Molecular Microbiology (1997) **25**(1), 175–187

Osmostress response in *Bacillus subtilis*: characterization of a proline uptake system (OpuE) regulated by high osmolarity and the alternative transcription factor sigma B

Carsten von Blohn, Bettina Kempf, Rainer M. Kappes and Erhard Bremer*

Philipps University Marburg, Department of Biology, Laboratory for Microbiology, Karl-von-Frisch Str., D-35032 Marburg, Germany.

Summary

Exogenously provided proline has been shown to serve as an osmoprotectant in *Bacillus subtilis*. Uptake of proline is under osmotic control and functions independently of the known transport systems for the osmoprotectant glycine betaine. We cloned the structural gene (*opuE*) for this proline transport system and constructed a chromosomal *opuE* mutant by marker replacement. The resulting *B. subtilis* strain was entirely deficient in osmoregulated proline transport activity and was no longer protected by exogenously provided proline, attesting to the central importance of OpuE for proline uptake in high-osmolarity environments. The transport characteristics and growth properties of the *opuE* mutant revealed the presence of a second proline transport activity in *B. subtilis*. DNA sequence analysis of the *opuE* region showed that the OpuE transporter (492 residues) consists of a single integral membrane protein. Database searches indicated that OpuE is a member of the sodium/solute symporter family, comprising proteins from both prokaryotes and eukaryotes that obligatorily couple substrate uptake to Na^+ symport. The highest similarity was detected to the PutP proline permeases, which are used in *Escherichia coli*, *Salmonella typhimurium* and *Staphylococcus aureus* for the acquisition of proline as a carbon and nitrogen source, but not for osmoprotective purposes. An elevation of the osmolarity of the growth medium by either ionic or non-ionic osmolytes resulted in a strong increase in the OpuE-mediated proline uptake. This osmoregulated proline transport activity was entirely dependent on *de novo* protein synthesis, suggesting a transcriptional control mechanism. Primer extension analysis revealed the presence of two osmoregulated and tightly spaced *opuE* promoters. The activity of one of these promoters was dependent on sigma A and the second promoter was controlled by the general stress transcription factor sigma B.

Received 14 March, 1997; revised 6 May, 1997; accepted 12 May, 1997. *For correspondence. E-mail bremer@mailer.uni-marburg.de; Tel. (49) 6421 281529; Fax (49) 6421 288979.

Introduction

The intracellular accumulation of compatible solutes is of central importance for the defence of many microorganisms against the deleterious affects of high osmolarity (Csonka and Hanson, 1991; Galinski and Trüper, 1994; Lucht and Bremer, 1994). These organic osmolytes are polar, highly soluble molecules that comprise a limited number of amino acids, amino acid derivatives, sugars and polyols. In contrast to most ions, they do not interfere with vital cellular functions. Hence, they can be accumulated to high levels and consequently contribute to the maintenance of turgor within tolerable limits under conditions of low water activity. In addition, they protect the structure of proteins and cell components from denaturation in solutions of high ionic strength, an effect that appears to stem from their preferential exclusion from the hydration shell of macromolecules (Yancey, 1994). Important examples of such compatible solutes are the trimethylammonium compound glycine betaine and the amino acid proline. Both substances have been adopted by a wide variety of prokaryotic and eukaryotic organisms as effective osmoprotectants.

The Gram-positive bacterium *Bacillus subtilis* primarily colonizes the upper layers of the soil, a habitat that is subjected to frequent fluctuations in the supply of water (Miller and Wood, 1996). To cope with these osmotic changes and to ensure its survival and growth, *B. subtilis* uses several adaptive mechanisms. The initial response is the uptake of K^+, followed by the accumulation of large amounts of proline by *de novo* synthesis (Measures, 1975; Whatmore *et al.*, 1990; Whatmore and Reed, 1990). *B. subtilis* can also effectively use compatible solutes present in the environment, and glycine betaine plays a particularly important role (Boch *et al.*, 1994; Kappes *et al.*, 1996). Glycine betaine is accumulated via

three osmoregulated high-affinity uptake systems, which comprise both secondary and binding protein-dependent ABC transporters (Kempf and Bremer, 1995; Lin and Hansen, 1995; Kappes *et al*., 1996). In addition, *B. subtilis* can also synthesize glycine betaine from exogenously provided choline (Boch *et al*., 1994). A type III alcohol dehydrogenase and a glycine betaine aldehyde dehydrogenase act together to convert choline into glycine betaine enzymatically (Boch *et al*., 1996). Both uptake and synthesis of glycine betaine confer a considerable degree of osmotolerance.

In addition to these specific cellular adaptation mechanisms to high osmolarity, exposure of *B. subtilis* to high salt triggers the expression of a large general stress regulon (Boylan *et al*., 1993; Völker *et al*., 1994). Induction of this regulon also occurs in response to a wide variety of environmental challenges, including heat, acid shock, oxidative stress and starvation of nutrients (Hecker *et al*., 1996). The diverse inducing conditions suggest that the members of this regulon serve general and non-specific protective functions. It is, however, still unclear how the induced proteins protect *B. subtilis* from the detrimental effects of high osmolarity. Many members of this general stress regulon are under the control of the alternative transcription factor sigma B (σ^B) (Haldenwang, 1995; Hecker *et al*., 1996).

Here, we demonstrate that exogenously provided proline functions as an osmoprotectant in *B. subtilis*. We have identified a new member of the sodium/solute symporter superfamily as the transport system (OpuE) that directs proline uptake under high-osmolarity growth conditions. Expression of the *opuE* structural gene is under osmotic control, and different sigma factors contribute to its regulation.

Results

Exogenously provided proline functions as an osmoprotectant

B. subtilis accumulates large amounts of proline through *de novo* synthesis when subjected to high-osmolarity stress (Whatmore *et al*., 1990). To assess whether exogenously provided proline would also protect the cells from the detrimental effects of high osmolarity, we grew the wild-type *B. subtilis* strain JH642 in Spizizen's minimal medium (SMM) in the presence or absence of 1.2 M NaCl. The high-osmolarity growth conditions strongly impaired the proliferation of strain JH642, but the addition of 1 mM proline alleviated this growth inhibition to a significant degree (Fig. 1). Hence, proline functions as an osmoprotectant, but, in direct comparison with glycine betaine, proline is clearly less effective (Fig. 1). Proline uptake in high-osmolarity stressed *Escherichia coli* and *Salmonella typhimurium* cells is mediated by the ProP and ProU

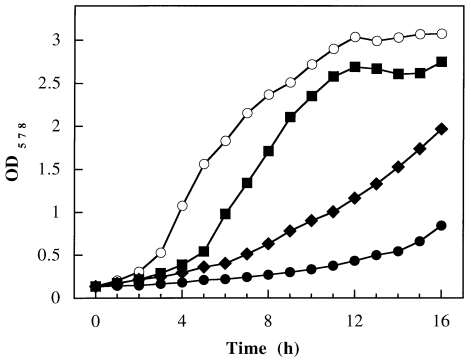


Fig. 1. Proline functions as an osmoprotectant. The *B. subtilis* strain JH642 was grown in SMM (○) and SMM with 1.2 M NaCl (●) in the presence of either 1 mM proline (♦) or 1 mM glycine betaine (■). The cultures (70 ml) were inoculated from an overnight culture pregrown in SMM and were grown in 500-ml Erlenmeyer flasks in a shaking water bath (200–220 r.p.m.) at 37°C.

glycine betaine transporters (Csonka and Hanson, 1991; Lucht and Bremer, 1994). We therefore tested whether any of the three glycine betaine uptake systems operating in *B. subtilis* (Kappes *et al*., 1996) would also mediate proline accumulation. However, we found that the degree of osmoprotection conferred by exogenously provided proline was the same in a wild-type strain and a mutant entirely deficient in glycine betaine uptake (data not shown). This finding indicated that proline transport under high-osmolarity growth conditions is mediated by an uptake system distinct from the *B. subtilis* glycine betaine transporters.

Proline uptake is osmotically controlled

To test whether proline transport was regulated in response to the environmental osmolarity, we measured the initial proline uptake in cells of JH642 grown to exponential phase in SMM or SMM with 0.4 M NaCl at a final substrate concentration of 10 μM. Uptake of radiolabelled proline was low in those cells grown in SMM, whereas proline transport was strongly stimulated in cells propagated in SMM with 0.4 M NaCl (Fig. 2A). Addition of the non-ionic osmolyte sucrose to the growth medium triggered an increase in proline uptake virtually identical to that observed in the presence of an equiosmotic concentration of NaCl (Fig. 2A). Hence, the stimulation of proline transport in *B. subtilis* is a true osmotic effect. In contrast, there was no significant stimulation of proline uptake when the osmolarity of the growth medium was raised by the addition of glycerol (Fig. 2A). This polyol is membrane permeable at high concentrations and is thus not able to establish an osmotically effective concentration gradient across the cytoplasmic membrane.

