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Osmoregulation in *Bacillus subtilis*: Synthesis of the Osmoprotectant Glycine Betaine from Exogenously Provided Choline

JENS BOCH, BETTINA KEMPF, AND ERHARD BREMER*

Max-Planck Institute for Terrestrial Microbiology, D-35043 Marburg, Federal Republic of Germany

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Exogenously provided glycine betaine functions as an efficient osmoprotectant for *Bacillus subtilis* in high-osmolarity environments. This gram-positive soil organism is not able to increase the intracellular level of glycine betaine through de novo synthesis in defined medium (A. M. Whatmore, J. A. Chudek, and R. H. Reed, J. Gen. Microbiol. 136:2527–2535, 1990). We found, however, that *B. subtilis* can synthesize glycine betaine when its biosynthetic precursor, choline, is present in the growth medium. Uptake studies with radiolabelled [*methyl*-^{14}C]choline demonstrated that choline transport is osmotically controlled and is mediated by a high-affinity uptake system. Choline transport of cells grown in low- and high-osmolarity media showed Michaelis-Menten kinetics with K_m values of 3 and 5 μM and maximum rates of transport (V_{max}) of 10 and 36 nmol min^{-1} mg of protein^{-1}, respectively. The choline transporter exhibited considerable substrate specificity, and the results of competition experiments suggest that the fully methylated quaternary ammonium group is a key feature for substrate recognition. Thin-layer chromatography revealed that the radioactivity from exogenously provided [*methyl*-^{14}C]choline accumulated intracellularly as [*methyl*-^{14}C]glycine betaine, demonstrating that *B. subtilis* possesses enzymes for the oxidative conversion of choline into glycine betaine. Exogenously provided choline significantly increased the growth rate of *B. subtilis* in high-osmolarity media and permitted its proliferation under conditions that are otherwise strongly inhibitory for its growth. Choline and glycine betaine were not used as sole sources of carbon or nitrogen, consistent with their functional role in the process of adaptation of *B. subtilis* to high-osmolarity stress.

The osmotic strength of the environment is an important physical parameter that influences the ability of microorganisms to grow and successfully compete for a given habitat. Bacteria maintain an osmotic pressure in the cytoplasm that is higher than that of the surrounding environment, resulting in an outward-directed pressure, the turgor. The proper maintenance of turgor is essential for cell division and growth. Since the cell envelope is permeable to water, changes in the environmental osmolarity trigger the flux of water across the cytoplasmic membrane. Thus, to avoid lysis under low-osmolarity or dehydration under high-osmolarity growth conditions, bacteria must possess active mechanisms that permit timely and efficient adaptation to changes in environmental osmolarity (7, 23).

The soil-living bacterium *Bacillus subtilis* occupies a habitat that is characterized by constant changes in the availability of water. Salt stress is a very efficient inducer of a set of general- and salt-specific-stress proteins in *B. subtilis*, suggesting that desiccation occurs frequently in the soil (14, 36). The alternative transcription factor σ^B plays a central role in regulating the expression of many genes in this general-stress regulon (5, 35); however, it is unclear whether any of the general- or salt-specific-stress proteins play a direct role in the adaptation of *B. subtilis* to high-osmolarity environments. *B. subtilis* can grow in media containing 7% NaCl (6). The intensive study of *Escherichia coli* and *Salmonella typhimurium* in recent years has shown that specific genetic and physiological adaptation processes are required to cope efficiently with high-osmolarity stress conditions (7, 23). Despite the importance of changes in the environmental osmolarity to the growth of *B. subtilis*, the physiological and genetic reactions of this organism to such changes are poorly understood. In the absence of any exogenously provided osmoprotectants, K^+ ions are rapidly accumulated from the environment when *B. subtilis* is subjected to hypersaline treatment. Apparently, K^+ accumulation occurs via turgor-sensitive transport systems, and it is essential for the recovery of turgor after osmotic upshock (38). This initial response of *B. subtilis* to the increase in the external osmolarity is followed by the intracellular accumulation of organic osmolytes by either synthesis or uptake from the environment. Natural-abundance ^{13}C nuclear magnetic resonance spectroscopy experiments revealed that proline is the major organic osmolyte synthesized by *B. subtilis* in defined medium (37). Thus, *B. subtilis* does not appear to accumulate the disaccharide trehalose, which is the dominant organic osmolyte synthesized by *E. coli* under high-osmolarity stress (32). The osmoprotectant glycine betaine is accumulated from exogenous sources by *B. subtilis* under high-osmolarity growth conditions (15, 37). Glycine betaine, a trimethylated derivative of the amino acid glycine, is found widely in nature and is synthesized in species as distantly related as enterobacteria, plants, and humans. It has been adopted by a wide variety of organisms as an efficient osmoprotectant (7, 23). In osmotically stressed cells, the intracellular accumulation of glycine betaine counterbalances the high extracellular concentrations of osmolytes and consequently helps to maintain turgor. In addition, glycine betaine stabilizes the integrity of cell components and preserves the functioning of proteins in solutions of high ionic strength (2, 39). We have recently found that at least two osmotically controlled glycine betaine transport systems are present in *B. subtilis* (18). The uptake of glycine betaine and the intracellular synthesis of proline in response to high osmolarity are subjected to a finely tuned regulatory circuit, since endogenous proline accumulation is severely repressed

* Corresponding author. Mailing address: Max-Planck-Institut für Terrestrische Mikrobiologie, Karl-von-Frisch Str., D-35043 Marburg, Federal Republic of Germany. Phone: (49) 6421-286681. Fax: (49) 6421-285833. Electronic mail address: bremer@mailer.uni-marburg.de.

when glycine betaine is transported into the cell (37). Apparently, glycine betaine is the preferred osmoprotectant for *B. subtilis*.

