

## High-Affinity Transport of Choline-*O*-Sulfate and Its Use as a Compatible Solute in *Bacillus subtilis*

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**We report here that the naturally occurring choline ester choline-*O*-sulfate serves as an effective compatible solute for *Bacillus subtilis*, and we have identified a high-affinity ATP-binding cassette (ABC) transport system responsible for its uptake. The osmoprotective effect of this trimethylammonium compound closely matches that of the potent and widely employed osmoprotectant glycine betaine. Growth experiments with a set of *B. subtilis* strains carrying defined mutations in the glycine betaine uptake systems OpuA, OpuC, and OpuD and in the high-affinity choline transporter OpuB revealed that choline-*O*-sulfate was specifically acquired from the environment via OpuC. Competition experiments demonstrated that choline-*O*-sulfate functioned as an effective competitive inhibitor for OpuC-mediated glycine betaine uptake, with a  $K_i$  of approximately 4  $\mu$ M. Uptake studies with [1,2-dimethyl-<sup>14</sup>C]choline-*O*-sulfate showed that its transport was stimulated by high osmolality, and kinetic analysis revealed that OpuC has high affinity for choline-*O*-sulfate, with a  $K_m$  value of  $4 \pm 1 \mu$ M and a maximum rate of transport ( $V_{max}$ ) of  $54 \pm 3$  nmol/min · mg of protein in cells grown in minimal medium with 0.4 M NaCl. Growth studies utilizing a *B. subtilis* mutant defective in the choline to glycine betaine synthesis pathway and natural abundance <sup>13</sup>C nuclear magnetic resonance spectroscopy of whole-cell extracts from the wild-type strain demonstrated that choline-*O*-sulfate was accumulated in the cytoplasm and was not hydrolyzed to choline by *B. subtilis*. In contrast, the osmoprotective effect of acetylcholine for *B. subtilis* is dependent on its biotransformation into glycine betaine. Choline-*O*-sulfate was not used as the sole carbon, nitrogen, or sulfur source, and our findings thus characterize this choline ester as an effective compatible solute and metabolically inert stress compound for *B. subtilis*. OpuC mediates the efficient transport not only of glycine betaine and choline-*O*-sulfate but also of carnitine, crotonobetaine, and  $\gamma$ -butyrobetaine (R. Kappes and E. Bremer, *Microbiology* 144:83–90, 1998). Thus, our data underscore its crucial role in the acquisition of a variety of osmoprotectants from the environment by *B. subtilis*.**

Rapid fluxes of water along the osmotic gradient are triggered by the exposure of microorganisms to high osmolality environments, which in turn imposes considerable strain on all the physiological processes of the cell (57). Microorganisms actively respond to such increases in the osmolality of their habitat to prevent dehydration of the cytoplasm and to maintain turgor within acceptable boundaries. Since bacteria do not possess active transport mechanisms for water, turgor is adjusted by controlling the pool of osmotically active solutes within the cell (12, 15, 34, 45, 54).

Most nonhalophilic bacteria do not use ions to permanently increase the osmotic potential of their cytoplasm when they are faced by high osmotic growth conditions (15, 67). Instead, large amounts of organic osmolytes, the so-called compatible solutes, are accumulated. These compounds are well tolerated by enzymes and cell components and are thus highly congruous with the entire cellular physiology, even when they are amassed at molar concentrations (72). The intracellular accumulation of compatible solutes as a strategy of adaption to high osmolality has been widely adopted not only by *Bacteria* and *Archaea*

but also by fungal, plant, animal, and even human cells (4, 8, 39, 42, 58) and is thus a remarkable example of convergent evolution. Moreover, the nature of the organic osmolytes that are amassed during water stress is maintained across the kingdoms, reflecting fundamental constraints on the kinds of solutes that are compatible with macromolecular and cellular functions (39, 72).

One of the most effective and widely used osmoprotectants in both the prokaryotic and eukaryotic world is the trimethylammonium compound glycine betaine (12, 15, 58). It can be amassed by microorganisms through either synthesis or uptake, and its intracellular accumulation confers a considerable degree of osmotic tolerance. A few bacteria produce this compatible solute by a stepwise methylation of glycine (15, 60). However, in most bacterial species glycine betaine synthesis proceeds via a two-step enzymatic oxidation of choline with glycine betaine aldehyde as the intermediate (6, 38, 61). Direct glycine betaine uptake from the environment has been observed in a wide variety of gram-negative and gram-positive *Bacteria* (12, 34, 45, 54) and a few *Archaea* (56). Molecular analysis of glycine betaine uptake systems initially focused on the gram-negative enterobacteria *Escherichia coli* and *Salmonella typhimurium*, for which two osmoregulated high-affinity transporters have been characterized in detail. These are the secondary transporter ProP and the binding-protein-dependent ATP-binding cassette (ABC) transporter ProU (11).

Recently, the genetics and physiology of the osmotic stress response in various gram-positive microorganisms have attracted wider attention (3, 16, 29, 52, 55, 64, 68). *Bacillus subtilis* is a ubiquitous inhabitant of the soil and is exposed to frequent osmotic changes due to flooding and desiccation of the upper

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layers of its habitat (45). Upon exposure to high-osmolality conditions, this bacterium produces large amounts of proline via de novo synthesis as an endogenous osmoprotective response (70). In addition, *B. subtilis* makes considerable effort to acquire compatible solutes or their precursors from the environment, and five osmoregulated transport systems have so far been characterized at the molecular level (34). OpuE (Opu denotes osmoprotectant uptake), a member of the sodium/solute symporter family, is used to efficiently scavenge proline from the habitat for osmoprotective purposes (69). In addition, three systems serve as effective glycine betaine transporters: OpuA, OpuC, and OpuD (31, 33, 35). OpuA and OpuC are multicomponent systems and are members of the ABC superfamily of transporters; both are related to the ProU transporter from *E. coli*. The secondary transporter OpuD is a representative of a new class of glycine betaine uptake systems (31) which also comprises BetP from the gram-positive soil bacterium *Corynebacterium glutamicum* (52). Acquisition of choline for the synthesis of glycine betaine (5, 6) is accomplished by both OpuC and the structurally closely related, substrate-specific ABC transporter OpuB (32). The OpuC system also serves for the high-affinity uptake of the betaines L-carnitine, crotonobetaine, and  $\gamma$ -butyrobetaine, all highly effective osmoprotectants for *B. subtilis* (30), and with low affinity (28), for the uptake of the frequently used osmoprotectant ectoine (15, 67). Hence, like other microorganisms, *B. subtilis* can rely on a spectrum of exogenously provided compatible solutes for osmoprotective purposes.