The increased proline transport observed in *B. subtilis* cells grown at high osmolarity could be caused by the

osmotic activation of pre-existing transport components, could depend on *de novo* protein synthesis, or could be a combination of both mechanisms. To distinguish between these possibilities, we subjected cultures of strain JH642 grown in SMM to a sudden osmotic upshock with 0.4 M NaCl in the absence or presence of the protein synthesis inhibitor chloramphenicol. The sudden rise in the osmolarity of the growth medium triggered a rapid and strong increase in the initial rate of proline uptake (Fig. 2B). This stimulation was entirely dependent on *de novo* protein synthesis, since the presence of chloramphenicol before the osmotic upshock prevented the osmotic induction completely (Fig. 2B). Taken together, these data strongly suggest that the expression of the structural gene(s) for this proline transport system of *B. subtilis* is controlled by the osmolarity of the environment.

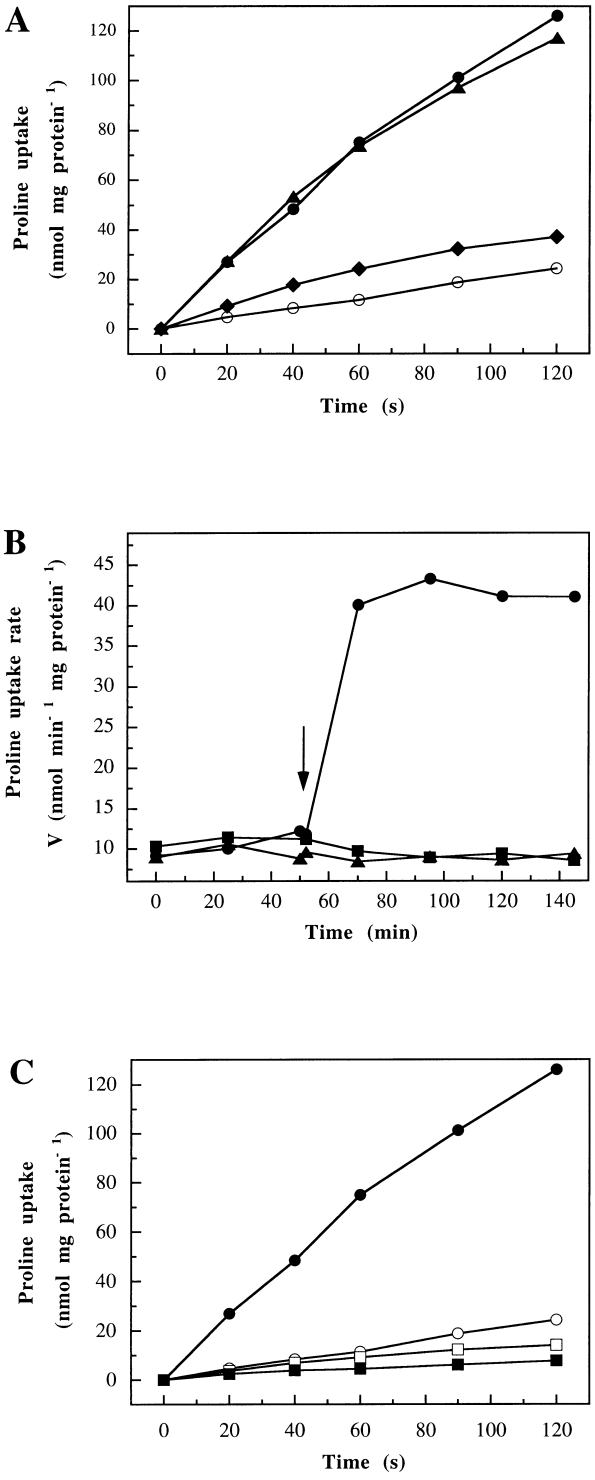


Fig. 2. Characteristics of proline uptake. The uptake of L-[^{14}C]-proline at a final substrate concentration of 10 μM was assayed in cultures of the *B. subtilis* strains grown in high- or low-osmolarity minimal medium (SMM). The cells were harvested at mid-exponential growth phase ($OD_{578} = 0.3–0.4$) and used immediately for the transport assays.
A. The *opuE*$^+$ strain JH642 was assayed for proline uptake in SMM (340 mosmol kg^{-1}) (○) or in SMM whose osmolarity had been increased to 1100 mosmol kg^{-1} by the addition of either 0.4 M NaCl (●), 0.67 M sucrose (▲) or 1.1 M glycerol (♦).
B. An exponentially growing culture (SMM) of strain JH642 (*opuE*$^+$) was divided into three portions. One culture was treated with 100 μg ml^{-1} chloramphenicol at time zero, two cultures were subjected to a sudden osmotic upshock (arrow) after 50 min by adding NaCl to a final concentration of 0.4 M NaCl. Symbols: ■, no addition; ●, addition of 0.4 M NaCl; ▲, addition of 0.4 M NaCl and chloramphenicol.
C. Cultures of strain JH642 (*opuE*$^+$) (○, ●) and BLOB9 (*opuE*) (□, ■) were assayed for proline uptake in SMM (○, □) and SMM with 0.4 M NaCl (●, ■).

Cloning of the gene encoding the osmoregulated proline transporter

The gene encoding the osmoregulated proline transporter was cloned by chance during our attempts to identify the genes for the glycine betaine uptake systems from *B. subtilis* by functional complementation of an *E. coli* mutant (Kempf and Bremer, 1995; Kappes *et al.*, 1996). Strain MKH13 (*putPA proP proU*) is defective for both glycine betaine and proline uptake (Haardt *et al.*, 1995), and this *E. coli* mutant was transformed with various plasmid-encoded *B. subtilis* gene libraries. The resulting transformants were screened for colonies that could grow on high-osmolarity minimal agar plates (MMA with 0.8 M NaCl) containing 5 mM glycine betaine. In addition to plasmids carrying the structural genes for the glycine betaine transport systems OpuA and OpuD (Kempf and Bremer, 1995; Kappes *et al.*, 1996), we recovered from this selection a group of colonies that could grow on these plates in the presence of glycine betaine but not in its absence. In contrast to the *opuA*$^+$ and *opuD*$^+$ plasmids, there was no detectable transport activity for radiolabelled glycine betaine (final substrate concentration 10 μM) in any of these strains. However, we discovered that these cells

exhibited uptake of radiolabelled proline even at very low external substrate concentration (10 μM). It thus appeared that these recombinant strains produced proline uptake systems that also permitted low-level glycine betaine accumulation as a side-reaction when the *E. coli* cells were subjected to a long-term osmotic stress in the presence of considerable amounts (5 mM) of glycine betaine.

The plasmids recovered from this group of strains (27 isolates) all carried chromosomal inserts that were related to each other, as judged from their restriction pattern and DNA hybridization experiments. Three plasmids (Fig. 3) were chosen for further analysis, and we demonstrated by Southern hybridization that their inserts originated from chromosomal DNA of *B. subtilis* (data not shown). The size (1.9 kb) of the *B. subtilis* DNA present in pORT2 (Fig. 3) suggested that the encoded transporter was a single-component system. We designate its structural genes as *opuE* (osmoprotectant uptake) and refer in the following to its gene product as the OpuE protein.

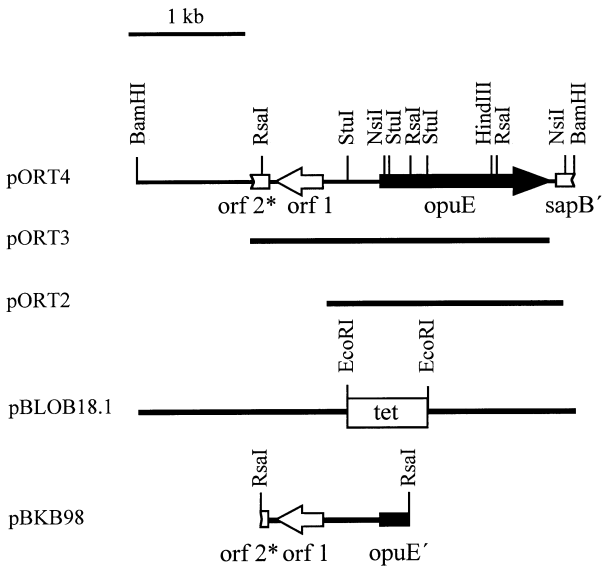


Fig. 3. Genetic organization of the *opuE* region. A restriction map of the chromosomal fragment present in plasmid pORT4 and the genetic organization of the *opuE-sapB* region is shown. Plasmid pORT4 was isolated from a gene library of chromosomal *Bam*HI fragments inserted into the low-copy-number vector pHSG575. Plasmids pORT2 and pORT3 were recovered from gene libraries of partially *Sau*3A-digested chromosomal *B. subtilis* DNA inserted into the unique *Bam*HI site of plasmid pACYC177; no *Bam*HI site was restored by the insertion of the genomic DNA segments. Plasmid pBLOB18.1 carries a tetracycline resistance cassette (not drawn to scale) replacing two adjacent *Stu*I fragments from pORT4 and was used to isolate the chromosomal Δ(*opuE::tet*)*1* mutation present in strain BLOB9. The *Stu*I sites were destroyed during the *in vitro* manipulations and replaced by *Eco*RI sites. Plasmid pBKB98 is an *E. coli–B. subtilis* shuttle vector and harbours a 1091-bp *Rsa*I fragment carrying the *opuE* promoter region.

