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# Enumeration of *Escherichia coli* in Frozen Samples After Recovery from Injury[1]

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More than 90% of the surviving cells of *Escherichia coli* NCSM were injured after freezing in water at −78 C. Injury was determined by the ability of cells to form colonies on Trypticase soy agar with yeast extract but not on violet red-bile agar and deoxycholate-lactose agar. Exposure of the injured cells to Brilliant Green-bile broth and lauryl sulfate broth prevented subsequent colony formation on Trypticase soy agar with yeast extract. The freeze-injury could be repaired rapidly in a medium such as Trypticase soy broth with yeast extract (TSYB). The repaired cells formed colonies on violet red-bile agar and deoxycholate-lactose agar and were not inhibited by Brilliant Green-bile broth and lauryl sulfate broth. At least 90% of the cells repaired in TSYB within 30 min at 20 to 45 C and began multiplication within 2 h at 25 C. When the cells were frozen in different foods, 60 to 90% of the survivors were injured. Repair of the injured cells occurred in foods during 1 h at 25 C, but generally repair was greater and more reproducible when the foods were incubated in TSYB. The study indicated that the repair of freeze-injured coliform bacteria should be accomplished before such cells are exposed to selective media for their enumeration.

Selective solid and liquid media are used for the enumeration of coliform bacteria from frozen as well as unfrozen foods (1, 2, 4, 6, 9). Cells of *Escherichia coli* are known to suffer sublethal injury from freezing (8, 10). One of the manifestations of freeze-injured *E. coli* cells is their increased sensitivity to many selective agents including those present in media used for their enumeration and detection (7, 10, 11). These selective agents presumably interfere with the reversible repair process of the injured cells and prevent their subsequent multiplication (11). How much the selective media adversely affect the enumeration of *E. coli* and other coliforms present in frozen food is not clearly known. Some workers reported less than 25% recovery of coliforms on violet red-bile agar (VRBA) (3), whereas other workers observed good recovery on both VRBA and deoxycholate-lactose agar (DLA) (6, 9). This study reports on the repair of freeze-injured cells of *E. coli* and their subsequent enumeration by selective media.

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## MATERIALS AND METHODS

**Freezing and thawing of cells.** *E. coli* strain NCSM, from the department stock collection, was grown on Trypticase soy broth supplemented with 0.3% yeast extract (TSYB) for 16 to 20 h at 35 C. The cells were harvested, washed, and suspended in sterile glass distilled water to a population of about $2 \times 10^8$ cells/ml. Cell suspensions in 10-ml portions were frozen in dry-ice acetone (−78 C) for 10 min and thawed at 8 C for 30 min. Details of these procedures have been described in a previous paper (10). Although other strains also were used in certain experiments, comparable results were obtained; consequently, only the data obtained for strain NCSM have been reported.

**Enumeration procedures.** Samples were serially diluted in sterile distilled water, and 0.1 ml of the dilution containing about 100 to 200 cells was used per plate. Samples were plated in triplicate on Trypticase soy agar containing 0.3% yeast extract (TSYA), VRBA, and DLA by surface and pour plate methods (10, 11). The plates were incubated at 35 C for 24 h after which the colonies were counted. For the most-probable-number (MPN) estimate, a total of five tubes in each of three successive decimal dilutions were used for each medium. A 1-ml portion from the appropriate dilution was used per tube containing 9 ml of Brilliant Green-bile broth (BGB)

or lauryl sulfate broth (LSB). The tubes were incubated for 48 h at 35 C, and the results were recorded by recommended methods (1, 2).

When the effects of the nonselective and selective media were studied, 1 ml of the frozen sample was first diluted in 9 ml of sterile water from which 1 ml was added to 9 ml of the respective medium to give about $10^6$ to $2 \times 10^6$ cells/ml. Enumeration was done by the methods described above. All the media were obtained from BBL and were prepared according to the manufacturer's direction.

**Effect of freezing E. coli in foods.** *E. coli* NCSM cells, harvested as described above, were suspended in four foods to a level of $2 \times 10^8$ cells/ml. Samples in 10-ml portions were frozen and thawed as before. The four foods used were: 10% reconstituted nonfat dry milk; meat broth (5% solids, prepared by boiling chopped veal and then filtering through cheese cloth); crab meat (1 part suspended in 9 parts of water and blended); and whole egg liquid. Milk, meat broth, and crab meat suspensions were autoclaved (121 C for 15 min). Whole egg liquid was removed from clean eggs (11).

