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FEMS Microbiology Letters 154 (1997) 325–330

FEMS MICROBIOLOGY LETTERS

# Ectoine functions as an osmoprotectant in *Bacillus subtilis* and is accumulated via the ABC-transport system OpuC

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Received 23 July 1997; accepted 24 July 1997

**Abstract**

We report here that the cyclic amino acid ectoine functions as an osmoprotectant for the soil bacterium *Bacillus subtilis*. Growth experiments with a set of *B. subtilis* strains that carry defined mutations in the glycine betaine transport systems OpuA, OpuC and OpuD and the choline transport system OpuB revealed that ectoine was specifically accumulated via the ABC-transport system OpuC. Competition experiments employing unlabeled ectoine and radiolabeled glycine betaine showed that the OpuC transport system has a low affinity for ectoine with a $K_i$ value of approximately 1.5 mM. Ectoine was identified by $^1$H NMR spectroscopy in the solute pool of cells grown in the presence of ectoine. Ectoine could not be used by *B. subtilis* as sole carbon or nitrogen source. Our data thus characterise ectoine as a metabolically inert stress compound for *B. subtilis* and establish a crucial role for the ABC-transport system OpuC for the acquisition of the osmoprotectant ectoine from the environment.

*Keywords:* *Bacillus subtilis*; ABC-transporter; Osmoprotectant; Ectoine; Glycine betaine

## 1. Introduction

The intracellular accumulation of organic osmoprotectants is a central response of the soil bacterium *Bacillus subtilis* to increases in the osmolarity of its habitat [1,2]. The amassing of these compatible solutes enables *B. subtilis* to reacquire water from the environment to restore turgor and to protect proteins and cell components from the detrimental effects of high ionic strength [3,4]. Proline is one of these compatible solutes and its de novo synthesis [1] and uptake from the environment through the OpuE system (osmoprotectant uptake) [5] is strongly increased in *B. subtilis* after a hyperosmotic shock. Increased production of proline and the uptake of $K^+$ [6] allows *B. subtilis* to adapt to moderate increases in the environmental osmolarity. A high level of osmotic tolerance and growth over a wide range of osmolarities is achieved either through the synthesis of the potent osmoprotectant glycine betaine from its precursor choline [2,7] or through its uptake

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*PII* S0378-1097(97)00349-2

from the environment [8]. Three high-affinity glycine betaine transport systems operate in *B. subtilis*. The OpuA and OpuC systems are multi-component transporters [8–10] and are members of the ABC-superfamily [11]. OpuC, together with the ABC-transport system OpuB, is also involved in choline uptake by *B. subtilis* (Kappes et al., unpublished results). The third glycine betaine uptake system, OpuD, is a single-component transporter and in concert with OpuA and OpuC ensures the effective scavenging of glycine betaine from the environment.

Screening of a large number of halophilic and halotolerant bacilli has revealed that the majority of these species produce the cyclic amino acid ectoine either alone or in combination with proline, as the major compatible solute [3]. *B. subtilis* thus seems to represent a minority of proline producers among the bacilli that are unable to synthesise ectoine in response to high osmolarity stress. The fact that many bacilli can accumulate this tetrahydropyrimidine by biosynthesis has prompted us to test whether exogenously provided ectoine would protect the non-producer *B. subtilis* from the detrimental effects of high osmolarity. We report here that ectoine serves as a compatible solute for this soil bacterium and we have identified the ABC-transport system OpuC as the sole uptake route for this osmoprotectant by *B. subtilis*.

## 2. Materials and methods

### 2.1. Bacterial strains

The *B. subtilis* strain JH642 (*trpC2 pheA1*; BGSC 1A96) is a derivative of the wild-type 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*)], 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.

### 2.2. Growth conditions, chemicals and transport assays

The *B. subtilis* strains were grown in Spizizen's minimal medium (SMM) with 0.5% glucose as the carbon source and supplemented with L-tryptophan (20 mg $l^{-1}$) and L-phenylalanine (18 mg $l^{-1}$) and a solution of trace elements [9]. The osmotic strength of media was increased by the addition of NaCl from stock solutions. Glycine betaine and ectoine were purchased from Sigma (Deisenhofen, Germany) and Bitop (Witten, Germany), respectively. Radiolabeled [1-$^{14}$C]glycine betaine (55 mCi $mmol^{-1}$) was obtained from ACR (American Radiolabeled Chemicals Inc., St Louis, MO). Uptake of glycine betaine by bacterial cells grown in minimal medium at 37°C was measured by using radiolabeled [1-$^{14}$C]glycine betaine at a final substrate concentration of 10 μM [8,9].

