

NOTES

Staphylococcus aureus Osmoregulation: Roles for Choline, Glycine Betaine, Proline, and Taurine

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Choline, glycine betaine, and L-proline enhanced the growth of *Staphylococcus aureus* at high osmolarity (i.e., they acted as osmoprotectants) on various liquid and solid defined media, while an osmoprotective effect of taurine was shown only for cells growing on high-NaCl solid medium that lacked other osmoprotectants. Potassium pool levels were high, and there was little difference in levels in cells grown at different osmolarities. Glycine betaine accumulated to high levels in osmotically stressed cells, and choline was converted to glycine betaine. Proline and taurine also accumulated in response to osmotic stress but to lower levels than glycine betaine.

Staphylococcus aureus is among the most osmotolerant of the nonhalophilic eubacteria, growing at water activities as low as 0.86 (equivalent to 3.5 M NaCl) (35). Although much early work on osmoregulation concerned *S. aureus* (5, 26), very little is known about the underlying mechanisms. Osmoregulation in this organism is inherently interesting for several reasons. Unlike many other organisms which grow in environments of elevated osmolarity, *S. aureus* also grows well at low osmolarities, suggesting an efficient means of regulating cytoplasmic osmolarity. *S. aureus* is also probably the major cause of food poisoning in Western countries (16), and reduction of water activity is commonly used as a method of food preservation. The expression of a variety of bacterial virulence factors has recently been linked to changes in environmental osmolarity (7). It is worth noting that the pathogenicity of *S. aureus* involves colonization of a high-salt environment, the mammalian skin.

In this laboratory, studies of staphylococcal methicillin resistance (24) and the discovery of a unique, highly specific, Na⁺-dependent transport system for taurine in staphylococci (2, 11, 36) were two lines of investigation that led to the present study of staphylococcal osmoregulation.

It was our intention to obtain a broad picture of the *S. aureus* osmotic stress response for future, more detailed analyses of various aspects (see reference 38). Some of this work has been presented previously in preliminary form (12).

Culture conditions. *S. aureus* 8325 was used. It was maintained on tryptic soy agar (Difco) slants at 4°C. Complex medium consisted of Bacto Peptone (Difco) (5 g/liter), yeast extract (Difco) (5 g/liter), and K₂HPO₄ (3 g/liter), pH 7.2 (PYK medium). When sucrose was used to adjust medium osmolarity, it was filter sterilized. The basic defined medium was that of Giehl et al. (11), which was based upon that of Pattee and Neveln (31), with the proline content

lowered to 20 μM. A modified defined medium, referred to as 10× defined medium, was also used in some studies because of relatively poor growth in the defined medium under high levels of osmotic stress. The major carbon source was changed from glucose to glycerol (4.75 g/liter) supplemented with sodium pyruvate (110 mg/liter), cystine and tyrosine were maintained at the same levels as in defined medium, aspartic acid was increased to 450 mg/liter, glutamic acid was increased to 850 mg/liter, and the remaining 13 amino acids were increased in concentration 10-fold. Proline was added to the level desired in particular experiments. Generally, 20-ml volumes of medium in 50-ml Erlenmeyer flasks were cultured at 37°C with shaking (200 rpm). A 1% (vol/vol) inoculum from an 18-h culture in low-osmolarity medium was the standard inoculum. Cell clumping was a problem in some experiments using defined medium, and clumps were dispersed by brief, low-frequency sonication with a sonicator probe flamed with ethanol. In some experiments, osmoprotective effects were conveniently visualized on solid medium (23, 37) by using 10× defined medium containing 10 μM proline, 1.5 or 1.7 M NaCl, Noble agar (Difco), and osmoprotectant. In order to measure the overall reduction in water activity caused by various solutes in the medium, a dew point depression osmometer was used (Wescor model 5100B vapor pressure osmometer; Wescor Inc., Logan, Utah).

Pool extraction and analysis. Solute pool metabolite fractions were obtained by extraction with 5% (wt/vol) trichloroacetic acid at 4°C for 15 min (36). Trichloroacetic acid was removed by five extractions with ether. Residual ether was removed by bubbling with N₂, and the sample was lyophilized. In the determination of K⁺ and amino acid pool levels, cells were grown to exponential phase (*A*₅₈₀ = 0.6) in PYK medium supplemented with various amounts of NaCl or sucrose and were washed with saline solution isotonic with the growth medium. Cells grown in medium without the addition of osmolarity-adjusting solutes were washed in a 0.5 M NaCl solution to protect against leakage of pool constituents (4, 13).

