Proline Transport in *Staphylococcus aureus*: a High-Affinity System and a Low-Affinity System Involved in Osmoregulation

DAVID E. TOWNSEND AND BRIAN J. WILKINSON*

Microbiology Group, Department of Biological Sciences, Illinois State University, Normal, Illinois 61761

Received 28 August 1991/Accepted 5 February 1992

L-Proline enhanced the growth of *Staphylococcus aureus* in high-osmotic-strength medium, i.e., it acted as an osmoprotectant. Study of the kinetics of L-[¹⁴C]proline uptake by *S. aureus* NCTC 8325 revealed high-affinity ($K_m = 1.7 \mu\text{M}$; maximum rate of transport [$V_{\max}$] = 1.1 nmol/min/mg [dry weight]) and low-affinity ($K_m = 132 \mu\text{M}$; $V_{\max} = 22 \text{ nmol/min/mg}$ [dry weight]) transport systems. Both systems were present in a proline prototrophic variant grown in the absence of proline, although the $V_{\max}$ of the high-affinity system was three to five times higher than that of the high-affinity system in strain 8325. Both systems were dependent on Na^+ for activity, and the high-affinity system was stimulated by lower concentrations of Na^+ more than the low-affinity system. The proline transport activity of the low-affinity system was stimulated by increased osmotic strength. The high-affinity system was highly specific for L-proline, whereas the low-affinity system showed a broader substrate specificity. Glycine betaine did not compete with proline for uptake through either system. Inhibitor studies confirmed that proline uptake occurred via Na^+ -dependent systems and suggested the involvement of the proton motive force in creating an Na^+ gradient. Hyperosmotic stress (upshock) of growing cultures led to a rapid and large uptake of L-[¹⁴C]proline that was not dependent on new protein synthesis. It is suggested that the low-affinity system is involved in adjusting to increased environmental osmolarity and that the high-affinity system may be involved in scavenging low concentrations of proline.

The physiology and genetics of bacterial responses to osmotic stress have been most extensively studied with *Escherichia coli* and *Salmonella typhimurium* (8). Important roles for K^+ and trehalose as osmolytes and for choline, glycine betaine, and proline as osmoprotectants and osmolytes have been demonstrated previously (8, 14). In contrast, although some of the pioneering studies in bacterial osmoregulation were carried out with the gram-positive coccus *Staphylococcus aureus* (7, 21), *S. aureus* osmoregulation has been neglected for more than a decade.

S. aureus is the most halotolerant, best-known, nonhalophile eubacterium. The generally accepted lowest medium water activity permitting growth of this organism is 0.86 (equivalent to 3.5 M NaCl) (27). The organism is probably the most common cause of food poisoning outbreaks in Western countries, and the salt tolerance of staphylococci means that they grow in foods at water activities lower than those of any other pathogenic bacterium (15).

Growth studies have shown that choline, glycine betaine, proline, and taurine are osmoprotectants for *S. aureus* (13). The proline content of the pool of *S. aureus* increases upon osmotic stress with NaCl (2, 13, 21). Graham et al. (12) and Koujima et al. (19) showed rapid uptakes of radiolabeled proline from complex medium upon hyperosmotic shock (upshock) with NaCl. These authors suggested that this was due to activation of a preexisting transport system rather than induction of it. Relatively little is known about proline transport in *S. aureus*. A high-affinity transport system ($K_m = 3.5 \mu\text{M}$) not shared by other amino acids was demonstrated with vesicles prepared from organisms grown in a low-osmolarity medium (28). In this paper, we report on two distinct transport systems with high and low affinities for proline and the probable involvement of the low-affinity system in osmoregulation in this organism.

MATERIALS AND METHODS

Bacterial strains. *S. aureus* PS47 (NCTC 8325) of the International Phage Typing set was used for most of these studies. This is the source of the 8325-4 series of strains (22) used as the standard background for most genetic studies (16) and for which a chromosome map exists (24). 8325 Pro⁺, a variant of 8325 which is prototrophic for proline, was also used in these studies. 8325 Pro⁺ was obtained by growing 8325 on defined medium (see below) plates lacking proline. After 3 days of incubation at 37°C, a large portion (79%) of the cells plated on the medium lacking proline grew, and one colony of 8325 Pro⁺ was selected for study. 8325 Pro⁺ was maintained on defined medium plates without proline. Strain 8325 was maintained on tryptic soy agar (Difco Laboratories, Detroit, Mich.) plates.

