Glycine betaine transport by *Staphylococcus aureus*: evidence for two transport systems and for their possible roles in osmoregulation

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The transport of glycine betaine by *Staphylococcus aureus* was investigated. Two transport systems were found that could be differentiated on the basis of their affinity for glycine betaine and their activation by osmotic pressure. The high-affinity system was relatively independent of osmotic pressure and exhibited a K_m of approximately 3 μM . This system was not inhibited by proline, for which a separate high-affinity transport system has been recently discovered. The low-affinity system was activated approximately 35-fold by an increase in osmotic pressure and exhibited a K_m of approximately 130 μM for glycine betaine. This system is partially inhibited by excess proline and may be identical to the low-affinity system recently described for proline. Both glycine betaine transport systems are Na^+ -dependent.

Introduction

One of the characteristic features of *Staphylococcus aureus* is its tolerance of low water activities. The strategies adopted by micro-organisms for survival of salt stress are well established (Imhoff, 1986; Csonka, 1989). Gram-negative bacteria adapt to high osmotic stress by a two-phase process, commencing with the accumulation of potassium glutamate (Epstein, 1986), followed by the activation of enzyme and transport systems and of gene expression leading to the accumulation of compatible solutes (Booth *et al.*, 1988). The most widespread compatible solute is glycine betaine (Imhoff, 1986). In Gram-positive bacteria, the situation is less clear since these cells possess a higher turgor pressure arising from their high cytoplasmic concentrations of solutes (Gould & Measures, 1976). Whether the simple model developed for the Gram-negative bacteria can be extrapolated to Gram-positive organisms has not been established.

Recent studies on *S. aureus* have begun to clarify our understanding of osmotic adaptation in this organism. That *S. aureus* can accumulate proline in response to osmotic stress has been established (Koujima *et al.*, 1978; Anderson & Witter, 1982). The stimulation of transport activity rather than *de novo* synthesis of the amino acid was reported to be responsible for the five-fold change in the proline pool of this organism after an increase in the external osmolarity (Koujima *et al.*, 1978). It has been

shown that glycine betaine can also be accumulated to high levels (Miller *et al.*, 1991; Graham & Wilkinson, 1992). In defined medium, betaine was shown to be more effective than proline in enhancing the growth of cells in the presence of 15% (w/v) NaCl (Miller *et al.*, 1991; Graham & Wilkinson, 1992). Subsequently, it was shown that there are two proline transport systems in *S. aureus*: a high-affinity system insensitive to osmotic stress, and a low-affinity transport system activated by osmotic stress (Bae & Miller, 1992; Townsend & Wilkinson, 1992).

This work was undertaken to establish the mechanisms that regulate the accumulation of compatible solutes in *S. aureus*. We demonstrate the presence of two betaine transport systems that differ in their affinity for betaine, their sensitivity to proline and its analogues, and their activation by osmotic pressure. We infer from the data that the low-affinity betaine transport system might be identical to the osmotically-activated proline transport system already described (Bae & Miller, 1992).

Methods

Bacterial strain and culture conditions. *Staphylococcus aureus* RN4220 (from Dr G. S. A. B. Stewart, University of Nottingham) was grown in the defined medium of Pattee & Neveln (1975). In addition to approximately 140 μM - Na^+ , this medium contains a defined mixture of amino acids, vitamins, purines and pyrimidines and glucose as the major carbon source. Cultures were grown in 250 ml Erlenmeyer flasks at 37 °C overnight using a low culture volume to ensure good aeration.

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Transport assays. Cultures were grown overnight in the defined medium and were diluted 25-fold and grown at 37 °C until the OD₆₀₀ reached 0.7 (LKB Spectronic). Cells were harvested by centrifugation and washed twice by resuspension and centrifugation in 0.12 M-potassium phosphate buffer pH 7, and stored on ice until required.

Transport assays were conducted at 30 °C in 45 mM-phosphate buffer (potassium salt) pH 7, unless stated. Cells (2 ml) were incubated at approximately 60 µg protein ml⁻¹ (OD₆₀₀ = 0.5) for 5 min prior to the addition of the radiolabelled proline and glycine betaine ([*N*-methyl-¹⁴C]glycine betaine 55 µCi µmol⁻¹ [2.035 MBq µmol⁻¹]; [U-¹⁴C]proline 257 µCi µmol⁻¹ [9.5 MBq µmol⁻¹]) at the concentrations indicated; 100 µl samples were removed, filtered through GFF (Whatman) filter discs under vacuum and the filtered cells washed with 3 ml of phosphate buffer. After drying, the filters were inserted into disposable plastic scintillation vials with 2 ml Ultima Gold scintillant (Packard) and counted for at least 10 min on a preset programme on a Packard 300C scintillation counter. Controls were analysed using boiled cells to establish the background binding of betaine to cells and filters and appropriate corrections were applied.

