The effects of osmotic upshock on the intracellular solute pools of *Bacillus subtilis*

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The effects of hypersaline treatment (osmotic upshock) on solute accumulation have been studied in the Gram-positive bacterium *Bacillus subtilis*. Natural abundance ^{13}C NMR spectroscopy studies revealed only proline as a major organic osmoticum in cells grown in defined medium (no exogenous organic solutes) and this finding was confirmed by amino acid analysis. Intracellular concentrations of both K^+ and proline rose markedly after osmotic upshock. K^+ influx from the medium was rapid (<1 h) but proline synthesis was a slower process (5–9 h). Proline synthesis appeared to be dependent on the prior accumulation of K^+ and it is possible that K^+ serves in some manner as the signal for increased proline synthesis. In cells upshocked in medium enriched in glycine betaine the endogenous synthesis of proline was repressed and glycine betaine served as the sole organic osmoticum. K^+ was also accumulated under these conditions.

Introduction

The responses of bacterial cells to a hyperosmotic environment have received much attention in recent years (see Csonka, 1989). All organisms must be able to adapt to fluctuations in environmental osmolality in order to maintain a positive turgor pressure. When a cell is subjected to a sudden osmotic upshock (increase in external osmolality) the internal osmotic pressure falls due to the rapid outflow of water. To restore turgor pressure and allow growth to continue, a cell must restore its internal osmotic pressure to a level exceeding that of the environment. This is achieved by the accumulation of various solutes by uptake and/or metabolic interconversion, often to very high concentrations leading to an influx of water into the cells and the restoration of turgor (Brown, 1976). The term 'compatible solute' was introduced by Brown & Simpson (1972) to describe accumulated solutes which are non-inhibitory to enzyme function at high concentration, i.e. compatible with cellular metabolism.

A diverse range of organic solutes are accumulated as compatible solutes in various micro-organisms; these solutes include carbohydrates, amino acids, imino acids, quaternary ammonium compounds and tertiary sulphonium compounds. Osmotic responses of bacteria have been best studied in Gram-negative enteric bacteria, notably *Escherichia coli* and *Salmonella typhimurium* (Booth *et al.*, 1988; Higgins *et al.*, 1987). A complex, integrated response occurs in these organisms involving accumulation of K^+, glutamic acid, glycine betaine and trehalose depending on the degree of upshock and the chemical nature of the environment (Imhoff, 1986; Dinnibier *et al.*, 1988). There is little similar data regarding the responses of Gram-positive bacteria to osmotic upshock. The neutral amino acids proline, glutamine and alanine are synthesized in *Streptomyces* (Killham & Firestone, 1984) while glutamine is synthesized and proline taken up in *Staphylococcus aureus* at the expense of glutamic and aspartic acids (Anderson & Witter, 1982). Additionally, the use of complex media has often made previous studies difficult to interpret: it is often unclear whether solutes are being synthesized *de novo* or transported into the cell from the medium.

Bacillus subtilis has been shown to accumulate proline in response to osmotic upshock (Measures, 1975; Gould & Measures, 1977), although the nature of the medium used in these studies is unclear. Specific chemical assays designed to quantify a specific osmolyte or class of osmolyte were used, with the possibility of overlooking other solutes. A more powerful technique is natural abundance ^{13}C nuclear magnetic resonance spectroscopy (NMR) – this technique provides a means of

identifying all of the major organic solutes in biological material from their characteristic NMR profiles. The only potential osmoregulatory solutes not detected are inorganic ions. Natural abundance ^{13}C NMR has been used to identify the organic osmotica in filamentous fungi (Gadd *et al.*, 1984), cyanobacteria (Reed *et al.*, 1985*b*), algae (Reed *et al.*, 1985*a*) and certain Gram-negative (Larsen *et al.*, 1987) and Gram-positive (Hutkins *et al.*, 1987) bacteria. Such use of NMR enables preliminary identification of all major organic solutes which then permits the selection of specific chemical assays or other rapid quantitative techniques for further work, examining the characteristics of accumulation.

This paper provides the first unequivocal data for the intracellular solute profile of *B. subtilis* and the effect of osmotic upshock on this profile, based on NMR analysis, and the first evidence of K^+-dependent osmoregulatory organic solute synthesis in a Gram-positive bacterium.

Methods

Organism, media and growth conditions. Bacillus subtilis NCIB 1650, obtained from the National Collection of Industrial Bacteria, Aberdeen, UK, was routinely maintained on nutrient agar at 25 °C. The defined liquid medium used in this study is described in the preceding paper (Whatmore & Reed, 1990); the K^+-free medium used was as above with the omission of the KCl. All experimental flasks were inoculated with exponentially growing cultures (10 h). All experiments were done at 25 °C unless stated otherwise. Unless stated otherwise all measurements of internal solute concentrations represent the mean ± 95% confidence limits ($1.96 \times$ SE) of three or four replicates.

