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Effect of Salt Concentration in the Recovery Medium on Heat-injured *Streptococcus faecalis*[1]

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Properties relating to the recovery of three heat-injured strains of *Streptococcus faecalis* were studied. All strains were cultured in all purpose plus Tween broth (APT) at 30 C for 24 hr before being subjected to heat in fresh APT broth. APT recovery medium containing various added amounts of NaCl, KCl, $MgCl_2$, or KCl and $MgCl_2$ was used to assess the effect of salts on the recovery of thermally injured *S. faecalis*. It was evident that, upon exposure to heat, *S. faecalis* cells became sensitive to increased salt concentrations. Analyses to determine the ribonucleic acid (RNA) content of heated cells showed a reduction of cellular RNA, but the per cent reduction was not directly proportional to the per cent reduction of the viable cells.

The viability of damaged organisms, as noted by Harris (7), is often markedly affected by environmental changes which do not influence undamaged cells. The assumption that similar environmental conditions are satisfactory for the growth of unheated bacteria, as well as the growth of bacteria which have been subjected to heat treatment of sublethal intensity, is questioned by Nelson (10). He states that heated bacteria are more demanding in their requirements of media for growth, and that the dormancy of heated organisms can be reduced or eliminated by use of suitable recovery media.

Leakage of ribonucleic acid (RNA) from cells is thought to be partly responsible for the death of heat-treated microorganisms. Haight and Morita (6) studied the leakage of RNA from heated *Vibrio marinus*. Both high and low molecular weight RNA were noted in the heating menstruum. The possibility was suggested that heating induced degradation of RNA inside the cell before leakage occurred, or that the RNA was hydrolyzed enzymatically after leaving the cell. The shape of death and leakage curves for heated *Aerobacter aerogenes* provides evidence that RNA breakdown does occur during heating (14).

The effect of adding various concentrations of salts to recovery media on the survival of heated organisms has been investigated by many workers (2, 8, 9, 11, 13). This paper discusses the effect of heat on RNA content of *Streptococcus faecalis* and the effect of adding NaCl, KCl, $MgCl_2$, or KCl and $MgCl_2$ to a recovery medium on the survival of heat-treated *S. faecalis*.

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MATERIALS AND METHODS

Test organisms. Several unidentified bacterial isolates from raw and partially processed meat samples of commercial origin were supplied by R. A. Greenberg of Swift and Co., Chicago, Ill. Three isolates were selected and identified. The organisms were grown in all purpose plus Tween broth (APT), a Difco formulation of a medium described by Evans and Niven (4). Cells from 24-hr cultures were used for all heating experiments, and incubation was at 30 C.

Heating procedure. A 1-ml amount of the 24-hr culture was inoculated into screw-cap test tubes (16 × 150 mm) containing 15 ml of sterile APT broth preheated to 60 C in a heater-stirrer water bath (Bronwill Scientific Div., Will Corp., Rochester, N.Y.). Tubes were removed from the water bath after appropriate periods of heat exposure and were plunged into an ice bath to obtain rapid cooling. Proper dilutions were made in sterile deionized water, and the heated organisms were plated in duplicate or triplicate in APT agar containing salt by the pour-plate technique. Na^+, K^+, and Mg^{++} were added as chlorides to obtain the desired final concentrations.

RNA analyses. A procedure for the isolation of RNA, as described by Clark (3), was modified slightly to determine the RNA content of the three strains of heated and unheated *S. faecalis*. Cultures of *S. faecalis* were grown in APT broth for 23 to 24 hr at 30 C. One liter of the culture was centrifuged at 4,080 × *g*. (Sorvall superspeed, model RC-2, rotor no. GSA, Ivan Sorvall, Inc., Norwalk, Conn.) for 20 min. The supernatant fluid was removed after centrifugation, and the cells were resuspended in 100 ml of distilled water. A 10-ml amount of the cell suspension was

dried at 140 C for 4 hr to determine the dry weight per ml of the cell concentrate. Concentrate (10 ml) was added to each of the six 250-ml screw-cap Erlenmeyer flasks containing 90 ml of APT broth preheated at 60 ± 0.25 C in a Metabolyte Water Bath Shaker (New Brunswick Scientific Co., New Brunswick, N.J.); the remainder of the cell concentrate was appropriately diluted in cool APT broth and was used as a sample representing zero heating time. The speed of the shaker was maintained at 100 rev/min to avoid settling of the suspension. Since the resistance of the three streptococci used was not the same, appropriate heating times were chosen for each strain in order to reduce the viable population of that particular strain by approximately the same percentage of 2 and 4 log cycles. S23 was heated 12 and 20 min, S21 was heated 20 and 40 min, and S28 was heated 40 and 70 min. When the required heating times were reached, three flasks (300 ml or one sample) of the suspension were immediately cooled. From this point on in the analyses, samples were handled separately but identically. Reference will be made to only one sample.

