Molecular Microbiology (1998) **29**(1), 285–296

Osmoregulation of the *opuE* proline transport gene from *Bacillus subtilis*: contributions of the sigma A- and sigma B-dependent stress-responsive promoters

Frank Spiegelhalter and Erhard Bremer*
Philipps University Marburg, Department of Biology, Laboratory for Microbiology, Karl-von-Frisch Str., D–35032 Marburg, Germany.

Summary

The *opuE* gene from *Bacillus subtilis* encodes a transport system (OpuE) for osmoprotective proline uptake and is expressed from two osmoregulated promoters: *opuE* P-1 recognized by the vegetative sigma factor A (σ^A) and *opuE* P-2 dependent on the stress-induced transcription factor sigma B (σ^B). The contributions of these two promoters to osmoregulation of *opuE* were analysed. Genetic studies using chromosomal *opuE–treA* operon fusions revealed that *opuE* transcription is rapidly induced after an osmotic upshock. The strength of *opuE* expression is proportionally linked to the osmolarity of the growth medium. Deletion analysis of the *opuE* regulatory region identified a 330 bp DNA segment carrying all sequences required *in cis* for full and osmoregulated transcription. The proper rotational orientation of the upstream region present within this fragment was essential for the functioning of both *opuE* promoters. Mutant *opuE–treA* fusions with defects in either the σ^A- or the σ^B-dependent promoters revealed different contributions of these sequences to the overall osmoregulation of *opuE*. *opuE* P-2 (σ^B) activity increased transiently after an osmotic upshock and did not significantly contribute to the level of *opuE* expression in cells subjected to long-term osmotic stress. In contrast, transcription initiating from *opuE* P-1 (σ^A) rose in proportion to the external osmolarity and was maintained at high levels. Moreover, both promoters exhibited a different response to the osmoprotectant glycine betaine in the medium. Our results suggest that at least two different signal transduction pathways operate in *B. subtilis* to communicate osmotic changes in the environment to the transcription apparatus of the cell.

Received 13 February, 1998; revised 14 April, 1998; accepted 17 April, 1998. *For correspondence. E-mail bremer@mailer.uni-marburg.de; Tel. 6421 281 529; Fax 6421 288 979.

Introduction

Frequent fluctuations in the availability of water is characteristic of the upper layers of the soil, thus soil-living bacteria must cope with the concomitant osmotic changes in their habitat (Miller and Wood, 1996). Increases in the environmental osmolarity and high salinity negatively affect the entire physiology of *Bacillus subtilis* (Boch *et al*., 1994; Kunst and Rapoport, 1995; Wong *et al*., 1995; Alice and Sanchesz-Rivas, 1997) and necessitate both general and specific adaptation processes to ensure survival and growth (Boch *et al*., 1994; Hecker *et al*., 1996). The most severe consequence of a sudden rise in the external osmolarity is a drop in turgor that inhibits cell division (Whatmore and Reed, 1990). Turgor is restored in *B. subtilis* by an initial rapid uptake of K^+ and the subsequent intracellular accumulation of compatible solutes; i.e. organic osmolytes that are highly congruous with the physiological functions of the cell (Whatmore and Reed, 1990; Whatmore *et al*., 1990; Boch *et al*., 1994). In addition to their major contribution to the osmotic balance, compatible solutes are thought to serve as stabilizers of enzymes and cell components against the deleterious effects of high ionic strength (Galinski and Trüper, 1994; Yancey, 1994). The intracellular amassing of compatible solutes is not restricted to the prokaryotic world (Galinski and Trüper, 1994; Csonka and Epstein, 1996) but is also widely used as response to osmo-stress in fungal, plant, animal and even human cells (Rhodes and Hanson, 1993, Blomberg, 1997; Burg *et al*., 1997).

In addition to the potent osmoprotectant glycine betaine (Kempf and Bremer, 1995; Lin and Hansen, 1995; Boch *et al*., 1996; Kappes *et al*., 1996), a variety of structurally related trimethylammonium compounds and the cyclic amino acid derivative ectoine (Jebbar *et al*., 1997; Kappes and Bremer, 1998), proline plays a very important role in the adaptation reaction of *B. subtilis* to high osmolarity. Large amounts of proline are accumulated by the cells through *de novo* synthesis in response to growth in high osmolarity environments (Whatmore *et al*., 1990). Furthermore, the uptake of proline is triggered by high osmolarity that permits the proliferation of *B. subtilis* under otherwise growth-inhibiting osmotic conditions. This osmoregulated proline import activity relies on OpuE (osmoprotectant uptake), a member of the sodium solute symporter superfamily (SSF) of transporters (von Blohn *et al*., 1997). The

OpuE-mediated proline transport increases strongly from a low basal level subsequent to osmotic upshifts with either ionic or non-ionic osmolytes. This rise in proline transport activity depends entirely on *de novo* protein synthesis, suggesting an osmotic control of *opuE* transcription.

Primer extension analysis identified two tightly spaced and osmoregulated *opuE* promoters recognized by different sigma transcription factors (von Blohn *et al*., 1997). The sequence of the downstream *opuE* P-1 promoter resembles that of promoters dependent on the vegetative sigma factor A (σ^A) from *B. subtilis*. The activity of the upstream *opuE* P-2 promoter requires the alternative transcription factor sigma B (σ^B). σ^B transiently activates the expression of a large stress regulon in response to a variety of environmental challenges, growth-limiting conditions and stationary-phase signals (Haldenwang, 1995; Hecker *et al*., 1996). Its members are thought to prepare *B. subtilis* cells for various unfavourable circumstances, regardless of the type of stress that was initially responsible for the induction of the σ^B-dependent regulon. A complex network of regulatory proteins allows the integration of both cellular and environmental signals into the control of the activity of the σ^B protein (Voelker *et al*., 1995; Akbar *et al*., 1997).

The addition of salt (Bernhardt *et al*., 1997) triggers the enhanced transcription of many genes of the σ^B regulon but *sigB* deletion mutants are not at a survival disadvantage when exposed to osmotic shock or extreme desiccation under laboratory conditions (Boylan *et al*., 1993). Three members of the σ^B regulon have been implicated in the cellular response to high osmolarity and desiccation. GsiB and Csb40 exhibit homologies to plant desiccation proteins, but no clear osmoprotective function has yet been demonstrated for these proteins (Varón *et al*., 1996). Likewise, a role for the *gtaB*-encoded 5′ diphosphate (UDP)-glucose pyrophosphorylase in the synthesis of the osmoprotectant trehalose has been discussed (Varón *et al*., 1993), but it is uncertain whether *B. subtilis* can produce this disaccharide. Trehalose is not detected by ^{13}C-NMR spectroscopy in extracts prepared from cells grown in high osmolarity glucose minimal media (Whatmore *et al*., 1990; Jebbar *et al*., 1997; Kappes and Bremer, 1998). *opuE* is thus the first example of a σ^B-responsive gene with a demonstrated physiological function for the osmoadaptive processes in *B. subtilis*. This has prompted us to investigate the contributions of the σ^A- and σ^B-dependent *opuE* promoters to the overall osmotic control of this proline transport gene. Our findings demonstrate that the *opuE* P-1 and *opuE* P-2 promoters play distinct physiological roles in osmoregulating cells.

Results

Northern analysis of opuE *transcription*

Transport studies with radiolabelled proline and primer extension experiments have suggested that regulation of *opuE* expression occurs at the level of transcription (von Blohn *et al*., 1997). To extend and confirm these studies, we analysed the transcription of the chromosomal *opuE* locus by Northern blot analysis. Total RNA was isolated from cultures of strain JH642 (*opuE*$^+$) that had been grown to mid-log phase in minimal medium (SMM) or minimal medium with elevated osmolarity (SMM with 0.4 M NaCl) and was probed with a radiolabelled *opuE* restriction fragment (probe 1; Fig. 1A). This probe detected a 1.6 kb transcript whose amount was strongly enhanced in cells grown at high osmolarity (Fig. 1B). Its size corresponded to the distance (1.62 kb) between the *opuE* promoters and a putative factor-independent transcription terminator immediately after the *opuE* coding region (Fig. 1A) (von Blohn *et al*., 1997). This mRNA species was absent in strain BLOB9 [Δ(*opuE*::*tet*)*1*], which has a deletion covering both *opuE* promoters and part of the *opuE* coding region (Fig. 1B).

