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Arsenobetaine: an ecophysiologically important organoarsenical confers cytoprotection against osmotic stress and growth temperature extremes

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Summary

Arsenic, a highly cytotoxic and cancerogenic metalloid, is brought into the biosphere through geochemical sources and anthropogenic activities. A global biogeochemical arsenic biotransformation cycle exists in which inorganic arsenic species are transformed into organoarsenicals, which are subsequently mineralized again into inorganic arsenic compounds. Microorganisms contribute to this biotransformation process greatly and one of the organoarsenicals synthesized and degraded in this cycle is arsenobetaine. Its nitrogen-containing homologue glycine betaine is probably the most frequently used compatible solute on Earth. Arsenobetaine is found in marine and terrestrial habitats and even in deep-sea hydrothermal vent ecosystems. Despite its ubiquitous occurrence, the biological function of arsenobetaine has not been comprehensively addressed. Using *Bacillus subtilis* as a well-understood platform for the study of microbial osmostress adjustment systems, we ascribe here to arsenobetaine both a protective function against high osmolarity and a cytoprotective role against extremes in low and high growth temperatures. We define a biosynthetic route for arsenobetaine from the precursor arsenocholine that relies on enzymes and genetic regulatory circuits for glycine betaine formation from choline, identify the uptake systems for arsenobetaine and arsenocholine, and describe crystal structures of ligand-binding proteins from the OpuA and OpuB ABC transporters complexed with either arsenobetaine or arsenocholine.

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Introduction

Arsenic is an abundant constituent of the Earth crust and is also an important constituent of the biosphere (Mukhopadhyay *et al.*, 2002; Oremland and Stolz, 2003; Li *et al.*, 2016; Zhu *et al.*, 2017). Millions of humans suffer from its cytotoxic and cancerogenic effects through their exposure to contaminated water sources (Oremland and Stolz, 2005). This situation is exacerbated by the widespread use of man-made organoarsenicals for disease-prevention and growth promotion of animals (Yoshinaga and Rosen, 2014). As a result of its wide distribution and abundance in the environment, microorganisms have been exposed to arsenic essentially since the origin of life. A global arsenic biotransformation cycle exists in nature, to which microbial

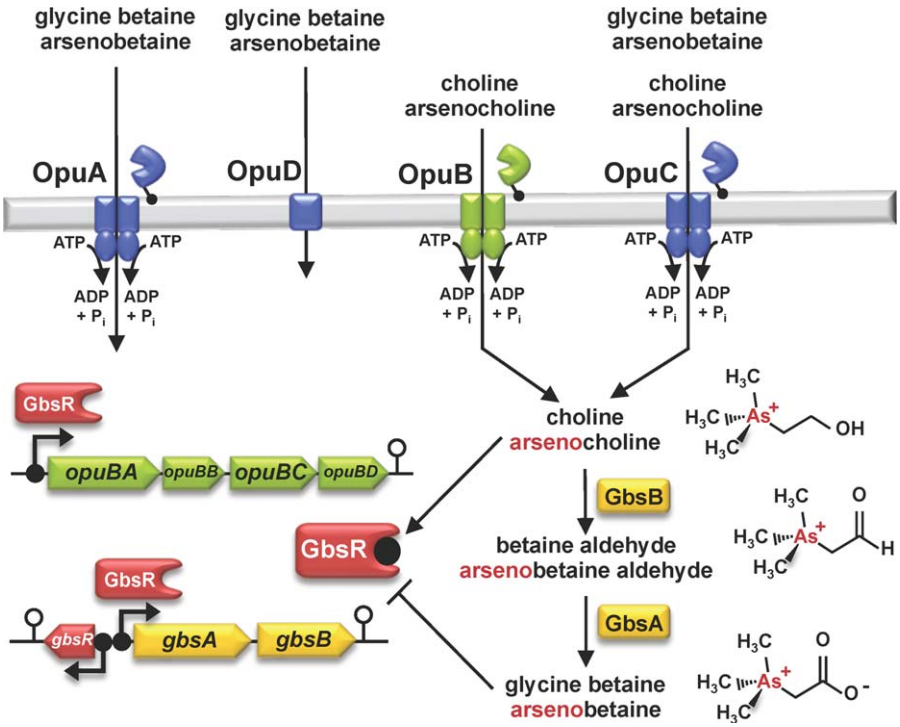

Fig. 1. Osmoprotectant uptake (Opu) systems in *B. subtilis* and the pathway used for the synthesis of glycine betaine and arsenobetaine from their precursor molecules.
The transcription of the operons encoding the choline/arsenocholine-specific ABC transporter (OpuB) and the enzymes for glycine betaine/arsenobetaine synthesis (GbsB/GbsA) is regulated via the GbsR repressor protein. The promoters and terminators of the corresponding operons are indicated by arrows and lollipops respectively. Choline and arsenocholine bound to GbsR are represented by a black sphere.

metabolic activities contribute greatly (Oremland and Stolz, 2003; Slyemi and Bonnefoy, 2012; Li *et al.*, 2016; Zhu *et al.*, 2017). Microorganisms have not only developed an impressive array of systems to resist and detoxify arsenic, but they have also learned to exploit it as a means for energy-generation through oxidative and reductive biochemical transformations (Slyemi and Bonnefoy, 2012; Li *et al.*, 2016; Zhao, 2016; Chen *et al.*, 2017; Edwardson and Hollibaugh, 2017; Zhang *et al.*, 2017; Zhu *et al.*, 2017).

There is one notable exception to the severe cytotoxicity of inorganic and organic arsenic-containing compounds, the organoarsenical arsenobetaine (Fig. 1); it has an estimated median lethal dose (LD_{50}) in mice of about 10 g kg^{-1} body weight (Kaise *et al.*, 1985). As arsenobetaine is widely found in marine ecosystems, humans ingest arsenobetaine primarily through the consumption of seafood and they excrete it again through their urine (Molin *et al.*, 2015; Thomas and Bradham, 2016; Taylor *et al.*, 2017). Since its discovery 40 years ago (Edmonds *et al.*, 1977), studies monitoring arsenic species in environmental and food samples consistently find arsenobetaine, often in substantial quantities (Caumette *et al.*, 2012; Molin *et al.*, 2015; Popowich *et al.*, 2016; Thomas and Bradham, 2016; Taylor *et al.*, 2017). Evidence for its production by microorganisms (Ritchie *et al.*, 2004), terrestrial fungi (Nearing *et al.*, 2015) and phytoplankton (Caumette *et al.*, 2012; Foster and Maher, 2016), has been presented, but the details of arsenobetaine synthesis are still incompletely understood (Popowich *et al.*, 2016). The current models for its formation rely mostly on the detection of potential biosynthetic precursors and intermediates. Two proposed biosynthetic pathways envision the formation of arsenobetaine from di- or tri-methylated arsenosugars that are primarily produced by eukaryotic organisms at the bottom of the aquatic food chain. The breakdown of these organoarsenicals lead either to the formation of arsenocholine as an intermediate that then could be further oxidized to arsenobetaine, or to the synthesis of dimethylarsinoylethanol, which could serve through several biotransformation reactions as precursor for arsenobetaine production. An alternative route for its synthesis proposes dimethylarsenite as the starting compound (Caumette *et al.*, 2012; Foster and Maher, 2016; Taylor *et al.*, 2017; Zhu *et al.*, 2017).

Despite the fact that arsenobetaine is an environmentally ubiquitous organoarsenical (Ciulla *et al.*, 1997; Caumette *et al.*, 2012; Molin *et al.*, 2015; Foster and Maher, 2016; Popowich *et al.*, 2016; Taylor *et al.*, 2017) its potential biological function has not yet been addressed systematically. The chemical relatedness of arsenobetaine to the nitrogen-containing and environmentally abundant glycine betaine molecule (Yancey, 2005) (Fig. 1) fostered speculations about its possible function as an osmostress protectant (Peddie *et al.*, 1994; Ciulla *et al.*, 1997; Devesa *et al.*, 2005; Nearing *et al.*, 2015), as glycine betaine prominently plays such a role in many *Prokarya* and *Eukarya* (Csonka and Hanson, 1991; da Costa *et al.*, 1998; Kempf and Bremer, 1998; Wood *et al.*, 2001; Yancey, 2005; Burg and Ferraris, 2008). However, the available evidence for

such a biological role of arsenobetaine is mostly indirect (Popowich *et al*., 2016).

Increases in the external osmolarity triggers water efflux from microbial cells, and as a result, vital turgor and growth will be impaired (Csonka and Hanson, 1991; Kempf and Bremer, 1998; Wood *et al*., 2001; Wood, 2011). Many members of the *Bacteria* and *Archaea* either import or synthesize specific organic osmolytes, the so-called compatible solutes, under these stressful conditions to balance the osmotic gradient across their cytoplasmic membrane and to optimize the solvent properties of the cytoplasm for biochemical reactions (Csonka and Hanson, 1991; da Costa *et al*., 1998; Kempf and Bremer, 1998; Roesser and Müller, 2001; Wood *et al*., 2001; Wood, 2011). Glycine betaine is such a compatible solute and can be synthesized under aerobic conditions either through the sequential methylation of glycine (Nyyssölä *et al*., 2000), or through the oxidation of prior imported choline (Lamark *et al*., 1991; Boch *et al*., 1996; Salvi *et al*., 2014). There is no evidence that bacteria can synthesize glycine betaine from precursors other than glycine or choline for the purpose of osmostress protection, but glycine betaine is formed when they catabolize environmentally abundant L-carnitine as a nutrient source (Meadows and Wargo, 2015). In addition to the synthesis of glycine betaine, microorganisms can use osmotically induced high-affinity transporters to scavenge glycine betaine and its biosynthetic precursor choline from environmental sources and they can thereby achieve a considerable degree of osmostress tolerance (Bremer and Krämer, 2000; Wood *et al*., 2001; Wood, 2011; Hoffmann and Bremer, 2016; 2017).

Previous efforts to define the biological function(s) of arsenobetaine in microorganisms (Peddie *et al*., 1994; Ciulla *et al*., 1997; Devesa *et al*., 2005; Nearing *et al*., 2015) suffer because no model system has been systematically studied that is amendable to genetic analysis so that physiological effects can be critically assessed, and the import and biosynthetic route(s) of this organoarsenical can be identified reliably. Here, we exploited *Bacillus subtilis* as a well-understood platform for the genetics, physiology and molecular biology of microbial osmostress adjustment systems (Hoffmann and Bremer, 2016; 2017) to comprehensively address these issues. Our data show that arsenobetaine and its biosynthetic precursor arsenocholine enter the *B. subtilis* cell via the same set of osmotically controlled Opu-type transporters that are also used by their nitrogen-containing counterparts (Fig. 1), confer effective osmostress protection and arsenobetaine serves as cytoprotectant against the restricting effects of extremes in either low or high growth temperatures. As uptake systems for glycine betaine and for its synthesis from choline are widely distributed in members of the *Bacteria*, the data presented here will shed new light on the physiological roles played by the ubiquitously distributed arsenobetaine molecule, and its biosynthetic precursor arsenocholine, in terrestrial and marine ecosystems.