The choline ester choline-*O*-sulfate (Fig. 1) is widespread in nature and plays an important role in the microbial transformation of sulfur in the soil (1, 41). It is synthesized by a variety of plants, lichens, algae, and fungi and by several bacterial species (10, 14, 20–24, 26). Its formation is catalyzed by sulfur transferases that use 3'-phosphoadenosine-5'-phosphosulfate and choline as their substrates (59). Production of choline-*O*-sulfate probably serves in the detoxification of  $\text{SO}_4^{2-}$ , and this ester can also function as a source of choline and sulfur after hydrolysis by choline sulfatases (41). An additional role for choline-*O*-sulfate as an osmoprotective compound was suggested by the observation that the intracellular pool of this betaine increased in various taxa of halophytic plants subjected to salt stress (21, 22, 59). Indeed, in bioassays choline-*O*-sulfate proved to be osmoprotective both for several gram-negative bacterial species (9, 19, 21, 44) and for the osmotolerant and xerotolerant fungus *Penicillium fellutanum* (51). However, the wider function of this choline ester as an effective osmoprotectant for the microbial world and its mechanisms for uptake from the environment and its intracellular accumulation either as a compatible solute or as a precursor for glycine betaine synthesis (50) have remained largely unexplored. Here, we report on the identification of a high-affinity choline-*O*-sulfate transport system at the molecular level in *B. subtilis* and on the role of this betaine as an effective compatible solute for this soil bacterium.

#### MATERIALS AND METHODS

**Bacterial strains.** Strain JH642 (*trpC2 pheA1*; BGSC 1A96; gift from J. Hoch) is a derivative of the wild-type *B. subtilis* strain 168 and is the parent of all mutants used in the study. The construction and properties of strains RMKB20 [ $\Delta(\text{opuA}::\text{erm})4 \text{ opuC-20}::\text{Tn10}(\text{spc}) \Delta(\text{opuD}::\text{neo})2$ ], RMKB22 [ $\Delta(\text{opuA}::\text{erm})4 \text{ opuB-20}::\text{Tn10}(\text{spc}) \Delta(\text{opuD}::\text{neo})2$ ], RMKB25 [ $\text{opuC-20}::\text{Tn10}(\text{spc})$ ], RMKB33 [ $\Delta(\text{opuA}::\text{erm})4 \Delta(\text{opuB}::\text{tet})23 \text{ opuC-20}::\text{Tn10}(\text{spc})$ ], and RMKB34 [ $\Delta(\text{opuB}::\text{tet})23 \text{ opuC-20}::\text{Tn10}(\text{spc}) \Delta(\text{opuD}::\text{neo})2$ ] have been described (30). Strain JBB5 [ $\Delta(\text{gbsAB}::\text{neo})2$ ] is defective in glycine betaine synthesis since it lacks the GbsA and GbsB enzymes (6).

**Growth conditions, media, and chemicals.** The *B. subtilis* strains were grown in Spizizen's minimal medium (SMM) with 0.5% (wt/vol) glucose as the carbon source and supplemented with L-tryptophan (20 mg/liter), L-phenylalanine (18

mg/liter), and a solution of trace elements (25). When it was used as the sole carbon source, choline-*O*-sulfate was added to SMM to a final concentration of 30 mM and the growth yield of the cultures was monitored spectrophotometrically (optical density at 578 nm [ $\text{OD}_{578}$ ]) after 24 h. Use by *B. subtilis* of choline-*O*-sulfate as the sole sulfur source was tested by substituting  $(\text{NH}_4)_2\text{SO}_4$  with  $\text{NH}_4\text{Cl}$  and substituting  $\text{MgSO}_4$  with  $\text{MgCl}_2$  in the SMM salts. The  $(\text{NH}_4)_2\text{SO}_4$  present in SMM was substituted with 30 mM  $\text{K}_2\text{SO}_4$  to test the use of choline-*O*-sulfate as the sole nitrogen source by *B. subtilis* JH642. The cultures used to probe the function of choline-*O*-sulfate as the sole carbon, nitrogen, or sulfur source were inoculated to an  $\text{OD}_{578}$  of approximately 0.05 from SMM-grown overnight cultures which had been carefully washed with the modified SMM to remove residual traces of  $(\text{NH}_4)_2\text{SO}_4$ ,  $\text{MgSO}_4$ , and glucose from the inoculum. The osmotic strength of SMM was increased by the addition of NaCl from stock solutions. The osmolality values of these media were determined with a vapor pressure osmometer (model 5500; Wescor); the osmolality values of SMM, SMM with 0.4 M NaCl, and SMM with 1.2 M NaCl were 340, 1,100, and 2,700 mosmol/kg of water, respectively. Growth conditions for *B. subtilis* cells cultured in SMM and SMM with increased osmolality (1.2 M NaCl) and testing for the osmoprotective effects of various compounds were as described previously (30). [1-*methyl*- $^{14}\text{C}$ ]glycine betaine (2.03 GBq/mmol) was custom synthesized by American Radiolabeled Chemicals Inc. (St. Louis, Mo.). [1,2-*dimethyl*- $^{14}\text{C}$ ]choline-*O*-sulfate was prepared from [1,2-*dimethyl*- $^{14}\text{C}$ ]choline (259 MBq/mmol) (New England Nuclear, Boston, Mass.) by the protocol of Stevens and Vohra (63) and was a kind gift from D. Le Rudulier and M. Østerås (Université de Nice, France) (50). [*methyl*- $^3\text{H}$ ]acetylcholine (3.15 TBq/mmol) was obtained from American Radiolabeled Chemicals Inc. Choline, glycine betaine, acetylcholine, and phosphorylcholine were purchased from Sigma (Steinheim, Germany).  $^2\text{H}_2\text{O}$  containing  $\text{D}_4$ -3-(trimethylsilyl)propionate was obtained from Aldrich (Deisenhofen, Germany).

**Synthesis of choline-*O*-sulfate.** Nonradiolabeled choline-*O*-sulfate was synthesized by heating 2 moles of choline chloride with an excess (5.2 mol) of concentrated  $\text{H}_2\text{SO}_4$  at 95°C overnight. The ester produced was then precipitated by adding the reaction mixture (500 ml) to four liters of 95% ethanol. The precipitated choline-*O*-sulfate was collected by filtration and was recrystallized from 60% ethanol as described by Bellenger et al. (2). Production of choline-*O*-sulfate was confirmed by  $^1\text{H}$  nuclear magnetic resonance (NMR) spectroscopy (10) by using a Bruker AC300 spectrometer operating at 300 MHz. The purity of choline-*O*-sulfate was checked by elementary analysis in a CHN-Rapid apparatus from Heraeus (Osterode, Germany) by the analytical service laboratory of the Chemistry Department of the Philipps University of Marburg, Germany.

**Transport assays.** The uptake of [1,2-*dimethyl*- $^{14}\text{C}$ ]choline-*O*-sulfate was measured at 37°C at various final substrate concentrations in bacterial cultures grown to log phase ( $\text{OD}_{578} = 0.3$  to 0.5) in minimal media of high osmolality (SMM with 0.4 M NaCl) with glucose as the carbon source, as detailed previously for the uptake of radiolabeled glycine betaine (31, 33). The choline-*O*-sulfate concentration in the uptake assay was varied from 1 to 40  $\mu\text{M}$  for kinetic studies. To determine the degree of inhibition of glycine betaine transport via the OpuC system by choline-*O*-sulfate, the concentration of the unlabeled choline-*O*-sulfate was kept at 50  $\mu\text{M}$  and the substrate concentration of the radiolabeled [1-*methyl*- $^{14}\text{C}$ ]glycine betaine was varied from 1 to 40  $\mu\text{M}$ .

