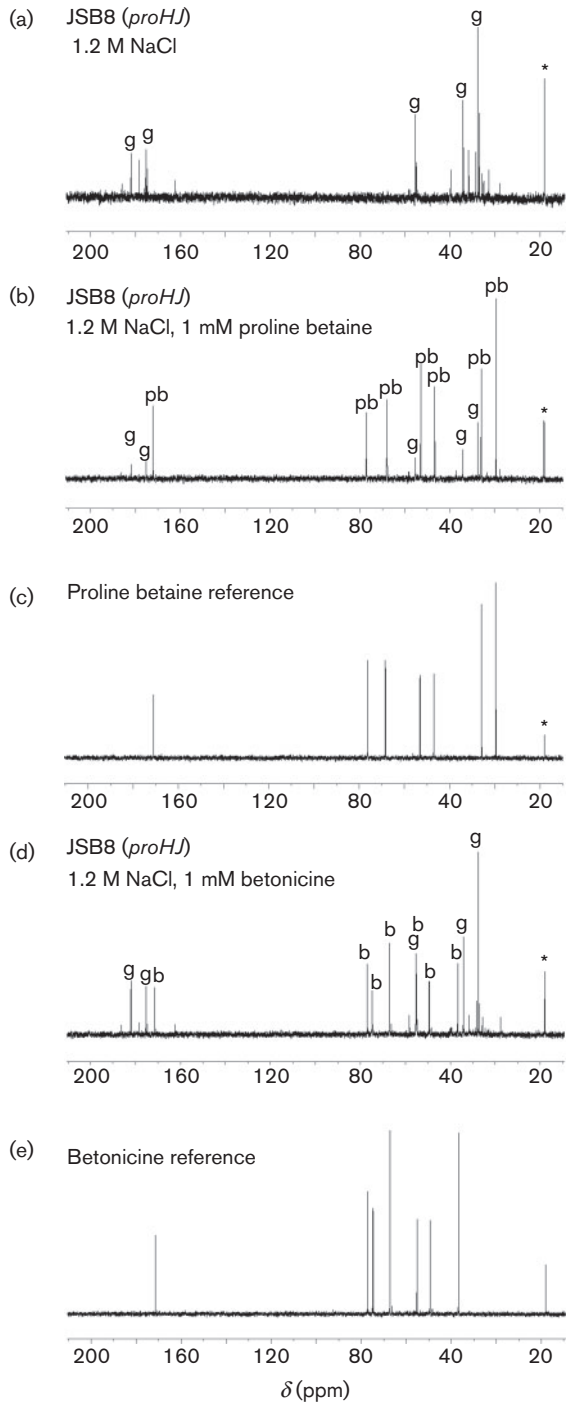

Fig. 4. L-Proline betaine and betonicine are accumulated in unmodified form by *B. subtilis*. (a–e) ^{13}C-NMR spectra of ethanolic cell extracts of the *B. subtilis proHJ* mutant strain JSB8 grown in SMM with 1.2 M NaCl (a) without compatible solute, (b) in the presence of 1 mM proline betaine or (d) in the presence of 1 mM betonicine. ^{13}C-NMR spectra of L-proline betaine (c) and betonicine (e) were recorded as references. The resonance signals for L-glutamate (g), L-proline betaine (pb), betonicine (b) and the internal standard D_4-3-(trimethylsilyl) propionate (*) are indicated.

