Interaction of pH and NaCl on Enumeration of Heat-Stressed Staphylococcus aureus

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The effect of pH level and NaCl concentrations, alone and in combination, on the enumeration of unstressed and heat-stressed cells of three strains of Staphylococcus aureus was determined. A definite narrowing of the optimum pH range for enumeration of both unstressed and heat-stressed cells was observed as the NaCl concentration was increased from 0.0 to 7.5%. Counts of unstressed cells diminished only slightly with increases in NaCl, whereas heat-stressed cells showed a marked sensitivity to NaCl concentrations of 4% and above, regardless of the pH level. Because of this sensitivity to NaCl, recoveries were far poorer than with unstressed cells at NaCl concentrations of 4% and above.

The ability of Staphylococcus aureus to grow at greater NaCl concentrations in comparison to many other bacteria is well documented (10, 12). A number of media that use NaCl as the basis of their selectivity have been developed for the quantitative enumeration of this organism (3, 4). In some situations, enumeration of S. aureus by such selective media may be difficult if the organism has been subjected to heat treatment or other forms of physical stress sufficient to cause sublethal injury. However, the assumption is often made that conditions satisfactory for the enumeration of unstressed bacteria are equally applicable to heat-damaged bacteria.

Busta and Jezeski (2) found that S. aureus cells heated at 60°C for various lengths of time lost their ability to multiply on Staphylococcus medium 110 containing the normal concentration of 7.5% NaCl. Since then other investigators (8, 13, 15, 17) have reported similar observations, indicating a definite NaCl sensitivity by S. aureus after thermal injury. Such a change in NaCl tolerance obviously reduces the effectiveness of media that are designed to selectively enumerate S. aureus on the assumption that the organism is tolerant to high concentrations of NaCl.

In addition to a loss of NaCl tolerance after thermal injury, F. E. Nelson (Bacteriol. Proc., p. 40, 1956) noted that the optimum pH range necessary for the recovery of heat-stressed S. aureus was reduced in comparison to that for the unstressed organism.

Other reports on the effects of pH alone or pH in combination with NaCl have been concerned with unstressed S. aureus. For example, Lechowich et al. (11) found that pH 5.6 reduced both aerobic and anaerobic growth of S. aureus in pickling brines used for curing meats, and pH 4.8 prevented growth. Iandolo et al. (9) demonstrated that the effects of NaCl on unstressed cells were greater either when pH deviated from the optimum or incubation temperature was raised to 45°C. Genigeorgis and Sadler (7) concluded that growth and enterotoxin production were better when initial pH of the medium was increased and salt concentration was decreased. Growth of organisms tended to raise the pH so that the pH at time of enterotoxin production unquestionably was higher than when growth was initiated. Scheusner et al. (14) obtained growth when the initial pH of the broth was 4.96 to 9.02.

Genigeorgis et al. (5) reported growth over the pH range from 4.00 to 9.83 in the absence of NaCl, but increasing concentrations of NaCl narrowed the range of both acid and alkaline values, the limits being pH values of 4.50 and 8.0 at 12% NaCl.

Nelson (Bacteriol. Proc., p. 20, 1971) reported that the inhibitory effects of pH and NaCl were additive when enumerating heat-stressed coliform bacteria, maximum counts being obtained only over a pH range of 6.0 to 7.0 in the presence of 3% NaCl, whereas unstressed organisms were
unaffected over a pH range from 5.0 through 9.2 in the absence of NaCl in the enumeration medium.

**MATERIALS AND METHODS**

**Test organisms.** Three strains of coagulase-positive *S. aureus* were employed. Strain UA-112 was obtained from the Department of Microbiology and Medical Technology at the University of Arizona. Strains S-6(B) and FRI-100, known producers of enterotoxin, were obtained from M. S. Bergdoll of the Food Research Institute of the University of Wisconsin. The organisms were maintained on brain heart infusion agar (Difco) slants held at 5 C prior to use. Cells were grown in 5 ml of nutrient broth (Difco) for 24 h at 37 C, after which 1 loopful was transferred to 50 ml of nutrient broth contained in 125-ml flasks and incubated for 24 h at 37 C.

**Thermal stressing and enumeration.** A 1-ml sample of a 24-h culture was placed into 5 ml of sterile reconstituted milk (110 g of milk solids-not-fat/liter) contained in screw-cap test tubes (16 by 125 mm). A sample serially diluted in phosphate buffer was plated on unmodified nutrient agar (Difco) by the procedure outlined in *Standard Methods* (1). Cells of UA-112 were plated on nutrient agar containing NaCl concentrations of 0, 2, 3, 5, 4, 5, and 7.5%. Counts at each NaCl concentration were made at pH levels of 5.0, 5.5, 6.0, 7.0, 8.0, 8.5, 9.0, and 10.0. Cells of strains S-6(B) and FRI-100 were plated on nutrient agar containing fewer NaCl concentrations accompanied by fewer pH levels to check only those points of greatest importance. NaCl concentrations of 0, 2, 3, 4, and 5% were used, along with pH levels of 5.0, 5.5, 7.0, 7.5, and 9.0.