**Preparation of cell extracts for  $^{13}\text{C}$ -NMR spectroscopy.** Cultures (300 ml) of different strains (JH642 and JBB5) were grown overnight in 1-liter Erlenmeyer flasks at 37°C on a rotary shaker (220 rpm) in SMM or SMM with 1.2 M NaCl and 0.5% (wt/vol) glucose as the carbon source in the absence or presence of choline-*O*-sulfates; these stationary-phase cultures reached  $\text{OD}_{578}$  between 2.5 and 3. The cells were harvested by centrifugation and washed once with 150 ml of SMM containing 1.2 M NaCl but no glucose, and the cell pellet was extracted with 10 ml of 80% (vol/vol) ethanol. The cellular debris was removed by centrifugation, and the supernatant was evaporated to dryness; ethanolic cell extracts from two 300-ml cultures were then dissolved in 1 ml of  $^2\text{H}_2\text{O}$  supplemented with 1.2 mg of  $\text{D}_4$ -3-(trimethylsilyl)propionate as a standard.  $^{13}\text{C}$ -NMR spectra were recorded with a Bruker AC300 spectrometer operating at 75 MHz (65).  $^{13}\text{C}$ -NMR tracings from choline-*O*-sulfate, glycine betaine, choline, and acetylcholine relative to  $\text{D}_4$ -3-(trimethylsilyl)propionate were recorded to identify unambiguously resonances from these compounds present in the  $^{13}\text{C}$ -NMR spectra from the ethanolic cell extracts.

#### RESULTS

**Choline-*O*-sulfate functions as an osmoprotectant.** To examine a possible osmoprotective effect of choline-*O*-sulfate (Fig. 1) for the proliferation of osmotically stressed *B. subtilis* cells, we grew the wild-type strain JH642 in SMM (340 mosmol/kg) and high-osmolality minimal medium (SMM with 1.2 M NaCl; 2,700 mosmol/kg) in the absence or presence of 1 mM choline-*O*-sulfate. High osmolality strongly impaired the growth of the cultures, and choline-*O*-sulfate largely reversed this growth inhibition (Fig. 2A). A concentration as low as 20  $\mu\text{M}$  of choline-*O*-sulfate was sufficient to exert a notable os-

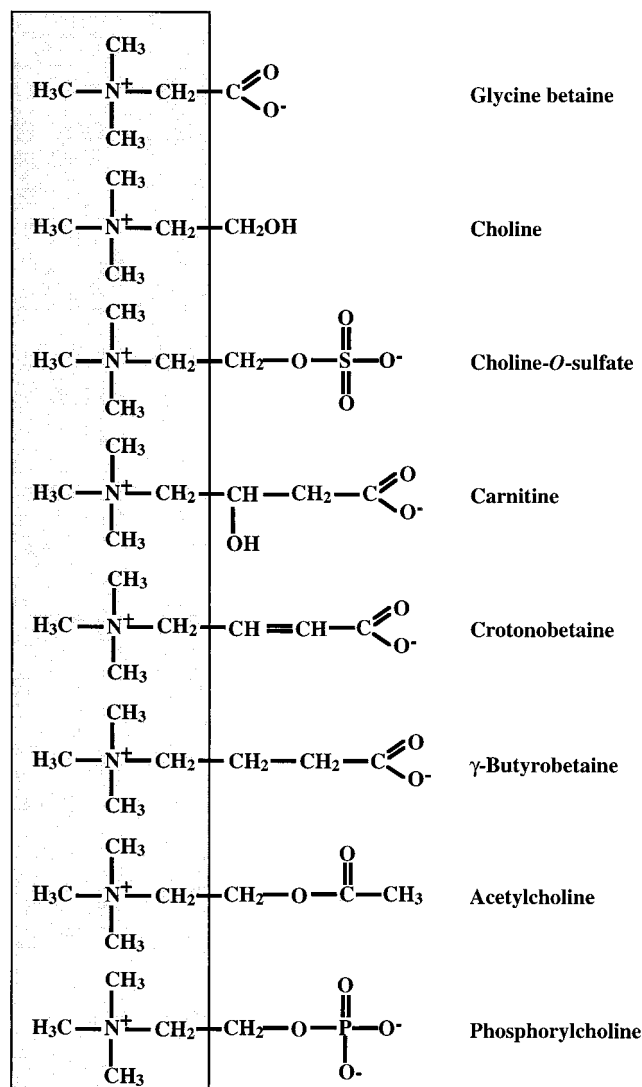


FIG. 1. Osmoprotective compounds for *B. subtilis* and chemically related substances.

moprotective effect on the growth of *B. subtilis* in the high-osmolality medium, and approximately 100 to 200  $\mu\text{M}$  provided full osmoprotection (Fig. 3A). The osmoprotective effects of choline-*O*-sulfate thus closely match those of the potent osmoprotectant glycine betaine and its biosynthetic precursor choline (Fig. 2 and 3) (5, 31). These data establish that choline-*O*-sulfate can serve as an effective osmoprotectant for *B. subtilis*.

#### Identification of the choline-*O*-sulfate transport system.

Bacteria frequently employ multiple transport systems with different substrate specificities to acquire compatible solutes from the environment (11, 34). We therefore investigated the involvement of the known *B. subtilis* transporters for glycine betaine (OpuA, OpuC, and OpuD) (30, 33) or its biosynthetic precursor choline (OpuB and OpuC) (32) in the uptake of choline-*O*-sulfate from the environment. In a preliminary growth experiment, we used an isogenic set of mutants expressing only one of these uptake systems (30). This survey revealed that only *B. subtilis* strains synthesizing the OpuC transporter (31) could use choline-*O*-sulfate as an osmoprotectant. OpuC