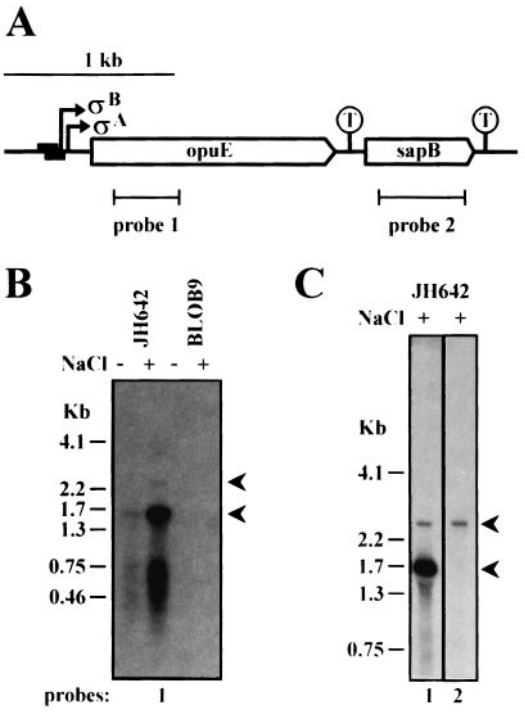

Fig. 1. Northern blot analysis of the *opuE–sapB* region.
A. Genetic and physical organization of the *opuE–sapB* locus. The positions of the σ^A- (*opuE P-1*) and σ^B-dependent (*opuE P-2*) promoters and putative factor independent transcription terminators [T] are indicated as are the *opuE* and *sapB* specific hybridization probes used for the Northern blot analysis.
B. Northern blot analysis of total RNA isolated from the *opuE*$^+$ *sapB*$^+$ strain JH642 and its Δ(*opuE*::*tet*)*1* derivative BLOB9 grown in SMM without or with 0.4 M NaCl was performed with an *opuE*-specific DNA probe.
C. Total RNA isolated from high osmolarity (SMM with 0.4 M NaCl) grown cultures of strain JH642 was probed either with the *opuE*- (1), or *sapB* (2)-specific DNA probes.

The *opuE* probe also detected small amounts of an osmoregulated 2.4 kb transcript that was not present in the RNA prepared from the *opuE* mutant BLOB9. *opuE* is followed by *sapB* (Fig. 1A), a gene of unclear physiological function (Whalen and Piggot, 1997). Fusion analysis with a *lacZ* reporter system has suggested that *sapB* transcription initiates either within *opuE* or that both genes are co-transcribed (Whalen and Piggot, 1997). We probed total RNA isolated from high osmolarity grown cells of strain JH642 with a radiolabelled fragment covering most of *sapB* (probe 2; Fig. 1A). This restriction fragment hybridized to the 2.4 kb mRNA species but not to the 1.6 kb *opuE* mRNA (Fig. 1C). As judged from its size and its absence in BLOB9, the longer mRNA apparently spans the sequence from the *opuE* promoters to a transcription termination signal located downstream of *sapB* (Whalen and Piggot, 1997). Hence, the *opuE* and *sapB* genes are organized in an operon whose expression is increased in cells grown at high osmolarity. However, the mRNA species coding only for *opuE* is accumulated to substantially higher levels than the *opuE–sapB* polycistronic transcript. There is currently no physiological explanation for this co-transcription as *sapB* is dispensable for osmoregulated proline uptake via OpuE (C. v. Blohn and E. Bremer, unpublished) and further possible physiological functions of SapB are unclear (Whalen and Piggot, 1997).

TreA fusion analysis of opuE *expression in response to high osmolarity*

To facilitate the further characterization of *opuE* regulation, we constructed an operon fusion between *opuE* and the reporter gene *treA*, which encodes a highly salt-tolerant phospho-α(1,1)-glucosidase (TreA) from *B. subtilis* (Gotsche and Dahl, 1996; Schöck *et al*., 1996). For this purpose, a 1.09 kb DNA fragment carrying part of the *opuE* coding region, both *opuE* promoters and 0.67 kb of upstream sequences (Fig. 2A) was cloned in front of a promoterless *treA* reporter gene. This *opuE–treA* fusion was integrated via homologous recombination between flanking *amyE* sequences as a single copy into the *amyE* locus of the *B. subtilis* strain FSB1 [(*treA*::*neo*)*1*], which harbours a defective *treA* gene. The resulting fusion strain TRB2 was then used to monitor *opuE–treA* expression in response to changes in the environmental osmolarity. TreA activity was low in cells of strain TRB2 grown in SMM and rapidly increased subsequent to an osmotic upshock with 0.4 M NaCl (Fig. 2B). This pattern of osmotic control in *opuE–treA* expression mirrors the strong increase in OpuE-mediated proline transport activity observed under the same experimental conditions (von Blohn *et al*., 1997). The extent of the increase in *opuE–treA* expression was dependent on the magnitude of the osmotic upshift (data not shown), indicating that *B. subtilis* cells can sensitively

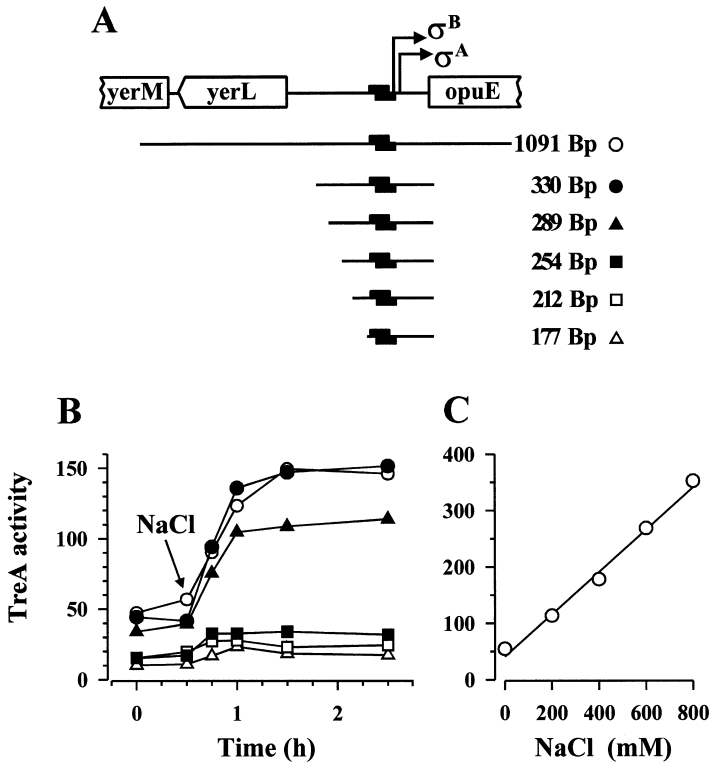

Fig. 2. Deletion analysis of the *opuE* promoter region.
A. DNA segments covering various 5′regions of *opuE* were fused to *treA* and the resulting operon fusions were integrated into the *amyE* locus of strain FSB1 [(*treA*::*neo*)1]. The positions and transcriptional initiation sites for the σ^B-dependent *opuE* P-2 and the σ^A-dependent *opuE* P-1 are indicated. *yerL* (ORF1) and *yerM* (ORF2*) are open reading frames of unknown function (von Blohn et al., 1997).
B. Cultures of the various fusions strains were grown in SMM and the regulation of the *opuE–treA* fusions was studied in response to a sudden osmotic upshift with 0.4 M NaCl (arrow). The following fusion strains were used: TRB2 (○); TRB6 (●); TRB10 (▲); TRB9 (■); TRB11 (□) and RB7 (△). A culture of strain TRB6 was grown in parallel in SMM and the TreA activity of this culture did not change over the course of the experiment. Strain TRB0 [(*treA*::*neo*)1] carrying the promoterless *treA* gene inserted into the *amyE* locus (*amyE*::*treA*) was used as a control and yielded less than three units of TreA activity (data not shown).
C. Cultures of strain TRB2 (○) were pregrown in SMM of different osmolarities and then assayed for TreA activity at mid-log phase under the same growth conditions.

monitor their degree of osmotic stress. Consistent with this suggestion, we found that the expression of the *opuE–treA* operon fusion was linearly correlated over a considerable range to the osmotic strength of the growth medium (Fig. 2C). We also tested whether the presence of the substrate of the OpuE proline transporter would induce the expression of the *opuE–treA* fusion. The addition of 1 mM proline to the growth medium (SMM) did not result in a derepression of *opuE* expression (data not shown).