All experiments were done in triplicate and the results represent the average of two separate sets of data points generated with the same batch of cells.

The relationship between optical density and protein was established by serial dilution of exponential phase cultures and digestion of the cells of known OD₆₀₀ with 0.1 M-NaOH. The protein content of the digests was measured by the Folin-Ciocalteu method adapted for Microtitre plates using standards of bovine serum albumin treated as the cell samples. A cell suspension giving OD₆₀₀ = 1 was equivalent to approximately 120 µg ml⁻¹ total protein.

Chemicals. Biochemicals were purchased from Sigma and Boehringer; inorganic components and buffers were AnalaR grade and were purchased from BDH. Complex media, for the maintenance of cell cultures, were purchased from Oxoid. Radioactive materials were purchased from Amersham and ICN.

Results and Discussion

Glycine betaine and proline transport in S. aureus

The accumulation of glycine betaine and proline was stimulated by the addition of sodium ions. Only very low transport rates were observed with cells grown and incubated in low osmolarity media (0.12 osm) in the absence of added sodium (Table 1). Addition of 10 mM-NaCl stimulated transport 50 to 100-fold. By varying the NaCl concentration, an approximate *K_m* for Na⁺ of 4 mM was obtained (data not shown). In the majority of studies, a stimulation by Na⁺ has reflected coupling to the transmembrane Na⁺ gradient and we interpret this Na⁺ stimulation in the same way (Cairney *et al.*, 1984).

Kinetic analysis of glycine betaine accumulation revealed two kinetic components. The initial rate of uptake of glycine betaine was linear for 4 min. An Eadie-Hofstee kinetic plot (Fig. 1) revealed the presence of two saturable components with *K_m* values of 3 µM and 130 µM and *V_{max}* values of 26.5 and 155 nmol glycine betaine transported per min per mg cell protein, respectively (assayed with cells suspended in 45 mM-

Fig. 1. Eadie-Hofstee kinetic plot of the variation of transport rate as a function of glycine betaine concentration. Transport assays were conducted over the range 1 µM to 5 mM at a range of specific activities (0.4–20 µCi µmol⁻¹) in the presence of 10 mM-NaCl and at a final osmolarity of approximately 0.12 osm.

Table 1. *Effect of Na⁺ and proline on glycine betaine transport via the low- and high-affinity transport systems of S. aureus*

Transport was assayed at either 10 µM (high-affinity system) or 500 µM (low-affinity system) glycine betaine as described in Methods.

System	[Na ⁺]	[Proline]	Transport rate [nmol min ⁻¹ (mg protein) ⁻¹]
High affinity	140 µM	–	ND
	100 mM	–	21 ± 0.9
	100 mM	1 mM	21 ± 0.9
Low affinity	140 µM	–	ND
	100 mM	–	106 ± 11
	100 mM	50 mM	23 ± 5

ND, not detectable.

potassium phosphate buffer, pH 7, approximately 0.12 osm). We refer to these as the high-affinity and low affinity systems, respectively.

The ProP and ProU proline transport systems of the enteric bacteria transport glycine betaine with high efficiency (Cairney *et al.*, 1985*a,b*). However, the two proline transport systems identified in *S. aureus* have been reported to be very specific for proline (Townsend & Wilkinson, 1992; Bae & Miller, 1992). In confirmation, we observed that the high-affinity proline transport system was only slightly inhibited by a 100-fold excess of glycine betaine (approx. 30% inhibition at 10 µM-proline and 1 mM-glycine betaine; data not shown). The high-affinity glycine betaine transport system (assayed with 10 µM-glycine betaine) of *S. aureus* was not significantly inhibited by the proline analogue 3,4-dehydropoline or by a 100-fold excess of proline [activity of 30 nmol min⁻¹ (mg cell protein)⁻¹ in the absence and presence of 1 mM-proline]. Thus, the high-affinity glycine betaine transport system is very specific for this solute.

Fig. 2. Activation of the low-affinity glycine betaine transport system by osmotic pressure. Transport was assayed at 500 μ M-glycine betaine and 10 mM- Na^+ ; osmolarities below 0.12 osm were achieved by dilution of the incubation medium with distilled water. Osmolarities above 0.12 osm were achieved by addition of glucose. Similar data were obtained when NaCl was used to raise the osmotic pressure of the growth medium, but this salt was not used routinely as it would cause large changes in the Na^+ -motive force that drives the glycine betaine transport system.