Adjustment of pH was made prior to plating by the addition of either 1 N H2SO4 or 1 N KOH to the warm NaCl-nutrient agar. After the plating of the unstressed cells, the remaining milk sample was immersed, along with a control milk sample containing a thermometer, in a water bath maintained at 56 ± 0.1 C. Timing of the sample commenced when the temperature reached 56 C in the control tube. Exposure time of the sample was designed so as to reduce the viable population as determined on nutrient agar by 99% and was 7 min for strain UA-112 and 6 min for strains S-6(B) and FRI-100. A high level of kill was selected to maximize the observed effects of stress. After exposure, samples were cooled in ice water and plated on the same series of media on which the unstressed cells had been plated. Plates were incubated at 37 C for 48 h. All platings of strain UA-112 were made in duplicate, and two runs were made for each NaCl-pH combination. Duplicate platings for strains S-6(B) and FRI-100 were used only for counts on the control unmodified nutrient agar. Two trials were run for each NaCl-pH combination.

**Statistics.** Counts of stressed cells on modified media were expressed as a percentage of the count obtained on unmodified nutrient medium, and this percentage then converted to logarithms to the base 10 to give log percent recovery.

Recovery values for unheated and heated *S. aureus* UA-112 were subjected to a completely randomized two-way factorial arrangement (16) by using NaCl and pH as the factors. A similar experimental analysis for unheated and heated cells of strains S-6(B) and FRI-100 involved a completely randomized three-way factorial analysis of variance, with NaCl, pH, and strain constituting the factors.

**RESULTS**

Data on recovery of unheated cells of *S. aureus* strain UA-112 at different levels of pH in the presence of increasing concentrations of NaCl are shown in Fig. 1. A gradual narrowing of the optimal pH was shown as the NaCl concentration increased from 0 to 7.5%. At lower NaCl concentrations, maximum recoveries were obtained over a pH range of 5.0 through 9.0. At NaCl concentrations of 3.5 and 4% recoveries at pH 9.0 were significantly less than maximum, whereas recoveries at 5 and 7.5% were significantly reduced at pH levels of 5.0, 5.5, and 6.0. Maximum recoveries at higher NaCl concentrations were only slightly lower than recoveries at the same pH levels in the absence of NaCl.

Data on recovery of heat-stressed *S. aureus* strain UA-112 at different pH levels in the presence of increasing concentrations of NaCl are shown in Fig. 2. These heat-stressed cells also showed a gradual narrowing of their optimal pH range as the NaCl concentration increased. Recoveries at 4% NaCl showed an overall decrease from counts obtained at 3.5% regardless of the pH level. Recoveries at 5 and 7.5% NaCl were quite similar to those obtained at 4% in that recoveries were only approximately 10% of the optimum.

Recoveries of unheated and heat-stressed cells of strains S-6(B) and FRI-100 at various pH levels in the presence of increasing NaCl concentrations were so similar to those of strain UA-112 that the data are not presented.

Analysis of variance for all three unstressed strains of *S. aureus* revealed significant first-order interactions between NaCl and pH, indicating that changes in the level of one factor modify the effects of the other factor on the enumeration of unstressed cells. In addition, comparison of unstressed cells of strains S-6(B) and FRI-100 showed no significant differences between these two strains in their responses to NaCl and pH. Heat-stressed cells of all three strains also showed significant first-order interactions between NaCl and pH, indicating an interactive effect of these two factors on enumeration. Furthermore, comparison of strains S-6(B) and FRI-100 to one another revealed two additional first-order interactions. Significant interactions were noted with the pH and strain.
Fig. 1. Recovery of unstressed cells of S. aureus VA-112 at various levels of pH in the presence of increasing concentrations of NaCl.
Fig. 2. Recovery of heat-stressed cells of S. aureus VA-112 at various levels of pH in the presence of increasing concentrations of NaCl.
and also NaCl and strain factors. These interactions indicate that the two strains reacted slightly differently to pH and over all NaCl concentrations and to NaCl over all pH levels. In comparison to unstrained cells, heat-stressed cells showed more variation from strain to strain in their responses to NaCl and pH, even though both groups show a narrowing of their optimum pH range as the NaCl concentration increases.

DISCUSSION

Although three strains do not constitute a large sample, the indications are that the responses to pH and NaCl demonstrated here are characteristic of the species. Such responses to pH and NaCl indicate an interaction effect by the two factors on recovery of S. aureus. As the degree of adversity, especially pH, was increased, a gradual decline in count occurred; this decline was accentuated by increasing NaCl concentration. Both unstrained and heat-stressed cells showed a narrowing of their optimum pH ranges as the NaCl concentration increased. The degree of the observed effects very probably could be altered appreciably by variations in the degree of stress. These findings were in close agreement with the results obtained by Genigeorgis et al. (5, 6) on unstrained cells of S. aureus, indicating a decrease in count of the organism at any pH level as the NaCl concentration increases. However, due to the marked sensitivity of heat-stressed cells to NaCl, overall recoveries at 4% NaCl and above were far poorer, regardless of pH, in comparison to unstrained cells.

Maximum counts of unstrained cells of S. aureus can be obtained quite effectively in a medium containing 7.5% NaCl adjusted to a pH range of 7.0 through 8.5. Maximum counts of heat-stressed cells cannot be attained on media containing 5% or more NaCl. The pH of the medium plus sample should not be outside a range of 5.5 through 8.5, even with an NaCl concentration below 4%. Although dropping the NaCl concentration of a selective medium would obtain higher counts of staphylococci that had been subjected to thermal stress, it would do away with much of the selective character of such a medium. Due to the NaCl sensitivity of S. aureus after thermal stress, it would appear that no combination of pH and NaCl would offer promise of improving recovery of such an organism from mixed populations of bacteria. Improvements of media for this purpose must be sought in other directions.

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