Growth conditions. Unless otherwise noted, all experiments were carried out with cells grown in a defined medium which contained glycerol as the primary carbon source and which was developed from that of Pattee and Neveln (11, 25). The medium contained (in milligrams per liter) glycerol (5,645), $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$ (7,000), $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ (2,300), $(\text{NH}_4)_2\text{SO}_4$ (1,000), $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (128), citric acid $\cdot \text{H}_2\text{O}$ (210), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (493), thiamine (1), niacin (1.2), sodium pantothenate (2.5), biotin (0.005), L-alanine (60), L-arginine (50), L-aspartic acid (90), L-cystine (20), L-glutamic acid (100), glycine (50), L-histidine (20), L-isoleucine (30), L-leucine (90), L-lysine (50), L-methionine (10), L-phenylalanine (40), L-proline (10), L-serine (30), L-threonine (30), L-tryptophan (10), L-tyrosine (50), and L-valine (80). In some early experiments, MgCl_2 (50 mg/liter) was used instead of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. When 0.5 M K_2HPO_4 was used to adjust the osmotic strength of the medium, the pH of the medium was adjusted to 7.0. Typically, cells were grown to mid-exponential phase ($A_{580} = 0.5$) with shaking (200 rpm) at 37°C in 300-ml nephelometer flasks containing 75 ml of medium. The cells were harvested by centrifugation ($13,000 \times g$, 5 min, 4°C). The pellet was washed by resuspension and centrifugation.

* Corresponding author.

gation once in 50 mM Tris-HCl buffer (pH 7.0) containing 20 mM D-glucose. The washed cell pellet was suspended in half the original culture volume in 50 mM Tris-HCl-20 mM glucose and kept on ice.

Transport assay. Prior to each assay, 3 ml of cell suspension was transferred to a 50-ml Erlenmeyer flask and equilibrated to 37°C by shaking at 200 rpm for 5 min. Each assay was initiated by adding 100 μ l of cell suspension to a 7-ml plastic minivial (Denville Scientific, Denville, N.J.) that contained 100 μ l of reaction mixture. A typical reaction mixture contained 50 mM Tris-HCl (pH 7.0), 20 mM D-glucose, and various concentrations of NaCl and L-[U- 14 C]proline (260 mCi/mmol; ICN Radiochemicals, Irvine, Calif.). Uptake was terminated by adding 5 ml of a stopping buffer to the reaction vial. The stopping buffer contained 50 mM Tris-HCl (pH 7.0), 20 mM D-glucose, and 2 mM HgCl₂. After stopping buffer was added, the samples were filtered under vacuum through a Millipore type HA filter (0.45- μ m pore size) (Millipore Corp., Bedford, Mass.) or a cellulosic filter (0.45- μ m pore size) (Micron Separations Inc., Westboro, Mass.) and then rinsed with another 5 ml of stopping buffer. The filter was then placed in a minivial and left to dry for several hours. No loss of radioactivity occurred during this drying period. A 5-ml volume of Cytosint scintillation fluid (ICN Biomedicals, Costa Mesa, Calif.) was then added to each vial, and the samples were counted in a Beckman LS 8100 scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.). The initial rate of uptake was determined from a time course of uptake with samples taken after 0, 5, 10, 15, 20, 25, 30, 45, and 60 s. The rate of uptake was linear for at least 60 s. The A_{580} of the cell suspension was measured to determine the concentration of bacteria (in milligrams [dry weight] per milliliter) by using a calibration curve, as described previously by Bieber and Wilkinson (6). In order to study the specificity of proline transport, various compounds were tested for their ability to compete for uptake of L-[14 C]proline. Proline uptake by the high-affinity system (see below) was studied by using 1 μ M L-[14 C]proline and 1 mM test compound, and proline uptake by the low-affinity system (see below) was studied by using 100 μ M L-[14 C]proline and 10 mM test compound, each in the presence of 0.1 M NaCl. Uptake was determined after 30 s. The effects of various inhibitors on transport by the two systems were also studied by using 0.1 M NaCl and 1 and 100 μ M L-[14 C]proline. The inhibitors were preincubated with the cell suspension for 5 min at 37°C with shaking (200 rpm) before transport was initiated. Necessary inhibitors were dissolved in 95% (vol/vol) ethanol and compared with controls with ethanol alone (maximum final concentration of ethanol, 10% [vol/vol]). Uptake was determined after 30 s.

Hyperosmotic stress (upshock) studies. Cells were grown in 300-ml nephelometer flasks containing 50 ml of defined medium to mid-exponential phase (A_{580} = 0.5) at 37°C. The cells were harvested by centrifugation (13,000 $\times$ g, 5 min, 4°C) and resuspended in defined medium lacking proline to a final volume of 2 ml. The cell suspension was used to inoculate 48 ml of defined medium containing 1 mM L-proline (0.0625 or 0.4 μ Ci of L-[14 C]proline per ml) in a 300-ml Erlenmeyer flask. Flasks, each containing no added substance (control), 0.5 M and 1.0 M NaCl, 0.5 M KCl, or 1 M sucrose as its source of osmotic stress, were set up. At intervals, samples (0.5 ml) of cell suspension from each culture were filtered and radioactivity was determined as described above. The A_{580} of the cultures were also determined. Upshock studies were also carried out with cells in which protein synthesis was inhibited by preincubating the

cells for 30 min with 100 μ g of chloramphenicol per ml prior to upshock and by carrying out the upshock in medium containing chloramphenicol (100 μ g/ml). The stopping buffers were adjusted to the same osmolality as the cultures to prevent leakage of intracellular proline upon the termination of uptake (7). The effect of hyperosmotic stress on L-[3,4- 3 H]glutamic acid (33 Ci/mmol; ICN Radiochemicals) uptake (1 mM glutamic acid and 0.1 μ Ci of L-[3 H]glutamic acid per ml) was studied in the same way.