DNA sequence analysis and map location of opuE

We determined the nucleotide sequence of a 2781-bp DNA segment from plasmid pORT4 (Fig. 3). Inspection of the DNA sequence (accession number U92466) revealed the presence of a long open reading frame, which represents the *opuE* coding region. The GTG start codon of this reading frame is preceded at an appropriate distance by a DNA sequence complementary to the 3′ end of the 16S RNA of *B. subtilis* and is thus likely to function as the ribosome-binding site of *opuE*. We found a divergently oriented small open reading frame (*orf*1; Fig. 3) upstream of *opuE* whose gene product shows sequence identity to putative proteins with unknown functions from *Synechocystis* spp. and *Staphylococcus aureus*. The *orf*1 reading frame is immediately followed by an incomplete open reading frame (*orf*2*; Fig. 3) whose deduced protein sequence shows similarity to amidases from a variety of organisms (data not shown). Downstream of the *opuE* stop codon, there is an inverted repeat (ΔG [25°C] = −67.8 kJ) that could possibly function as a factor-independent transcription terminator. This stem-loop structure is followed by the beginning of an open reading frame representing the *sapB* gene. Whalen and Piggot (1997) have recently determined the entire nucleotide sequence of *sapB* and, for the region of overlap (724 bp), the two sequences agree completely. Mutations in *sapB* have been genetically located near 56° on the genetic map of *B. subtilis* (Biaudet *et al.*, 1996), thus positioning *opuE* at this map location as well. Our DNA sequence has recently been used in the course of the *B. subtilis* genome sequencing project to place the *opuE* gene at position 754 kb on the physical map (R. Borriss, personal communication; Borriss *et al.*, 1996).

Features of the OpuE protein

The *opuE* gene encodes a 492-residue protein with a calculated molecular mass of 53.26 kDa. A hydrophobicity plot of OpuE showed that it is a hydrophobic polypeptide and revealed an alteration of hydrophilic and hydrophobic segments characteristically found in integral cytoplasmic membrane proteins (Henderson and Maiden, 1989). OpuE has a strongly charged and highly hydrophilic carboxy-terminal end: of its 22 residues, one is negatively charged and nine are positively charged. The BLAST algorithms (Altschul *et al.*, 1990) were used to search the databases for proteins homologous to OpuE. These searches revealed a striking sequence identity of OpuE to proteins from the sodium/solute symporter family. This superfamily comprises proteins from both prokaryotes and eukaryotes that mediate the symport of Na^+ with a variety of substrates, including pantothenat, nucleosides, myo-inositol, glucose and amino acids (Reizer *et al.*, 1994). In

full agreement with our physiological characterization of the *B. subtilis* OpuE protein as a proline transporter, we found in our database searches the highest sequence identity to a number of proline transport proteins (PutP) from both Gram-negative and Gram-positive bacteria. The sequence identity extends over the entire length of these polypeptides, and the proteins homologous to OpuE comprise a predicted proline permease from *Haemophilus influenzae* (48% identity) and the PutP proline uptake system from *E. coli* (50% identity), *S. typhimurium* (50% identity) and *Staphylococcus aureus* (55% identity). The alignment of these protein sequences shows that each of these polypeptides is of similar size, displays a closely related hydrophobicity profile and probably exhibits a similar membrane topology. Symport of proline and Na^+ has been shown directly for the PutP permeases of *E. coli* and *S. typhimurium* (Cairney *et al*., 1984; Chen *et al*., 1985; Myers and Maloy, 1988; Wood, 1988). The *B. subtilis* OpuE protein and each of its homologues feature the proposed ion-binding (SOB) motif (Deguchi *et al*., 1990). Both genetic studies and chemical modification experiments (Hanada *et al*., 1992) have suggested that Cys-344 of the *E. coli* PutP protein is functionally involved in both proline and cation binding. One would therefore expect that this Cys residue is evolutionarily highly conserved within the group of bacterial Na^+/proline symporters, but this is not the case. We note that the *B. subtilis* OpuE protein is devoid of cysteine residues.

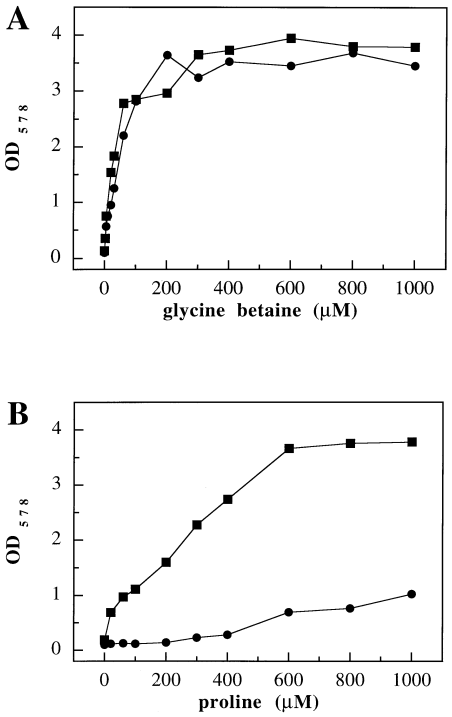


Fig. 4. Osmoprotection by proline is dependent on OpuE. Strains JH642 (opuE$^+$) (■) and its *opuE* mutant derivative strain BLOB9 (●) were grown in SMM with 1.2 M NaCl in the presence of various concentrations of (A) glycine betaine or (B) L-proline on a shaker platform at 37°C. The growth yield of the cultures (20 ml in 100-ml Erlenmeyer flasks) was determined spectrophotometrically (OD_{578}) after 24 h.

An opuE *mutant strain lacks the osmotically regulated proline transport activity*

To prove that OpuE was responsible for the osmotically controlled proline uptake in *B. subtilis*, we isolated an *opuE* mutant derivative of the wild-type strain JH642 and assayed its proline transport activity. For this purpose, we first constructed a plasmid-encoded mutant *opuE* gene [Δ(*opuE*::*tet*)*1*], in which the entire regulatory region and part of the coding sequence is replaced by a tetracycline resistance cassette (Fig. 3). The defective *opuE* gene was then inserted into the chromosome of JH642 by marker replacement, resulting in the isolation of strain BLOB9 [Δ(*opuE*::*tet*)*1*]. This mutant lacked the osmotically stimulated proline activity entirely (Fig. 2C). However, in SMM, strain BLOB9 showed a basal rate of proline uptake similar to that of the wild-type strain JH642 (Fig. 2C). Thus, in addition to the osmoregulated OpuE system, at least one other proline transporter is operating in *B. subtilis*. In agreement with this conclusion, we found that the *opuE* mutant BLOB9 can still use proline as the sole nitrogen, carbon and energy source (data not shown).

To test whether the OpuE system mediated osmoprotection by exogenously provided proline, we grew strains JH642 (*opuE*$^+$) and BLOB9 (*opuE*) under high-osmolarity conditions (SMM with 1.2 M NaCl) in the presence of various amounts of proline or glycine betaine (0–1 mM). We then determined the growth yield of these cultures after 24 h (Fig. 4). Both strains were protected by glycine betaine, but only the *opuE*$^+$ strain could grow efficiently in the high-osmolarity medium in the presence of proline. The properties of the *opuE* mutant thus show that the OpuE system of *B. subtilis* both mediates osmoregulated proline transport (Fig. 2) and is essential for the use of proline present in the environment as an osmoprotective compound (Fig. 4).

