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Response of *Bacillus subtilis* to high osmolarity: uptake of carnitine, crotonobetaine and γ-butyrobetaine via the ABC transport system OpuC

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It was found that low concentrations of the naturally occurring and structurally related betaines L-carnitine, crotonobetaine and γ-butyrobetaine conferred a high degree of osmotic tolerance to *Bacillus subtilis*. Kinetic analysis of L-[*N-methyl*-^{14}C]carnitine uptake in cells grown in minimal medium revealed the presence of a high-affinity transport system with a K_m value of 5 μM and a maximum rate of transport (V_{max}) of 41 nmol min^{-1} (mg protein)$^{-1}$. A rise in medium osmolarity moderately increased the maximum velocity [V_{max} 71 nmol min^{-1} (mg protein)$^{-1}$] of this transport system, but had little effect on its affinity. Growth and transport studies with a set of strains that carried defined mutations in the previously identified glycine betaine transport systems OpuA, OpuC and OpuD allowed the identification of the ATP-binding cassette (ABC) transport system OpuC as the only uptake route for L-carnitine in *B. subtilis*. Competition experiments with crotonobetaine and γ-butyrobetaine revealed that the OpuC system also exhibited a high affinity for these trimethylammonium compounds with K_i values of 6·4 μM. Tracer experiments with radiolabelled L-carnitine and ^{13}C-NMR tracings of cell extracts demonstrated that these betaines are accumulated by *B. subtilis* in an unmodified form. In contrast, the β-substituted acylcarnitine esters acetylcarnitine and octanoylcarnitine both functioned as osmoprotectants for *B. subtilis* but were found to be accumulated as carnitine by the cells. None of these trimethylammonium compounds were used as sole carbon or nitrogen sources. The results thus characterize L-carnitine, crotonobetaine and γ-butyrobetaine as effective compatible solutes for *B. subtilis* and establish a crucial role of the ABC transport system OpuC for the supply of *B. subtilis* with a variety of osmoprotectants.

Keywords: compatible solutes, osmoprotection, ABC transporters, carnitine, acylcarnitines

INTRODUCTION

A decrease in the water content of soil imposes a considerable stress on soil-living micro-organisms: water exits from the cells, resulting in decreased turgor and cessation of growth (Miller & Wood, 1996). *Bacillus subtilis* actively modulates the osmolarity of its cytoplasm under these adverse circumstances through uptake of K^+ (Whatmore & Reed, 1990) and by amassing organic osmolytes (Boch *et al.*, 1994; Whatmore *et al.*, 1990) that are highly compatible with cellular functions (Yancey, 1994). Widely used compatible solutes are the amino acid proline and the trimethylammonium compound glycine betaine (Csonka & Epstein, 1996; Galinski & Trüper, 1994). *B. subtilis* can use both solutes for osmoprotection, and their intracellular accumulation allows cell growth over a wide range of osmolarities (Boch *et al.*, 1994; von Blohn *et al.*, 1997).

A rise in the environmental osmolarity triggers a large increase in *de novo* proline synthesis (Measures, 1975; Whatmore *et al.*, 1990) and also results in a marked stimulation of proline uptake via the osmoregulated

Abbreviation: ABC, ATP-binding cassette.

proline transport system, OpuE (osmoprotectant uptake) (von Blohn *et al.*, 1997). Similarly, high osmolarity causes high-level accumulation of glycine betaine through either enhanced uptake from the environment (Kappes *et al.*, 1996; Kempf & Bremer, 1995; Lin & Hansen, 1995) or increased synthesis from the precursor choline (Boch *et al.*, 1994, 1996). Glycine betaine is brought into the soil by root exudates and decaying plant material (Goldmann *et al.*, 1991) and is taken up by *B. subtilis* via three effective transport systems (Kappes *et al.*, 1996). One of these, OpuD, is a secondary transporter that exhibits over its entire length a significant degree of sequence identity to the carnitine transporter, CaiT (24% identity) (Eichler *et al.*, 1994), and the choline transporter, BetT (35% identity) (Lamark *et al.*, 1991), from *Escherichia coli* (Kappes *et al.*, 1996). A glycine betaine transporter closely related to OpuD has recently also been described in the soil bacterium *Corynebacterium glutamicum* (Peter *et al.*, 1996). The other two uptake systems, OpuA and OpuC (Kappes *et al.*, 1996; Kempf & Bremer, 1995; Lin & Hansen, 1995), are binding-protein-dependent transporters and are members of the ATP-binding cassette (ABC) superfamily of transporters in which ATP hydrolysis is coupled to solute uptake (Boos & Lucht, 1996; Higgins, 1992). OpuA and OpuC are related to the periplasmic binding-protein-dependent glycine betaine transport system ProU from *E. coli* (Csonka & Epstein, 1996). Each of the three *B. subtilis* glycine betaine uptake systems has a high affinity for its substrate, with K_m values in the low micromolar range, but their transport capacity differs (Kappes *et al.*, 1996). OpuC, together with the ABC transport system OpuB, is also involved in choline uptake by *B. subtilis* (R. M. Kappes and others, unpublished results). The intracellular accumulated choline is then enzymically converted by the cells in a two-step oxidation process into the osmoprotectant glycine betaine (Boch *et al.*, 1994, 1996).