In contrast, when the transport assay was conducted at 500 μ M-glycine betaine (thus measuring both high-affinity and low-affinity transport components), uptake of glycine betaine was significantly reduced by the presence of 5 mM-proline (Table 1). The residual rate of glycine betaine uptake in the presence of proline was only 23 $\text{nmol min}^{-1} (\text{mg cell protein})^{-1}$ which is similar to the maximum transport rate observed when the primary route of betaine uptake is the high-affinity system. In a parallel experiment, greater than 75% inhibition of the low-affinity proline transport (assayed at 500 μ M-proline) was observed in the presence of a 100-fold excess of glycine betaine. From these data we conclude that the low-affinity transport system may also transport proline.

Osmotic modulation of glycine betaine transport

The accumulation of glycine betaine serves the function of protecting cytoplasmic components from the deleterious effects of high concentrations of potassium glutamate during growth at high osmolarity (Booth *et al.*, 1988). Thus, it is expected that the transport systems will show osmotic modulation of their activity by high osmotic pressure (Booth *et al.*, 1988). The low-affinity glycine betaine transport system varied in activity approximately 35-fold when the osmolarity of the medium was altered either by dilution of the incubation buffer or by addition of an osmolyte (either glucose or NaCl) to the incubation buffer (Fig. 2). Glucose and NaCl were found to be equivalent activators of the low-affinity system (rates of 102 ± 19 and 99 ± 6 nmol

glycine betaine transported $\text{min}^{-1} (\text{mg cell protein})^{-1}$ in the presence of 100 mM-NaCl and 140 mM-glucose plus 10 mM-NaCl, respectively). At high osmolarities (above 1.5 osm), the activity of the transport system was progressively inhibited. In contrast to the glycine betaine transport systems of the enteric bacteria (Cairney *et al.*, 1985*a,b*), the low-affinity system in *S. aureus* was active even at low osmolarities. The osmotic modulation of the low-affinity glycine betaine transport system is remarkably similar to that recently described for the low-affinity proline transport system in *S. aureus* (Bae & Miller, 1992; Townsend & Wilkinson, 1992). In contrast, the high-affinity glycine betaine transport system was found not to be strongly activated by osmotic stress. Transport was only slightly altered by additions of either glucose or NaCl (maximum stimulation 5–7 fold), and the changes observed could not be conclusively separated from changes in the low-affinity system which is strongly activated under these conditions.

We have recently shown that the enteric bacteria possess a separate glycine betaine efflux system that is independent of the uptake systems, and which is activated by the thiol reagent *p*-chloromercuribenzoate (PCMB) (Koo *et al.*, 1991). The addition of PCMB (0.5 mM final concentration) prior to the addition of radioactively-labelled glycine betaine completely inhibited the uptake of this solute via both the low- and high-affinity systems of *S. aureus* (Fig. 3). When PCMB was added after glycine betaine accumulation had reached the steady state, there was a slight efflux of the accumulated material. However, the extent of efflux was much smaller than that seen in the enteric bacteria and suggests that, although the uptake systems are equally sensitive to PCMB, the equivalent efflux system is either absent or cannot be activated by PCMB.

The evidence supports two glycine betaine transport systems in *S. aureus*, only one of which is significantly regulated by osmotic pressure. Unlike other bacteria studied to date, betaine accumulation occurs via the high-affinity system even under conditions of low osmolarity. In addition, however, the pool of glycine betaine is modulated via activation of the low-affinity transport system which then accounts for most of the steady-state pool. At low osmolarity (approximately 0.12 osm) in the presence of 500 μ M-glycine betaine, the cells establish a steady state of 200 nmol glycine betaine $(\text{mg cell protein})^{-1}$ and, assuming a cytoplasmic water content of approximately 3.4 $\mu\text{l} (\text{mg cell protein})^{-1}$ (Koujima *et al.*, 1978), this would yield an internal concentration of around 50 mM. These data are compatible with other studies on *S. aureus* (Kunin & Rudy, 1991; Graham & Wilkinson, 1992). At higher osmolarities, the cytoplasmic level of glycine betaine increases such that at 500 μ M-glycine betaine and approximately 0.27 osm the

Fig. 3. Inhibition of glycine betaine accumulation by PCMB. Cells were incubated with either (a) 10 µM-glycine betaine or (b) 500 µM-glycine betaine. The effect of PCMB on accumulation of glycine betaine was investigated both by prior incubation of the cells with 0.5 mM-PCMB for 1 min prior to the addition of the radioactive glycine betaine (■) and by allowing cells to accumulate glycine betaine before the addition of PCMB (arrow) (●). Control incubations (○) were carried out under identical conditions with no addition of PCMB.

cells establish a steady state of 900 nmol glycine betaine (mg cell protein)⁻¹, equivalent to a concentration of approximately 270 mM. Whether simple activation of the low-affinity uptake system is sufficient to account for the regulation of the pool is the subject of further investigation.

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