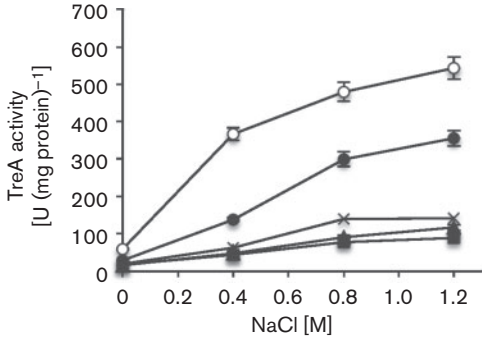

Fig. 5. Externally provided compatible solutes repress *opuA* promoter activity. Cultures of the *opuA–treA* fusion strain MBB9 were grown in SMM with increasing NaCl concentrations in the absence of a compatible solute (○), or with 1 mM (final concentration) of either glycine betaine (■), carnitine (×), L-proline betaine (▲) or betonicine (●). Cells were harvested and assayed for TreA activity when the cultures reached mid-exponential growth phase (OD_{578} 1.5). The data shown are the mean ± SD of two independent replicates.

Heat and cold stress protection by L-proline betaine and betonicine

Previous studies have shown that most of the compatible solutes conferring cellular protection to *B. subtilis* cells against high osmolarity (Bremer, 2002) also serve as protectants against extremes in either high or low growth temperatures (Hoffmann & Bremer, 2011; Holtmann & Bremer, 2004). The beneficial effects of the uptake of these solutes become most notable at the very edges of the upper (52–52.2 °C) and lower (13 °C) boundaries of growth. We found that betonicine was a very good heat stress protectant at 52 °C, with a degree of effectiveness that matched that of glycine betaine; in contrast, L-proline betaine exhibited no heat stress protection (Fig. 6a). Heat adaptation of the cells was improved to an extent that the lag phase of the culture was shortened for ~3 h by betonicine and 4.5 h by glycine betaine (Fig. 6a). At 52.2 °C, a temperature at which the *B. subtilis* WT laboratory strain JH642 could no longer grow in a chemically defined medium (Fig. 6b), betonicine still afforded cell growth, but it was much less effective than glycine betaine (Fig. 6b). Uptake of betonicine under heat stress conditions (52 °C) was mediated primarily by the OpuA ABC transporter, whereas each of the glycine betaine uptake systems (OpuA, OpuC and OpuD) of *B. subtilis* (Kappes *et al.*, 1996) contributed to the import of glycine betaine in high-temperature-challenged cells (Fig. S3a).

When we tested the cold stress protection potential of betonicine and L-proline betaine, we found that L-proline betaine was an excellent cold protectant at a growth temperature of 13 °C, with effectiveness similar to that of glycine betaine. In contrast, betonicine did not confer cold stress protection (Fig. 6c). Under sustained cold stress growth conditions, OpuA served as the major uptake

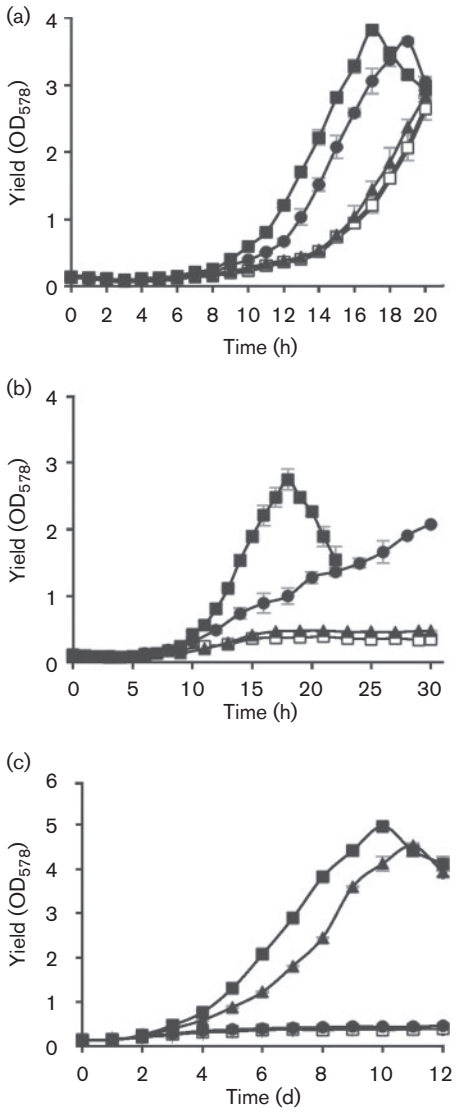

Fig. 6. Temperature stress protection by compatible solutes. Growth curves of *B. subtilis* WT strain JH642 grown at (a) 52 or (b) 52.2 °C, and of the WT strain 168 grown at 13 °C (c) in SMM without a compatible solute (□), or with 1 mM (final concentration) of glycine betaine (■), L-proline betaine (▲) or betonicine (●). The data shown are the mean ± SD of three independent replicates.

