Repair of Injury Induced by Freezing *Escherichia coli* as Influenced by Recovery Medium

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Freezing an aqueous suspension of *Escherichia coli* NCSM at −78 C for 10 min, followed by thawing in water at 8 C for 30 min, resulted in the death of approximately 50% of the cells, as determined by their inability to form colonies on Trypticase soy agar containing 0.3% yeast extract (TSYA). Among the survivors, more than 90% of the cells were injured, as they failed to form colonies on TSYA containing 0.1% deoxycholate. Microscope counts and optical density determinations at 600 nm suggested that death from freezing was not due to lysis of the cells. Death and the injury were accompanied by the loss of 260- and 280-nm absorbing materials from the intracellular pool. Injury was reversible as the injured cells repaired in many suitable media. The rate of repair was rapid and maximum in a complex nutrient medium such as Trypticase soy broth supplemented with yeast extract. However, inorganic phosphate, with or without $MgSO_4$, was able to facilitate repair. Repair in phosphate was dependent on the pH, the temperature, and the concentration of phosphate.

Cellular injury of gram-negative bacteria induced by freezing and thawing and other sublethal stresses has been reported (6, 8–12, 16). The various manifestations of the injury are impaired permeability, activation of certain enzymes, degradation of ribosome, and increased sensitivity to many selective agents (3, 4, 6, 13, 15). The injury is reversible because the injured cells are able to repair when exposed to a suitable environment (4–7, 9). During repair, the nutrient requirements are more exacting, and certain small peptides and amino acids expedite the repair of injury induced by freezing (4, 6). Certain simpler compounds are also reported to facilitate repair of freeze-injury in *Salmonella anatum* (9). This study reports nutrient requirements of *Escherichia coli* for the repair of freeze injury.

MATERIALS AND METHODS

Test organism. *E. coli* NCSM was propagated weekly on nutrient agar slants and stored at 4 C. Trypticase soy broth containing 0.3% yeast extract (TSY broth) was inoculated with this culture and incubated at 35 C overnight. The cells were centrifuged for 10 min at 2,500 × *g*, washed three times with an amount of sterile water equal to the volume of the growth medium, and then suspended in water

to contain about 2×10^8 cells/ml.

Freezing and thawing. The cell suspension in 10-ml quantities was frozen statically in dry ice-acetone (−78 C) for 10 min, unless otherwise mentioned. The frozen sample was then thawed in a circulating water bath at 8 C for 30 min.

Plating medium. Nonselective and selective media were used. The nonselective medium was Trypticase soy agar containing 0.3% yeast extract (TSYA), and the selective medium was TSYA with 0.1% sodium deoxycholate (TSYDA). TSYA was sterilized at 121 C for 15 min, and TSYDA was prepared by steaming for 30 min. The pH of the media was between 7.1 to 7.3. The media were poured into petri dishes and allowed to dry at 25 C for 48 hr before use.

Suspending medium. TSY broth, minimal salt broth (M broth), and different components of the M broth, singly or in combinations, and sodium pyruvate (0.1%) were used as suspending media. The composition of M broth was K_2HPO_4, 0.5%; $MgSo_4$, 0.01%; $(NH_4)_2SO_4$, 0.25%; NaCl, 0.01%, and glucose, 0.2%. In preparing this medium, glucose was dissolved in 20% of the water, and all other components were dissolved in 50% of the water; these portions were sterilized separately (121 C for 15 min), cooled, and then mixed. After adjusting the medium to pH 7.0, the final volume was adjusted with sterile water to 90% of the final volume. Then 9 ml of this was used with 1 ml of the cell suspension to obtain the required concentration of ingredients. When indi-

vidual ingredients of M broth were tested, 10-fold concentrated aqueous solutions were prepared and 1 ml was mixed with 8 ml of H_2O, followed by the addition of 1 ml of cell suspension. Similarly, 1 ml of 1% sodium pyruvate (pH 7.0) was used with 1 ml of cell suspension and 8 ml of H_2O for the repair study.

Injury repair studies. To study repair, 1 ml of thawed cells was suspended in 9 ml of sterile water. From this, 1-ml portions were inoculated into 9 ml of suspending medium (at 8 C, unless otherwise mentioned). For a control, 9 ml of sterile water was used. The suspensions were incubated at 20 C in a water bath. Samples were withdrawn at intervals, serially diluted, and 0.1 ml was surface-plated in triplicate on TSYA and TSYDA. The plates were incubated at 35 C for 24 hr, and the colonies were enumerated. Further incubation did not increase the colony counts significantly.