Identity of radioactivity taken up from L-[14 C]proline in transport assays and upshock experiments. Radioactivity was extracted from cells collected on filters in transport assays (100 μ M L-[14 C]proline, 100 mM NaCl, 60 s) and upshock experiments (1 mM L-[14 C]proline, 1 M NaCl, 1 h) with 5% (wt/vol) trichloroacetic acid for 15 min at 0°C (6). The trichloroacetic acid was removed by ether extraction, and the samples were lyophilized. The samples and authentic L-[14 C]proline and L-[3 H]glutamic acid in the presence of unlabeled carrier L-proline and L-glutamic acid were subjected to chromatography. Whatman 3MM paper with a solvent system of 1-propanol-NH₄OH (3:1, vol/vol) (9) and Eastman Chromagram 13255 cellulose without fluorescent indicator sheets with a solvent system of 95% (vol/vol) ethanol-H₂O (2:1, vol/vol) were used for paper and thin-layer chromatography, respectively. The chromatograms were cut into strips, and radioactivity was determined by liquid scintillation counting as described by Smiley and Wilkinson (30). Unlabeled L-proline and L-glutamic acid were chromatographed alone and were detected by using ninhydrin (30). The unlabeled and labeled amino acids chromatographed identically. In addition, the incorporation of radioactivity into cold trichloroacetic acid-insoluble material was measured (6, 30).

FIG. 1. Influence of various concentrations of L-proline on the growth of *S. aureus* in defined medium.

FIG. 2. Influence of L-proline on the growth of *S. aureus* in defined medium with and without 1 M NaCl (A) and 0.5 M K₂HPO₄ (B).

Chemicals. Potential competitors and inhibitors were purchased from Sigma Chemical Co., St. Louis, Mo.

RESULTS

Proline functions as an osmoprotectant in *S. aureus*. In low-osmotic-strength medium (defined medium), L-proline was necessary for growth to be initiated without a long lag phase, and it was determined that 30 μ M L-proline supported the maximum growth rate and stationary-phase yield of cells (Fig. 1). The inclusion of 1 M NaCl in defined medium reduced the rate of growth and the final cell yield of cells (Fig. 2A). Various amounts of L-proline were added to defined medium containing 1 M NaCl, and increasing the amounts of L-proline increased the growth rate and the stationary-phase cell population sizes (Fig. 2A). Similar results were obtained when 0.5 M K₂HPO₄ replaced NaCl as the stressing agent (Fig. 2B). This indicates that growth inhibition is not due to Na⁺ or Cl⁻ toxicity and is most likely due to the increased osmotic strength of the medium. Graham and Wilkinson (13) showed that proline alleviated growth inhibition in sucrose-stressed cultures. Thus, L-proline acted as an osmoprotectant. The inclusion of 1 M NaCl or 0.5 M K₂HPO₄ in defined medium lacking L-proline resulted in a very long lag phase before growth was initiated (Fig. 2A and B, respectively).

Kinetics of L-proline transport. Initial transport velocities over a wide range of L-proline concentrations (1 to 500 μ M) in the presence of 100 mM NaCl were determined (Fig. 3a). Preliminary investigations had revealed that L-proline transport was Na⁺ dependent. An Eadie-Hofstee plot of the data was biphasic, suggesting the presence of two proline transport systems with high and low affinities for L-proline. The kinetics of the two systems were investigated in more detail by using L-proline concentrations in the range of 0.5 to 14 μ M for the high-affinity system and 25 to 1,500 μ M for the low-affinity system (Fig. 3b and c). Eadie-Hofstee plots revealed that the high-affinity system had a K_m of 1.7 μ M and a maximum rate of transport (V_{max}) of 1.1 nmol/min/mg (dry weight), whereas the low-affinity system had a K_m of 132 μ M and a V_{max} of 22 nmol/min/mg (dry weight). A Hill plot of the data for these two transport systems indicated that both of the proline carriers had only one binding site for proline ($n_{app} = 1.1$ for the high-affinity system and 0.9 for the low-affinity system). Altering the pH from 5.5 to 8.5 had little effect on the transport rate of L-proline through either system.

