

Microbial Injury Reviewed For the Sanitarian

"Since the potential exists for the injury of microorganisms during food processing and the subsequent repair of these injured cells in foods, it is imperative that food microbiologists and sanitarians be aware of the phenomenon of microbial injury and repair."

Knowledge of stress-induced microbial injury is important to food microbiologists and sanitarians because injured cells are not detected on the usual bacteriological selective media. Failure to detect injured microorganisms present in food may be hazardous, because under the proper conditions, the injured cells may repair the injury, grow and produce food poisoning. The site of injury,

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* * Abbreviations used in text: Tryptic Soy agar, TSA; TSA + 7% NaCl, TSAS; TSA + 1% sodium pyruvate, TSAP; TSA + 9% NaCl, TSAX; Tryptone Soya agar, T(one)SA; T(one)SA + 0.075% sodium deoxycholate, T(one)SAD; Levine's Eosin Methylene Blue agar + 2% NaCl, EMBS; Trypticase Soy agar, T(ase)SA; Trypticase Soy broth, T(ase)SB; colony forming unit, CFU.

regardless of the type of stress, is usually considered to be the microbial membrane; concomitant with membrane damage, cellular constituents leak out of the cell which, if repair is prevented, eventually leads to death. RNA synthesis is necessary for repair of injured cells but additional requirements may be essential to the repair process depending on the microbial species in question. In order to determine the microbiological status of a processed food, detection of both injured and non-injured cells is necessary; therefore, the injured microorganisms must be resuscitated prior to placing them in a selective medium. The sample, containing both injured and non-injured microorganisms, is placed in a non-selective medium for a short period to effect repair of the injured cells and is then placed in a selective medium. Examples of resuscitation media used for food borne pathogens are described.

Microbial injury caused by sub-lethal stress is a phenomenon that every sanitarian—and indeed all concerned with food safety—should understand and be alert for in the surveillance of the food and water supply. Microorganisms, are considered to be injured when they: 1) cannot grow on a medium which is otherwise satisfactory for the growth of non-injured cells; and 2) can recover under appropriate culture conditions and resume normal growth and biochemical activity. The laboratory technician, using a particular medium on which injured cells cannot grow, may report that a heated food is free of a particular pathogen when actually it contains large numbers of injured organisms.

Injured pathogens undetected in foods present a potential hazard because these injured cells could repair, grow, and produce food poisoning. Indeed, detection media now used to determine the presence of food and waterborne pathogens will not support the growth of injured cells. These media have no apparent effect on non-injured cells.

There appears to be no documented case in which microorganisms, injured during food processing, have repaired the injury and subsequently caused food poisoning. However, stress induced injury and its repair have been demonstrated repeatedly in the laboratory under conditions simulating actual food processing operations (8,11). Therefore, the potential for food poisoning caused by food products containing injured but repaired microorganisms is very real. However, it is difficult to distinguish between this type of food poisoning (or food spoilage, for that matter) and that caused by post processing contamination of the food with a similar microorganism. Since the potential exists for the injury of microorganisms during food processing and the subsequent repair of these injured cells in foods, it is imperative that food microbiologists and sanitarians be aware of the phenomenon of microbial injury and repair.

Stresses That Induce Microbial Injury

Certain environmental and chemical stresses applied at sub-lethal levels can produce injury in micro-

organisms (4,5,7). Such stresses include (a) heat, (b) refrigeration temperatures, (c) drying, (d) irradiation, (e) changes in nutritional environment, (f) chemicals, such as sanitizers, food preservatives, and acids, (g) freeze-drying, and (h) freezing and thawing. Many of the unit operations of food processing, as well as combinations of them, may also lead to injury.

Many species of bacteria, yeast, and fungi show injury when subjected to sub-lethal stress. Bacteria important in public health situations which show stress-induced injury include: *Escherichia coli*, *Salmonella* species, *Vibrio parahaemolyticus*, *Yersinia enterocolitica*, *Staphylococcus aureus*, *Bacillus cereus*, *Clostridium botulinum*, and *C. perfringens*.

Detection of Injured Cells

Injured cells escape detection because they are sensitive to inhibitory compounds present in some bacteriological media. For example, sodium chloride is added to media used to isolate *Staphylococcus aureus* from food products because the microorganism is resistant to high levels of salt (>10%); however, after injury, the cells lose their salt tolerance. A complete picture of the extent of injury in *S. aureus* can be obtained by comparing counts on a medium lacking NaCl with counts on a medium containing a high level of salt (6).