Determination of cell number and volume. Cells were sized and counted using a Coulter counter (ZBI) and C1000 Channelyzer linked to an Acorn microcomputer as described by Whatmore & Reed (1990).

Nuclear magnetic resonance (NMR) spectroscopy. Samples for ^{13}C NMR were cultured in 12 l flasks containing 10 l of medium with constant sparging using sterile air. Samples were harvested in the mid-exponential phase by continuous centrifugation and rinsed in isotonic salt solution to remove any traces of adhering glucose from the growth medium. Harvested cells were extracted twice overnight in 80% (v/v) ethanol and centrifuged to remove cellular debris; the residue was then dried in a rotary evaporator. Samples were freeze-dried overnight, dissolved in 1 ml D_2O and filtered (Millipore cellulose nitrate, pore size 0.45 µm). Natural abundance ^{13}C NMR spectra were obtained using a Bruker WP360 FT spectrometer. Values are expressed as $\delta =$ observed shift/spectrometer frequency (p.p.m.).

Intracellular glycine betaine was identified and quantified by ^{1}H NMR (Chudek *et al.*, 1987). Samples varying from 320 to 100 ml were harvested and rinsed in isotonic salt solution and prepared as for ^{13}C NMR with sodium acetate added as an internal standard. Estimates of the sample size required to generate a suitable signal were based on concentrations of proline previously seen after upshock. Since 1 mg glycine betaine is equivalent to 2·1 mg sodium acetate in terms of ^{1}H NMR peak area, the amount of glycine betaine present in each sample could be quantified from the peak area, relative to that of the internal standard. Samples were analysed with a Bruker HX90 continuous wave spectrometer operating at 90 MHz. Line positions were measured relative to 3-(trimethylsilyl)propanesulphonic acid (sodium salt). Measurements were made using the trimethyl resonance of glycine

betaine at $\delta_H = 3.26$ p.p.m. and for acetate at $\delta_H = 1.29$ p.p.m. The methylene signal of glycine betaine was not used due to weak signal intensity.

Extraction and measurement of amino acid pools. Samples for amino acid analysis were harvested by centrifugation and extracted overnight in 80% (v/v) ethanol. After removal of debris by centrifugation, samples were evaporated to dryness on a photographic warmplate and then redissolved in 200 mM-lithium hydroxide/sodium citrate buffer, pH 2·2. After filtration (Millipore cellulose nitrate, pore size 0·45 µm), amino acids were analysed on a LKB-4400 Amino Acid Analyzer using a lithium citrate buffer system. n-Leucine was used as the internal standard.

Determination of intracellular free proline. A modification of the method of Bates *et al.* (1973) was used for rapid determination of free proline. The method is a simple colorimetric determination which proved adequate to quantify the relatively large amounts of proline found in this organism. The colour yield of interfering amino acids is small: glutamine, the major interfering compound, yields less than 1·5% of the colour given by an equivalent proline concentration. In addition, amino acid analysis showed that the amounts of other amino acids (notably glutamine) present in salt-stressed *B. subtilis* were negligible when compared with proline. Samples were harvested by centrifugation and extracted overnight in 1 ml 3% (w/v) aqueous 5-sulphosalicylic acid. Precipitated protein and other debris were removed by microcentrifugation. Acid ninhydrin was prepared by dissolving 1·25 g ninhydrin in 30 ml glacial acetic acid and 20 ml 6 M-H_3PO_4. The cellular extract was reacted with 0·5 ml acid ninhydrin and 0·5 ml glacial acetic acid at 100 °C for 1 h. Samples were then plunged into ice and the colour extracted by mixing vigorously with 2 ml toluene. After 15 min the absorbance was read at 520 nm against a toluene blank.

A standard curve was prepared using 0·02–0·4 µmol ml⁻¹ authentic proline in 3% 5-sulphosalicylic acid. Intracellular concentrations of proline could be calculated since the total intracellular volume of all samples was determined by Coulter counter before harvesting.

Determination of intracellular K^+. Following particle size analysis, cells were harvested either by centrifugation or by passage through glass-microfibre filters (Whatman GF/F). Samples were then rinsed thoroughly in isotonic $CaCl_2$ to remove extracellular K^+ in the cell wall and the growth medium. The inorganic ions were then extracted overnight in 1 ml concentrated HNO_3. Each sample was then diluted as appropriate in distilled water and analysed for K^+ using a flame photometer (EEL, model 100). Quantification was with respect to an external standard curve (0·25–1·00 mM-KCl).