Identification of a minimal opuE *regulatory DNA fragment*

To identify the DNA sequences required *in cis* for osmoregulated *opuE* transcription, we carried out a deletion analysis of both the 5′ and the 3′ DNA segments flanking the *opuE* promoters. We first constructed an *opuE–treA* operon fusion carrying a 330 bp DNA *opuE* fragment with its 3′ fusion junction within the fourth codon of the *opuE* reading frame (Fig. 2A; Fig. 3A). Integration of this fusion as a single copy at the chromosomal *amyE* site of FSB1 [(*treA*::*neo*)*1*] resulted in strain TRB6. TRB6 exhibited the same pattern and level of osmoregulated reporter gene expression as strain TRB2, which carries the 1.09 kb *opuE* fragment initially used for the *treA* fusion construction (Fig. 2B). Hence, the 330 bp DNA fragment (Fig. 3A) contains all DNA sequences required *in cis* for full and osmoregulated *opuE* expression. Furthermore, these data indicate that the *opuE* coding region does not harbour any elements participating in the genetic control of *opuE* transcription.

To delineate the 5′ sequences necessary for proper expression of the *opuE* gene, we systematically shortened the DNA region located upstream of the σ^B- and σ^A-dependent *opuE* promoters in the *treA* fusion present in strain TRB6. For these new fusion constructs, the original 3′ junction between *opuE* and *treA* was maintained (Figs 2A and 3A). The deletion of 41 bp from the 330 bp promoter fragment resulted in an *opuE–treA* fusion (TRB10) that still showed osmoregulated gene expression but no longer reached the level of TreA activity exhibited by the parental strain TRB6 (Fig. 2B). Thus, DNA sequences required for maximal expression are impaired by this deletion in the 5′ region of *opuE* (Fig. 3A). Further shortening

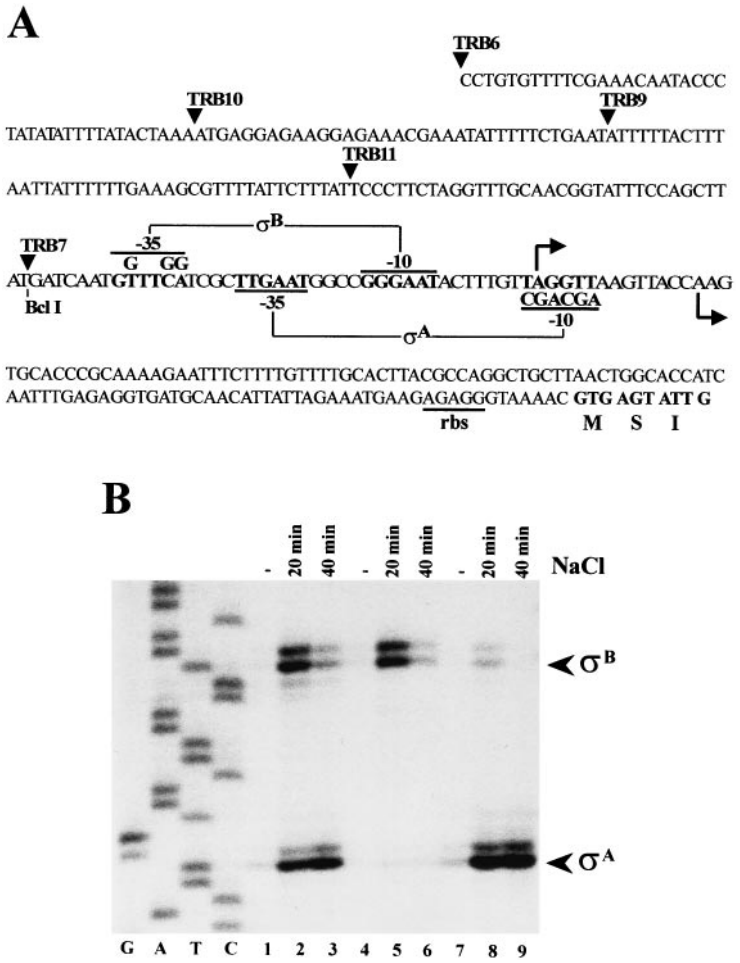

Fig. 3. A. Nucleotide sequence of the 330 bp DNA fragment (662 bp to 991 bp; accession number U92466) containing all sequences required *in cis* for full and osmoregulated *opuE* promoter activity. The end points of the deletions in the various *opuE–treA* fusion strains are indicated by ▼. The positions of the σ^A-dependent *opuE* P-1 and the σ^B-dependent *opuE* P-2 promoters and the transcription initiation sites are indicated. The mutations introduced by site-directed mutagenesis into the −10 region of *opuE* P-1 and the −35 region of *opuE* P-2 are given. The ribosome binding site (rbs) is underlined and the first codons of the *opuE* gene are shown in bold. The *Bcl*I site was used for the insertion of short DNA segments to alter the rotational orientation of the *opuE* upstream region.
B. Primer extension analysis of the *opuE* transcripts was carried out on total RNA isolated from cultures of strains JH642 grown in SMM (−) or cultures subjected to osmotic upshocks with 0.4 M NaCl for either 20 min or 40 min. The cells either harboured the *opuE′* plasmid pFSB20 (wild-type *opuE* promoters; lanes 1, 2, 3), pFSB21 (mutations in the σ^A-dependent *opuE* P-1 promoter; lanes 4, 5, 6) or pFSB34 (mutations in the σ^B-dependent *opuE* P-2 promoter; lanes 7, 8, 9).

of the 289 bp *opuE* promoter fragment resulted in operon fusion strains with a considerably reduced basal level of *opuE–treA* expression (TRB7, TRB9, TRB11) and a rudimentary response to osmotic upshifts (Fig. 2B). A comparison of the properties of strains TRB6 and TRB10 reveals that more than 119 bp upstream of the −35 region of the σ^B-dependent *opuE* P-2 promoter are required for the full functioning of the *opuE* promoters. It is thus apparent that the presence of the σ^B- and the σ^A-dependent *opuE* promoters by themselves on these DNA segments is insufficient to yield full and osmoregulated *opuE–treA* expression.

Rotational orientation of the upstream DNA segment affects opuE *promoter function*

Noticeably, many short A/T or T/A stretches are present in the DNA sequences located upstream of the *opuE* promoters (Fig. 3A). Such highly AT-rich DNA segments frequently exhibit intrinsic DNA curvature (Pérez-Martín and de Lorenzo, 1997). DNA fragments originating from the *opuE* upstream region were analysed on polyacrylamide gels under conditions known to enhance (4°C) or reduce (60°C) abnormal electrophoretic migration of bent DNA (Diekmann and Wang, 1985). These experiments (data not shown) suggested that the *opuE* upstream region has an intrinsically curved DNA structure. The extent of the curvature seems to be moderate, however, as judged from the variation in electrophoretic mobility of the analysed DNA fragments.

The rotational orientation of curved DNA fragments located upstream of transcription initiation sequences frequently affects promoter activity in *B. subtilis* (McAllister and Achberger, 1989). We therefore tested whether the spatial orientation between the *opuE* promoters and their upstream region was important for the overall level of *opuE* expression. Starting with the *opuE–treA* fusion TRB6 carrying all regulatory sequences required *in cis* (Fig. 2), we used PCR mutagenesis to insert short DNA segments at a *Bcl*I site present immediately upstream of the −35 region of the σ^B-dependent *opuE* P-2 promoter (Fig. 3A). This altered the rotational orientation of the upstream region by approximately half or full helical turns relative to the *opuE* promoters. The insertion of either 5 bp (TRB15) or 16 bp (TRB17) strongly reduced the basal level of *opuE–treA* expression and practically abolished the osmotic regulation of the operon fusion (Fig. 4A). The insertion of half helical turns thus results in a low level of *opuE–treA* transcription similar to that observed in the fusion strain TRB7, in which the entire *opuE* upstream region is lacking (Fig. 2B). In contrast, the displacement of the upstream region by approximately one (TRB16) or two (TRB18) helical turns still permitted osmoregulated *opuE* expression, albeit at reduced levels (Fig. 4A). We conclude from these experiments that the rotational orientation of the upstream DNA segment is an important determinant for the activity of both *opuE* promoters.