Figure 1 shows an example of detection of injured *S. aureus* from a food product. At timed intervals, *S. aureus* present in sausages undergoing a lactic fermentation were plated on both TSA and TSAS. Injured and non-injured cells form colonies on TSA (injured cells re-

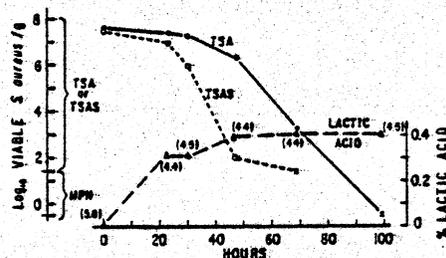


Fig. 1. Acid injury to *Staphylococcus aureus* in sausages, with 1% glucose and lactic acid starter culture, during fermentation at 35 C; numbers in parentheses are pH values (data from reference 34). (Final cell counts were determined using a 3-tube MPN to detect small numbers of cells. These values are designated by "X".) Difference in cell counts on TSA and TSAS indicates magnitude of injury.

paired their injury rapidly in the absence of salt and formed colonies); only non-injured cells form colonies on TSAS. The difference in counts between TSA and TSAS measures bacterial injury. Data in Fig. 1 show that little injury occurred at 20 h fermentation; as lactic acid accumulated (indicated by pH decrease), the acid-induced injury increased. At 50 h, the number of injured cells (difference in count between TSA and TSAS) was quite large. After 70-80 h, however, colony counts on TSA and TSAS converge, indicating that the acid was killing the staphylococci (since TSA supports the growth of both non-injured and injured cells, a decrease in the count on TSA means that the cells are dead; i.e., unable to form a colony on any medium). Therefore, sausages fermented for relatively short times have the potential to contain large numbers of injured *S. aureus*.

In a study of freeze-thaw injury, beef contaminated with *E. coli* was placed in frozen storage and sampled at intervals (Fig. 2). After thawing,

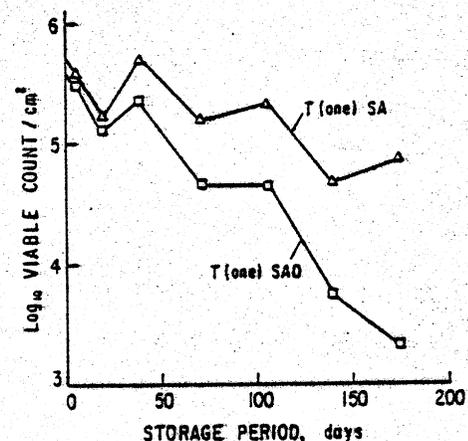


Fig. 2. Freeze-thaw injury in *Escherichia coli* during storage at -5 C in beef (data adapted from reference 22). Difference in cell counts on T(one)SA and T(one)SAD indicates magnitude of injury.

aliquots were plated on T(one)SA and T(one)SAD. Injured cells did not form colonies on T(one)SAD; however, non-injured cells grew well. Both non-injured and injured *E. coli* formed colonies on T(one)SA. Difference in counts between T(one)SA and T(one)SAD indicate a substantial freeze-thaw injury to *E. coli* in frozen beef stored for more than 150 days. Some cell death was evident as shown by a decrease in count on T(one)SA. The use of a detection medium containing deoxycholate will thus indicate low numbers of *E. coli* and not give a true indication of the extent of bacterial contamination of the frozen beef.

Other plating systems have been proposed for the detection and quantitation of injured cells. Lee and Goepfert (21), working with heat-stressed *S. typhimurium*, used Trypticase Soy agar plus 0.2% yeast extract for both injured and non-injured cells and EMBS for non-injured and injured cells) and plate count agar plus 2.5% NaCl (for

non-injured cells) were used for heat-stressed vegetative cells of *B. cereus* (30). Other examples can be found in the references cited by Busts (4) and Tomlins and Ordal (39).