Characteristics of the OpuE proline transport system

We determined the kinetic parameters for proline uptake in the *opuE*$^+$ strain JH642 and its *opuE* derivative BLOB9. For these experiments, we prepared overnight cultures of both strains in SMM, inoculated the precultures into fresh SMM and SMM with 0.4 M NaCl to induce the OpuE system, and grew the cells for approximately 4 h to reach mid-exponential phase ($OD_{578} = 0.3–0.4$). The initial velocity of L-[^{14}C]-proline uptake in these cultures was then determined over a range of substrate concentrations (1–40 μM). L-[^{14}C]-proline uptake in both JH642 and

BLOB9 showed saturation kinetics. The proline uptake system operating in the *opuE* mutant strain BLOB9 exhibits high affinity for its substrate but shows a moderate transport capacity both in SMM ($K_m = 5.4\ \mu M$; $V_{max} = 19\ nmol\ min^{-1}\ mg^{-1}$ protein) and in SMM with 0.4 M NaCl ($K_m = 4\ \mu M$; $V_{max} = 20\ nmol\ min^{-1}\ mg^{-1}$ protein). As expected, the *opuE*$^+$ strain JH642 also displayed high affinity for proline ($K_m = 5.4\ \mu M$ in SMM and 5.5 μM in SMM with 0.4 M NaCl). However, the V_{max} was influenced by the osmolarity of the medium: it increased from $30\ nmol\ min^{-1}\ mg^{-1}$ protein in SMM-grown cells to $92\ nmol\ min^{-1}\ mg^{-1}$ protein in cells grown in SMM with 0.4 M NaCl.

To characterize the osmotically modulated proline uptake in *B. subtilis* further, we subjected cultures of strains JH642 and BLOB9 to a long-term osmotic stress by growing them for approximately 20 h to allow the cells to adapt fully to high-osmolarity environments. We also varied the osmolarity of the growth medium between 340 mosmol kg^{-1} (SMM) and 2240 mosmol kg^{-1} (SMM with 1 M NaCl) in these cultures to determine the activity profile of the *B. subtilis* proline transport systems. By elevating the osmolarity of the growth medium from 340 mosmol kg^{-1} (SMM) to 1480 mosmol kg^{-1} (SMM with 0.6 M NaCl), the initial rate of L-[^{14}C]-proline uptake in the wild-type strain JH642 was stimulated approximately fourfold in response to the rise in osmolarity of the growth medium. Further increases in the external osmolarity led to a reduction in proline transport, resulting in a distinctive activity profile (Fig. 5). Proline uptake in *B. subtilis* in high-osmolarity environments is primarily determined by the characteristics of the OpuE system. This is evident from the proline transport activity

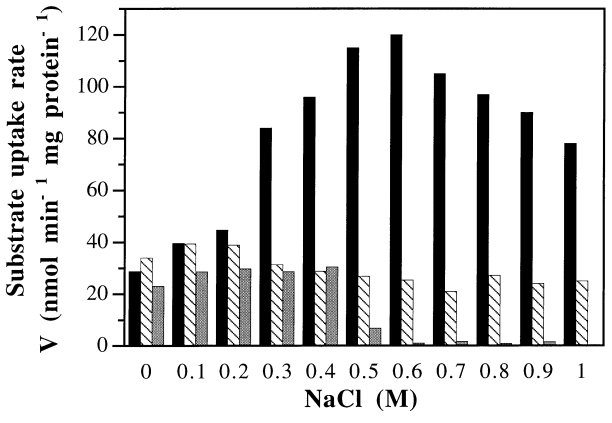


Fig. 5. Transport of radiolabelled proline and glutamate in osmotically adapted cells. The initial uptake rate of L-[^{14}C]-proline in strain JH642 (*opuE*$^+$) (black columns) and strain BLOB9 (*opuE*) (grey columns) was assayed in log-phase cells from cultures adapted to various osmolarities. The osmolarity of the SMM was changed by adding various amounts of NaCl. L-[^{14}C]-glutamate uptake (stippled columns) was assayed as a control in strain JH642.

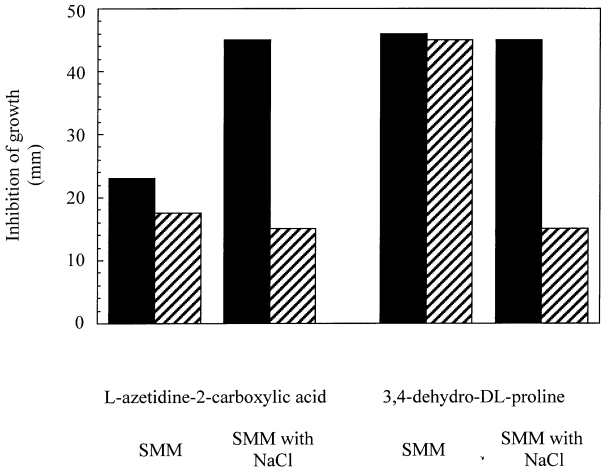


Fig. 6. Sensitivity against toxic proline analogues. Growth inhibition by the proline analogues L-azetidine-2-carboxylic acid (AC) and 3,4-dehydro-DL-proline (DHP) was tested by placing a paper filter disk (5 mm) impregnated with 10 μl of a stock solution (12.5 mg ml^{-1}) of either AC or DHP onto lawns of strains JH642 (*opuE*$^+$) (black columns) or BLOB9 (*opuE*) (stippled columns). The plates were incubated at 37°C for 2 days, and the zone of growth inhibition was then recorded on several plates.

found in the *opuE* mutant strain BLOB9. Proline transport operated at a modest level in unstressed cells and was not significantly influenced by increases in medium osmolarity up to 1100 mosmol kg^{-1} (SMM with 0.4 M NaCl). It was then reduced practically to background levels by further increases in the external osmolarity (Fig. 5). Changes in medium osmolarity did not influence amino acid uptake in *B. subtilis* indiscriminately: there was no significant effect of high osmolarity on L-[^{14}C]-glutamate transport in strain JH642 (Fig. 5).

In *E. coli* and *S. typhimurium*, the three proline uptake systems, PutP, ProP and ProU, show different sensitivities to the toxic proline analogues, L-azetidine-2-carboxylic acid (AC) and 3,4-dehydro-DL-proline (DHP), in response to the osmolarity of the growth medium (Csonka, 1982; Grothe *et al.*, 1986). To investigate whether OpuE was also involved in the uptake of these toxic proline analogues, we tested the sensitivity of the wild-type strain JH642 and its *opuE* mutant derivative BLOB9 on SMM and SMM with 0.4 M NaCl agar plates, using filter disks impregnated with either AC or DHP solutions. An increase in the osmolarity resulted in increased sensitivity towards AC in the wild-type strain but not in the *opuE* mutant (Fig. 6). The wild-type strain JH642 was highly sensitive against DHP at both low and high osmolarity. DHP sensitivity at high osmolarity, but not at low osmolarity, was largely dependent on the presence of OpuE (Fig. 6). Hence, these data demonstrate the uptake of both AC and DHP in *B. subtilis* via the osmoregulated OpuE system and reflect the presence of additional transporters for these toxic proline analogues.

Identification of the opuE *transcription initiation sites*

To identify the *opuE* promoter(s), we mapped the transcription initiation site(s) by primer extension analysis. For these experiments, we inserted a 1091-bp *Rsa*I restriction fragment carrying the entire *opuE* upstream region and part of its structural gene (Fig. 3) into the *E. coli–B. subtilis* shuttle vector pRB373, which yielded plasmid pBKB98. Total RNA was prepared from JH642 (pBKB98) grown to mid-exponential phase in SMM or SMM with 0.4 M NaCl. An *opuE*-specific primer labelled at its 5′ end with an infrared dye was annealed to the RNA and extended with reverse transcriptase. The reaction products were then analysed using an automatic DNA sequencer. We detected two *opuE*-specific mRNA species whose production was under osmotic control (Fig. 7A). Inspection of the DNA sequence (Fig. 7B) upstream of the initiation site for the shortest transcript (*opuE*-1) revealed the presence of putative −10 and −35 sequences that resemble the consensus sequence of promoters recognized by the main vegetative sigma factor (σ^A) of *B. subtilis* (Helmann, 1995). The −10 and −35 sequences of the *opuE*-P1 promoter are separated by 17 bp, a typical spacing for promoters recognized by σ^A. The *opuE*-P1 promoter directed the production of two mRNA species that differed by one nucleotide; the main transcription initiation site corresponds to an A residue at 869 bp (Fig. 7B).

No DNA sequences are present upstream of the *opuE*-2 transcript that resemble a σ^A-dependent promoter. However, this DNA region (*opuE*-P2) contains an appropriately spaced DNA segment with a high degree of homology to promoters controlled by the alternative transcription factor sigma B (σ^B) (Fig. 7C), suggesting that the synthesis of this *opuE* transcript was under σ^B control. Indeed, the *opuE*-2 transcript was no longer produced when the RNA was prepared from the *sigB* mutant strain BLOB22 (pBKB98), whereas the σ^A-dependent *opuE* mRNA was still made in the full amount and in an osmoregulated fashion (Fig. 7A). In addition to the *opuE*-P1- and *opuE*-P2-directed mRNAs, we detected a weak *opuE* transcript (Fig. 7A), but its initiation site is not preceded by sequences that resemble any of the established consensus sequences for *B. subtilis* promoters (Haldenwang, 1995). In contrast to the σ^A- and σ^B-dependent *opuE* transcripts, its production was highly variable in different RNA preparations.

