Research Note

Effects of Growth at Low Water Activity on the Thermal Tolerance of *Staphylococcus aureus*

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ABSTRACT

*Staphylococcus aureus* is the most osmotolerant foodborne pathogen, and outbreaks of staphylococcal food poisoning are often associated with foods containing reduced water activity. To determine the effect of growth medium water activity, precultures of *S. aureus* were grown in medium containing no added solute, a_w 0.997) or TSB medium adjusted to a final a_w value of 0.97 (using 5% NaCl) or 0.94 (using

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

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, although a few other compounds, such as proline betaine, taurine, and carnitine, have been reported to act as compatible solutes in this organism. In addition to compatible solute accumulation, the synthesis of cellular proteins is also altered when cells encounter a decrease in growth medium a_w. Although the rate of synthesis of most cellular proteins is decreased under these conditions, the rate of synthesis of certain cellular proteins, which may represent osmotic stress proteins or general stress proteins, increases in response to a decrease in growth medium a_w. Curiously, despite the great importance of the stress tolerance response of *S. aureus*, the identity of only a few potential stress proteins of *S. aureus* has been established.

In the present study, we have examined the effects of growth at low a_w on the thermal tolerance of *S. aureus*. Our results demonstrate that cells grown at low a_w have a markedly greater thermal tolerance than cells simply heated in a low a_w menstruum. Furthermore, our study reveals that the identity of the accumulated compatible solute within cells grown at low a_w can strongly influence the development of thermal tolerance in this bacterium. We also show that the development of enhanced thermal tolerance at low a_w is dependent upon protein synthesis, suggesting the involvement of osmotic stress and/or general stress proteins.

MATERIALS AND METHODS

Strains, media, and culture conditions. The *S. aureus* type strain, ATCC 12600, was used in the present study. Trypticase soy broth (TSB) was obtained from Difco Laboratories (Detroit, Mich.). Trypticase soy agar was prepared by adding Bacto Agar (Difco) to a concentration of 1.5% (wt/vol) to TSB. The defined medium (DFM) was that of Pattee and Neveln (27) with the following modifications: purines, pyrimidines, and agar were omitted, and the basal proline concentration used was 10 μM. Water activity of media was measured in duplicate at room temperature in an Aqua Lab CX-1 water activity meter (Decagon, Pullman, Wash.).

Precultures were prepared by inoculating 6 ml of liquid TSB medium with an isolated colony from a trypticase soy agar plate. Precultures were incubated at 37°C in a water bath shaker for 18 to 24 h, or until an optical density at 650 nm of 1.4 to 1.6 had been obtained. One milliliter of preculture was then used to inoculate flasks containing 50 ml of a high a_w medium (TSB medium containing no added solute, a_w 0.997) or TSB medium adjusted to a final a_w value of 0.97 (using 5% NaCl) or 0.94 (using

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10% NaCl or 15% KCl). The inoculated flasks were incubated at 37°C in a water bath shaker until the midexponential growth phase, at which time thermal tolerance was assessed as described below.

**Assessment of thermal tolerance.** The thermal tolerance of *S. aureus* cell suspensions (1 ml) was assessed at 60°C within 1.5-ml capacity microfuge tubes that were fully submerged in a Lauda MS circulating water bath (Brinkmann Instruments, Westbury, N.Y.). In some experiments, 1-ml aliquots of culture were directly transferred to microfuge tubes and heated. In other experiments, 50-μl aliquots of culture grown in TSB medium were transferred to 950 μl of TSB medium containing added solute (NaCl or KCl) immediately prior to heating. In other experiments, cells grown at aw 0.94 were centrifuged and resuspended in high aw TSB medium (containing no added solutes) prior to heating. Cell suspensions were heated for various times (typically between 0 and 25 min).