Differential regulation of the opuE *P-1 and* opuE *P-2 promoters*

The activity of the alternative transcription factor σ^B is controlled by a network of regulatory proteins that are responsive to a variety of environmental challenges and stationary-phase signals (Haldenwang, 1995; Hecker *et al*., 1996). We tested whether in addition to salt stress other known inducers of the σ^B-dependent general stress regulon, such as ethanol and heat shock, would also

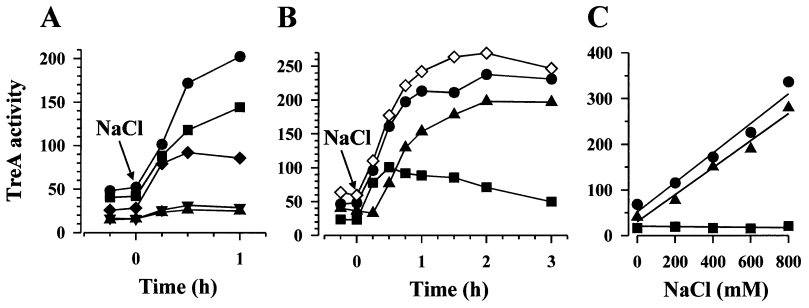

Fig. 4. Osmoregulation of *opuE* expression.
A. TreA activities in *opuE–treA* fusion strains with various short DNA insertions into the *Bcl*I site (see Fig. 3A) were determined in cultures grown in SMM and subjected to a sudden (arrow) osmotic upshock with 0.4 M NaCl. Strain TRB6 (●), wild-type fusion; mutant fusion strains carrying a 5 bp (TRB15; ▲), 11 bp (TRB16; ■), 16 bp (TRB17; ▼), 21 bp (TRB18; ♦) insertion.
B. Influence of *opuE* P-1 (σ^A-dependent) and *opuE* P-2 (σ^B-dependent) promoter mutations on *opuE–treA* expression. Expression of the chromosomally encoded wild-type and mutant *opuE–treA* fusions was monitored in strains TRB6 (wild-type fusion; ●), TRB19 (*opuE* P-1 mutant; ■) and TRB20 (*opuE* P-2 mutant; ▲). The cultures were grown in SMM whose osmolarity was suddenly increased (arrow) by the addition of 0.4 M NaCl. The symbols (◇) represent the sum of the TreA activities of strains TRB19 and TRB20.
C. TreA activities of *opuE–treA* fusion strains TRB6 (●), TRB19 (■) and TRB20 (▲) were assayed in SMM grown cultures adapted to different osmolarities.

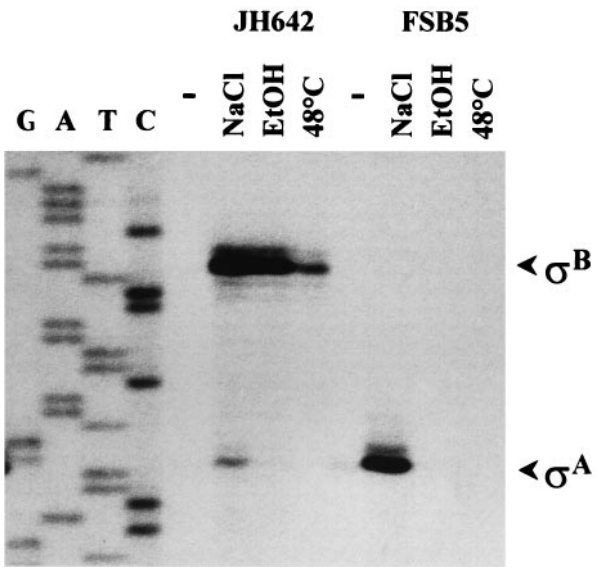

Fig. 5. Primer extension analysis of *opuE*-specific transcripts. Total RNA was prepared from cultures of JH642 (*sigB*$^+$) and FSB5 (*sigB*) carrying the *opuE′* plasmid pBKB98 grown in SMM (−), or cultures briefly exposed to either 0.4 M NaCl, 4% (v/v) ethanol (EtOH) or a heat shock from 37° to 48°. Transcripts initiating from the σ^B-dependent *opuE* P-2 and the σ^A-dependent *opuE* P-1 were identified by primer extension analysis.

increase the activity of the σ^B-controlled *opuE* P-2 or the σ^A-dependent *opuE* P-1 promoter. Log-phase cultures of strains JH642 (*sigB*$^+$) and its *sigB* mutant derivative strain FSB5 (*sigBΔ2*::*spc*) carrying the *opuE′* plasmid pBKB98 were challenged briefly (10 min) with either 0.4 M NaCl, 4% ethanol (v/v) or a sudden temperature upshift from 37°C to 48°C. The influence of these stresses on the activity of the *opuE* promoters was then monitored by primer extension analysis (Fig. 5). As observed previously (von Blohn *et al*., 1997), salt stress induced the transcription initiating from both *opuE* P-1 and *opuE* P-2 (see also Fig. 3B). Ethanol and heat stress induced σ^B-dependent transcription initiating from *opuE* P−2. In contrast, these stresses failed to activate transcription directed by the *opuE* P-1 promoter in either the wild type or its *sigB* mutant derivative (Fig. 5). As judged from these primer extension experiments, the activity of the σ^A-dependent *opuE* P-1 promoter is increased in a *sigB* mutant in comparison with its activity in a wild-type strain subsequent to an osmotic upshock.

Construction and primer extension analysis of opuE *promoter mutants*

To study the contributions of each of the two *opuE* promoters to the overall pattern of *opuE* transcription in response to osmotic changes, we changed either the −10 region of the σ^A-dependent *opuE* P-1 promoter or the −35 region of the σ^B-dependent *opuE* P-2 promoter by site-directed mutagenesis (Fig. 3A). The −10 region of *opuE* P-1 (5′ TAGGTT) was replaced by 5′ CGACGA, a sequence completely different from the canonical −10 regions of σ^A-dependent promoters. The −35 sequence of the *opuE* P-2 (5′ GTTTCA) was altered to 5′ GGTTGG, thereby changing a highly conserved T at the second position in −35 regions of σ^B-dependent promoters to a G (Hecker *et al*., 1996; von Blohn *et al*., 1997). These alterations were introduced into the 330 bp *opuE* promoter fragment, which contains all *cis* regulatory elements necessary for *opuE* expression (Fig. 3A). The resulting *opuE* promoter mutants and the equivalent wild-type DNA fragment were subcloned into the *B. subtilis*–*E. coli* shuttle vector pRB373 (Brückner, 1992) to assess their functionality using primer extension analysis. The mutations in the σ^A-dependent *opuE* P-1 completely eliminated transcription initiating at this promoter, whereas transcription mediated by the σ^B-dependent *opuE* P-2 was not affected. Conversely, the multiple mutations introduced into the σ^B-dependent *opuE* P-2 strongly reduced the activity of this promoter and did not affect the activity of *opuE* P-1 (Fig. 3B).

The activity of the alternative transcription factor σ^B occurs only transiently subsequent to the imposition of environmental stresses (Varón *et al*., 1993; Haldenwang, 1995; Voelker *et al*., 1995; Hecker *et al*., 1996; Akbar *et al*., 1997). Transcription mediated by *opuE* P-2 reflects this temporal pattern. The amount of the *opuE* P-2-directed mRNA species is strongly elevated within 20 min after an osmotic upshock with 0.4 M NaCl and then drops back to almost basal levels. In contrast, the amount of the *opuE* P-1-directed mRNA species remained at a high level during this time period (Fig. 3B). These different characteristics of the two *opuE* promoters are retained in both promoter mutant constructs.

