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Humectant Permeability Influences Growth and Compatible Solute Uptake by *Staphylococcus aureus* Subjected to Osmotic Stress

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ABSTRACT

The effects of different humectants (sodium chloride, sucrose, and glycerol) on the growth of and compatible solute (glycine betaine, proline, and carnitine) uptake by the osmotolerant foodborne pathogen *Staphylococcus aureus* were investigated. While growth in the presence of the impermeant humectants sodium chloride and sucrose induced the accumulation of proline and glycine betaine by cells, growth in the presence of the permeant humectant glycerol did not. When compatible solutes were omitted from low-water-activity media, growth was very poor in the presence of impermeant humectants. In contrast, the addition of compatible solutes had essentially no effect on growth when cells were grown in low-water-activity media containing glycerol as the humectant. Carnitine was found to accumulate to high intracellular levels in osmotically stressed cells when proline and glycine betaine were absent, making it a potentially important compatible solute for this organism.

Staphylococcus aureus is described by Sheagren *(36)* as "the most important bacterial pathogen of humans." It is a common cause of food poisoning *(4, 41)* and skin infections *(31)* and can cause other, more serious illnesses such as toxic shock syndrome *(8)* and infective endocarditis *(16)*. It is also the leading cause of nosocomial infections *(6, 36)*, being the single most frequent isolate of positive blood cultures from hospitalized patients *(36)*.

An estimated 30% of all cases of foodborne illness worldwide are caused by staphylococci *(4)*. As the most osmotolerant of the foodborne pathogens *(44)*, *S. aureus* is able to grow in foods that are inhospitable to other pathogens and normally considered "safe." Indeed, outbreaks of staphylococcal food poisoning are often associated with food items of reduced water activity (a_w), such as various cured and/or fermented fish and meat products, custards, and cream-filled pastry *(4, 5, 34, 41, 44)*. The minimal a_w for the growth of *S. aureus* is usually taken to be 0.86 *(9, 41)*, although a few early studies suggested an even lower minimal a_w (0.83) *(23, 38)*.

A primary physiological response of *S. aureus* to lowered a_w is the accumulation of compatible solutes to high intracellular concentrations. The most prominent compatible solutes in *S. aureus* are proline and glycine betaine (hereinafter referred to as betaine) *(29)*, although a few other compounds, such as proline betaine *(2)* and taurine *(14)*, have been reported to act as compatible solutes in this organism. Carnitine, which is found in various foods, particularly meats *(12)*, has been shown to be a compatible solute for a number of bacterial species, including several gram-positive bacteria *(18, 20, 39, 45)*. To our knowledge, its role in the osmoadaptation of *S. aureus* has not previously been investigated.

Although the growth of *S. aureus* in low-a_w environments has been studied periodically since at least the 1930s *(15)*, some aspects of its growth kinetics at low a_w, particularly with regard to different humectants and compatible solutes, remain unclear. Many bacteria show pronounced differences, termed "specific solute effects," in their responses to lowered a_w, depending on the humectant used *(9, 10, 37, 44)*. Although Scott's *(35)* pioneering study in the early 1950s suggested that the growth response of *S. aureus* to osmolarity did not significantly depend on the nature of the humectant, later studies have suggested otherwise. Specifically, Li and Torres *(24)* found that the use of sucrose as the humectant gave rise to lower growth rates than did the use of sodium chloride in this capacity, and a few investigators have found glycerol to be more inhibitory to this bacterium than sodium chloride is *(17, 28, 33)*. Curiously, this is the opposite of what has been found for many gram-negative bacteria *(9, 10, 37)*. However, there are a few discrepancies in the literature concerning the effects of glycerol on *S. aureus*. For example, the growth-limiting a_w value reported for glycerol varies from 0.865 *(33)* to 0.89 *(28)*. Furthermore, Troller *(42, 43)* found that cultures in glycerol at an a_w of 0.90 gave rise to viable cell counts in excess of 10^9 CFU/ml, while other humectants gave rise to significantly fewer colonies at the same a_w level. One important goal of the present study was to further examine the effects of high concentrations of glycerol on the growth of *S. aureus,* partly as an attempt to resolve

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these discrepancies in the earlier literature, and partly because of the recent surge of interest in the use of glycerol as an alternative humectant in the food industry *(34).*

The work reported herein represents the initial phases of our investigation into specific solute effects in *S. aureus.* In this study, we examined the effects of three humectants (sodium chloride, sucrose, and glycerol) and three compatible solutes (carnitine, betaine, and proline) on the growth response of *S. aureus* at low a_w.

MATERIALS AND METHODS

Strains and media. The *S. aureus* type strain, ATCC 12600, was used in this study. The defined medium (DFM) was that of Pattee and Neveln *(32)* with the following modifications: purines, pyrimidines, and agar were omitted; 1 ml of a trace mineral solution containing $FeCl_2 \cdot 6H_2O$ (11 mM), $MnCl_2 \cdot 4H_2O$ (5.1 mM), $Na_2MoO_4 \cdot 2H_2O$ (0.046 mM), $ZnSO_4 \cdot 7H_2O$ (0.035 mM), $CuSO_4 \cdot 5H_2O$ (0.040 mM), H_3BO_4 (0.13 mM), and $CoCl_2 \cdot 6H_2O$ (0.042 mM) was added per liter of medium; and the proline concentration was varied (from 0.05 to 5 mM). Trypticase soy broth (TSB) and Baird Parker agar were obtained from Difco Laboratories (Detroit, Mich.). Trypticase soy agar (TSA) was prepared by adding Bacto agar (Difco) to a concentration of 1.5% (wt/vol) to TSB. A medium's a_w was measured in duplicate at room temperature with an Aqua Lab CX-2 water activity meter (Decagon, Pullman, Wash.) calibrated against a series of saturated salt solutions according to the manufacturer's instructions.