The amount of death was determined from the differences in colony-forming units (CFU) on TSYA before and after the freezing. The amount of injury was estimated from the differences in CFU on TSYA and TSYDA at any particular sampling. The plated dilutions were selected so that approximately 100 to 300 viable cells were added to each plate before and after freezing.

RESULTS

Death and injury due to freezing. E. coli cells frozen by using different cooling agents showed different levels of killing (Table 1). Although the range of death at each temperature varied considerably, the average maximum cell death occurred as a result of freezing in dry ice-acetone (56%). Ethylene glycol freezing produced a minimum average amount of killing. Among the survivors, a large percentage of cells, usually greater than 90%, were injured at all three freezing temperatures. The amount of injury at each temperature was more consistent than the amount of death. The results in Table 2 show that although TSYA counts were reduced approximately 50% after freezing, essentially there was no change in microscope count and in optical density at 600 nm. Cell death, as measured by the differences in colony counts on TSYA before and after freezing, was not related to lysis of the cells. After freezing, a considerable amount of 260- and 280-nm absorbing materials appeared in the suspending medium which presumably leaked from the damaged cells.

Repair and growth of the freeze-injured cells. The ability of injured cells to repair and grow was observed by suspending injured cells in TSY broth and M broth. During incubation at 20 C, samples were plated on TSYA and TSYDA at 1-hr intervals. For a control, unfrozen cells were also tested in the same manner. The results (Fig. 1) showed that the

unfrozen cells from both TSY and M broth initially had approximately the same counts on TSYA and TSYDA, and they started to multiply after 3 hr. The frozen cells in both liquid media intially showed approximately 90% lower count on TSYDA. The counts on TSYDA increased rapidly up to 1 hr and then remained essentially constant until the cells had been in TSY broth for 3 hr and in M broth

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

Method of freezing	Death (%)		Injury (%)[b]	
	Average	Range	Average	Range
Liquid nitrogen (−196 C)	49	24 to 60	94	87 to 98
Dry ice-acetone (−78 C)	56	34 to 66	97	94 to 99
Ethylene glycol (−20 C)	38	15 to 53	94	91 to 98

[a] The cell suspensions in water were frozen by holding 5 min for liquid nitrogen, 10 min for dry ice-acetone, and 2 hr for ethylene glycol in a freezer. Thawing was done following freezing in a circulating water bath at 8 C for 30 min (time for complete thawing). The results are the average of four experiments. With unfrozen cells, the count on TSYDA was 15% lower than the count on TSYA.

[b] Data are for cells surviving freezing.

TABLE 2. *Effect of freezing E. coli cells on plate count, microscope count, and optical density (OD)*[a]

Determination	Fresh	Frozen
Plate count (/ml)		
TSYA	245×10^7	127×10^7
TSYDA	210×10^7	54×10^6
Microscope count (/ml)	300×10^7	290×10^7
OD at 600 nm	2.6	2.7
OD of supernatant at		
280 nm	0.058	0.12
260 nm	0.082	0.27

[a] Appropriate dilutions were used for plating, microscope count, and OD at 600 nm. Plating was done on Trypticase soy agar with 0.3% yeast extract (TSYA) and TSYA + 0.1% sodium deoxycholate (TSYDA). Microscope count was done in a Petroff-Hauser counting chamber. OD at 600 nm was taken in a Bausch and Lomb Spectronic 20 colorimeter. OD at 260 nm and 280 nm was taken in a Bausch and Lomb Spectronic 600 spectrophotometer. To determine the OD at 260 nm and 280 nm, the cell suspension was centrifuged twice at 12,000 × g for 15 min in a Sorvall RC 2-B automatic refrigerated centrifuge in a SS-34 rotor (Ivan Sorvall Inc; Newton, Conn.), and the supernatant fluid was filtered through a 0.45-μm membrane filter (Millipore Corp.).

for 4 hr. During this period, counts on TSYA remained about the same. The increase in counts on TSYDA is due to repair of the injured cells in the TSY and M media. The amount of repair was greater in TSY, and multiplication of the frozen cells started after 3 hr in TSY broth and after 4 hr in M broth. The greater amount of repair of injury and increased growth rates of cells in the TSY was presumably a reflection of the available nutrients in this medium.

To determine the rate of repair from freeze injury more closely, the samples were plated from TSY broth, M broth, and water (control) at shorter intervals up to 120 min (Fig. 2). In both TSY and M at 20 C, the number of cells detected by TSYDA increased rapidly up to 20 min and slowed until 2 hr. Cells suspended in water showed very little repair. The counts on TSYA in all three suspending media remained nearly the same during the 2-hr test period. Similar studies with 0.1% sodium pyruvate at pH 7.0 and 20 C also indicated rapid rate of repair (data not presented).