Carnitine, crotonobetaine and γ-butyrobetaine are ubiquitous in nature and are structurally related to the compatible solute glycine betaine (Bieber, 1988; Jung *et al.*, 1993; Kleber, 1997). In this study, we have focused on a possible role of these trimethylammonium compounds as osmoprotectants for *B. subtilis* and we show that each of these betaines can confer a considerable degree of osmotic tolerance. Genetic and physiological experiments allowed us to identify the ABC transporter OpuC as the sole and high-affinity uptake system for carnitine, crotonobetaine and γ-butyrobetaine in *B. subtilis*.

METHODS

Bacterial strains. Strain JH642 (*trpC2 pheA1*; J. Hoch, Scripps Research Institute, CA, USA; BGSC 1A96) is a derivative of the wild-type *B. subtilis* strain 168. Strains RMKB20 [Δ(*opuA*::*erm*)*4* *opuC-20*::Tn*10*(*spc*) Δ(*opuD*::*neo*)*2*], RMKB22 [Δ(*opuA*::*erm*)*4* *opuB-20*::Tn*10*(*spc*) Δ(*opuD*::*neo*)*2*], RMKB33 [Δ(*opuA*::*erm*)*4* Δ(*opuB*::*tet*)*23* *opuC-20*::Tn*10*(*spc*)] and RMKB34 [Δ(*opuB*::*tet*)*23* *opuC-20*::Tn*10*(*spc*) Δ(*opuD*::*neo*)*2*] are derivatives of strain JH642 and each expresses only one of the glycine betaine and choline transport systems operating in *B. subtilis*. Strain RMKB25 [*opuC-20*::Tn*10*(*spc*)] is also a derivative of strain JH642 and lacks only the OpuC uptake system. The *Pseudomonas aeruginosa* strain PAO1 was kindly provided by D. Jahn (University of Freiburg, Germany).

Growth conditions, media and chemicals. The bacterial strains were grown in Spizizen's minimal medium (SMM) with 0·5% (w/v) glucose as the carbon source and supplemented with L-tryptophan (20 μg ml^{-1}), L-phenylalanine (18 μg ml^{-1}) and a solution of trace elements (Harwood & Archibald, 1990). The osmotic strength of SMM was increased by the addition of NaCl from stock solutions. The osmolarity of growth media was determined with a vapour pressure osmometer (model 5500; Wescor) and the osmolarity of SMM, SMM with 0·4 M NaCl and SMM with 1·2 M NaCl was 340 mosmol kg^{-1}, 1100 mosmol kg^{-1} and 2700 mosmol kg^{-1}, respectively. For experiments where we wanted to determine the growth yield of cultures in high osmolarity media, we inoculated from a preculture 20 ml medium in 100 ml Erlenmeyer flasks to an OD_{578} of 0·06 and grew these cultures in the presence or absence of an osmoprotectant on an orbital shaker at 37 °C for 21 h. Under these conditions, there is essentially no cell growth of strains inoculated in SMM with 1·2 M NaCl in the absence of an osmoprotectant. For experiments where we continuously monitored the growth of cultures, we inoculated 75 ml prewarmed medium in a 500 ml Erlenmeyer flask with a late-exponential-phase preculture to an OD_{578} of 0·13 and grew these cultures in a shaking water bath at 37 °C. Under these conditions, high osmolarity (SMM with 1·2 M NaCl) also strongly impairs the growth of cells propagated in the absence of an osmoprotectant; however, the cultures will eventually reach an optical density similar to that of cultures grown in the presence of an osmoprotectant. When used as sole carbon sources, L-carnitine, D-carnitine, crotonobetaine and γ-butyrobetaine were added to the SMM medium to final concentrations of 24 mM and the growth yield of the cultures was monitored after 26 h. The use of proline as a carbon or nitrogen source by *B. subtilis* was monitored in parallel as a control. When used as sole nitrogen sources, L-carnitine, D-carnitine, crotonobetaine, γ-butyrobetaine and proline were added to a modified nitrogen-free SMM medium containing K_2SO_4 instead of $(NH_4)_2SO_4$ to final concentrations of 30 mM. Radiolabelled [1-^{14}C]glycine betaine [55 mCi mmol^{-1} (2·03 GBq mmol^{-1})] was purchased from American Radiolabeled Chemicals and L-[*N-methyl*-^{14}C]carnitine [53 mCi mmol^{-1} (1·96 GBq mmol^{-1})] was obtained from DuPont de Nemours. Glycine betaine (*N,N,N*-trimethylglycine), L-carnitine (β-hydroxy-γ-trimethylaminobutyrate), D-carnitine, acetyl-L-carnitine and DL-octanoylcarnitine were from Sigma, γ-butyrobetaine (γ-trimethylaminobutyrate) was obtained from Aldrich, and crotonobetaine was a kind gift from J. Brass (Lonza, Visp, Switzerland).