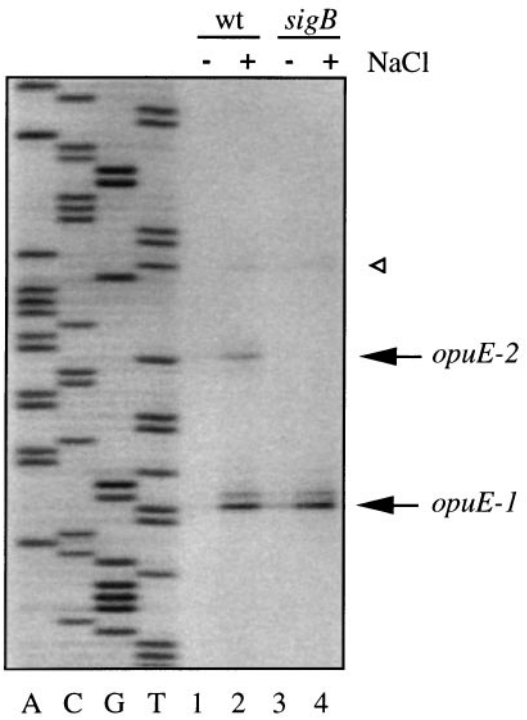
The involvement of σ^B in *opuE* expression prompted us to test the influence of a *sigB* deletion on the OpuE-dependent proline transport and the use of exogenously provided proline as an osmoprotectant. There was no difference in the initial rate of proline uptake between strain JH642 and its *sigB* derivative, BLOB22, in cells grown in either SMM or SMM with 0.4 M NaCl. Consequently, proline was also able to protect both strains from the detrimental effects of high osmolarity equally (data not shown). Thus, the σ^A-dependent *opuE*-P1 promoter can apparently mediate *opuE* transcription in high-osmolarity grown cultures at a level that is fully sufficient for the acquisition of proline as an osmoprotectant.

Discussion

The pioneering work of Measures (1975) demonstrated that *B. subtilis* accumulates large amounts of proline as a central stress response when the cells are grown in high-osmolarity rich media. Analysis of proline biosynthesis in defined media revealed a strong increase in *de novo* synthesis of this amino acid subsequent to an osmotic upshock (Whatmore *et al.*, 1990). Our data show that *B. subtilis* can also use exogenously provided proline as an osmoprotectant. Hence, the originally observed intracellular accumulation of proline in high-osmolarity rich media (Measures, 1975) results from both increased synthesis and uptake from the environment. We carried out a physiological and genetic characterization of the transport system (OpuE), which mediates proline uptake for osmoprotective purposes, and identified it as an osmoregulated member of the sodium/solute symporter family. After exposure to osmotic stress, many plants accumulate large quantities of proline through *de novo* synthesis from glutamate (Peng *et al.*, 1996). Substantial amounts of proline are released into the environment by wilting plants (Kemble and Macpherson, 1954), and decaying plant tissues are thus probably the major source for this osmoprotectant in the natural habitat of *B. subtilis*. Since the amount of proline is probably highly variable in the soil, effective uptake systems are necessary to ensure an appropriate supply for the osmotically stressed *B. subtilis* cells. The high affinity of the OpuE system for its substrate, its substantial transport capacity and the osmotic induction of the transcription of its structural gene make this transporter well suited for such a physiological task.

Exogenously provided proline is used by a wide spectrum of Gram-negative and Gram-positive bacteria as an osmoprotectant (Csonka and Hanson, 1991; Galinski and Trüper, 1994). Two proline uptake systems with different affinities are present in *S. aureus*, a food-borne pathogen phylogenetically closely related to the soil bacterium *B. subtilis* (Townsend and Wilkinson, 1992; Pourkomailian and Booth, 1994; Wengender and Miller, 1995). The high-affinity system is apparently used primarily for the scavenging of proline for catabolic purposes, whereas the low-affinity transporter is an osmotically activated proline uptake system, which also mediates transport of glycine betaine. In the Gram-negative enterobacteria, *E. coli* and *S. typhimurium*, uptake of proline for osmoprotective purposes is mediated by the osmoregulated glycine betaine transport systems, ProP and ProU (Wood, 1988;

A



B

```
                                                        opuE-2          opuE-1
811  TTATGATCAATGTTTCATCGCTTGAATGGCCGGGAATACTTTGTTAGGTTAAGTTACCAAGTGCACCCGC
                -35        -35        -10          -10

881  AAAAGAATTTCTTTTGTTTTGCACTTACGCCAGGCTGCTTAACTGGCACCATCAATTTGAGAGGTGATGC

951  AACATTATTAGAAATGAAGAGAGGGTAAAACGTGAGTATTGAAATTATTATATCTTTAGGAATTTACTTT
                opuE              M  S  I  E  I  I  I  S  L  G  I  Y  F
```

C

	-35		-10		
CAAT	GTTT	CATCGCTTGAATGGCC	GGGAAT	ACT	opuE
TGGT	GATT	GATTTTGGCTGAAAAG	GGGTAT	GGT	csbA
AAAG	GTTT	AACGTCTGTCAGACGA	GGGTAT	AAA	sigB
CGAG	GTTT	AAATCCTTATCGTTAT	GGGTAT	TGT	ctc
ACAG	GTTT	AAACGACTTTAAAAAA	AGGAAT	AGC	csbB
AAAT	GTGT	AAAAACATATTGAAAA	GGGTAA	ATA	gtaB
ACGT	GTTT	ATTTTTTTGAAAAA	GGGTAT	GTA	gspA
AGCA	GTTT	ATATGAAGAACGCCAC	GGGTAA	ATG	katE
TCAG	GTTT	TGTGGACCGGGAAAA	TGGAAA	TAA	clpC
GTTT	GTTT	AAAAGAATTGTGAGC	GGGAAT	ACA	gsiB
ACAT	GTTT	ATGATTGAAGAAAAC	GGGTAA	ACA	csb40

Csonka and Hanson, 1991; Lucht and Bremer, 1994). Glycine betaine transporters related to the ProU system operate in *B. subtilis* (Kempf and Bremer, 1995; Lin and Hansen, 1995; Kappes *et al.*, 1996), and one would therefore have expected the involvement of these systems in proline uptake as well. However, our data show that these transporters play no physiologically significant role in proline uptake. Loss of OpuE in an otherwise wild-type background completely eliminated the osmotically stimulated proline transport activity and practically abolished the ability to use this amino acid as an osmoprotectant. Thus, genetic, physiological and transport studies unambiguously establish the central role of OpuE in the acquisition of proline for osmoprotective purposes. Expression of *opuE* is under osmotic control, and OpuE activity is not enhanced in response to increased osmotic pressure. The *B. subtilis* OpuE system shares these features with OusA, a ProP-related proline transporter from the plant pathogen, *Erwinia chrysanthemi*. However, in contrast to OpuE, the OusA transporter is also involved in glycine betaine uptake (Gouesbet *et al.*, 1996).

OpuE is closely related in sequence to the PutP proteins from *E. coli* (50% identity), *S. typhimurium* (50% identity) and *S. aureus* (55% identity). This is surprising, since these transporters mediate proline uptake when this amino acid is used as a carbon, nitrogen or energy source, but are not involved in the acquisition of proline for osmoprotective purposes (Wood, 1988; Wengender and Miller, 1995). PutP activity of *E. coli* and *S. typhimurium* is actually reduced when the cells are grown under high-osmolarity conditions (Wood, 1988). In striking contrast, OpuE activity is strongly enhanced in hypertonic media, highlighting the distinct physiological functions of the PutP and OpuE proteins. The *opuE* mutant strain, BLOB9, is still sensitive against toxic proline analogues and can use proline as a sole carbon and nitrogen source. These characteristics demonstrate the presence of at least one additional proline uptake system in *B. subtilis*. Transport studies with BLOB9 uncovered the activity of such a system, and we observed that this transporter was inhibited by high osmolarity. Hence, the previously detected high-affinity ($K_m = 2.3\,\mu M$) proline transport in *B. subtilis* (Ordal *et al.*, 1978) clearly reflects the activity of at least two transport systems. Recent data from the *B. subtilis* genome sequencing project (Yamane *et al.*, 1996) give a clue to the nature of this second proline uptake system, since the predicted product of the *ycgO* reading frame exhibits 63% identity to the *B. subtilis* OpuE transporter. The involvement of YcgO in proline metabolism is further suggested by the proximity of *ycgO* to a reading frame (*ycgN*) that encodes a protein with significant identities to a portion of PutA, an enzyme mediating the first steps in proline degradation in *E. coli* and *S. typhimurium*. Because a similar genetic organization is also found for the *putPA* genes in *E. coli* and *S. typhimurium* (Wood, 1988), it is tempting to speculate that the *B. subtilis* YcgO and YcgN proteins serve in the acquisition and catabolic degradation of proline. No genes with obvious functions in the catabolism of proline are located in the vicinity of *opuE*. It is thus apparent that closely related proline transport proteins have been tailored in the course of evolution for different physiological functions by endowing their structural genes with special regulatory patterns.

