Glycine betaine transport by *Staphylococcus aureus*: evidence for feedback regulation of the activity of the two transport systems

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The regulation of glycine betaine accumulation by *Staphylococcus aureus* was investigated. The accumulation of glycine betaine was regulated by the osmotic pressure of the medium and the low affinity transport system played the major role in this regulation. Mutants were isolated that lack the low affinity, osmotically activated glycine betaine/proline transport system. Such mutants accumulated glycine betaine via the high affinity system but the glycine betaine pool was smaller and responded poorly to osmotic pressure changes. The regulation of glycine betaine transport has revealed that at the steady state net influx is reduced and that this is achieved by inhibition of both the low affinity and the high affinity transport systems. Cells pre-loaded with glycine betaine exhibited a reduced $V_{\max}$ for both systems: the low affinity system was reduced in activity fivefold and the high affinity system was reduced 10-fold and became virtually undetectable. Although glycine betaine transport at the steady state is reduced, retention of the compatible solute is an active process since addition of an uncoupler provokes rapid release of the accumulated material. These data suggest that feedback regulation of the activity of the uptake systems is a major mechanism for controlling the level of compatible solute accumulation.

Keywords: Glycine betaine, transport, osmoregulation, *Staphylococcus aureus*

INTRODUCTION

One of the characteristic features of *Staphylococcus aureus* is its tolerance of low water activities. This organism, which can grow in media containing up to 3 M NaCl, is among the most osmo-tolerant bacterial species and is a major cause of food poisoning. Recent studies on *S. aureus* have begun to clarify our understanding of osmotic adaptation in this organism. Proline and glycine betaine are the two major osmoprotectants, although choline can also be utilized for this purpose after conversion to glycine betaine in the cytoplasm. Unlike the glycine betaine and proline transport systems, which are constitutive, that for choline is inducible by osmotic stress (Kaenjak *et al.*, 1993). In cells grown and incubated at low osmolarity the proline and betaine transport systems display a low activity. The activation of proline transport system(s) in response to osmotic stress is well established (Koujima *et al.*, 1978; Anderson & Witter, 1982). 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).

The accumulation of the compatible solute glycine betaine is also activated by osmotic stress (Miller *et al.*, 1991; Graham & Wilkinson, 1992; Pourkomialian & Booth, 1992). In defined medium, glycine betaine was shown to be more effective than proline in enhancing the growth of cells in the presence of 15% NaCl (Miller *et al.*, 1991; Graham & Wilkinson, 1992). Analysis of the transport systems that mediate glycine betaine accumulation has yielded different results in two laboratories. We demonstrated the presence of two glycine betaine transport systems that differ in their K_m values for glycine betaine, in their degree of inhibition by proline and its toxic analogues and in their activation by osmotic pressure (Pourkomialian & Booth, 1992). The low affinity glycine betaine transport system was shown to be inhibited by proline and was activated by osmotic pressure in a manner that was similar to that previously reported for the low

Abbreviations: AC, azetidine carboxylate; CCCP, carbonyl cyanide *m*-chlorophenylhydrazine.

affinity proline transport system. We inferred from these data that the low affinity glycine betaine transport system might be identical to the osmotically activated proline transport system already described by others (Bae & Miller, 1992; Townsend & Wilkinson, 1992). In contrast, the same strain of *S. aureus* was subsequently reported to contain a single, moderate affinity (K_m 90 μ M) transport system that was activated by osmotic pressure (Bae *et al.*, 1993).

The current studies were undertaken to investigate the mechanism of regulation of the glycine betaine pool and to determine whether regulation of the transport activities is the basis of the discrepancy between the various studies on glycine betaine transport in *S. aureus*.

METHODS

Bacterial strain and culture conditions. *Staphylococcus aureus* RN4220 (from Dr G. S. A. B. Stewart, University of Nottingham, UK) was grown in the defined medium of Pattee & Neveln (1975). 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 (50 ml) to ensure good aeration. Mutant strain BP100 is a spontaneous streptomycin resistant mutant isolated by plating 0.1 ml of an overnight culture of RN4220 on minimal medium plates containing 100 μ g streptomycin ml⁻¹. Strain RN4220 carrying plasmid pTV1ts and bacteriophage ϕ 85 were the generous gift of Professor T. J. Foster (Trinity College, Dublin, Ireland).