Growth experiments. Cultures were prepared in 96-well microtiter plates by adding 2.5 μl of a stationary-phase DFM preculture to each well. The final volume of DFM medium in each well was 250 μl. Compatible solutes (carnitine, proline, and glycine betaine) were added to wells for a final concentration of 5 mM. Humectants (sodium chloride, sucrose, and glycerol) were added to wells at various concentrations for final a_w values of 0.95, 0.92, and 0.89. The final a_w value for wells containing no added humectant was 1.00. The plates were covered with a SealPlate sealing film (Sigma Chemical Company, St. Louis, Mo.) to minimize evaporation and were incubated without shaking at 20, 30, 37, 42, and 46°C for 150 h. Growth was monitored by scanning the plates at 630 nm with a Microplate Autoreader spectrophotometer (Biotek Instruments, Winooski, Vt.) at 1- to 3-h intervals. Triplicate cultures were used for all growth conditions.

The minimal a_w for growth was determined by inoculating 10 ml of TSB containing sodium chloride, sucrose, and glycerol at a_w values of 0.89, 0.88, 0.87, and 0.86 with 50 μl of a TSB preculture and incubating at 37°C at 175 rpm for 360 h. Growth was monitored turbidometrically at 650 nm with a Bausch & Lomb Spectronic 21 spectrophotometer (Milton Roy Company, Rochester, N.Y.). In order to verify that the observed growth was that of *S. aureus,* samples were taken after the incubation period, serially diluted in 0.1% peptone, and plated in duplicate on Baird Parker agar.

To determine the effect of the preculture growth phase, two cultures were grown in TSB at 37°C at 175 rpm until the late exponential phase (4.5 h; optical density at 650 nm [OD_{650}] = 1.35), at which time the translation-blocking agent chloramphenicol was added to one of the precultures to a concentration of 50 μg/ml (approximately six times the MIC for this strain as determined in the present study). The precultures were then incubated for another 12 h, and 50 μl of each preculture was transferred to 10 ml of TSB or TSB containing sodium chloride, sucrose, or glycerol at a_w values of 0.92 and 0.89. The subcultures were incubated at 37°C at 175 rpm for approximately 120 h. Growth was monitored turbidometrically as described above.

Uptake of compatible solutes. Since *S. aureus* is auxotrophic for proline *(13),* it was necessary to include this amino acid in all growth media. A concentration of 50 μM has been found to be sufficient to satisfy auxotrophic requirements while being low enough as to not permit significant accumulation of free intracellular proline as a compatible solute *(40).* Precultures were grown at 37°C at 175 rpm to the early stationary phase in DFM containing 1 mM proline. Fifty microliters of the preculture was transferred to 5 ml of DFM containing 50 μM proline, humectant (glycerol, sodium chloride, or sucrose at an a_w of 0.95), or no humectant (a_w = 1.00), and 1.0 mM L-[2,3-^{3}H]proline (33 cpm/nmol) or 1.0 mM [*methyl*-^{14}C]glycine betaine (107 cpm/nmol). Cultures were grown further at 37°C at 175 rpm. The OD of the cultures was monitored turbidometrically as described above, and viable cell numbers were estimated from standard curves generated by plating duplicate nonradiolabeled samples onto TSA from DFM cultures containing the appropriate amounts of humectants and compatible solutes. Samples (250 μl) were taken during exponential growth and filtered through a Millipore GS filter (pore size, 0.22 μm) that had been prewetted with water or the appropriate humectant at a_w 0.95. The filters were washed twice with 1.0 ml of water or humectant and then placed into a scintillation vial containing 2.0 ml of ethylene glycol monomethyl ether. Ten milliliters of Ecoscint H scintillation fluid (National Diagnostics, Atlanta, Ga.) was added, and the samples were counted in a Beckman LS1701 scintillation counter (Beckman Instruments, Palo Alto, Calif.) for 10 min.

For the determination of ^{3}H-proline that had been incorporated into cellular protein, 500-μl culture samples were treated with trichloroacetic acid as described by Daniels et al. *(11),* except that the final precipitate was collected by centrifugation in a Beckman microfuge rather than by filtration. Radiolabeled pellets were resolubilized in 1.5 ml of 0.5 M Tris free base containing 1% (wt/vol) Triton X-100. Aliquots of these samples were then added to scintillation vials containing 10 ml of Ecoscint H scintillation fluid and counted for 20 min. Trichloroacetic-acid-precipitable ^{3}H counts were subtracted from total ^{3}H-proline counts.

Thin-layer chromatography. Cells from 10-ml DFM cultures grown in the presence of ^{3}H-proline or ^{14}C-glycine betaine and in the presence of sodium chloride, glycerol, or sucrose (final a_w value, 0.95) were collected by filtration through Millipore GS filters (pore size, 0.22 μm). The filters were washed twice with 1.0 ml of the appropriate humectant at an a_w of 0.95. The filtered cells were then extracted with approximately 3 ml of 70% (vol/vol) ethanol. The extracts were concentrated under nitrogen to a volume of approximately 100 μl. Five-microliter samples of extracts were applied to silica gel 60 plates (E. Merck, Darmstadt, Germany) and chromatographed in 70% (vol/vol) ethanol. Lanes were cut into 5-mm pieces that were placed into scintillation vials containing 10 ml of Ecoscint H scintillation fluid and counted for 10 min. Relative mobilities (R_f) of radiolabeled spots were compared with ^{3}H-proline (R_f = 0.47) and ^{14}C-glycine betaine (R_f = 0.32) standards.