The effect on the transport systems of growth in the absence of L-proline was studied by using the proline prototroph 8325 Pro⁺ isolated by culturing 8325 in the absence of proline (see Materials and Methods). Assay of proline

FIG. 3. Kinetics of L-proline transport by *S. aureus*. In all cases, initial transport velocity is plotted versus substrate concentration and the insets are Eadie-Hofstee plots of the data. (a) 1 to 500 μM L-proline; (b) 0.5 to 14 μM L-proline; (c) 25 to 1,500 μM L-proline.

transport in this strain with 1 and 100 μM L-[^{14}C]proline for the high- and low-affinity systems, respectively, revealed a rate of transport via the high-affinity system three- to fivefold higher than that of the high-affinity transport system in 8325,

whereas the rate of transport via the low-affinity system was very similar for both strains. The kinetic parameters of the high- and low-affinity transport systems in strain 8325 Pro⁺ were $K_m = 1.7 \mu\text{M}$ and $V_{\max} = 5.6 \text{ nmol/min/mg (dry weight)}$

FIG. 4. Effects of different Na^+ concentrations on proline transport via the two systems. Initial transport rates were determined in the presence of various Na^+ concentrations by using 1 and 100 μM L-[^{14}C]proline for the high- and low-affinity systems, respectively.

and $K_m = 62 \mu\text{M}$ and $V_{\max} 23 \text{ nmol/min/mg}$ (dry weight), respectively. These observations suggest that proline is not necessary for the induction of these systems and that the absence of proline leads to increased expression of the high-affinity system.

Na^+ requirements of the high- and low-affinity transport systems. Transport through both systems was dependent on Na^+ . As with other Na^+ -linked bacterial transport systems, Li^+ supported transport through both systems but at a lower rate (Fig. 4). In the absence of Na^+ , no transport occurred with 0.1 M KCl or 1 M sucrose (Fig. 4). The effects of different Na^+ concentrations on L-[^{14}C]proline uptake through the high- and low-affinity systems are shown in Fig. 4. The high-affinity system was more responsive to low Na^+ concentrations than the low-affinity system. Of the concentrations of Na^+ tested, 100 mM Na^+ supported the fastest rate of proline transport and the rate of transport declined at higher Na^+ concentrations. The low-affinity system was appreciably less stimulated by low Na^+ concentrations. Maximum uptake rates were observed with 250 and 500 mM NaCl, and 1 M NaCl allowed 20% of the maximum rate, compared with less than 8% with the high-affinity system.

With the low-affinity system, it seems likely that the rate of transport was affected by the concentration of NaCl by providing Na^+ as a coupling ion for transport and Na^+ and Cl^- as sources of osmotic stress. The initial rates of transport supported by different concentrations of NaCl were compared with those of reaction mixtures maintained at 1 osM by using a combination of NaCl and sucrose and those of reaction mixtures containing NaCl and no additional osmotic stress. It was found that rates of transport were higher at low Na^+ concentrations with osmotic stress than without (Fig. 5). A similar effect was not seen with the high-affinity system (data not shown).

Cells were grown in defined medium containing 0.5 M NaCl, and the kinetic parameters of the low-affinity system were determined in the presence of 0.5 M NaCl. A K_m of 233 μM and a $V_{\max}$ of 34 nmol/min/mg (dry weight) were obtained, suggesting that growth under conditions of osmotic stress did not have much effect on the expression of this system.

Specificities of the proline transport systems. The specificities of the systems were studied by observing the initial rates of L-[^{14}C]proline uptake in the presence of 1,000-fold excess and 100-fold excess unlabeled compounds for the high- and low-affinity systems, respectively. The results are shown in Table 1. The low-affinity system may have a broader substrate specificity than the high-affinity system. D-Proline, L-azetidine-2-carboxylic acid, 3,4-dehydro-DL-proline, and to a lesser degree *cis*-hydroxy-L-proline were all able to compete with L-[^{14}C]proline for transport through the low-affinity system when present at only 100-fold excess. When these compounds were present at 1,000-fold excess, only L-azetidine-2-carboxylic acid and 3,4-dehydro-DL-proline strongly inhibited transport of L-[^{14}C]proline via the high-affinity system. None of the other amino acids tested, representing the 13 amino acid transport systems in *S. aureus* (28), competed for proline uptake. Glycine betaine, a compound known to play a role in osmotic tolerance in many bacteria, including *S. aureus* (13), did not compete for proline uptake through either system.

Effects of various inhibitors on proline transport. The effects of various agents that disrupt protein structure, ATPase activity, proton motive force, and Na^+ electrochemical gradients on the initial rates of L-[^{14}C]proline uptake via the high- and low-affinity transport systems were

FIG. 5. Proline transport supported by different Na^+ concentrations in the presence and absence of osmotic stress. Initial transport rates were determined in the presence of various NaCl concentrations and 100 μM L-[^{14}C]proline in two ways: (i) by keeping the osmotic strength of the reaction mixture constant at 1 osM (○) by adding the appropriate concentrations of sucrose and (ii) by using NaCl without sucrose (●).

studied. The results are shown in Table 2. Similar patterns of inhibition were noted with both systems. The inhibitors *p*-chloromercuribenzoate (SH reagent), carbonyl cyanide *m*-chlorophenylhydrazone and dinitrophenol (proton motive force), and gramicidin D and monensin (Na^+ gradients) each inhibited transport through both systems by more than 50% with both inhibitor concentrations tested. The only notable difference in inhibitor sensitivities between the two systems occurred with *N*-ethylmaleimide and *p*-chloromercuribenzoate, which inhibited transport via the low-affinity system more than through the high-affinity system.