Freeze-injury repair in different components of the M broth and in other media. The ability of the individual components to aid in repair was studied by suspending the freeze-injured cells in each of the ingredients in the same concentration and pH as in the M medium. The inoculated test solutions were incubated at 20 C, and samples were plated at time intervals to 2 hr. Among the different components, only phosphate (K_2HPO_4) was able to aid in the repair process (Fig. 3a). However, both the rate of repair and the total amount of repair after 2 hr were much lower in phosphate than in the M medium. $MgSO_4$,

$(NH_4)_2SO_4$, NaCl, and glucose, when present separately, did not help in the repair process significantly. Each of the last four ingredients was mixed with phosphate to determine any possible additive effect on repair of injury and was tested as before (Fig. 3b). The results indicated that $(NH_4)_2SO_4$ and NaCl had no such effect. Glucose provided some additive effect on the rate of repair, but not on the total amount of repair after 2 hr. However, $MgSO_4$ added to phosphate increased both the rate and the amount of repair over that in phosphate alone. From the results, it appeared that phosphate and $MgSO_4$ were the two essential components necessary for the freeze-injury repair in M broth. The relative effectiveness of several recovery media, including sodium pyruvate, is presented in Fig. 4. After 2 hr at 20 C, about 90% of the cells were repaired in TSY. In M medium, phosphate, and pyruvate, uninjured cells after 2 hr were 70, 40, and 50%, respectively. A combination of phosphate and $MgSO_4$ produced about the same amount of repair as the M medium.

Effect of pH, phosphate concentration and temperature on phosphate repair of freeze injury. Freeze-injured cells were suspended in phosphate solution (0.5% K_2HPO_4) at pH values from 4 to 10. The test solutions were incubated at 20 C, and samples were plated on TSYA and TSYDA at 0 hr and after 2 hr. The results show (Fig. 5) that, during the 2-hr test period, the counts on TSYA remained essentially the same at each pH. Counts on TSYDA were the same at all pH values at 0 hr; after 2 hr the counts on TSYDA increased between pH 6 to 10. Maximum increase was observed in the pH 8 to 9 range.
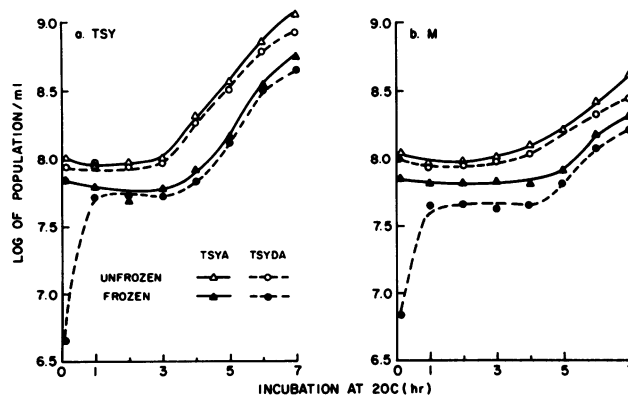

FIG. 1. *Growth of unfrozen and frozen E. coli in Trypticase soy yeast extract broth (TSY) and minimal broth (M) at 20 C. Samples were surface plated on TSYA (TSY + agar) and TSYA + 0.1 sodium deoxycholate (TSYDA).*

The freeze-injured cells were suspended in different concentrations of K_2HPO_4 at pH 7.0, incubated at 20 C, and tested for repair at 0 hr and 2 hr as described above. The results in different experiments were not entirely consistent, but maximum repair occurred generally at about 0.5 to 0.75% concentration (data not presented).

The influence of temperature on the repair of freeze injury was studied by suspending the injured cells in tempered (0 to 45 C) phosphate solution (0.5% K_2HPO_4, pH 7.0). The test solutions were incubated for 2 hr and observed for repair. TSYA counts were the same at 0 and 2 hr at all temperatures, except at 0 and 45 C (Fig. 6). Some reduction in counts occurred at 45 C after 2 hr. The counts on TSYDA were minimum at 45 C at 0 hr. The cells showed repair above 15 C with maximum repair between 25 to 35 C.
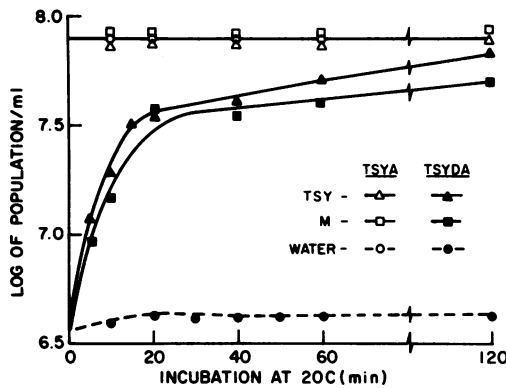