system for L-proline betaine. Again, the OpuA, OpuC and OpuD uptake systems imported glycine betaine that was used as control for this experiment (Hoffmann & Bremer, 2011), but the physiological relevance of these transporters for the acquisition of this compound varied in the low-temperature-stressed cells (Fig. S3b).

Binding of L-proline betaine and betonicine by the OpuAC solute receptor protein

The three glycine betaine transporters operating in *B. subtilis* all possess a high affinity for their substrate with K_m values in the low micromolar range. However, the OpuA system dominates glycine betaine import due to its high capacity (V_{max}) (Kappes *et al.*, 1996). This property is probably also the reason why L-proline betaine and betonicine were imported primarily via the OpuA system under temperature stress conditions (Fig. S3). The functionality and substrate specificity of the OpuA transporter are dependent on an extracellular ligand-binding protein (OpuAC) tethered with a lipid anchor to the cytoplasmic membrane of *B. subtilis* (Horn *et al.*, 2006; Kempf & Bremer, 1995).

We overexpressed a recombinant version of the *B. subtilis* OpuAC protein in *E. coli* and purified it to apparent homogeneity using previously described procedures (Bashir *et al.*, 2014; Smits *et al.*, 2008). Ligand binding by OpuAC is reflected by changes in the intrinsic Trp fluorescence and these changes can be used to quantify the affinity of the OpuAC protein for its various ligands (Bashir *et al.*, 2014; Horn *et al.*, 2006; Smits *et al.*, 2008). Ligand binding of glycine betaine and L-proline betaine by OpuAC resulted in an increase in the fluorescence intensity (Smits *et al.*, 2008), whereas the newly tested betonicine caused a decrease. Using fluorescence spectroscopy, we measured the stability constant (K_d) of OpuAC–ligand complexes, and K_d values of 38 ± 3, 135 ± 23 and 324 ± 65 μM were obtained for glycine betaine, betonicine and L-proline betaine, respectively (Fig. 7). The K_d values for glycine betaine (Fig. 7a) and L-proline betaine (Fig. 7b) agreed quite well with previous measurements (Horn *et al.*, 2006), whereas that of betonicine (Fig. 7c) had not been determined previously.

In silico docking of betonicine into the OpuAC ligand-binding site

Crystal structures of OpuAC in complex with either glycine betaine (PDB ID: 2B4L) or L-proline betaine (PDB ID: 2B4M) have been reported (Horn *et al.*, 2006), and the observed contacts between these ligands and the OpuAC protein have been buttressed via site-directed mutagenesis experiments (Smits *et al.*, 2008). As L-proline betaine and betonicine are chemically closely related (Fig. 1), we were able to use the crystal structure of the OpuAC–L-proline betaine complex (Fig. 8a) as a template for *in silico* modelling studies. The aim of this modelling approach was to (i) reveal the likely position of betonicine within the OpuAC ligand-binding pocket and (ii) understand the molecular underpinnings for the somewhat higher affinity of OpuAC for betonicine in comparison with the non-hydroxylated L-proline betaine (Fig. 1).