Repair of injury was studied by adding 1 ml of the frozen food sample containing *E. coli* to either 9 ml of the respective food or to 9 ml of TSYB; the mixtures were incubated for 1 h at 25 C before enumeration. Frozen cells added to water and incubated as above were used as controls.

**Calculations and assumptions.** Percent dead equals {1 − (TSYA counts after freezing)/(TSYA counts before freezing)} × 100. Percent injured equals {1 − (VRBA or DLA counts)/(TSYA count)} × 100.

**Repair.** Any increase in VRBA (or DLA) counts but not in TSYA counts at any time was assumed to be due to repair of the injured cells.

**Multiplication.** Simultaneous increase in counts on TSYA and VRBA (or DLA) was assumed to be due to cell multiplication.

## RESULTS

**Effect of freezing on the detection of E. coli.** *E. coli* NCSM in aqueous suspension was enumerated by surface plating on TSYA, VRBA, and DLA before and after freezing. Unfrozen cells showed essentially comparable counts in the three media (Table 1). After freezing, about 40% of the cells failed to form colonies on TSYA and were assumed dead. More than 90% of the survivors formed colonies on TSYA, but not on VRBA and DLA. These cells were considered injured.

The frozen cells were enumerated on three solid media by surface and pour plate methods (Table 2). Counts on TSYA varied little among methods. However, counts on VRBA and DLA showed wide differences; much higher numbers of cells were detected by surface plating than by pour plating on either of the two media. In both methods injured cells failed to form colonies on the selective solid media, but not on the nonselective solid medium.

The effects of nonselective and selective liquid media on injured cells were determined by adding frozen cells to TSYB, BGB, and LSB at 35 C. After mixing, samples were withdrawn within 1 to 2 min, serially diluted, and surface plated on TSYA, VRBA, and DLA. Cells suspended in water were used as controls (Table 3). Counts from water on VRBA and DLA indicated that more than 90% of the survivors were injured. Exposure to the selective liquid media caused death in about 60% of the surviving cells since about only 40% were detected from these media on TSYA. No such effect was observed with cells suspended in TSYB. Counts on VRBA and DLA from all three liquid media were higher than the counts from the control. This could be due to repair of some of

TABLE 1. *Effect of freezing on the survival and injury of E. coli cells*[a]

| Plating medium | Plate counts | |
|---|---|---|
| | Unfrozen | Frozen |
| TSYA | $21 \times 10^7$ | $12 \times 10^7$ |
| VRBA | $19 \times 10^7$ | $11 \times 10^6$ |
| DLA | $18 \times 10^7$ | $10 \times 10^6$ |

[a] Results are the average of three trials.

TABLE 2. *Effect of plating methods on the recovery of frozen E. coli cells*[a]

| Plating medium | Plate counts | |
|---|---|---|
| | Surface plating | Pour plating |
| TSYA | $96 \times 10^6$ | $88 \times 10^6$ |
| VRBA | $85 \times 10^5$ | $15 \times 10^5$ |
| DLA | $74 \times 10^5$ | $11 \times 10^5$ |

[a] Results are the average of three trials.

TABLE 3. *Effects of selective and nonselective suspending media on the recovery of frozen E. coli cells*[a]

| Plating medium | Percent[b] detected from | | | |
|---|---|---|---|---|
| | Water (control) | BGB | LSB | TSYB |
| TSYA | 100 | 40 | 45 | 113 |
| VRBA | 9 | 18 | 18 | 26 |
| DLA | 6 | 13 | 17 | 23 |

[a] Results are the average of three trials.
[b] As percentage of counts on TSYA from water.

the injured cells in the liquid media. Unfrozen cells of this culture did not show susceptibility to these two selective media (data not presented).