In defined medium, no glycine betaine can be detected in the solute pools of *B. subtilis* cells grown at high osmolarity (37), indicating that no de novo synthesis of this osmoprotectant occurs under these conditions. Although many organisms lack the ability to produce glycine betaine de novo, glycine betaine can often be synthesized from exogenously provided choline with glycine betaine aldehyde as an intermediate (7, 23). We therefore investigated whether *B. subtilis* can use choline as a precursor for glycine betaine synthesis in order to adapt to a high-osmolarity environment. Our results show that exogenously provided choline is accumulated inside the cell by an efficient and osmotically modulated transport system and that it is used as a precursor for glycine betaine synthesis. This choline-glycine betaine pathway is an integral part of the physiological response of *B. subtilis* to a high-osmolarity environment.

MATERIALS AND METHODS

Bacterial strains, media, and chemicals. *B. subtilis* JH642 (*trpC2 pheA1*; BGSC 1A96), a derivative of the *B. subtilis* wild-type strain 168, was constructed by J. Hoch and was obtained from M. Marahiel (University of Marburg, Marburg, Federal Republic of Germany). This *B. subtilis* strain was used throughout this study and was maintained on Luria-Bertani agar plates (25). Spizizen's minimal medium (SMM), consisting of $(NH_4)_2SO_4$ (2 g/liter), K_2HPO_4 (14 g/liter), KH_2PO_4 (6 g/liter), Na_3-citrate · 2 H_2O (1 g/liter), and $MgSO_4$ · 7 H_2O (0.2 g/liter), with 0.5% glucose as the carbon source was used as defined growth medium (31) and was supplemented with tryptophan (20 mg/liter) and phenylalanine (18 mg/liter) as required. To determine whether *B. subtilis* could use choline or glycine betaine as the sole nitrogen source, we used a modified SMM in which the $(NH_4)_2SO_4$ had been replaced by K_2SO_4 (13). The osmolarity of the SMM was determined with a vapor pressure osmometer (model 5.500; Wescor Inc., Logan, Utah) and was found to be 340 mosmol/kg of H_2O. The osmotic strength of the SMM was increased by addition of salts (NaCl and KCl), sugars (glucose and maltose), or glycerol from highly concentrated stock solutions. *B. subtilis* cultures were grown aerobically in Erlenmeyer flasks at 37°C with shaking (200 to 220 rpm). Bacterial growth was monitored spectrophotometrically as the optical density at 578 nm (OD_{578}). Glycine betaine, glycine betaine aldehyde, choline, and compounds used as inhibitors for [*methyl*-^{14}C]choline uptake were purchased from Sigma Chemie GmbH (Deisenhofen, Federal Republic of Germany). The antibiotics kanamycin and spectinomycin were used in Luria-Bertani agar plates at final concentrations of 30 and 50 μg/ml, respectively.

Genetic construction of *E. coli* mutants. We constructed a ProP$^-$ ProU$^-$ derivative of the Bet$^+$ ProP$^+$ ProU$^+$ *E. coli* strain W3110 [F$^-$ λ$^-$ IN(*rrnD-rrnE*)*1*] (3). The Φ(*proU-lacZ*) hyb2 (λp*lac*Mu15; Kan^r) protein fusion from strain GM37 (24) was introduced by P1*vir*-mediated transduction (25) into strain W3110 by selecting for Kan^r colonies. In one of these transductants, strain BK103, we replaced the Φ(*proU-lacZ*)hyb2 fusion with the Δ(*proU*::spc^r)*608* deletion, using a P1*vir* lysate prepared on strain MKH13 (12) and selecting for Spc^r Kan^s colonies. We then constructed from one of the resulting transductants, strain BK104, a ProP$^-$ derivative. This was done by crossing with phage P1*vir* the Φ(*proP-lacZ*)2 (λp*lac*Mu55; Kan^r) operon fusion from strain EF074 (10) into strain BK104; the transductants were selected as Kan^r colonies. The resulting strain, BK105, is deficient in both the low-affinity and the high-affinity glycine betaine transport systems ProP and ProU, respectively, but is Bet$^+$ and hence can transport choline and convert it enzymatically into glycine betaine.

Transport assays. Uptake of choline in *B. subtilis* was measured by using [*methyl*-^{14}C]choline (53.0 mCi/mmol; DuPont, NEN Research Products, Boston, Mass.) as a substrate. The cells were grown to mid-exponential phase (OD_{578}, 0.4 to 0.8) and used immediately for the transport assay. A typical uptake assay contained 10 μM [*methyl*-^{14}C]choline (5.3 mCi/mmol) in a total reaction volume of 2 ml. Samples (0.3 ml) were taken at various times (20, 40, 60, 90, and 120 s) and filtered through 0.45-μm-pore-size filters (Schleicher & Schuell GmbH, Dassel, Federal Republic of Germany), and the cells were washed with 20 ml of isotonic SMM minimal salts. The radioactivity retained on the filters was determined in a scintillation counter. Protein concentrations were estimated from the OD_{578} of the culture (25), and uptake velocities were calculated as initial rates of transport when transport rates were linear with time. Compounds used as competitors for [*methyl*-^{14}C]choline uptake were added to the transport assay to a final concentration of 1 mM (100-fold excess). For kinetic studies, the choline concentration in the uptake assay was varied from 0.5 to 200 μM (1.4 to 53.0 mCi/mmol).