Water bath temperature was monitored with type T thermocouples attached to a digital thermometer (Omega Engineering, Stamford, Conn.). One thermocouple probe was placed in the water bath, and a second probe was submerged within a microfuge tube containing 1 ml of medium. Heating time for each sample was calculated from the time that the sample was placed into the water bath until the time the sample was removed from the water bath. The come-up time was consistently 5 min and was determined in each experiment using the thermocouple probe. Upon removal from the water bath, the samples were rapidly cooled to room temperature on ice. After cooling, aliquots were removed from the tubes, diluted in 0.1% peptone diluent (Difco), and spread plated onto TSA medium. The plates were incubated at 37°C for 24 to 48 h. Following incubation, CFU were determined and CFU/ml of sample were calculated. Average CFU/ml values or % survivors were then plotted versus time for each evaluation conducted.

Experiments were generally performed a minimum of three times. In a typical experiment, one flask was used for each treatment. Duplicate samples were removed from each flask for each time point, and a minimum of two spread plates were used for each sample dilution examined. Results from representative experiments are shown in Figures 1 through 4.

**Role of protein synthesis.** In additional experiments, the role of protein synthesis during the development of thermal tolerance was examined by adding chloramphenicol to cultures. In a typical experiment, a 50-ml culture of *S. aureus* was grown in TSB medium to the midexponential phase of growth. The culture was centrifuged and resuspended in 50 ml of TSB medium containing 10% NaCl. Equal volumes (25 ml each) of the resuspended cells were placed into sterile flasks. Chloramphenicol (50 μg/ml) was added to one flask, and both flasks were incubated at 37°C in a water bath shaker at 175 rpm. One-milliliter samples were removed periodically from the cultures (e.g., between 0 and 200 min), placed directly into microfuge tubes, and assessed for thermal tolerance at 60°C as described above.

**RESULTS AND DISCUSSION**

**Thermal tolerance is enhanced when cells are grown at low aw.** The thermal tolerance of *S. aureus* ATCC 12600 was found to be influenced by the aw of the heating menstrum. As shown in Figure 1, cells grown in TSB medium and subsequently heated in TSB medium containing either 10% NaCl or 15% KCl had a greater thermal tolerance than cells heated in TSB medium containing no added solutes. These results are consistent with those reported previously by other investigators who have demonstrated that the aw of the heating menstrum can have an important influence on the thermal tolerance of bacterial cells (7, 9, 16, 32–34, 38).

Based on our finding that the aw of the heating menstrum can have such a dramatic effect on the overall thermal tolerance of cells, it is surprising that there has been very little prior study of this phenomenon. Two prior studies that examined the effect of growth at low aw on the development of thermal tolerance in *S. aureus* appear to give somewhat contradictory results. While Hurst and Hughes (17) reported results similar to those found in the present study, our results would appear to contradict those of Calhoun and Frazier (6), who reported that prior growth at low aw has
FIGURE 2. Survival of S. aureus ATCC 12600 heated at 60°C following growth in TSB or TSB medium containing various solutes. S. aureus was grown to the midexponential growth phase in TSB medium (○), TSB containing 5% NaCl (■), TSB containing 10% NaCl (□), or TSB containing 15% KCl (▲). One-milliliter samples were placed directly into microfuge tubes and heated at 60°C as indicated.

only a relatively small effect on the heat resistance of S. aureus. It should be noted, however, that Calhoun and Frazer only examined cultures from the late stationary phase in contrast to our study that utilized cultures in the mid-logarithmic growth phase. This difference in culture age could offer an explanation for the differences between the two studies because cultures in the stationary phase have been reported to have generally a higher thermal tolerance than cultures in the logarithmic growth phase (14, 24, 25, 29).

Based on the results of the present study, we propose that physiological changes that occur during the growth of S. aureus at low aw values confer enhanced thermal tolerance to the cell. In order to explore further the types of physiological changes that occur within cells grown at low aw and that may contribute to enhanced thermal tolerance, two experimental approaches were pursued. First, the possible role of the accumulated compatible solute was examined using cells grown in defined media. Second, the possible role of osmotic stress and/or general stress proteins was explored through the use of the protein synthesis inhibitor chloramphenicol.