Results

Natural abundance ^{13}C NMR spectroscopy

Fig. 1 shows the ^{13}C NMR spectra obtained from *B. subtilis* grown in basal medium with and without added NaCl (400 mM). The resonances from *B. subtilis* grown in an increased salt concentration were identified as glutamic acid ($\delta = 23.5$, 30·0, 51·4, 171·3 and 177·8 p.p.m.) and proline ($\delta = 20.6$, 25·6, 42·9, 58·0 and 171·3 p.p.m.) by reference to spectra from authentic compounds. As all other resonances were negligible, glutamic acid and proline are the only two organic compounds accumulated in osmotically significant quan-
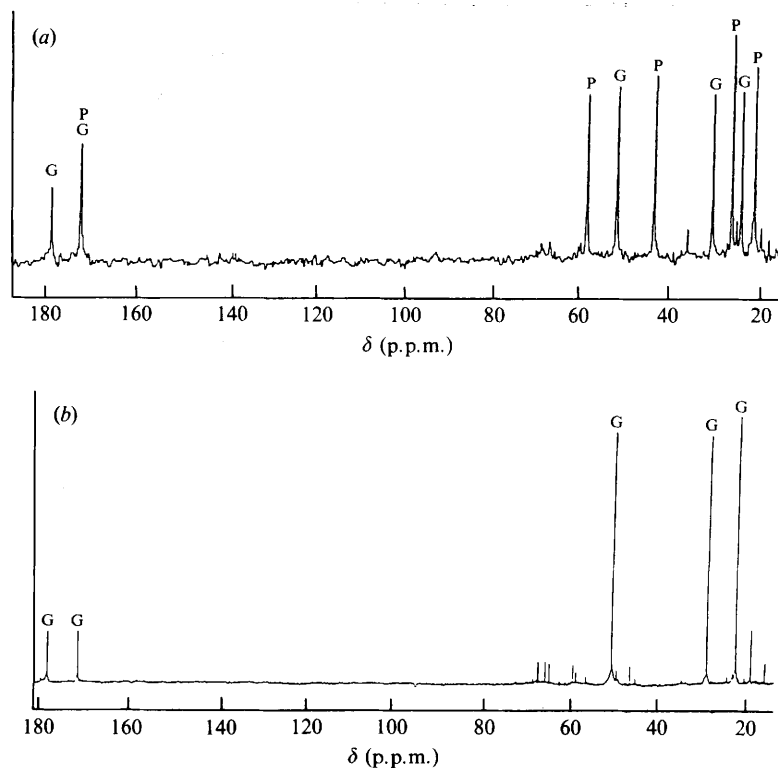
Fig. 1. ^{13}C NMR spectra of *B. subtilis* grown in the presence (*a*) and absence (*b*) of 400 mM-NaCl in defined medium at 25 °C. Resonances are not directly comparable, between the two spectra, due to differences in biomass and the absence of a reference (internal standard). G, glutamic acid; P, proline.

tities under these growth conditions. The spectrum obtained from *B. subtilis* grown in basal medium showed glutamic acid was a major solute but no proline was detected. Hence it appears that proline may be the sole osmotically responsive endogenous organic solute in *B. subtilis*.

Determination of free amino acid pools

Table 1 gives details of the amino acid pools of *B. subtilis* at points prior to upshock, during recovery (i.e. at 2 h, before growth had resumed), once exponential growth had resumed (7 h) and during the stationary phase (25 h). The results confirmed the NMR findings, since glutamic acid and proline were the only amino acids present in significant quantities. The total amino acid pool increased from 138 mM to 893 mM 7 h after upshock; at this point cells had recovered turgor (Whatmore & Reed, 1990) and growth had resumed. By 25 h the total amino acid pool had fallen considerably, to 398 mM. In unstressed cells, glutamic acid formed approximately 75% of the total amino acid pool but 7 h after upshock the glutamic acid proportion had fallen to 19% and the proportion of proline had risen to 80%. Although the total amino acid pool declined in the stationary phase,

Table 1. *Free amino acid pools of B. subtilis after osmotic upshock (400 mM-NaCl) in defined medium at 25 °C*

All concentrations are expressed in mM. Other amino acids were not present in sufficient quantities to be detected.