Conversion of intracellular accumulated choline into glycine betaine. To monitor the fate of exogenously provided choline, cultures of *B. subtilis* JH642 were grown in low- or high-osmolarity SMM to mid-exponential growth phase (OD_{578}; 0.4 to 0.8). Radiolabelled [*methyl*-^{14}C]choline was added to 10 ml of cells at a final substrate concentration of 10 μM, and the culture was incubated at 37°C in a shaking water bath. Samples (0.5 ml) were taken at various intervals, and the cells were pelleted by centrifugation for 2 min in an Eppendorf tabletop centrifuge. From each sample, 0.3 ml of the supernatant was used for scintillation counting to determine the fraction of [*methyl*-^{14}C]choline remaining in the medium, and the cell pellet was frozen at −20°C. The cells were then broken for analysis of the intracellular derivatives of [*methyl*-^{14}C]choline by thin-layer chromatography. The cell pellet was thawed, resuspended in a solution (50 μl) containing Tris-EDTA (50 mM Tris, 50 mM EDTA; pH 8.0) and freshly prepared lysozyme (3 mg/ml), and incubated for 10 min in a water bath (37°C); 2 μl of a 10% sodium dodecyl sulfate solution was then added to lyse the cells. Cellular debris was removed by centrifugation, and 5 μl of the supernatant was spotted onto thin-layer chromatography plates (Silica Gel G; Macherey-Nagel, Düren, Federal Republic of Germany). Compounds were separated by using a methanol–0.88 M ammonia (75:25) solution as the running solvent, and radiolabelled compounds were subsequently visualized by autoradiography with Fuji RX film. This thin-layer chromatography system allows the separation of choline and glycine betaine, but glycine betaine aldehyde cannot be distinguished from choline.

Synthesis of radiolabelled glycine betaine. [*methyl*-^{14}C]glycine betaine was synthesized from [*methyl*-^{14}C]choline (53.0 mCi/mmol; DuPont, NEN Research Products) by using choline oxidase from an *Alcaligenes* sp. (Sigma Chemie GmbH) and purified from the remaining [*methyl*-^{14}C]choline on ion-exchange resins essentially as described by Peters et al. (26). The purity of the synthesized [*methyl*-^{14}C]glycine betaine was checked by thin-layer chromatography (on Silica Gel G plates with a methanol–0.88 M ammonia [75:25] solution as the running solvent) and subsequent autoradiography. In addition, uptake assays were performed with the synthesized [*methyl*-^{14}C]glycine betaine, using the Bet$^+$ ProP$^+$ ProU$^+$ *E. coli* strain W3110 and the Bet$^+$ ProP$^-$ ProU$^-$ strain BK105. Strain

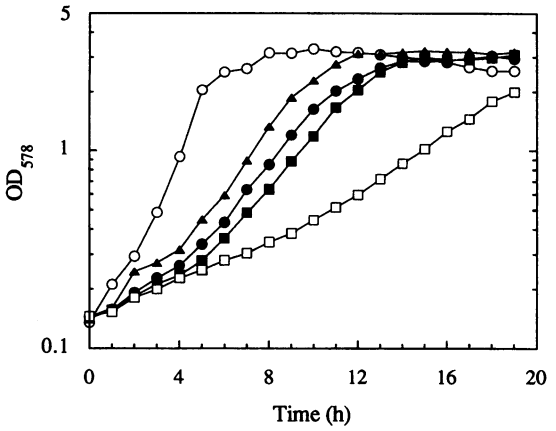

FIG. 1. Choline functions as an osmoprotectant. *B. subtilis* JH642 was grown in SMM (○) and minimal medium with 1.2 M NaCl (□). Glycine betaine (▲), choline (●), and glycine betaine aldehyde (■) were added to the high-osmolarity growth medium in a final concentration of 1 mM. Cultures (75 ml) were inoculated from an overnight culture of strain JH642 and were grown in 500-ml Erlenmeyer flasks in a shaking water bath (200 to 220 rpm) at 37°C.

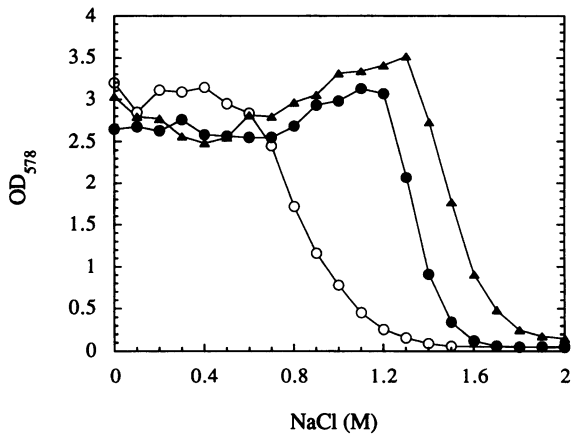

FIG. 2. Choline protects *B. subtilis* from the detrimental effects of high osmolarity. SMM of different osmolarities (20-ml amounts) was inoculated with 0.4 ml of an overnight culture of strain JH642 grown in SMM. The cultures were grown on a shaker platform at 37°C for 16 h, and the optical densities of the cultures were then determined and plotted against the concentration of NaCl in the growth medium. SMM alone (○) or with 1 mM choline (●) or 1 mM glycine betaine (▲) was used.

W3110 readily accumulated the synthesized [*methyl*-^{14}C]glycine betaine, whereas strain BK105 did not show glycine betaine uptake at a low substrate concentration (0.5 μM final substrate concentration). Conversely, both strains transported the [*methyl*-^{14}C]choline used to synthesize the radiolabelled glycine betaine. The final substrate concentration of [*methyl*-^{14}C]choline in the uptake assay was 0.5 μM.