The identity of the accumulated compatible solute within S. aureus can strongly influence the development of thermal tolerance. Because complex growth media, such as TSB, contain a variety of compatible solutes that may be accumulated by S. aureus during growth at low aw, it was necessary to use a defined medium (DFM) for these experiments. As shown in Table 1, the addition of glycine betaine to the defined medium containing 10% NaCl resulted in a dramatic decrease in the thermal tolerance of S. aureus. Indeed, the thermal tolerance of cultures grown in the presence of glycine betaine was reduced approximately 100-fold when compared to cultures grown in the same medium containing proline or no added compatible solute. Interestingly, Fletcher and Csonka (8) reported a similar finding for the nonosmotolerant bacterium Salmonella Typhimurium.

The data described above provide evidence that the identity of the accumulated compatible solute can strongly influence the thermal tolerance of S. aureus. Furthermore, it seems likely that such physiological changes may be of general significance for other bacterial species. For example, recent studies have documented a greater thermal tolerance of S. enterica serovar Typhimurium (15) and Escherichia coli (27, 28, 29) with decreased osmotic stress.

**TABLE 1. Effect of compatible solute identity on the development of thermal tolerance of S. aureus ATCC 12600 in DFM medium containing 10% NaCl.

<table>
<thead>
<tr>
<th>Compatible solute</th>
<th>% survival</th>
</tr>
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<tbody>
<tr>
<td>None</td>
<td>12.2 ± 3.5</td>
</tr>
<tr>
<td>Proline</td>
<td>11.9 ± 3.4</td>
</tr>
<tr>
<td>Glycine betaine</td>
<td>0.2 ± 0.1</td>
</tr>
</tbody>
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*S. aureus* was grown to the midexponential phase of growth in DFM medium containing 10% NaCl and the indicated compatible solute (none, proline, or glycine betaine) at a final concentration of 5 mM. One-milliliter samples were added directly to microfuge tubes and heated at 60°C for a total of 20 min. The percent survival represents the average and standard deviation of values determined in at least two separate experiments.
influence the development of thermal tolerance of S. aureus cultures. How the identity of the accumulated compatible solute influences the overall thermal tolerance of cells remains unclear and requires further study. However, there is evidence suggesting that the effects of compatible solutes on thermal tolerance may be both direct and indirect. For example, in the present study, we have shown that the enhanced thermal tolerance of cells grown at low $a_w$ is not evident when cells are heated in a high $a_w$ menstrum (Fig. 3). This result would appear to argue for a direct role for compatible solutes because intracellular compatible solutes are rapidly effluxed when cells are transferred from a low $a_w$ medium into a high $a_w$ medium (21, 31). However, it is also possible that compatible solutes may influence the development of thermal tolerance within S. aureus cultures indirectly. For example, Vijaranakul et al. (39, 40) have shown that the addition of glycine betaine to low $a_w$ cultures of S. aureus can influence both peptidoglycan structure and the relative levels of at least one NaCl stress protein. It is therefore possible that these compositional alterations could influence the overall thermal tolerance of this bacterium.

Protein synthesis is required for the development of enhanced thermal tolerance during exposure of cells to low $a_w$. Chloramphenicol was used to assess the role of protein synthesis during the development of thermal tolerance of S. aureus cultures upon exposure to low water activity conditions. As shown in Figure 4, cells incubated in the absence of chloramphenicol at $a_w$ 0.94 developed increasing thermal tolerance over time, whereas those incubated in the presence of chloramphenicol at $a_w$ 0.94 showed no increase in thermal tolerance. This result suggests that the synthesis of general stress and/or osmotic stress proteins during adaptation of S. aureus to low $a_w$ is crucial for the development of enhanced thermal tolerance.

It is well established that organisms synthesize a variety of stress proteins upon exposure to environmental stress and that the synthesis of stress proteins in response to one type of stress may provide cross protection of cells toward a second type of stress (8, 18–20, 23, 30, 43). Thus, it is possible that certain osmotic stress proteins and/or general stress proteins synthesized by S. aureus during growth at low $a_w$ may be, in part, responsible for the enhanced level of thermal tolerance of these cultures. However, it becomes difficult to explain why enhanced thermal tolerance is not evident in cultures grown at low $a_w$ upon heat treatment in a high $a_w$ menstrum (Fig. 3). Further research is clearly warranted because the greatly enhanced thermal tolerance of this foodborne pathogen in low $a_w$ foods has important implications concerning the safety of these foods.

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REFERENCES