NMR analysis. A 1,000-ml DFM culture containing NaCl at an a_w of 0.92, 5 mM carnitine, and 20 μM proline was grown to the early stationary phase at 30°C, harvested by centrifugation at 9,000 × *g,* and extracted with 70% ethanol as described previously *(29).* The cell density of the culture was determined by plating in duplicate on TSA as well as by a total cellular protein assay following the method of Lowry et al. *(26)* as modified by

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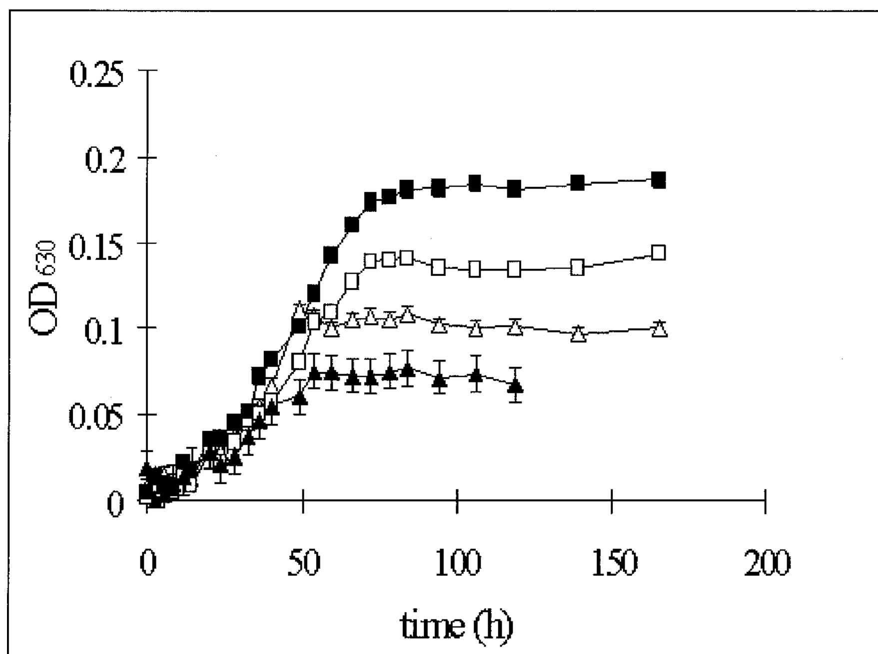

FIGURE 1. *The growth of* S. aureus *in DFM containing sucrose at an* a_w *of 0.95. Cultures were grown in microtiter plates at 30°C without shaking and contained (■) betaine, (□) proline, or (△) carnitine at a concentration of 5 mM. Growth was also tested in the presence of no added compatible solutes (▲). The proline concentration of the basal medium was 50 μM. The error bars represent the standard error for triplicate experiments.*

Markwell et al. *(27).* The ethanol extract was dried under nitrogen at 25°C, and the dry residue was suspended in nuclear magnetic resonance (NMR) buffer *(29).* ^{1}H NMR spectra were obtained as previously described *(29),* and peak integrals were used to quantify the carnitine concentration by comparison with integrals obtained using a standard solution of 100 mM. Peak identities were confirmed by spiking samples with a carnitine standard solution and obtaining a second spectrum.

Statistical analysis. Growth data were analyzed by a one-way analysis of variance with Minitab version 11.1 for Windows (Minitab Inc., State College, Pa.). Results were considered statistically significant when $P < 0.05$.

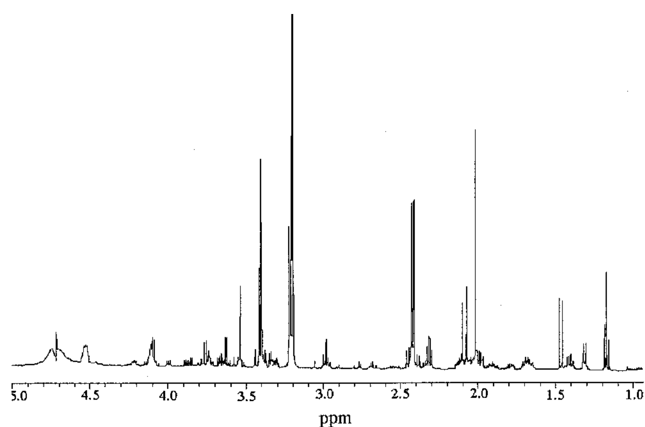

FIGURE 2. *^{1}H NMR spectrum of an* S. aureus *cell extract derived from cells grown in DFM containing NaCl at an* a_w *of 0.92 and 5 mM carnitine. Cells were extracted with 70% ethanol as described in the text. Comparison with a 100 mM standard solution of carnitine revealed that the peaks at approximately 2.4, 4.5, 3.4, and 3.2 ppm belonged, respectively, to the α, β, γ, and methyl protons of carnitine. Peak integrals indicated that carnitine was present at an intracellular concentration of 670 mM, assuming an average cell radius of 0.5 μm and a spherical cell shape (giving a cell volume of* 5×10^{-13} *ml).*

