

Death and Injury in *Staphylococcus aureus* 196E: Effect of Growth Temperature

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The incubation temperature at which Staphylococcus aureus 196E was grown affected its degree of thermal injury and death upon subsequent heat treatment. Using a heat treatment of 52°C for 1 h, lethality was low (one log or less) and injury was high (>3 logs) for cells grown at 37 or 42°C under aerobic conditions. However, for S. aureus grown aerobically at 12 or 19°C, lethality was high (>3 logs) but injury was reduced. Shifting cells grown at 12, 19 or 28°C to 37°C for 5 h yielded cells that were less readily killed by the 52°C heat treatment. Growth at 28, 37 or 42°C under anaerobic conditions produced cells that were thermally resistant (approximately one log or less of killing). Cells grown anaerobically at 19°C were more resistant than aerobic cultures. Injury was greater with 19 and 28°C cells grown anaerobically than aerobically; the reverse was true with 37 and 42°C cells. The presence of nalidixic acid or rifamycin during the shift-up period (28°C grown cells shifted to 37°C for 5 h) inhibited the gain in thermotolerance but chloramphenicol was only slightly inhibitory. The data suggest that S. aureus growing in foods stored at lower temperatures should be killed more readily by lower processing temperatures. Since temperature abuse of foods would lead to staphylococci with increased heat resistance, foods should be stored at refrigerated temperatures until they are heat processed.

Introduction

The temperature at which microorganisms are grown can have a profound effect on heat resistance. Microorganisms grown at higher temperatures show greater resistance to heat stress (1-3,9,13,20). Also, when cells are shifted from a lower growth temperature to a higher non-lethal temperature for short periods, these cells demonstrate increased heat resistance (4,5,11,12,18,22). However, there does not appear to be literature available concerning the effect of either growth temperature or temperature shift-ups on heat resistance in *Staphylococcus aureus*. Using *S. aureus* 196E as a representative strain, the effect of growth temperature and the shifting from a low temperature to a higher one on heat resistance of the organism was studied.

Materials and Methods

Microorganism

Staphylococcus aureus 196E was maintained in tryptic soy broth (TSB; Difco) stored at 5°C. A seed culture was prepared by inoculating 50 ml TSB with *S. aureus* and incubating on a rotary shaker (150 rpm) at 28°C for 18-20 h. The 28°C grown cells were used as a source of inoculum for 50 ml TSB in 250 ml flasks. Flasks were incubated by shaking (150 rpm) at temperatures ranging from 42 to 12°C. Stationary phase cells ($1.0-2.5 \times 10^9$ /ml) were harvested by centrifugation, washed twice with sterile distilled water and resuspended in 5 ml sterile distilled water. In some experiments, *S. aureus* was grown at different temperatures under N₂ (<85 ppm O₂). Trypsinizing flasks (250 ml) with side arms fitted with rubber septa and containing 50 ml TSB were inoculated with 28°C grown cells

and aseptically gassed with nitrogen for 15 min. The loose screw cap (to allow venting) was replaced with a sterile rubber bung at the end of the gassing period. The anaerobic flasks were incubated at various temperatures with shaking. Cells from 10 ml of anaerobic growth, at stationary phase, were washed by centrifugation as described above and resuspended in 5 ml of sterile distilled water. For temperature shift studies, *S. aureus* cells grown at 28°C were collected by centrifugation, resuspended in fresh TSB and incubated at 37°C for 5 h before harvesting. In some of the shift-up experiments, chloramphenicol, sodium nalidixate, or rifamycin (Sigma Chemical Co.) were added to the growth flasks just before transferring them to 37°C. The harvesting step for antibiotic-treated cells was similar to that of untreated cells.

To study the effect of chloramphenicol on synthesis of phospho-beta-galactosidase, *S. aureus* was grown in TSB with added glucose (final concentration of glucose was 1.25%, w/v) at 37°C on a shaker (150 rpm). At 18 h, cells were removed from the medium by centrifugation and then added to fresh TSB w/o glucose (Difco), containing 1% galactose (w/v) with and without the addition of 200 µg/ml chloramphenicol; the flasks were then incubated shaken at 37°C for 5 h. The cells were harvested and subjected to the phospho-beta-galactosidase assay outlined by Smith *et al* (16).