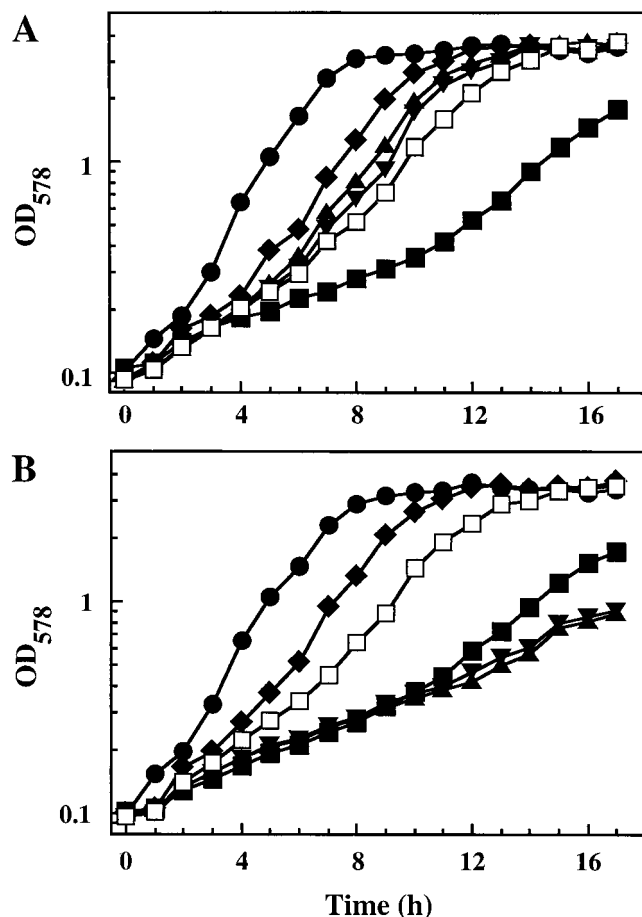


FIG. 2. Osmoprotective effects of choline-*O*-sulfate and acetylcholine for *B. subtilis*. The wild-type strain JH642 (A) and its  $\Delta(\textit{gbsAB}::\textit{neo})2$  derivative JBBS (B) were grown in SMM (●) and SMM with 1.2 M NaCl in the absence (■) or presence of 1 mM glycine betaine (◆), choline (▲), acetylcholine (▼), or choline-*O*-sulfate (□). Cultures (75 ml) were inoculated to an  $\text{OD}_{578}$  of 0.1 from overnight cultures pregrown in SMM and were propagated in 500-ml Erlenmeyer flasks in a shaking water bath (220 rpm) at 37°C. Cell growth was monitored over time by measuring the  $\text{OD}_{578}$ .

exhibits the characteristic features of a bacterial binding-protein-dependent transport system (7) and is a member of the ABC superfamily of transporters. It consists of an evolutionarily highly conserved ATPase (OpuCA), two sequence-related integral cytoplasmic membrane proteins (OpuCB and OpuCD), and a hydrophilic lipoprotein (OpuCC). This latter polypeptide is likely to serve as an extracellular substrate-binding protein tethered by its N-terminal lipid modification to the cytoplasmic membrane (32).

Disruption of the OpuC transporter in an otherwise wild-type background completely abolished osmoprotection by choline-*O*-sulfate (Fig. 3B). In contrast, this mutant strain was still fully protected by glycine betaine (Fig. 3B), which can be accumulated not only by OpuC but also via the high-affinity ABC transporter OpuA (33) and the secondary uptake system OpuD (31). These growth experiments unequivocally demonstrate that *B. subtilis* possesses only one choline-*O*-sulfate uptake system and establish that the multicomponent ABC transporter OpuC mediates the uptake of this betaine from exogenous sources.

**Competitive inhibition of glycine betaine transport by choline-*O*-sulfate.** Transport of radiolabeled glycine betaine via

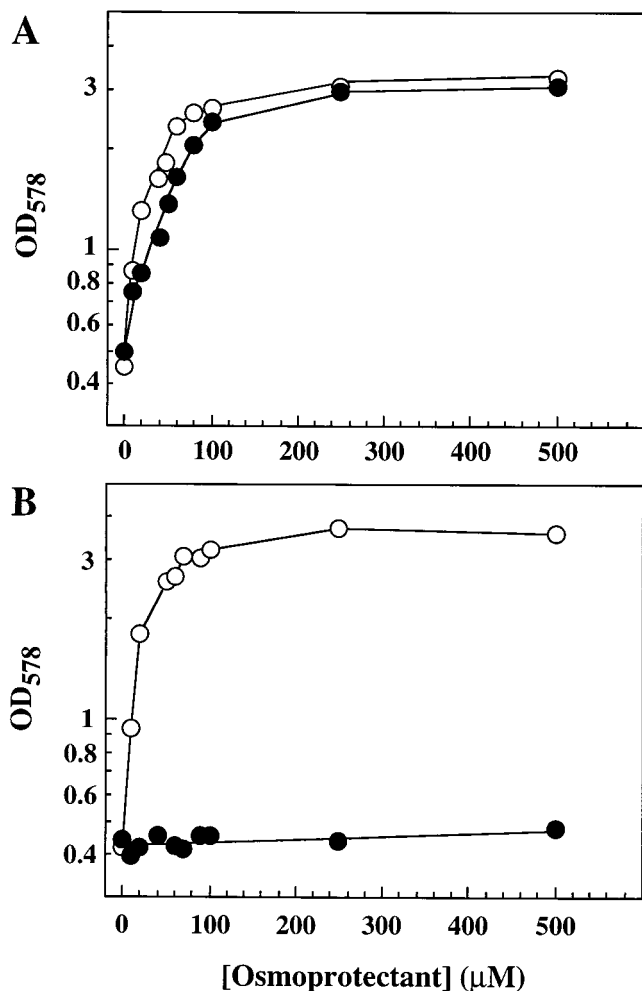


FIG. 3. Uptake of choline-*O*-sulfate is mediated by the ABC transport system OpuC. Strains RMKB22 (OpuA<sup>-</sup> OpuB<sup>-</sup> OpuC<sup>+</sup> OpuD<sup>-</sup>) (A) and RMKB25 (OpuA<sup>+</sup> OpuB<sup>+</sup> OpuC<sup>-</sup> OpuD<sup>+</sup>) (B) were grown in high osmolality minimal medium (SMM with 1.2 M NaCl) in the presence of various concentrations of glycine betaine (○) and choline-*O*-sulfate (●). The cells were inoculated to an OD<sub>578</sub> of 0.05 from an overnight SMM culture and were grown in 20 ml of medium in a 100-ml Erlenmeyer flask on an orbital shaker (220 rpm) at 37°C. The growth yield of each culture was determined spectrophotometrically by measuring the OD<sub>578</sub> after 22-h incubation. Under these circumstances, there is essentially no cell growth of strains inoculated in SMM with 1.2 M NaCl in the absence of an osmoprotectant, in contrast to cells grown in 75-ml cultures in 500-ml Erlenmeyer flasks, conditions that provide a much better aeration for the cells (30).