Results

Osmostress protection by arsenobetaine and its dependence on the OpuA, OpuC and OpuD osmolyte transporters

To assess the osmostress protective potential of arsenobetaine for the *B. subtilis* wild-type strain JH642, we challenged cultures grown in a chemically defined medium (SMM) with sustained high salinity (1.2 M NaCl) and then monitored the influence of both glycine betaine and arsenobetaine on cell growth. The addition of 1.2 M NaCl to the SMM medium confers severe osmotic stress to *B. subtilis* (Boch *et al*., 1994) (Fig. 2A). When added to the salt-challenged cultures at a final concentration of 1 mM, both glycine betaine and arsenobetaine strongly enhanced growth. Arsenobetaine provided the same level of cellular osmostress protection to *B. subtilis* as that conferred by glycine betaine (Fig. 2A). Both glycine betaine (Wargo, 2013) and arsenobetaine (Hanaoka *et al*., 1995; Khokiattiwong *et al*., 2001; Jenkins *et al*., 2003; Devesa *et al*., 2005; Huang *et al*., 2007; Harrington *et al*., 2008) can be metabolized by microorganisms under oxic growth conditions, but *B. subtilis* lacks the ability to degrade glycine betaine and use it as a nutrient (Boch *et al*., 1994). As expected, arsenobetaine could not be used as sole carbon and energy source (Supporting Information Fig. S1) indicating that *B. subtilis* can also not metabolize arsenobetaine.

A genetically isogenic set of mutants (Supporting Information Table S1) possessing only one of the three glycine betaine transporters (OpuA, OpuC, OpuD) (Fig. 1) operating in *B. subtilis* (Hoffmann and Bremer, 2016; 2017) is a convenient tool to assess the transport system through which a given osmostress protectant is imported (Holtmann and Bremer, 2004; Hoffmann and Bremer, 2011). We conducted such an experiment to define the entry routes of arsenobetaine into the *B. subtilis* cell and found that the OpuA, OpuC and OpuD transporters each served for its import. The simultaneous genetic disruption of these import system abolished osmostress protection by both glycine betaine and arsenobetaine (Supporting Information Fig. S2A).

Affinity of the OpuA, OpuC and OpuD transporters for arsenobetaine

To determine the apparent affinity of the *B. subtilis* OpuA, OpuC and OpuD transporters for arsenobetaine, we initially measured the K_m-values of these transport systems using radiolabelled [1-^{14}C]glycine betaine as the substrate,

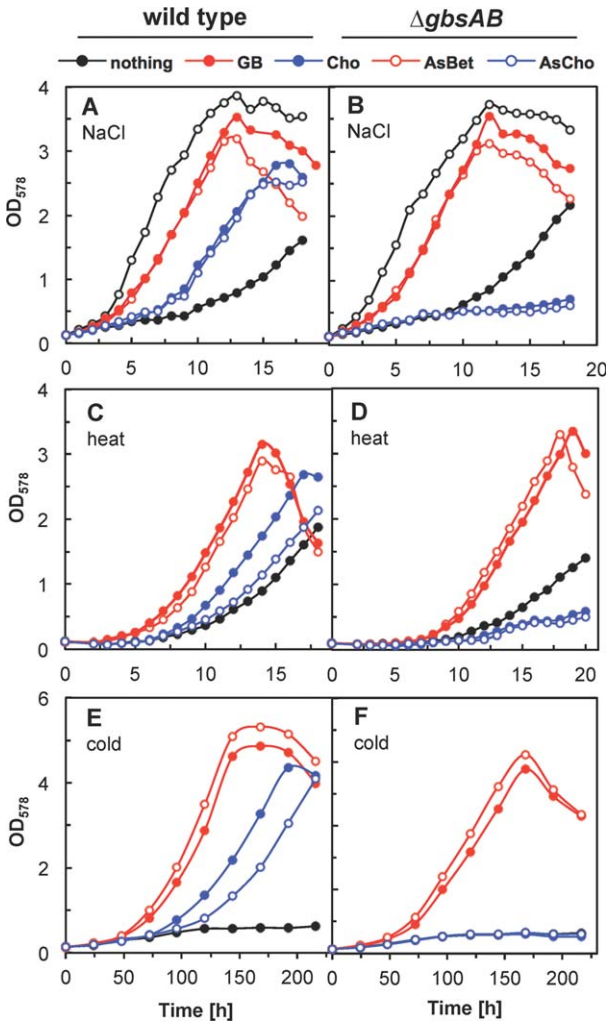

Fig. 2. Influence of high salinity stress (1.2 M NaCl; A,B) and high (52.1°C; C,D) and low (13°C; E, F) temperature stress on the growth of the *B. subtilis* wild-type strains JH642 (A,C) and 168 (E) in comparison with their corresponding *gbsAB* mutants JBB5 (B,D) and SOB9 (F) respectively.
The cultures were grown in minimal medium (SMM) in the absence or the presence of 1 mM (final concentration) of the indicated compounds. The osmostress protection growth assays (A,B) were conducted at 37°C and growth of these cultures were compared with that of a wild-type strain grown in the SMM in the absence of additional NaCl (open black circels). The shown growth curves represent the averages of three independently grown *B. subtilis* cultures.

and subsequently determined the apparent inhibitory constant (K_i) for arsenobetaine. Consistent with previous data (Kappes *et al.*, 1996), the OpuA, OpuC and OpuD systems each transported glycine betaine with high affinity with K_m-values in the low μM range in cells that were grown in SMM with moderately increased osmolarity (with 0.4 M NaCl) to induce the expression of the *opuA*, *opuB* and *opuC* operons (Kempf and Bremer, 1995; Kappes *et al.*, 1999; Hoffmann and Bremer, 2016) (Table 1). Arsenobetaine was also taken up with high affinity (as evidenced through their K_i-values) via the OpuA, OpuC and OpuD systems but the corresponding K_i values were in general about fourfold to fivefold lower than that for glycine betaine.

Osmotic stress triggers high-level arsenobetaine accumulation

The severity of the osmotic stress imposed onto the *B. subtilis* cell dictates the intracellular pool size of glycine betaine (Hoffmann *et al.*, 2013). We used Liquid Chromatography-Electrospray Ionization-Mass Spectrometry (LC-ESI-MS) to measure the glycine betaine and arsenobetaine content in cells that were grown either under non-stressed (in SMM) or osmotically stressed (SMM with 1.2 M NaCl) conditions. SMM-grown cells had glycine betaine and arsenobetaine pools of about 103 mM, and 82 mM respectively (Fig. 3). Osmotic stress triggered a strong increase in the cellular content of glycine betaine (4.8-fold) and arsenobetaine (6.5-fold), leading to comparable pools of these compatible solutes (between 495 mM and 549 mM respectively) in the *B. subtilis* cells (Fig. 3).

Osmostress protection by arsenocholine is dependent on its enzymatic conversion into arsenobetaine

As in other microorganisms (Lamark *et al.*, 1991; Kempf and Bremer, 1998), glycine betaine is synthesized in *B. subtilis* from the precursor choline in a two-step enzymatic reaction (Boch *et al.*, 1994; Boch *et al.*, 1996). We note, that choline does not possess osmostress protective properties *per se*; instead, osmostress protection by imported choline relies on its oxidation to glycine betaine by the *B. subtilis* GbsB/GbsA enzymes with glycine betaine aldehyde as the biosynthetic intermediate (Fig. 1) (Boch *et al.*, 1996). We found that arsenocholine affords the same level of osmostress protection to *B. subtilis* as that provided through the addition of 1 mM choline (Fig. 2A). However, disruption of the glycine betaine synthesis pathway (Boch *et al.*, 1996) in the Δ(*gbsAB*) mutant JBB5 leads to a loss of osmostress protection by both choline and arsenocholine but preserved that for glycine betaine and arsenobetaine (Fig. 2B). As shown previously for choline (Boch *et al.*, 1996), the absence of the GbsB/GbsA enzymes results in growth inhibition of *B. subtilis* cells by arsenocholine (Fig. 2B), probably as the result of the strong accumulation of the positively charged choline and arsenocholine molecules.

Natural abundance ^{13}C-nuclear magnetic resonance (NMR) spectroscopy can be used to assess the dominant compatible solutes in osmotically stressed cells (Kuhlmann and Bremer, 2002; Bashir *et al.*, 2014b). We used this technique to confirm the enzymatic transformation of

Table 1. Substrate affinities of the Opu transporters for glycine betaine, arsenobetaine, choline and arsenocholine.

Transport system	K_m (μM) Glycine betaine	K_i (μM) Arsenobetaine	K_m (μM) Choline	K_i (μM) Arsenocholine
OpuA	4.6 ± 0.9	17 ± 2	–	–
OpuB	-[a]	–	2.5 ± 0.4	9 ± 2
OpuC	4.7 ± 0.4	22 ± 2	50 ± 7	382 ± 80
OpuD	4.4 ± 0.4	22 ± 2	–	–

Cultures of *B. subtilis* mutant strains that express only one of the OpuA, OpuB, OpuC or OpuD uptake systems were grown in SMM at 37°C containing 0.4 M NaCl to an OD_{578} of 0.3 and were then used for the transport assay. The substrate concentrations were varied between 3 μM and 50 μM for the glycine betaine and between 1 μM and 400 μM for the choline uptake assays. The unlabelled compounds were spiked with 1 μM of the radiolabelled substrate used in the particular transport assays. The data shown for the various transport assays were derived from two to three independently grown cultures.
a(-): no transport activity.

externally provided arsenocholine into intracellular arsenobetaine. For these experiments, we used a *B. subtilis* mutant (strain JSB8) unable to produce the large amounts of the compatible solute proline that are synthesized by osmotically stressed *B. subtilis* wild-type cells (Whatmore *et al*., 1990; Brill *et al*., 2011; Hoffmann *et al*., 2013) and fed these cultures either 1 mM arsenobetaine or 1 mM arsenocholine. Cells cultivated in the presence of arsenobetaine accumulated it in an unmodified form, whereas those grown in the presence of arsenocholine converted most of it into arsenobetaine; however, arsenocholine was also detected (Supporting Information Fig. S3).

We further validated and extended these data by measuring the pool sizes of glycine betaine, arsenobetaine, choline and arsenocholine by LC-ESI-MS in cells that were grown either in the presence of 1 mM choline or arsenocholine (Fig. 3). In cultures grown in the presence of choline, choline was present at a concentration of 10 mM (in SMM) and 4 mM (in SMM with 1.2 M NaCl), whereas the corresponding derived glycine betaine pools were 111 mM and 599 mM respectively (Fig. 3). Cells grown in the presence of arsenocholine under salt stress conditions contained mostly (about 77%) arsenobetaine (462 mM), but also 141 mM arsenocholine were detected (Fig. 3). Hence, in full agreement with the genetic data on the GbsB/GbsA-dependent osmostress protection by arsenocholine (Fig. 2B), the ^{13}C-NMR spectroscopy data (Supporting Information Fig. S3) and pool measurements (Fig. 3) directly demonstrate the conversion of externally supplied arsenocholine into an intracellular arsenobetaine pool. Our ^{13}C-NMR and LC-ESI-MS data did not reveal any break-down products of arsenobetaine, an observation fully consistent with our finding that *B. subtilis* cannot use arsenobetaine as a nutrient (Supporting Information Fig. S1).