### 2.3. Preparation of cytoplasmic pool extracts for $^{1}$H NMR spectroscopy

Cells used for nuclear magnetic resonance (NMR) spectroscopy [12] were grown in 300 ml of SMM or SMM with 0.4 M NaCl in the absence or presence of 1 mM ectoine in 1-l Erlenmeyer flasks at 37°C with shaking (200 rpm) to late exponential phase. The cells were harvested by centrifugation, the cell pellets were extracted twice with 80% ethanol, and cellular debris was removed by centrifugation. The supernatant was evaporated to dryness, and dissolved in 0.8 ml $D_2O$ containing 3 mg of 2-methylpropanol (2) as internal standard. $^{1}$H NMR spectra were recorded on a Bruker AM300WB-300 spectrometer.

## 3. Results

### 3.1. Ectoine functions as an osmoprotectant for B. subtilis

To determine whether ectoine could protect *B. subtilis* from the detrimental effects of high osmolarity, we grew strain JH642 in low (SMM; 340 mosmol $kg^{-1}$) or high osmolarity (SMM with 1.2 M NaCl; 2700 mosmol $kg^{-1}$) minimal medium in the absence or presence of 1 mM ectoine and determined the

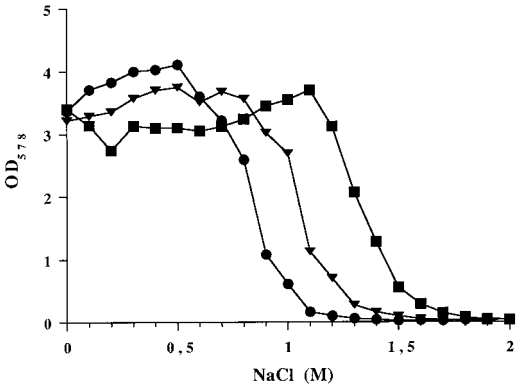


Fig. 1. Ectoine improves cell growth in media of different osmolarities. SMM of different osmolarities (20 ml culture in 100-ml Erlenmeyer flasks) was inoculated with 0.5 ml of an overnight culture of strain RMKB22 (OpuA$^-$ OpuB$^-$ OpuC$^+$ OpuD$^-$) grown in SMM. The cultures were grown at 37°C for 22 h without an osmoprotectant (●), in the presence of 1 mM ectoine (▼) or 1 mM glycine betaine (■).

growth yield of the cultures spectrophotometrically. A culture containing 1 mM of the osmoprotectant glycine betaine was used as a control. High osmolarity strongly impaired the growth of strain JH642 and this growth inhibition was relieved by both ectoine and glycine betaine. To determine in more detail the beneficial effect of ectoine on the proliferation of *B. subtilis* under elevated osmotic conditions, we performed growth studies with cultures of different osmolarities. The addition of more than 0.5 M NaCl to the minimal medium resulted in a gradual decrease in the growth yields of those cultures propagated in the absence of an osmoprotectant. The presence of 1 mM ectoine protected the cells from the deleterious effects of high osmolarity to a significant degree and permitted cell growth over a wide range of osmolarities. In direct comparison with ectoine, glycine betaine was the more effective osmoprotectant (Fig. 1).

### 3.2. Uptake of ectoine is mediated by the OpuC transport system

Since uptake systems for compatible solutes are often involved in the transport of more than one osmoprotectant, we tested a possible role for the glycine betaine (OpuA, OpuC, OpuD) and the choline (OpuB) transport systems in the uptake of ectoine. For these experiments we used an isogenic set of strains, each of which synthesised only one of the three glycine betaine uptake systems or the choline transporter OpuB. We grew the *B. subtilis* wild-type strain JH642 and its mutant derivatives in SMM with 1.2 M NaCl in the absence or presence of 1 mM ectoine or 1 mM glycine betaine and determined the growth yield of the cultures after 22 h at 37°C. Osmoprotection by ectoine occurred only in the wild-type strain JH642 and the OpuC$^+$ strain RMKB22 (OpuA$^-$ OpuB$^-$ OpuD$^-$). Loss of the OpuC activity in an otherwise wild-type background, strain RMKB25 (*opuC*::Tn*10*; OpuA$^+$ OpuB$^+$ OpuD$^+$), completely abolished osmoprotection by ectoine but not by glycine betaine, which can be