OpuC is moderately osmotically stimulated and exhibits a  $K_m$  of 6  $\mu\text{M}$  and a  $V_{\text{max}}$  of 65 nmol/min · mg of protein in cultures grown in SMM in the presence of 0.4 M NaCl (31). To analyze the entry of choline-*O*-sulfate into the *B. subtilis* cells through the OpuC system, we performed competition experiments with [1-*methyl*-<sup>14</sup>C]glycine betaine and unlabeled choline-*O*-sulfate. We grew strain RMKB22 (OpuA<sup>-</sup> OpuB<sup>-</sup> OpuC<sup>+</sup> OpuD<sup>-</sup>) in SMM with 0.4 M NaCl to mid-exponential phase and measured the uptake of [1-*methyl*-<sup>14</sup>C]glycine betaine over a range (1 to 40  $\mu\text{M}$ ) of substrate concentrations in either the absence or the presence of a fixed concentration (50  $\mu\text{M}$ ) of the competitor choline-*O*-sulfate. In close agreement with the previously reported data (31) we found a  $K_m$  of 2.8  $\mu\text{M}$  for glycine betaine uptake in the absence of choline-*O*-sulfate, and an apparent  $K_m$  of 34  $\mu\text{M}$  in its presence (Fig. 4A). From these

kinetic data we calculated a  $K_i$  value of  $4.5 \pm 1 \mu\text{M}$ . The lines in the Hanes plot were linear and parallel and yielded very similar  $V_{\text{max}}$  values for [1-*methyl*-<sup>14</sup>C]glycine betaine uptake in the absence ( $V_{\text{max}}$  was 89 nmol/min · mg of protein) or presence ( $V_{\text{max}}$  was 92 nmol/min · mg of protein) (Fig. 4A) of the inhibitor. These transport characteristics indicated that choline-*O*-sulfate behaves as a competitive inhibitor (71) for glycine betaine uptake via OpuC and that this ABC transporter serves as a high-affinity uptake system for this osmoprotectant.

**Kinetic parameters for OpuC-mediated choline-*O*-sulfate transport.** To determine the parameters for choline-*O*-sulfate transport directly, we measured the initial uptake of [1,2-*dimethyl*-<sup>14</sup>C]choline-*O*-sulfate at a final substrate concentration of 10  $\mu\text{M}$  in the wild-type strain JH642 in cultures grown in SMM or SMM with elevated osmolality (SMM with 0.4 M NaCl). Like the OpuC-mediated transport of glycine betaine and L-carnitine (30, 31), choline-*O*-sulfate uptake was moderately stimulated (three- to fourfold) by increases in the osmolality of the growth medium (Fig. 4B). Disruption of the OpuC system in an otherwise wild-type background by the *opuC-20::Tn10(spc)* mutation (strain RMKB25) completely abolished radiolabeled choline-*O*-sulfate transport at both low and high osmolality (Fig. 4B). This observation corroborates the finding that osmoprotection by choline-*O*-sulfate is eliminated in an OpuC-lacking strain that still possesses the OpuA, OpuB, OpuD, and OpuE osmoprotectant uptake systems (Fig. 3B).

We then determined the initial velocities of [1,2-*dimethyl*-<sup>14</sup>C]choline-*O*-sulfate uptake over a range of substrate concentrations (1 to 40  $\mu\text{M}$ ) in cells of strain JH642 grown in SMM with 0.4 M NaCl. Choline-*O*-sulfate uptake was saturable and showed typical Michaelis-Menten kinetics. Transformation of the transport data according to the Lineweaver-Burk method suggested that there is a single, high-affinity choline-*O*-sulfate transport activity in *B. subtilis*. This uptake system exhibits a  $K_m$  of  $4 \pm 1 \mu\text{M}$  and a  $V_{\text{max}}$  of  $54 \pm 3$  nmol/min · mg of protein (Fig. 4C). These kinetic parameters for choline-*O*-sulfate transport are comparable to those for the OpuC-mediated glycine betaine ( $K_m = 6 \mu\text{M}$ ;  $V_{\text{max}} = 65$  nmol/min · mg of protein) and L-carnitine ( $K_m = 5.8 \mu\text{M}$ ;  $V_{\text{max}} = 71.5$  nmol/min · mg of protein) transport (30, 31).

**Osmoprotective effects of choline-*O*-sulfate are not dependent on its enzymatic conversion into glycine betaine.** Choline-*O*-sulfate is a derivative of choline (Fig. 1), and hydrolysis of the ester bond would liberate the precursor for glycine betaine biosynthesis (5). Consequently, the osmoprotective effects of choline-*O*-sulfate for *B. subtilis* (Fig. 2A and 3A) could be indirect and dependent on its enzymatic conversion to glycine betaine. To determine whether choline-*O*-sulfate acted as a compatible solute per se or whether its osmoprotective effects resulted from its biotransformation into glycine betaine, we tested its osmoprotective capacity in strain JBB5 [ $\Delta(gbsAB::neo)2$ ]. This mutant cannot synthesize glycine betaine from choline because it lacks the choline dehydrogenase (GbsB) and the glycine betaine aldehyde dehydrogenase (GbsA) (6). Unlike choline, both choline-*O*-sulfate and glycine betaine were fully osmoprotective for strain JBB5 (Fig. 2B). Hence, osmoprotection of *B. subtilis* by choline-*O*-sulfate clearly does not depend on its conversion into glycine betaine.

**Osmoprotection by acetylcholine requires its conversion into glycine betaine.** We also observed that the choline ester acetylcholine (Fig. 1) protected *B. subtilis* cells from the detrimental effects of high osmolality with an efficiency similar to those of choline and choline-*O*-sulfate (Fig. 2A). Acetylcholine is found widely in plants and thus could reach soil bacteria through decaying plant material. However, its concentration in