Injury procedure

To determine heat injury, 160 ml screw-cap dilution bottles containing 20 ml sterile potassium phosphate buffer (pH 7.2; 0.1 M) were equilibrated to 52°C in a constant temperature circulating water bath. One milliliter of washed *S. aureus* was added to give approximately 1×10^9 cfu/ml buffer. The buffer-cell suspension was agitated (150 agitations/min) using a Burrell Wrist-Action Shaker. At zero time and at 1 h, 0.1 ml of culture was removed from the bottles and added to 9.9 ml sterile 0.1% (w/v) Bacto peptone (Difco) water tubes and

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Table 1 Effect of growth temperature under aerobic conditions on *Staphylococcus aureus* 196E death and injury at 52°C in phosphate buffer

Growth temperature (°C)	N ^a	Log ₁₀ cfu/ml at zero time		Log ₁₀ cfu/ml at 1 h		Log ₁₀ cfu/ml	
		TSAP	TSAS	TSAP	TSAS	Death ^a	Injury ^b
42	12	8.93 (0.12) ^c	8.94 (0.11)	8.39 (0.11)	5.23 (0.33)	0.54 (0.09) ¹	3.14 (0.36) ⁵
37	26	8.98 (0.13)	8.96 (0.13)	8.00 (0.54)	4.55 (0.68)	0.98 (0.52) ³	3.42 (0.86) ⁵
28	21	9.26 (0.11)	9.29 (0.12)	5.28 (0.36)	3.68 (0.12)	3.98 (0.40) ⁶	1.66 (0.57) ¹
28-37 ^d	13	9.30 (0.09)	9.35 (0.09)	8.07 (0.54)	4.05 (0.81)	1.22 (0.59) ³	3.97 (0.74) ⁶
19	19	8.96 (0.15)	8.89 (0.19)	5.96 (0.54)	3.35 (0.14)	3.00 (0.58) ⁴	2.51 (0.51) ⁴
19-37 ^d	8	9.22 (0.12)	9.18 (0.08)	8.33 (0.31)	6.08 (0.51)	0.89 (0.34) ²	2.21 (0.35) ³
12	8	8.95 (0.09)	8.61 (0.14)	5.51 (0.37)	3.32 (0.05)	3.45 (0.38) ⁵	1.85 (0.44) ²
12-37 ^d	8	9.17 (0.07)	9.19 (0.12)	6.42 (0.60)	4.82 (0.24)	2.75 (0.59) ⁴	1.60 (0.46) ¹

^aN = number of replicates.

^bMeans followed by the same superscripted number in a column are not significantly different at $P = 0.05$ (Fisher's LSD Comparison).

^cNumbers in parentheses are standard deviation of means.

^d*S. aureus* was grown at 28, 19, or 12°C and then shifted to 37°C for 5 h

Table 2 Effect of growth temperature under anaerobic conditions on *Staphylococcus aureus* 196E death and injury at 52°C in phosphate buffer

Growth temperature (°C)	N ^a	Log ₁₀ cfu/ml at zero time		Log ₁₀ cfu/ml at 1 h		Log ₁₀ cfu/ml	
		TSAP	TSAS	TSAP	TSAS	Death ^b	Injury ^b
42	10	8.74 (0.08) ^c	8.78 (0.10)	7.98 (0.46)	7.17 (0.30)	0.75 (0.41) ¹	0.90 (0.40) ¹
37	10	9.06 (0.37)	8.83 (0.21)	8.37 (0.56)	6.66 (0.34)	0.69 (0.37) ¹	1.48 (0.44) ¹
28	10	9.11 (0.08)	9.13 (0.10)	8.08 (0.35)	4.88 (0.95)	1.07 (0.37) ¹	3.10 (1.09) ²
19	10	9.08 (0.05)	9.15 (0.11)	6.55 (0.76)	3.32 (0.05)	2.54 (0.75) ²	3.30 (0.78) ²

For explanation of footnotes ^a, ^b, and ^c, see **Table 1**

successive dilutions prepared. Using a spiral plater (Spiral Systems Instruments, Inc., Bethesda, MD), appropriate dilutions were plated onto tryptic soy agar + 1% (w/v) Na pyruvate (TSAP) and TSA + 7% (w/v) added NaCl (TSAS). Plates were incubated at 37°C and counted after 2 days.

Death and injury were monitored by the use of a differential plating technique consisting of TSAP and TSAS. Both non-injured cells and injured-repaired cells form colonies on TSAP whereas only non-injured cells form colonies on TSAS; repair of injured cells does not take place in the presence of sodium chloride (14). Increase in death was defined as bacterial counts on TSAP_{t=0} minus that on TSAP_{t=1}. Increase in injury was defined as counts on TSAS_{t=0} minus that on TSAS_{t=1} minus increase in death.