Cellular Damage Resulting from Sub-Lethal Stress

The primary site of sub-lethal heat damage in bacteria appears to be the limiting cell membrane (i.e., the membrane responsible for selective permeability) because early indications of injury are leakage of cellular components from the cell into the external milieu (16) and damage to membrane transport mechanisms (18). Cellular materials that leak out of the cell include species that absorb ultraviolet radiation at 260 nm (nucleic acids) and 280 nm (proteins); sodium, potassium, and magnesium ions; membrane lipids and phospholipids; and amino acids. Degradation of cellular macromolecules also occurs during injury and includes breakdown of ribosomes, breakage of single strand DNA, and destruction or inactivation of enzymes which impair bacterial metabolism.

Membrane damage also occurs following low temperature and freeze-thaw stress (23), freeze-drying, and exposure to chemicals such as solvents or cationic detergents (2). Leakage of ultraviolet-absorbing materials, however, was not observed in acid-injured *E. coli* (29) or *S. aureus* (Smith and Palumbo, unpublished observations). Stresses may differ in their effects on bacterial membranes. Stevenson and Graumlich (36) indicated that yeast and fungi also suffer membrane damage after injury with heat, low temperature, or freeze-thaw stress.

Bacterial spores, though more resistant to stresses than are vegetative cells, also can be injured. The effect of sub-lethal heat on bacterial spores appears to be more complicated than heat stress on vegetative cells (1,16). Sub-lethal heat stress can affect the germination and outgrowth of the spore into a multiplying, vegetative cell. Heat injury at the germination stage probably involves inactivation of a germination initiation enzyme. Injury of the outgrowth process involves damage to the spore membrane: germination occurs but outgrowth to a multiplying, vegetative cell does not.

Factors That Affect Microbial Injury

A variety of factors can change the pattern of bacterial injury, including the presence of solutes, cell age, cell growth temperature, injury medium composition, as well as other environmental, chemical, and physiological factors (2,4).

Cryoprotectants such as glycerol, sucrose, or NaCl protected gram-negative bacteria from freeze-thaw injury (2,23). Yeast were not injured by freeze-thaw stress when glycerol was present in the freezing menstruum (36).

S. aureus was protected from heat injury when heated in the presence of solutes (Fig. 3). TSAP, which allows growth of both injured and non-injured cells, and TSAX, which allows the growth only of non-injured cells, were used to measure the extent of injury. The presence of NaCl, glycerol, or sucrose protected *S. aureus* cells from heat injury, as shown by increased counts on TSAX in the presence of solutes (Smith and Palumbo, unpublished observations).

Patterson and Jackson (26) showed that the cells of *S. aureus* or *E. coli*

grown to the exponential stage of growth (young cells) were more susceptible to injury by low temperature storage (4 C) than cells grown to the stationary stage (old cells). Similar results were obtained with a variety of gram-negative species (2).

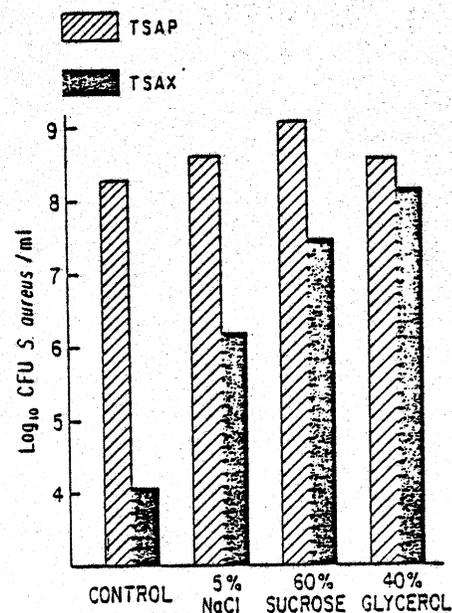


Fig. 3. Protection by solutes of *S. aureus* in 25% ground beef slurry heated for 90 min at 49 C.

S. aureus became increasingly more resistant to acid injury with increasing growth temperature of the culture (Fig. 4). As the growth temperature increased from 25 to 43 C, the amount of injury decreased as shown by an increase in count on TSAS (Smith and Palumbo, unpublished observations).

The composition of the suspending medium in which injury is to be detected may be important in determining the extent of injury. For example, *Salmonella anatum* subjected to freeze-thaw stress showed fewer injured cells in milk than in water (20).

