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Revival and Subsequent Isolation of Heat-Injured Bacteria by a Membrane Filter Technique[1]

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Cultures containing mixed flora from raw milk were heated at 62.8 C for 15, 20, 25, and 30 min. Dilutions were filtered through membrane filters, and the filters were incubated on Trypticase soy broth (TSB) and on TSB plus NaCl (TSBS). The TSB count indicated the total population which survived heating and included injured and uninjured cells. The colonies on TSBS indicated the uninjured cells and were marked by perforating the membrane near the colony. This membrane was then transferred to fresh TSB and incubated further. The injured organisms recovered and formed colonies which could be distinguished from previous colonies of uninjured organisms. Transfer counts on TSB were not substantially different from the initial TSB counts at 15, 20, 25, and 30 min of heating.

During a study on heat injury and recovery of bacteria, it was speculated that the membrane filter technique (1) with modification could be used to detect and separate colonies developing from injured and uninjured cells. With some methods, there is a question as to whether designated organisms are noninjured survivors or recovered injured cells.

Heat-injured cells usually will grow in complex media containing a variety of supplemental nutrients, but are suppressed in media containing a selective or inhibitory agent that does not affect uninjured cells. Busta and Jezeski (2) noted that NaCl tolerance was reduced by heat injury. Iandolo and Ordal (5) used Trypticase soy agar (TSA) and TSA containing 7.5% NaCl (TSAS) as differential media for determining the number of injured cells of *Staphylococcus aureus* MF31. Heat injury caused the cells to lose their ability to grow on TSAS. However, cells in duplicate portions were able to recover and grow on TSA. Furthermore, incubation in Trypticase soy broth (TSB) allowed the injured cells to recover salt tolerance and grow on TSAS. Similarly, Clark et al. (3) used 6% TSAS to evaluate heat injury in *Streptococcus faecalis* R57. Heat-injured cells would not grow on TSAS but recovered salt tolerance when incubated in TSB.

Although a growing body of information has been collected regarding the injury and repair of bacteria, little attention has been paid to the development of methods to differentiate injured from uninjured cells. In this study it was postulated that, with the membrane filter procedure and TSB plus NaCl (TSBS) medium, the detectable colony growth of heat-treated cultures would reflect the uninjured population. Subsequent transfer of the membrane to TSB medium would allow injured cells to revive and form additional colonies. These could then be differentiated from other colonies and enumerated and isolated for further study.

The objective of this investigation was to evaluate the proposed procedure as a technique for segregating revived heat-injured from uninjured bacteria.

MATERIALS AND METHODS

All equipment and media were sterilized by appropriate means, and aseptic techniques were em-

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ployed.

Since the original study was related to milk pasteurization, it would have been preferred to conduct the work with milk and a natural milk flora. However, bacterial membrane filtration of whole milk presented problems even when the milk was diluted and surfactant filtering aids were used. Furthermore, surfactants have been shown by Maxcy (6) to inhibit growth of heat-injured organisms. Scheusner et al. (7) noted the same effect with organisms injured by sanitizers. Accordingly, the study was conducted with TSB cultures. To maintain a mixed flora somewhat representative of milk, 1% of raw milk was used as inoculum in the broth.

The broth cultures were incubated at 32 C for 24 hr. Unheated cultures were tested for salt tolerance. Others were heat-treated and subsequently tested for heat injury. Media used were TSB (prepared from individual ingredients) and TSBS (TSB containing various concentrations of NaCl).

Determination of salt sensitivity. Petri plates (glass, 50-mm diameter) with absorbant pads were charged with 2 ml of TSBS containing 0.5, 1, 2, 3, 4, and 5% concentrations of salt. Broth cultures were appropriately diluted and filtered through membrane filters (Millipore Corp., Bedford, Mass.; Type HAWG, 0.45-μm pore size, 47-mm) in 10-ml quantities, and the filters were placed on the absorbant pads. These were incubated at 32 C for 48 hr. The incubator was maintained at a suitable humidity level to prevent drying of the filters.