Time after	Amino acid pool			
upshock (h) ...	0	2	7	25
Glutamate	103·2	157·6	167·4	61·6
Proline	16·4	188·2	705·4	318·2
Glycine	10·4	12·9	10·1	7·3
Alanine	2·6	5·9	7·5	7·0
Aspartate	0·64	0·43	0·80	1·28
Ornithine	4·9	0·60	1·85	2·33
Total free amino acid pool	138·1	365·6	892·9	397·7
Glutamate (% total)	74·7	43·1	18·7	15·5
Proline (% total)	11·9	51·5	79·0	80·0

the proportion of glutamic acid to proline remained similar. The concentration of proline rose some 43-fold after upshock while the glutamic acid pool increased by little more than half. The pools of other amino acids were
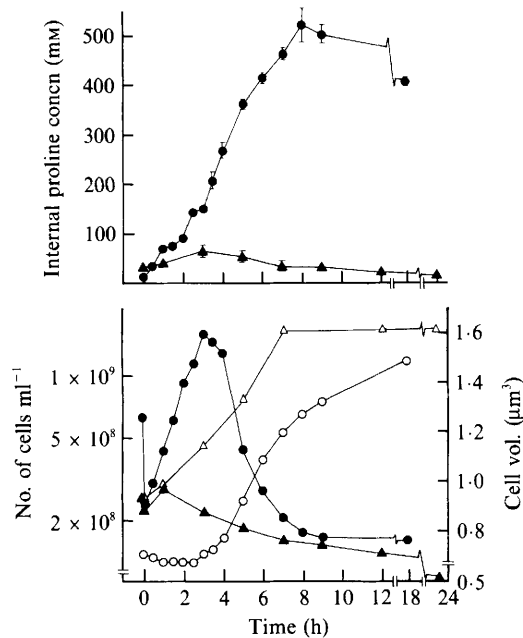
Fig. 2. (*a*) Effect of upshock with 400 mM-NaCl on the internal proline concentration of *B. subtilis* in defined medium at 25 °C. Mean ± 95% confidence limits of three or four replicates. ●, Upshocked cells; ▲, non-stressed cells. (*b*) Corresponding mean cell volume and cell count data. Mean of four to six replicates. Mean cell volume: ●, upshocked cells; ▲, non-stressed cells. Cell number: ○, upshocked cells; △, non-stressed cells.

too small to be regarded as osmotically significant. It was noted that a significant quantity of an unknown ninhydrin-reactive compound was detected in the stationary phase sample.

Accumulation of proline after upshock

In all of the subsequent experiments the intracellular proline concentration was determined by the chemical assay in conjunction with biovolume density measurements obtained from the Coulter counter.

The effect of upshock with 400 mM-NaCl on exponentially growing *B. subtilis* is shown in Fig. 2. The intracellular proline concentration rose steadily after upshock, reaching a peak of 520 mM after 8 h before falling somewhat as the cells entered stationary phase. Control cells, which were diluted by the same factor as shocked cells in basal medium (without added NaCl) maintained a relatively constant proline level. The salt-shocked cells showed a lag period of 3 h before growth resumed, while control cells showed no significant lag, with growth maintained throughout (Fig. 2*b*). Growth resumed prior to peak proline accumulation in cells and therefore maximal proline accumulation does not appear to be necessary for growth at high osmolality. As predicted by osmotic theory the mean cell volume fell
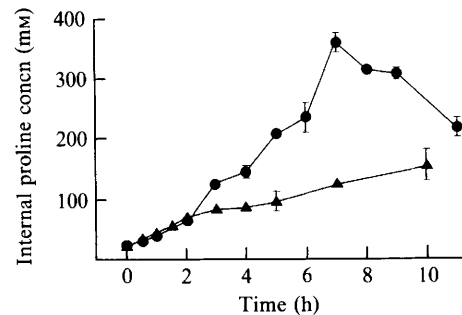

Fig. 3. Effect of upshock with 400 mM-NaCl on the internal proline concentration after chloramphenicol treatment of *B. subtilis* grown in defined medium at 25 °C. Mean ± 95% confidence limits of three or four replicates. ▲, Chloramphenicol-treated cells (6 μg ml⁻¹, 60 min); ●, control cells (salt-stress only).

sharply immediately after upshock but then rose rapidly over the next 3 h to a level considerably higher than that prior to upshock. After this point the mean cell volume of NaCl-treated cells fell rapidly – the peak mean cell volume corresponded with the point at which cell division was resumed. Control cells maintained a less variable volume throughout, although the cells became somewhat smaller as they approached the stationary phase.