FIG. 2. *Rate of repair of freeze-injured E. coli in different suspending media at 20 C. Legends are same as in Fig. 1.*

DISCUSSION

Injury in *E. coli* after freezing can be manifested by the response of cells to a restricted supply of nutrients or to the presence of compounds in media that impose additional stresses on the cells. The cause of such injury must be understood if detection of debilitated cells in foods is to be accomplished.

E. coli NCSM, after freezing and thawing in a water suspension, consisted of three kinds of cells: dead, injured, and uninjured. The cells which lost their ability to multiply in a nutritionally rich medium, such as TSYA, were considered dead. However, these cells maintained their cell integrity as the microscope counts and optical density of the frozen and unfrozen samples remained essentially the same. Similar observations have been reported before (4, 6). Among the survivors of freezing and thawing, more than 90% of the cells failed to multiply in a medium which was nutritionally adequate but contained deoxycholate. Gram-negative bacteria, after freezing and freeze-drying, were known to develop sensitivity to many surface active agents including deoxycholate at a concentration which otherwise is ineffective to uninjured cells (9, 10, 11). This increased sensitivity to surface active agents thus could be used for measuring injury induced by sublethal stresses in gram-negative bacteria. Some workers have used minimal agar media for measuring injury and observed a far lower percentage of injury (3, 4, 12, 14). This may be due to the possibility that the latter method measures only "metabolic injury," whereas the former method measures injury in a structure which normally protects cells from bactericidal effect of the surface ac-
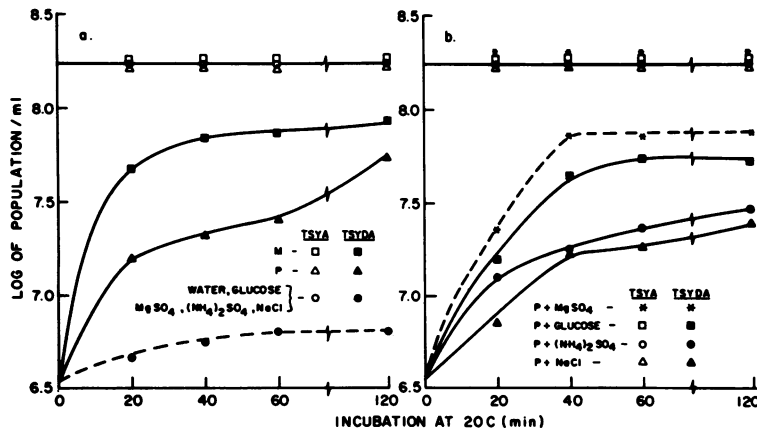

FIG. 3. *Repair of freeze-injured E. coli in minimal broth (M), K_2HPO_4 (P) and other components of M at 20 C. The ingredients were used in single (a) or in combination with P (b). Legends are same as in Fig. 1.*
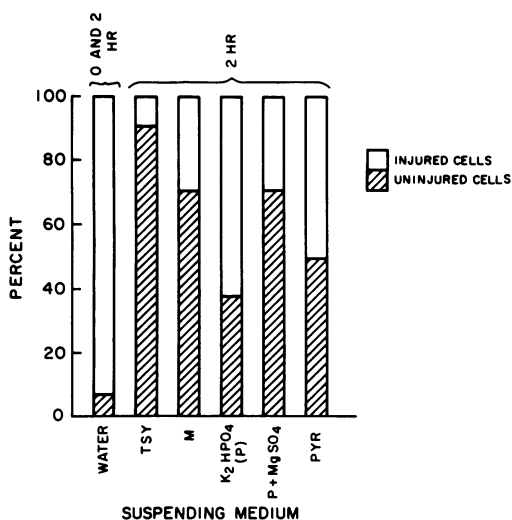
FIG. 4. *Percentage of injured and uninjured E. coli suspended in different media for 2 hr at 20 C. Legends are same as in Fig. 1 and 3. Pyr is 0.1% sodium pyruvate.*
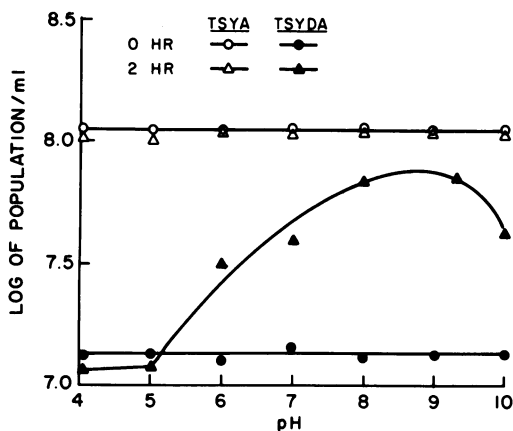