After growth, most colonies were small, and some were difficult to see under normal room light. These colonies were counted by lighting the filter with a microscope illuminator placed at an angle of about 15 degrees to the surface. Shadows behind the raised colonies then were counted. This procedure was followed throughout the study. Count data were statistically analyzed to determine the lowest concentration at which salt inhibition occurred. After the inhibitory concentration was determined, that level of salt was used in TSBS in subsequent trials to detect bacteria uninjured in heating trials.

Determination of heat injury. Sealed 3-ml ampoules containing 2.6 ml of the TSB broth cultures were immersed in a water bath held at 62.8 C (vat pasteurization temperature for milk). Heating periods were varied to obtain different degrees of heat injury among survivors. Cultures were held for 15, 20, 25, and 30 min with 1 min allowed for come-up time. The ampoules then were cooled in an ice bath and filtered within 10 min.

Detection of heat-injured organisms. After cooling, duplicate 1-ml samples were taken from each ampoule and placed in 9-ml water blanks. Each dilution was shaken and passed through a membrane filter. One of the filters then was placed on TSB and the other on TSBS. Plates were incubated for 48 hr at 32 C, and the colonies were counted. The filters from TSBS then were transferred to fresh TSB plates. These plates were incubated for an additional 48 hr at 32 C, and the colonies were counted again. The original TSB count was considered to represent the total population of each suspension. The TSBS count indicated the uninjured population. After the TSBS plates were counted, each colony was marked by perforating the filter near the colony with a sterile needle. The colonies thus marked were disregarded when counted after transfer to TSB. The new colonies which were produced during the second incubation on TSB gave a direct count of the revived organisms.

Description of organisms. In some heat-injury trials following the second incubation, both marked and unmarked colonies were transferred to litmus milk and incubated for 48 hr at 32 C. Cultures were observed for litmus milk reactions and examined microscopically for Gram stain and morphology. This allowed a general description of the types of organisms which were injured or uninjured by the heat treatment.

RESULTS AND DISCUSSION

Level of salt sensitivity. Membrane filters from unheated cultures were incubated on TSBS media with NaCl content ranging from 0.5% (normal TSB) to 5.0%. Fifteen replications were performed. Counts were expressed as 10^8 per ml, and the resultant means were 28.9, 27.5, 26.6, 27.7, 21.4, and 17.7 for NaCl concentrations of 0.5, 1, 2, 3, 4, and 5%, respectively. The New Multiple Range Test (4) showed 4 and 5% NaCl to be the significant treatments, as the counts were reduced in these media. Three per cent TSBS was considered to be the highest NaCl content which could be tolerated by the unheated cells and accordingly was used in all subsequent experiments.

Determination of heat injury. Duplicate portions of cultures heated for four different periods were filtered and incubated as described. Twenty-seven replications of each of the four heating times were performed. Count data for trials with heating periods of 15 min at 62.8 C are presented in Table 1. As noted in the table, some plates were uncountable due to spreading colonies, molds, or contamination. These plates were not used in compiling data for statistical analyses. The TSB counts represented both heat-injured and uninjured organisms. The counts on TSBS are from uninjured bacteria with the difference representing injured cells unable to develop on the TSBS. The variation in count level among trials is not unexpected due to differences in numbers of organisms in the initial inocula. The number of injured bacteria also varied among trials. For the 27 trials, the mean count decreased from 110.8 to 23.2 per ml, indicating 79.2% injured cells. The remaining 20.8% that retained salt tolerance were considered to be uninjured.

TABLE 1. *Effect of membrane filter transfer technique on bacterial counts per ml of heated[a] cultures*

Trial	TSB count[b]	TSBS 3% count[b]	Transfer count[c]	Recovery count[d]
1	97	61	97	36
2	—[e]	17	26	9
3	—	57	117	60
4	147	10	136	126
5	53	60	79	19
6	174	20	186	166
7	17	8	21	13
8	138	1	111	110
9	—	17	39	22
10	—	12	66	54
11	40	9	54	45
12	20	18	25	7
13	43	33	42	9
14	44	10	39	29
15	24	10	22	12
16	37	12	33	21
17	107	19	98	78
18	74	67	69	2
19	356	16	340	324
20	98	20	84	64
21	113	18	106	88
22	232	22	196	174
23	46	8	43	35
24	224	31	188	157
25	162	20	158	138
26	127	17	118	101
27	175	34	165	131
Means	110.8[f]	23.2	98.4[f]	75.2

[a] Heated for 15 min at 62.8 C.