RESULTS AND DISCUSSION

Carnitine is a compatible solute for *S. aureus.* The growth experiments reported herein reveal that *S. aureus* is capable of using carnitine as a compatible solute. Typical results are shown in Figure 1, which shows that the addition of carnitine to a low-a_w medium containing sucrose as the humectant allowed cells to grow to a higher overall yield than was obtained in the absence of compatible solutes. This stimulation of growth rate and yield by carnitine was also evident, albeit to a lesser extent, in cultures grown in the presence of NaCl as the humectant. NMR analysis revealed that carnitine did indeed accumulate to high intracellular levels under these growth conditions (Fig. 2). An example of the effect of carnitine addition on the growth rate of *S. aureus* in a variety of media is presented in Table 1. Data for other growth parameters and growth at other

TABLE 1. *Effects of compatible solutes on growth rate*[a] *of* S. aureus *at 42°C in media with* a_w *adjusted to 0.92*[b]

Compatible solute	Value for humectant		
	Sucrose	NaCl	Glycerol
Carnitine	0.04	0.23	0.18
Proline	0.06 ± 0.02	0.25 ± 0.03	0.19
Betaine	0.04 ± 0.01	0.30	0.18
None	NG[c]	0.13 ± 0.04	0.18

[a] Maximum growth rate (μ_{max}, h^{-1}) was calculated from the natural logarithm (ln_e) of the observed OD_{630} according to $d(ln_e\ OD_{630})/dt = \mu$. The time intervals ($dt$) were 1 to 3 h (ca. 1 h at exponential growth of most cultures). The largest value of μ obtained for each culture was taken as μ_{max}.

[b] Each value presented represents the average of at least triplicate measurements. Except where otherwise indicated, standard deviations were within 10% of the determined value.

[c] NG, no growth.

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temperatures and at other a_w values gave similar results (not shown). Under many of the conditions tested, the addition of carnitine gave rise to a slower growth rate, a lower maximal cell density, and a longer lag time than did the addition of the other two compatible solutes (proline and betaine). However, statistical analysis (as described in "Materials and Methods") of these results revealed that the difference was not significant ($P > 0.05$) except when sodium chloride was the humectant. In this case, growth rates were found to be significantly ($P < 0.05$) slower in the presence of carnitine than in the presence of betaine; however, growth rates were significantly higher than those obtained in the absence of compatible solutes.

These results reveal that *S. aureus* is capable of using carnitine as a compatible solute. Although carnitine has been shown to act as a compatible solute in several gram-positive bacteria, such as *Listeria monocytogenes (39, 45), Lactobacillus plantarum (20),* and *Bacillus subtilis (18),* to our knowledge the present study is the first to show that carnitine has this function in *S. aureus.* Furthermore, our results demonstrate that, as is the case for other gram-positive bacteria *(18, 45),* carnitine per se is accumulated in *S. aureus* and is not converted to γ-butyrobetaine as it is in the *Enterobacteriaceae (21).* It is noteworthy that although carnitine demonstrably acts as a compatible solute (i.e., leads to increased growth rates and yields and shorter lag times) when the a_w of the growth medium is lowered with sucrose, this effect is less clear when NaCl is the humectant, even though this compound is accumulated to high levels by cells grown in the presence of NaCl.

Because carnitine is accumulated as a compatible solute by *S. aureus* in low-a_w broth, it is possible that carnitine may be important for the growth of *S. aureus* in low-a_w foods. Indeed, this compound is widely distributed in biological materials, with mammalian skeletal muscle being a particularly rich source *(12).* Relevant to this role for carnitine, Tombras Smith *(39)* found that *L. monocytogenes* is capable of accumulating carnitine when grown on processed meats.

***S. aureus* displays specific solute effects.** As has been pointed out by Chirife and coworkers *(3, 9),* the identity of the humectant when present in high concentrations influences several key physical characteristics of the growth medium, such as viscosity, oxygen solubility, and the dielectric constant. In addition, humectants differ in terms of permeation across the cell membrane and in terms of compatibility with cellular metabolism when present in the cytoplasm. It seems, therefore, that specific solute effects would not be entirely unexpected, particularly for such a highly osmotolerant organism as *S. aureus,* which is capable of growing in the presence of humectant concentrations on the molar level. Nevertheless, ever since Scott's *(35)* pioneering work in the early 1950s, it has been a prevailing opinion in the literature that, unlike the case for many other bacterial species, humectant identity is of marginal importance in the growth response of *S. aureus* to low a_w. However, a few studies that appear to support the hypothesis that specific solute effects can be observed in *S. aureus* have been carried out *(24, 28, 42, 43),* as discussed in the introduction.

TABLE 2. *Effects of humectant identity on growth rate*[a] *of* S. aureus *in DFM with glycine betaine at 5 mM and with a_w adjusted to 0.95*[b]

Growth temperature (°C)	Value for humectant		
	Sucrose	NaCl	Glycerol
20	0.11	0.14	0.27
30	0.14	0.35	0.28
37	0.32	0.31	0.31
42	0.30	0.29	0.41
46	0.29	0.10	0.14

[a] Determined as described in footnotes to Table 1.

[b] Each value presented represents the average of at least triplicate measurements. Standard deviations were within 10% of the determined value.