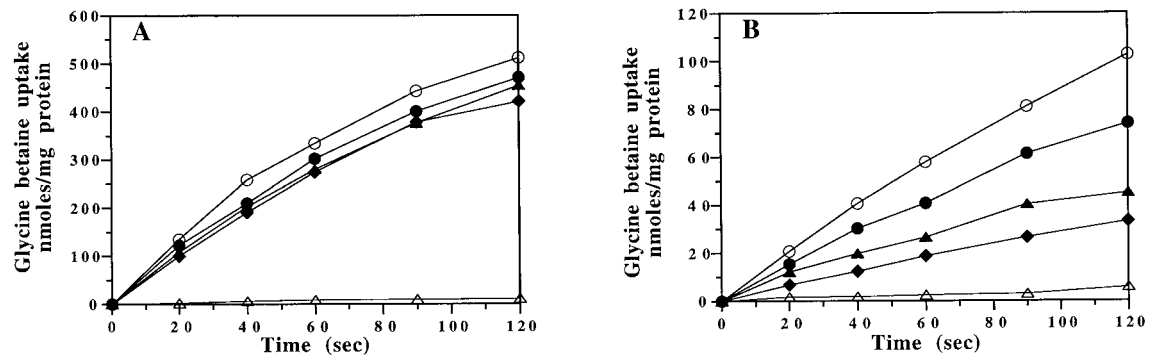


Fig. 2. Inhibition of glycine betaine uptake by ectoine. Strains (A) JH642 (OpuA$^+$ OpuB$^+$ OpuC$^+$ OpuD$^+$) and (B) RMKB22 (OpuA$^-$ OpuB$^-$ OpuC$^+$ OpuD$^-$) were assayed for [1-$^{14}$C]glycine betaine uptake at a final substrate concentration of 10 μM in the absence (○) or presence of a 100-fold (●), 500-fold (▲) or 1000-fold (◆) excess of unlabeled ectoine. Uptake activity for [1-$^{14}$C]glycine betaine in the presence of 100-fold unlabeled glycine betaine (△) was determined as a control.

accumulated via the OpuA and OpuD transport systems [8]. These results clearly identify the ABC-transport system OpuC as the only physiologically relevant uptake route for ectoine in *B. subtilis*.

### 3.3. Ectoine competes with glycine betaine uptake via OpuC

To analyse the entry of ectoine into *B. subtilis*, we performed competition experiments between unlabeled ectoine and radiolabeled glycine betaine. [1-$^{14}$C]Glycine betaine was used at a final substrate concentration of 10 μM, and ectoine was added to the transport assays in 100-, 500- and 1000-fold excess. Even a 1000-fold excess of ectoine over glycine betaine had very little effect on the initial glycine betaine uptake activity of the wild-type strain JH642 (Fig. 2A). In marked contrast, the accumulation of [1-$^{14}$C]glycine betaine by JH642 was completely blocked by the addition of a 100-fold excess of unlabeled glycine betaine (Fig. 2A). This lack of competition between ectoine and glycine betaine transport in a wild-type strain is readily understood since OpuA, the high-capacity and dominating glycine betaine transport system of *B. subtilis* [8], does not participate in ectoine uptake. As predicted from the pattern of osmoprotection afforded by ectoine for the various *B. subtilis* mutants, we found that the transport of radiolabeled glycine betaine via OpuC was inhibited by ectoine (Fig. 2B), whereas transport through the OpuA and OpuD systems was not affected (data not shown). A 100-, 500- or 1000-fold excess of ectoine resulted in the inhibition of the OpuC-mediated [1-$^{14}$C]glycine betaine transport by 27%, 53% and 68%, respectively (Fig. 2B).

To estimate the affinity of OpuC for ectoine, we measured the $K_i$ value for this osmoprotectant. We grew strain RMKB22 (OpuA$^-$ OpuB$^-$ OpuC$^+$ OpuD$^-$) in SMM with 0.4 M NaCl to mid-exponential phase and measured the uptake of [1-$^{14}$C]glycine betaine over a range of substrate concentrations (1–20 μM) in the absence or presence of a fixed concentration (5 mM) of the unlabeled competitor ectoine. In the absence of the inhibitor, we found a $K_m$ of 2.5 μM for the uptake of glycine betaine via OpuC, a value in agreement with the previously reported $K_m$ of 6 μM for the OpuC system [8]. In the presence of ectoine the apparent $K_m$ of the OpuC system was approximately 10.5 μM and from these kinetic data we calculated a $K_i$ value of 1.56 mM for ectoine. Hence, OpuC clearly functions in ectoine uptake, but it does not recognise this osmoprotectant with high affinity.