Transport assays. The uptake of L-[*N-methyl*-^{14}C]carnitine was measured at 37 °C at a final substrate concentration of 10 μM in bacterial cultures grown in minimal media of low (SMM) or high (SMM with 0·4 M NaCl) osmolarity and with glucose as the carbon source as detailed previously for the uptake of radiolabelled glycine betaine (Kappes *et al.*, 1996; Kempf & Bremer, 1995). Four independent experiments were carried out to determine the kinetic parameters of carnitine uptake in *B. subtilis* and the standard deviation of the values was determined. Compounds used as competitors for L-[*N-methyl*-^{14}C]carnitine uptake were added to the transport assays in 10-fold excess (100 μM). For kinetic studies, the

carnitine concentration in the uptake assays was varied from 1 μM to 30 μM. To determine the K_i values for crotonobetaine and γ-butyrobetaine via OpuC, the concentration of the unlabelled competitor was kept at 20 μM and the substrate concentration of the radiolabelled L-[*N-methyl*-^{14}C]carnitine was varied from 1 μM to 30 μM. These experiments were repeated twice.

Fate of intracellular accumulated L-[*N-methyl*-^{14}C]carnitine. Cultures (6 ml) of the *B. subtilis* strain JH642 and *P. aeruginosa* PAO1 were grown to mid-exponential phase (OD_{578} 0·6) in SMM with 0·4 M NaCl and glucose as the carbon source. Radiolabelled L-[*N-methyl*-^{14}C]carnitine was added to the cultures to a final concentration of 20 μM [0·1 μCi ml^{-1} (3·7 kBq ml^{-1})], and samples (0·5 ml) were taken at time intervals. The cells were collected by centrifugation and immediately frozen at −80 °C until further use. The cell pellets were thawed at room temperature and 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^{-1}). The mixture was incubated for 10 min in a water bath (37 °C), and 2 μl 10% (w/v) SDS solution was then added to lyse the cells. Cellular debris was removed by centrifugation, and 5 μl supernatant was spotted onto TLC plates (Polygram SIL G; Macherey-Nagel). Radiolabelled L-[*N-methyl*-^{14}C]carnitine and [*methyl*-^{14}C]glycine betaine were used as standards. The compounds were separated by using a methanol/0·88 M ammonia (75:25) solution as the running solvent. The radiolabelled compounds were subsequently visualized by autoradiography with Fuji RX film.

Preparation of cell extracts for ^{13}C-NMR spectroscopy. Cultures (300 ml) of strain JH642 were grown in 1 l Erlenmeyer flasks at 37 °C on a shaker (220 r.p.m.) in SMM with 1·2 M NaCl and 0·5% (w/v) glucose as the carbon source in the absence or presence of 1 mM L-carnitine, crotonobetaine, γ-butyrobetaine, acetylcarnitine or octanoylcarnitine. After the cultures had reached an OD_{578} of 2·5, the cells were harvested by centrifugation, the cell pellet was extracted with 12 ml 80% (v/v) ethanol, and the cellular debris was removed by centrifugation. The supernatant was evaporated to dryness and dissolved in 1 ml ^2H_2O supplemented with 1·2 mg D_4-3-(trimethylsilyl)propionate as standard. ^{13}C-NMR spectra (Talibart *et al.*, 1994) were recorded with a Bruker AC300 spectrometer operating at 300·13 MHz with a dual probe heading using a 8 μs pulse length. To identify resonances unambiguously in ^{13}C-NMR spectra from cell extracts, we recorded ^{13}C-NMR tracings from glutamate, proline, glycine betaine, carnitine, crotonobetaine, γ-butyrobetaine, acetylcarnitine and octanoylcarnitine relative to D_4-3-(trimethylsilyl)propionate as the standard.

RESULTS

Carnitine, crotonobetaine and γ-butyrobetaine function as osmoprotectants

To test whether any of the trimethylammonium compounds L-carnitine, D-carnitine, crotonobetaine and γ-butyrobetaine could function as osmoprotectants in *B. subtilis*, we grew the wild-type strain JH642 in SMM (340 mosmol kg^{-1}) or high-osmolarity minimal medium (SMM with 1·2 M NaCl; 2700 mosmol kg^{-1}) in the absence or presence of 1 mM of each of these betaines and determined the growth yield of the cultures after incubation for 21 h. Growth of strain JH642 was strongly impaired by the high-osmolarity medium, and the presence of L-carnitine, D-carnitine, crotonobetaine and γ-butyrobetaine each relieved this growth inhibition to an extent similar to that of the osmoprotectant glycine betaine. Hence, each of these betaines serves as an effective osmoprotectant for *B. subtilis*. A concentration as low as 100 μM was sufficient to protect the cells from the detrimental effects of high osmolarity (Fig. 1).