Stimulation of proline uptake in growing cultures by hyperosmotic stress. Strain 8325 was subjected to various osmotic upshocks, and proline uptake was measured (Fig. 6A). Proline uptake occurred rapidly after upshock. A total of 1.0 M NaCl supported the largest uptake of L-[^{14}C]proline, followed by 0.5 M NaCl, 1 M sucrose, 0.5 M KCl, and the control (898, 632, 512, 413, and 124 nmol/mg [dry weight] per 30 min, respectively). Maximum proline uptake occurred 3 h after upshock, which was followed by a slow decrease in the cell-associated proline. Similar experiments were carried out with cells upshocked with 0.5 M NaCl in the presence and absence of chloramphenicol (100 $\mu\text{g/ml}$). Identical amounts of L-[^{14}C]proline were transported in the presence or absence of chloramphenicol. This suggests that new protein synthesis is not necessary for rapid proline uptake and that hyperosmotic shock activates a preexisting transport system. L-[^3H]glutamic acid uptake was not stimulated by

TABLE 1. Effects of potential competitors on the initial rates of proline transport through high- and low-affinity systems

Compound ^a	% Inhibition	
	High-affinity system	Low-affinity system
L-Proline	97	99
D-Proline	33	80
<i>trans</i> -4-Hydroxy-L-proline	5	6
<i>cis</i> -4-Hydroxy-L-proline	30	34
L-Azetidine-2-carboxylic acid	84	91
3,4-Dehydro-DL-proline	84	80
L-Alanine	5	NT ^b
L-Arginine	5	5
L-Asparagine	5	NT
L-Cysteine	5	5
L-Glutamic acid	5	4
Glycine	5	5
L-Histidine	5	4
L-Isoleucine	5	NT
L-Leucine	5	NT
L-Lysine	5	5
L-Methionine	5	5
L-Phenylalanine	5	5
L-Serine	5	5
L-Threonine	5	NT
L-Tryptophan	5	NT
L-Valine	5	5
Glycine betaine	5	5
Taurine	5	5

^a Substrate concentrations were 1 and 100 μ M L-[¹⁴C]proline, and potential competitor concentrations were 1 and 10 mM for the high- and low-affinity systems, respectively.

^b NT, not tested.

upshock (Fig. 6B), suggesting that the observed proline uptake is not a nonspecific response to hyperosmotic stress.

Identity of radioactivity accumulated from L-[¹⁴C]proline in transport assays and upon hyperosmotic stress of growing cultures. The accumulated radioactivity was extracted from cells that had been allowed to take up L-[¹⁴C]proline for 1 min in a transport assay and for 1 h in an upshock experiment. In both cases, virtually all of the radioactivity migrated as proline upon paper chromatography and when subjected to thin-layer chromatography on cellulose sheets with a different solvent system (data not shown). In the transport assay, 18% of the cell-associated radioactivity was present in cold trichloroacetic acid-insoluble material, presumably representing protein. After 1 h in an upshock experiment, 4% of the radioactivity was present in cold trichloroacetic acid-insoluble material in cells shocked with 1 M NaCl, compared with 24% in unshocked control cells. There were very similar absolute amounts of cold trichloroacetic acid-insoluble radioactivity in upshocked and control cells.

DISCUSSION

Previous studies (2, 19, 21) have suggested that the accumulation of proline in response to osmotic stress is important for *S. aureus* to adjust to the stress. In accordance with these findings, we and Graham and Wilkinson (13) have shown that L-proline is an osmoprotectant for *S. aureus* in a high-osmolarity medium.

Kinetic studies revealed two transport systems for L-proline that had high and low affinities and low and high capacities for proline transport, respectively. Two transport systems for proline in *S. aureus* ATCC 12600 with K_m s of 8

TABLE 2. Effects of various inhibitors on the initial rates of proline transport through high- and low-affinity systems