Although, as shown in Table 2, growth rates at most growth temperatures were generally similar among cultures grown in the presence of the different humectants, several of our observations, discussed below, hint at fundamental differences in growth responses for different humectants. First, when growth rates were analyzed by one-way analysis of variance, significant differences were found in growth rates ($P < 0.05$), with glycerol giving rise to the highest growth rates. Although significant, the difference in growth rates between sodium chloride- and glycerol-grown cultures was slight, except at extreme temperatures. In Table 2, a subset of results (for cultures grown at an a_w of 0.95 with 5 mM betaine) is presented. Cultures grown at other a_w values and in the presence of other compatible solutes displayed similar trends. Lag times were not significantly affected by humectant identity, and maximal densities were not compared between humectants, since it was found that the relationship between OD and cell density varied with humectant (Fig. 3). As expected, growth rate, maximal density, and lag time all varied significantly with both a_w and temperature (data not shown).

Growth in the presence of high levels of glycerol does not induce compatible solute accumulation. A second observation supporting our hypothesis that specific humectant effects can indeed be observed for *S. aureus* is that, unlike the case in which the impermeant humectants (NaCl and sucrose) were present, when the permeant humectant glycerol was present in the growth medium, no significant differences in growth rate could be observed in cultures with compatible solutes and those without compatible solutes (Table 1, Fig. 4, and data not shown). The same held true for growth yield and lag time (data not shown). To investigate whether compatible solutes were in fact accumulated when glycerol was present as the humectant at high levels, intracellular levels of proline and betaine were measured as described in "Materials and Methods." As expected, proline and betaine were found to accumulate to high intracellular levels (Table 3) in cells grown in media containing NaCl or sucrose as the humectant at an a_w of

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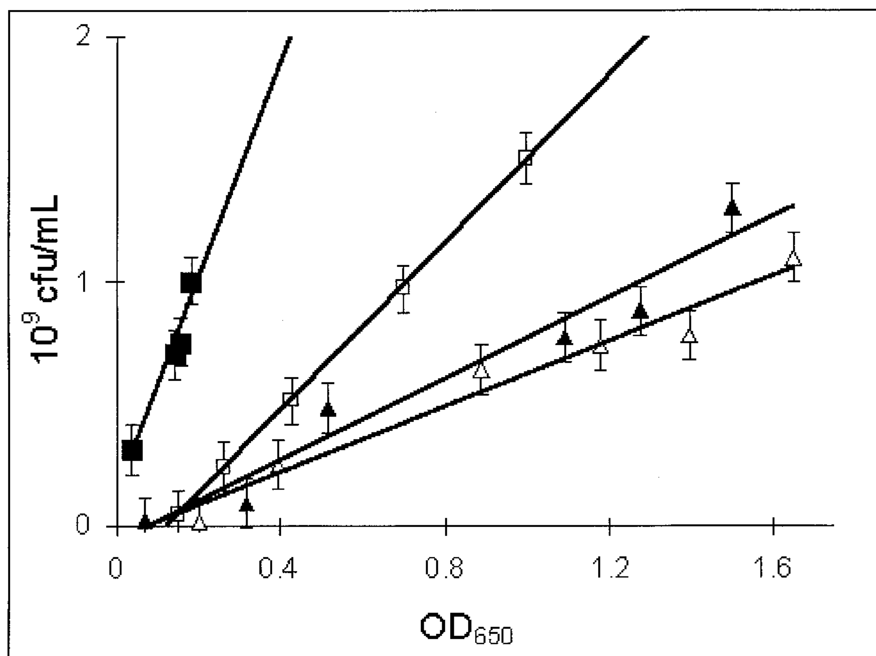

FIGURE 3. *The relationship between cell density and OD_{650} for* S. aureus *cultures in DFM containing various humectants. Samples from cultures containing (■) sucrose, (□) glycerol, and (△) NaCl (each at an a_w of 0.95) and (▲) no humectant were taken at the indicated OD_{650} values, plated in duplicate on TSA, and incubated at 37°C for 48 h. The error bars represent the standard error for duplicate experiments.*

0.95. However, when glycerol was used as the humectant (also at an a_w of 0.95), proline and betaine accumulation by cells was significantly reduced ($P < 0.05$). Indeed, intracellular proline levels of cultures grown in the presence of high glycerol were not significantly higher than levels detected for cells grown at high a_w values ($a_w = 1.00$, DFM medium with no added humectants). These results are consistent with the growth studies described above and indicate that proline and betaine are not used as compatible solutes when *S. aureus* cultures are grown in the presence of high concentrations of glycerol.

It would be expected that glycerol, which is highly permeant toward bacterial membranes *(1, 10),* would rapidly equilibrate across the cell membrane and therefore be present intracellularly at a high concentration when used as a humectant. Interestingly, high intracellular concentrations of glycerol have been observed in a variety of halotolerant eukaryotes, which do in fact accumulate glycerol as a compatible solute against a steep concentration gradient (reviewed in Brown et al. *(7)* and Nevoigt and Stahl *(30)*). It thus seems reasonable to assume that glycerol at high levels is compatible with cellular metabolism. This leads us to propose that glycerol can act as a compatible solute for *S. aureus,* specifically under conditions where it is present in the environment at high concentrations. Under these conditions, there would be no need for the cells to accumulate additional compatible solutes. It is worth noting that Kets et al. *(19),* who carried out an exhaustive high-performance liquid chromatography analysis of osmotically shocked *Pseudomonas putida* cells, were unable to find any com-