Import of arsenocholine depends on the OpuB and OpuC ABC transport systems

We studied the import routes for arsenocholine in osmostress protection experiments. As reported for choline (Kappes *et al*., 1999), the uptake of arsenocholine was dependent on the OpuB and OpuC ABC transporters (Fig. 1). The simultaneous genetic disruption of the *opuB* and *opuC* operons abolished osmostress protection by these compounds, but preserved it for glycine betaine and arsenobetaine (Supporting Information Fig. S2A). Kinetic studies using radiolabelled [*methyl*-^{14}C]-choline as the substrate and arsenocholine as the inhibitor were then

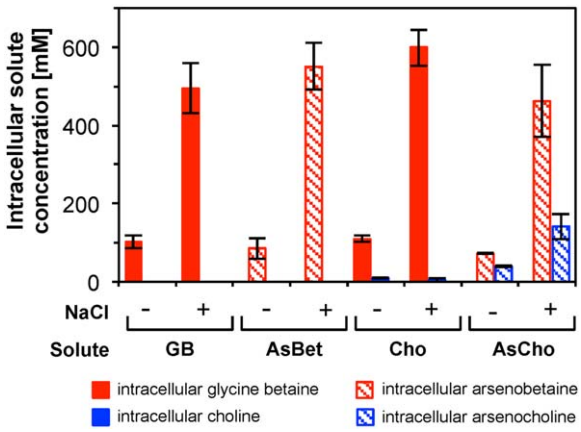

Fig. 3. Influence of high salinity on the cellular pools of glycine betaine, choline, arsenobetaine and arsenocholine.
The *B. subtilis* wild-type strain JH642 was grown either in SMM or in SMM containing 1.2 M NaCl in the presence of glycin betaine, arsenobetaine, choline or arsenocholine (1 mM each) until the cultures reached an OD_{578} between 2 and 2.5. These compounds were extracted with 80% ethanol from the cells and their intracellular pools were measured by LC-ESI-MS. Strain TMB118 was used as a control for the true import of glycin betaine, arsenobetaine, choline or arsenocholine as it lacks the OpuA, OpuB, OpuC and OpuD transporters (Teichmann *et al*., 2017). The TMB118 control strain had apparent pool sizes of 18 mM glycine betaine, and 3 mM arsenobetaine respectively. These values represent the incomplete removal of glycine betaine and arsenobetaine from the cells during processing of the samples, as such a mutant cannot import glycine betaine or choline (Teichmann *et al*., 2017). Therefore, we subtracted these values from the pool sizes observed in the $OpuA^+$ $OpuC^+$ $OpuD^+$ wild-type strain JH642. GB: glycine betaine; AsBet: arsenobetaine; Cho: choline; AsCho: arsenocholine.

used to assess the properties of these transporters with respect to arsenocholine uptake. Both the OpuB and OpuC systems exhibited high affinity for choline, and in agreement with previous data (Kappes *et al*., 1999), OpuB had a greater affinity for this compound than OpuC (Table 1). There was an approximately 3.6-fold reduction in apparent affinity with respect to arsenocholine transport via OpuB, while the affinity of this compound for OpuC was reduced by about 7.6-fold (Table 1). These kinetic parameters characterize the OpuC transporter, an uptake system with very broad substrate specificity for osmostress protectants (Hoffmann and Bremer, 2016; 2017; Teichmann *et al*., 2017), as a low-affinity transporter for arsenocholine. In contrast, OpuB, an import system with a very restricted substrate specificity (Teichmann *et al*., 2017), that is genetically co-regulated with the *gbsAB* glycine betaine biosynthetic operon (Nau-Wagner *et al*., 2012; Hoffmann and Bremer, 2016) (Fig. 1), is able to scavenge this organoarsenical effectively.

Crystal structures of the OpuAC::arsenobetaine and OpuBC::arsenocholine complexes

We asked if the architecture of the substrate-binding sites in extracellular ligand-binding proteins operating in conjunction with the *B. subtilis* glycine betaine/arsenobetaine and choline/arsenocholine ABC-type uptake system (Fig. 1) would match. To this end, we focused on a structural analysis of the OpuAC and OpuBC ligand-binding proteins (Horn *et al*., 2006; Pittelkow *et al*., 2011) (Fig. 1). The obtained crystal structures of the OpuAC::arsenobetaine and OpuBC::arsenocholine complexes (Fig. 4) had resolutions of 2.2 Å and 1.9 Å respectively (the relevant crystallographic statistics are summarized in Supporting Information Table S2). The overall fold of the OpuAC::arsenobetaine and OpuBC::arsenocholine proteins adhere to that of a typical type I ligand-binding protein with their two lobes and the connecting hinge region (Supporting Information Fig. S5A,C,D,F) (Berntsson *et al*., 2010; Scheepers *et al*., 2016). As expected, single arsenobetaine and arsenocholine molecules are trapped in the deep cleft formed by the two lobes of the OpuAC and OpuBC binding-proteins (Horn *et al*., 2006; Pittelkow *et al*., 2011), respectively (Supporting Information Fig. S5C,F).

The OpuAC::glycine betaine (Protein Data Bank entry 2B4L) and OpuAC::arsenobetaine (Protein Data Bank entry 5NXX) complexes are structurally closely related, as indicated by an RMSD value of 0.4 Å over 260 Cα atoms (Supporting Information Fig. S5B). As observed for glycine betaine (Horn *et al*., 2006), the bulky, positively charged trimethylated head-group of arsenobetaine is wedged into an aromatic cage formed by the side-chains of Trp-72, Trp-178 and Trp-225; cation-π and van der Waals interactions stabilize it within this hydrophobic environment (Fig. 4A).

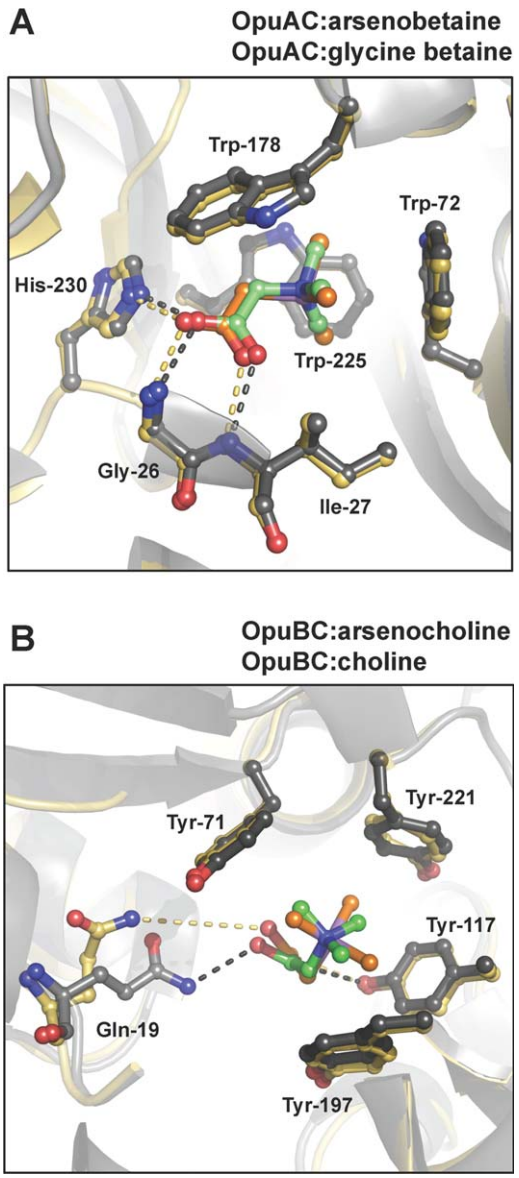

Fig. 4. Architecture of the substrate-binding site for arsenobetaine and arsenocholine in the ligand-binding proteins of the OpuA and OpuB ABC transporters.
A. Overlay of the arsenobetaine-binding site in OpuAC (yellow; PDB entry 5NXX) with that for glycine betaine (black; PDB entry 2B4L) (Horn *et al*., 2006). The glycine betaine molecule is depicted in green and arsenobetaine is shown in orange.
B. Overlay of the OpuBC arsenocholine-binding site in OpuBC (yellow; PDB entry 5NXY) with that for choline (black; PDB entry 3R6U) (Pittelkow *et al*., 2011). The choline molecule is depicted in green and arsenocholine is shown in orange. For parts (A) and (B), we superimposed the OpuAC::glycine betaine and OpuAC::arsenobetaine structures (A) and the OpuBC::choline and OpuBC::arsenocholine structures (B) using the Cα atoms and not according to the position of their ligands. This allows the ready visualization of any conformational changes in these crystal structures.

Glycine betaine and arsenobetaine differ only in the type of the atom at the centre of the tri-methylated head-group (Fig. 1). The radii of the nitrogen and arsenic atoms are 65 pm and 115 pm, respectively, which will make the tri-methylated head-group of arsenobetaine somewhat bulkier than that of glycine betaine. The carboxylate moiety of the arsenobetaine ligand protrudes out of the aromatic cage and forms hydrogen bonds with the backbone amides of Gly-26 and Ile-27 and the side-chain of His-230, molecular determinants for substrate binding also observed in the OpuAC::glycine betaine complex (Horn *et al*., 2006; Smits *et al*., 2008). This is reflected in an almost perfect overlay of the architecture of the substrate binding sites and that of the ligands in the OpuAC::glycine betaine and OpuAC::arsenobetaine crystal structures (Fig. 4A).