RESULTS

Choline functions as an osmoprotectant in *B. subtilis*. We investigated the influence of high-osmolarity media on the growth of the *B. subtilis* wild-type strain JH642 (*trpC2 pheA1*). When the osmolarity of the standard SMM (340 mosmol/kg of H_2O) was increased by the addition of 1.2 M sodium chloride, the growth of strain JH642 was strongly impaired. The addition of 1 mM glycine betaine largely relieved the detrimental effects of high osmolarity on the growth of strain JH642 (Fig. 1). We tested the ability of exogenously provided choline and glycine betaine aldehyde to function as osmoprotectants in *B. subtilis*. Both compounds serve as precursors for glycine betaine synthesis in *E. coli* (22). We found that choline and glycine betaine aldehyde functioned as efficient osmoprotectants when they were added at a concentration of 1 mM to the high-osmolarity growth medium and strongly stimulated the growth of osmotically stressed cells (Fig. 1). Choline and glycine betaine aldehyde were somewhat less efficient osmoprotectants than glycine betaine (Fig. 1).

We tested how much choline was required to serve as an osmoprotectant. Cultures of strain JH642 were grown in high-osmolarity minimal medium containing various concentrations (1 μM to 10 mM) of choline. The addition of 100 μM choline to the growth medium was sufficient to cause partial osmoprotection, whereas lower choline concentrations had no stimulating effect. An increase in the concentration of exogenously provided choline to 1 mM further improved the growth of the osmotically stressed cells, but the presence of 10 mM choline did not result in a concomitant stimulation of growth (data not shown). Thus, a relatively low concentration (100 μM) of choline suffices to protect *B. subtilis* from the deleterious effects of a high-osmolarity environment.

To elucidate the extent to which choline can stimulate the growth of *B. subtilis* in high-osmolarity media, we performed growth studies with cultures of increasing osmolarities. Cultures of different osmolarities were inoculated either in the absence or in the presence of the osmoprotectant choline or glycine betaine (1 mM each) and grown for 16 h; the optical densities of the cultures were then determined (Fig. 2). The increase in the osmolarity of the medium resulted in a gradual decline in the optical density reached by the cultures grown without osmoprotectant when the osmolarity was raised by adding >0.6 M NaCl. The presence of choline and glycine betaine strongly protected the cells from the detrimental effects of osmotic stress and permitted their growth under conditions that were strongly inhibitory for cultures grown in the absence of these osmoprotectants. Again, glycine betaine was a more efficient osmoprotectant than choline (Fig. 2). It should be noted that this experiment does not distinguish between a reduction in the cell yield and a decrease in the growth rate of the osmotically stressed cultures. However, these data show that choline not only stimulates the growth of *B. subtilis* in a high-osmolarity environment but also enables this soil bacterium to multiply under osmotic conditions which are otherwise inhibitory for its proliferation.

Uptake of choline is osmotically controlled. Osmoprotection by a given compound requires its intracellular accumulation. We therefore measured the initial rates of choline uptake in cells grown at low and high osmolarities, using [*methyl*-^{14}C]choline as a substrate. There is substantial choline transport in cultures of strain JH642 grown at low osmolarity, and this choline transport activity is stimulated three- to fourfold when the osmolarity of the growth medium is raised by the addition of NaCl (Fig. 3A). The osmolarity of the medium has a distinct influence on choline transport activity. An increase in medium osmolarity of up to 0.4 M added NaCl resulted in a concomitant increase in choline transport. When the osmolarity of the growth medium was raised to >0.5 M added NaCl, a decrease in choline uptake was found (Fig. 3A). This reduction in choline transport activity occurs in parallel to the inhibition of cell growth in high-osmolarity environments (Fig. 2).

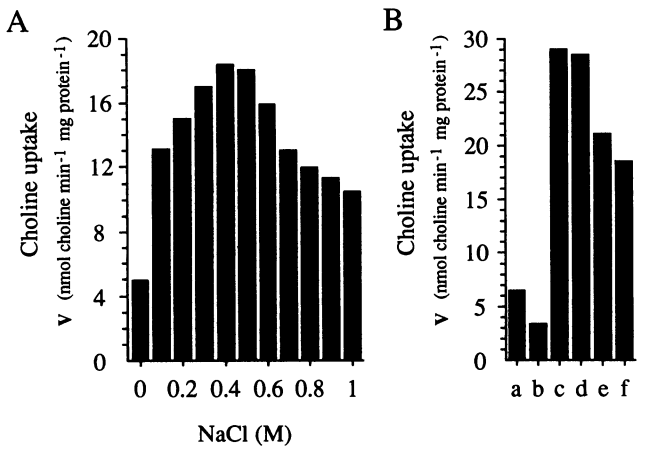

FIG. 3. Osmotically modulated transport of radiolabelled choline. (A) *B. subtilis* JH642 was grown in minimal media of various osmolarities. Choline transport into the cells was determined by measuring the uptake of radiolabelled [*methyl*-^{14}C]choline at a final substrate concentration of 5 μM. The uptake velocity was plotted against the NaCl concentration of the growth medium. (B) *B. subtilis* JH642 was grown in minimal medium (a) and minimal medium whose osmolarity was increased by adding 0.68 M glycerol (b), 0.4 M NaCl (c), 0.4 M KCl (d), 0.61 M maltose (e), or 0.67 M glucose (f). The uptake velocities of [*methyl*-^{14}C]choline were determined in transport assays with 10 μM radiolabelled choline. Aliquots (2 ml) of the culture were withdrawn, and choline uptake was initiated by adding [*methyl*-^{14}C]choline. The uptake assays were performed in low- or high-osmolarity growth medium. Samples (300 μl) were taken at various intervals, and the cells were collected by filtration and washed with 20 ml of the medium used for cell growth. The radioactivity retained by the cells was then determined in a scintillation counter.