Characteristics of the σ^A- and σ^B-dependent opuE *expression*

Both mutant *opuE* promoter fragments were used for the construction of *B. subtilis treA* operon fusion strains. Strain TRB19 carries the *opuE* P-1 mutation and thus expresses the *treA* fusion under sole control of the σ^B-dependent *opuE* P-2 promoter. Strain TRB20 expresses this fusion under the primary control of the σ^A-dependent *opuE* P-1 promoter because *opuE* P-2 activity is greatly reduced (Fig. 3B). Both mutant strains and their corresponding wild-type parent TRB6 were subjected to a sudden osmotic upshift, and TreA activity in these cells was followed for several hours (Fig. 4B). TreA activity in strain TRB19 rose immediately after the addition of 0.4 M NaCl to the growth medium but subsequently declined again. In contrast, TreA activity in strain TRB20 did not increase immediately

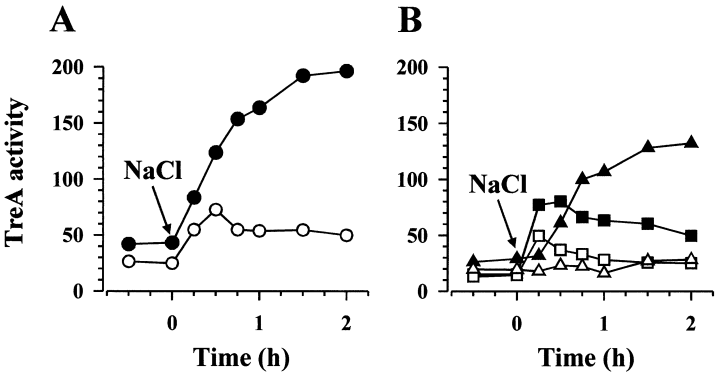

Fig. 6. Modulation of *opuE–treA* expression by glycine betaine. Expression of the chromosomally encoded wild-type (A) and mutant (B) *opuE–treA* fusions was monitored in strain TRB6 (wild-type fusion; ● ○), TRB19 (*opuE* P-1 mutant; ■ □) and TRB20 (*opuE* P-2 mutant; ▲ △).The cultures were grown in SMM in the absence (● ■ ▲) or presence (○ □ △) of 1 mM glycine betaine and were subjected to a sudden osmotic upshock with 0.4 M NaCl (arrow).

after the osmotic upshift, but after a delay it rose rapidly and was maintained at high levels (Fig. 4B). When the TreA activities exhibited by the *opuE* promoter mutant strains TRB19 and TRB20 are summed up, the time course and level of the resulting enzyme activities closely resemble those measured in parallel in the fusion strain TRB6 with intact *opuE* P-1 and *opuE* P-2 promoters (Fig. 4B). These findings indicate that the individual contributions of these two promoters to the overall level of *opuE* transcription are additive.

We also monitored the expression of the mutant *opuE–treA* operon fusions in cells grown over longer time periods (at least 16 h) in media of various osmolarities. The wild-type strain TRB6 showed the expected linear relationship between medium osmolarity and *opuE–treA* expression (Fig. 4C). Such a correlation was also manifested in the fusion strain TRB20 carrying an intact σ^A-dependent *opuE* P-1 promoter and a mutated σ^B-dependent *opuE* P-2. In contrast, regardless of the osmotic strength of the growth medium, strain TRB19 with a mutated *opuE* P-1 and an intact *opuE* P-2 promoter exhibited a low level of TreA activity (Fig. 4C). It is thus apparent that under conditions of long-term osmotic stress, the σ^B-dependent *opuE* P-2 promoter makes only a minor contribution to the total level of *opuE* expression and moreover does not participate in the osmoregulation of this proline transport gene.

Influence of the osmoprotectant glycine betaine on opuE *expression*

The addition of the compatible solute glycine betaine to the growth medium can ameliorate the effects of high salt on osmoregulated gene expression in *Escherichia coli* and *Salmonella typhimurium* (Barron *et al*., 1986; Sutherland *et al*., 1986; Lucht and Bremer, 1994). We observed that the addition of 1 mM glycine betaine to the growth medium strongly reduced both the basal level and the degree of *opuE–treA* induction in strain TRB6 subsequent to an osmotic upshift with 0.4 M NaCl (Fig. 6A). The presence of glycine betaine had different effects on transcription mediated by the osmoregulated *opuE* P-1 and *opuE* P-2 promoters: in strain TRB20 *opuE–treA* expression was reduced and its induction was practically prevented, whereas in strain TRB19 expression of the fusion was only reduced to around 50% (Fig. 6B).

Discussion

B. subtilis can acquire the osmoprotectant proline from exogenous sources, such as decaying plant material and root exudates, in its natural habitat (Kemble and Macpherson, 1954) via the high-affinity and substrate-specific OpuE transporter (von Blohn *et al*., 1997). The data presented here show that the expression of the *opuE* structural gene is tailored to the physiological needs of *B. subtilis* cells to sensitively adjust the intracellular level of this compatible solute to the extent of osmotic stress. After an osmotic upshock, *opuE* transcription rises rapidly, and the level reached is maintained and proportionally linked to the environmental osmolarity (Fig. 2). This pattern of gene expression distinguishes *opuE* from other members of the σ^B regulon where transcription is only transiently induced after osmotic upshifts (Voelker *et al*., 1995; Hecker *et al*., 1996; Varón *et al*., 1996; Akbar *et al*., 1997). It also reflects the physiological role of the OpuE transporter. *B. subtilis* cells growing under high osmolar conditions require a continuous uptake of proline (von Blohn *et al*., 1997), and consequently *opuE* expression must be kept at elevated levels for as long as the osmotic stimulus persists. The osmotically induced increase in OpuE-mediated proline transport is entirely dependent on the *de novo* synthesis of protein (von Blohn *et al*., 1997). Consistent with this observation, Northern blot analysis detected a massive accumulation of *opuE* specific transcripts in high osmolarity grown cells (Fig. 1). Hence, in contrast to various other uptake systems for osmoprotectants (Milner *et al*., 1988; Peter *et al*., 1996), transport activity of the OpuE protein is not osmotically controlled.

opuE–treA operon fusion studies identified a 330 bp

DNA fragment containing all sequences required *in cis* for maximal and osmoregulated *opuE* promoter activity (Fig. 2). As this DNA segment comprises only the first three codons of the *opuE* reading frame (Fig. 3A), the coding region contains no regulatory sequences such as those reported for the first structural gene (*proV*) of the osmoregulated *proU* operon from *E. coli* and *S. typhimurium* (Overdier *et al*., 1989; Dattananda *et al*., 1991; Owen-Hughes *et al*., 1992; Lucht *et al*., 1994). This minimal regulatory fragment includes a 160 bp region upstream of the −35 region of the σ^B-dependent *opuE* P-2 promoter. Both deletion of the upstream region (Fig. 2) and the disruption of its proper rotational orientation with respect to the *opuE* promoter elements (Fig. 4A) resulted in a similar loss of promoter activity and effective osmoregulation. This AT-rich region shows features of curved DNA. Its activating properties could result either from positive effects of the intrinsically bent DNA on promoter use (McAllister and Achberger, 1989; Pérez-Martín and de Lorenzo, 1997) or from the binding of transcription activators. The demand for an upstream region distinguishes the σ^B-dependent *opuE* P-2 promoter from the σ^B-controlled *ctc* gene in which only 11 bp of upstream DNA are sufficient for full promoter function (Igo and Losick, 1986).

The *opuE* P-1 and *opuE* P-2 promoters contribute additively to the level of *opuE* expression but have different roles in the osmotic control of this gene (Fig. 4B and C). Activity of the σ^B-dependent *opuE* P-2 promoter rose transiently in response to an osmotic upshock, consistent with the temporal pattern of σ^B activation after sudden environmental challenges (Voelker *et al*., 1995; Varón *et al*., 1996; Akbar *et al*., 1997). This promoter also allowed the input of other typical σ^B-inducing stimuli (e.g. heat and ethanol stress) into the genetic control of *opuE*. Both in the wild-type and in a *sigB* deletion mutant, heat and ethanol stress did not induce transcription initiating from the σ^A-dependent *opuE* P-1 promoter (Fig. 5). Hence, the *sigB* deficiency is not compensated by endowing additional regulatory circuits onto *opuE* P-1. This is different from the stress-induced transcription mediated by the σ^A- and σ^B-dependent *clpC* promoters for which such a compensation at the σ^A-controlled promoter occurs in a *sigB* mutant strain (Krüger *et al*., 1996). The σ^B regulon is thought to prepare *B. subtilis* cells in transition from a growing to a non-growing state for a variety of adverse circumstances (Haldenwang, 1995; Hecker *et al*., 1996). The way in which transient proline accumulation might contribute to this σ^B-mediated general stress resistance of *B. subtilis* remains to be elucidated.