In our *in silico* model, the betonicine ligand fitted well into the binding pocket of the OpuAC protein, with a spatial orientation that was comparable with the L-proline betaine molecule (Fig. 8). As observed in the crystal structures of the OpuAC–glycine betaine and OpuAC–L-proline betaine complexes (Horn *et al.*, 2006), the positively charged head group of betonicine resides in

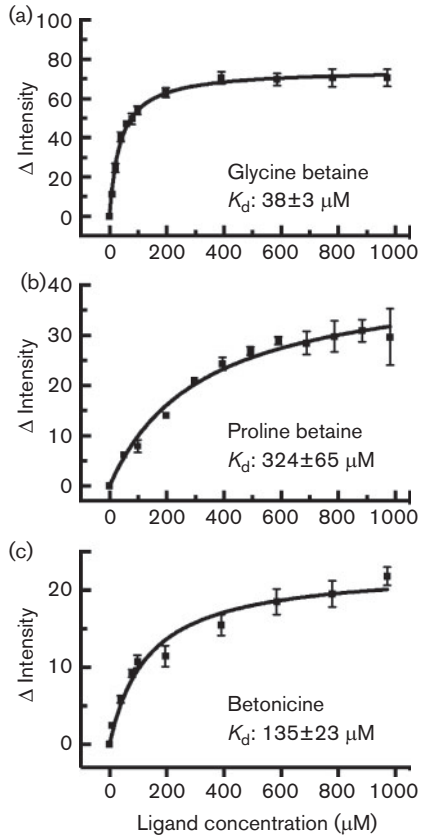

Fig. 7. Binding of glycine betaine, L-proline betaine and betonicine by the OpuAC solute receptor protein. Ligand binding by OpuAC is reflected by corresponding changes in the maximal intensity of the intrinsic Trp fluorescence of this protein; the extent of these changes (△ intensity) is dependent on the ligand concentration (Horn *et al.*, 2006; Smits *et al.*, 2008) and was used to determine the affinity of the OpuAC protein to its ligand (K_d). The Michaelis–Menten fittings for OpuAC binding to (a) glycine betaine, (b) L-proline betaine and (c) betonicine are shown.

an aromatic cage created by the side-chains of three Trp residues and is stabilized via cation–π interactions (Trp72, Trp178 and Trp252) (Horn *et al.*, 2006). To accommodate the hydroxyl group at position C-4 within the L-proline ring of betonicine (Fig. 1), our model suggests that this ligand is slightly rotated in comparison with the position of L-proline betaine within the ligand-binding site (Fig. 8). This rotational movement by ~20° is needed to firmly accommodate the positively charged dimethlyammonium head group as well as the negatively charged hydroxyl group of betonicine within the OpuAC ligand-binding site. As a further result of this slight rotational movement, the hydroxyl group of betonicine was now able to interact with the positively charged nitrogen in the ring of the Trp178 side-chain and the carboxylate of betonicine could interact with the backbone amide groups of Gly26. Further contacts were via electrostatic interactions with the side-chain of His230. This latter protein–ligand interaction has also been observed in the OpuAC–glycine betaine complex and is a key determinant for the higher affinity of OpuAC for glycine betaine than for L-proline betaine (Horn *et al.*, 2006; Smits *et al.*, 2008). Taken together, the interaction of the hydroxyl group of betonicine at position C-4 in the proline ring structure, as well as the additional interaction of its carboxyl group with the side-chain of His230, not only compensated for the loss of the interaction with the backbone of Ile27 (Fig. 8), but also fostered stronger interactions of the ligand with the OpuAC protein. Our *in silico* model thus provided an explanation for the experimentally observed two- to threefold higher binding affinity of OpuAC for betonicine (K_d=135±23 μM) in comparison with L-proline betaine (K_d=324±65 μM).