Statistics

Data were analyzed by one-way analysis of variance using the Ecstatic (Someware in Vermont, Montpelier, VT) and Number Crunching Statistical System (J. L. Hintze, Kaysville, UT) statistical software programs.

Results

When grown under aerobic conditions at temperatures ranging from 12 to 42°C, *S. aureus* 196E showed a decreased heat resistance (increase in death) to 52°C as the temperature at which the cells were grown was decreased (**Table 1**). The effect on heat induced injury was more erratic but, in general, growth temperatures under aerobic conditions did not have as great an effect on injury as on death (**Table 1**). Under anaerobic conditions, the pattern of death induced by the 52°C heat treatment was similar to that of aerobically grown *S. aureus* except that anaerobically grown cells were more heat resistant (**Table 2**). There was also an increase in injury as the anaerobic growth temperature was decreased (**Table 2**).

S. aureus, grown at 12, 19 or 28°C and then shifted up to 37°C for 5 h, showed a significant increase in heat tolerance when the cells were heated at 52°C, i.e., fewer cells were killed by

heat treatment (**Table 1**). The level of injured cells was greater with 28°C grown cells shifted to 37°C than with unshifted 28°C cells. However, this was not true with either 19 or 12°C grown cells—the cells pretreated at 37°C for 5 h demonstrated less injury (**Table 1**).

The presence of antibiotics during the 5 h shift-up period from 28 to 37°C indicated that nalidixic acid and rifamycin inhibited the shift-induced increase in heat tolerance (**Fig. 1**) whereas chloramphenicol had little inhibitory effect. There was approximately 400-fold decrease in the number of surviving *S. aureus* cells when 100 µg/ml nalidixic acid was present during the 5 h shift-up period before heating the cells. Rifamycin at 100 µg/ml gave approximately 60-fold decrease in survivors whereas 200 µg/ml chloramphenicol gave only a six-fold decrease in surviving cells. Since the results obtained with chloramphenicol were surprising, it was tested for its effect on the synthesis of phospho-beta-galactosidase. *S. aureus*, in the presence of galactose, produced the protein phospho-beta-galactosidase (16). Chloramphenicol, at 200 µg/ml, completely inhibited the synthesis of the enzyme (data not shown) indicating that chloramphenicol inhibited protein synthesis in this strain of *S. aureus*.

Discussion

The data presented here indicate that growth of *S. aureus* 196E at low temperatures (≤28°C) generates cells with decreased thermotolerance. Shift of cells grown at a low temperature to 37°C for 5 h resulted in staphylococci with increased heat resistance. The presence of either nalidixic acid or rifamycin during the shift-up period inhibited the gain in thermotolerance. Chloramphenicol was much less inhibitory, thereby suggesting that DNA or RNA synthesis may be more important than protein synthesis during the shift-up period. Previous workers have demonstrated that growing both gram-positive or gram-negative bacteria at low temperatures leads to cells with decreased heat resistance (1-3, 9, 13, 15, 20). While

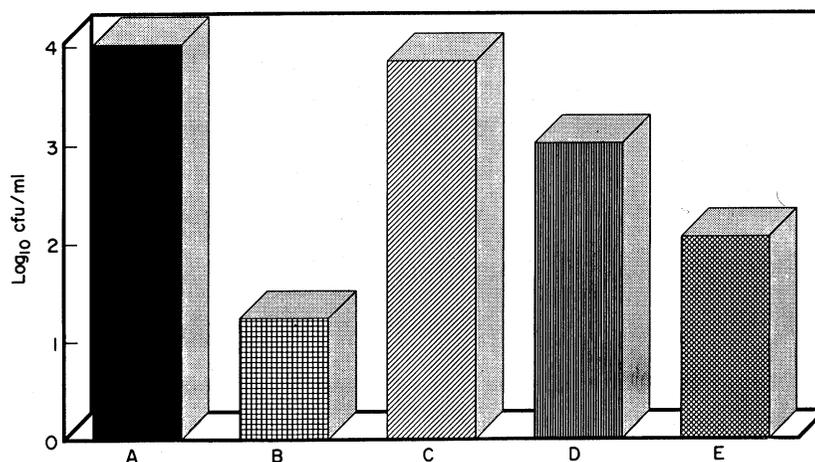


Fig. 1 The effect of antibiotics on death of 28°C grown *Staphylococcus aureus* shifted to 37°C for 5 h followed by a 52°C heat treatment. Each bar represents the mean of three to four determinations. A, cells grown at 28°C; B, cells grown at 28°C and then shifted to 37°C for 5 h; C, as B + 100 µg/ml nalidixic acid; D, as B + 100 µg/ml rifamycin; E, as B + 200 µg/ml chloramphenicol