[b] Colony counts on membrane filter after 48 hr at 32 C. TSB, Trypticase soy broth.

[c] Colony counts on membrane filter from TSB plus NaCl (TSBS) following an additional 48 hr of incubation on TSB.

[d] Colonies which were unmarked following final incubation.

[e] Uncountable plates due to spreaders, molds, or unexplained contamination; data not used to calculate paired *t* test.

[f] Means are not significantly different ($P = 0.05$).

When the filters from TSBS were transferred to TSB, the counts increased and reached the same general level as the original TSB counts. The means for the 27 trials showed the difference not to be significant ($P = 0.05$). Colonies formed as a result of the repair and growth of injured bacteria were distinguishable from the original uninjured population and could be counted separately.

Similar cell suspensions were heated for longer periods of time; the mean values from the trials for each of the four heating periods are given in Table 2. Although the count levels generally were lower after 25 min, similar results were obtained. There were no substantial differences between the means of the original TSB counts and those of the transfer counts within each of these exposure tests.

Effect of incubation time on TSBS. Although no marked differences between the original TSB counts and transfer count were observed up to 30 min of heat treatment, it was evident from the data in Table 2 that there was a trend for such counts to be less than the controls. A possible explanation was that contact of the injured cells with NaCl in TSBS caused death of the more severely injured cells and resulted in a lower transfer count. To test this possibility, the following study was conducted. Cultures were heated in 6-ml quantities for 30 min at 62.8 C to induce maximum variability. Five replicates from each heated culture were then filtered as described. The filters were placed in prepared TSBS plates in a randomized order and incubated at 32 C for periods of time ranging from 0 to 48 hr before transfer to TSB. The results of 12 trials of this experiment are presented in Table 3. Decreasing counts with increasing NaCl exposure time would indicate that NaCl had a lethal effect on the heat-injured population. However, no significant differences were found ($P = 0.05$) when these data were collected, regardless of the length of incubation on TSBS; that is, rather than killing the injured cells, incubation in NaCl-containing media merely prevented repair and subsequent growth.

Effect of incubation time on counts. Three

TABLE 2. *Membrane filter counts per milliliter of heated culture for four heating periods (means of 27 trials for each period)*

Heating time (62.8 C) (min)	TSB[a]	TSBS[a]	Transfer[b] count	Recovery[c] count	Injured[d] cells (%)
15	110.8	23.2	98.4	75.2	79.2
20	111.6	20.5	96.4	75.9	81.7
25	84.4	21.0	79.0	58.0	75.2
30	74.9	16.3	66.4	50.1	78.2

[a] Colony count on membrane filter after 48 hr at 32 C. TSB, Trypticase soy broth.

[b] Colony count on membrane filter from TSB plus NaCl (TSBS) following an additional 48 hr of incubation on TSB.

[c] Colonies which were unmarked following final incubation.

[d] Difference between TSB and TSBS counts in per cent of TSB count.

TABLE 3. *Effect of incubation time in Trypticase soy broth plus NaCl (TSBS) on bacterial counts following transfer to Trypticase soy broth (TSB) for 48 hr at 32 C. Cultures were heated for 30 min at 62.8 C.*

Trial	Period of incubation on TSBS before transfer to TSB (hr)				
	0	6	18	24	48
1	85[a]	87	81	85	84
2	83	81	80	85	80
3	180	242	257	260	275
4	61	50	66	70	67
5	84	81	78	77	75
6	99	93	97	102	95
7	50	49	43	46	52
8	160	164	159	155	152
9	82	81	84	77	74
10	20	23	16	18	24
11	192	198	202	192	182
12	58	53	49	47	46
Means[b]	104.5	100.2	101.0	101.3	100.5