The cells of the sample were pooled by centrifugation at 4,080 × *g* for 20 min, washed twice with distilled water, and mechanically ruptured by means of a cell disintegrator (type 2876, Bronwill Scientific Inc.). Larger cellular components were separated from the supernatant fluid by centrifugation at 7,970 × *g* for 20 min. Glass beads used for disrupting cells were washed twice, retaining the supernatant fluid each time. An equal volume of 88% phenol was added to the chilled supernatant fluid, and the mixture was stirred at room temperature for 30 min. The emulsion was then cooled in an ice bath for 5 min, before breaking by centrifugation at 3,300 × *g* for 15 min at 0 to 5 C. The upper aqueous layer of supernatant fluid and most of any intermediate layer containing denatured protein were decanted from the brown phenol phase. Removal of the denatured protein from the aqueous phase was accomplished by centrifugation at 7,970 × *g* for 5 min at 0 to 5 C. A volume of 20% (w/v) potassium acetate (*p*H 5.0), 10% of the volume of the aqueous fraction, was added to the aqueous fraction, followed by precipitation of RNA with the addition of two volumes of cold absolute ethyl alcohol. The suspension was chilled in an ice bath for 5 min before the precipitate was collected by centrifugation at 1,020 × *g* for 10 min at 0 to 5 C. The precipitate was washed once with each of the following: ethyl alcohol-water (3:1), absolute ethyl alcohol, and anhydrous ether; it was then air dried.

The dried precipitate was dissolved in 10 ml of 0.5 N potassium hydroxide, and hydrolysis was allowed to proceed at room temperature for 24 to 48 hr. Sufficient 20% (v/v) hydrogen perchlorate was added to the chilled hydrolyzed solution to reduce the *p*H to about 2, followed by removal of any precipitated potassium perchlorate, DNA, or protein or all three by centrifugation at 1,020 × *g* for 10 min. The *p*H of the supernatant fluid was adjusted to 3.5 with 1.0 N KOH. Any additional precipitate was removed by centrifugation at 1,020 × *g* for 10 min.

A modification of the Ashwell (1) procedure for colorimetric analysis of sugars was used to determine the RNA content of heated and unheated *S. faecalis*. The acidified hydrolyzed sample of RNA was diluted 1:10 with deionized water, and suitable samples were placed in screw-cap test tubes (16 × 150 mm). Similarly, samples of a 10^{-4} M nucleic acid standard (ribonucleic acid; Nutritional Biochemicals Corp., Cleveland, Ohio) were placed in test tubes. To each tube was added enough deionized water to bring the total volume to 3 ml, followed by the addition of 6 ml of orcinol acid reagent (0.1% $FeCl_3$ in concentrated HCl) and 0.4 ml of 6% (w/v) alcoholic orcinol. The solutions were boiled for 20 min in a water bath and cooled, and the absorbancies at 660 mμ were measured with a Spectronic-20 colorimeter (Bausch & Lomb, Rochester, N.Y.).

RESULTS AND DISCUSSION

Classification. The three unknown bacterial isolates were identified according to the physiological and biochemical tests described by Gibbs and Skinner (5). Two of the bacterial isolates were identified as *S. faecalis* var. *liquefaciens* (S21 and

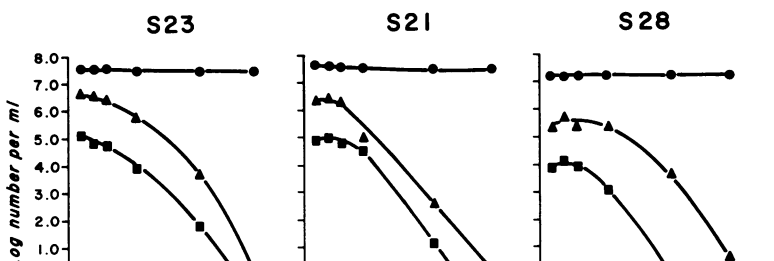

FIG. 1. *Effect of adding Na^+ to APT recovery medium on the survival of Streptococcus faecalis heated for various lengths of time. Key: S23, ●, 0 min at 60 C; ▲, 12 min at 60 C; ■, 20 min at 60 C; S21, ●, 0 min at 60 C; ▲, 20 min at 60 C; ■, 40 min at 60 C; S28, ●, 0 min at 60 C; ▲, 40 min at 60 C; ■, 70 min at 60 C.*

S23), and the third was identified as *S. faecalis* (S28).

Effect of salts. The effect of adding Na^+, K^+, and Mg^{++} to APT recovery medium on the survival of heated and unheated *S. faecalis* is shown in Fig. 1 through 4. Since the three strains used in this study were not equally resistant to heat, each strain had to be exposed for various lengths of time to obtain comparable reductions in the viable number of organisms.

