The role of trehalose in the osmoadaptation of *Escherichia coli* NCIB 9484: interaction of trehalose, K⁺ and glutamate during osmoadaptation in continuous culture

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Natural abundance ¹³C nuclear magnetic resonance spectroscopy identified the disaccharide trehalose as the major organic osmolyte synthesized by *Escherichia coli* grown in continuous culture under nitrogen limitation in the presence of 0.5 M-NaCl. Trehalose accumulation was dependent on both the growth phase of the culture and the osmolality of the growth medium, but independent of the solute used to increase the osmolality as long as the solute was non-penetrant. The penetrant solute glycerol did not induce trehalose synthesis indicating that the loss of cell turgor rather than increasing medium osmolality *per se* was the mechanism stimulating trehalose synthesis. Under conditions of either carbon or nitrogen limitation osmoadaptation was distinctly biphasic. The initial response consisted of a rapid (within 30 min) accumulation of K⁺ and a concurrent synthesis of the amino acid glutamate; trehalose synthesis occurred during the second slower phase of osmoadaptation. Chloramphenicol severely inhibited trehalose accumulation indicating that the enzyme(s) involved in trehalose synthesis were inducible.

Introduction

The ability of bacteria to adapt to fluctuations in the osmolarity of the growth medium is of fundamental importance for their survival. *Escherichia coli* adapts to changes in osmolarity by a series of mechanisms under the control of osmoregulated genes, enzymes and transport systems (Le Rudulier *et al.*, 1984; Higgins *et al.*, 1987). These mechanisms include such adaptations as: (1) alterations in protein composition of the outer membrane (Van Alphen & Lugtenberg, 1977), (2) K⁺ transport (Epstein & Schultz, 1965; Laimins *et al.*, 1981; Epstein, 1986), (3) the active intracellular accumulation of exogenous organic compatible solutes such as proline (Gowrishanker, 1985), betaines (fully *N*-methylated amino acid derivatives) (Perroud & Le Rudulier, 1985; Larsen *et al.*, 1987) and dimethylthetin (Chambers *et al.*, 1987), and/or (4) synthesis of glutamic acid (Measures, 1975; Larsen *et al.*, 1987), trehalose (Strøm *et al.*, 1986; Larsen *et al.*, 1987) and glycine betaine, if its precursors choline or glycine betaine aldehyde are present (Landfald & Strøm, 1986; Styrvold *et al.*, 1986). These organic solutes can be accumulated to high intracellular concentrations by *E. coli* subjected to osmotic stress in order to restore cell turgor and so allow growth.

Until recently, most studies investigating osmoadaptation of *E. coli* have concentrated on the characterization of the osmoregulated uptake systems for K⁺, proline and betaines (see review by Csonka, 1989). The endogenous synthesis of compatible solutes by *E. coli* has been investigated during osmoadaptation in batch cultures by Larsen *et al.* (1987) and Dinnibier *et al.* (1988). These workers identified glutamate and α - α trehalose (a non-reducing disaccharide of glucose) as the dominant osmolytes synthesized by *E. coli* when subjected to osmotic stress in glucose mineral salts media in the absence of exogenous osmoprotective compounds. These results were extended by Dinnibier *et al.* (1988) who demonstrated in time course experiments a transient accumulation of K⁺ and glutamate after osmotic upshock. Subsequently, K⁺ and glutamate concentrations decreased to almost pre-stress levels and trehalose became the dominant intracellular osmolyte. These data are at variance with those of Epstein & Schultz (1965) and Richey *et al.* (1987) who demonstrated respectively that the intracellular concentrations of K⁺ and glutamate in exponentially growing cultures of *E. coli* were proportional to the osmolarity of the growth medium. This relationship would not hold if the concentration of these osmolytes rapidly returned to pre-stress levels

following osmoadaptation. The reasons for this disparity are unclear but need to be resolved because of the central role proposed for K^+ as an intracellular osmoregulatory signal (Sutherland *et al.*, 1986; Booth & Higgins, 1990).

In the present study, *E. coli* was grown in both batch and continuous culture in a defined glucose mineral salts medium and its ability to adapt to changes in the osmolarity of the growth medium was investigated. The intracellular concentrations of K^+ , glutamate and trehalose were also followed during osmoadaptation of *E. coli* in continuous culture under conditions of either carbon or nitrogen limitation in order to investigate the roles of the individual osmolytes during osmoadaptation.