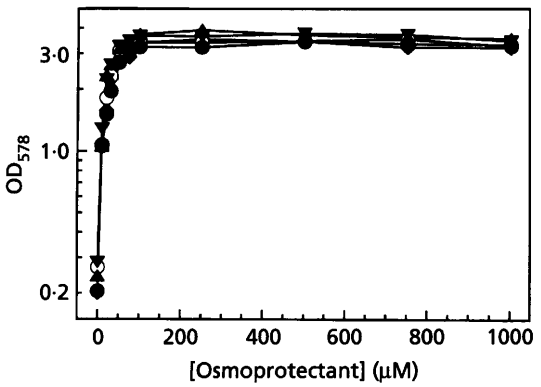

Fig. 1. Carnitine, crotonobetaine and γ-butyrobetaine improve the growth yield of *B. subtilis* in high osmolarity medium. The wild-type strain JH642 was grown in SMM with 1·2 M NaCl in the presence of various concentrations of L-carnitine (○), D-carnitine (●), crotonobetaine (▼), γ-butyrobetaine (▲) and glycine betaine (◆). The cells were grown in 20 ml medium in a 100 ml Erlenmeyer flask on a rotary shaker at 37 °C and the growth yield of each culture was determined spectrophotometrically by measuring the OD_{578} after 21 h incubation.

The ABC transport system OpuC mediates carnitine, crotonobetaine and γ-butyrobetaine uptake

Transport systems involved in the uptake of compatible solutes often mediate the uptake of more than one compound (Csonka & Epstein, 1996). We therefore tested for a possible role of the known transport systems in *B. subtilis* for glycine betaine (OpuA, OpuC and OpuD) and choline (OpuB and OpuC) in the uptake of L-carnitine, D-carnitine, crotonobetaine and γ-butyrobetaine. For these growth studies we used an isogenic set of strains, each of which synthesized only one of these transporters, and grew the strains overnight in SMM with 1·2 M NaCl in the absence or presence of 1 mM of these betaines. Only strains with an intact OpuC transport system permitted osmoprotection by L-carnitine, D-carnitine, crotonobetaine and γ-butyrobetaine. This finding was corroborated in experiments where the growth of the cultures was continuously monitored. Each of these compounds afforded about the same degree of osmoprotection in the OpuC$^+$ strain RMKB22 (OpuA$^-$ OpuB$^-$ OpuD$^-$), and the osmoprotective effect was similar to that conferred by glycine betaine (Fig. 2a). Loss of the OpuC activity in an otherwise wild-type background completely abolished osmoprotection by L-carnitine, D-carnitine, crotonobetaine and γ-butyrobetaine but not by glycine betaine (Fig. 2b), which can also be accumulated via the high-affinity OpuA and OpuD transport systems (Kappes *et al.*, 1996; Kempf &

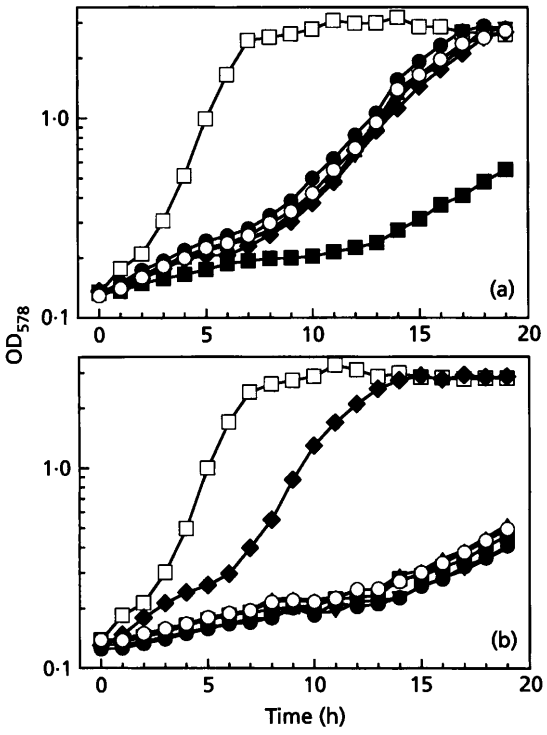

Fig. 2. Osmoprotective effects of carnitine, crotonobetaine and γ-butyrobetaine depend on the OpuC system. Strains RMKB22 (OpuA⁻ OpuB⁻ OpuC⁺ OpuD⁻) (a) and RMKB25 (OpuA⁺ OpuB⁺ OpuC⁻ OpuD⁺) (b) were grown in SMM (□) and SMM with 1·2 M NaCl in the absence (■) or presence of 1 mM L-carnitine (○), D-carnitine (●), crotonobetaine (▼), γ-butyrobetaine (▲) and glycine betaine (◆). Cultures (75 ml) were inoculated from overnight cultures pregrown in SMM and were grown in 500 ml Erlenmeyer flasks in a shaking water bath at 37 °C. Cell growth was monitored by measuring the OD_{578}.