K⁺ concentrations were determined by using a valinomy-

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TABLE 1. Influence of various solutes on stationary-phase population levels of *S. aureus* grown in media of differing osmolarities^a

Medium	Maximum stationary-phase population level (A_{580}) at the following concn (M) of NaCl added (medium osmolarity) ^b			
	0 (0.204)	0.5 (1.09)	1.0 (2.11)	1.5 (3.06)
Defined	2.2	0.27	0.16	0.05
Defined plus 1 mM glycine betaine	1.9	1.60	1.31	0.89
Defined plus 1 mM L-proline	2.3	2.3	2.1	0.48
Defined plus 1 mM taurine	1.7	0.5	0.21	0.21
Defined plus peptone (5 g/liter) plus yeast extract (5 g/liter)	4.7	3.3	2.1	1.9

^a Cells were grown in defined medium containing different amounts of NaCl supplemented with different potential osmoprotectants. A 0.2% (vol/vol) inoculum was used.

^b Maximum stationary-phase population levels in cultures incubated up to 96 h were measured. The values represent the averages of 10 experiments for defined medium and defined medium supplemented with glycine betaine and proline. For taurine, the values are the averages of two experiments; one experiment with peptone plus yeast extract is shown. Medium osmolarity is expressed as osmoles per kilogram of H₂O.

cin electrode with a Beckman E4A electrolyte analyzer. Individual amino acid pool levels were determined with a Beckman 119CL amino acid analyzer. A physiological amino acid program was used to distinguish between glutamine and glutamic acid. In addition, osmolytes accumulating in response to osmotic stress were measured radiochemically. [*methyl*-¹⁴C]choline (58.5 mCi/mmol), [1,2-¹⁴C]taurine (112.2 mCi/mmol), and [2,3-³H]L-proline (35.2 Ci/mmol) were purchased from DuPont, NEN Products. [*methyl*-¹⁴C]glycine betaine was enzymatically synthesized from [*methyl*-¹⁴C]choline as described previously by Landfald and Strom (22). The identity of labeled glycine betaine was confirmed by thin-layer chromatography on 0.25-mm-thick Silica Gel G plates by using the solvent systems described by Blunden et al. (3). The enzymatically synthesized [¹⁴C]glycine betaine cochromatographed with authentic glycine betaine. *S. aureus* was grown for 72 h in the 10× medium adjusted to various osmolarities with NaCl or sucrose. Intracellular solute accumulation was determined as described previously by Smiley and Wilkinson (36).

Growth studies. (i) **Liquid media.** The probability that cells will survive transfer to media of increased osmotic strength is related to inoculum size (10) and medium composition (30). Defined media containing 0, 0.5, 1.0, and 1.5 M NaCl with and without glycine betaine, proline, taurine, and peptone plus yeast extract were inoculated with a 0.2% (vol/vol) inoculum from a 24-h culture in defined medium. Growth occurred only in the control and the 0.5 M NaCl flasks in the absence of osmoprotectants after 24 h, indicating that higher concentrations of NaCl inhibited the ability of *S. aureus* to initiate growth (Table 1). Supplementation with 1 mM glycine betaine enhanced growth in that 0.5 M NaCl caused only a slight inhibition of growth and growth occurred in the presence of 1 M NaCl. Proline also had an osmoprotective effect, but glycine betaine was more effective than proline at the highest level examined (1.5 M). No osmoprotective effect of taurine was observed under these conditions. Peptone and yeast extract allowed the cells to grow in 1.5 M NaCl. These constituents are known to

contain the osmoprotectants glycine betaine and proline (8). In liquid medium, the exponential growth rate (mean generation time) in unstressed cultures was increased from 1.25 to 2 and 2.5 h by 0.5 and 1.0 M sucrose, respectively. Supplementation of medium with 1 mM choline or 1 mM glycine betaine had little effect on the unstressed growth rate but increased the growth rate in the presence of sucrose to the unstressed level. Proline at 1 mM increased the growth rate even in the absence of osmotic stress. However, no decrease in growth rate was seen upon the addition of sucrose.