Methods

Bacterial strain and growth media. *E. coli* NCIB 9484 (a wild-type strain) was maintained by monthly subculture on nutrient agar plates (Oxoid CM3) stored at 4 °C.

Liquid cultures were grown in the medium of Evans *et al.* (1970) under conditions of either carbon or nitrogen limitation as stated in the text (osmolarity approximately 195 and 105 mosmol kg^{-1} during nitrogen and carbon limitation respectively). The composition of the medium was as follows: glucose, 140 mM (nitrogen limitation) or 10 mM (carbon limitation); NH_4Cl , 5 mM (nitrogen limitation) or 15 mM (carbon limitation); KCl , 5 mM; Na_2SO_4 , 1.8 mM; citric acid, 1 mM; $MgCl_2$, 0.3 mM; $CaCl_2$, 0.5 mM; NaH_2PO_4 , 5.6 mM, Na_2HPO_4 , 20 mM; $ZnSO_4$, 3.8 μM ; $FeCl_2$, 50 μM ; $MnCl_2$, 25 μM ; $CuCl_2$, 2.5 μM ; H_3BO_3 , 2.5 μM ; $CoCl_2$, 0.5 μM . $NaCl$ and other solutes used to raise the osmolarity of the growth medium were added prior to autoclaving to the concentration indicated in the text. Chloramphenicol, when required, was added aseptically to autoclaved medium as a filter-sterilized aqueous solution, to give a final concentration of 50 mg l^{-1} .

Growth of *E. coli* in a 25 l fermenter. A 400 ml overnight culture of *E. coli* grown in Evans' medium under nitrogen limitation was used to inoculate 25 l of modified Evans' medium (10 g glucose l^{-1} , 3.5 g NH_4Cl l^{-1}) containing 0.5 M- $NaCl$ in a 25 l New Brunswick CMF128S fermenter. The culture was grown at 37 °C, at an aeration rate of 20 $l\ min^{-1}$. The pH was automatically maintained at 7.0 using sterile 1 M- $NaOH$. Samples for gas liquid chromatography (GLC) and protein analysis were aseptically removed at selected time intervals over a 72 h period.

Effects of exogenous $NaCl$ and other solutes on trehalose accumulation. Samples (150 ml) of a chemostat culture of *E. coli* grown under carbon limitation were aseptically transferred to pre-sterilized 250 ml conical flasks containing 1 g glucose and sufficient $NaCl$ to give the final concentrations indicated in the text, or sufficient solute to give a concentration equivalent to that of 0.5 M- $NaCl$. Cultures were incubated at 37 °C for 4 h in an orbital incubator at 100 cycles min^{-1} and samples harvested for GLC and protein analysis.

Growth of *E. coli* in continuous culture. *E. coli* was grown in a 2 l (1.5 l working volume) single-stage glass chemostat (Baker, 1968). The culture was aerated at a rate of 500 $ml\ min^{-1}$ and mixed by means of a magnetic stirrer linked to a variable speed motor. The temperature of the chemostat was maintained at 37 °C using a 'cold finger' connected to a Churchill heater/cooling unit and the culture grown at a dilution rate of 0.05 h^{-1} (mean generation time 13.9 h^{-1}). Steady-state cultures were pulsed with 0.5 M- $NaCl$ by adding 150 ml of sterile 5 M- $NaCl$ to the chemostat vessel and simultaneously replacing the medium

reservoir with a reservoir of the appropriate Evans' medium containing 0.5 M- $NaCl$. Samples were harvested to determine intracellular levels of protein, trehalose, amino acids and potassium as described below.

Determination of protein. Samples (10 ml) of culture were harvested by centrifugation at 5000 g for 5 min; the cell pellet was resuspended in 2 ml imidazole/HCl buffer at pH 7.15 and subjected to sonic cavitation (MSE Soniprep 150) for a total of 2 mins in 20 s bursts at 30 s intervals to lyse the cells. The protein content of 0.1 ml samples of the lysate was determined using the method of Bradford (1976).

Determination of intracellular trehalose by GLC. Samples (50 ml) of culture were harvested by filtration through 55 mm diameter Whatman GF/F filters and rinsed with 50 ml of isotonic $NaCl$. The filters were extracted overnight in 10 ml 70% (v/v) ethanol containing 0.5 mg sucrose as an internal standard. The ethanol extract was decanted and rotary evaporated to dryness at 40 °C. The residue was dissolved in 1 ml of anhydrous dimethylsulphoxide and trimethylsilyl derivatives were prepared by the addition of 80 μl hexamethyldisilazane and 50 μl trimethylchlorosilane.