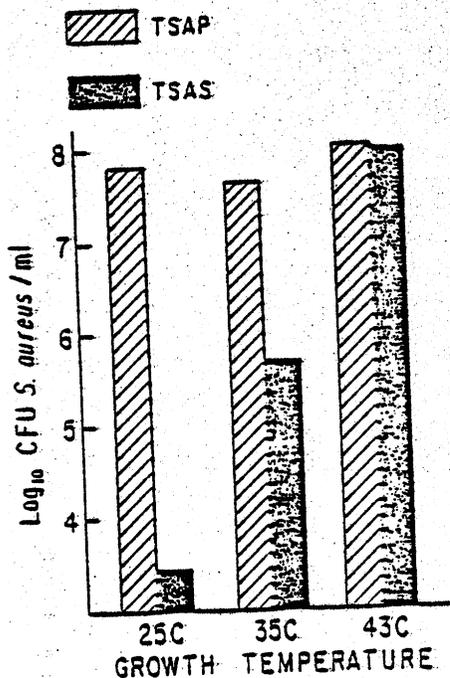


Fig. 4. Effect of growth temperature of *S. aureus* on susceptibility of cells to acid injury (acetate buffer, 0.2 M, pH 4.6-4.7, 90 min at 40 C).

Thus, in processed food, the effect of stress on contaminating microorganisms may be modified by the pH, presence of additives, water activity, ionic strength, age of the cells, and cell growth temperature. The effect of a specific stress on bacterial injury in a food product, therefore, may differ markedly from that in a simple laboratory system.

Restoration of the Normal Activities of Injured Cells

Injured cells can repair the damage incurred during stress, then grow and divide normally. Upon repair, cells of *S. aureus* injured by heat or freeze-drying have been shown to regain the salt tolerance lost during the stress treatment, to grow, and to produce enterotoxin similar to un-

Repair is measured by observing the restoration of tolerance for the restrictive agent(s) utilized in the media for detection of injured cells. Repaired cells of *S. typhimurium* regain their tolerance for dyes and salt and form colonies on EMBS (40). Data in Fig. 5 show the repair of heat-injured *S. typhimurium*. Cells heated in phosphate buffer at 48 C were injured as shown by the decrease in count on EMBS (Fig. 5a). After 30 min of heating, the cells were centrifuged and then resuspended in T(ase)SB incubated at 37 C to allow repair. After 3 h in the broth, the count on EMBS was similar to that on T(ase)SA, indicating that the injured cells had repaired the damage and regained tolerance to the inhibitory substances

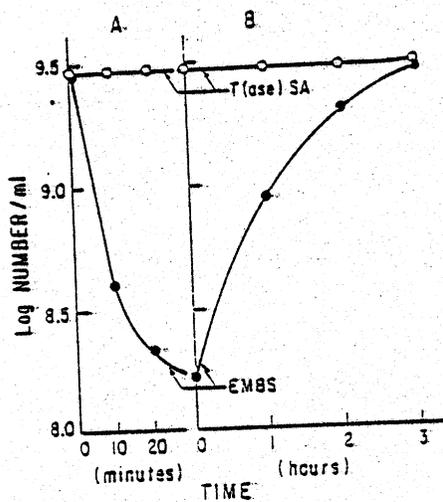


Fig. 5. Injury and repair in heated *Salmonella typhimurium*. A. Cells were heated in 0.1 M phosphate buffer, pH 6.0, at 48 C; at intervals, samples were plated on T(ase)SA and EMBS. B. Cells in A were concentrated by centrifugation and added to T(ase)SB incubated at 37 C on a rotary shaker; at intervals, samples were plated on T(ase)SA and EMBS (data modified from reference 40).

in EMBS (Fig. 5b). Cell division of non-injured cells was not responsible for regaining inhibitor tolerance because no increase in total cells, as measured by counts on T(ase)SA, occurred during the 3 h repair period in broth (Fig. 5b).

Tomlins and Ordal (39) and Pierson et al. (27) have reported that there is an extended lag (with no cell division) during repair; during this extended lag, the cells synthesize membrane lipids and phospholipids, protein, ATP, ribosomal RNA, ribosomes, and repair breaks in single strand DNA. Specific details of the repair process differ with different bacterial species. Actinomycin D, which interferes with DNA-mediated RNA synthesis, prevented return to salt tolerance in heat-injured *S. aureus* (i.e., prevented repair). However, chloramphenicol, penicillin, and cycloserine did not prevent repair (18). Chloramphenicol interferes with protein synthesis, whereas penicillin and cycloserine inhibit bacterial cell wall synthesis. Thus, synthesis of RNA, but not of protein or cell wall(s), is necessary for repair of heat-injured *S. aureus*.