(ii) **Agar surface growth.** Figure 1 reveals the extent of surface growth on agar plates containing 1.5 and 1.7 M NaCl. Choline and glycine betaine stimulated growth when present at concentrations as low as 0.25 mM. Colonies on choline-containing plates grew more slowly and were fewer in number than those on plates containing L-proline or glycine betaine. The effect of taurine was not as dramatic (Fig. 1A), but an enhancement of growth was seen, especially on plates containing higher concentrations of NaCl (Fig. 1B).

Plates containing 1.01 mM L-proline showed the greatest growth stimulation. Again, it was difficult to distinguish between its nutrient value and its osmoprotective capacity. However, when plates containing 1.5 M NaCl, 260 μM proline, or 1.01 mM proline were compared (Fig. 1A), a large increase in colony diameter was noted at the higher proline concentration. According to the report of Nagamachi et al. (29) and confirmed by Townsend and Wilkinson (38), the lower proline content should be sufficient to satisfy an auxotrophic requirement, indicating an additional beneficial effect on cell growth in the presence of 1.5 M NaCl.

Analysis of pool constituents in osmotically stressed cells. The results of analyses of the K⁺ and amino acid pools of exponential-phase cells in complex medium are shown in Table 2. Results are expressed as nanomoles per milligram (dry weight) and as adjusted pool concentrations, correcting for the loss of cell water that occurs during water stress (5). K⁺ pools increased by only 26% under the water stress imposed by the addition of 2 M NaCl, which represents a 15-fold increase in medium osmolarity. This small increase

FIG. 1. Osmoprotective activities of choline, glycine betaine, L-proline, and taurine on the growth of *S. aureus* on solid media of increased osmolarities. All plates contained 10× defined medium containing 10 μM L-proline and 2% (wt/vol) Noble agar plus 1.5 M NaCl (A) or 1.7 M NaCl (B). The plates were supplemented with 0, 0.25, or 1.0 mM osmoprotectant and incubated for 48 h (A) and 56 h (B) at 37°C.