Inhibitor	Concn	% Inhibition	
		High-affinity system	Low-affinity system
<i>N</i> -Ethylmaleimide	0.5 mM	18	40
	5.0 mM	35	71
Sodium	0.5 mM	53	80
<i>p</i> -Chloromercuribenzoate	5.0 mM	82	92
Sodium iodoacetate	0.5 mM	6	18
	5.0 mM	35	31
Sodium arsenate	1.0 mM	0	0
	10.0 mM	0	0
Sodium fluoride	1.0 mM	0	0
	10.0 mM	0	0
Potassium cyanide	1.0 mM	35	31
	10.0 mM	29	44
Dinitrophenol	1.0 mM	33	41
	10.0 mM	90	82
Carbonyl cyanide <i>m</i> -chlorophenylhydrazide	0.01 mM	67	67
	0.10 mM	72	77
<i>N,N'</i> -Dicyclohexylcarbodiimide	0.01 mM	8	5
	0.10 mM	49	33
Monensin	0.015 mM	77	67
	0.150 mM	82	73
Gramicidin D	1 μ g/ml	87	73
	10 μ g/ml	87	84

μ M and 1 mM have been reported previously by others (3). Anderson (1) has also described two transport systems for proline in *S. aureus* MF-31. The high-affinity system had a K_m of 2.2 μ M and a V_{max} of 0.22 nmol/min/mg (dry weight), and the low-affinity system had a K_m of 33.2 mM and a V_{max} of 31.4 nmol/min/mg (dry weight). In the presence of 1 M NaCl, Lineweaver-Burk plots were monophasic, with a K_m of 183 μ M and a V_{max} of 5.6 nmol/min/mg (dry weight). These three independent reports strongly support the occurrence of at least two proline transport systems in *S. aureus*. The differences in determined kinetic parameters are probably due to transport being assayed in the presence of different concentrations of Na⁺, in that Na⁺ often has its major effect on the affinity of the transport protein for its substrate (20). Short and Kaback (28) described a K_m of 3.5 μ M for proline transport in membrane vesicles of *S. aureus*, which probably represents the high-affinity system.

Neither transport system required proline as an inducer. Proline deprivation increased the activity of the high-affinity system, suggesting that this system may be involved in scavenging low concentrations of proline from the environment. Growth in high-osmotic-strength medium was not required for the induction of these transport systems. The high-affinity system appeared to be quite specific for proline. The low-affinity system may have a broader specificity for proline, in that competition comparable to that observed

FIG. 6. Effects of hyperosmotic stress on the uptake of L-[^{14}C]proline (A) and L-[^3H]glutamic acid (B) in growing cultures. Various solutes were added to exponential-phase cultures and the uptakes of L-[^{14}C]proline and L-[^3H]glutamic acid were measured at intervals.

with 1,000-fold excess in the high-affinity system was noted with 100-fold excess unlabeled compound in this system.

Both transport systems were dependent on Na^+ for their activity, and the high-affinity system had an apparent higher affinity for Na^+ than the low-affinity system. High concentrations of Na^+ (0.25 to 0.5 M) were required for maximum activity of the low-affinity system. The reagents gramicidin D and monensin, which collapse Na^+ gradients, both inhibited proline transport through both systems. Transport through either system showed low susceptibility to inhibition of glycolysis (NaF, sodium iodoacetate) or ATP formation (sodium arsenate). However, the uncouplers carbonyl cyanide *m*-chlorophenylhydrazone and dinitrophenol were potent inhibitors. It may be that an Na^+ gradient is created in exchange for the proton gradient (Na^+ - H^+ antiport). However, a variety of mechanisms for creating Na^+ gradients are

known for different bacteria (20, 29), and little information is available on how this may occur in *S. aureus*. The transport of taurine by staphylococci was Na^+ dependent (6, 11), and it seems likely that the transport of other compounds is dependent on Na^+ in these bacteria.

When the inhibitor susceptibilities of the two systems were compared, the only notable difference was the higher susceptibility of the low-affinity system to *N*-ethylmaleimide and *p*-chloromercuribenzoate. One possible explanation is that Na^+ is better able to inhibit the access of these reagents to the active site of the high-affinity transport system because of its probable higher affinity for that carrier. Recently, Yamada et al. (33) described higher-than-normal levels of proline transport inhibition by *N*-ethylmaleimide in *putP* mutants which possessed a lower affinity for Na^+ .

The low-affinity system appears to be responsible for the

accumulation of large amounts of proline upon hyperosmotic stress. The $V_{\max}$ of the system is compatible with the large uptake of proline that occurs upon osmotic upshock. Also, maximum activity of this transport system occurred with 0.25 to 0.5 M NaCl. The most detailed knowledge of bacterial proline transport exists from studying the enteric bacteria (see reference 32 for a review). Proline porter I (PutP) is induced by proline for proline catabolism, has a high affinity for proline, and is an Na^+ -dependent system. Proline porter II (ProP) is expressed constitutively, but its activity is increased by nutritional or hyperosmotic stress. Proline porter III (ProU) is induced by osmotic stress and is primarily involved in tolerance to hyperosmotic stress. Proline porter III has a higher affinity for glycine betaine than for proline, whereas proline porter II has approximately equal affinities for proline and glycine betaine. ProU (proline porter III) is a member of the periplasmic substrate-binding class of transport systems (4, 14). Glycine betaine did not inhibit proline uptake by either the high- or low-affinity system of *S. aureus*. Proline porter II is integral to the *E. coli* cytoplasmic membrane and is energized by respiration. When the *E. coli* and *S. aureus* proline transport systems are compared, most similarities in regulatory and functional properties are seen between the *S. aureus* low-affinity system and proline porter II of *E. coli*.