The characteristics of the σ^B-dependent *opuE* P-2 promoter permit a rapid osmotic induction of the OpuE transport system. In growing cells subjected to long-term osmotic stress, regulation of *opuE* expression depends entirely on the σ^A-dependent *opuE* P-1 promoter (Fig. 4B and C). Its activity is proportionally linked to the environmental osmolarity and is retained in a *sigB* mutant background (our unpublished results), suggesting that osmotic control of this promoter occurs independently from any member of the σ^B regulon. The mechanisms governing the regulation of the σ^A-dependent *opuE* P-1 promoter are currently unknown. However, the osmotic upshock experiments with *opuE–treA* fusion strains in the presence of the osmoprotectant glycine betaine give an important clue. Overall induction of *opuE* transcription is drastically reduced when low concentrations (1 mM) of glycine betaine are available to the cells (Fig. 6). This is mainly caused by the loss of the *opuE* P-1 response to the rise in external osmolarity. As the increase in medium osmolarity is the same in the absence or presence of glycine betaine, it is apparent that to induce transcription at *opuE* P-1, the bacterial cell does not sense osmolarity *per se*. Consistent with this conclusion, high concentrations of the membrane-permeable glycerol fail to induce OpuE-mediated proline uptake in contrast to ionic or non-ionic compounds capable of establishing osmotically effective concentration gradients across the cytoplasmic membrane (von Blohn *et al*., 1997). Thus, osmotic control of *opuE* P-1 activity rather depends on consequences of the rise in the environmental osmolarity, such as the drop in turgor, uptake of K^+, changes in the ionic and physical composition of the cytoplasm, or alterations in the supercoiling of the chromosomal DNA (Sutherland *et al*., 1986; Higgins *et al*., 1988; Dattananda *et al*., 1991; Csonka and Epstein, 1996; Conter *et al*., 1997). The independence of the osmoregulation of the σ^A-dependent *opuE* P-1 promoter from the σ^B regulon implies that at least two different signal transduction pathways operate in *B. subtilis* to communicate osmotic changes in the environment to the transcription apparatus of the cell.

Experimental procedures

Media, growth conditions and chemicals

E. coli and *B. subtilis* strains were maintained and propagated on Luria–Bertani (LB) agar plates. Spizizen's minimal medium (SMM) with 0.5% glucose as the carbon source, L-tryptophane (20 mg l^{-1}), L-phenylalanine (18 mg l^{-1}) and a solution of trace elements (Harwood and Archibald, 1990) was used for the growth of *B. subtilis* strains. The osmolarity of SMM is 340 mosmol kg^{-1}; the osmolarity of SMM with 0.2 M, 0.4 M, 0.6 M and 0.8 M NaCl was 710 mosmol kg^{-1}, 1100 mosmol kg^{-1}, 1480 mosmol kg^{-1} and 1860 mosmol kg^{-1} respectively. Osmolarities of the various growth media were determined with a vapour pressure osmometer (model 5.500; Wescor). For osmotic upshock experiments, 75 ml cultures (in 500 ml Erlenmeyer flasks) of the various *B. subtilis opuE–treA* fusion strains were grown aerobically to mid-log phase (OD_{578} 0.4) at 37°C in a shaking water bath set at 220 r.p.m. The osmolarity of the cultures was then suddenly increased by the addition

of 0.4 M NaCl from a stock of NaCl dissolved in SMM. The *treA* fusion strains used for long-term osmotic stress experiments were pregrown overnight in SMM with various osmolarities; aliquots were used to inoculate fresh 20 ml SMM cultures (in 100 ml Erlenmeyer flasks) with the appropriate osmolarity and were then grown to mid-log phase (OD_{578} 0.5–0.8) on an orbital shaker (220 r.p.m) at 37°C. The antibiotics kanamycin, spectinomycin and chloramphenicol were used at final concentrations of 5 μg ml^{-1}, 100 μg ml^{-1} and 5 μg ml^{-1}, respectively, for *B. subtilis* strains. Ampicillin and chloramphenicol were used at final concentrations of 100 μg ml^{-1} and 30 μg ml^{-1}, respectively, for *E. coli* cultures. Production of the extracellular α-amylase AmyE by *B. subtilis* strains was tested by flooding the colonies grown on LB plates containing 1% starch with Gram's iodine stain [0.5% (w/v) iodine, 1% potassium iodide] for 1 min and scoring for zones of clearing around the colonies after decanting the stain (Cutting and Vander Horn, 1990). The chromogenic substrate for TreA, *para*-nitrophenyl-α-D-glucopyranoside (PNPG), was purchased from Sigma. [α-^{32}P]-dCTP (3000 Ci mmol^{-1}) was from Hartmann Analytic, Braunschweig.

Bacterial strains and construction of B. subtilis *mutants*

The *E. coli* strains DH5α (Gibco BRL) and XL1-Blue (Stratagene) were used for the routine propagation of plasmids. Strain GM2159 (*dam*$^-$) (Marinus *et al*., 1983) was used for the preparation of the plasmid DNA used for restriction digestions with *Bcl*I. The *B. subtilis* wild-type strain JH642 [(*trpC2 pheA1*), J. Hoch, BGSC 1A96] and its mutant derivatives BLOB9 [Δ(*opuE*::*tet*)*1*] and BLOB22 [*sigBΔ2*::*cat*] have been described previously (von Blohn *et al*., 1997). Strain FSB5 is derived from the *sigB* mutant BLOB22 and carries the *sigBΔ2*::*spc* allele. The *cat* antibiotic resistance marker present in the chromosomal *sigBΔ2*::*cat* mutation was replaced *in vivo* through homologous recombination with a 4.3 kb *Eco*RI restriction fragment derived from plasmid pCm::Sp carrying a *spc* resistance cassette (Steinmetz and Richter, 1994). The physical structure of the *sigBΔ2*::*spc* mutation in strain FSB5 was verified by Southern hybridization using the pCm::Sp-derived *Eco*RI fragment as a hybridization probe. The *treA* gene present in strain JH642 was disrupted by transformation with an ≈4.5 kb *Not*I–*Stu*I restriction fragment from pJMB6 [(*treA*::*neo*)*1*] (M. Jebbar and E. Bremer, unpublished) and subsequent selection for kanamycin-resistant colonies; pJMB6 is a derivative of the *treA*$^+$ plasmid pCH5 (Helfert *et al*., 1995). The neo cassette in the (*treA*::*neo*)*1* allele is inserted into a unique *Hin*dIII site present, close to the 5′ end in the *treA* coding region (Helfert *et al*., 1995). The correct integration of the (*treA*::*neo*)*1* mutation into the chromosome in one of the resulting transformants, strain FSB1, was verified by Southern hybridization, with the *Not*I–*Stu*I fragment from pJMB6 used as the probe. Operon fusions between *opuE* and *treA* were constructed in plasmid pJMB1 [*amyE–treA–cat–amyE*]. These *opuE–treA* operon fusions were integrated as single copies into the chromosome of strain FSB1 [(*treA*::*neo*)*1*] by homologous recombination between the genomic *amyE* sequences and the *amyE* sequences flanking the plasmid-encoded *treA* gene fusion (Schöck *et al*., 1996). Transformants in which the *opuE–treA* operon fusions were integrated into the chromosomal *amyE* gene were selected as chloramphenicol-resistant colonies. The correct integration of the *opuE–treA* fusions was verified by scoring the AmyE phenotype on starch plates and by Southern hybridization using an *amyE*-specific probe. Strain TRB0 carries a promoterless *treA* gene from pJMB1 inserted into *amyE* and was used as a control for the *opuE–treA* fusion experiments.

Methods used with nucleic acids and plasmid constructions

Routine manipulations of plasmid DNA, PCR, the construction of recombinant plasmids, the isolation of chromosomal DNA from *B. subtilis* and the detection of homologous sequences by Southern hybridization using digoxigenin (DIG)-labelled DNA probes were carried out using standard techniques (Sambrook *et al*., 1989). The nucleotide sequence of cloned PCR *opuE* promoter fragments was verified by the chain termination method with the Thermo Sequenase fluorescent-labelled primer cycle sequencing kit (Amersham). The reactions were primed with synthetic oligonucleotides labelled at their 5′ end with the infrared dye IRD-41 (MWG) and the products were analysed on a LI-COR DNA sequencer (model 4000; MWG). The intrinsic curvature of DNA fragments originating from the *opuE* regulatory region was studied by monitoring their relative electrophoretic mobilities on 15% polyacrylamide gels (37:1 acryl/bis) run in TBE buffer (90 mM Tris-HCl, 90 mM H_3BO_3, 2.5 mM Na_2EDTA) at either 4°C or 60°C.