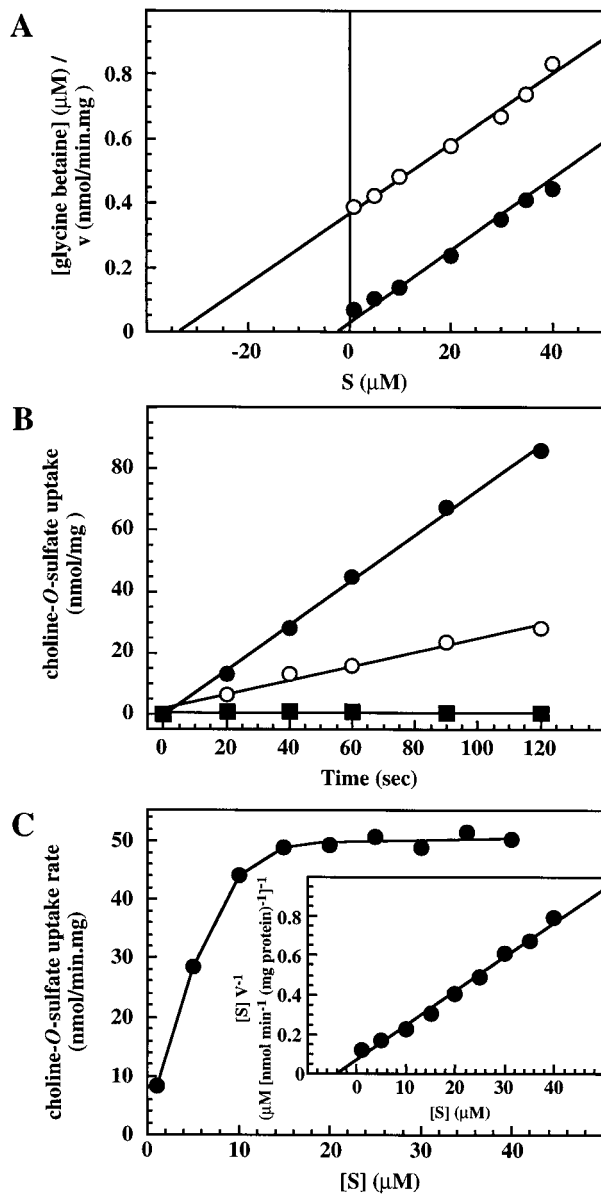


FIG. 4. Transport of choline-*O*-sulfate in *B. subtilis*. (A) Inhibition of radiolabeled glycine betaine by choline-*O*-sulfate in strain RMKB22 (OpuA<sup>-</sup> OpuB<sup>-</sup> OpuC<sup>+</sup> OpuD<sup>-</sup>). [1-*methyl*-<sup>14</sup>C]glycine betaine uptake was measured at various substrate concentrations in cells grown in SMM with 0.4 M NaCl in the absence (●) or the presence (○) of 50  $\mu\text{M}$  unlabeled choline-*O*-sulfate. In the absence of the inhibitor the apparent  $K_m$  of glycine betaine transport via OpuC is 2.8  $\mu\text{M}$  and the  $V_{max}$  is 89 nmol/min · mg of protein. In the presence of choline-*O*-sulfate the apparent  $K_m$  of glycine betaine transport is 34  $\mu\text{M}$ , the  $V_{max}$  is 92 nmol/min · mg of protein, and the  $K_i$  is 4.5  $\mu\text{M}$ . (B) Osmotically stimulated choline-*O*-sulfate transport. Cells of the wild-type strain JH642 (OpuA<sup>+</sup> OpuB<sup>+</sup> OpuC<sup>+</sup> OpuD<sup>+</sup>) (○, ●) and its OpuC<sup>-</sup> derivative RMKB25 (■) were grown in SMM (○) or SMM with 0.4 M NaCl (●, ■) and [1,2-*dimethyl*-<sup>14</sup>C]choline-*O*-sulfate uptake was assayed at a final substrate concentration of 10  $\mu\text{M}$ . (C) Kinetics of choline-*O*-sulfate transport. Strain JH642 was grown in SMM with 0.4 M NaCl, and the initial velocities of [1,2-*dimethyl*-<sup>14</sup>C]choline-*O*-sulfate uptake were measured at various substrate concentrations (1 to 40  $\mu\text{M}$ ).

plants is rather low compared with that of choline (46, 66). In contrast to choline-*O*-sulfate, acetylcholine could not serve as an osmoprotectant for strain JBB5 [ $\Delta(gbsAB::neo)2$ ] (Fig. 2B). Hence, for the *gbsAB*<sup>+</sup> *B. subtilis* strain JH642 (Fig. 2A), osmoprotection by acetylcholine was entirely dependent on its

hydrolysis to choline and acetate and the subsequent enzymatic conversion of the choline moiety to glycine betaine. As observed previously for choline, the presence of acetylcholine was detrimental to cell growth for strain JBB5 (Fig. 2B), an effect possibly connected with the accumulation of a large number of positively charged choline molecules inside the cells (6). Thus, the mechanism of osmoprotection by acetylcholine in *B. subtilis* is like that of *Pseudomonas aeruginosa*, where acetylcholine serves as a precursor for glycine betaine synthesis (40), but it differs from that of the gram-positive bacterium *Lactobacillus plantarum*, where both acetylcholine and choline are accumulated as moderately effective cationic osmolytes (37).

We also conducted preliminary experiments on the uptake of acetylcholine in *B. subtilis* by measuring the transport of [methyl-<sup>3</sup>H]acetylcholine at different substrate concentrations in cultures grown in SMM with 0.4 M NaCl. However, its uptake was very low and variable (data not shown), suggesting either that acetylcholine transport is very inefficient or that the hydrolysis of the ester bond and the subsequent transport of [methyl-<sup>3</sup>H]choline into the cell is rather slow. We previously observed the cleavage of the  $\beta$ -substituted acylesters acetylcarnitine and octanoylcarnitine by *B. subtilis* and their intracellular accumulation in the form of L-carnitine in osmotically stressed cells (30). Although it is not clear where the hydrolysis of acetylcholine and the acylesters of L-carnitine occurs, in its natural habitats *B. subtilis* might use such compounds as additional sources for generating compatible solutes.

In addition, we also investigated a possible osmoprotective effect of the choline ester phosphorylcholine (Fig. 1) for the growth of *B. subtilis* in high-osmolality media, since this trimethylammonium compound can serve as a precursor for glycine betaine synthesis in *P. aeruginosa* under hyperosmotic conditions (13, 40). However, this negatively charged choline ester did not display any osmoprotection for the *B. subtilis* wild-type strain JH642. Hence, not all choline esters serve as a source of precursor for glycine betaine synthesis in *B. subtilis*.

**Choline-*O*-sulfate is accumulated in the cytoplasmic solute pool.** To further substantiate the result that choline-*O*-sulfate serves as a compatible solute per se for *B. subtilis*, we monitored the fate of this compound in cultures grown at high osmolality by natural abundance <sup>13</sup>C-NMR spectroscopy, a technique that is frequently used to assess the spectrum of compatible solutes inside bacterial cells (15, 30, 65, 70). Crude ethanol extracts were prepared from cells of strain JH642 grown in SMM or in SMM with 1.2 M NaCl in the presence or absence of choline-*O*-sulfate and were analyzed by <sup>13</sup>C-NMR spectroscopy. As observed previously (28, 30, 70), glutamate and proline were the predominant osmolytes in salt-stressed cells grown in the absence of an osmoprotectant. The presence of 1 mM choline-*O*-sulfate in the growth medium resulted in the accumulation of this trimethylammonium compound in the cytoplasmic solute pool at both low and high osmolality (Fig. 5). Resonances from glycine betaine could not be detected in these spectra, although signals from this compound were readily found when it was added (1 mM) to the growth medium (data not shown).

The addition of 1 mM choline or acetylcholine, in contrast to choline-*O*-sulfate, to cultures of strain JH642 resulted in their complete conversion into glycine betaine as assessed by <sup>13</sup>C-NMR spectroscopy. When the production of glycine betaine from either choline or acetylcholine was blocked in the *gbsAB* mutant strain JBB5, choline (but no glycine betaine) was detected in the cytoplasmic solute pools (data not shown). Thus, the <sup>13</sup>C-NMR spectroscopy analysis confirmed the results of our growth experiments (Fig. 2) and revealed that choline-*O*-sulfate serves as a compatible solute per se in *B. subtilis* and