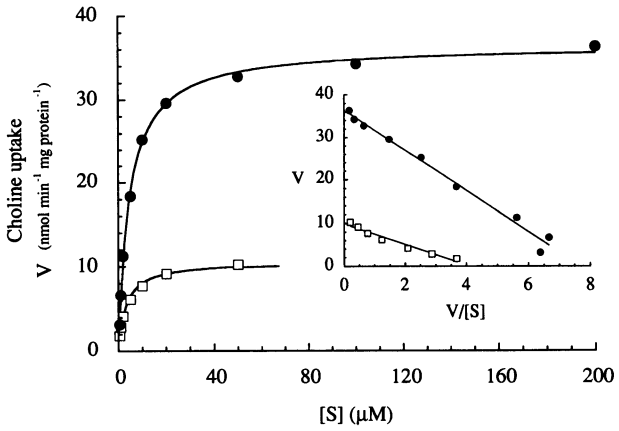

FIG. 4. Kinetics of the osmotically controlled choline transport. The uptake of [*methyl*-^{14}C]choline in *B. subtilis* JH642 was determined at various substrate concentrations for cultures grown either in minimal medium (SMM), (□) or in minimal medium with increased osmolarity (SMM plus 0.4 M NaCl) (●). Initial transport velocities were plotted against the substrate concentration. An Eadie-Hofstee plot (insert) was used to determine the kinetic parameters (K_m and V_{max}) of the choline transport at low and high osmolarities.

To investigate whether the enhanced rate of choline transport in high-osmolarity media reflects a true osmotic stimulus or is merely an NaCl-dependent effect, we used a number of compounds to raise the osmotic strength of the medium prior to the [*methyl*-^{14}C]choline uptake assays. Strain JH642 was grown in minimal media in which osmolarities were raised to 1,100 mosmol/kg of H_2O by the addition of either salts (NaCl and KCl), sugars (maltose and glucose), or glycerol. The cultures grown in the presence of increased concentrations of either salts or sugars showed a strong stimulation in choline uptake activity in comparison with the culture grown in the standard minimal medium (Fig. 3B). In contrast, there was no stimulation of choline transport when the osmolarity of the growth medium was raised by the addition of glycerol. Glycerol can freely pass the cytoplasmic membrane at high substrate concentrations and is thus not capable of exerting an osmotic pressure on the cells. We thus conclude that the observed stimulation of choline transport activity in cells grown at high osmolarity is truly an osmotic effect. Even in the low-osmolarity minimal medium (SMM; 340 mosmol/kg of H_2O), there was a substantial choline transport activity (Fig. 3). The rate of choline transport was not reduced by diluting the SMM with H_2O to an osmolarity of 200 mosmol/kg of H_2O (data not shown).

Kinetics of choline transport. The initial velocities of [*methyl*-^{14}C]choline uptake were determined over a wide range of substrate concentrations (0.5 to 200 μM) in cells grown in either low- or high-osmolarity minimal media. For these experiments, we chose high-osmolarity growth conditions in which the osmolarity of the minimal medium was increased by the addition of 0.4 M NaCl, conditions under which we had observed maximum choline transport rates (Fig. 3A). Choline uptake showed saturation kinetics, and Eadie-Hofstee plots of the data indicate that only one high-affinity and efficient choline transport system is present in *B. subtilis* (Fig. 4). The maximum rate of substrate uptake through this choline transport system is modulated by the osmolarity of the growth medium, with V_{max} values of 10 and 36 nmol min^{-1} mg of protein^{-1} for cells grown in low- and high-osmolarity media, respectively. In contrast, the affinity of this choline transporter for its substrate is essentially unaffected by medium osmolarity, since the K_m values are 3 and 5 μM for cells grown at low and high osmolarity, respectively.

Osmotic induction and activation of choline transport activity. The data presented above show that the uptake of choline by *B. subtilis* is osmotically controlled. To test whether increased choline transport at high osmolarity depends on de novo protein synthesis or on osmotic activation of preexisting transport proteins, we measured choline uptake in the presence or absence of chloramphenicol. Exponentially growing cells of strain JH642 were subjected to a sudden osmotic upshock by adding NaCl to the SMM, to a final concentration of 0.4 M. This environmental challenge resulted in an immediate stimulation of the rate of choline uptake which was followed by an additional increase in choline transport activity over a period of 50 min (Fig. 5). Chloramphenicol-treated cells showed a comparable initial stimulation in choline uptake following osmotic upshock, but there was no further increase in choline transport activity (Fig. 5). Thus, these data strongly suggest that the increase in choline transport activity exhibited by cells grown in high-osmolarity media depends on both de novo protein synthesis and the activation of preexisting choline transporters.

Substrate specificity of the choline transport system. A number of unlabelled compounds were used to study the relative specificity of the osmotically controlled choline transport system of *B. subtilis*. Uptake assays with [*methyl*-^{14}C]choline were performed in the presence of a 100-fold excess of substances with structures related to that of choline. The measured uptake velocities of choline transport showed that