Fig. 3 shows the effects of pretreating cells with chloramphenicol before upshock in an attempt to determine whether proline synthesis was genetically induced or resulted from activation of existing enzymes by osmotic upshock. Cells were treated with 6 μg chloramphenicol ml⁻¹ for 1 h prior to upshock. The accumulation of proline in untreated and chloramphenicol-treated cells was virtually identical for the first 2 h after upshock. After this point proline levels in chloramphenicol-treated cells increased at a considerably slower rate than those of untreated cells. The mean cell volume of chloramphenicol-treated cells showed little recovery over the time course (data not shown) in contrast to that of control cells which showed the same characteristic peak seen previously (Fig. 2*b*). There was no increase in the cell number of chloramphenicol-treated cells over the course of the experiment.

Accumulation of K⁺ after upshock

Fig. 4 shows the effect of upshock on the internal K⁺ levels of exponentially growing *B. subtilis*, in minimal medium both with and without the addition of 1 mM-glycine betaine. In upshocked cells the K⁺ level rose from a basal value of approximately 350 mM to approximately 650 mM within 1 h. After this point the K⁺ concentration remained relatively steady, at 600–725 mM, until the stationary phase when it fell sharply to around 200 mM.
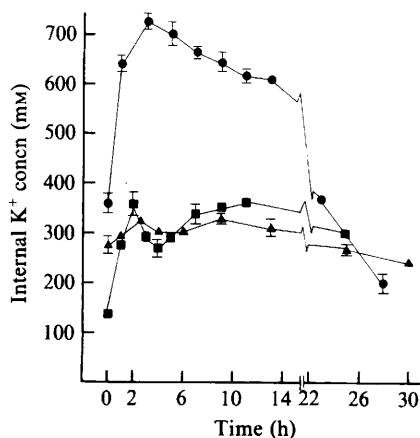
Fig. 4. Effect of upshock with 400 mM-NaCl on the internal K⁺ concentration of *B. subtilis* grown in defined medium with and without 1 mM-glycine betaine at 25 °C. Mean ± 95% confidence limits of three or four replicates. ●, Upshocked cells (no glycine betaine); ▲, non-stressed cells (no glycine betaine); ■, upshocked cells (1 mM-glycine betaine).

Fig. 5. (*a*) Effect of exogenous K⁺ on proline accumulation after 400 mM-NaCl upshock of *B. subtilis* in defined medium at 25 °C. Cells were harvested and rinsed in isotonic CaCl₂ before being resuspended in growth medium (± KCl, as required) with 400 mM-NaCl. Mean ± 95% confidence limits of three or four replicates. × Marks the point at which K⁺ (5 mM was added). (*b*) Corresponding cell volume data. (*c*) Corresponding cell number data. Symbols for (*a*), (*b*) and (*c*): ●, 5 mM-KCl in medium; ■, 5 mM-KCl added 22 h after upshock; ▲, K⁺-free medium.

In unstressed cells the K⁺ level remained relatively constant during growth with a mean value of about 300 mM, with a slow decline during the stationary phase. Cell volume and cell number (data not shown) showed the same patterns as seen in the proline accumulation experiments, with a 3 h lag phase for salt-stressed cells, corresponding to a peak in cell volume at 3 h.

Effects of K⁺ starvation on proline accumulation

The effects of K⁺ starvation on proline accumulation are shown in Fig. 5. This experiment was done by harvesting a large volume of exponentially growing cells, rinsing them in isotonic CaCl₂ to remove all external K⁺ and then resuspending them in a small volume of K⁺-free basal medium. This cell suspension was then split into three equal volumes and resuspended in three flasks of medium containing 400 mM-NaCl. One flask contained complete (K⁺-sufficient medium) while the remaining flasks contained K⁺-free medium. KCl (5 mM) was added to one of the latter flasks after 22 h.

In the presence of exogenous K⁺ proline was accumulated as in previous experiments, with a steady rise over 9 h to approximately 420 mM before declining in the stationary phase to 45 mM. In the absence of K⁺ there was relatively little accumulation of proline, with a maximum level of 100 mM. In the remaining culture there was similarly no accumulation of proline until KCl was added after 22 h. Within 1 h of this addition the proline level rose sharply and peaked at approximately 500 mM after 7 h. Growth and volume data (Fig. 5*b, c*) showed the responses that would be predicted from previous experiments. The culture in K⁺-sufficient
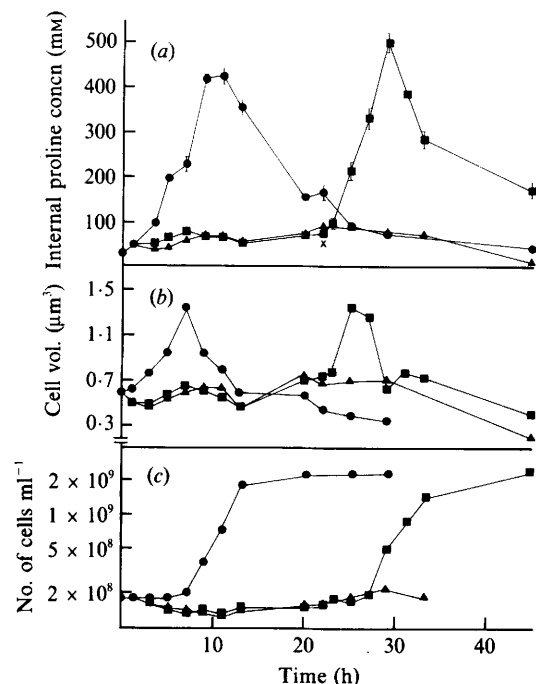
medium showed a lag of approximately 4 h before growth resumed. The culture devoid of K⁺ showed no significant increase in cell number over the course of the experiment while in the remaining culture growth resumed at 25 h. The K⁺-sufficient culture showed the normal pattern of volume recovery while the K⁺-deplete culture showed no volume recovery. The remaining culture showed a rapid rise in volume after addition of KCl at 22 h, peaking at 24 h.