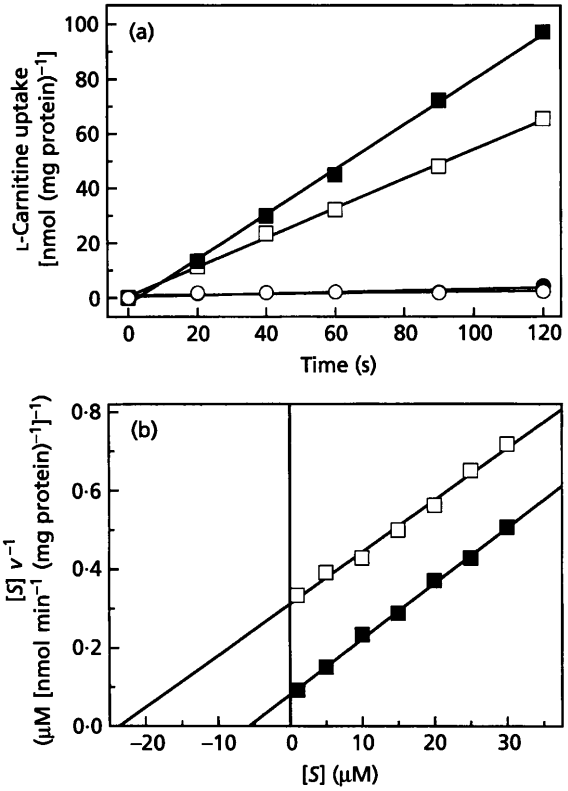

Fig. 3. L-Carnitine transport. (a) Cultures of the OpuC⁺ strain JH642 (□,■) and the OpuC⁻ mutant strain RMKB25 (○,●) were grown in SMM (□,○) and SMM with 0·4 M NaCl (■,●) to mid-exponential phase (OD_{578} 0·4) and assayed for L-[*N-methyl*-^{14}C]carnitine uptake at a final substrate concentration of 10 μM. (b) Determination of apparent K_m and V_{max} values for L-carnitine, crotonobetaine and γ-butyrobetaine. L-[*N-methyl*-^{14}C]Carnitine uptake was measured in cells grown in SMM with 0·4 M NaCl in the absence (■) or presence (□) of 20 μM of either unlabelled crotonobetaine or γ-butyrobetaine. In the absence of the inhibitors, the K_m is 5·8 μM and the V_{max} is 71 nmol min^{-1} (mg protein)$^{-1}$. In the presence of either crotonobetaine or γ-butyrobetaine, the V_{max} is 76 nmol min^{-1} (mg protein)$^{-1}$. Identical values were obtained when L-carnitine was used as the unlabelled inhibitor.

Bremer, 1995). Hence, these results identified the ABC transport system OpuC as the only physiologically important uptake route for the osmoprotectants L-carnitine, D-carnitine, crotonobetaine and γ-butyrobetaine in *B. subtilis*.

Kinetics of L-carnitine transport

We measured the initial transport of L-[*N-methyl*-^{14}C]carnitine in strain RMKB22 (OpuA⁻ OpuB⁻ OpuC⁺ OpuD⁻) in cultures grown in SMM or SMM with 0·4 M NaCl at a final substrate concentration of 10 μM. Uptake of L-[*N-methyl*-^{14}C]carnitine was readily detectable at this low substrate concentration, and high osmolarity moderately stimulated its transport (Fig. 3a). L-Carnitine uptake activity was abolished by the presence of an *opuC* mutation in an otherwise wild-type background (Fig. 3a). The initial velocities of L-[*N-methyl*-^{14}C]carnitine uptake were determined over a range of substrate concentrations (1–30 μM), and we found that L-carnitine transport showed saturation kinetics. The uptake assays revealed the presence of a high-affinity transport system with a K_m of 5·1 ± 0·5 μM and a maximum rate of transport (V_{max}) of 41 ± 3 nmol min^{-1} (mg protein)$^{-1}$ in cultures grown in SMM. High-osmolarity growth conditions (SMM with 0·4 M NaCl) did not significantly affect the affinity of the transport system (K_m 5·8 ± 0·5 μM), but the V_{max} of L-carnitine uptake was moderately increased to 71 ± 5 nmol min^{-1} (mg protein)$^{-1}$. These parameters closely match those for the OpuC-mediated glycine betaine uptake, with a K_m of 5·1 μM and a V_{max} of 41 nmol min^{-1} (mg protein)$^{-1}$ in SMM-grown cultures and a K_m of 6 μM and a V_{max} of 65 nmol min^{-1} (mg protein)$^{-1}$ in cultures grown in SMM with 0·4 M NaCl (Kappes *et al.*, 1996).

D-Carnitine, crotonobetaine, γ-butyrobetaine and glycine betaine compete with L-carnitine uptake

To analyse the entry of D-carnitine, crotonobetaine and γ-butyrobetaine into the cells via OpuC, we performed competition experiments with these unlabelled com-