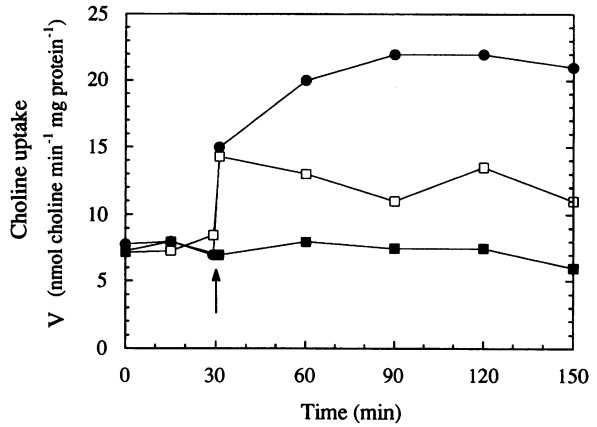

FIG. 5. Activation and induction of choline transport activity. Cells of strain JH642 were grown in SMM to mid-exponential phase (OD_{578} = 0.3), and the culture was then divided into three portions. One culture was treated with 100 μg of chloramphenicol per ml at time zero, and samples were taken at various intervals and assayed for [*methyl*-^{14}C]choline uptake activity. Two of the cultures were subjected to a sudden osmotic upshock after 30 min (arrow) by adding NaCl to the growth medium to a final concentration of 0.4 M. Symbols: ■, no addition; ● and □, addition of 0.4 M NaCl alone or with chloramphenicol (100 μg/ml), respectively.

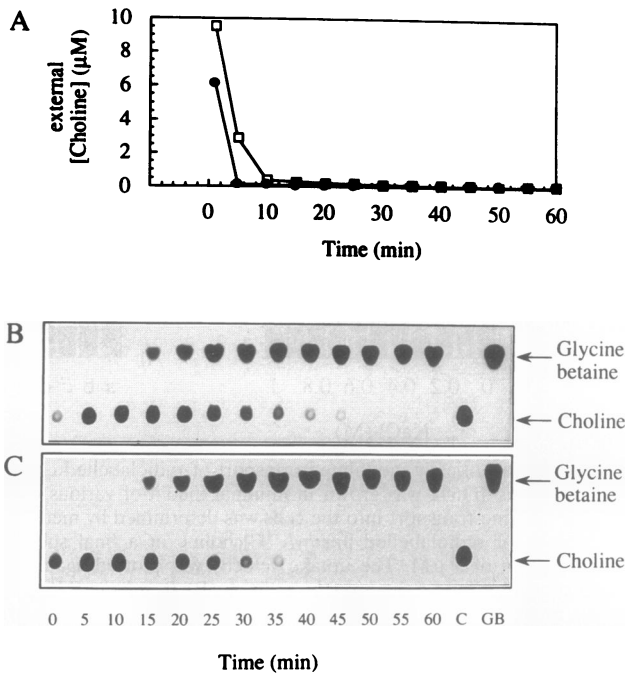

FIG. 6. Choline is converted into glycine betaine. (A) *B. subtilis* JH642 was grown to mid-log phase (OD_{578}, 0.7) in minimal medium (SMM) (□) and minimal medium with increased osmolarity (SMM plus 0.4 M NaCl) (●). Choline uptake was initiated by adding [*methyl*-^{14}C]choline to a final substrate concentration of 10 μM. Samples (500 μl) were taken at various times, the cells were pelleted by centrifugation, and the radioactivity in the supernatant was determined in a scintillation counter. (B and C) The cell pellets from the samples taken at the various time points were disrupted, and the soluble components were separated by thin-layer chromatography on Silica Gel G plates. The radiolabelled choline and its product were visualized by autoradiography. The samples analyzed in panels B and C are from the cells grown at low and high osmolarity, respectively. C and GB, radiolabelled choline and glycine betaine, respectively, used as standards. Choline does not migrate on the Silica Gel G plates when run with methanol–0.88 M ammonia (75:25) solution as the solvent system.

this transport system has a high degree of substrate specificity (Table 1). The strong inhibition of choline uptake by glycine betaine aldehyde (Table 1) indicates that the *B. subtilis* choline transport system also mediates the uptake of this osmoprotectant (Fig. 1). The choline analogs *N*,*N*-dimethylethanolamine, *N*-methylethanolamine, and ethanolamine had only a weak inhibitory effect on choline uptake, indicating that the completely methylated quaternary ammonium group is a key feature for substrate recognition by the choline transporter. This view is supported by the strong inhibition of choline uptake by trimethylamine, which features a fully methylated ammonium group (Table 1). None of these compounds at a substrate concentration of 1 mM had any osmoprotective effect on the growth of *B. subtilis* in high-osmolarity media. Although phosphorylcholine possesses a fully methylated ammonium group, it inhibited choline uptake only weakly (Table 1) and had no osmoprotective effect (data not shown). Glycine betaine inhibited choline transport to a certain degree, whereas its derivatives *N*,*N*-dimethylglycine, *N*-methylglycine, and glycine had only marginal effects on choline uptake (Table 1). The inhibition of choline uptake by glycine betaine raises the possibility that a certain part of total glycine betaine uptake at high osmolarity (Fig. 1 and 2) is mediated by the choline transporter described here. We found that *N*,*N*-dimethylglycine can serve as a weak osmoprotectant in *B. subtilis* (data not shown).

TABLE 1. Effects of various compounds used as competitors for [*methyl*-^{14}C]choline uptake[a]

Inhibitor	% Inhibition of choline uptake velocity
N,*N*-Dimethylethanolamine	28
N-Methylethanolamine	2
Ethanolamine	5
Trimethylamine	49
Phosphorylcholine	18
Glycine betaine aldehyde	84
Glycine betaine	38
N,*N*-Dimethylglycine	19
N-Methylglycine	9
Glycine	3

[a] Strain JH642 was grown to mid-exponential phase (OD_{578}, 0.4 to 0.8) in high-osmolarity medium (SMM plus 0.4 M NaCl), and the transport assay was initiated by adding [*methyl*-^{14}C]choline to a final substrate concentration of 10 μM. Nonlabelled compounds were added to the uptake assays at a final substrate concentration of 1 mM (100-fold excess). The transport activity of the uninhibited sample (100%) was 25 nmol of choline · mg of protein^{-1} · min^{-1}.