Transcription of the *opuE* structural gene is sensitively adjusted to the osmolarity of the environment and, consequently, OpuE activity is strongly enhanced after an osmotic upshock. Since this increase in proline uptake is entirely dependent on *de novo* protein synthesis, the activity of the OpuE protein is not stimulated by elevated osmolarity. Mapping of the initiation sites for the *opuE* transcripts by high-resolution primer extension reactions revealed the presence of two different *opuE* mRNA species whose amount is regulated in response to the osmolarity of the environment. In some experiments, we detected the production of a third mRNA species, but its transcription initiation site is not preceded by sequences that resemble any of the established consensus sequences for *B. subtilis* promoters (Haldenwang, 1995), and its physiological relevance is thus unclear.

The osmoregulated *opuE*-P1 and *opuE*-P2 promoters are recognized by the housekeeping sigma factor σ^A and the alternative transcription factor σ^B respectively. Such a dual control of gene expression has already been found for a number of σ^B-responsive genes (Akbar and Price, 1996; Krüger *et al.*, 1996; Varón *et al.*, 1993; 1996), indicating that many members of the *B. subtilis* general stress regulon are part of a redundantly regulated system (Hecker *et al.*, 1996). The σ^B-dependent control of

Fig. 7. The *opuE* transcription initiation sites.
A. Total RNA was prepared from cells of the wild-type (*sigB*$^+$) strain JH642 (pBKB98) and its *sigB* mutant derivative BLOB22 (pBKB98) grown in either SMM (−) or SMM with 0.4 M NaCl (+). The transcription initiation sites were identified by primer extension analysis. The positions of the σ^A- and σ^B-dependent *opuE*-1 and *opuE*-2 transcripts are marked by arrows, and the transcript originating from an unknown promoter is indicated by a triangle.
B. Nucleotide sequence of the *opuE* promoter region. The transcription initiation sites for the two *opuE* mRNA species are indicated by arrows. The −10 and −35 sequences of the σ^A-dependent promoter *opuE*-P1 are underlined, and those of the σ^B-dependent *opuE*-P2 promoter are boxed. The *opuE* start codon is boxed and the potential ribosome-binding site is underlined.
C. Sequences of σ^B-dependent *B. subtilis* promoters. The DNA sequence of these promoters was compiled from the literature (Akbar and Price, 1996; Hecker et al., 1996; Varón et al., 1996).

opuE-P2 activity links the OpuE-mediated uptake of an osmoprotectant to the general stress regulon of *B. subtilis*. Although the addition of salt triggers enhanced transcription of many genes of the σ^B regulon (Hecker *et al.*, 1996), no clear function in osmoadaptation has emerged for any of its members (Varón *et al.*, 1993). OpuE is thus the first example of a σ^B-responsive gene with a demonstrated physiological role in the adaptation process of *B. subtilis* to a high-osmolarity environment. However, σ^B is dispensable for the induction of the OpuE system under high-osmolarity growth conditions, indicating that the activity of the σ^A-dependent *opuE*-P1 promoter is sufficient for the overall osmotic control of *opuE*. Apparently, two independent signal transduction pathways operate in *B. subtilis* to control the level of *opuE* expression osmotically. The *opuE* gene might thus serve as a useful model system for unravelling the mechanism of osmosensing in *B. subtilis* and the processing of this information by the cells into a genetic signal that governs gene expression in response to a changing environment.

Experimental procedures

Media, growth conditions and chemicals

E. coli and *B. subtilis* strains were routinely maintained and propagated on Luria–Bertani (LB) agar plates. Spizizen's minimal medium (SMM) with 0.5% glucose as the carbon source, L-tryptophan (20 mg l^{-1}), L-phenylalanine (18 mg l^{-1}) and a solution of trace elements was used as defined medium for growth and transport experiments with *B. subtilis* strains (Kempf and Bremer, 1995). The osmolarity of SMM was determined by a vapour pressure osmometer (model 5.500; Wescor) and is 340 mosmol kg^{-1}. When required, the osmolarity of this medium was increased to 1100 mosmol kg^{-1} by the addition of 400 mM NaCl, 1100 mM glycerol or 670 mM sucrose from concentrated stock solutions. When proline was used as the sole carbon source, the SMM was supplemented with 0.6% L-proline instead of 0.5% glucose. To test whether *B. subtilis* can use L-proline as the sole nitrogen source, we replaced $(NH_4)_2SO_4$ by K_2SO_4 and added 30 mM L-proline to the growth medium. The bacteria were grown aerobically at 37°C in a shaking water bath set at 200–220 r.p.m. The antibiotics tetracycline and chloramphenicol were used at final concentrations of 10 μg ml^{-1} and 5 μg ml^{-1} for *B. subtilis* strains. Tetracycline, chloramphenicol and ampicillin were used at final concentrations of 5 μg ml^{-1}, 30 μg ml^{-1} and 100 μg ml^{-1} for *E. coli* cultures respectively. The sensitivity of *B. subtilis* strains against the toxic proline analogues L-azetidine-2-carboxylic acid (AC) and 3,4-dehydro-DL-proline (DHP) was tested using a filter disk procedure (Csonka, 1982; Grothe *et al.*, 1986). Both proline analogues were purchased from Sigma Chemie. Radiolabelled [1-^{14}C]-glycine betaine (55 mCi $mmol^{-1}$) was obtained from American Radiolabeled Chemicals Inc. L-[^{14}C(U)]-proline (40 mCi $mmol^{-1}$) and L-[1-^{14}C]-glutamate (45 mCi $mmol^{-1}$) were purchased from DuPont de Nemours. [^{35}S]-dATP (1000 Ci $mmol^{-1}$) was from Amersham Buchler.

Bacterial strains, plasmids and construction of B. subtilis *mutants*

The *B. subtilis* gene libraries used to identify the *opuE* gene were propagated in *E. coli* DH5α (Gibco BRL). The *E. coli* strain MKH13 [Δ(*betTIBA*)*U169* Δ(*putPA*)*101* Δ(*proP*)*2* Δ(*proU*)*608*] is a derivative of the *E. coli* K-12 strain MC4100 and is deficient in both proline and glycine betaine uptake (Haardt *et al.*, 1995). Strain BKB4 [Δ(*opuA*::*neo*)*1*] is a derivative of the *B. subtilis* strain JH642 [(t*rpC2 pheA1*), J. Hoch, BGSC 1A96] (Kempf and Bremer, 1995). The *B. subtilis* strains BLOB9 (Δ(*opuE*::*tet*)*1* and BLOB22 (*sigB*Δ*2*::*cat*) are derivatives of JH642 as well and were constructed in the course of this study. Strain PB153 (Boylan *et al.*, 1991) was used as the source of the *sigB*Δ*2*::*cat* mutation and was introduced into JH642 by transformation with chromosomal DNA and selection for chloramphenicol-resistant colonies. BLOB9 was isolated after transformation of strain JH642 with DNA of plasmid pBLOB18.1 (Fig. 3) linearized with the restriction enzyme *Sal*I and selection for tetracycline-resistant transformants. The presence and structure of the *sigB*Δ*2*::*cat* and Δ(*opuE*::*tet*)*1* mutations in strains BLOB22 and BLOB9 were verified by Southern hybridization. The *sigB*$^+$ plasmid (pKSsigB1) (Völker *et al.*, 1994), the *E. coli–B. subtilis* shuttle vector pRB373 (Brückner, 1992), the low-copy-number cloning vector pHSG575 (Takeshita *et al.*, 1987) and the construction of a *B. subtilis* gene library consisting of chromosomal *Sau*3A fragments cloned into pACYC177 (Kappes *et al.*, 1996) have been described previously. The plasmid DNA of the *B. subtilis* gene library was transformed into the *E. coli* strain MKH13 to search for osmotolerant clones capable of growing on high-osmolarity (0.8 M NaCl) minimal medium A agar plates (MMA), containing the appropriate antibiotic and 5 mM glycine betaine (Kempf and Bremer, 1995; Kappes *et al.*, 1996).