[a] Values expressed as colony counts.
[b] Means are not significantly different (P = 0.05).

trials were conducted to determine whether incubation in excess of 48 hr on TSBS could produce additional colonies. Data from duplicate sets of filters are shown in Table 4. Additional significant growth of cells did not occur on TSBS after 48 hr. On the other hand, the count following transfer of the 96-hr TSBS filter to TSB for an additional 48 hr of incubation indicated that no lethal effect was present even after extended periods of exposure to NaCl. No significant differences (P = 0.05) existed between 48 hr of TSBS incubation followed by 48 hr of TSB incubation and 96 hr of TSBS incubation followed by 48 hr of TSB incubation. In addition, although not shown in Table 4, significant differences were not found between controls incubated initially on TSB and final TSB transfer counts. The incubation on media containing 3% NaCl did not affect the injured population except for temporarily inhibiting colony formation while on that medium.

Effect of source of raw milk inocula. The foregoing results were obtained with milk inocula from mixed bulk milk obtained from one producer. To determine whether different sources of milk would produce different results due to variation in the natural flora, trials were repeated with milk from two other sources. Bulk milk from a single producer and comingled tanker milk from a number of producers were heated and examined with this technique. Results are presented in Table 5. In these trials there was a somewhat greater percentage of heat injury than in previous trials. Because no salt sensitivity tests were made initially on the mixed culture from these sources, it is possible that the cultures were more salt sensitive. Other data were essentially the same as in the previous work. The membrane transfer technique gave the same recovery as before with no statistically significant difference between TSB counts and transfer counts on the heated cultures. The effectiveness of the recovery

TABLE 4. *Effect of extended incubation periods on colony counts of cultures heated at 62.8 C for 30 min*

Trial no.	Colony counts on duplicate membrane filters after[a]			
	48 hr TSBS	96 hr TSBS	48 hr TSBS; +48 hr TSB	96 hr TSBS; +48 hr TSB
1	8	7	52	50
2	13	14	74	68
3	11	10	46	47
Means	10.6[b]	10.3[b]	58.6[c]	55.0[c]

[a] Abbreviations: TSB, Trypticase soy broth; TSBS, TSB plus NaCl.
[b] Means are not significantly different (P = 0.05).
[c] Means are not significantly different (P = 0.05).

TABLE 5. *Effect of source of raw milk inocula on counts of cultures heated at 62.8 C for 30 min*

Raw milk source	TSB count[a]	TSBS count[a]	Transfer count[b]	Recovery count[c]
Bulk milk				
Trial				
1	90	14	86	72
2	71	11	69	58
3	107	19	104	85
Means	89.3[d]	14.7	86.3[d]	71.6
Comingled tanker milk				
Trial				
1	114	14	109	95
2	123	19	99	80
Means	118.5[d]	16.5	104.0[d]	87.5

[a] Colony count per milliliter on membrane filter after 48 hr at 32 C. TSB, Trypticase soy broth; TSBS, TSB plus NaCl.
[b] Colony count per milliliter on membrane filter from TSBS after additional 48 hr of incubation on TSB.
[c] Colonies which were unmarked after final incubation.
[d] Within each source, means are not significantly different (P = 0.05).

technique was not affected by the milk source of the flora.

Figure 1 shows the colonial growth on a membrane filter after incubation on TSBS and after transfer and subsequent incubation on TSB. The perforation marking technique was satisfactory in allowing colonies to be differentiated as injured and uninjured. Other methods of marking colonies were tried without success; either stains washed off the colonies or the dyes inhibited the organism. The punch marks did not photograph well and cannot be seen in the photographs.