The structures of the OpuBC::choline (1.6 Å) (Protein Data Bank entry 3R6U) and OpuBC::arsenocholine (1.9 Å) (Protein Data Bank entry 5NXY) complexes are closely related as well, as indicated by an RMSD value of 0.3 Å over 260 Cα atoms (Supporting Information Fig. S5E). The side-chains of Tyr-71, Tyr-117, Tyr-197 and Tyr-221 form the aromatic cage that accommodates the fully methylated head-groups of either choline (Pittelkow *et al*., 2011), or arsenocholine (Fig. 4B). As observed for glycine betaine, arsenobetaine and choline, the positive charge of this part of the arsenocholine molecule is delocalized over all three methyl groups and interacts through cation-π interactions and van der Waals contacts with the four tyrosine residues forming the aromatic cage. The side-chains of these four residues in the OpuBC::choline and OpuBC::arsenocholine ligand-binding site are superimposable. However, the arsenocholine molecule is slightly rotated within the aromatic cage with reference to choline (Fig. 4B). The hydroxyl tail of choline protrudes out of the aromatic cage and makes a direct interaction with the side-chain of Tyr-117 and Gln-19 (Fig. 4B). The distance of the alcohol group of choline to the side-chain of Gln-19 is 3.1 Å, while it is lengthened to 5.4 Å in the OpuBC::arsenocholine complexes due to the rotated position of the ligand with the binding site and a concomitant repositioning of the Gln-19 side-chain (Fig. 4B). Replacement of Gln-19 by an Ala residue exerts a major influence on choline binding by the purified OpuBC protein (an 11-fold drop in affinity) (Pittelkow *et al*., 2011). The substantial lengthening of the distance between the OH-group of arsenocholine and the side-chain of Gln-19 should therefore contribute significantly to the observed reduction in affinity of the OpuB transporter for arsenocholine (Table 1). It should be noted in this context that the slight rotation of the arsenocholine molecule within the OpuBC ligand-binding site is made possible through the flexibility offered by the aromatic cage (Fig. 4B) to position the positively charged trimethylated head-group via through cation-π interactions (Mahadevi and Sastry, 2013).

In Fig. 5, we show a representation of the binding-site of the OpuAC::arsenobetaine and OpuBC::arsenocholine complexes along with the corresponding 2Fo-Fc and the Fo-Fc electron density maps. It is apparent that both the arsenobetaine and arsenocholine molecules fit nicely into the observed electron densities of the OpuAC::arsenobetaine and OpuBC::arsenocholine complexes, attesting to the overall quality of the high-resolution (2.2 Å and 1.9 Å respectively) crystal structures of these two substrate-binding proteins.

Arsenobetaine modulates the osmotic stress response of B. subtilis

The accumulation of glycine betaine at high osmolarity rescues cellular hydration, stabilizes turgor and optimizes the solvent properties of the cytoplasm (Csonka and Hanson, 1991; Kempf and Bremer, 1998; Roesser and Müller, 2001; Wood *et al*., 2001; Wood, 2011). In addition, it also affects gene expression of the cell's osmostress response systems in a global fashion by downregulating those encoding systems for the synthesis and uptake of compatible solutes once osmotic equilibrium has been attained (Hoffmann and Bremer, 2016; 2017). We tested whether the addition of arsenobetaine to the growth medium would influence the pool size of proline, the only compatible solute *B. subtilis* can synthesize *de novo* (Whatmore *et al*., 1990; Brill *et al*., 2011; Hoffmann and Bremer, 2016). Severely osmotically stressed cells [grown in the presence of 1.2 M NaCl (Boch *et al*., 1994)] had a proline pool of 580 ± 28 mM, and the presence of either 1 mM glycine betaine or 1 mM arsenobetaine in the growth medium reduced it about 30-fold (down to 19 ± 1 mM) and about 22-fold (down to 26 ± 2 mM) respectively (Table 2).

The accumulation of glycine betaine by salt-stressed *B. subtilis* cells also influences the expression level of the osmotically regulated *opuA* operon (Hoffmann *et al*., 2013) and other osmotically controlled genes (Hoffmann and Bremer, 2016). The addition of arsenobetaine to the growth medium downregulated the osmotically induced expression of an *opuA-treA* reporter fusion to a degree similar to that afforded by the addition of glycine betaine (Table 3).

Genetic control of arsenobetaine synthesis through the arsenocholine-responsive repressor GbsR

Choline serves as osmostress protectant for *B. subtilis* but not as a nutrient (Boch *et al*., 1994). It is therefore physiologically fitting to strongly induce the transcription of the genes (*gbsAB*) encoding the enzymes for the two-step oxidation of choline to the metabolically inert glycine betaine molecule (Boch *et al*., 1994; Boch *et al*., 1996) (Fig. 1) and those from the choline-specific OpuB transporter only

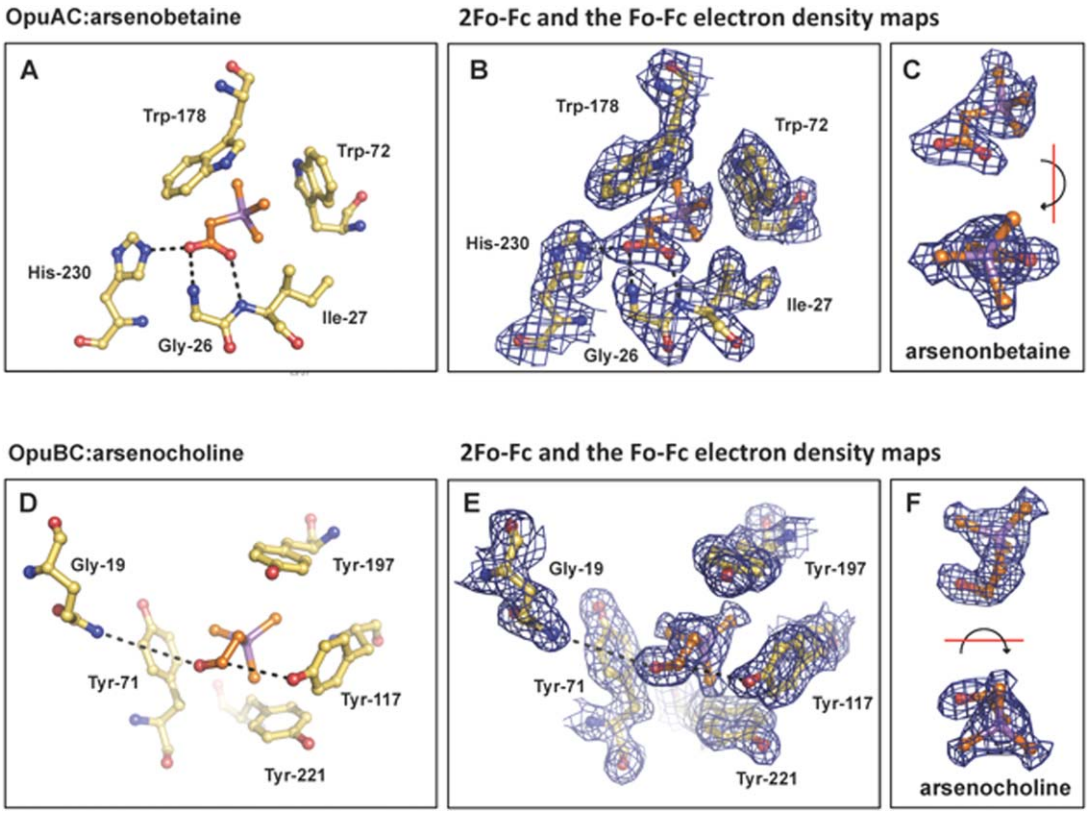

Fig. 5. Representation of the binding-sites of the OpuAC::arsenobetaine and OpuBC::arsenocholine complexes along with the corresponding 2Fo-Fc and the Fo-Fc electron density maps.
A. The OpuAC ligand-binding site in complex with arsenobetaine is shown in a stick representation.
B. The same orientation of the architecture of the substrate-binding site depicted in (A) is shown together with the 2Fo-Fc and the Fo-Fc electron density map contoured at 1 and 2 sigma respectively. For reasons of clarity of the presentation, both in (A) and (B) Trp-225 [a residue part of the aromatic cage; see Fig. 4A)] was omitted from the figures.
C. The arsenobetaine substrate present with the OpuAC ligand-binding site is represented with the same electron density in two different orientations showing the quality of the electron density surrounding the substrate.
D. The OpuAC ligand-binding site in complex with arsenobetaine is shown in a stick representation.
E. The same orientation of the architecture of the substrate-binding site depicted in (D) is shown together with the 2Fo-Fc and the Fo-Fc electron density map contoured at 1 and 2 sigma, respectively (E), and (F) depicts the same electron density in two different orientations of the arsenocholine molecule trapped within the OpuB substrate-binding site.

when choline is present in the growth medium (Nau-Wagner *et al.*, 2012). The MarR-type regulator GbsR serves as a choline-sensing repressor that allows enhanced induction of *opuB* and *gbsAB* expression in the presence of choline but it does not control *opuC* transcription (Nau-Wagner *et al.*, 2012; Ray *et al.*, 2002) as the OpuC transporter possesses a very broad substrate specificity (Hoffmann and Bremer, 2016; 2017; Teichmann *et al.*, 2017). In addition to the involvement of GbsR in controlling the transcription of the choline import and oxidation genes, expression of the *opuB* and *opuC* operons is enhanced by high environmental osmolarity to provide increased choline

Table 2. Influence of glycine betaine and arsenobetaine on the intracellular proline pool.

	Intracellular proline pool (mM)			
Solute present	Without NaCl	With 0.4 M NaCl	With 0.8 M NaCl	With 1.2 M NaCl
None	13 ± 1	223 ± 2	285 ± 14	580 ± 28
Glycine betaine	9 ± 1	12 ± 1	10 ± 1	19 ± 1
Arsenobetaine	9 ± 1	11 ± 1	9 ± 1	36 ± 2

The *B. subtilis* wild type JH642 was grown in minimal medium with the indicated salinities either in the absence or the presence of 1 mM (final concentration) of the indicated compatible solute. The proline content of the cells was measured after the cultures reached exponential growth phase (OD_{578nm} 2). The values given are the means and standard deviations of three independent biological replicates.

Table 3. Influence of glycine betaine and arsenobetaine on *opuAA* promoter activity.

	TreA activity [U (mg protein)$^{-1}$]	
Solute	Without NaCl	With 1.2 M NaCl
Without	37 ± 6	492 ± 34
Glycine betaine	32 ± 1	113 ± 14
Arsenobetaine	25 ± 6	127 ± 4

The *B. subtilis opuA-treA* reporter fusion strain MBB9 was grown in minimal medium with the indicated salinity either in the absence or the presence of 1 mM (final concentration) of glycine betaine or arsenobetaine. After the cultures reached mid-exponential growth phase (OD_{578} of about 1.5), cells were harvested by centrifugation and assayed for TreA reporter enzyme activity. The values given are the means and standard deviations of three independent biological replicates.

uptake capacity to osmotically challenged *B. subtilis* cells (Kappes *et al*., 1999).

Given that arsenocholine serves as the precursor for arsenobetaine synthesis (Fig. 2B), we wondered if arsenocholine would also serve as an inducer for GbsR. To test this idea genetically, we used a GbsR-controlled *gbsA-treA* reporter fusion (Nau-Wagner *et al*., 2012) as a read-out system for the response of this repressor to arsenocholine. The *gbsA-treA* reporter fusion was inserted into the chromosome of either the *B. subtilis* wild-type strain JH642 [*gbsR*$^+$ *gbsAB*$^+$; strain GNB45], its isogenic *gbsR* mutant derivative [(*gbsR::neo*)1 *gbsAB*$^+$; strain GNB48], or a mutant strain that carries a disruption of the *gbsAB* operon [*gbsR*$^+$ Δ(*gbsAB::neo*)2; strain GNB46]. If arsenocholine would function as an inducer for the GbsR repressor, *gbsA-treA* reporter expression should be enhanced by the presence of arsenocholine in the growth medium. In contrast, the responsiveness of this reporter fusion system to arsenocholine should be lost in the *gbsR* mutant strain, and transcription should instead occur at a high level. High-level *gbsA-treA* expression is also expected for the *gbsAB* mutant strain, as the inducer will accumulate in the cells and can no longer be removed from the system through its enzymatic conversion into arsenobetaine (Nau-Wagner *et al*., 2012). The data summarized in Fig. 6A and in Table 4 show that arsenocholine serves indeed as an inducer for the GbsR repressor protein.