To study the effect of incubation of the injured cells for a longer period in liquid media, frozen cells were suspended in tempered TSYB and BGB and water ($10^6$ cells/ml) and incubated at 35 C for 180 min. Another sample was first incubated in TSYB ($10^7$ cells/ml) for 60 min at 25 C and then transferred to BGB ($10^6$ cells/ml) at 35 C and incubated for another 120 min. At selected intervals, samples were withdrawn, serially diluted, and surface plated on TSYA and VRBA. The results in relation to the initial counts in frozen samples ($10^8$ cells/ml) are presented in Fig. 1. During the first 30 min in TSYB, the cell counts on VRBA increased rapidly, which was a reflection of repair. Between 30 to 60 min the cells started multiplication as indicated by an increase in counts in both plating media. Cells in BGB showed a sharp initial reduction on TSYA counts but a simultaneous increase in VRBA counts (Fig. 1b). We assumed that cells with various degrees of injury were present in the population; those which were less injured were not susceptible to BGB and thus showed repair, whereas those which were relatively more injured were susceptible and showed loss in viability. However, when the cells were allowed to repair in TSYB for 60 min at 25 C and then transferred to BGB at 35 C, the repaired cells showed resistance to BGB. Population levels in BGB after 180 min were higher when the cells were first allowed to repair in TSYB rather than when they were transferred to BGB directly.

**Effect of temperature of incubation on the repair and growth of injured E. coli.** The amount of repair in TSYB as a function of incubation temperature was studied by incubating the injured cells in tempered TSYB at temperatures between 0 to 45 ± 1 C. Samples were withdrawn at 30 and 60 min after incubation, diluted, and surface plated on TSYA and VRBA. For initial counts, cells suspended in water (control) were plated. The results (Fig. 2) indicate that the number of uninjured cells increased from an initial 6% (control) to more than 90% when incubation was for 30 min at temperatures between 20 to 45 C; this remained essentially the same from 30 to 60 min. At 15 C and below, very little repair occurred in 30 min; after 60 min about 65% of the cells repaired at 15 C. At 35 and 45 C cell multiplication was initiated at 60 min (data not presented).

For proper enumeration of *E. coli* the period for repair should be completed before cell multiplication starts. The time of initiation of multiplication was determined by incubating

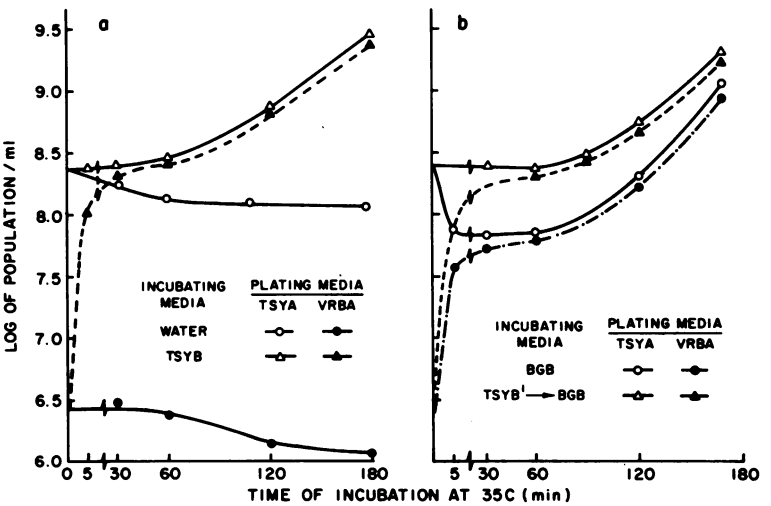


FIG. 1. *a, Effect of incubation of frozen E. coli in water and in TSYB with 0.3% yeast extract on repair and growth at 35 C. For other explanations, see Materials and Methods. b, Effect of incubation of frozen E. coli in BGB on survival, repair, and growth at 35 C. TSYB¹ indicates studies in which the freeze-injured cells were incubated in TSYB for 60 min at 25 C prior to their transfer to BGB and subsequent incubation for another 120 min at 35 C.*

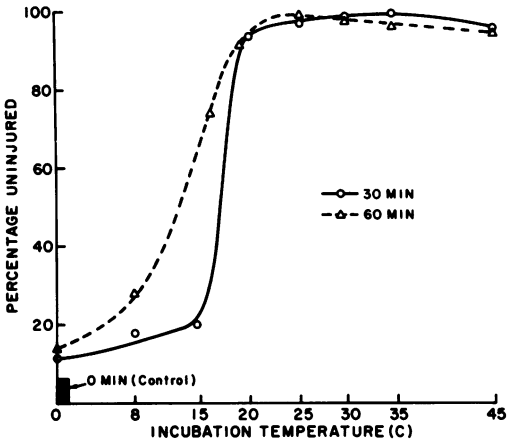


FIG. 2. *Injury repair by frozen E. coli in TSYB as influenced by incubation temperature. At 0 min there was about 6% uninjured cells (plate counts on VRBA per plate counts on TSYA) × 100; at 30 and 60 min the percentage of uninjured cells increased (an indication of repair).*