Transport assays. Cultures were grown overnight in defined medium, 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 re-suspension and centrifugation in 0.045 M potassium phosphate buffer, pH 7, and stored on ice until required. Transport assays were conducted at 30 °C in 0.045 M phosphate buffer (potassium salt), pH 7, containing 10 mM NaCl. Aliquots of cells (2 ml) were incubated at approximately 60 μ g protein ml⁻¹ (OD₆₀₀ = 0.5) for 5 min prior to the addition of [*N*-methyl-¹⁴C]glycine betaine (55 μ Ci μ mol⁻¹; 2.035 MBq μ mol⁻¹) or [U-¹⁴C]proline (257 μ Ci μ mol⁻¹; 9.5 MBq μ mol⁻¹) at the concentrations indicated; 100 μ l samples were removed, filtered through GFF (Whatman) filter discs under vacuum and the filtered cells washed with 3 ml 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 pre-set programme on a Packard 300C scintillation counter. Controls were analysed using boiled cells to establish the background binding of glycine betaine to cells and filters and appropriate corrections were applied. Exchange reactions at the steady state were assayed by allowing cells to accumulate unlabelled glycine betaine (500 μ M), as in a normal transport experiment, for 30–40 min. A small volume of radioactive glycine betaine (specific activity 55 mCi mmol⁻¹; stock concentration 3.8 mM) was then added and the rate of radioactive solute accumulation determined as described above.

To pre-load cells with glycine betaine, the cells were harvested as above and incubated in assay buffer at 30 °C with 500 μ M glycine betaine for 45 min. The cell suspension was then filtered and washed with 3 ml assay buffer to remove the external betaine and the cells re-suspended in 2 ml pre-warmed assay buffer and the uptake experiment carried out as described. The rate of loss of glycine betaine from such cells was determined by

loading cells with 500 μ M radiolabelled glycine betaine (2 μ Ci μ mol⁻¹), filtering, washing the cells as above and resuspension in medium at the same osmolarity and incubation at 30 °C. Retention of radioactivity was measured as described for transport assays. All experiments have been carried out in triplicate and data points represent the mean of two separate sets of data points generated with the same batch of cells.

The relationship between optical density and protein concentration was established by serial dilution of exponential phase cultures and lysis of the cells of known OD₆₀₀ with 0.1 M NaOH. The protein content was measured by the Folin–Ciocalteu method adapted for microtitre plates using a bovine serum albumin standard. A cell suspension giving an OD₆₀₀ = 1 was equivalent to approximately 120 μ g total protein ml⁻¹.

Analogue toxicity. Analogue toxicity was determined by the filter disc method. Aliquots (10 μ l) of a toxic proline analogue were pipetted onto sterile filter discs (5 mm diameter) placed in the centre of an agar plate spread with 0.1 ml of an overnight culture. After incubation for 48 h the zone of inhibition was measured across two diameters at 90° to each other. Two concentrations of azetidine carboxylate (AC) were used: 3 M and 5 M. *S. aureus* has a requirement for proline for growth and we have previously shown that proline and AC are transported by the same system. Thus, the basic growth medium was modified by the exclusion of free proline and the incorporation of the peptide Pro-Gly (1 mM) to supply proline via the peptide uptake system.