Intracellular conversion of choline into glycine betaine. In *E. coli*, the osmoprotective function of choline depends on its intracellular oxidation to glycine betaine (33). We therefore analyzed the fate of exogenously provided [*methyl*-^{14}C]choline in *B. subtilis*. The addition of radiolabelled [*methyl*-^{14}C]choline to exponentially growing cultures of *B. subtilis* in low- and high-osmolarity minimal media resulted in a rapid and complete uptake of choline by the cells under both growth conditions (Fig. 6A). We collected these cells by centrifugation, lysed them, and analyzed the soluble part of the cell extracts by thin-layer chromatography and subsequent autoradiography. Initially, choline can be detected in an unmodified form inside

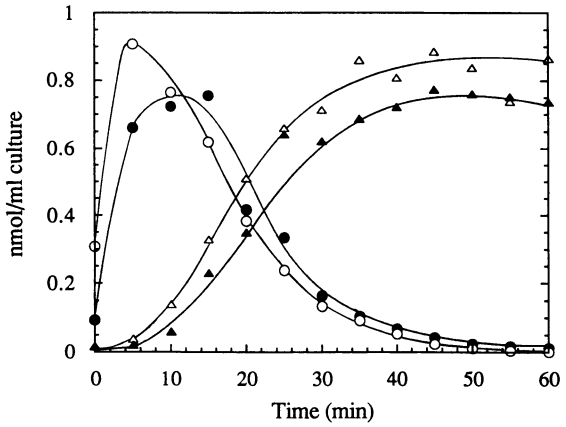

FIG. 7. Quantification of the conversion of choline into glycine betaine by *B. subtilis*. The distribution of radiolabelled choline and glycine betaine on the thin-layer chromatogram of the culture of strain JH642 grown in low-osmolarity (SMM; see Fig. 6B) (● and ▲) or high-osmolarity (SMM plus 0.4 M NaCl; see Fig. 6C) (○ and △) medium was analyzed with a PhosphorImager (Molecular Dynamics, Krefeld, Federal Republic of Germany). Symbols: ● and ○, choline; ▲ and △, glycine betaine.

the cells, but it is subsequently completely converted into glycine betaine (Fig. 6B and C). Thus, *B. subtilis* uses exogenous choline as a precursor for glycine betaine synthesis. A quantitative analysis (Fig. 7) of the kinetics of glycine betaine synthesis from choline using the data shown in Fig. 6B and C indicates that approximately 20 min after the addition of [*methyl*-^{14}C]choline to the cells grown at high and low osmolarities, approximately 50% of the radiolabelled choline has been oxidized into glycine betaine by the cell. The conversion of the entire amount of exogenously provided choline into glycine betaine is completed within 50 min (Fig. 6 and 7). A comparison of the effect of medium osmolarity on the uptake of choline and its enzymatic conversion into glycine betaine shows that choline uptake is under osmotic control (Fig. 3), whereas glycine betaine synthesis from choline is not strongly stimulated by the osmolarity of the growth medium (Fig. 7).

Choline and glycine betaine are not used as sole carbon or nitrogen sources. We tested whether choline or glycine betaine could be used by *B. subtilis* as the sole carbon source. For this experiment, we replaced the glucose (28 mM) in our standard minimal medium with an equal amount of either choline or glycine betaine. Neither of these compounds was able to support the growth of strain JH642. Likewise, choline and glycine betaine could not serve as the sole nitrogen source in a modified SMM in which the $(NH_4)_2SO_4$ had been replaced by K_2SO_4, indicating that glycine betaine is metabolically inert in *B. subtilis* and that choline is not metabolized beyond glycine betaine.

DISCUSSION

In its soil environment, *B. subtilis* encounters frequent osmotic challenges due to the drying and wetting of its habitat. Active processes that permit adaptation to changes in the osmolarity are consequently needed to ensure survival and growth of the bacteria. The data presented in this study firmly establish that the choline-glycine betaine pathway enables *B. subtilis* to cope efficiently with high-osmolarity growth conditions. The intracellular accumulation of the osmoprotectant glycine betaine is an integral part of the process of adaptation of many microorganisms to high-osmolarity stress (7, 23). *B. subtilis* can accumulate glycine betaine from exogenous sources (15, 37), and our recent genetic analysis has shown that it possesses at least two osmotically controlled transport systems for this osmoprotectant (18). The presence of glycine betaine strongly stimulated the growth of *B. subtilis* under high-osmolarity conditions and allowed its proliferation in a high-osmolarity environment that is normally strongly inhibitory for its growth (Fig. 1 and 2). The assessment by ^{13}C nuclear magnetic resonance spectroscopy of the intracellular solute pools of *B. subtilis* by Whatmore et al. (37) has revealed that no de novo synthesis of glycine betaine occurs in response to osmotic upshock. Our results demonstrate that *B. subtilis* has the capacity to synthesize glycine betaine when choline is present in the growth medium. Exogenously provided choline stimulated cell growth in high-osmolarity media and conferred a considerable osmotic tolerance (Fig. 1 and 2). Uptake and tracer studies with radiolabelled [*methyl*-^{14}C]choline showed that the intracellular accumulated choline is quantitatively converted into glycine betaine (Fig. 6 and 7). Thus, the osmoprotective effects of choline appear to depend on its enzymatic conversion to glycine betaine. Indeed, a detailed genetic and biochemical analysis of the choline-glycine betaine pathway in *E. coli* has proven that choline has no osmoprotective properties per se (33). *B. subtilis* thus shares the ability to oxidize choline to glycine betaine for osmoprotective purposes with a number of gram-negative and gram-positive bacteria (1, 4, 7, 8, 17, 22, 23, 29). This oxidation is a two-step process with glycine betaine aldehyde as an intermediate. In microorganisms, it can involve either a soluble choline oxidase that is proficient for both enzymatic reactions or a combination of a membrane-bound choline dehydrogenase (which also can oxidize glycine betaine aldehyde to glycine betaine) and a soluble glycine betaine aldehyde dehydrogenase with a high degree of substrate specificity (29). Further biochemical and genetic studies are required to analyze the types and numbers of enzymes involved in the conversion of choline into the osmoprotectant glycine betaine in *B. subtilis*.