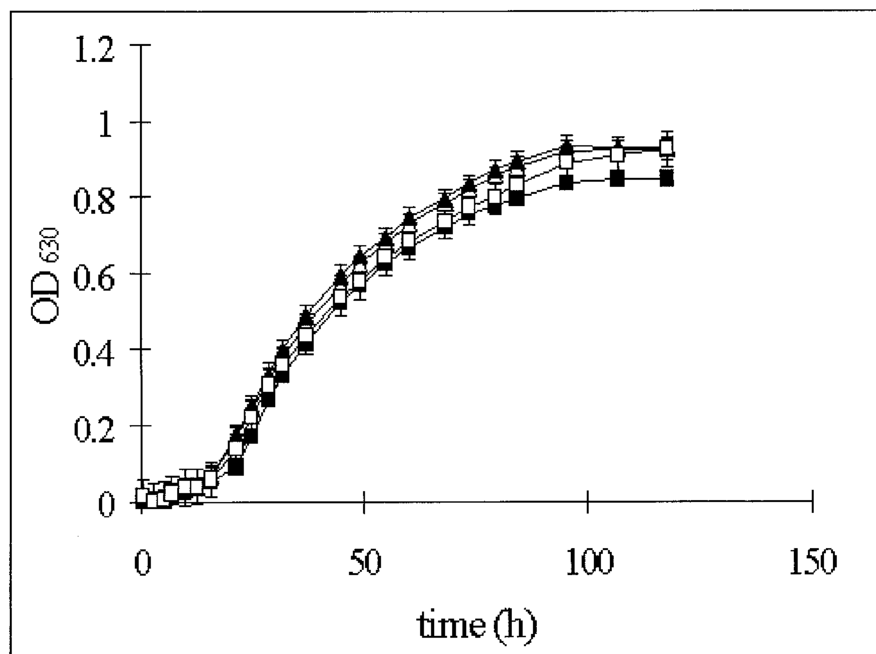

FIGURE 4. *The growth of* S. aureus *in DFM containing glycerol at an a_w of 0.92. Cultures were grown in microtiter plates at 30°C without shaking and contained (■) betaine, (□) proline, or (△) carnitine at a concentration of 5 mM. Growth was also tested in the presence of no added compatible solutes (▲). The proline concentration of the basal medium was 50 μM. The error bars represent the standard error for triplicate experiments.*

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TABLE 3. *Intracellular levels of compatible solutes in* S. aureus *cultures*[a]

Growth medium humectant	Level for compatible solute (mM)	
	Proline	Betaine
No humectant	206 (±19)	171 (±21)
Glycerol	200 (±28)	264 (±34)
NaCl	904 (±21)	1,106 (±33)
Sucrose	612 (±61)	1,061 (±50)

[a] Cultures were grown in DFM containing various humectants at a_w 0.95 or in DFM with no added humectant (a_w = 1.00). The media contained either ^{3}H-proline (33 cpm/nmol) or ^{14}C-betaine (107 cpm/nmol) at a final concentration of 5 mM. Cells were harvested during exponential growth, and levels of accumulated radiolabeled solute were determined by liquid scintillation counting as described in "Materials and Methods." Each value represents the average of quadruplicate measurements. Values are expressed as intracellular concentrations (±SE), which were calculated from turbidometric data at 650 nm as shown in Figure 3 assuming an average cell radius of 0.5 μm and a spherical cell shape (for a cell volume of 5 × 10^{-13} ml).

patible solutes accumulated within cells when glycerol was used to lower the a_w of the growth medium.

Minimal a_w values for growth further support specific solute effects. To further explore the possibility that specific humectant effects can be observed for *S. aureus,* we tested whether the minimum inhibitory a_w value for growth depends on the identity of the humectant. Although similar studies had previously been performed by other investigators *(17, 28, 33),* the results of these studies were somewhat contradictory, warranting their repetition. Our study revealed that the minimal a_w for the growth of *S. aureus* ATCC 12600 in the presence of glycerol was 0.88, while growth in the presence of sucrose or NaCl was possible at an a_w value of 0.87. These results are in accord with those of Plitmann et al. *(33)* and Kamman et al. *(17),* but not with those of Marshall et al. *(28),* who reported that *S. aureus* could not grow at a_w values below 0.89 in the presence of glycerol. Considerable osmotic tolerance variation has been demonstrated among different strains of *S. aureus (22, 25),* and therefore this discrepancy may be due to the use of different strains or media. Another possible explanation is that different preculture conditions, which we found to be an important determinant of growth in glycerol (as discussed below), were used.

Stationary-phase protein synthesis in precultures is required for subsequent growth in the presence of high levels of glycerol. During the growth studies reported above, it was observed that the growth phase of the preculture had a substantial effect on the subsequent growth characteristics of cells in media containing glycerol as the humectant. Specifically, cultures derived from mid- to late-exponential-phase inocula displayed significantly longer lag times, lower growth rates, and higher growth-inhibitory a_w values than did those derived from stationary-phase inocula (Table 4). To explore these findings further, the effect of

TABLE 4. *Maximal growth rate, maximal optical density at 630 nm (OD_{max}) and lag time for* S. aureus *cultures in the presence of glycerol at a_w 0.92, as influenced by the growth phase of the preculture*

Preculture growth phase[a]	Compatible solute[b]	μ_{max} (h^{-1})	OD_{max}	Lag time (h)
Mid-exponential	None	0.07	0.08	50
	Proline	0.08	0.07	50
	Carnitine	0.08	0.09	50
	Betaine	0.08	0.09	50
Stationary	None	0.221	1.10	12
	Proline	0.185	1.12	12
	Carnitine	0.193	1.15	12
	Betaine	0.174	1.07	12

[a] Precultures were grown in DFM in sidearm flasks in a shaker bath at 37°C at 150 rpm to the mid-exponential phase (8 h, 2.1 × 10^7 CFU/ml) or to the stationary phase (32 h, 2.0 × 10^9 CFU/ml), after which 5 μl was transferred to 245 μl of DFM containing glycerol at an a_w of 0.92 with or without added compatible solutes in microtiter wells. The cultures were incubated at 37°C for 86 h. Growth was monitored at 630 nm as described in "Materials and Methods."