Data obtained by the use of inhibitors of repair indicate that thermally-injured bacteria from different species show different sites of stress damage. Heat-injured *Vibrio parahaemolyticus* required synthesis of cell wall ribosomal RNA and protein for repair (14). Tomlins and Ordal (37) showed that repair in heat-injured *S. typhimurium* depended on synthesis of ribosomal RNA, ATP, and new protein but did not require DNA synthesis (and cell division). They used a citrate-containing repair medium in which 2,4-dinitrophenol was used to un-

couple microbial oxidative phosphorylation. They could thus demonstrate a need of ATP synthesis for repair of heat-injured *S. typhimurium*. Use of glucose instead of citrate would have permitted microbial synthesis of ATP through substrate-level phosphorylation which is not sensitive to 2,4-dinitrophenol. A medium containing citrate, phosphate, NH_4^+ , and trace metals permitted repair of heat-injured *S. typhimurium*; a complex source of nitrogen was not required (38). Heat-injured *S. aureus* required a nitrogen source (complex mixture of amino acids), phosphate, and an energy source (glucose) in the repair medium (19). However, Hughes and Hurst (15) could not show a requirement for glucose during the repair of heat-injured *S. aureus* but did indicate absolute requirements for phosphate and a complex mixture of amino acids. The addition of magnesium ions facilitated repair. The nutritional requirements for repair thus vary greatly and probably depend on the bacterial species (or even strain) as well as on the type and extent of injury.

Resuscitation of Injured Cells for Food and Water Analysis

Many of the selective media currently in use for the detection, isolation, and quantitation of microorganisms from food and water are not suitable for the examination of foods that have undergone drying, heating, freezing, or some other such treatment or for water that has been treated chemically. An appropriate resuscitation (i.e., to restore from apparent death) medium for stressed microorganisms is necessary for

microbiological analyses of food. During the food processing water treatment and other kinds of purification technology, microbial contaminants are exposed to a variety of stresses which may cause cell injury. The level of chlorine added to drinking and recreational water can decrease as a result of its reaction with organic matter or by dilution and can cause injury instead of death to microorganisms. The nutritionally adequate resuscitation medium must consider any cellular damage which the stress produced in the microorganism and support the repair of any stress-induced damage (e.g., regeneration of ribosomes and enzymes, synthesis of proteins and membrane lipids, and repair of damaged DNA).

Among factors that must be considered in the formulation are: type of stress, species and/or strain of microorganism, physical and chemical conditions before and during stress, and the chemical and physical environment of resuscitation. These factors have been discussed in an excellent review by van Schothorst (33). Other reviews giving specific details for resuscitation of injured bacteria from food are: injured coliforms in frozen foods (35), injured spores (32), stressed staphylococci (9), injured coliforms and salmonellae (13), and stressed coliforms and *Vibrio parahaemolyticus* (31). The importance of resuscitation of injured cells is being recognized increasingly by food microbiologists. The Compendium of Methods for the Microbiological Examination of Foods (25) has a chapter detailing specific procedures to be followed for detecting and enumerating injured bacteria in foods. Articles in recent issues of

Journal of Food Protection, Applied and Environmental Microbiology, Journal of Applied Bacteriology, and Journal of Food Science may then be consulted for further examples of resuscitation procedures for various stressed microorganisms.

There are two approaches for resuscitation and detection of stressed microorganisms in foods: liquid-repair or solid-repair (31). In the liquid-repair method, the food sample is blended in a non-selective broth followed by incubation at optimum repair temperature for a suitable length of time to allow repair of injured cells. An aliquot of the repair broth is transferred to selective liquid medium or diluted and plated on selective agar. The liquid-repair system is suitable for Most Probable Number (MPN) determinations, for presence/absence tests, and for isolation of organisms. It may not be suitable for regulatory purposes when enumeration is done on selective agar because the count reflects not only repair of injured cells in the repair broth but also multiplication of non-injured cells in that broth. Thus, an accurate bacterial count suitable for regulatory purposes cannot be made on solid media. Another disadvantage of liquid-repair is the addition of potential inhibitors (e.g., salt or acids) from the food to the repair broth.