Characterization of the choline uptake activity in *B. subtilis* revealed the presence of a high-affinity transport system. Choline transport is under osmotic control, and its maximum rate of substrate uptake is stimulated approximately fourfold under high-osmolarity growth conditions (Fig. 3A). This stimulation is a true osmotic effect, since it can be triggered by increases in medium osmolarity with either ionic or nonionic osmolytes (Fig. 3B). The increase in choline transport activity in cells grown at high osmolarity is under dual control (Fig. 5). A sudden osmotic upshock stimulates immediately the activity of preexisting choline transporters, and subsequent de novo protein synthesis is required to achieve maximal choline uptake activity. Thus, the expression of the structural gene for the *B. subtilis* choline transporter is most likely regulated in response to changes in medium osmolarity. Both a modulation of the activity of the choline transporter and a stimulation at the level of gene expression contribute also to the increase in choline transport observed in *E. coli* under high-osmolarity growth conditions (9, 21). A number of environmental factors (osmolarity, availability of oxygen, and temperature) and the presence of choline in the growth medium all influence the transcription of the *betT* gene, which encodes the *E. coli* choline transport protein (9). High osmolarity, low phosphate concentrations, and the availability of choline are also known to stimulate the expression of the gene(s) for an effective choline transport system in *Staphylococcus aureus* that serves to scavange choline from the environment for the synthesis of the osmoprotectant glycine betaine (17). The *B. subtilis* choline

transport system shows a marked substrate specificity and, as for choline transporters from other organisms (17, 27, 28), the fully methylated quaternary ammonium group is a key feature for substrate recognition (Table 1). Exogenously provided glycine betaine aldehyde is an effective inhibitor for choline uptake (Table 1) and also functions as a potent osmoprotectant in *B. subtilis* (Fig. 1). This indicates that the *B. subtilis* choline transporter also functions in glycine betaine aldehyde uptake, which is then used as a substrate for glycine betaine synthesis.

The ability to decompose choline is widespread among microorganisms, and many of these choline-utilizing bacteria can also use glycine betaine as the sole carbon or nitrogen source (19, 34). For instance, in *Rhizobium meliloti*, choline serves as the precursor in the synthesis of glycine betaine, which then can be further metabolized under low-osmolarity growth conditions. However, degradation of glycine betaine is inhibited by high osmolarity, showing that choline serves a dual role in this soil bacterium (30). In contrast, in *B. subtilis*, neither choline nor glycine betaine can be used as a carbon or nitrogen source. This observation is consistent with the report of Kortstee (19) that bacilli were not found among the choline-utilizing bacteria that can be readily isolated from the soil. Since *B. subtilis* is dependent on exogenous choline as a precursor for glycine betaine synthesis, the question of the source of choline in its natural habitat arises. Only a small amount of free choline is present in the soil, but considerable quantities of phosphatidylcholine and other choline-containing lipids are frequently brought into this habitat by the degradation of plant and animal tissues (34). Choline can be released from phosphatidylcholine either by the action of phospholipase D or in the form of phosphorylcholine by the action of phospholipase C, which then can be further cleaved to choline and P_i. Tracer studies have established that phosphatidylcholine is actually degraded in the soil in a stepwise process ultimately releasing P_i, a diglyceride, and choline (34). To the best of our knowledge, it is unknown whether *B. subtilis* itself produces a phospholipase to directly liberate choline or phosphorylcholine from phosphatidylcholine or whether it is dependent on the enzymatic activities of other microorganisms for the provision of choline for glycine betaine synthesis. In contrast to choline, phosphorylcholine showed no osmoprotective activity for cultures of *B. subtilis* grown in SMM with a high concentration of NaCl.

Since both choline and glycine betaine are metabolically inert in *B. subtilis*, we were surprised to find that even in low-osmolarity media, choline is taken up by the cells and oxidized to glycine betaine (Fig. 3 and 6). Hence, the choline-glycine betaine pathway in *B. subtilis* appears to serve a physiological function in addition to its role in adaptation to extreme osmolarities. Glycine betaine synthesis at low osmolarity might be involved in the maintenance of the very high turgor found in *B. subtilis*, which has been estimated at 1.9 MPa (38). To maintain this high turgor, it might be beneficial for *B. subtilis* to accumulate intracellularly osmotically active substances that are compatible with the normal structure and function of cell components and proteins instead of ionic osmolytes which are deleterious at high concentrations (2, 7, 23, 39). Thus, *B. subtilis* would benefit physiologically from the uptake and synthesis of compatible solutes even in a low-osmolarity environment. Indeed, significant quantities of glycine betaine are accumulated from exogenous sources when *B. subtilis* is grown in low-osmolarity media (15, 37). In contrast, in *E. coli*, which maintains a much lower turgor (300 kPa; see reference 16), there is very little glycine betaine uptake and synthesis when the cells are grown in low-osmolarity media (24, 33). However, similar to *B. subtilis*, the highly salt-tolerant gram-positive *S. aureus* is known to maintain a high turgor and to accumulate compatible solutes even in the absence of osmotic stress (11, 20).

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