Mutant isolation. Mutants of *S. aureus* resistant to AC were isolated in two different ways. Spontaneous mutants (strains BP101–105) were isolated as colonies growing within the zone of inhibition created by AC (3 M) placed on a sterile filter disc in the centre of a plate spread with 0.1 ml of an overnight culture (as above). Strain BP106 is a derivative of strain BP105 and was isolated from the zone of inhibition created by a filter disc carrying 5 M AC. Transposon Tn917 mutants were created using plasmid pTV1ts. A transposon pool was created by overnight incubation of RN4220/pTV1ts at 42 °C in defined medium [at this temperature the plasmid cannot replicate and erythromycin resistant survivors arise by transposition of Tn917(Erm) into the chromosome]. Aliquots (100 μ l) of the overnight survivors were plated onto defined medium plates containing: Pro-Gly peptide (1 mM), 0.7 M NaCl to activate the glycine betaine/proline transport system, and erythromycin (10 μ g ml⁻¹). A filter disc carrying 10 μ l 5 M AC was placed in the centre of the plate and the plates incubated at 37 °C. Resistant colonies were picked from the inhibition zone, purified and the resistance of each strain to AC was determined as described above. The most resistant strain, BP107, was taken for transduction into a clean genetic background to verify that the mutant phenotype was linked with the transposon insertion. The Tn917 insertion was transduced into strain BP100 using bacteriophage ϕ 85. An aliquot (100 μ l) of an overnight culture of strain BP107 was inoculated into complex medium LK (Rowland *et al.*, 1984) containing 1 mM CaCl₂ and incubated in a shaking incubator at 37 °C for 4 h. A dilution (10⁻³) of the ϕ 85 stock was prepared using LK as diluent and 200 μ l was added to 300 μ l of cells. Molten LK agar (10 ml, 0.5%, w/v) containing 1 mM CaCl₂ was added to the cells/phage mixture and immediately poured onto LK agar plates containing 1 mM CaCl₂ and the plates incubated at 37 °C overnight in a plastic bag to retain moisture. The top layer of the lysed cells and phage was scraped off the plate into a sterile Oakridge centrifuge tube and was spun in a bench centrifuge at 10000 r.p.m. for 10 min. The supernatant was removed, was filtered through a 0.45 μ m filter and was stored at 4 °C for use in transduction. An aliquot

(500 µl) of a mid-exponential phase culture of BP100 was harvested and suspended in 1 ml LK containing 1 mM CaCl₂ and 500 µl of the fresh phage preparation added. The mixture was incubated for 10 min at room temperature and then 1 ml ice-cold 0.02 M sodium citrate was added and the cells harvested and re-suspended in 1 ml 0.02 M sodium citrate and incubated on ice for 2 h. Aliquots (100 µl) were plated onto LK plates containing: streptomycin (100 µg ml⁻¹), erythromycin (10 µg ml⁻¹) and sodium citrate (2 mM). The plates were incubated at 37 °C for 72 h to recover transductants. The transductants were purified on LK medium containing streptomycin (100 µg ml⁻¹) and erythromycin (10 µg ml⁻¹) and were then tested for resistance to AC by measuring the zone of inhibition caused by 10 µl 5 M AC.

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.

Table 1. Inhibition of growth of *S. aureus* by AC

Sensitivity to AC was determined as described in Methods. The peptide was Pro-Gly and was present at a final concentration of 1 mM.

Strain	Zone size with 5 M AC (mm)	
	+ Proline	+ Peptide
RN4220	2.0 ± 1.0	53.0 ± 1.5
BP101	1.0 ± 1.0	5.5 ± 0.5
BP102	1.5 ± 0.5	8.0 ± 1.0
BP103	1.5 ± 1.0	8.0 ± 1.5
BP104	1.5 ± 1.0	8.0 ± 1.0
BP106	0.0	0.0
BP107	0.0	0.0
BP108	0.0	0.0

RESULTS

Mutants deficient in the low affinity transport system

We have previously shown that the low affinity glycine betaine/proline uptake system also transports the toxic proline analogue, azetidine carboxylic acid (AC). In contrast, the high affinity system was unaffected by AC (Pourkomialian & Booth, 1992). We isolated AC-resistant mutants to determine whether the low affinity transport of betaine and proline was carried out by the same protein. *S. aureus* requires proline for growth and this interferes with isolation of mutants resistant to AC due to the competition between the substrate and the analogue for a single transport system. However, if the proline requirement was supplied via a peptide, such as Pro-Gly, AC sensitivity was readily demonstrated (Table 1). Mutants resistant to AC were isolated as spontaneous isolates (BP101–104) from the zone of inhibition around a disc containing 3 M AC. Strains BP106 and BP107 (a Tn917 insertion mutant) were isolated from plates carrying discs containing 5 M AC. When the transposon insertion in strain BP107 was transduced into a clean genetic background all of the transductants were found to be AC resistant, thus confirming the genetic linkage between the transposon and the resistance to AC. In general, the spontaneous mutants were more sensitive to AC than was the transposon mutant, suggesting impaired transport function in these strains rather than complete elimination of the transport system.