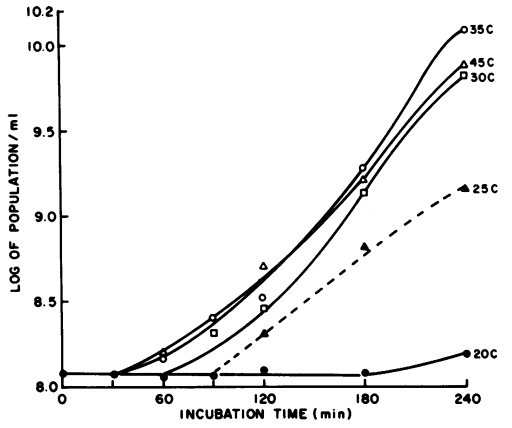


FIG. 3. *Initiation of cell multiplication in freeze-injured E. coli as influenced by incubation temperatures. The frozen cells were incubated in TSYB and plated on TSYA.*

the injured cells in tempered TSYB between 20 to 45 C for 240 min and plating on TSYA (Fig. 3). Cell multiplication started between 30 to 60 min at 35 and 45 C, 60 to 90 min at 30 C, 90 to 120 min at 25 C, and 180 to 240 min at 20 C.

**Death, injury, and repair of E. coli frozen in foods.** *E. coli* cells after freezing and thawing in sterile milk, liquid whole egg, meat broth, and crab meat were serially diluted and surface plated on TSYA and VRBA. The results (Table 4) indicated that cell death was 40% or higher in foods other than milk. However, in all foods 75% or more of the survivors were injured. After 1 h of incubation at 25 C in the respective food (water for control), the number of injured cells decreased in all four foods, suggesting that the cells had repaired. But with the exception of milk, the amount of repair in the other three foods was low, since about 50% of the cells remained injured. Repair in TSYB under the same conditions was much higher in all cases including the control.

The enumeration efficiencies of the surface and pour plate procedures on TSYA, VRBA, and DLA, and by MPN in BGB and LSB were observed on unfrozen, frozen, and repaired *E. coli* cells in liquid whole egg. TSYA counts by surface plating were assumed to be 100% (Table 5). MPN estimates in BGB for the stressed and repaired cells produced good recovery. However, in individual tests wide variation and poor reproducibility occurred by the MPN method. For example, with TSYB-repaired cells the detection on BGB varied between 40 to 200%, with an average of 99%. Among the two plating methods, surface plating definitely produced much higher detection, especially after the 1 h of TSYB treatment. This method also yielded reproducible results with the unstressed and repaired cells on VRBA. Similar results were obtained with milk, meat broth, and crab meat.

## DISCUSSION

A large percentage of *E. coli* and other coliform bacteria in frozen materials may be present in an injured state. Microbiological analysis of frozen foods for coliform bacteria on selective media may fail to detect these injured cells, and could affect the evaluation of the sanitary quality of such foods. The injured bacteria are able to repair rapidly in a nonselective nutritionally rich liquid media, such as TSYB. The repaired cells became insensitive to

TABLE 4. *Death, injury, and repair of frozen E. coli cells in several kinds of food*[a]

| Suspending medium | Dead (%) | Percent injured at[b] | | |
|---|---|---|---|---|
| | | 0 h | 1 h at 25 C in suspending medium | 1 h at 25 C in TSYB |
| Water (control) .... | 55 | 95 | 95 | 10 |
| Milk ..... | 15 | 75 | 20 | 10 |
| Liquid whole egg ... | 42 | 80 | 50 | 18 |
| Meat broth (5%) ... | 40 | 86 | 40 | 15 |
| Crab meat (1:10) .. | 44 | 78 | 50 | 16 |

[a] Results are the average of four trials.
[b] As percentage of survivors.