Figure 1 shows the effect of Na^+, in concentrations of up to 1.39 molal, on the survival of heated and unheated organisms. When the three strains were not subjected to heat, they grew equally well in media containing all Na^+ concentrations. Concentrations above 0.27 molal Na^+ decreased the total recovery of all three strains of heated *S. faecalis*. The effect of various concentrations of NaCl on the recovery of heated *Staphylococcus aureus* MF-31 has been investigated by many workers. After subjecting *S. aureus* MF-31 to 55 C for 15 min, Sogin and Ordal (12) noted an extended lag phase and loss of the ability to reproduce on a recovery medium containing 7.5% NaCl. The same exposure left only 1% of the viable population able to reproduce on a medium containing 7.5% NaCl (8). It was concluded that thermal injury was partly due to changes in the cell membrane, which allowed soluble components to be released into the menstruum. The extended lag phase was attributed to readjustment in the form of cell repair.

Figure 2 shows the effect of adding K^+ to a recovery medium on the survival of heated and unheated S23, S21, and S28. At concentrations of up to 1.34 molal K^+, the growth of unheated *S. faecalis* was not affected. A decrease in total survival was observed when the three heated *S. faecalis* strains were plated on a medium containing concentrations greater than 0.25 molal K^+.

The effects of adding Mg^{++} to APT recovery medium in concentrations up to 0.35 molal are shown in Fig. 3. Again, total growth of unheated *S. faecalis* was not affected by the highest cation

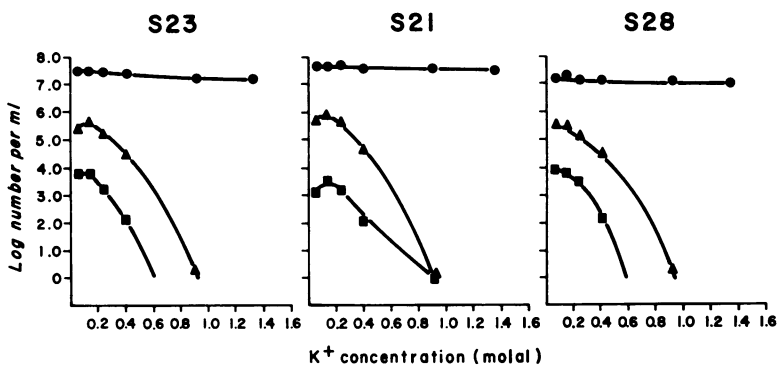

FIG. 2. *Effect of adding K^+ to APT recovery medium on the survival of Streptococcus faecalis heated for various lengths of time. Key: S23, ●, 0 min at 60 C; ▲, 12 min at 60 C; ■, 20 min at 60 C; ●, 0 min at 60 C; ▲, 20 min at 60 C; ■, 40 min at 60 C; S28, ●, 0 min at 60 C; ▲, 40 min at 60 C; ■, 70 min at 60 C.*

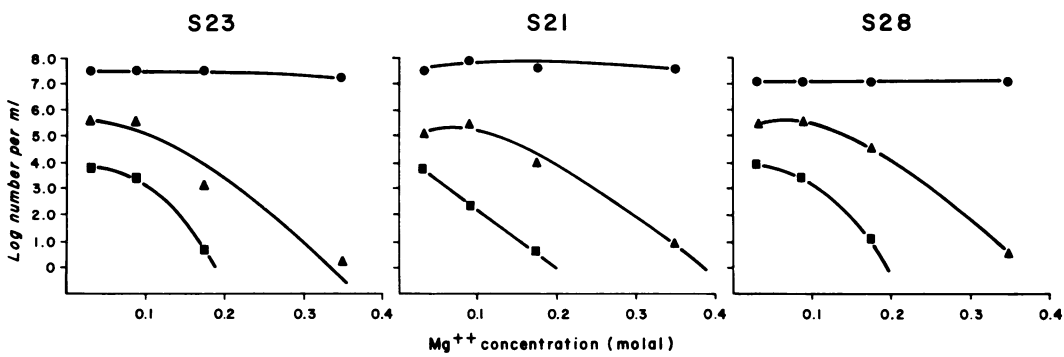

FIG. 3. *Effect of adding Mg^{++} to APT recovery medium on the survival of Streptococcus faecalis heated for various lengths of time. Key: S23, ●, 0 min at 60 C; ▲, 12 min at 60 C; ■, 20 min at 60 C; S21, ●, 0 min at 60, C; ▲, 20 min at 60 C; ■, 40 min at 60 C; S28, ●, 0 min at 60 C; ▲, 40 min at 60 C; ■, 70 min at 60 C.*