Methods used with nucleic acids

Routine manipulations of plasmid DNA, the construction of recombinant plasmids, the isolation of chromosomal DNA from *B. subtilis* and the detection of homologous sequences by Southern hybridizations were all carried out using standard techniques (Sambrook *et al.*, 1989). DNA sequencing of *opuE* and its flanking segments was determined by the chain termination method with the Sequenase 2.0 kit (Amersham). DNA sequencing reactions were primed with a series of synthetic oligonucleotides spaced along the *opuE* region. To prepare a new gene library, chromosomal DNA of the *B. subtilis* strain BKB4 was cut with *Bam*HI. The resulting restriction fragments were separated by electrophoresis, and DNA segments in the range between 2 and 5 kb were eluted from the agarose gel and cloned into the *Bam*HI site of plasmid pHSG575. The *opuE*$^+$ plasmid pORT4 (Fig. 3) was digested with *Stu*I; a 6.7 kb restriction fragment was then isolated from a preparative 1% agarose gel and ligated with a 1.9 kb *Eco*RI tetracycline resistance cassette prepared from plasmid pBEST307 (Itaya, 1992) to obtain the Δ(*opuE*::*tet*)*1* plasmid pBLOB18.1 (Fig. 3). Before the ligation of the two DNA segments, the ends of the *Eco*RI restriction fragment were filled in with Klenow enzyme. These manipulations destroyed the originally present *Stu*I sites (Fig. 3) and created *Eco*RI sites at the

junctions between the *opuE* sequences and the tetracycline resistance cassette. A 1091-bp *Rsa*I fragment carrying the *opuE* regulatory region was isolated from pORT4 (Fig. 3), cloned into the *Sma*I site in the polylinker of the *lacZ* fusion vector pGP22 (P. Gerlach and E. Bremer, unpublished results); this fragment was subsequently recovered from the resulting plasmid pBKB97 as an *Eco*RI–*Pst*I fragment. It was then inserted into the *Eco*RI and *Pst*I sites present in the *E. coli–B. subtilis* shuttle vector pRB373 (Brückner, 1992), yielding plasmid pBKB98 (Fig. 3). The non-radioactive digoxigenin (DIG)–DNA labelling and detection kit (Boehringer Mannheim) was used for the labelling of restriction fragments with DIG-dUTP and the detection of DNA segments by Southern hybridization. The DNA probe for the verification of the *sigBΔ2*::*cat* mutation in the chromosome of BLOB22 was prepared from plasmid pKSsigB1 and consisted of a 1.9 kb *Pst*I restriction enzyme fragment. The structure of the Δ(*opuE*::*tet*)*1* mutation in strain BLOB9 was verified by hybridizing appropriate chromosomal digests with DIG-labelled restriction fragments (a 2.1 kb *Bam*HI–*Nsi*I and a 1.6 kb *Nsi*I) prepared from plasmid pORT4 (Fig. 3).

Mapping of the 5′ ends of the opuE *mRNA*

Total RNA was prepared from cultures of the *B. subtilis* strain JH642 (pBKB98) and BLOB22 (pBKB98) grown to exponential phase ($OD_{578} = 0.3$) in either SMM or SMM with 0.4 M NaCl using the Total RNA Midi Kit from Qiagen. The total amount of RNA isolated was determined spectrophotometrically (A_{260}); an A_{260} of 1 corresponds to approximately 40 μg ml^{-1} (Sambrook *et al.*, 1989). For the primer extension reaction, 5 μg of total RNA was hybridized with 2 pmol of a synthetic primer complementary to the *opuE* mRNA (5′-CAATACTCACGTTTTACCCTC-3′; position 991 bp to 971 bp). This oligonucleotide was labelled at its 5′ end with the infrared dye IRD-41 (MWG). The primer was extended with avian myoblastosis virus reverse transcriptase (Promega) in the presence of 0.32 mM of each dNTP at 42°C for 1 h. The reaction products were purified by phenol extraction; the nucleic acids were then precipitated with ethanol and resuspended in 6 μl of 40% sequencing stop solution (US Biochemical). One microlitre of this solution was applied to a 6% DNA sequencing gel, and the reaction products were analysed on a LI-COR DNA sequencer (model 4000; MWG). A sequencing ladder produced by using the same primer was run in parallel to determine the exact position of the 5′ ends of the *opuE* mRNA.

Transport assays

Uptake of radiolabelled glycine betaine, L-proline and L-glutamate by bacterial cells was measured at room temperature in cell cultures grown to exponential phase in SMM or SMM with increased osmolarity as described previously (Kempf and Bremer, 1995). For kinetic studies, the L-proline concentration in the uptake assay was varied from 1 to 40 μM, and the transport assays were performed at 37°C. To study the activation and induction of the proline transport mediated by the OpuE system, cells of strain JH642 were grown in SMM to exponential phase ($OD_{578} = 0.3$). The culture was then divided into three portions. One portion was treated with 100 μg ml^{-1} chloramphenicol at time zero, and the samples were assayed for L-[^{14}C]-proline uptake at various time intervals. The chloramphenicol-treated culture and one of the untreated cultures were then subjected after 50 min to a sudden osmotic upshock by adding NaCl from a concentrated stock solution (4 M NaCl in SMM) to the growth medium to a final concentration of 0.4 M. To measure L-[^{14}C]-proline uptake in *B. subtilis* strains adapted to various osmolarities, strains JH642 (*opuE*$^{+}$) and BLOB9 (*opuE*) were grown overnight in 20-ml cultures of SMM whose osmolarities were varied by adding different amounts of NaCl (between 0.1 and 1 M). Fresh cultures of identical osmolarity were then inoculated from these precultures, and the cells were grown to exponential phase ($OD_{578} = 0.3–0.6$). The initial rate of L-[^{14}C]-proline or L-[1-^{14}C]-glutamate transport was subsequently determined at 37°C for each of these cultures.

Computer analysis

DNA and protein sequences were assembled and analysed with the LASERGENE program (DNASTAR) on an Apple Macintosh computer. Searches for homologies were performed at the National Center for Biotechnology Information using the BLAST programs (Altschul *et al.*, 1990).

Nucleotide sequence accession number

The nucleotide sequence of *opuE* and its flanking regions has been deposited in GenBank and has been assigned the accession number U92466.

Acknowledgements

We thank R. Brückner, M. Hecker, M. Itaya, C. Price and D. Zeigler for generously providing plasmids and bacterial strains. We are grateful to R. Borriss, P. Piggot and M. Whalen for communicating data before publication. We appreciate the help of V. Koogle in preparing the manuscript. Financial support for this study was provided by the Deutsche Forschungsgemeinschaft through SFB-395, the Graduiertenkolleg Enzymchemie and Fonds der Chemischen Industrie.

References

Akbar, S., and Price, C.W. (1996) Isolation and characterization of *csbB*, a gene controlled by *Bacillus subtilis* general stress transcription factor σ^B. *Gene* **177:** 123–128.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. (1990) Basic local alignment search tool. *J Mol Biol* **215:** 403–410.

Biaudet, V., Samson, F., Anagnostopoulos, C., Ehrlich, S.D., and Bessières, P. (1996) Computerized genetic map of *Bacillus subtilis*. *Microbiology* **142:** 2669–2729.

Boch, J., Kempf, B., and Bremer, E. (1994) Osmoregulation in *Bacillus subtilis*: synthesis of the osmoprotectant glycine betaine from exogenously provided choline. *J Bacteriol* **176:** 5364–5371.

Boch, J., Kempf, B., Schmid, R., and Bremer, E. (1996) Synthesis of the osmoprotectant glycine betaine in *Bacillus subtilis*: characterization of the *gbsAB* genes. *J Bacteriol* **178:** 5121–5129.

Borriss, R., Porwollik, S., and Schroeter, R. (1996) The 52°–55° segment of the *Bacillus subtilis* chromosome: a region devoted to purine uptake and metabolism, and containing the genes *cotA*, *gabP* and *guaA* and the *pur* gene cluster within a 34960 bp nucleotide sequence. *Microbiology* **142:** 3027–3031.

Boylan, S.A., Thomas, M.D., and Price, C.W. (1991) Genetic method to identify regulons controlled by nonessential elements: isolation of a gene dependent on alternate transcription factor σ^B of *Bacillus subtilis*. *J Bacteriol* **173:** 7856–7866.

Boylan, S.A., Redfield, A.R., Brody, M.S., and Price, C.W. (1993) Stress-induced activation of the σ^B transcription factor of *Bacillus subtilis*. *J Bacteriol* **175:** 7931–7937.

Brückner, R. (1992) A series of shuttle vectors for *Bacillus subtilis* and *Escherichia coli*. *Gene* **122:** 187–192.