[b] The compatible solute concentration was 5 mM. "None" indicates no added compatible solutes. Proline was present at a concentration of 50 μM in the basal medium.

the blocking of protein synthesis during the late exponential phase of the preculture was investigated. Subsequent growth of *S. aureus* occurred in TSB containing glycerol at a_w values of 0.92 and 0.89 when inocula that had not been treated with chloramphenicol were used. However, when inocula were derived from precultures exposed to chloramphenicol, subsequent growth was not detected at these a_w values. This was not the case when sucrose or NaCl was used as the humectant. Growth at a_w values of 0.92 and 0.89 in TSB containing either of these two humectants was observed regardless of whether precultures were treated with chloramphenicol. Thus, it would appear that stationary-phase protein synthesis by precultures is critical for the subsequent growth of *S. aureus* in low-a_w media containing glycerol as the humectant. The identity of the proteins involved has not yet been determined, but studies on humectant-specific protein synthesis have been initiated in our laboratory.

CONCLUSIONS

The results of the present study reveal that *S. aureus* is capable of using carnitine as a compatible solute. Our findings also provide clear evidence for specific solute effects on several growth characteristics of this organism, such as the growth rate and the minimal a_w value permitting growth. While we were able to demonstrate that the compatible solutes proline and betaine were accumulated by cells grown in the presence of NaCl or sucrose as the humectant, accumulation did not occur when cells were grown in the presence of high concentrations of glycerol, suggesting that glycerol itself may serve as a compatible solute for this organism. Finally, our results provide evidence that

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stationary-phase protein synthesis by precultures is required for subsequent growth in media containing high concentrations of glycerol. This latter finding provides a possible explanation for earlier discrepancies in the literature concerning the inhibitory effects of glycerol on *S. aureus*.