pounds and radiolabelled L-carnitine. Uptake of L-[*N-methyl*-^{14}C]carnitine was strongly inhibited (> 80%) by a 10-fold excess of these betaines. As expected from the function of OpuC in glycine betaine transport (Kappes *et al.*, 1996), a low concentration (10-fold excess) of glycine betaine was also strongly inhibitory (> 80%) for L-[*N-methyl*-^{14}C]carnitine accumulation. These competition experiments thus indicated that OpuC also serves as a high-affinity transport system for D-carnitine, crotonobetaine and γ-butyrobetaine. To estimate the affinity of OpuC for crotonobetaine and γ-butyrobetaine, we measured the K_i values for these osmoprotectants. We grew strain RMKB22 (OpuA$^-$ OpuB$^-$ OpuC$^+$ OpuD$^-$) in SMM with 0·4 M NaCl to mid-exponential phase and measured the uptake of L-[*N-methyl*-^{14}C]carnitine over a range of substrate concentrations (1–30 μM) in the absence or presence of a fixed concentration (20 μM) of the unlabelled competitors. The transport rates for carnitine uptake in the presence of either crotonobetaine or γ-butyrobetaine varied by approximately 4% for each substrate concentration between different experiments. In the absence of the inhibitors, we found a K_m of 5·8 μM for carnitine uptake, and in the presence of either crotonobetaine or γ-butyrobetaine the apparent K_m of the OpuC system was 24 μM. From the kinetic data we calculated K_i values of 6·4 μM for both crotonobetaine and γ-butyrobetaine. The curves in the Hanes plot (Fig. 3b) were linear and yielded very similar V_{max} values for L-carnitine uptake in the absence [71 nmol min^{-1} (mg protein)$^{-1}$] or presence [76 nmol min^{-1} (mg protein)$^{-1}$] of an inhibitor. This indicated that both crotonobetaine and γ-butyrobetaine behave as competitive inhibitors (Xavier *et al.*, 1996) for L-carnitine uptake via OpuC. Thus the OpuC system has similar affinities and kinetic properties for the structurally related osmolytes glycine betaine, L-carnitine, crotonobetaine and γ-butyrobetaine. Carnitine detected in natural environments is always found in the L-configuration (Bieber, 1988; Kleber, 1997). The *B. subtilis* OpuC system apparently does not differentiate between the enantiomers of carnitine since D-carnitine strongly competed for L-carnitine uptake and also functioned effectively as an osmoprotectant (Figs 1 and 2).

L-Carnitine does not serve as a precursor for glycine betaine

Different *Pseudomonas* strains can use L-carnitine as a precursor for the synthesis of glycine betaine (Jung *et al.*, 1993; Kleber, 1997; Lucchesi *et al.*, 1995). A plausible explanation for the osmoprotective effect of L-carnitine in *B. subtilis* would thus entail its biotransformation into glycine betaine. We tested this possibility by monitoring the fate of exogenously provided L-[*N-methyl*-^{14}C]carnitine in cells of strain JH642 grown in SMM with 0·4 M NaCl. L-[*N-methyl*-^{14}C]Carnitine was added at a final concentration of 20 μM to exponential-phase cells, aliquots of the cultures were collected by centrifugation at various time intervals, the cells were lysed, and portions of the soluble fractions were then used for TLC. L-Carnitine and its possible metabolic product glycine betaine were subsequently visualized by autoradiography. Radiolabelled L-carnitine was rapidly taken up by the *B. subtilis* wild-type strain, but it was not converted into glycine betaine (Fig. 4). In contrast, conversion of L-[*N-methyl*-^{14}C]carnitine into glycine betaine was readily detectable in *P. aeruginosa* strain PAO1 (Fig. 4). Hence, L-carnitine does not serve as a precursor for glycine betaine synthesis in *B. subtilis*.

Accumulation of crotonobetaine and γ-butyrobetaine in the cytoplasmic solute pools of *B. subtilis*

Some micro-organisms can enzymically convert crotonobetaine and γ-butyrobetaine into L-carnitine (Jung *et al.*, 1993; Kleber, 1997). We questioned whether *B. subtilis* could use these compounds as precursors for the synthesis of carnitine, which would then act as a compatible solute. Since radiolabelled crotonobetaine and γ-butyrobetaine are not commercially available, we monitored the fate of both compounds in osmoregulating *B. subtilis* cells by ^{13}C-NMR spectroscopy. Cells of the wild-type strain JH642 were grown in high-osmolarity minimal medium (SMM with 1·2 M NaCl) in the absence or presence of 1 mM crotonobetaine or γ-butyrobetaine until the cultures reached an OD_{578} of 2·5. A crude ethanolic extract was prepared from these cells and analysed by ^{13}C-NMR spectroscopy. Corroborating a previous report (Whatmore *et al.*, 1990), we found that proline and glutamate were the predominant cytoplasmic osmolytes when cells were grown at high osmolarity in the absence of an exogenously provided osmoprotectant. Depending on the osmoprotectant added to the growth medium, we found either crotonobetaine or γ-butyrobetaine in the cytoplasmic solute pool in addition to the endogenously synthesized osmolytes (data not shown). L-Carnitine was not detected in these cell extracts, but was readily found when the culture was grown in the presence of this osmoprotectant. Thus, crotonobetaine, γ-butyrobetaine and L-carnitine are accumulated by *B. subtilis* under high-osmolarity growth conditions but undergo no further enzymic conversion. Likewise, neither of these compounds was metabolized in cells grown in SMM.