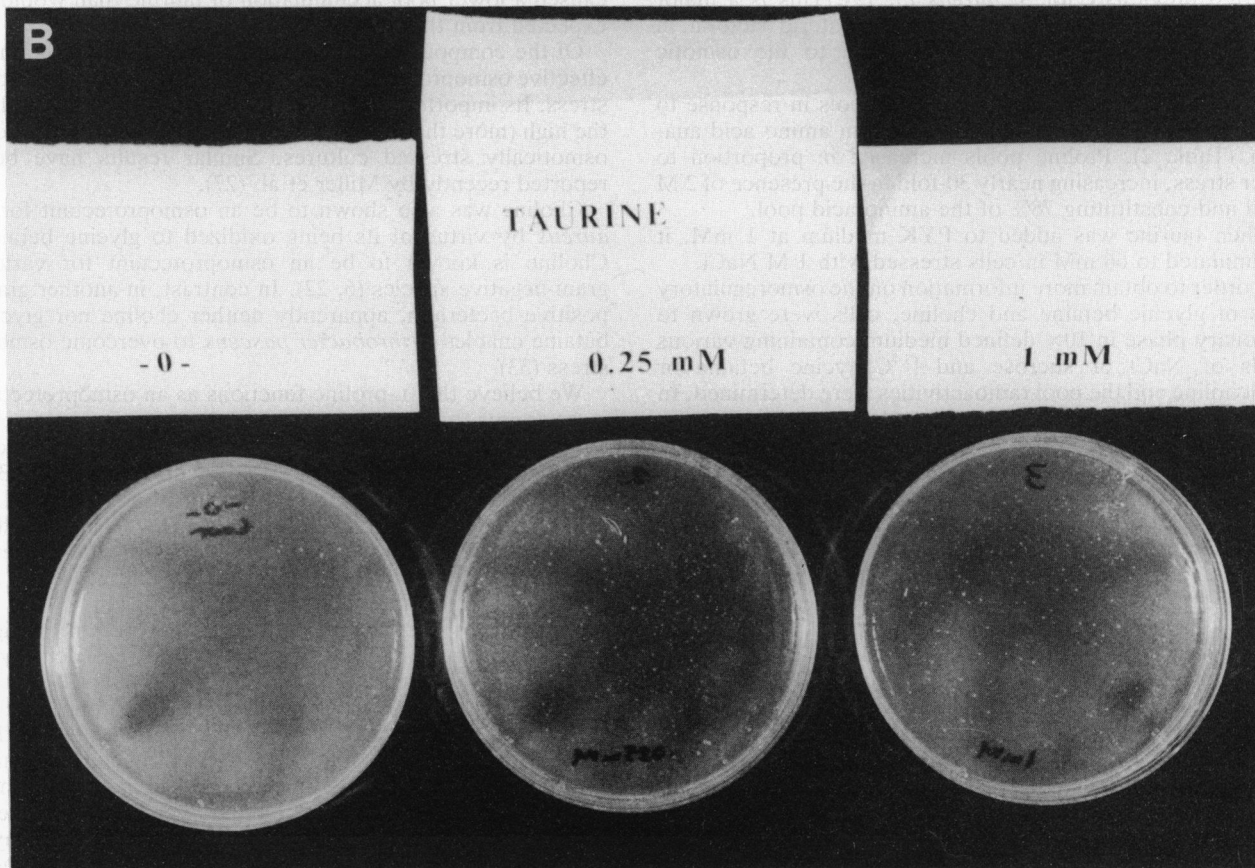
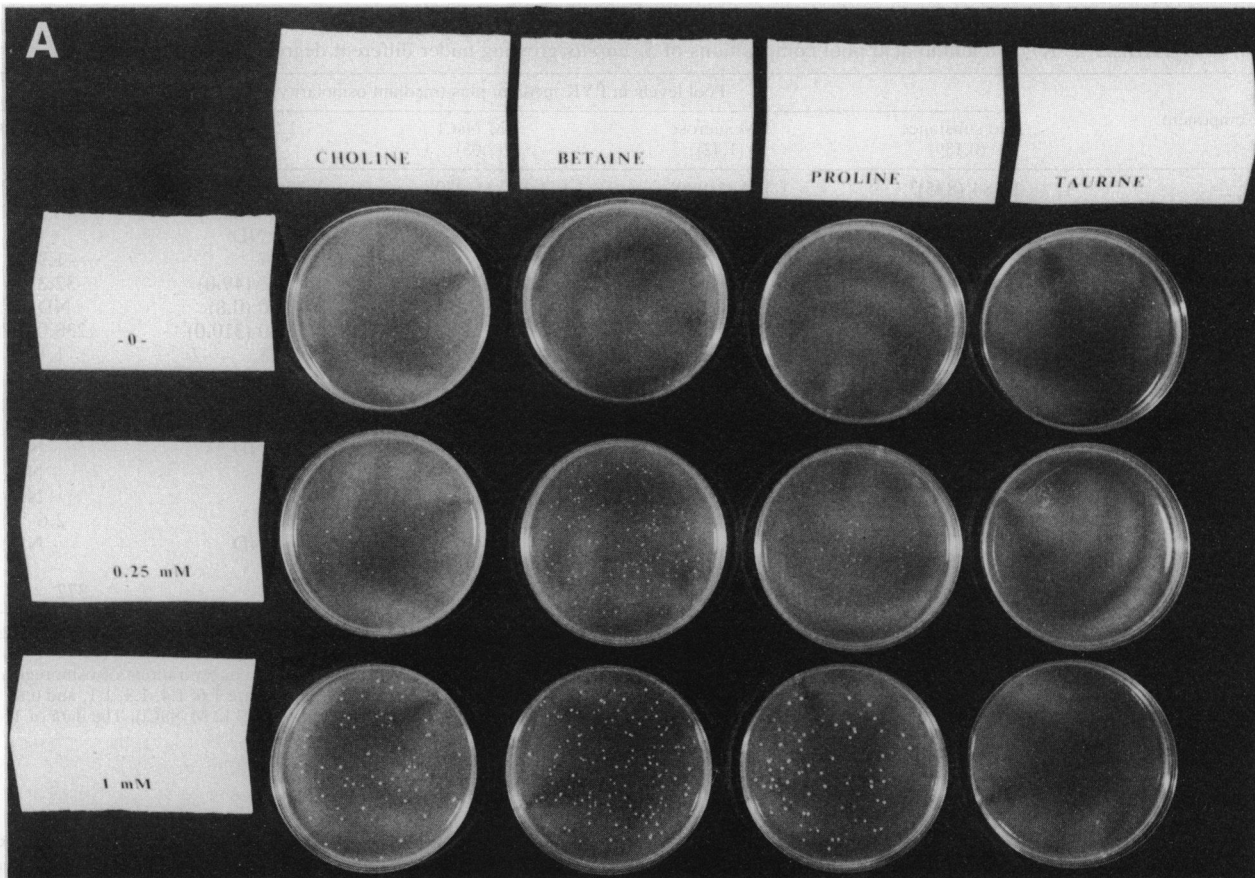