Kinetic analysis of a number of AC-resistant mutants revealed that glycine betaine transport via the low affinity uptake system was severely impaired (Table 2) compared with the wild-type strain RN4220. The $V_{\max}$ of the low affinity system was reduced in all the mutants (Table 2). Strain BP101, a spontaneous AC-resistant mutant, appears to be affected in both the K_m and $V_{\max}$ of the low affinity system, an observation typical of missense mutations that affect carrier activity (Table 2). Mutant strain BP108 was created by transduction of the Tn917 mutation of strain

Table 2. Kinetic parameters for betaine and proline transport by *S. aureus*

Initial rate determinations were performed as described in Methods. All determinations were completed at least twice and the data shown are the mean and standard deviation. HA, High affinity; LA, low affinity; ND, not detected.

Strain	Betaine uptake system				Proline uptake system			
	K_m (µM)		$V_{\max}$ [nmol (mg total protein) ⁻¹ min ⁻¹]		K_m (µM)		$V_{\max}$ [nmol (mg total protein) ⁻¹ min ⁻¹]	
	HA	LA	HA	LA	HA	LA	HA	LA
RN4220	3.0 ± 0.3	133 ± 14.6	26.5 ± 2.9	155 ± 17.1	2.4 ± 0.2	1022 ± 332	2.8 ± 0.8	84.0 ± 4.0
BP101	4.8 ± 0.4	433 ± 39.0	24.5 ± 2.2	62 ± 8.3	2.0 ± 0.4	801 ± 14	2.1 ± 0.1	38.3 ± 4.3
BP106	5.8 ± 1.2	ND	27.0 ± 6.3	ND	18.0	ND	5.5	ND
BP108	10.6 ± 0.1	ND	29.3 ± 10.8	ND	8.9	ND	4.1	ND

BP107 into the streptomycin-resistant strain BP100. The low affinity transport system was undetectable in this strain and in strain BP106 a spontaneous mutant isolated as resistant to 5 M AC. In contrast, the high affinity transport system was fully active and comparable with that observed in strain RN4220 (Table 2). Thus, mutants that had acquired resistance to AC lost the low affinity glycine betaine transport system. Similarly, such mutants were found to have reduced activity of the low affinity proline transport system (Table 2). As was observed for glycine betaine transport, the low affinity proline transport system could not be detected in strain BP108, but the high affinity system was unaffected (Table 2). These data strongly support the hypothesis that the low affinity osmotically activated glycine betaine/proline transport systems are mediated by the same gene product.

Regulation of accumulation of glycine betaine in *S. aureus*

Glycine betaine accumulation is regulated by the osmotic pressure of the incubation medium, but it is not clear whether both systems contribute to the regulation of the pool of the compatible solute. We have previously shown that there are two glycine betaine transport systems in *S. aureus* that differ in their affinity for the substrate and in their activation by osmotic pressure (Pourkomailian & Booth, 1992). The high affinity system is only slightly activated by osmotic pressure and this activity is expected to be dominant when the glycine betaine concentration is low (1–20 μM , initial concentration). Under these conditions the glycine betaine accumulation was found to be relatively independent of the osmotic pressure and was predominantly determined by the amount of glycine betaine in the medium, since it can be calculated that the external glycine betaine concentration at the steady state is reduced to close to zero (Fig. 1a). In the mutant strain BP108 the glycine betaine pool was always lower than in the parent strain, suggesting that both transport systems contribute to accumulation even at very low glycine betaine concentrations. However, when the osmolarity was raised to 0.82 osm by the addition of NaCl the activity of the high affinity system was sufficient to accumulate virtually all of the glycine betaine present in the incubation mixture. This demonstrates the immense scavenging capacity of this transport system.

At higher concentrations of glycine betaine (100–750 μM , initial concentration) the accumulation of the compatible solute was determined by the osmolarity of the growth medium (Fig. 1b). Under these conditions the steady state concentration in the cells was independent of the external concentration and was dependent upon the osmolarity of the incubation medium. Although the external concentration of glycine betaine is reduced significantly below the initial concentration, the steady state external concentration is between 60% and 90% of the initial concentration (data calculated from Fig. 1b). Glycine betaine accumulation in strain BP108, which only possesses the high affinity transport system, was found to be substantially lower than that in strain RN4220 even at