Accumulation of solutes in the presence of exogenous glycine betaine

The effect of upshock on solute levels of cells grown in the presence of 1 mM-glycine betaine was examined. Glycine betaine is not synthesized by *B. subtilis* grown in defined medium under these conditions, as shown by ¹³C NMR (Fig. 1). However, it is known to act as a compatible solute in many bacteria if available externally (Imhoff & Rodriguez-Valera, 1984).

Fig. 6 shows the effect of osmotic upshock on levels of glycine betaine and proline in cells grown and upshocked
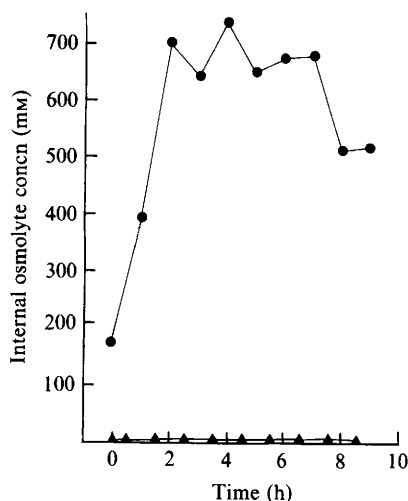
Fig. 6. Effect of upshock with 400 mM-NaCl on internal glycine betaine and proline concentrations of *B. subtilis* grown in defined medium with 1 mM-glycine betaine at 25 °C. ●, Glycine betaine (data from a single sample); ▲, proline (mean of three or four replicates).

in the presence of 1 mM-glycine betaine. From a level of approximately 175 mM in basal medium glycine betaine rose within 2 h of upshock to approximately 700 mM and remained relatively steady for the next 5 h before falling somewhat in the late exponential phase. Experiments done with killed cells confirmed that the rather high levels of glycine betaine detected did not result from significant carry over from the growth medium (Whatmore, 1989). The level of proline detected in these cells was very low: in basal medium before upshock there was only 6 mM-proline, somewhat less than the 10–20 mM generally seen in cells grown in basal medium without glycine betaine. After upshock, proline levels showed no substantial increase remaining below 10 mM throughout.

Fig. 4 shows the effect of upshock on internal K⁺ concentration for cells shocked in a medium with added glycine betaine. As before, there was a considerable rise in internal K⁺ concentration after upshock, though the initial level of 135 mM and the maximum level (360 mM) attained were considerably lower than those found in cells grown without glycine betaine. After 2 h, K⁺ levels remained at 275–375 mM throughout the experiment.

Discussion

Natural abundance ^{13}C NMR revealed only proline as a major endogenous organic osmolyte in *B. subtilis* grown in minimal medium. Amino acid analysis confirmed NMR findings: glutamic acid was not substantially accumulated in response to upshock in contrast to Gram-negative bacteria (Measures, 1975). The small increase

seen at 2 h (Table 1) may be due to increased activity in the biosynthetic pathway leading to proline (Hayzer & Leisinger, 1980). No evidence was obtained of significant accumulation of any of the other neutral amino acids found in other Gram-positive bacteria. The unidentified ninhydrin-reactive compound, which did not correspond to any of the known amino acid standards, appeared in significant quantities only in stationary phase cells, suggesting its role was probably not osmotic; it may be a storage compound or a metabolite involved in sporulation.