Cairney, J., Higgins, C.F., and Booth, I.R. (1984) Proline uptake through the major transport system of *Salmonella typhimurium* is coupled to sodium ions. *J Bacteriol* **160:** 22–27.

Chen, C.-C., Tsuchiya, T., Yamane, Y., Wood, J.M., and Wilson, T.H. (1985) Na^+ (Li^+) proline cotransport in *Escherichia coli*. *J Membr Biol* **84:** 157–164.

Csonka, L.N. (1982) A third L-proline permease in *Salmonella typhimurium* which functions in media of elevated osmotic strength. *J Bacteriol* **151:** 1433–1443.

Csonka, L.N., and Hanson, A.D. (1991) Prokaryotic osmoregulation: genetics and physiology. *Annu Rev Microbiol* **45:** 569–606.

Deguchi, Y., Yamato, I., and Anraku, Y. (1990) Nucleotide sequence of *gltS*, the Na^+/glutamate symport carrier of *Escherichia coli*. *J Biol Chem* **265:** 21704–21708.

Galinski, E.A., and Trüper, H.G. (1994) Microbial behaviour in salt-stressed ecosystems. *FEMS Microbiol Rev* **15:** 95–108.

Gouesbet, G., Trautwetter, A., Bonnassie, S., Wu, L.F., and Blanco, C. (1996) Characterization of the *Erwinia chrysanthemi* osmoprotectant transporter gene *ousA*. *J Bacteriol* **178:** 447–455.

Grothe, S., Krogsrund, R.L., McClellan, D.J., Milner, J.L., and Wood, J.M. (1986) Proline transport and osmotic stress response in *Escherichia coli* K-12. *J Bacteriol* **166:** 253–259.

Haardt, M., Kempf, B., Faatz, E., and Bremer, E. (1995) The osmoprotectant proline betaine is a major substrate for the binding-protein-dependent transport system ProU of *Escherichia coli* K-12. *Mol Gen Genet* **246:** 783–786.

Haldenwang, W.G. (1995) The sigma factors of *Bacillus subtilis*. *Microbiol Rev* **59:** 1–30.

Hanada, K., Yoshida, T., Yamato, I., and Anraku, Y. (1992) Sodium ion and proline binding sites in the Na^+/proline symport carrier of *Escherichia coli*. *Biochim Biophys Acta* **1105:** 61–66.

Hecker, M., Schumann, W., and Völker, U. (1996) Heat-shock and general stress response in *Bacillus subtilis*. *Mol Microbiol* **19:** 417–428.

Helmann, J.D. (1995) Compilation and analysis of *Bacillus subtilis* σ^A-dependent promoter sequences: evidence for extended contact between RNA polymerase and upstream promoter DNA. *Nucleic Acids Res* **23:** 2351–2360.

Henderson, P.J.F., and Maiden, M.C. (1989) Homologies between sugar transporters from eukaryotes and prokaryotes. *Annu Rev Physiol* **51:** 459–471.

Itaya, M. (1992) Construction of a novel tetracycline resistance gene cassette useful as a marker of the *Bacillus subtilis* chromosome. *Biosci Biotech Biochem* **56:** 685–686.

Kappes, R., Kempf, B., and Bremer, E. (1996) Three transport systems for the osmoprotectant glycine betaine operate in *Bacillus subtilis*: characterization of OpuD. *J Bacteriol* **178:** 5071–5079.

Kemble, A.R., and Macpherson, H.T. (1954) Liberation of amino acids in perennial rye grass during wilting. *Biophys J* **58:** 46–59.

Kempf, B., and Bremer, E. (1995) OpuA: an osmotically regulated binding protein-dependent transport system for the osmoprotectant glycine betaine in *Bacillus subtilis*. *J Biol Chem* **270:** 16701–16713.

Krüger, E., Msadek, T., and Hecker, M. (1996) Alternate promoters direct stress-induced transcription of the *Bacillus subtilis clpC* operon. *Mol Microbiol* **20:** 713–723.

Lin, Y., and Hansen, J.N. (1995) Characterization of a chimeric *proU* operon in a subtilin-producing mutant of *Bacillus subtilis* 168. *J Bacteriol* **177:** 6874–6880.

Lucht, J.M., and Bremer, E. (1994) Adaptation of *Escherichia coli* to high osmolarity environments: osmoregulation of the high-affinity glycine betaine transport system ProU. *FEMS Microbiol Lett* **14:** 3–20.

Measures, J.C. (1975) Role of amino acids in osmoregulation of non-halophilic bacteria. *Nature* **257:** 398–400.

Miller, K.J., and Wood, J.M. (1996) Osmoadaptation by rhizosphere bacteria. *Annu Rev Microbiol* **50:** 101–136.

Myers, R.S., and Maloy, S.R. (1988) Mutations of *putP* that alter the lithium sensitivity of *Salmonella typhimurium*. *Mol Microbiol* **2:** 749–755.

Ordal, G.W., Villani, D.P., Nicolas, R.A., and Hamel, F.G. (1978) Independence of proline chemotaxis and transport in *Bacillus subtilis*. *J Biol Chem* **253:** 4916–4919.

Peng, Z., Lu, Q., and Verma, D.P.S. (1996) Reciprocal regulation of Δ^1-pyrroline-5-carboxylate synthase and proline dehydrogenase genes controls proline levels during and after osmotic stress in plants. *Mol Gen Genet* **253:** 334–341.

Pourkomailian, B., and Booth, I.R. (1994) Glycine betaine transport by *Staphylococcus aureus*: evidence for feedback regulation of the activity of the two transport systems. *Microbiology* **140:** 3131–3138.

Reizer, J., Reizer, A., and Saier, M.H. (1994) A functional superfamily of sodium/solute symporter. *Biochim Biophys Acta* **1197:** 133–166.

Sambrook, J., Fritsch, E.F., and Maniatis, T.E. (1989) *Molecular Cloning. A Laboratory Manual*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Takeshita, S., Sato, M., Toba, M., Masahashi, W., and Hashimoto-Gotoh, T. (1987) High-copy-number and low-copy-number plasmid vectors for *lacZ* α-complementation and chloramphenicol- or kanamycin-resistance selection. *Gene* **61:** 63–74.

Townsend, D.E., and Wilkinson, B.J. (1992) Proline transport in *Staphylococcus aureus*: a high-affinity system and a low-affinity system involved in osmoregulation. *J Bacteriol* **174:** 2702–2710.

Varón, D., Boylan, S.A., Okamoto, K., and Price, C.W. (1993) *Bacillus subtilis gtaB* encodes UDP-glucose pyrophosphorylase and is controlled by stationary-phase transcription factor σ^B. *J Bacteriol* **175:** 3964–3971.

Varón, D., Brody, M.S., and Price, C.W. (1996) *Bacillus subtilis* operon under the dual control of the general stress transcription factor σ^B and the sporulation transcription factor σ^H. *Mol Microbiol* **20:** 339–350.

Völker, U., Engelmann, S., Maul, B., Riethdorf, S., Völker, A., Schmidt, R., Mach, H., and Hecker, M. (1994) Analysis of the induction of general stress proteins of *Bacillus subtilis*. *Microbiology* **140:** 741–752.

Wengender, P.A., and Miller, K.J. (1995) Identification of a PutP proline permease gene homologue from *Staphylococcus aureus* by expression cloning of the high-affinity proline transport system in *Escherichia coli*. *Appl Environ Microbiol* **61:** 252–259.

Whalen, M.B., and Piggot, P.J. (1997) Gain-of-function mutations of *sapB* that affect formation of alkaline phosphatase by *Bacillus subtilis* in sporulation conditions. *Microbiology* **143:** 577–583.

Whatmore, A.M., and Reed, R.H. (1990) Determination of turgor pressure in *Bacillus subtilis*: a possible role for K^+ in turgor regulation. *J Gen Microbiol* **136:** 2521–2526.

Whatmore, A.M., Chudek, J.A., and Reed, R.H. (1990) The effects of osmotic upshock on the intracellular solute pools of *Bacillus subtilis*. *J Gen Microbiol* **136:** 2527–2535.

Wood, J.M. (1988) Proline porters effect the utilization of proline as nutrient or osmoprotectant for bacteria. *J Membr Biol* **106:** 183–202.

Yamane, K., Kumano, M., and Kurita, K. (1996) The 25°–36° region of the *Bacillus subtilis* chromosome: determination of sequence of a 146 kb segment and identification of 113 genes. *Microbiology* **142:** 3047–3056.

Yancey, P.H. (1994) Compatible and counteracting solutes. In *Cellular and Molecular Physiology of Cell Volume Regulation,* Strange, K. (ed.). Boca Raton, USA: CRC Press, Inc., pp. 81–109.