In the solid-repair method, the food sample is blended with a phosphate diluent, and aliquots (0.1-3.3 ml) are transferred to petri dishes. A non-selective agar is poured into the plate (approximately 5 ml). The plates are incubated at a suitable temperature and time to obtain repair. A selective agar (approximately 10 ml) is then poured

over the non-selective agar. Plates are incubated at a suitable temperature until countable colonies are formed. The solid-repair method allows a direct count suitable for the use of regulatory agencies, since each colony represents either an original injured cell that had repaired or a non-injured cell. However, the method is not suitable for foods with low bacterial counts (<10/g).

Freeze-thaw injured *E. coli* were enumerated by use of a solid-repair system in which the suspension of *E. coli* was pour-plated with 10-12 ml of T(ase)SA. Plates were incubated at 35 C for 2 h to allow repair; then 10-12 ml of Violet Red Bile agar was poured over the T(ase)SA. Plates were incubated at 45.5 C for 24 h. Typical colonies were counted and confirmed as *E. coli* (28). A similar method was used by Hackney et al. (12) for determination of coliforms from seafoods and marine environments.

Enterococci were recovered from marine environments and frozen seafoods by pour-plating the sample with 5 ml T(ase)SA and incubating the plates at room temperature for 2 h to effect repair. Ten to twelve ml of selective KF Streptococcal agar was poured over the T(ase)SA, and plates were incubated at 35 C for 48 h. Typical colonies were counted and confirmed as enterococci (12).

Low temperature and freeze-thaw stressed *V. parahaemolyticus* were recovered from seafood by a liquid-repair method (31). The sample was blended with T(ase)SB and incubated at 35 C for 2 h. Sufficient sterile NaCl (20%) was then added to make the final salt concentration to 3%. Salt is necessary for optimum growth of *V. parahaemolyticus*, but must be added after some repair has

occurred because injured cells are sensitive to it. The T(ase)SB + NaCl tubes were incubated overnight at 35 C. A portion was transferred to selective Glucose Salt Teepol broth and incubated at 35 C for 6 h; then a loopful of this broth was streaked onto Thiosulfate Citrate Bile Salts Sucrose agar plates and incubated at 35 C. Plates were examined for typical colonies of *V. parahaemolyticus*, and the MPN was determined.

Other workers have managed to plate injured cells directly onto selective agars by neutralizing the inhibitory effect of the selective agent on injured cells but still retain the selectivity of the agars for the desired organisms. Hydrogen peroxide accumulation appears to be associated with cellular injury due to sub-lethal stress (24). Addition of catalase or pyruvate (both act by decomposing peroxide) to TSA + 7% NaCl or Vogel Johnson agar (containing 0.02% tellurite) permitted repair and growth of heat-injured *S. aureus* even though stressed *S. aureus* cannot repair and grow on such media in which catalase or pyruvate are omitted (24). Baird-Parker agar is a pyruvate-containing selective medium for stressed and unstressed staphylococci which permits plating of food samples without a prior repair period in a nonselective medium and is available commercially. Martin et al. (24) also demonstrated that addition of catalase to the selective agars used for detection of *S. typhimurium*, *Pseudomonas fluorescens*, and *E. coli* permitted direct enumeration of stressed forms of these bacteria.

As indicated above, direct plating is not suitable for the detection of low numbers of cells in foods. A liquid MPN medium is necessary to

determine small populations. Brewer et al. (3) modified the MPN procedure for *S. aureus* by adding catalase or pyruvate to T(ase)SB + 10% NaCl. They demonstrated that the use of the modified T(ase)SB permitted direct MPN determinations of *S. aureus* in foods without use of a prior repair medium. Confirmation of the MPN tubes was done on Baird-Parker agar. The Bacteriological Analytical Manual for Foods (10) recommends T(ase)SB + 10% NaCl for the MPN determination of *S. aureus* but neglects the possibility of injured cells. Addition of catalase or pyruvate to the broth should improve the MPN determination of staphylococci.

The addition of peroxide decomposing agents such as catalase or pyruvate to selective media should find wide-spread use in the food industry and regulatory agencies. It would permit inoculation of food or water samples containing stressed organisms directly into the selective media without the use of non-selective repair media. Use of such procedures would result in a savings in time, money, and equipment and permit more widespread monitoring of the bacteriological quality of processed foods.

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