The accumulation of proline after upshock was somewhat less rapid than previously reported for osmotically stressed *B. subtilis* (Gould & Measures, 1977), when growth recommenced after a few minutes. The differences between the present study and previous work in the time-scale of proline accumulation and the resumption of growth may be due to variations in cultural conditions/medium composition, strain differences or variation in other environmental determinants. Adding exogenous proline (1 mM) or raising the growth temperature to 37 °C reduced the lag period to under 2 h (Whatmore, 1989). Interestingly, the level of proline synthesized after shock at 37 °C (180 mM) was considerably lower than that seen at 25 °C (520 mM, Fig. 2). Similar phenomena have been reported in both cyanobacteria (Warr *et al.*, 1985) and in heterotrophic halophilic eubacteria of the family *Halomonadaceae* (Wohlfarth *et al.*, 1990), where the nature of the osmolyte pool has also been shown to vary with temperature. It seems that at more favourable temperatures *B. subtilis* can tolerate osmotic stress with a reduced level of intracellular proline, although it is not known whether this is due to the accumulation of alternative solutes or to changes in the relationship between turgor and growth with temperature, e.g. due to variations in cell wall composition.

The addition of chloramphenicol prior to upshock produced an effect similar to that on the osmotically regulated trehalose phosphate synthase of *E. coli*, where a slow accumulation of trehalose relative to that of control cells was seen after upshock (Dinnibier *et al.*, 1988). Proline synthesis in chloramphenicol treated and untreated upshocked *B. subtilis* cells was identical over the first 2 h; accumulation in chloramphenicol-treated cells was then severely curtailed. This observation, and the fact that throughout this study no lag was observed between upshock and the onset of proline synthesis, suggests that at least some of the accumulation was due to activation of pre-existing enzyme. However, some protein synthesis appears to be necessary for maximal sustained synthesis of proline.

Data presented in Fig. 4 imply that K⁺ also plays a role in osmotic adjustment in *B. subtilis*, since the K⁺ level

more than doubled after upshock. This effect was more rapid than for proline accumulation and must reflect a stimulation of (active) net K$^+$ influx by increased external osmotic pressure. Hence the restoration of turgor pressure in *B. subtilis* appears to be the result of the accumulation of two principal osmolytes (in the absence of exogenous organic solutes). An initial rapid accumulation of K$^+$ from the external medium is followed by a slower accumulation of proline synthesized by the cell. It appears that the short-term accumulation of K$^+$ followed by long-term accumulation of more compatible organic solutes may be a common osmoregulatory mechanism employed by a range of micro-organisms (Epstein, 1986; Booth *et al.*, 1988). Similar mechanisms are known to operate in enteric Gram-negative bacteria (Dinnibier *et al.*, 1988) and in a number of cyanobacteria (Reed & Stewart, 1985; Reed *et al.*, 1985*b*). In these organisms there is an energy-dependent K$^+$ uptake system which responds directly to changes in turgor. The present study has provided novel evidence for the involvement of K$^+$ in the turgor regulation of *B. subtilis*. Data presented in Fig. 5 show that K$^+$ is essential for proline synthesis and consequent recovery of growth following osmotic upshock. In addition, we demonstrated in the preceding paper (Whatmore & Reed, 1990) that K$^+$ is essential for recovery of turgor pressure after upshock. Hence it can be postulated that *B. subtilis* has a turgor-sensitive K$^+$ uptake system similar in function to the system characterized for *E. coli* (Epstein, 1986).

A model similar in principle to that which operates in *E. coli* can be proposed for the response of *B. subtilis* to osmotic upshock. After upshock the outflow of water and subsequent loss of turgor will activate K$^+$ uptake. If upshock is only slight, the accumulation of K$^+$ alone may be sufficient to restore turgor. Thus cells grown in 200 mM-NaCl showed negligible increases in internal proline concentration but significant increases in K$^+$ concentration over the same range (Whatmore, 1989). However, if upshock is more substantial, accumulation of K$^+$ will lead to induction and/or activation of proline biosynthetic enzymes, since accumulation of excessive K$^+$ may be potentially deleterious to cellular metabolism (Sutherland *et al.*, 1986; Booth *et al.*, 1988). K$^+$ has now been shown to exert its effects in upshocked enteric bacteria via the induction of glycine betaine uptake (*proU*-mediated) due to alteration of DNA structure. Increased osmotic strength leads to an increase in DNA supercoiling which results in activation of transcription from the *proU* promoter (Higgins *et al.*, 1988).