DISCUSSION

The soil-dwelling bacterium *B. subtilis* lives in a challenging habitat in which desiccation processes lead to increases in the environmental osmolarity (Bremer, 2002). Organic matter, including compatible solutes, is primarily brought into the soil via root exudates and decaying plant tissues

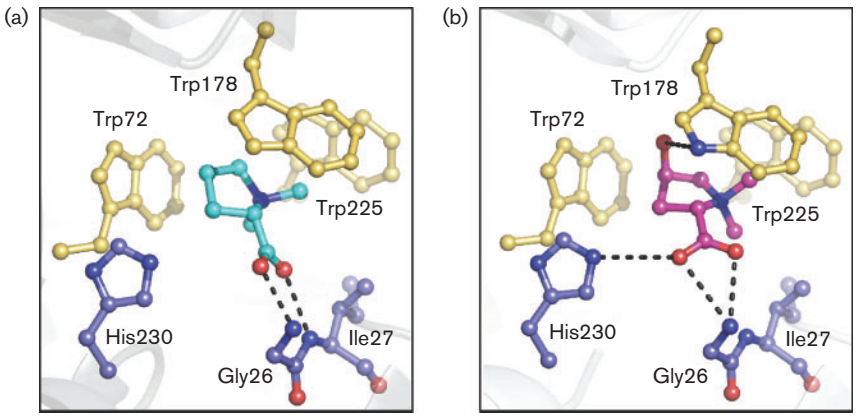

Fig. 8. Coordination of L-proline betaine and betonicine within the ligand-binding site of the OpuAC solute receptor protein. (a) The structural data for the OpuAC–L-proline betaine complex were taken from the PDB database (PDB ID: 2B4M) (Horn *et al.*, 2006). (b) *In silico* model for the OpuAC–betonicine complex.

(Moe, 2013). The release of newly synthesized osmoprotectants by osmotically down-shocked or decayed microbial cells is also a key contributor to the compatible solute cocktail found in the soil (Warren, 2013, 2014). Consequently, the uptake of compatible solutes provides soil micro-organisms such as *B. subtilis* with the opportunity to derive protection against osmotic (Bremer, 2002; Kappes *et al.*, 1999) or temperature challenges (Hoffmann & Bremer, 2011; Holtmann & Bremer, 2004). The data presented here add the OpuA/OpuC/(OpuD)-mediated import of the plant-derived L-proline derivatives L-proline betaine and betonicine (Hanson *et al.*, 1994; Rhodes & Hanson, 1993) to the physiological defence arsenal of *B. subtilis* against high salinity and growth-restricting extremes in temperatures (Bremer, 2002; Hoffmann & Bremer, 2011; Holtmann & Bremer, 2004). In contrast to L-proline (Moses *et al.*, 2012), L-proline betaine and betonicine cannot be catabolized by this soil bacterium (Fig. 2), regardless of the fact that L-proline betaine can serve as an inducer (Table 2) for the L-proline catabolic *putBCP* operon (Moses *et al.*, 2012).

Despite the close chemical relatedness of L-proline betaine and betonicine to L-proline (Fig. 1), both compounds are not imported through OpuE – the dominating uptake system for L-proline when it is acquired by *B. subtilis* as an osmoprotectant (von Blohn *et al.*, 1997; Zaprasis *et al.*, 2013). Instead, L-proline betaine and betonicine are taken up by transporters mediating the uptake of various di- or trimethylated osmoprotectants, OpuA/OpuC/(OpuD) (Bremer, 2002; Hoffmann & Bremer, 2011). Crystallographic analysis has revealed that cation–π interactions between the fully methylated and positively charged head group of L-proline betaine (Fig. 1) and the side-chains of aromatic residues present in the OpuAC proteins from *B. subtilis* (Horn *et al.*, 2006; Smits *et al.*, 2008) and *Lactococcus lactis* (Wolters *et al.*, 2010), and the ProX proteins from *E. coli* (Schiefner *et al.*, 2004a) and the archaeon *Archaeoglobus fulgidus* (Schiefner *et al.*, 2004b), are key contributors to ligand binding. Our modelling study of the OpuAC–betonicine complex suggests that such an aromatic cage is also involved in the recognition and capturing of this ligand by the OpuAC substrate-binding protein (Fig. 8b). Furthermore, this *in silico* model provides hints as to why OpuAC can bind betonicine with a higher affinity than L-proline betaine (Fig. 7).