To prevent an over-accumulation of imported or newly produced glycine betaine at a given level of osmotic stress (Hoffmann *et al*., 2013), the cellular glycine betaine pool formed through biosynthesis from choline downregulates the expression of the *gbsAB* biosynthetic genes (Nau-Wagner *et al*., 2012). This effect can also be triggered by an external supply of glycine betaine and is dependent on the GbsR regulatory protein (Nau-Wagner *et al*., 2012). We found that this modulating influence of glycine betaine on *gbsA-treA* expression is also exerted by an external supply of arsenobetaine (Fig. 6A and Table 4).

GbsR binds arsenocholine

The genetic data described above show that arsenocholine is a GbsR-dependent inducer of the glycine betaine/arsenobetaine biosynthetic pathway of *B. subtilis* (Fig. 1). Hence, arsenocholine should bind the GbsR protein directly, as has been shown for choline through fluorescence spectroscopic ligand-binding assays with the purified GbsR protein (Nau-Wagner *et al*., 2012). A K_d value of 165 ± 15 μM has previously been determined for the GbsR-choline complex (Nau-Wagner *et al*., 2012). We measured binding of arsenocholine to the purified GbsR protein by fluorescence spectroscopy through the choline and arsenocholine-induced changes in the intrinsic tryptophan fluorescence spectra (Fig. 6B) and determined a K_d of 180 ± 20 μM for choline (Fig. 6C) and a K_d value of 2.1 ± 0.2 mM for arsenocholine (Fig. 6D).

Heat and cold stress protection by arsenobetaine and arsenocholine

In addition to serving as an effective osmostress protectant (Csonka and Hanson, 1991; Kempf and Bremer, 1998; Bremer and Krämer, 2000; Roesser and Müller, 2001; Wood *et al*., 2001), glycine betaine is also an excellent temperature stress protectant for various microorganisms. It bestows cytoprotection to *B. subtilis* cells grown either at the very upper (about 52°C) and lower (about 13°C) boundaries of growth in a chemically defined medium (Holtmann and Bremer, 2004; Hoffmann and Bremer, 2011). Arsenobetaine proved to be a very effective heat and cold stress protectant as well (Fig. 2C and E). Temperature protection assays performed with a set of *B. subtilis* mutants expressing only one of the Opu compatible solute uptake systems revealed that OpuA, and with minor contributions OpuC and OpuD as well, are responsible for the uptake of arsenobetaine under heat- or cold-stress conditions (Supporting Information Fig. S2B,C). The temperature-stress relieving properties of choline and arsenocholine were dependent on the conversion of these compounds into their corresponding betaine derivatives by the GbsBA enzymes. This was ascertained by analysing the growth properties of a strain [JBB5 (Δ(*gbsAB::neo*)] (Boch *et al*., 1996) that is defective in the glycine betaine/arsenobetaine biosynthetic route (Fig. 2D and F).

While no significant difference exists in our growth assays between the osmostress protective potential of choline and arsenocholine (Fig. 2A), arsenocholine is a less efficient temperature stress protectant, a shortfall that becomes particularly notable under heat stress conditions (Fig. 2C and D). Our temperature stress protection assays are conducted at the cutting upper and lower temperature boundaries of the *B. subtilis* growth window in minimal media, conditions under which the cells are already

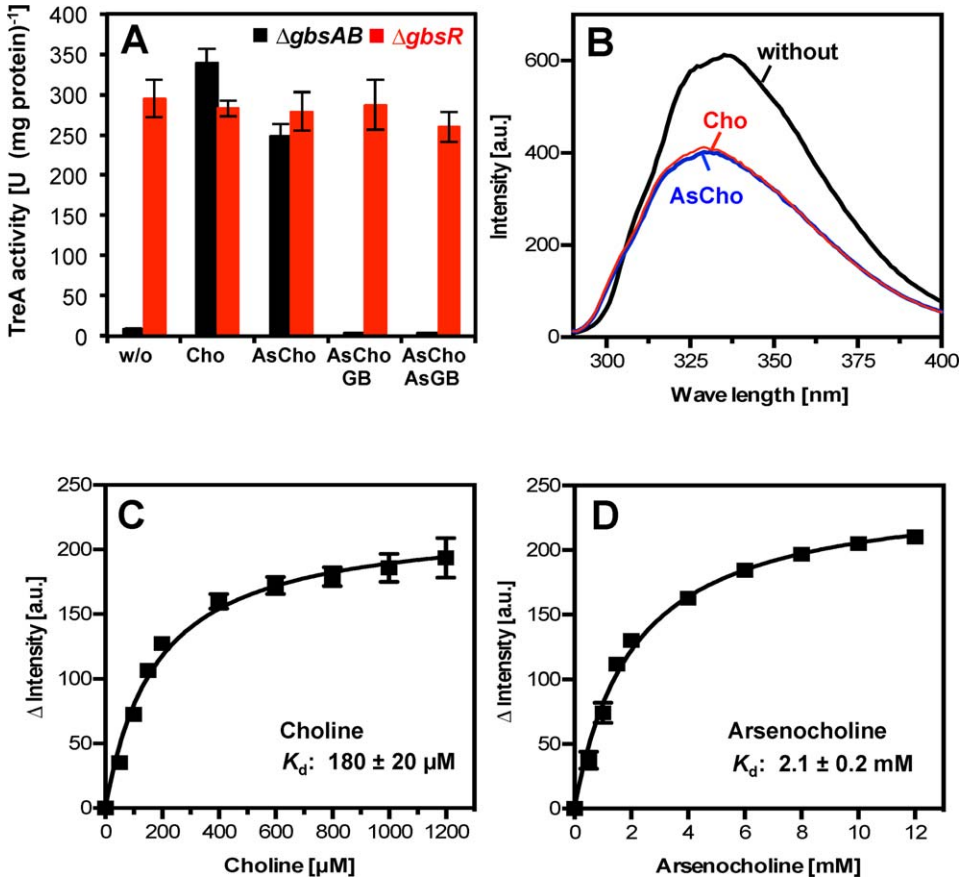

Fig. 6. GbsR-dependent control of the *gbsA* promoter activity in response to choline, arsenocholine, glycine betaine and arsenobetaine and ligand binding assays with the GbsR repressor.
A. The *gbsA-treA* reporter fusion strains GNB46 [Δ(*gbsAB::neo*)2 Φ(*gbsA′-treA*)1; black bars] and GNB48 [(*gbsR::neo*)1 Φ(*gbsA′-treA*)1; red bars] were grown in minimal medium (SMM) in the absence or the presence of 1 mM of the indicated compounds. After the cultures reached early exponential growth phase (OD_{578} 1–1.5), the cells were harvested by centrifugation and assayed for TreA reporter enzyme activity. The values given are the means and standard deviations of four independent biological replicates. The data shown were extracted from a more complex dataset summarized in Table 4.
B. Binding of choline and arsenocholine by the purified GbsR protein. Intensity changes of the intrinsic fluorescence of the GbsR repressor incubated in the absence (black curve), or the presence of 1.2 mM choline (Cho; red curve) or 12 mM arsenocholine (AsCho; blue curve) reflect binding of the compound under study to the GbsR protein. Intensity changes in intrinsic fluorescence in response to the solute concentration were used to determine the affinity of GbsR to its ligands (K_d).
Shown are the Langmuir 1:1 binding curves for GbsR binding to (C) choline and (D) arsenocholine. The values shown are the means and standard deviations derived from two independently purified and assayed GbsR protein batches, that were each measured three times.

tinkering with death (Holtmann and Bremer, 2004; Hoffmann and Bremer, 2011). Hence, the arsenocholine import system OpuB and OpuC (Supporting Information Fig. S2B,C) and/or the GbsBA glycine betaine biosynthetic enzymes (Fig. 1) might not be able to handle their substrate (arsenocholine for GbsB) and the predicted intermediate (arsenobetaine aldehyde for GbsA) (Fig. 1) with the necessary efficiencies in these severely temperature challenged *B. subtilis* cells.

Discussion

We attribute here to the environmentally relevant organoarsenical arsenobetaine (Caumette *et al.*, 2012; Molin *et al.*, 2015; Foster and Maher, 2016; Popowich *et al.*, 2016; Taylor *et al.*, 2017) effective cytoprotective functions against osmotic and temperature stress, define the importers for this compound in *B. subtilis*, assess the pool size of arsenobetaine in osmotically stressed cells, delineate the genetics and physiology of arsenobetaine synthesis from the precursor arsenocholine, and provide crystal structures of extracellular ligand-binding-proteins from two ABC transporters in complex with either arsenobetaine (OpuAC) or arsenocholine (OpuBC). In addition, our data show that arsenobetaine closely matches the physiological properties of glycine betaine not only with respect to osmotic and temperature stress protection (Boch *et al.*, 1994; Holtmann and Bremer, 2004; Hoffmann and Bremer, 2011) but also

Table 4. Influence of arsenocholine and arsenobetaine on *gbsAB*-promoter activity.

Strain	*gbsAB*	*gbsR*	Solute	TreA activity [U (mg protein)$^{-1}$]
GNB45	+	+	Without	9 ± 0
GNB45	+	+	Choline	56 ± 3
GNB45	+	+	Arsenocholine	39 ± 4
GNB45	+	+	Arsenocholine + glycine betaine	4 ± 0
GNB45	+	+	Arsenocholine + arsenobetaine	4 ± 0
GNB46	–	+	Without	9 ± 1
GNB46	–	+	Choline	339 ± 18
GNB46	–	+	Arsenocholine	248 ± 15
GNB46	–	+	Arsenocholine + glycin betaine	4 ± 0
GNB46	–	+	Arsenocholine + arsenobetaine	4 ± 0
GNB48	+	–	Without	295 ± 23
GNB48	+	–	Choline	283 ± 10
GNB48	+	–	Arsenocholine	279 ± 24
GNB48	+	–	Arsenocholine + glycine betaine	287 ± 31
GNB48	+	–	Arsenocholine + arsenobetaine	260 ± 19

The *gbsAB-treA* reporter fusion strains GNB45 (Φ(*gbsA′-treA*)1 *gbsR*$^+$], GNB46 [Δ(*gbsAB*::*neo*)2 Φ(*gbsA′-treA*)1 *gbsR*$^+$] and GNB48 Φ(*gbsA′-treA*)1 [(*gbsR*::*neo*)1] were grown in minimal medium in the absence, or the presence of 1 mM of the indicated solutes. After the cultures reached early exponential growth phase (OD_{578} 1–1.5), the cells were harvested by centrifugation and assayed for TreA reporter enzyme activity. In all strains, the authentic chromosomal *treA* gene is inactivated by a gene disruption mutation [(*treA::neo*)1]. The values given are the means and standard deviations of four independent biological replicates.

with respect to its influence on the pool size of endogenously synthesized compatible solutes (e.g., proline in *B. subtilis*) (Whatmore *et al*., 1990; Brill *et al*., 2011), and its dampening impact on the expression of osmotically induced genes (e.g., *opuA* and *proHJ*) (Brill *et al*., 2011; Hoffmann *et al*., 2013; Hoffmann and Bremer, 2016).