Acetylcarnitine and octanoylcarnitine are accumulated by the cells as carnitine

Carnitine is present in nature either in a free form or as β-substituted acylesters with side-chains of different length (Bieber, 1988). We tested whether acetylcarnitine or octanoylcarnitine could function as osmoprotectants for *B. subtilis*. We found that both compounds conferred osmotolerance but their osmoprotective capacity was less than that of L-carnitine (data not shown). Osmoprotection by acetylcarnitine and octanoylcarnitine was dependent on an intact OpuC transport system. To test whether these acylesters were intracellularly accumulated by the *B. subtilis* cells in an unmodified form, we performed ^{13}C-NMR experiments with cell extracts prepared from high-osmolarity cultures grown in the

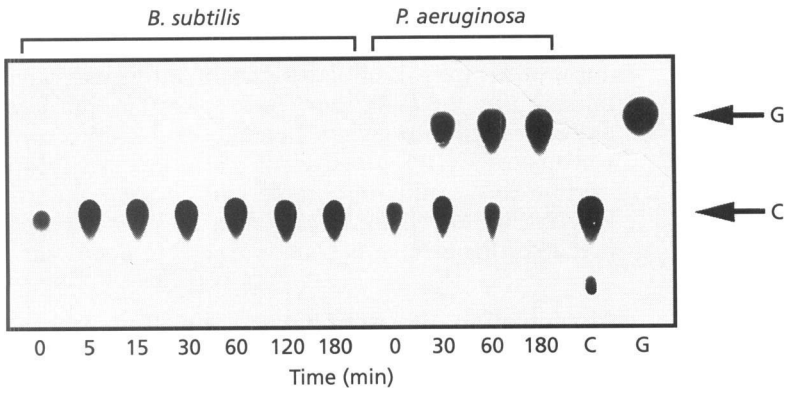

Fig. 4. Fate of L-[*N-methyl*-^{14}C]carnitine. The *B. subtilis* wild-type strain JH642 and *P. aeruginosa* PAO1 were grown in SMM with 0·4 M NaCl to mid-exponential phase (OD_{578} 0·6), and L-[*N-methyl*-^{14}C]carnitine was added to 6 ml of these cultures at a final concentration of 20 μM. Samples were taken at the indicated time intervals, and the soluble intracellular components were analysed by TLC. The radiolabelled compounds were visualized by autoradiography. G, Glycine betaine; C, L-carnitine.

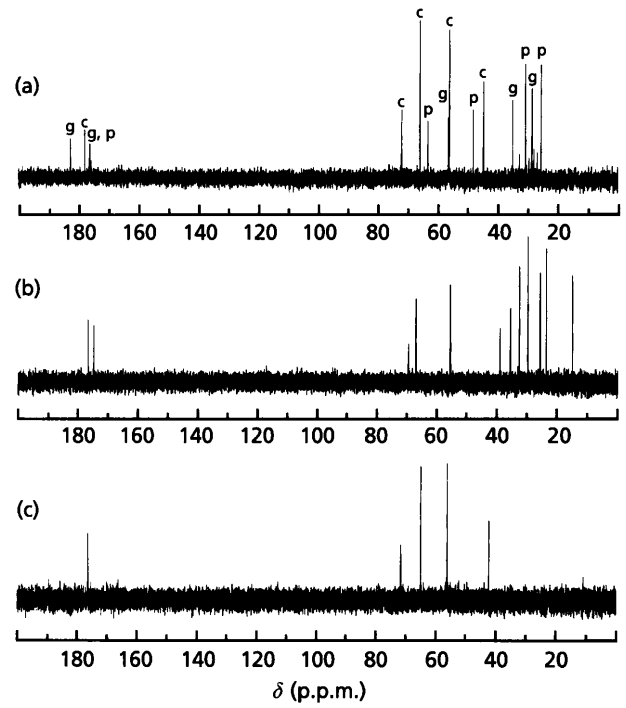

Fig. 5. ^{13}C-NMR spectra of ethanolic cell extracts. Strain JH642 was grown in SMM with 1·2 M NaCl in the presence of 1 mM octanoylcarnitine (a). ^{13}C-NMR tracings for octanoylcarnitine (b) and L-carnitine (c) were recorded as references. The resonance signals for glutamate (g), proline (p) and carnitine (c) are identified in the spectrum shown in (a).

presence of either 1 mM acetylcarnitine or octanoylcarnitine. In addition to the resonances of the endogenously synthesized amino acids proline and glutamate (Whatmore *et al.*, 1990), we detected the characteristic resonances of carnitine but not those of the side-chains from acetylcarnitine and octanoylcarnitine. This is documented for octanoylcarnitine in Fig. 5. Hence the β-substituted acylesters acetylcarnitine and octanoylcarnitine serve as precursors for the production of carnitine, which then acts as an osmoprotectant in *B. subtilis*.

Carnitine, crotonobetaine and γ-butyrobetaine are not used as sole carbon or nitrogen sources

We tested whether *B. subtilis* degrades the carbon backbones of L-carnitine, D-carnitine, crotonobetaine and γ-butyrobetaine for use as the sole carbon source. None of these substances supported growth of the wild-type strain JH642 in minimal medium. Likewise, none of these compounds served as sole nitrogen source in a modified SMM in which the $(NH_4)_2SO_4$ had been replaced by K_2SO_4, whereas L-proline was readily used by JH642 as sole nitrogen and carbon source.

DISCUSSION

The data presented here identify the structurally related trimethylammonium compounds L-carnitine, D-carnitine, crotonobetaine and γ-butyrobetaine as osmoprotectants for *B. subtilis* and characterize the ABC transport system OpuC as their uptake route. Based upon the sequence homology of the *B. subtilis* glycine betaine transporter OpuD (Kappes *et al.*, 1996) to the L-carnitine transporter CaiT from *E. coli* (Eichler *et al.*, 1994), one would have expected that this secondary transport system might be involved in L-carnitine uptake. Our data rule out this possibility, since a mutation in *opuD* had no influence on the level of osmoprotection conferred by L-carnitine, D-carnitine, crotonobetaine and γ-butyrobetaine, whereas the disruption of the OpuC system abolished the use of these compounds as osmoprotectants.