Fig. 1. Glycine betaine accumulation by osmotic pressure in strains RN4220 and BP108. Transport assays were conducted at 30 °C in 45 mM potassium phosphate buffer, pH 7, containing 10 mM NaCl (approximately 0.12 osm). NaCl (0.5 M) was added to raise the osmolarity to 0.82 osm. Aliquots of cells (2 ml) were incubated at approximately 60 μg protein ml^{-1} ($\text{OD}_{600} = 0.5$) for 5 min prior to the addition of [*N*-methyl-¹⁴C]glycine betaine, specific activity 0.4–55 $\mu\text{Ci } \mu\text{mol}^{-1}$ (1–750 μM), and the incubation continued until the steady state was reached (approximately 45 min), at which time duplicate samples were taken and filtered and the radioactivity in the cells determined by scintillation counting as described in Methods. (a) Incubation with low glycine betaine concentrations (1–20 μM). (b) Incubation with high glycine betaine concentrations (100–750 μM).

high osmolarity (Fig. 1). All of the spontaneous mutants were also found to exhibit reduced rates of glycine betaine transport and failed to accumulate high levels of this compatible solute when incubated at high osmolarity (data not shown). Thus, osmotically regulated glycine betaine accumulation is primarily due to the activity of the osmotically activated low affinity system.

Regulation of transport activity

The regulation of the glycine betaine pool by osmotic pressure suggested that the transport systems might be subject to control mechanisms in addition to activation of the low affinity system by osmotic pressure, as demonstrated previously (Pourkomailian & Booth, 1992). To investigate this possibility, the initial rate and the exchange rate at the steady state were investigated. Cell

Fig. 2. Comparison of steady state exchange rate and the initial rate of glycine betaine transport. Cell suspensions were treated as described in Fig. 1 at a final osmolarity of 0.26 osM. The initial glycine betaine concentration was 500 μ M (specific activity 2 μ Ci μ mol $^{-1}$). For initial rate determinations (○) radiolabelled glycine betaine was added at zero time and the rate of transport determined in the normal manner. To determine the steady state rate of uptake (●) cells were incubated with unlabelled glycine betaine and 200 nCi radiolabelled glycine betaine (55 μ Ci μ mol $^{-1}$ stock) added at the steady state (arrow). The final specific activity was calculated from the known addition of radioactivity and the estimated steady state external glycine betaine concentration calculated from the observed accumulation (○). Uptake of the radiolabelled material was assayed as described in Methods.

Fig. 3. Glycine betaine retention. Cells were loaded with 500 μ M glycine betaine as described in Fig. 2; the suspension was harvested by filtration, washed and re-suspended in iso-osmotic medium lacking glycine betaine. Samples were taken as described in Methods and the retained glycine betaine pool determined by scintillation counting. ○, No additions; ●, 25 μ M CCCP added at arrow.

suspensions were incubated with 500 μ M glycine betaine, such that the pool achieved the maximum level attainable (Fig. 1b), and the exchange rate determined by addition of a trace amount of radiolabelled glycine betaine (Fig. 2). The rate of glycine betaine exchange was reduced to approximately 20% of the initial rate of transport observed in a parallel incubation (Fig. 2). Thus, when

Fig. 4. Eadie-Hofstee plots of glycine betaine accumulation by *S. aureus* RN4220. Cells were pre-loaded with unlabelled glycine betaine as described in Fig. 2 and the cells incubated with radiolabelled glycine betaine (0.5–2000 μ M, specific activity 0.4–20 μ Ci μ mol $^{-1}$) and the initial rate of transport determined over 2 min. The final osmolarity of the incubation was 0.26 osM. (a) Cells pre-loaded with 500 μ M glycine betaine. (b) Cells pre-loaded with 20 μ M glycine betaine.

cells have achieved a satisfactory glycine betaine pool, one or more of the transport systems is inhibited.

Since either transport system could be subject to inhibition, the kinetic parameters of the two transport systems were investigated in cells that had been pre-loaded with glycine betaine. Cells were pre-incubated with 500 μ M unlabelled glycine betaine for 30–40 min, collected by filtration, washed and resuspended with isotonic buffer, and the K_m and V_{max} of the two transport systems were determined. Since the cells contained high levels of glycine betaine a control experiment was conducted to ensure that leakage of unlabelled glycine betaine from the cells did not interfere with the assay of the influx rate. Thus, cells incubated with radiolabelled glycine betaine and harvested by filtration were incubated in medium free of glycine betaine (Fig. 3). Glycine betaine did not exit from the cells over the 6 min period equivalent to that used for assay of the uptake rate in K_m and V_{max} determinations. Retention was energy-dependent since addition of CCCP elicited rapid glycine betaine efflux (Fig.