Clues derived from the inspection of the arsenobetaine chemical structure (Fig. 1) has previously inspired speculations about an osmoregulatory function of this glycine betaine homologue in both *Prokarya* and *Eukarya*, but so far only indirect evidence for such a role has been reported [recently summarized by Popowich *et al*. (2016)]. Lack of clarity in this issue, as far as microbes are concerned, stems from the facts that (i) bacteria have only been cursorily studied and (ii) mostly in species in which the systems for glycine betaine uptake and synthesis are not well defined. We circumvented these shortcomings by relying in our analysis on the model system for Gram-positive bacteria, *B. subtilis*, a microorganism where the properties of three osmotically inducible glycine betaine transporters have been intensively characterized and where the systems for the import of the glycine betaine biosynthetic precursor choline and its oxidation to glycine betaine are clearly defined (Hoffmann and Bremer, 2016; 2017). Collectively, the arsenic-homologs are taken up through the same transport systems that are also used by *B. subtilis* to acquire glycine betaine and/or choline from environmental sources (Fig. 1). Our crystallographic data demonstrate that the architectures of the arsenobetaine- and arsenocholine-binding sites in extracellular substrate-binding proteins (OpuAC and OpuBC) of ABC transporters for these osmoprotectants closely match that for their nitrogen-containing counterparts (Figs 4 and 5). The structural data thus show that the molecular determinants allowing the binding of types of organic osmolytes that are otherwise preferentially excluded from protein surfaces (Bolen and Baskakov, 2001; Street *et al*., 2006) are preserved for arsenobetaine and arsenocholine alike (Fig. 4A and B). It follows from our physiological, kinetic and structural analysis that in all likelihood essentially every microbial glycine betaine and choline transport system should be able to accommodate the corresponding arsenic homologue as substrates. Indeed, in a mutant derivative of the OpuD-related BCCT-type glycine betaine transporter BetP from *Corynebacterium glutamicum* (Ressl *et al*., 2009) that can transport choline in a side-reaction with moderate affinity, an arsenocholine binding site has been detected through crystallographic analysis (Perez *et al*., 2014). We note with interest that substantial intracellular arsenobetaine pools have been found in several microorganisms isolated from Mono Lake (California, USA) (Ciulla *et al*., 1997), a hypersaline soda lake that naturally contains high levels of arsenic (Edwardson and Hollibaugh, 2017). In this challenging ecosystem, arsenobetaine is possibly produced by brine shrimp that feed on algae synthesizing arsenosugars and its release into the water column then probably provides osmostress protection to different types of microorganisms (Ciulla *et al*., 1997). Our own data on the pools of arsenobetaine attained through uptake or synthesis from arsenocholine show that this organoarsenical can be amassed to cellular levels closely matching those of glycine betaine (Fig. 3).

We have studied the production of arsenobetaine from the precursor arsenocholine and report for the first time genetic and physiological data that unambiguously prove the existence of a synthesis route in nature that relies on established enzymes for glycine betaine formation from choline (Fig. 1) (Boch *et al*., 1994; Boch *et al*., 1996). In *B. subtilis*, it is genetically controlled via a regulatory circuit that accepts both choline (Nau-Wagner *et al*., 2012) and arsenocholine (this study) as an inducer for the GbsR repressor protein (Fig. 6A and Table 4). Bioinformatics and modelling studies with GbsR, a member of the MarR family of transcriptional regulators, have suggested that it possesses an aromatic ligand-binding cage positioned in the vicinity of a flexible linker region that connects the N-terminal DNA reading head with the C-terminal dimerization domain (Nau-Wagner *et al*., 2012). The architecture of this inducer-binding cage probably resembles that of choline binding sites present in extracellular ligand-binding proteins operating in conjunction with microbial choline ABC transporters (Oswald *et al*., 2008; Du *et al*., 2011; Pittelkow *et al*., 2011) (Supporting Information Fig. S4A,B,C,D). Hence, the accommodation of the trimethylated head-groups of choline and arsenocholine (Fig. 1) within this putative ligand-binding site in GbsR (Supporting Information Fig. S4E,F) via cation-π interactions can readily be envisioned (Nau-Wagner *et al*., 2012). Indeed, the positively charged head-group of arsenocholine is positioned in such an aromatic cage in the OpuBC substrate-binding protein (Fig. 4B). However, our ligand-binding studies (Fig. 6C and D) show that the presumed aromatic cage in GbsR is certainly not optimally configured for arsenocholine.

As observed with choline (Boch *et al*., 1996; Holtmann and Bremer, 2004; Hoffmann and Bremer, 2011), the osmotic and temperature stress-protective properties of arsenocholine are strictly dependent on its enzymatic conversion into arsenobetaine (Fig. 2B, D and F). The ability to synthesize glycine betaine under oxic conditions from choline is found widely in bacteria (Lamark *et al*., 1991; Boch *et al*., 1996; Kempf and Bremer, 1998; Salvi *et al*., 2014) and corresponding enzymes are also present in fungi (Lambou *et al*., 2013). We therefore surmise that biotransformation of arsenocholine into arsenobetaine will very likely occur frequently in the microbial world.

Like glycine betaine (Holtmann and Bremer, 2004; Hoffmann and Bremer, 2011), arsenobetaine serves as a cytoprotectant against extremes in both low and high growth temperatures for *B. subtilis*. While the accumulation of glycine betaine (and by inference arsenobetaine as well) has a major impact on the hydration status of the cytoplasm and magnitude of turgor under osmotic stress conditions (Cayley *et al*., 1992; Kempf and Bremer, 1998; Bremer and Krämer, 2000; Wood, 2011), its cytoprotective effects under temperature stress might primarily stem from its chemical chaperone activity, which *in vivo* ameliorates protein aggregation, and promotes proper protein folding in strained microbial cells (Caldas *et al*., 1999; Bourot *et al*., 2000; Diamant *et al*., 2001; Stadmiller *et al*., 2017). We contend that these effects of glycine betaine will be mirrored, at least to some extent, by arsenobetaine.

It is thought that life on earth has arisen in hydrothermal settings (Weiss *et al*., 2016). Interestingly, arsenobetaine has been detected in marine deep-sea hydrothermal vent ecosystems (Larsen *et al*., 1997; Taylor *et al*., 2012). Hence, arsenobetaine should be able to function as a stress protectant against the detrimental effects of high salinity and high temperature for microbes that populate such extreme habitats in abundance. It might additionally serve as a piezolyte (Martin *et al*., 2002; Oger and Jebbar, 2010), as such a function has already been ascribed to glycine betaine (Smiddy *et al*., 2004). We note, however, that this concept has recently been challenged through *in vitro* studies (Papini *et al*., 2017).

Finally, arsenobetaine enters the human food chain, often in substantial amounts, primarily through the consumption of seafood (Caumette *et al*., 2012; Molin *et al*., 2015; Thomas and Bradham, 2016; Taylor *et al*., 2017). Previous studies have shown that ingested arsenobetaine is rapidly excreted by humans through their urine in a non-altered form (Molin *et al*., 2015; Thomas and Bradham, 2016; Taylor *et al*., 2017), thereby lessening health concerns (Kaise *et al*., 1985). However, one needs to keep in mind that human gut microbiota are metabolically versatile and are capable to chemically alter a broad spectrum of xenobiotics, thereby modifying their biological effects (Koppel *et al*., 2017). Microorganisms with the capacity to hydrolyse arsenic-carbon bonds are well-known (Yoshinaga and Rosen, 2014; Li *et al*., 2016), and the metabolism of arsenobetaine by both marine and soil bacteria has been described (Hanaoka *et al*., 1995; Khokiattiwong *et al*., 2001; Jenkins *et al*., 2003; Devesa *et al*., 2005; Huang *et al*., 2007; Harrington *et al*., 2008). Our comprehensive data on the arsenocholine and arsenobetaine transporters from *B. subtilis* (Table 1 and Fig. 4) indicate that human gut microbiota should be able to import these compounds as transport system for glycine betaine and choline are widely distributed in bacteria (Lucht and Bremer, 1994; Bremer and Krämer, 2000; Wood *et al*., 2001; Ziegler *et al*., 2010; Wood, 2011). Indeed, microorganisms populating the human gastrointestinal tract have been shown to transform arsenobetaine into various methylated arsenic species (Harrington *et al*., 2008), a process that could potentially lead to the formation of toxic metabolites of this organoarsenical (Devesa *et al*., 2005; Huang *et al*., 2007).

Experimental procedures

Bacterial strains

All *B. subtilis* strains used in this study are derivatives of the wild-type strains 168 or JH642; their genotypes are listed in Supporting Information Table S1. Except for strain SOB9, these strains all have been described in former studies. The *gbsAB* gene deletion present in the JH642 derived strain JBB5 [JH642 Δ(*gbsAB*::*neo*)2] (Supporting Information Table S1) was moved via DNA transformation of chromosomal DNA into the *B. subtilis* 168 wild type genetic background by selecting for kanamycin-resistant transformants; this yielded strain SOB9 [*B. subtilis* 168 Δ(*gbsAB*::*neo*)2] (Supporting Information Table S1). Maintenance and genetic manipulation of *B. subtilis* strains followed routine procedures (Harwood and Archibald, 1990). The *Escherichia coli* strain DH5α (Invitrogen, Carlsbad, CA, USA) was used for the construction of plasmids, and for their routine maintenance.

Chemicals, media and growth conditions

Glycine betaine, choline, the chromogenic substrate *para*-nitrophenyl-α-D-glucopyranoside (α-PNPG), and the ninhydrin reagent, were purchased from Sigma-Aldrich (Steinheim, Germany). Arsenobetaine and arsenocholine were obtained from Argus Chemical (Verino, Italy). Radiolabelled [1-^{14}C]glycine betaine (55 mCi mmol^{-1}) was purchased from American Radiolabelled Chemicals Inc. (St. Louis, MO, USA) and [*methyl*-^{14}C]choline chloride (55 mCi mmol^{-1}) was obtained from PerkinElmer LAS GmbH (Rodgau, Germany). Anhydrotetracyline-hydrochloride (AHT), desthiobiotin and Strep-Tactin Superflow chromatography material were purchased from IBA GmbH (Göttingen, Germany). D_4-3-(trimethylsilyl)-propionate and antibiotics were purchased from Carl Roth GmbH (Karlsruhe, Germany). The Factor Xa protease was obtained from Merck (Darmstadt, Germany).