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REFERENCES

1. Alemohammad, M. M., and J. C. Knowles. 1974. Osmotically induced volume and turbidity changes of *Escherichia coli* due to salts, sucrose and glycerol, with particular reference to the rapid permeation of glycerol into the cell. J. Gen. Microbiol. 82:125–142.
2. Amin, U. S., T. D. Lash, and B. J. Wilkinson. 1995. Proline betaine is a highly effective osmoprotectant for *Staphylococcus aureus*. Arch. Microbiol. 163:138–142.
3. Ballesteros, S. A., J. Chirife, and J. P. Bozzini. 1993. Specific solute effects on *Staphylococcus aureus* cells subjected to reduced water activity. Int. J. Food Microbiol. 20:51–66.
4. Barg, N. L., and T. Harris. 1997. Toxin-mediated syndromes, p. 527–543. *In* K. B. Crossley and G. L. Archer (ed.), The staphylococci in human disease. Churchill Livingstone, New York.
5. Baross, J. A., and L. M. Lenovich. 1992. Halophilic and osmophilic microorganisms, p. 199–212. *In* C. Vanderzant and D. F. Splittstoesser (ed.), Compendium of methods for the microbiological examination of foods, 3rd ed. American Public Health Association, Washington, D.C.
6. Boyce, J. M. 1997. Epidemiology and prevention of nosocomial infections, p. 309–329. *In* K. B. Crossley and G. L. Archer (ed.), The staphylococci in human disease. Churchill Livingstone, New York.
7. Brown, A. D., K. F. Mackenzie, and K. K. Singh. 1986. Selected aspects of microbial osmoregulation. FEMS Microbiol. Rev. 39:31–36.
8. Chesney, P. J. 1997. Toxic shock syndrome, p. 509–525. *In* K. B. Crossley and G. L. Archer (ed.), The staphylococci in human disease. Churchill Livingstone, New York.
9. Chirife, J. 1994. Specific solute effects with special reference to *Staphylococcus aureus*. J. Food Eng. 22:409–419.
10. Christian, J. H. B. 1981. Specific solute effects on microbial water relations, p. 825–854. *In* L. B. Rockland and G. F. Stewart (ed.), Water activity: influences on food quality. Academic Press, New York.
11. Daniels, L., R. S. Hanson, and J. A. Phillips. 1994. Chemical analysis, p. 512–554. *In* P. Gerhardt, R. G. A. Murray, W. A. Wood, and N. R. Krieg (ed.), Methods for general and molecular bacteriology. ASM Press, Washington, D.C.
12. Fraenkel, G. 1954. The distribution of vitamin BT (carnitine) throughout the animal kingdom. Arch. Biochem. Biophys. 50:486–495.
13. Gladstone, G. P. 1937. The nutrition of *Staphylococcus aureus*: nitrogen requirements. Br. J. Exp. Pathol. 19:208–226.
14. Graham, J. E., and B. J. Wilkinson. 1992. *Staphylococcus aureus* osmoregulation: roles for choline, glycine betaine, proline, and taurine. J. Bacteriol. 174:2711–2716.
15. Hucker, G. J., and W. C. Haynes. 1937. Certain factors affecting the growth of food poisoning micrococci. Am. J. Public Health 27:590–594.
16. Ing, M. B., L. M. Baddour, and A. S. Bayer. 1997. Bacteremia and infective endocarditis: pathogenesis, diagnosis, and complications, p. 331–354. *In* K. B. Crossley and G. L. Archer (ed.), The staphylococci in human disease. Churchill Livingstone, New York.
17. Kamman, J. F., S. R. Tatini, and T. P. Labuza. 1978. Effect of water activity on nuclease production by *Staphylococcus aureus*. J. Food Sci. 43:1284–1286, 1292.
18. Kappes, R. M., and E. Bremer. 1998. Response of *Bacillus subtilis* to high osmolarity: uptake of carnitine, crotonobetaine and γ-butyrobetaine via the ABC transport system OpuC. Microbiology 144:83–90.
19. Kets, E. P. W., J. A. M. de Bont, and H. J. Heipieper. 1996. Physiological response of *Pseudomonas putida* S12 subjected to reduced water activity. FEMS Microbiol. Lett. 139:133–137.
20. Kets, E. P. W., E. A. Galinski, and J. A. M. de Bont. 1994. Carnitine: a novel compatible solute in *Lactobacillus plantarum*. Arch. Microbiol. 162:243–248.
21. Kleber, H. P. 1997. Bacterial carnitine metabolism. FEMS Microbiol. Lett. 147:1–9.
22. Kunin, C. M., and J. Rudy. 1991. Effect of NaCl-induced osmotic stress on intracellular concentrations of glycine betaine and potassium in *Escherichia coli, Enterococcus faecalis,* and staphylococci. J. Lab. Clin. Med. 118:217–224.
23. Labuza, T. P., S. Cassil, and A. J. Sinskey. 1972. Stability of intermediate moisture foods. 2. Microbiology. J. Food Sci. 37:160–162.
24. Li, K.-Y., and J. A. Torres. 1993. Water activity relationships for selected mesophiles and psychrotrophs at refrigeration temperature. J. Food Prot. 56:612–615.
25. Louch, H. A., M. L. Eck, and K. J. Miller. 1997. Osmoadaptation by *Staphylococcus aureus*: analysis of several strains linked to food poisoning outbreaks. J. Food Prot. 60:139–143.
26. Lowry, O. H., N. J. Rosebrogh, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193:265–275.
27. Markwell, M. A., S. M. Haas, L. L. Bieber, and N. E. Tolbert. 1978. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Anal. Biochem. 87:206–210.
28. Marshall, B. J., D. F. Ohye, and J. H. B. Christian. 1971. Tolerance of bacteria to high concentrations of NaCl and glycerol in the growth medium. Appl. Microbiol. 21:363–364.
29. Miller, K. J., S. C. Zelt, and J.-H. Bae. 1991. Glycine betaine and proline are the principal compatible solutes of *Staphylococcus aureus*. Curr. Microbiol. 23:131–137.
30. Nevoigt, E., and U. Stahl. 1997. Osmoregulation and glycerol metabolism in the yeast *Saccharomyces cerevisiae*. FEMS Microbiol. Rev. 21:231–241.
31. Noble, W. C. 1997. Staphylococcal carriage and skin and soft tissue infection, p. 401–412. *In* K. B. Crossley and G. L. Archer (ed.), The staphylococci in human disease. Churchill Livingstone, New York.
32. Pattee, P. A., and D. S. Neveln. 1975. Transformation analysis of three linkage groups in *Staphylococcus aureus*. J. Bacteriol. 124:201–211.
33. Plitman, M., Y. Park, R. Gomez, and A. J. Sinskey. 1973. Viability of *Staphylococcus aureus* in the intermediate moisture range. J. Food Sci. 38:1004–1008.
34. Rao, D. N. 1997. Intermediate moisture foods based on meats—a review. Food Rev. Int. 13:519–551.
35. Scott, W. J. 1953. Water relations of *Staphylococcus aureus* at 30°C. Aust. J. Biol. Sci. 6:549–564.
36. Sheagren, J. N. 1998. Staphylococcal infections, p. 1546–1553. *In* J. H. Stein (ed.), Internal medicine, 5th ed. Mosby, St. Louis, Mo.
37. Sperber, W. H. 1983. Influence of water activity on foodborne bacteria—a review. J. Food Prot. 46:142–150.
38. Tatini, S. R. 1973. Influence of food environments on growth of *Staphylococcus aureus* and production of various enterotoxins. J. Milk Food Technol. 36:559–563.
39. Tombras Smith, L. 1996. Role of osmolytes in adaptation of osmotically stressed and chill-stressed *Listeria monocytogenes* grown in liquid media and on processed meat surfaces. Appl. Environ. Microbiol. 62:3088–3093.
40. Townsend, D. E., and B. J. Wilkinson. 1992. Proline transport in *Staphylococcus aureus*: a high-affinity system and a low-affinity system involved in osmoregulation. J. Bacteriol. 174:2702–2710.
41. Trantner, H. S. 1990. Foodborne staphylococcal illness. Lancet 336:1044–1046.
42. Troller, J. A. 1971. Effect of water activity on enterotoxin B pro-

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duction and growth of *Staphylococcus aureus.* Appl. Microbiol. 21: 435–439.

43. Troller, J. A. 1972. Effect of water activity on enterotoxin A production and growth of *Staphylococcus aureus.* Appl. Microbiol. 24: 440–443.
44. Troller, J. A. 1986. Water relations of foodborne bacterial pathogens—an updated review. J. Food Prot. 49:656–670.
45. Verheul, A., E. Glaasker, B. Poolman, and T. Abee. 1997. Betaine and L-carnitine transport by *Listeria monocytogenes* Scott A in response to osmotic signals. J. Bacteriol. 179:6979–6985.

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