The level of osmostress protection afforded by L-proline betaine is similar to that of glycine betaine, whereas that conferred by betonicine is more modest and resembles that of L-proline. A correlation seems to exist between the osmoprotective effects of these solutes (Fig. 3a) and their influence on (i) the size of the L-proline pool build-up through *de novo* synthesis (Fig. 3b) and (ii) the transcriptional activity of the osmotically controlled *opuA* promoter (Fig. 5). This set of data can most easily be interpreted within the physiological context of osmotically stressed *B. subtilis* cells (Bremer, 2002) if we assume that the Opu-mediated uptake processes attain an intracellular betonicine pool smaller than that of L-proline betaine. However, such presumed differences in the pool sizes of these compounds remain to be verified experimentally. Factors other than the actual intracellular concentrations of L-proline betaine and betonicine also need to be taken into account when assessing the data. The physico-chemical properties of these solutes, their influence on the functionality of macromolecules, the transcriptional machinery of the cell and the solvation status of the cytoplasm might be sufficiently dissimilar to cause different physiological effects with respect to cell growth under osmotically challenging conditions (Cayley *et al.*, 1992; Jackson-Atogi *et al.*, 2013; Street *et al.*, 2006; Wood, 2011).

B. subtilis adapts to decreases or increases in temperatures suboptimal for growth by inducing a set of complex stress management systems, e.g. cold-shock and heat-shock proteins, the induction of the SigB-controlled general stress response system, and the production of a lipid-modifying enzyme that prevents the rigidification of the cytoplasmic membrane at low temperature (Budde *et al.*, 2006; Graumann & Marahiel, 1996; Hecker *et al.*, 2007; Martín & de Mendoza, 2013; Schumann, 2003). All these well-studied temperature stress response systems fail completely to ensure growth at the very cutting upper (52–53 °C) and lower (11–13 °C) boundaries of the temperature spectrum that *B. subtilis* cells can populate. Remarkably, for temperature-challenged cells tinkering with death, the uptake of compatible solutes permits cell proliferation (Bashir *et al.*, 2014; Hoffmann & Bremer, 2011; Holtmann & Bremer, 2004). The molecular and biochemical underpinning(s) of this type of temperature stress protection are far from clear (for a discussion of this issue, see Hoffmann & Bremer, 2011), but studies with glycine betaine have shown that the intracellular concentrations required for *B. subtilis* to sustain growth at very high or very low temperatures (Hoffmann & Bremer, 2011; Holtmann & Bremer, 2004) are far lower than those needed to achieve osmostress protection at high salinity (Hoffmann *et al.*, 2013). Hence, it seems possible that the temperature stress protection afforded by L-proline betaine and betonicine is routed in the physico-chemical properties of these molecules (Cayley *et al.*, 1992; Jackson-Atogi *et al.*, 2013; Street *et al.*, 2006) and the ensuing chemical chaperone function of compatible solutes that preserves the functionality of macromolecules and biosynthetic processes (Bourot *et al.*, 2000; Chattopadhyay *et al.*, 2004; Diamant *et al.*, 2001; Fisher, 2006; Ignatova & Gierasch, 2006; Jackson-Atogi *et al.*, 2013; Manzanera *et al.*, 2002). The chemical differences between L-proline betaine and betonicine appear to be rather minor (Fig. 1), but their stress-protective activities at high and low growth temperatures are strikingly different (Fig. 6). Unless these disparate physiological effects are rooted in different steady-state intracellular pool sizes that result from a different efficiency in L-proline betaine and betonicine import, it will be a challenge to understand in biophysical and molecular terms the foundation(s) for their dissimilar cell-protective properties. Collectively, the data presented here highlight the

notion that small differences in the chemical structure of a given compatible solute can make a big difference with respect to its physiological properties for a given micro-organism.

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