Selective Luria-Bertani (LB) agar plates used for the propagation of plasmid-harbouring bacterial strains contained the antibiotics ampicillin and gentamycin for *E. coli* cells (final concentrations 100 μg ml^{-1} and 20 μg ml^{-1} respectively), or kanamycin (10 μg ml^{-1}) for the genetic construction of *B. subtilis* mutant strains. Wild-type *B. subtilis* cells and their isogenic mutant derivatives were grown in Spizizen's minimal medium (SMM) with 0.5% (wt/vol) glucose as the carbon source, a solution of trace elements (Harwood and Archibald, 1990) and L-tryptophan (20 mg l^{-1}) and L-phenylalanine (18 mg l^{-1}) to satisfy the auxotrophic needs of the *B. subtilis* strains genetically related to JH642 (*trpC*2 *pheA*1) and 168 (*trpC*2) (Supporting Information Table S1). For growth experiments, liquid cultures of *B. subtilis* cells were grown in 100-ml Erlenmeyer flasks containing 20-ml of medium in a water bath with vigorous shaking (220 r.p.m.). Bacterial growth was monitored photometrically as the optical density of cultures at 578 nm (OD_{578}). Precultures were propagated in SMM to mid-exponential growth phase (OD_{578} about 1.5) and were then used to inoculate fresh SMM to an OD_{578} of 0.1. The osmolarity of growth media was increased by the addition of NaCl from 5 M NaCl stock solutions to the final NaCl concentrations indicated with the individual experiment. Cultures that were inoculated for temperature-stress experiments were shifted from precultures grown at 37°C to 13°C (cold stress) or 52°C (heat stress), respectively, following the formerly described temperature shift procedure (Holtmann and Bremer, 2004; Hoffmann and Bremer, 2011; Bashir *et al.*, 2014a). To test the stress protective properties of glycine betaine, arsenobetaine, choline and arsenocholine against the detrimental effects of high osmolarity and high and low extremes in growth temperature, these solutes were added to the *B. subtilis* cultures at a final concentration of 1 mM.

Reporter gene assays

The transcriptional fusions (*gbsAB-treA, opuA-treA*) used in this study rely on the salt-tolerant reporter enzyme TreA [phospho-α-(1,1)-glucosidase], whose activity can be quantified using the chromogenic substrate α-PNPG (Schöck *et al.*, 1996). All *treA* reporter fusion constructs used in this study are present in a single copy stably integrated into the genome of *B. subtilis* strains at the non-essential *amyE* gene via a double homologous recombination event, and they all carry a chromosomal *treA* gene disruption mutation (Supporting Information Table S1). The details of the construction of these reporter fusion strains have been previously reported (Nau-Wagner *et al.*, 2012; Hoffmann *et al.*, 2013). TreA enzyme activities of *B. subtilis* cultures carrying these reporter genes were assayed as described (Hoffmann *et al.*, 2013). One unit of TreA activity is defined as 1 μmol of the colorimetric substrate (α-PNPG) converted by the phospho-α-(1,1)-glucosidase enzyme per min per mg of protein [U (mg protein)$^{-1}$].

Colorimetric quantification of cellular L-proline pools

To measure the intracellular L-proline content of *B. subtilis* cells, 20-ml cultures were grown either in SMM, or in SMM with increased NaCl concentrations until they reached an OD_{578} of about 1.8. In some experiments, 1 mM glycine betaine or arsenobetaine were added to the cultures. The free L-proline content of the cells were quantified with a colorimetric proline assay that detects L-proline as a coloured proline-ninhydrine complex that can be quantified by measuring the absorption of the solution at 480 nm in a photometer. The harvesting and processing of the cells, the details of the assay conditions and the calculation of the intracellular volume of *B. subtilis* cells have been described (Hoffmann *et al.*, 2013). The *B. subtilis* cytoplasmic volume [0.67 μl per 1 OD_{578} unit of cell culture] has previously been calculated from the determination of the internal and total water spaces of cultures incubated with membrane-permeable ^3H_2O and membrane-impermeable inulin-[^{14}C]carboxylic acid after the cells were centrifuged through silicon oil (Bakker and Mangerich, 1981; Holtmann and Bremer, 2004). The same value for the intracellular volume of *B. subtilis* cells [0.67 μl per 1 OD_{578} unit of cell culture] was also used for the calculation of glycine betaine, arsenobetaine, choline and arsenocholine pools.

^{13}C-NMR analysis of arsenobetaine synthesized from arsenocholine and measurements of intracellular arsenobetaine pools

To prepare cell extracts for natural abundance ^{13}C-NMR spectroscopy, cultures (600 ml) of the *B. subtilis* strains JSB8

[Δ(*proHJ*)] (Supporting Information Table S1) were grown overnight in 2-liter Erlenmeyer flasks on a rotary shaker (set to 220 r.p.m.) in SMM that contained 1.2 M NaCl and that had been supplemented with either 1 mM glycine betaine, arsenobetaine, choline or arsenocholine. The cells were grown at 37°C until the cultures reached an OD_{578} between 2 and 2.5, and were then harvested by centrifugation and processed as described before (Bashir *et al.*, 2014b). The dried samples were dissolved in 0.6 ml ^2H_2O together with 3 mg D_4-3-(trimethylsilyl) propionate as the internal standard. ^{13}C-NMR spectra (at 75 MHz) were recorded on a Bruker Avance II 300 MHz spectrometer equipped with a 5 mm BBFO probe. The spectra were processed with the program Topspin 3.1 (Bruker). ^{13}C-NMR tracings of glycine betaine, arsenobetaine, choline, arsenocholine, proline and glutamate were recorded as references to permit the unambiguous identification of the resonances originating from these compounds in the ^{13}C-NMR spectra recorded from the ethanolic cell extracts (Bashir *et al.*, 2014b).

To determine intracellular solute pools of glycine betaine, choline and their arsenic analogues, the *B. subtilis* strain JH642 was grown in SMM or in SMM with 1.2 M NaCl in the presence of either 1 mM glycine betaine, arsenobetaine, choline or arsenocholine until the cultures reached an OD_{578} between 2 and 2.5. The cells were harvested by centrifugation at 37°C, washed once with iso-osmotic prewarmed growth media (at 37°C) lacking the osmostress protectants. Cell extracts were obtained using 80% (vol/vol) ethanol, followed by centrifugation to remove cellular debris. The ethanolic supernatant was stored at −80°C until the measurements of their choline, glycine betaine, arsenocholine and arsenobetaine content. These compounds were detected and quantified using LC-ESI-MS. Cholines and betaines were baseline separated on a weak cation exchanger [C6–150/2.0 (Metrohm AG)] using a 70/30 (vol/vol) mixture of ammonium formiate (100 mM formic acid, pH 3.77 in water) and acetonitrile at a flow rate of 0.2 ml min^{-1}. The detection of these compounds was accomplished using an Agilent MSD G1946D system with an electrospray ionization source and by monitoring of the analyte cations at m/z 104, 118, 165 and 179 in the SIM mode. The cell extracts were diluted 1:100 with ultrapure water and 5 μl injection volumes were used. The calibration was performed using aqueous multi-analyte standards in the range between 1 and 60 mg L^{-1}. All samples were measured in triplicates.

Competition assays of radiolabelled glycine betaine and choline uptake by the corresponding arsenic derivatives

To determine kinetic parameters of different Opu transporters for arsenobetaine and arsenocholine, the uptake of [1-^{14}C]glycine betaine or [*methyl*-^{14}C]choline was measured in the absence or presence of the arsenic-derivatives of these radiolabelled substrates. Cultures of *B. subtilis* mutant strains that express only one of the OpuA, OpuB, OpuC or OpuD uptake systems (Supporting Information Table S1) were grown in SMM at 37°C containing 0.4 M NaCl to an OD_{578} of 0.3 and were then used for the transport assay. The substrate concentrations were varied between 3 μM and 50 μM for the glycine betaine and between 1 μM and 400 μM for the choline uptake assays. The unlabelled compounds were spiked with 1 μM of the radiolabelled substrate used in the particular transport assays. To measure competition of glycine betaine uptake by arsenobetaine, the arsenic-derivative was added to a final concentration of 70 μM. For competition of choline uptake by arsenocholine, this compound was added to a final concentration of 100 μM for uptake via the OpuB transporter, and to final concentrations of 50 μM, 400 μM and 600 μM for uptake experiments involving the OpuC transport system. Uptake assays were conducted at 37°C. The import of [1-^{14}C]glycine betaine or [*methyl*-^{14}C]choline was followed over time, and the amount of radiolabelled substrate that was taken up by the *B. subtilis* cells was determined by scintillation counting as described (Kappes *et al.*, 1996).

Overproduction and purification of recombinant OpuAC and OpuBC proteins

The overexpression of recombinant versions of the *B. subtilis* OpuAC and OpuBC proteins were performed with the *E. coli* strain Arctic express (DE3) RIL (Agilent Technologies, Waldbronn, Germany). For the overproduction of these recombinant proteins, *E. coli* cells carrying the appropriate expression plasmids were grown at 37°C in MMA that was supplemented with the following ingredients: 0.2% casamino acids (wt/vol), 1 mg l^{-1} thiamine and 1 mM $MgSO_4$; it contained 0.5% glucose (wt/vol) as the carbon source and 100 μg ml^{-1} of the antibiotic ampicillin to select for the presence of the overexpression plasmids pMH24 [*opuAC*$^+$] (Amp^R) (Smits *et al.*, 2008) and pMP31 [*opuBC*$^+$] (Amp^R) (Pittelkow *et al.*, 2011) respectively. Plasmids pMH24 and pMP31 were initially introduced into the overproduction strain Arctic express (DE3) RIL by selecting simultaneously for ampicillin and gentamycin resistance to assure the presence of the plasmid that encodes the cold-active chaperone in the recombinant strains (Agilent Technologies). Plasmids pMH24 and pMP31 are derivatives of the overexpression vector pASK-IBA6 (IBA, Göttingen, Germany) and express the *opuAC* and *opuBC* coding regions under the control of the TetR-responsive and AHT-inducible *tet* promoter present in the backbone of the pASK-IBA6 vector. In plasmids pMH24 and pMP31, the coding region (without that for the authentic signal sequence) of the *opuBC* and *opuCC* gene (Kappes *et al.*, 1999), respectively, is inserted into the pASK-IBA6 vector in such a way that it is fused to DNA sequences encoding an OmpA signal sequence for export into the periplasm of *E. coli*, followed by DNA sequences coding for a *Strep*-tag-II affinity peptide and a few amino acids providing a cleavage site for the factor Xa protease. This allows the recovery of the *Strep*-tag-II-OpuAC and *Strep*-tag-II-OpuBC proteins from extracts of the overproducing *E. coli* cells via affinity chromatography on Strep-Tactin Superflow material and the subsequent removal of the affinity peptide through cleavage with the factor Xa protease to yield the mature OpuAC and OpuBC proteins. The details of the growth conditions for the OpuAC and OpuBC overproduction cultures, triggering *opuAC* and *opuBC* over-expression with the inducer AHT, the processing and disruption of the cells in a French pressure cell (at 1000 psi), and the purification and cleavage of the *Strep*-tag II affinity peptide from the recombinant OpuAC and OpuBC proteins have all been described (Smits *et al.*, 2008; Pittelkow *et al.*, 2011).