It was previously noted that in transport assays for proline uptake by either the high- or low-affinity system in cells incubated in buffer, 1 M NaCl was inhibitory. In contrast, in upshock experiments with growing cultures in defined medium 1 M NaCl caused more proline uptake than 0.5 M NaCl. The reason for this discrepancy is not known, but it could be due to some component of the defined medium not present in transport assays.

High-affinity, energy-dependent proline uptake has been demonstrated with a variety of bacterial species (32), including *Bacillus subtilis* (23). Proline transport had a K_m of 2.3 μM in this organism, which is phylogenetically closely related to staphylococci (31). A high- and a low-affinity proline transport system in the gram-positive bacterium *Streptomyces clavuligerus* (5) have recently been reported. Proline was not required for the induction of either system, and the low-affinity system had broader substrate specificities. The possible relations of these systems to osmoregulation were not reported.

The results of our upshock studies show that proline accumulates very rapidly during the first moments after osmotic upshock. The addition of 1 M NaCl to exponentially growing cultures of 8325 resulted in a 21-fold increase in cell-associated proline 150 s after upshock. This indicates that proline is one of the first osmolytes to respond to sudden shifts in environmental osmolality. Chromatography revealed that radioactivity from $\text{L-}[^{14}\text{C}]\text{proline}$ accumulated as proline. Perhaps the accumulation of proline is linked to a decrease in cell turgor, much like the Kdp transport system of *E. coli*, which is responsible for a rapid accumulation of K^+ in an effort to restore osmotic balance (10). The Kdp system is believed to function as a membrane-bound osmotic sensor and appears to be involved in osmoregulatory gene expression (14). Potassium glutamate has recently been identified as the molecular signal stimulating *proU* expression in *in vitro* systems (17, 26). Analysis of the K^+ pool in upshocked cultures of *S. aureus* 8325 did not show large increases in cell K^+ (18), which was in contrast to the situation with *E. coli* (10). This suggests that K^+ may not play the same key role in the osmotic response of *S. aureus* that it does in those of *E. coli* and *S. typhimurium*. Further-

more, proline uptake occurred more rapidly than glycine betaine uptake upon hyperosmotic stress (18). This suggests that proline uptake may be very important in the initial response to osmotic stress in *S. aureus*.

ACKNOWLEDGMENTS

This work was supported in part by a Biomedical Research Support Grant and by grant number 1 R15 GM42080-01A2 from the National Institutes of Health.