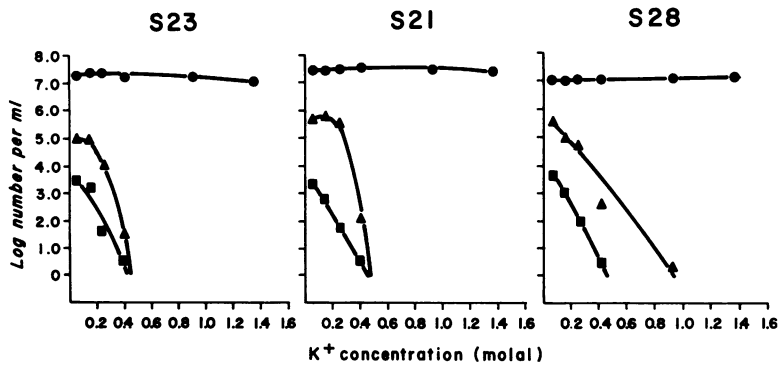

FIG. 4. *Effect of adding K^+ and 0.089 molal Mg^{++} to APT recovery medium on the survival of Streptococcus faecalis heated for various lengths of time. Key: S23, ●, 0 min at 60 C; ▲, 12 min at 60 C; ■, 20 min at 60 C; S21, ●, 0 min at 60 C; ▲, 20 min at 60 C; ■, 40 min at 60 C; S28, ●, 0 min at 60 C; ▲, 40 min at 60 C; ■, 70 min at 60 C.*

TABLE 1. *Percentage of RNA in Streptococcus faecalis cells (dry weight basis) before and after heating at 60 C*

Strain	Heat sufficient to reduce the viable population by approximately		
	0.0%	99.0%	99.99%
S21	10.41	7.13	5.76
S23	11.97	4.84	3.44
S28	11.61	4.92	2.78

concentration. In suspensions in which the total viable number of organisms was reduced to about 1.0%, concentrations of greater than 0.089 molal Mg^{++} tended to decrease the total survival. Concentrations greater than 0.067 molal Mg^{++} decreased the total survival of organisms subjected to thermal treatment sufficient to reduce the viable population to 0.01%.

Figure 4 shows the effect of adding K^+ and 0.089 molal Mg^{++} to APT recovery medium on the survival of heated *S. faecalis*. Unheated cells were not affected in a medium containing up to 1.34 molal K^+ plus 0.089 molal Mg^{++}. A comparison of the results shown in Fig. 2, 3, and 4 indicates that the presence of elevated amounts of K^+ and Mg^{++} in the recovery medium had a lethal effect, but that the effect was not strictly additive. Induction of sensitivity was brought about by heat exposure, and a decrease in total survival was observed when a recovery medium with greater than 0.086 molal K^+ plus 0.089 molal Mg^{++} was used.

To summarize the effect of salts, little difference was observed in the total counts of unheated organisms when concentrations of up to 1.39 molal Na^+, 1.34 molal K^+, 0.35 molal Mg^{++}, and 1.34 molal K^+ plus 0.089 molal Mg^{++} were present in a recovery medium. It is evident from the figures, however, that a reduction in total recovery of *S. faecalis* in a medium containing fixed levels of Na^+, K^+, Mg^{++}, and K^+ and Mg^{++} was brought about by exposing the organism to heat of a sublethal intensity, and that this reduction was magnified by additional heat treatment. The same additional heat treatment has been shown to reduce RNA (Table 1). The percentage of reduction of cellular RNA brought about by heating was not directly proportional to the percentage of reduction of the viable cells. For example, in the case of S21, a 99% reduction in viable cells showed a 31% reduction in cellular RNA. This would indicate that not all of the nonviable cells became sufficiently disrupted to allow extrusion of the total cellular RNA into the menstruum. In fact, comparison of unheated and heated cells with phase-contrast microscopy revealed little difference in gross appearance. It is possible that in *S. faecalis* cells there are multiple sites on the cell membrane vulnerable to inactivation or disruption, a minimum of which must be acted upon to cause death. When cells which have been subjected to heat are exposed to a less than optimal recovery environment, the critical number of sites seems to be decreased. Disruption of the cell permeability upon subjection to heat could result in leakage of RNA from the cell. Further loss in permeability control might be brought about by the exposure of the weakened cells to unfavorably high salt concentrations. If the loss of cellular RNA by leakage was extensive, recovery would not be possible, not even under favorable conditions. Whether the inability to recover which is caused by Na^+, K^+, or Mg^{++} is due to unfavorable osmotic conditions, disruption of the transport mechanism for transfer of metabolites across the

cell membrane (into or out of the cell), coagulation of cell protein, or some other physical or chemical phenomenon is not clearly understood.

ACKNOWLEDGMENT

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