Overproduction and purification of recombinant GbsR protein and determination of the dissocaiation constant of the GbsR::choline and GbsR::arsenocholine complexes

Overproduction of the *B. subtilis* GbsR repressor protein (Nau-Wagner *et al*., 2012) was carried out in the *E. coli* strain BL21 harbouring plasmid pSTH02. This plasmid is a derivative of the expression vector pASG-IBA3 (IBA, Göttingen, Germany) and is similar in its genetic structure to the previously described *gbsR* overproduction plasmid pDH1 (Nau-Wagner *et al*., 2012), except that the *Strep*-tag II affinity peptide is now positioned at the C-terminal end of the GbsR protein. The recombinant *gbsR* gene in plasmid pSTH02 is expressed under the control of the TetR/AHT-responsive *tet* promoter. Overproduction of the GbsR-*Strep*-tag II protein, its affinity purification on Strep-Tactin Superflow chromatography material, and the further processing of the purified protein followed previously reported procedures (Nau-Wagner *et al*., 2012). The purified GbsR protein was concentrated with VivaSpin 6 columns (Sartorius AG, Göttingen, Germany) to a concentration of about 2 mg ml^{-1} and was maintained in a buffer containing 10 mM Tris-HCl (pH 7.5). The dissociation constant of GbsR for choline and arsenocholine was determined by intrinsic tryptophan fluorescent spectroscopy (Pittelkow *et al*., 2011; Nau-Wagner *et al*., 2012) using a Carry Eclips fluorescense spectrometer (Varian, Surry, United Kingdom). GbsR (5 μM) was titrated with various concentrations of choline (from 0 to 1.25 mM), and arsenocholine (0 to 12.5 mM). In these experiments, the excitation wavelength was set to 280 nm, the slit width was 5 nm, the emission spectrum was recorded from 290 nm to 400 nm, and the photomultiplier-tube (PMT) voltage of the fluorescence detector was set to 800 V. The difference in intrinsic fluorescence intensity caused by ligand binding to the GbsR protein was recorded at 333 nm and used to calculate the apparent K_D (equilibrium dissociation constant) value as described (Pittelkow *et al*., 2011; Nau-Wagner *et al*., 2012).

Crystallization, data collection and structural determination of the OpuAC and OpuBC ligand-binding proteins

Crystals of the OpuAC::arsenobetaine complex were obtained under conditions similar to the ones described for the glycine betaine and proline betaine complexes formed by OpuAC (Horn *et al*., 2006; Smits *et al*., 2008). Prior to crystallization, OpuAC (at a concentration of 12 mg ml^{-1}) was incubated with 5 mM arsenobetaine (dissolved in H_2O). Subsequently, 1 μl of protein solution was mixed with 1 μl of reservoir solution containing 100 mM Tris–HCl (pH 8.25–8.5), 150 mM NH_4OAc and 12%-22% (wt/vol) PEG 4000. Crystal plates appeared at room temperature after several weeks. Crystals were transferred into cryo-buffer (150 mM Tris-HCl (pH 8.3), 20% (wt/vol) ethylene glycol, 200 mM NH_4OAc, 20% (wt/vol) PEG 4000) and flash-frozen in liquid nitrogen.

Crystals of the OpuBC::arsenocholine complex were obtained using the hanging-drop vapor diffusion technique at 12°C. Purified OpuBC was concentrated to 10 mg ml^{-1} and incubated with 15 mM arsenocholine (dissolved in H_2O) prior to crystallization. Protein solution of 1 μl was mixed with 1 μl of reservoir solution containing 160–220 mM potassium acetate and 16%–21% PEG3350. Crystals appeared after 3 days and were harvested after 2 weeks in 200 mM potassium acetate, 20% PEG3350, and 30% ethylene glycol and were flash frozen in liquid nitrogen.

The final datasets of the OpuAC::arsenobetaine and OpuBC::arsenocholine complexes were collected at beamline ID23-eh1 (ESRF, Grenoble, France) and processed using XDS (Kabsch, 1993). Initial phases were obtained by molecular replacement using the program PHASER (McCoy *et al*., 2007) with the crystal structure of the OpuAC::glycine betaine (PDB entry 2B4L) (Horn *et al*., 2006), or OpuBC::-choline (PDB entry 3R6U) (Pittelkow *et al*., 2011) complexes as the search template respectively. Model building and refinement were performed using coot and REFMAC5 (Murshudov *et al*., 1997). Data refinement statistics and model content are summarized in Supporting Information Table S2.

Preparation of figures of crystal structures

Figures of the experimentally determined crystal structure of the OpuAC and OpuBC proteins with their ligands were prepared using the PyMOL software package (hppt://www.pymol.org).

Deposition of crystallographic data

The crystallographic information related to the OpuAC::arsenobetaine and OpuBC::arsenocholine complexes were deposited in the RCSB Protein Data Bank (PDB) (http://www.rcsb.org/pdb/home/home.do) under accession codes 5NXX and 5NXY respectively.

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Conflict of Interest

The authors declare that they have no financial conflict of interest with regard to the data presented in this study.

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Supporting information

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

Fig. S1. Glycine betaine, choline, or their arsenic derivatives cannot be used as sole carbon source by *B. subtilis*. Cells of the wild-type strain JH642 were cultivated in shake flasks containing SMM without a carbon source, or with 28 mM (final concentration) of either glucose (Gluc), glycine betaine (GB), arsenobetaine (AsBet) choline (Cho) or

arsenocholine (AsCho) respectively. Growth yields of the cultures were determined after 12 hours of incubation in a shaking water bath set to 220 r.p.m. at 37°C. The data represent the mean and the standard deviation of three independently grown cultures.

Fig. S2. Genetic identification of the Opu transport systems responsible for the uptake of glycine betaine (GB), arsenobetaine (AsBet), choline (Cho) or arsenocholine (AsCho) under osmotic or temperature stress conditions. (A) Growth yields of cultures grown in the absence (grey bars), or presence of 1.2 M NaCl without (black bars) or with (coloured bars) the addition of 1 mM of the indicated compatible solutes. The growth yields of the cultures were measured after 14 hours of incubation at 37°C. (B) Growth yields of cultures grown at 52°C without (black bars) or with (coloured bars) the addition of 1 mM of the indicated compatible solutes; the growth yields were measured after 16 hours of incubation. (C) Growth yields of cultures grown at 13°C without (black bars) or with (coloured bars) the addition of 1 mM of the indicated compatible solutes measured after seven days of cultivation. The growth yields were measured for a set of *opu* mutants derived either from the *B. subtilis* wild-type strain JH642 (A,B), or from the wild-type strain 168 (C). The used strains express only one of the indicated Opu transporters; all strains possess the osmotically controlled proline-specific uptake system OpuE (von Blohn *et al*., 1997). The values shown are the means and standard deviations of three independently grown cultures.

Fig. S3. Detection of accumulated arsenobetaine and arsenocholine in the cytoplasm of *B. subtilis*. ^{13}C-NMR spectra of ethanolic cell extracts of the *B. subtilis* wild-type strain JH642 and its *proHJ* mutant derivative JSB8. Cells were grown in SMM containing 1.2 M NaCl either without compatible solute (A,B), in the presence of glycine betaine (C), arsenobetaine (D), choline (E), or arsenocholine (F). These solutes were added to the cultures at a final concentration of 1 mM. ^{13}C-NMR spectra of arsenobetaine (G) and arsenocholine (H) were recorded as references. The resonance signals for glycine betaine (GB), arsenobetaine (AB), arsenocholine (AC), or glutamate (g) are indicated. The ^{13}C-NMR spectrum of an extract of the *B. subtilis* wild type strain JH642 grown at 1.2 M NaCl (A) serves as reference for the detection of glutamate (g) and proline (p). Strain JSB8 is a *proHJ* mutant and is therefore unable to synthesize osmostress protective proline pools.

Fig. S4. Architecture of aromatic ligand binding boxes for choline and arsenocholine. Shown are the crystal structures of the ligand binding protein OpuBC of *B. subtilis* in complex with either (A) choline (PDB code 3R6U), or (B) arsenocholine (PDB code 5NXY), and of the ligand binding protein OpuCC of *B. subtilis* (C) (PDB code 3PPQ), and of ChoX from *Sinorhizobium meliloti* (D) (PDB code 2REG) with bound choline respectively. The aromatic residues involved in cation-π interactions with the various ligands are presented as sticks. In (E) and (F), an *in silico* derived model of a monomer of the dimeric GbsR repressor protein from *B. subtilis* is shown. This *in silico* model (Nau-Wagner *et al*., 2012) was obtained through a comparison with the crystal structure of the *Methanococcus jannaschii* Mj223 protein (PDB code 1KU9) (Ray *et al*., 2002). An overview (E) and the central region (F) of the model are shown. Those aromatic amino acid residues that might interact via cation-π interactions with the fully methylated head-groups of either choline or arsenocholine are represented as sticks. This region of the GbsR protein is not present in the *M. janaschii* Mj223 protein whose crystal structure (Ray *et al*., 2002) was used as the template to model the presumed tertiary structure of the *B. subtilis* GbsR protein (Nau-Wagner *et al*., 2012). The winged helix-turn-helix DNA-binding region present in the N-terminal domain of the GbsR repressor is shown in light blue.

Fig. S5. Crystal structures of the solute binding proteins OpuAC and OpuBC. The OpuAC protein structures in complex with either (A) bound glycine betaine (green) or (C) arsenobetaine (orange); an overlay of both structures is shown in (B). The three tryptophan residues forming the aromatic ligand-binding boxes for either glycine betaine or arsenobetaine are presented as sticks. The OpuBC protein structures in complex with either (D) choline (green) or (F) arsenocholine (orange); an overlay of both structures is shown in (E). The four tyrosine residues forming the aromatic ligand-binding boxes for either choline or arsenocholine are presented as sticks. Structural data for these crystal structures are deposited at the RCSB Protein Data Bank (PDB) with the following accession codes: 2B4L (OpuAC::glycine betaine), 5NXX (OpuAC::arsenobetaine), 3R6U (OpuBC::choline) and 5NXY (OpuBC::arsenocholine).

Table S1. Bacterial strains used in this study.

Table S2. Data collection, phasing and refinement statistics for OpuAC::arsenobetaine and OpuBC::arsenocholine complexes.