Derivatives were analysed on a Varian 3700 gas-liquid chromatograph fitted with a glass column (2 m $\times$ 4 mm internal diameter) packed with 2% (w/w) SE52 methyl phenyl silicon gum on a diatomite support. The injection temperature (140 °C) was maintained for 1 min and then increased at a rate of 20 °C min^{-1} to a final temperature of 280 °C, which was maintained for 6 min.

Amino acid analysis. Samples (50 ml) of culture were harvested onto 55 mm diameter Whatman GF/F filters as described above. The intracellular amino acid pool was extracted overnight in 10 ml 70% (v/v) ethanol containing 175 nmol nor-leucine as an internal standard. The ethanolic extract was rotary evaporated to dryness at 40 °C and the residue dissolved in 1 ml 0.2 M-lithium citrate loading buffer (pH 2.2) and passed through a 13 mm diameter cellulose acetate filter (0.22 μm pore size) to remove remaining cell debris. Samples (40 μl) were analysed on an LKB 4400 Amino Acid Analyzer utilizing a series of lithium citrate buffers (LKB).

Determination of intracellular potassium. Samples (50 ml) of culture were harvested by filtration as described above, rinsed with 50 ml of isotonic $CaCl_2$ and digested overnight in 2 ml of concentrated nitric acid. Extracts were suitably diluted and analysed on an EEL flame photometer.

Preparation of samples for NMR spectroscopy. One litre of a steady-state nitrogen-limited chemostat culture grown in the presence of 0.5 M- $NaCl$ was harvested by continuous centrifugation and the cell pellet rinsed with 1 l 0.5 M- $NaCl$. The cell pellet was suspended in 50 ml 70% (v/v) ethanol and incubated overnight at 28 °C to extract the intracellular low- M_r organic solutes pool. The cell suspension was centrifuged at 5000 g for 10 min to remove cell debris and the supernatant ethanolic extract was rotary evaporated to dryness at 40 °C. The residue was dissolved in 1 ml D_2O and analysed using a Bruker WH360 FT nuclear magnetic resonance spectrometer.

Results and Discussion

Identification of organic osmolytes by natural abundance ^{13}C NMR spectroscopy

Natural abundance ^{13}C NMR spectra of extracts of *E. coli* grown in continuous culture under osmotic stress generated with 0.5 M- $NaCl$ showed strong signals at 60.8, 69.9, 71.3, 72.4, 72.8 and 93.0 p.p.m. These signals

Fig. 1. Effect of increasing extracellular NaCl concentration on the mean (two replicates) intracellular trehalose content of *E. coli* grown in batch culture at 37 °C in an orbital incubator at 100 cycles min^{-1} .

correspond to those given by authentic trehalose. Trehalose was never detected in cultures of *E. coli* grown in media containing 0 and 100 mM-NaCl respectively (Fig. 1), indicating that a threshold level of osmotic stress is required before trehalose synthesis is initiated. In cultures grown in the presence of 200 to 500 mM-NaCl, the intracellular trehalose concentration increased progressively with increasing medium osmolarity up to a maximal level of 0.4 $\mu\text{mol trehalose (mg protein)}^{-1}$ in the culture grown in the presence of 500 mM-NaCl. However, no further increase in trehalose concentration was observed in the presence of 600 mM-NaCl. This result may be due to an effect of growth rate on trehalose accumulation as growth of *E. coli* was severely inhibited by 600 mM-NaCl in Evans' medium.

The accumulation of trehalose by *E. coli* was examined in the presence of a range of exogenous solutes at a concentration equivalent to that of 0.5 M-NaCl. Cultures grown in the presence of the ionic solutes NaCl, KCl and CaCl_2 all accumulated trehalose to a similar concentration [0.9 $\mu\text{mol trehalose (mg protein)}^{-1}$], indicating that trehalose accumulation was not dependent on the presence of a specific cation or the concentration of Cl^- in the growth medium. However, a culture in which the osmolarity of the medium was raised using $(\text{NH}_4)_2\text{SO}_4$

Fig. 2. Intracellular accumulation of trehalose during growth of *E. coli* in batch culture in a 25 l fermenter at 37 °C in modified Evans' medium (10 g glucose l^{-1} , 3.5 g $\text{NH}_4\text{Cl l}^{-1}$) containing 0.5 M-NaCl. ●, Protein concentration; ○, intracellular trehalose content. Data points represent the mean of two replicate samples.