FIG. 5. *Effect of pH on repair of freeze-injured E. coli suspended in K_2HPO_4 for 2 hr at 20 C. Legends are same as in Fig. 1.*

FIG. 6. *Effect of temperature on repair of freeze-injured E. coli suspended in K_2HPO_4 for 2 hr at 20 C. Legends are same as in Fig. 1.*

tive agents.

One of the manifestations of freezing and thawing of *E. coli* cells is their impaired permeability, as indicated by leakage of various cellular components including 260- and 280-nm absorbing materials. Impairment of the permeability barrier allows leakage of cellular components such as proteins, peptides, amino acids, and nucleic acids, and entrance of various ions from the environment into the cells (2-4, 6, 15). The changes which facilitate leakage of 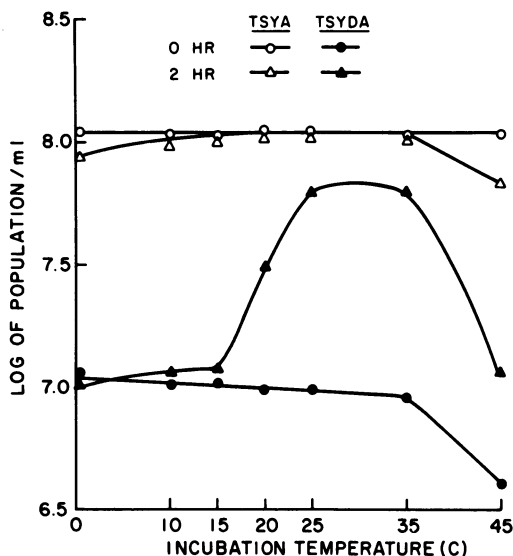various components essential for cellular integrity have been believed to be the cause of injury and subsequent death of frozen gram-negative bacteria (4, 6).

The injury of *E. coli* induced by freezing and thawing was reparable. The repaired cells regained the ability to multiply in a medium containing deoxycholate. The cells were able to repair their injury at a fairly rapid rate in the presence of complex as well as simple compounds. A considerable percentage of cells was able to repair injury under conditions which were insufficient to support growth, such as exposure to K_2HPO_4 and pyruvate. However, a higher initial rate and a maximum amount of repair, as well as a shorter lag for subsequent multiplication, were observed in a nutritionally rich medium. These results indicated that complex nutrients were probably required for maximum repair of damage to freeze-injured cells. Several other workers have also reported the ability of various complex nutrients to repair metabolic damage of frozen gram-negative bacteria (4-8, 9, 12). The repair process of freeze injury in *E. coli* appeared to commence immediately after the cells were exposed to nutrients, including K_2HPO_4 and pyruvate. Also, the rate was very rapid initially and was completed essentially in 1 to 2 hr at 20 C. Similar observations have been reported for frozen and freeze-dried *Salmonella anatum* (9, 10). Using freeze-dried *E. coli* (11) or heat-shocked *Salmonella typhimurium* (16), much

longer periods were required for completion of repair.

The repair observed in the presence of K_2HPO_4 was dependent on the pH, temperature, and phosphate concentration and, thus, might conceivably involve the need for some essential enzymatic activity. K_2HPO_4 and probably pyruvate could be utilized by the enzyme(s) for the synthesis of energy-rich compounds, which may be required for the repair process. Mg^{2+} might be necessary for the activity of enzyme involved in the repair process or for maintaining stability of the cell wall, or both. Mg^{2+} has been reported to be necessary for the stability of the cell wall, cell membrane, and ribosomes of normal and cold-shocked gram-negative bacteria (1, 13). However, glucose, when present alone, was ineffective in the repair of freeze injury in E. coli. This could be due at least partially to the damage of some components of glucose transport system from freezing. It is known that enzymes involved in transport are released from sublethally stressed gram-negative cells (4).

In conclusion, it appears that freezing and thawing produce some kind of damage to the cell envelope of E. coli and render them sensitive to deoxycholate. This damage in some cells (injured cells) is reversible and can be repaired in the presence of simple compounds such as K_2HPO_4. The repair process in K_2HPO_4 appears to involve enzymatic activity.

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