REFERENCES

- Anderson, C. B. 1982. Ph.D. thesis. University of Illinois, Urbana.
- Anderson, C. B., and L. D. Witter. 1982. Glutamine and proline accumulation by *Staphylococcus aureus* with reduction in water activity. Appl. Environ. Microbiol. **43**:1501-1503.
- Bae, J. H., and K. J. Miller. 1990. Characterization of two proline transport systems in *Staphylococcus aureus* and their possible roles in osmotic adaptation, abstr. P-30, p. 283. Abstr. 90th Annu. Meet. Am. Soc. Microbiol. 1990. American Society for Microbiology, Washington, D.C.
- Barron, A., J. U. Jung, and M. Villarejo. 1987. Purification and characterization of a glycine betaine binding protein from *Escherichia coli*. J. Biol. Chem. **262**:11841-11846.
- Bascaran, V., C. Hardisson, and A. F. Brana. 1990. Proline uptake in *Streptomyces clavuligerus*. FEMS Microbiol. Lett. **57**:27-30.
- Bieber, E. J., and B. J. Wilkinson. 1984. Sodium-dependent uptake of taurine in encapsulated *Staphylococcus aureus* strain M. Biochim. Biophys. Acta **770**:127-135.
- Christian, J. H. B., and J. A. Waltho. 1964. The composition of *Staphylococcus aureus* in relation to the water activity of the growth medium. J. Gen. Microbiol. **35**:205-218.
- Csonka, L. N. 1989. Physiological and genetic responses of bacteria to osmotic stress. Microbiol. Rev. **53**:121-147.
- Deutch, C. D., J. M. Hasler, R. M. Houston, M. Sharma, and V. J. Stone. 1989. Nonspecific inhibition of proline dehydrogenase synthesis in *Escherichia coli* during osmotic stress. Can. J. Microbiol. **35**:779-785.
- Epstein, W. 1986. Osmoregulation by potassium transport in *Escherichia coli*. FEMS Microbiol. Rev. **39**:73-78.
- Giehl, T. J., M. W. Qoronfleh, and B. J. Wilkinson. 1987. Transport, nutritional and metabolic studies of taurine in staphylococci. J. Gen. Microbiol. **133**:849-856.
- Graham, J. E., K. W. Stimeling, and B. J. Wilkinson. 1989. Taurine, choline, betaine, and proline function in *Staphylococcus aureus* osmoregulation, abstr. I-138, p. 240. Abstr. 89th Annu. Meet. Am. Soc. Microbiol. 1989. American Society for Microbiology, Washington, D.C.
- Graham, J. E., and B. J. Wilkinson. 1992. *Staphylococcus aureus* osmoregulation: roles for choline, glycine betaine, proline, and taurine. J. Bacteriol. **174**:2711-2716.
- Higgins, C. F., J. Cairney, D. A. Stirling, L. Sutherland, and I. R. Booth. 1987. Osmotic regulation of gene expression: ionic strength as an intracellular signal. Trends Biochem. Sci. **12**:339-344.
- Hurst, A., and D. L. Collins-Thompson. 1979. Food as a bacterial habitat. Adv. Microb. Ecol. **3**:79-134.
- Iandolo, J. J. 1989. Genetic analysis of extracellular toxins of *Staphylococcus aureus*. Annu. Rev. Microbiol. **43**:375-402.
- Jovanovich, S. B., M. T. Record, Jr., and R. R. Burgess. 1989. In an *Escherichia coli* coupled transcription-translation system, expression of the osmoregulated gene *proU* is stimulated at elevated potassium concentrations and by an extract from cells grown at high osmolality. J. Biol. Chem. **264**:7821-7825.
- Kaenjak, A., and B. J. Wilkinson. 1991. Roles of various osmoregulating molecules in the *Staphylococcus aureus* response to hyperosmotic stress, abstr. K-122, p. 90. Abstr. 91st Annu. Meet. Am. Soc. Microbiol. 1991. American Society for Microbiology, Washington, D.C.
- Koujima, I., H. Hayashi, K. Tomochika, A. Okabe, and Y. Kanemasa. 1978. Adaptational change in proline and water

- content of *Staphylococcus aureus* after alteration of environmental salt concentration. *Appl. Environ. Microbiol.* **35**:467–470.
20. **Maloy, S.** 1989. Sodium coupled cotransport, p. 203–224. In T. A. Krulwich (ed.), *The bacteria*, vol. XII. Bioenergetics. Academic Press, New York.
 21. **Measures, J. C.** 1975. Role of amino acids in osmoregulation of non-halophilic bacteria. *Nature (London)* **257**:398–400.
 22. **Novick, R. P.** 1967. Properties of a cryptic high frequency transducing phage in *Staphylococcus aureus*. *Virology* **33**:155–166.
 23. **Ordal, G. W., D. P. Villani, R. A. Nicholes, and F. G. Hamel.** 1978. Independence of proline chemotaxis and transport in *Bacillus subtilis*. *J. Biol. Chem.* **253**:4916–4919.
 24. **Pattee, P. A.** 1987. *Staphylococcus aureus*, p. 148–154. In S. J. O'Brien (ed.), *Genetic maps 1987*, vol. 4. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
 25. **Pattee, P. A., and D. S. Neveln.** 1975. Transformation analysis of three linkage groups in *Staphylococcus aureus*. *J. Bacteriol.* **124**:201–211.
 26. **Ramirez, R. M., W. S. Prince, E. Bremer, and M. Villarejo.** 1989. *In vitro* reconstitution of osmoregulated expression of *proU* of *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **86**:1153–1157.
 27. **Scott, W. J.** 1953. Water relations of *Staphylococcus aureus* at 30°C. *Aust. J. Biol. Sci.* **6**:549–564.
 28. **Short, S. A., and H. R. Kaback.** 1974. Amino acid transport and staphylococcal membrane vesicles. *Ann. N.Y. Acad. Sci.* **23**:123–143.
 29. **Skulachev, V. P.** 1987. Bacterial sodium transport: bioenergetic functions of sodium ions, p. 131–164. In B. P. Rosen and S. Silver (ed.), *Ion transport in prokaryotes*. Academic Press, New York.
 30. **Smiley, D. W., and B. J. Wilkinson.** 1983. Survey of taurine uptake and metabolism in *Staphylococcus aureus*. *J. Gen. Microbiol.* **129**:2421–2428.
 31. **Woese, C. R.** 1987. Bacterial evolution. *Microbiol. Rev.* **51**:221–271.
 32. **Wood, J. M.** 1988. Proline porters affect the utilization of proline as nutrient or osmoprotectant for bacteria. *J. Membr. Biol.* **106**:183–202.
 33. **Yamada, I., M. Ohsawa, and Y. Anraku.** 1990. Defective cation coupling mutants of *Escherichia coli* Na⁺/proline symport carrier. *J. Biol. Chem.* **265**:2450–2455.