Measures (1975) showed that the glutamate biosynthetic enzymes (GDH & GOGAT) of many bacteria were K$^+$-stimulated and Giaever *et al.* (1988) showed that the trehalose phosphate synthase of *E. coli* is both osmotically induced and K$^+$-activated. The genes and enzymes

of proline biosynthesis have been characterized in a number of micro-organisms including *E. coli* (Hayzer & Leisinger, 1980), *Pseudomonas aeruginosa* (Krishna & Leisinger, 1979) and *Saccharomyces cerevisiae* (Tomenchok & Brandriss, 1987). Proline biosynthesis in all of these organisms occurs via a series of three enzymic reactions and regulation of synthesis is exerted primarily through feedback inhibition of γ-glutamyl kinase, the first enzyme of the pathway, by proline. Since the γ-glutamyl kinase of Gram-negative organisms is inhibited by relatively low proline concentrations (50% inhibition by 5 mM-proline: Krishna & Leisinger, 1979) the regulation of this pathway in proline-producing, salt tolerant Gram-positive bacteria must be somewhat different. Little is known about proline synthesis in Gram-positive bacteria but it is noteworthy that Yoshinaga *et al.* (1975) purified a γ-glutamyl kinase from the Gram-positive bacterium *Brevibacterium flavum* which was unaffected by proline.

The induction of stress proteins in salt-shocked *B. subtilis* has recently been examined by Hecker *et al.* (1988). Salt stress was extremely effective in the induction of at least 14 general stress proteins as well as three proteins induced only by osmotic stress. The nature of these three proteins was not examined but it is possible that they may represent the three enzymes of the proline biosynthetic pathway.

An obvious question concerns the nature of the counter-ion to K$^+$ in *B. subtilis*. Glutamate is known to be accumulated as the counter-ion to K$^+$ in *E. coli* (Larsen *et al.*, 1987). However, glutamate levels increased only slightly after upshock and could account only for a small proportion of the required counter-ion (about 15%, based on Table 1 and Fig. 4). Natural abundance ^{13}C NMR spectroscopy showed that any other counter-ion present could not be an organic molecule. The intracellular levels of inorganic anions were not measured in this study but Cl$^-$ is the most likely candidate for the counter-ion. Killham & Firestone (1984) have shown that Cl$^-$ increases correspondingly as K$^+$ rises in *Streptomyces* sp. transferred to a higher osmolality medium. It is also possible that efflux of Na$^+$ may help balance the influx of K$^+$, as in some cyanobacteria (Reed *et al.*, 1985*b*). It should be noted that methods employed in this study measured the total potassium content of cells rather than free K$^+$ alone – only free ions will act as osmolytes. If cation solutes such as K$^+$ are bound to negatively charged components of high molecular mass within the cell their osmotic significance will be greatly reduced. Richey *et al.* (1987) concluded that, due to binding to cellular components, the cytoplasmic K$^+$ concentration was only half the total K$^+$ in *E. coli*. Tempest *et al.* (1970) have also reported that osmotic stress leads to alkalinization of the cytoplasm, though Dinnibier *et al.* (1988)

reported that this was a transient phenomenon, and hence the expulsion of H$^+$ could account for a proportion of the accumulated K$^+$.

The osmolytes accumulated by *B. subtilis* in response to upshock with 400 mM-NaCl can be shown to be sufficient to regenerate turgor, though we believe such calculations to be a somewhat simplistic approach to a complex system. Upshock with 400 mM-NaCl will raise the external osmolality by 0·740 osmol kg^{-1}. Results in the preceding paper (Whatmore & Reed, 1990) showed that turgor is restored within 5 h of upshock; hence if the proline concentration is taken as 360 mM (from Fig. 2) and the increase in K$^+$ concentration as 340 mM (from Fig. 4) the resulting osmolalities generated by these solutes are 0·380 osmol kg^{-1} and 0·306 osmol kg^{-1} respectively (by use of a cryoscopic osmometer). The sum of the osmolalities generated by these two solutes, at 0·686 osmol kg^{-1}, approaches the external osmolality increase of 0·740 osmol kg^{-1}. No account is taken of the necessary counter-ion of K$^+$ in this analysis, but equally there may be unaccounted effluxes as noted above. If the solute concentrations are expressed in terms of cell osmotic volume at 60–70% of total cell volume (Whatmore & Reed, 1990), the net increase in internal osmolality would be considerably higher.

The response of *B. subtilis* to upshock was markedly different in the presence of glycine betaine. Glycine betaine was accumulated as a compatible solute with no synthesis of proline. This may reflect the status of glycine betaine as a more effective osmoprotective solute due to an enhanced compatibility with enzyme function (Warr *et al.*, 1988). The K$^+$ level was elevated immediately after upshock of *B. subtilis* in the presence of glycine betaine. However, the K$^+$ pool was consistently smaller in the presence of glycine betaine. Hence it appears that the intracellular K$^+$ concentration may be involved in both the regulation of proline biosynthesis and the uptake of exogenous compatible solute, if available, after osmotic upshock.

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