TABLE 5. *Enumeration of E. coli in liquid whole egg by direct plate count and by MPN*

| Plating method | Percent[a] detected | | | |
|---|---|---|---|---|
| | Unfrozen | Frozen | | |
| | | Unrepaired (0 h) | Repaired 1 h at 25 C in egg | Repaired 1 h at 25 C in TSYB |
| Surface plating | | | | |
| TSYA | 100 | 100 | 100 | 100 |
| VRBA | 88 | 12 | 58 | 80 |
| DLA | 69 | 10 | 61 | 82 |
| Pour plating | | | | |
| TSYA | 90 | 88 | 89 | 92 |
| VRBA | 53 | 10 | 33 | 66 |
| DLA | 38 | 2 | 27 | 51 |
| MPN (5 tubes) | | | | |
| BGB | 145 | 91 | 95 | 99 |
| LSB | 68 | 28 | 110 | 158 |

[a] As percentage of counts on TSYA surface plates for each kind of cells. Results are the average of five trials.

the selective media and are able to multiply. Thus, for better enumeration of coliforms from frozen samples, a period of treatment in a nonselective liquid medium seems to be necessary. Though food materials can aid in repair, much higher and more uniform amounts of repair were obtained in TSYB. For a generalized procedure, treatment of the frozen samples in TSYB is necessary for accurate enumeration of viable cells. The rate of repair and the initiation of multiplication in TSYB are dependent on the time and temperature of treatment. To separate repair from multiplication, time and temperature of such treatment should be standardized. In our laboratory with laboratory strains of *E. coli*, 1 h of treatment at 25 C in TSYB has been found to be optimum.

A greater degree of variability in the detection of *E. coli* by the MPN was observed in this study; this was also observed with unfrozen cells (11). Several other workers have reported poor reproducibility of the MPN method and have preferred the use of direct plating on VRBA and DLA for the enumeration of coliforms from foods (5, 6, 9). Direct plating on VRBA has been reported not only to give higher reproducible results and quantitative isolation of low numbers of coliforms from foods, but also to require less time (4, 5). Other workers have found that VRBA and DLA are equally satisfactory (5, 9). We obtained relatively higher and more reproducible enumeration of *E. coli* on VRBA than on DLA from both unfrozen and frozen samples, especially by the pour plate method with certain sensitive strains (11). Surface plating reduced the inhibitory effects of both selective solid media and allowed higher detection of *E. coli*. This was observed more with VRBA than DLA. Studies currently are in progress to evaluate the effect of the repair process on coliform counts of naturally contaminated foods.

ACKNOWLEDGMENT

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LITERATURE CITED

1. American Public Health Association. 1967. Standard methods for the examination of dairy products, 12th ed. Amer. Pub. Health Ass., Inc. New York. p. 53–67.
2. American Public Health Association. 1966. Recommended methods for the microbiological examination of foods, 2nd ed. Amer. Pub. Health Ass., Inc., New York. p. 133–141.
3. Gunderson, M. F., and K. D. Rose. 1948. Survival of bacteria in a precooked fresh frozen food. Food Res. **13**:254–263.
4. Hall, H. E. 1964. Methods for isolation and enumeration of coliform organisms, p. 13–19. *In* K. H. Lewis and R. Angelotti (ed.), Examination of foods for enteropathogenic and indicator bacteria. U.S. Dept. of Health, Education and Welfare. Public Health Service Publication No. 1142. Washington, D.C.
5. Kereluk, K., and M. F. Gunderson. 1959. Studies on the bacteriological quality of frozen meat pies. II A comparison of methods for the enumeration of coliforms. J. Milk Food Technol. **22**:176–178.
6. Machala, W. E. 1961. A bacteriological investigation of frozen foods in the Oklahoma area. J. Milk Food Technol. **24**:45–48.
7. Maxcy, R. B. 1970. Nonlethal injury and limitation of recovery of coliforms organisms on selective media. J. Milk Food Technol. **33**:445–448.
8. Moss, C. W., and M. L. Speck. 1966. Release of biologically active peptides from *Escherichia coli* at subzero temperature. J. Bacteriol. **91**:1005–1111.
9. Nickerson, J. T. R., G. J. Silverman, M. Salberg, D. W. Duncan, and M. M. Joselow. 1962. Microbial analysis of commercial frozen fish sticks. J. Milk Food Technol. **25**:45–47.
10. Ray, B., and M. L. Speck. 1972. Repair of injury induced by freezing *Escherichia coli* as influenced by recovery medium. Appl. Microbiol. **24**:258–263.
11. Ray, B., and M. L. Speck. 1972. Discrepancies in the enumeration of *Escherichia coli*. Appl. Microbiol. **25**: 494–498.