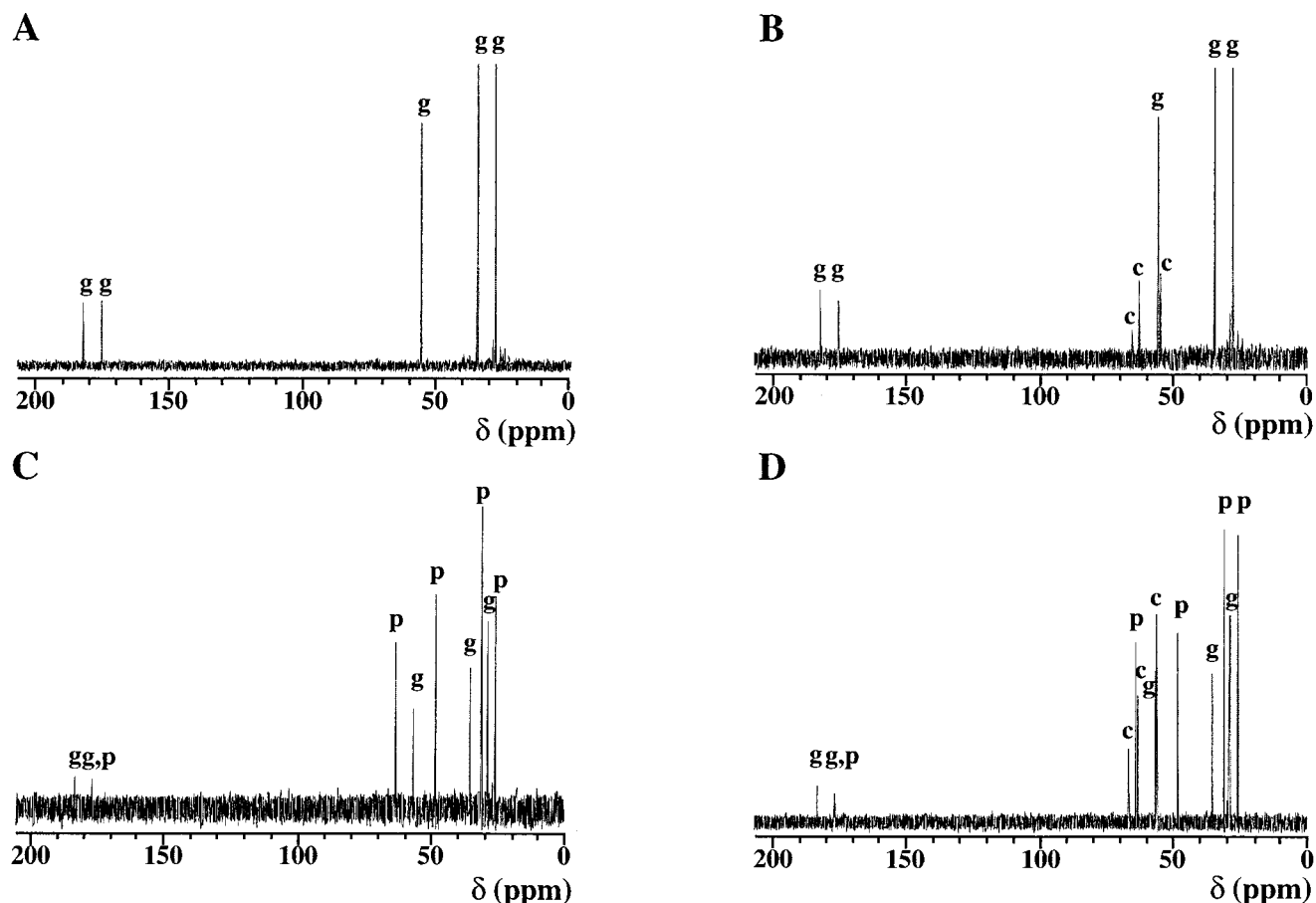


FIG. 5.  $^{13}\text{C}$ -NMR spectra of ethanol cell extracts. Strain JH642 was grown in SMM (A), in SMM with 1 mM choline-*O*-sulfate (B), in SMM with 1.2 M NaCl (C), and in SMM with 1.2 M NaCl in the presence of 1 mM choline-*O*-sulfate (D). Resonances originating from glutamate (g), proline (p), and choline-*O*-sulfate (c) are indicated. Chemical shifts for glycine betaine [given in ppm relative to the standard  $\text{D}_4$ -3-(trimethylsilyl)propionate] are 56.1, 69.0, and 171.8.

that the osmoprotective effect of the structurally related compound acetylcholine depends on its enzymatic conversion into glycine betaine.

**Choline-*O*-sulfate is metabolically inert in *B. subtilis*.** We tested whether *B. subtilis* degrades choline-*O*-sulfate for use as a carbon, nitrogen, or sulfur source. Choline-*O*-sulfate did not support the growth of the *B. subtilis* wild-type strain JH642 when supplied at a concentration of 30 mM as the sole carbon source. Likewise, it did not serve as a nitrogen source (provided at a concentration of 30 mM) in a modified SMM medium in which  $(\text{NH}_4)_2\text{SO}_4$  had been replaced by  $\text{K}_2\text{SO}_4$  at the equivalent concentration, whereas proline (30 mM) served as an effective nitrogen source in this  $(\text{NH}_4)_2\text{SO}_4$ -depleted medium. In an SMM medium where  $(\text{NH}_4)_2\text{SO}_4$  and  $\text{MgSO}_4$  were replaced by  $\text{NH}_4\text{Cl}$  and  $\text{MgCl}_2$  at equivalent concentrations, choline-*O*-sulfate (15 mM) did not support the growth of strain JH642, but methionine at a concentration of 15 mM readily served as a sole sulfur source. Hence, choline-*O*-sulfate is not used by *B. subtilis* for anabolic purposes.

## DISCUSSION

The use of a mixture of osmoprotectants by a given microorganism is frequently observed. It enhances the ability of bacteria to adapt flexibly to different ecological niches and to varying conditions in their habitats (12, 15, 34, 45). The data

presented here show that choline-*O*-sulfate, which is produced by a wide variety of plants, lichens, algae, fungi, and bacteria, serves as an effective and metabolically inert compatible solute for *B. subtilis*. By using a set of strains with mutations in previously defined systems for uptake of osmoprotectants (34), we identified the osmotically stimulated ABC transporter OpuC as the uptake system for choline-*O*-sulfate. To our knowledge this is the first report in which the kinetic parameters and molecular details of a bacterial choline-*O*-sulfate uptake system have been characterized.

Compatible solutes are highly soluble molecules; they do not carry a net charge at physiological pH, and often they also stabilize proteins in vitro and protect against denaturation in solutions of high ionic strength (72). Choline-*O*-sulfate fulfills the criteria for a compatible solute. This zwitterion (Fig. 1) is amassed in osmotically stressed *E. coli* and *S. typhimurium* cells to levels exceeding 0.5 M (21). Such high concentrations do not inhibit the enzymatic function of purified barley leaf malate dehydrogenase, and they protect the enzyme against inhibition by NaCl (53). Depending on the degree of osmotic stress, osmoregulating *B. subtilis* cells amass glycine betaine from exogenous sources at intracellular levels ranging between 0.5 and 1 M (47, 70). Since choline-*O*-sulfate and glycine betaine have similar osmoprotective effects for *B. subtilis* when accumulated via OpuC (Fig. 3), one can expect that both com-

pounds are amassed to comparable intracellular concentrations.