The *treA* fusion vector pJMB1 is a derivative of pMD476 (Schöck *et al*., 1996) and carries the origin of replication from the low-copy-number plasmid pACYC177 (M. Jebbar and E. Bremer, unpublished). The *opuE–treA* operon fusions isolated in the course of this study were, in general, constructed by cloning *opuE* promoter fragments amplified by PCR into pBluescript SK− (Stratagene); the fragments were then recovered from the resulting plasmids as restriction fragments carrying a blunt end at their 5′ end and a *Bam*HI overhang at their 3′ end. These DNA segments were inserted into pJMB1 cleaved with *Sma*I and *Bam*HI, which positioned the *opuE* promoter fragments in the desired orientation in front of the promoterless *treA* gene. The junction of the *opuE–treA* operon fusion present in strain TRB2 (Fig. 2A) is at position 1234 bp of *opuE* (accession number U92466), and the junctions present in strains TRB6, TRB7, TRB9, TRB10, TRB11 are at position 991 bp of *opuE*. The *opuE* material present in each of these operon fusions has a different 5′ end (Fig. 3A). Additional fusion strains are derived from the *opuE–treA* operon fusion inserted into the genome of TRB6 and carry either oligonucleotide insertions at a *Bcl*I site in the *opuE* material or mutations in the *opuE* P-1 or *opuE* P-2 promoters (Fig. 3A). Plasmids pFSB20, pFSB21 and pFSB34 are derivatives of the *E. coli–B. subtilis* shuttle vector pRB373 (Brückner, 1992) and carry a 431 bp *opuE* fragment (561 bp to 991 bp) with either the wild-type *opuE* promoter sequence (pFSB20), mutations in the *opuE* P-1 (pFSB21) or the *opuE* P-2 promoters (pFSB34). The promoter mutations (Fig. 3A) present in pFSB21 and pFSB34 (and their corresponding *opuE–treA* fusion strains TRB19 and TRB20 respectively) were generated by PCR using mutant primers. PCR mutagenesis was also used for the insertion of short DNA sequences [5 bp, 5′-GATCA; 11 bp, 5′-GATCAAGATCA;

16 bp, 5′-GATCAATCATGGATCA; 21 bp, 5′-GATCAATCATGGATCAGATCA] into the *Bcl*I site present in the 330 bp *opuE* promoter fragment (Fig. 3A). These mutant *opuE* promoter fragments were fused to *treA* in plasmid pJMB1, and the resulting operon fusions were used for construction of the *opuE–treA* fusion strains TRB15, TRB16, TRB17 and TRB18 (Fig. 4A). Part of the *sapB* gene (Whalen and Piggot, 1997) was amplified by PCR, and a 493 bp *Nsi*I–*Acc*I restriction fragment (658 bp to 1150 bp; accession number U59128) was cloned into pBluescript SK− cleaved with *Acc*I and *Pst*I, yielding plasmid pFSB25.

Northern and primer extension analysis

Total RNA was isolated from *B. subtilis* log-phase (OD_{578} 0.5–0.8) cell cultures grown in SMM or SMM with 0.4 M NaCl using the Total RNA Midi Kit (Qiagen). Approximately 10 μg of total RNA was electrophoretically separated on a denaturing 1.5% agarose gel, transferred to a Schleicher & Schuell NY13N membrane and hybridized with [α-^{32}P]-dCTP radiolabelled double-stranded DNA probes specific for *opuE* or *sapB* (Fig. 1A) (Sambrook *et al*., 1989). The *opuE* probe (a 366 bp *Stu*I fragment) was prepared from plasmid pORT4 (von Blohn *et al*., 1997), and the *sapB* probe (a 526 bp *Xho*I–*Xba*I fragment) was isolated from pFSB25. Approximately 20 ng of each DNA restriction fragment was labelled using the Rediprime random primer labelling kit (Amersham). They were subsequently purified with Nuc Trap columns (Stratagene) to remove the unincorporated [α-^{32}P]-dCTP. DNA–RNA hybridization was performed at 68°C overnight using ≈10^6 c.p.m. of each radiolabelled probe per ml of hybridization solution (5× SSC, 5× Denhardt's, 0.5% sodium dodecyl sulphate and 10 μg ml^{-1} salmon-sperm DNA). The membrane filters were washed and exposed to radiograph films according to routine procedures (Sambrook *et al*., 1989). Primer extension analysis of the *opuE* P-1- and *opuE* P-2-mediated transcription was carried out as described previously (von Blohn *et al*., 1997) using 10 μg of total RNA and an infrared dye IRD-41-labelled *opuE*-specific primer (5′-CAATACTCACGTTTTACCCTC; position 991 bp to 971 bp). The reaction products were analysed on a LI-COR DNA sequencer (MWG). Total RNA from cell cultures of the *B. subtilis* strains JH642 or FSB5 (*sigB*) harbouring the *opuE′* plasmid pBKB98 (von Blohn *et al*., 1997) was isolated after ethanol, salt or heat stress (Fig. 5). Ethanol stress was imposed on SMM-grown cultures (OD_{578} 0.5) by adding the alcohol to a final concentration of 4% (v/v); salt stress was imposed by adding 0.4 M NaCl; and heat stress was imposed by pouring the culture into an Erlenmeyer flask in a shaking water bath preheated to 48°C (Bernhardt *et al*., 1997). For the primer extension experiments with the mutant *opuE* P-1 and *opuE* P-2 promoters, total RNA was prepared from the *B. subtilis* strain JH642 carrying either plasmids pFSB20, pFSB21 or pFSB34 (Fig. 3B). Although the strains used for the primer extension experiments are *opuE*$^+$, no reaction product was detected in the absence of plasmid-borne *opuE* promoters (data not shown).

TreA activity assays

Aliquots (0.5–1.5 ml) from cultures of *opuE–treA B. subtilis* fusion strains were harvested by centrifugation for 2 min in an Eppendorf micro centrifuge and resuspended in 0.25 ml of Z-buffer (Helfert *et al*., 1995; Gotsche and Dahl, 1996) adjusted to pH 6.0 containing 1 mg ml^{-1} lysozyme. After incubation for 5 min at 37°C in an Eppendorf thermomixer, cellular debris was removed by centrifugation and the supernatant was then used for TreA activity assays. A 0.2 ml sample of this cleared cell extract was added to 0.6 ml of Z-buffer (pH 6.0) and 0.2 ml of a solution of PNPG (4 mg ml^{-1} dissolved in 10 mM K^+ phosphate buffer; pH 7.5) and was incubated at 28°C. The enzyme reaction was terminated by the addition of 0.5 ml of a 1 M solution of sodium carbonate, and the optical density of the assay mixture was then immediately recorded at 420 nm in a spectrophotometer. TreA [phospho-α-(1,1)-glucosidase] specific activity is expressed in Miller units per mg of protein (Gotsche and Dahl, 1996; Helfert *et al*., 1995). Protein concentrations were determined using the Bio-Rad protein assay with acetylated bovine serum albumin as the standard.

Acknowledgements

We are grateful to R. Brückner, M. Dahl and M. Jebbar for generously providing plasmids and appreciate our discussions with M. Hecker, U. Völker and C. von Blohn. We thank B. Kempf for critical reading of the manuscript and V. Koogle for her help in editing the paper. Financial support for this study was provided by the Deutsche Forschungsgemeinschaft through SFB-395 and the Fonds der Chemischen Industrie.

References

Akbar, S., Kang, C.M., Gaidenko, T.A., and Price, C.W. (1997) Modulator protein RsbR regulates environmental signalling in the general stress pathway of *Bacillus subtilis*. *Mol Microbiol* **24:** 567–678.

Alice, A.F., and Sanchesz-Rivas, C. (1997) DNA supercoiling and osmoresistance in *Bacillus subtilis* 168. *Curr Microbiol* **35:** 309–315.