accumulated only 0.5 $\mu\text{mol trehalose (mg protein)}^{-1}$. When organic solutes were used to increase the osmolarity of the growth medium, large variations in intracellular trehalose concentration were detected. The penetrant solute glycerol did not cause trehalose accumulation, indicating that it is loss of cell turgor rather than an increase in medium osmolarity *per se* which stimulates trehalose synthesis. The cell membrane of *E. coli* is freely permeable to glycerol (Alemohammad & Knowles, 1974), and thus the high glycerol concentration would not affect cell turgor. The non-penetrant solute sorbitol caused the accumulation of 0.96 $\mu\text{mol trehalose (mg protein)}^{-1}$, a level similar to that observed with inorganic solutes. Glucose stimulated trehalose accumulation when it was used to increase the osmolarity of medium resulting in a mean concentration of approximately 1.5 $\mu\text{mol trehalose (mg protein)}^{-1}$.

Effect of growth phase on trehalose accumulation

In this experiment *E. coli* was grown in a 25 l fermenter and the intracellular trehalose concentration followed during batch growth. The protein concentration of the culture was used as a measure of biomass concentration and this followed a typical bacterial growth curve (Fig. 2); the extended lag phase was due to the initial inhibitory effect of the high NaCl concentration in the growth medium. Trehalose accumulation was initiated approximately 2 h after inoculation and continued throughout the lag and exponential phases of growth attaining a maximum concentration of 1.4 $\mu\text{mol (mg protein)}^{-1}$ approximately 38 h after inoculation. This accumulation profile for trehalose during cell growth would be expected for a compatible solute, since growing cells must maintain a positive turgor pressure to drive

Fig. 3. (a) Accumulation of K⁺ (○), glutamate (●) and trehalose (□) by *E. coli* grown in continuous culture at 37 °C in Evans' medium under nitrogen limitation at a dilution rate of 0.05 h⁻¹ and pulsed with 0.5 M-NaCl. (b) Accumulation of trehalose by *E. coli* grown in continuous culture at 37 °C in Evans' medium under nitrogen limitation at a dilution rate of 0.05 h⁻¹ and simultaneously pulsed with 0.5 M-NaCl and chloramphenicol (50 mg l⁻¹). (c) Accumulation of K⁺ (○), glutamate (●) and trehalose (□) by *E. coli* grown in continuous culture at 37 °C in Evans' medium under carbon limitation at a dilution rate of 0.05 h⁻¹ and pulsed with 0.5 M-NaCl. Data points represent the mean of at least two replicate experiments.

cell expansion and ultimately cell division. The intracellular trehalose concentration decreased rapidly once the culture entered stationary phase. Again, this is consistent with a role in osmotic adjustment, as in non-growing cells the requirement for a turgor pressure sufficient to allow cell expansion has been removed. Consequently, intracellular trehalose may be respired or converted into carbon storage products such as glycogen. Decreased turgor pressures in stationary phase cells have been reported previously for *Saccharomyces cerevisiae* (Meikle *et al.*, 1989) and *Bacillus subtilis* (Whatmore, 1989). In both studies the decrease in cell turgor pressure correlated with lower intracellular concentrations of osmolytes as compared to those recorded for exponential phase cultures.

Osmotic adjustment of *E. coli* in continuous culture

The accumulation of trehalose by *E. coli* and the interaction of trehalose with the other intracellular solutes accumulated (i.e. K⁺ and glutamate) were followed during osmotic upshock in continuous culture. Results presented in Fig. 3(a) show the effect of

osmotically shocking a nitrogen-limited continuous culture of *E. coli* with 0.5 M-NaCl. Osmotic adjustment under these conditions was distinctly biphasic, the initial phase consisting of a rapid accumulation of K⁺ from the growth medium and a concomitant synthesis of the amino acid glutamate as a counter ion, reaching maximal levels of approximately 3.0 and 1.0 μmol (mg protein)⁻¹, respectively, after 30 min. However, the increase in cytoplasmic glutamate is only equivalent to approximately half the increase in K⁺ concentration, and so other systems to maintain electroneutrality must be present. Munro *et al.* (1972) proposed that the unaccounted K⁺ may be balanced by the efflux of putrescine, each molecule of putrescine being exchanged for two K⁺ ions, thereby allowing an increase in the osmotic strength of the cytoplasm without effecting the overall ionic balance. It has also been reported (Castle *et al.*, 1986) that osmotic stress causes an increase in the pH of the cytoplasm. Richey *et al.* (1987) calculated that this rise in pH would represent the efflux of sufficient H⁺ to account for the excess K⁺. Dinnibier *et al.* (1988), however, reported that this increase in the pH of the cytoplasm is transient and corresponds to the period of rapid K⁺ accumulation, but thereafter returned to pre-stress levels, although this was accompanied by a loss of K⁺ and glutamate. In this study, no subsequent loss of K⁺ was observed (Fig. 3), although the intracellular glutamate concentration returned to pre-stress levels during the second phase of osmoadaptation. We have no plausible explanation for this apparent electrical imbalance.