In addition to the chemical and biophysical properties of compatible solutes, the kinetic parameters of their transport systems greatly determine their effectiveness as osmoprotectants for bacteria. A telling case is the uptake of the cyclic amino acid derivative ectoine, an effective compatible solute for a wide range of bacterial species (15, 67). An exogenous supply (1 mM) of ectoine is only moderately osmoprotective for *B. subtilis* due to its inefficient import by OpuC ( $K_i = 1.5$  mM) (28). However, this tetrahydropyrimidine has an osmoprotective capacity similar to that of glycine betaine for *E. coli* (27) and the plant pathogen *Erwinia chrysanthemi* (17), bacteria which possess high-affinity ectoine and glycine betaine uptake systems. Although OpuC shows only low affinity for ectoine, this ABC transporter exhibits high-affinity uptake for the compatible solutes choline-*O*-sulfate, glycine betaine, L-carnitine, crotonobetaine, and  $\gamma$ -butyrobetaine, with  $K_m$  and  $K_i$  values in the low micromolar range (4 to 7  $\mu$ M) and a substantial transport capacity ( $V_{max}$  of 54 to 71 nmol/min  $\cdot$  mg of protein) (30, 31). With the exception of glycine betaine (31, 33), these compounds are taken up only via OpuC. Thus, this transporter plays a pivotal role in supplying *B. subtilis* with a variety of structurally related osmoprotectants.

Choline-*O*-sulfate, L-carnitine, crotonobetaine, and  $\gamma$ -butyrobetaine each serve as effective inhibitors for glycine betaine uptake via OpuC (Fig. 4A) (30), suggesting that these trimethylammonium compounds compete for the same binding site(s). The substrate-binding proteins of binding-protein-dependent ABC transporters serve as the primary site for high-affinity substrate recognition (7). The OpuC system possesses a lipoprotein (OpuCC) that is likely to function as an extracellular substrate-binding protein which is tethered by its N-terminal lipid tail to the cytoplasmic membrane (32). Unfortunately, it has not yet been possible to isolate the OpuCC protein in a functional form (36), which precludes a direct biochemical analysis of substrate binding. We have successfully performed such experiments with purified OpuAC, which is the lipid-modified substrate-binding protein from the glycine betaine ABC transporter OpuA (35). Consideration of the chemical nature of the substrates transported with high affinity via OpuC reveals that all of them possess a fully methylated ammonium group and that the main carbon chains exhibit considerable variation with respect to length, chemical bonds, and branching (Fig. 1). Each of these substrates is a zwitterion at physiological pH, whereas uptake of the positively charged choline via OpuC proceeds with a somewhat reduced affinity ( $K_m = 38$   $\mu$ M) (32).

Choline-*O*-sulfate is a major product of sulfate metabolism in a variety of plant, fungal, and bacterial species (14, 22, 24, 26) and is thus likely to be released from decaying cells of these primary producers into the soil. Several species of the genus *Pseudomonas* are even known to excrete choline-*O*-sulfate into the medium (14). Like other osmoprotectants, this betaine is probably present only at low and variable concentrations in the natural habitats of *B. subtilis*. Therefore, the ability to scavenge compatible solutes efficiently from the environment and to accumulate them to very high intracellular levels against a steep concentration gradient is a distinctive requirement for a transport system involved in the acquisition of osmoprotectants. The ABC transporter OpuC meets these physiological demands, and the osmoprotective effects of choline-*O*-sulfate (Fig. 3A), glycine betaine, L-carnitine, crotonobetaine, and  $\gamma$ -butyrobetaine were clearly noticeable when these compounds were supplied at concentrations as low as 20  $\mu$ M to an OpuC-positive *B. subtilis* strain (30). The presence of choline-*O*-sulfate in physiologically relevant concentrations in the soil

is substantiated by reports demonstrating the presence of transport systems for this ester in barley roots (49), in soil fungi (2, 24), and in strains of *Corynebacterium* and of the rhizosphere bacteria *Pseudomonas*, *Agrobacterium*, and *Rhizobium* (48). Although the details on the type(s) of transport system involved and the kinetic parameters for choline-*O*-sulfate uptake by most of these organisms have not been documented, in barley roots (49), *Neurospora crassa* (43), and *Penicillium notatum* (2) choline-*O*-sulfate transport activities with  $K_m$  values of 7.7, 25, and 300  $\mu$ M, respectively, have been reported. Choline-*O*-sulfate from the environment is accumulated in each of these cells as a source of sulfur which becomes available after intracellular hydrolysis of choline-*O*-sulfate by choline sulfatases. The OpuC-mediated choline-*O*-sulfate transport in *B. subtilis* for osmoprotective purposes reported here compares favorably ( $K_m$  value of 4  $\mu$ M) with that described for anabolic functions.

Choline sulfatases are present in various microbial species (18, 23, 43, 62). They may play a role in providing the precursor for glycine betaine synthesis. However, biotransformation of choline-*O*-sulfate into glycine betaine is clearly not the cause for its osmoprotective effects in *B. subtilis*. As we have demonstrated here, choline-*O*-sulfate was fully osmoprotective for a *B. subtilis* mutant defective in the glycine betaine biosynthetic pathway (Fig. 2B) and was readily detected as an osmolyte in the cytoplasmic solute pool of osmotically stressed cells (Fig. 5). These findings contrast with the observations made with acetylcholine, whose osmoprotective effects for *B. subtilis* were entirely dependent on its enzymatic conversion into glycine betaine (Fig. 2). The accumulation of choline-*O*-sulfate as a metabolically inert stress compound is common to the bacteria *B. subtilis*, *E. coli*, and *S. typhimurium* (21), and its amassing at high intracellular levels is also observed in the fungus *P. fellutanum* (51). However, the osmoprotective effect of choline-*O*-sulfate does not always depend on its accumulation in an unmodified form. Recently, Østerås et al. (50) reported the presence of a choline sulfatase activity (BetC) in the rhizosphere bacterium *Sinorhizobium meliloti* that catalyzed the hydrolysis of choline-*O*-sulfate into choline and sulfate; the produced choline was then further oxidized into glycine betaine by the BetA and BetB enzymes. The structural gene (*betC*) for this choline sulfatase is part of the *S. meliloti betICBA* operon. The fact that both choline-*O*-sulfate and choline serve as inducers for this gene cluster thus further underscores the physiological relevance of the choline-*O*-sulfate to glycine betaine biosynthetic pathway. In contrast to *B. subtilis* (5), *S. meliloti* can use both glycine betaine and choline-*O*-sulfate not only for osmoprotection but also as sole carbon and nitrogen (sulfur) sources for growth (50).

The previously reported osmoprotective effects of choline-*O*-sulfate for several plant, fungal, and bacterial species (9, 19, 21, 44, 51) and the function of this choline ester as a precursor for glycine betaine synthesis in *S. meliloti* (50) coupled with our discovery of a high-affinity choline-*O*-sulfate transport system in *B. subtilis* for osmoprotective purposes all suggest that this betaine plays a much wider and physiologically more important role for osmoprotection than has previously been assumed.

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