3). Similarly, addition of an excess of unlabelled glycine betaine elicited rapid exchange of the accumulated material (data not shown).

Kinetic plots of glycine betaine transport into cells that had been pre-incubated with glycine betaine showed greater experimental error than studies with cells not pre-loaded, but the data revealed only a single transport system, with K_m 450–525 μM and a $V_{\max}$ of 18–21 $\text{nmol min}^{-1} (\text{mg protein})^{-1}$ (Fig. 4a). This represents a threefold reduction of the affinity for glycine betaine and a fivefold reduction in the $V_{\max}$. The high affinity transport system was not detected in cells pre-treated with 500 μM glycine betaine (Fig. 4a). These data are consistent with the reduced exchange rate seen at the steady state in cells incubated with 500 μM glycine betaine (Fig. 2b). When cells were pre-loaded with an intermediate glycine betaine concentration (20 μM) both transport systems were still active, but the high affinity system had a reduced $V_{\max}$ of 2.6, compared with 26 $\text{nmol min}^{-1} (\text{mg protein})^{-1}$ in cells not pre-incubated with glycine betaine. The $V_{\max}$ of the low affinity transport system was reduced slightly to 88, compared with 150 $\text{nmol min}^{-1} (\text{mg protein})^{-1}$ in cells not pre-incubated with glycine betaine (Fig. 4b). These data are consistent with both the high affinity and the low affinity transport systems being subject to feedback inhibition by the pre-accumulated glycine betaine pool.

DISCUSSION

The data presented in this paper confirm the existence of two separate glycine betaine transport systems in *S. aureus*. Mutants isolated for their resistance to the toxic analogue AC were impaired in the low affinity transport system, but the high affinity system was unaffected. The analysis of these mutants also provides further support for our previous assertion that a single system is responsible for the low affinity, osmotically regulated transport activity for proline and glycine betaine (Pourkomailian & Booth, 1992), since both activities were lost in the mutants. Data obtained with point mutations can often be misleading, but the isolation of single transposon insertions that eliminate both the osmotically activated glycine betaine and proline transport systems is most simply explained by their being conducted by the same protein. It is possible that the transposon inactivates a regulatory gene that controls the expression of separate proline and glycine betaine transport systems. However, while we cannot definitively eliminate this possibility, both systems are expressed constitutively by cells and there are no data to suggest regulation of their expression that would necessitate this more complex model. Further, the transposon data are consistent with the previous demonstration that glycine betaine uptake via the low affinity system is inhibited by proline and vice versa (Pourkomailian & Booth, 1992).

We have established that both of the glycine betaine transport systems are subject to feedback inhibition by pre-accumulated solute. This represents a novel mechanism of regulation of compatible solute accumulation.

The high affinity system is more seriously affected by the regulatory mechanism and is almost undetectable in cells pre-incubated with 500 μM glycine betaine. However, the low affinity system is also subject to regulation since the $V_{\max}$ of this system is significantly reduced in cells pre-loaded with glycine betaine. These data may also explain why other groups had suggested a single glycine betaine transport system. Their studies were conducted primarily with cells grown in broth, which contains glycine betaine and proline. Accumulation of these compatible solutes by cells would cause the inactivation of the high affinity glycine betaine transport system. In our experiments we found that kinetic plots obtained with pre-loaded cells showed much greater experimental variability than similar data obtained with cells that had not been pre-incubated with glycine betaine. This observation has also been made with assays conducted on cells grown in broth (Stimeling *et al.*, 1994). Recent studies comparing the transport kinetics of cells grown in different media have concluded that cells grown in broth show a single glycine betaine transport system, whereas two systems were found in cells grown in defined medium (Stimeling *et al.*, 1994). Further, those studies revealed that cells grown in defined medium and pre-incubated with glycine betaine exhibited similar kinetics to those grown in broth and confirm our observation of feedback regulation of glycine betaine transport activity (Stimeling *et al.*, 1994).