Types of organisms from heated cultures. Since the culture flora was originally from raw milk it was of interest to note the types of organisms which survived heat treatments uninjured and those that were injured and subsequently recovered. During the study, about 200 colonies were selected and transferred to litmus milk. The organisms that survived heating uninjured were gram-positive rods, small gram-negative rods, and gram-positive cocci. The gram-positive rods produced rapid proteolysis of litmus milk. Spores were observed microscopically, and the organism was identified as a *Bacillus* type. The small gram-negative rods were gas-producing, lactose fermenters in litmus milk and resembled coliform types. The gram-positive cocci resembled *S. faecalis* var. *liquefaciens* in litmus milk reactions producing acid, coagulation, reduction, and typical proteolysis. The injured and subsequently recovered organisms were all gram-positive cocci again resembling *S. faecalis* var. *liquefaciens* in litmus milk reactions. Pseudomonads and other psychrotrophic types normally associated with raw milk were not present in injured or uninjured groups and apparently were killed in the heating treatment. However, the incubation temperature of 32 C for broth cultures and membrane filters may have inhibited some of these types. This aspect is being investigated further.

In using a mixed flora it should be recognized that different types of organisms have different NaCl tolerances. However, the use of a mixed flora is thought to be important in investigations of this type since milk and other foods rarely contain pure cultures. Nevertheless, this may account partly for the fact that the transfer TSB count on filters previously incubated on TSBS tended to be lower than the original TSB count of a duplicate portion. Another possible source of variation may have been the heating temperature. Results reported in this study were obtained with pasteurization temperatures which were relatively severe, and perhaps lower temperatures would result in less lethality and more consistent injury counts.

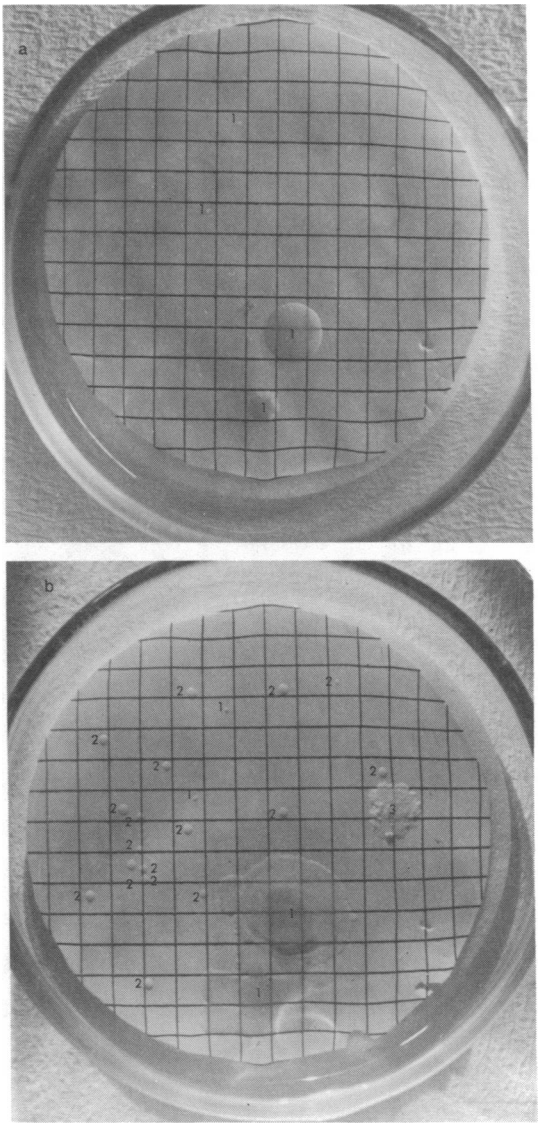

FIG. 1. a, *Membrane filter with colonies which developed during 48 hr incubation on TSBS.* b, *Same membrane filter as in a after transfer to TSB with an additional 48 hr of incubation. 1, Colonies which grew during initial incubation on TSBS; 2, colonies which grew after transfer to TSB; 3, mold.*

The need for a procedure to differentiate between injured and uninjured bacteria in a population has been pointed out recently by Speck and Cowman (8). The membrane filter technique described provides a method that facilitates further study of either uninjured or injured and revived organisms.

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