### 3.4. Accumulation of ectoine in the cytoplasmic pool of B. subtilis

We used $^1$H NMR spectroscopy to test if ectoine was present in the cytoplasmic solute pool of *B. subtilis*. Cells of strain RMKB22 (OpuC$^+$) were grown in SMM or SMM with 0.4 M NaCl in the absence or presence of 1 mM ectoine and crude ethanolic extracts of these cells were analysed by $^1$H NMR spectroscopy. In complete agreement with previous reports [1,3], we identified both glutamate and proline as major organic osmolytes in extracts prepared from cells grown in SMM or SMM with 0.4 M NaCl (Table 1). When the cells were grown in the presence of ectoine, this osmoprotectant was detected in the cytoplasmic solute pool of *B. subtilis* (Table 1) and ectoine was found in an unmodified form (data not shown). The accumulation of ectoine

Table 1
Accumulation of ectoine in the cytoplasmic pool of *B. subtilis*

| | SMM | | SMM with 0.4 M NaCl | |
|---|---|---|---|---|
| | no addition | +1 mM ectoine | no addition | +1 mM ectoine |
| Glutamate | 3189 | 2670 | 4580 | 4031 |
| Proline | 255 | 276 | 1410 | 885 |
| Ectoine | – | 442 | – | 753 |

The solutes are expressed in nmol mg protein$^{-1}$.
The amount of each compound present in the ethanolic cell extracts was determined by $^1$H NMR spectroscopy and calculated using the concentration of the added standard 2-methylpropanol (2). The values given represent the average of two independent experiments.

by cells grown in SMM is a reflection of the substantial transport activity exhibited by OpuC in standard laboratory minimal media [8].

### 3.5. Ectoine is not used as sole carbon or nitrogen source

To determine whether ectoine is metabolised by *B. subtilis* we tested its ability to serve as a carbon or nitrogen source. When the $OpuC^+$ strain RMKB22 was cultured for 24 h at 37°C in SMM with 10 mM ectoine as the sole carbon source, no growth was observed. Likewise, strain RMKB2 did not grow when 10 mM ectoine was used as nitrogen source in a modified SMM in which $(NH_4)_2SO_4$ had been replaced by $K_2SO_4$.

## 4. Discussion

The tetrahydropyrimidine ectoine is synthesised by a wide range of microorganisms [13] and is accumulated to high intracellular levels in response to osmotic stress [3,14–16]. The presence of an inducible, binding-protein-dependent transport system for ectoine in *Rhizobium meliloti* attests to the availability of this compound in the soil [12]. Many bacilli can produce ectoine but *B. subtilis* is unable to synthesise it [3]. The results presented in this study demonstrate that preformed ectoine can protect the non-producer *B. subtilis* from the detrimental effects of high osmolarity, allowing cell growth over a range of osmotic conditions (Fig. 1). The use of a set of strains with defined mutations in the glycine betaine transport systems OpuA, OpuC and OpuD [8] was critical for the identification of the *B. subtilis* ectoine uptake system. Loss of OpuC activity in an otherwise wild-type background completely abolished osmoprotection by ectoine thus pinpointing OpuC as the sole ectoine transporter of physiological relevance in *B. subtilis*.

The OpuC system is a binding-protein-dependent transporter [8,10] and a member of a large family of uptake systems in which ATP hydrolysis is coupled to solute accumulation [11]. OpuC exhibits a high affinity for glycine betaine [8] but it serves only as a low-affinity transport system for ectoine (Fig. 2B). When both ectoine and glycine betaine are accumulated by the cells via the OpuC system, ectoine is the less effective osmoprotectant (Fig. 1). This difference reflects the different kinetic parameters of the OpuC transporter for ectoine and glycine betaine rather than differences in the osmoprotective capacities of these compatible solutes per se. Effective ectoine transport systems are present in *Escherichia coli* and the plant pathogen *Erwinia chrysanthemi* and in these bacteria, ectoine has an osmoprotective capacity similar to that of glycine betaine [17,18]. Using $^1H$ NMR spectroscopy, we detected ectoine in the cytoplasmic solute pool of *B. subtilis* when this amino acid was present in the growth medium (Table 1) but ectoine was not used as sole carbon or nitrogen source. Our results thus identify ectoine as a metabolically inert stress compound for *B. subtilis* that is accumulated by the cell via the binding-protein-dependent transport system OpuC to cope with a high osmolarity environment.

## Acknowledgments

We thank E. Galinski for the initial supply of ectoine, R.M. Kappes for providing bacterial strains and B. Kempf for critically reading of the manuscript. We are grateful to J. Hamelin (University of Rennes) for his kind advice on the NMR spectroscopy. We appreciate the help of V. Koogle in preparing the manuscript. Financial support for this study was provided by the Max-Planck Society, the Deutsche Forschungsgemeinschaft through SFB-395 and the Graduiertenkolleg 'Enzymchemie' and the Fonds der Chemischen Industrie.

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