Incubation of the cells with glycine betaine resulted in the inactivation of the high affinity system and reduced activity of the low affinity system. Previous work has shown that the two transport systems are essentially constitutive, since they are present in cells grown under conditions of low osmolarity. Our data suggest that the transport systems are inactivated rather than repressed, since the studies were conducted with non-growing cells that had been incubated for only a short time with the unlabelled glycine betaine prior to the kinetic analysis. This system of regulation is unique in so far as no evidence for feedback regulation of the activity of the compatible solute transport systems has been seen in this or other organisms (Booth *et al.*, 1988; Csonka, 1989). The model, based primarily on our data, is that the high affinity transport system exists to scavenge for glycine betaine and its efficiency is indicated by the ability of cell suspensions to reduce the external glycine betaine concentration from 20 μM to close to zero, particularly when the cells are incubated at high osmolarity (0.82 osm; Fig. 1b). This ability might constitute a hazard to normal osmoregulation when cells are in environments with significant glycine betaine or proline pools. Consequently, once sufficient glycine betaine has been accumulated this system is closed down. If the organism is subjected to osmotic stress the low affinity system is activated and if sufficient glycine betaine is available a large pool is established in the cytoplasm. The magnitude of the pool is determined primarily by the degree of osmotic stress (Fig. 1b). When sufficient glycine betaine has been accumulated the activity of the uptake system is reduced. Thus, the low affinity system must be capable of sensing both the internal glycine betaine pool (either directly or indirectly)

and the external osmolarity. In contrast, the high affinity system is not activated by osmotic stress (Pourkomailian & Booth, 1992) but is inhibited by the accumulation of glycine betaine by osmotically stressed cells. In many ways this pattern of regulation compares with that for potassium transport in enteric bacteria. The constitutive Trk systems have been shown to be activated by osmotic stress and there are suggestions that they are also subject to feedback inhibition (Rhoads & Epstein, 1978; Meury *et al.*, 1985; Epstein, 1986; Booth *et al.*, 1988). In this instance the regulation is via changes in the turgor pressure. In *S. aureus* there is no clear involvement of potassium transport in turgor regulation and it may be that it is the flux of glycine betaine that restores turgor. Control of the low affinity system would be exerted through activation by low turgor followed by inactivation after accumulation of sufficient glycine betaine to restore turgor. The intermediate transport kinetics seen in cells loaded with 20 μ M glycine betaine, which is insufficient to fulfil the osmotic requirements of the cell (Fig. 1a, b), would be consistent with this model.

We have previously shown that the *Escherichia coli* glycine betaine transport system ProP, which is the nearest equivalent of the low affinity system in *S. aureus*, is not subject to feedback inhibition and that it is inhibited, rather than activated, by low turgor (Koo *et al.*, 1991). *S. aureus* and *E. coli* belong to different subdivisions of the bacterial kingdom and this is in part reflected by their respective turgor pressures, which have been estimated to lie in the 20 and 3–4 atmosphere ranges, respectively (Mitchell & Moyle, 1956). One consequence of these different turgor pressures may be that these two organisms have adopted very different initial responses to osmotic stress. The enteric bacteria are able to take advantage of the ubiquitous potassium ion for their turgor restoration without initially impairing cell function. In contrast, the high turgor of *S. aureus*, which derives from high solute pools in the cytoplasm, may have precluded the evolution of potassium transport mechanisms to regulate cell turgor and have led to the dependence upon glycine betaine (or compounds that can be converted to this osmolyte). If turgor is the main regulator of the low affinity glycine betaine/proline transport system then this would represent a major evolutionary adaptation to the possession of high turgor.

NOTE ADDED IN REVISION

This analysis was extended to proline transport, which is dependent upon two transport systems, a proline-specific high affinity system and the osmotically activated, low affinity, proline/betaine system (Bae & Miller, 1992; Pourkomailian & Booth, 1992). The kinetics of proline transport were analysed in cells of strain RN4220 that had been pre-loaded by incubation with 500 μ M glycine betaine as described above (see legend to Fig. 4). In cells that had been pre-loaded with glycine betaine the high affinity proline transport system was undetectable and the $V_{\max}$ of the low affinity system was reduced almost 30-fold, from 84 to 3 nmol min⁻¹ (mg cell protein)⁻¹. This

suggests that the high affinity proline and betaine transport systems, though separate proteins, are regulated in the same manner by compatible solute accumulation.

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