The second phase of osmoadaptation consisted of a slow accumulation of the compatible solute trehalose which replaced glutamate as the major organic osmolyte after 4 h. The profile of trehalose accumulation in chemostat-grown populations of *E. coli* differs markedly from that recorded for equivalent batch grown cultures. Most notably, the intracellular trehalose concentration [1.0 μmol (mg protein)⁻¹] remained stable for the duration of the time course (Fig. 3a, b) in chemostat-grown cultures. In contrast, trehalose concentrations in batch-grown cultures never stabilized and exhibited a progressive decline during stationary phase. These data indicate that growth rate and phase of growth may substantially influence the intracellular trehalose concentration in *E. coli* populations subjected to osmotic stress. These results are in agreement with those of Larsson *et al.* (1990), who demonstrated that growth rate influenced the intracellular concentrations of glycerol and arabitol in the yeast *Debaryomyces hansenii* when grown in continuous culture and exposed to osmotic stress. Data presented in Fig. 3(a) show that trehalose accumulation was severely inhibited (~90%) when *E. coli* cultures were simultaneously pulsed with 0.5 M-NaCl

and 50 mg chloramphenicol l^{-1} indicating that *de novo* protein synthesis is required prior to trehalose synthesis. These results confirm the results of Dinnibier *et al.* (1988), although these workers only achieved 50% inhibition of trehalose synthesis with a chloramphenicol concentration of 100 mg l^{-1} . This disparity in inhibition of trehalose synthesis by chloramphenicol probably reflects differences in growth conditions.

Whilst the results presented in this study are broadly in agreement with those of Dinnibier *et al.* (1988), in that the response to osmotic upshock was the rapid accumulation of K^+ and glutamate prior to trehalose synthesis, there are important differences. Data presented in this paper show that the elevated intracellular K^+ concentrations produced in response to osmotic shock remain stable and do not decrease to pre-stress levels following osmoadaptation. These results are consistent with those of Epstein & Schultz (1965), who demonstrated that the intracellular K^+ concentration in *E. coli* cells was proportional to the osmolarity of the growth medium. In contrast, Dinnibier *et al.* (1988) showed that the intracellular K^+ concentration decreased to pre-stress levels following osmoadaptation.

When carbon-limited continuous cultures of *E. coli* were shocked with 0.5 M-NaCl (Fig. 3*b*), the initial response was the rapid accumulation of K^+ and concomitant synthesis of glutamate, achieving maximal levels of approximately 2.0 and 1.1 $\mu\text{mol (mg protein)}^{-1}$, respectively, 30 min after osmotic upshock. The lower level of K^+ accumulation in this culture may reflect a lack due to carbon limitation of a cytoplasmic anion or of a cation such as putrescine, which could be exchanged for K^+ from the growth medium. The accumulation profile for glutamate also differs from that observed under conditions of nitrogen limitation, as the elevated intracellular glutamate concentration was maintained throughout the time course. These data indicate that the decrease in glutamate concentration observed during growth under conditions of nitrogen limitation was a result of either nitrogen limitation itself or an inability of the cells to accumulate an anion with which to replace glutamate whilst growing under carbon limitation (Fig. 3*b*). The rate of trehalose accumulation during upshock under conditions of carbon limitation was also slower than that observed during nitrogen limitation, although a similar steady-state concentration of 1.0 $\mu\text{mol (mg protein)}^{-1}$ was achieved (Fig. 3*b*). These results would be expected as the carbon source (glucose) is the growth-limiting substrate in this culture whereas it was present in excess under nitrogen limitation. In summary, the data presented in Fig. 3(*a*, *b*) show a similar biphasic system of osmoadaptation, the initial phase consisting of a rapid accumulation of K^+ and concomitant synthesis of glutamate. These systems appear to function as a short-

term survival strategy, to counteract plasmolysis and restore cell turgor quickly, prior to the synthesis of trehalose during the second phase of osmoadaptation.

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