Barron, A., May, G., Bremer, E., and Villarejo, M. (1986) Regulation of envelope protein composition during adaptation to osmotic stress in *Escherichia coli*. *J Bacteriol* **167:** 433–438.

Bernhardt, J., Völker, U., Völker, A., Antelmann, H., Schmid, R., Mach, H., and Hecker, M. (1997) Specific and general stress proteins in *Bacillus subtilis* – a two-dimensional protein electrophoresis study. *Microbiology* **143:** 999–1017.

Blomberg, A. (1997) Osmoresponsive proteins and functional assessment strategies in *Saccharomyces cerevisiae*. *Electrophoresis* **18:** 1429–1440.

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.

Boylan, S.A., Redfield, A.R., Brody, M.S., and Price, C.W.

(1993) Stress-induced activation of the σB 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.

Burg, M.B., Kwon, E.D., and Kültz, D. (1997) Regulation of gene expression by hypertonicity. *Annu Rev Physiol* **59:** 437–455.

Conter, A., Menchon, C., and Gutierrez, C. (1997) Role of DNA supercoiling and RpoS Sigma factor in the osmotic and growth phase-dependent induction of the gene *osmE* of *Escherichia coli* K12. *J Mol Biol* **273:** 75–83.

Csonka, L.N., and Epstein, W. (1996) Osmoregulation. In *Escherichia coli and Salmonella: Cellular and Molecular Biology.* Neidhard, C.F., Curtis, R., III, Ingraham, J.L., Lin, E.C.C., Low, K.B., Magasanik, B., Reznikoff, W.S., Riley, M., *et al.* (eds). Washington DC: American Society for Microbiology, pp. 1210–1223.

Cutting, S.M., and Vander Horn, P.B. (1990) Genetic analysis. In *Molecular Biological Methods for Bacillus.* Harwood, C.R., and Cutting, S.M. (eds). Chichester, UK: John Wiley & Sons, pp. 27–74.

Dattananda, C.S., Rajkumari, K., and Gowrishankar, J. (1991) Multiple mechanisms contribute to osmotic inducibility of *proU* operon expression in *Escherichia coli*: demonstration of two osmoresponsive promoters and of a negative regulatory element within the first structural gene. *J Bacteriol* **173:** 7481–7490.

Diekmann, S., and Wang, J.C. (1985) On the sequence determinants and flexibility of the kinetoplast DNA fragment with abnormal gel electrophoretic mobilities. *J Mol Biol* **186:** 1–11.

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

Gotsche, S., and Dahl, M.K. (1996) Purification and characterization of the phospho-α(1,1)glucosidase (TreA) of *Bacillus subtilis* 168. *J Bacteriol* **177:** 2721–2726.

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

Harwood, C.R., and Archibald, A.R. (1990) Growth, maintenance and general techniques. In *Molecular Biological Methods for Bacillus.* Harwood, C.R., and Cutting, S.M. (eds). Chichester, UK: John Wiley & Sons, pp. 1–26.

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

Helfert, C., Gotsche, S., and Dahl, M.K. (1995) Cleavage of trehalose-phosphate in *Bacillus subtilis* is catalyzed by a phospho-α(1,1)-glucosidase encoded by the *treA* gene. *Mol Microbiol* **16:** 111–120.

Higgins, C.F., Dorman, C.J., Stirling, D.A., Waddell, L., Booth, I.R., May, G., and Bremer, E. (1988) A physiological role for DNA supercoiling in the osmotic regulation of gene expression in *S. typhimurium* and *E. coli*. *Cell* **52:** 569–584.

Igo, M.M., and Losick, R. (1986) Regulation of a promoter that is utilized by minor forms of RNA polymerase holoenzyme in *Bacillus subtilis*. *J Mol Biol* **191:** 615–624.

Jebbar, M., von Blohn, C., and Bremer, E. (1997) Ectoine functions as an osmoprotectant in *Bacillus subtilis* and is accumulated via the ABC-transport system OpuC. *FEMS Microbiol Lett* **154:** 325–330.

Kappes, R.M., and Bremer, E. (1998) Response of *Bacillus subtilis* to high osmolarity: uptake of carnitine, crotonobetaine and γ-butyrobetaine via the ABC transport system OpuC. *Microbiology* **144:** 83–90.

Kappes, R.M., 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.

Kunst, F., and Rapoport, G. (1995) Salt stress is an environmental signal affecting degradative enzyme synthesis in *Bacillus subtilis*. *J Bacteriol* **177:** 2403–2407.

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.

Lucht, J.M., Dersch, P., Kempf, B., and Bremer, E. (1994) Interactions of the nucleoid-associated DNA-binding protein H-NS with the regulatory region of the osmotically controlled *proU* operon of *Escherichia coli*. *J Biol Chem* **269:** 6578–6586.

McAllister, C.F., and Achberger, E.C. (1989) Rotational orientation of upstream curved DNA affects promoter function in *Bacillus subtilis*. *J Biol Chem* **264:** 10451–10456.

Marinus, G.M., Carraway, M., Frey, A.Z., Brown, L., and Arraj, A.J. (1983) Insertion mutations in the *dam* gene of *Escherichia coli* K-12. *Mol Gen Genet* **192:** 288–289.

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

Milner, J.L., Grothe, S., and Wood, J.M. (1988) Proline porter II is activated by a hyperosmotic shift in both whole cells and membrane vesicles of *Escherichia coli* K12. *J Biol Chem* **263:** 14900–14905.

Overdier, D.G., Olson, E.R., Erickson, B.D., Ederer, M.M., and Csonka, L.N. (1989) Nucleotide sequence of the transcriptional control region of the osmotically regulated *proU* operon of *Salmonella typhimurium* and identification of the 5′ endpoint of the *proU* mRNA. *J Bacteriol* **171:** 4694–4706.

Owen-Hughes, T.A., Pavitt, G.D., Santos, D.S., Sidebotham, J.M., Hulton, C.S.J., Hinton, J.C.D., and Higgins, C.F. (1992) The chromatin-associated protein H-NS interacts with curved DNA to influence DNA topology and gene expression. *Cell* **71:** 255–265.

Pérez-Martín, J., and de Lorenzo, V. (1997) Clues and consequences of DNA bending in transcription. *Annu Rev Microbiol* **51:** 593–628.

Peter, H., Burkovski, A., and Krämer, R. (1996) Isolation, characterization and expression of the *Corynebacterium glutamicum betP* gene, encoding the transport system for the compatible solute glycine betaine. *J Bacteriol* **178:** 5229–5234.

Rhodes, D., and Hanson, A.D. (1993) Quaternary ammonium and tertiary sulfonium compounds in higher plants. *Annu Rev Plant Physiol Plant Mol Biol* **44:** 357–384.

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

Schöck, F., Gotsche, S., and Dahl, M.K. (1996) Vectors using the phospho-α(1,1)-glucosidase-encoding *treA* of *Bacillus subtilis* as a reporter. *Gene* **170:** 77–80.

Steinmetz, M., and Richter, R. (1994) Plasmids designed to alter the antibiotic resistance expressed by insertion mutations in *Bacillus subtilis*, through *in vivo* recombination. *Gene* **142:** 79–83.

Sutherland, L., Cairney, J., Elmore, M.J., Booth, I.R., and Higgins, C.F. (1986) Osmotic regulation of transcription: induction of the *proU* betaine transport gene is dependent on accumulation of intracellular potassium. *J Bacteriol* **168:** 805–814.

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.

Voelker, U., Voelker, A., Maul, B., Hecker, M., Dufour, A., and Haldenwang, W.G. (1995) Separate mechanisms activate σ^B of *Bacillus subtilis* in response to environmental and metabolic stress. *J Bacteriol* **177:** 3771–3780.

von Blohn, C., Kempf, B., Kappes, R.M., and Bremer, E. (1997) Osmostress response in *Bacillus subtilis*: characterization of a proline uptake system (OpuE) regulated by high osmolarity and the alternative transcription factor sigma B. *Mol Microbiol* **25:** 175–187.

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.

Wong, L.S., Johnson, M.S., Sandberg, L.B., and Taylor, B.L. (1995) Amino acid efflux in response to chemotactic and osmotic signals in *Bacillus subtilis*. *J Bacteriol* **177:** 4342–4349.

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, pp. 81–109.