the mechanism as to why microorganisms grown at low temperatures are more susceptible to heat killing is unknown, the general microbial response to low growth temperatures is increased production of unsaturated fatty acids in the phospholipids present in the cytoplasmic membrane (1, 8, 17, 19, 21). The increased unsaturation results in a more fluid, less viscous membrane. The increase in unsaturated fatty acids with lowered growth temperatures has been found in both gram-positive and gram-negative species (1, 8, 17, 19, 21). However, *S. aureus* behaves anomalously because it does not respond to lowered growth temperature by incorporating unsaturated fatty acids into the cytoplasmic membrane (7). Instead, *S. aureus*, when grown at low temperatures, shows a marked increase in the synthesis of rubixanthin (7). Rubixanthin may have a function similar to the unsaturated fatty acids found in other organisms which are grown at low temperatures.

Beuchat and Worthington (1) demonstrated that the increased heat resistance that correlated with higher growth temperature was also correlated with the decreased unsaturation of the fatty acids present in the membrane. They suggested that a microbial cytoplasmic membrane with decreased unsaturation induced by higher growth temperatures was important in maintaining the resistance of the microorganism to heat stress.

Yatvin (23) agreed with Beuchat and Worthington that increased fluidity of membrane lipids due to growth at low temperatures is a major factor contributing to death of these cells during thermal stress.

Shift-up of bacteria grown at a lower temperature (usually cells grown at temperatures ranging from 30 to 37°C) to a higher incubation temperature results in microorganisms that are more heat resistant. The protective effect against heat injury and death due to a short incubation of microorganisms at a temperature higher than the growth temperature has been observed with both bacterial media and foods (4, 5, 11, 12, 22).

Heat shock proteins are synthesized when eucaryotic or pro-caryotic cells are exposed to sublethal stresses (10). These proteins appear to protect cells against stress damage and may be important in returning the cells to their normal physiological state following the stress (14).

Yamamori and Yura (22) demonstrated that the shift-up of 30°C grown *E. coli* to 42°C increased the resistance of the cells to heating at 55°C; they felt that the effect was due to heat shock proteins. The presence of chloramphenicol during the shift-up period inhibited the acquisition of heat resistance suggesting that protein synthesis was necessary (22).

The data presented in **Tables 1** and **2** indicated that a substance, responsible for increased thermotolerance in *S. aureus*, is synthesized at 37 or 42°C but is not synthesized at lower temperatures. Shifting the cells from a lower growth temperature to 37°C permitted the synthesis of the substance with resultant increase in thermotolerance. However, the increase in thermotolerance was inhibited by nalidixic acid (a DNA synthesis inhibitor) or rifamycin (a RNA synthesis inhibitor) but is inhibited only to a small extent by chloramphenicol (a protein synthesis inhibitor). The results obtained with the use of antibiotics during the shift-up period suggest that DNA and/or RNA synthesis may be more important than protein synthesis. Jones *et al.* (6) have demonstrated that *S. aureus* 196E grown at 37°C and then heated at 50°C resulted in cells with depleted ribosomal material. As the extent of injury increased, the loss of ribosomes was greater. It may be possible that the ribosomes found in low temperature ($\leq 28^\circ\text{C}$) grown *S. aureus* are more thermolabile than those found in cells grown at 37 or 42°C. Therefore, when low temperature grown *S. aureus* is subjected to temperatures that are normally sublethal, the more labile ribosomes are destroyed with resultant increase in injured and dead cells. However, the nature of the substance synthesized during shift-up that leads to thermotolerance in *S. aureus* is unknown. We were unable to find data in the literature concerning the presence of heat shock proteins and/or their protective effect in *S. aureus*. Since the response to stress in organisms is to produce heat shock proteins, it would be surprising if *S. aureus* did not do so.

The mechanism by which high growth temperature or shift of cells to a higher but non-lethal temperature leads to thermotolerance is not understood. The results presented here suggest that the temperature at which *S. aureus* is grown will profoundly influence the heat requirement of a food process that is used to ensure the destruction of the organism. In addition, if a food containing *S. aureus* undergoes a short period of temperature abuse, there is potential for the organism to increase its thermotolerance and the heating process would have to be changed to reflect that increase in heat resistance. Therefore, foods should be kept refrigerated continuously until they are heat processed.

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