TABLE 2. K⁺ and amino acid pool compositions of *S. aureus* growing under different degrees of osmotic stress

Component	Pool levels in PYK medium plus (medium osmolarity) ^a :				
	No substance (0.139)	1 M sucrose (1.42)	1 M NaCl (2.05)	1.5 M NaCl (3.09)	2 M NaCl (4.05)
K ⁺	1,584 (835) ^b	1,104 (1,080)	1,152 (1,070)	1,376 (895)	992 (1,127)
Asp	96.7 (60.6)	148.5 (106.0)	94.4 (73.0)	50.6 (46.0)	48.3 (55.0)
Thr	3.7	2.4	1.3	ND ^c	ND
Ser	10.4	19.7	5.0	2.7	1.9
Glu	37.9 (23.7)	197.6 (141.2)	67.7 (52.0)	53.8 (49.0)	32.3 (37.0)
Gln	2.6 (1.6)	10.6 (7.5)	1.0 (0.7)	1.0 (0.8)	ND (ND)
Pro	16.2 (10.1)	92.8 (66.0)	243.2 (187.0)	341.0 (310.0)	238.0 (270.0)
Gly	3.2	36.8	0.6	1.8	1.1
Ala	2.9	24.0	1.9	8.3	1.9
Ile	ND	4.0	0.8	ND	ND
Leu	1.1	2.6	0.5	1.1	ND
Tyr	0.5	ND	0.6	ND	ND
Pag	1.3	2.7	1.1	0.5	ND
His	3.4	27.2	2.9	0.5	ND
Lys	4.0	10.4	6.7	1.8	2.6
Arg	0.5	ND	ND	ND	ND
Total amino acids	115.2	406.2	328.2	419.8	372

^a Cells were grown to exponential phase in complex medium and extracted with cold trichloroacetic acid. Pool levels are expressed in nanomoles per milligram (dry weight). Medium osmolarity is expressed as osmoles per kilogram of H₂O. All dry weights were estimated from A₅₈₀ measurements.

^b Parenthetical data represent adjusted pool concentrations (in millimolar). In order to report pool contents in terms of concentrations of osmoregulatory solutes, the values for cell water reported by Christian and Waltho (5) and confirmed by others (19, 21, 28) were used. These are 1.6, 1.4, 1.3, 1.1, and 0.88 ml/g (dry weight) at water activities of 0.993 (unadjusted medium), 0.98 (1 M sucrose), 0.97 (1 M NaCl), 0.95 (1.5 M NaCl), and 0.93 (2 M NaCl). The data of Troller and Christian (39) were used to establish these water activities for NaCl and sucrose solutions.

^c ND, not detected.

in pool K⁺ was attributable to cell dehydration and not K⁺ accumulation. Similar levels and fluctuations have been reported previously for *S. aureus* (5, 19). This is a major point of difference between *S. aureus* and enteric bacteria, in which pool K⁺ increases in proportion to the osmotic strength of the medium (9).

Alterations in individual amino acid pools in response to water stress were determined by using an amino acid analyzer (Table 2). Proline pools increased in proportion to water stress, increasing nearly 30-fold in the presence of 2 M NaCl and constituting 78% of the amino acid pool.

When taurine was added to PYK medium at 1 mM, it accumulated to 66 mM in cells stressed with 1 M NaCl.

In order to obtain more information on the osmoregulatory roles of glycine betaine and choline, cells were grown to stationary phase in 10× defined medium containing various levels of NaCl or sucrose and [¹⁴C]glycine betaine or [¹⁴C]choline and the pool radioactivities were determined. In both cases, 99% of the cell-associated radioactivity was in the pool metabolite fraction. Glycine betaine pool levels rose from 76 mM in unstressed cells to 1,167 mM in cells grown with 2 M NaCl (Table 3). There was a direct proportionality between the degree of osmotic stress and glycine betaine accumulation. The radioactivity from [¹⁴C]choline accumulated as [¹⁴C]glycine betaine, as revealed by thin-layer chromatography, to similar but somewhat lower levels than when [¹⁴C]glycine betaine was supplied.