OpuC was previously recognized as one of the three glycine betaine uptake systems operating in *B. subtilis*. It is a member of the ABC superfamily of transporters (Kappes *et al.*, 1996; Lin & Hansen, 1995) and thus can mediate solute accumulation against a steep concentration gradient (Boos & Lucht, 1996; Higgins, 1992). OpuC should thus be well suited to scavenge glycine betaine, carnitine, crotonobetaine and γ-butyrobetaine effectively from the environment, even when these compounds are present only in trace amounts. Consistent with this expectation, we found that these betaines afforded a strong degree of osmoprotection, even at external concentrations as low as 20 μM (Fig. 1).

The kinetic parameters for glycine betaine, L-carnitine, crotonobetaine and γ-butyrobetaine uptake via OpuC are very similar and hence the multicomponent OpuC transporter serves a crucial role for the efficient acquisition of a spectrum of structurally related osmoprotectants from the environment. The OpuC system functions also in high-affinity choline transport (R. M. Kappes and others, unpublished results) and serves, albeit with low affinity (K_i value of 1·5 mM), as an uptake route for the compatible solute ectoine, a cyclic amino acid (Jebbar *et al.*, 1997).

An ATP-dependent L-carnitine transporter involved in osmoprotection has also been identified in the foodborne human pathogen *Listeria monocytogenes*, but the molecular details of this transport system are not yet known (Verheul *et al.*, 1995). The kinetic parameters of this transporter closely resemble those of OpuC. However, these two transport systems differ in substrate specificity since the L-carnitine transporter from *L. monocytogenes* does not mediate the uptake of glycine betaine (Verheul *et al.*, 1995), which is an effective osmoprotectant and cryoprotectant for this pathogen (Ko *et al.*, 1994). L-Carnitine is ubiquitous in the biosphere since it functions as a carrier of activated fatty acids and activated acetate across the inner mitochondrial membrane (Bieber, 1988). It is therefore not surprising that micro-organisms can utilize L-carnitine and the metabolically and structurally related crotonobetaine and γ-butyrobetaine for various cellular processes (Jung *et al.*, 1993; Kleber, 1997). The presence of an inducible active transport system for L-carnitine in *P. aeruginosa* (Kleber & Aurich, 1967) and a high-affinity binding-protein-dependent transport system for L-carnitine, crotonobetaine and γ-butyrobetaine in an *Agrobacterium* isolate (Nobile & Deshussen, 1986) attest to the availability of these compounds in the soil. Several soil bacteria, including *Pseudomonas*, *Agrobacterium* and *Rhizobium* species, have the ability to utilize L-carnitine, crotonobetaine and γ-butyrobetaine as sole sources of carbon and nitrogen (Kleber, 1997). As previously observed for glycine betaine (Boch *et al.*, 1994), *B. subtilis* cannot catabolize any of these compounds under aerobic growth conditions and hence accumulates them only for osmoregulatory purposes. L-Carnitine and γ-butyrobetaine can also function as osmoprotectants in *E. coli* and *Salmonella typhimurium* and are taken up with low efficiency via the osmoregulated glycine betaine transport systems ProP and ProU (Gouesbet *et al.*, 1994; Gutierrez & Csonka, 1995; Jung *et al.*, 1990). *Klebsiella pneumoniae*, *Lactobacillus plantarum* and various *Rhizobium* strains can use exogenously provided carnitine as an osmoprotectant as well, but the route of transport in these micro-organisms is unknown (Bernard *et al.*, 1986; Kets *et al.*, 1994; Le Rudulier *et al.*, 1984).

Some micro-organisms can enzymically convert crotonobetaine and γ-butyrobetaine into L-carnitine, and L-carnitine can serve as the precursor for glycine betaine synthesis (Jung *et al.*, 1993; Kleber, 1997). Such enzymic pathways do not operate in *B. subtilis* but the naturally occurring β-substituted acylcarnitine esters acetylcarnitine and octanoylcarnitine, both of which exhibit osmoprotective activity, were accumulated inside the *B. subtilis* cells as carnitine. Hydrolysis of acetylcarnitine has also been observed in osmoregulating *E. coli* cells (Peddie *et al.*, 1994), whereas propionylcarnitine and acetylcarnitine are accumulated in an unmodified form in osmotically stressed *Lb. plantarum* cells (Kets & de Bont, 1997). Our data demonstrate that the osmoprotective effects of acetylcarnitine and octanoylcarnitine for *B. subtilis* are dependent on the OpuC transport system, but we currently cannot tell whether these carnitine esters are hydrolysed on the cell surface or inside the cell. The uptake of carnitine and glycine betaine considerably enhances the survival of lactic acid bacteria subjected to drying (Kets & de Bont, 1997), and the accumulation of these trimethylammonium compounds via OpuC might prove to be beneficial for *B. subtilis* under such stressful conditions as well.

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