For comparative purposes, proline and taurine accumulations under these conditions were studied. Sixty-four percent of the cell-associated radioactivity from [³H]proline was found in the pool metabolite fraction, and proline pools rose approximately 16-fold with a 15-fold increase in medium osmolarity, confirming the osmoregulatory role of proline indicated by the amino acid analyzer measurements. Ninety-one percent of the radioactivity from [¹⁴C]taurine was present in the pool fraction, and taurine pools increased

25-fold at the highest osmotic stress level examined, suggesting a role for taurine in *S. aureus* osmoregulation. Sucrose caused a lower pool accumulation of taurine than would be expected from the corresponding NaCl osmolarity.

Of the compounds studied, glycine betaine was the most effective osmoprotectant, especially at high levels of osmotic stress. Its important role in osmotic balancing is revealed by the high (more than 1 M) cellular concentrations achieved in osmotically stressed cultures. Similar results have been reported recently by Miller et al. (27).

Choline was also shown to be an osmoprotectant for *S. aureus* by virtue of its being oxidized to glycine betaine. Choline is known to be an osmoprotectant for various gram-negative species (6, 22). In contrast, in another gram-positive bacterium, apparently neither choline nor glycine betaine enables *Arthrobacter pascens* to overcome osmotic stress (33).

We believe that L-proline functions as an osmoprotectant in *S. aureus*, but interpretations of the growth experiments are complicated by the possible contributions of exogenous proline to protein synthesis and energy metabolism (20, 38). Proline has also been shown to be an osmoprotectant by Miller et al. (27). Analyses of proline pool levels indicate that this amino acid plays a significant but secondary role in osmotic balancing in *S. aureus* (Tables 2 and 3) (1, 21, 26, 27).

Taurine was not shown to be an osmoprotectant in high-osmotic-strength liquid medium but had a weak activity in solid 10× defined medium containing 1.7 M NaCl and no other added osmoprotectants. Taurine may contribute to osmotic balancing, possibly in combination with biosynthesized osmolytes, when no other exogenous osmoprotectants are available. Taurine pool levels increased in proportion to medium osmolarity in osmotically stressed cells. Taurine is an excellent compatible solute (3), and it is known to play a role in osmoregulation in a wide range of marine species,

TABLE 3. Radiochemical estimations of osmolyte pools in *S. aureus*

Component ^a	Adjusted pool levels in 10× defined medium plus (medium osmolarity) ^b :				
	No substance (0.250)	1 M sucrose (1.66)	1 M NaCl (2.24)	1.5 M NaCl (2.90)	2 M NaCl (4.13)
Glycine betaine ^c	32	171	445	689	1,260
Glycine betaine	76	421	588	805	1,167
Proline	21	69	171	139	309
Taurine	8	21	98	158	209

^a All cultures were supplemented with a minimum of 1 mM proline and 1 mM glycine betaine and 1 μ Ci of the radiolabeled solute under study per 20 ml of culture. When [¹⁴C]glycine betaine, [¹⁴C]choline, [³H]proline, and [¹⁴C]taurine accumulations were studied, their concentrations were raised to 5, 5, 2.02, and 2 mM, respectively.

^b Cells were grown to stationary phase in 10× defined medium and extracted with cold trichloroacetic acid, and pool levels of osmolytes (in millimolar) were estimated by measuring radioactivity. Medium osmolarity is expressed as osmoles per kilogram of H₂O.

^c Supplied as [¹⁴C]choline.

Ehrlich ascites mouse tumor cells, and the myocardium (15, 17, 18, 34). Reports of taurine in prokaryotic osmoregulation are limited to those of Graham et al. (12) and McLaggan and Epstein (25). Taurine accumulated in *Escherichia coli* via the ProU and ProP transport systems and showed weak osmo-protectant activity only in those cells unable to synthesize trehalose (25).

For *E. coli*, K⁺ levels have been proposed to play critical roles in the induction of *proU*, which encodes a transport system for glycine betaine (14, 32). Our study indicates that changes in K⁺ levels are less important in *S. aureus* osmoregulation. However, the substantial K⁺ pools in unstressed cells may make a major contribution to the osmotolerance of *S. aureus*.

It is possible to get an estimate of the total intracellular osmolyte pools of *S. aureus* by combining the data in Tables 2 and 3. It seems that *S. aureus* cells growing in high-osmotic-strength media have much lower turgor pressures than cells growing in low-osmolarity media.

In summary, K⁺ and glycine betaine pool levels appear to play very important roles in osmotic balancing in *S. aureus*, whereas taurine and proline play secondary roles.

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